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Nuclear Localization of *Schizosaccharomyces pombe* Mcm2/Cdc19p Requires MCM Complex Assembly

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The minichromosome maintenance (MCM) proteins MCM2–MCM7 are conserved eukaryotic replication factors that assemble in a heterohexameric complex. In fission yeast, these proteins are nuclear throughout the cell cycle. In studying the mechanism that regulates assembly of the MCM complex, we analyzed the *cis* and *trans* elements required for nuclear localization of a single subunit, Mcm2p. Mutation of any single *mcm* gene leads to redistribution of wild-type MCM subunits to the cytoplasm, and this redistribution depends on an active nuclear export system. We identified the nuclear localization signal sequences of Mcm2p and showed that these are required for nuclear targeting of other MCM subunits. In turn, Mcm2p must associate with other MCM proteins for its proper localization; nuclear localization of MCM proteins thus requires assembly of MCM proteins in a complex. We suggest that coupling complex assembly to nuclear targeting and retention ensures that only intact heterohexameric MCM complexes remain nuclear.

INTRODUCTION

The minichromosome maintenance (MCM) proteins are a family of essential eukaryotic replication proteins with six distinct members (MCM2–MCM7) (reviewed by Chong *et al.*, 1996; Romanowski and Madine, 1996; Kearsey and Labib, 1998). Initially identified in *Saccharomyces cerevisiae*, they have been characterized in a wide variety of eukaryotes, including human, mouse, *Xenopus*, *Drosophila*, and *Arabidopsis*, as well as in the fission yeast *Schizosaccharomyces pombe*. The MCM proteins are essential for the initiation of replication and normal S phase progression. Although closely related, each MCM protein is essential for viability. This suggests that they contribute unique activities to their common function.

The MCM proteins form heteromeric complexes with the proteins in a 1:1 stoichiometry (reviewed by Kearsey and Labib, 1998). The hexameric complex is apparently composed of a subcomplex consisting of a tightly associated core complex (MCM4/6/7) that is loosely associated with MCM2 and a more loosely associated subcomplex, the peripheral dimer (MCM3/5) (Table 1) (Burkhart *et al.*, 1995; Kimura *et al.*, 1996; Schulte *et al.*, 1996; Adachi *et al.*, 1997; Kubota *et al.*, 1997; Thommes *et al.*, 1997; Sherman and Forsburg, 1998; Sherman *et al.*, 1998). Although the precise molecular function of the MCM protein complex remains to be determined, a recent report showed weak ATPase and DNA helicase activities in the core complex of human MCM4/6/7 (Ishimi, 1997), which may be regulated by MCM2 (Ishimi *et al.*, 1998). To identify the *cis* and *trans* factors required for complex assembly, we have examined in detail a single fission yeast subunit, Mcm2p (encoded by the *cdc19*$^+$ gene). Previously, we carried out an extensive mutational analysis of the protein to define its functional domains (Forsburg *et al.*, 1997). Using our panel of mutant derivatives, we used coimmunoprecipitation analyses to demonstrate that multiple regions of Mcm2p are required for its association with other proteins in the MCM complex (Sherman *et al.*, 1998). In this study, we investigate a mechanism for regulating the assembly of the functional hexameric MCM complex in the nucleus.

MCM proteins are located in the nucleus throughout the cell cycle in most organisms (reviewed by Kearsey and Labib, 1998). The exception is the budding yeast *S. cerevisiae*, in which most MCM proteins exhibit cell cycle–regulated nuclear localization and are found in the nucleus only during G1 and S phase; one recent report disputes this (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995; Young *et al.*, 1997; Young and Tye, 1997). However, the MCM proteins in mammalian, *Xenopus*, and *Drosophila* systems remain in the nucleus throughout interphase, although intranuclear distribution changes. The proteins are chromatin associated in late mitosis but are displaced progressively during S phase. Chromatin association appears to correlate with the phosphorylation state of the MCM proteins (reviewed by Kearsey and Labib, 1998).

In fission yeast, all six MCM proteins are constitutively in the nucleus (Maiorano *et al.*, 1996; Okishio *et al.*, 1996; Sherman and Forsburg, 1998; this work; Liang and Forsburg, unpublished data), as are their metazoan homologues. In the

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Table 1. *S. pombe* MCM proteins and their positions in the complex

MCM protein	*S. pombe* gene	MCM complex component
MCM2	*cdc19*$^+$/*nda1*$^+$	Peripheral (forms subcomplex with core complex)
MCM3	*mcm3*$^+$	Peripheral dimer
MCM4	*cdc21*$^+$	Core complex
MCM5	*nda4*$^+$	Peripheral dimer
MCM6	*mis5*$^+$	Core complex
MCM7	*mcm7*$^+$	Core complex

Designation of membership in the MCM complex is based on previous studies (Burkhart *et al.*, 1995; Kimura *et al.*, 1996; Schulte *et al.*, 1996; Adachi *et al.*, 1997; Kubota *et al.*, 1997; Thommes *et al.*, 1997; Sherman and Forsburg, 1998; Sherman *et al.*, 1998).

current study, we present evidence demonstrating for the first time that this nuclear localization depends on the assembly of an intact hexameric MCM complex. We show that nuclear localization of wild-type MCM proteins is disrupted in *mcm* mutant strains, suggesting that localization of each MCM protein depends on the other MCM proteins. Our data further suggest that wild-type MCM subunits are actively exported from the nucleus when MCM function is abrogated. Focusing on the Mcm2p subunit, we show that mutant Mcm2 proteins defective in binding to other MCM proteins are also defective for nuclear localization but that the NLS-defective mutant Mcm2p is still capable of binding other MCM proteins. Interestingly, the NLS sequences we define in Mcm2p are necessary but not sufficient for its localization. We propose that MCM protein localization requires both targeting to and retention in the nucleus and that these events require assembly of an MCM complex.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast cultures were grown in rich (yeast extract with supplements) or Edinburgh minimal medium supplemented with leucine, adenine, and uracil as needed at 25°C unless indicated otherwise (Moreno *et al.*, 1991). Culture medium was purchased from Difco Laboratories (Detroit, MI). Repression of the *nmt* promoter was maintained by addition of thiamine to 15 μM. For monitoring the localization of mutant Mcm2 proteins, low-level expression was induced by growing cells in selective medium supplemented with 0.05 μM thiamine for 20 h (Javerzat *et al.*, 1996). For monitoring the localization of the chimeric jellyfish green fluorescent protein and β-galactosidase fusion protein (GFP-βgal), cells were induced for expression in the absence of thiamine for 24 h at 25°C. Congenic *S. pombe* strains were originally derived from the strain 972 (h^-). The fission yeast strains used in this study are listed in Table 2. Strains FY831, FY832, FY835, FY836, FY837, and FY979 were generated by crossing the temperature-sensitive mutants to FY798. The *mis5-268* allele used to construct FY979 and FY961, and the *crm1-809* allele used to construct FY894 and FY1102, were a gift from M. Yanagida (Kyoto University, Kyoto, Japan). The *crm1-11R* allele used to construct strains FY1139 and FY1152 was a gift from T. Toda (Imperial Cancer Research Fund Laboratories, London, United Kingdom). NLS reporter strains were generated by transformation of the wild-type fission yeast strain FY6 with each *GFP-lacZ* integrating plasmid digested with *Nru*I to target integration to the *leu1* locus. Stable integration in Leu$^+$ prototrophs was confirmed by random spore analysis. Plasmids pSGP581 (no NLS), pSGP583 (SV40 NLS), pSGP584 (mutant SV40 NLS), pSGP585 (NLS1), pSGP586 (NLS2), and pSGP587 (NLS1-M9) were integrated to generate the strains FY838 (no NLS), FY840 (SV40 NLS), FY841 (mutant SV40 NLS),

