Changes in Heterogeneous Nuclear RNP Core Polypeptide Complements during the Cell Cycle

George P. Leser and Terence E. Martin
Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

Abstract. Mammalian heterogeneous nuclear RNP (hnRNP) subcomplexes are shown to be comprised of 14-17 basic A and B core group polypeptides (chrp) when subjected to two-dimensional immunoblot analysis. These proteins are normally confined to the nucleus but are distributed throughout the cell during mitosis. However, not all of the 17 protein spots are observed for all stages of the cell cycle. HeLa cell populations have been synchronized and the basic hnRNP core protein complement examined during S, G2, mitosis, and G1. During cell division several distinct chrp polypeptide species at 35 and 37 kD appear, while another of 37 kD and a chrp of 38 kD are diminished. These altered chrp complements are not due to any effects induced by thymidine treatment but appear to be physiological changes in the chrp polypeptide modification state. The new charge isomers found during mitosis are not the result of selective phosphorylation of the chrp polypeptides. However the nature of the modifications has yet to be determined. The mitosis-specific modified forms of the chrp polypeptides are found in the cytoplasmic fraction derived from mitotic cell populations. When this fraction is centrifuged upon sucrose density gradients the modified chrp polypeptides sediment from 30–200S in a distribution similar to that of hnRNP complexes isolated from the nuclei of randomly dividing cell populations. RNase digestion experiments indicate that the general substructure of the RNA/protein complexes in mitotic cell cytoplasm is similar to that of nuclear hnRNP isolated from unsynchronized cells or tissue.

Ascent heterogeneous nuclear RNA (hnRNA)1 of higher eukaryotes is complexed with a relatively simple set of major protein species (7, 20, 25, 26, 38). The resulting RNP chain or fibril yields, upon mild RNase digestion, subcomplexes that sediment as a relatively homogeneous peak at 30–40S in a sucrose density gradient (3, 25, 33, 38). Ultrastructural analyses of transcriptionally active chromatin are consistent with these biochemical data; Miller spreads of amphibian lambrush chromosomes show nascent hnRNA molecules, still attached to RNA polymerase, associated with protein to form chains of discrete particles of a size similar to that of 30S particles (2, 30). Furthermore, polyclonal antibodies specific for biochemically isolated RNP polypeptides strongly stain the RNP matrix of lampbrush loops in indirect immunofluorescent studies (15, 23, 24). Presumably these RNA:protein complexes serve as substrates for subsequent processing events, including polyadenylation and splicing, to form mature mRNA.

The major heterogeneous nuclear RNP (hnRNP) polypeptides range in molecular weights from 33,000 to 45,000 and number approximately five to seven when examined in one dimension by SDS gel electrophoresis (4, 7, 26). The hnRNP-associated proteins with molecular masses of 33–40 kD are a biochemically and antigenically related subset equivalent to the previously termed A and B proteins, and distinct from the two hnRNP polypeptides in the range of 40–45 kD, the so-called C proteins of Beyer et al. (3; see 18 for discussion). The 33–40 kD subset is termed the core hnRNP polypeptides (chrp). They are themselves sufficient to reconstitute 30S hnRNP substructure, they possess basic isoelectric values, have similar amino acid compositions, and they contain the unusual modified amino acid N2,N6-dimethylarginine (3, 5, 8, 35). When examined by two-dimensional gel electrophoresis as many as 17 chrp species have been detected (19). All except the lower molecular mass chrp (the 34,000-D species for mouse RNP particles) possess long half-lives, comparable to histones and ribosomal proteins (14, 27). They appear to be highly conserved among vertebrates (3, 4, 25). RNP particles isolated from diverse species, including birds, rodents, amphibians, and humans, reveal similarities in the number of chrps of comparable, relatively narrow ranges of molecular weight (3, 4, 19, 25). Most recently, isolation of cDNA clones has begun to yield primary sequence data for this protein group (37, 39).

We have previously characterized monoclonal antibodies formed against embryonic chick liver RNP particles that cross react with chrps from a wide range of vertebrate species (19). Significantly, the majority of our hybridoma clones

1. Abbreviations used in this paper: chrp, core heterogeneous RNP protein; hnRNA, heterogeneous nuclear RNA; hnRNP, heterogeneous nuclear RNP; PLP, periodate-lysine-paraformaldehyde.
Materials and Methods

Secreted immunoglobulins that reacted with all of the chrp polypeptides; in fact, only two of approximately 20 clones examined reacted with a single protein or a subset of the group. An analysis of the hnrnps proteins by two-dimensional immunoblots revealed 13-17 A and B chrp polypeptides sharing common antigenic determinants (19). The cumulative evidence therefore suggests that chrps are the products of a gene family or are posttranslational modifications of a few gene products.

