SELECTIVE DETERMINATION OF mRNA SPECIFIC RADIOACTIVITY IN HELA CELLS WITHOUT THE USE OF INHIBITORS

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SUMMARY:

Polysomes from (^{3}H)-uridine pulse-labeled HeLa cells were isolated and the specific radioactivity of polysome-associated mRNA was determined by selective enzymic hydrolysis at 0°C of the interribosomal mRNA sections. Intraribosomal mRNA protected from hydrolysis during ribonuclease treatment and subsequently isolated by the proteinase K method (1) exhibited the same specific radioactivity as the interribosomal mRNA split products.

When labeled polysomes were subjected to ribonuclease treatment at 25°C instead of 0°C a higher specific radioactivity of the interribosomal split products resulted, while intraribosomal sections still exhibited the same values as after 0°C treatment. The labeled polysomes used as substrate exhibited one single A_{260} and radioactivity peak in CsCl density gradients. No RNP material banding at ρ = 1.35 - 1.45 could be detected. However, the radioactivity maximum banded at slightly lower densities than the A_{260} peak (ρ = 1.55 versus 1.57). The shift appears to be caused by a contaminant RNA. These findings as well as the radioactivity pattern of pulse-labeled polysomes in sucrose gradients may indicate the presence of newly synthesized mRNA associated with monosomes (and oligosomes) protected from ribonuclease action at 0°C by (transport ?) proteins.

Up to now, the kinetic analysis of (polysome-associated) mRNA formation has been hampered as no adequate methods were available. Actinomycin commonly used to measure mRNA formation or decay (cf. 2 - 4) is not reliable (5, 6). In a previous publication (7), therefore, a new approach to the determination of specific radioactivity of polysomal mRNA avoiding the use of inhibitors has been put forward. This paper describes additional experiments proving the validity of the method and demonstrating the absence of RNP particles sedimenting at ρ = 1.35 - 1.45 in the polysomal preparation.

MATERIAL AND METHODS

(^{3}H)-uridine (uridine-5-T; spec. act. 30 Ci/mmole) was purchased from the Radiochemical Centre, Amersham, England; modified Joklik medium (F-13) from Grand Island Biological Comp., USA; NP 40 was obtained from Shell Comp.; Pancreatic ribonuclease (chromatographically pure) was obtained from E. Merck (Darmstadt, Germany). Proteinase K (chromatographically pure) was a generous gift of Dr. H. Lang (E. Merck, Darmstadt).

HeLa S3 cells were grown in suspension culture as described previously (8).

Isolation of free polysomes from pulse-labeled cells: 1 mCi (^{3}H)-uridine was added to 1800 ml suspension culture (5 - 6 x 10^5 cells/ml) for 75 min. Cells were harvested by centrifugation and broken up at a concentration of 1 x 10^8/ml in 'cell buffer' containing 10^{-4} M cycloheximide and 0.5% NP-40 (1, 9) by 2 - 3 strokes in a Dounce homogenizer. Nuclei were sedimented at 700 g. The supernatant was layered on 5 ml 0.25 M sucrose in TKMC buffer (0.05 M Tris-HCl, pH = 7.4, 0.1 M KCl, 0.01 M $MgCl_2$, 0.1 mM cycloheximide) and centrifuged at 10 000 g for 20 min. Polysomes were isolated from the postmitochondrial supernatant by centrifugation through a discontinuous sucrose gradient, consisting of 8 ml 1.0 M sucrose and 8 ml 1.85 M sucrose in TKMC in a 60 Ti Spinco rotor for 120 min at 50 000 rpm. The polysomes were stored at -30°C. For further analysis, polysomes were suspended in 0.1 M sucrose in TKMC at a concentration of 10 - 50 A_{260} units/ml and centrifuged at 2 000 g to remove non-suspended material.

