Transcription-Dependent and Transcription-Independent Nuclear Transport of hnRNP Proteins

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Heterogeneous nuclear RNAs and specific nuclear proteins form heterogeneous nuclear ribonucleoprotein complexes (hnRNPs), one of the most abundant components of the nucleus. In mitosis, as the nuclear envelope breaks down, hnRNPs disperse throughout the cell. At the end of mitosis, hnRNPs dissociate and their proteins are transported into the daughter cell nuclei separately. Some are transported immediately (early group), while others are transported later (late group). Transport of the late group appears to require transcription by RNA polymerase II, in that inhibitors of this polymerase cause the late proteins to remain in the cytoplasm. Thus, there are two modes, transcription-dependent and transcription-independent, for the transport of nuclear proteins.

THE COMPLEXES OF HETEROGENEOUS nuclear RNA with specific nuclear hnRNA-binding proteins (hnRNPs) are among the most abundant components of the eukaryotic nucleus. The protein composition of these complexes, the structure of their proteins, and the arrangement of the proteins on the RNA, as well as the possible functions of hnRNP proteins in mRNA biogenesis, have been extensively investigated (*1*). Immunocytochemical studies with antibodies to the major hnRNP proteins have indicated that the complexes are confined to the nucleus in interphase and that during mitosis, after the nuclear envelope breaks down, they are found throughout the entire cellular space (*2–4*) as hnRNP complexes similar to those of interphase cells (*4, 5*).

HnRNPs can be purified with the use of monoclonal antibodies to individual hnRNP proteins (*6, 7*). To examine in detail the fate of hnRNP proteins during mitosis, we compared the protein composition of hnRNPs purified from asynchronous and mitotic HeLa cells. In order to circumvent the difficulty of metabolic labeling of proteins in mitotic cells, which are transcriptionally and translationally quiescent, we examined the composition of the isolated hnRNPs by two-dimensional nonequilibrium gel electrophoresis (NEPHGE) (Fig. 1). The protein composition of hnRNP complexes from asynchronous and mitotic cells is similar, although many of the hnRNP proteins, including the A, B, and C groups, have slightly different mobilities during mitosis as a result of post-translational modifications (*4*), which include mitosis-specific phosphorylation of the C proteins (*8*). The integrity of these mitotic complexes, like that of the interphase complexes, is dependent on the presence of RNA (*8*). The lack of contamination of the hnRNP proteins by other abundant cellular proteins confirms the specificity of the immunopurification procedure and indicates that these complexes remain as discrete entities during mitosis without apparent stable associations with other cellular structures.

Immunofluorescence microscopy with monoclonal antibodies to the hnRNP C and A1 proteins, 4F4 and 4B10, respectively, showed that these proteins are restricted to the nucleoplasm of cells that have fully formed nuclei, whereas during mitosis the same proteins are dispersed throughout the cell. The hnRNP proteins that were dispersed in mitosis returned to the nucleus after it re-formed (Fig. 2, A to C). That these are preexisting hnRNP proteins is demonstrated by the fact that the protein synthesis inhibitors emetine (Fig. 2, D to F) or cycloheximide (*8*) do not diminish accumulation of hnRNP proteins in the newly formed nucleus.

Examination of cells by double-label immunofluorescence microscopy revealed differences in the transport of the C and A1 proteins to the nucleus. In recently divided cells, the C proteins accumulated in the daughter cell nuclei as soon as the nuclei were formed, whereas most of the A1 protein was still in the cytoplasm (Fig. 2, G to I). Thus, although hnRNP proteins remain associated as hnRNP complexes during mitosis (Fig. 1), they dissociate from each other prior to their transport to the nucleus and return to the nucleus separately.

