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Saccharomyces cerevisiae Mod5p-II Contains Sequences Antagonistic for Nuclear and Cytosolic Locations

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ABSTRACT

MOD5 encodes a tRNA modification activity located in three subcellular compartments. Alternative translation initiation generates Mod5p-I, located in the mitochondria and the cytosol, and Mod5p-II, located in the cytosol and nucleus. Here we study the nucleus/cytosol distribution of overexpressed Mod5p-II. Nuclear Mod5p-II appears concentrated in the nucleolus, perhaps indicating that the nuclear pool may have a different biological role than the cytoplasmic and mitochondrial pools. Mod5p contains three motifs resembling bipartite-like *n*uclear *l*ocalization *s*equences (NLSs), but only one is sufficient to locate a passenger protein to the nucleus. Mutations of basic residues of this motif cumulatively contribute to a cytosolic location for the fusion proteins. These alterations also cause decreased nuclear pools of endogenous Mod5p-II. Depletion of nuclear Mod5p-II does not affect $tRNA^{Tyr}$ function. Despite the NLS, most Mod5p is cytosolic. We assessed whether Mod5p sequences cause a karyophilic reporter to be located in the cytosol. By this assay, Mod5p may contain more than one region that functions as cytoplasmic retention and/or nuclear export sequences. Thus, distribution of Mod5p results from the presence/absence of mitochondrial targeting information and sequences antagonistic for nuclear and cytosolic locations. Mod5p is highly conserved; sequences responsible for subcellular distribution appear to reside in "accessory" motifs missing from prokaryotic counterparts.

SORTING isozymes are located in multiple subcellular locations but they are encoded by a single gene (Gillman *et al.* 1991). They are widely distributed in nature and generally differ at their amino termini via differential transcription and/or translation initiations, and the different forms of the gene products have different subcellular distributions (for reviews, see Martin and Hopper 1994; Danpure 1995). Studies of how the subcellular distribution of sorting isozymes is achieved provide the opportunity to identify *cis*- and *trans*-acting factors involved in the delivery of proteins to appropriate organelles (for an example, see Zoladek *et al.* 1995).

Among genes encoding sorting isozymes, the *Saccharomyces cerevisiae MOD5* and *CCA1* genes are unusual in encoding isozymes located in three, rather than two, locations: mitochondria, nuclei, and the cytosol (Hopper *et al.* 1982; Boguta *et al.* 1994; Wolfe *et al.* 1994, 1996). *CCA1* encodes Cca1p-I, Cca1p-II, and Cca1p-III, the amino termini of which correspond to the first, second, and third translational initiation codons, respectively (Wolfe *et al.* 1994). Cca1p-I is predominantly mitochondrial, whereas Cca1p-II and Cca1p-III are predominantly cytosolic with a smaller nucleus-located pool (Wolfe *et al.* 1996). The nuclear and cytosolic Cca1p pools serve different biological functions; the nucleus-located form adds CCA nucleotides to tRNA 3′ ends during biogenesis whereas the cytosolic pool repairs tRNA 3′ ends (Wolfe *et al.* 1996). The *cis*-acting signals that regulate the nucleus/cytosol Cca1p-II and Cca1p-III distributions have not been elucidated.

MOD5 encodes two isozymes, Mod5p-I and Mod5p-II, initiating at codons 1 and 12 of the open reading frame (ORF), respectively (Gillman *et al.* 1991). Mod5p-I is mitochondrial and cytosolic whereas Mod5p-II is cytosolic and nuclear (Boguta *et al.* 1994). *MOD5* products catalyze the formation of isopentenyl adenosine (i^6A) located at position 37 of some tRNAs (Dihanich *et al.* 1987). Modification of A to i^6A has been reported to occur only in the cytosol in Xenopus (Nishikura and De Robertis 1981). Thus, it is not clear why there should be a nuclear pool of this protein in yeast. One goal of this work was to determine whether, as is the case for Cca1p, the nucleus and cytosolic pools of Mod5p have different biological functions.

Several different types of *cis*-acting sequences can de-

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liver karyophilic proteins to the nucleus. These include the SV40 large T antigen *n*uclear *l*ocalization *s*equence (NLS) PKKKRKV (Kalderon *et al.* 1984), the bipartite NLS motif consisting of two regions of basic amino acids separated by ~10 nonspecific amino acids (Robbins *et al.* 1991; for a review, see Dingwall and Laskey 1991), and the M9-like sequences that function also in nuclear export (Michael *et al.* 1995, 1997). Inspection of the *MOD5* ORF revealed the presence of three sequences resembling the bipartite-like NLS (Boguta *et al.* 1994). A goal of this work was to determine which, if any, of the bipartite-like sequences suffice to deliver a passenger protein to the yeast nucleus and whether the sequence(s) is necessary for the nucleus location of Mod5p-II.

Many eukaryotic proteins are found in both the nucleus and the cytosol. Several mechanisms regulate the protein distribution of these two subcellular compartments (for a review, see Jans and Hübner 1996). One is masking of an NLS. For example, although sufficient to target large proteins to the nucleus, the SV40 large T antigen NLS is flanked by sequences that affect the rate of SV40-NLS-driven nuclear import. Another mechanism involves the regulation of a *cis*-acting domain involved in cytosolic retention [called the *c*ytoplasmic *r*etention *d*omain (CRD) or the *c*ytoplasmic *r*etention *s*equence (CRS)] of an otherwise karyophilic protein. Two well-studied retention signals, the xnf7 CRD and the cyclin B1 CRS, bear little sequence similarity to each other (Pines and Hunter 1994; Shou *et al.* 1996). There are no apparent CRD or CRS-like motifs in Mod5p. A third mechanism that can regulate nucleus/cytosol pools of proteins is protein shuttling. Proteins that shuttle into and out of the nucleus contain *s*equences that function in protein *n*uclear *e*xport (NES; Fischer *et al.* 1995; Michael *et al.* 1995; Wen *et al.* 1995; for a review, see Gerace 1995). One type of NES contains a conserved leucine-rich motif (Fischer *et al.* 1995; Wen *et al.* 1995; Murphy and Wente 1996). Although the Mod5p-II sequence contains leucine-rich regions, none are exact matches to the leucine-rich NES. *MOD5* mRNA 3′ ends in the vicinity of the motif most closely resembling the bipartite-like NLS (Najarian *et al.* 1987) are heterogeneous and the shorter mRNAs would code for isozymes lacking the complete putative NLS. Therefore, mRNA 3′ end heterogeneity could account for the cytosol/nucleus distribution of Mod5p-II. The final goal of this work was to explore whether the cytosolic pool of Mod5p-II is due to 3′ end mRNA heterogeneity, NLS occlusion, or the presence of CRD, CRS, or NES *cis*-acting sequences.

MATERIALS AND METHODS

Strains, media, and transformation: The *Saccharomyces cerevisiae* strain MT-8 [*MAT*α *ura3-1 leu2-3,112 ade2-1 trp1 lys1-1 lys2-1 SUP7 can1-100 mod5::TRP1* (Gillman *et al.* 1991)] was used for all studies except that strains BJ5465 (Yeast Genetic Stock Center) and RS453 (from Dr. Ed Hurt) were used to characterize the monoclonal antibody 32D6. Yeast strains were maintained on YEPD medium or synthetic defined media lacking the appropriate nutritional ingredient. Yeast cells were transformed with plasmid DNA by the one-step lithium acetate procedure (Chen *et al.* 1992) or by modification of the TRAFCO protocol (Gietz *et al.* 1992).

Escherichia coli RR1 and DH5α were used for propagation of recombinant DNA constructs. RZ1032 was used for propagation of M13 phage, and RR1 and JM109 were recipients for mutagenesis reactions. *E. coli* were maintained on yeast extract tryptone (YT) media or YT media containing the appropriate antibiotic to select for plasmid expression. *E. coli* were transformed using the calcium chloride procedure (Maniatis *et al.* 1982), the frozen storage buffer (FSB)-based protocol (Hanahan *et al.* 1991), or by electrotransformation.

Oligonucleotide generation and PCR amplification: Oligonucleotides were prepared by the Pennsylvania State University College of Medicine Macromolecular Core Facility. Table 1 lists the sequences of the oligonucleotides that were used as primers. Table 2 lists the PCR products used in this study and the primer pairs and templates used in their synthesis.

