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Messenger RNA in HeLa Cells:

An Investigation of Free and Polyribosome-Bound Cytoplasmic Messenger Ribonucleoprotein Particles by Kinetic Labelling and Electron Microscopy

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Messenger RNA (mRNA) and messenger like RNA (mlRNA) were investigated in the cytoplasm of HeLa cells while ribosomal RNA synthesis was arrested. Under these conditions, functional mRNA associated in polyribosomes and cytoplasmic free mlRNA are formed and can be labelled selectively to steady state.

All cytoplasmic non-ribosomal RNA sedimenting at more than 6–7 S exists in the form of ribonucleoprotein complexes which pre-exist in the cell, and are stable upon cell lysis, sedimentation and (after fixation) CsCl density gradient analysis. The functional, true mRNA is contained in a complex of mRNA and protein which bands in association with ribosomes ($\varrho = 1.52$ to 1.60 g/cm³) in CsCl density gradients or, released by EDTA, at its own intrinsic density of 1.40–1.48 g/cm³. The cytoplasmic free mlRNA bands as a particle of mlRNA-containing ribonucleoprotein at an identical low density. The molecular weight spectrum of mRNA is identical to that of mlRNA and the sedimentation pattern of the mRNA · protein complex released from polyribosomes is similar to that of the free mlRNA · protein complex.

The physico-chemical separation of mRNA · and mlRNA · protein complexes allowed us to follow their relative kinetics of synthesis and decay. Each type of ribonucleoprotein obeys a different, strictly time-dependent pattern. Label enters the pool of free mlRNA · protein complexes first and may, in a pulse-chase experiment, be partially chased into polyribosomes. At steady state (6 h) 40–60% of the labelled RNA remains in the form of free mlRNA · protein particles. These cannot be chased into polyribosomes, the kinetics of mRNA · and mlRNA · protein complexes decay following identical patterns.

These findings are in agreement with a model according to which mlRNA from the nucleus first joins the pool of free ribonucleoprotein. Then, the activated mRNA · protein complexes attach to ribosomal subunits and form polyribosomes whereas inactivated mlRNA · protein complexes remain free in the cytoplasm.

In order to further strengthen the evidence in favour of the real existence of mRNA · and mlRNA · protein complexes in the cells, the corresponding fractions from sedimentation or CsCl density gradients were observed in the electron microscope. By this method it was possible to see small cytoplasmic particles which have not before been identified. The rounded structures, with diameters ranging from 100 Å to 200 Å, seem to consist of the coiling of a 35 Å wide pearl-like chain which may also be identified in polyribosomes. The frequency of these particles is highest in the mlRNA · protein band ($\varrho = 1.40–1.48$ g/cm³). Thus they may correspond to the mRNA · protein complex. However since they share some morphological features with other known biological structures the evidence is not conclusive.

† We are desolated to announce to our friends and collegues that Nicole Granbouland died in an accident shortly before the completion of this manuscript. We dedicate this paper to her memory.

Unusual Abbreviations. mRNA, messenger RNA (this term is restricted to functional mRNA isolated from active polyribosomes); mlRNA, messenger-like RNA; rRNA, ribosomal RNA; A_{260} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a 1-cm path length cell.

The existence in animal cells of messenger ribonucleoprotein particles, formed by the association of nascent and functional messenger RNA with specific protein, has long been considered possible on theoretical grounds. It was an enigma how messenger RNA in eukariotic cells is protected against non-specific degradation on its way from the chromatin to the polyribosome, especially in cells such as avian erythroblasts, which are rich in ribonucleases.

Furthermore, the existence of messenger RNA species with different half lives in the same cell led to the postulation of factors confering specific stability to particular types of messenger [1].

The demonstration that messenger-like RNA (mlRNA) (*cf.* [2—4]) forms complexes with proteins in the nucleus [5] and in the cytoplasm [6] not associated with ribosomal particles was the first experimental evidence for the existence of such structures. However, conclusive evidence was lacking that true messenger RNA was present in these complexes.

The experiments of Perry and Kelley [7], Henshaw [8], Burny *et al.* [9] and Cartouzou *et al.* [10] demonstrated similar ribonucleoprotein complexes in functional polyribosomes and thus gave the first hint to the existence of specific ribonucleoprotein particles involving true mRNA.

However the interpretation of these data became uncertain since the discovery by Girard and Baltimore [11] of non-specific RNA—protein associations in cytoplasmic extracts of animal cells.

Control experiments carried out in our laboratory showed that the formation of artificial complexes between RNA and proteins of cytoplasmic extracts of HeLa cells may indeed occur but depend on the kind of purified RNA added. Ribosomal RNA forms few such artifacts but messenger-like RNA fractions engage in them to a large extent.

However, theoretical arguments were still in favour of the existence of messenger-protein complexes. Their supposed function in messenger stability and possibly regulation, as well as the experimental evidence in favour of the existence of mRNA · protein complexes encouraged us to find other lines of evidence for their real existence and functions.

A reproducible, time dependent pattern of synthesis and decay of particular ribonucleoprotein particles excluding random aggregations would be a strong argument in favour of their real existence. Thus, we chose the possibility to distinguish artifacts from reality by kinetic labelling. Furthermore, we decided to attempt the direct demonstration of these particles by electron microscopy.

It was possible to demonstrate a reproducible pattern of labelling and decay of free and polyribosome-bound messenger ribonucleoprotein particles in our experiments. They show the existence of two classes of mRNA · protein particles in the cytoplasm: those which engage with polyribosomes having passed through the pool of free particles and another class which remains always free in the cytoplasm, apparently unable to attach to ribosomes and thus to express its message. By electron microscopy we have found a hitherto unidentified cytoplasmic structure which, although sharing some morphological features with other biological entities may possibly represent the mRNA · protein complex.

MATERIAL AND METHODS

Substances

Sucrose: RNAse free preparations from Mann Research Laboratories (U.S.A.). Actinomycin D: two preparations graciously given by Rhône-Poulenc (France) and by Merck, Sharp and Dohme (U.S.A.) were used. Sodium deoxycholate: from Fluka (Switzerland). It was purified from an ethanol solution by precipitation with hexane or a hexane/acetone mixture. Sodium dodecylsulfate: from Serva (Germany). Triton X-100: from Mann Research Laboratories (U.S.A.). [^{3}H]Uridine: from the Radiochemical Center (Amersham, England) with a specific activity of more than 20 C/mmole. Media for tissue culture: from Gibco (U.S.A.).

Solutions

Lysis buffer: 0.01 M KCl, 0.001 M $MgCl_2$, 0.005 M 2-mercaptoethanol, 0.01 M triethanolamine, pH 7.4. Suspension buffer: 0.01 M KCl, 0.001 M $MgCl_2$, 0.01 M triethanolamine pH 7.4. Gradient buffer: 0.01 M triethanolamine, 0.01 M NaCl, 0.001 M $MgCl_2$. Fixation buffer: 0.01 M KCl, 0.003 M $MgCl_2$, 0.03 Triethanolamine pH 7.8. Dialysis buffer: 0.01 M KCl, 0.003 M $MgCl_2$, 0.01 Triethanolamine pH 7.8. Sucrose gradients where not specified 10—40 % sucrose in suspension buffer. The percentage of sucrose solutions are always given in weight per volume.

METHODS

Cell Culture and Labelling Techniques

HeLa cells (clone S_3) were grown in suspension with a generation time of about 24 h in Eagle's spinner medium, supplemented with 10 % calf serum. Actinomycin D was added at a concentration of 0.05 μg/ml 30 min prior to labelling [12]. Where not specified, tritiated uridine was added diluted to a final concentration of 0.5 μM.

Cell Fractionation

Labelling was stopped by the addition of about one third the volume of frozen (−20°) Earle's saline (without magnesium and calcium). The cells were centrifuged at 280 × *g* for 5 min, washed first with 20 times their volume of Earle's saline and then with 20 volumes of isotonic sucrose in lysis buffer. Then the washed cells were suspended in 8 times their volume of hypotonic lysis buffer.

After 3 min of swelling, Triton X-100 was added to a concentration of 0.25 %; 2 min later isotonicity was restored by the addition of 2 M sucrose (in lysis

buffer) to 0.25 M; 10 min after suspension, the cells were disrupted by 6—12 measured strokes of a tight Dounce glass homogenizer. In order to keep nuclear breakage negligible, homogenization was stopped when about 70% of the nuclei were free.

Unbroken cells, nuclei, mitochondria and lysosomes were sedimented for 20 min at $10000 \times g$. The postmitochondrial supernatant (S-10000) was adjusted to a concentration of 0.5% sodium deoxycholate and immediately layered on a sucrose gradient. Alternatively, all cytoplasmic ribosomal and messenger ribonucleoprotein particles were sedimented for 3 h at $360000 \times g$ through a cushion of 15% sucrose in suspension buffer into the "cytoplasmic particle pellet". Under these conditions more than 90% of the labelled RNA heavier than 6 S sediments associated with proteins. This pellet was resuspended in 0.25 M sucrose in suspension buffer with the help of a small Dounce homogenizer. The suspension was clarified by a 15 min centrifugation at $4500 \times g$. All operations were performed at temperatures between 0° and 4°.

Caesium Chloride and Density-Gradient Centrifugation

Buoyant density centrifugation was carried out in the presence of formaldehyde according to Spirin *et al.* [13,14].

Usually 2 to 4 units of absorbance at 260 nm of particles were adjusted to 6% formaldehyde in 1.0 ml fixation buffer and kept at 4° for at least 20 h. Subsequently, the sample was dialysed exhaustively against dialysis buffer containing formaldehyde at 1%.

The dialysed sample, in a final volume of 1.6 ml, was adjusted with solid CsCl to a density of $\varrho = 1.40$ g/cm³ and the solution was layered over 1.9 ml of CsCl at a density of 1.60 in fixation buffer. Centrifugation was for 24 to 48 h at 32000 rev./min in the SW-56 Spinco Rotor.

Alternatively, the Spinco SW-65 Rotor was used. In this case, heavy and light solutions (2 ml each) had a density of 1.65 and 1.30 g/cm³, respectively. Centrifugation was for 18 h at 50000 rev./min.

6 drop-fractions were collected from the bottom of the tubes and absorbance was determined at 260 nm; the density of every 5th fraction was determined by refractive index and corrected for the error induced by formaldehyde.

General Methods

RNA extraction, sucrose gradient analysis and radioactivity assays were carried out according to Scherrer [15].

Electron Microscopy Techniques

Gradient samples (sucrose or caesium chloride) were treated with 6% formaldehyde in fixation buffer (if not already fixed) and prepared for electron microscopy as follows: one droplet was deposited on a grid covered with a thin collodion membrane stabilized by a carbon film. After about 15 sec the excess of liquid was removed by absorption with a filter paper. The grid was immediately rinsed with the buffer used for the preparation of the samples (without sucrose or CsCl). Without allowing it to dry, the material was negatively stained with a freshly prepared aqueous solution of uranyl acetate (0.5%), and then immediately examined in the electron microscope. As known, when the concentration of the biological material on the grid is very low, the stain obtained is positive. In some cases sodium silicotungstate (1% in distilled water containing Mg^{++} at the same molarity as the buffer) was also used.

For shadowing, the excess buffer used to rinse the grids was removed with a filter paper and the grids were dried in air. Then they were shadowed with platinum-carbon at an angle of 7° by rotation under a vacuum of 5×10^{-5} mm Hg. All specimens were examined with a Hitachi Electron Microscope at a nominal magnification of 40000 for stained preparations and 20000 or 25000 for shadowed grids.

RESULTS

The Influx of mRNA to Cytoplasm and Polyribosomes under Conditions of Arrested rRNA Synthesis

In order to follow the influx of messenger-like RNA to the cytoplasm in the absence of ribosomal RNA synthesis we took advantage of the observation of Perry [16—17] and Georgiev *et al.* [18] that low doses of actinomycin D specifically inhibit 45 S RNA formation in the nucleolus without seriously affecting mlRNA synthesis in the nucleoplasm. Exploratory experiments showed that the dose of 0.05 μg/ml actinomycin D, close to that chosen by Penman *et al.* [12], was optimal for our purpose.

Earlier reports in the literature [19] claim that the rate of protein synthesis is not affected under these conditions. We found that it drops slowly to about 60% of the initial rate after 8 h of exposure (Fig. 1). Since the half lives of polyribosomes and mRNA in HeLa cells after complete inhibition of RNA synthesis by a high actinomycin dose are about 3 h [20] it is evident that under our conditions new messenger RNA must reach the polyribosomes and be translated in order to maintain this level of protein synthesis. This labelling system is adequate to our purpose, since our main goal is to investigate the pattern of influx of mRNA into the various ribo-

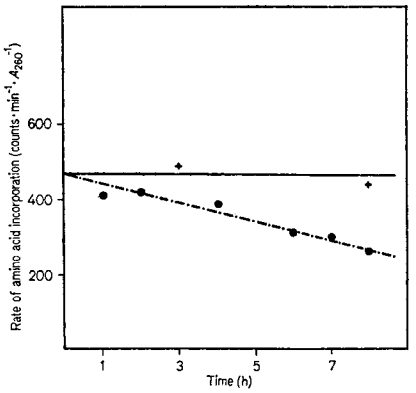

Fig. 1. *Rate of protein synthesis in HeLa cells exposed to a low dose of actinomycin D.* A cell suspension (34×10^4 cells/ml, 500 ml) in complete medium was treated with actinomycin (0.05 μg/ml). At each time point a 25 ml aliquot was removed and incubated for 30 min with a mixture of ^{14}C-labelled amino acids, 1 μC each of valine (260 mC/mmole), leucine (311 mC/mmole), and isoleucine (308 mC/mmole). Labelling was stopped by addition of crushed frozen Earle's saline, the cells were centrifuged at $280\times g$ and washed twice with cold Earle's saline. The cell pellet was suspended in hypotonic buffer and lysed by homogenization. The lysate was incubated with DNAse (20 μg/ml, 20°, 15 min) to reduce viscosity, and lysis was completed by the addition of sodium-dodecylsulfate. To control self-absorption, 3 aliquots of different volumes were precipitated in 10% trichloroacetic acid. Radioactivity was determined as described in methods and normalized relative to absorbance at 260 nm. ●, actinomycin treated cultures; +, control culture

nucleoprotein fractions in a given physiological condition, and also to investigate the possibility of a regulatory discrimination between messenger types before their entry into polyribosomes.

