STUDIES ON THE MECHANISM AND PRODUCTS OF TRANSCRIPTION OF THE NUCLEAR GENOME IN ANIMAL CELLS

Thesis by

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TO PATTIE

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ABSTRACT

Part I

Chapter 1. The metabolic stability of pulse labeled or long-term labeled mRNA from cytoplasmic free polysomes has been measured in HeLa cells, using chase conditions which do not involve inhibitors of RNA synthesis, and chromatography on benzoylated-DEAE-cellulose or poly(T)-cellulose for the isolation of mRNA. For these studies, a new chase technique has been developed which allows the analysis of the stability of mRNA labeled during a short ^{3}H-uridine pulse. Pulse labeled and long-term labeled mRNA have been found to decay with an estimated average half-life of about 2 and 3 days, respectively, much longer than hitherto assumed.

Chapter 2. Polyadenylated messenger RNA extracted from HeLa cells was hybridized with a mass excess of HeLa DNA. The kinetics of the hybridization reaction demonstrated that most of the mRNA is transcribed from nonrepetitive DNA. The amount of mRNA hybridized to DNA was measured both with and without prior RNase treatment. Comparison of the results indicates that within the limits of detection, HeLa mRNA does not contain repetitive sequence elements covalently linked to nonrepetitive sequence transcripts. However, a small fraction of the HeLa mRNA preparation is transcribed entirely from repetitive DNA sequences. This fraction represents about 6% of the total polyadenylated mRNA preparation.

Chapter 3. The sedimentation properties of pulse-labeled and long-term labeled mRNA from HeLa cell free-polysomes, selected for poly(A) content by two successive passages through poly(T)-cellulose columns, was analyzed under native and denatured conditions. The sedimentation profile of the mRNA on both sodium dodecyl SO_4-sucrose gradients and formaldehyde-sucrose gradients showed a broad distribution of components with estimated molecular weights ranging from 2×10^5 to 5.5×10^6 daltons and a weight-average molecular weight of 8.5×10^5 daltons.

Part II

The > 50 S HnRNA, isolated from either HeLa cells or immature duck erythrocytes labeled for different times with [5-^{3}H]uridine, was examined for the presence of complementary transcripts capable of forming RNase-resistant duplexes. After extensive self-annealing of the HnRNA, carried out under conditions such that complementary RNA sequences present once or a few times in the RNA population would have formed hybrids, no evidence was found for the existence of symmetrical transcripts in either cell system. However, 2-3% and 4-5% of the purified duck and HeLa HnRNA, respectively, did form RNase-resistant hybrids. These hybrids resulted from base-pairing of complementary regions within the HnRNA molecule, as judged from the lack of concentration dependence and from the kinetics of formation of the RNA-RNA duplexes. The weight-average length of the RNase-resistant fragments from

the duck HnRNA was found to be approximately 125 nucleotide pairs; however, shorter double-stranded segments as well as longer duplexes, up to 2000 nucleotide pairs, were also observed. Annealing of the duck HnRNA in the presence of an excess of 10 S hemoglobin mRNA showed that 2% of the HnRNA formed RNase-resistant hybrids in excess of those expected from intramolecular homology. The RNase-resistant complexes formed between the 10 S mRNA and HnRNA had about the same size range as the intramolecular duplexes.

The failure to detect any intermolecular hybridization in the short-pulse labeled HnRNA from either actively growing cells or highly differentiated, non-dividing cells, strongly suggests that the mechanism for the synthesis of HnRNA in animal cells does not involve the production of high molecular weight complementary transcripts.

Part III

This report describes the use of purified rDNA to map by electron microscopy the relative position of the 18 S and 28 S RNA regions within the duck rRNA precursor and their relationship to the nonconserved portions of the precursor molecule. In the first part, the purification from duck erythrocytes of rDNA sequences suitable for use in the electron microscopic mapping of the rRNA precursor is discussed. By repeated fractionation of the total DNA, based on the relative reassociation rates of the DNA sequences with different degrees of repetition, a fraction of the rapidly renaturing

DNA was obtained which comprised only 6% of the total DNA, but contained 71% of the rRNA cistrons. Further purification of the rDNA was achieved by saturation hybridization with rRNA and separation of the rRNA-rDNA hybrids by banding in CsCl. In this manner, an rDNA-rRNA fraction was obtained which had a buoyant density of 1.805 gm/cm^3, an RNA to DNA ratio of 1.01, and a base composition for the RNA present in the hybrid identical to that of an equimolar mixture of 18 S and 28 S rRNA. The final yield of rDNA isolated by this procedure is 32%. When the purified rDNA was annealed with a mixture of 18 S and 28 S rRNA and the hybrids spread for electron microscopy, they appeared as two distinct populations with a number-average length of 0.62 ± 0.13 μm and 1.37 ± 0.18 μm, respectively. Likewise, hybrids between the rRNA precursor, isolated from duck embryo fibroblasts, and the rDNA appeared as structures containing two duplex regions of lengths 0.60 ± 0.11 μm and 1.38 ± 0.15 μm, separated from each other by a single-stranded region appearing as a large bush: this represents the portion of the precursor molecule not conserved during processing of the parent molecule. From these observations a model of the structure of the avian rRNA precursor is proposed.

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STUDIES ON THE PHYSICAL AND METABOLIC PROPERTIES OF MESSENGER RNA IN HELA CELLS

CHAPTER 1

THE STABILITY OF CYTOPLASMIC MESSENGER RNA IN HELA CELLS

BIOCHEMISTRY

The Stability of Cytoplasmic Messenger RNA in HeLa Cells

[actinomycin D/benzoylated-DEAE-cellulose/poly(T)-cellulose/ nucleoside triphosphate pools/cold chase]

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Running title: Stability of Messenger RNA in HeLa Cells

ABSTRACT The metabolic stability of pulse labeled or long-term labeled mRNA from cytoplasmic free polysomes has been measured in HeLa cells, using chase conditions which do not involve inhibitors of RNA synthesis, and chromatography on benzoylated-DEAE-cellulose or poly(T)-cellulose for the isolation of mRNA. For these studies, a new chase technique has been developed which allows the analysis of the stability of mRNA labeled during a short ^{3}H-uridine pulse. Pulse labeled and long-term labeled mRNA have been found to decay with an estimated average half-life of about 2 and 3 days, respectively, much longer than hitherto assumed.

Until now, because of the lack of an effective cold chase technique and of methods for purification of mRNA, the main approach used for the study of the metabolic stability of cytoplasmic mRNA in animal cells has been the analysis of the quantitative behavior and synthetic capacity of cytoplasmic polysomes after further mRNA synthesis has been arrested by actinomycin D. The results of these experiments have suggested that, while in differentiated cell types such as the erythrocyte (1,2) or liver cells (3) the bulk of the mRNA is considerably stable, the half-life of the cytoplasmic mRNA in rapidly growing cells is relatively short, 3-4 hr (4,5). The basic premise upon which these experiments rely is that the polysome breakdown is a direct measurement of the normal degradation of pre-existing mRNA. Recent studies on the secondary toxic effects of actinomycin D raise serious doubts as to the validity of this assumption (6-8).

Because most of these toxic effects of the drug would lead to an underestimate of the stability of the mRNA in rapidly growing cells, it was felt to be essential that this question be re-examined under physiological conditions. In the present work, we report experiments by which we have measured the half-life of mRNA of cytoplasmic free polysomes in HeLa cells, using chase conditions which do not involve inhibitors of RNA synthesis, and chromatography on benzoylated-DEAE-cellulose or poly(T)-cellulose for isolation of mRNA. It has been found that the stability of the bulk of the free polysome mRNA population is much greater than previously reported, with an estimated average half-life of approximately 3 days.

MATERIALS AND METHODS

Cells and Method of Growth. HeLa cells were grown in suspension in modified Eagle's medium (9) with 5% calf serum.

Labeling Conditions. For short term labeling of the RNA or DNA, HeLa cells (1-2 x 10^5/ml) were exposed to [5-^{3}H]uridine (20-30 Ci/mmol; 0.1 µCi/ml in the kinetic experiments, 2.5-5.0 µCi/ml in the experiments for RNA analysis), or to [8-^{3}H]adenosine (28 Ci/mmol, 1.0 µCi/ml), or to [methyl-^{3}H]thymidine (29 Ci/mmol, 1 µCi/ml), for the times indicated below.

Long term labeling of the mRNA for stability measurements was performed by growing the cells (0.5-1.0 x 10^5/ml) in the presence of 2.5 µCi/ml [5-^{3}H]uridine (20-30 Ci/mmol) for 24 hr. In order to ensure, as much as possible, a uniform incorporation of the precursor into the mRNA over the entire 24 hr period, additional label was administered at 8, 16 and 22 hr of incubation to bring the amount of radioactivity in the medium to its original level (10). As an internal control to monitor the recovery of mRNA from various samples in the analysis of mRNA stability, a constant amount of cells grown for 1 or 2 generations in the presence of 0.03 µCi/ml [2-^{14}C]uridine (62 mCi/mmol), with an additional 0.03 µCi/ml of label being administered 2-3 hr prior to harvesting, was added to the sample for each time point.

To measure the rate of protein synthesis, HeLa cells, resuspended at 1 x 10^5/ml in modified Eagle's medium with 2 x 10^{-4} M leucine and 5% dialyzed serum, were incubated with 0.2 µCi/ml [^{3}H]leucine (40 Ci/mmol). Total acid precipitable, alkali resistant, radioactivity incorporated was determined as previously described (11).

For the analysis of labeling of nucleotide pools, HeLa cells, resuspended at 1 x 10^5/ml in modified Eagle's medium with 10^{-3} M phosphate and 5% dialyzed serum, were grown in the presence of carrier-free [^{32}P]orthophosphate (1 μCi/ml) for 12-24 hr. By this time, the nucleoside triphosphate pools are uniformly labeled with ^{32}P (12). After ^{32}P labeling, [5-^{3}H]uridine (2.5-5.0 μCi/ml), or [methyl-^{3}H]thymidine (1 μCi/ml), or [8-^{3}H]adenosine (1.0 μCi/ml) was added to the cultures for the times indicated below.

Chase Conditions. "Cold" chase: After labeling cells for 30 min or 24 hr with [5-^{3}H]uridine, unlabeled 10^{-2} M uridine and 5 x 10^{-3} M cytidine were added. The culture was then cooled in an ice bath to 4°C (in a 12 min period), allowed to remain at this temperature for 3 hr under gentle stirring, rewarmed in a water bath to 37°C (in 12 min) and kept thereafter at this temperature. Warm chase: It was carried out by addition of 10^{-2} M unlabeled uridine (in some experiments, also of 5 x 10^{-3} M unlabeled cytidine) to the growth medium, or by centrifuging down the cells and resuspending them in fresh medium, as specified below.

Free Polysome Isolation. A HeLa cell extract was prepared as previously described (13), using 1.5 x 10^{-3} M $MgCl_2$ in the homogenization medium. The free polysomes were isolated from the postmitochondrial supernatant by centrifugation through a discontinuous sucrose gradient (14). The polysome pellet was resuspended in 1.0 ml TKM (0.05 M Tris buffer, pH 6.7 (25°C), 0.025 M KCl, 0.0025 M $MgCl_2$), layered over a 15-30% sucrose gradient in TKM, and centrifuged for 90 min at 25,000 rpm, 2°C, in the SW 25.1 Spinco rotor. For analysis of the EDTA sensitivity of the polysomes, the polysome pellet was dissolved in TKV (=TKM, with 0.01 M EDTA in place of $MgCl_2$) and centrifuged, as described above, through a 15-30% sucrose gradient in TKV.

RNA Extraction and Analysis. RNA was extracted from the polysomes by the sodium dodecylsulfate (SDS)-pronase-phenol method, as described previously (15). For sedimentation analysis, the RNA was centrifuged for 15 hr at 26,000 rpm at 20°C in the SW 25.3 Spinco rotor through a 15-30% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in SDS buffer.

Benzoylated-DEAE-Cellulose (BDC) Chromatography. The polysomal RNA was analyzed on BDC columns, as described by Sedat et al. (16). The resin was either prepared in this laboratory or purchased from Schwarz/Mann or Boehringer-Mannheim Corp.

Poly(T)-Cellulose Chromatography. It was performed according to Kates et al. (17), except that the RNA was applied to the column in 0.12 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA, and the column washed with the same buffer.

Nucleotide Pool Analysis. The nucleoside triphosphates were isolated from cells long-term labeled with [^{32}P]orthophosphate, and labeled for different times with tritiated precursors, by two-dimensional thin layer chromatography on PEI-cellulose, as previously described (11).

RESULTS

Effectiveness and Physiological Effects of the "Cold" Chase Technique. The conventional pulse-chase technique, involving the use of a large excess of unlabeled precursor to dilute the intracellular pools, has proven to be ineffective in the study of RNA metabolism in animal cells, because of the continued RNA labeling from the large triphosphate pools, which are fed by the turnover of unstable RNA species. In view of the need for an effective

chase technique in the present work, an effort was made to develop chase conditions which would overcome this difficulty.

Since it has been reported that at least some of the enzymes involved in nucleotide phosphorylation may remain active at reduced temperatures (18,19), a chase procedure was devised in which the addition of an excess of unlabeled precursors was coupled with a relatively brief cold treatment, in an attempt to wash out the triphosphate pools while RNA synthesis and degradation are arrested.[1] A comparison of this "cold" chase with a warm chase using an equal concentration of exogenous unlabeled uridine is shown in Fig. 1A. After a 30 min [^{3}H]uridine pulse, the warm chase fails to stop further incorporation of label into RNA, which continues for at least 2 hr. By contrast, after the "cold" chase, there is an immediate decay of the incorporated label, with a reduction of about 35% during a 40 min period after the culture is rewarmed. The initial kinetics of decline in acid precipitable cpm is similar to that found after blocking further RNA synthesis with 5 µg/ml actinomycin D; this decay represents in most part the turnover of heterogeneous nuclear RNA (20), which in the warm chase is masked by a continued high level of incorporation. The basis for the effectiveness of the "cold" chase procedure is shown in Fig. 1B. During the three hr "cold" treatment, there is no change in the level of incorporated radioactivity, while the specific activities of the UTP and CTP pools are reduced to 13 and 38%, respectively, of their original value. This reduction in specific activity is the result of two cooperative effects: the increase by a factor of about 2.5 in the UTP pool size, due to the high uridine concentration in the medium,[2] and a loss of 70% of the total acid soluble radioactivity from the cells.

In Fig. 2, the kinetics of net synthesis of RNA, DNA, and protein after the "cold" chase are compared to control kinetics. The net incorporation of

Figure 1. (a) Kinetics of net incorporation of [^{3}H]uridine by HeLa cells and decay of labeled RNA using three different pulse-chase techniques.

Three HeLa cell cultures were labeled for 30 min with [^{3}H]uridine; one was cooled for 3 hr in the presence of 10^{-2} M-uridine and 5 x 10^{-3} M-cytidine, and then rewarmed to 37°C (■——■), the other two were chased at 37°C by addition of 10^{-2} M-uridine (Δ——Δ), or 5 μg/ml actinomycin D (O——O). The data plotted are the acid precipitable cpm in aliquots of the cultures, after washing the cells with a salt solution. The labeling data for the latter two cultures are displaced on the axis of abscissae, so as to compare the effectiveness of the chase at 37°C with that of the cold treated culture. The actinomycin experimental data are redrawn from Houssais and Attardi (20).

(b) Specific activities of the UTP and CTP acid soluble pools in cells labeled for 30 min with ^{3}H uridine and, either subjected to a "cold" chase and then rewarmed, or directly chased at 37°C with 10^{-2} M-uridine and 5 x 10^{-3} M-cytidine. The cells had been prelabeled for 24 hr with [^{32}P]orthophosphate, and the specific activity data are expressed as ^{3}H to ^{32}P ratios (11). "Cold chase: ■——■ UTP, ▼——▼ CTP; warm chase: □——□ UTP, ▽——▽ CTP.

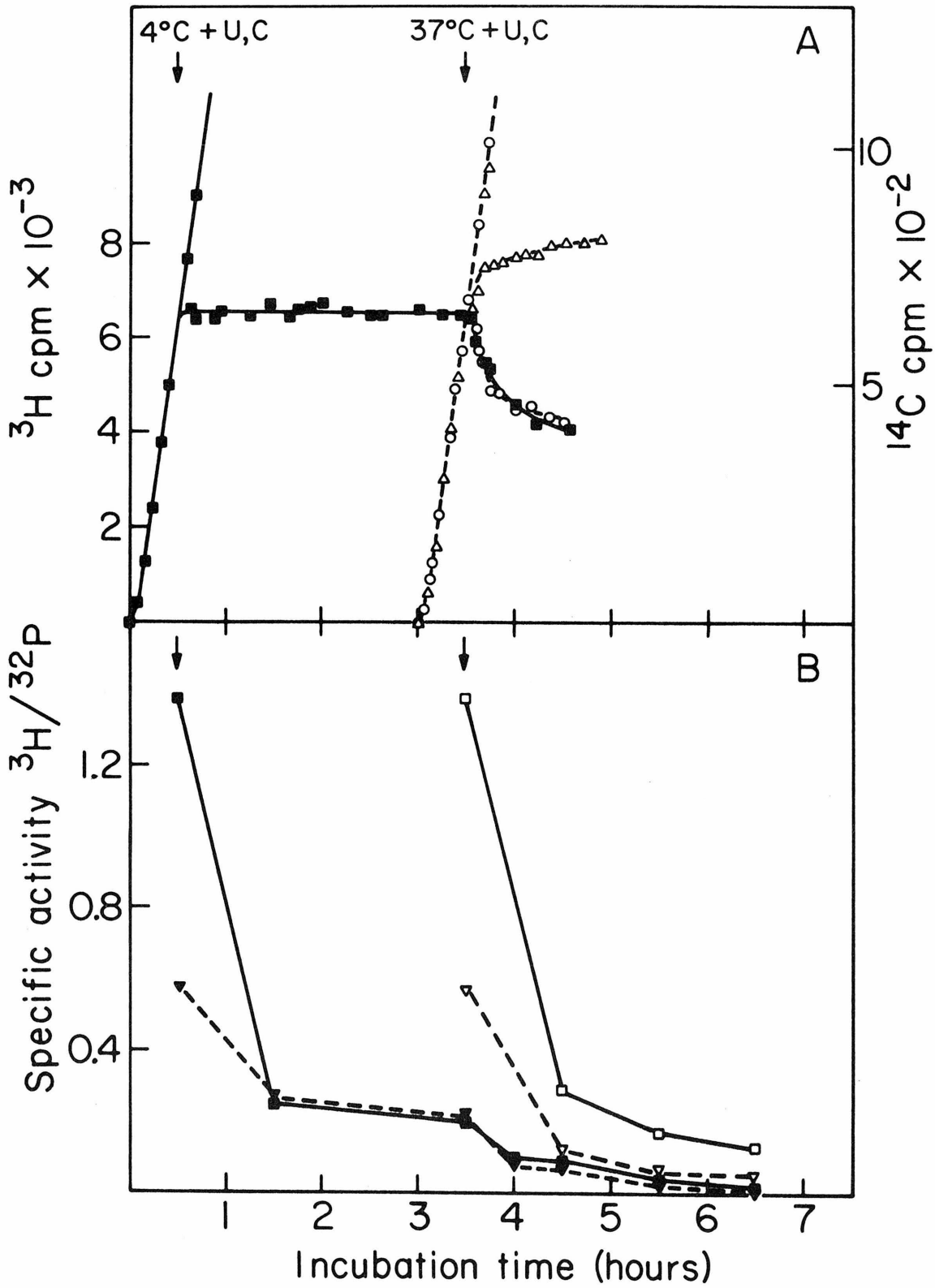
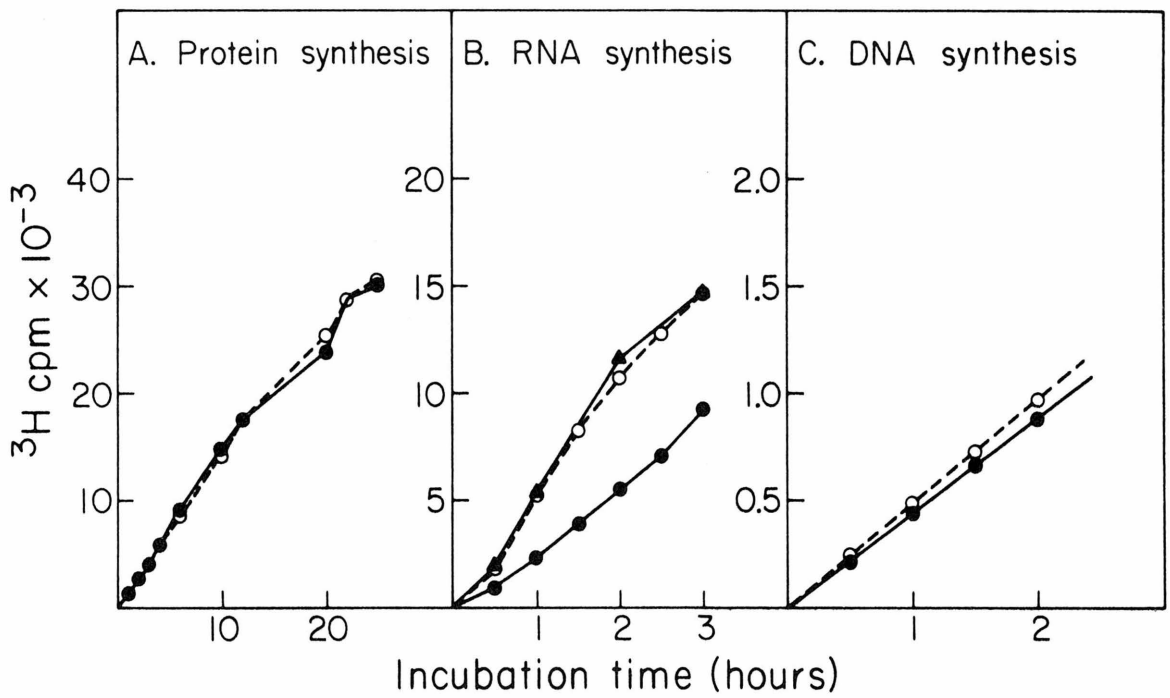

4°C + U,C
37°C + U,C
A
B
^{3}H cpm x 10^{-3}
^{14}C cpm x 10^{-2}
Specific activity ^{3}H/^{32}P
Incubation time (hours)

Figure 2. Kinetics of net protein, RNA or DNA synthesis in cells subjected to "cold" chase.

The time courses of synthesis measured in "cold" chased cultures immediately after the cooling period, ●——● or, in the case of RNA, also after 3 hr rewarming ▲——▲, are compared to those of untreated cultures at the same cell concentration O——O. The incorporation of [8-^{3}H]adenosine into RNA was measured as acid precipitable cpm solubilized by treatment with 0.5 N-NaOH at 30°C for 22 hr. The [8-^{3}H]adenosine and [^{3}H]thymidine labeling data are corrected for differences in specific activity of the ATP and TTP pools, respectively.


A. Protein synthesis
B. RNA synthesis
C. DNA synthesis
^{3}H cpm x 10^{-3}
40
30
20
10
20
15
10
5
2.0
1.5
1.0
0.5
10
20
1
2
3
1
2
Incubation time (hours)

[^{3}H]leucine into protein starts immediately after rewarming (within 2 min from transfer of the culture to the 37°C water bath), and proceeds at a rate identical to that of the control culture, when analyzed both within the first few minutes after rewarming (not shown), and over an extended period (Fig. 2). These observations suggest that not only does the "cold" chase not impair polysome protein synthesis, but also it allows the arrival of mRNA at the polysomes to occur at a normal rate. Similarly, the measured rate of DNA synthesis is about 90% that of control, the difference being presumably not significant. This result, coupled with an observed steady exponential increase in cell number and a normal generation time (about 24 hr), indicates that no significant degree of synchronization was induced by the cold shock. There does exist, however, a transitory inhibition of about 35% of the rate of net RNA synthesis in the cell. This inhibition, which is caused by the high concentration of exogenous uridine, gradually disappears until, by 3 hr, the rate of net RNA synthesis returns to normal.

Isolation of Free Polysome mRNA. Since benzoylated-DEAE-cellulose (BDC) chromatography has been successfully used in the purification of bacterial mRNA (16), isolation of HeLa cell mRNA was attempted by such a procedure. Free polysomes from cells pulse labeled for 20 min with [^{3}H]uridine and uniformly labeled with [^{14}C]uridine were isolated as described in Materials and Methods. These polysomes are more than 95% pure, as judged by the sensitivity to EDTA of both the UV absorbing and the radioactive material (21), as shown in Fig. 3. Fig. 4A shows the sedimentation profile of polysomal RNA. Clear [^{14}C]uridine peaks corresponding to rRNA and tRNA are seen, while the pulse labeled mRNA shows a heterogeneous sedimentation profile extending from about 8S to more than 40S, with a broad peak centered around

Figure 3. Sedimentation patterns in sucrose gradients of free polysomes from 20 min [^{3}H]uridine labeled cells before and after disruption with EDTA.

The free polysome pellet from 1.6 x 10^{8} cells was divided into two equal parts: one half was analyzed in sucrose gradient in TKM, the other half was analyzed in sucrose gradient in TKV. 0----0, A_{260} TKM; Δ----Δ, A_{260} TKV; ●——●, ^{3}H cpm TKM; ▲——▲, ^{3}H cpm TKV.