Oligonucleotide-directed mutagenesis: Four mutant alleles of *MOD5* were generated essentially as described by Kunkel *et al.* (1987) using the following oligonucleotides: (1) *mod5-M1-SV40* with oligonucleotide 5′-GTTGCTTAAATATGTCTC CTAAAAAAAAAAGAAAAGTTATAGTGATTG-3′, and pBlueScriptSK(+)mod5-M1 as the template, (2) *mod5-M1-KRK* using oligonucleotide 5′-GAAAAATGGAAATGAAACAAGAAG GAG-3′ and pBlueScriptSK(+)mod5-M1, (3) mutant allele *mod5-M1-KRNNN* using the oligonucleotide 5′-TGAAAAATGG AACATAAACAACAACGAGACTGTGGAA-3′ and pBlueScript SK(+)mod5-M1, and (4) the mutant allele *mod5-M1-NNNNN* using the template pBlueScriptSK(+)mod5-M1-KRNNN, which was previously mutated, and oligonucleotide 5′-GACATAAATC CAACGTTAACAACAACACTCGTCAAGC-3′.

Plasmid construction: Sequences of *MOD5* PCR products and mutated alleles were determined by the dideoxy-chain termination method (Sanger *et al.* 1977) using Sequenase T7 DNA polymerase (USB) or automated cycle sequencing in the Pennsylvania State University College of Medicine Macromolecular Core Facility. All sequences were correct with the following exceptions: for MOD5(12–234) two bases differed from those of the *MOD5* sequence found in the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/); one nucleotide change translated into a change of codon 22 from threonine to lysine. The other change did not translate into an amino acid alteration. For MOD5(230–403) one nucleotide difference changed codon 278 from leucine to methionine; another nucleotide difference changed codon 329 from glutamine to lysine. For MOD5(12–427) a change at codon 155 altered lysine to glutamic acid.

MOD5 PCR products were initially ligated into pGEM-T (Promega, Madison, WI), except for MOD5(12–234) and MOD5(230–403), which were ligated into pBlueScriptSK(−) (Stratagene, La Jolla, CA). These intermediate constructs were then digested with *Bam*HI and the subcloned PCR fragments were released and isolated. DNA fragments MOD5(102–203), MOD5(375–427), mod5-KRKNN, mod5-KRNNN, and mod5-NNNNN were each ligated into the *Bam*HI site of the vector pFB1-7a (Moreland *et al.* 1987). This resulted in plasmids pFB1-7aMOD5(102–203), pFB1-7aMOD5(375–427), pFB1-7amod5-KRKNN, pFB1-7amod5-KRNNN, and pFB1-7amod5-NNNNN, in which *MOD5* sequences are fused in-frame with *lacZ.* MOD5(12–234), MOD5(12–109), MOD5(102–234), MOD5(12–427), MOD5(230–403), MOD5(230–326), MOD5-

TABLE 1

Oligonucleotide sequences of primers

Primer	Sequence
HUNT01	5′-GTGCGGATCCCGCAACGTCTGTCGTAAC-3′
HUNT02	5′-GCCCGGATCCCACAGTCTCCTTCTTG-3′
HUNT06	5′-CTAGCGGATCCAGGGGTAAGATACCCATC-3′
HUNT07	5′-TATAGGGATCCAAATAGGGTATCAAACTTC-3′
BENK03	5′-GCCCGGATCCCACAGTCTCGTTGTTG-3′
BENK04	5′-AAAGGTGGATCCAATATGTCT-3′
BENK05	5′-ATAGGATCCCTTGATTTCTTG-3′
BENK15	5′-AGTGGATCCAACAACGATGGGTATC-3′
BENK06	5′-AGAGGATCCTTGCAAGAAATCAA-3′
BENK07	5′-AGTTGGATCCATGTCTTCTGCTAC-3′
BENK16	5′-TTGGGATCCATTCGTGTCCCAT-3′
BENK17	5′-TCTGGATCCGACACGAATGCATCT-3′
BENK18	5′-AATGGATCCAGGAGGGTACAACGA-3′
BENK19	5′-TTGGATCCCTATACAGTAAGCCGGAG-3′
BENK20	5′-CGGCGTGGATCCATTTTGACTGTA-3′
BENK21	5′-CTCGGATCCTACTGTGTTGTCATC-3′
BENK22	5′-GATGGATCCACAGTAAAATTGGAG-3′
MOD5GFP5′	5′-GGCGGCCGCATGAGTAAAGGAGAAGAA-3′
MOD5GFP3′	5′-CGCGGCCGCTTATTTGTATAGTTC-3′

(324–403), MOD5(170–243), MOD5(206–243), MOD5(206–276), MOD5(230–276), and MOD5(275–326) were ligated into the *Bam*HI site of the vector pFB1-67a (Morel and *et al.* 1987). This resulted in plasmids pFB1-67aMOD5(12–234), pFB1-67aMOD5(12–109), pFB1-67aMOD5(102–234), pFB1-67aMOD5(12–427), pFB1-67aMOD5(230–403), pFB1-67a-MOD5(230–326), pFB1-67aMOD5(324–403), pFB1-67aMOD-5(170–243), pFB1-67aMOD5(206–243), pFB1-67aMOD5-(206–276), pFB1-67aMOD5(230–276), and pFB1-67aMOD-5(275–326) in which the *MOD5* sequences are fused in-frame between the coding region for the histone H2B NLS and *lacZ.* For a complete list of plasmids, see Table 3.

MOD5 mutant alleles were released from pBlueScriptSK(+) by digestion with *Bst*YI and were cloned into the *Bam*HI site of YCf50 (Table 3). All constructs were subsequently cloned into the unique *Bam*HI site of pJDB207 (Table 3).

The plasmids used for the green fluorescent protein (GFP) studies were constructed as follows. The open reading frame for *Aequorea victoria* GFP from pSEY18/GAL1-10/GFP was PCR-amplified using primers MOD5GFP5′ and MOD5GFP3′,

TABLE 2

Primers and templates used to create PCR products

PCR product	Primer pair	Template
MOD5(102–203)	HUNT06/HUNT07	YCfmod5-M2
MOD4(375–427)	HUNT01/HUNT02	YCfmod5-M2
mod5-KRKNN	HUNT01/BENK03	YCfmod5-M2
mod5-KRNNN	HUNT01/BENK03	YCfmod5-M1-KRNNN
mod5-NNNNN	HUNT01/BENK03	pBlueScriptSK(+)mod5-M1-NNNNN
MOD5(12–427)	BENK04/HUNT02	YCfmod5-M1
MOD5(12–234)	BENK04/BENK05	YCfmod5-M1-KRK
MOD5(12–109)	BENK04/BENK15	YCfmod5-M1-KRK
MOD5(102–234)	HUNT06/BENK05	YCfmod5-M1-KRK
MOD5(230–403)	BENK06/BENK07	YCfmod5-M1-KRK
MOD5(230–326)	BENK06/BENK16	YCfmod5-M1-KRK
MOD5(324–403)	BENK17/BENK07	pBSmod5-M2[a]
MOD5(170–243)	BENK18/BENK20	YCfmod5-M1-KRK
MOD5(206–243)	BENK19/BENK20	YCfmod5-M1-KRK
MOD5(206–276)	BENK19/BENK21	YCfmod5-M1-KRK
MOD5(230–276)	BENK06/BENK21	YCfmod5-M1-KRK
MOD5(275–326)	BENK22/BENK16	YCfmod5-M1-KRK
GFP/NotI	MOD5GFP5′/MOD5GFP3′	pSEY18/GAL1-10/GFP[b]

[a] P. Artz, N. C. Martin and A. K. Hopper, unpublished data.
[b] Gift from Roger Y. Tsien.

