The heteronuclear RNP (hnRNP) proteins comprise a group of ~20 abundant proteins that associate with pre-mRNA molecules immediately upon their emergence from the transcription apparatus (for review see Dreyfuss et al., 1993; Kiledjian et al., 1994). Pre-mRNA/mRNA remains associated with hnRNP proteins throughout its lifetime in the nucleus. Many of the human hnRNP proteins have been cloned and sequenced, and some have been studied in detail. These studies have indicated that hnRNP proteins function in the extensive pre-mRNA processing reactions required to generate mRNA, e.g., splicing (Mayeda and Krainer, 1992; Choi et al., 1986; Munroe and Dong, 1992; Portman and Dreyfuss, 1994; Yang et al., 1994; Cáceres et al., 1994) and export of mRNA from the nucleus (Piñol-Roma and Dreyfuss, 1992; Michael et al., 1995b). Some of the hnRNP proteins are restricted to the nucleus in interphase cells. These include hnRNP C1, C2, and U (Piñol-Roma and Dreyfuss, 1992, 1993a). However, a number of them, although predominantly nuclear at steady state, shuttle continuously between the nucleus and the cytoplasm. These shuttling hnRNP proteins include hnRNP A1, K, and E (Piñol-Roma and Dreyfuss, 1992, 1993a; Michael et al., 1995b). While in the cytoplasm, hnRNP A1 is associated with mRNA, suggesting that shuttling hnRNP proteins accompany mRNA from the point of its emergence from the transcription machinery, through the nucleoplasm to the nuclear pore complex (NPC), and during translocation through the NPC (Piñol-Roma and Dreyfuss, 1992, 1993a). The question of whether mRNA is carrying, or is being carried by, the shuttling hnRNP proteins has now been addressed with the identification of a protein nuclear export signal (NES) within the shuttling hnRNP protein A1 (Siomi and Dreyfuss, 1995; Michael et al., 1995a). The A1 NES (a 38-amino acid segment of the protein termed M9) is not involved in RNA binding and, when fused to a protein that is normally restricted to the nucleus, is capable of promoting its export to the cytoplasm, in a temperature-sensitive manner (Michael et al., 1995a). Therefore, like mRNA export (for review see Izaurralde and Mattaj, 1995; Nakielny et al., 1996), shuttling hnRNP protein export is a signal-dependent, temperature-sensitive process, suggesting that shuttling hnRNP proteins carry mRNA to the cytoplasm. This conclusion is supported by recent observations of mRNA export in salivary gland cells of the insect *Chironomus tentans.* In these cells, export of very large, abundant transcripts, called Balbiani ring transcripts, can be visualized by EM (for review see Mehlin and Dancho, 1993).
translocation through the NPC to the cytoplasm has been shown to be an A1-like hnRNP protein (Visa et al., 1996).

The mounting evidence that mRNA export is mediated by hnRNP proteins highlights the importance of understanding the molecular mechanisms of hnRNP protein transport and localization. In this study, we address the molecular mechanism of retention of nucleus-restricted hnRNP proteins. We demonstrate that the nonshuttling hnRNP C proteins are restricted to the nucleus not because they lack an export signal, but rather because they bear a retention sequence. This nuclear retention sequence (NRS) overrides the NESs of shuttling hnRNP proteins. We have delineated the NRS within hnRNP C1. We suggest that NRSs, such as those present in the hnRNP C proteins, can mediate nuclear retention of pre-mRNA, and that removal of NRS-bearing hnRNP proteins from RNA is critical to allow mRNA to be exported from the nucleus.