In addition these experiments show that rRNA synthesis and the formation of new ribosomal subunits are not necessary for the transport of mRNA from the nucleus to the cytoplasmic polyribosomes as it was proposed by Joklik and Becker [21] and recently by Sidebottom and Harris [22].

Free and Polyribosome-Bound Cytoplasmic Messenger Ribonucleoprotein Particles

The experiment shown in Fig. 2 demonstrates the existence of free and polyribosome-bound messenger ribonucleoprotein particles. Furthermore it shows that it is possible to distinguish free messenger ribonucleoprotein particles from those bound in polyribosomes, since little cross contamination occurs in a CsCl density gradient.

The uridine labelled mRNA associated with polyribosomes bands after fixation exclusively in the area of ribosomal material ($\varrho = 1.50-1.57$ g/cm³). But labelled cytoplasmic mlRNA not associated with polyribosomes, cosedimenting in sucrose gradients with monoribosomes and subribosomal particles, bands in CsCl exclusively at a density lower than 1.48 g/cm³, outside the band of ribosomal material.

HeLa cells were labelled with uridine for 3.5 h in the presence of actinomycin as described in Material and Methods. Polyribosomes and monoribosomes of the cytoplasmic extract were pelleted by a 45 min centrifugation at $360000\times g$ through a cushion of 15% sucrose. The redissolved pellet was fractionated on a sucrose gradient according to Fig. 2A. The fraction of polyribosomes containing 3 to about 30 ribosomal units was sedimented quantitatively and resuspended. One half of this polyribosome suspension was fixed immediately with formaldehyde (Fig. 2B), the other half was fixed after addition of EDTA (Fig. 2C) and both were banded in CsCl. It is obvious that the labelled RNA bound to polyribosomes bands exclusively in an area with the ribosomal material at a density of 1.50—1.60 g/cm³ with little or no contamination in the zone of free messenger-like ribonucleoprotein particles ($\varrho = 1.45$ g/cm³). The release of messenger RNA from polyribosomes by EDTA (Fig. 2C) results in a quantitative shift of the polyribosome bound label to the area of free messenger ribonucleoprotein particles with a density of about 1.45 g/cm³. No label remains associated with the 1.57 g/cm³ density band.

This result represents a confirmation of the experiments of Perry and Kelley [7], Henshaw [8] and Cartouzou *et al.* [10], indicating that the mRNA in animal polyribosomes may not be free but is associated with proteins. The density of 1.45 g/cm³ corresponds to about 75% protein and 25% RNA in the complex.

To demonstrate the existence of free messenger-like ribonucleoprotein particles, the particles remaining in the polyribosome supernatant were pelleted by 2 h centrifugation at $360000\times g$. The resuspended pellet was fixed with formaldehyde without EDTA treatment and banded in a CsCl gradient as shown in Fig. 2D. This fraction, which is slightly contaminated with monoribosomes, but does not contain polyribosomes, shows the wider absorbance distribution characteristic for native ribosomal subunits. These are completely unlabelled in our experiment. However, the labelled RNA, which corresponds to messenger-like fractions, bands under these conditions at a density of 1.45 g/cm³ and thus in the same area as the messenger ribonucleoprotein particles released from polyribosomes (Fig. 2C).

The density of this fraction corresponds to that of the particles, termed informosomes [23], isolated by Spirin and Nemer [6] from sea urchin embryos, and by Spirin *et al.* [14] from fish embryos.

The radioactivity liberated from polyribosomes by EDTA treatment (Fig. 2C) must be attributed to functional messenger RNA associated with proteins. This follows from the fact that protein synthesis

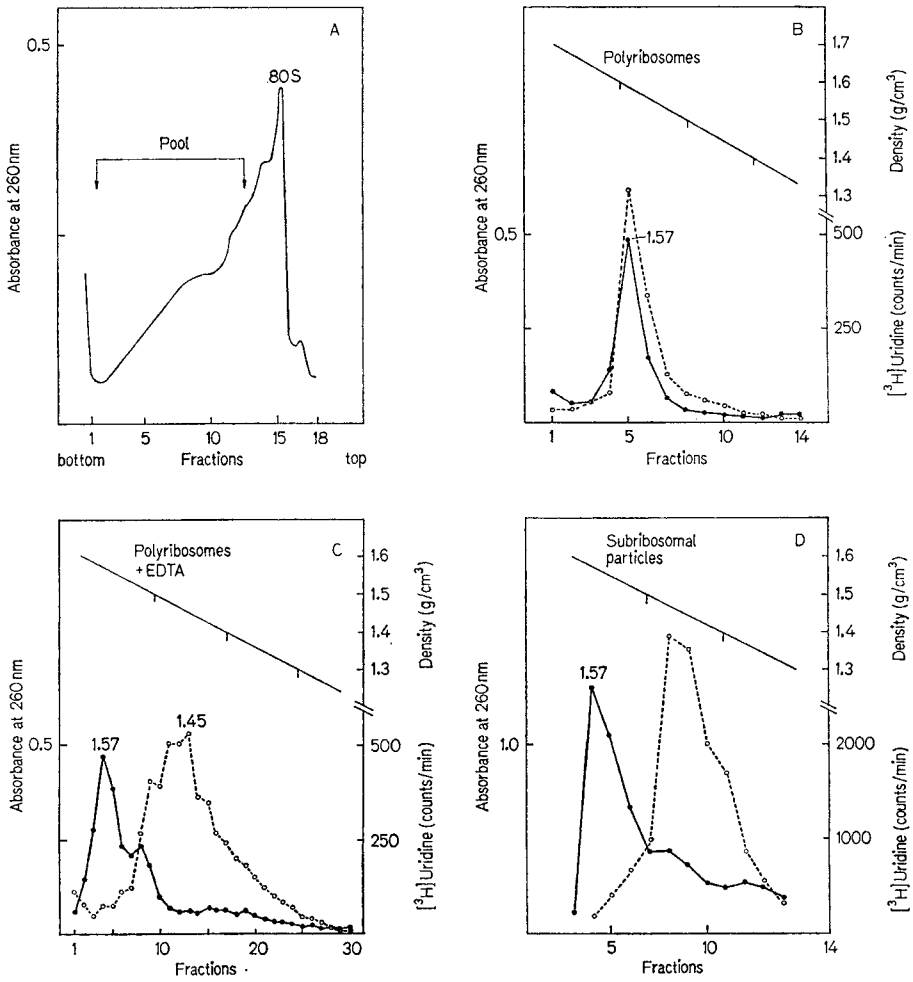

Fig. 2. *Density profiles of polyribosome bound messenger ribonucleoprotein and of free cytoplasmic messenger-like ribonucleoprotein*: (A) Cells were labelled for 3.5 h (200 μC [^{3}H]uridine diluted to 0.05 μM, 29×10^4 cells/ml, 800 ml, actinomycin 0.05 μg/ml) and the postmitochondrial cytoplasmic extract prepared according to methods. The polyribosomal pellet obtained by centrifugation (30 min, 360000 × *g*) through a 15 % sucrose cushion in buffer was resuspended and fractionated on a sucrose gradient (15 to 30 %, 27000 rev./min, 120 min, 2°, Spinco SW 27). (B) and (C) Polyribosomes were pooled according to (A) and collected by centrifugation (2 h, 360000 × *g*), the pellet was resuspended, fixed and analysed on a CsCl density gradient according to methods (50000 rev./min, 18 h, 4°, Spinco SW 65). (B): without EDTA, (C): treated before fixation with EDTA (20 μmoles/mg ribosomes). (D): the supernatent of the polyribosomes fraction prepared for (A) was sedimented by centrifugation (2 h, 360000 × *g*) and the pellet was fixed and analysed on CsCl gradients according to (B). ●, absorbance at 260 nm; ○, [^{3}H]uridine activity

continues under the given experimental conditions at a rate superior to that attributable to pre-existing mRNA. Thus newly formed messenger RNA which can be represented in our experiment only by the labelled RNA must associate with polyribosomes.

The attribution of messenger quality to the RNA contained in the fractions of cytoplasmic free messenger-like ribonucleoprotein is more questionable. However there are lines of evidence confirming such an assumption. We have been able to demonstrate that analogous fractions isolated from HeLa cells contain biological activity in protein synthesis *in vitro* [24]. In another experimental approach we could demonstrate by molecular hybridization that competition takes place between the RNA from polyribosome-bound and free cytoplasmic ribonucleoprotein fractions labelled under analogous conditions [25]. However, there is no evidence that all the label in the free messenger ribonucleoprotein fraction represents true mRNA. Thus, we will continue to term this fraction messenger-like RNA (mlRNA).

Before analysing in more detail the messenger ribonucleoprotein particles, we have to confront two possibilities of artifacts which could falsify our

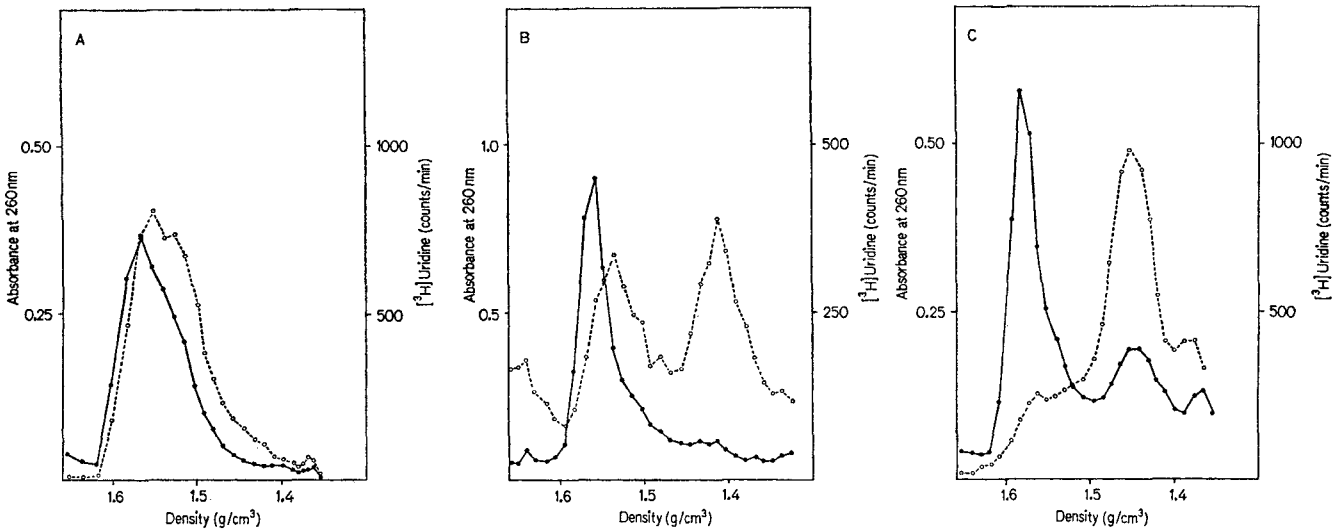

Fig. 3. *Buoyant density profiles of pure polyribosomes (A) and of cytoplasmic particle fractions, pure (B) or contaminated (C) with nuclear ribonucleoprotein particles.* (A) *Purified polyribosomes free of cytoplasmic or nuclear particles.* Cells were labelled for 6 h (200 μC [^{3}H]uridine, 34×10^4 cells/ml, 200 ml, 0.05 μg/ml actinomycin) and a cytoplasmic extract was prepared according to Methods. After sucrose gradient fractionation, the polyribosome area was pooled (*cf.* Fig. 2) sedimented (3 h, 360000 × *g*), resuspended, prepared for CsCl density centrifugation and banded according to Methods (32000 rev./min, 24 h, 2°, Spinco SW 56). (B) *Cytoplasmic particle fraction.* Cells were labelled for 6 h (100 μC [^{3}H]uridine, 33×10^4 cells/ml, 250 ml, 0.05 μg/ml actinomycin) and a cytoplasmic extract was prepared, the pellet obtained by centrifugation (3 h, 360000 × *g*) through a 15% sucrose cushion was resuspended, prepared and banded in CsCl as described in Methods (32000 rev./min, 24 h, 2°, Spinco SW 56). (C) *Cytoplasmic particle fraction contaminated with nuclear ribonucleoprotein particles.* Cells were labelled for 16 h (100 μC [^{3}H]uridine, 31×10^4 cells/ml, 300 ml, 0,05 μg/ml actinomycin). Preparation and analysis in a CsCl density gradient as described in (B). ●, absorbance at 260 nm; ○, [^{3}H]uridine activity

conclusions: the presence of particles due to nuclear leakage and the formation of artificial associations of RNA with protein.

