The Fate of Heterogeneous Nuclear Ribonucleoprotein Complexes during Mitosis*

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Using immunochemical techniques, we have examined the macromolecular state of association of the major heterogeneous nuclear ribonucleoprotein (hnRNP) core proteins in mitotic HeLa cells. We find that these proteins are not free but are associated with high-molecular-weight RNA in the form of particles that sediment as a broad band between 80 and 200 S. We have termed these complexes MhnRNP for mitotic hnRNP protein-containing particles. Their quantity, composition, sedimentation coefficients, buoyant density, and sensitivity to dissociating conditions suggest that they are closely related to the MhnRNP complexes of interphase cells and may represent hnRNP complexes containing unprocessed or partially processed heterogeneous nuclear RNA that have been released into the cytoplasm during mitosis. Exogenously added RNA does not associate with the MhnRNP nor does it compete for the major MhnRNP proteins. The MhnRNP remain distinct from other ribonucleoprotein complexes and do not associate with ribosomes even though these structures are not separated by a nuclear envelope during mitosis.

During mitosis, cells undergo drastic alterations in both morphology and metabolic activity. Among these, several dramatic changes in RNA metabolism take place. The synthesis of both hnRNA1 and rRNA is completely inhibited although 5 and 4 S RNAs apparently continue to be synthesized (Zyliber and Penman, 1971), rRNA processing is inhibited (Fan and Penman, 1971; Gelfand and Smith, 1983), and the rate of translation is reduced to about 30% of the average interphase rate, probably because of a block in initiation (Fan and Penman, 1976; Tarnowka and Begliomini, 1979). During mitosis, rRNA that is synthesized immediately before cell division remains in an unprocessed form, associated with the chromosomes but in an altered ribonucleoprotein structure, until it returns to the nucleus during late telophase (Fan and Penman, 1971; Abramova and Neyfakh, 1973; Gelfand and Smith, 1983). Little, however, is known about the metabolism of hnRNA during mitosis.

During interphase, hnRNA associates, as it is being synthesized, with a set of proteins to form hnRNP complexes (Beyer et al., 1981). Although the exact composition of the hnRNP particles is not known, a set of proteins with molecular weights between 30,000 and 45,000 accounts for most of the protein mass. The major hnRNPs from HeLa cells can be resolved into six bands by SDS-polyacrylamide gel electrophoresis (Beyer et al., 1977) and a larger number by two-dimensional separations (Peters and Comings, 1980). These proteins are probably closely related; they cross-react immunologically, even when monoclonal antibodies are used (Leser et al., 1984), and peptide-mapping experiments suggest that the multiple species arise from post-translational modifications of a smaller set of precursor proteins (Fuchs et al., 1980). The hnRNP proteins of widely divergent species also cross-react immunologically, suggesting that they are evolutionarily conserved (Leser et al., 1984). No enzymatic function for the major core proteins has been identified although a number of enzymatic activities are associated with the hnRNP complexes. Rather, these proteins probably function to package the hnRNA. The best characterized hnRNP protein is HD40, the major hnRNP protein of the brine shrimp Artemia. This protein binds to RNA, unwinding most of its secondary structure, and then folds the unwound RNA into a somewhat fluid "beads-on-a-string" structure (Thomas et al., 1983).

In interphase cells, the hnRNP proteins are, as shown by immunofluorescence, restricted to the nucleus (Jones et al., 1980), the mature mRNA being associated with a different set of proteins to form cytoplasmic mRNP complexes. As cells enter mitosis, the hnRNP proteins distribute throughout the cell (but are not associated with chromosomes), returning to the nucleus as it reforms during telophase (Martin and Oka- muru, 1981). Very little, however, is known about the molecular state of association of these proteins or the fate of hnRNA in mitotic cells. In this report, we explore these problems and show that during mitosis the hnRNP core proteins are stably associated with high-molecular-weight RNA in the form of complexes that we have termed MhnRNP.