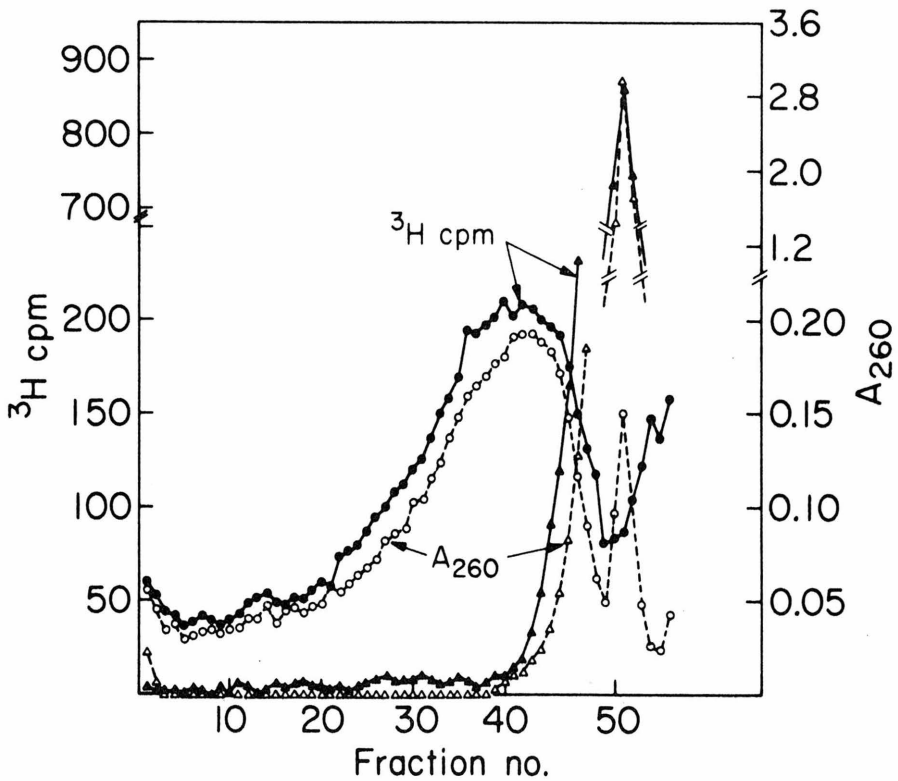
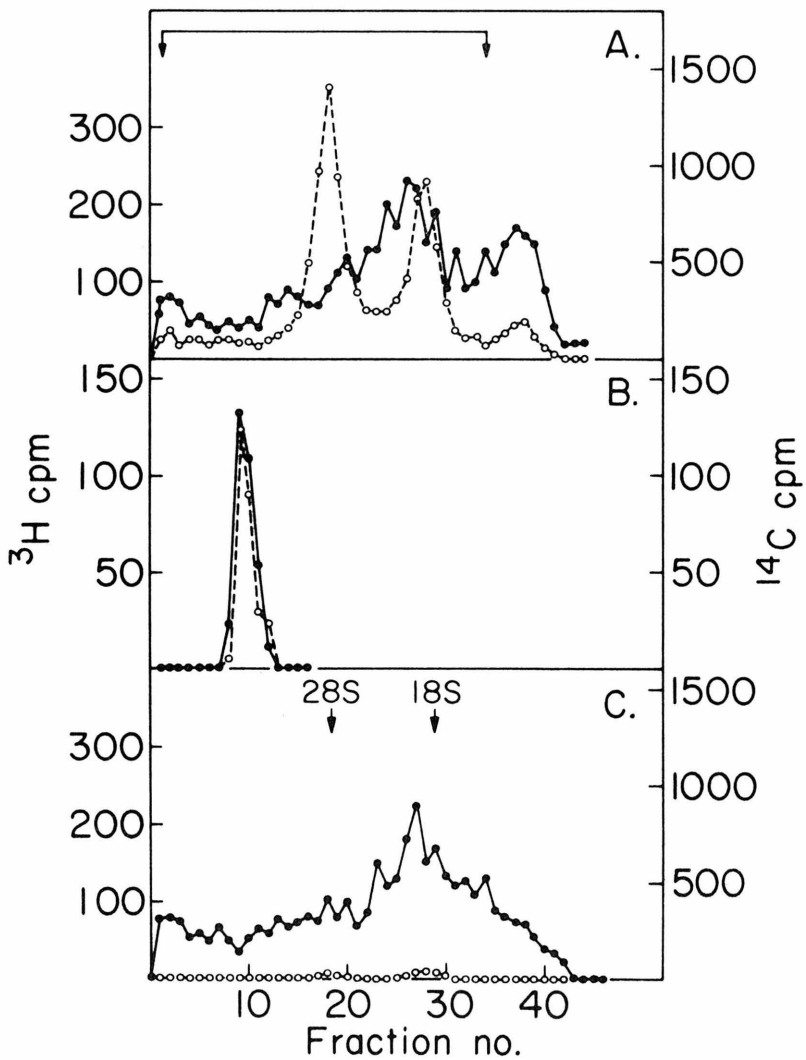

900
800
700
200
150
100
50
^{3}H cpm
3.6
2.8
2.0
1.2
0.20
0.15
0.10
0.05
A_{260}
^{3}H cpm
A_{260}
10
20
30
40
50
Fraction no.

Figure 4. Sedimentation patterns in sucrose gradients of HeLa cell polysomal RNA before and after BDC chromatography.

(a) Sedimentation profile of RNA extracted from a mixture of cells labeled for 20 min with [^{3}H]uridine and cells labeled for 48 hr with [^{14}C]uridine and chased for 24 hr with 10^{-3} M-uridine.

(b) The portion of the gradient indicated by arrows in (a) was collected by ethanol precipitation and centrifugation, and chromatographed on BDC, pH 3.5. The RNA eluted with 1 M-NH_4Cl in urea buffer is shown.

(c) A portion of the RNA eluted from the BDC column was run in sucrose gradient as in (a). ●——●, ^{3}H cpm; 0----0, ^{14}C cpm.


A.
B.
C.
300
200
100
150
100
50
1500
1000
500
^{3}H cpm
^{14}C cpm
28S
18S
10
20
30
40
Fraction no.

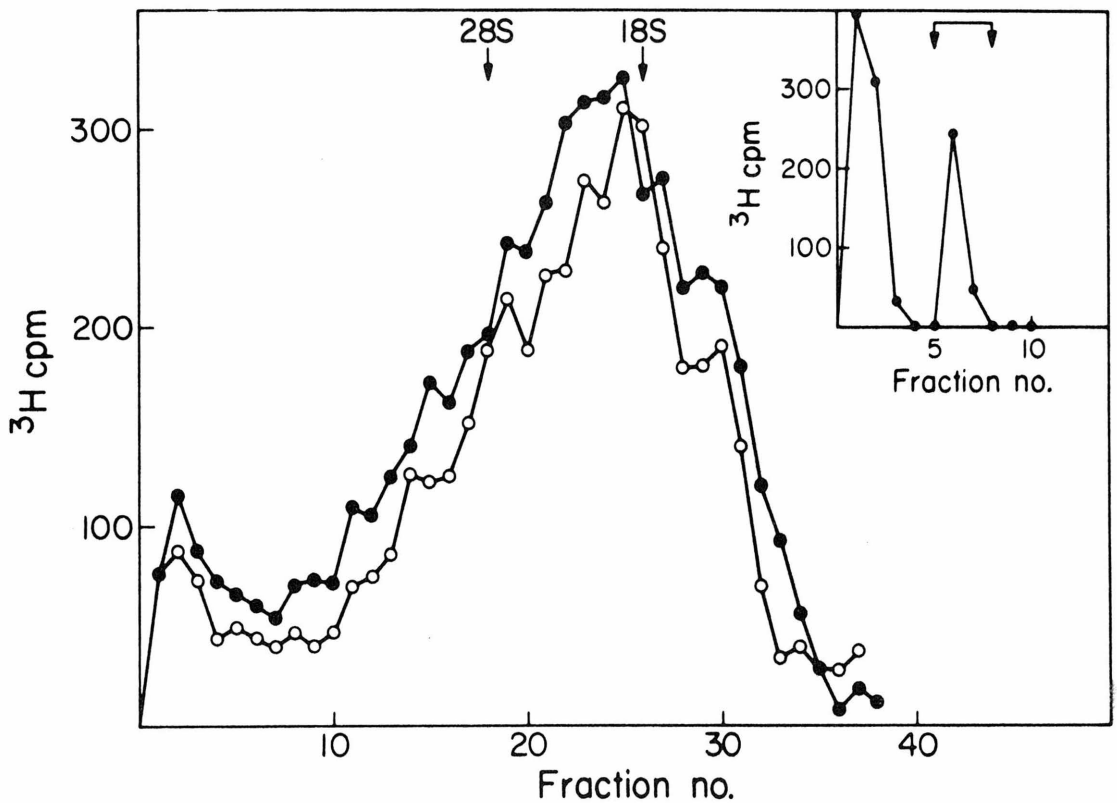
20S. The small peak of ^{3}H label found at the bottom of the tube is RNA which has been prevented from pelleting by the cushion of 64% sucrose.

The portion of the gradient indicated by arrows in Fig. 4A was pooled and chromatographed on BDC at pH 3.5. Fig. 4B shows the RNA eluted, as a single peak, with 1 M NH_4Cl in urea buffer (8 M urea, 0.1 M acetic acid, pH 3.5): this RNA includes about 90% of the ^{3}H-labeled mRNA (in different experiments, the yield of mRNA varied between 70 and 95%) and only about 5% of the ^{14}C-labeled RNA. As shown in Fig. 4C, the sedimentation profile of the eluted pulse labeled material is identical to that shown in the original gradient, showing that there was no degradation nor preferential isolation of any size class of mRNA. The ^{14}C curve shows a small peak at 18S and a smaller one at 28S: these peaks presumably represent a small amount of eluted rRNA, corresponding to about 2% of that originally present in the preparation.

The basis of the separation on BDC at pH 3.5 of mRNA and rRNA is not clear (16). Thus, although this procedure appeared to be effective for the isolation of HeLa cell mRNA, it was felt to be desirable to check the results with an independent technique. For this purpose, the recently described procedure for isolation of mRNA from mammalian and other eukaryotic polysomes on poly(U)- or poly(T)-cellulose columns (22, 23) (exploiting the existence of poly-A stretches covalently linked to the mRNA molecules (22-24), was used. The sedimentation patterns of 30 min pulse labeled mRNA isolated on a BDC and a poly(T)-cellulose column (insert) are compared in Fig. 5. The ^{3}H profiles of the mRNA are substantially identical, with the recovery of the ^{3}H label being about 15% higher with the poly(T)-cellulose column. A similar sedimentation pattern of HeLa mRNA separated on poly(T)-cellulose

Figure 5. Comparison of sedimentation patterns of pulse labeled polysomal RNA isolated by BDC or poly(T)-cellulose chromatography.

A portion of polysomal RNA from 30 min [^{3}H]uridine labeled cells was run on a poly(T)-cellulose column (insert), and an equal portion on a BDC column. The material eluted with 0.01 M-Tris, pH 7.4, 0.001 M-EDTA from the poly(T)-cellulose column (indicated by arrows in insert) and that eluted from the BDC column were collected by ethanol precipitation and centrifugation, and run on sucrose gradients, as in Figure 4. ●——●, poly(T)-cellulose isolated RNA; O——O, BDC isolated RNA.


28S
18S
300
200
100
^{3}H cpm
10
20
30
40
Fraction no.
300
200
100
^{3}H cpm
5
10
Fraction no.

column has been recently published (25). In the present work, both BDC and poly(T)-cellulose chromatography were used to isolate mRNA for analysis of its metabolic stability.

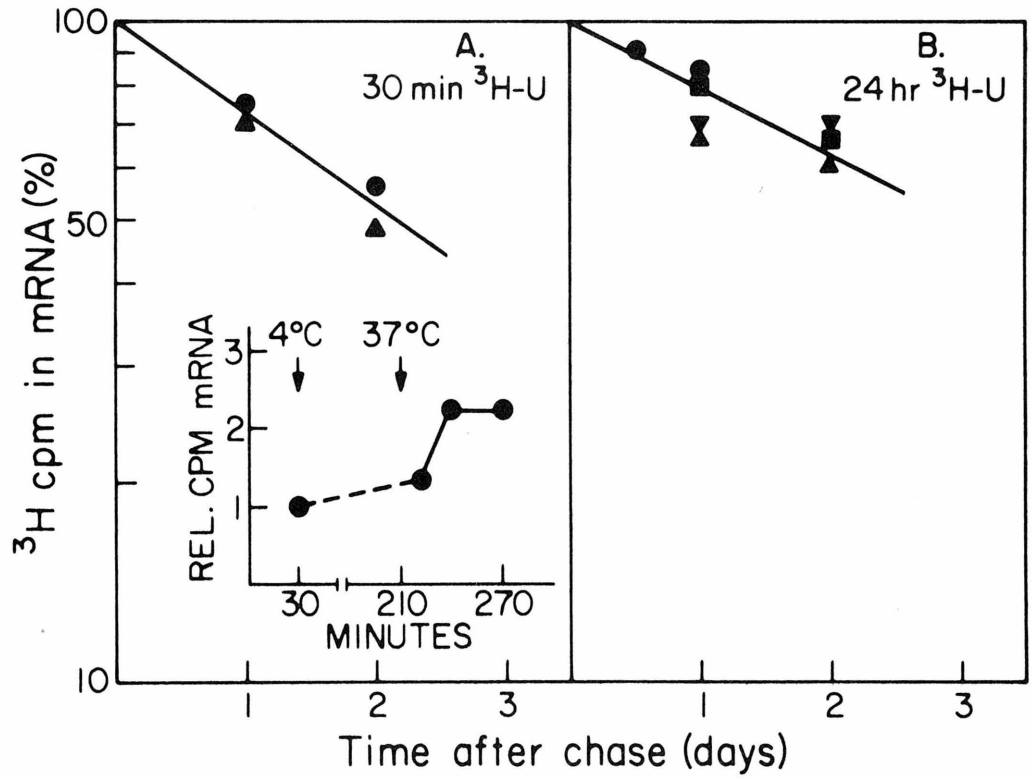
The Metabolic Stability of Free Polysome mRNA. Using the techniques described above, the metabolic stability of free polysome mRNA was investigated. In order to be able to follow the fate of both the faster turning over and the more stable components of mRNA, the cells were labeled either for a short time (30 min) or for 24 hr.

Fig. 6 summarizes the results of several experiments measuring the rate of decay, after "cold" chase, of free polysome mRNA labeled for 30 min or for 24 hr with [^{3}H]uridine, and isolated by either BDC or poly(T)-cellulose chromatography. The zero time point in these experiments was measured immediately after the temperature of the rewarmed culture reached 37°C. In the experiments involving a 30 min pulse, this zero time value was corrected, as described in the legend of the figure, both for the arrival at the polysomes of mRNA labeled during the pulse (insert) and for continued incorporation of the label remaining in the nucleotide pools. No such correction was applied to the 24 hr labeled mRNA, because of its small value. As shown in Fig. 6A, the 30 min [^{3}H]uridine labeled mRNA isolated by BDC or poly(T)-cellulose chromatography decays with an approximately first order kinetics, with a half-life of about 2 days. The mRNA labeled with [^{3}H]uridine for 24 hr and isolated by BDC chromatography also appears to decay exponentially with a half-life which, by extrapolation of the curve, has been estimated to be about 3 days (Fig. 6B).

Figure 6. Kinetics of decay of free polysome mRNA.

(a) Equal samples of HeLa cells, labeled for 30 min with [^{3}H]uridine and subjected to the "cold" chase, were mixed, at different times, with the same amount of long-term [^{14}C]uridine labeled cells; free polysome mRNA was extracted and purified by BDC (●) or poly(T)-cellulose (▲) chromatography. The amounts of [^{3}H]uridine labeled mRNA at different times have been normalized for recovery on the basis of the ^{14}C label. The zero time value (determined after rewarming the culture to 37°C) has been corrected: (1) for the continued arrival at the polysomes of mRNA labeled during the 30 min pulse, on the basis of the increment in labeled mRNA isolated on poly(T)-cellulose 1 hr after rewarming (insert), after subtraction of the small contribution of new synthesis from the labeled pools, estimated as described below [the increment measured relative to the amount at the end of the pulse would correspond to a mRNA pool equivalent to 12 min of synthesis, in good agreement with previous observations (21)]; (2) for the continued synthesis from the labeled uridine and cytidine nucleotides: the fractional increase due to the latter was estimated from the ratio of the integral of the UTP and CTP pool labeling in the 3 hr interval after rewarming (Figure 1b) to that in the interval 0 to 30 min (estimated from Figure 1 ref. 26). No account was taken of the 35% depression in rate of RNA synthesis; its effect would be small and in any case would increase the estimate of mRNA stability.

(b) Equal samples of HeLa cells were labeled for 24 hr with [^{3}H]uridine and subjected either to the "cold" chase, with the mRNA being isolated by BDC (●,■), or to resuspension in fresh medium at 37°C, with the mRNA being isolated by BDC (▼), or poly(T)-cellulose (▲) chromatography. The data have been normalized for recovery of the mRNA on the basis of long-term ^{14}C-labeled mRNA from cells added as internal control, as described above.


100
50
10
A.
30 min ^{3}H-U
B.
24 hr ^{3}H-U
^{3}H cpm in mRNA (%)
4°C
37°C
REL. CPM mRNA
3
2
1
30
210
270
MINUTES
1
2
3
1
2
3
Time after chase (days)

As a control for any possible side effects of the "cold" chase which might lengthen the life-time of the mRNA, HeLa cells were labeled for 24 hr with [^{3}H]uridine and resuspended in fresh medium without added uridine or cytidine. Fig. 6B shows that the metabolic stability of the mRNA chased in this manner, isolated by either BDC or poly(T)-cellulose chromatography, is close to that obtained after "cold" chase.

DISCUSSION

The main observation reported here is that in HeLa cells cytoplasmic mRNA is endowed with a much greater stability than up to now has been assumed. Using chase conditions which do not involve inhibitors of RNA synthesis, and chromatography on BDC or poly(T)-cellulose for the isolation of mRNA, the average half-life of the bulk of free polysome mRNA in these cells has been estimated to be approximately 3 days.

The similarity of half-life between the 30 min and 24 hr labeled mRNA suggests that there is no large subpopulation of unstable mRNA in HeLa cells. At present, it cannot be said, however, whether the measured decay results from a random degradation of the various molecular species of mRNA, or whether there are different classes of mRNA with different half-lives. The existence of mRNA species less stable then the bulk is indeed hinted at by the somewhat shorter lifetime measured for the 30 min as compared to the 24 hr labeled mRNA. One component of this shorter-lived mRNA fraction is possibly the histone messenger (5,27). Likewise, the analysis of the decay of mRNA has not been extended for a long enough time to exclude that there exists a fraction of the mRNA population with a half-life considerably

longer than the average. Although there is a slight suggestion of a leveling off of the decay curve of the 24 hr-labeled mRNA, further work is needed to verify this point.

The considerable stability shown here for the bulk of free polysome mRNA in a rapidly growing mammalian cell line provides a striking contrast to the situation in bacteria, where the majority of mRNA is short-lived (28). This difference presumably underlies a fundamental difference in the regulation of genetic expression in the two types of organisms. While in bacteria this is known to operate mainly at the level of transcription (29), in animal cells translational mechanisms of control are likely to play a prominent role.

The average half-life of cytoplasmic mRNA previously estimated in HeLa and L cells using actinomycin D (4,5) (3-4 hr) was much shorter than that measured here. This implies that the assumption, that the polysome breakdown caused by actinomycin D is the consequence of normal degradation of pre-existing mRNA, was not correct. The actual mechanism of drug-induced polysome decay is not known. This polysome breakdown may represent only one aspect of the general cellular deterioration which actinomycin has been shown to induce in HeLa cells (6-8). An alternative explanation is that the cells may possess a labile RNA or protein factor needed for polysome stability, whose continued synthesis or function is inhibited by the drug.

The new chase procedure described here has allowed the analysis of the stability of mRNA labeled during a short [^{3}H]uridine pulse. The mechanism by which the intracellular pools become depleted of radioactivity during the "cold" chase has not been studied in detail, although the experimental evidence indicates that expansion of the pools and release of the labeled

precursors into the medium, presumably after dephosphorylation, play a role here. With the exception of a transient depression by 35% of the rate of net RNA synthesis, no side effects of the "cold" chase were observed. A possible cause for this depression are the changes induced by the excess of uridine in the intracellular concentration of several nucleotides, which persist for at least 5 hr after rewarming the culture. The 3 hr recovery period may represent the time needed for the cells to adapt to these pool changes. The chase procedure described here provides a valuable alternative to the use of inhibitors of RNA synthesis for the study of the stability or processing of RNA species in animal cells under more physiological conditions.

In the present work, chromatography on BDC columns, first used for purification of bacterial mRNA (16), has been applied with success to the isolation of mammalian mRNA. The observation that messenger of animal and bacterial cells behaves alike on this resin may point to a common property of mRNA of all organisms. The recovery of 30 min or 24 hr [^{3}H]uridine or [^{14}C]uridine labeled mRNA was slightly better (about 15%) after poly(T)-cellulose than after BDC chromatography. In the light of this observation, the finding that the BDC gave consistently a better yield of mRNA (6-9%) than the poly(T)-cellulose from the 1-day chased samples, and even better (16-18%) from the 2-day chased samples, may be significant as a possible indication of changes occurring in the mRNA with ageing, which affect its retention on poly(T)-cellulose columns.

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FOOTNOTES

[1]A detailed description of this procedure will be published elsewhere.

[2]No such effect was observed for the CTP pool (its size was about one-tenth of that of UTP after the chase), while the ATP pool was reduced to one half.

CHAPTER 2

DISTRIBUTION OF REPETITIVE AND NONREPETITIVE SEQUENCE TRANSCRIPTS IN HELA MESSENGER RNA

Distribution of Repetitive and Nonrepetitive Sequence Transcripts in HeLa mRNA

(polyadenylated mRNA / hydroxyapatite / RNA·DNA hybridization / repetitive and nonrepetitive sequence)

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Running title: HeLa mRNA·DNA hybridization

Classification: Biochemistry

Abbreviations: HAP, hydroxyapatite; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; PB, phosphate buffer (pH 6.8); (polyA)mRNA, polyadenylated mRNA.

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ABSTRACT Polyadenylated messenger RNA extracted from HeLa cells was hybridized with a mass excess of HeLa DNA. The kinetics of the hybridization reaction demonstrated that most of the mRNA is transcribed from nonrepetitive DNA. The amount of mRNA hybridized to DNA was measured both with and without prior RNase treatment. Comparison of the results indicates that within the limits of detection, HeLa mRNA does not contain repetitive sequence elements covalently linked to nonrepetitive sequence transcripts. However, a small fraction of the HeLa mRNA preparation is transcribed entirely from repetitive DNA sequences. This fraction represents about 6% of the total polyadenylated mRNA preparation.

INTRODUCTION

Recent studies indicate that most eucaryotic mRNA sequences are transcribed from DNA sequences which occur only once per genome (1-9). Thus in a variety of systems, ranging from insect to sea urchin and rodent, most of the structural genes are in the class of nonrepetitive DNA sequence. A notable exception is the set of histone structural genes (10, 11), and other exceptions may well exist. In order to avoid cumbersome phrases we will occasionally use the terms repetitive and nonrepetitive transcript to describe RNA sequences that have been transcribed from repetitive and nonrepetitive DNA sequences. The demonstration that mRNAs are complementary to nonrepetitive DNA sequences has generally involved measurements of the kinetics of hybrid formation between trace quantities of labeled mRNA and excess cellular DNA. Assay of hybrid formation in such experiments customarily requires the use of RNase to destroy nonhybridized RNA. Experiments utilizing labeled cDNA (DNA transcribed from mRNA using an RNA-dependent DNA polymerase) and excess cellular DNA have involved treatment of S_1 nuclease for analysis of hybrids. Such measurements, however, preclude investigation of the possibility that a large fraction of mRNA molecules contains repetitive sequence "tags" covalently associated with the nonrepetitive coding sequences. Repetitive tags could be relatively short and, though present on a large fraction of mRNA molecules, might include only a small fraction of the total RNA nucleotides. On the basis of filter hybridization measurements, Dina *et al.* (12) claimed recently that the repetitive

"tag" model applies to a major fraction of the mRNA of Xenopus embryos.

To investigate this question further, we recently developed a procedure for the assay of RNA·DNA hybrids on HAP columns in which ribonuclease is not utilized. This procedure depends on the observation that 8 M urea prevents the binding of non-hybridized RNA to hydroxyapatite but permits the binding of molecules containing hybrid regions (2, 13). Using this method we recently showed (2) that total sea urchin gastrular mRNA is transcribed almost exclusively from single copy DNA sequences and that no appreciable portion of the mRNA molecules contains repetitive sequence "tags."

In the present communication we describe essentially similar observations on the mRNA of a human cell type, HeLa. We find that most (polyA)-containing mRNA of HeLa cells is transcribed from unique DNA sequences. However, a small class of RNAs transcribed from repetitive sequences can also be detected. Furthermore, we show that within the limits of detection none of the mRNA molecules transcribed from single copy DNA have repetitive sequence "tags" covalently linked to them.

MATERIALS AND METHODS

Cell Culture and Labeling of mRNA. HeLa cells (clonal strain S_3) were grown in suspension culture in modified Eagle's medium (14) containing 5% calf serum. The cultures were free of any detectable contamination with PPLO (Mycoplasma). The mRNA was labeled to approximately constant specific activity by growing the cells (1×10^5/ml)

in the presence of [5-^{3}H]uridine (26 Ci/mmol) for 48 hr. Isotope was added at zero time to a concentration of 1.25 μCi/ml, and additional label was added at 25 hr (1.25 μCi/ml) and 35.5 hr (0.9 μCi/ml) in order to ensure approximately uniform incorporation of the precursor into the mRNA over the entire 48-hr period. Under these conditions the supply of exogenous radioactive precursor varies less than twofold (15). During the last 12 hr of the labeling period the concentration of radioactive precursor in the medium dropped to about 60% of its value at 36 hr. Therefore the specific activity of any mRNA species which turns over more rapidly than the bulk of the mRNA could be as much as 1.7-times lower than that of the bulk mRNA (16). The specific activity of the purified mRNA preparation used for these experiments was 50,000 cpm/μg under our scintillation counting conditions.

Purification of HeLa mRNA. Free HeLa cell polysomes were isolated in essentially pure form, as described earlier (16). The polysomes displayed greater than 99% sensitivity to EDTA. Polysomal RNA was extracted and the mRNA was purified by poly(T)-cellulose chromatography (16). In the present experiments the mRNA was passed two times through 0.5 x 5.0 cm columns of poly(T)-cellulose.

HeLa DNA Isolation. DNA was isolated from HeLa cells by standard procedures. The DNA was bound to a HAP column in the presence of 8 M urea, which we found to be of aid in removing RNase contamination. After elution the DNA was sheared in a Virtis blender to about 300 nucleotides (17).

mRNA·DNA Hybridization. mRNA and DNA were mixed and denatured by immersion in a boiling water bath for 5 min. The samples were incubated at 60° in Kontes microflex vials in 0.12 M PB or 0.48 M PB. Equivalent $C_o$ts were calculated in the latter medium by correcting for the increase in reassociation rate compared to the rate in 0.12 M PB due to monovalent cation concentration (17). The fraction of RNA molecules containing hybrid regions was measured by binding to HAP (Biorad DNA grade HTP lot #9404) in the presence of 0.2 M PB, 8 M urea, 1% SDS at 40° ("urea-phosphate HAP" assay system). The incubation mixtures destined for the urea phosphate HAP analysis usually contained 0.1% SDS. In the other half of the procedure the fraction of RNA nucleotides actually residing in hybrid regions was measured by treating the sample with Worthington RNase A (10 µg/ml in 0.24 M PB), followed by passage over a Sephadex G-200 column equilibrated in 0.12 M PB. These assay procedures have been standardized and are described in detail in previous studies (2, 13).

RESULTS

Assay of hybrids without nucleases

Since many of the observations reported below are obtained by the urea-phosphate HAP method of hybrid analysis, it is useful to review briefly the evidence that this is a valid procedure for assay of RNA molecules containing regions of DNA·RNA duplex. The results of prior studies (2, 13) have shown that: a) Binding of non-hybridized RNA to HAP is almost completely suppressed in 8 M urea-0.2 M PB-1% SDS. This has been found to be true for sea urchin mRNA and hnRNA,

and for HeLa mRNA (Table 1). b) Optical measurements show that 8 M urea lowers the T_m of DNA by about 20°. DNA duplexes are also thermally eluted from hydroxyapatite in 0.2 M PB, 8 M urea at about 20° lower than in 0.12 M PB in the absence of urea. c) Measurements of DNA reassociation kinetics on HAP in the urea-phosphate buffer at 40° yield essentially the same results as standard measurements at 60°, 0.12 M PB. A small discrimination against lower stability repetitive duplexes is the only difference noted [6% less of the total DNA is bound in urea after incubation to $C_o t$ 40 (i.e., 40%) than is bound under standard conditions (46%)] (13). d) The evidence most directly relevant to the studies reported here comes from observations on sea urchin nuclear RNA (13). Hybridization of this RNA at low $C_o t$ yields structures consisting of 1000 to 2000 nucleotide long RNA fragments paired on the average over about 1/3 of their length with DNA, while 2/3 of their length remains single-stranded. These molecules, which are about the same size as the HeLa mRNAs, are bound efficiently to HAP in the urea-phosphate buffer, due to their duplex regions. The amount of RNA bound in this assay system is about the same as the amount estimated to contain hybridized sequences by isopycnic centrifugation in Cs_2SO_4 (13) or CsCl gradients (unpublished data). These experiments show that the urea-phosphate method adequately recognizes RNA molecules which contain even a minor portion of their length as RNA·DNA duplex. These are the structures which would be formed by low $C_o t$ hybridization if the repetitive "tag" hypothesis of mRNA structure is correct. The minimum length of duplex required for binding has not been determined

TABLE 1. Hybridization of HeLa mRNA with HeLa DNA

DNA	$C_o t$	% mRNA obtained as hybrid	
		Urea-phosphate hydroxyapatite	RNase-Sephadex
None	-	0.6, 1.7	-
Sea urchin	40	2.4	-
HeLa	40	5.6, 7.0, 7.2	7.6, 7.1
HeLa + 12.5 µg rRNA*	40	7.1	-
HeLa (50° incubation)	40	8.1 (30° HAP)	5.8
HeLa	13,000	31.0	24.2

Hybridization reactions and analyses were carried out as described in Materials and Methods. The reaction mixtures each contained 2500 cpm of HeLa mRNA (50,000 cpm/µg) and 250 µg of HeLa DNA or sea urchin DNA sheared to 300 nucleotides in length. At $C_o t$ 40 about 1% of the single copy DNA fraction has reacted.

