*HIGH MOLECULAR WEIGHT NONRIBOSOMAL-TYPE NUCLEAR RNA AND CYTOPLASMIC MESSENGER RNA IN HELA CELLS**

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As reported elsewhere,[1] the study of RNA synthesis *in vitro* in immature duck erythrocytes has revealed the occurrence in the nucleus of fast turning-over RNA of high molecular weight (from 2×10^6 to 10^7, or possibly more) and with base composition different from that of ribosomal RNA, and characterized by a high U and relatively low GC content. No relationship of precursor-to-product type—or, at any rate, not a simple one—appears to exist between this RNA and the messenger RNA fraction associated with cytoplasmic polysomes. In this paper, evidence is presented indicating that this class of nuclear RNA molecules is not exclusive of immature erythrocytes, or in general of nondividing cells undergoing differentiation, but occurs also in exponentially growing cells.

Materials and Methods.—(a) *Cells:* HeLa cells growing in suspension in a modified Eagle's medium[2] supplemented with 5% calf or horse serum were utilized throughout. The cultures used here were free of any detectable contamination with PPLO (Mycoplasma).

(b) *Labeling conditions:* Exponentially growing HeLa cells, at a concentration of $1–3 \times 10^5$ cells/ml, were exposed for various times to H^3-uridine (0.01 mM, 0.2–0.3 mc/μM) or C^{14}-uridine (0.01 mM, 5 μc/μM). In the experiments involving the use of P^{32}-orthophosphate, the cells were washed twice in phosphate-free Eagle's medium (with dialyzed serum) and then resuspended in the same medium, supplemented, in most cases, with 10^{-5} *M* phosphate; carrier-free P^{32}-orthophosphate was utilized at a concentration of 20–30 μc/ml.

Long-term labeling of HeLa cell protein was carried out by growing the cells for 48 hr in leucine- and lysine-free Eagle's medium (+5% dialyzed serum) supplemented with L-H^3-leucine (1.25 μc/ml; 16.7 μc/μM) and L-H^3-lysine (0.63 μc/ml; 8.3 μc/μM).

(c) *Isolation of polysomes:* The labeled cells to be utilized for polysome extraction were washed three times with 0.13 *M* NaCl, 0.005 *M* KCl, 0.0075 *M* $MgCl_2$, resuspended in 6 vol of 0.01 *M* tris buffer, pH 7.4, 0.01 *M* KCl, and 0.0015 *M* $MgCl_2$, left 5 min at 0–2°C, then homogenized with an A. H. Thomas homogenizer. The homogenate was centrifuged for 10 min at $600 \times g$ at 2°C to separate the nuclear from the cytoplasmic fraction; ribosomes and polysomes were isolated from the latter by a method previously described.[1]

(d) *Extraction and analysis of RNA:* Total RNA was routinely isolated by a procedure involving cold phenol-sodium dodecylsulfate extraction of total nucleic acids followed by digestion of DNA by RNase-free DNase.[1]

Conditions for RNA extraction from polysomes, sedimentation analysis of RNA, enzymatic tests, isotope determinations, and base composition analysis are described in detail elsewhere.[1]

Results.—(a) *Sedimentation pattern of rapidly labeled RNA after different times of exposure of the cells to a labeled RNA precursor:* After a short exposure (up to 30 min) Eo H^3- or C^{14}-uridine or P^{32}-orthophosphate, only a small proportion of the total radioactivity incorporated into RNA is found to be associated with the cytoplasmic fraction, under conditions of cell breakage which release into this fraction 50–70 per cent of the total RNA. This result confirms previous findings in this and other animal cell types,[3–6] indicating a nuclear localization of the earliest-labeled RNA components. An analysis of the sedimentation behavior of this fast-labeled RNA shows that, after a 5-min pulse, the labeled components are distributed in the region of the sucrose gradient corresponding to *S* values of about 10 to 100 or possibly more, with a greater accumulation in the 30–65*S* region (see, e.g., top graph of Fig. 4).