Materials and Methods

Plasmid Constructions

All expression plasmids used in this study are derived from a modified form of pcDNA3 (Invitrogen, San Diego, CA) termed pcDNA3-myc, which has a myc epitope (MGEQKLISEEDL) just upstream of the EcoRI site (Siomi and Dreyfuss, 1995). Myc-C1 was made by PCR amplification of C1 cDNA pHCI2 (Swanson et al., 1987) to generate at the 5' end an EcoRI site immediately upstream of the second codon of C1, and at the 3' end an XhoI site downstream of the translation termination codon of C1. This PCR fragment was digested with EcoRI and XhoI and subcloned into pcDNA3-myc. Myc-A1 was made as described previously (Michael et al., 1995a). Myc-C1-A1 was made by gene splicing by overlap extension (Horton et al., 1990). Briefly, PCR was used to generate C1 with an EcoRI site immediately upstream of the second codon of C1 at the 5' end, and at the 3' end an 18-base region identical to the 5' end of codons 2-7; Buvoli et al., 1990). PCR was used to make A1 with an 18-base region identical to the 3' end of codons 285-290 at the 5' end of the fragment, and an XhoI site downstream of the A1 translation termination codon at the 3' end of the fragment. The C1 and A1 fragments were mixed and subjected to two cycles of PCR to form the recombinant C1-A1 fusion. Addition of the 5' C1 primer and the 3' A1 primer to the PCR reaction allowed amplification of C1-A1. This PCR fragment was digested with EcoRI and XhoI and subcloned into pcDNA3-myc. Myc-C1-K (hnRNP K cDNA; Matunis et al., 1992) and myc-A1 fused to portions of C1 or hRaly (hRaly cDNA; Vaughan et al., 1995; full-length clone kindly provided by V. Pollard [Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia]) were made in the same way as myc-C1-A1. Myc- pyruvate kinase (PK) and myc-PK-A1 (kindly provided by H. Siomi, Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia), were made as described previously (Siomi and Dreyfuss, 1995). To make myc-PK fused to C1 sequences, myc-PK-NLS (described in Michael et al., 1995a) was digested with HindIII and EcoRI, and the resulting myc-PK fragment was cloned into pcDNA3. Fragments of C1 with an EcoRI site at the 5' end and an XhoI site downstream of a translation termination codon at the 3' end were generated by PCR amplification of C1 cDNA, and these C1 fragments were subcloned into pcDNA3-myc-PK.

Cell Culture and Transfection

HeLa cells were cultured in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (GIBCO BRL). Cells were transfected with cesium chloride-ethidium bromide density gradient centrifugation-purified plasmid DNA, using the calcium phosphate coprecipitation method as described previously (Siomi and Dreyfuss, 1995).

Heterokaryon Formation

This was performed as described previously (Michael et al., 1995a) with minor modifications. Briefly, HeLa cells growing on 100-mm dishes were seeded 24 h after transfection onto glass coverslips in 30-mm dishes. After overnight incubation, the HeLa cells were seeded with an equal number of mouse NIH 3T3 cells that had been incubated for 30 min in the presence of 75 μg/ml cycloheximide (Calbiochem-Novabiochem Corp., La Jolla, CA). The cocultures were incubated for 3 h in the presence of 75 μg/ml cycloheximide, and then fused exactly as described previously (Pihol-Roma and Dreyfuss, 1992). The heterokaryons were returned to media containing 100 μg/ml cycloheximide and incubated for 4 h before fixation for immunostaining.

Immunofluorescence Microscopy

Fixation and permeabilization were performed as described previously (Siomi and Dreyfuss, 1995). Immunostaining with primary mAb 9E10 that recognizes the myc tag (Evan et al., 1985) was done at 1:3,000 dilution in PBS containing 3% BSA. Secondary antibody incubation was as described previously (Siomi and Dreyfuss, 1995) except, for analysis of heterokaryons, Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) was included at 1 μg/ml. After staining, cells were visualized and photographed with an Axiopt microcope (Carl Zeiss, Inc., Thornwood, NY).

Results

The hnRNP C Proteins Contain Nuclear Retention Sequences that Override Nuclear Export Signals of Shuttling hnRNP Proteins

To examine the nucleocytoplasmic transport properties of hnRNP proteins, a transient transfection interspecies heterokaryon assay similar to previously published procedures was used (Pihol-Roma and Dreyfuss, 1992; Schmidt-Zachmann et al., 1993; Michael et al., 1995a). Briefly, HeLa cells were transfected with DNA encoding the epitope-tagged protein of interest, and then fused to mouse NIH 3T3 cells to form heterokaryons, in which human and mouse nuclei share a common cytoplasm. 4 h after fusion, heterokaryons were fixed and stained for immunofluorescence microscopy to examine the distribution of the tagged proteins. A protein scores positive for retention if a signal is detected in only the human nucleus of the heterokaryons, and negative for retention if the protein is detected in both the human and the mouse nuclei (indicating it has migrated between the heterologous nuclei in the 4 h postfusion incubation period).