EXPERIMENTAL PROCEDURES

RESULTS

Sedimentation Properties of Mitotic hnRNP Proteins—By using a sensitive immunochemical assay system (ELISA) that is described in the Miniprint Section, we have investigated...
the sedimentation properties of the major hnRNP proteins in mitotic cells. When mitotic cell extracts are prepared by gentle homogenization of hypotonically swollen cells (80-90% lysis), we find that most of the unlysed cells are nucleated. Appar-ently, gentle lysis preferentially disrupts the mitotic cells, in effect increasing the mitotic index. Unlysed cells, nuclei from nonmitotic cells, chromatin, and other cellular debris are removed by low-speed centrifugation and the supernatant fractionated by centrifugation through sucrose gradients. As shown in Fig. 3A, the hnRNP proteins, as detected by ELISA, sediment in a broad band ranging from about 80 to about 200 S, centered at about 130 S with very few core hnRNP proteins remaining at the top of the gradient. Thus, during mitosis very few, if any, of the hnRNP proteins are present as free proteins. Rather, they are associated with large macromolecular complexes. We refer to these complexes as MhnRNP for mitotic hnRNP protein-containing particles. Electrophoretic analysis of the rapidly sedimenting material followed by immunochemical detection of the proteins after transfer to a nitrocellulose sheet gives a qualitative, but not quantitative, picture of which hnRNP core proteins are associated with the MhnRNP. Fig. 3, C and D, shows that most of the 30,000-45,000 molecular weight core hnRNP proteins sediment together with the complexes. The A and C proteins are clearly seen, and B1 can be occasionally seen on the transfers. B2 is not detected by the antibodies used (Fig. 1D). When unsynchronized cells (which are about 98% nonmitotic) or unsynchronized cells treated with colcemid are treated in the same way, no immunologically cross-reacting material is found in the cytoplasmic supernatant (not shown); it all remains associated with the nuclear pellet. The MhnRNP are, therefore, not simply contaminants derived from the nuclei of the 10-15% nonmitotic cells in the preparation.

MhnRNP Contain High-molecular-weight RNA—The MhnRNP are sensitive to ribonuclease digestion. When a mitotic cell extract is treated with ribonuclease, the hnRNP proteins are no longer seen as rapidly sedimenting structures but remain at the top of the sucrose gradient (Fig. 3B).

Immunochernical detection of proteins transferred to nitrocellulose from SDS-12% polyacrylamide gels or of gradient fractions applied directly to nitrocellulose sheets (not shown) also give no indication of any hnRNP proteins sedimenting at 80-200 S; the hnRNP proteins are all found at the top of the gradient. These results suggest that the MhnRNP contain RNA and that their structure is dependent on the presence of this RNA.

The RNA that is associated with the MhnRNP has been analyzed by immunoprecipitating the fractions from a sucrose gradient of a mitotic HeLa cell extract, isolating the RNA by phenol/chloroform/isoamyl alcohol extraction, and separating the RNAs by agarose gel electrophoresis. As shown in Fig. 4, the RNA in the MhnRNP that sediments faster than about 80 S is mostly high-molecular-weight RNA. Based on a long extrapolation of the mobilities of MS2 RNA, 23 S rRNA, and 16 S rRNA, we estimate that most of the RNA is in the 6,000-10,000 nucleotide size range. The average size of the RNA is somewhat larger in the more rapidly sedimenting complexes. The fact that the antibodies do not precipitate ribosomes (as judged by the absence of 18 and 28 S RNA in the gel of Fig. 5) suggests that the RNA is not rRNA or rRNA precursors. The fractions that sediment at about 60 S contain a large amount of much smaller RNA, ranging in size from about 200 to 1000 nucleotides, with some degree of periodicity in the size distribution being evident. Since this material is sensitive to RNase A, these bands are not DNA contaminants. It is possible that these smaller particles may result from limited nucleolytic digestion of the larger complexes. Supporting this argument is the observation that when cytoplasmic extracts are allowed to stand, the average sedimentation coefficient of the complexes gradually decreases with time to a sedimentation coefficient of about 40 S.\(^3\)  

Two experiments show that the RNA found in the MhnRNP complexes does not result simply from a fortuitous association of the hnRNP proteins with free RNA during cell lysis. When the mitotic cell extract is treated with ribonuclease, the hnRNP proteins are no longer seen as rapidly sedimenting structures but remain at the top of the sucrose gradient (Fig. 3B).