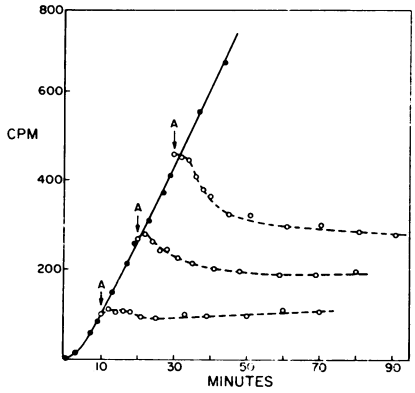

FIG. 1.—Kinetics of net incorporation of C^{14}-uridine by HeLa cells and decay of newly synthesized RNA in the presence of actinomycin D. C^{14}-uridine was added to four HeLa cell suspensions; after 10, 20, and 30 min incubation, actinomycin D was added (5 μg/ml) to each of three suspensions, while the fourth was kept as a control. At different times, aliquots were removed in duplicate from each suspension and precipitated with 5% TCA containing 200 μg/ml uridine, and the precipitates collected on Millipore filters for radioactivity determination.

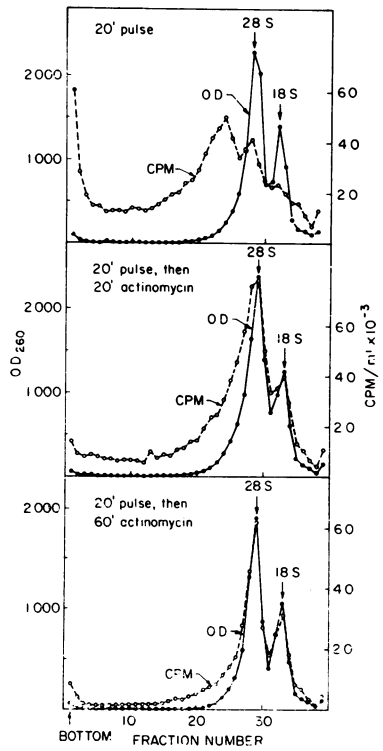

FIG. 2.—Effect of actinomycin D on the sedimentation pattern of total rapidly labeled RNA in HeLa cells. H^3-uridine was added to 900 ml of HeLa cell suspension. After 20 min, 300 ml of the culture were withdrawn, and actinomycin D was added to the remainder (5 μg/ml); 300-ml samples were taken 20 min and 60 min after addition of actinomycin. Total RNA was extracted from each sample and analyzed in a sucrose gradient.

After a 10- to 30-min pulse (see, e.g., top graph of Fig. 2 and middle graph of Fig. 4), a prominent 45*S* peak, corresponding, presumably, to the large-size ribosomal RNA precursor,[4, 5] is recognizable over a background of heterogeneous RNA; the small peak on the light side of the 45*S* peak in Figure 2 probably represents the 35*S* RNA species, described as an intermediate in the formation of 28*S* RNA.[5, 6] After the first hour's incubation, while the labeling of the 45*S* peak and of the heavier components in the gradient does not appreciably increase, there is a progressive accumulation of radioactivity in the two ribosomal RNA components. All the labeled material in the gradient is RNase-sensitive.

The radioactivity recovered in RNA after a 30-min H^3-uridine pulse accounts for 40–50 per cent of the total label incorporated by the cells into acid-precipitable, alkali-sensitive products. A hot phenol extraction[7] of the insoluble residue remaining after cold phenol extraction yields additional labeled material (15–20 %), forming a 45*S* peak with small amounts of heavier components.

(b) *Fate of rapidly labeled RNA:* The fate of the early-labeled RNA was investigated by blocking further RNA synthesis with the antibiotic actinomycin D. After addition of this drug, about 35 per cent of the total radioactivity incorporated during a 20- or 30-min exposure to C^{14}-uridine becomes acid-soluble on further incubation in the presence of the antibiotic, with a half life of about 10 min (Fig. 1), as already observed by Scherrer *et al.*[5] The decay after actinomycin is apparently