*rRNA was isolated from human liver. The rRNA mass was 100 times in excess of the rDNA present in the reaction mixture.

but it is probably similar to the minimum length of DNA·DNA duplex recognized by hydroxyapatite under standard conditions, which is less than 20 nucleotides (21).

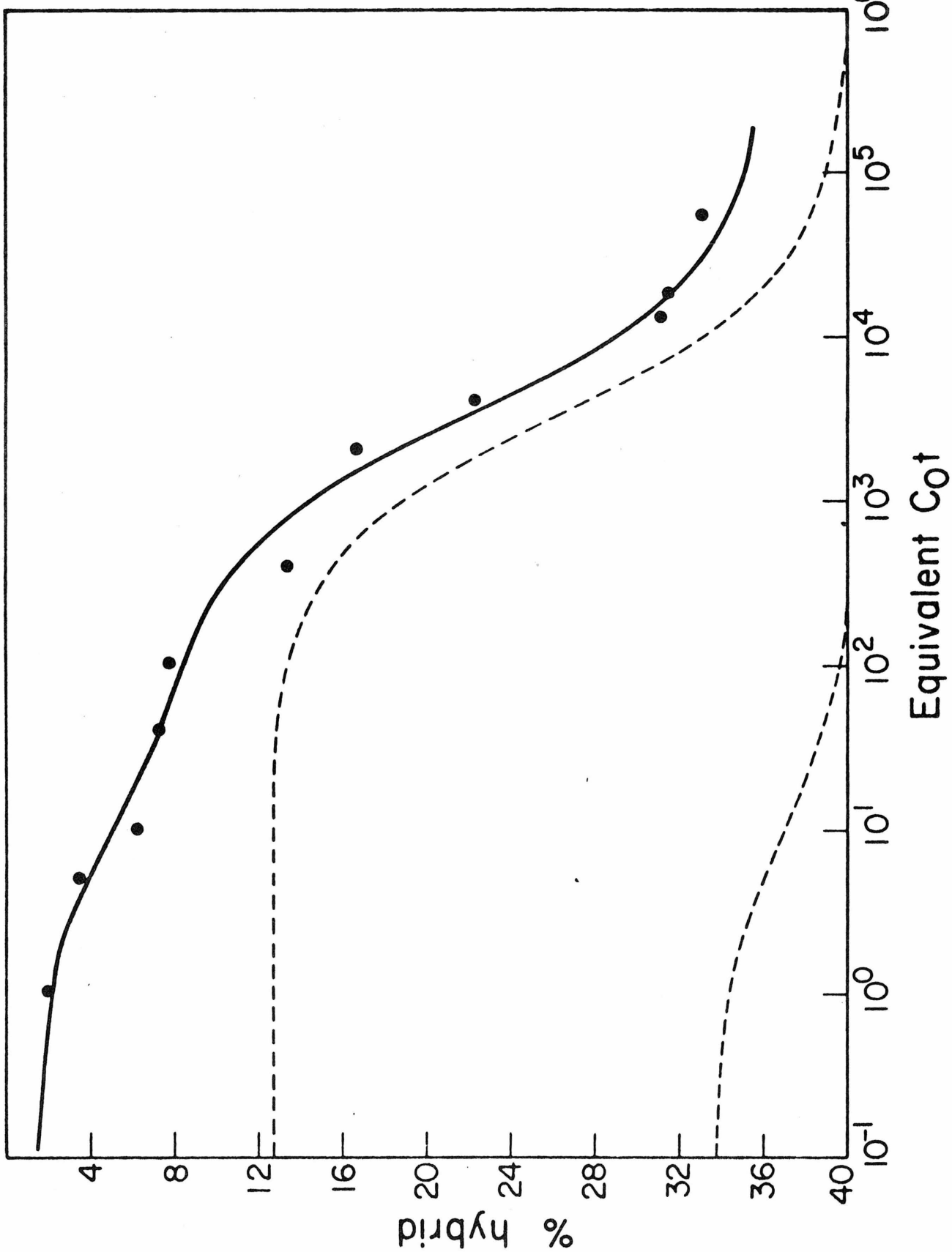
We emphasize that the urea-phosphate HAP system measures the fraction of RNA in molecules which contain hybrid regions. In contrast, the procedures using RNase measure the fraction of the RNA nucleotides which are actually in duplex regions.

Kinetics of hybrid formation between HeLa mRNA and DNA

The (polyA)mRNA was hybridized with a 5000-fold excess of DNA to various DNA $C_o$ts, and the hybrid content assayed by the urea-phosphate HAP procedure. Fig. 1 shows the kinetics of hybrid formation as a function of DNA $C_o t$. By least squares analysis the reaction is best fit assuming two components. The slow component (about 30% of the input RNA) reacts with a rate constant of 3.0×10^{-4} M^{-1} sec^{-1}, which is close to the calculated rate constant for the single copy fraction of human DNA. The faster component (about 6% of the input RNA) reacts with a rate constant for which the best estimate is 1.1×10^{-1} M^{-1} sec^{-1}. Though a rate constant for this component cannot be evaluated with any great accuracy, it is evident that the faster hybridizing RNA component is transcribed from a class of sequences present about 10^2-10^3 times per genome.

Figure 1. Hybridization kinetics of HeLa mRNA to DNA.

Reactions contained 2500 cpm of HeLa mRNA (50,000 cpm/μg) and 250 μg of HeLa DNA. The reactions were incubated in 0.12 M PB or 0.48 M PB at 60°C for appropriate times. Reaction volumes were 0.1 ml. Following incubation the hybrids were analyzed by the urea-phosphate HAP procedure. For details of incubation conditions and hybrid analysis see Materials and Methods. The solid line represents a least squares fit to the data. The root mean square error for the computer fit is 2.7%. The broken lines represent the resolved kinetic components. The fast component reacts with a rate constant of 1.1×10^{-1} M^{-1} sec^{-1} and the slow component reacts with a rate constant of 3.0×10^{-4} M^{-1} sec^{-1}.

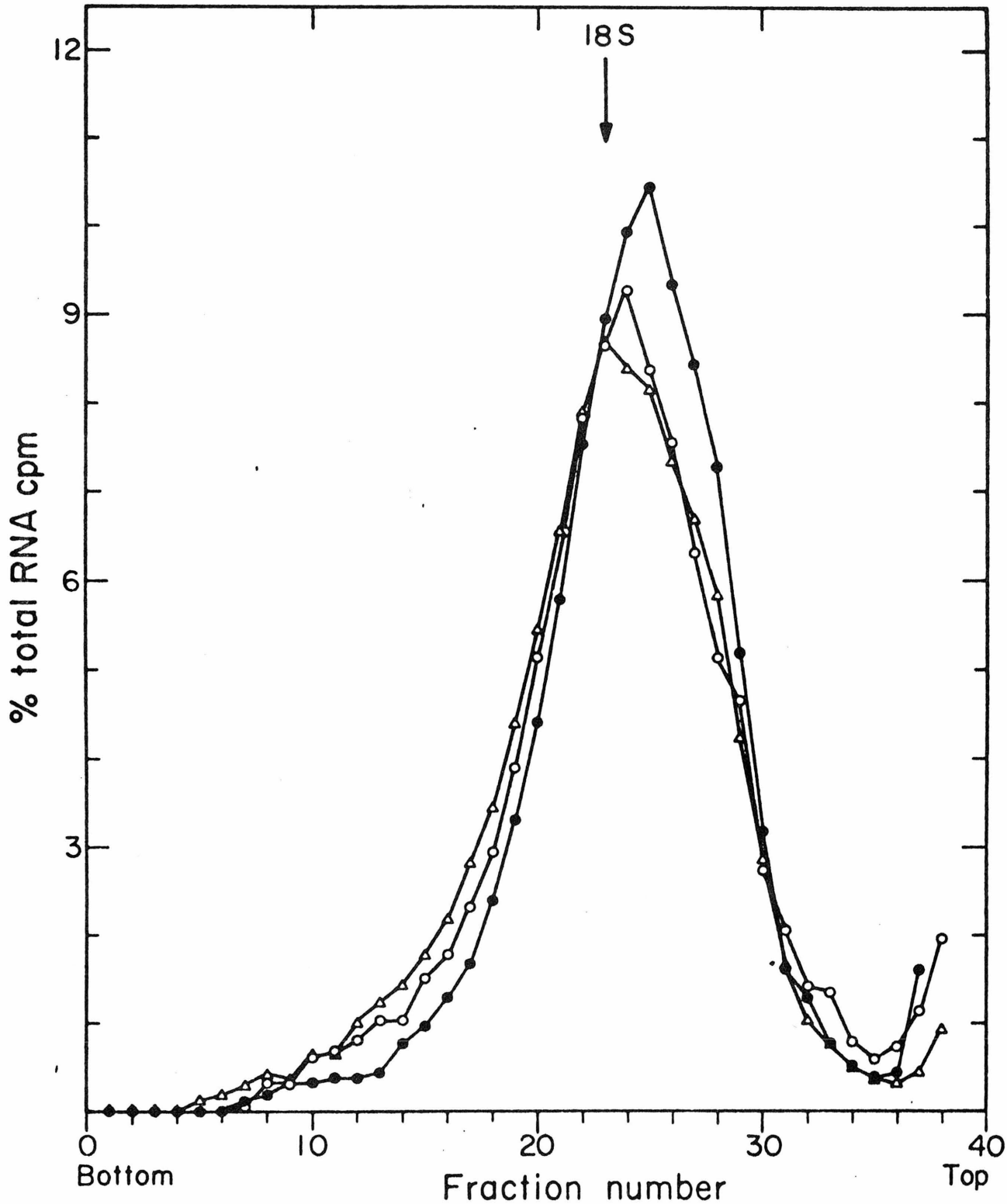

% hybrid
4
8
12
16
20
24
28
32
36
40
Equivalent Cot
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
10^{6}

Repetitive sequences are confined to a small fraction of entirely repetitive transcripts.

The next experiments are aimed at determining whether individual molecules in the mRNA preparation contain sequences transcribed from repetitive as well as single copy DNA. Alternatively repetitive and nonrepetitive sequences could be represented on separate sets of mRNA molecules. In the former case mRNA molecules, after hybridization to low $C_o t$ would contain many single-strand "tails" of unhybridized RNA. The two methods yield almost exactly the same results (Table 1). About 6-7% of the RNA molecules appear to contain repetitive regions but this is also the fraction of mRNA nucleotides which are present in repetitive regions. It follows that the repetitive sequences in the mRNA preparation must be confined to molecules which consist entirely of repetitive sequence transcript, since these molecules lack RNase-sensitive single-stranded tails after low $C_o t$ hybridization. For this measurement the size of the RNA fragments is important. If the fragments have been reduced in size, a smaller fraction of any possible nonrepetitive sequences would remain linked to the repetitive sequences. To test this possibility, RNA·DNA hybrids formed at $C_o t$ 40 were bound to HAP in urea phosphate, the RNA and DNA eluted, denatured, and the RNA size measured on formaldehyde sucrose gradients. Fig. 2 reveals no significant difference in the sedimentation profile between the hybridized and the input RNA. That is, essentially no degradation of the RNA molecules has taken place. The unbound RNA (RNA transcribed from nonrepetitive DNA sequences) sediments slightly more slowly than do the

Figure 2. Effect of annealing and HAP chromatography on the size of HeLa mRNA.

50,000 cpm of HeLa mRNA (1 µg) was incubated with 500 µg of HeLa DNA. The reaction was carried out for 3.5 hr (to $C_o t$ 40) following which the hybrids were bound to HAP in the urea-phosphate buffer (see Materials and Methods). The hybrids were eluted from the HAP columns with 0.5 M PB. Both the bound RNA and the unbound RNA were dialyzed against water, sodium acetate was added to 0.3 M and the RNA was precipitated in ethanol. The mRNA was then denatured by incubation at 63°C for 15 min in 0.1 M-sodium phosphate buffer (pH 7.7) containing 3% neutralized formaldehyde (22) and then centrifuged through a 5-20% sucrose gradient in formaldehyde-phosphate buffer (0.1 M-NaCl, 0.02 M-potassium phosphate buffer pH 7.4, 1% formaldehyde) for 24 hr at 26,000 rpm, 2°C, in a SW25.3 Spinco rotor. RNA which was bound to HAP (O——O); RNA which was not bound to HAP (●——●); control RNA, no incubation or HAP chromatography, (Δ——Δ).


18S
12
9
6
3
% total RNA cpm
0
Bottom
10
20
30
40
Top
Fraction number

other fractions, but the extent of degradation of these molecules is also negligible.

Table 1 also demonstrates that little if any additional hybrid is formed at low $C_o$ts when HeLa DNA is reacted with the HeLa mRNA under less stringent conditions. Lowering the temperature of incubation from 60° to 50° and the temperature of assay in urea-phosphate from 40° to 30° causes little change in the amount of RNA which binds to the HAP column. This shows that after low C_ot hybridization there is not a large population of mRNA·DNA hybrids which are only marginally stable in the usual hybridization conditions.

We conclude from the experiments so far presented that HeLa mRNA molecules in general lack any recognizable tags of repetitive sequence transcript covalently linked to nonrepetitive sequence transcript. Most of the hybridizing molecules thus contain only nonrepetitive sequence transcript. However, it is apparent that a small class of RNA molecules transcribed entirely or almost entirely from repetitive DNA sequence is also present in the preparation. This RNA does not appear to be rRNA, since the binding to HAP is not reduced by the presence of large amounts of unlabeled human rRNA (Table 1).

Size and amount of the repetitive component of HeLa (polyA)mRNA

Analysis of the hybridization kinetics presented in Fig. 1 indicates that about 6% of the mRNA is transcribed from repetitive sequences. However this interpretation requires that sufficient excess of DNA was present in the experiments of Fig. 1 to hybridize all of the repetitive

sequence transcripts. A limit estimate of the DNA/RNA ratio needed to hybridize all the RNA transcribed from sequences present about 10^2-10^3 times per genome (Fig. 1) can be obtained by assuming the lowest reasonable complexity for the RNA. For this calculation we can assume that there is only one species of such RNA present, the complexity of which is equal only to the transcript length, i.e. about 2000 nucleotides (Fig. 2). The DNA/RNA ratio needed to hybridize the repetitive RNA is given by the expression

$$\frac{\text{DNA}}{\text{RNA}} = \frac{\text{genome size}}{\text{reiteration frequency x complexity}}$$

and even for this extreme case, such a calculation indicates that a total DNA/RNA ratio of about 4-40 x 10^3 would suffice. In accordance with this calculation, Table 2 shows that no change in the amount of hybridization at low $C_o t$ is observed over a range of two orders of magnitude in DNA/RNA ratio. Even at a DNA/RNA ratio of 5 x 10^4, no more than 6% (background subtracted) of the RNA reacts at $C_o t$ 40. We conclude that no more than 6% of the HeLa (polyA)mRNA preparation is actually transcribed from repeated regions on the HeLa DNA, since this value has not been underestimated due to insufficient DNA/RNA ratio.

Table 3 presents an experiment in which the HeLa (polyA)mRNA was divided into four size fractions on the basis of sedimentation in sucrose gradients. Each of the fractions was then reacted with excess DNA to low $C_o$ts and the amount of hybrid measured. The data suggest a slight enrichment for repetitive transcripts in the heavier classes of RNA. It is clear that some molecules > 35S in size belong to the repetitive sequence class.

TABLE 2. Hybridization of HeLa mRNA with DNA at various DNA/RNA ratios

$C_o t$	DNA/RNA	% Hybrid
40	500	6.1
40	5,000	5.6, 7.0, 7.2
40	50,000	7.3
13,000	5,000	31.0 (35.2)*
13,000	50,000	35.7 (40.6)*

Hybridization reactions contain 2500 cpm of HeLa mRNA and appropriate amounts of HeLa DNA. The hybrids were analyzed by binding to HAP in urea-phosphate buffer as described in Materials and Methods.

*Corrected for kinetic completion of DNA reassociation. At $C_o t$ 13,000 81% of the nonrepetitive DNA has reacted. As estimated from the DNA reassociation (data not shown), 57% of the DNA fragments react as single copy sequence, 38% react as repetitive DNA and 5% are unreacted. Thus the correction factor for kinetic completeness is $\frac{(0.81)(0.57) + 0.38}{0.95} = 0.88$.

TABLE 3. Size of RNA hybridizing at $C_o t$ 40

mRNA fraction	% of total cpm	% of HeLa mRNA bound		
		+HeLa DNA	-HeLa DNA	Δ
Unfractionated	100.0	7.2, 7.0, 5.6	1.7	3.9-5.5
>35S	8.4	10.2	0.6	9.6
25-35S	25.4	9.6	0.6	9.0
15-25S	49.3	4.9	0.7	4.2
<15S	16.9	2.8	0.6	2.2

The mRNA was centrifuged on a 15-30% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in SDS buffer for 16 hr, 26,000 rpm, 20°, in an SW 25.3 Spinco rotor. The mRNA was pooled into the four size classes indicated. 10 μg of E. coli rRNA was added as carrier, and the RNA precipitated with ethanol. Hybridization reactions contained 2500 cpm (0.05 μg) of the indicated RNA fraction and where indicated, 250 μg of HeLa DNA. Reactions were incubated to $C_o t$ 40 and the hybrids were analyzed by the urea-phosphate HAP method.

Single copy sequence in HeLa mRNA

According to the hybridization kinetics in Fig. 1 about 30% of the input RNA reacts with single copy DNA at DNA/RNA = 5000. The amount of hybrid observed after a high $C_o t$ incubation is shown in Table 1. The difference between the values obtained with the urea-phosphate HAP assay method as compared to the RNase-Sephadex assay method (31% vs. 24%) is probably due to the fact that some regions of the relatively long RNA are not completely covered by DNA at this DNA/RNA ratio and therefore more RNA is bound to HAP in the urea-phosphate system. Earlier studies (13) showed that as the DNA/RNA ratio is increased, averaged coverage of an RNA molecule also increases, and the difference between RNase and urea-phosphate value decreases.

At the highest DNA/RNA ratio used in these studies, about 41% of the mRNA molecules contain hybridized regions when the reaction has terminated kinetically (Table 2). Since, as shown above, the 59% of the RNA which remains unhybridized cannot represent repetitive transcripts, this RNA must also be derived from nonrepeated DNA sequences. The question then remains why all the RNA did not hybridize at high $C_o t$. From calculations of the number of copies of each nonrepetitive DNA sequence in the reaction mixtures and the yield of (polyA)mRNA/cell[‡], it is apparent that at the highest DNA/RNA ratio used (5×10^4), mRNAs transcribed from single copy sequences and occurring less than 5×10^3 times per cell should have hybridized. This suggests that the 59% unhybridized RNA represents transcripts present in large numbers of copies, i.e. $> 5 \times 10^3$/cell. Experiments utilizing higher

ratios would have required higher specific activity mRNA than was available.

An alternative explanation for lack of complete hybridization is that some form of artefact interferes with hybridization and is responsible for the unhybridized RNA. We have carried out many control experiments in an effort to demonstrate such an artefact (Smith and Davidson, unpublished). In these experiments we have utilized mRNA from sea urchin embryos, which we have studied by methods similar to those used here (2). As with HeLa mRNA, the fraction of the sea urchin mRNA which can be hybridized with nonrepetitive DNA sequence increases as the DNA/RNA ratio is increased, but over half (55%) of the mRNA remains unhybridized at the highest DNA/RNA ratios attempted. The RNA was not significantly degraded during the hybridization reaction. Nor was the rate of the hybridization reaction found to be slower at very high DNA/RNA ratios (e.g., 300000/1) compared to more moderate DNA/RNA ratios (3000/1). When the unhybridized fractions of the mRNA were re-reacted with fresh DNA there was no significant increase in the amount of hybrid formed. That is, all the RNA which can hybridize appears to do so on first exposure to DNA. With the same methods of reassociation and assay as used in the present work, furthermore, up to 80% of sea urchin hnRNA molecules can be recovered as hybrid-containing molecules at very high DNA/RNA ratios. This shows that in themselves these methods neither inhibit hybridization nor cause substantial losses in the amounts of hybrid measured. These data suggest, though they do not prove, that the correct explanation for the failure of all the mRNA to hybridize lies in the high frequency with which some of the sequences are represented.

DISCUSSION

The experiments described in this report lead to three main conclusions regarding the nature of the sequences present in the (polyA)mRNA isolated from free cytoplasmic polyribosomes of HeLa cells. First, most of the mRNA molecules appear to be transcribed from nonrepetitive DNA sequence. This conclusion is based mainly on measurement of the rate of mRNA·DNA hybrid formation, which is consistent with that expected for single copy DNA sequence transcripts. However, our data are not sufficiently extensive to preclude the (unlikely) alternatives that the sequences from which these mRNAs are transcribed are present an average of 0.5 or 2 times per genome. Nor can we say whether the rate of hybrid formation is less than a factor of two different from the rate of DNA·DNA duplex formation (18, 19). Using RNase treatment for assay of the hybrids, Penman and Bishop also reached the conclusion that HeLa mRNA is mainly nonrepetitive sequence transcript (unpublished data). Thus in human as well as other animal cell types (1-9), most of the structural genes are single copy DNA sequences.

The second conclusion is that a small but non-negligible component of the HeLa mRNA preparation (about 6%) is evidently transcribed entirely from repetitive sequence elements [or slightly more if this RNA fraction turns over rapidly compared to the bulk of the mRNA (16)]. Assuming that this RNA is indeed mRNA, there must exist an as yet unidentified class of repetitive structural genes. Histone mRNA is excluded as a possibility, since the mRNA was selected on the basis

of its poly(A) content. Other workers have also noticed some repetitive sequence transcript present in total tissue culture cell mRNA preparations (e.g., ref. 7; Penman and Bishop, unpublished data), but it has previously been impossible to distinguish between a small class of RNAs totally transcribed from repetitive sequence or a larger class of RNAs transcribed mainly from single copy DNA but containing a fraction of repetitive sequence. Only the former case requires the postulation of repetitive structural genes. This argument, however, turns on the certainty with which the hybridizing radioactivity can be ascribed to mRNA. The following data are relevant for the HeLa mRNA preparation used here: a) After two passages over poly(T)-cellulose, less than 1% of the radioactivity can be attributed to rRNA, according to both formaldehyde and SDS-sucrose gradient analysis, and to the yield of RNA recovered from the column after long term labeling (2% of the total polysomal radioactivity); b) The size distribution of the RNA species hybridizing at low $C_o t$ (Table 3) includes species larger than rRNA, i.e. > 35S. c) Addition of a large excess of unlabeled human rRNA to the hybridization reactions results in no decrease in the amount of labeled RNA bound (Table 1). d) The structures from which the ^{3}H-mRNA is extracted are > 99.7% sensitive to EDTA disaggregation. This is of course a diagnostic characteristic of polysomes, and contrasts to the behavior expected of structures which contain hnRNA. Furthermore, the extracted RNA displays the turnover rates and labeling characteristics of polysomal rather than hnRNA (16). e) The hybridization behavior of the RNA is distinct from that of hnRNA. Both in HeLa (20) and in sea urchin cells (13), hnRNA contains

interspersed repetitive sequence elements, and the fraction of nucleotides present in repetitive regions is far smaller than the fraction of molecules containing them. In contrast, our measurements yield identical values for the fraction of RNA nucleotides present in the repetitive sequence and the fraction of HeLa mRNA molecules containing these sequences. This is a strong argument that the poly(A)-containing repetitive transcripts actually represent mRNA rather than an hnRNA contaminant.

Our experiments argue strongly against the existence of recognizable repetitive sequence "tags" on a large portion of the mRNA molecules. Prior studies (13) have shown the urea-phosphate HAP assay system on which we rely for this conclusion to be capable of binding with high efficiency RNA molecules which are 1-2 x 10^3 nucleotides long, but contain less than one-third their length as RNA·DNA duplex. The present data show that such structures are not detectable in HeLa mRNA. This result was reported earlier for total mRNA of sea urchin gastrulae (2). Our conclusions are thus inconsistent with Dina et al. (12) who reported that 80% of Xenopus embryo mRNAs contain short repetitive sequence elements recognizable in hybridization experiments carried out at much the same annealing criterion that we have used. While it is possible that phylogenetic differences account for this discrepancy, it seems far more likely to be technical in origin.

It remains of course possible that mRNAs contain repetitive sequence regions shorter than the recognizable limit. For hydroxyapatite columns operated under standard conditions in 0.12 M PB this limit is known to be very small (Wilson and Thomas report 85% binding

of 17 nucleotide long duplexes under our conditions; 21 and unpublished data). Though we see no reason why there should be a substantial difference we do not know exactly what this limit is for hydroxyapatite columns operated in 8 M urea, 0.2 M PB, 1% SDS. However the recovery of short DNA·DNA duplexes in the urea-phosphate medium leads to the conclusion that duplex sequences 100 nucleotides or longer would be bound quantitatively under these conditions. Nontranslated sequences now known on several specific mRNAs appear to exceed this short length. Thus we provisionally conclude that at least part of the nontranslated as well as the coding sequences of the mRNA must in general be transcribed from a single copy DNA.

Acknowledgments

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Footnote

‡ 3.8 pg is the mass of the haploid HeLa genome. At a DNA/RNA ratio of 5×10^{4}, a reaction mixture contains 2500 µg of DNA and 0.05 µg of RNA. The number of copies of each nonrepetitive sequence per µg of RNA is $2500/0.05 \times 1/3.8 \times 10^{-6} = 1.3 \times 10^{10}$. The amount of mRNA/cell is 4×10^{-7} µg (based on direct measurements of mRNA yield) and $1.3 \times 10^{10} \times 4 \times 10^{-7} = 5.2 \times 10^{3}$ copies of a given mRNA sequence per cell.