CsCl density gradient centrifugation: Polysomes were fixed in PMN buffer (0.01 M Na phosphate, pH = 7.4, 0.0015 $MgCl_2$, 0.01 M NaCl) with 2.7% neutralized formaldehyde for 24 hrs at 0°C according to conventional methods (10 - 12). Samples were layered on 6.5 ml of a CsCl solution (ρ = 1.5 g/ml, containing 2% neutralized formaldehyde and 0.5% brij 58). The gradients were run for 60 hrs in a SW 40 Spinco rotor at 35 000 rpm and 10°C. Fractions were collected, and aliquots were taken for density determination. 200 µl of each fraction were hydrolyzed with 500 µl 0.5 N KOH for 18 hrs at 25°C, 500 µl 8% $HClO_4$ were added, and A_{260} and cpm were determined in the supernatant after low-speed centrifugation.

Gel electrophoresis: Polyacrylamide gel electrophoresis was performed in an analytical apparatus according to conventional methods (13, 14). To analyze low molecular weight RNA, 50 - 500 µg RNA were applied to 12.5% polyacrylamide gels and run for 4.5 hrs at 5 mA/gel at 4°C.

For analytical determinations gels were stained with methylene blue according to Peacock (13). The migration distances of the RNA bands were measured, and the gels were cut into 1.5 mm slices. The slices were incubated in scintillation counter vials with 500 µl 30% H_2O_2 for 18 hrs at 50°C. Radioactivity was determined after addition of 15 ml

dioxane scintillation mixture (Scintimix III, E. Merck, Darmstadt).

For quantitative determinations the gels were cut into 1.5 mm slices immediately after electrophoresis and incubated with 1 ml 0.25 N KOH for 18 hrs at 25°C. After the addition of 500 µl 6% $HClO_4$ the samples were kept at 0°C for 4 hrs. Salt and gel slice were then centrifuged off, and A_{260} and cpm were determined in the supernatant. Two control gels (no RNA applied) to determine the A_{260} (and cpm) background were always analyzed in parallel. Cpm were corrected for quenching by the internal standard method.

Determination of specific radioactivity of polysome-associated mRNA:

a. Interribosomal strands: Polysomes (6 A_{260}-units/ml) were incubated at 0°C and at 25°C with pancreatic ribonuclease at a final concentration of 2×10^{-1} µg/ml and 10^{-2} µg/ml, respectively, for the times indicated. 500 µl of the incubation mixture were precipitated with 500 µl of 8% icecold $HClO_4$. After 10 min in ice samples were centrifuged. A_{260} and radioactivity were determined in the supernatant. All determinations were run in duplicate.

b. Intraribosomal strands: Polysomes (6 A_{260}-units/ml) were incubated with pancreatic ribonuclease as described above. Ribonuclease was immediately inactivated by incubation at 37°C with proteinase K (final conc. 500 µg/ml) (1). Further incubation for 60 min led to the digestion of ribosomal proteins. RNA was then precipitated by the addition of 2 volumes of abs. ethanol. Samples were allowed to stand at -30°C for at least 8 hrs. RNA was taken up in sterile water and separated on a sucrose gradient (5% to 20% sucrose in 0.01 M Tris-HCl, pH = 7.4, 0.1 M NaCl, 0.001 M EDTA) in a SW 27 Spinco rotor for 15 hrs at 27 000 rpm. Low molecular weight RNA (4 S region) was precipitated from the gradient with ethanol (2 vol) and analyzed by polyacrylamide gel electrophoresis.

RESULTS

1. Identical specific radioactivity of interribosomal and intraribosomal mRNA sections

When HeLa suspension cultures were labeled with (^{3}H)-uridine for 75 min and cells broken up in isotonic medium in the presence of a non-ionic (9) detergent, free polysomes could be isolated from the post-mitochondrial supernatant by centrifugation through 1.85 M sucrose. Low concentrations of pancreatic ribonuclease (2×10^{-1} µg/ml x 6 A_{260}-units) at 0°C selectively hydrolyzed interribosomal mRNA to acid-soluble split products. The selectivity of the enzymic degradation was indicated by two observations:

Conditions of RNase digestion	Interribosomal mRNA (dpm/A_{260} of split products)	Intraribosomal mRNA (dpm/A_{260} in gel fractions)
0°C		
0 - 10 min	114 000	-
10 - 20 min	116 000	-
20 - 40 min	117 000	-
0 - 20 min	115 000	114 500
25°C		
0 - 10 min	147 000	-
10 - 20 min	265 000	-
20 - 40 min	263 000	-
0 - 20 min	187 000	108 300