Using this assay, transfected hnRNP C1 was found to be retained in the human nuclei and therefore behaves like endogenous C1. Transfected hnRNP A1 also behaves like its endogenous counterpart and shuttles between the heterologous nuclei (data not shown; Pihol-Roma and Dreyfuss, 1992).

The hnRNP proteins that are restricted to the nucleus may be retained because they lack an NES, or because they contain nuclear retention sequences. To distinguish between these possibilities, an expression plasmid encoding hnRNP C1 fused at its carboxyl terminus to hnRNP A1 was constructed, and the nucleocytoplasmic transport properties of the expressed fusion protein were examined using the heterokaryon assay (Fig. 1 A). The C1–A1 fusion protein behaved identically to C1 and was completely retained in the human nuclei (Fig. 1 B). The hnRNP C2 protein, which is identical to C1 except for a 13-amino acid insertion near the middle of the protein (Burd et al., 1989), was expressed as a fusion with A1, and like C1–A1, C2–A1 was restricted to the human nuclei (data not shown). Fusion of C1 to a different shuttling hnRNP protein, hnRNP K,
Figure 1. The hnRNP C protein can retain shuttling hnRNP proteins in the nucleus. (A) Schematic representation of the fusion proteins used to study the effect of C protein on the nucleocytoplasmic transport properties of shuttling hnRNP proteins. Functional domains are indicated by RBD (RNA-binding domain), AUX (auxiliary domain), RGG (ArgGlyGly box), M9 (nuclear localization sequence/nuclear export sequence), and KH (K homology domain). (B) Plasmids encoding the myc-tagged fusion proteins shown above were transfected into HeLa cells. After expression of the transfected DNA, the cells were fused to NIH 3T3 cells to form heterokaryons. The heterokaryons were incubated for 4 h in the presence of 100 μg/ml cycloheximide, and then fixed and stained for immunofluorescence microscopy with mAb 9E10 (anti-myc tag) to localize the proteins, and with Hoechst 33258 to distinguish the human and mouse nuclei. (Arrows) Mouse nuclei in heterokaryons. The phase panels show the phase-contrast image of the heterokaryons, whose approximate outlines are shown by dashed lines.
also resulted in nuclear retention of this shuttling protein (Fig. 1). In contrast, when PK was fused to hnRNP A1, this had no effect on its ability to shuttle (Fig. 1). Western blot analysis showed that all fusion proteins are expressed as full-length proteins (data not shown). From these results we concluded that hnRNP C1 contains an NRS that is capable of retaining shuttling hnRNP proteins in the nucleus.

The Auxiliary Domains of Nonshuttling hnRNP Proteins Contain Nuclear Retention Sequences

The hnRNP C1 protein is shown schematically in Fig. 1 A. The major features of the protein are an RNP motif RNA-binding domain at the amino terminus (RBD) and a carboxyl-terminal auxiliary domain (AUX) rich in acidic residues (Swanson et al., 1987; Dreyfuss et al., 1993). The auxiliary domain also contains sequences that localize C1 to the nucleus (Siomi and Dreyfuss, 1995). To identify the region or regions of hnRNP C1 that have nuclear retention activity, portions of C1 were fused to A1, and the fusion proteins were tested for nuclear retention in the heterokaryon assay. Fusing the minimal RBD of C1 (amino acids 1–87; Burd and Dreyfuss, 1994) to A1 had no effect on its shuttling activity, whereas fusing the auxiliary domain of C1 (amino acids 88–290) to A1 prevented A1 from shuttling (Fig. 2). The auxiliary domain of C1 therefore contains sequences capable of retaining A1 in the nucleus.