Table 2. *S. pombe* strains used in this study

Strain	Genotype	Source
FY6	*h$^-$ leu1-32 ade6-704 ura4-294*	P. Nurse
FY243	*h$^-$ cdc19-P1 ade6-M210 ura4-D18 leu1-32*	Forsburg *et al.* (1997)
FY254	*h$^-$ ura4-D18 leu1-32 ade6-M210 can1-1*	Forsburg *et al.* (1997)
FY798	*h$^-$ Δcdc19::[cdc19HA::leu1$^+$] ura4-D18 leu1-32 ade6-M210*	Snaith and Forsburg (1999)
FY831	*h$^+$ pol1-1 Δcdc19::[cdc19HA leu1$^+$] ura4-D18 leu1-32*	Derived from *pol1-1* D'Urso *et al.* (1995)
FY832	*h$^+$ cdc22-M45 Δcdc19::[cdc19HA leu1$^+$] ura4-D18 leu1-32 ade6-M210*	Forsburg *et al.* (1997)
FY835	*h$^+$ orp1-4 Δcdc19::[cdc19HA leu1$^+$] ura4-D18 leu1-32 ade6-M210*	Derived from *orp1-4* Grallert and Nurse (1996)
FY836	*h$^+$ cdc21-M68 Δcdc19::[cdc19HA leu1$^+$] ura4-D18 leu1-32 ade6-M210*	Forsburg *et al.* (1997)
FY837	*h$^+$ cdc17-K42 Δcdc19::[cdc19HA leu1$^+$] ura4-D18 leu1-32 ade6-M210*	Forsburg *et al.* (1997)
FY838	*h$^-$ leu1-32::leu1$^+$/nmt/GFP-lacZ ura4-294 ade6-704*	This study
FY840	*h$^-$ leu1-32::leu1$^+$/nmt/SV40 NLS-GFP-lacZ ura4-294 ade6-704*	This study
FY841	*h$^-$ leu1-32::leu1$^+$/nmt/mutSV40 NLS-GFP-lacZ ura4-294 ade6-704*	This study
FY842	*h$^-$ leu1-32::leu1$^+$/nmt/NLS1-GFP-lacZ ura4-294 ade6-704*	This study
FY843	*h$^-$ leu1-32::leu1$^+$/nmt/NLS2-GFP-lacZ ura4-294 ade6-704*	This study
FY844	*h$^-$ leu1-32::leu1$^+$/nmt/M9-GFP-lacZ ura4-294 ade6-704*	This study
FY894	*h$^-$ crm1-809 leu1-32 ura4-D18 ade6-M210*	Derived from *crm1-809* (Nishi *et al.*, 1994)
FY961	*h$^+$ mis5-268 ura4-D18 leu1-32 ade6-M210*	Derived from Y107 (Takahashi *et al.*, 1994)
FY979	*h$^-$ mis5-268 Δcdc19::[cdc19HA leu1$^+$] leu1-32 ura4-D18 ade6-M210*	This study
FY1023	*h$^-$ Δmcm3::[mcm3HA leu1$^+$] ura4-D18 leu1-32 ade6-M216 can1-1*	This study
FY1100	*h$^+$ cdc19-P1 Δmcm3::[mcm3HA leu1$^+$] ura4-D18 leu1-32 ade6-M216*	This study
FY1101	*h$^-$ cdc21-M68 Δmcm3::[mcm3HA leu1$^+$] ura4-D18 leu1-32 ade6-M216 can1-1*	This study
FY1102	*h$^-$ crm1-809 Δmcm3::[mcm3HA leu1$^+$] ura4-D18 leu1-32 ade6-M216*	This study
FY1139	*h$^-$ cdc21-M68 crm1-11R leu1-32 ura4-D18 ade6-M210 his3-D1*	This study
FY1152	*h$^-$ cdc19-P1 crm1-11R leu1-32 ura4-D18 ade6-M216*	This study

FY842 (NLS1), FY843 (NLS2), and FY844 (NLS1-M9). The strain FY1023 was constructed by digesting the pJK148-derived vector containing the triple hemagglutinin (HA) epitope tag fused to the carboxy terminus of Mcm3p (pDS97) with *Sph*I and integrating it into a wild-type fission yeast diploid. Leu^+ diploids were sporulated to obtain Leu^+ haploid prototrophs. Leu^+ haploids were cold sensitive (restrictive temperature, 17°C). Backcross of the Leu^+ haploid to Leu^- confirmed linkage of the cold-sensitive phenotype to the Leu^+ marker. Genomic *mcm3*$^+$ rescued the cold-sensitive phenotype. Double mutant strains FY1100, FY1101, FY1102, FY1139, and FY1152 were all constructed by tetrad dissection. Double mutant *cdc19-P1 mcm3-HA* was a rare segregant. In tetrad analysis, most double mutants arrested as microcolonies with 12–40 cells, with some cells elongated. The *cdc19-P1 mcm3-HA* strain we recovered contains an uncharacterized suppressor of the cold-sensitive phenotype of *mcm3-HA*. The strain behaves similarly to *cdc19-P1 mcm3*$^+$.

Plasmid DNA Construction

Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). A *GFP-lacZ* reporter vector, pSGP502, allows insertion of a test NLS cassette at the amino terminus of GFP-βgal. pSGP500 (*leu1*$^+$, *ars1*, *lacZ*) contains *ars1* as a 1.2-kilobase (kb) *Eco*RI/end-filled fragment cloned into *Not*I/end-filled pSLF107 (*lacZ* gene cloned into pJK148-derived vector) (Forsburg, 1993; Keeney and Boeke, 1994). pSGP501 (*leu1*$^+$, *ars1*, *GFP-lacZ* with no NLS) contains the *nmt* promoter and *GFP* gene (Haseloff *et al.*, 1997) from pSGP573 as a 2-kb *Pst*I/*Bgl*II (blunt) fragment placed upstream and in frame with *lacZ* of pSGP500 digested with *Pst*I/*Sal*I (end filled). pSGP502 (*leu1*$^+$, *ars1*, *GFP-lacZ* with no initiation ATG, unique *Kpn*I site) was constructed by swapping the *nmt* promoter and the part of *GFP* from pSGP572K (GFP carboxy-terminal tagging vector with unique *Kpn*I site at the amino terminus of GFP) as a 1.3-kb *Pst*I/*Msc*I fragment. Thus, pSGP502 can be used to assay the functionality of a putative NLS by subcloning the test NLS with its own initiation ATG into the unique *Kpn*I site in frame with the *GFP-lacZ* fusion. pSGP572 and pSGP573 are GFP-tagging vectors (Sherman, Pasion, and Forsburg, unpublished data).

Various NLS sequences were assayed by insertion at the *Kpn*I site. The NLS linker fragments were constructed by annealing pairs of oligonucleotides. The SV40 NLS (SV40 large T antigen residues 126–132, MAPKKKRKV) was constructed by annealing oligonucleotides 147 (5′ACCATGGCTCCTAAGAAGAAGCGTAAGGTTGTAC3′) and 148 (5′AACCTTACGCTTCTTCTTAGGAGCCATGGTGTAC3′). Mutant SV40 NLS (MAPKEKDKV) was constructed by annealing oligonucleotides 149 (5′ACCATGGCTCCTAAGGAGAAGGACAAGGTTGTAC3′) and 150 (5′AACCTTGTCCTTCTCCTTAGGAGCCATGGTGTAC3′). NLS1 (Mcm2p residues 5–10, MARKRGRR) was constructed by annealing oligonucleotides 151 (5′ACCATGGCTCGGAAAAGGGGTCGCCGCGTAC3′) and 152 (5′GCGGCGACCCCTTTTCCGAGCCATGGTGTAC3′). The NLS1-M9 (MARKEGDR) was constructed by annealing oligonucleotides 153 (5′ACCATGGCTCGGAAAGAGGGTGACCGCGTAC3′) and 154 (5′GCGGTCACCCTCTTTCCGAGCCATGGTGTAC3′). NLS2 (Mcm2p residues 114–118, MARLRRR) was constructed by annealing oligonucleotides 155 (5′ACCATGGCTAGGTTGAGACGACGTGTAC3′) and 156 (5′ACGTCGTCTCAACCTAGCCATGGTGTAC3′). Subcloning of each NLS linker into pSGP502 digested with *Kpn*I generated pSGP503 (SV40 NLS), pSGP504 (mutant SV40 NLS), pSGP505 (NLS1), pSGP506 (NLS2), and pSGP507 (NLS1-M9). To integrate each reporter construct into fission yeast, each *GFP-lacZ* sequence was subcloned as a *Pst*I/*Nru*I fragment into the integrating vector pJK148 (Keeney and Boeke, 1994) to generate the plasmids pSGP581 (no NLS, derived from pSGP501), pSGP583 (SV40 NLS), pSGP584 (mutant SV40 NLS), pSGP585 (NLS1), pSGP586 (NLS2), and pSGP587 (NLS1-M9).

The Mcm2p expression vectors pSLF176, pSLF196 series, and pDS196 series were described previously (Forsburg *et al.*, 1997). To construct fission yeast vectors that expressed Mcm2p-M9 with the wild-type or mutant SV40 NLS on the amino terminus, we constructed the Mcm2p-M9 expression vectors pSGP86-M9 and pSGP96-M9. A *Kpn*I linker was inserted at the unique *Sal*I site of pSLF187-M9 (Forsburg *et al.*, 1997) and at the unique *Xho*I site of the HA-tagging vector pSLF172 (Forsburg and Sherman, 1997) to generate pSGP187K-M9 and pSLF172K. The fragment encoding Mcm2p-M9 was subcloned from pSGP187K-M9 as a 3-kb *Kpn*I/*Not*I fragment into pSLF172K digested with *Kpn*I/*Not*I to generate pSGP187-M9. Either the wild-type SV40 NLS (oligonucleotides 147/148) or mutant SV40 NLS (oligonucleotides 149/150) was ligated into pSGP187-M9 digested with *Kpn*I to generate pSGP86-M9 or pSGP96-M9, respectively. To express wild-type Mcm2p or NLS mutant Mcm2p-M9, we constructed pSGP56 and pSGP56-M9, which are essentially pSLF176 and pSLF196-M9 lacking the HA epitope. The 0.7-kb *Bgl*II/*Sma*I fragment (carboxy terminus of Mcm2p with no HA epitope) was subcloned from pSLF167 into *Bgl*II/*Sma*I-digested pSLF176 or pSLF196-M9 to generate pSGP56 and pSGP56-M9. pSLF167 contains the *cdc19*$^+$ cDNA (Forsburg *et al.*, 1997).

Antibodies and Immunofluorescence Analysis

For immunofluorescence, anti-HA antibody was used at a 1:2500 dilution (mAb 16B12; BAbCO, Richmond, CA) and affinity-purified anti-Mcm4/Cdc21p and anti-Mcm6/Mis5p (Sherman *et al.*, 1998) were used at a 1:500 dilution. Secondary antibodies (donkey anti-mouse immunoglobulin G–Cy3 and donkey anti-rabbit immunoglobulin G–Cy3; Jackson Immunoresearch Laboratories, West Grove, PA) for immunofluorescence were used at a 1:250 dilution.

