

# THE TURNOVER OF NUCLEAR DNA-LIKE RNA IN HELA CELLS

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## ABSTRACT

The subcellular distribution of various types of RNA in HeLa cells is described. In addition, the relative rate of synthesis of the major classes of nuclear RNA has been determined. From these experiments it can be deduced that the heterogeneous nuclear RNA fraction is rapidly synthesized and degraded within the cell nucleus.

## INTRODUCTION

Two major types of RNA in animal cell nuclei have been identified by the rapid incorporation of isotopically labeled RNA precursors. One of these types is a monodisperse molecular species that is the precursor to ribosomal RNA (45S r-pre-RNA) (1-5). Many laboratories have now demonstrated a second class of polydisperse nuclear RNA which is comprised of molecules which sediment from 20S-100S (6-11). The base composition of this second type of nuclear RNA, which we have called HnRNA (heterogeneous nuclear RNA) (8), is similar to that of cellular DNA (42-47% G + C) and is very different from that of rRNA (over 60% G + C). In duck erythroblasts (6, 7), where ribosome synthesis proceeds slowly if at all, it has been possible to show that most of the HnRNA does not enter the cytoplasm. Thus in these cells, since HnRNA is rapidly labeled but does not contribute to cytoplasmic RNA, it must be rapidly synthesized and degraded, i.e. "turning over" (6, 7). Experiments with growing cultures also have suggested that HnRNA is turning over, but because of the rapid synthesis of ribosomal RNA in growing cells, this point was not definitely settled (8, 11). The present experi-

ments were therefore designed to investigate further the question of HnRNA turnover in growing HeLa cells. From the results, it appears that at least 90% of the HnRNA of HeLa cells turns over in the cell nucleus.

## MATERIALS AND METHODS

### *Cell Culture and Labeling Techniques*

Suspension cultures of HeLa cells were grown in Eagle's spinner medium supplemented with 7% horse serum (12). Labeling of RNA was accomplished by centrifuging the cells and resuspending them in one of the following: (a) phosphate-free medium containing dialyzed serum in which the cells were washed three times, and then resuspended at a normal concentration in the same medium to which 100  $\mu\text{C}/\text{ml}$  carrier-free orthophosphate- $^{32}\text{P}$  was added for various times (incubation in this low phosphate medium was restricted to 40 min or less); (b) an equal volume of medium with normal phosphate content, supplemented with normal serum and 1  $\mu\text{C}/\text{ml}$  carrier-free  $^{32}\text{PO}_4$ , in which incubation was carried out for four generations; or (c) fresh complete medium containing uridine- $^{14}\text{C}$  (0.02 mM, 5  $\mu\text{C}/\text{mmole}$ ) in which cells were also grown for four generations.

TABLE I  
Cellular Distribution of HeLa Cell RNA

			Total Cellular RNA					
			Uridine- <sup>14</sup> C		<sup>32</sup> P		Orcinol	
			%	%	%	%	%	%
Cytoplasm	Ribosomal RNA	28S	53					
		18S	24					
	Transfer RNA	14.2						
	mRNA	≈3.0						
	Total		94.2		95		95	
Nucleus	Nuclear pellet (nucleolus)		3.5		3.4		3.7	
	Nuclear supernate		2.3		1.6		1.3	
	Total		5.8		5.0		5.0	

RNA was extracted from cells which had been labeled for four generations with either uridine-<sup>14</sup>C or orthophosphate-<sup>32</sup>P and subjected to zonal sedimentation for analysis of amounts of various types of RNA. \* Transfer RNA includes not only tRNA but also 5S and 7S RNA which exist in ribosomes in the cell (33, 34).

† mRNA was estimated indirectly as described in the text.

### RNA Isolation

In experiments with brief <sup>32</sup>P labels, detergent-cleaned nuclei (5, 13) were isolated and then lysed by exposure to cold (4°C) high-salt buffer (0.5 M NaCl, 0.05 M Mg<sup>++</sup>, and 0.01 M tris pH 7.4) containing DNase (50 γ/ml, electrophoretically purified, Worthington Corporation, Harrison, N. J.) (13). The total nuclear RNA was isolated by hot-phenol extraction (60°) of the nuclear lysate after the addition of EDTA to 0.05 M, acetate buffer, pH 5.1, to 0.05 M, and SDS (sodium dodecyl sulfate) to 1%. Nuclear lysates from cells labeled for 24–48 hr were subfractionated into nuclear supernatant and nucleolar fractions, as has been described by Penman et al. (13).