The closest known relative of hnRNP C1 is a protein termed Raly, which was originally cloned in mouse (Michaud et al., 1993). Recently, the human homologue was cloned (Vaughan et al., 1995). Human Raly (hRaly) has a similar overall structure to C1, with a single RBD at the amino terminus of the protein, which is 70% identical to C1 RBD, and an auxiliary domain, which is 30% identical to the corresponding domain of C1 (Vaughan et al., 1995). To test the nucleocytoplasmic transport properties of hRaly, DNA encoding myc-tagged hRaly was trans-
ected into HeLa cells. The expressed protein was completely localized to the nucleus, and it was restricted to the human nuclei when analyzed in the heterokaryon assay (data not shown). Fusion of the auxiliary domain of hRaly to A1 prevented A1 from shuttling (Fig. 2). Therefore, like C1, the auxiliary domain of hRaly contains sequences that can retain A1 in the nucleus.

A 78-Amino Acid Segment of hnRNP C Proteins Is an NRS

To delineate the sequences within C1 auxiliary domain that function as NRSs, this region of the protein was subjected to amino- and carboxyl-terminal deletions. The smallest region capable of retaining A1 in the nucleus of >80% of heterokaryons examined was found to span amino acids 88-165 of C1 (Fig. 3). The smallest region that retains A1 in >60% of heterokaryons examined was residues 98-155. In the remaining heterokaryons examined for both of these fusions, the proteins show very weak shuttling. Further deletions impaired the retention activity of C1 sequences (Fig. 3). We conclude that C1 contains a core NRS of 58 amino acids (residues 98-155), which requires ~10 amino acids at both the amino and the carboxyl terminus for full NRS function (see Fig. 5 A).

The NES of A1 lies at the carboxyl terminus of the protein (Fig. 1 A). It is possible that fusing C1 sequences to A1 may obscure the NES, and therefore block its export nonspecifically. The observations that PK-A1 shuttled as readily as A1 under the assay conditions used (Fig. 1), and that fusing residues 88-190 of C1 to the amino terminus of A1 or fusing residues 88-175 to the carboxyl terminus of A1 retained it in the nucleus, argue that C1 NRS functions by a specific mechanism, rather than by occlusion of NESs.

The hnRNP C1 NRS and NLS Overlap

Sequences within C1 that are required to localize it to the nucleus have previously been mapped to the region between residues 145 and 174 (Siomi and Dreyfuss, 1995). Since the NRS includes part of this region and contains clusters of basic residues, the ability of the NRS and several amino- and carboxyl-terminal deletions of the NRS to localize the cytoplasmic protein PK to the nucleus was tested. The 78-amino acid C1 NRS (residues 88-165) was able to localize PK to the nucleus (Fig. 4 A, a and b). The smallest portion within this segment, which completely localized PK to the nucleus in all cells, was the 58-amino acid segment (residues 98-155) that corresponds to the core NRS (Fig. 4 A, c). Removal of five amino acids from the carboxyl terminus slightly impaired NLS function, and further deletions had a more dramatic effect (Fig. 4 A, d-f). Comparison of nuclear retention activity and nuclear localization activity of C1 sequences revealed a strong coincidence of these two activities (Fig. 4 B).

Discussion

In this work we have addressed the mechanism by which nonshuttling hnRNP C proteins are restricted to the nucleus. We found that these proteins remain in the nucleus not because they lack an NES, but because they contain an NRS. When an NES and an NRS are present in the same RNA-binding protein, e.g., in an hnRNP C1-A1 fusion protein, the NRS overrides the NES, so that C1-A1 is retained in the nucleus.

By fusing portions of the C1 protein to A1 and testing the shuttling activity of the fusion proteins using the heterokaryon assay, we delineated the NRS in hnRNP C1 that is sufficient to retain A1 in the nucleus. The C1 NRS comprises ~78 amino acids (residues 88-165), which contains a core sequence of ~58 amino acids (residues 98-155). The core sequence has considerable NRS activity, but it requires an additional ~10 residues at both termini for full NRS activity. Most of the NRS lies within the auxiliary domain of C1, although the 17 amino-terminal residues overlap the RNA-binding domain (Fig. 5 A). The C1 NRS contains a proline-rich region, clusters of basic residues, potential phosphorylation sites for casein kinase II and protein kinase C, and a potential glycosylation site. Whether any of these motifs are important for NRS function is not yet known.