The immunofluorescence protocol was basically as described (Demeter *et al.*, 1995) with a modification in cell wall digestion: 0.2 mg of Novozyme 234 (BiosPacific, Emeryville, CA) and 0.5 mg of Zymolyase 20T (Seikagaku, Tokyo, Japan) per milliliter of buffer for 5–7 min at 25°C. For the double mutant strains FY1139 and FY1152, cells were digested for 15 min. DNA was stained with DAPI. For photography, cells were briefly heat fixed onto microscope slides and mounted in 4 μl of antifade (1 mg of *p*-phenylenediamine per milliliter of glycerol) and covered with glass coverslips. Microscopy was performed with a Leitz Laborlux S microscope (Leica, Wetzlar, Germany). Images were captured on Kodak (Rochester, NY) Ektachrome 400 film or on a SPOT2 charge-coupled device digital camera (Diagnostics Instruments, Sterling Heights, MI) directly into Adobe (Mountain View, CA) Photoshop. Slides were scanned by a Nikon (Garden City, NY) slide scanner.

RESULTS

MCM Protein Localization Is Compromised in mcm Mutants

Fission yeast has six MCM proteins found in the nucleus throughout the cell cycle. For simplicity, we will refer to each protein by its MCM family name rather than by its gene name in this report (Table 1). We monitored their cellular localization using indirect immunofluorescence analyses. Endogenous Mcm2p and Mcm3p were tagged at the carboxy terminus with a triple HA epitope and detected with anti-HA antibody. We detected Mcm4p and Mcm6p with affinity-purified polyclonal antibodies (Sherman *et al.*, 1998). Figure 1A verifies that our reagents give results consistent with previous reports and also shows that Mcm6p is nuclear at all stages of the cell cycle (Maiorano *et al.*, 1996; Okishio *et al.*, 1996; Sherman and Forsburg, 1998).

Mcm2p localization was disrupted in *mcm* mutant strains. At the restrictive temperature, Mcm2p nuclear localization diminished and cytoplasmic staining increased in both the *cdc21ts* and *mis5ts* strains (Figure 1, Bi and Bii). This is not

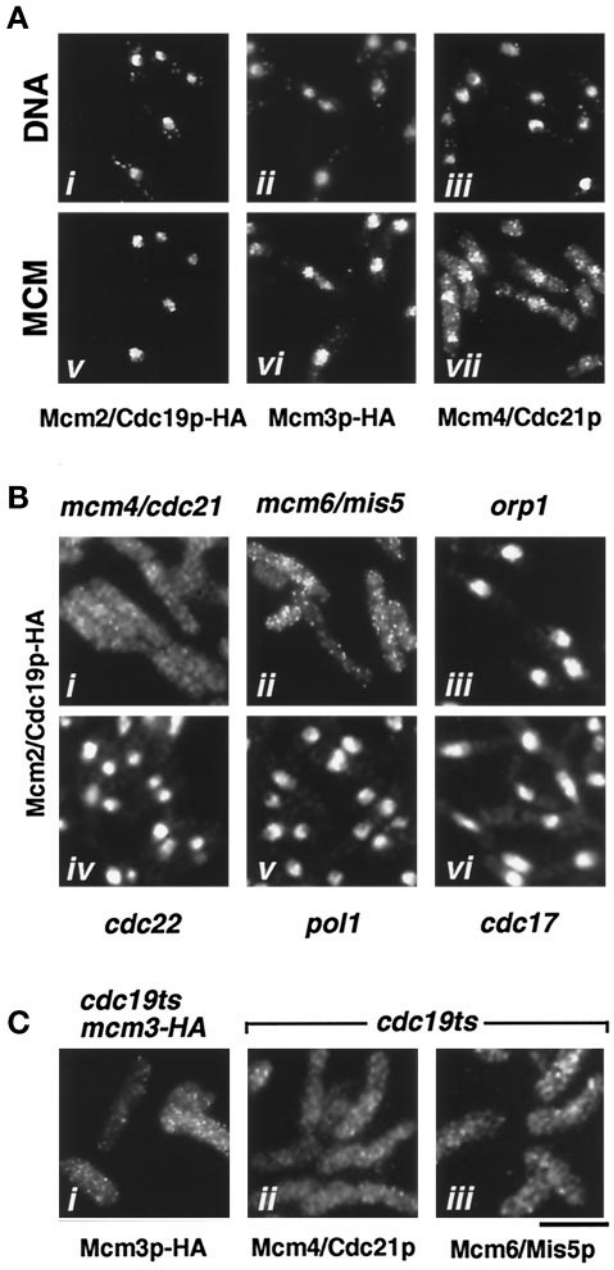

Figure 1. MCM protein localization is disrupted in *mcm* mutant strains. (A) The MCM proteins are constitutively nuclear proteins in wild-type cells. Fission yeast cells FY798 (for Mcm2p-HA) and FY1023 (for Mcm3p-HA) were incubated at 32°C and prepared for immunofluorescence as described in MATERIALS AND METHODS. Cells are shown stained with DAPI (i–iv) or antibodies (v–viii) against Mcm2p-HA (v), Mcm3p-HA (vi), Mcm4p (vii), and Mcm6p (viii). (B) Mcm2 protein localization is compromised in *mcm* mutant strains but not in other S phase mutant strains. *mcm* mutant strains FY836 (*cdc21-M68*, i) and FY979 (*mis5-268*, ii) and other S phase mutants FY835 (*orp1-4*, iii), FY832 (*cdc22-M45*, iv), FY831 (*pol1-1*, v), and FY837 (*cdc17-K42*, vi) were prepared for immunofluorescence as described in MATERIALS AND METHODS after incubation for 4 h at 36°C. Antibody staining is shown for Mcm2p-HA. (C) MCM protein localization is perturbed in *cdc19* mutant strains. Strains FY243 (*cdc19-P1*) and FY1100 (*cdc19-P1 mcm3-HA*), for detection of Mcm3p-HA, were prepared for immunofluorescence as described in MATERIALS AND METHODS after incubation for 4 h at 36°C. Antibody staining is shown for Mcm3p-HA (i), Mcm4p (ii), and Mcm6p (iii). Bar, 10 μm.

simply a result of blocking cells in S phase, because there is no effect on Mcm2p localization in other S phase mutants with defects in the *S. pombe* origin recognition complex (*orp1-4*), ribonucleotide reductase (*cdc22-M45*), DNA polymerase α (*pol1-1*), or DNA ligase (*cdc17-K42*) (Figure 1, Biii–Bvi). Mutants causing cell cycle arrest at start (*cdc10-V50*) or G2/M (*cdc25-22*) similarly had no effect on Mcm2p localization (our unpublished results). Thus, Mcm2p nuclear localization depends on other MCM proteins.

A reciprocal experiment showed that the localization of wild-type MCM proteins was affected in a *cdc19* temperature-sensitive strain. At the restrictive temperature, Mcm3p, Mcm4p, and Mcm6p nuclear localization was strikingly decreased and cytoplasmic staining was increased (Figure 1C). Thus, not only does Mcm2p localization require other MCM proteins, but localization of other MCM proteins requires Mcm2p.

We completed our analysis by assessing the localization of multiple MCM proteins in different *mcm* mutant backgrounds (Table 3A; Figure 1, B and C). At the nonpermissive temperature for each *mcm* mutant strain, every wild-type MCM protein analyzed demonstrated an increase in cytoplasmic staining and a decrease in nuclear staining. Together, these results are consistent with each MCM protein

Table 3. MCM protein localization in *mcm* mutant strains

A. *mcm* allele (phenotype[a])	Temperature (°C)[b]	Mcm2p-HA[c]	Mcm4p	Mcm6p
cdc19-P1 (ts)	36	n.d.	C	C
cdc21-M68 (ts)	36	C/N	n.d.	C
mis5-268 (ts)	36	C/N	C	n.d.
cdc19-P1 crm1-11R (ts)	36	n.d.	n.d.	N
cdc21-M68 crm1-11R (ts)	36	n.d.	n.d.	N
B.		Mcm3p-HA	Mcm4p	Mcm6p
mcm3-HA (cs)	17	N	C/N	C/N
cdc19-P1 mcm3-HA (ts)	36	C	C	C/N
cdc21-M68 mcm3-HA (cs, ts)	36	C	C	C
crm1-809 mcm3-HA (cs)	17	N	N	N

MCM protein localization determined in *mcm* mutant strains at the restrictive temperature.

[a] Temperature-sensitive phenotype: *ts,* thermosensitive; *cs,* cold sensitive.

[b] Restrictive temperature at which MCM protein localization was assayed.

[c] Localization was assayed by immunofluorescence analyses: N, predominantly nuclear; C, predominantly cytoplasmic; C/N, cytoplasmic and nuclear; n.d., not determined.

depending on other MCM subunits for appropriate nuclear localization. We next examined whether these phenotypes reflected defects in targeting, in retention, or both.

MCM Proteins Are Actively Exported from the Nucleus

The loss of the wild-type MCM proteins from the nucleus in *mcm* temperature-sensitive strains could reflect protein turnover and failure to import newly synthesized molecules, or it could indicate a defect in protein retention in the nucleus. Previously, we reported that steady-state levels of wild-type MCM proteins are unchanged in cells arrested by *mcm* temperature-sensitive mutations, even though assembly of the MCM complex is disrupted (Sherman *et al.*, 1998). Because there is no evidence for any changes in protein level, we examined the role of nuclear export in redistribution of MCM proteins in wild-type and *mcm* mutant cells. The *crm1*$^+$ gene encodes a nuclear export receptor (Fukuda *et al.*, 1997). To determine whether MCM relocalization requires active nuclear export, we simultaneously inactivated *crm1* and the MCM complex. If MCM proteins require *crm1*-dependent active export, they should be trapped in the nucleus under these restrictive conditions.