Preparation of Cytoplasmic Extracts Free of Nuclear Material

A prerequisite for the continuation of our investigation was to exclude, or to reduce to a negligible amount, the contamination of cytoplasmic extracts with nuclear fractions due to extraction and/or breakage of nuclei. The contaminating giant nuclear RNA is associated with proteins also [5], and possibly with chromatin, which confer to it, according to Penman *et al.* [12] sedimentation values of up to 3000 S. It may band in CsCl gradients at densities from 1.40 to 1.45 g/cm^3 [26], together with free cytoplasmic mesenger-like ribonucleoprotein particles [23]. Thus the analysis of free cytoplasmic messenger-like ribonucleoprotein particles in CsCl density gradients is possible only if nuclear contaminations are reduced to a negligible amount.

We control nuclear leakage by several methods. As shown by Perry and Kelley [7], the pool of purified polyribosomes does not contain any free messenger ribonucleoprotein particles banding at the density of 1.40 to 1.45 g/cm^3. Thus the absence of label at this density is a proof of the absence of nuclear contamination. Fig. 3A shows the density profile of a polyribosome pool with little or no nuclear contamination.

Fig. 3B shows the banding pattern in a CsCl gradient of the cytoplasmic material sedimented for 3 h at 360000 × *g* (cytoplasmic particle pellet) containing RNA labelled for 6 h. Polyribosome-associated messenger ribunocleoprotein bands slightly lighter than single ribosomes at densities between 1.50 and 1.58 g/cm^3 (the monoribosomes band at $\varrho = 1.55$ to 1.60) the free messenger-like ribonucleoprotein bands at a density below 1.48 g/cm^3.

Evident nuclear leakage is shown in Fig. 3C (due in this experiment to the fragility of nuclei after 16 h, too long an incubation with actinomycin): not only does a large amount of radioactivity appear at densities of 1.42 to 1.46 g/cm^3 but also a slight amount of material absorbing at 260 nm is visible in the same area as a separate peak. Thus not only the amount of radioactivity but also the presence of a peak in absorbance serves as an indication of nuclear leakage.

Another argument for the distinction of cytoplasmic and nuclear ribonucleoproteins in cytoplasmic extracts is the kinetic pattern of labelling and decay itself, the nuclear mlRNA being characterized by a particularly high rate of turnover [28]. The incorporation kinetics of tritiated uridine into cytoplasmic messenger ribonucleoprotein has shown

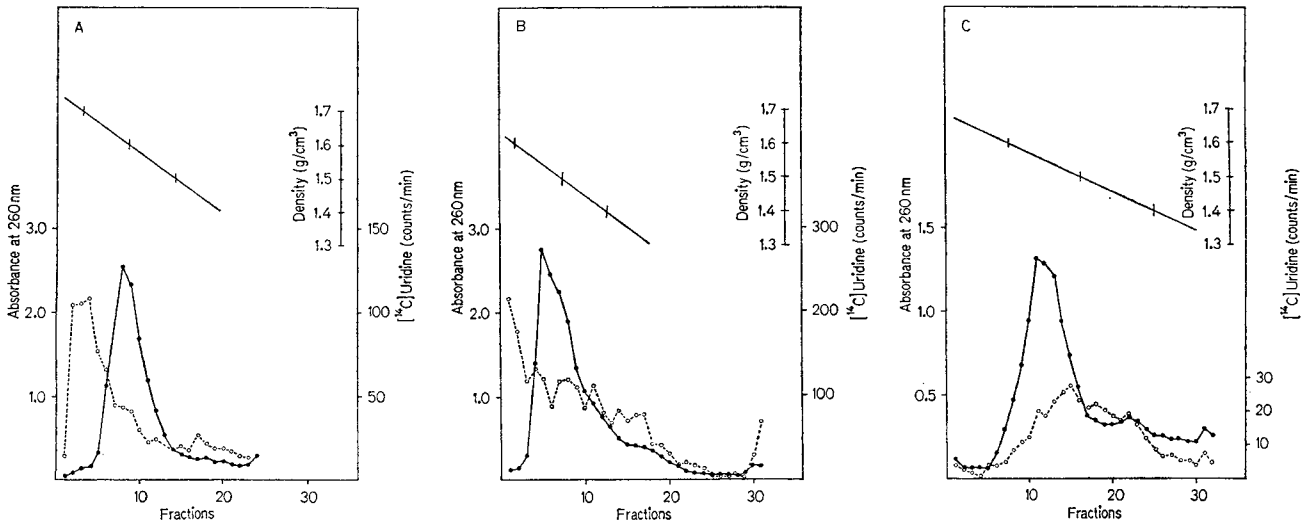

Fig. 4. *Artificial association of purified RNA with proteins present in the cytoplasmic particle fraction.* Polyribosomes, ribosomes and subribosomal particles were sedimented into one pellet and resuspended in buffer according to Methods. Purified [^{14}C]-uridine-labelled RNA was added and incubated in the cold for 15 min, the mixture was fixed with formaldehyde and banded in CsCl density gradients (50000 rev./min, 18 h, 2°, Spinco SW 65). (A) 28 S ribosomal RNA added, (B, C) nuclear mlRNA added

that a steady state of synthesis and decay is reached at approximately 6 h (*cf.* Fig. 11). On the contrary, the nucleoplasmic heterogeneous RNA approaches steady state already after 2 h [12].

This extensive discussion should point out the importance we give to the control of the cytoplasmic origin of the complexes subject to this type of analysis. The method finally adopted for preparing cytoplasmic extracts is described in Methods. The restoration of isotonicity before cellular lysis limited the extraction of nuclei. (Hypotonicity during cell homogenisation led inevitably to nuclear leakage.) The limitation of homogenization to a level where about 70% of nuclei only are liberated reduced nuclear breakage to a negligible level.

Control of Artificial Association of RNA with Protein in the Cytoplasmic Extracts

Girard and Baltimore [11] had shown that labelled poliomyelitis virus RNA added to cytoplasmic extracts of HeLa cells forms artificial ribonucleoprotein complexes. However, Perry and Kelley [7] claimed in their demonstration of the presence of mRNA · protein complexes in polyribosomes that labelled cellular RNA did not form such artifacts.

Our control experiments (Fig. 4A) show that ribosomal type RNA indeed binds to a limited extent only to proteins contained in the cytoplasmic particle fraction. However, mRNA and mlRNA associate with proteins contained in the cytoplasmic particle fraction and band in CsCl at densities between 1.40 and 1.55 g/cm^3 (Fig. 4B and 4C).

The nature of these artificial complexes is so far not known. Their definition as artifacts is ambiguous. Indeed if specific proteins exist which bind to RNA it is reasonable to expect similar complexes to form *in vitro* as those present *in vivo*. However experimental criteria allow one to distinguish them.

Sedimentation Characteristics of Polyribosome Bound mRNA · protein Complexes and Free Cytoplasmic mlRNA · protein Particles

A comparison of the sedimentation profile of polyribosome bound mRNA · protein complexes and free cytoplasmic mlRNA · protein complexes demonstrates that both types of Ribonucleoproteins are represented with the same spectrum of apparent sizes. Thus both may contain RNA with the same spectrum of molecular weights.

The cytoplasmic extract of cells labelled for 6 h with [^{3}H]uridine was fractionated on a sucrose gradient into polyribosomes, monoribosomes (70 to 100 S) and the subribosomal zone (30 to 70 S) as shown in Fig. 5A and B. The particles contained in the respective zones were collected by a 3 h centrifugation at 360000 $\times g$ and the resuspended clarified material was analysed by sedimentation after the addition of 2 μmoles EDTA per absorbance unit at 260 nm.

The sedimentation pattern of the free labelled ribonucleoprotein fractions is shown in Fig. 5D—E. The mRNA · protein particles extracted from polyribosomes by addition of EDTA sediment between 20 and 70 S (Fig. 5C). A lighter peak may represent

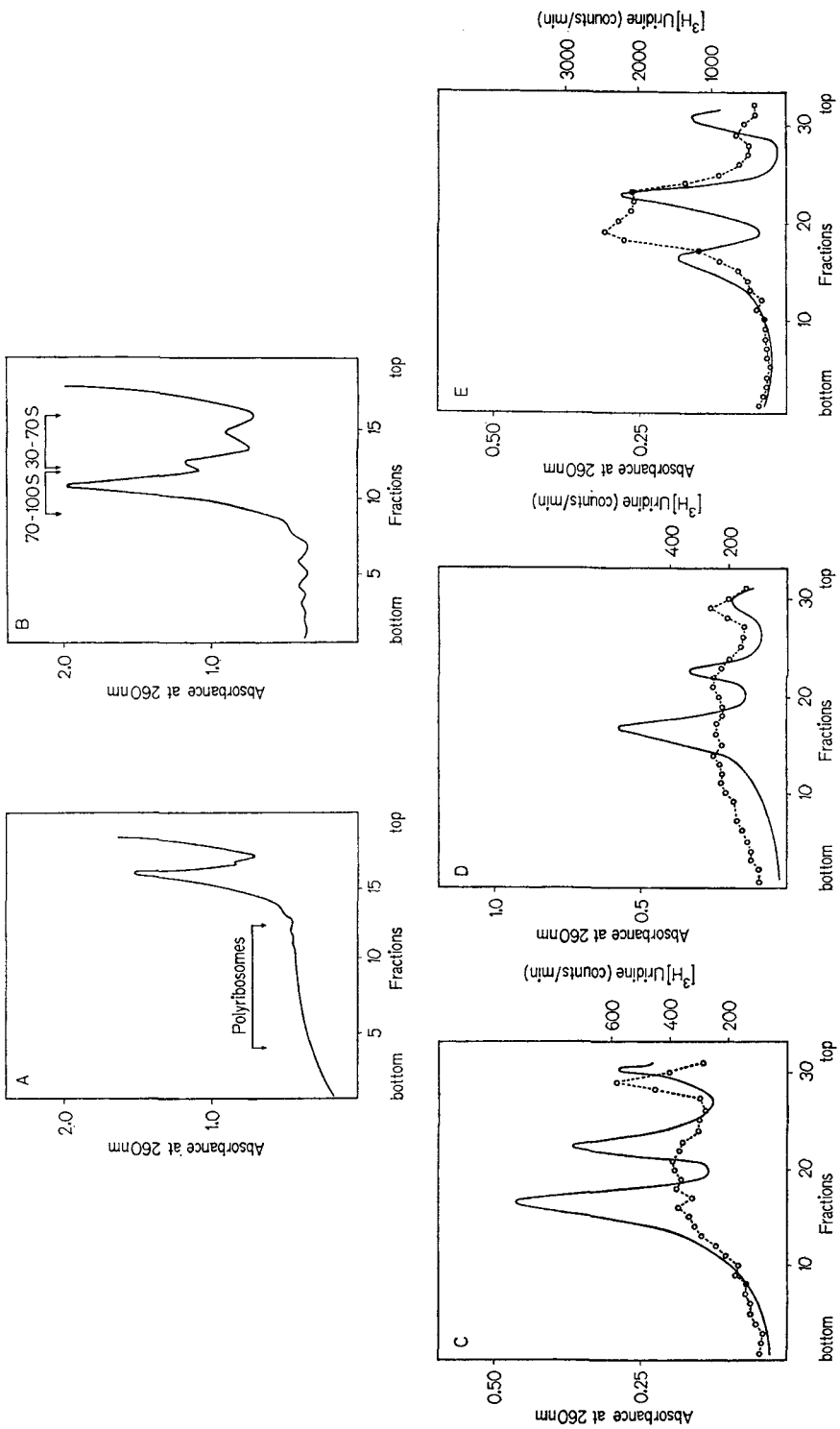

Fig. 5. *Sedimentation profile of messenger ribonucleoprotein contained in polyribosomes, monoribosomes and the subribosomal fraction.* The cells were labelled for 6 h (800 μC [^{3}H]uridine, $3{,}4 \times 10^4$ cells/ml, 1.2 l, 0.05 μg/ml actinomycin). The postmitochondrial extract prepared according to Methods was fractionated on sucrose gradients (10 to 40%, 27000 rev./min, 2 h (A) or 6 h (B), Spinco SW 27). Polyribosomes, monoribosomes (70 to 100 S) and the subribosomal fraction (30 to 70 S) were pooled according to (A) and (B) collected by centrifugation (3 h, 360000×*g*). The pellets were resuspended in 0.25 M sucrose in suspension buffer, treated with EDTA (20 μmoles/mg of ribosomes plus compensation of Mg^{++} in buffer) and analysed on a sucrose gradient 5—20% in Mg^+ free suspension buffer (40000 rev./min, 3 h, 2°, Spinco SW 40). (C) Polyribosomes; (D) monoribosomal zone; (E) Subribosomal zone. ——, absorbance at 260 nm; O–––O, [^{3}H]uridine activity

tRNA bound to activating enzymes as well as small mRNA · protein particles. The mlRNA · protein particles of the 70 to 100 S zone (Fig. 5D) which, as demonstrated in Fig. 7B are essentially not bound to ribosomal material, sediment also between 10 and 70 S. The free mlRNA · protein particles from the subribosomal zone show a predominance of particles in the sedimentation zone below 50 S (Fig. 5E). The gradient analysis of free mlRNA · protein particles shown in Fig. 5D and E was carried out in the presence of EDTA in analogy to Fig. 5C. Control experiments showed that EDTA does not alter the sedimentation velocity of labelled RNA in the free messenger-like ribonucleoprotein fraction. Thus the particles of the entire 30 to 100 S area (Fig. 5D + E) which contains the free mlRNA · protein show the same size distribution as the mRNA · protein dissociated from polyribosomes by EDTA treatment.

