SIZE DISTRIBUTION OF POLY(A)-CONTAINING MESSENGER RNA FROM FREE POLYSOMES OF HELA CELLS

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SUMMARY. The sedimentation properties of pulse-labeled and long-term labeled mRNA from highly purified HeLa cell free-polysomes, selected for poly(A) content by two successive passages through poly(T)-cellulose columns, were 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 (1,2,3) 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 poly(A)-containing HeLa mRNA purified by poly(T)-cellulose chromatography (3,4,5). However, none of these analyses was carried out under denaturing conditions so as to exclude the possible aggregation of the RNA, nor were adequate criteria used to assess the purity of polysomes, especially with respect to hnRNA containing structures. This has prevented, so far, a reliable estimate of the largest size of the mRNA. This information is particularly important for an evaluation of the evidence relating mRNA to hnRNA. In the present work, the size distribution of pulse-labeled and long-term labeled poly(A)-containing mRNA from highly purified 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.

Abbreviations: mRNA, messenger RNA; hnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; RNP, ribonucleoprotein particle.

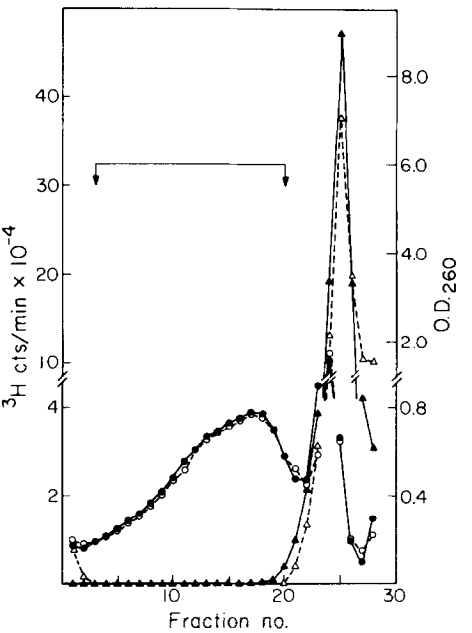

Figure 1. Sedimentation patterns in sucrose gradients of free polysomes from HeLa cells before and after disruption with EDTA. HeLa cells, at an initial concentration of 5 x 10^4/ml, were uniformly labeled for 48 hr with ^{3}H-uridine (7), the free cytoplasmic polysomes were isolated from the post-mitochondrial supernatant by centrifugation through a discontinuous sucrose gradient (6), 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 at 25° C), 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 15-30% sucrose gradient in a SW 25.1 Spinco rotor at 25,000 rev./min at 2° C (3). O---O, O.D.$_{260}$, Tris-K-Mg; Δ---Δ, O.D.$_{260}$, Tris-K-EDTA; O---O, ^{3}H cts/min, Tris-K-Mg; Δ---Δ, ^{3}H cts/min, Tris-K-EDTA.

MATERIALS AND METHODS

HeLa cell growth, labeling conditions, isolation of polysomes and purification of polysomal mRNA was carried out as previously described (3).

RESULTS AND DISCUSSION

A prerequisite for this analysis was the preparation of free polysomes not contaminated by heterogeneous RNAs. It has previously been shown that 20 min [5-^{3}H]uridine pulse-labeled free polysomes, when isolated by pelleting through a discontinuous sucrose gradient (6), are pure to the extent of more