TABLE 1

NUCLEOTIDE COMPOSITION OF RAPIDLY LABELED NUCLEAR RNA IN HELA CELLS

Fraction	Expt. no.	P^{32} pulse (min)	Moles Per Cent A	C	U(T)	G	GC, %	G/A	C/U
*Ribosomal RNA**									
28S			16.5	31.6	17.4	34.6	66.2	2.10	1.82
18S			20.0	25.5	22.3	32.2	57.7	1.61	1.14
A. *Rapidly labeled nuclear RNA*									
>75S (I)	1	5	21.3	26.4	31.0	21.1	47.5	0.99	0.85
45–75S (II)		5	20.6	27.3	26.5	25.4	52.7	1.23	1.03
30–45S (III)		5	19.8	28.0	26.9	25.1	53.1	1.27	1.04
15–30S (IV)		5	22.2	27.9	30.2	19.5	47.4	0.88	0.92
>70S	2	15	22.1	25.9	29.5	22.3	48.2	1.01	0.88
30–55S		15	22.0	25.7	23.7	28.4	54.1	1.29	1.08
30–65S†	3	15	17.0	28.1	27.6	27.3	55.4	1.61	1.02
>100S (I)	1	30	23.2	23.5	30.5	22.5	46.0	0.97	0.77
84–100S (II)		30	23.4	25.5	29.5	21.4	46.9	0.91	0.86
72–84S (III)		30	23.1	24.7	28.2	23.8	48.5	1.03	0.87
63–72S (IV)		30	20.7	27.1	27.1	24.9	52.0	1.20	1.00
54–63S (V)		30	19.2	28.5	24.0	28.1	56.6	1.46	1.19
41–54S (VI)		30	17.6	30.9	22.2	29.1	60.0	1.65	1.39
32–41S (VII)		30	18.7	30.0	22.9	28.2	58.2	1.51	1.31
20–32S (VIII)		30	21.6	26.6	25.3	26.3	52.9	1.22	1.05
3–20S (IX)		30	22.8	25.3	27.9	23.8	49.1	1.04	0.91
B. *Fraction stable to actinomycin*‡									
>80S (I)		30	24.3	21.9	32.6	21.1	43.0	0.87	0.67
40–50S (II)		30	22.3	27.1	23.8	26.7	53.8	1.20	1.14
30S (III)		30	20.0	30.5	20.6	28.8	59.3	1.44	1.48
18S (IV)		30	24.5	25.2	25.8	24.2	49.4	0.98	0.98
DNA[11]			29.8	20.0	30.1	20.1	40.1	0.67	0.66

* Isolated from uniformly P^{32}-labeled cells (the data represent averages of two analyses).
† Residual fraction extracted with hot phenol (see text).
‡ Fraction of 30-min pulse-labeled RNA persisting after a 60-min actinomycin treatment. The RNA fractions analyzed in expt. 1 are portions of the sedimentation patterns shown in Fig. 4.

less pronounced (~15%) after a 10-min pulse: this may be due, at least in part, to the compensatory effect of the continuing incorporation of label into the terminal cytidylic acid residues in sRNA.[8] This labeling accounts also, presumably, for the slow rise in the incorporated radioactivity which follows the initial decay.

Figure 2 shows that after 60 min actinomycin treatment, the bulk of the heavy RNA components (>28S) labeled in a 20-min pulse have disappeared, and there has been a transfer of a part of the radioactivity to the 28S and 18S RNA.

(c) *P^{32}-nucleotide composition of fast-labeled nuclear RNA:* The "apparent" base composition of various fractions of P^{32}-labeled RNA was determined by measuring the P^{32} in the 2′,3′-nucleotides isolated by Dowex 1-X8 chromatography[9] after alkaline hydrolysis.[10] Almost all radioactivity was found to be associated with the four main 2′,3′-nucleotides. As appears in Table 1, the heaviest RNA components (>70S) have a base composition characterized by high U and relatively low GC content, which differs from that of DNA in that there is a lower A level. The components sedimenting in the region 30–50S, after relatively long pulses (30 min), have base ratios similar to those expected for ribosomal RNA precursors consisting of equimolar amounts of 28S and 18S sequences; after shorter pulses (5–15 min), the base composition of the 30–50S components resembles less the ribosomal type and tends to be closer to the high U type, the more so the shorter the exposure to the label. Finally, the labeled components in the intermediate portion of the sedimentation pattern (50–70S) and, less clearly, the slower-sedimenting material (3–30S) have a base composition which corresponds to that expected for