To our knowledge, C1 NRS is the first protein nuclear retention sequence that can retain NES-bearing proteins in the nucleus to be described. Other proteins have been found to contain regions that appear to mediate their retention in the nucleus. For example, the very weak shuttling ability of the nucleolar protein nucleolin can be enhanced by deletion of domains that target it to the nucleolus; the nonshuttling protein lamin B2 is converted to a shuttling protein by removal of regions required for its polymerization at the nuclear membrane (Schmidt-Zachmann et al., 1993; discussed in Laskey and Dingwall, 1993); and it was recently reported that the La protein contains a region of ~200 amino acids that restricts it largely to the nucleus (Simons et al., 1996). However, it has not been shown that these proteins have transferable NRSs capable of conferring nuclear retention on NES-bearing proteins.

The sequence of the C1 NRS region is conserved between human and Xenopus C1 proteins (Fig. 5 B). The auxiliary domain of human Raly, the closest relative of hnRNP C proteins, also has the ability to retain shuttling hnRNP proteins in the nucleus. The NRS of Raly has not been delineated, but alignment of Raly and C1 reveals a modest homology between C1 NRS and the corresponding region of Raly (Fig. 5 B). Screening sequence data bases with C1 NRS showed that it bears no obvious homology to other proteins, except around the proline-rich region. However, we suggest that other nonshuttling nuclear RNA-binding proteins (e.g., hnRNP U) contain functionally equivalent sequence motifs that retain them in the nucleus and that can override NESs.

A strong correlation between NRS and NLS activity of C1 sequences was observed, suggesting that the same region of C1 confers both nuclear localization and nuclear retention on the protein. Although C1 NRS contains clusters of basic residues, it does not conform to the rather loose consensus for classical basic NLS motifs (Dingwall and Laskey, 1991; Boulkas, 1993). However, C1 NRS does interact with Rch1 (Nakielny, S., and G. Dreyfuss, unpublished results), a component of the import machinery that localizes classical basic NLS-containing proteins to the nucleus (Cuomo et al., 1994; Sweet and Gerace, 1995; Görlich and Mattaj, 1996). Since the NRS and NLS of C1 appear to overlap, it is possible that components of
Figure 3. Delineation of the nuclear retention sequence in the auxiliary domain of hnRNP C1. (A) Plasmids encoding myc-tagged A1 fused to portions of the auxiliary domain of C1 were transfected into HeLa cells, and the transport properties of the expressed proteins were analyzed using the heterokaryon assay exactly as described in Fig. 1 B. (B) Summary of the heterokaryon analyses. The auxiliary domain of C1 (aa 88–290), and portions of it, are represented by the black bars. ++, +, and +/-, nuclear retention in ~80, ~60, and ~40% of heterokaryons examined, respectively. -, negligible nuclear retention activity.
the import machinery that interact with the NLS may have a second function of retaining C1 in the nucleus. However, this hypothesis remains to be tested.