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\(^3\) D. K. Lahiri and J. O. Thomas, unpublished observation.
MS2 RNA
23S RNA
16S RNA

tRNA

Fig. 4. Analysis of RNA from MhnRNP complexes. A mitotic HeLa cell extract was centrifuged through a sucrose gradient. Fractions with the indicated $s_{20,w}$ were immunoprecipitated with RNase-free IgG (300 pg/ml) that was purified from anti-hnRNP serum. The IgG did not precipitate free RNA. RNA was isolated from the immunoprecipitates and analyzed on a 1.2% agarose-formaldehyde gel. Experimental details are given under "Experimental Procedures".

Fig. 5. Sedimentation of $[^{3}H]$hnRNA in the presence of MhnRNP. $[^{3}H]$Uridine-labeled hnRNA (specific activity about 700,000 cpm/μg), obtained from a HeLa cell nuclear extract, was added to mitotic HeLa cells immediately before homogenization and centrifugation as described under "Experimental Procedures." $[^{3}H]$hnRNA; O—O, ELISA; (-----), $A_{260}$.

During homogenization of the cells, then tRNA-hnRNP protein complexes should be formed since there is very little specificity in the nucleic acid-binding properties of the major hnRNP proteins (Wilk et al., 1983; Pullman and Martin, 1983; Nowak et al., 1980). Because of the small size of the tRNA, these complexes would be expected to sediment near the top of the gradient. This clearly does not occur. Nor does the tRNA associate with the rapidly sedimenting MhnRNP complexes. This is demonstrated by polyacrylamide gel electrophoresis of RNA isolated from the gradient fractions (Fig. 6B) which shows that the tRNA remains at the top of the gradient. There are several discrete bands of RNA that sediment throughout the gradient. These discrete RNAs are easily distinguished from the added tRNA which is heterogeneous in size. The discrete RNA bands are also present in the absence of added tRNA (not shown). These experiments show that exogenously added free tRNA neither associates with MhnRNP complexes nor competes with the MhnRNP for the major hnRNP proteins.

Fig. 6. Sedimentation of MhnRNP in the presence of added tRNA. E. coli tRNA (50 μg/5 x $10^{7}$ cells in 1 ml) was added to mitotic HeLa cells immediately before homogenization and centrifugation as described under "Experimental Procedures." A, ELISA (O—O) and $A_{260}$ (-----); B, RNA separated on a 10% polyacrylamide-urea gel.
**MhnRNP Are Distinct from Other Cytoplasmic RNPs**—Although the MhnRNP co-sediment with polysomes, they are probably not associated with them. As shown in Fig. 7A, the presence of 20 mM EDTA completely dissociates the 80 S ribosomes and polysomes to subunits but does not dissociate the MhnRNP complexes. The complexes are, however, sensitive to dissociation by 0.5% sodium deoxycholate (Fig. 7B), which has no effect on the sedimentation of ribosomes but does dissociate hnRNP complexes (Pullman and Martin, 1983). Elevated concentrations of sodium chloride also lead to the dissociation of MhnRNP (Fig. 7C). Others have shown that sodium chloride causes a progressive dissociation of hnRNP complexes at concentrations between 0.4 and 1 M (Beyer et al., 1977; Stevenin and Jacob, 1974). The effects of salt on the sedimentation of hnRNP complexes are similar to the effects on MhnRNP complexes that are reported here.