We used two *crm1* alleles for this experiment. First, we combined a cold-sensitive allele of *crm1* (*crm1-809*) (Nishi *et al.*, 1994; Kudo *et al.*, 1997) and a cold-sensitive allele of *mcm3* (*mcm3-HA*). Fortuitously, the carboxy-terminal HA epitope tag on Mcm3p confers cold sensitivity (Figure 2A); the cells arrest with a *cdc* phenotype and a 2C DNA content (our unpublished results) typical of other *mcm* conditional mutants (Coxon *et al.*, 1992; Miyake *et al.*, 1993; Forsburg and Nurse, 1994; Takahashi *et al.*, 1994). Interestingly, Mcm3p-HA itself remains in the nucleus at the restrictive temperature (Figure 2B). The same effect has been observed for other cold-sensitive MCM proteins, accompanied by enhanced detection by immunofluorescence (Okishio *et al.*, 1996). However, Mcm3p-HA is able to exit the nucleus under some conditions because it is redistributed in *cdc19* and *cdc21* temperature-sensitive strains grown at 36°C (Table 3B). Consistent with our observations for all the other mutant *mcm* strains, arrest of cold-sensitive *mcm3-HA* at 17°C resulted in redistribution of wild-type MCM proteins to the cytoplasm (Figure 2C; Table 3B). In the double mutant *crm1-809 mcm3-HA*, the wild-type MCM proteins remained in the nucleus instead of accumulating in the cytoplasm after incubation at 17°C (Figure 2D; Table 3B).

Next, we combined a temperature-sensitive allele of *crm1* (*crm1-11R*) (Kumada *et al.*, 1996) with the temperature-sensitive allele of *mcm2* (*cdc19-P1*). The results were the same: the wild-type MCM proteins now remained nuclear (Figure 2D; Table 3A). These results suggest that an active nuclear export system is required for relocalization of the MCM proteins when a single subunit is inactivated. Together, these data are consistent with a model in which localization of any one MCM protein is influenced by the activity of all other members of the complex. When MCM function is abrogated, the wild-type MCM subunits are removed from the nucleus via an active nuclear export system.

Nuclear Localization of Mcm2p Requires Multiple Domains

The only fission yeast MCM proteins reported to have identifiable nuclear localization signals are Mcm2p and Mcm3p (Forsburg and Nurse, 1994; Sherman and Forsburg, 1998). Mcm2p has two sequences with homology to the SV40 NLS (Kalderon *et al.*, 1984b): Mcm2p residues 5–10 (RKRGRR), designated NLS1, and residues 114–118 (RLRRR), designated NLS2 (Forsburg and Nurse, 1994). Nuclear localization signals of this type have been characterized in several other fission yeast proteins (Shiozaki and Yanagida, 1992; Birkenbihl and Subramani, 1995; Bouvier and Baldacci, 1995). In addition, there are two potential nuclear export sequences (NESs) (reviewed by Kim *et al.*, 1996; Nakielny and Dreyfuss, 1997; Mattaj and Englmeier, 1998) at amino acid residues 627–638 (IVTTLQARCTII) and 771–780 (VRHLESAIRL). We have previously analyzed point muta-

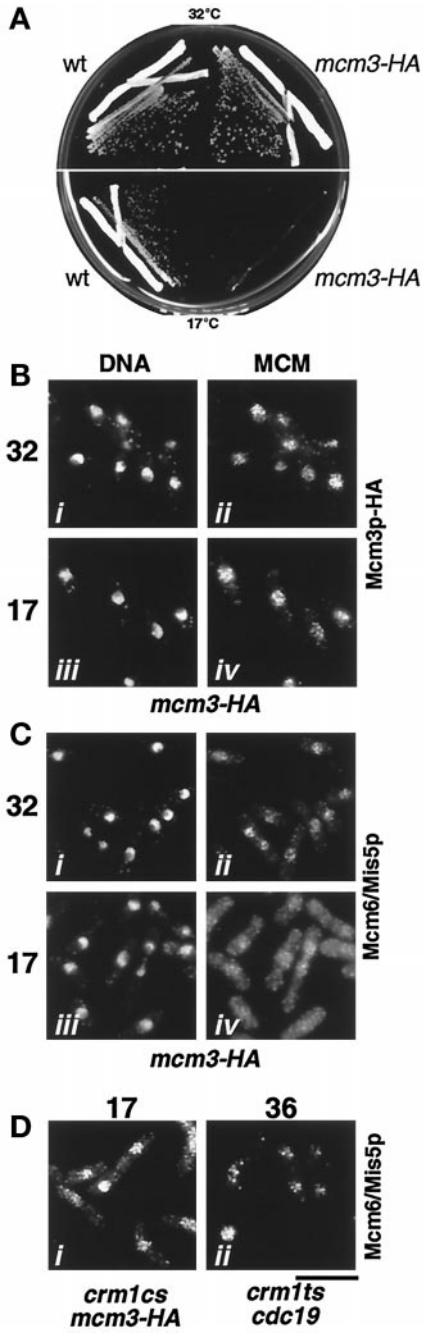

Figure 2. Relocalization of MCM proteins in *mcm* mutant is *crm1* dependent. (A) *mcm3-HA* cells fail to form colonies at 17°C. Assay of colony formation of wild-type and *mcm3-HA* cells at 32°C for 4 d or 17°C for 13 d. (B and C) MCM protein localization in *mcm3-HA*. Cells were incubated at 32°C or the restrictive temperature (17°C) for 6 h before fixation for immunofluorescence. Cells were visualized with DAPI staining (i and iii) or antibody staining (ii and iv) for Mcm3p-HA (B, ii and iv) or Mcm6p (C, ii and iv). (D) MCM protein localization in *crm1-809 mcm3-HA* (i) and *crm1-11R cdc19-P1* (ii) mutant strains. Double mutant cells were incubated at the restrictive temperature (17°C) for 6 h or at 36°C for 4 h before fixation for immunofluorescence. Antibody staining is shown for Mcm6p. MCM proteins remain in the nucleus in wild-type or *crm1* mutants at 17°C (our unpublished results). Bar, 10 μm.

tions and sequence deletions in these regions of Mcm2p for their effect on complementation (Forsburg *et al.*, 1997) and complex assembly (Figure 3K; Table 4) (Sherman *et al.*, 1998). We used indirect immunofluorescence to determine the effect of these mutations on Mcm2p localization. Wild-type and mutant proteins were expressed at low levels in wild-type cells under the control of the thiamine-regulatable *nmt* promoter. Each mutant protein was tagged with an HA epitope at the carboxy terminus, which allowed the episomally encoded protein to be distinguished from the endogenous wild-type Mcm2p. The epitope tag itself has no effect on normal Mcm2p function or localization in a wild-type strain background (Forsburg *et al.*, 1997).

Wild-type HA-tagged Mcm2p expressed at low levels from the plasmid (Figure 3A) showed similar localization to that seen with Mcm2p-HA integrated under its own promoter (Figure 1A), although with a slightly higher cytoplasmic background. However, when the promoter was fully induced and expression was ~100-fold that of endogenous Mcm2p (Forsburg *et al.*, 1997), the cells stained heavily throughout the cytoplasm for Mcm2p-HA and the nuclei appeared dark (Figure 3B). Interestingly, this overproduction has no toxic effect (Forsburg *et al.*, 1997). That excess Mcm2p accumulates in the cytoplasm suggests that there may be some mechanism or limiting factor regulating the level of free Mcm2p in the nucleus. The phenotype of Mcm2p overexpression in wild-type cells contrasts with the phenotype of Mcm4p overproduction, which is toxic and results in the accumulation of spots or aggregates of Mcm4p in the cytoplasm (Maiorano *et al.*, 1996; Forsburg *et al.*, 1997).

Using this episomal expression assay, we tested our panel of mutant Mcm2 proteins for defects in localization. The results of this assay are shown in Figure 3 and summarized in Table 4. Only functional versions of Mcm2p (wild type or mutants with small amino-terminal deletions) were clearly detected as nuclear proteins (Figure 3, A and G) (our unpublished results). These proteins are all able to complement *cdc19-P1* and form MCM complexes with wild-type affinity, as assayed previously by coimmunoprecipitation analyses (Forsburg *et al.*, 1997; Sherman *et al.*, 1998). Mcm2p-M6 and Mcm2p-M7 (NTP-binding site mutant; Figure 3D) (our unpublished results) showed weak nuclear localization. These proteins have mutations in the same residue and have weak MCM-binding ability; only Mcm2p-M7 can complement (Forsburg *et al.*, 1997; Sherman *et al.*, 1998). The remaining mutant proteins all showed widespread cytoplasmic staining and failed to accumulate in the nucleus. In general, functional Mcm2p mutants were able to localize properly, whereas mutants that failed to complement *cdc19ts* also failed to localize to the nucleus.