TABLE 3

Plasmids

Name	Description	Source
pBlueScriptSK(+)mod5-M1	pBlueScriptSK(+) with Mod5p aa 12–428; encodes Mod5p-II	This study
pBlueScriptSK(+)mod5-M1-KRNNN	pBlueScriptSK(+)mod5-M1 with aa 420, 423, 424 altered	This study
pBlueScriptSK(+)mod5-M1-NNNNN	pBlueScriptSK(+)mod5-M1-KRNNN with aa 408, 409 altered	This study
pBSmod5-M2	pBlueScriptSK(+) with Mod5p aa 1–428 but 12 altered; encodes Mod5p-I	P. Artz, N. C. Martin and A. K. Hopper (unpublished data)
pFB1-7a	Histone H2B aa 1–14 fused to β-galactosidase	Moreland *et al.* (1987)
pFBI-7aMOD5(102–203)	pFB1-7a with Mod5p aa 102–203	This study
pFB1-7aMOD5(375–427)	pFB1-7a with Mod5p aa 375–427	This study
pFB1-7amod5-KRKNN	pFB1-7aMOD5(375–427) with aa 423, 424 altered	This study
pFB1-7amod5-KRNNN	pFB1-7aMOD5-KRKNN with aa 420 altered	This study
pFB1-7amod5-NNNNN	pFB1-7aMOD5-KRNNN with aa 408, 409 altered	This study
pFB1-67a	Histone H2B aa 1–67 fused to β-galactosidase	Moreland *et al.* (1987)
pFB1-7aMOD5(12–427)	pFB1-67a with Mod5p aa 12–427	This study
pFB1-67aMOD5(12–234)	pFB1-67a with Mod5p aa 12–234	This study
pFB1-67aMOD5(12–109)	pFB1-67a with Mod5p aa 12–109	This study
pFB1-67aMOD5(102–234)	pFB1-67a with Mod5p aa 102–234	This study
pFB1-67aMOD5(230–403)	pFB1-67a with Mod5p aa 230–403	This study
pFB1-67aMOD5(230–326)	pFB1-67a with Mod5p aa 230–326	This study
pFB1-67aMOD5(324–403)	pFB1-67a with Mod5p aa 324–403	This study
pFB1-67aMOD5(170–243)	pFB1-67a with Mod5p aa 170–243	This study
pFB1-67aMOD5(206–243)	pFB1-67a with Mod5p aa 206–243	This study
pFB1-67aMOD5(206–276)	pFB1-67a with Mod5p aa 206–276	This study
pFB1-67aMOD5(230–276)	pFB1-67a with Mod5p aa 230–276	This study
pFB1-67aMOD5(275–326)	pFB1-67a with Mod5p aa 275–326	This study
YCf50	Low-copy vector	Gillman *et al.* (1991)
YCfMOD5	YCf50 with *MOD5*; encodes Mod5p-I and Mod5p-II	Gillman *et al.* (1991)
YCfmod5-M1	YCf50 with Mod5p aa 12–428; encodes Mod5p-II	Gillman *et al.* (1991)
YCfmod5-M1-KRNNN	YCfmod5-M1 with aa 420, 423, 424 altered	This study
YCfmod5-M1-KRK	YCfmod5-M1 with aa 421–428 deleted	This study
YCfmod5-M1-NNNNN	YCfmod5-M1 with aa 408, 409, 420, 423, 424 altered	This study
YCfmod5-M1-SV40	YCfmod5-M1 with the SV40 NLS added at codon 15	This study
YCfmod5-M2	YCf50 with Mod5p aa 1–428 but 12 altered; encodes Mod5p-I	Gillman *et al.* (1991)
pJDB207	High-copy vector (∼100 copies/cell)	Beggs (1991)
pJDBMOD5	*MOD5* in pJDB207; encodes Mod5p-I and Mod5p-II	Gillman *et al.* (1991)
pJDBmod5-M1	pJDB207 with Mod5p aa 12–428; encodes Mod5p-II	Gillman *et al.* (1991)
pJDBmod5-M1-KRNNN	pJDBmod5-M1 with aa 420, 423, 424 altered	This study
pJDBmod5-M1-KRK	pJDBmod5-M1 with aa 421–428 deleted	This study
pJDBmod5-M1-NNNNN	pJDBmod5-M1 with aa 408, 409, 420, 423, 424 altered	This study
pJDBmod5-M1-SV40	pJDBmod5-M1 with the SV40 NLS added at codon 15	This study
pRS416mod5-M1-GFP	Centromeric vector expressing *mod5-M1* fused to GFP ORF	This study
pRS426mod5-M1-GFP	Episomal plasmid expressing *mod5-M1* fused to GFP ORF	This study
pRS426mod5-M2-GFP	Episomal plasmid expressing *mod5-M2* fused to GFP ORF	This study

which contain *Not*I sites. The resulting product, GFP/NotI, was subcloned into the pGEM-T vector (Promega). GFP was isolated from the vector through a *Not*I restriction digest and inserted into the *Not*I site of pBlueScribemod5-M2KR6.Not (Zoladek *et al.* 1995). A *Sac*I/*Sal*I fragment encoding mod5-M2KR6.Not-GFP was ligated to the 2-μm vector pRS426 (Christianson *et al.* 1992) cut with *SacI*/*Sal*I. A *Sac*I/*Eco*RI fragment of the resulting plasmid containing the 5′ end of the mod5-M2KR6.NotI-GFP ORF was replaced with a *Sac*I/*Eco*RI fragment from YCfmod5-M1 or YCfmod5-M2 (Gillman *et al.* 1991) to form pRS426mod5-M1-GFP and pRS426mod5-M2-GFP, respectively. A *Sac*I/*Kpn*I fragment encoding mod5-M1-GFP was ligated to the centromeric vector pRS416 (Sikorski and Hieter 1989) cut with *Sac*I/*Kpn*I to form pRS416mod5-M1-GFP.

Sequence analysis: The *S. cerevisiae* Mod5p (GenBank accession no. 2507067) sequence was BLASTed (Altschul *et al.* 1997) at the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). Similar proteins were identified, retrieved, and used to search for additional matches. A consensus *miaA* protein was derived from a CLUSTAL W alignment (Higgins *et al.* 1996) of 33 prokaryotic *miaA* peptides. The organisms and their respective GenBank accession numbers for the *miaA* peptides are as follows: *E. coli*, 1790613; *Shigella flexneri*, 2506034; *Salmonella typhimurium*, 585484; *Haemophilus influenzae*, 1170952; *Pseudomonas putida*, 2353324; *Agrobacterium tumefaciens*, 585483; *Synechocystis* PCC6803, 2498562; *Mycobacterium tuberculosis*, 2292961; *Mycobacterium leprae*, 1170953; *Helicobacter pylori*, 2314590; *Borrelia burgdorferi*, 2688758; *Bacillus subtilis*, 2634117; *Streptomyces coelicolor*, 2995299; *Aquifex aeolicus*, 2983550; *Chlamydia trachomatis*, 3329229; *Treponema pallidum*, 3322935; from The Institute for Genomic Research (TIGR) at http://www.tigr.org/: *Deinococcus radiodurans*, TIGR|gdr_54; *Enterococcus faecalis*, TIGR|gef_6326 and GEFAG05R; *Neisseria meningitidis*, TIGR|GNMCM05F, GNMCH28F, and GNMCF04R; *Streptococcus pneumoniae*, TIGR|stp_4233; *Thermotoga maritima*, TIGR|BTMDZ23R, BTMDX01F, and BTMBL35R; *Vibrio cholerae*, TIGR|GVCCC29F, GVCCQ59F, and GVCDG60R; two homologs in *Porphyromonas gingivalis*, TIGR|P.gingivalis_37 and TIGR|P.gingivalis_50; from the University of Oklahoma's Advanced Center for Genome Technology (OUACGT) at http://www.genome.ou.edu/: *Actinobacillus actinomycetemcomitans*, OUACGT|Contig636 and Contig506; *Neisseria gonorrhoeae*, OUACGT|Contig237; *Streptococcus pyogenes*, OUACGT|Contig112; *Staphylococcus aureus*, OUACGT|Contig551; from the Pseudomonas Genome Project, University of Washington Genome Center and PathoGenesis Corporation at http://www.pathogenesis.com/: *Pseudomonas aeruginosa*, PAGP|Contig276; from The Sanger Center at http://www.sanger.ac.uk/: *Campylobacter jejuni*, cam8c1.p1b; *Yersinia pestis*, contigs 592 and 943; *Bordetella pertussis*, bord62f1.p1c, bord116c2.p1t, and bord1h6.q1c; and from Genome Therapeutics at http://www.genome.corp.com/: *Clostridium acetobutylicum*, contigs 127I and 128I. Five additional eukaryotic homologs were also identified (*Caenorhabditis elegans*, 532094; *Plasmodium falciparum*, TIGR|PF2IR07F and Sanger|M3J2d4.r1t; *Schizosaccharomyces pombe*, cosmid-c343 from the Sanger Center; *Drosophila melanogaster* from GenBank EST AA816785; and human from several GenBank ESTs, AA332152, F07677, AA356092, N89342, AA204763, AA309660, and AA833930).

Characterization of monoclonal antibody 32D6: The monoclonal antibody 32D6 was generated against a nucleolus-enriched fraction from yeast nuclei consisting of nucleoli with attached nuclear envelopes that contain nuclear pore complexes (Dove *et al.* 1998). Immunization of mice, isolation of hybridoma cell lines, screening methods, and production of ascites fluid were as described (Chen *et al.* 1997). Whole cell protein extracts were prepared by lysing yeast cells with glass beads in the presence of 10% trichloroacetic acid (TCA) to minimize proteolysis (de Beus *et al.* 1994). TCA precipitates were washed with 1% TCA, boiled for 5 min in SDS-PAGE sample buffer containing 250 mm Tris base, bath sonicated briefly, and microcentrifuged for 5 min. Protein extracted from 0.1 OD_{600} unit of cells was loaded per lane, separated on a 10.5% SDS-polyacrylamide gel and transferred to membranes. The blot was probed with mAb 32D6, followed by secondary goat anti-mouse horseradish peroxidase conjugate (both diluted 1:20,000), and detection was done by enhanced chemiluminescence (ECL) according to the manufacturer (Amersham, Arlington Heights, IL).