The buoyant density of the MhnRNP complexes is also distinct from that of ribosomes and polysomes. The peak MhnRNP-containing fractions of a sucrose gradient such as the one shown in Fig. 3 were pooled, fixed with 5% glutaraldehyde, mixed with CsCl to an average density of 1.35 g/ml, and centrifuged to near equilibrium. As seen in Fig. 8, the MhnRNP have a rather broad range of buoyant densities, averaging about 1.45 g/ml. This is slightly more dense (and the peak is broader) than hnRNP particles prepared by nuclear pH 8 extraction that were analyzed at the same time in a similar gradient. An examination of MhnRNP fractions having different sedimentation coefficients (not shown) does not show any appreciable differences in buoyant density between the slower and more rapidly sedimenting complexes. The density of the MhnRNP is significantly less than that of polysomes and ribosomes, but comparable to that of free cytoplasmic mRNP, mRNP released from polysomes by EDTA, and hnRNP (Preobrazhensky and Spirin, 1974). The peaks of interphase cells. The heterogeneous sedimentation properties of the MhnRNP to refer to mitotic hnRNP protein-containing particles. The physical properties of the MhnRNP suggest that they are closely related to the hnRNP particles of interphase cells. The heterogeneous sedimentation properties of the MhnRNP, averaging about 130 S, are similar to those of hnRNP particles obtained by sonication of nuclei.

**FIG. 7. The effect of dissociating solvent conditions on the sedimentation of MhnRNP complexes.** A, EDTA (20 mM) was added to a mitotic HeLa cell extract and centrifuged through a sucrose gradient containing 20 mM EDTA as otherwise described under "Experimental Procedures." B, EDTA (20 mM) was added to a mitotic HeLa cell extract and centrifuged through a sucrose gradient containing 20 mM EDTA as otherwise described under "Experimental Procedures." C, sodium deoxycholate (0.5%) was added to a mitotic HeLa cell extract and centrifuged through a sucrose gradient (not containing deoxycholate) as described under "Experimental Procedures" and analyzed by ELISA (●), and A~w (----). D, C, sodium chloride, 0.5 M (O--O), 0.75 M (●—●), was added or not (C——C) to portions of a mitotic HeLa cell extract, centrifuged through sucrose gradients containing the same concentration of NaCl but as otherwise described under "Experimental Procedures," and analyzed by ELISA.

**FIG. 8. Buoyant density of MhnRNP complexes.** The peak MhnRNP fractions of a sucrose gradient such as the one shown in Fig. 3A were pooled, dialyzed against 10 mM Tris, pH 7.4, 1.5 mM MgCl₂, 10 mM NaCl, and fixed for 15 min at 4 °C by the addition of bicarbonate-neutralized (pH 7.4) glutaraldehyde to a final concentration of 5%. Solid CsCl was added to a final density of 1.35 g/ml, and the samples were centrifuged for 40 h at 35,000 rpm in a Beckman SW 50.1 rotor at 4 °C. An identical experiment was done at the same time with hnRNP obtained from a HeLa cell nuclear pH 8 extract (Samarina et al., 1968) partially purified on a sucrose gradient. Fractions were analyzed by ELISA. ———MhnRNP; ——C, hnRNP; ————, density.
from unsynchronized cells (Pederson, 1974). They both appear to be sensitive to limited RNA digestion which gives rise to 40 S particles (Samarina et al., 1968; Walker et al., 1980), and their sensitivity to dissociating conditions is similar. The MhnRNP have a buoyant density in CsCl of 1.45 g/ml. This is close to that of hnRNP (Pederson, 1974) and corresponds to a protein:RNA ratio of about 4:1. The exact relationship between MhnRNP and hnRNP in terms of composition and metabolic activity, however, remains to be established.

From the ELISA we estimate that there is about 14 μg of MhnRNP in 10⁵ cells or about 2.8 μg of RNA. This is roughly the same as the amount of hnRNA that is present in this number of interphase cells (Pederson, 1974). This suggests that the RNA that is present in the nucleus prior to the onset of cell division is retained in the cell in the form of MhnRNP during mitosis, and its size suggests that it is largely unprocessed. The 45 and 32 S RNA precursors are also present in mitotic cells in roughly the same amount as in interphase cells, and their processing is inhibited during mitosis but resumes as cell division is completed (Fan and Penman, 1971; Gelfand and Smith, 1983).