When we correlated nuclear localization to binding and complementation data, the noncomplementing cytoplasmic mutants fell into two classes. The majority of these were defective not only in nuclear localization but also in MCM complex assembly (Table 4) (Forsburg *et al.*, 1997; Sherman *et al.*, 1998). These included most mutations constructed throughout the Mcm2 protein (Table 4; Figure 3) (our unpublished results): the zinc finger mutants Mcm2p-M1, -M2, -M3, and -M4 (Figure 3C) (our unpublished results); the carboxy-terminal truncation Mcm2p-D6 (Figure 3H); the large amino-terminal truncation Mcm2p-D7 (Figure 3I); and

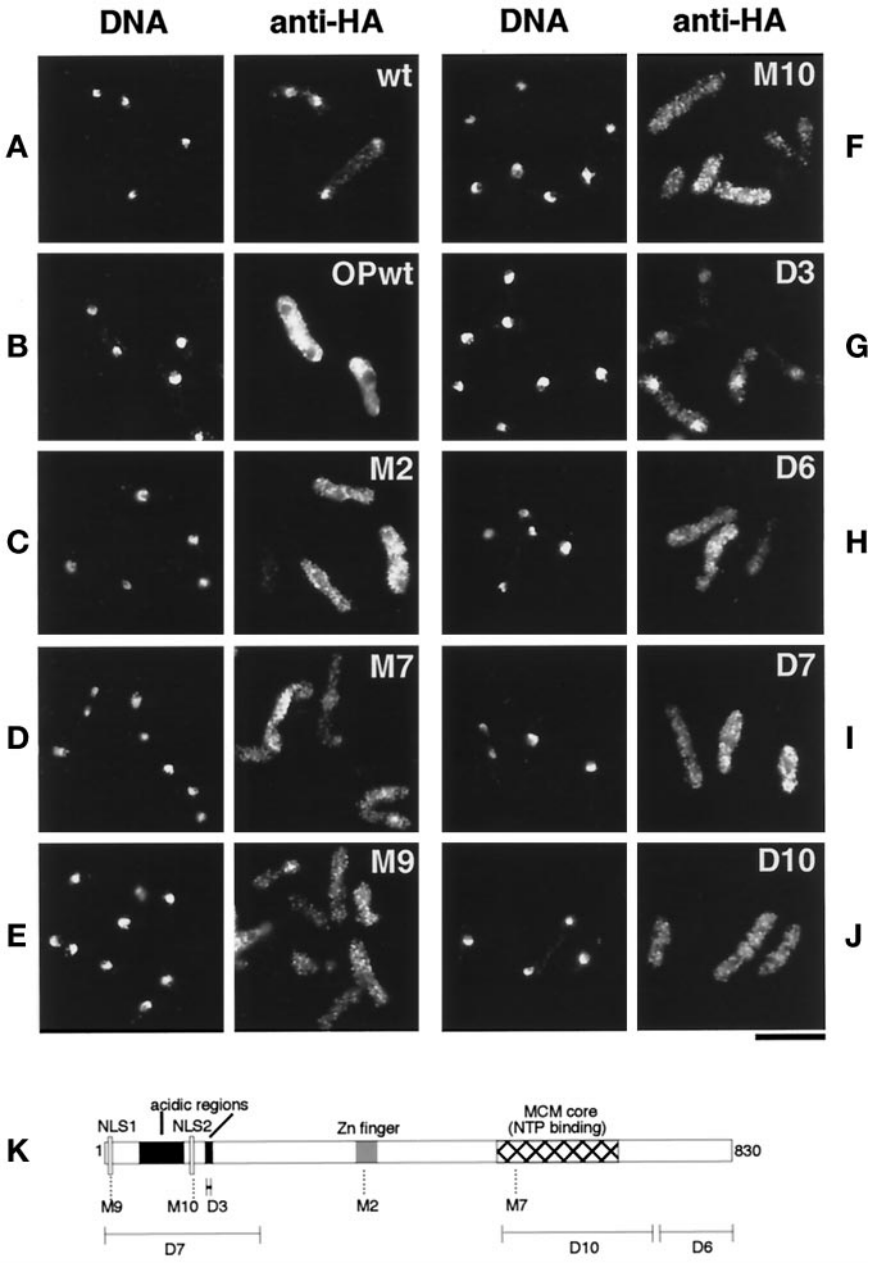

Figure 3. Multiple regions of Mcm2p affect nuclear localization. (A–J) Wild-type fission yeast cells expressing either wild-type or mutant Mcm2p were prepared for immunofluorescence as described in MATERIALS AND METHODS. Note that some cells fail to stain because of variation in the plasmid copy number or the loss of the episome. DAPI (left panel) and Mcm2p-HA (right panel) antibody staining are shown for wild-type Mcm2p (A), overexpressed wild-type Mcm2p (B), zinc finger point mutant Mcm2p-M2 (C), NTP-binding point mutant Mcm2p-M7 (D), NLS1 point mutant Mcm2p-M9 (E), NLS2 point mutant Mcm2p-M10 (F), small amino-terminal deletion Mcm2p-D3 (G), carboxy-terminal truncation Mcm2p-D6 (H), amino-terminal truncation Mcm2p-D7 (I), and MCM core deletion Mcm2p-D10 (J). Bar, 10 μm. (K) Scheme of Mcm2p structural motifs and location of mutations. Mcm2p structural motifs as well as positions of point and deletion mutations are indicated (Forsburg *et al.*, 1997). See Table 4 for description of mutations.

the deletions spanning the MCM core homology domain, Mcm2p-D8, -D9, and -D10 (Figure 3J) (our unpublished results). Thus, in most cases, mutations that result in defective MCM protein interaction also disrupt Mcm2p nuclear localization.

However, two mutant proteins retained the ability to bind other MCM proteins but remained cytoplasmic (Figure 3, E and F) (Forsburg *et al.*, 1997; Sherman *et al.*, 1998). Mcm2p-M9 and Mcm2p-M10 contain mutations in the two putative NLS elements. Thus, under some conditions, binding of Mcm2p to other MCM proteins can be uncoupled from nuclear localization. We further characterized these mutant proteins to determine whether they define true NLS sequences and used them as tools to determine whether Mcm2p binds other MCM proteins in the cytoplasm.

NLS1 Is a Functional Fission Yeast NLS

The mutants Mcm2p-M9 and Mcm2p-M10 fail to localize properly, suggesting that they define sequences necessary for nuclear localization. We asked whether these putative NLS elements are sufficient for nuclear localization. We fused NLS1 or NLS2 to a chimeric GFP-βgal fusion protein. This reporter strategy has been used successfully to assay NLS function in budding yeast (Lee *et al.*, 1996; Yoon *et al.*,

Table 4. Summary of mutant Mcm2p phenotypes

Plasmid	Mutation/affected region	Rescues *cdc19ts*[a]	MCM complex formation[b]	Localization[c]
pSLF176	None/wild type	+	++	N
pSLF196-M1	C334S/zinc finger	−	−	C
pSLF196-M2	C337S/zinc finger	−	−	C
pSLF196-M3	C357S/zinc finger	−	−	C
pSLF196-M4	C360S/zinc finger	−	−	C
pSLF196-M6	K540A/putative NTP-binding site	−	+	N/C
pSLF196-M7	K540R/putative NTP-binding site	+	+	N/C
pSLF196-M9	R7E R9D/NLS1 mutant	−	++	C
pSLF196-M10	A113T R116E R117E/NLS2 mutant	−	++	C
pSLF196-D1	Δ50-79/acidic region	+	++	N
pSLF196-D2	Δ80-104/acidic region	+	++	N
pSLF196-D3	Δ137-143/acidic region	+	++	N
pSLF196-D4	Δ45-126/acidic region	−	−	C
pDS196-D6	Δ736-830/carboxy terminus	−	−	C
pDS196-D7	Δ1-204/amino terminus	−	−	C
pDS196-D8	Δ528-613/MCM homology core	−	−	C
pDS196-D9	Δ615-724/MCM homology core	−	−	C
pDS196-D10	Δ528-724/MCM homology core	−	−	C

[a] Forsburg *et al.* (1997); +, rescues *ts* phenotype; −, no rescue of *ts* phenotype.

[b] Sherman *et al.* (1998); −, no binding; +, weak binding; ++, binding; based on coimmunoprecipitation analyses.

[c] Localization assayed in wild-type strain (FY254): N, nuclear; C, cytoplasmic; N/C, nuclear and cytoplasmic.

1997). We integrated the *GFP-lacZ* reporter gene at the *leu1* locus in fission yeast. For positive and negative controls, we fused the reporter to the wild-type SV40 NLS, which has been shown to function in fission yeast (Shiozaki and Yanagida, 1992), and to a nonfunctional mutant SV40 NLS (Kalderon *et al.*, 1984a). GFP-βgal requires a functional NLS for nuclear localization (Figure 4B). Absence of an NLS or insertion of the mutant SV40 NLS in place of the wild-type NLS generated a cytoplasmic protein (Figure 4, A and C). We determined that the NLS1 sequence from Mcm2p also targets the reporter protein to the nucleus (Figure 4D), whereas point mutations corresponding to Mcm2p-M9 disrupt NLS function in this reporter assay (Figure 4E). Thus, NLS1 fulfills the criteria for a bona fide nuclear localization sequence, being both necessary and sufficient for nuclear targeting.

Neither NLS2 nor the mutant NLS2-M10 was able to target the reporter to the nucleus (Figure 4F) (our unpublished results). In this protein context, the NLS2 sequence is insufficient for activity. It is possible that the NLS2 sequence as defined is incomplete and requires additional flanking sequence. The sequence is still necessary for localization of Mcm2p (Figure 3F), even though it is not sufficient for targeting a reporter protein.