Indirect immunofluorescence and microscopic imaging: Indirect immunofluorescence experiments were carried out essentially as described by Kilmartin and Adams (1984) and Pringle *et al.* (1991) with the modifications previously reported (Boguta *et al.* 1994). MT-8 cells harboring plasmids containing *MOD5-lacZ* gene fusions were fixed for 1 hr. H2B/Mod5p/β-galactosidase antigens were detected using either a 1:5000 dilution of a mouse monoclonal anti-β-galactosidase antibody (Promega) with a 1:10,000 dilution of FITC-conjugated goat-anti-mouse secondary antibody (Jackson ImmunoResearch Labs., Inc., West Grove, PA) or using a 1:750 or 1:1000 dilution of an affinity-purified rabbit anti-β-galactosidase antibody (Hopper *et al.* 1990) and a 1:750 or 1:1000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch Labs., Inc.).

A 1:5 dilution of affinity-purified rabbit-anti-Mod5p antibody (Gillman *et al.* 1991) was used to detect the products of the various *mod5* mutant alleles on high-copy pJDB207 vector in MT-8. The secondary antibody was FITC-conjugated goat-anti-rabbit (Jackson ImmunoResearch Labs., Inc.) in a 1:150 dilution. Cells stained only with anti-Mod5p antibody were fixed for 20 min.

For double staining procedures, cells expressing wild-type *MOD5* on pJDB207 were fixed for 30 min and were stained with a 1:5 dilution of anti-Mod5p antibody in addition to either 32D6 or anti-Nop1p antibodies. Antibody 32D6 was used in a 1:20,000 dilution to detect nuclear pore complex proteins. The anti-Nop1p antibody (Henriquez *et al.* 1990) is a mouse monoclonal antibody that was used in a 1:100,000 dilution to detect the Nop1p nucleolar protein. The secondary antibodies were a 1:150 dilution of FITC-conjugated goat-anti-rabbit antibody to detect Mod5p immunoreactive complexes and a 1:400 dilution of Cy3-conjugated goat-anti-mouse antibody (Jackson ImmunoResearch Labs., Inc.) to detect either the 32D6 antibody or the anti-Nop1p antibody.

To observe moderately overexpressed GFP fusion protein and DNA in the same cells, MT-8 cells containing either pRS426mod5-M1-GFP or pRS426mod5-M2-GFP were grown overnight in selective media containing 10 ng/ml 4′,6-diamidino-2-phenylindole (DAPI). To visualize GFP fusion protein encoded by pRS416mod5-M1-GFP in MT-8, cells were grown overnight on plates at 23° and then suspended in phosphate-buffered saline prior to microscopy.

Fluorescence images were obtained by using either a Nikon Microphot-FX or a Nikon Optiphot-2 microscope equipped with a SenSys CCD camera (Photometrics Ltd., Tucson, AZ) and a Nikon 35-mm camera. Image processing for Figures 1, 6, 7, and 8 was done using IPLab Spectrum software (Signal Analytics Corp., Vienna, VA); image processing for Figures 3 and 9 employed QED software (Pittsburgh). Images for Figure 5 were obtained using a 35-mm camera and TMAX ASA 400 film (Eastman Kodak Co., Rochester, NY).

β-Galactosidase assay: β-Galactosidase activity was measured with a modified version of the method of Reynolds *et al.* (1997).

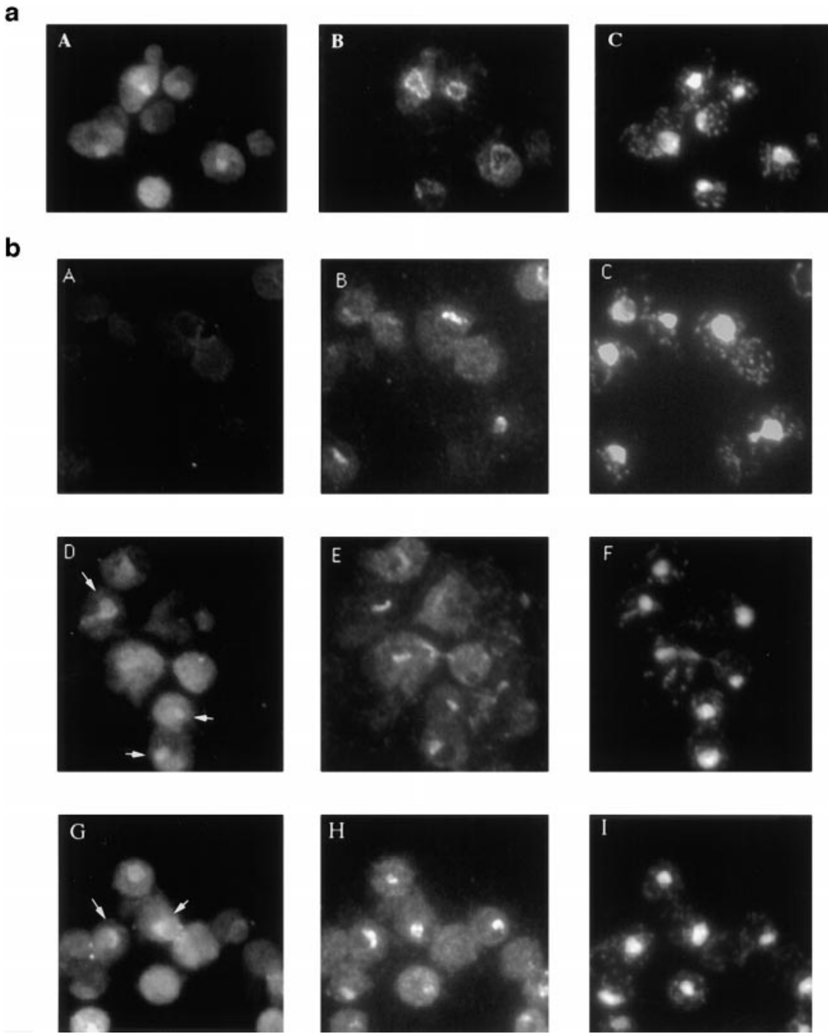

Figure 1.—Subnuclear localization of highly overexpressed Mod5p-II. Indirect immunofluorescence microscopy of MT-8 yeast cells double stained with antibodies against Mod5p and either Nop1p or a nuclear pore complex protein. (a) Cells harboring pJDB-mod5-M1. (A) Affinity-purified anti-Mod5p primary antibody with FITC-conjugated anti-rabbit secondary antibody. (B) Detection of nuclear pore complex proteins by mAb 32D6 with Cy3-conjugated goat-anti-mouse secondary antibody. (C) Detection of nuclear and mitochondrial DNA by DAPI staining. (b) A–C display cells harboring pJDB207, D–F display cells harboring pJDBmod5-M1, and G–I display cells harboring pJDBMOD5. (A, D, and G) Affinity-purified anti-Mod5p antibody with FITC-conjugated anti-rabbit secondary antibody. (B, E, and H) Detection of nucleolar protein Nop1p by mAb A66 and Cy3-conjugated goat-anti-mouse secondary antibody. (C, F, and I) Detection of nuclear and mitochondrial DNA by DAPI staining. Arrows point to cells with clear nucleolar staining.

RESULTS

Nucleus-located Mod5p-II appears to be concentrated in the nucleolus: Previously, employing cell fractionation procedures using yeast cells containing endogenous levels of Mod5p or immunofluorescence procedures using yeast cells containing overexpressed Mod5p, we reported that Mod5p-II was located in both the cytosol and nucleus of yeast cells (Boguta *et al.* 1994). Closer inspection of the noncytoplasmic (nuclear) staining indicated that the protein was not uniformly distributed (Figure 1). A portion of the protein often appeared to be nonoverlapping with nuclear DNA as assessed by DAPI staining. This raised the possibility that Mod5p-II may be located outside of the nuclear membrane or within a subcompartment of the nucleus. To resolve the location, we used an antibody to a component of the nuclear pore complex to view the boundaries of the nuclear membrane and Mod5p in the same cells.