Even after the addition of [3H]hnRNA or large amounts of tRNA, the hnRNP proteins do not exchange but remain associated with the MhnRNP complexes. The stability of these complexes may be of functional significance to the mitotic cell. During interphase, the hnRNP proteins are restricted to the nucleus while cytoplasmic mRNAs are associated with different sets of proteins to form mRNP complexes. During mitosis, however, there is no physical or gross spatial compartmentalization of the nuclear and cytoplasmic components. It is possible that the hnRNAs and mRNAs may be distinguished by the proteins with which they associate, a molecular compartmentalization provided by their associated proteins. In this capacity, the MhnRNP proteins might assist in the return of the hnRNA to the nucleus during telophase and may function to inhibit the interaction of hnRNA with the machinery of protein synthesis. The tight association of the RNA with the hnRNP proteins in these complexes also suggests that the exchange of hnRNP and mRNP proteins that accompanies the translocation of RNA from the nucleus to the cytoplasm in interphase cells must be a catalyzed, and perhaps energy-requiring, process.

In mitotic cells, only a small fraction of the RNA is actively translated (Fan and Penman, 1970), probably because of a block of the initiation of translation (Tarnowska and Baglioli, 1979). Using CsCl density gradient centrifugation, Fan and Penman (1970) analyzed the rapidly labeled RNA that sediments with polysomes and found that a small portion of the RNA bands at 1.54 g/ml, the density of ribosomes and polysomes, but the majority bands at a lower density. They speculated that this low-density RNP is of nuclear origin. Our results, which show that the MhnRNP complexes sediment in the polysomal region of a sucrose gradient and have a buoyant density in CsCl of about 1.45 g/ml, support this. These observations, along with the insensitivity of the MhnRNP to dissociation by EDTA, suggest that a substantial portion of the rapidly labeled RNA is present in the form of MhnRNP complexes that do not associate with ribosomes.

It is likely that an examination of the properties of MhnRNP complexes will provide some insight into the structure and metabolism of hnRNA. In the experiments described here, the cells are gently lysed and the cytoplasm layered onto a sucrose gradient after a low-speed centrifugation to remove chromatin and other large structures. It is unlikely that these particles are contaminated by vestiges of nuclear structure such as nuclear matrix. This relatively direct approach to cellular fractionation is considerably different than those used for the isolation of hnRNP complexes from interphase cells which require either sonication of isolated nuclei or extraction of nucleolytically degraded hnRNP particles from intact nuclei and then purification by sucrose gradient centrifugation. We are currently examining the composition, structure, and metabolic properties of the MhnRNP complexes in greater detail.

REFERENCES
Heterogeneous Nuclear Ribonucleoprotein Complexes in Mitosis

Deborah N. Lustrin AND John D. Thomas

EXPERIMENTAL PROCEDURES

Cell Growth and Synchronization

The "glass" with nuclei suspension culture in Julkis medium was supplemented with 18 fetal calf serum (GIBCO). The cells were grown in a double tissue culture dish and then exposed to a xiphid platelet. All cultures were added to a final concentration of 0.6 µM HMDP (殇 and Potter, 1966), and the nuclei suspension culture was obtained by centrifugation at 5000 rpm for 10 min. The cell nuclei on the filter were washed three times with 100 Nagl. The centrifugation of the nuclei was repeated and the nuclei were finally suspended in 5 mL of 0.5 M sucrose cushion. The nuclei suspension culture was centrifuged at 9000 rpm for 10 min.

Chromosomal Mapping

Chromosomes were isolated with the major hnRNP core protein isolated from human brain nuclei. Nuclei were isolated with the method of Brandt and Potter (1966), and the hnRNP core protein was obtained by centrifugation at 5000 rpm for 10 min. The nuclei suspension culture was added to a final concentration of 0.6 µM HMDP (殇 and Potter, 1966). The combined 18 nuclei suspension culture was layered onto a 10-35% (w/v) sucrose gradient and centrifuged for 15 min at 2,000 × g. The 18 nuclei suspension culture was added to a final concentration of 0.6 µM HMDP (殇 and Potter, 1966). The combined 18 nuclei suspension culture was layered onto a 10-35% (w/v) sucrose gradient and centrifuged for 15 min at 2,000 × g. The 18 nuclei suspension culture was added to a final concentration of 0.6 µM HMDP (殇 and Potter, 1966). The combined 18 nuclei suspension culture was layered onto a 10-35% (w/v) sucrose gradient and centrifuged for 15 min at 2,000 × g. 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