CHAPTER 3

SIZE DISTRIBUTION OF MESSENGER RNA FROM FREE POLYSOMES OF HELA CELLS

ABSTRACT

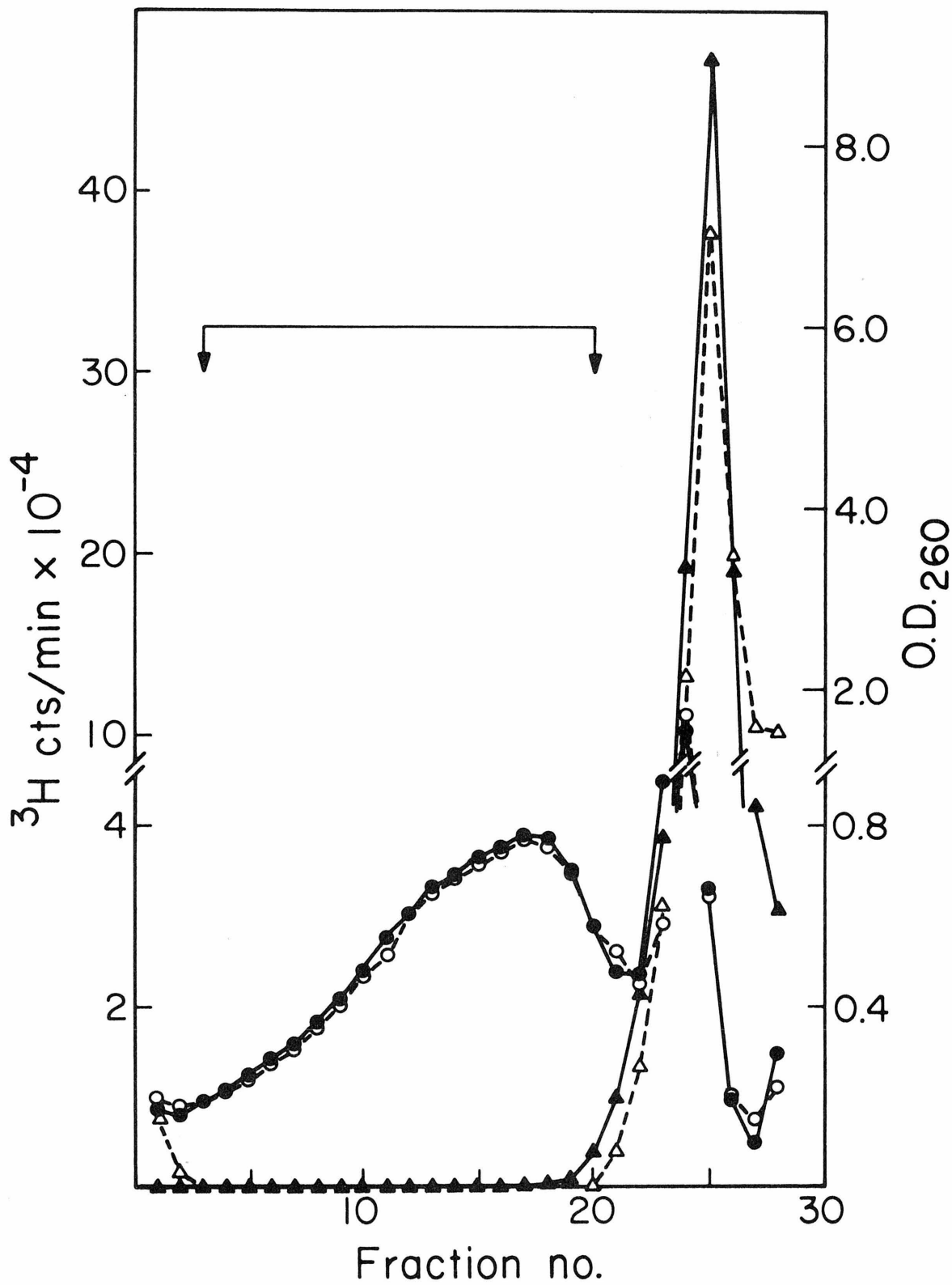
The sedimentation properties of pulse-labeled and long-term labeled mRNA from HeLa cell free-polysomes, selected for poly(A) content by two successive passages through poly(T)-cellulose columns, was analyzed under native and denatured conditions. The sedimentation profile of the mRNA on both sodium dodecyl SO_4-sucrose gradients and formaldehyde-sucrose gradients showed a broad distribution of components with estimated molecular weights ranging from 2×10^5 to 5.5×10^6 daltons and a weight-average molecular weight of 8.5×10^5 daltons.

The recent development of techniques for the purification of mRNA from animal cells (Kates, 1970; Edmonds et al., 1971, Murphy & Attardi, 1973) has made possible the study of some properties of the mRNA which were not previously amenable to analysis. Several laboratories have described the steady state size distribution, as estimated from sedimentation analysis, of long-term labeled HeLa mRNA purified by poly(T)-cellulose chromatography (Nakazato & Edmonds, 1972; Murphy & Attardi, 1973; Singer & Penman, 1973). However, none of these analyses was carried out under denaturing conditions so as to exclude the possible aggregation of the RNA: this has prevented, so far, a reliable estimate of the largest size of the mRNA. In the present work the size distribution of pulse-labeled and long-term labeled mRNA from HeLa cell free polysomes was analyzed under native and denaturing conditions with particular attention paid to the largest mRNA species which are normally synthesized in exponentially growing HeLa cells.

A prerequisite for this analysis was the preparation of free polysomes not contaminated by heterogeneous RNAs. It has previously been shown that 20 minute [5-^{3}H]uridine pulse-labeled free polysomes, when isolated by pelleting through a discontinuous sucrose gradient according to the procedure of Attardi et al., (1970), are pure to the extent of more than 95% as judged by the sensitivity to EDTA of both the UV absorbing and the radioactive material (Murphy & Attardi, 1973). Figure 1 shows a comparison of the sedimentation patterns of control and EDTA-treated free polysomes from

Figure 1. Sedimentation patterns in sucrose gradients of free polysomes from HeLa cells before and after disruption with EDTA.

HeLa cells, at an initial concentration of 5 x 10^4/ml, were uniformly labeled for 48 hr with ^{3}H-uridine according to the procedure of Aloni & Attardi (1971b). Free cytoplasmic polysomes were isolated from the postmitochondrial supernatant by centrifugation through a discontinuous sucrose gradient (Attardi, et al., 1970) and the polysome pellet was divided into two equal parts: one half was resuspended in 1 ml of Tris-K-Mg buffer (0.05 M-Tris buffer (pH 6.7), 0.025 M-KCl, 0.0025 M-$MgCl_2$) and analyzed in sucrose gradient in the same buffer; the other half was dissolved in 1 ml of Tris-K-EDTA buffer (0.05 M-Tris buffer (pH 6.7), 0.025 M-KCl, 0.01 M-EDTA) and centrifuged in sucrose gradient in this buffer. The polysomes were centrifuged through a 15 to 30% sucrose gradient in a SW25.1 Spinco rotor at 25,000 rev./min for 100 min at 2°C (Murphy & Attardi, 1973). 0---0, O.D. $_{260}$Tris-K-Mg; Δ---Δ, O.D. $_{260}$Tris-K-EDTA; ● - ●, ^{3}H cts/min Tris-K-Mg; ▲ -▲ , ^{3}H cts/min Tris-K-EDTA.


40
30
20
10
4
2
^{3}H cts/min x 10^{-4}
8.0
6.0
4.0
2.0
0.8
0.4
O.D.260
10
20
30
Fraction no.

48 hour labeled HeLa cells isolated by the above mentioned procedure. From the residual UV absorbing material and radioactivity sedimenting in the polysome regions after EDTA treatment (less than 1%), it can be estimated that the polysomes are substantially free of other ^{3}H-uridine labeled cellular components.

The RNA was extracted from the polysomes shown in Figure 1 by the sodium dodecyl SO_4-pronase-phenol method (Aloni & Attardi, 1971a), and the mRNA purified by two successive runs through poly(T)-cellulose columns: the size distribution was then analyzed by centrifugation through a sodium dodecyl SO_4-sucrose gradient as shown in Figure 2a. The sedimentation profile shows a broad distribution of components with molecular weights ranging from 2×10^5 to greater than 4.5×10^6 daltons, with a weight-average molecular weight of approximately 8.5×10^5 daltons. The small peak of radioactivity near the bottom of the gradient represents the heaviest mRNA components which have been prevented from pelleting by a cushion of dense sucrose. A similar size distribution was observed when the mRNA was denatured by heating at 63°C for 15 minutes in the presence of 3% formaldehyde and then centrifuged in a formaldehyde-sucrose gradient, according to the method of Boedtker (1968) (Figure 2c). The fact that the sedimentation behavior of the RNA remains unchanged after denaturation indicates that no significant aggregation of the mRNA has occurred in the sedimentation analysis under native conditions shown in Figure 2a.

Figure 2. Sedimentation analysis of HeLa mRNA.

(a),(c) RNA was extracted by the sodium dodecyl SO_4-pronase-phenol procedure (Aloni & Attardi, 1971a) from 48 hr ^{3}H-uridine labeled free polysomes, isolated as described in the legend of Figure 1, and the mRNA purified by chromatographing twice the polysomal RNA on 0.5 x 5.0 cm columns of poly(T)-cellulose, according to the procedure previously described (Murphy & Attardi, 1973). (a) 0.1 μg of the purified mRNA was dissolved in 1 ml of sodium dodecyl SO_4 buffer (0.01 M-Tris buffer (pH 7.0), 0.1 M-NaCl, 0.001 M-EDTA, 0.5% sodium dodecyl SO_4), and centrifuged through a 15 to 30% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in the same buffer in a SW27.1 Spinco rotor at 26,000 rev./min for 15.5 hr at 20°C. (c) 0.1 μg of RNA was dissolved in 1 ml of 0.1 M-sodium phosphate buffer (pH 7.6), containing 3% formaldehyde, and the solution was heated at 63°C for 15 min and then fast-cooled (Boedtker, 1968). The RNA was centrifuged through a 5 to 20% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in 0.1 M-NaCl, 0.02 M-K_2HPO_4, pH 7.4, 1% formaldehyde, in a SW27.1 Spinco rotor, at 26,000 rev./min for 24 hr at 2°C.

(b),(d) mRNA was isolated, as described above, from free polysomes purified from a mixture of 2.5 x 10^8 HeLa cells labeled for 25 min with ^{3}H-uridine and 2.5 x 10^8 cells labeled for 48 hr with ^{14}C-uridine. (b) Sedimentation analysis of the mRNA in a sodium dodecyl SO_4-sucrose gradient was carried out as described in (a). (d) Sedimentation analysis in a formaldehyde-sucrose gradient was carried out as described in (c).

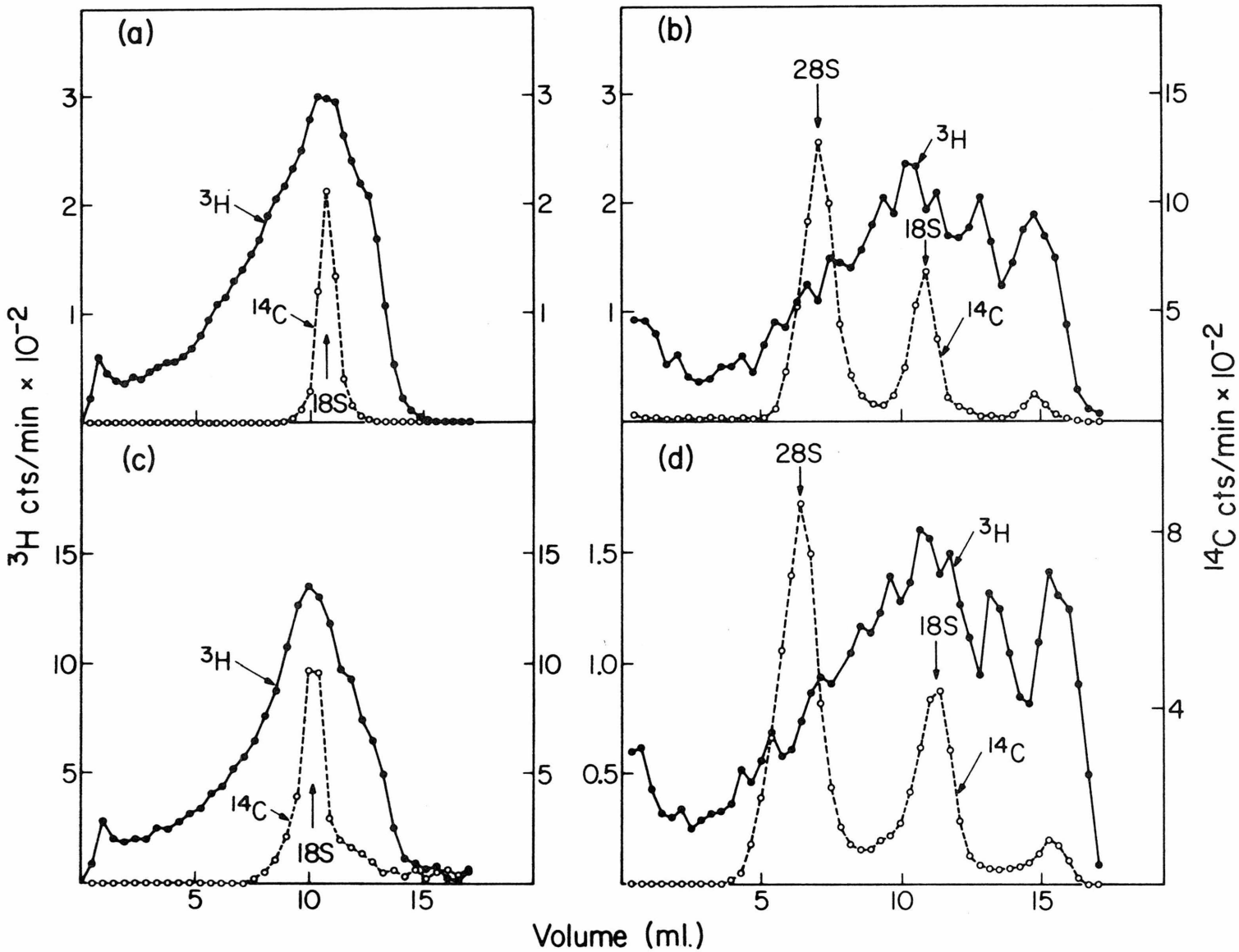

(a)
(b)
(c)
(d)
28S
18S
^{3}H
^{14}C
^{3}H cts/min × 10^{-2}
^{14}C cts/min × 10^{-2}
Volume (ml.)

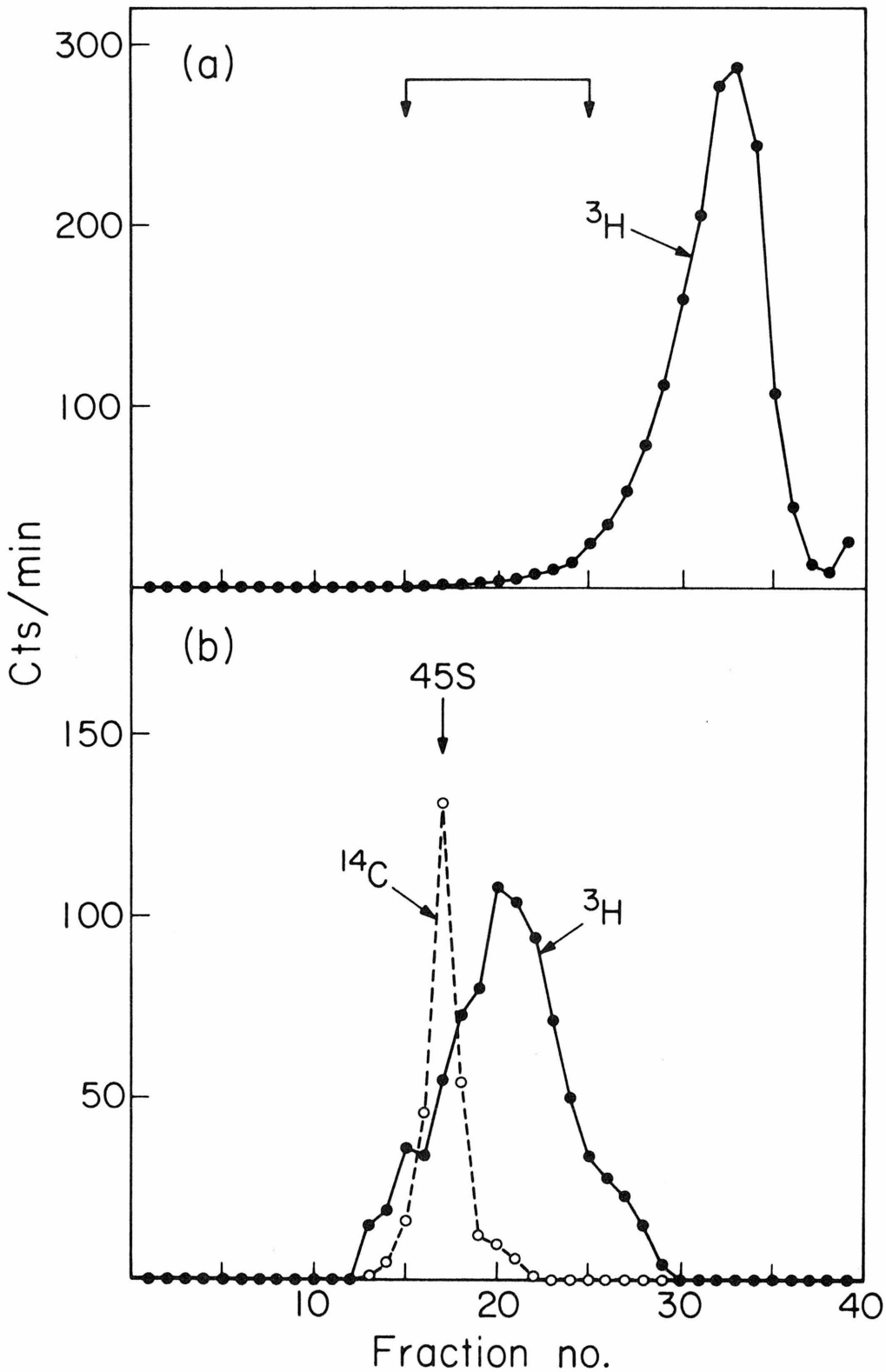
Figure 2b shows the sedimentation pattern in a sodium dodecyl SO_4-sucrose gradient of mRNA isolated from free polysomes purified from a mixture of an equal number of HeLa cells labeled for 25 minutes with [5-^{3}H]uridine and for 48 hours with [2-^{14}C]uridine. The pulse-labeled mRNA exhibits approximately the same size range as the 48 hour labeled mRNA. However, several discrete peaks or shoulders of radioactivity, which are reproducible from preparation to preparation, can be seen in the 25 minute labeled mRNA; among them are two prominent peaks centered around 4-5 S and 9 S. These peaks are also clearly recognizable when the RNA is centrifuged under denaturing conditions (Figure 2d). As the labeling time increases these discrete peaks become less distinct, and eventually disappear as the total mRNA pattern assumes the smoother profile seen in panels 2a and c. These peaks probably represent species of mRNA which are either more rapidly synthesized or more quickly transported to the cytoplasm than the bulk of the mRNA; in particular, the 9 S mRNA peak presumably represents histone mRNA.

To estimate more accurately the maximum size of the long-term labeled mRNA, the heaviest RNA components in a sedimentation velocity run in a sodium dodecyl SO_4-sucrose gradient were pooled, and rerun in a second gradient together with ^{14}C-labeled HeLa 45 S rRNA as an internal marker (Figure 3). Panel 3b shows that the heaviest mRNA components sediment with a sedimentation constant of about 50 S, corresponding to approximately 5.5×10^6 daltons (Spirin, 1961). Although this centrifugation was not

Figure 3. Sedimentation analysis of the fastest-sedimenting components of 48 hr [5-^{3}H]uridine labeled mRNA.

(a) The mRNA was centrifuged through a 15 to 30% sucrose gradient in sodium dodecyl SO_4 buffer in a SW27.1 Spinco rotor at 27,000 rev/min for 6 hr at 20°C. The fractions indicated by arrows were pooled, 10 µg of 4 S RNA was added as a carrier, and the RNA collected by ethanol precipitation and centrifugation.

(b) The RNA from (a) was dissolved in 1 ml of sodium dodecyl SO_4 buffer and rerun, together with an internal ^{14}C-labeled HeLa 45 S rRNA marker, through a 15 to 30% sucrose gradient in the same buffer in a SW27.1 Spinco rotor at 27,000 rev./min for 7 hr at 20°C.


(a)
300
200
100
^{3}H
(b)
45S
150
100
50
^{14}C
^{3}H
Cts/min
10
20
30
40
Fraction no.

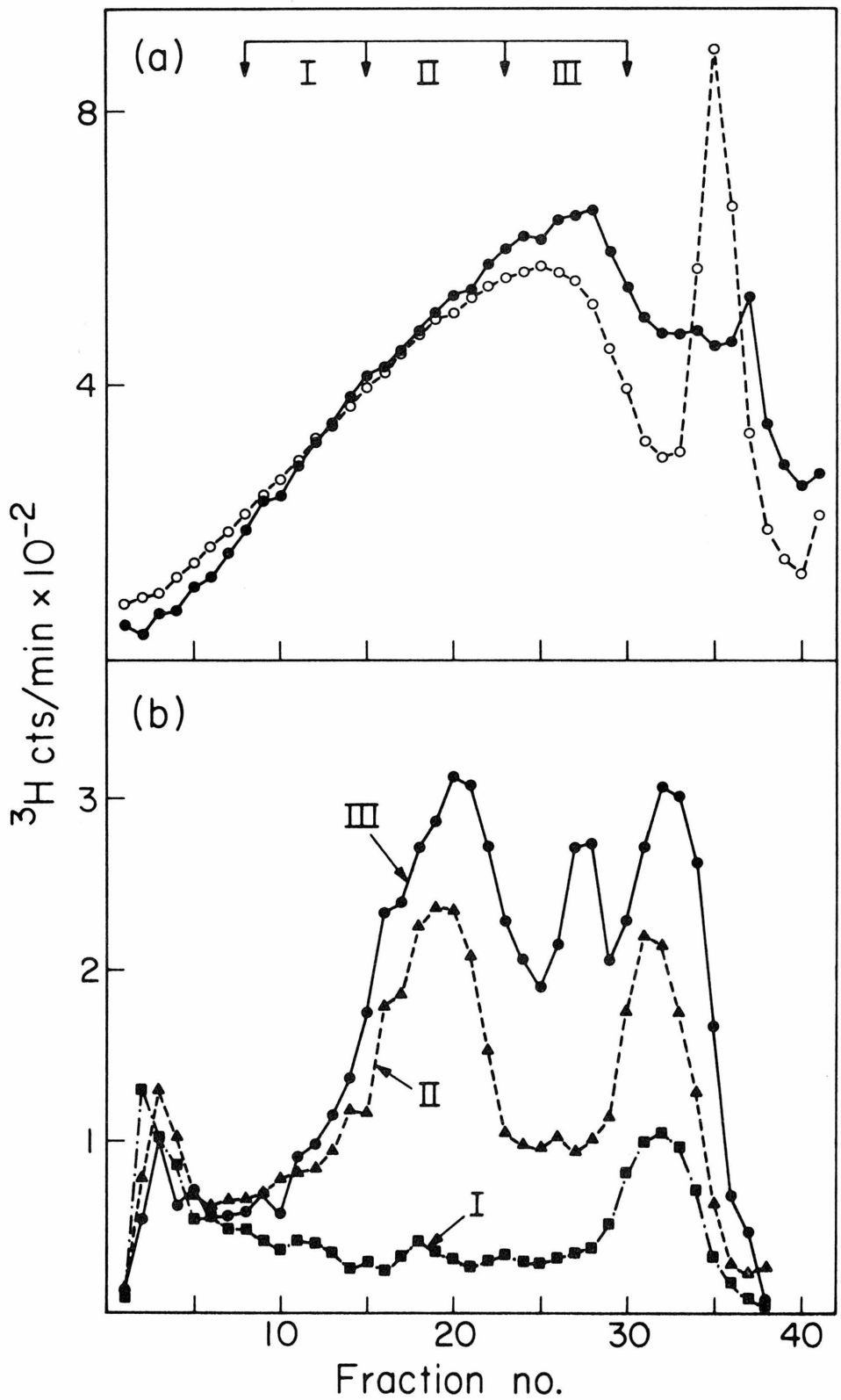
carried out in formaldehyde, the above described similarity in size distribution of the total mRNA, (including the fastest sedimenting components) under native and denaturing conditions (Figure 2) strongly suggests that the molecular weight estimated above represents the true molecular weight of the heaviest mRNA components.

Figure 4 illustrates the results of an experiment in which polysomes isolated from 25 minute pulse-labeled HeLa cells were run through a sucrose gradient and divided into three size classes (Figure 4a); the RNA was extracted from each polysome cut by the sodium dodecyl SO_4-pronase-phenol procedure (Aloni & Attardi, 1971a) and centrifuged separately on a sodium dodecyl SO_4-sucrose gradient. Figure 4b shows the sedimentation patterns thus obtained. It is clear from the radioactivity profiles that the heaviest polysome cut(I) is greatly enriched in very large mRNA components, while there is a progressive increase in the proportion of the lighter mRNA as the polysomes become smaller (cuts II and III). As expected, the RNA from the lightest polysome fraction contains a distinct peak of radioactivity at about 9 S, which probably represents the histone mRNA. The presence of some large mRNA even in the lightest polysome cut(III), confirms earlier findings (Latham & Darnell, 1965) indicating that in HeLa cells many mRNA molecules are never fully loaded with ribosomes. In any case, the observation that the fastest-sedimenting components in the mRNA preparation are preferentially associated with the heaviest polysomes is in agreement with the idea that these components are true mRNA.

Figure 4. Size distribution of the 25 min [5-^{3}H]uridine labeled mRNA isolated from free polysomes of different size classes.

(a) Free polysomes were isolated from 3 x 10^{8} HeLa cells, labeled for 25 min with ^{3}H-uridine, and centrifuged through a 15 to 30% sucrose gradient in TKM buffer in a SW25.1 Spinco rotor at 24,000 rev./min for 100 min at 2°C. The polysome region of the gradient was divided into three sections as shown, and the RNA extracted from each section by the sodium dodecyl SO_4-pronase-phenol procedure. ●, ^{3}H cts/min; 0, O.D.$_{260}$

(b) The RNA from each section was dissolved in 1 ml of sodium dodecyl SO_4 buffer and centrifuged through a 15 to 30% sucrose gradient (prepared over a 2 ml cushion of 64% sucrose) in the same beffer in a SW27.1 Spinco rotor at 26,000 rev./min for 16 hr at 20°C.


(a)
I
II
III
8
4
(b)
III
II
I
3
2
1
10
20
30
40
Fraction no.
^{3}H cts/min x 10^{-2}

The purity of the isolated mRNA, especially the exclusion of any HnRNA contamination, is extremely important in interpreting the results reported here. The following observations are pertinent in assessing the purity of the mRNA preparations used in this work: (1) The radioactive RNA sedimenting with the free polysomes is completely EDTA sensitive, as shown in Figure 1. (2) No RNA of a size larger than about 5.5×10^6 daltons was found. If the large RNA components were contaminated with HnRNA, then RNAs much larger in size should have been observed, especially since the procedures used for isolating the polysomes would have selected for the largest RNP particles containing HnRNA (Samarina et al., 1973). (3) The stability of the > 35 S mRNA is identical to the bulk of the mRNA (half-life two to three days, Murphy & Attardi, 1973) in both pulse-labeled and long-term labeled preparations (unpublished observation). In view of the much shorter half-life of the HnRNA (Weinberg, 1973), if significant contamination by this RNA class existed the stability of the large mRNA fraction would be markedly reduced.