The specificity of the nuclear pore complex antibody, mAb 32D6, was demonstrated as follows: first, it recognizes a nuclear pore complex antigen by immunofluorescent localization; second, in a λgt11 screen 32D6 identified a single clone with ~66% of the C-terminal portion of *NSP1*; third, we show here that proteins from strain RS453, which expresses a short form of Nsp1p, and from the wild-type strain BJ5465 migrate on SDS gels as expected (Figure 2). *NSP1* in RS453 contains an internal deletion that removes the coding sequences for six nonessential FXFG repeats, encoding a protein migrating at ~85 kD on SDS gels (E. Hurt, personal communication). Wild-type Nsp1p migrates on SDS gels at ~100 kD (Hurt 1988). The apparent molecular mass of wild-type Nsp1p on our SDS gel was 108 kD, whereas the short isoform of Nsp1p in RS453 migrated at 91 kD (Figure 2). This demonstrates that 32D6 recognizes the pore complex protein Nsp1p. Longer exposures of the immunoblot did not reveal additional proteins (data not shown), indicating that 32D6 is highly selective for the nucleoporin Nsp1p.

As endogenous Mod5p cannot be detected by immunofluorescence (Boguta *et al.* 1994), *mod5-M1* (Figure 1a) was expressed on the high-copy pJDB207 shuttle vector in the yeast MT-8. Cells were stained with mAb 32D6 and affinity-purified Mod5 antibody (Gillman *et*

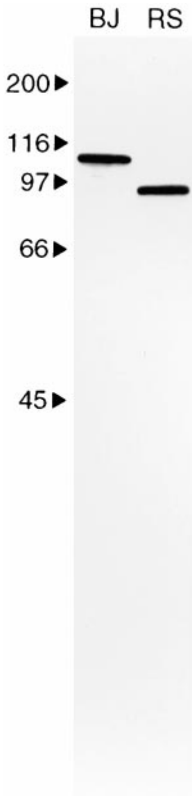

Figure 2.—mAb 32D6 monoclonal antibody is specific to Nsp1p. Whole cell protein extracts from BJ5465 (BJ) and RS453 (RS) were analyzed by immunoblotting with mAb 32D6. Positions of molecular weight markers are shown (in kilodaltons). Strain BJ5465 encodes wild-type pore complex protein Nsp1p, whereas RS453 expresses a shortened isoform of Nsp1p that is missing six FXFG repeats.

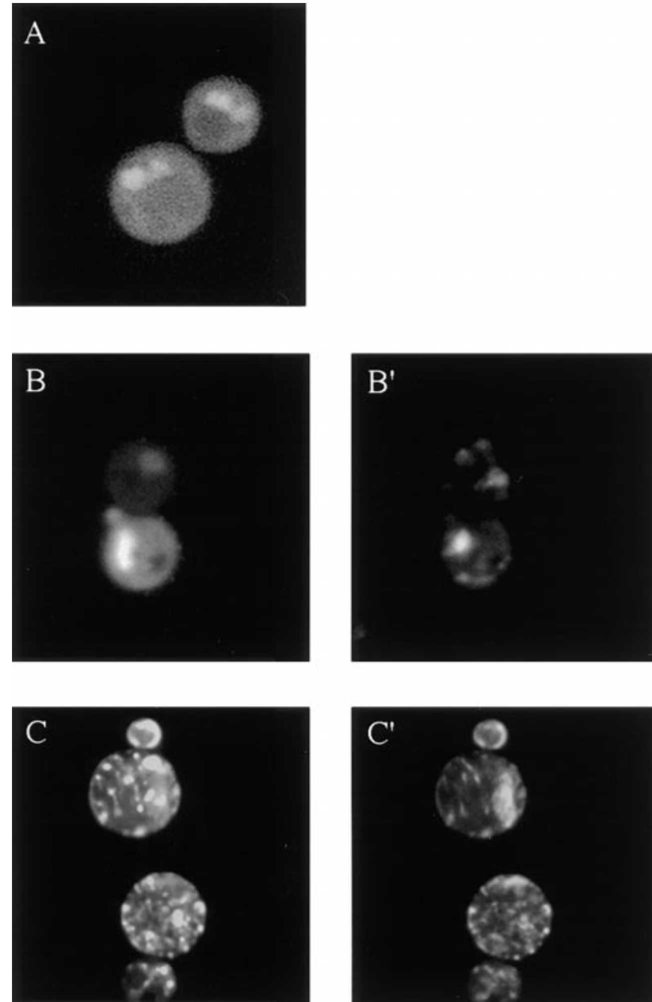

Figure 3.—Location of moderately overexpressed levels of Mod5p in living cells by fluorescence microscopy of live MT-8 cells expressing Mod5p-GFP fusion proteins. (A) Cells expressing centromeric pRS416mod5-M1-GFP. (B and B′) Cells expressing episomal pRS426mod5-M1-GFP. (C and C′) Cells expressing episomal pRS426mod5-M2-GFP. A, B, and C show subcellular locations of Mod5p-GFP fusion protein. B′ and C′ show DAPI staining of mitochondrial and nuclear DNA.

al. 1991). We found Mod5p-II to be within the nuclear boundaries as delineated by mAb 32D6 nuclear membrane staining. This result shows that much of the nucleus-localized Mod5p-II appears not to overlap with the region of the nucleus occupied by DNA.

Since a portion of the Mod5p-II pool is inside the nuclear border, but apparently adjacent to DNA, we hypothesized that it may be located in the nucleolus. To determine whether Mod5p staining coincides with the nucleolus, yeast cells harboring pJDB207 (Figure 1b, A–C), pJDBmod5-M1 (Figure 1b, D–F), or pJDBMOD5 (Figure 1b, G–I) were double stained with anti-Mod5p antibody and anti-Nop1p, the latter an established nucleolar protein (mAb A66; Aris and Blobel 1988). Cells harboring pJDB207 displayed no detectable Mod5p staining and clear staining of the Nop1p nucleolar protein (Figure 1b, A–C). DAPI staining of nuclear DNA is next to that of Nop1p (Figure 1b, compare B and C). Cells with pJDBmod5-M1 (Figure 1b, D–F) or pJDBMOD5 (Figure 1b, G–I) showed Mod5p staining throughout the cells, with an accumulation in an area that overlaps with Nop1p (see arrows). Thus, under these conditions Mod5p-II appears to be concentrated in the nucleolus.

As overexpression of Mod5p-II could lead to accumulation in an inappropriate subcellular compartment, we attempted to view Mod5p-II in cells expressing lower levels. Plasmids pRS416mod5-M1-GFP, pRS426mod5-M1-GFP, and pRS426mod5-M2-GFP encode Mod5p-GFP fusion proteins that complement the *mod5::TRP1* disruption allele (data not shown). Fusion proteins encoded by episomal plasmid pRS426mod5-M1 are located in the cytosol and the nucleus. Often two putative nuclear bodies are visualized (Figure 3B), only one of which overlaps with DAPI staining of DNA (compare Figure 3, B and B′). The pool of Mod5p that appears nuclear but does not overlap with DNA is likely nucleolar. However, the identity of the nucleolus cannot be confirmed because it is not possible to double stain living cells with two different fluorochromes.

The centromere-containing vector pRS416mod5-M1-GFP construct generated very faint signals (Figure 3A) that could be viewed only when amplified with a CCD camera and could not be counterstained due to UV signal overlapping in the GFP channel. However, the results indicate that in moderate-copy and in low-copy vectors, Mod5p-II is nucleoplasmic and nucleolar in liv-

SV40 large T antigen	P**KKKRK**V
nucleoplasmin	**KR**PAATKKAGQA**KKKK**
N1	**KR**KTEEESPLKD**K**DA**KK**
Mod5p(122-136)	**KR** VDTKSSER **K**LT**RK**
Mod5p(170-185)	**RR**VQRMLEITT **K**TG**KK**
Mod5p(408-424)	RRHKSNL**KR**NTRQADFEKW**K**IN**KK**
Mod5p(408-424) KRKNN	RRHKSNL**KR**NTRQADFEKW**K**IN**NN**
Mod5p(408-420) KRK	RRHKSNL**KR**NTRQADFEKW**K**
Mod5p(408-424) KRNNN	RRHKSNL**KR**NTRQADFEKW**N**IN**NN**
Mod5p(408-424) NNNNN	RRHKSNL**NN**NTRQADFEKW**N**IN**NN**

Figure 4.—Native and mutated NLS-like sequences of Mod5p compared to known localization sequences. The nuclear localization sequences of SV40 large T antigen (Kalderon *et al.* 1984), nucleoplasmin (Robbins *et al.* 1991), and N1 (Dingwall and Laskey 1991) are aligned with putative NLS-like sequences of Mod5p and mutated sequences of the Mod5p C-terminal NLS. Residues relevant to conserved NLS motifs are in bold. Basic residues in the vicinity of the C-terminal bipartite NLS are underlined.

ing cells, as it is when expressed from high-copy vectors in fixed cells. As previously reported for fixed cells (Boguta *et al.* 1994), Mod5p-I is located in mitochondria and the cytosol (Figure 3, C and C′).