Despite numerous biochemical characterizations of hnRNA-associated proteins from a variety of organisms, comparatively little is known of the state of these polypeptides during the individual phases of the cell cycle. Perhaps most interesting is the period of cell division where nuclear proteins encounter the environment of the cytoplasm. How the cessation of hnRNA synthesis during this time affects the interactions of RNP polypeptides with other core proteins and RNA is not known. Lahiri and Thomas (17) have described a complex containing chrps in cells arrested at metaphase, however, the structural organization of hnrnp complexes during normal mitosis is not clear. Also, the question arises as to whether during mitosis all chrps are found in complex form or if they dissociate into free proteins. Furthermore the associations that chrps maintain could have a bearing on their subsequent return to the nucleus after cell division. Our laboratory has previously used specific polyclonal antisera to determine the cellular distribution of core proteins in interphase and mitosis (23, 24). The antigens, which are restricted to the interphase nucleus, become generally distributed throughout the cell during division and are not associated with any specific structures identifiable at the resolution of the light microscope. These results have been confirmed using hnrnnp protein-specific monoclonal antibodies (19). We have found no differences in the distribution determined by an immunoglobulin reactive with all the core polypeptides or by one selective for a subset of the group (19). In the present study we use a monoclonal antibody specific for all of the basic A and B core chrp polypeptides (i.e., those of 33-40 kD and possessing basic isoelectric points) in a study of the hnrnnp core protein complements in synchronized HeLa cell cultures. The results indicate substantial modifications occur among the core group polypeptides and that the degree of modification changes during the course of the cell cycle.

Sample Preparation, Electrophoresis, and Blotting

Whole cell sonicates were prepared by washing HeLa cells in Earle's balanced salt solution (Gibco), placed briefly in distilled water then re-suspended in 8 mM KCl, 1.5 mM MgCl2, 15 mM Tris-HCl, 0.25 mM sucrose, pH 7.6 (1/10 TKM-0.25 M sucrose). The cells were disrupted in a Dounce homogenizer by eight strokes of a B pestle. After washing the isolated nuclei once in 1/10 TKM-0.25 M sucrose they were washed successively in 0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M MgCl2, pH 7 (STM 7), then washed in the same buffer adjusted to pH 9 (STM 9). Next, nuclei were extracted in STM 9 buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mg/ml apronin with gentle agitation at 0°C for 3-4 h. A low speed centrifugation to remove nuclei, the nuclear pellet was layered onto a 35-ml 15-30% sucrose density gradient prepared with STM 8 (pH 8) and centrifuged for 17 h at 22,000 rpm in a rotor (SW27; Beckman Instruments, Inc., Palo Alto, CA). The gradient fractions containing the 30S peak were collected and either used immediately or pooled and stored at -20°C.

Preparation of hnrnnp Particles from HeLa Cells

The 30S hnrnnp subcomplexes were isolated from HeLa cells by a modification of the procedure of Martin and McCarthy (25). HeLa cells from an exponentially growing cell culture were washed several times with Earle's balanced salt solution (Gibco), placed briefly in distilled water then re-suspended in 8 mM KCl, 1.5 mM MgCl2, 5 mM Tris-HCl, 0.25 mM sucrose, pH 7.6 (1/10 TKM-0.25 M sucrose). The cells were disrupted in a Dounce homogenizer by eight strokes of a B pestle. After washing the isolated nuclei once in 1/10 TKM-0.25 M sucrose they were washed successively in 0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M MgCl2, pH 7 (STM 7), then washed in the same buffer adjusted to pH 9 (STM 9). Next, nuclei were extracted in STM 9 buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mg/ml apronin with gentle agitation at 0°C for 3-4 h. After low speed centrifugation to remove nuclei, the nuclear pellets was layered onto a 35-ml 15-30% sucrose density gradient prepared with STM 8 (pH 8) and centrifuged for 17 h at 22,000 rpm in a rotor (SW27; Beckman Instruments, Inc., Palo Alto, CA). The gradient fractions containing the 30S peak were collected and either used immediately or pooled and stored at -20°C.

Sample Preparation, Electrophoresis, and Blotting

Whole cell sonicates were prepared by washing HeLa cells in Earle's balanced salt solution several times finally resuspending and sonicating the cell pellet in 2% SDS, 5% mercaptoethanol, 1 mM PMSF. The total cellular protein from ~3 x 10⁶ cells was used for two-dimensional protein blot analysis, while a third of that amount was loaded on single dimension SDS gels. In one instance, where cells were collected by the mitotic shake-off procedure, the total cellular protein from 1.5 x 10⁶ cells was used.