Finally, we demonstrated that the only defect in Mcm2p-M9 is in nuclear localization. When fused to the SV40 NLS, this protein was able to complement *cdc19ts* and Δ*cdc19* and was correctly localized to the nucleus (Figure 5A) (our unpublished results). There was no complementation when we used a mutant derivative of SV40 NLS (Figure 5A) (our unpublished results). Thus, the only defect in Mcm2p-M9 is its failure to localize to the nucleus. Similar results were obtained for Mcm2p-M10 (our unpublished results).

MCM Subcomplexes Can Form in the Cytoplasm

Because Mcm2p-M9 and Mcm2p-M10 cannot localize to the nucleus (Figure 3, E and F) but can associate with other MCM proteins (Sherman *et al.*, 1998), we predicted that binding of Mcm2p to the core complex (Mcm4/6/7) occurs in the cytoplasm at a step that precedes nuclear import. To confirm this, we sought an in vivo assay independent of immunoprecipitation analyses. We reasoned that if Mcm2p-M9 binds the core complex in the cytoplasm, then increasing the dose of Mcm2p-M9 might trap wild-type MCM proteins in the cytoplasm. At sufficiently high levels, this redistribution of wild-type MCM proteins should inhibit growth as the MCM proteins become limiting in the nucleus. Previously, we showed that overexpression of Mcm2p-M9 is not toxic in wild-type cells but that overexpression of Mcm2p-M9 in *cdc19ts* cells is lethal at the permissive temperature (Forsburg *et al.*, 1997). This "synthetic dosage lethality" is also observed on overexpression of Mcm2p-M9 in the double mutant *cdc19-P1 mcm3-HA* strain (Figure 5B). The amount of Mcm2 temperature-sensitive protein is reduced in these strains (Sherman *et al.*, 1998; Liang *et al.*, 1999), and the protein has a reduced affinity for other MCM proteins compared with wild-type Mcm2p (Sherman *et al.*, 1998). This suggests that the Mcm2-M9 protein outcompetes the Mcm2 temperature-sensitive protein for binding to the wild-type MCM proteins in this strain. If so, we predict that the wild-type MCM proteins should be redistributed to the cytoplasm when Mcm2p-M9 is overproduced.

This proved to be correct. As shown in Figure 5C, the toxic phenotype associated with overproduction of Mcm2p-M9 in *cdc19-P1 mcm3-HA* is indeed accompanied by redistribution of wild-type MCM proteins to the cytoplasm. Localization is normal in strains overproducing

panel	strain	NLS	source	NLS sequence	localization
A	FY838	none			C
B	FY840	SV40	SV40 NLS aa 126-132	MA**PKKKRKV**	N
C	FY841	SV40mut		MA**PKEKDKV**	C
D	FY842	NLS1	Cdc19p aa 5-10	MA**RKRGRR**	N
E	FY844	M9	NLS1 point mutant	MA**RKEGDR**	C
F	FY843	NLS2	Cdc19p aa 114-118	MA**RLRRR**	C

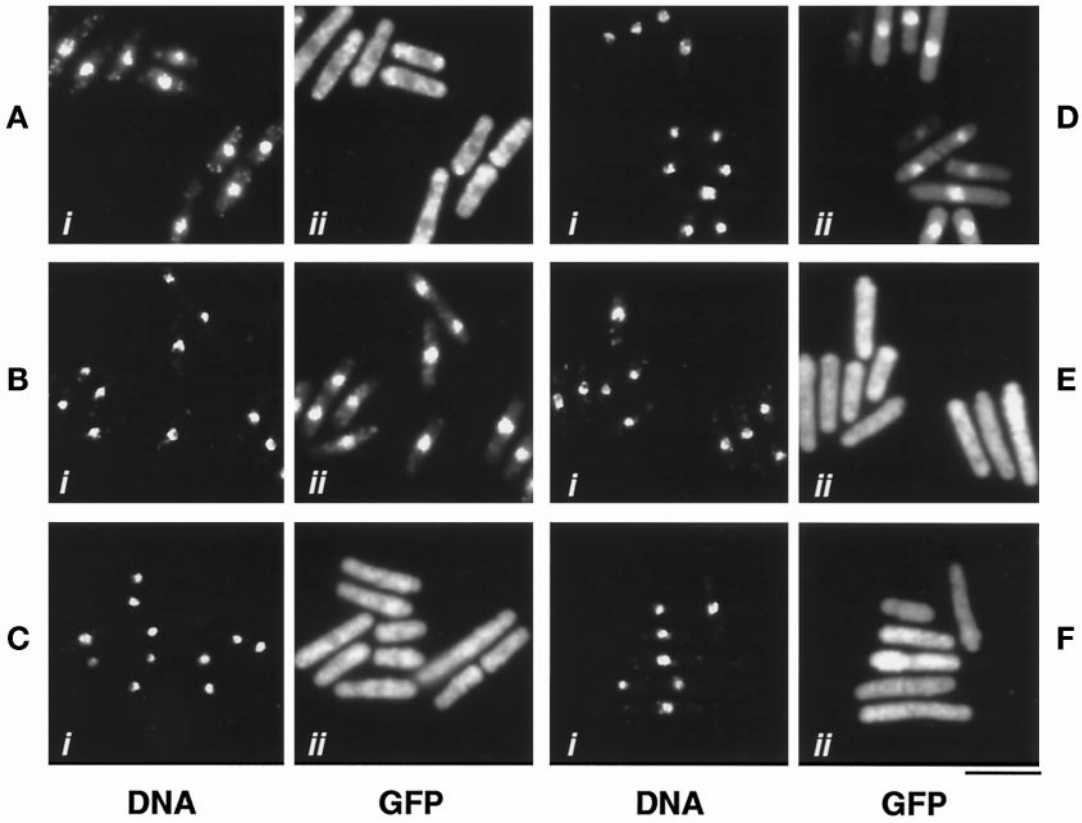

Figure 4. Mcm2p NLS1 is a function of NLS. Upper panel lists the fission yeast strains used for the NLS reporter assay as described in MATERIALS AND METHODS. Localization of GFP-βgal is summarized as C for cytoplasmic or N for nuclear. Lower panels show localization of NLS reporter proteins in fission yeast. Fission yeast cells expressing the GFP-βgal fusion protein were visualized by DAPI staining (i) or GFP fluorescence (ii). (A) No NLS; (B) the SV40 NLS; (C) the mutant SV40 NLS; (D) NLS1 from Mcm2p; (E) the point mutant version of NLS1 from Mcm2p-M9; and (F) NLS2 from Mcm2p. Bar, 10 μm.

wild-type Mcm2p (Figure 5, Ci–Ciii). However, cells overproducing Mcm2p-M9 were elongated and unable to form colonies, accumulating a 2C DNA content (our unpublished results). Nuclear immunofluorescence of the core complex components Mcm4p and Mcm6p was significantly reduced, and cytoplasmic immunofluorescence was increased (Figure 5, Civ–Cvi). In addition, overexpression of Mcm2p-M9 also disrupted the localization of the peripheral dimer component Mcm3p-HA. Even at low levels of expression of Mcm2p-M9, endogenous Mcm4p nuclear localization was compromised (our unpublished results). This not only indicates that MCM complexes can assemble in the cytoplasm but also suggests that the NLS defect of Mcm2p-M9 is sufficient to keep them there.

Mutant Mcm2 Proteins with an Intact NLS1 Fail to Accumulate in the Nucleus

These observations present us with a paradox. NLS1 is required for the normal localization of Mcm2p and is clearly sufficient to target a reporter protein to the nucleus. However, it is not sufficient to target Mcm2p; most Mcm2p mutants that are defective for both MCM binding and nuclear localization have an intact NLS1. The NLS mutants are defective for localization but retain the ability to bind other MCM proteins. In no case did we find a mutant that was able to localize to the nucleus but unable to associate with other MCM proteins. Therefore, we suggest that complex assembly precedes, and is required for, nuclear localization.