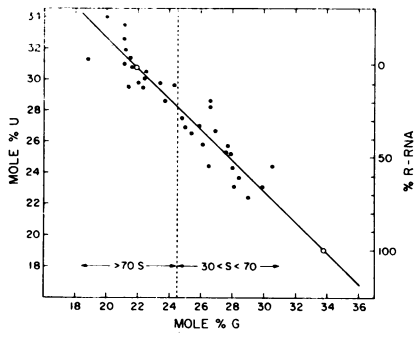

FIG. 3.—Plot of U versus G content of different fractions of rapidly labeled RNA separated by sucrose gradient centrifugation. Each point pertains to a different experiment. The upper open circle corresponds to an RNA with base composition equal to the calculated average for the heaviest components so far studied; the lower open circle, to an RNA with equimolar amounts of 28*S* and 18*S* sequences.

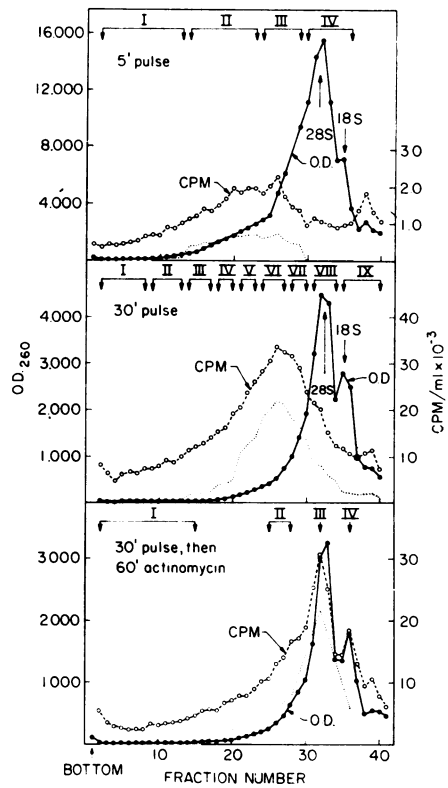

FIG. 4.—Identification of ribosomal-type components in the sedimentation pattern of total fast labeled RNA in HeLa cells. An HeLa cell suspension was exposed to P^{32}-orthophosphate for 5 min (*top graph*). Another suspension was labeled for 30 min: then one half was withdrawn, to the remainder actinomycin D was added (5 μg/ml), and incubation continued for 60 additional min. Total RNA was extracted from each sample and analyzed in a sucrose gradient.

In this experiment, the effect of actinomycin appears to be somewhat reduced or retarded, as compared to that observed in the experiments shown in Figs. 1 and 2, possibly due to the influence of the phosphate limitation in the medium on cell permeability to actinomycin or on RNA turnover rate. —O—O—, Total fast-labeled RNA; . . . . . , ribosomal-type fast-labeled RNA, as determined on the basis of the plot shown in Fig. 3 (see text).

mixtures consisting of high U-type and ribosomal-like RNA. The RNA isolated with hot phenol from the insoluble residue remaining after cold phenol extraction appears likewise to represent a mixture of the two RNA classes. The pattern of base ratios suggests that the various fractions of fast-labeled RNA consist of ribosomal-type (high GC) RNA and high U-type RNA, mixed in varying relative amounts in different portions of the sucrose gradient pattern and after different labeling times. If this is the case, a plot of the U content versus G content[12] of the individual RNA fractions should show a linear relationship, with parameters defined by the G and U values pertaining to the two RNA species. Figure 3 represents this kind of plot for the fast-labeled RNA components isolated from various portions of the sucrose gradient pattern (>30*S*), in many experiments carried out under different conditions of labeling. It appears that most points fall indeed relatively close to the expected theoretical line. The plot shown in Figure 3 pointed out a way for estimating, in each portion of the sucrose gradient pattern, the contribution of ribosomal-like RNA. The results of this type of analysis are shown in Figure 4. It appears that after a 5-min pulse, the majority of the labeled components belong

to the high U type, with a relatively small contribution of the ribosomal-like RNA in the 30–70*S* region. After a 30-min pulse, a prominent ribosomal-type peak can be seen sedimenting at 45*S;* this is undoubtedly the large-size ribosomal RNA precursor. After 60 min actinomycin treatment, the radioactivity incorporated in a 30-min pulse which pertains to the ribosomal RNA precursors is substantially conserved and transferred to the mature ribosomal RNA species (see also Table 1), whereas there is a marked reduction (~45%) of the heavier high U-containing RNA species. This suggests strongly that the fraction of 30-min pulse-labeled RNA labile after actinomycin treatment is in its majority high U-type RNA.