Nuclear and cytoplasmic fractionation was accomplished by treating washed HeLa cells with distilled water for 15 min, underlaying the cell suspension with 1/10 volume 5% TKM, 20% sucrose, and then rupturing the swollen nuclei in a Dounce homogenizer with a B pestle; the extent of cell breakage was monitored by phase microscopy. Nuclei were pelleted by low speed centrifugation and the supernatant was retained as the cytoplasmic fraction after the addition of 1 mM PMSF and 10 µg/ml apronin. The nuclei were washed then sonicated in 1/2 TKM, 1 mM PMSF, 10 µg/ml apronin. Human placental RNase inhibitor (Amersham Corp., Arlington Heights, IL) was added to the nuclear and cytoplasmic fractions, except where indicated, at a concentration of 100 U/ml in the presence of 2 mM diithiothreitol. After centrifugation as described above the samples were either loaded onto gradients or saved for electrophoretic analysis. Samples (3 ml) were layered onto 35 ml 15-40% sucrose density gradients prepared with 1/10 TKM and centrifuged for 14 h at 18,000 rpm in a rotor (SW27; Beckman Instruments, Inc.). Gradients were fractionated into 3-ml aliquots which were saved for subsequent immunoblot analysis. Approximately 25 µl of nuclear or the equivalent of 800 µl of cytoplasmic gradient fractions were loaded per lane. In one set of experiments nuclear and cytoplasmic fractions without added RNase inhibitor were treated with RNase A at a concentration of 0.1 and 0.5 µg/ml, respectively. The samples were incubated on ice for 30 min and centrifuged on sucrose density gradients as described above. Where nuclear or cytoplasmic samples were ex-
amined directly the amount loaded on gels was approximately equivalent to the material obtained from $3 \times 10^9$ cells.

Samples examined in a single dimension were separated by electrophoresis on 0.8-mm-thick SDS polyacrylamide slab gels (16). Two-dimensional nonequilibrium pH gradient/SDS electrophoresis was carried out essentially as described by O’Farrell and co-workers (31). Cell fractions or sonicates of whole cells were first separated by electrophoresis on polyacrylamide tube gels made using ampholines with a pH range of 3-10 (Bio-Rad Laboratories, Richmond, CA) for 1,600-2,000 Vh. The second dimension was run on 0.8-mm-thick SDS slab gels. The relative pI range of the hnRNP core polypeptides was determined by directly comparing the electrophoretic mobilities of chrips in nonequilibrium pH gradient gels with that of pI marker proteins (U.S. Biochemical Corp., Cleveland, OH) run on parallel gels under identical conditions. These results were compared with those obtained by extracting the ampholines from slices of a pH gradient gel with distilled water and directly reading the pH with an electrode.

Proteins were electrophoretically transferred onto nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH) as described previously (39). Blots were hydrated briefly in distilled water then incubated for 1-2 h at room temperature in 1% BSA, 0.15 M NaCl, 0.05 M Tris-HCl, 0.005 M EDTA, 0.05% Tween-20, pH 7.4 to block remaining protein-binding sites. Blots were incubated for 2 h in hybridoma culture supernatant, followed by several rinses in 0.15 M NaCl, 0.05 M Tris-HCl, 0.25% gelatin, 0.005 M EDTA, 0.05% Tween-20, pH 7.4 over the period of 1 h. Antibody binding was visualized by incubating blots with 5 μg/ml affinity-purified goat anti-mouse IgG-IgM (Jackson Immuno Research Laboratories, Inc., Avondale, PA) directly labeled with [14C]formaldehyde (ICN Biomedicals Inc., Irvine, CA) by the method of Dottario-Martin and Ravel (9). Blots were washed with three changes of gelatin buffer over 1 h, rinsed three times with distilled water, and air-dried. The nitrocellulose blots were exposed to Kodak X-ray film for 1-5 d. Blots used for direct comparisons were reacted simultaneously using the same antibody solutions; they were exposed the same length of time, and where practical, upon the same sheet of film.

**Indirect Immunofluorescent Studies of Synchronized HeLa Cells**

Aliquots of HeLa cell spinner cultures were taken at the indicated time points and placed on poly-L-lysine-coated glass coverslips and incubated at room temperature for 15 min. After three washes with PBS the cells were fixed with periodate-lysine-parafformaldehyde (PLP) fixative (29) for 15 min. Alternatively some coverslip preparations were fixed with 4% formaldehyde in acetone for 15 min. Then the coverslips were washed several times with PBS and stored at 4°C in PBS containing azide. Many of the antibodies used in this study are of the IgM subclass (39), and the preparation of small, immunologically active fragments proved necessary to ensure even and reproducible penetration into all compartments of cells fixed with the PLP fixative. This was accomplished by a mild tryptic digestion of monoclonal antibodies fractionated from ascitic fluid (28). Before staining, PLP-fixed cells were permeabilized with 0.1% Triton X-100 for 15 min to facilitate antibody penetration. Formaldehyde-acetone-treated cells could be stained directly without prior permeabilization or fragmentation of IgM antibodies. Coverslips were then incubated with an appropriate dilution of the cleaved immunoglobulin in PBS for 30 min, rinsed with several changes of PBS over 30 min, then reacted with a 1:50 dilution in PBS of affinity-purified FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.). Again the preparations were washed with several changes of PBS over 30 min and then mounted on glass slides with elvanol containing n-propyl gallate to minimize fading (13). Slides were examined with a Nikon Optiphot fitted for phase contrast and epifluorescence. Photographs were taken on Kodak tri-X pan film.