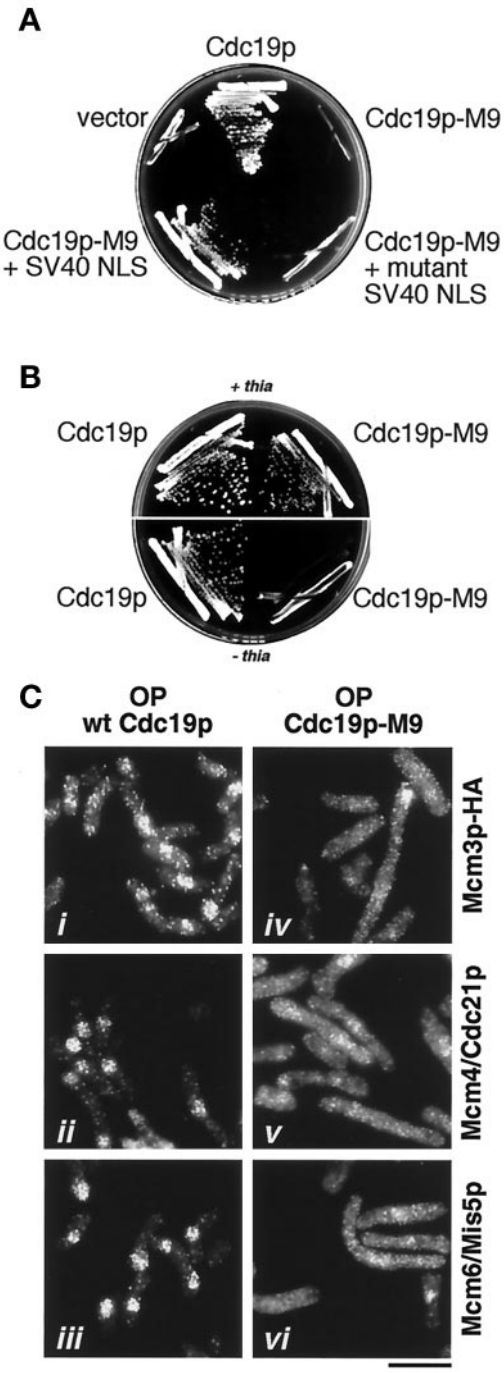

Figure 5. Overexpression of NLS mutant Mcm2p-M9 sequesters wild-type MCM proteins in cytoplasm. (A) Mcm2p-M9 with a heterologous NLS is functional. The mutant *cdc19-P1* cells harboring a control vector (pREP4X), wild-type Mcm2/Cdc19p (pSLF176), NLS mutant Mcm2/Cdc19p-M9 (pSLF196-M9), mutant SV40NLS-Mcm2/Cdc19p-M9 (pSGP96-M9), or SV40NLS-Mcm2/Cdc19p-M9 (pSGP86-M9) were streaked out on minimal medium plates and incubated for 4 d at 36°C before photography. (B) Overproduction of the NLS mutant protein Mcm2p-M9 is toxic in the *cdc19-P1 mcm3-HA* strain. The strains expressing wild-type Mcm2/Cdc19p (pSGP56) or mutant Mcm2/Cdc19p-M9 (pSGP56-M9) were streaked out on minimal medium plates with (+ thia) or without (− thia) thiamine and incubated at the permissive temperature (29°C) for 4 d before photography. (C) Localization of MCM proteins in *cdc19-P1 mcm3-HA* strain when mutant Mcm2p-M9 is overproduced. *cdc19-P1 mcm3-HA* cells expressing wild-type Mcm2/Cdc19p or mutant Mcm2/Cdc19p-M9 were prepared for immunofluorescence analysis after induction in the absence of thiamine for 20 h at 29°C. Antibody staining is shown for Mcm3p-HA (i and iv), Mcm4p (ii and v), and Mcm6p (iii and vi). Bar, 10 μm.

We reasoned that NLS1, and perhaps NLS2, is not functional in its native context unless Mcm2p is assembled in an MCM complex. If this is true, the mutant Mcm2/Cdc19 proteins should never enter the nucleus, despite the presence of an intact NLS1. Alternatively, they might enter the nucleus but be immediately exported because of a failure to assemble with other MCM proteins. To distinguish between these alternatives, we used the cold-sensitive *crm1* strain to block nuclear export. If mutant Mcm2p derivatives are capable of nuclear import, they should be trapped in the nucleus in *crm1* cells at the restrictive temperature.

crm1 cells expressing wild-type or mutant Mcm2 proteins were incubated at the restrictive temperature for 6 h. Wild-type Mcm2p remained in the nucleus (Figure 6A). The NLS mutants Mcm2p-M9 (Figure 6D) and Mcm2p-M10 (Figure 6E) and the amino-terminal truncation Mcm2p-D7 (Figure 6G), which lacks both NLS1 and NLS2, were found in the cytoplasm. This is the expected result because they lack functional targeting sequences. We tested two deletion mutants, Mcm2p-D6 and Mcm2p-D10, and a zinc finger point mutant, Mcm2p-M2, each of which contains NLS1 and NLS2 but fails to associate with other MCM proteins. In the nuclear export–defective strain, Mcm2p-D6 remained cytoplasmic (Figure 6F). This mutant protein is a carboxy-terminal truncation (Table 4). Similar cytoplasmic localization was observed for Mcm2p-M2 (Figure 6C). This result suggests that mutant Mcm2/Cdc19 proteins that are unable to associate with other MCM proteins are not imported into the nucleus despite the presence of NLS1 and NLS2. This is consistent with subcomplex assembly being required for Mcm2p NLS function.

Interestingly, Mcm2p-D10 did accumulate in the nucleus in ~10% of *crm1* cells (Figure 6H). This mutant protein contains a large deletion in the MCM core domain and is a cytoplasmic protein when expressed in a wild-type strain. We also examined the localization of excess wild-type Mcm2p in the *crm1* mutant strain. In wild-type cells, Mcm2p accumulated in the cytoplasm when overproduced. If it were shuttling in and out of the nucleus, we would expect the protein to accumulate in the nucleus in a *crm1* mutant that blocks export. However, this was not observed (Figure 6B).

DISCUSSION

In fission yeast and metazoa, the conserved MCM proteins are found in the nucleus throughout the cell cycle. We have examined the localization of MCM proteins in fission yeast and found that there is a role for the nuclear envelope in regulating these proteins even though their localization is

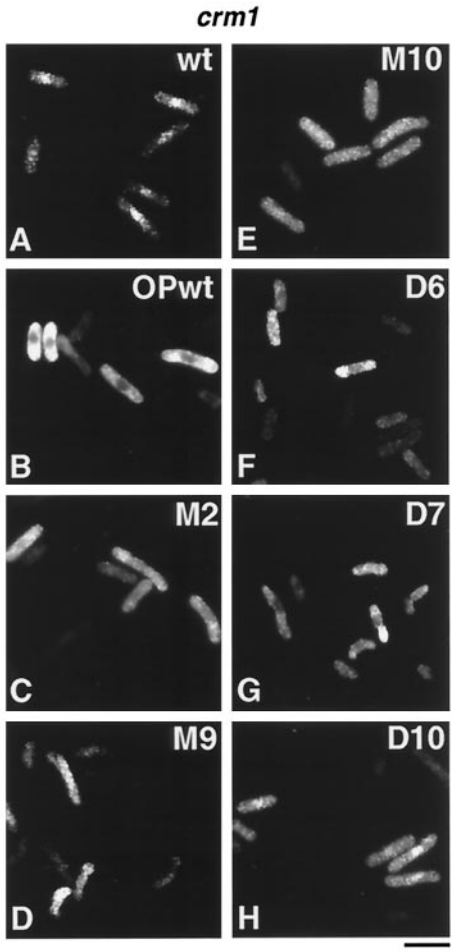

Figure 6. Mutant Mcm2p-HA localization in *crm1* strain. Cytoplasmic mutant Mcm2p-HA proteins do not accumulate in the nucleus in the nuclear export–defective strain *crm1-809*. Cells harboring a plasmid encoding wild-type or mutant Mcm2 proteins were grown at the restrictive temperature (17°C) for 6 h before fixation for immunofluorescence analysis. Wild-type Mcm2p was overproduced by induction in the absence of thiamine for 16 h followed by incubation at 17°C for 6 h. Cells were visualized with anti-HA antibody. (A) Wild-type Mcm2p; (B) overexpressed wild-type Mcm2p; (C) zinc finger mutant Mcm2p-M2; (D) NLS mutant Mcm2p-M9; (E) NLS mutant Mcm2p-M10; (F) carboxy-terminal truncation Mcm2p-D6; (G) amino-terminal truncation Mcm2p-D7; and (H) MCM core domain deletion Mcm2p-D10. Bar, 10 μm.

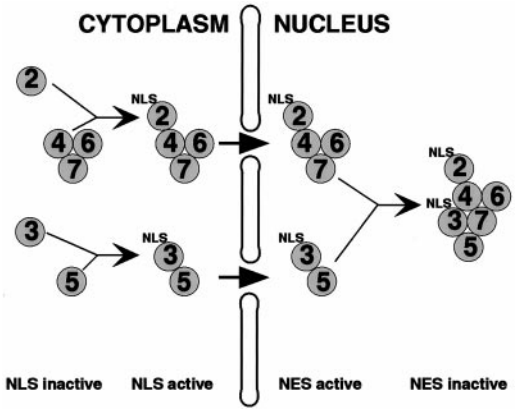

Figure 7. MCM complex assembly and nuclear localization. A speculative model coupling MCM complex assembly and nuclear localization. The six MCM proteins depend on NLS sequences supplied by MCM2 and MCM3 for nuclear import. The NLS sequences on MCM2 and MCM3 are inactive until association with the core complex, MCM4/6/7 and MCM5, respectively. Alternatively, the assembly of the full hexameric complex in the cytoplasm may be required for nuclear targeting. Once imported into the nucleus, the MCM subcomplexes have active NESs that are inactivated by the association of the two subcomplexes as an intact hexameric MCM complex. Inactivation of the conditionally mutant MCM proteins results in disassembly of the hexameric complex, exposure of the active NESs, and subsequent export of the MCM subcomplexes.

not cell cycle dependent: it serves to maintain intact hexameric MCM complexes inside the nucleus. We propose that the nuclear targeting of Mcm2p and associated proteins requires assembly of the MCM proteins to activate the Mcm2p NLS. In contrast, when Mcm2p is not associated with other MCM proteins in the nucleus, the NLS is inaccessible and the protein is exported. Thus, assembly of an intact hexameric MCM complex is linked to the nuclear localization of individual MCM subunits.