Treatment of cells for 45 min with actinomycin D at doses that inhibit RNA polymerase II transcription (5 μg/ml) (*9*) did not affect the return to the nucleus of the C proteins but blocked the transport of A1 to the nucleus (Fig. 3, A and B). Double-label immunofluorescence microscopy of actinomycin D–treated cells showed, therefore, many post-mitotic cells in which the C proteins were found in the nucleus and most of the A1 protein was found in the cytoplasm. Similar results were obtained with cells treated with 5,6-dichlorobenzimidazole riboside (DRB), an RNA polymerase II inhibitor whose structure and mechanism of action are different from those of actinomycin D (Fig. 3, C and D) (*10*). When cells were treated with actinomycin D (0.04 μg/ml), which inhibits RNA polymerase I but not RNA polymerase II, transport of A1 to the daughter cell nuclei was not affected (*8*). The interphase cells seen in the same fields do not show detectable leaching of the A1 protein out of the nucleus after treatment with these inhibitors (Fig. 3). Therefore, these results indicate that transcription by RNA polymerase II is required for the return of hnRNP protein A1, but not the C proteins, to the nucleus after mitosis. Other hnRNP proteins, including A2, B1, B2, E, H, and L, are also transported to the nucleus in a transcription-dependent mode (Fig. 4). In contrast, the 120-kD U protein, proteins of small nuclear ribonucleoprotein (snRNP) complexes, and nuclear lamins, like the C proteins, are rapidly transported to the nucleus even in the presence of transcriptional inhibitors (Fig. 4). The insensitivity of the nuclear transport of snRNP proteins to inhibitors of transcription was previously demonstrated (*11*).

These results indicate that there are at least two distinct modes of transport of hnRNP proteins to the nucleus: an early, transcription-independent mode and a late, transcription-dependent mode. Both modes of transport appear to be part of the normal process of nuclear assembly after mitosis. The transport of the late group is probably

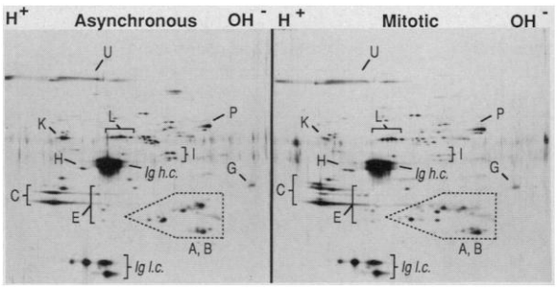

Fig. 1. The composition of hnRNP complexes in mitosis. HnRNP complexes were immunopurified from asynchronous (left) or from mitotic (right) HeLa cells, with a monoclonal antibody to the C proteins, 4F4, and the proteins were resolved by two-dimensional gel electrophoresis (*17*). The positions of some hnRNP proteins are indicated. The nomenclature used is described in (*7*). Ig h.c. and Ig l.c. indicate immunoglobulin heavy and light chains, respectively.

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delayed because transcription resumes only in late telophase. The return of proteins to the nucleus after mitosis provides a general system for the study of nuclear transport. Also hnRNP complexes are dynamic structures in that the hnRNP proteins can dissociate from the complexes and be transported separately. Although nuclear localization signals (*12*) have been defined in some nuclear proteins and some of the components of the nuclear transport machinery have been identified (*13*), many of the hnRNP proteins, including A1, have no obvious candidate nuclear localization signal (*1, 14*).

Several different mechanisms can be envisioned for transcription-dependent transport of proteins to the nucleus. One possibility is that an RNA molecule is required to function as a shuttle to carry these proteins to the nucleus. The synthesis of this RNA would then be a prerequisite for the transport of the late group of hnRNP proteins. Another possibility is that an RNA-dependent modification of these proteins or their RNA-dependent assembly with other proteins in the cytoplasm must occur before they can be transported to the nucleus. Modifications that occur in mitosis may affect the state of assembly and the transportability of these proteins. That proteins can be transported from the cytoplasm to the nucleus as complexes with RNA has been demonstrated for snRNPs (*15*). It is also possible that a signaling pathway exists that senses free binding sites for these proteins in the form of RNA polymerase II transcripts in the nucleus and that delivery of this information to the cytoplasm is required to allow the proteins to translocate to the nucleus.