**Results**

When examined by electrophoresis in a single dimension with SDS, the major A and B chrip polypeptides of a HeLa hnRNP preparation migrate in a narrow molecular mass range. The predominant group of polypeptides appear as four to six bands between 33 and 40 kD in Coomassie-stained gels. However, the exact number of species was difficult to determine from stained one-dimensional gel patterns. A detailed study of possible cell cycle-specific changes in these proteins requires a greater degree of resolution; therefore we have used two-dimensional immunoblot analysis. Approximately 60 μg of protein from the sucrose gradient fraction containing the 30S hnRNP subcomplex isolated from a randomly dividing HeLa cell population was subjected to nonequilibrium pH gradient electrophoresis with a second dimension in SDS and finally stained with Coomassie Brilliant Blue (Fig. 1 Co). The C group hnRNP polypeptides migrate at ~43 and 45 kD (C) and are relatively more acidic than chrips. The blot was stained with monoclonal antibody from hybridoma id2 which cross reacts with all the basic chrip polypeptides (id2).

![Figure 1](https://jcb.rupress.org/)

**Figure 1.** Two-dimensional immunoblot analysis of hnRNP core proteins using a specific monoclonal antibody. hnRNA-associated proteins separated by nonequilibrium pH gradient electrophoresis and run on a 10% SDS-polyacrylamide second dimension were blotted onto nitrocellulose and reacted as described. A duplicate gel was stained with Coomassie Blue for comparison (Co). The C group hnRNP polypeptides migrate at ~43 and 45 kD (C) and are relatively more acidic than chrips. The blot was stained with monoclonal antibody from hybridoma id2 which cross reacts with all the basic chrip polypeptides (id2).
Figure 2. Localization of hnRNP core polypeptides in HeLa cells populations. Cells harvested during mitosis were allowed to adhere to coverslips, fixed with PLP, and stained with a chrp-specific monoclonal antibody. The iD2 immunoglobulin, an IgM, was cleaved by mild trypsin digestion. When these cleaved antibodies are used to stain a mitotic cell population the chrp polypeptides are found distributed throughout dividing cells while in interphase cells staining is restricted to nuclei (M). Only dividing cells exhibit fluorescence when intact antibodies are used to stain a mitotic cell population (U). The intact IgM molecule is unable to penetrate the nuclear membrane of PLP-fixed cells. Randomly dividing HeLa cells fixed with formaldehyde-acetone and stained with intact antibodies (FA) show a similar fluorescent pattern in both interphase and mitotic cells. Examples of interphase (i), metaphase (m), anaphase (a), and telophase (t) cells are indicated. Bar, 20 nm.
Figure 3. hnRNP core protein complements of synchronized HeLa cells. Spinner cultures were synchronized, subjected to two-dimensional gel electrophoresis, blotted, and reacted with specific monoclonal antibodies as described. Identical numbers of cells were used to prepare the protein samples. The blots were reacted simultaneously and similarly exposed to minimize procedural differences. A Coomassie-stained gel showing total cellular proteins prepared from cells harvested during mitosis is shown (MC0) along with a representative immunoblot of this sample (M/b). For greater detail only the stained portions of the blots of S, G2, mitosis (M), and G1 are shown. The pI range for the chrp polypeptides is from 8.3 to 9.2 (M/b).

Localization of chrp Polypeptides during Cell Cycle

The ease with which HeLa spinner cultures can be synchronized makes them useful for studies that require populations enriched for a specified stage of the cell cycle (34, 36). The cells were synchronized by the double thymidine block method; aliquots were taken at the times indicated. The cells were counted and protein samples prepared for electrophoresis. In addition, at each time point HeLa cells were prepared for indirect immunofluorescence as described. This allowed the visualization of the distribution of chrp proteins in the cell samples used for subsequent biochemical analyses. During S phase there were only interphase cells visible with the nuclei exhibiting strong fluorescence and the absence of staining in the cytoplasm and nucleoli. While the majority of cells in the G1 sample showed a fluorescent pattern typical of interphase, a small number of dividing cells were apparent judging from the distribution of antigens in the cells.

The mitotic sample demonstrated a sharp increase in cells undergoing division (results not shown). During the period of cell division the RNP core proteins become distributed throughout the cell, however the highly condensed chromat-in present throughout prometaphase, metaphase, anaphase, and early telophase does not stain (Fig. 2 M). The antigens enter the reformed nucleus in late telophase as evidenced by the return to the typical interphase pattern seen in the G1 sample (results not shown). There were a few mitotic cells in G1 most likely due to a slight loosening of population synchrony occurring by the time this sample was collected, 16.5 h after release of the second block.

Synchronized HeLa cells, as well as randomly dividing PtK2 cells, have been stained with a variety of chrp-specific monoclonal antibodies including those that recognize a subset of the core group (results not shown; see 19). No differences were observed in the immunofluorescent-staining pat-
terns with these different antibodies thus suggesting that the polypeptides comprising the chrp complement localize similarly during the cell cycle or that differences are too subtle to be detected at the level of resolution of the light microscope.