Table 1: Comparison of specific radioactivities of interribosomal and intraribosomal mRNA produced by ribonuclease treatment at 0° and at 25°.
Polysomes from HeLa suspension cultures were pulse-labeled with (^{3}H)-uridine for 75 min. During the first 20 min of ribonuclease treatment 0.022 ± 0.002 A_{260} and 2531 ± 150 dpm at 0°, and 0.026 ± 0.002 A_{260} and 4865 ± 200 dpm at 25° were rendered acid-soluble. Specific radioactivity of intraribosomal mRNA was determined as mean value from 10 fractions corresponding to fractions No. 37 - 47 in fig. 1b. Experimental details are described in methods.

The RNA rendered acid-soluble exhibited the same specific radioactivity throughout the incubation period (table 1, upper part) indicating hydrolysis of a single RNA species. Furthermore, these degradation products of the interribosomal mRNA had exactly the same specific radioactivity as the intraribosomal mRNA sections protected from ribonuclease digestion by the ribosomes. It seems very unlikely that eventual interfering RNA species should contribute to both mRNA sections (split products and ribosome-protected moieties) to the same degree. In these experiments the intraribosomal sections were isolated from ribonuclease-treated polysomes by separation of monosomes in a sucrose gradient, inactivation of ribonuclease and digestion of ribosomal proteins by the

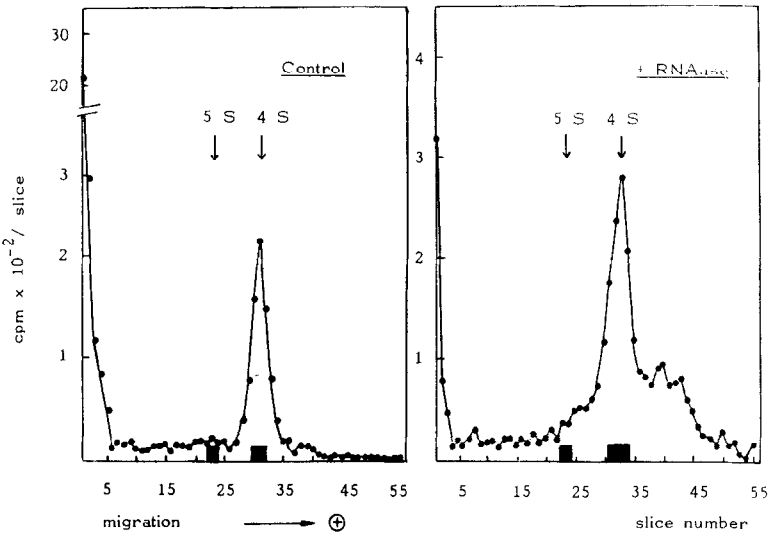
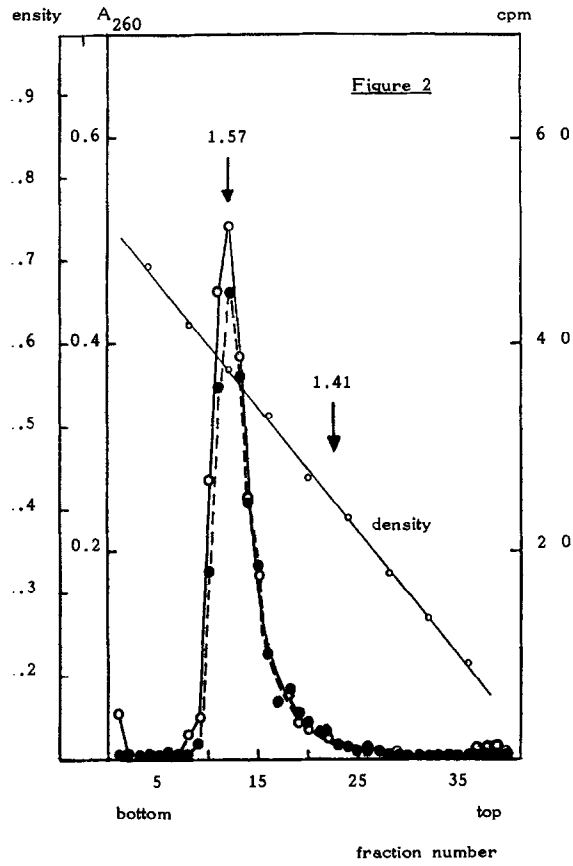