(d) *Significance of the high sedimentation constants of the heavy nonribosomal-type RNA components:* Several lines of evidence tend to exclude that the heavy high U-containing RNA components detected in the present study result from aggregation of smaller molecules by divalent cations, by residual DNA or protein, or by hydrogen bonds. Thus, the presence of 10^{-3} to 10^{-2} *M* EDTA during extraction or sedimentation analysis did not affect in the least their sedimentation properties. An aggregation by DNA seems unlikely in view of the fact that the RNA extraction procedure used here involved a DNase digestion step; in addition, a second treatment with DNase (10 μg/ml, 20 min, 25°C) of the heavy components (>60*S*), isolated by sucrose gradient centrifugation of total RNA, had no effect on their sedimentation behavior (Fig. 5), while making 99 per cent acid-soluble a tritiated DNA marker.

The introduction of three additional phenol extractions or the use, for deproteinization, of chloroform-phenol mixtures at room temperature[13] did not cause any change in the sedimentation profile of the total RNA.

In cells which had been labeled to a high specific activity with H^3-leucine and H^3-lysine, and then pulse-labeled with C^{14}-uridine, no labeled protein or amino acids were detected in association with the heaviest RNA components. From the expected specific activity in the cell protein of the labeled amino acids used, and from the theoretical maximum specific activity of uridine in the intracellular pool, it can be calculated that the presence of a polypeptide 12,000 in mol wt, containing 17 per cent of residues as leucine + lysine (as in the average HeLa cell protein[2]), per polynucleotide chain of 2.5×10^6 mol wt could have been detected here.

Sedimentation analysis of the heaviest labeled components ($S > 80$) in 0.01 *M* tris buffer, 0.001 *M* EDTA, pH 7.4, revealed a 30–40 per cent reduction in their sedimentation rate, in comparison to that observed in 0.10 *M* salt, as expected for single-stranded RNA. In order to examine whether the heaviest RNA molecules consist of shorter chains held together by hydrogen bonds, the effect of thermal denaturation on their sedimentation properties was investigated. RNA components sedimenting with $S > 80$ in 0.10 *M* salt were heated, in the presence of 28*S* RNA marker, in 0.10 *M* NaCl, 0.01 *M* Na citrate for 5 min at 80°C (which is well above the denaturation temperature in this solvent for an RNA of the same GC content as that found here[14, 15]), quickly cooled, and then rerun in sucrose gradient in the same buffer. After this treatment, their sedimentation rate appeared to be reduced with respect to the 28*S* marker; a broad band centered around 50*S* was observed, with components sedimenting as fast as 65*S* and possibly faster, which were represented, presumably, by continuous polynucleotide chains (Fig. 6).

(e) *Analysis of cytoplasmic messenger RNA fraction:* A preliminary investigation was carried out on the possible relationship between heavy high U-containing nu-