The availability of a variety of specific monoclonal antibodies provided an unanticipated advantage in assessing the number of mitotic cells present in a given sample by a direct examination of the characteristic distribution of the hnRNP core proteins. Due to the relatively small size and round shape of HeLa cells grown in suspension their cell cycle state was difficult to judge solely by the use of phase contrast microscopy. Performing immunofluorescent staining allowed not only a definite appraisal of the mitotic index but at the same time showed the localization of the proteins being examined. The mitotic index of a population fixed with PLP could be rapidly determined by staining with intact IgM immunoglobulins. In these PLP preparations of a mitotic cell population only the core proteins of dividing cells were accessible to the antibodies since the pentameric IgM molecules could not penetrate the nuclear membranes of interphase cells (Fig. 2 U). When PLP-fixed cell preparations from S, G2, and G1 phases were stained in a similar manner, no fluorescence was visible (results not shown). However, PLP-fixed cells in late telophase with reformed nuclei frequently exhibited some staining. This was probably due to the fragility of the newly assembled nuclear membrane (results not shown).

The fixation of cells with PLP provides a broad maintenance of protein antigenicity yet yields a degree of structural preservation compatible with electron microscopy. Formaldehyde-acetone-treated cells from a random population reacted with id2 (Fig. 2 F4) show penetration and staining of nuclei without prior permeabilization of the cells or cleavage of the immunoglobulins. The fluorescent pattern observed is essentially the same as that seen with PLP, however, fixation with formaldehyde-acetone causes partial dissolution of cell fine structure.

**hnRNP Core Protein Complements during the Cell Cycle**

Past studies characterizing the major constituents of hnRNP have used randomly dividing cells from cultures or tissues (20, 25, 26, 38). In this study we have used synchronized HeLa cell cultures, thereby allowing the examination of chrp polypeptide complements during the course of the cell cycle. No differences were detected when total cellular proteins of synchronized HeLa cells were separated by electrophoresis in a single SDS dimension, blotted, and reacted with various hnRNP-specific monoclonal antibodies (data not shown). We have now used whole cell sonicates prepared from synchronized cultures for analysis by two-dimensional immunoblotting. In this way we have avoided fractionation or enrichment procedures that might result in the preferential recovery of one form, for example polypeptides involved in complex formation, over another, such as free core group proteins. Aliquots of a synchronized HeLa spinner cell culture were harvested at various times after release of the final thymidine block; then sonicated in sample buffer. Two-dimensional electrophoresis, transfer onto nitrocellulose, and subsequent reaction with hnRNP-specific monoclonal antibodies were as described. Immunoglobulin from hybridoma id2, which reacts with all the basic core group proteins, was used to stain these blots. A gel of the protein sample prepared from cells harvested at mitosis (M) stained with Coomassie Brilliant Blue is shown in Fig. 3 MCo. The corresponding immunoblot of the whole gel of the mitotic sample (Fig. 3 Mf) is shown as a representative example of these results: only the areas that demonstrated antibody binding are shown for S (Fig. 3 S), G2 (Fig. 3 G2), M (Fig. 3 M), and G1 (Fig. 3 G1).

The relative pi values of the chrp polypeptide array extend from ~8.3 to 9.2 (Fig. 3 Mf). Although not present throughout every phase, 17 major and minor proteins were visible during the course of the cell cycle. The chrp polypeptide pattern is represented diagramatically in Fig. 4. Those spots undergoing marked changes are denoted with a star. The most prominent variations that we found took place during mitosis. During cell division two chrp species of 35 (Fig. 4, 35") and 37 kD (Fig. 4, 37") were observed which were not apparent in other phases. During interphase there were three chrp species at 35 kD ranging in relative pi from 8.8 to 8.4, upon cell division a fourth 35-kD polypeptide appeared with a pi of 8.3. The 37-kD chrp (Fig. 4, 37") that became apparent during mitosis was also more acidic relative to the other 37-kD core protein species with an apparent pi of ~8.8. Occasionally in both G2 and G1 samples these mitotic specific spots could be faintly observed (for example, see Fig. 3 G2). Synchrony decreases with time; in both G2 and G1, a small percentage of dividing cells slightly ahead (G0) or lagging behind (G1) the general population could be commonly observed by phase contrast microscopy. It is likely that these mitotic cells account for the weak spots sometimes seen. No dividing cells were ever observed in the S phase population. In addition to the appearance of these chrps during mitosis, another protein spot of 37 kD (Fig. 4, 37") decreased with the onset of cell division as did a 38-kD chrp (Fig. 4, 38). Both of these protein spots were lost by G1 (Fig. 3 G1). These phase-specific variations were consistently observed in identical configurations on two-dimensional immunoblots of cell sonicates (derived from over 10 separate cell cycle synchronization experiments).