We cannot distinguish by our experiments whether the required component is for mass accumulation of hnRNA or for the transcription of certain RNAs by RNA polymerase II. The lack of nuclear staining by antibodies to A1 after treatment with actinomycin D or DRB suggests that A1 is excluded from the daughter cell nuclei as they re-form, and that A1 cannot freely diffuse into the nuclei. Whatever the mechanism of the transcription-dependent transport of hnRNP proteins to the nucleus, control of that process may serve to couple RNA polymerase II transcription with post-transcriptional regulation, for which assembly of hnRNP complexes is probably required.

The exchange of hnRNP proteins for proteins that bind mRNA accompanies nucleocytoplasmic transport of mRNA (*16*) and is reminiscent of and may be mechanistically similar to the dissociation of hnRNPs that occurs prior to the return of their hnRNP proteins to the nucleus after mitosis. The transcription-dependent mechanism of transport of hnRNP proteins to the nucleus after mitosis may also be operational in

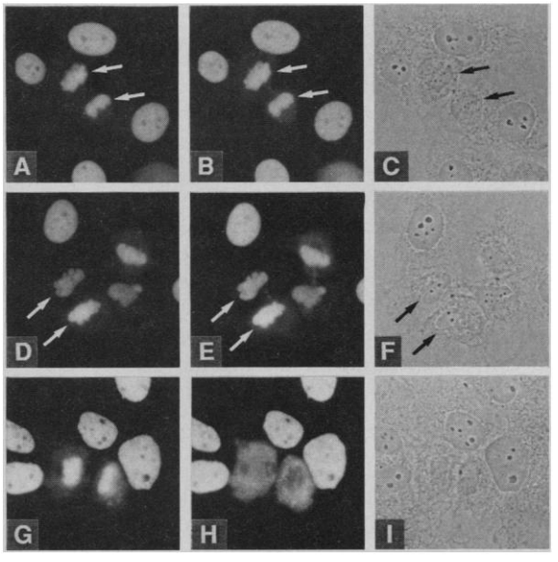

Fig. 2. Preexisting hnRNP proteins return to the nucleus after mitosis. HeLa cells grown on glass cover slips were immunostained with FITC-conjugated 4F4 (antibody to the C proteins) and TRITC-conjugated 4B10 (antibody to the A1 protein) (*19*). The intracellular localization of the proteins was then examined by immunofluorescence microscopy on a Zeiss Axiophot with the fluorescein channel or the rhodamine channel, respectively. In order to enrich for cells in mitosis and early G1, cells were partly synchronized by exposure to 2 mM thymidine for 20 hours. The cells were fixed 13 hours after the removal of thymidine from the culture medium. (**A** to **C** and **G** to **I**) Control cells and (**D** to **F**) cells incubated with emetine (20 μg/ml) for 1 hour prior to fixation in order to inhibit synthesis of new hnRNP proteins. Arrows indicate cells that have just exited mitosis, identified by their pairwise arrangement and their symmetry with respect to each other. C proteins accumulate in newly formed nuclei, whereas staining with 4B10 reveals a greater concentration of A1 in the cytoplasm of the same cells. Most likely the cells in (G) to (I) are at an earlier stage of nuclear hnRNP transport than those shown in (A) to (F). Cells stained with FITC-conjugated 4F4 (A, D, and G); TRITC-conjugated 4B10 (B, E, and H); and phase-contrast view of the same field (C, F, and I).