We conclude that free mlRNA · protein particles and polysome-bound mRNA · protein-containing particles, or in other terms inactive messenger-like ribonucleoprotein and active messenger ribonucleoprotein, show the same sedimentation behaviour. Thus they should contain the same size classes of RNA.

Sedimentation Characteristics of Labelled RNA Extracted from Free and Polyribosome-Bound mRNA · protein Particles

The sedimentation analysis of phenol extracted RNA from the various cytoplasmic fractions demonstrates that mRNA · protein particles contain RNA species with a spectrum of molecular weights analogous to that we found earlier by pulse labelling in HeLa cell polyribosomes [20].

HeLa cells were labelled to steady state (6 h) with uridine as described in Methods and the postmitochondrial cytoplasmic particles were fractionated on sucrose gradients into polyribosomes, monoribosomes and a subribosomal fraction. The RNA of the free mlRNA · protein particles and polysome-bound mRNA · protein contained in these fractions was extracted by dodecylsulfate dissociation (and directly sedimented [29]) or by the hot phenol method. Both methods gave comparable results.

Fig. 6 demonstrates that RNA extracted from the polyribosome-bound messenger ribonucleoprotein and from free messenger-like ribonucleoprotein gives rise to the same overall sedimentation profile spreading from 6 S to about 30 S. As expected, the separately extracted heavy free messenger-like ribonucleoprotein particles cosedimenting with monoribosomes contain RNA of higher molecular weight then those in the subribosomal zone (Fig. 6B and C resp.). Polyacrylamide gel analysis of the phenol extracted RNA leads to essentially the same results (Fig. 6D—F).

The sedimentation of mRNA and mlRNA relative to rRNA can be compared to that of the corresponding messenger ribonucleoprotein particles relative to ribosomal subunits (Fig. 5). The overall pattern of the RNA or ribonucleoprotein spectra are the same: polyribosomes contain the full spectrum of sizes whereas the free messenger-like ribonucleoprotein particles or their RNA have higher sedimentation values in the monoribosomes area compared to those in the subribosomal zone.

The fact that in this experiment the monoribosome pool contains not only mlRNA with high sedimentation values corresponding to large mlRNA particles but also smaller types may be attributed either to the presence of some messenger ribonucleoprotein bound to small polyribosomes ("mono-or di-polyribosomes"), since the entire 70 to 100 S area was pooled, or to the fact that in the absence of EDTA or of high salt some association between messenger-like ribonucleoprotein and ribosomal material may possibly occur.

Our general conclusion is that in the cytoplasm of HeLa cells (free of mitochondria) mRNA and mlRNA types exist, polyribosome-bound or in free ribonucleoprotein particles, with molecular weights ranging from 50000 to about 1 million daltons. Heavier RNA was found in negligible amounts in experiments where no nuclear leakage had occurred.

Density Profiles of Individual Messenger and Messenger-Like Ribonucleoprotein Fractions

The shallow CsCl density gradients obtained at lower speed in the SW-56 Rotor allow analysis of the particulate fraction of cytoplasmic extracts in more detail (Fig. 7). Postmitochondrial cytoplasmic extracts from cells labelled for 3 or 6 h were fractionated on sucrose gradients into pure polyribosomes (more than 150 S), monoribosomes (70 to 100 S) and subribosomal particles (30—70 S). The pooled fractions were analysed on CsCl gradients after fixation. The polyribosomal material bands in an area at a density of 1.50—1.60 g/cm^3, the free messenger-like ribonucleoprotein present in the 70 S to 100 S pool bands at a buyoant density of approximately 1.40—1.50 g/cm^3 and that of the 30 S to 70 S sedimentation zone bands at a density lower than 1.45 g/cm^3. The 70—100 S zone contains little labelled messenger ribonucleoprotein bound to monoribosomes.

The fact that the free ribonucleoprotein particles containing mlRNA from the heavier sedimentation zone band at a slightly higher density than those of the subribosomal area may be attributed to intrinsic differences in composition of different classes of these particles or perhaps to the attachment of small messenger ribonucleoprotein particles to ribosomal subunits. However, as shown in Fig. 5D the 70—100 S zone contains mainly large, messenger ribonucleo-

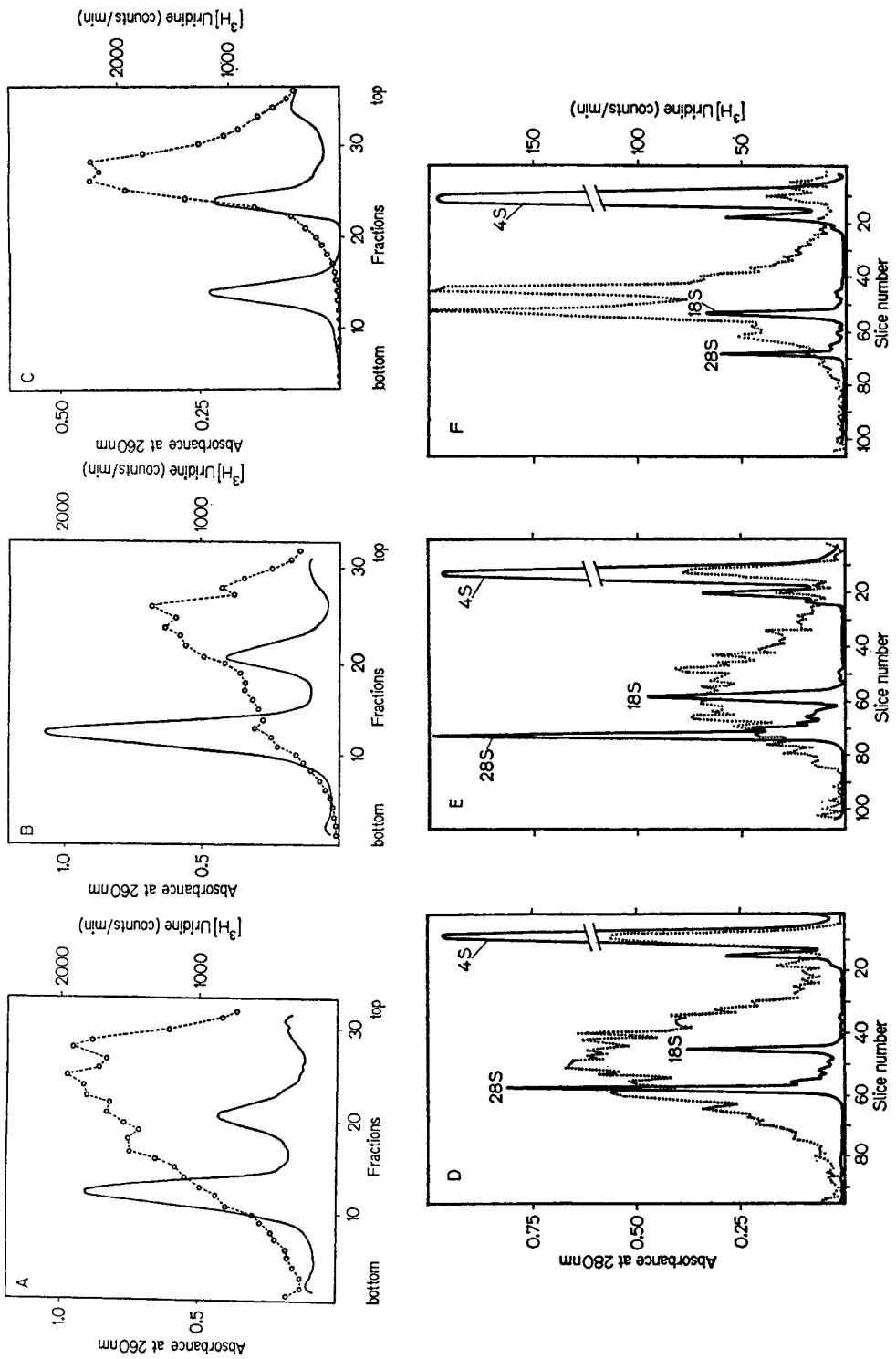

Fig. 6. *Analysis of RNA extracted from polyribosomes, monoribosomes and the subribosomal fraction.* Cells were labelled for 6 h according to Fig. 5 and fractionated into the polyribosome, monoribosome and subribosomal particle zone (*cf.* Fig. 5 A and B). (A—C) The pooled fractions were sedimented (3 h, 360000 × *g*) resuspended in 0.25 M sucrose in gradient buffer, adjusted to 1% sodium dodecylsulfate, 0.5% deoxycholate and 0.5% urea, diluted with buffer to a sucrose concentration of 3.5% and analysed on a sucrose gradient (0.01 M triethanolamine, pH 7.4, 0.01 M NaCl, 0.01 M EDTA) containing 0.2% sodium deoxycholate and 0.2% sodium dodecylsulfate (5 to 20%, 40000 rev./min, 6 h, 2°, Spinco SW 40). (D—F) The pooled fractions were extracted with hot phenol and analysed according to Mirault *et al.* [30] on polyacrylamide exponential gels (2—15% gel in E-RNA buffer, 9 h, 2°, 10 volts/cm, 3 mA/tube). (A, D) RNA of the polyribosome zone; (B, E) RNA of the monoribosome zone (70 to 100 S); (C, F) RNA of the subribosomal particles zone (30 to 70 S). ——, absorbance at 260 nm; O——O and ·····, [^{3}H]uridine activity

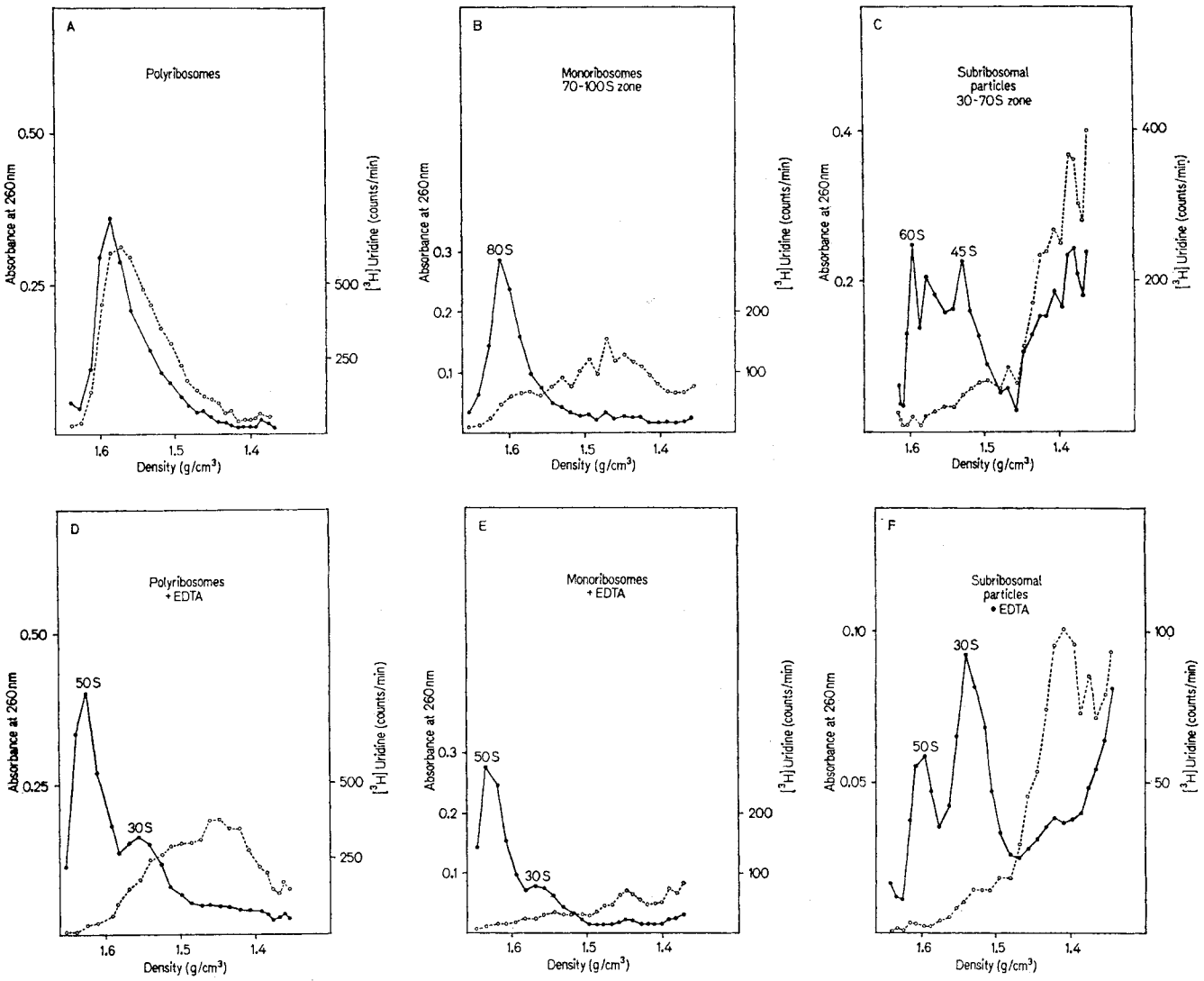

Fig. 7. *Buoyant density profiles of messenger ribonucleoprotein particles contained in polyribosomes, monoribosomes and the subribosomal fraction before and after EDTA treatment.* Cells were labelled for 3 (C and F) or 6 (A, D, and B, E) h (800 μC, [^{3}H]-uridine, 33×10^4 cells/ml, 1.5 l, 0.05 μg/ml actinomycin), the postmitochondrial extract was prepared according to Methods and fractionated on a sucrose gradient into polyribosomes, monoribosomes and the subribosomal particle zone (*cf.* Fig. 5). The pooled fractions were sedimented (3 h, $360000\times g$) resuspended in 0.25 M sucrose in suspension buffer, treated or not with EDTA (20 μmoles/mg ribosome plus compensation for Mg in buffer). CsCl density analysis according to Methods (32000 rev./min, 24 h, 2°, Spinco SW 56). (A, D) polyribosomes; (B, E) monoribosomes; (C—F) subribosomal particles. (A—C) controls; (D—F) EDTA treated. ●——●, absorbance at 260 nm; O----O, [^{3}H]uridine activity

protein particles. This favours the first interpretation rather than the second.