The Mod5p-II carboxyl terminus contains a bipartite-like NLS sequence sufficient to deliver a passenger protein to the nucleus: Mod5p-II possesses three sequences that resemble the bipartite NLS motif, which consists of two basic clusters separated by a 10-amino-acid spacer. The first two sequences, $_{122}$**KR**VDTKSSER**K**LT**RK**$_{136}$ and $_{170}$**RR**VQRMLEIYY**K**TG**KK**$_{185}$, are located in the N-terminal half of Mod5p. The third sequence, $_{408}$**KR**NTRQADFEKW**K**IN**KK**$_{424}$, is located near the C terminus of the protein (Figure 4). To determine whether the Mod5p sequences that resemble the bipartite NLS motif have NLS activity, we tested whether regions of Mod5p containing them were sufficient to target a passenger protein to the nucleus. pFB1-7a encodes a cytoplasmic protein with the first 14 amino acids of histone H2B fused in-frame to β-galactosidase (Moreland *et al.* 1987). We inserted regions of *MOD5* that include the putative NLSs at the H2B/β-galactosidase junction of pFB1-7a. The resulting plasmids encoding H2B-Mod5p-β-galactosidase fusion proteins were transformed into yeast strain MT-8 and the fusion protein was localized by indirect immunofluorescence microscopy using an anti-β-galactosidase antibody. The two putative N-terminal NLSs of Mod5p were amplified together in a DNA encoding amino acids 102–203 to create pFB1-7aMOD5(102–203) (Table 3). The third putative NLS was amplified in a separate DNA encoding amino acids 375–427 creating pFB1-7aMOD5(375–427) (Table 3). pFB1-67a encoding 67 amino acids of H2B histone, containing the histone NLS, fused in-frame with β-galactosidase served as a positive control for nuclear staining.

β-Galactosidase in cells harboring pFB1-7a (Figure 5, A and B) was distributed throughout the cells, with no nuclear accumulation. Cells expressing pFB1-67a (Figure 5, C and D) displayed nearly exclusive nuclear staining. Expression of pFB1-7aMOD5(102–203) (Figure 5, E and F), encoding the two N-terminal putative Mod5p NLSs, resulted in staining indistinguishable from that obtained for plasmid pFB1-7a. In contrast, cells expressing pFB1-7aMOD5(375–427) (Figure 5, G and H), which encodes the C-terminal putative Mod5p NLS, displayed nuclear staining indistinguishable from the results obtained for pFB1-67a. Thus, pFB1-7aMOD5(375–427) encodes a protein with NLS activity.

To determine whether the NLS activity of the protein encoded by pFB1-7aMOD5(375–427) maps to the sequence that resembles the bipartite NLS motif, we altered the candidate Mod5p NLS consensus sequence and determined the locations of the resulting fusion proteins. Previous studies have shown that substitution of asparagines for basic amino acids of the bipartite motif destroys NLS activity and that the two halves of the NLS are interdependent (Robbins *et al.* 1991; for a review, see Dingwall and Laskey 1991). We generated three variants of pFB1-7aMOD5(375–427). pFB1-7amod5-KRKNN and pFB1-7amod5-KRNNN have the two or three, respectively, most carboxyl lysines of the Mod5p motif changed to asparagine, whereas pFB1-7amod5-NNNNN has all five consensus basic amino acids altered (Table 3 and Figure 4). The consequences upon the location of the H2B-Mod5p-β-galactosidase fusion proteins were assessed by indirect immunofluorescence. Each plasmid coded for similar amounts of fusion protein as assessed by β-galactosidase activity (Table 4). Alteration of the two (Figure 6, C and D) or three most carboxyl lysines (Figure 6, E and F) of the Mod5p-II bipartite-like NLS motif to asparagines produced a small increase in the quantity of cytosolic β-galactosidase antigen compared to control MT-8 cells harboring pFB1-7aMOD5(375–427) (Figure 6, A and B). Alteration of all five basic residues to asparagine resulted in a much-increased cytoplasmic pool and a significantly smaller nuclear pool of Mod5p-II in most cells (Figure 6, G and G′) in comparison with control cells; however, a few cells still displayed nuclear staining (Figure 6, H and H′). We conclude that the amino acids in the region 375–427 that have NLS activity include those that resemble the consensus amino acids of bipartite NLSs and that the five basic amino acids of the Mod5p NLS function in an additive fashion to target β-galactosidase to the nucleus.

The carboxyl terminal bipartite-like NLS is necessary for the nuclear location of Mod5p-II: The above studies show that Mod5p amino acids 408–424 have NLS activity. However, they do not show that this motif is important for the nuclear location of authentic Mod5p. Nor do they eliminate the possibility that the two other Mod5p sequences (at 122–136 and 170–185) that resem-

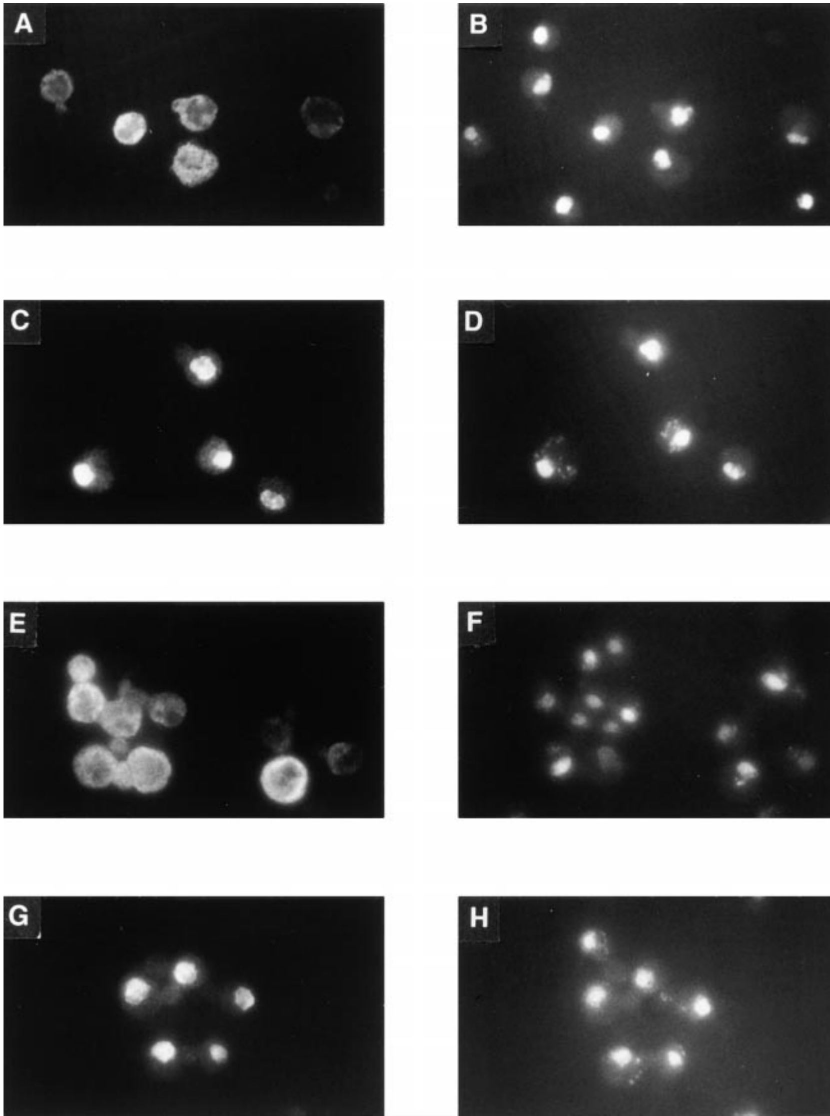

Figure 5.—A carboxyl-terminal Mod5p sequence is sufficient to deliver a surrogate protein to the nucleus. Indirect immunofluorescence microscopy of MT-8 yeast cells expressing H2B-Mod5p-β-galactosidase fusion proteins, which were detected with an anti-β-galactosidase mAb. A, C, E, and G display the FITC detection of anti-β-galactosidase immunoreactive complexes, while B, D, F, and H display DAPI staining of nuclear and mitochondrial DNA. (A and B) Cells harboring pFB1-7a. (C and D) Cells harboring pFB1-67a. (E and F) Cells expressing pFB1-7aMOD5 (102–203). (G and H) Cells expressing pFB1-7aMOD5(375–427).

ble the bipartite NLS consensus but did not appear to have NLS activity by the passenger protein assay do have NLS activity in endogenous Mod5p. It is also possible that sequences other than those we tested could be important for the nuclear pool of Mod5p. Therefore, we tested whether the motif located at 408–424 was necessary for the nucleus location of Mod5p-II by altering the same basic amino acids of this motif in Mod5p-II. Mutations of the three lysines of Mod5p at 420, 423, and 424 to asparagines generated a mutated sequence Mod5p-KRNNN and mutations of lysines at 408, 409, 420, 423, and 424 generated Mod5p-NNNNN. The altered sequences were subcloned to create plasmids that have the first ATG destroyed and, therefore, encode only the Mod5p-II variant.