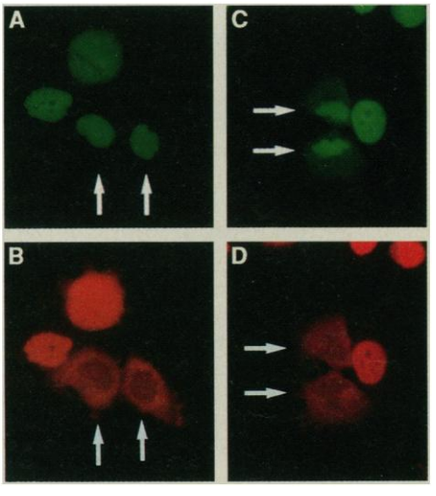

Fig. 3. Effect of inhibition of RNA polymerase II transcription on the return of A1 or C proteins to the nucleus after mitosis. HeLa cells grown on glass cover slips were partly synchronized as described in Fig. 2. Before the cells were fixed, actinomycin D (5 μg/ml) or DRB (75 μM) was added to parallel cultures, and the cells were incubated for 45 min. All samples were then fixed and immunostained with FITC-conjugated 4F4 and TRITC-conjugated 4B10 simultaneously. (**A** and **B**) Immunofluorescence staining pattern with FITC-4F4 and TRITC-4B10, respectively, of cells exposed to actinomycin D (5 μg/ml). The fluorescent signal observed with 4F4 is indistinguishable from that observed in the control cells (compare Fig. 2). Immunostaining of the same cells with 4B10 shows cytoplasmic staining in those cells that have just completed mitosis, in contrast to the exclusive nuclear localization of the C proteins that were immunostained with 4F4. (**C** and **D**) Immunofluorescence staining pattern with FITC-4F4 and TRITC-4B10, respectively, of cells exposed to DRB at 75 μM shows a similar relative distribution of C proteins and A1 to that observed in cells treated with actinomycin D.

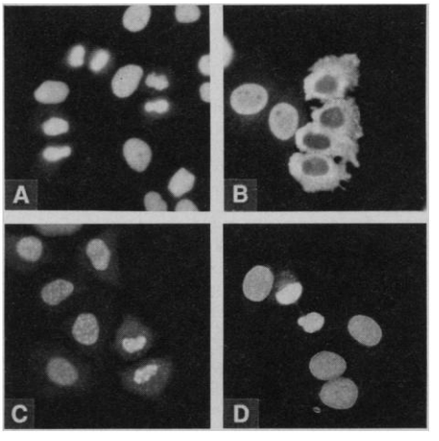

Fig. 4. Effect of actinomycin D on return to the nucleus of hnRNP, snRNP, and nuclear lamins. HeLa cells grown on cover slips were partially synchronized and exposed to actinomycin D at 5 μg/ml as described in Fig. 3. The cells were stained for immunofluorescence microscopy with the following antibodies: (**A**) 3G6, antibody to the hnRNP U protein (*19*); (**B**) 7A9, antibody to the hnRNP proteins A, B, E, G, H, and L (*8*); (**C**) Y12, antibody to snRNP proteins (*20*); (**D**) Ls1, antiserum to lamin (*21*). The staining pattern observed with 7A9 in the absence of transcriptional inhibitors is indistinguishable from that observed with 4B10 (see Fig. 2).

the nuclear transport of proteins during interphase.