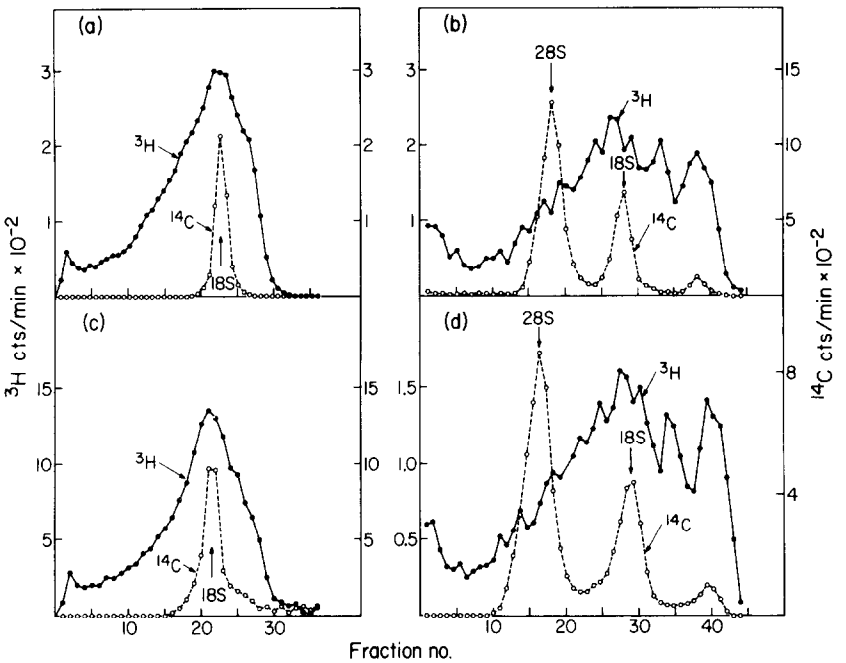

Figure 2. Sedimentation analysis of HeLa mRNA. (a), (c) RNA was extracted by the sodium dodecyl SO_4-pronase-phenol procedure (8) from 48 hr ^{3}H-uridine labeled free polysomes, isolated as described in the legend of Figure 1, and the mRNA purified by poly(T)-cellulose chromatography. (a) 0.1 µg of 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, in the presence of ^{14}C-uridine labeled HeLa 18 S rRNA marker, through a 15-30% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in the same buffer in a SW 27.1 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 (9). The RNA was centrifuged through a 5-20% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in 0.1 M-NaCl, 0.02 M-KPO_4 buffer, pH 7.4, 1% formaldehyde, in a SW 27.1 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).

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than 95%, as judged by the sensitivity to EDTA of both the UV absorbing and the radioactive material (3). Figure 1 shows a comparison of the sedimentation patterns of control and EDTA-treated free polysomes from 48 hr 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 (8), 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 min in the presence of 3% formaldehyde and then centrifuged in a formaldehyde-sucrose gradient, according to the method of Boedtker (9) (fig. 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 con/itions shown in figure 2a.

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 min with [5-^{3}H]uridine and for 48 hr with [2-^{14}C]uridine. The pulse-labeled mRNA exhibits approximately the same distribution as the 48 hr labeled mRNA. However, several discrete peaks or shoulders of radioactivity, which are reproducible from preparation to preparation, can be seen in the 25 min 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

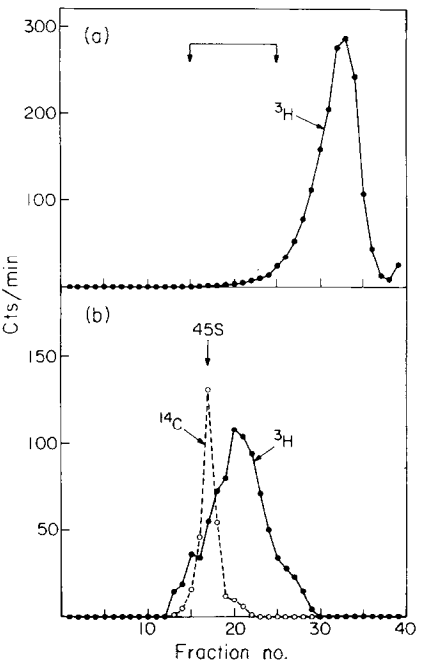

Figure 3. Sedimentation analysis of the fastest-sedimenting components of 48 hr [5-^{3}H]uridine labeled mRNA. (a) The mRNA was centrifuged through a 15-30% sucrose gradient in sodium dodecyl SO_4 buffer in a SW 27.1 Spinco rotor at 27,000 rev/min for 6 hr at 20° C. The fractions indicated by arrows 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 15-30% sucrose gradient in the same buffer in a SW 27.1 Spinco rotor at 27,000 rev/min for 7 hr at 20° C.