It is interesting to note that the ribosomal material of the polyribosome zone bands at densities slightly lighter than the monoribosomes (Fig. 7 A and B). This partial separation can be confirmed by electron microscopy, investigating the material contained in each zone as shown below. The binding of messenger ribonucleoprotein confers a slight decrease in density to ribosomes, as would be expected of a complex formed by association of a component containing 75% protein with one containing 60% protein. This also explains why in the cytoplasmic particle pellet the radioactivity associated with polyribosomes has a lower mean buoyant density than the absorbancy which corresponds to inactive monoribosomes. The fact that the monoribosome zone bands heavier than polyribosomes and carries little radioactivity under steady state conditions demonstrates that monoribosomes are not engaged in protein synthesis. Furthermore, this serves as a control that during the isolation procedure polyribosomes have not been degraded to monoribosomes associated with messenger ribonucleoprotein.

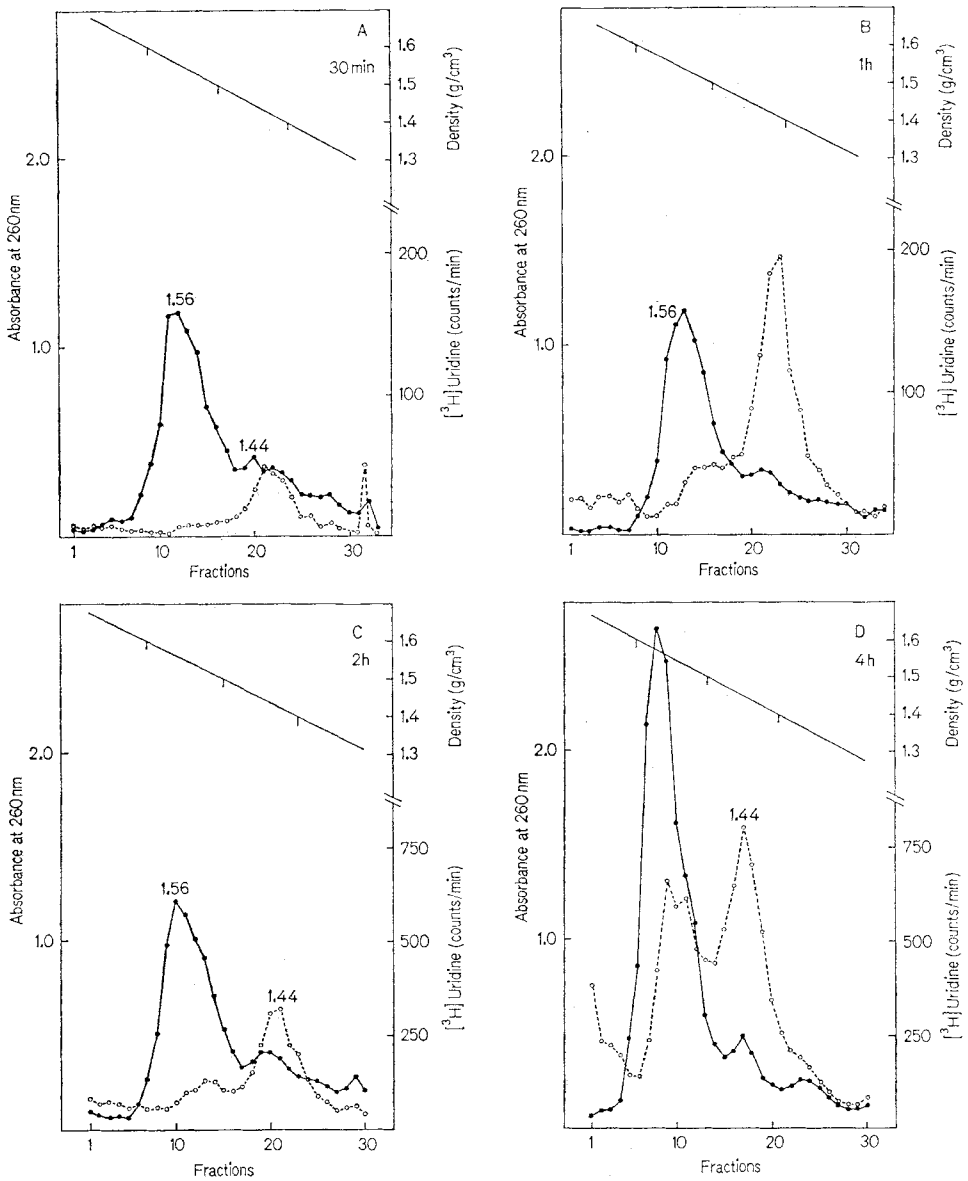

Fig. 8. *Labelling pattern in density profiles of free messenger-like ribonucleoprotein and polyribosome bound messenger ribonucleoprotein.* Cells were labelled for the indicated time periods (150 μC [³H]uridine diluted to 0.1 μM, 66×10^4 cells/ml, 600 ml, 0.05 μg/ml actinomycin) as indicated in Methods. Aliquots of 125 ml were chilled, the cytoplasmic particle pellet was prepared and analysed in CsCl density gradients as indicated in Methods (50000 rev./min, 18 h, 2°, Spinco SW 65). ●, absorbance at 260 nm; ○, [³H]uridine activity

Kinetics of Synthesis and Decay of Ribonucleoprotein Complexes of mlRNA and Polyribosome Bound mRNA

The incorporation of labelled uridine into polyribosomal mRNA · protein and free mlRNA · protein was determined for up to 12 h under conditions of blocked rRNA synthesis. After 6 h a steady state of labelling and decay had been reached and a high concentration of actinomycin D (5 μg/ml) was added to follow the decay of the ribonucleoprotein particles containing mRNA or mlRNA, respectively.

The labelling pattern after various times of synthesis and decay of mRNA · and mlRNA · protein was analysed in steep (Fig. 8) and in the better resolving shallow CsCl gradients (Fig. 9). For every time point the entire particulate fraction was sedimented for 3 h at $360000 \times g$ from a postmitochondrial cytoplasmic extract. The resuspended and clarified pellets were directly analysed in CsCl gradients after fixation with formaldehyde.

After a 30 min exposure to [³H]uridine free mlRNA · protein is labelled almost exclusively

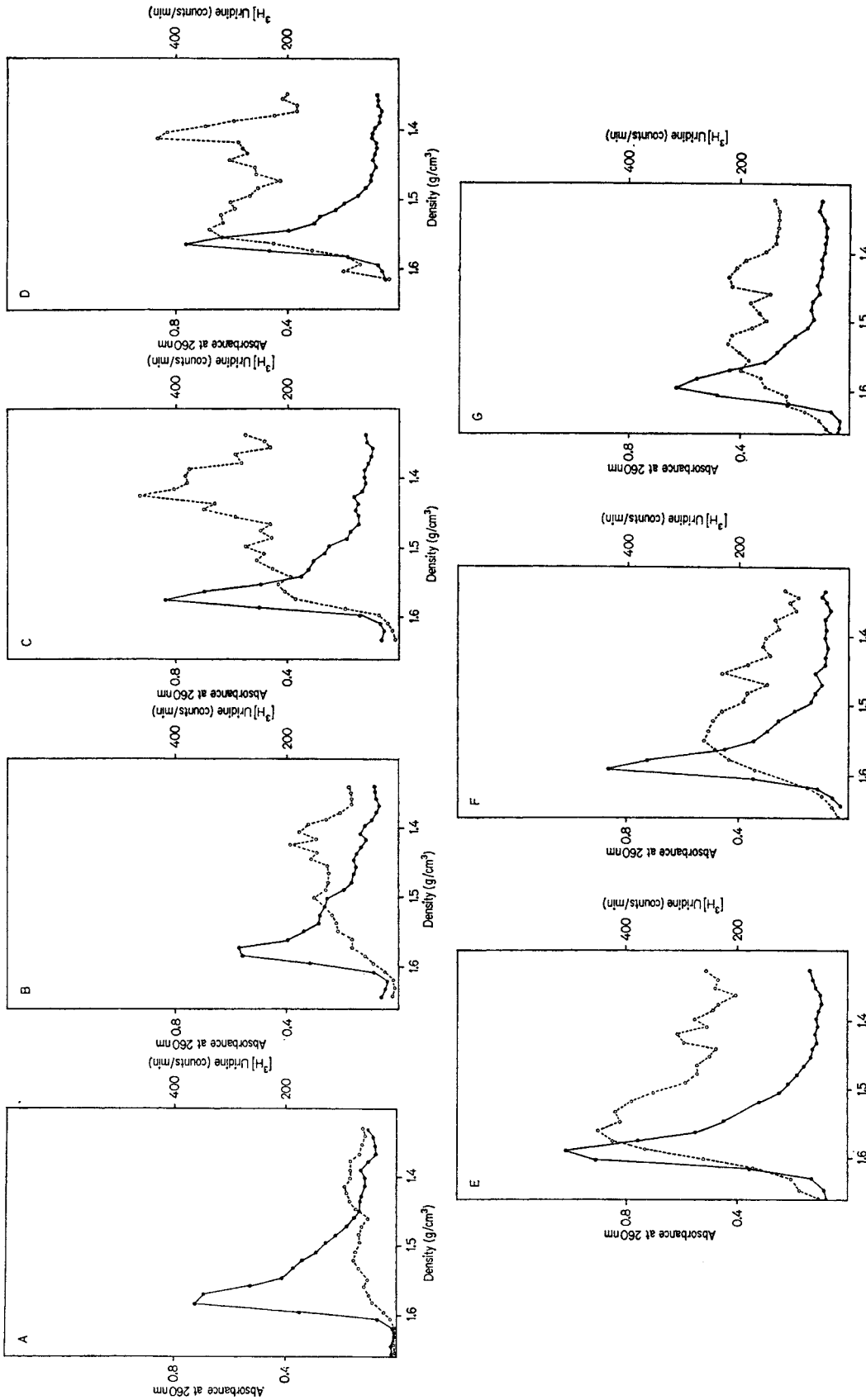

Fig. 9. *Pattern in density profiles of labelling and decay of messenger ribonucleoprotein particles.* Cells were labelled for 6 h (500 μC [^{3}H]uridine, 33×10^4 cells/ml, 1300 ml, 0.05 μg/ml actinomycin), according to Methods; after 6 h the culture was adjusted to 5 μg/ml actinomycin in order to induce the chase. After the indicated times 150 ml aliquots were chilled and the cytoplasmic particle pellet prepared and analysed in CsCl density gradients (32000 rev./min 24 h, 2°, Spinco SW 56) as indicated in Methods. (A) 1 h; (B) 2 h; (C) 4 h; (D) 6 h; (E) 6 h and 1 h chase; (F) 6 h and 3 h chase; (G) 6 h and 6 h chase. ●——●, absorbance at 260 nm; ○- - -○, [^{3}H]uridine activity

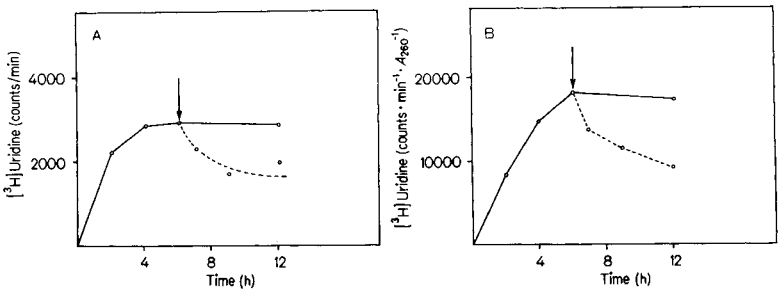

Fig. 10. *Kinetics of RNA labelling in whole cells and in the cytoplasmic particle fraction.* Cells were labelled as indicated in Fig. 9. To determine the radioactivity in whole cells 1 ml aliquots of the culture were sedimented at $280 \times g$, washed twice with cold Earle's saline and lysed in isotonic saline with 0.5% sodium dodecylsulfate. 10% trichloroacetic precipitable radioactivity was determined as described in Methods. The specific activity of the cytoplasmic particle fraction prepared as indicated in Fig. 9 was determined according to Methods. (A): whole cells; (B): cytoplasmic particle pellet. (——) control; (----) 5 μg/ml actinomycin added at 6 h, as indicated by the arrow

(Fig. 8 A). Subsequently more enters the cytoplasm and some polyribosome-bound label appears (Fig. 8 B and C). After 4 h of labelling (Fig. 8 D) approximatively the same amount of radioactivity is present in mRNA · protein and free mlRNA · protein.