REFERENCES AND NOTES

1. G. Dreyfuss, *Annu. Rev. Cell Biol.* **3**, 459 (1986); ———, M. S. Swanson, S. Piñol-Roma, *Trends Biochem. Sci.* **13**, 86 (1988); R. J. Bandziulis, M. S. Swanson, G. Dreyfuss, *Genes Dev.* **3**, 431 (1989).
2. T. E. Martin and C. S. Okamura, in *The Cell Nucleus*, H. Busch, Ed. (Academic Press, New York, 1981), vol. 9, p. 119.
3. Y. D. Choi and G. Dreyfuss, *J. Cell Biol.* **99**, 1997 (1984).
4. G. P. Leser and T. E. Martin, *ibid.* **105**, 2083 (1987).
5. D. K. Lahiri and J. O. Thomas, *J. Biol. Chem.* **260**, 598 (1985).
6. Y. D. Choi and G. Dreyfuss, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7471 (1984).
7. S. Piñol-Roma, Y. D. Choi, M. J. Matunis, G. Dreyfuss, *Genes Dev.* **2**, 215 (1988).
8. S. Piñol-Roma and G. Dreyfuss, unpublished results.
9. R. P. Perry and D. E. Kelley, *J. Cell. Physiol.* **76**, 127 (1970).
10. E. Egyhazi, A. Pigon, L. Rydlander, *Eur. J. Biochem.* **122**, 445 (1982).
11. G. W. Zieve and B. E. Slitzky, *J. Cell Physiol.* **128**, 85 (1986).
12. L. Gerace and B. Burke, *Ann. Rev. Cell Biol.* **4**, 335 (1988); C. Dingwall and R. A. Laskey, *ibid.* **2**, 367 (1986).
13. J. W. Newport and D. J. Forbes, *Ann. Rev. Biochem.* **56**, 535 (1987).
14. M. Buvoli *et al.*, *Nucleic Acids Res.* **16**, 3751 (1988); F. Cobianchi, D. N. SenGupta, B. Z. Zmuda, S. H. Wilson, *J. Biol. Chem.* **261**, 3536 (1986); K. R. Williams, K. L. Stone, M. B. LoPresti, B. M. Merrill, S. R. Planck, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5666 (1985). C. G. Burd, M. S. Swanson, M. Görlach, G. Dreyfuss, *ibid.* **86**, 9788 (1989).
15. E. M. De Robertis, S. Lienhard, R. F. Parisot, *Nature* **295**, 572 (1982); R. Zeller, T. Nyffeneger, E. M. De Robertis, *Cell* **32**, 425 (1983); R. A. Sauterer, R. J. Feeney, G. W. Zieve, *Exp. Cell Res.* **176**, 344 (1988); U. Fischer and R. Lührmann, *Science* **249**, 786 (1990); J. Hamm, E. Darzynkiewicz, S. M. Tahara, I. W. Mattaj, *Cell* **62**, 569 (1990).
16. G. Dreyfuss, Y. D. Choi, S. A. Adam, *Mol. Cell. Biol.* **4**, 1104 (1984).
17. Immunopurification of hnRNP complexes was done essentially as described [*6, 7,* and S. Piñol-Roma, Y. D. Choi, G. Dreyfuss, *Methods Enzymol.* **181**, 317 (1990)] except that the nuclear isolation step was omitted. Asynchronously growing HeLa cells and mitotic HeLa cells accumulated in the presence of nocodazole (*18*) were suspended in RSB-100 (10 mM tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$) containing 0.5% Triton X-100, 0.5% aprotinin, and 1 μg/ml each of leupeptin and pepstatin A. The cells were disrupted by two 5-s exposures to sonication on ice with the use of a microtip sonicator (model w-220F, Heat System Ultrasonics, Plainview, NY) set at scale 2. The disrupted cells were layered on a 30% sucrose cushion in RSB-100 and centrifuged at 4000*g* for 15 min. The supernatant fraction was incubated with protein A–Sepharose beads that had been coated with monoclonal antibody to the hnRNP C proteins, 4F4 (*3*), for 10 min at 4°C with gentle rocking. The beads were washed five times by resuspension in 1-ml portions of RSB-100 containing 0.5% Triton X-100. Under these lysis and washing conditions, the hnRNP complexes remain essentially intact. Bound proteins were eluted from the beads with 25 μl of electrophoresis sample buffer, resolved by two-dimensional gel electrophoresis [nonequilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension, and SDS-PAGE in the second dimension] as described (7), and visualized by silver staining.
18. G. W. Zieve, D. Turnbull, J. M. Mullins, J. R. McIntosh, *Exp. Cell Res.* **126**, 397 (1980).
19. Monoclonal antibodies 4F4 and 4B10 were purified from ascites fluid by anion exchange column chromatography on a PEI-HPLC column (Rainin) as suggested by the manufacturer. The purified antibodies were concentrated, 4F4 was coupled to fluorescein isothiocyanate (FITC; Sigma), and 4B10 was coupled to tetramethylrhodamine isothiocyanate (TRITC; Sigma). Coupling was carried out as described [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)].
20. E. A. Lerner, M. R. Lerner, C. A. Janeway, J. A. Steitz, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2737 (1981).
21. F. D. McKeon, D. L. Tuffanelli, K. Fukuyama, M. W. Kirschner, *ibid.* **80**, 4374 (1983).