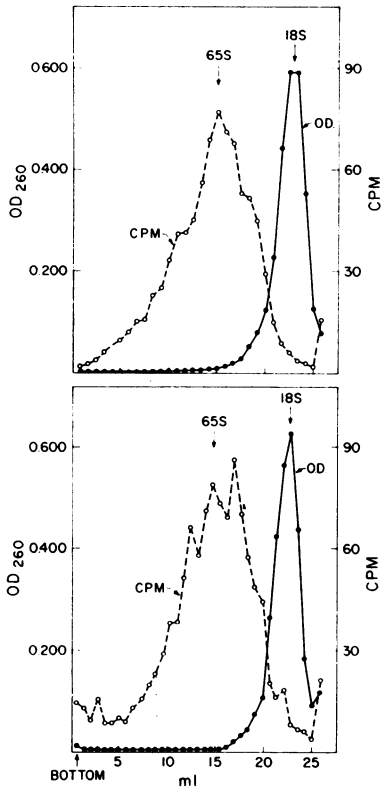

FIG. 5.—Sedimentation pattern of the heavy components of fast-labeled RNA after a second DNase treatment. Fast-labeled RNA components corresponding to $S > 60$ (in 0.10 M NaCl) were precipitated with 2 vol ethanol in the presence of carrier 18S RNA, centrifuged down, and dissolved in 2.0 ml tris buffer 0.05 M, pH 7.4, KCl 0.025 M, $MgCl_2$ 0.0025 M: one half of the solution was treated with RNase-free DNase (10 μg/ml, 25°C, 20 min) (*lower graph*); the other half was left as a control (*upper graph*). Both samples were then run on a 5–20% sucrose gradient in 0.15 M NaCl, 0.015 M Na citrate, 0.001 M EDTA, pH 7.0, for $3^1/_2$ hr at 24,000 rpm, 4°C.

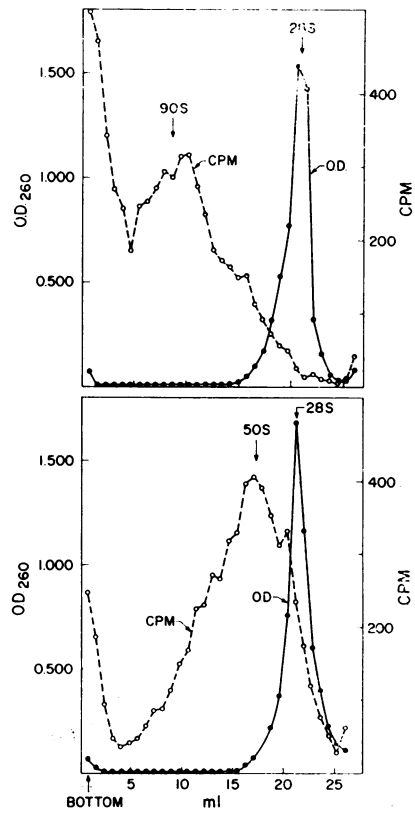

FIG. 6.—Effect of heating on the sedimentation properties of the heavy components of fast-labeled RNA. Fast-labeled RNA components corresponding to $S > 80$ and carrier 28S RNA were dissolved in 2.0 ml 0.10 M NaCl, 0.01 M Na citrate, pH 7.0: one half of the solution was heated at 80°C for 5 min and fast-cooled (*lower graph*); the other half was left as a control (*upper graph*). Both samples were then run on a 5–20% sucrose gradient in the same buffer as described above for $3^1/_2$ hr at 24,000 rpm, 4°C.

clear RNA and cytoplasmic messenger RNA. The ribosome-polysome fraction was isolated from HeLa cells pulse-labeled with P^{32}-orthophosphate, and analyzed in a sucrose gradient. About 60 per cent of the ribosomal material in the gradient was found to sediment as a broad band corresponding to sedimentation constants from 130 to 450S. After a 20- to 30-min P^{32}-pulse, the RNA phenol-extracted from this polysomal band contained only a few per cent of the total radioactivity incorporated into RNA. Sedimentation analysis of polysomal RNA showed no or very little (less than 5%) label associated with the two ribosomal RNA components, the great majority of radioactivity being in the form of a heterogeneous RNA fraction, presumably of the messenger type, sedimenting between 6 and 40S, with