A similar pattern may be observed in more shallow gradients (Fig. 9 A to D). Incorporation of label into free mlRNA · protein precedes always incorporation into polyribosomes. In the presence of the low actinomycin concentration used a steady state of synthesis and decay is reached after approximately 6 h. The synthesis of mRNA · and mlRNA · protein was followed for up to 12 h. This represents the practical time limit for these experiments due to the increased fragility of the nuclei after prolongued exposure to actinomycin.

If after 6 h of labelling a high dose of actinomycin (5 μg/ml) was added to inhibit completely further RNA synthesis, the decay of both polysome-bound mRNA · protein and free mlRNA · protein could be observed.

In these shallow gradients a different pattern of synthesis and decay was evident in the various density zones. In several experiments it was observed that the light mlRNA · protein of the 1.40 g/cm³ density zone disappear more rapidly than others (Fig. 9 E—G). If in the first hour of actinomycin chase the disappearance of free mlRNA · protein seems to be more rapid than in polyribosome-bound mlRNA · protein, a continued chase reveals approximately the same rate of decay in both fractions.

In order to express quantitatively the pattern of mRNA · and mlRNA · protein synthesis and decay observed, the radioactivities corresponding to the free and polyribosome bound ribonucleoprotein zones were integrated. Specific activities were calculated by normalisation relative to the total ribosomal material present in a gradient.

For comparison, Fig. 10 A shows the radioactivity pattern during labelling and chase in whole cells, and Fig. 10 B in the total cytoplasmic particle fraction. In both cases a steady state is reached after 6 h. It is established slightly faster in the total cell since, according to Penman *et al.* [12], nuclear label is already maximal after 3 h. The radioactivity in the entire cytoplasmic particle fraction enters steady state after 6 h. The half life of the labelled RNA is also about 6 h. However it has to be considered that this analysis includes the metabolically stable soluble RNA which is continuously labelled in the presence of a low actinomycin dose [31].

The kinetics of mRNA · protein labelling in several typical experiments are shown in Fig. 11. These patterns demonstrate the general finding that the labelling of free mlRNA · protein always precedes that of the mRNA · protein bound in polyribosomes to a more or less pronounced degree. However, the specific activities of both fractions finally reach approximately the same value. Under the chosen experimental conditions in steady state only 50% of the newly synthesized ribonucleoprotein particles are engaged in active polyribosomes and thus carry functional mRNA. The label remaining in free particles raises the problem if these contain inactivated mRNA species not able to bind ribosomal subunits, or if they represent precursors to the polyribosome-bound mRNA · protein containing the same messenger which accumulate as a consequence of a limitation in ribosomes available.

In order to answer this question chase experiments were carried out (Fig. 9 E—G and Fig. 11 A, B). They demonstrated clearly that on the average the free mlRNA · protein particles decay as rapidly as the mRNA · protein particles engaged in polyribosomes, although specific mlRNA · protein particles of one density class may disappear more rapidly than those of an other class. Thus on the average inactive mlRNA · protein species have the same stability as mRNA · protein-containing messenger particles and cannot be chased into polyribosomes.

This pattern of decay indicates that at steady state either a majority of mlRNA in free particles is inactivated and does not represent precursors of the polyribosome-bound mRNA, or that the equilibrium

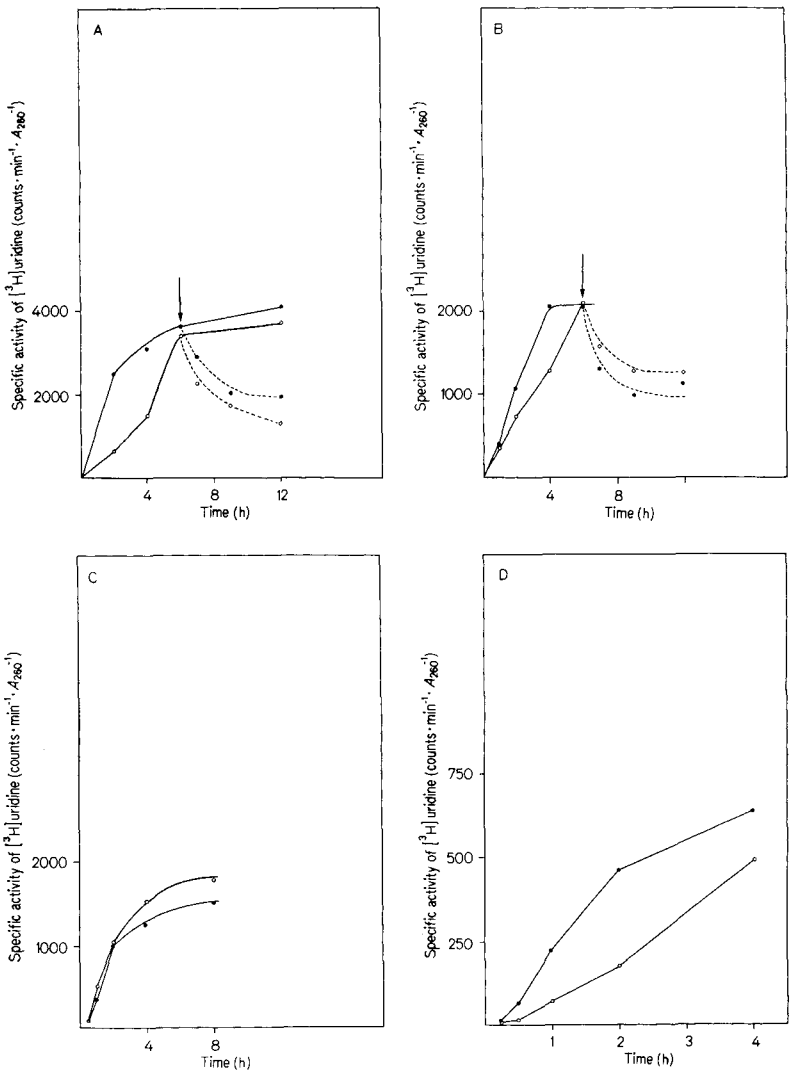

Fig. 11. *Kinetics of uridine incorporation into polyribosome bound messenger ribonucleoprotein and free cytoplasmic messenger-like ribonucleoprotein.* Cells were labelled in the presence of actinomycin (0.05 μg/ml) and cytoplasmic particle pellets were prepared analysed and as reported in Methods and in the legends of Fig. 8 and 9. The radioactivity of fractions with densities from 1.36 to 1.46 g/cm³ was attributed to free messenger-like ribonucleoprotein (●) and between 1.48—162 to polyribosome bound messenger ribonucleoprotein (○), the values were integrated and normalized relative to the ribosomal material in the profile. Fig. (A) to (D) refer to four different experiments. Note the different time scale in D. The arrow indicates the addition of actinomycin D (5 μg/ml) to induce the chase (----)

between active and inactive forms of mRNA is disturbed by the high dose of actinomycin added for the chase.

In the attempt to answer this question pulse-chase experiments were carried out and will be published in detail elsewhere. They tend to show that a fraction of mlRNA had been shifted during the chase from the free mlRNA · protein particle pool to the polyribosomes, being thus a precursor of the functional mRNA. However, the major part of cytoplasmic mlRNA labelled in 40 min cannot be chased into polyribosomes and may be inactivated. The possibility has to be taken into account that limiting factors may be necessary to activate free mlRNA · protein complexes and enable them to become bound to ribosomes.

It was evident in these experiments that the stability of mRNA or mlRNA is an intrinsic property of the ribonucleoprotein particle itself, quite independent of its eventual association with ribosomes and its function in the active complex during protein synthesis.

The time limitation in this type of experiment which is the consequence of the presence of actinomycin at a very low dose, made it impossible to follow the fate of the messenger ribonucleoprotein beyond 6 h of chase. The rate of decay during this period of both types of particles corresponds to an

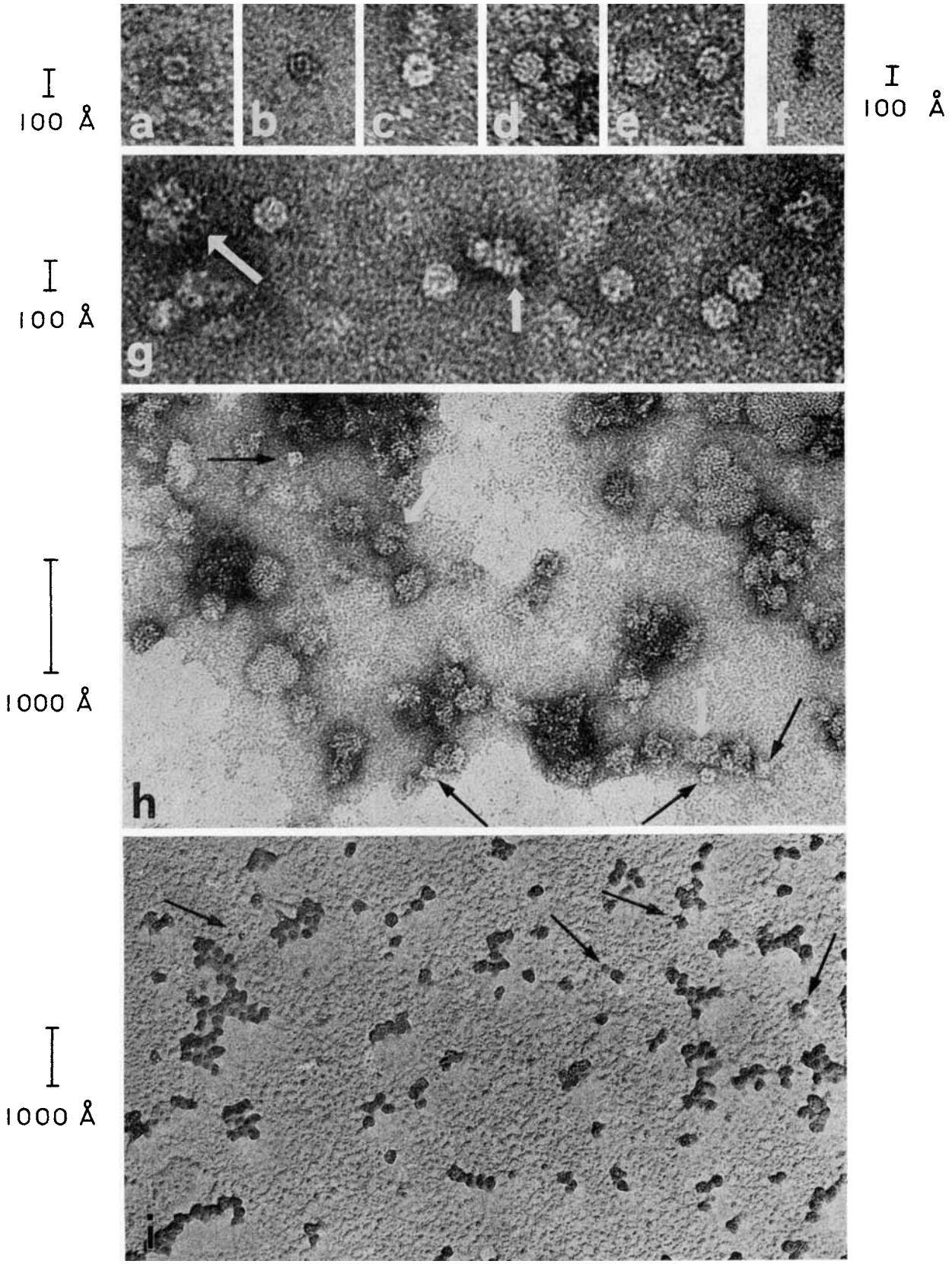

Fig. 12. *The visualisation of non-ribosomal small particles in the cytoplasmic particle fractions.* (a, b, c, d, e, g). Negatively stained small particles found in CsCl gradients of the cytoplasmic particle fraction. From (a) to (e) the size and the more or less compact aspect of these particles seem to depend on the length of the strand 35 Å in diameter, forming them by coiling. In (g) their morphological feature can be compared to that of 30S and 50S ribosomal subunits. (Magnification: 450000×.) (⇩) 30S, (→) 50S ribosomal subunits. (f) Three particles in positive staining. (Magnification: 300000.) (h) Negatively stained sample of the polyribosomal zone in a CsCl gradient of the cytoplasmic particle fraction. (Density: 1.55 g/cm³; tube: 12; Fig. 13B.) (→) Small particles located close to polyribosomes. (Magnification: 200000×.) The large amorphous area of 300 Å diameter and larger are staining artefacts. (i) Shadowed preparation of polyribosomal fraction of a sucrose gradients. (→) Small particles which seem to be linked to polyribosomes. Note the fine strand linking the ribosomes in the upper right corner. (Magnification: 100000×)

average half life of 3 h. In an earlier investigation we had shown that the half life of polyribosomes and thus of messenger RNA function is also of the order of 3 h in HeLa cells [20]. In view of these experiments the disappearance of active polyribosomes and thus the arrest of messenger RNA function is the consequence of the decay of the messenger or of the mRNA · protein complex itself.

The Visualisation of Ribonucleoprotein Particles Containing mRNA and mlRNA in the Electron Microscope

Besides easily identified ribosomal material such as polyribosomes, monoribosomes and ribosomal subunits, electron micrographs of the cytoplasmic particle fraction (15 to 400 S) from HeLa cells show a distinct type of particle. We observed small, generally round particles, with a diameter varying from 110 to 200 Å. They seem to be formed by the coiling of a thin continuous strand presenting regularly located small knobs along its length (Fig. 12a, b, c, d, e, g). This strand measures about 35 Å in thickness. The size of the particles and their more or less compact aspects seem to vary with the length of the coiled strand. Thus, some of them appear with a small central hole (Fig. 12a, c) and, most frequently, they are compact and electron dense (Fig. 12d, e, g).