Clearly, the metabolic and physical properties of the largest components in the mRNA population appear to be identical to those of the bulk of the mRNA, and unlike the known properties of the HnRNA. Therefore, it can

be concluded that classes of mRNA at least as large as 5.5×10^6 daltons are normally expressed in exponentially growing HeLa cells.

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Abbreviations

mRNA, messenger RNA; HnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; RNP, ribonucleoprotein particle.

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PART II

FAILURE TO DETECT HIGH MOLECULAR WEIGHT COMPLEMENTARY TRANSCRIPTS IN THE NUCLEAR RNA FROM ANIMAL CELLS

Failure to Detect High Molecular Weight Complementary Transcripts in the Nuclear RNA from Animal Cells

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ABSTRACT

The > 50 S HnRNA, isolated from either HeLa cells or immature duck erythrocytes labeled for different times with [5-^{3}H]uridine, was examined for the presence of complementary transcripts capable of forming RNase-resistant duplexes. After extensive self-annealing of the HnRNA, carried out under conditions such that complementary RNA sequences present once or a few times in the RNA population would have formed hybrids, no evidence was found for the existence of symmetrical transcripts in either cell system. However, 2-3% and 4-5% of the purified duck and HeLa HnRNA, respectively, did form RNase-resistant hybrids. These hybrids resulted from base-pairing of complementary regions within the HnRNA molecule, as judged from the lack of concentration dependence and from the kinetics of formation of the RNA-RNA duplexes. The weight-average length of the RNase-resistant fragments from the duck HnRNA was found to be approximately 125 nucleotide pairs; however, shorter double-stranded segments as well as longer duplexes, up to 2000 nucleotide pairs, were also observed. Annealing of the duck HnRNA in the presence of an excess of 10 S hemoglobin mRNA showed that 2% of the HnRNA formed RNase-resistant hybrids in excess of those expected from intramolecular homology. The RNase-resistant complexes formed between the 10 S mRNA and HnRNA had about the same size range as the intramolecular duplexes.

The failure to detect any intermolecular hybridization in the short-pulse labeled HnRNA from either actively growing cells or highly differentiated, non-dividing cells, strongly suggests that the mechanism for the synthesis of HnRNA in animal cells does not involve the production of high molecular weight complementary transcripts.

1. Introduction

Recent investigations on the mode of *in vivo* transcription of HeLa mitochondrial DNA, SV_{40}DNA, and polyoma virus DNA have shown that both strands are transcribed into RNA over a considerable portion, if not the entire length, of these circular DNA molecules (Aloni & Attardi, 1971; Aloni & Attardi, 1972; Aloni, 1972; Aloni, 1973; Aloni & Locker, 1973). One consequence of this symmetrical transcription is the ability of the RNA transcripts produced from these templates to form, either *in vivo* or after isolation and self-annealing, RNA-RNA hybrids which are resistant to ribonuclease. Both in the mitochondrial system and in the two above mentioned virus systems, it has been found that the products of transcription of one of the two strands have a much shorter half-life than those of the other strand. One implication of this is that the proportion of labeled complementary transcripts is greater after short pulse-labeling of the RNA.

To date, it is not known whether the symmetric transcription demonstrated for mitochondrial DNA and the SV_{40} and polyoma DNA is related to the closed circular structure of these DNAs, or whether it is a particular case of a more general mechanism of transcription in eukaryotic cells.

Many laboratories have reported the existence of an RNase-resistant, double-stranded RNA fraction which is confined to the nucleus of a variety of animal cell types, and which, at least in part, is complementary to the nuclear DNA (Montagnier, 1968; Stern & Friedman, 1970; Harel & Montagnier, 1971; Kronenberg & Humphreys, 1972; Jelinek & Darnell, 1972; Ryskov et al., 1972; Ryskov et al., 1973). At least in some cases, this double-stranded RNA appears to derive from intramolecular base-pairing of complementary portions of HnRNA molecules (Jelinek & Darnell, 1972; Ryskov et al., 1972; Ryskov et al., 1973). However, the possibility that the synthesis of at least a part of the nuclear RNA may result from symmetrical transcription involving labile species or sequences present in low frequency has not been directly examined.

In order to investigate the above possibility, we have analyzed pulse-labeled, high molecular weight HnRNA from both a permanent mammalian cell line, HeLa cells, and a differentiating cell system, immature duck erythrocytes, for the presence of intermolecular complementarity. Although a small amount of intramolecularly complementary sequences was detected in the HnRNA molecules, no evidence was found for the existence of high molecular weight complementary RNA transcripts in either cell system, even when the analysis was extended to include transcripts present in one or a few copies per cell.

2. Materials and Methods

(a) Animals

Nine to fourteen month old male Pekin ducks, made anemic by daily intramuscular injections of 25 mg of neutralized phenylhydrazine for five to seven consecutive days, were used in these experiments. Blood was drawn by cardiac puncture on the day following the last injection; to prevent coagulation, heparin was added to a final concentration of 20 µg/ml of blood.

(b) Cells and method of growth

HeLa cells (S_3 clonal strain) were grown in suspension in modified Eagle's medium (Levintow & Darnell, 1960) supplemented with 5% calf serum. The cultures used in these experiments were free of any detectable contamination with PPLO (Mycoplasma).

(c) Labeling conditions

Exponentially growing HeLa cells were pulse-labeled by exposure for either 2 minutes or 15 minutes to [5-^{3}H]uridine (10 µCi/ml; 26 Ci/m-mol)

in modified Eagle's medium containing 5% dialized calf serum.

In vitro labeling of the duck erythrocytes was carried out by incubation of the cells with [5-^{3}H]uridine (100 μCi/ml; 29 Ci/m-mol) for 15 minutes or 3 hours in modified Eagle's medium supplemented with 10% dialyzed duck serum, as previously described (Attardi et al., 1966).

(d) Buffers

(1) Tris-K-Mg: 0.05 M-Tris buffer (pH 6.7, 25°C), 0.025 M-KCl, 0.0025 M-MgCl; (2) Tris-K-EDTA: 0.05 M-Tris buffer (pH 6.7), 0.025 M-KCl, 0.01 M-EDTA; (3) sodium dodecyl SO_4 buffer: 0.01 M-Tris buffer (pH 7.0), 0.1 M-NaCl, 0.001 M-EDTA, 0.5% sodium dodecyl SO_4; (4) Urea buffer: 0.01 M-Tris buffer (pH 7.4), 0.35 M-NaCl, 0.001 M-EDTA, 7 M-Urea (Mann Ultrapure), 2% sodium dodecyl SO_4; (5) formaldehyde-phosphate buffer: 0.1 M-NaCl, 0.02 M-potassium phosphate buffer (pH 7.4), 1% formaldehyde; (6) PB: sodium phosphate buffer (pH 6.7).

(e) RNA extraction and isolation of the HnRNA

(i) Immature duck erythrocytes. The total RNA from 7 x 10^{10} cells was extracted as previously described (Attardi et al., 1966). The efficiency of extraction of the total acid precipitable radioactivity for the 15 minute and 3 hour labeling period was 91 and 93%, respectively. The RNA was centrifuged through a 15 to 30% (w/w) sucrose gradient in sodium dodecyl SO_4 buffer, prepared over a 6 ml cushion of 64% sucrose, in a

Spinco SW27 rotor at 27,000 rev/min for 4 hours at 20°C. The fractions of the gradient corresponding to RNA with a sedimentation constant greater than 50 S were pooled, the RNA precipitated with ethanol, collected by centrifugation, and rerun in a sucrose gradient under the same conditions. This procedure was repeated and the HnRNA sedimenting faster than 50 S in the third gradient was collected by ethanol precipitation and centrifugation, dissolved in Tris-K-Mg (100 µg/ml), digested a second time (The first DNase treatment was carried out during the extraction of the RNA) with RNase-free DNase (30 µg/ml, 20°C, 1 hour), sodium dodecyl SO_4-pronase-phenol extracted (Aloni & Attardi, 1971) and passed through a Sephadex G-100 column to remove any digested DNA fragments. The peak of excluded RNA was pooled, precipitated with ethanol and dissolved in 10^{-3} M PB, pH 6.7, at a final concentration of 1 mg/ml.

(ii) HeLa cells. (a) 2 minute [5-^{3}H]uridine pulse labeled cells. Three aliquots of 5 x 10^8 cells were pulse labeled, and utilized for RNA extraction by the following three procedures: (1) total cell RNA was extracted as described in (i); (2) the cells were homogenized as previously described (Attardi et al., 1966), the nuclear pellet was lysed with 15 volumes of sodium dodecyl SO_4 buffer, and the lysate digested with 200 µg/ml pronase for 90 minutes and phenol extracted; the remainder of the extraction procedure was identical to (i); (3) whole cells were lysed at 22°C in 25 volumes of urea buffer (Holmes & Bonner, 1973), phenol extracted, and

the RNA isolated as in (i). (b) 15 minute [5-^{3}H]uridine labeled cells. Total RNA was extracted from 5 x 10^8 cells as described in section (i). In all extractions of HeLa RNA the final recovery of total acid precipitable radioactivity was 90 to 95%. Centrifugation of the RNA, isolation of the HnRNA sedimenting faster than 50 S and DNase treatment were carried out as described in section (i). The purified > 50 S HnRNA was dissolved in 10^{-3} M PB, pH 6.7, at a final concentration of 1 mg/ml.

(f) Purification of 10 S hemoglobin mRNA from immature duck erythrocytes

The erythrocytes were homogenized and the free polysomes isolated by pelleting through a discontinuous sucrose gradient as previously described (Attardi et al., 1970). The polysomes were disrupted by resuspension in Tris-K-EDTA supplemented with 2 x 10^{-2} M-EDTA, and centrifuged through a 15 to 30% (w/w) sucrose gradient in Tris-K-EDTA in a Spinco SW27 rotor at 26,000 rev/min for 17 hours at 20°C. The fractions of the gradient corresponding to the 20 S ribonucleoprotein particles, which contain the 10 S mRNA (Pemberton et al., 1972), were pooled, and the RNA was extracted by the sodium dodecyl SO_4-pronase-phenol procedure. The 10 S mRNA was isolated by three successive centrifugations through sodium dodecyl SO_4-sucrose gradients, and by pooling each time the fractions corresponding to the 10 S region of the gradient; final purification was carried out by

chromatography on poly(T)-cellulose, as previously described (Murphy & Attardi, 1973).

(g) Annealing of the RNA

Prior to annealing, the RNA was denatured by heating at 90°C for 5 minutes in 10^{-3} M PB, pH 6.7, or at 37°C for 15 minutes in recrystallized formamide. The conditions of incubation of the HnRNA alone and in the presence of 10 S mRNA, for RNA-RNA hybrid formation, are described in the Table Legends. After incubation, the RNA was diluted to a final concentration of 10 to 25 µg/ml in 1 ml of 0.27 M PB, pH 6.7, and the extent of RNase resistance of the RNA was tested by incubation with 10 µg/ml of pancreatic RNase and 10 units/ml of T_1 ribonuclease for 1 hour at 25°C. Following digestion, the RNA was precipitated with 10% trichloroacetic acid in the presence of 100 µg bovine serum albumin, filtered through Millipore membranes and counted.

(h) Size analysis of the RNase-resistant fraction

After treatment of the RNA with RNase under the conditions described in section (g), the RNA was sodium dodecyl SO_4-pronase-phenol extracted and centrifuged through a 15 to 30% (w/w) sucrose gradient in sodium dodecyl SO_4 buffer in a Spinco SW41 rotor at 40,000 rev/min for 20 hours at 20°C.

(i) Analysis of the size of HnRNA during incubation at 66°C

A 0.1 ml sample of the 15 minute [5-^{3}H]uridine pulse-labeled HeLa HnRNA, incubated for 1 to 6 days under the same conditions as described in the legend of Table 1, was diluted with 0.9 ml of 0.1 M-sodium PO_4 buffer, pH 7.6, containing 3% formaldehyde, the solution was heated at 63°C for 15 minutes and fast cooled, and the RNA centrifuged through a 5 to 20% (w/w) sucrose gradient (prepared over a 3 ml cushion of 64% sucrose) in formaldehyde-phosphate buffer in a Spinco SW41 rotor at 40,000 rev/min for 22 hours at 2°C (Boedtker, 1968). Size analysis of the original RNA, which had been denatured twice by heating at 90°C for 5 minutes, was performed as described above, with the exception that the centrifugation was through a 5 to 20% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in formaldehyde-phosphate buffer in the SW41 rotor, 37,000 rev/min, for 3 hours at 2°C.

3. Results

(a) Isolation of the > 50 S HnRNA from immature duck erythrocytes and HeLa cells

It has been shown for the RNA transcribed *in vivo* from both the mitochondrial and SV_{40}DNA, that, after very short pulses with the RNA

precursor, an appreciable proportion of the labeled RNA is in the form of RNase-resistant RNA-RNA hybrids and that this proportion increases after self-annealing. However, the percentage of labeled RNA capable of forming such hybrids decreases with increasing labeling times, due to the metabolic instability of the products of transcription of one of the two strands. Therefore, in the present work, in order to achieve the greatest sensitivity of detection of a self-complementary RNA fraction in the HnRNA and at the same time to compare the extent of any such self-complementarity after various labeling times, the RNA was labeled in HeLa cells for either 2 or 15 minutes. It has been shown that the time needed for transcription of the HeLa 45 S rRNA precursor is 2.3 minutes (Greenberg & Penman, 1966). If the same rate of transcription applies to HnRNA, it is clear that the majority of the radioactivity incorporated into the > 50 S HnRNA during a 2 minute labeling is associated with nascent RNA chains. In duck erythrocytes, because of the lower rate of RNA synthesis, longer labeling times, i.e. 15 minutes and 3 hours, had to be used. Since a quantitative recovery of the pulse-labeled RNA in intact form was essential for proper evaluation of the results, three different procedures for the isolation of HnRNA were compared as to efficiency of extraction and sedimentation properties of the extracted RNA.

Figure 1(a & c) shows the sedimentation patterns in sodium dodecyl SO_4-sucrose gradients of the RNA from immature duck erythrocytes (15 minute and 3 hour pulses) and HeLa cells (2 and 15 minute pulses), respectively. From a comparison of the sedimentation patterns of the 2 minute pulse-labeled HeLa RNA extracted by the three procedures (Figure 1c) it can be seen that these yield RNA with the same size distribution and approximately the same efficiency of extraction, i.e. 90 to 95%. A similar efficiency of extraction was found for the 15 minute labeled HeLa HnRNA and for both duck HnRNA preparations. Since the recovery of the labeled RNA was the same for all labeling times, it can be concluded that no selection has occurred, during isolation of the RNA, for either the earliest or latest synthesized RNA species.

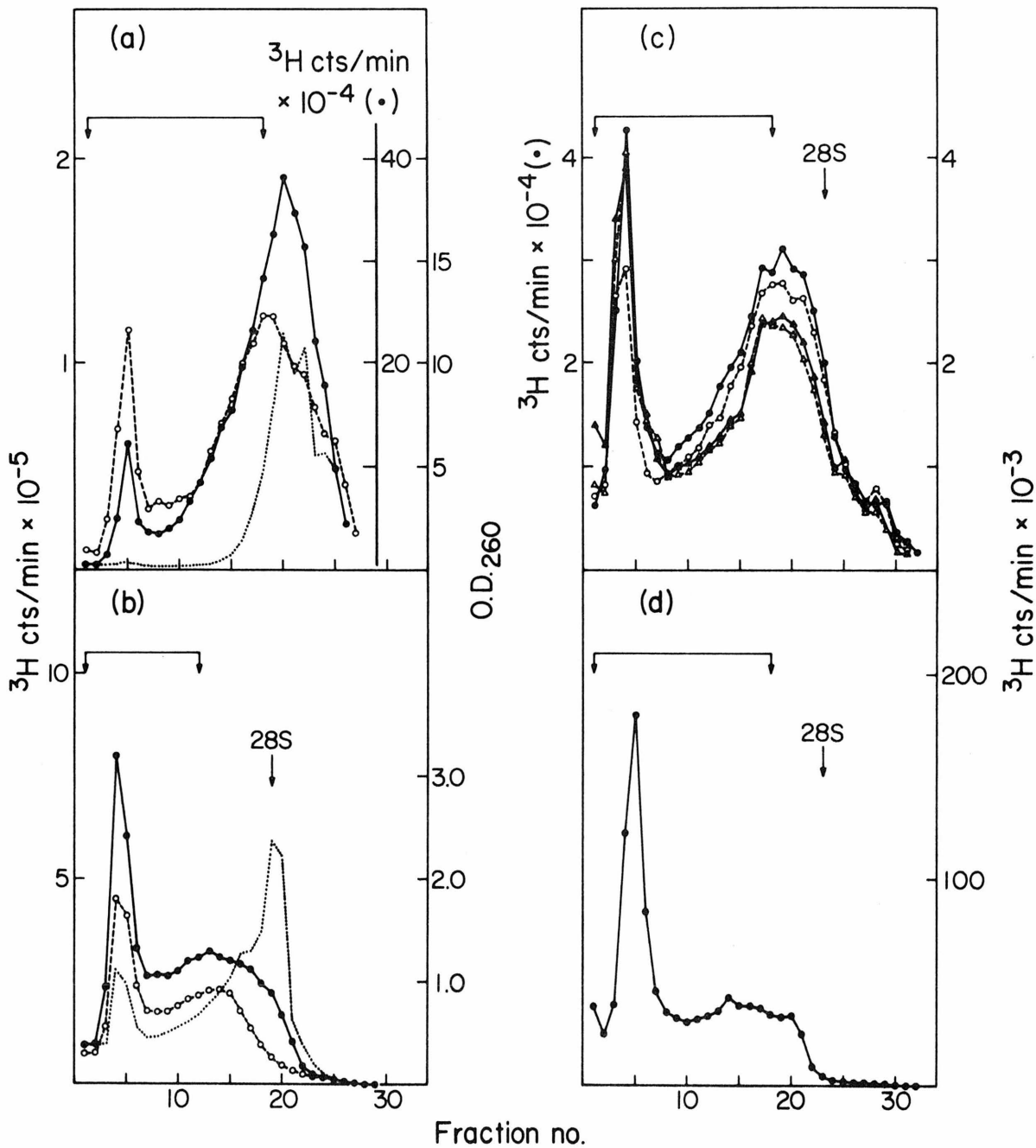
The > 50 S RNA in each gradient was collected by ethanol precipitation and centrifugation and further purified by twice rerunning it through sodium dodecyl SO_4-sucrose gradients. The sedimentation patterns from the final gradients for the 15 minute and 3 hour labeled erythrocyte HnRNA and the 15 minute labeled HeLa HnRNA are shown in Figure 1b and d, respectively. The peak of radioactivity near the bottom of the gradients represents the heaviest components of the HnRNA, which have been prevented from pelleting by a cushion of dense sucrose. The > 50 S HnRNA was pooled as shown, collected by ethanol precipitation and centrifugation, subjected to a second treatment with DNase, and used for the study of the self-complementarity of the HnRNA, as described below.

Figure 1. Isolation of the > 50 S HnRNA from immature duck erythrocytes and HeLa cells.

(a),(b) Total RNA was isolated from 7 x 10^{10} erythrocytes, which had been labeled with [5-^{3}H]uridine for 15 minutes (0) or 3 hours (●), as described in Materials and Methods, Section e(i). (a)Each RNA sample was centrifuged through a 15 to 30% (w/w) sucrose gradient in sodium dodecyl SO_4 buffer (prepared over a 6 ml. cushion of 64% sucrose) in a SW27 Spinco rotor at 27,000 rev/min for 4 hours at 20°C. The fractions corresponding to the > 50 S HnRNA were pooled as shown, the RNA was collected by ethanol precipitation and centrifugation, and rerun through a second sodium dodecyl SO_4-sucrose gradient under the same conditions (sedimentation pattern not shown). (b) The > 50 S HnRNA from the second gradient was rerun through a third 15 to 30% sucrose gradient in sodium dodecyl SO_4 buffer (prepared over a 6 ml. cushion of 64% sucrose) in a SW27 Spinco rotor at 26,000 rev/min for 5.5 hours at 20°C. The > 50 S HnRNA was collected by ethanol precipitation and centrifugation, treated a second time with DNase, and dissolved in 10^{-3}M PB, pH 6.7, at a final concentration of 1 mg/ml.

(c),(d) RNA was isolated from three samples of 5 x 10^{8} HeLa cells, labeled for 2 minutes with [5-^{3}H]uridine, by the procedures 1(0), 2(▲), or 3(Δ) described in Materials and Methods, Section e (ii, a), and from 5 x 10^{8} HeLa cells, labeled for 15 minutes with [5-^{3}H]uridine (●), according to the procedure 1. (c) Each RNA sample was centrifuged through a sodium

dodecyl SO_4-sucrose gradient, as described in (a). The fractions corresponding to >50 S HnRNA were pooled as shown, and the RNA was rerun through a second sucrose gradient under the same conditions. (d) The >50 S HnRNA from the second sucrose gradient was rerun through a third sodium dodecyl SO_4-sucrose gradient, as described in (b) (only the sedimentation pattern for the 15 minute labeled HnRNA is shown). The >50 S HnRNA was collected by ethanol precipitation and centrifugation, treated a second time with DNase, and dissolved in 10^{-3} M PB, pH 6.7 at a final concentration of 1 mg/ml.


(a)
^{3}H cts/min
× 10^{-4} (•)
(b)
28S
(c)
28S
(d)
28S
^{3}H cts/min × 10^{-5}
^{3}H cts/min × 10^{-4}(•)
^{3}H cts/min × 10^{-3}
O.D.$_{260}$
Fraction no.

(b) Self-annealing of the HnRNA from immature duck erythrocytes and HeLa cells

When the purified HnRNA was subjected to RNase treatment without prior denaturation and self-annealing,2 to 3% and 4 to 5% of the duck and HeLa HnRNA, respectively, was found to be resistant to RNase digestion. In order to investigate the nature of this RNase-resistant fraction and the possible presence of complementary transcripts in the pulse-labeled >50 S HnRNA, the RNA was denatured, self-annealed for different lengths of time, and the extent of RNA-RNA hybrid formation measured by the resistance of the RNA to ribonuclease digestion, as detailed in Materials and Methods(g). Immediately after denaturation, the RNase resistance of the RNA decreased to approximately 0.1% and 0.3% for the duck and HeLa RNA, respectively (Table 1). However, after incubation of the RNA for 15 minutes, the percentage of RNase resistance returned to 2-3% for duck erythrocytes, and 4-5% for HeLa cells. Increasing the RNA and salt concentrations in the hybrid mixture and extending the incubation time up to 6 days failed to produce any increase in the amount of RNase-resistant radioactivity. Thus, an examination of Table 1 shows that by 15 minutes of hybridization the amount of RNA-RNA duplex formation has reached a maximum. Also, a comparison of the behavior of duck erythrocyte or HeLa RNA which had been labeled for varying lengths of time showed no detectable difference in the percent of RNase resistance, indicating that the proportion

TABLE 1

RNase Resistance (% of input cts/min)

Incubation Conditions*

RNA	(1) 0' Undenatured RNA	(2) 0' Control	(3) 15' 0.27 M PB	(4) 1 Day 0.27 M PB	(5) 2 Days 0.27 M PB	(6) 2 Days 1.0 M PB	(7) 4 Days 0.4 M PB	(8) 6 Days 1.0 M PB	(9) 6 Days 1.0 M PB	(10)** 6 Days 1.0 M PB
duck-15' label	2.6	0.1	2.6	2.3	1.8	2.1	2.8	2.5	2.9	100
duck-3 hr label	2.5	0.1	3.0	2.9	2.6	2.8	2.5	3.0	2.4	97
HeLa-2' label(1)+	4.3	0.2	4.7	5.2	4.5	4.6	4.9	4.2	4.3	99
HeLa-2' label(2)	4.3	0.3	4.4	5.4	4.3	4.7	4.7	4.2	4.8	98
HeLa-2' label(3)	4.8	0.5	5.3	5.1	4.6	5.0	5.1	4.6	4.9	100
HeLa-15' label	5.0	0.3	4.8	5.2	4.8	5.2	5.3	4.6	5.2	99

* Prior to hybridization, the RNA preparations(except #1)were denatured by heating at 90°C for 5 min in 10^{-3} M PB, pH 6.7, and quick cooled. All incubations were carried out, for the times and at the salt concentrations indicated above, at 66°C in sealed 50 or 100 lambda Corning pipettes. After incubation, the samples were cooled,diluted to 1.0 ml at a final concentration of 0.27 M PB and RNase digested as described in Materials & Methods,Section(g). The RNA concentrations for the individual samples were:2=10 µg/ml;3,4,5,10=100 µg/ml;8=250 µg/ml;6,7,9=500 µg/ml.

** No RNase treatment

\+ 1,2,3 represents the RNA isolated according to procedures 1,2,or3, as described in Section e(ii) of Materials & Methods.

of labeled complementary RNA molecules was not greater after short as compared to relatively long labeling times.

Jelinek and Darnell (1972) have shown that the double-stranded, RNase-resistant portion of the HeLa HnRNA results from base-pairing of complementary sequences within the same HnRNA molecule. The results presented in Table 1 strongly suggest that the RNase-resistant fraction detected here in the HnRNA from both HeLa cells and duck erythrocytes, which becomes RNase-sensitive after denaturation and reappears rapidly after annealing, represents the same kind of double-stranded RNA resulting from intra-molecular complementarity within the HnRNA molecules. In order to obtain additional evidence on this question another series of experiments was carried out with duck erythrocyte HnRNA, to test its ability to form double-stranded structures as a function of decreasing RNA concentrations in the incubation mixture. It was expected that if the formation of the double-stranded RNA is a unimolecular reaction, due to intramolecular base-pairing, then the rate of duplex formation should be independent of the RNA concentration; on the contrary, if the duplexes are composed of RNA segments from separate molecules, then hybrid formation would be a bimolecular reaction and its rate should decrease with decreasing concentration of the RNA in the incubation mixture. Table 2 shows the formation of the RNase-resistant RNA duplexes with decreasing RNA concentration. The data indicate that, for both 15 minute and 3 hour labeled RNA, maximum duplex formation,

TABLE 2

RNase Resistance (% of input cts/min)

Time of Incubation	Concentration of RNA	(A) 66°C, 0.27 M PB, pH 6.7				(B) 37°C, 40% Formamide, 0.27 M PB, pH 6.7		
		0.01 µg/ml	0.1 µg/ml	5 µg/ml	100 µg/ml	0.1 µg/ml	5 µg/ml	100 µg/ml
					15' labeled duck HnRNA			
0' Control		0.08	0.1	0.06	0.04	0.05	0.08	0.08
15'		2.3	2.6	2.2	3.0	2.1	1.9	2.3
24 hr		2.8	2.0	1.9	2.4	2.6	2.3	2.1
48 hr		2.5	2.9	2.1	3.1	2.5	2.7	2.8
					3 hour labeled duck HnRNA			
0' Control		0.2	0.04	0.05	0.1	0.1	0.05	0.04
15'		3.1	2.2	2.6	3.3	2.6	2.1	3.1
24 hr		2.8	2.6	2.6	2.1	2.8	3.2	2.1
48 hr		2.2	2.5	2.1	2.8	3.0	2.5	2.8

All the incubations at 66°C were carried out in a volume of 1 ml, with the exception of the experiments with 100 µg/ml RNA in which the volume was 0.1 ml. All the experiments at 37°C in 40% formamide were carried out in a total volume of 0.1 ml. After incubation, the samples were cooled, brought to a final volume of 1 ml (if necessary) in 0.27 M PB and RNase digested as described in Materials & Methods, Section (g). Prior to self-annealing, the RNA was denatured either by heating for 5 min at 90°C in 10^{-3} M PB and fast-cooling (A), or by dissolving the RNA in recrystallized formamide, heating at 37°C for 15 min and cooling to room temperature (B).