### Analysis of RNA

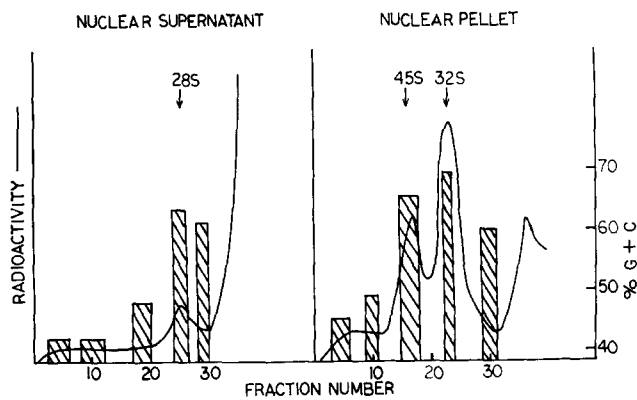
Uridine-<sup>14</sup>C-labeled RNA was analyzed by sucrose-gradient zonal sedimentation (4). <sup>32</sup>P-labeled RNA was fractionated for base composition analysis by zonal sedimentation followed by precipitation from appropriate fractions by cold 2.5% perchloric acid along with 1 mg carrier yeast RNA; the precipitates were rinsed with ethanol and hydrolyzed with 0.3 N KOH at 37° for 18 hr. The resulting 2'-3'-nucleotides were separated by electrophoresis for determination of the base composition (14). Total RNA in various samples was measured by the orcinol technique (15).

## RESULTS

### Steady-State Distribution of RNA

The cell fractionation techniques which have been developed by Penman and collaborators

(13) allow the fractionation of HeLa cells into nuclear and cytoplasmic fractions with little cross-contamination. We therefore wished to take advantage of these techniques to measure the steady-state distribution of the various classes of RNA in the nucleus and the cytoplasm of the HeLa cell and to relate these measurements to the apparent rate of synthesis of nuclear components. The distribution of labeled RNA in various cellular compartments of cells labeled for four generations with uridine-<sup>14</sup>C or <sup>32</sup>PO<sub>4</sub> is shown in Table I, and the sedimentation diagrams of <sup>32</sup>P-labeled nuclear RNA are shown in Fig. 1. Although various species of RNA may be partially separated by sucrose-density gradient sedimentation, an accurate assessment of the relative contents of two species which overlap in zonal sedimentation is not possible. Such is the case with HnRNA and r-pre-RNA in the nucleus (see Fig. 1, nuclear pellet fractions 15–30). However, the relative amount of HnRNA and r-pre-RNA in mixtures can be estimated by another method. Highly purified r-pre-RNA has a base composition of 70% G + C (16, 17, 18, and Soeiro, R. Unpublished observations), whereas the base composition of HnRNA is about 44% G + C (8). Thus, base ratio determinations of nuclear RNA samples should reveal the relative proportions of the two major species present. For example, Fig. 1 shows that the total RNA in the 45S region has a composition of 66% G + C, which would represent a mixture of 7 parts r-pre-RNA (70% G + C)



C	21.2	22.3	24.1	30.4	30.6	21.9	23.3	28.2	32.8	29.2
A	27.5	26.2	24.4	17.4	20.5	25.6	24.3	15.1	14.9	19.1
G	21.3	21.9	23.9	33.0	31.0	24.1	26.0	37.2	36.0	31.8
U	29.6	29.5	27.4	18.1	19.0	30.4	26.7	19.5	16.2	19.8

FIGURE 1 Equilibrium labeling of HeLa nuclear RNA with  $^{32}\text{P}$ . Cells were sedimented, resuspended, and grown in normal medium containing  $^{32}\text{P}$  for four generations (see Materials and Methods). RNA was isolated from fractionated nuclei and analyzed by sucrose-gradient centrifugation. Appropriate fractions from the gradient were pooled, and the RNA was hydrolyzed and subjected to base ratio analysis (14) which is shown at the bottom of the figure.

and 1 part HnRNA (44% G + C). This technique has been applied later in this paper (see Fig. 2 and Table II) to assess the distributions of HnRNA and r-pre-RNA in samples of nuclear RNA.