The presence of these particles can be demonstrated as well by negative staining, uranyl acetate giving best results, as by shadowing. They are also detectable by positive staining but not so easily as ribosomal material (Fig. 12f).

Observations on CsCl Gradient Fractions

The material corresponding to a density of 1.58 g/cm³ (Fig. 13B, fraction 9) is composed of monoribosomes. Very few small particles were found attached to monoribosomes: exceptionally, free particles may be seen. In the density zone from 1.49 to 1.56 g/cm³ (Fig. 13A, fractions: 8, 12; Fig. 13B, fractions 12, 13, 14, 16) the material consists principally of polyribosomes including from two to more than ten ribosomes. Some particles can be distinguished attached to polyribosomes.

It was especially difficult to view the particles attached to polyribosomes due to their small size and the possibility of a superposition effect (Fig. 12h). They are better demonstrated by shadowing (Fig. 12i). These polyribosomal fractions also contain monoribosomes and very rare free small particles.

At densities from 1.44 to 1.48 g/cm³ (Fig. 13A: fractions 18, 19; Fig. 13B: fractions 18, 20) the observed material consists of ribosomal subunits, monoribosomes and free small particles. These latter particles are more frequent than in the preceding fractions. In the density fractions of 1.39 to 1.44 g/cm³ (Fig. 13A; fractions 22, 23; Fig. 13B, fractions 22, to 24) which correspond to the region of mlRNA ·

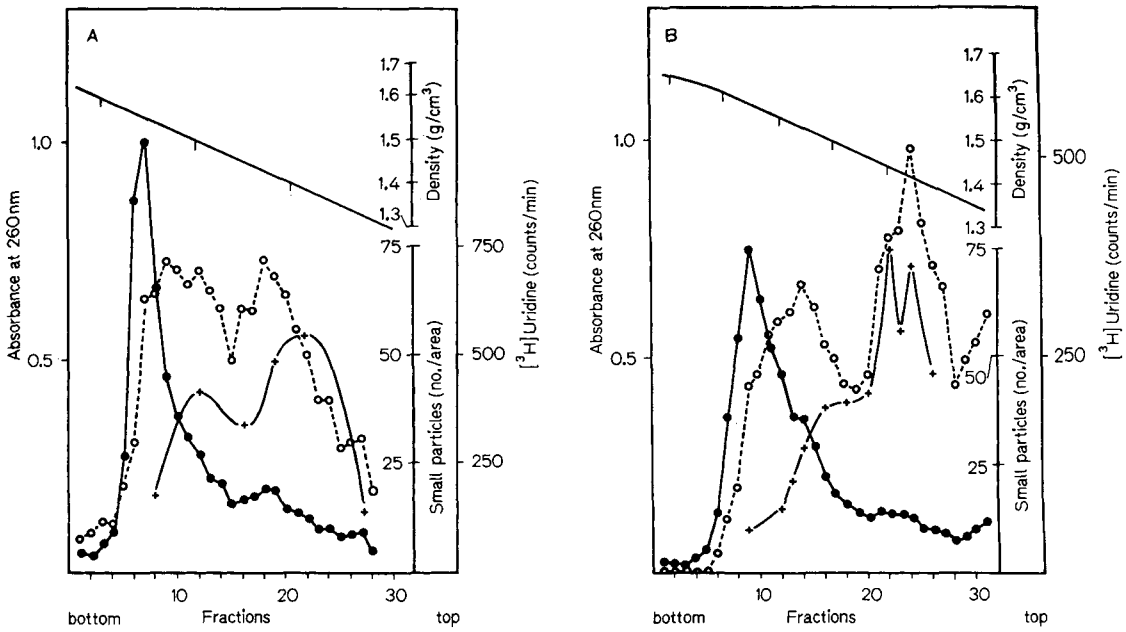

Fig. 13. *Frequency in buoyant density gradients of the small particles detected by electron microscopy.* Cells were labelled for 6 h according to Fig. 9 and the cytoplasmic particle fraction was analysed on CsCl density gradient as described in Methods. (32000 rev./min, 24 h, 2°, Spinco SW 56.) (A) and (B) refer to two different experiments. (●——●), absorbance at 260 nm; (O----O), radioactivity; (+——+), number of small particles detected by electron microscopy

protein complexes as defined in the biochemical experiments, the free small particles are found more frequently than in fractions of higher density or those of lower density. These fractions contain also some more or less degraded ribosomal subunits. The frequency of the free small particles decreases in the following fractions corresponding to $\varrho = 1.33$ to 1.38 g/cm³ (Fig. 13 A: fraction 27; Fig. 13 B, fraction 26) where the zone of pure proteins free of RNA begins.

Since the small particles did not appear as the only material in the mlRNA · protein region of the gradients, an attempt was made to count them on the micrographs and to compare their mean number per electron microscopical area to the pattern of radioactivity. These data are given with some reservation due to the problem of regular distribution on electron microscope grids. Unfortunately, the material was not abundant enough to use the filtration technique which is known to give a better distribution [32].

It can be seen (Fig. 13) that the frequency distribution of the small particles roughly follows the curve of radioactivity. The richest tubes are found in the zone of the labelled mlRNA · and mRNA · protein complexes. It has to be taken into consideration that many small particles attached to polyribosomes may not be seen due to the above mentioned superposition effect or their unwinding into the 35 Å wide strand.

Observation of Pure Polyribosomes

In a pure fraction of polyribosomes isolated on a sucrose gradient some small particles attached to polyribosomes are visible. Free particles are exceptional (Fig. 12 i and 14 a).

When this fraction is treated with EDTA (0.5 or 0.1 μmoles per A_{260} unit, 30 min, 0°) before formaldehyde fixation and preparation for electron microscopy, some free small particles can be detected either by negative staining or by shadowing (Fig. 14 b and c). The ribosomal material is principally in the form of 30 S and 50 S subunits. Some of them present a "tail" (Fig. 14 c) which may be interpreted as an elongated piece of the 35 Å diameter strand which would, after complete condensation, form the small particles by coiling. The "knobs" are clearly visible in the developed 35 Å strand.

After exposure of the polyribosomal fraction to 3 M urea (30 min, 20°), free small particles are detectable (Fig. 14 d). The ribosomal structures are profoundly altered by this treatment presenting extended structures distinctly larger than the tails mentioned above. Thus the small particles seem to be more resistant than ribosomes although their structure has become looser, as can be detected in negatively stained preparations.

DISCUSSION

This investigation of cytoplasmic messenger RNA in animal cells was started in order to find new lines of evidence for the physicochemical reality *in vivo* of messenger-ribonucleoprotein complexes, which have been described by several laboratories [6—10, 23]. Their biochemical characterisation is crucial in view of the role such ribonucleoprotein associations may play in messenger RNA stability, transport, function, and possibly regulation.

In the following we will discuss some of the experimental evidence and present our conclusions.

Control of Artificial RNA-Protein Association

The artificial association of proteins with RNA observed first by Girard and Baltimore [11] and recently by Baltimore and Huang [34] constitutes a major argument which could cast doubt on the reality and significance of the mRNA · protein complexes observed *in vivo*.

Perry and Kelly [7] demonstrated that purified mlRNA did not bind cytoplasmic proteins in L cells. However artificial associations were observed under our conditions in HeLa cells (*cf.* Fig. 4) in agreement with Baltimore and Huang [34]. We did not observe the formation of a specific band at $\varrho = 1.45$ g/cm³ which Spirin [23] demonstrated to form in particle free cytoplasmic extracts mixed with purified RNA, but found rather a non-specific absorption of RNA to preexisting structures across the zone of active polyribosomes and mlRNA · protein particles.

Two considerations should be taken into account:

a) If mRNA · protein complexes play a physiological role in the cell, then the formation of ribonucleoprotein complexes upon mixing of their constituents must be expected *a priori*. The extent of their formation on addition of purified RNA to a lysate or particle fraction will depend on the availability of the corresponding proteins and their binding equilibrium, *i. e.* the physicochemical stability of the complex.

b) If some cytoplasmic proteins may bind purified RNA it does not follow necessarily that ribonucleoprotein particles already present will bind RNA to the same extent and with equal stability. The two phenomena may be of very different nature. The resistance of native mRNA · protein particles to EDTA or to 3 M urea (a treatment which destroys ribosomes) speaks in favour of a relatively tight binding of the protein in the complex, a situation that may not necessarily apply to the absorption of externally added RNA.

Thus the problem is to develop a biochemical distinction between the native particle and a non-specific absorption of RNA.

Such an experimental distinction may be derived from the specific time dependent pattern of syn-

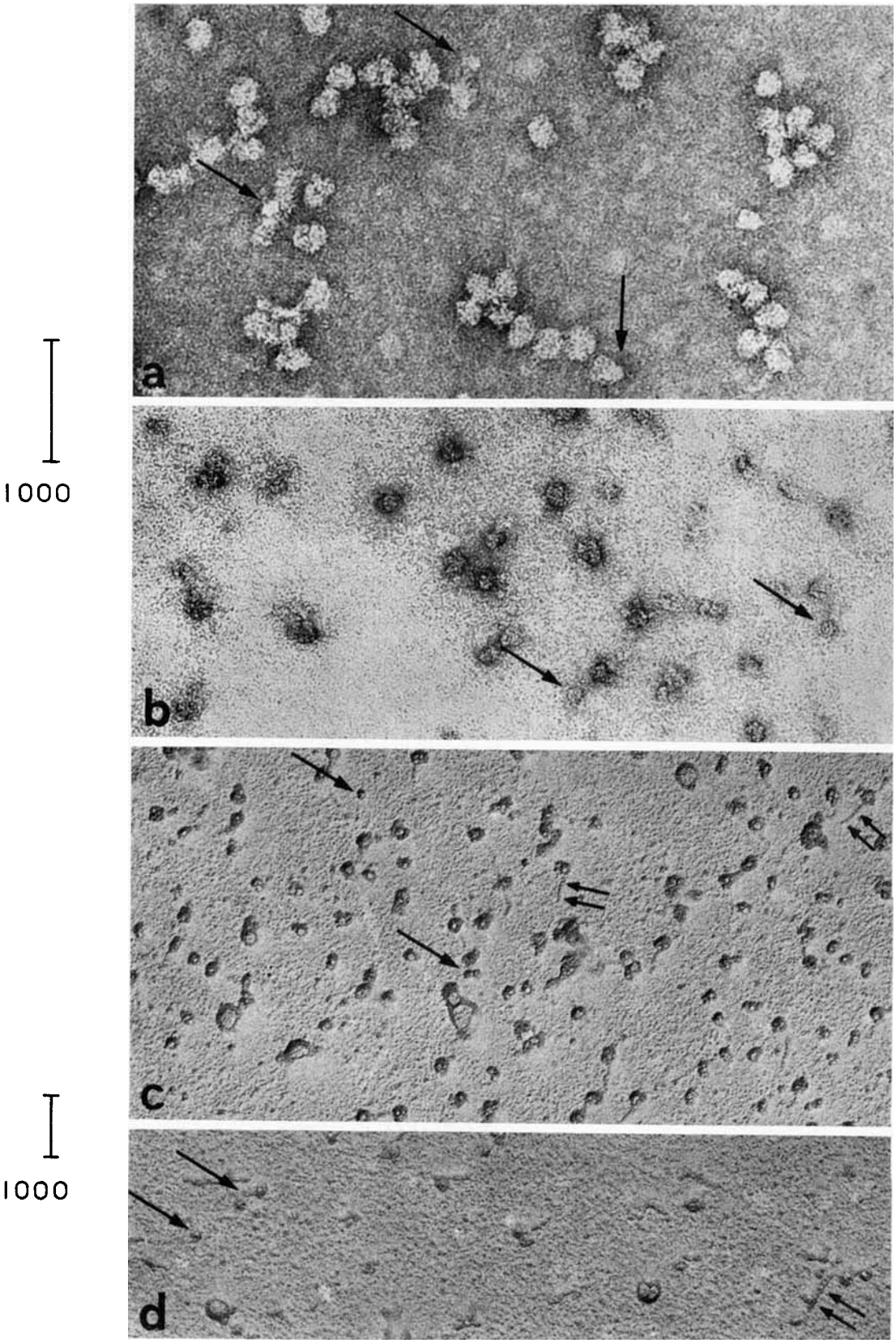

Fig. 14a–d

thesis of two distinct types of mRNA · protein particles. Furthermore the demonstration of Orchinnikov *et al.* [35] and Henshaw and Loebenstein [36] that unlabelled purified RNA added to the lysis buffer will not reduce the specific activity of the complex labelled *in vivo* shows that a differentiation is possible. All these arguments are in favour of the real existence of mRNA · protein complexes in the cell.

Messenger Characteristics of the RNA in Ribonucleoprotein Particles

One of the critical questions in the interpretation of our experimental results is the attribution of messenger quality to the labelled RNA contained in ribonucleoprotein complexes.

In the absence of a direct test *in vitro* producing a specific protein in a cell free ribosomal system after addition of the specific mRNA, the only RNA to which messenger quality can be attributed is by definition the functional mRNA in the polyribosome. However, a labelled RNA in the polyribosome can only be identified with the messenger if it can be demonstrated that the continuation of protein synthesis depends on formation and attachment to ribosomes of mRNA newly synthesized during the labelling period. This condition is fulfilled in tissue culture-cells, even in a condition of arrested growth, as could be shown in this investigation.