Figure 1: Analysis by polyacrylamide gel of protected mRNA chains produced by ribonuclease treatment of polysomes. - Isolated polysomes from pulse-labeled (60 min) HeLa cells were incubated with pancreatic ribonuclease as described in methods. 80 S monosomes were isolated by sucrose gradient recentrifugation. RNA was extracted by the proteinase K method (1) and analyzed in 12.5% polyacrylamide gels. RNA of untreated polysomes was run in parallel as a control. For further details see methods. - Solid blocks correspond to stained RNA bands.

proteinase K method (1), separation of low molecular weight RNA by sucrose gradient centrifugation, and electrophoretic analysis in 12.5% polyacrylamide gels (fig. 1). While control runs showed one single peak of radioactivity coinciding with tRNA, monosomes obtained from ribonuclease-treated (0°C) polysomes revealed additional, highly labeled, low molecular weight material with a minimum size of < 4 S. This accumulation of intraribosomal mRNA sections protected from enzymic digestion is paralleled by an almost quantitative conversion of polysomes to monosomes (not shown).

When polysomes were subjected to low ribonuclease treatment at 25°C, a distinctly different result was obtained (table 1, lower part). While RNA rendered acid-soluble in the first 10 min had a moderately higher specific radioactivity than mRNA hydrolyzed at 0°C, the split products at prolonged incubation showed a specific radioactivity more than twice



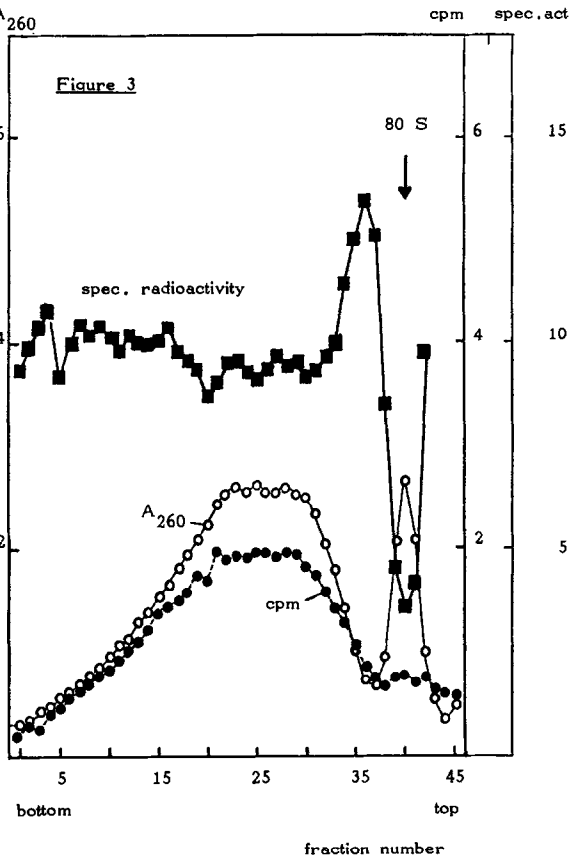


Figure 2: CsCl density gradient analysis of isolated polysomes. - Polysomes (2 A_{260}-units) from cells labeled for 75 min with (^{3}H)-uridine were isolated and analyzed as described in methods.
o—o A_{260}, ●- -● cpm x 10^{-2}/ fraction, o——o density (g/ml)

Figure 3: Sucrose gradient analysis of isolated polysomes. - Isolated polysomes (6 A_{260}-units) were analyzed on a linear sucrose gradient (0.3 - 1.3 M sucrose in TKMC buffer, SW 40 Spinco rotor, 90 min at 27.000 rpm and 4°C).
o—o A_{260}, ●- -● cpm x 10^{-2}/ fraction, ■- -■ spec. radioact. (cpm/A_{260}).