An indirect method must be used to obtain an estimate of the quantity of mRNA in the cytoplasmic polyribosomes. Four previously determined experimental facts allow an estimation of the fraction of the total cellular RNA which is represented by mRNA in the cytoplasmic poly-

ribosomes. These are: (a) 77% of the cellular RNA is in cytoplasmic ribosomes (see Table I); (b) 85% of the ribosomes are in polysomes (19); (c) the average polysome has six ribosomes (20); and (d) the average messenger RNA molecule has a molecular weight of about  $6 \times 10^5$  (19, 21).

Since the RNA of a ribosome has a molecular weight of  $2.2 \times 10^6$  (22), the maximum amount of the total RNA that is mRNA is  $(0.77 \times 0.85 \times 6 \times 10^5) / (6 \times 2.2 \times 10^6) = 2.9\%$

#### Relative Rates of Synthesis of RNA Species

Thus in the simplest situation where both 28S and 18S rRNA and mRNA are the only cellular RNA species and are equally stable, the synthesis of rRNA should exceed mRNA about 30/1. The situation is not simple, however, and in order to evaluate the possible role of nuclear molecules as precursors to cytoplasmic molecules the following considerations must be made.

(a) Mature ribosomal RNA appears to be stable in growing cultures, at least for a period equivalent to several generations (1, 23). In contrast, the r-pre-RNA in HeLa cells is not all stable (17, 18, 24, 25). The molecular weight of the 45S r-pre-RNA is about  $4-4.5 \times 10^6$  (24 and McConkey, E. H. and J. Hopkins. In preparation.), about half of which is degraded during formation of a 28S ( $1.7 \times 10^6$ ) and an 18S ( $0.6 \times 10^6$ ) molecule.

(b) Cytoplasmic mRNA appears to be unstable, turning over with a half-life of at least 3-4

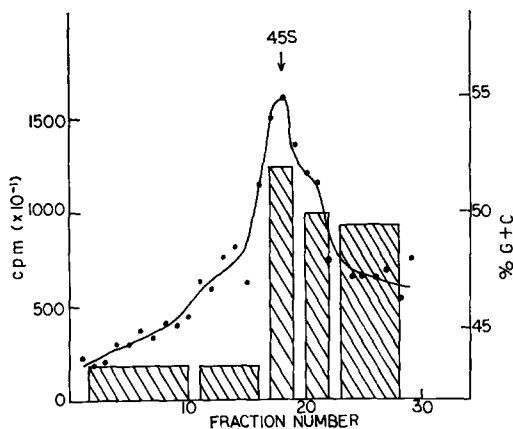


FIGURE 2 Pulse labeling of HeLa nuclear RNA with  $^{32}\text{P}$ . Cells were labeled with  $^{32}\text{P}$  in low phosphate medium for 10 min. RNA was isolated from purified nuclei and analyzed on a sucrose gradient. Appropriate fractions from the gradient were pooled, and the RNA was hydrolyzed and subjected to base ratio analysis (14). Results of this analysis are shown in Table II.

TABLE II  
*Estimation of HnRNA and 45S r-pre-RNA Content of Rapidly Labeled Nuclear RNA*

Lable time	Regions of sucrose gradient	<sup>32</sup> P recovered					Total	HnRNA*	45s
		C	A	G	U	G + C			r-pre-RNA*
			%	%	%	%		cmp‡	cmp‡
10 min	1-10	23.4	23.2	19.9	32.5	43.3	633	633	—
	11-16	23.1	22.8	19.9	32.6	43.0	925	925	—
	17-19	27.3	19.2	24.1	28.2	51.4	930	620	307
	20-22	25.1	19.7	24.5	29.0	49.6	634	444	190
	23-29	27.1	22.4	22.0	24.1	49.4	799	600	200
	Pellet						152	152	—
Total							4374	697	
20 min	1-10	23.6	24.6	17.1	34.1	40.7	2109	2109	—
	11-17	25.6	23.8	18.0	33.0	43.6	3808	3808	—
	18-19	28.2	19.1	25.4	26.7	53.6	2393	1355	1080
	20-21	26.4	19.7	26.3	27.6	52.7	1829	1220	609
	22-24	26.7	21.4	21.7	29.6	48.4	1730	1440	290
	25-29	30.2	22.9	23.7	23.0	53.9	1574	890	685
	Pellet						608	608	—
Total							11,430	2664	

Data derived from experiment shown in Fig. 2.