Furthermore the size of mRNA is a crucial feature since, in a given type of cells and assuming a prevalence of monocistronic translation, the mRNA molecules should correspond in size to the types of polypeptide chains being synthesized in this cell.

The observed sedimentation spectrum of mRNA in HeLa cells is in agreement with the expectation: the vast majority of polyribosomal labelled mRNA molecules corresponds to molecular weights of 0.1 to 1.0×10^6 daltons and thus to polypeptide chains of 10000 to 100000 daltons, prevalent in animal cells.

It may be pointed out that essentially no mRNA or mlRNA sedimenting more rapidly than 35 S could be found in polyribosomes. The presence of RNA with higher molecular weights was correlated with the occurrence of nuclear contamination.

The attribution of messenger quality to the RNA contained in the free mlRNA · protein particles is much more ambigous than the identification of the mRNA in polyribosomes. Therefore we classify this RNA as messenger-like in order to distinguish it from true mRNA. However several arguments can be brought forward favouring the presence of true messenger in free mlRNA · protein particles. (a) As will be shown elsewhere [24] biological activity can be detected in subribosomal fractions, free of polyribosomes and ribosome bound mRNA · protein, by incubation *in vivo* under the conditions for cell-free protein synthesis. (b) By competitive hybridization we could show that common sequences must exist in labelled RNA extracted from polyribosomes and from free mlRNA · protein particles [25]. (c) The spectrum of mRNA sizes is essentially the same in polyribosomes and in free mlRNA · protein complexes (*cf.* Fig. 6). (d) In pulse chase experiments a fraction of the RNA in free mlRNA · protein can be chased into polyribosomes.

We may thus conclude that true mRNA is present both in polyribosome bound mRNA · protein and to some extent in free mlRNA · protein particles.

The Ribonucleoprotein Complex and Messenger RNA Transport

The results presented in this paper also permit some conclusions relative to the mechanism of messenger RNA transport. It was suggested that mRNA is transported associated with 45 S native ribosomal subunits [21, 37, 38] or with incomplete ribosomal particles [39]. Sidebottom and Harris [22] proposed recently that nucleolar ribosome formation was essential for mRNA transport. Spirin and coworkers [40] showed on the contrary that newly synthesized vaccinia virus-specific mRNA has all the characteristics of a free mlRNA · protein complex and is not associated with polyribosomes.

Our experimental evidence supports Spirin's model by contradicting the existence of a linkage between messenger transport and the transfer of newly synthesized ribosomal material into the cytoplasm:

a) Protein synthesis and transfer of mRNA to the cytoplasm and into polyribosomes proceeds in the absence of rRNA synthesis; this was arrested in our experiment by a low dose of actinomycin D as visible in Fig. 2C and D.

b) The labelled mlRNA unattached to polyribosomes sediments in the form of ribonucleoprotein complexes which spread over the entire 10 S to 80 S zone of a sucrose gradient (*cf.* Fig. 5D, E). EDTA does not influence this pattern. The presence of labelled material in the 50 S or in the 30 S sedimentation

Fig. 14. *Polyribosomal fraction isolated from a sucrose gradient and treated with EDTA or urea.* (a) Negatively stained sample of a polyribosomal sucrose gradient fraction (→) small particles attached to polyribosomes (Magnification: 200000×). (b, c) Same preparation of polyribosomes treated with EDTA (20 μmoles/mg, 30 min, 0°) before formaldehyde fixation. The polyribosomal structure is destroyed; only monoribosomes and ribosomal subunits are present. On shadow-casted preparations some of them present a "tail" (⇉); (→) free small particles. (Magnification: (b) 200000×; (c) 100000×). (d) Same preparation of polyribosomes as in (a) treated with 3 M urea (30 min 20°) before formaldehyde fixation: Destruction of the polyribosomal structure. Alterations in the morphological appearance of ribosomal material. (→) Free small particles; tails (⇉) (Magnification: 100000×)

zone is due to a fortuitous superposition effect. Furthermore, EDTA treatment, which breaks up the mRNA-ribosome association (*cf.* Fig. 7 A and D) does not alter the density distribution of free mlRNA · protein particles (*cf.* Fig. 7 C and F). Thus no association of free mlRNA · protein with ribosomal material could be observed.

The presence of newly synthesized ribosomal 28 S and 18 S RNA in a CsCl gradient at densities below 1.50 g/cm³ shown by Lissitzky *et al.* [39] to be associated mainly with the "clarification pellet" of redissolved cytoplasmic particles may be due to denatured and agglomerated ribosomal material. We could observe such material in the electron microscope along with the mlRNA · protein particles in the density zone of 1.40—1.45 g/cm³.

It may be concluded that cytoplasmic mlRNA is transported in the form of a ribonucleoprotein complex as proposed by Spirin *et al.* [14]. Ribosome synthesis is not necessary for mRNA transport.

However, there is a limitation to this conclusion. We cannot exclude experimentally whether preexisting ribosomal subunits of a nuclear pool may play a role in a very short lived, transient phase [41], nor that ribosome formation may be essential for mRNA transfer during a phase of regulational adaptation.

The Visualisation of mRNA · Protein Particles

The electron microscopical investigation presented here in a preliminary form and which will be extended in a forthcoming paper [42], reveals the presence of small rounded particles in cytoplasmic fractions containing ribosomal material, like polyribosomes, monoribosomes or ribosomal subunits. The detailed morphological appearance and their size is variable. The electron microscopical pictures suggest a spectrum of morphological variations from the smallest type (110 Å diameter) with a central hole or excentric cleft, to the largest (more that 200 Å in diameter) which seems to be full and more electron dense; this form is the most frequent found in HeLa cells. These variations can be attributed to differences in the length of the 35 Å wide strand which forms the rounded particles by coiling.

A possible interpretation of the structure seen is that the messenger ribonucleoprotein strand seen in *polyribosomes* (*cf.* Fig. 12 i), upon complete liberation from (or in between) ribosomes, would coil up into a random coil and be fixed by formaldehyde as the spherical particles we observe.

However, we cannot exclude at this stage of the investigation that some different biological materials may give similar images. In this respect the ultrastructural resemblance of the smallest particle having a central hole with RNA polymerase [43, 44] or a contaminant of RNA polymerase preparations [45] should be noted. These enzymes, which however, should not occur in quantity in cytoplasmic extracts, are more homogenous in size and rod shaped rather than spherical. This morphological feature generally leads to the stacking of individual molecules, an effect which we could never observe in our preparations.

The possibility of a viral contamination can be excluded by considerations of morphology, size and cellular localisation.

Ferritin molecules resembles closely the smallest of the structures we observed and it cannot fully be excluded that some of the small particles shown (Fig. 12 a, b) may be ferritin indeed. However ferritin molecules are more homogenous in size (about 100 Å in diameter) whereas the full particles we describe have diameters of up to more than 200 Å. They are also less regular in shape (ferritin appears often rectangular) and we never observed the typical tetrad structure. A further consideration is that, although in HeLa cells ferritin exists, it could not be demonstrated in our preparations.

Similar small particles can be observed in electron micrographs illustrating earlier papers on ribosomal subunits [46] or polyribosomes from mammalian cells [47] but no description of these particles was given. Slayter *et al.* [48] mention the presence of particles 100 Å in diameter in rabbit reticulocyte polyribosomes without giving any identification.

Considering the detailed description of polyribosomes, monoribosomes and ribosomal subunits given by Shelton and Kuff [49] the morphological appearance of the small particles with diameters ranging from 110 Å to 200 Å is easy to distinguish from ribosomal material: the 50 S ribosomal subunit has a diameter of 270 Å and the 30 S subunit is ellipsoidal with axes 270 Å and 150 Å in length (Fig. 12 g).

The difficulties encountered in seeing mRNA · protein particles associated with polyribosomal structures may have two explanation. (a) Many may be masked because of their small size by a superposition effect with ribosomes in negative staining as well as after shadowing. (b) The partial or complete uncoiling of the 35 Å wide strand when engaged in polyribosomes is another interesting and suggestive possibility for the difficulty of their identification in the active complex of translation. In this respect it should be recalled that they could be identified again as free particles if the polyribosomal fraction was treated with EDTA or 3 M urea.

In summary we consider the electron microscopical evidence as preliminary only and suggestive but in no way conclusive. The morphological identity of the free mlRNA · protein complex remains to be proven.

The Role of mRNA · and mlRNA · Protein Complexes

The essential result of this investigation is the finding that messenger RNA in the cytoplasm of

HeLa cells is present in the form of ribonucleoprotein complexes. This observation is in agreement with those of Perry and Kelly [7] in L cells, of Henshaw [8] in liver, of Burny *et al.* [9] in rabbit reticulocytes and of Cartouzou *et al.* [10] in thyroid tissue, that mRNA in polyribosomes of animal cells is associated with protein.

Furthermore, our results are in agreement with those of Spirin and coworkers [6, 14, 23] and of Kafatos [50] concerning the existence of free ribonucleoprotein particles containing mlRNA in the cytoplasm of developing embryos, of tissue culture cells and of insects. Our findings support the "Informosome" model [14, 23] of cytoplasmic mRNA transport and fit into the general scheme of "cascade regulation" in animal cells [3, 4, 51].

Such a mechanism calls for modulating agents which could confer to mRNA specificity in respect to stability, transport and the capability to attach to ribosomal subnits in the process of initiation of protein synthesis. It is evident that protein molecules would be ideal for such a mechanism in view of their capability to interact with macromolecules like DNA, RNA and the constituents of cell membranes, as well as with small molecular weight effectors inducing allosteric transitions. Samarina *et al.* [5, 26] and others [53, 54], have shown that nascent mlRNA in the nucleus is already associated with proteins. Much of these ribonucleoprotein complexes, which exist also in HeLa cells [12, 55], remain in the nucleus [52, 56]. However, some of them are transferred to the cytoplasm constituting the pool of free mlRNA · protein particles described in this investigation.

This transfer from the nucleus to the cytoplasm is paralleled by an apparent reduction in size of the giant mlRNA molecules: although we can detect in the nucleus of HeLa cells, under the labelling conditions used during this investigation, a majority of mlRNA molecules in a broad sedimentation zone from 28 S up to an estimated 150 S (as will be shown elsewhere [52]) we never could detect in the cytoplasm appreciable amounts of RNA with sedimentation constants of more than 35 S. Since the appearance of heavier molecules in the cytoplasm could be correlated always with nuclear leakage, we have to consider that, at least in HeLa cells, the "non-translatable, pre-ribosomal informosomes" described by Spirin [23] as co-sedimenting with polyribosomes might be of nuclear origin.

However we found during this investigation that non-translatable, messenger-like ribonucleoprotein complexes indeed do exist in the cytoplasm of HeLa cells and contain mlRNA spread over sedimentation zones from 6 to 30 S, comparable thus in size to the mRNA in polyribosomes. 40 to 60 % of the cytoplasmic free mlRNA does not associate with ribosomes in steady state: only a small fraction of this free pool can be chased into polyribosomes. The majority of free mlRNA · protein complexes decays as free particles at the same rate as the polyribosomal mRNA · protein complexes. This average rate of decay is not exponential in time and shows a kinetic heterogeneity for the mlRNA · protein particles as well as the polyribosomal mRNA · protein particles. We can conclude that they disappear with half-lives of about 1.5 h for the rapidly decaying and of up to about 6 h for the more stable fractions.

We propose that the stability of the translatable messenger RNA and of the non-translated messenger-like RNA of the free pool is an intrinsic property of the ribonucleoprotein complex. The stability of an mRNA may thus be a function of the equilibrium constant of an RNA—protein association which is in dynamic equilibrium with its constituents. According to such a model, every protecting protein molecule would dissociate from the complex with a given statistical frequency. The size of the non-protected area and the duration of the periods of non-protection against hydrolytic or enzymatic attacks would determine the life span of an RNA molecule of specific sequence. Local nucleotide sequences are the basis of regions with specific secondary structure and would determine *per se* or by a process of "induced fit" the binding of the protein molecules.

The same kind of consideration may be justified in regard to the activation or inactivation of an mRNA: in fact, the binding constant of the protein moiety in the mRNA · protein complex might be allosterically influenced by cytoplasmic agents. Translational regulation could be understood assuming that the binding energy of this protein would be in competition with the energy provided by the translational system for translocation since ribosomes moving along the mRNA would have to displace temporarily the protein from the complex.

In fact it is a surprising finding that about 50 % of mlRNA in the cytoplasm does not enter the translation process. This amount may possibly be explained by the low actinomycin dose used for this investigation. However, since the rate of protein synthesis is affected less, such an explanation cannot account for the amount of free mRNA. Thus a more physiological interpretation of the equilibrium between ribonucleoprotein complexes of mRNA and mlRNA may be justified. Further work will have to be done in order to determine the actual equilibrium between these complexes under normal conditions.

In embryonic tissue large amounts of maternal messenger RNA are present which had been completely "masked" in the oocyte prior to fertilisation [57]. They may account for the large amount of non-translatable mRNA in early development. The large fraction of inactivated messenger ribonucleoprotein in tissue culture cells points to the possibility that a more general system of regulation not limited to developing systems may be involved. It would ex-

plain many of the experimental facts brought forward by Tomkins *et al.* [58] and others in favour of post-transcriptional controls operating in animal protein synthesis, possibly within the frame of a multilevel regulational system [51].

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