The generally used A, B, and C nomenclature of Beyer et al. (3) for core proteins (for example see Fig. 4), which

![Figure 4. Diagrammatic representation of the hnRNP core protein array visualized by two-dimensional immunoblotting. The proteins are represented by their molecular mass with the nomenclature commonly used to describe one-dimensional SDS gels shown at the left. (Star) The chrp polypeptides that undergo alterations during the course of the cell cycle. The pi range of the chrp polypeptides is indicated at the top.]
was based on one-dimension electrophoresis, does not ade-
quately identify the species revealed in these two-dimen-
sional chrp arrays. The greater specificity and resolving
power of the two-dimensional immunoblotting technique re-
veals subspecies not accounted for by the nomenclature; for
example the A1 region is composed of at least two (33 and
34 kD) ranks. An additional complication is that several
polypeptide species, most notably 35 and 38 kD, can some-
times be resolved as a doublet (for example see Fig. 3 G7,
M, and G1, and Figs. 7 and 8). Furthermore, a description
of this group of related proteins must allow the identification
of a variable number of charge isomers. Our data as well as
those of others (7, 10) show that taken together the A and B
groups have basic relative pI values and are immunologically
distinct from the more acidic C group polypeptides. There
are also minor immunoreactive polypeptides at 40 kD. These
spots are reproducibly present, but are difficult to see on au-
toradiographs that have been optimally exposed for the major
chrp species. Examples of these 40-kD proteins, migrating
where indicated in Fig. 4, can be best observed in Fig. 3 G7
(see also Fig. 5, S2 and I). These polypeptides previously
might have been termed C group proteins on the basis of their
electrophoretic mobility in one dimension; however, they are
basic and possess antigenic determinants in common with
the other basic core proteins. These clearly are not members
of the acidic so-called C group family.

Effect of Alternative Synchronization Procedures

We have been able to show that these observed modifications
were not an artifact of the means of cell synchronization.
Various other methods for inducing synchrony in cell popu-
lations have been described (21). These include inhibitory
agents (such as aphidicolin which inhibits DNA synthesis
effectively, therefore preventing entry into S phase, as does
thymidine), disruptive agents (i.e., the action of colcemid
upon microtubules prevents the separation of chromosomes
at metaphase, thus causing an accumulation of mitotic cells),
and mechanical selection procedures.

We first attempted to minimize the possible effects of the
double thymidine block by following cells a second cycle.
When HeLa cells were harvested 21.5 h after the release of
the last thymidine block, thus approximating a second S
phase (S2), from a HeLa monolayer culture mitotic cells were
harvested (I). Thymidine-synchronized HeLa cells were collected
at both S and M, and incubated with colcemid as described (CS and
CM, respectively). In all cases whole cell sonicates were subjected
to two-dimensional immunoblot analysis with a monoclonal anti-
body, iD2, specific for all chrp polypeptides. Identical numbers of
cells (except for panel M) were used to prepare protein samples.
All blots were treated similarly, and in some cases (CS and CM),
exposed on the same sheet of film.

Figure 5. Effects of the means of inducing cell synchrony on chrp
modification. HeLa cells were harvested 21.5 h after release of the
second thymidine block, approximating a second S phase (S2).
From a HeLa monolayer culture mitotic cells were collected by
mechanical selection (M), the interphase cells remaining were also

Figure 6. Sedimentation of hnRNP core proteins of mitotic and S phase cells in sucrose density gradients. Synchronized HeLa cell cultures were harvested and fractionated into cytoplasmic and nuclear samples. Aliquots were centrifuged on 15–40% sucrose density gradients. Gradient fractions were analyzed by single dimension immunoblots with antibody iD2. Gradients were run with cytoplasmic fractions of cells in S (SC) and mitosis (MC), as well as nuclear samples prepared from S (SN) and mitotic (MN) samples. The first lane of each gel was loaded with 30S hnRNP subcomplexes as markers (30S).

The use of a mechanical "mitotic shake-off" procedure, while not as convenient for large scale experiments, provided an additional means for substantiating those results obtained with the double thymidine block. This technique does not involve the alteration of any metabolic pathways. As expected the mitotic pattern was enhanced in this sample (Fig. 5 M) since >80% of the cells were undergoing division. The protein array is very similar to that seen for the mitotic cells synchronized with thymidine (Fig. 3 M). The cells still adherent to the flasks after harvesting were mildly trypsinized and subsequently immunoblotted (Fig. 5 I). The two-dimensional polypeptide pattern of the remaining interphase cells was identical to those observed with nonmitotic samples from thymidine synchronized cultures.

To examine the effect of antimicrotubule agents on the hnRNP core protein complement, which lead to mitotic arrest, we incubated thymidine-synchronized HeLa cells for a relatively short period of time in the presence of 0.5 μg/ml colcemid. In both S phase (Fig. 5 CS) and mitosis (Fig. 5 CM) the protein pattern was altered from that observed when cells were synchronized with thymidine alone (i.e., Fig. 3, S and M in text). Our data show that after cells had been exposed to colcemid there was a change in the core protein complement resulting in fewer polypeptide species detected in both S phase and arrested mitotic samples resolved on two-dimensional immunoblots of total cellular proteins.