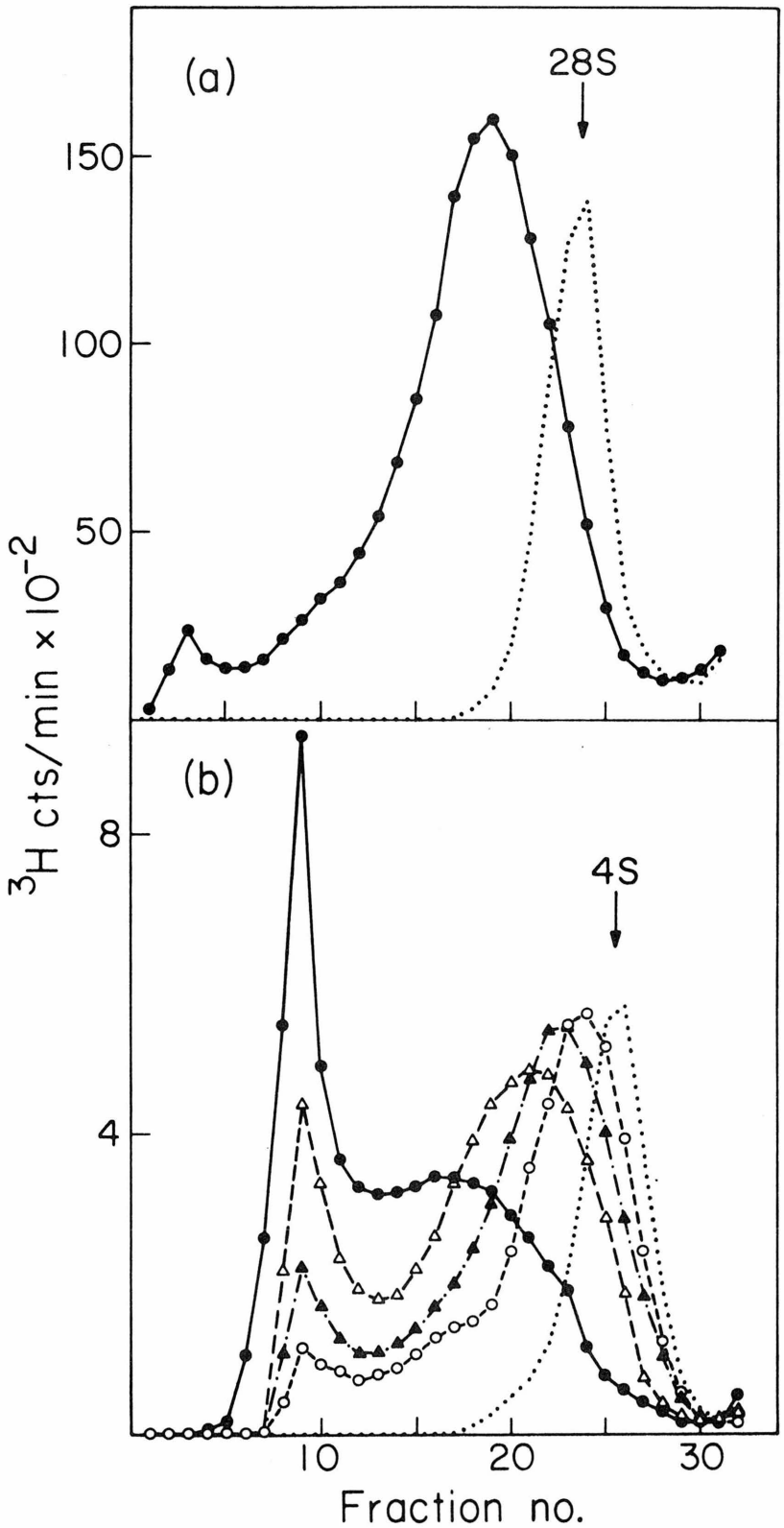
corresponding to 2 to 3% of the RNA, again occurred by 15 minutes of incubation over a range of concentrations of RNA varying by a factor of 10,000. This is in agreement with the idea that the formation of the double-stranded RNA segments in duck erythrocyte HnRNA, as in the case of HeLa HnRNA, results from intramolecular complementarity within the HnRNA molecules.

Proper evaluation of the extent and time course of RNA duplex formation under the conditions used in the above described experiments demands the analysis of the rate of breakdown of the HnRNA during the prolonged incubation at elevated temperatures. Figure 2a shows the sedimentation profile of the 15 minute pulse-labeled HeLa HnRNA in a formaldehyde-sucrose gradient. The HnRNA shows a broad distribution corresponding to a size range between 2.5×10^6 and 2×10^7 daltons, with a weight-average molecular weight of approximately 8.5×10^6 daltons (Boedtker, 1968). Figure 2b shows the changes in sedimentation profile of the RNA during incubation for 1 to 6 days at 66°C under conditions identical to those used for the annealing of the RNA in the experiments of Table 1. The RNA is gradually degraded during the incubation with a progressive transfer of radioactivity from the heavier RNA to the lighter components, corresponding in size to approximately 3×10^4 to 3×10^5 daltons (100-1000 nucleotides). After 24 hours of incubation 65%, and after 48 hours > 80%, of the radioactivity had been transferred to the lighter components. An analysis of

Figure 2. Thermal degradation of the 15 minute [5-^{3}H]uridine pulse-labeled >50 S HeLa HnRNA during incubation at 66°C.

(a) The sedimentation behavior of the HnRNA prior to incubation at 66°C was analyzed under denaturing conditions in a formaldehyde-sucrose gradient in the presence of HeLa [2-^{14}C]uridine labeled 28 S rRNA marker, as described in Materials and Methods, Section (i).

(b) Sedimentation patterns in formaldehyde-sucrose gradients of the HnRNA, which had been incubated at 66°C, under the conditions described in the legend of Table 1, for 1 day (●), 2 days (Δ), 4 days (▲), or 6 days (0). [5-^{3}H]uridine labeled HeLa 4 S RNA marker was run in a parallel gradient.


(a)
28S
150
100
50
(b)
4S
8
4
^{3}H cts/min x 10^{-2}
10
20
30
Fraction no.

the rate of breakdown of the RNA under the conditions of pH and temperature used in these experiments gives a value of 1 scission/10^8 nucleotides/second. This estimate is in agreement with the values reported by Eigner et al., (1961) for somewhat different experimental conditions. Even after six days of incubation, all radioactivity remains acid insoluble, and the estimated size of the smallest RNA chains is about 3 x 10^4 daltons.

The extent of thermal degradation observed for the HnRNA was not such as to effect the kinetics and extent of RNA duplex formation in the annealing experiments described above. In particular, the fact that maximum RNA duplex formation occurred in 15 minutes, i.e. at a time when no appreciable HnRNA degradation was expected to occur, suggests that the extent of intramolecular homology of HnRNA was not significantly underestimated as a result of thermal breakage of HnRNA. This interpretation is supported by a series of self-annealing experiments similar to those already described in Table 2, but carried out in 40% formamide at 37°C (McConaughy et al., 1969). As shown in Table 2, no difference in the extent and time course of duplex formation was observed. From this one can conclude that if the amount of the duplex regions was underestimated due to breakage of the HnRNA molecules and, therefore, to separation of the originally intramolecular complementary segments, this breakage must have occurred during isolation of the RNA. Against this, however, speaks the fact that no difference was observed in either the sedimentation profiles or the percentage of RNase resistance of

the 2 minute labeled HeLa HnRNA preparations, isolated by three different procedures (Table 1); furthermore, no increase in RNase resistance was found under the more extensive hybridization conditions, as expected for intermolecular hybrid formation.

(d) Size of the RNase-resistant material from the HnRNA of duck erythrocytes

The size of the RNase-resistant segments of the HnRNA from duck erythrocytes was examined by sodium dodecyl SO_4-sucrose gradient centrifugation. Figure 3 shows the sedimentation profiles of the duplex regions formed either at 66°C(b) or in 40% formamide at 37°C(a). In both cases, the weight-average molecular weight of the double-stranded RNA molecules corresponds in length to approximately 125 nucleotide pairs (estimated from their sedimentation rate, relative to the 4 S RNA marker, by using the equation for native DNA of Studier, 1965); however, the distributions are rather broad and include much smaller double-stranded segments, as well as duplexes up to 2000 nucleotide pairs. This size distribution of the RNase-resistant structures from duck erythrocyte HnRNA is very similar to that previously reported for the HnRNA from HeLa cells (Jelinek & Darnell, 1972).

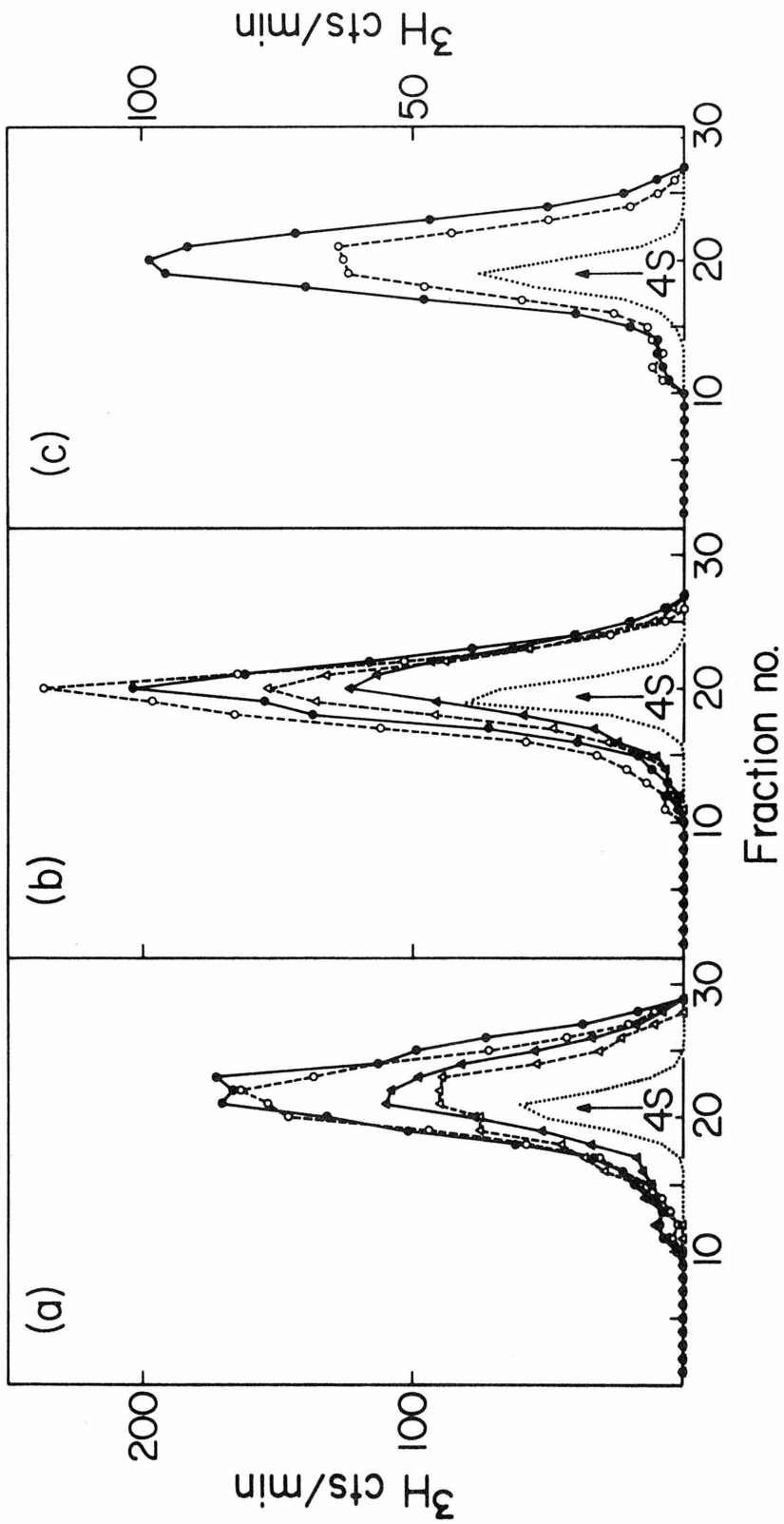
Figure 3. Sedimentation analysis of the RNase-resistant fraction from duck HnRNA.

(a) 15 minute (▲) or 3 hour (●) [5-^{3}H]uridine labeled duck HnRNA, which had not been denatured nor self-annealed, and 15 minute (Δ), or 3 hour (0) labeled duck HnRNA, which had been denatured and self-annealed for 15 minutes at 37°C in 40% formamide, as described in the legend of Table 2, was digested with ribonuclease, sodium dodecyl SO_4-pronase-phenol extracted, and analyzed by sedimentation through sodium dodecyl SO_4-sucrose gradients, as described in Materials and Methods, Section (h).

(b) 15 minute (▲), or 3 hour (●) [5-^{3}H]uridine labeled duck HnRNA, which had been denatured and self-annealed at 66°C for 15 minutes, and 15 minute (Δ) or 3 hour (0) labeled duck HnRNA, which had been self-annealed at 66°C for 24 hours, as described in the legend of Table 2, was digested with ribonuclease and sodium dodecyl SO_4-pronase-phenol extracted, and the RNase-resistant fraction was sedimented through sodium dodecyl SO_4-sucrose gradients.

(c) A mixture of 0.1 μg of 15 minute (0) or 3 hour (●) [5-^{3}H]uridine labeled duck HnRNA and 10 μg of 10 S mRNA was denatured by heating at 37°C for 15 minutes in recrystallized formamide, and annealed for 24 hours in 40% formamide, 0.27 M PB, pH 6.7, at 37°C. After incubation, the RNA was digested with ribonuclease as described in Materials and Methods, Section (g),

sodium dodecyl SO_4-pronase-phenol extracted, and the RNase-resistant fraction was analyzed by sedimentation through a 15 to 30% sucrose gradient in sodium dodecyl SO_4 buffer (prepared over a 1 ml cushion of 64% sucrose) in a SW41 Spinco rotor at 40,000 rev/min for 24 hours at 20°C.


(a)
(b)
(c)
4S
4S
4S
200
100
100
50
10
20
30
10
20
30
10
20
30
^{3}H cts/min
^{3}H cts/min
Fraction no.

(e) Sequence homology between 10 S hemoglobin mRNA and HnRNA from immature duck erythrocytes

It has recently been reported that a portion of the mRNA from HeLa cells contains sequences which are complementary to the HnRNA (Stampfer et al., 1972). It seemed of interest to extend these observations to the investigation of the homology between the 10 S hemoglobin mRNA and the HnRNA from duck erythrocytes, because of the greater homogeneity of this system.

The HnRNA from duck erythrocytes was annealed with purified 10 S mRNA present in 100-fold excess. Table 3 shows that an additional 2% of the HnRNA forms RNase-resistant RNA duplexes with the 10 S mRNA above the background of intramolecular homology. This increase in RNase-resistant material is specific for the mRNA, since it was not observed with ribosomal RNA under identical conditions. The relative increase in double-stranded RNA formation upon annealing of HnRNA with 10 S mRNA was about the same with both the 15 minute and 3 hour labeled HnRNA preparations. Figure 3c shows that the overall size distribution of the RNase-resistant regions, formed by annealing HnRNA in the presence of 10 S mRNA, is similar to that found for the intramolecular RNA duplexes in HnRNA: this indicates that the RNase-resistant complexes formed between 10 S mRNA and HnRNA have about the same size range as the intramolecular duplexes.

TABLE 3

RNase Resistance (% of input cts/min)

RNA	0' Control	24 hour incubation		
		No Addition	+10 µg 10 S mRNA	+10 µg E.coli rRNA
15' labeled duck HnRNA	0.05	2.4	4.6	2.6
3 hr labeled duck HnRNA	0.08	2.7	4.5	3.0

The RNA was first denatured at 90°C for 5 min in 10^{-3} M PB, pH 6.7, fast-cooled, brought to 0.27 M PB in a final volume of 0.1 ml, and incubated at 66°C for 24 hr. After incubation, the samples were cooled, diluted with 0.9 ml of 0.27 M PB, pH 6.7, and RNase digested as described in Materials & Methods, Section (g).

4. Discussion

The main purpose of this report has been to examine the > 50 S HnRNA, labeled after short [5-^{3}H]uridine pulses, from both HeLa cells and immature duck erythrocytes for the presence of complementary transcripts capable of forming RNase-resistant duplexes. It was hoped that duck erythrocytes, because of the expected restriction in genome expression and greater homogeneity of the RNA population, as compared to HeLa cells, would provide a more favorable system for the detection of a self-complementary RNA fraction. It was found that 2-3% and 4-5% of highly purified, untreated duck and HeLa HnRNA, respectively, was resistant to extensive RNase digestion. The RNase resistance was almost completely abolished by heat denaturation of the RNA, indicating that double-stranded RNA was responsible for it; furthermore, the RNase resistance could be restored to its original level by self-annealing the HnRNA in high salt at 66^{o}C for 15 minutes, pointing to the reformation of the RNA duplexes. Further incubation of the RNA, even under the most extensive hybridization conditions employed, failed to show any increase in the amount of radio-activity present in the hybrids. When RNA duplex formation was examined in denatured duck HnRNA at very low concentration, the rate of reassociation was found to be much faster than that reported for purified mouse satellite DNA and slightly faster than that found for poly(A-T) (Britten & Kohne, 1968).

Therefore, it is extremely unlikely that this double-stranded RNA in duck HnRNA is formed by association of separate RNA strands, but rather the most plausible explanation is that it results from intramolecular base-pairing. These results agree with observations by Jelinek & Darnell (1972), who reported that approximately 3% of the HeLa HnRNA occurs as RNase-resistant structures formed by intramolecularly complementary regions.

The failure to detect any significant amount of RNA duplex formation in addition to the intramolecular base-pairing, even after 6 day incubation at high RNA concentration (500 μg/ml) should be contrasted with the situation described for the pulse-labeled HeLa mitochondrial RNA. When mitochondrial RNA, greater than 60 S, was self-annealed at 66°C at a concentration estimated to be <0.1 μg/ml, it formed RNase-resistant hybrids to the extent of 48% during a 24 hour incubation (Aloni & Attardi, 1972). The initial large size and broad distribution of the HnRNA used in the hybridization precludes the determination of the rate of duplex formation by the $C_o t$ analysis technique of Britten and Kohne (1968). However, from the analysis of the size distribution of the HnRNA during incubation, and from the RNA and salt concentrations used, it can be estimated that the majority of, if not all, the complementary RNA sequences present once or a few times in the population would have formed hybrids during the most prolonged incubations. Therefore, the failure to detect any significant level of intermolecular hybridization in the HnRNA from either an actively

dividing cell line or differentiating cells suggests that the cellular mechanism for transcribing the HnRNA does not produce high molecular weight complementary transcripts. However, the experiments reported here would not exclude the possibility that symmetrical transcription might occur, but for some reason the RNA transcribed from one of the two DNA strands never exists, or only for extremely short time, in the form of high molecular weight RNA molecules. In this connection, it should be noticed that no difference was found in the precent of radioactivity associated with RNase-resistant structures when RNA labeled for different times was used. In HeLa HnRNA the 2 minute labeling time was presumably sufficiently short for a substantial portion, if not the majority, of the radioactivity to be incorporated into nascent RNA chains, which were quantitatively extracted from the cell. This excludes the possibility of an extensive symmetrical transcription with the transcripts of one strand being rapidly degraded after their release from the template, since, then, an increase in the proportion of labeled RNA duplexes after the 2 minute, as compared to the 15 minute, labeling time should have been observed.

The weight-average size of the RNase-resistant fragments from the duck HnRNA is approximately 125 nucleotide pairs in agreement with the observations by Jelinek and Darnell for HeLa cells (1972). However, longer RNA duplexes, up to 2000 nucleotide pairs, also were observed in the present work. No such large fragments were observed by the above cited

authors. In contrast, Kimball and Duesberg reported the presence in HeLa cells of double-stranded RNA sedimenting at 7 - 11 S, which would correspond to 500-1500 nucleotides. No information was obtained in the present work as to the distribution pattern of the complementary regions in the HnRNA molecules. The amount of double-stranded segments formed, assuming a weight-average size of 125 nucleotide pairs, and uniform distribution, would correspond to several such segments (1 to 10) for HnRNA molecules of a size ranging between 2.5×10^6 and 2×10^7 daltons.

Lastly, we have shown that approximately 2% of the duck HnRNA will form double-stranded RNA structures with purified duck 10 S mRNA, and that the size of these hybrids, within the experimental limits, is identical to that of the intramolecular hybrids. Although it is possible that a part of the regions involved in intramolecular base-pairing may also hybridize with the 10 S mRNA, at least a portion of the HnRNA molecules must contain segments which hybridize with the mRNA and which do not participate in the intramolecular hybridization. The possible functional significance of these observations warrants further investigation.

ABBREVIATIONS

HnRNA, heterogeneous nuclear RNA; mRNA, messenger RNA; rRNA, ribosomal RNA.

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PART III

USE OF A DNA PROBE FOR MAPPING BY ELECTRON MICROSCOPY THE RIBOSOMAL SEQUENCES IN RIBOSOMAL RNA PRECURSORS FROM AVIAN CELLS

Use of a DNA Probe for Mapping by Electron Microscopy the Ribosomal Sequences in Ribosomal RNA Precursors from Avian Cells

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ABSTRACT

This report describes the use of purified rDNA to map by electron microscopy the relative position of the 18 S and 28 S RNA regions within the duck rRNA precursor and their relationship to the nonconserved portions of the precursor molecule. In the first part, the purification from duck erythrocytes of rDNA sequences suitable for use in the electron microscopic mapping of the rRNA precursor is discussed. By repeated fractionation of the total DNA, based on the relative reassociation rates of the DNA sequences with different degrees of repetition, a fraction of the rapidly renaturing DNA was obtained which comprised only 6% of the total DNA, but contained 71% of the rRNA cistrons. Further purification of the rDNA was achieved by saturation hybridization with rRNA and separation of the rRNA-rDNA hybrids by banding in CsCl. In this manner, an rDNA-rRNA fraction was obtained which had a buoyant density of 1.805 gm/cm^3, an RNA to DNA ratio of 1.01, and a base composition for the RNA present in the hybrid identical to that of an equimolar mixture of 18 S and 28 S rRNA. The final yield of rDNA isolated by this procedure is 32%. When the purified rDNA was annealed with a mixture of 18 S and 28 S rRNA and the hybrids spread for electron microscopy, they appeared as two distinct populations with a number-average length of 0.62 $\pm$ 0.13 μm and 1.37$\pm$0.18 μm, respectively. Likewise, hybrids between the rRNA precursor, isolated from duck embryo fibroblasts, and the rDNA appeared as structures containing two duplex regions of lengths 0.60$\pm$0.11 μm and

1.38 ± 0.15 μm, separated from each other by a single-stranded region appearing as a large bush: this represents the portion of the precursor molecule not conserved during processing of the parent molecule. From these observations a model of the structure of the avian rRNA precursor is proposed.

1. Introduction

The results of work from many laboratories have shown that, in animal cells, the formation of several major classes of RNA involves the synthesis of larger RNA precursor molecules which are posttranscriptionally modified to the mature products (Bernhardt & Darnell, 1969; Jeanteur & Attardi, 1969; Weinberg & Penman, 1970; Firtel & Lodish, 1973; Darnell et al., 1973; for additional references see Weinberg, 1973). In these studies, our understanding of the relationship of the final RNA product to its precursor has derived mainly from a comparison of the chemical and physical properties of the precursor and product molecules, from the partial correspondence in their base sequence and their kinetics of labeling. Valuable insight into the nature of these processes could be more directly obtained if it were possible to map by electron microscopy the position of the final RNA products to their position within the parent molecule. In this manner, the topographical relationship which the RNA product has to the remainder of the molecule, not conserved during processing, could be studied in detail.

The rRNA system is especially suitable for developing such an approach for several reasons: the processing pathway for this class of RNA has been biochemically well defined (Weinberg & Penman, 1970; Choi & Busch, 1970; Perry & Kelly, 1972; Wellauer & Dawid, 1973); purification of both the precursor and final RNA products is relatively easy; and the size of the RNA species is especially well suited for electron microscopic analysis. Furthermore, techniques have been developed in recent years for the isolation of specific DNA sequences

from both bacteria and lower eukaryotes (Kohne, 1968; Marks & Spencer, 1970; Colli et al., 1971; Chattophadhysy et al., 1972; Spadari et al., 1972) and from animal cells (Wallace & Birnstiel, 1966; Brown & Weber, 1968; Becker et al., 1970) have been most successfully applied for the purification of DNA complementary to rRNA (rDNA).

In the present paper we report a modification of these techniques which has allowed the preparation of purified rDNA from duck erythrocytes with a relatively high yield. This DNA has been successfully used to map by electron microscopy the relative positions of the 18 S and 28 S RNA regions in the duck rRNA precursor[1] and their relationship to the nonconserved portions of the molecule.

2. Materials and Methods

(a) Animals

Nine- to fourteen-month-old male Pekin ducks, made anemic by intramuscular daily injections of 25 mg of neutralized phenylhydrazine for five to seven consecutive days, were used in these experiments. Blood was drawn by cardiac puncture on the day following the last injection; to prevent coagulation, heparin was added to a final concentration of 20 µg/ml of blood.

(b) Method of duck embryo fibroblast growth

Duck embryo fibroblasts (purchased from Microbiological Associates) were grown in plastic Petri dishes, at 37°C, in 10 ml Dulbecco-Vogt modified Eagle's medium containing 10% tryptose phosphate and 10% fetal calf serum.

(c) Buffers

(1) Tris-K-Mg: 0.05 M-Tris buffer (pH 6.7, 25°C), 0.025 M-KCl, 0.0025 M-$MgCl_2$; (2) Tris-K-EDTA: 0.05 M-Tris buffer (pH 6.7), 0.025 M-KCl, 0.01 M-EDTA; (3) Tris-Na-EDTA: 0.01 M-Tris (pH 7.4), 0.1 M-NaCl, 0.001 M-EDTA; (4) Tris-EDTA: 0.001 M-Tris (pH 7.0), 2.5×10^{-4} M-EDTA; (5) sodium dodecyl SO_4 buffer: 0.01 M-Tris buffer (pH 7.0), 0.1 M-NaCl, 0.001 M-EDTA, 0.5% sodium dodecyl SO_4; (6) acetate-Na-EDTA: 0.01 M-acetate buffer (pH 5.0), 0.1 M-NaCl, 0.001 M-EDTA; (7) alkaline buffer: 0.1 M-NaOH, 0.05 M-Na_3PO_4 (pH 12.5); (8) acetate-Na-Zn buffer: 0.03 M-sodium acetate (pH 4.6), 0.1 M-NaCl, 5×10^{-4} M-$ZnCl_2$; (9) Tris-Cs-EDTA: 0.01 M-Tris buffer (pH 7.4), 0.4 M-CsCl, 0.001 M-EDTA; (10) PB: Na phosphate buffer, pH 6.7.