The existence of two populations of hnRNP proteins, shuttling hnRNP proteins and nonshuttling hnRNP proteins, that differ in their nucleocytoplasmic transport properties invites speculation as to why this may be. As outlined in the Introduction, shuttling hnRNP proteins like A1 are strongly implicated as mediators of mRNA export (Piñol-Roma and Dreyfuss, 1992, 1993a; Michael et al., 1995a,b). Much less is understood of the role of nonshuttling hnRNP proteins like C1. However, it is an attractive possibility that this group of hnRNP proteins prevents transcripts that have not been fully processed from exiting the nucleus to the cytoplasm, where their translation would be detrimental to the cell. The majority of pre-mRNA molecules are not exported to the cytoplasm, despite being associated with NES-bearing hnRNP proteins. However, pre-mRNAs are also associated with hnRNP C proteins, whose NRS overrides at least two different NESs (the M9 NES of A1, and the novel NES of hnRNP K; Michael, W.M., and G. Dreyfuss, manuscript in preparation). Therefore, we speculate that NRS-bearing proteins, by overriding NESs, can retain pre-mRNA in the nucleus. A role for spliceosomes in nuclear retention of pre-mRNA has been described, although the molecular mechanism of spliceosome-mediated retention has not yet been characterized (Legrain and Rosbash, 1989; Chang and Sharp, 1989; Hamm and Mattaj, 1990). It is possible that hnRNP C proteins are components of this retention mechanism and/or that spliceosomal factors contain NRSs. We also note that hnRNP C proteins appear to specifically interact with a nuclear retention element in human immunodeficiency virus RNA, suggesting a role for the C proteins in retention of this RNA (Olsen et al., 1992).

We do not know at what point(s) the C proteins are removed from RNA during pre-mRNA maturation and transport. EM of Balbiani ring mRNA has revealed a dra-

Figure 4. Comparison of NLS and NRS activity of hnRNP C1 auxiliary domain sequences. (A) The C1 NRS is an NLS. Plasmids encoding myc-tagged PK or myc-tagged PK fused to portions of the auxiliary domain of C1 protein were transfected into HeLa cells, and the subcellular localizations of the expressed proteins were analyzed by fixing and staining the cells with mAb 9E10 for immunofluorescence microscopy. (B) Summary of the subcellular localization analyses of PK fused to portions of C1 auxiliary domain, and comparison of NLS and NRS activity of these regions of C1. The portions of C1 fused either to PK (to test NLS activity) or to A1 (to test NRS activity) are represented by the black bars. N, nuclear localization; C, cytoplasmic localization.
ing proteins are removed from pre-mRNA/mRNA will be important for understanding the fundamental eukaryotic cell process of mRNA export from the nucleus.

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Figure 5. Functional domains of hnrNRP C1. (A) Amino acid sequence of hnrNRP C1 and location of functional domains. The RNA-binding domain (RBD, residues 1--104; Gorlach et al., 1994) is outlined, the core NRS is boxed in black, and the additional regions that are required for full NRS activity are boxed in gray. (B) Alignment of human C1 NRS with the corresponding regions of Xenopus C1 and human Raly. Amino acids 85--168 of human C1, 86--167 of Xenopus C1, and 90--154 of human Raly are aligned. Identical residues are boxed in black.

mantic reorganization of the mRNA--protein complex during translocation through the NPC, which likely coincides with shedding of proteins from the mRNA (Mehlin and Daneholt, 1993). We suggest that these proteins are the insect counterparts of NRS-bearing hnrNRP C proteins. The removal of NRS-bearing hnrNRP proteins from pre-mRNA/mRNA is likely to be critical to allow mRNA to be exported from the nucleus, and we expect this to be an important posttranscriptional regulatory step in the pathway of gene expression.

The molecular mechanisms by which C1 NRS operates and by which NRS-bearing proteins are removed from RNA are currently unknown. Most likely, NRS function is mediated by its interaction with components of the nucleus. We have found that C1 NRS is active in the context of an RNA-binding protein. It will be of interest to determine whether the NRS is also functional in a protein fusion that does not bind RNA, i.e., outside the context of an hnRNP complex, and whether the retention machinery can recognize the NRS in other environments. Removal of C1 from mRNA may involve posttranslational modifications. The C proteins are phosphorylated in vivo (Holcomb and Friedman, 1984; Pi#ol-Roma and Dreyfuss, 1993b), and it appears that phosphorylation can decrease their RNA-binding activity (Mayrand et al., 1993).

In summary, the abundant nuclear pre-mRNA/mRNA-binding hnrNRP C proteins have been shown to contain a sequence that retains them in the nucleus. This protein element, which we term NRS, serves as a nuclear retention sequence that is capable of overriding the activity of NESs of shuttling hnrNRP proteins that are most likely mediators of mRNA export. Elucidating the molecular mechanism of NRS function and the mechanism by which NRS-contain-
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