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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 x 10^6

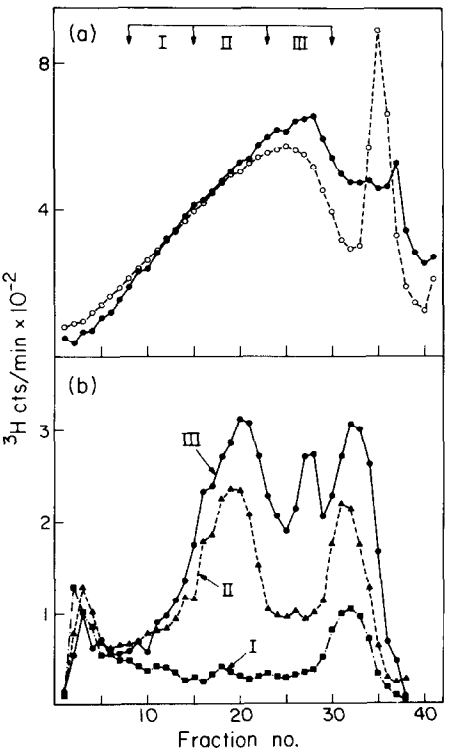

Figure 4. Size distribution of the 25 min [5-^{3}H]uridine labeled mRNA isolated from free polysomes of different size classes. (a) Free polysomes were isolated from 3 x 10^8 HeLa cells labeled for 25 min with ^{3}H-uridine, and centrifuged through a 15-30% sucrose gradient in TKM buffer in a SW 25.1 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. O, ^{3}H cts/min; O, O.D.$_{260}$.

(b) The RNA from each section was dissolved in 1 ml of sodium dodecyl SO_4 buffer and centrifuged through a 15-30% sucrose gradient (prepared over a 2 ml cushion of 64% sucrose) in the same buffer in a SW 27.1 Spinco rotor at 26,000 rev/min for 16 hr at 20° C.

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daltons (10). Although this centrifugation was not 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 min pulse-labeled HeLa cells were run through a sucrose

gradient and divided into three size classes (panel 4a); the RNA was extracted from each polysome cut by the sodium dodecyl SO_4-pronase-phenol procedure 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 histone mRNA. The presence of some large mRNA even in the lightest polysome cut(III) confirms earlier findings (11) 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.

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 preparation 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 (12). (3) The stability of the > 35S mRNA is identical to that found for the bulk of the mRNA (3) in both pulse-labeled and long-term labeled preparations (unpublished observation). In view of the much shorter half-life of the hnRNA (13), if significant contamination by this RNA class existed, the stability of the large mRNA fraction would be markedly reduced. (4) The hybridization behavior of this mRNA preparation with respect to

hybridization kinetics and pattern of RNase resistance of the RNA-DNA hybrids was distinct from that of hnRNA, and no evidence for contamination of the mRNA with either hnRNA or rRNA was detected (14). (5) Experiments involving electron microscopic visualization of isolated HeLa cell polysomes and direct counting of the number of ribosomes associated with individual polysomes have shown that HeLa polysomes contain an average of 20 ribosomes per polysome, and that polysomes containing as many as 125 ribosomes can be found (A. Bakken, personal communication). If one assumes that the average polysome contains 125 nucleotides of mRNA per ribosome [based on a molecular weight of hemoglobin mRNA of 2×10^5 daltons and 5 ribosomes per average hemoglobin polysome (15,16)], then the average molecular weight of HeLa mRNA calculated from the E.M. experiments is 8.3×10^5 daltons and the size of the largest mRNA is 5.2×10^6 daltons. These figures are in good agreement with our values derived by sedimentation analysis of the isolated mRNA.

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. It is interesting to note that a distribution of mRNA sizes similar to that determined here has been calculated from the size distribution of HeLa cell proteins, suggesting that most cytoplasmic polysomal mRNAs in HeLa cells are monocistronic (17).

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