Each construct was transferred to a centromere-containing vector to assess Mod5p activity when the protein was expressed in approximately endogenous quantities (Tables 3 and 5). Since i^6A modification affects the efficiency of suppression by the nuclear-encoded suppressor tRNA *SUP7*, inactivation of *MOD5* can be assessed by the levels of nonsense suppression of *lys1-1*, *lys2-1*, and *ade2-1.* Cells with the *ade2-1* allele accumulate a red pigment, generating red colonies on rich media, and cells with *lys1-1*, *lys2-1*, and *ade2-1* are unable to grow on media lacking lysine or adenine. Cells expressing vector alone (YCf50) produce no Mod5p and are unable to suppress the nonsense alleles. Conversely, cells expressing YCfMOD5 produce roughly endogenous levels of cytoplasmic Mod5p-I and Mod5p-II and completely suppress *lys1-1*, *lys2-1*, and *ade2-1.* Cells expressing YCfmod5-M1, which produces only Mod5p-II, have suppression indistinguishable from wild type. The suppression phenotypes of cells harboring the *mod5-KRNNN* and *mod5-NNNNN* mutant alleles were indistinguishable from the parental counterparts, demonstrating that proteins encoded by these constructs are fully functional.

Extracts were obtained from MT-8 cells expressing various forms of Mod5p-II in the high-copy vector,

TABLE 4

Activity and cellular location of β-galactosidase/Mod5p fusion proteins

Protein	Activity (units/mg protein × 10^4)	Protein location
pFB1-7a	20.5	Cytosol
pFB1-7aMOD5(375–427)	6.7	Nucleus
pFB1-7amod5-KRKNN	6.3	Primarily nucleus
pFB1-7amod5-KRNNN	7.4	Nucleus and cytosol
pFB1-7amod5-NNNNN	10.8	Primary cytosol
pFB1-67a	11.8	Nucleus
pFB1-67aMOD5(12–427)	4.0	Primarily cytosol
pFB1-67aMOD5(12–234)	3.3	Primarily cytosol
pFB1-67aMOD5(230–403)	4.5	Primarily cytosol
pFB1-67aMOD5(12–109)	5.8	Nucleus and cytosol
pFB1-67aMOD5(102–234)	5.1	Cytosol and nucleus
pFB1-67aMOD5(230–326)	10.3	Cytosol and nucleus
pFB1-67aMOD5(324–403)	5.5	Primarily nucleus
pFB1-67aMOD5(170–243)	3.2	Nucleus and cytosol
pFB1-67aMOD5(206–243)	14.0	Primarily nucleus
pFB1-67aMOD5(206–276)	7.2	Primarily cytosol
pFB1-67aMOD5(230–276)	9.5	Cytosol and nucleus
pFB1-67aMOD5(275–326)	14.3	Nucleus and cytosol

Plasmids were transformed into yeast strain MT-8 (relevant genotype *mod5::TRP1 leu2-3,112*; Gillman *et al.* 1991) and transformants were selected on −leu media. β-Galactosidase activity was determined by the method of Reynolds *et al.* (1997). The subcellular location of the fusion protein was determined by immunofluorescence using anti-β-galactosidase to locate the protein and DAPI to locate nuclear and mitochondrial DNA (see Figures 5, 6, 8, and 10).

pJDB207 (Table 3). The quantities and mobilities of proteins from each strain were detected by Western blot analysis using affinity-purified Mod5p antibody. The blot was counterstained with an actin-specific antibody to confirm equal loading of protein. By this assay each construct produced roughly equivalent levels of protein of the appropriate electrophoretic mobility (data not shown).

To determine the effects of mutating the putative NLS on the nuclear localization of Mod5p-II, the mutant constructs were expressed from the pJDB207 multicopy plasmid and mutant proteins were localized by indirect immunofluorescence. Cells expressing the pJDB207 vector alone revealed no Mod5p-specific staining (Figure 7, A and B). Cells harboring pJDBmod5-M1 (Figure 7, C and D), which produces only Mod5p-II, as expected, had both cytosol and nuclear (nucleolar) Mod5p. pJDBmod5-M1-KRNNN (Figure 7, E and F) resulted in only a small increase in the level of cytoplasmic Mod5p and a concomitant decrease in nuclear accumulation of the mutant isozyme, whereas the protein encoded by pJDB207mod5-M1-NNNNN (Figure 7, G and H) appeared to be located primarily in the cytosol. The data show that the C-terminal NLS-like sequence of Mod5p is important for nuclear accumulation of the overexpressed Mod5p-II isozyme and that the motif at 408–424 is the only NLS in the *MOD5* sequence.

Cytoplasmic retention/nuclear export sequence(s) may regulate the nucleus/cytosol distribution of Mod5p-II: Mod5p-II resides in both the nucleus and cytoplasm. Although the putative Mod5p NLS appears to be a "strong" targeting sequence, as it can quantitatively deliver a passenger protein to nuclei, it does not completely localize Mod5p-II to the nucleus. Several possibilities could account for the cytoplasmic pool. One is that the protein may fold in such a manner that the NLS is not optimally exposed or easily accessible to the import machinery, thereby decreasing its efficiency in nuclear import. To determine whether NLS occlusion might explain cytosolic Mod5p-II, we inserted DNA encoding the SV40 large T antigen NLS into plasmids harboring the *mod5-M1* allele just downstream of the second ATG of the *MOD5* ORF. The amino terminus was chosen since presumably it is an exposed area of the protein that is able to present the mitochondrial targeting information to the import machinery. The SV40 large T antigen NLS has been shown to function efficiently in yeast (Benton *et al.* 1990; Wolfe *et al.* 1996) and, as it is a hydrophilic and terminal sequence, should be exposed. The mutant allele was designated *mod5-M1-SV40* and it encodes the isozyme Mod5p-II-SV40 (Table 3). If occlusion of the Mod5p NLS prevents efficient interaction with the appropriate import molecules, the addition of a second exposed NLS should generate a protein that is located primarily in the nucleus. The mutant protein was determined to be functional as assessed by monitoring *SUP7*-mediated suppression of nonsense mutant alleles (Table 5). The