An attractive model is that the association of at least the core MCM proteins (MCM4/6/7) and MCM2 in the cytoplasm is required for their targeting of the subcomplex to the nucleus (Figure 7). Previously, we and others have shown that the MCM heterohexamer contains subcomplexes: a core of MCM4/6/7 bound by MCM2 and a peripheral dimer of MCM3/5 (Ishimi *et al.*, 1996; Kimura *et al.*, 1996; Adachi *et al.*, 1997; Kubota *et al.*, 1997; Thommes *et al.*, 1997; Sherman and Forsburg, 1998; Sherman *et al.*, 1998). That coassembly of subcomplexes of MCM proteins occurs in the cytoplasm is demonstrated by the ability of the NLS1 mutant, Mcm2p-M9, to trap wild-type MCM proteins in the cytoplasm. In fission yeast, as in budding yeast, only Mcm2p and Mcm3p homologues have consensus nuclear localization sequences. Thus, Mcm3p may similarly target Mcm5p to the nucleus. In this model, retention of all six MCM proteins in the nucleus would require binding of the peripheral dimer MCM3/5 to the subcomplex MCM2/4/6/7 to inactivate NESs. The disruption of Mcm3p localization in the *cdc19* mutant and the disruption of MCM core complex localization in the *mcm3* mutant may indicate that assembly of the full hexameric complex is required for nuclear retention. Alternatively, the intact complex might assemble in the cytoplasm and require both sets of NLSs to target the hexamer to the nucleus.

This model linking hexameric MCM complex assembly and nuclear localization in fission yeast is based on three major observations. First, MCM protein localization is interdependent: in *mcm* temperature-sensitive strains, the wild-type MCM proteins are lost from the nucleus at the restrictive temperature. MCM protein associations are also disrupted under these conditions (Sherman *et al.*, 1998). In either the *mcm3-HA* cold-sensitive or the *cdc19* temperature-sensitive mutant, this redistribution of MCM proteins requires an active nuclear export system. It remains to be determined if each MCM protein contains an NES, whether

MCM subcomplexes or individual subunits are targets for export, or if export depends on interaction with an NES-containing protein. There are potential NESs in Mcm2p at residues 627–638 and 771–780, based on comparison with consensus NESs (Kim *et al.*, 1996; Nakielny and Dreyfuss, 1997; Nigg, 1997; Mattaj and Englmeier, 1998). Notably, Mcm2p-D10 retains the putative NES at residues 771–780 (Table 4) and can be trapped in the nucleus in the nuclear export–defective strain. Masking of the NES has been proposed as a mechanism for regulating p53 subcellular localization (Stommel *et al.*, 1999). Tetramerization of p53 monomers blocks the NES elements, resulting in nuclear retention of the p53 complex.

Second, using a panel of mutations in Mcm2p, we show that mutants that disrupt complementation and MCM protein interactions (Forsburg *et al.*, 1997; Sherman *et al.*, 1998) also abolish nuclear localization, even in the presence of an intact NLS. Trivially, this could suggest that all mutant proteins fail to fold properly, even though they are all produced and stable (Forsburg *et al.*, 1997; Sherman *et al.*, 1998). However, several observations argue against this. (1) The NLS2 mutant Mcm2p-M10 also contains an intact NLS1, yet it fails to accumulate in the nucleus. In this case, however, the protein is still able to bind other MCM proteins (Sherman *et al.*, 1998) and can be rescued by adding a heterologous NLS, which suggests that its structure is still intact. (2) The protein tolerates deletions in the amino terminus (D1, D2, D3) that do not affect binding, localization, or complementation. (3) A mutation in the putative nucleotide-binding site, M7 (K540R), also produces a functional protein; an alanine mutation of the same lysine residue disrupts activity. Both mutant proteins exhibit reduced MCM protein binding and nuclear localization. These observations demonstrate that the protein can accommodate both point and deletion mutations. (4) Among the nearly 20 other mutant proteins with point and deletion mutations throughout the protein, all are produced and stable; none shows nuclear localization, despite the presence of NLS1 and NLS2. Although one can never prove that these proteins are folded normally, indirect evidence suggests that at least some of them are intact. We suggest that they are specifically deficient in targeting. An intriguing reason is that the NLS remains masked because the mutant proteins fail to interact with other MCM proteins. We also observed that overproduced Mcm2p does not accumulate in the nucleus in wild-type or *crm1* mutant cells. This result suggests that some factor is limiting for either its import or its retention. This is unlikely to be attributable to an effect on general import machinery because overexpression of wild-type Mcm2p has no deleterious phenotype. Because we never observed nuclear localization without MCM binding, we deduce that binding is required for localization.

Our third major observation is based on analysis of Mcm2p NLS sequences. Mcm2p contains at least one NLS (NLS1) that is sufficient to target a reporter protein to the nucleus, but that sequence is insufficient to target binding-defective Mcm2p mutants to the nucleus. Nor can NLS1 target an NLS2 mutant Mcm2p to the nucleus, even though its ability to assemble with other MCM proteins is normal. Mutations in either NLS have no effect on complex assembly (Sherman *et al.*, 1998) but abolish localization; this localization can be rescued by adding a heterologous NLS to the mutant protein. In addition, NLS mutants can be used to sequester wild-type MCM proteins in the cytoplasm. The NLS mutants allow us to uncouple complex assembly from localization and suggest that complex assembly precedes localization. Thus, we suggest that complex assembly is necessary but not sufficient for localization of Mcm2p.

We infer that the NLS sequences are not active or exposed unless Mcm2p is assembled with other MCM proteins. The Mcm2p-D10 mutant, which contains a large deletion but retains NLS1 and NLS2, shows weak nuclear localization in the *crm1* mutant, suggesting that NLS masking is defective for this mutant protein. One possible explanation for this observation is that the large deletion in Mcm2p-D10 not only prevents association of the mutant protein with other MCM proteins but also prevents complete inactivation of NLS1 and NLS2. In the wild-type strain, Mcm2p-D10 with partially active NLS function is inefficiently imported, but the functional nuclear export mechanism removes the mutant protein from the nucleus to the cytoplasm. In the *crm1* strain, Mcm2p-D10 is captured in the nucleus because of the failure of the nuclear export system, but its nuclear localization is substantially reduced compared with that of wild-type Mcm2p. There is precedent for intramolecular masking of NLS function. Recently, Humbert-Lan and Pieler (1999) reported that a carboxy-terminal transport regulatory domain may mask two NLS sequences in the *Xenopus* B-Myb transcription factor, thus restricting B-Myb to the cytoplasm. They propose that the domain acts via either intramolecular or intermolecular interactions to regulate NLS function.

Because the Mcm2/Cdc19 NLS mutants can sequester the other MCM proteins in the cytoplasm, and because localization of MCM subunits is interdependent, we propose that Mcm2p may be required for import of those MCM proteins that lack identifiable NLS elements. There have been several recent reports that complex assembly allows nuclear targeting of a protein that lacks a functional NLS. Piggyback mechanisms have been proposed for the Fanconi anemia protein complex (Naf *et al.*, 1998), mushroom homeodomain transcription factor complex (Spit *et al.*, 1998), cytomegalovirus capsid assembly (Plafker and Gibson, 1998), mouse DNA primase (Mizuno *et al.*, 1996), mammalian DNA repair enzymes (Boulikas, 1997), STAT proteins (Johnson *et al.*, 1998a,b), and IκB (Turpin *et al.*, 1999). Furthermore, two recent reports (Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999) have demonstrated that the subcellular localization of the *Drosophila* homeodomain protein Extradenticle depends on regulating the accessibility of its NLS and NES elements. Heterodimerization of Extradenticle with Homothorax blocks NES accessibility and leads to nuclear accumulation of the Extradenticle/Homothorax complex. In each case, the cell assembles a protein complex in the cytoplasm and then imports the complex into the nucleus. The latter case provides an example of the role of nuclear export in maintaining appropriate stoichiometry of complex subunits. In this way, only active, intact complexes are present in the nucleus. Our data suggest that fission yeast MCM complexes may also have this spatial control of their assembly.

Such a mechanism may be conserved in other eukaryotes. Kimura and colleagues (1996) showed that overexpression of murine MCM6 and MCM5 led to their accumulation in the cytoplasm unless MCM2 and MCM3, respectively, were coexpressed. In characterizing the NLS of budding yeast Mcm3p, Young and colleagues (1997) speculated that it may provide nuclear access for other MCM proteins. Furthermore, Maiorano and colleagues (1996) suggested that the cytoplasmic accumulation of overproduced fission yeast Mcm4p may indicate that Mcm4p depends on its association with an NLS-containing limiting factor for its nuclear import. Our interpretation is consistent with these data. It provides a mechanism for the cell to assemble intact hexameric MCM complexes in the nucleus and maintain the correct stoichiometry of the individual subunits. By requiring the subcomplexes to assemble in the cytoplasm before nuclear entry and actively exporting free MCM subunits or subcomplexes from the nucleus, the cell avoids having unassociated MCM proteins bind other factors or interfere with the function of the intact MCM complex. Interestingly, this active nuclear export may explain the shuttling behavior of MCM proteins reported in *S. cerevisiae* (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995; Young *et al.*, 1997; Young and Tye, 1997); uniquely in this organism, MCM proteins leave the nucleus during S phase. This may indicate that the MCM complex is normally disrupted during the cell cycle in this species.

Nuclear localization of MCM proteins thus appears to reflect a balance of nuclear import and export. Access to the requisite targeting sequences may be mediated by the interaction of the complex components or associated factors. Even though this localization is not cell cycle dependent in normal growth, it clearly imposes spatial regulation on the assembly and activation of these conserved proteins. We expect that such regulation will be a common feature of the assembly of complex protein structures in the nucleus.

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