Modified hnRNP Core Proteins Are Present in Mitotic Cell Cytoplasm

Although all the cells of a thymidine-synchronized population divide during the mitotic period, only 20–30% are actually undergoing mitosis at any given point during this time span (36). Therefore, the demonstration of the presence of modified proteins in cells physically undergoing mitosis and any examination of the nature of these chrs required the fractionation of mitotic hnRNP components from those of the general cell population. Synchronized HeLa cells taken
at various phases of the cell cycle were fractionated into nuclear and cytoplasmic fractions. Single dimension immunoblot analysis of the nuclear and cytoplasmic samples showed no major quantitative changes throughout the cell cycle; however, significant levels of RNP polypeptides were present in cytoplasmic fractions only during mitosis (data not shown). Small amounts were detectable in the cytoplasmic fractions of the G2 and G1 samples probably owing to a small percentage of cells either entering mitosis early or, in the case of G1, lagging behind the general population (for example, see Fig. 3 G2). Also, cells in early G1 could have fragile nuclear membranes increasing the likelihood of leakage of proteins during the fractionation procedure. This possibility is further suggested by immunofluorescent-staining patterns previously discussed.

Since it was possible using cytoplasmic extracts to examine the hnRNP of mitotic cells specifically, we proceeded to analyze the state of possible supramolecular association of the hnRNP core proteins during mitosis. Of particular interest was whether the polypeptides existed free or as components of a hnRNP complex, or whether a novel structure is formed during mitosis. Nuclear and cytoplasmic samples from both S phase and mitosis were centrifuged upon sucrose density gradients. After fractionation of the gradients into 3-ml aliquots these were run on single dimension SDS slab gels and immunoblotted. The cytoplasmic gradient of the S phase sample (Fig. 6 SC) showed only a small amount of chrp polypeptide at the top of the gradient probably the result of nuclear leakage during sample preparation. The immunoblot of the cytoplasmic fraction from the mitotic sample (Fig. 6 MC) showed core proteins extending down the gradient to ~200S (fraction 11). Both the nuclear samples from S phase (Fig. 6 SN) and mitosis (Fig 6 MN) show chrp polypeptides associated with broadly sedimenting complexes in a pattern similar to that observed for hnRNP isolated from nuclei of randomly dividing cell populations (data not shown). The staining at 68 kD in certain fractions of the mitotic nuclear sample is most likely due to the formation of multimers of the chrp polypeptides that are resistant to dissociation (26). However, the appearance of these multimers is not confined to samples prepared from mitotic cells (see 19). A portion of the chrp polypeptides seen in the mitotic cytoplasmic sample could have been released due to the fragility of the nuclear membranes of cells about to begin or just completing division. However, an examination of gradient fractions from the mitotic cytoplasm sample showed modified core proteins associated with complexes extending down the gradient from 30S through the 200S region. Here we show a representative analysis of fractions 4 (Fig. 7 4) and 8 (Fig. 7 8). While these fractions clearly contain the described mitosis-enhanced modifications, note that the two-dimen-

**Figure 7.** Two-dimensional immunoblot analysis of sucrose density gradient fractions of the mitotic cytoplasmic sample. Gradient fractions 4 (ca. 30S) and 8 (ca. 200S) of the mitotic cytoplasmic sample (see Fig. 7 MC) were subjected to electrophoresis on nonequilibrium pH gradient gels followed by a second dimension in SDS. The gels were blotted onto nitrocellulose and reacted as described with monoclonal antibody iD2.

**Figure 8.** Effect of RNase on RNP complexes found in the mitotic cytoplasm fraction. The cytoplasmic fraction isolated from a mitotic cell population was centrifuged on sucrose density gradients after incubation with either 0 or 0.5 µg/ml RNase. The gradients were fractionated, subjected to electrophoresis on a single SDS dimension, and immunoblotted. The blots were reacted with a monoclonal antibody, iD2, specific for chrp polypeptides.
The major proteins associated with hnRNA are normally retained telophase (23, 24). As such they represent a useful model for blot analysis presented here of total cellular proteins from the phase of the cell cycle or exposure to the cytoplasmic environment. These heterogeneous complexes yield RNP particles sedimenting as a homogenous peak around 30S. To examine the organization of the RNP complexes found in mitotic cells both nuclear and cytoplasmic fractions were incubated with a low concentration of RNase, centrifuged upon gradients, and fractionated as before. The hnRNPs complexes found in the cytoplasmic fraction from mitotic cells became concentrated in fraction 4 (ca. 30S) after mild RNase cleavage (Fig. 8 0.5) while the RNP complexes in the untreated sample sedimented down through 200S (Fig. 8 0). Similar results were obtained with the nuclear samples (results not shown). This suggests that hnRNPs complexes during mitosis maintain molecular associations and substructure similar to those in other stages of the cell cycle. However, more subtle alterations of hnRNPs structure in mitosis cannot be ruled out by these data and it is apparent that these complexes contain more chrp forms than do the nucleus-restricted hnRNPs of interphase cells.