(d) Labeling conditions

The general experimental conditions for *in vitro* labeling of the duck erythrocytes have been described previously (Attardi *et al.*, 1966). Labeling of the DNA was carried out by incubating the erythrocytes for 2 h in modified Eagle's medium (Levintow & Darnell, 1960) supplemented with 10% dialyzed duck serum in the presence of [*methyl*-^{3}H]thymidine (100 µCi/ml; 28 Ci/mmol). The 18 S and 28 S rRNAs were labeled by incubating the cells for 3 h in phosphate-free medium containing 500 µCi/ml carrier-free [^{32}P]orthophosphate.

In order to follow the rRNA precursor during purification, a portion of the duck embryo fibroblast cultures (10%) were pulse-labeled for 25 min with [5-^{3}H]uridine (1 µCi/ml; 29 Ci/mmol) using normal growth medium with dialyzed serum.

(e) RNA and DNA extraction procedures

For isolation of the 18 S and 28 S rRNAs, a cytoplasmic extract, prepared from the erythrocytes as previously described (Attardi et al., 1966), was centrifuged at 12,000 revs/min for 20 min in a SS-34 Servall rotor to remove mitochondria and other membranous material; the free-polysomes were isolated from the post-mitochondrial supernatant by pelleting through a discontinuous sucrose gradient (Attardi et al., 1970). The polysome pellet was resuspended in Tris-K-EDTA supplemented with 0.02 M-EDTA, and the ribosomal subunits were separated by centrifugation through a 15 to 30% (w/w) sucrose gradient in Tris-K-EDTA at 26,000 revs/min in a SW27 Spinco rotor for 17 h at 2°C (Amaldi & Attardi, 1968). The fractions corresponding to the 30 S and 50 S ribosomal subunit peaks were pooled separately, the RNA was extracted by the sodium dodecyl SO_4-pronase-phenol procedure (Aloni & Attardi, 1971), and the 18 S and 28 S RNAs were purified by sedimentation through sucrose gradients in sodium dodecyl SO_4 buffer. The isolated rRNA components were collected by ethanol precipitation and centrifugation, dissolved in Tris-K-Mg (500 µg/ml), digested with 50 µg/ml of RNase-free DNase (Worthington) for 30 min at 25°C, re-extracted by the sodium dodecyl SO_4-pronase-phenol method, precipitated with ethanol and passed through a Sephadex G-100 column (equilibrated with 0.3 M-NaCl, 0.01 M-Tris buffer, pH 6.7, 10^{-3} M-EDTA) to separate the RNA from any DNA digestion products. In order to isolate intact 18 S and 28 S molecules for hybridization with DNA, the RNA was dissolved, at a concentration of 250 µg/ml, in low ionic strength buffer (Tris-EDTA), heated at 80°C for 3 min, fast-cooled

and sedimented through 5 to 20% sucrose gradients in the same buffer in a SW41 Spinco rotor at 40,000 revs/min for 15 h at 2°C.

For the preparation of the rRNA precursor from duck embryo fibroblasts, the RNA was released by directly lysing the cells on Petri dishes with 1 ml sodium dodecyl SO_4 buffer + 50 µg/ml pronase per plate. The lysate was phenol extracted, precipitated with ethanol, resuspended in sodium dodecyl SO_4 buffer and centrifuged through a 15 to 30% sucrose gradient in the same buffer at 26,000 revs/min in a SW27 Spinco rotor for 5 h at 20°C. The fractions corresponding to the rRNA precursor were pooled, the RNA was collected by ethanol precipitation, and purified from contaminating HnRNA molecules by thermal denaturation and sedimentation in a sucrose gradient in low ionic strength buffer (Tris-EDTA), as previously described (Jeanteur et al., 1968).

Duck erythrocyte DNA was prepared from 5 x 10^{10} cells essentially according to the procedure of Marmur (1961), with the following modifications: the cells were lysed in sodium dodecyl SO_4 buffer and digested with 1 mg/ml pronase for 2 h at 37°C prior to the chloroform-isoamyl alcohol extraction; two additional pronase digestions were performed, one after the first chloroform-isoamyl extraction and the second immediately following the pancreatic RNase digestion; three phenol deproteinization steps were added after the final chloroform-isoamyl alcohol extraction.

(f) Fragmentation of the duck erythrocyte DNA with DNase II

The DNA was dissolved, at a concentration of 500 µg/ml, in acetate-Na-EDTA containing 1 µg/ml cytochrome C, and digested with 5 units/ml

of hog spleen DNase II (Sigma) for 30 min at 37°C. After incubation, the DNA was deproteinized by two extractions with chloroform-isoamyl alcohol, precipitated with ethanol, again treated with pancreatic RNase (50 µg/ml, 1 h at 20°C) to remove any residual RNA, and twice extracted by the sodium dodecyl SO_4-pronase-phenol technique. The size distribution of the DNA fragments was analyzed by sedimentation in a sucrose gradient in alkaline buffer under the conditions specified in the legend of Figure 1. The total DNA was used for the purification of the rDNA.

(g) Enrichment of the DNA for rDNA sequences

In order to enrich the erythrocyte DNA for sequences complementary to the rRNA, the DNA was dissolved in 10^{-3} M-EDTA, pH 7.0, denatured by heating at 100°C for 10 min, and incubated at 60°C in 0.12 M PB to a $C_o t = 50$. The renatured DNA sequences were separated from the single stranded DNA by hydroxyapatite chromatography (Davidson & Hough, 1971), dialyzed against Tris-Na-EDTA, collected by ethanol precipitation and centrifugation, denatured and reincubated under the above conditions to $C_o t = 4$. By submitting the DNA sequences renatured in the above step to a third cycle of denaturation and to incubation to $C_o t = 0.1$, a single stranded fraction of the DNA was isolated which represented 6% of the total DNA, but contained 71% of the rRNA cistrons: this material was used for the purification of rDNA sequences, as described below. The fractionation procedure is summarized in the scheme shown in Figure 2.

(h) Purification of the DNA complementary to rRNA on CsCl gradients

The fraction of the DNA which was single stranded at $C_o t = 0.1$ but double stranded at $C_o t = 4$ was dissolved in 0.001 M-EDTA, pH 7.0, and mixed with equimolar amounts of 18 S and 28 S duck rRNA in a volume of 5 ml. at a final concentration of 200 µg/ml of the DNA and 50 µg/ml of the total RNA. The mixture was denatured by heating at 100°C for 5 min, the ionic composition was adjusted to 0.3 M-NaCl, 0.01 M-Tris, pH 7.4, 0.001 M-EDTA, and the solution incubated for 8 h at 75°C. These conditions have previously been shown to produce complete hybridization of the rDNA with rRNA (Jeanteur et al., 1969). At the conclusion of the hybridization, the sample was digested with 100 µg/ml pancreatic RNase and 50 units/ml T_1 RNase for 1 h at 22°C to degrade the unreacted RNA, and then sodium dodecyl SO_4-pronase-phenol extracted. After removal of traces of phenol from the aqueous phase by shaking with ether, and of the residual ether by bubbling nitrogen, solid CsCl was added to give a density of 1.75 gm/cm^3, and the solution was then centrifuged in a Spinco SW41 rotor at 32,000 revs/min for five days at 20°C using a polyallomer tube.

The fractions corresponding to the hybrid peak in the CsCl gradient were pooled, precipitated with ethanol, and resuspended in 1 ml of acetate-Na-Zn buffer. Denatured HeLa DNA (100 µg) was added as a carrier and the mixture digested with 100 units of S_1 enzyme from Aspergillus orizae, which specifically attacks single-stranded DNA (Ando, 1966), (purified from Takadiastase by DEAE-cellulose chromatography, according to the procedure of Sutton, 1971) for 45 min at 37°C. The solution was made 10^{-2} M-EDTA and phenol extracted, and the hybrid twice precipitated with ethanol. The sample was dissolved in Tris-Cs-EDTA, and the hybrid again digested

with 5 µg/ml pancreatic RNase and 5 units/ml T_1 RNase for 30 min at 22°C, treated with 50 µg/ml pronase for 30 min and sodium dodecyl SO_4-phenol extracted. After the traces of phenol were removed by the ether-N_2 treatment, the solution was adjusted to density of 1.75 gm/cm^3 with CsCl in a total volume of 5.0 ml, and centrifuged at 33,000 revs/min for five days in a Spinco SW65 rotor at 20°C in a polyallomer tube.

The region of the CsCl gradient corresponding to the RNA-DNA hybrid was pooled, 15 µg of E. coli rRNA was added as carrier and the nucleic acids were precipitated with 2 vol ethanol. The precipitate was dissolved in 0.3 N-NaOH and incubated for 18 h at 37°C to digest the RNA portion of the hybrid and the carrier RNA; the solution was then neutralized by addition of an equal volume of 0.3 N-HCl and the pH adjusted to 8.0 with 0.1 M-Tris. This purified DNA fraction was used for RNA-DNA hybridization experiments, as described below. The size distribution of the DNA fragments in this fraction was analyzed in an alkaline sucrose gradient as described in the legend of Figure 1.

(i) Base composition of the RNA in the RNA-DNA hybrid

A portion of the RNA-DNA hybrid was taken from both the first and second CsCl gradients, and separated from any small fragments of RNA or DNA by passage through Sephadex G-100. Carrier RNA (1 mg) was added to the hybrid peak in the Sephadex eluate, the nucleic acids were precipitated with 2 vol of ethanol, resuspended in 0.5 N-NaOH and incubated at 30°C for 16 h. Base composition analysis of the RNA digestion products was performed on 0.5 x 3.5 cm columns of Dowex 1-X8, as previously described (Attardi et al., 1966).

(j) Hybridization of the purified rDNA with duck 18 S and 28 S rRNAs and rRNA precursor

A 0.01 µg sample of an equimolar mixture of 18 S and 28 S rRNA from duck erythrocytes, which had been denatured by heating to 80°C for 3 min in low ionic strength Tris-EDTA buffer, was mixed with 0.2 µg of purified rDNA in 50 µl of 0.3 M-NaCl, 0.1 M-Tris, pH 8.0 (final concentration), and incubated for 30 min at 66°C. The same procedure was followed for preparation of hybrids between the rDNA and rRNA precursor from duck embryo fibroblasts.

(k) Electron microscopy

Upon completion of the annealing reaction between rDNA and rRNA or rRNA precursor, 2 µl of 5 M-ammonium acetate, 1 M-Tris, pH 8.0, 0.005 M-EDTA, and 1 µl of a 5 mg/ml solution of cytochrome c in 0.1 M-Tris, pH 8.0, 0.005 M-EDTA, were added to the incubation mixture, and the preparation spread for examination in the electron microscope by the basic protein film technique, as described by Davis et al., 1971. The films were picked up from a hypophase of 0.25 M-ammonium acetate, 0.05 M-Tris, pH 8.0, 0.0025 M-EDTA onto 3% parlodion-coated 300-mesh copper grids, stained with uranyl acetate and shadowed with Pt-Pd. The grids were examined in a Philips EM 300 electron microscope at 60 kV with a 40 µ objective aperture. Photographs of the hybrid were taken on 35 mm film at an instrument magnification of 11,000. The length measurements were calibrated with a diffraction grating replica of 54,864 lines per inch (Ladd).

3. RESULTS

(a) Enrichment of the duck DNA for sequences complementary to rRNA

It has been reported that the DNA from duck erythrocytes contains approximately 150 copies each of the 18 S and 28 S rRNA cistrons per haploid genome, and that the $C_o t_{1/2}$ for the reassociation of the rDNA sequences is about 16 (Bishop et al., 1972). It should, therefore, be possible to partially purify the rDNA sequences from the bulk of the DNA by partial renaturation of the DNA and its fractionation into repetitive and unique components.

Purified erythrocyte DNA was fragmented with DNase II as described in Materials and Methods (f). Sedimentation analysis of the digested DNA in an alkaline sucrose gradient (Fig. 1) revealed a broad band extending from about 3 S to 12 S (estimated relative to that of 4 S RNA run in a parallel gradient), with a modal sedimentation coefficient of about 4.5 S. By applying Studier's formula and correcting for ionic strength (Studier, 1965), the size of the denatured DNA chains was estimated to range between 100 and 1500 nucleotides with a weight average of about 300 nucleotides. The total fragmented DNA was denatured, allowed to reassociate to a $C_o t = 50$, and the fraction containing double-stranded segments ("highly" and "middle repetitive" DNA) separated from the single-stranded material by HAT chromatography. Since the rDNA should be contained within the "middle repetitive" portion of the DNA, further fractionation of the repetitive material was performed according to the flow diagram shown in Figure 2, in order to remove the most rapidly and the most slowly reassociating DNA components. The percentage of the total rRNA cistrons present in each fraction was assayed by hybridization of a portion of each sample

Figure 1. Sedimentation analysis of duck erythrocyte DNA in an alkaline sucrose gradient.

A sample of DNA immediately after digestion with DNase II (o) and a sample of the final rDNA to be used for electron microscopy (●) were dissolved in 0.1 N-NaOH, 0.05 M-Na_3PO_4 (pH 12.5), and sedimented through a 5-20% sucrose gradient in the same buffer at 40,000 rev./min in a Spinco SW41 rotor for 2 hr at 20°C. The dotted line represents the 4 S RNA marker run on a parallel gradient in acetate-Na buffer.

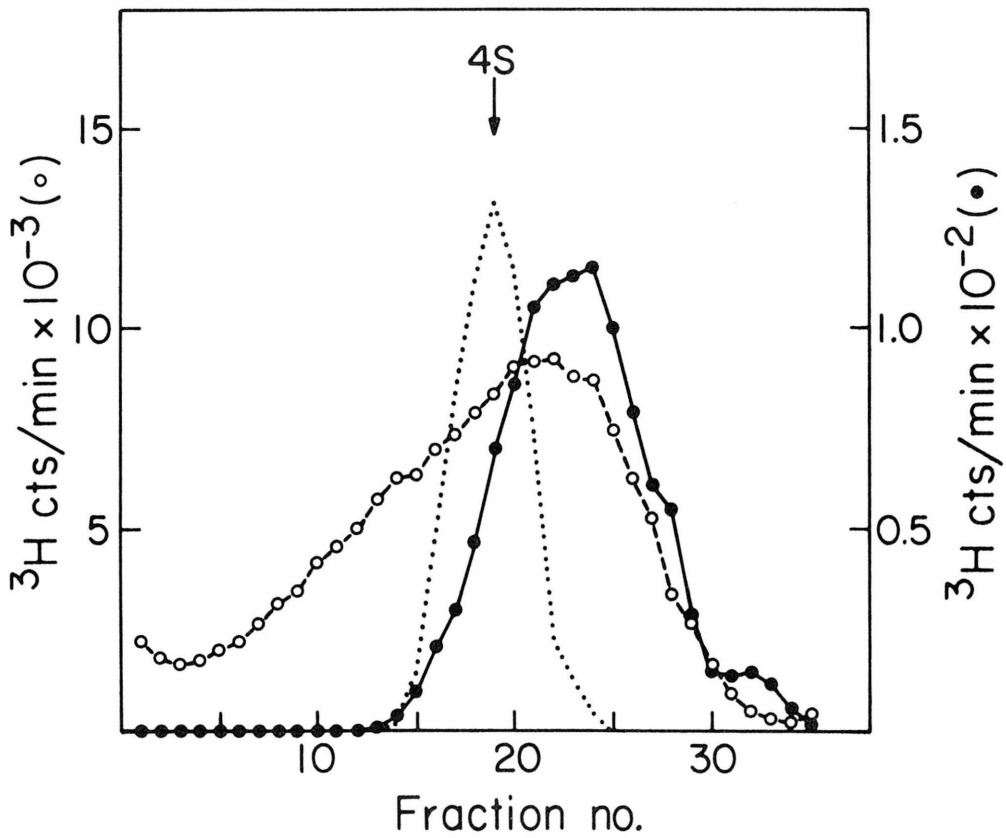
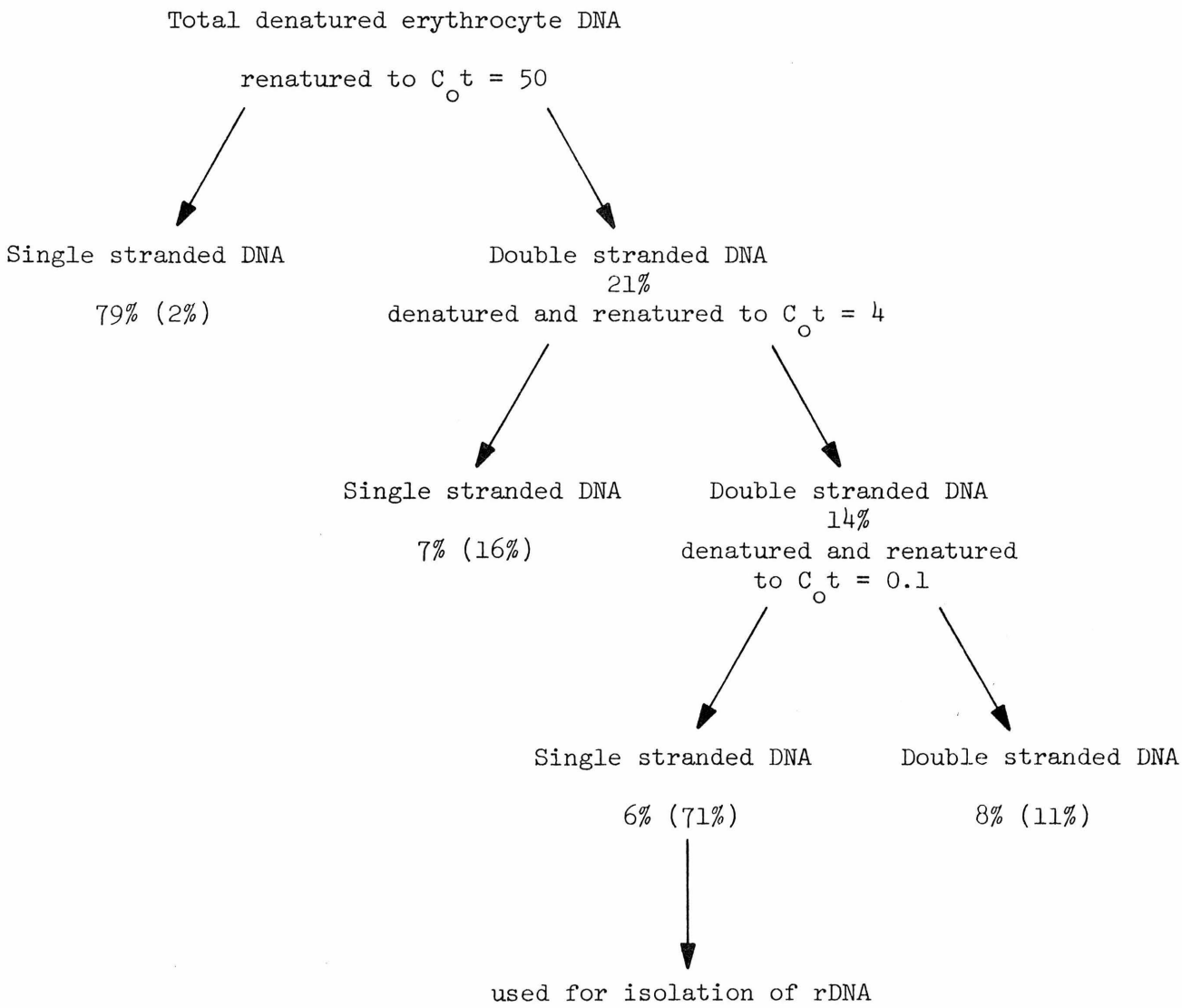

4S
15
10
5
1.5
1.0
0.5
^{3}H cts/min x 10^{-3}(○)
^{3}H cts/min x 10^{-2}(●)
10
20
30
Fraction no.

Figure 2. Flow diagram for the fractionation of duck erythrocyte DNA on the basis of reassociation rate ($C_o t$).

Denatured DNA was allowed to reassociate at 60°C in 0.12 M PB to the indicated $C_o t$ values. Single stranded and double stranded DNA were separated by chromatography on hydroxyapatite. The figures outside parentheses under each DNA fraction indicate the percentage of the original DNA preparation recovered in that fraction; the figures in parentheses indicate the percentage of the total rRNA cistrons.

FIG. 2

Scheme of fractionation of duck erythrocyte DNA



with an excess of rRNA, under conditions known to give saturation of the rRNA sites (Jeanteur & Attardi, 1969). The distribution of the rDNA sequences among the various DNA fractions is given by the figures in parentheses in Figure 2. These results show, that under our conditions of reassociation, the population of DNA which is single stranded at $C_o t = 0.1$ but double stranded at $C_o t = 4$ represents approximately 6% of the total DNA, but contains 71% of the sequences coding for rRNA. This DNA fraction was used to further purify the rDNA from the remainder of the extraneous DNA sequences, as described below. The specific activity of the DNA was found to remain constant throughout the purification procedure.

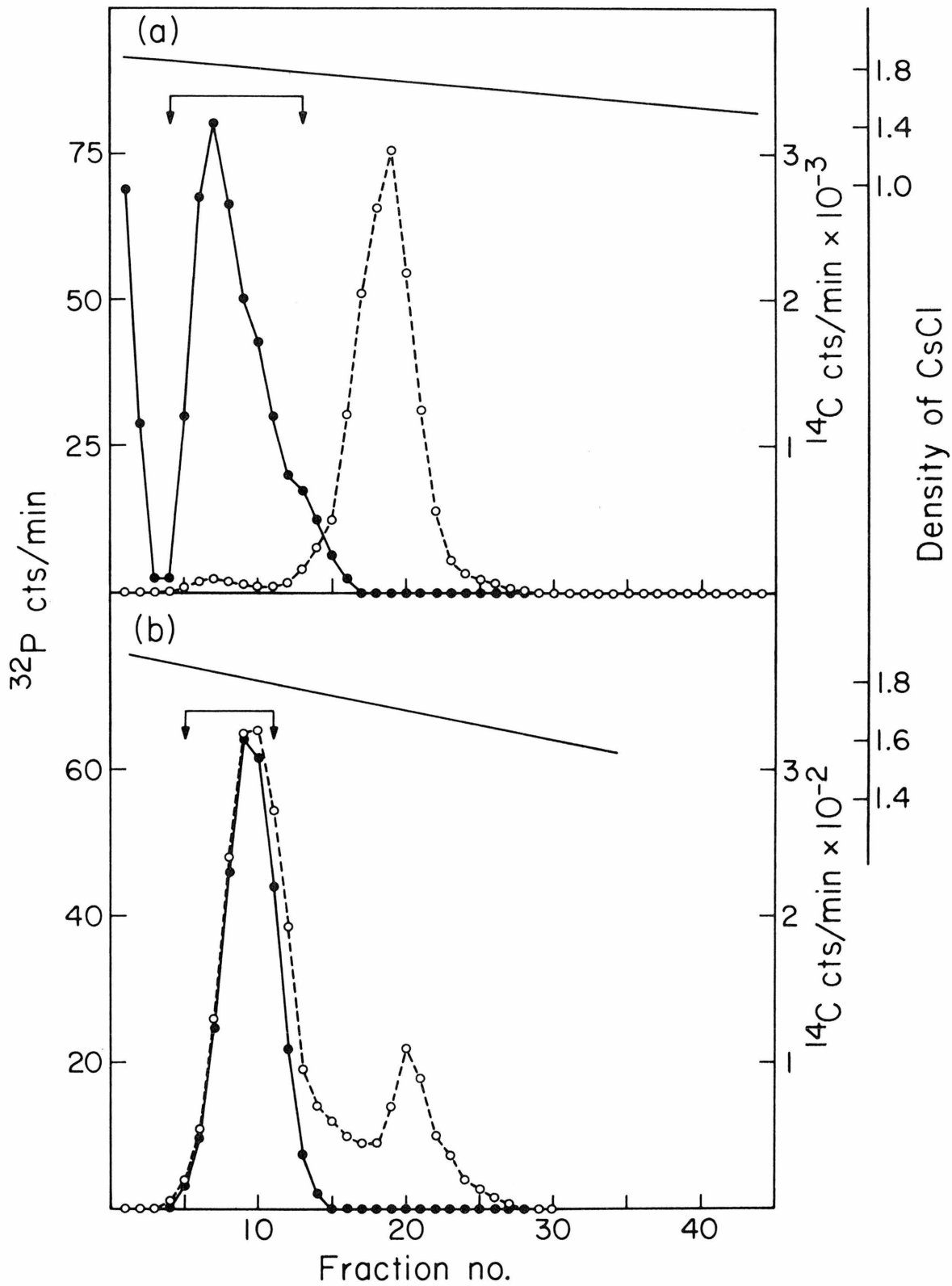
(b) Isolation of the DNA sequences complementary to rRNA

The DNA fraction which was enriched for the presence of rDNA (Results (a)) was hybridized with [^{32}P]18 S and 28 S rRNA, as detailed in Materials and Methods (h), under conditions known to saturate the rDNA sites, treated with pancreatic and T_1 RNase to remove unhybridized RNA, and centrifuged in a CsCl equilibrium density gradient. Figure 3a shows the density distribution of [^{3}H]DNA and [^{32}P]RNA in such an experiment. The majority of the DNA, which under the present conditions of hybridization has totally renatured, bands symmetrically around a buoyant density of 1.700 gm/cm^3. A minor portion of DNA, which represents approximately 0.4% of the total DNA in the gradient, bands at a position centered around a density of 1.805 gm/cm^3. The [^{32}P]rRNA exhibits one single peak at a position which is intermediate between that of the DNA and that expected for free rRNA, and which corresponds well to the smaller peak of DNA: the data indicate that this DNA peak and the [^{32}P]rRNA peak represent

Figure 3. Purification of rDNA sequences by CsCl equilibrium centrifugation.

(a) 1 mg of the ^{3}H-labeled "middle repetitive" DNA (o) was hybridized with 250 μg of an equimolar mixture of ^{32}P-labeled 18 S and 28 S rRNA (●), digested with pancreatic and T_1 RNase, sodium dodecyl SO_4-pronase-phenol extracted, and centrifuged to equilibrium in a CsCl gradient, as described in Materials and Methods (h).

(b) The fractions indicated by arrows in (a) were pooled, the hybrids were digested with S_1 nuclease and pancreatic and T_1 RNase and rerun in a second CsCl equilibrium gradient, as described in Materials and Methods (h). The portion of the gradient indicated by the arrows in (b) was pooled, the hybrids were collected by ethanol precipitation and, after alkali digestion of the RNA chains, the DNA was used for hybridization with the rRNA species and the rRNA precursor.


(a)
(b)
75
50
25
60
40
20
10
20
30
40
Fraction no.
32P cts/min
14C cts/min x 10-3
14C cts/min x 10-2
Density of CsCl
1.8
1.4
1.0
1.6

hybrids between rDNA and rRNA. From the ratio of RNA to DNA calculated from the specific activities of the two components (1.06), it can be estimated that the hybrid in the peak fraction is composed of approximately equal amounts of RNA and DNA.

To further purify the rDNA, especially from covalently attached DNA sequences not homologous to rRNA, the fractions corresponding to the hybrid band were pooled as shown, digested with S_1 nuclease from Aspergillus orizae, which specifically degrades single-stranded DNA (Ando, 1966), again digested with pancreatic and T_1 RNase, and rebanded in a CsCl density gradient as described in Materials and Methods (h). Panel b in Figure 3 shows the density profile of the rebanded material. A distinct peak of the hybrid is again seen at $\rho = 1.805$ gm/cm^3, now representing more than 70% of the DNA present in the gradient, with an RNA to DNA ratio of 1.01 in the peak fraction of ^{32}P. A small peak of DNA at 1.700 gm/cm^3, which represents the double-stranded DNA segments released from the hybrid by the S_1 nuclease treatment, is also observed.