\* Calculations of fraction of total cpm as HnRNA and 45S r-pre-RNA is described in the text.

‡ One-fiftieth of the fractions from the sucrose gradients was assayed for acid-precipitable radioactivity.

hr (26). Thus it is clear that the relative rate of synthesis of mRNA to rRNA must be greater than their mass ratios, expressed as  $m/r$ . In an earlier publication, the following equation was derived (8):

$$\frac{\text{rate of synthesis of mRNA}}{\text{rate of synthesis of stable rRNA}} = \frac{m}{r} (1 + T/Tm) \quad (1)$$

where  $T$  is the doubling time of the cells and  $Tm$  the half-life of mRNA. Since about  $1/2$  of the 45S r-pre-RNA is lost during maturation (16-18, 24, 25), the equation should be written

$$\frac{\text{rate of synthesis of mRNA}}{\text{rate of synthesis of r-pre-RNA}} = \frac{1}{2} \frac{m}{r} (1 + T/Tm) \quad (2)$$

Substituting  $m/r = 0.03$ ,  $T = 24$  hr, and  $Tm = 3$  hr, we can calculate the expected rate of synthesis of mRNA or any precursor to mRNA relative to

the rate of synthesis of r-pre-RNA by

$$\frac{dm}{dr} = \frac{1}{2} 0.03 \left( 1 + \frac{24}{3} \right) = 0.14$$

Therefore, if most of the HnRNA serves as precursor to cytoplasmic mRNA, it should be synthesized only about  $1/7$  as fast as r-pre-RNA.

By the analysis of RNA taken from cells which were briefly exposed to <sup>32</sup>P, the differential synthesis of r-pre-RNA and HnRNA in the nucleus can be measured. The data of Fig. 2 and Table II show that the rate of synthesis of HnRNA is more than four times the rate of synthesis of r-pre-RNA. If this value is compared with that obtained from equation 2, it is clear that the rate of synthesis of HnRNA far exceeds the rate of synthesis of any direct mRNA precursor. Thus very little, if any, of the HnRNA ever becomes mRNA, but rather the HnRNA must turn over in the nucleus.

The HnRNA turnover is also indicated by another type of analysis. If attention is confined to the 45S region of the sucrose gradient where some HnRNA sediments along with the 45S r-pre-RNA, it is found (Figs. 2, 3, and Table II) that the RNA of the 45S region has a base com-

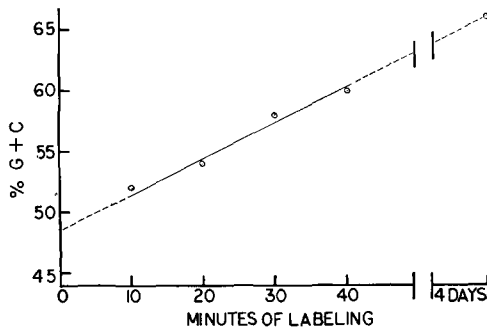


FIGURE 3 Base ratio of "45S region."  $^{32}\text{P}$ -labeled nuclear RNA was prepared, as in Fig. 2, from cells labeled for various periods of time. The 45S region was isolated, and base analysis was performed; results are plotted here as percent guanylic cytidylic + acid (% G + C) as function of length of label.

position of 51.4% G + C at 10' which rises to 53.6% by 20'. If the RNA from this region is analyzed after equilibrium labeling with  $^{32}\text{P}$ , the base composition is found to rise as high as 66% (see Fig. 1). As previously pointed out, a base composition of 66% G + C would arise from a mixture of 7 parts 45S r-pre-RNA and 1 part HnRNA. Thus the HnRNA, because it is rapidly synthesized and degraded, contributes a large fraction of the total radioactive RNA in the 45S region after short labeling times. The 45S r-pre-RNA, which is actually the predominant "45S" molecule in terms of mass, contributes proportionately more label with time. Therefore the average turnover time of the HnRNA must be faster than the processing of the 45S r-pre-RNA.