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as high as the acid-soluble material produced at 0°C. Intraribosomal mRNA, however, exhibited the same specific radioactivity independent of the incubation temperature.

These observations indicate the presence of a highly labeled RNA fraction in the polysomal preparation not identical with polysome-associated

mRNA. It does not interfere with the mRNA determination performed at 0°C, but will become sensitive to low ribonuclease concentrations at elevated temperatures.

2. Analysis of a contaminating highly labeled RNA fraction interfering with the mRNA determination at elevated temperatures only.

A likely contamination of polysomal preparations are RNP particles of nuclear or cytoplasmic origin (cf. 12) which could be resistant to ribonuclease at 0°C, but might become accessible at elevated temperatures. However, when polysomes prepared as described above were analyzed in a CsCl density gradient, no material banding in the region of ρ = 1.35 - 1.45 (fig. 2) could be detected. There is a significant amount of such material (< 60 S) in the postmitochondrial supernatant which can be separated by sucrose gradient centrifugation and banded by CsCl centrifugation at ρ = 1.41. However, the isolation of the polysomes by centrifugation through 1.85 M sucrose prevents contamination with these particles. Instead, a single peak of A_{260} absorbing material at ρ = 1.57 indicative of HeLa polysomes (11), and a single peak of radioactivity was found. It appeared, however, that the peak of radioactivity banded in a reproducible way at slightly lighter densities than the peak of the A_{260}-absorbing substances.

The contaminant of the polysomal preparation not identical with polysome-associated mRNA then should be "hidden" in the single peak. It could cause this slight shift of the peak radioactivity to lighter densities.

If this were true, ribonuclease treatment of polysomes at 0°C should affect the fraction 'left' of the A_{260} peak to a higher degree than the fractions to the 'right', while treatment at 25°C should affect the other (counter) fractions too. Such a difference could in fact be shown: Polysomes were treated with low ribonuclease concentrations at 0°C and at 25°C as described before, fixed with formaldehyde or glutardialdehyde, and analyzed in a CsCl density gradient. Mean specific radioactivities of three fractions 'left' ($\rho > 1.57$) of the A_{260} peak

Pooled fractions	Treatment with ribonuclease		
	none	0°	25°
	(mean specific radioactivities of 3 neighbor fractions)		
'left' of A_{260}-peak (ρ = 1.57 - 1.60)	6 400	3 950	4 080
'right' of A_{260}-peak (ρ = 1.53 - 1.57)	8 350	7 100	5 500

Table 2: Divergent disappearance of highly labeled RNA in polysomes by ribonuclease treatment at 0° vs. 25° (CsCl density gradients). -
Polysomes (2 A_{260}-units) from (^{3}H)-uridine-labeled cells (75 min) were incubated with ribonuclease at 0° or 25° for 20 min and analyzed as described in methods.

(ρ = 1.57), and of three fractions 'right' (ρ < 1.57) of the A_{260} peak were compared (table 2). It became apparent that, at 0°C, ribonuclease degraded material from both sides of the peak with a preponderance of the 'left' leg. On the other hand, when treated at 25°C, additional alterations occurred only on the fractions slightly lighter than the peak. This points to the presence of highly labeled material banding at the edge of polysomes, but not identical with normal polysome-associated mRNA.[1] - The presence of such material is also indicated in sucrose gradients of untreated pulse-labeled polysomes (fig. 3). As shown before (7), specific radioactivity over the gradient revealed a distinct peak at the oligosome region.

These 'features' of the material - its high specific radioactivity; its banding at slightly lower densities than polysomes and at S values corresponding to the monosome to pentasome region; its ribonuclease insensitivity at 0°C - point to a mRNA species associated with mono- and oligosomes, which might represent newly synthesized mRNA protected from ribonuclease action at 0°C by (transport ?) proteins.

1) In previous experiments (7), polysomes were degraded at 25°C with lower ribonuclease concentrations. Under these conditions, the contaminant does not appear to be attacked to a significant degree.