Analysis of the base composition of the RNA present in the hybrid region of the two gradients is shown in Table 1. It can be seen that the base composition is virtually identical to that expected for an equimolar mixture of 18 S and 28 S rRNA on the basis of the reported data for the rRNA species from duck erythrocytes (Attardi et al., 1966).

The size of the purified rDNA product to be used for electron microscopy was analyzed by sedimentation in an alkaline sucrose gradient, as shown in Figure 1. Both the range and the average size of the single-stranded DNA chains appeared to be reduced relative to those of the total DNase II-digested DNA preparation. The estimated length of the DNA chains

TABLE 1

Base composition of rRNA hybridized with rDNA

	Moles per cent					
	A	C	U	G	%GC	Pu/Pyr
Hybrid 1st CsCl	19.7	27.1	17.6	35.6	62.7	1.23
Hybrid 2nd CsCl	19.0	27.0	17.6	36.4	63.4	1.24
18 S*	21.4	24.1	21.4	33.0	57.1	1.20
28 S*	17.8	27.7	17.2	37.3	65.0	1.23
Expected‡	18.9	26.6	18.5	36.0	62.6	1.22

*From Attardi et al., J. Mol. Biol. 20, 145, 1966.

‡Assuming equimolar amounts of 28 S and 18 S present in hybrid.

ranged between 100 and 400 nucleotides, with a weight average of about 150 nucleotides.

The region of the gradient indicated by the arrows in Figure 3b was pooled, the RNA portion of the hybrids was digested with 0.3 N-NaOH, and the final DNA product adjusted to pH 8.0, for use in RNA-DNA hybridization experiments, as described below (section d).

(c) Isolation of the rRNA precursor from duck embryo fibroblasts

Although duck erythrocytes provide a convenient source for the preparation of large quantities of DNA, they synthesize relatively small amounts of rRNA, and, thus, are a very poor source for the isolation of the rRNA precursors (Attardi et al., 1966, 1970). We therefore isolated the rRNA precursors from cultures of duck embryo fibroblasts.

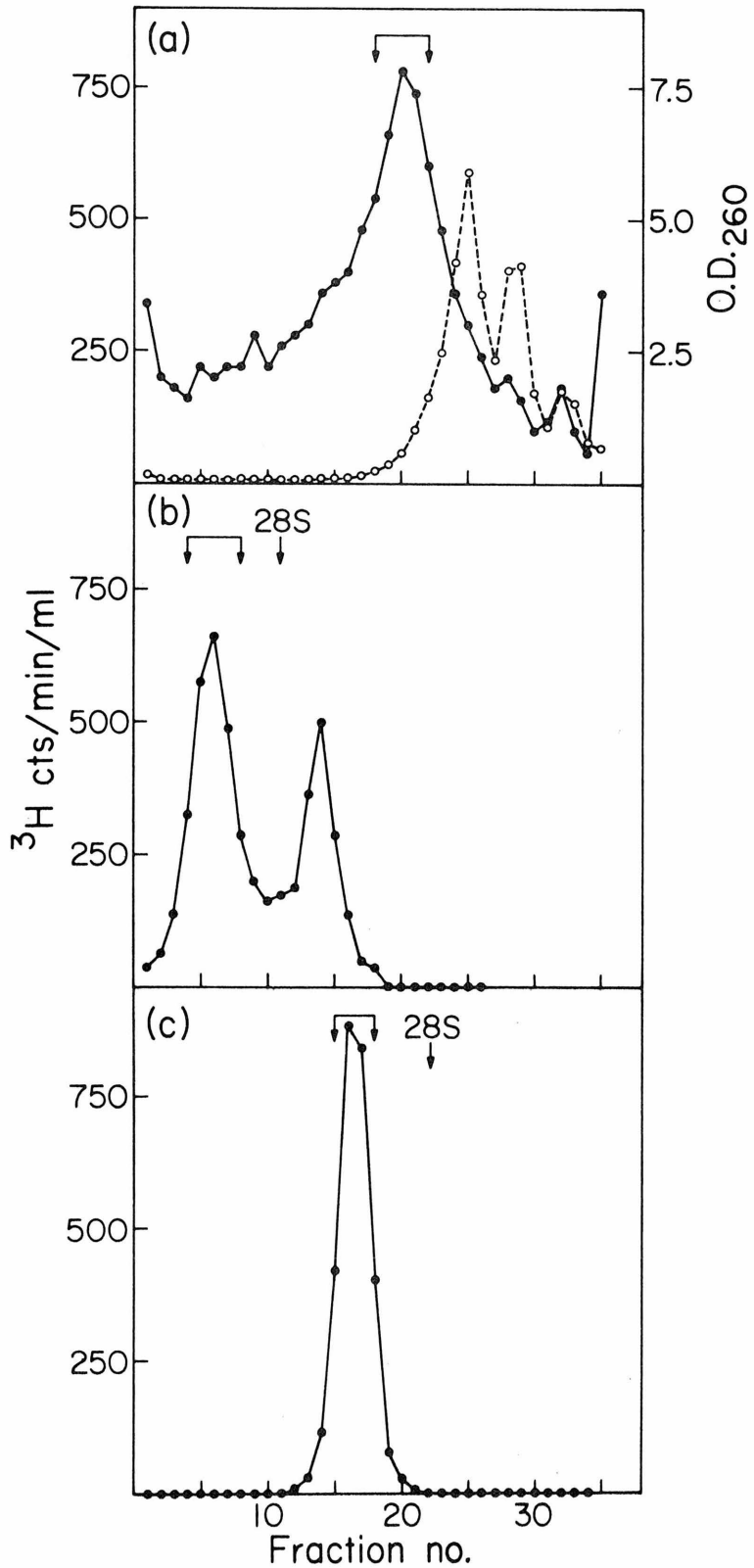
The isolation of the rRNA precursors was carried out as described in Materials and Methods (e), following essentially the procedure of Jeanteur et al. (1969), modified for cells grown on petri dishes. The sedimentation profile in a sucrose gradient in sodium dodecyl SO_4 buffer of total cell RNA pulse-labeled for 25 minutes with [^{3}H]uridine is shown in Figure 4a. A distinct broad peak of radioactivity is seen at a position centered around 41 to 42 S relative to the internal 28 S and 18 S RNA optical density markers, which represents the rRNA precursors emerging over a background of HnRNA. The material corresponding to this peak RNA was pooled in such a way as to include all components in the range 38 to 52 S, and therefore very little, if any, of the presumptive "32 S" intermediate, heat denatured and rerun in a sucrose gradient in low ionic strength Tris-EDTA buffer (Fig. 4b). The above procedure has been previously

Figure 4. Sedimentation analysis of [^{3}H]uridine pulse-labeled rRNA precursor from duck embryo fibroblasts at different stages of purification.

(a) Total cell RNA was extracted from 50 cultures of duck embryo fibroblasts, five of which had been pulse-labeled for 25 min with [^{3}H]uridine, as described in Materials and Methods (d), and sedimented through a 15 to 30% sucrose gradient in sodium dodecyl SO_4 buffer in a Spinco SW27 rotor at 26,000 rev./min for 5 hr at 20°C.

(b) The portion of the gradient indicated by arrows in (a) was pooled, the RNA collected by ethanol precipitation and centrifugation, heated at 80°C for 3 min in low ionic strength Tris-EDTA buffer and centrifuged through a 5 to 20% sucrose gradient in the same buffer in an SW41 Spinco rotor at 40,000 rev./min for 11.5 hr at 2°C.

(c) The material corresponding to the more rapidly sedimenting peak in (b) was collected by ethanol precipitation and centrifugation and rerun on a 15 to 30% sucrose gradient in sodium dodecyl SO_4 buffer in a Spinco SW41 rotor at 40,000 rev./min for 3 hr at 20°C.


(a)
750
500
250
7.5
5.0
2.5
O.D.260
(b)
28S
750
500
250
^{3}H cts/min/ml
(c)
28S
750
500
250
10
20
30
Fraction no.

shown to separate the intact HeLa cell 45 S rRNA precursor from the contaminating HnRNA (Jeanteur et al., 1969). In agreement with the findings in the HeLa cell system, one can recognize in Figure 4b two peaks, the faster sedimenting peak representing presumably intact rRNA precursors and the slower sedimenting peak, the HnRNA components. The material corresponding to the entire more rapidly sedimenting peak was pooled as shown and again centrifuged through a sucrose gradient in sodium dodecyl SO_4 buffer (Fig. 4c), where it formed a sharp symmetrical peak at 41 S, relative to a 28 S HeLa RNA marker. The peak, which was expected to include very little, if any, of "32 S" intermediate, was pooled and used for electron microscopy, as described below.

(d) Electron microscopic examination of hybrids formed between rDNA and 18 S rRNA, 28 S rRNA and the rRNA precursor

When the purified rDNA described in section (b) was hybridized with an equimolar mixture of 18 S and 28 S rRNA at a DNA:RNA ratio of 20:1, as described in Materials and Methods (j), and spread for electron microscopy by the basic protein film technique in an aqueous medium, the hybrid molecules observed appeared as shown in Plate 1(a&b). Extended smoothly curved filaments of the thickness expected for duplex nucleic acids and of two distinct lengths can be seen, with no indication of the presence within these molecules of single-stranded segments appearing as bushes. A histogram of the frequencies of the lengths found in the preparation (Fig. 5a) shows the expected bimodal distribution with the number-average lengths of the 18 S and 28 S rRNA species being 0.62 and 1.37 μm, respectively. Table 2 lists the number-average and weight-average lengths of the two species, together with the corresponding standard deviations. For both

PLATE LEGENDS

Plate 1.

(a) Electron micrographs of selected 18 S rRNA-rDNA hybrids.

(b) Electron micrographs of selected 28 S rRNA-rDNA hybrids.

(c) Electron micrographs of selected hybrids of rRNA precursors with rDNA.

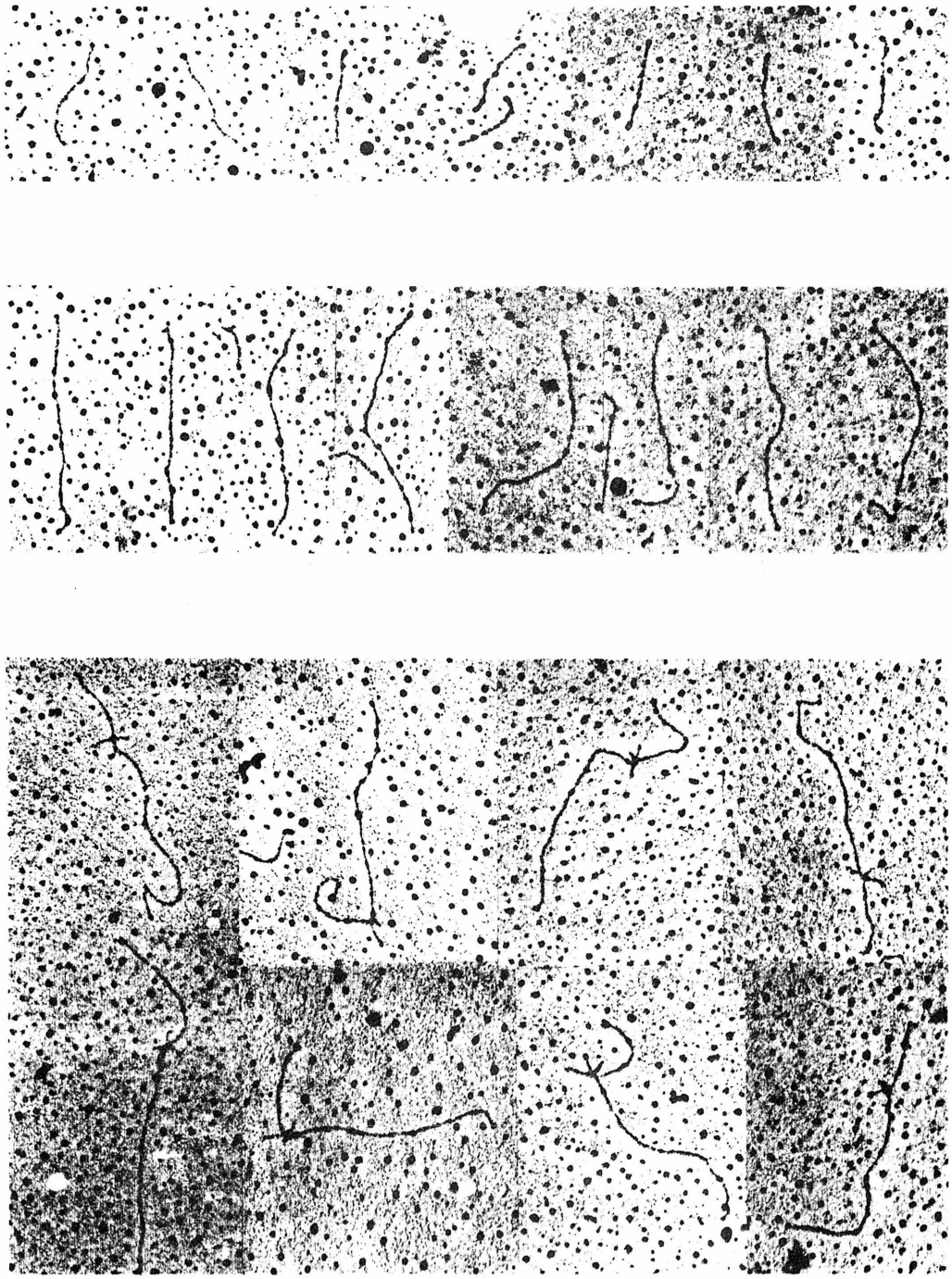

(a)
(b)
(c)
1μ

Figure 5. Length distributions of the rRNA-rDNA hybrids.

(a) 18 S and 28 S hybrids with rDNA. A total of 552 molecules were scored.

(b) rRNA precursor-rDNA hybrids. Only those hybrids which on visual examination appeared to contain intact 18 S and 28 S duplex regions were scored (37 molecules) (see text).

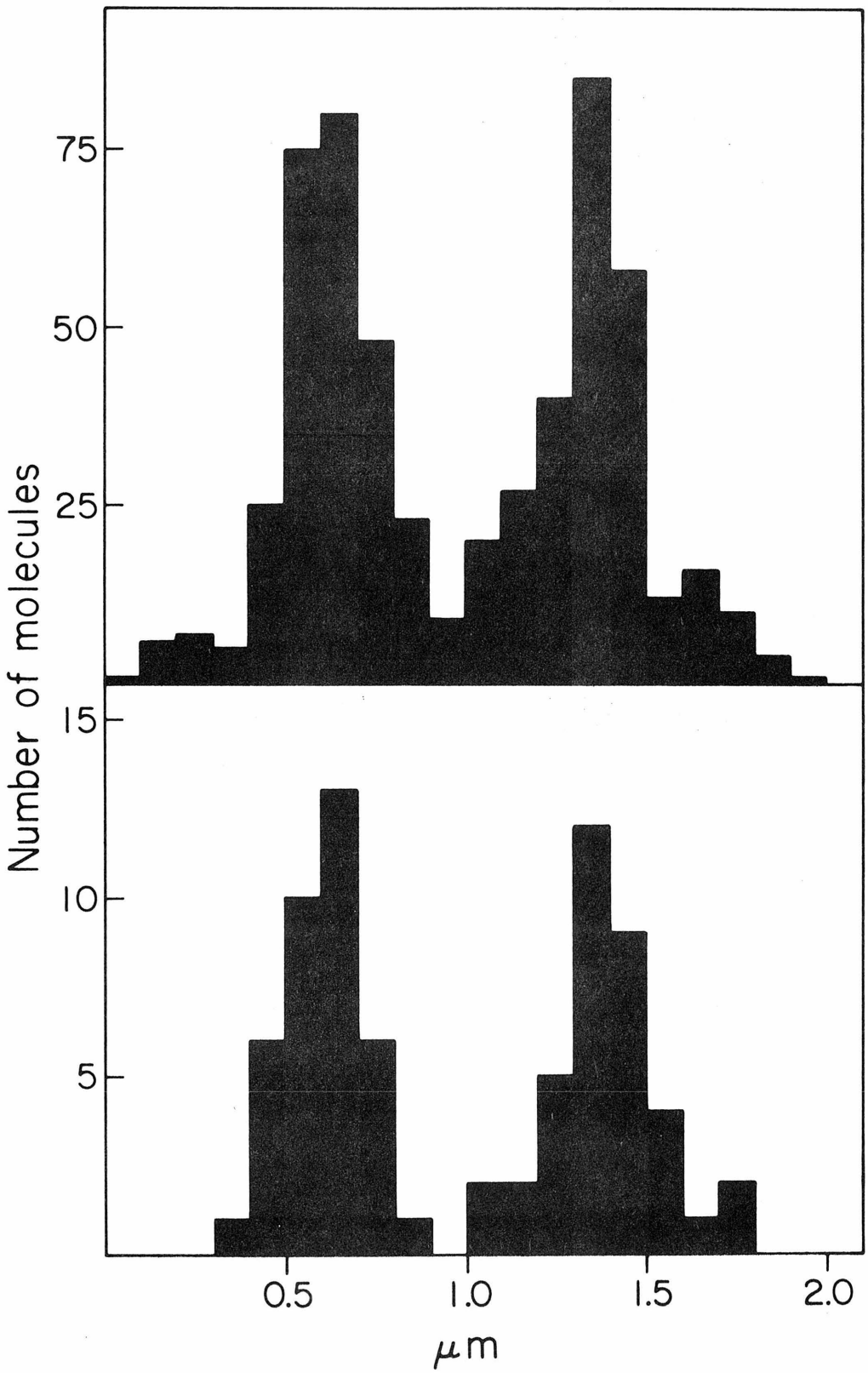

75
50
25
15
10
5
Number of molecules
0.5
1.0
1.5
2.0
μm

TABLE 2

Length measurements of hybrids between rDNA with rRNA species and rRNA precursors

		L_N	L_W	T_N	T_W	$\frac{L_N 28}{L_N 18}$	L_W Å/base
28 S		1.37	1.39	0.18	0.18		2.80
						2.2	
18 S		0.62	0.65	0.13	0.14		3.00
45 S:							
	28 S	1.38	1.41	0.15	0.16		2.84
						2.3	
	18 S	0.60	0.63	0.11	0.13		2.91

L_N = number average length (Tanford, 1963).

L_W = weight average length.

T_N = standard deviation based on L_N.

T_W = standard deviation based on L_W.

species, the number-average and weight-average lengths are about the same, and, in all cases, the standard deviation is between 10 and 20% of the average length of the molecules. Table 2 shows that the ratio of the number-average lengths of 28 S and 18 S RNA, measured by electron microscopy, is 2.2, which is close to the value of 2.3 calculated, from previously reported values, for the $O.D._{260}$ ratio of the two RNA components in polysomes (Attardi et al., 1966).

Plate 1(c) shows selected hybrids of the rRNA precursor with rDNA, spread by the aqueous technique. Only those molecules which, on visual examination, appeared to have a double-stranded structure over a total length roughly corresponding to the sum of a 28 S and 18 S region were scored. Such molecules, which all exhibited two distinct duplex regions of the expected length for the 28 S and 18 S segments, separated by an internal single-stranded region appearing as a bush, represented about 20% of the total molecules present in the fields examined. The hybrids which were not scored contained either one or both duplex segments shorter than those present in the selected hybrids, and presumably represented hybrids involving broken rRNA precursors. This breakage probably occurred during the incubation of the RNA at elevated temperature. The histogram in Figure 4b shows the length distributions for the duplex regions of the selected hybrids. There exists a bimodal distribution for the duplexes which is virtually identical to that observed for the mixture of 18 S and 28 S RNAs. The average lengths of the longer and shorter duplex segments of the ribosomal precursor are 1.38 and 0.60 μm, respectively, their ratio being 2.3, very close to that observed for the hybrid with the individual rRNA components. The mammalian 45 S rRNA precursor contains a non-conserved segment at the free end of the 18 S region (Wellauer &

Dawid, 1973). Any such segment would have appeared in the hybrids examined here as a bush. On the contrary, no clear bush was observed at the free terminus of the shorter duplex region in the 37 hybrid molecules scored. Although many hybrids showed at this end a knob or branched expansion, which could conceivably be a small bush, the lack of reproducibility of its occurrence, its similarity to the cytochrome c grains, and its frequent presence also at the other end of the hybrids of rDNA with 18 S or 28 S RNA (Plate 1a&b) make it impossible to assign with any confidence a single-stranded region at this end of the hybrid. The failure to detect this single-stranded region might conceivably be due to the relatively small number of hybrids examined, if the majority of the molecules had lost this segment during either the isolation of the rRNA precursor or the preparation of the hybrids for electron microscopy. Therefore, we expanded our observations to include any molecule which contained an internal or terminal large bush and at least one intact duplex region roughly equal in length to the 18 S region. In this manner we scored 153 additional molecules representing hybrids between rDNA and broken rRNA precursors: in no case was a second unambiguous bush observed.

4. DISCUSSION

The first part of this report describes the purification from duck erythrocytes of rDNA sequences suitable for use in the electron microscopic mapping of the rRNA precursor. By repeated fractionation of the total DNA, based on the relative reassociation rates of the DNA sequences with different degrees of repetition, a fraction of the rapidly renaturing DNA was obtained which comprised only 6% of the total DNA, but contained

about 70% of the rRNA cistrons. Further purification of the rDNA was achieved by hybridization with rRNA under saturating conditions and separation of the rRNA-rDNA hybrids from the remainder of the DNA by banding in CsCl. In this manner, an rDNA-rRNA fraction was obtained which had a buoyant density of 1.805 gm/cm^3, an RNA to DNA ratio of 1.01, and a base composition for the RNA present in the hybrid identical to that of an equimolar mixture of 18 S and 28 S rRNA. The latter observation indicates that the 18 S and 28 S rDNA was also purified in approximately equimolar amounts, and that no selection for one or the other DNA sequence occurred during isolation. The final yield of the rDNA isolated by the procedure described above was 32%, assuming a value of 4×10^{-4} for the fraction of the erythrocyte DNA complementary to rRNA (Bishop et al., 1972).

When the purified rDNA was annealed with a mixture of 18 S and 28 S rRNA, at a high DNA to RNA ratio, and the hybrids thus formed were spread for electron microscopy by the basic protein film technique in an aqueous medium, they appeared as duplex structures of relatively uniform thickness, which could be classified in two distinct populations with number-average lengths of 0.62 ± 0.13 μm and 1.37 ± 0.18 μm. Likewise, hybrids between the rRNA precursor and rDNA appeared as structures containing two duplex regions, with a length of 0.60 ± 0.11 μm and 1.38 ± 0.15 μm, separated from each other by a large single-stranded region appearing as a bush. The obvious interpretation is that these duplex regions correspond to the 18 S and 28 S RNA portions of the rRNA precursor molecule, while the internal bush is a portion of the precursor not conserved in the processing of the parent molecule.

It has been shown by secondary-structure mapping in the electron microscope that the 45 S rRNA precursor from mammalian cells contains two non-conserved portions, one situated between the 28 S and 18 S RNA regions, and other, slightly larger, at the 3' end of the molecule, contiguous to the 18 S region (Wellauer & Dawid, 1973). This observation is in agreement with the model proposed by Perry and Kelley for the rRNA precursor from mouse L cells (1972). In the present work, no single stranded region appearing as a bush was unambiguously identified at the 18 S RNA end of the 37, presumably intact, hybrids between duck rRNA precursor molecules and rDNA which were scored; nor was the presence of any such bush indicated by the analysis of an additional 153 molecules representing hybrids with fragments of the rRNA precursor. Although the possibility that the specific loss of the non-ribosomal portion at the 3' end of the rRNA precursor molecule occurred during the preparative procedures cannot be completely excluded, the extreme rapidity of RNA isolation, involving direct sodium dodecyl SO_4 lysis of the cells, and the lack of any indication of degradation in the sedimentation patterns argue strongly against it. Likewise, the possibility that the occurrance of larger size precursors may have escaped detection, both in the sedimentation analysis and in the electron microscope scoring of the hybrids, due to their low amount *in vivo* appears very unlikely. In fact, the cuts used to select the material from the sucrose gradient patterns were such as to include any possible component of 45-50 S size; furthermore, no single hybrid with a large size bush which could be unambiguously attributed to the 3' end of the rRNA precursor was observed among about 200 molecules scored.

The absence or marked reduction in size of the non-ribosomal segment at the 18 S RNA end of the duck rRNA precursor may in large part account for the

reported difference in size between the avian and mammalian ribosomal primary transcripts, based upon estimates of their respective molecular weights by polyacrylamide gel electrophoresis (Perry et al., 1970a,b; Grierson et al., 1970). Consistent with these observations is our finding that the duck rRNA precursor sediments under native conditions at 41-42 S, corresponding to a molecular weight of about 3.3×10^6 daltons (Wellauer & Dawid, 1973).

Apart from the absence or great reduction in size of the non-conserved portion at the 3' end of the molecule, the overall organization of the avian rRNA precursor closely resembles that of the mammalian 45 S rRNA (Wellauer & Dawid, 1973; Perry & Kelley, 1972). Since in the course of evolution the size of the rRNA precursor has increased (Perry et al., 1970a; Grierson et al., 1970), one can speculate that the addition of a non-conserved segment to the 3' end of the mammalian rRNA precursor may have resulted from the loss of the original terminator signal at the end of the 18 S sequence and from the inclusion of a portion of the spacer region in the precursor molecule.

Since a minimum of 15 and 33 DNA molecules of average size 150 nucleotides are required to completely cover the duck 18 S and 28 S RNAs, respectively, (assuming for these a molecular weight of 0.70 and 1.6×10^6 daltons, respectively, as for the chick components (Loening, 1968)), it is possible that there are in the RNA-DNA hybrids examined here short single stranded regions between adjacent DNA molecules, which are too small to be seen as bushes in the electron microscope. Since this would have the effect of shortening the observed lengths for the duplex molecules, the values obtained here for the size of the hybrids with the two rRNA species must be considered as minimum estimates of the lengths of the 18 S and 28 S RNAs. However, a comparison of the base to base distances calculated for these hybrid molecules, assuming

the above given molecular weights for the two rRNA species, (2.8-3.0 Å) with that reported for hybrids between mitochondrial 16 S rRNA and the mitochondrial DNA heavy strand spread under similar conditions (2.96 Å), or that reported for native DNA (3.27 Å) (Robberson et al., 1972), suggests that, under the conditions of hybrid formation used in the present experiments, the fraction of the length of the RNA chains remaining uncovered by the complementary DNA is very small.

The results reported in the present work have shown the suitability of the approach described here for studying the relationship between the mature rRNA components and their nuclear precursor, in particular, the topographical arrangement of the conserved and non-conserved portions in the parent molecule. This approach could be adapted for the analysis of the single stranded regions of the precursor by the formamide modification of the basic protein film technique (Wu et al., 1972). In light of the recent advances in the techniques for the isolation of repetitive DNA sequences and of the possibility of using reverse transcriptase to synthesize DNA complementary to purified RNA species, the present approach might be extended to the investigation of the precursor to product relationship of other classes of RNA molecules in eukaryotes. In particular, it might provide a critical test of the widely accepted but not rigorously proven hypothesis that the HnRNA is the precursor of mRNA.

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FOOTNOTE

[1]The expression "rRNA precursor" is used here to indicate the primary rRNA transcript.

ABBREVIATIONS

rRNA, ribosomal RNA; rDNA, DNA complementary to rRNA; HnRNA, heterogenous nuclear RNA; HAT, hydroxyapatite.