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Kinetic Characterization of Ribonuclease-Resistant 2′-Modified Hammerhead Ribozymes

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The incorporation of 2′-fluoro- and 2′-aminonucleotides into a hammerhead ribozyme was accomplished by automated chemical synthesis. The presence of 2′-fluorouridines, 2′-fluorocytidines, or 2′-aminouridines did not appreciably decrease catalytic efficiency. Incorporation of 2′-aminocytidines decreased ribozyme activity approximately by a factor of 20. The replacement of all adenosines with 2′-fluoroadenosines abolished catalysis in the presence of $MgCl_2$ within the limits of detection, but some activity was retained in the presence of $MnCl_2$. This effect on catalysis was localized to a specific group of adenines within the conserved single-stranded region of the ribozyme. The decrease in catalytic efficiency was caused by a decrease in the rate constant; the Michaelis constant was unaltered. The 2′-fluoro and 2′-amino modifications conferred resistance toward ribonuclease degradation. Ribozymes containing 2′-fluoro- or 2′-aminonucleotides at all uridine and cytidine positions were stabilized against degradation in rabbit serum by a factor of at least 10^3 compared to unmodified ribozyme.

THE HIGH SPECIFICITY OF ANtisense oligonucleotides and ribozymes has made them potential therapeutic agents for the treatment of diseases such as acquired immunodeficiency syndrome (*1, 2*). However, the instability of these molecules stands in the way of their practical application (*1, 3*). In this report we describe the kinetic characterization of a number of hammerhead ribozymes (*4*) that contain conservative nucleotide replacements within the RNA. These modifications render the RNA oligomers resistant to alkaline hydrolysis and ribonuclease degradation without causing major disruptions in the catalytic ability of the RNA enzymes.

RNA exists predominantly in the A-form, in which the ribose moiety adopts the 3′-endo (N) conformation, whereas B-DNA usually has the sugar in the 2′-endo conformation (S) (*5*). It was therefore not surprising that ribozymes that were partially substituted with 2′-deoxynucleotides exhibited large decreases in catalytic efficiency compared to an all-ribonucleic ribozyme (*6*). Structural studies of the 2′-deoxy-2′-fluoro analogs of the common nucleosides have revealed that they adopt an N conformation and polymers containing these analogs exhibit circular dichroism and nuclear magnetic resonance spectral properties that indicate they have an N conformation and an A-form structure [for a summary, see Williams and co-workers (7)]. The partition ratio of 2′-amino-2′-oligodeoxynucleosides between N and S conformations shows a slight preference for the S conformer compared to unmodified nucleosides (*8*). Therefore, hammerhead ribozymes in which the 2′-hydroxyl group was replaced by a fluorine atom or an amino group were prepared and kinetically characterized. The 34-nucleotide ribozyme described by Fedor and Uhlenbeck (*9*) (Fig. 1) was chosen for this systematic study because of its high turnover number.

The catalytic efficiency of hammerhead ribozymes has been thought to depend on the presence of a 2′-hydroxyl group at the site of cleavage and at specific positions throughout the ribozyme structure. The introduction of 2′-deoxynucleotides at the conserved positions E 13, 14, and 27 to 29 (Fig. 1) within the ribozyme sequence resulted in a 96% decrease of catalytic efficien-

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