#### Rate of Turnover of HnRNA

Since the large amount of HnRNA synthesized does not accumulate, it must be rapidly degraded. In order to estimate the average rate at which the HnRNA turns over, equation 2 was rearranged and values for HnRNA were used rather than values for mRNA. The half-life of HnRNA can then be computed:

$$T_h = \frac{T}{2 \frac{r}{h} \frac{dh}{dr} - 1}, \quad (4)$$

$$\text{or} \quad T_h \approx \frac{T}{2} \frac{h}{r} \frac{dr}{dh} \quad (5)$$

$$T_h = \frac{24}{2} \times 0.017 \times 0.25 = 0.05 \text{ hr} \approx 3 \text{ min}$$

It should be emphasized that this turnover time is an average value for all the HnRNA. It is possible that the HnRNA is a collection of molecules with a spectrum of turnover times. For example, let us consider an extreme case where half the molecules turned over every few hours and the remaining half turned over every 1.5 min. The molecules with the longer turnover time would represent relatively little of the total radioactivity in brief labels and more radioactivity as the label approached equilibrium. The average turnover time would still be 3 min.

The time required for the synthesis of individual chains of RNA has been estimated for two types of RNA in animal cells. 45S r-pre-RNA requires about 2 min for synthesis (27), whereas poliovirus RNA requires about 1 min (28). Since the HnRNA molecule is larger than either of these molecules, it may require longer than 2 min to be synthesized. If the average half-life is only 3 min, it is clear that many of the molecules exist for very short times.

One previous observation which conflicts with the conclusion of this work should be pointed out. It was noticed previously that briefly labeled cells contained an RNA fraction which decayed in the presence of actinomycin (1, 4). This decay represented, in large measure, the loss of HnRNA. The HnRNA was not lost in actinomycin, however, with a decay time of 3 min. It is possible therefore that the actinomycin stops the synthesis of some HnRNA chains before they leave the DNA template, which might slow the process of destruction of the HnRNA molecules. Alternatively, as suggested above, the HnRNA may have molecules with widely divergent turnover times. In any case, the apparent turnover of HnRNA in actinomycin-treated cells is slower than that in growing cells.

#### DISCUSSION

For some years the existence of a nuclear RNA fraction which failed to move to the cytoplasm has been a matter of speculation (29). The proof of such a fraction has been achieved in two quite different situations, (a) duck erythroblasts (6, 7) and (b) growing HeLa cells. The HnRNA turnover in duck erythroblasts is relatively easy to observe because no ribosomes are made in these cells. In growing cells or in tissues making ribosomes to replace those ribosomes lost by turnover, it is difficult to be certain that the turnover of HnRNA is taking place unless a careful analysis of

the proportions of various types of RNA in very brief labels is made. This type of analysis now confirms that HnRNA in growing HeLa cells is largely unstable; by implication, perhaps a great deal of the HnRNA which is known to exist in various mammalian cells is also unstable (30-32). At the present time we have no idea as to why so much RNA is transcribed only to be almost immediately degraded. Any future understanding of gene control in animal cells is likely to come only when the role of HnRNA is understood.

Two potential limitations of the measurements made in this paper should be recognized. First, we have made the assumption that both HnRNA and r-pre-RNA are synthesized from a single pool of nucleotide triphosphates. If separate pools exist, then the ratio of radioactivities taken up would not necessarily reflect the ratio of absolute rates of synthesis. Preliminary data, however, indicate that pyrimidines derived from an exogenous source (uridine) and an endogenous source (glutamine) flow into HnRNA and r-pre-

RNA in parallel, implying that the two species share a common pool (Soeiro, R., and J. E. Darnell. Unpublished data). Secondly, it is difficult to measure the uptake of  $^{32}\text{P}$  in RNA for times shorter than 10 min, the briefest exposure reported here. Since the average half-life of HnRNA appears to be only 3 min, we might be underestimating the relative rate of HnRNA synthesis. This is of little concern, however, since the major conclusion we wish to draw is that, even on the basis of the reported measurements, the rate of HnRNA synthesis greatly exceeds the rate of synthesis of a potential precursor to mRNA.

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