**Discussion**

The major proteins associated with hnRNA are normally restricted to the nucleus but become dispersed throughout the cell during mitosis, reentering the newly formed nucleus in late telophase (23, 24). As such they represent a useful model for the study of the fate of nuclear proteins during cell division; of particular interest is the question of whether there are special modifications of nuclear proteins dependent on the phase of the cell cycle or exposure to the cytoplasmic environment. In this context it is of interest that Celis and coworkers (6) showed a correlation between levels of certain chrp polypeptides and the growth state of cells. The immunoblot analysis presented here of total cellular proteins from synchronized HeLa cell populations revealed that modifications of the hnRN A and B core polypeptide (chrp) complement do occur during the cell cycle. The most dramatic changes take place during mitosis where several new species appear prominently and two decrease significantly. The changes most likely represent altered modification states of these proteins since the species appear to be variant charge isomers of a fundamental group of chrp polypeptides of Mr 33, 34, 35, 37, 38, and 40 kD in human cells.

Although some methods of synchronizing cell populations involve cytotoxic agents (11, 12), the modifications described here were observed (see Fig. 5) in proteins prepared from cells synchronized by both the thymidine block method (34, 36) and by mechanical selection (40). We feel that the changes observed in the core protein complement represent distinct cell cycle specific alterations not attributable to the method of cell synchronization or the means of protein visualization. Many past studies involving mitotic cells have used extended incubation with colcemid to obtain a population with >90% of the cells arrested at metaphase. Our data reemphasize the potentially cytotoxic effects of colcemid and similar agents when they are used for synchronizing cell populations.

From our data it appears that the core proteins may be modified while present in the foreign environment of the cytoplasm during cell division. The core proteins migrate in a consistent pattern during G1, S, and G2, while mitosis is characterized by marked modifications in the migration pattern. Conceivably these modifications of core proteins are a means for establishing or maintaining new protein/protein or RNA/protein interactions while in a cytoplasmic environment. They possibly serve to sequester nuclear components via mitosis-specific interactions or to facilitate reentry into the nucleus. Whether the altered state of these polypeptides is necessary for successful cell division or represents a superficial response to a changed environment remains to be determined. When cells were treated with colcemid, although core protein modifications were diminished, cells could continue through the cycle until becoming blocked at metaphase. Although Lahiri and Thomas (17) described large, RNA-containing, 120S particles found in cells arrested at metaphase, their use of colcemid necessitates an evaluation of associations involving hnRNPs core proteins with aberrant modifications. In the experiments described here we have shown that the modified chrp of the mitotic cytoplasm are found apparently associated with complexes sedimenting heterogeneously up to 200S in a sucrose density gradient. These complexes yield particles sedimenting at 30S upon mild nucleolytic cleavage indicating a supramolecular organization similar to hnRNPs complexes isolated from nuclei of a randomly dividing cell population.

From our data it would appear that the majority of chrp exist associated with a complex rather than free in the cell during mitosis. This raises an interesting question: If these proteins exist in a complex, by what means do they return to the nucleus after the reformation of the nuclear envelope? The chrp polypeptides could enter the reformed nucleus as components of a complex. However this would require a reverse of the usual transport direction across the nuclear envelope. Alternatively the complexes might disassemble just at the time of reentry yielding free chrp for transport through the nuclear pores. Okamura, C. S., and T. E. Martin (unpublished observations) have found that RNA synthesis is required for chrp to return to the nucleus. The mechanism for the requirement of RNA synthesis upon the redistribution of hnRNPs core proteins remains to be determined.

While the highly modified core protein pattern observed in cell division is not found during interphase, we have yet to determine whether the modifications themselves are reversible. The possibility of new synthesis of unmodified proteins to replace those altered during cell division, while less likely, has yet to be ruled out. However, the data presented suggest that the decrease in modified forms of the core proteins after mitosis is not merely a dilution of these proteins since the relative staining intensities of many of the chrp polypeptides present throughout the cell cycle remained unchanged.

An understanding of the alteration in hnRNPs structure during mitosis will require the elucidation of the biochemical
nature of these protein modifications. It is clear that some of the spots visualized on two-dimensional immunoblots are the result of posttranslational modifications of an unspecified number of gene products. Since the altered core proteins become more acidic during mitosis, phosphorylation or basic amino acid acetylation or methylation could potentially be involved. Ottaviano and Gerace (32) have recently described an increase during mitosis in the phosphorylation of laminins, proteins normally confined to the nuclear envelope of the interphase nucleus but dispersed throughout the cytoplasm in mitosis. Since the potential of RNP particle polypeptides to be phosphorylated has been documented by our laboratory and others (1, 12, 25, 41) phosphorylation was considered a possible source for the protein modifications that we observed. Studies of polypeptides prepared from synchronized HeLa cell cultures grown in the presence of 32P suggested that the altered patterns of hnRNP core proteins were not due to selective phosphorylation. In addition, incubation of extracts from synchronized cell populations with alkali phosphatase did not alter the migration of the chrp polypeptides on two-dimensional gels (Leser, G. P., and T. E. Martin, unpublished observations). While phosphorylation could still play an important role in chrp polypeptide modification these data suggest it does not account for the mass amount of alterations seen during mitosis. The hnRNP associated polypeptides have been shown to be methylated (5), however the stability of core protein methylation during the course of the cell cycle is unknown. The determination of the nature of these apparently transient protein modifications would appear to be important to the understanding of the behavior of normally nucleus-restricted proteins during cell division and possibly the events surrounding their return to the nucleus.

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