TABLE 2

NUCLEOTIDE COMPOSITION OF CYTOPLASMIC MESSENGER RNA IN HELA CELLS

Fraction	P^{32} pulse (min)	Moles Per Cent A	C	U	G	GC, %	U/A	A/G
Cytoplasmic messenger RNA								
6–40*S*	20	28.0	24.3	28.1	19.4	43.7	1.00	1.44
9–40*S*	20	27.2	24.5	28.3	19.9	44.4	1.04	1.37
6–35*S*	25	24.9	25.0	27.3	22.6	47.6	1.10	1.10
9–16*S*	30	25.7	26.9	28.2	19.1	46.0	1.10	1.35
22–40*S*	30	25.8	23.9	27.0	23.0	46.9	1.05	1.12
6–12*S*[16]	45	25.4	26.9	27.3	21.0	47.9	1.07	1.21
12–22*S*[16]	45	26.6	24.2	27.7	21.5	45.7	1.04	1.24
*Rapidly labeled nuclear RNA**								
>70*S* (1)	5	21.3	26.4	31.0	21.1	47.5	1.46	1.01
>70*S* (4)	15	22.3	25.1	30.7	21.7	46.8	1.38	1.03
>70*S* (5)	30	22.8	23.9	30.9	22.2	46.1	1.36	1.03

* The figure in parentheses indicates the number of analyses.

the bulk accumulated in the region 6–15*S*. As is shown in Table 2, the cytoplasmic messenger RNA isolated after 20- 25-, and 30-min pulses (this work), and after 45-min pulse (Penman *et al.*[16]), is not only clearly distinct from ribosomal RNA, but also reveals consistent differences in base ratios (especially U/A and A/G) from the heavy, high U-containing nuclear RNA.

Discussion.—The main result of this paper has been the demonstration of the occurrence in HeLa cells of a class of fast-labeled RNA molecules which had not been hitherto recognized in these cells. The fact that this heterogeneous, high U-containing RNA had not been found in the earlier studies by Scherrer *et al.*[5] may be due to their use of the hot phenol extraction procedure or to other technical differences. This class of RNA molecules has been found also in nondividing immature duck erythrocytes,[1] suggesting that it is of general occurrence in animal cells. A heavy (~50*S*) DNA-like RNA component has been reported in growing FL cells,[17] but it is not clear, because of the marked difference in the A content, whether it is related to that studied here. The same holds for the DNA-like RNA fractions described in other animal cells.[12, 18–20] The RNA detected in the present work appears to be confined to the nucleus and is characterized by a nonribosomal type of base ratios, with high U and relatively low GC content, and by a great heterogeneity in size, with estimated sedimentation constants ranging between about 10*S* and more than 100*S*. The inequality of A and U and of G and C and the dependence of the sedimentation rate on ionic strength indicate that these molecules are single-stranded. All the evidence accumulated in the present work along a variety of lines supports the conclusion that the high sedimentation constants of the bulk of these molecules are a reflection of their large size. By using Spirin's equation[14] for conversion of *S* values to mol wt, the heaviest nonribosomal-type RNA molecules detected here after thermal denaturation (50–65*S*) can be estimated to have a mol wt as high as 6×10^6 to 10^7. Whether the more rapidly sedimenting components found in nonheated preparations ($S > 70$) are aggregates, or represent, on the contrary, even larger molecules, which can be dissociated by heating into shorter chains, because of the existence of hidden breaks, remains to be established.

After actinomycin treatment, an appreciable fraction of the rapidly labeled RNA is degraded to acid-soluble products, as previously reported by others,[5, 21] and the evidence obtained in this work suggests that a major portion, if not all, of this labile fraction is represented by the high U-containing heavy nuclear RNA.

In duck erythrocytes maintained *in vitro*, the lability of this type of RNA could be demonstrated under conditions which did not involve the use of actinomycin, a drug for which various side effects have been described. It is therefore likely that, also in HeLa cells, the decay occurring after actinomycin treatment reflects a physiological turnover of a labile fraction. In agreement with this conclusion is the observation that the high U-containing nuclear RNA does not accumulate in the cell. Quantitatively, it appears that the synthesis of this type of RNA represents a major portion of the total RNA synthesis in both growing and nongrowing cells.

The difference in size and base composition detected in the present work between this RNA and the cytoplasmic messenger RNA and the reported observations concerning the half life of HeLa cell polysomes,[16] which appears to be considerably longer than that of the heavy nuclear RNA, suggest that there is no relationship, or at least no simple relationship of precursor-to-product type, between the two RNA classes. More work will be necessary to exclude any role of high U-containing nuclear RNA in transfer of information to cytoplasm, and to test the alternative possibilities that it may be involved in some nuclear messenger activity or in some other function not directly connected with protein synthesis.

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