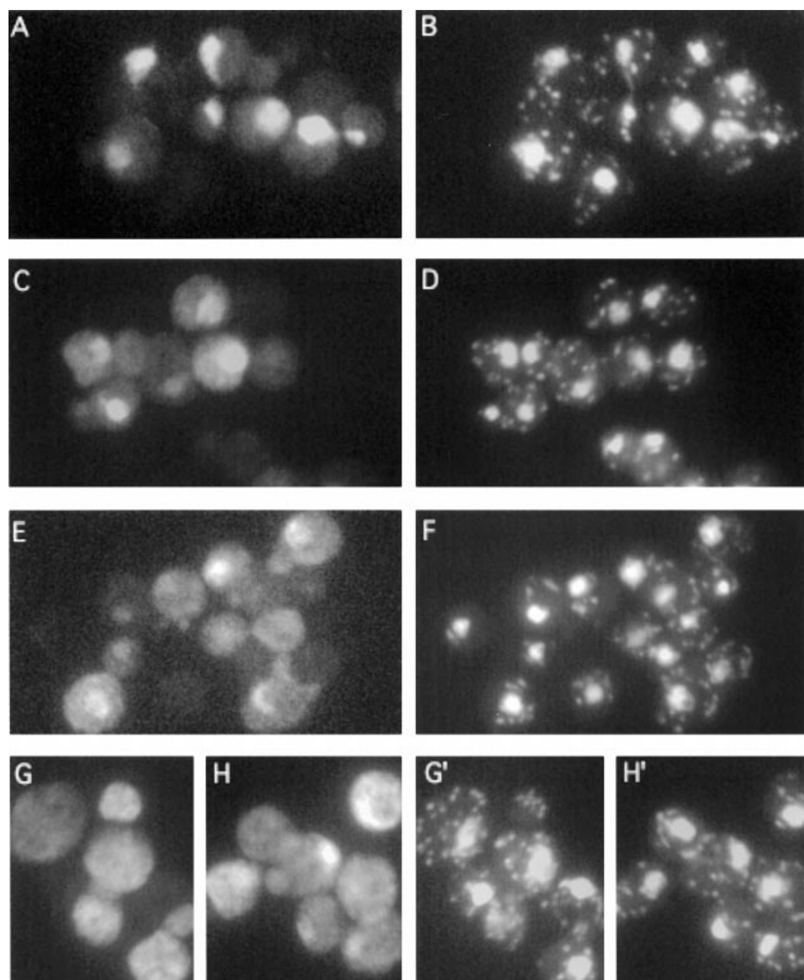

Figure 6.—Localization of reporter β-galactosidase by wild-type and mutant Mod5p sequences in MT-8. MT-8 cells expressing fusion proteins consisting of β-galactosidase and the wild-type Mod5p carboxyl terminus KRKKK (A and B) or mutant Mod5p carboxyl terminus KRKNN (C and D), KRNNN (E and F), or NNNNN (G, G′, H, and H′) from multicopy plasmids were subjected to immunofluorescence with rabbit anti-β-galactosidase and FITC-conjugated anti-rabbit IgG (A, C, E, G, and H). Cells were stained with DAPI to locate nuclear and mitochondrial DNA (B, D, F, G′, and H′).

single-copy expression of *mod5-M1-SV40* in MT-8 suppressed *ade2-1*, *lys1-1*, and *lys2-1* as displayed by growth on −ade media, white colonies on rich media, and growth on −lys media.

To determine the effect of the addition of the SV40 large T antigen NLS on nuclear import of Mod5p-II, the *mod5-M1-SV40* allele was expressed in multicopy in the yeast MT-8 and the location of the resulting protein was analyzed by indirect immunofluorescence (Figure 8, compare A–D with E and F). While there may be a slight increase in the nuclear accumulation of Mod5p-II-SV40, this isozyme was not efficiently delivered to the nucleus. The inability of Mod5p-II-SV40 to efficiently locate to the nucleus indicates that the cytoplasmic pool

TABLE 5

Effects of *MOD5* mutations on nonsense suppression

Plasmid	Suppression of *ade2-1*		Suppression of *lys1-1, lys2-1*: Growth on −lys
	Growth on −ade	Color on YEPD	
YCf50	−	Red	−
YCfMOD5	+	White	+
YCfmod5-M1	+	White	+
YCfmod5-M1-KRNNN	+	White	+
YCfmod5-M1-KRK	+	White	+
YCfmod5-M1-NNNNN	+	White	+
YCfmod5-M1-SV40	+	White	+

Plasmids were transformed into yeast strain MT-8 (relevant genotype *mod5::TRP1 ura3-52*; Gillman *et al.* 1991) and grown on selective media. Transformants were tested for complementation of the *mod5::TRP1* allele by assessing growth and/or colony color on the relevant media after 2 days at 30°.

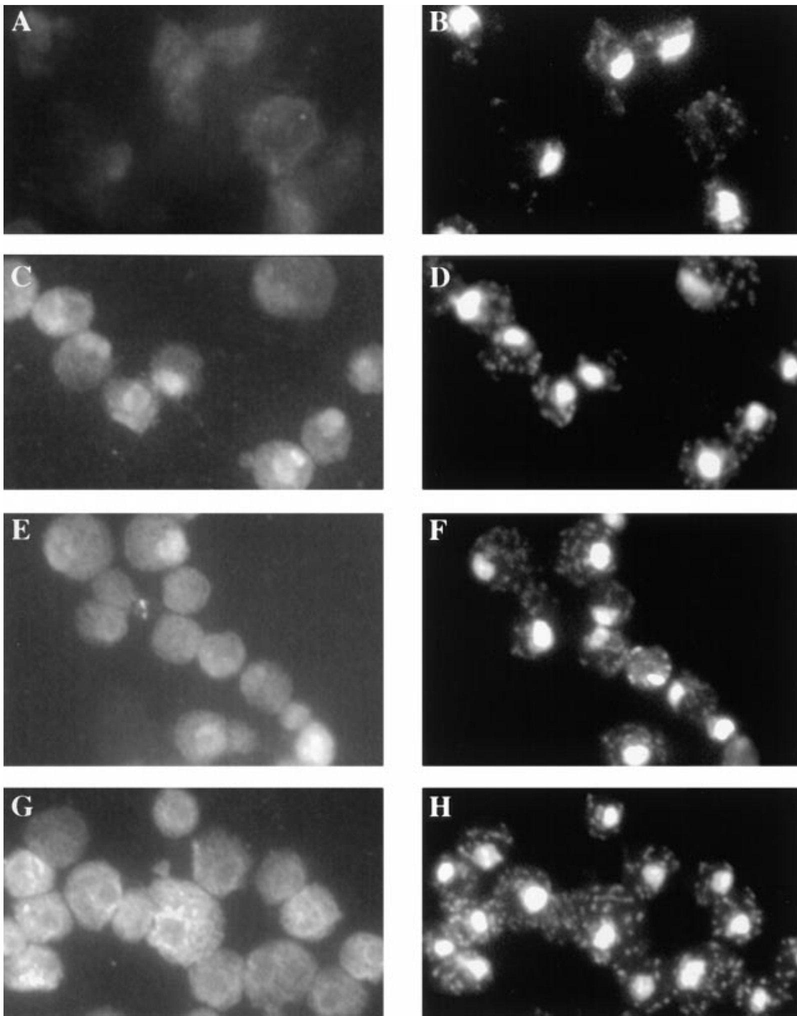

Figure 7.—A C-terminal bipartite-like NLS is necessary for the nuclear localization of Mod5p-II. Indirect immunofluorescence of MT-8 yeast cells expressing mutant forms of Mod5p. A, C, E, and G display FITC detection of anti-Mod5p antibody-antigen complexes, while B, D, F, and H display DAPI staining of nuclear and mitochondrial DNA. (A and B) Cells expressing pJDB207. (C and D) Cells expressing pJDBmod5-M1. (E and F) Cells harboring pJDBmod5-M1-KRNNN. (G and H) Cells harboring pJDBmod5-M1-NNNNN.

of this isozyme is unlikely to be due to masking of the Mod5p NLS.

We considered 3′ mRNA end heterogeneity (Najarian *et al.* 1987; P. Artz, N. C. Martin and A. K. Hopper, unpublished results) as another possible mechanism to account for the incomplete accumulation of Mod5p-II in nuclei. Protein encoded by transcripts with the shortest termini should lack a complete NLS (P. Artz, N. C. Martin and A. K. Hopper, unpublished results). To test this possible mechanism, we introduced a translational stop codon at the site of the shortest mRNA 3′ end and determined the consequences on Mod5p-II subcellular location. Isoleucine 421 of the Mod5p-II sequence was changed to a stop codon (TGA) creating *mod5-KRK* (Table 3). This mutated gene encodes an isozyme, Mod5p-II-KRK, possessing the KR amino acids of the upstream half of the NLS and only one of the three basic amino acids of the downstream half. The mutant allele produced appropriate levels of protein as determined by Western blot analysis of protein extracts from cells harboring the mutated gene (data not shown). The proteins were also functional as demonstrated by monitoring the *SUP7*-mediated suppression of nonsense-mutant alleles (Table 5).

Cells expressing pJDBmod5-M1-KRK did not show a decrease in nuclear staining (Figure 8, compare C and D with G and H). These results are consistent with our other studies (Figure 6) showing that mutations of the downstream basic amino acids of the Mod5p NLS have little effect on the ability of the NLS to function in the delivery of a passenger protein to the nucleus. The data indicate that 3′ mRNA heterogeneity is an unlikely mechanism to account for the cytosolic pool of Mod5p-II.

As NLS masking and 3′ mRNA heterogeneity do not seem to regulate the nucleus/cytosol distribution of Mod5p-II, we considered the possibility that Mod5p-II could possess sequences that act either to retain a portion of the pool of this protein in the cytosol (CRD or CRS) or, alternatively, that act to redistribute nucleus-located Mod5p-II to the cytosol (NES). Even though Mod5p-II does not possess motifs identical to characterized CRD, CRS, or NES sequences, Mod5p-II may possess *cis*-regions of different sequence that fulfill similar functions. To identify such putative sequences we employed an assay to test for their function. Plasmid pFB1-67a contains the codons for the first 67 amino acids of histone H2B that include the histone NLS fused in-frame to β-galactosidase. The resulting H2B/β-galacto-

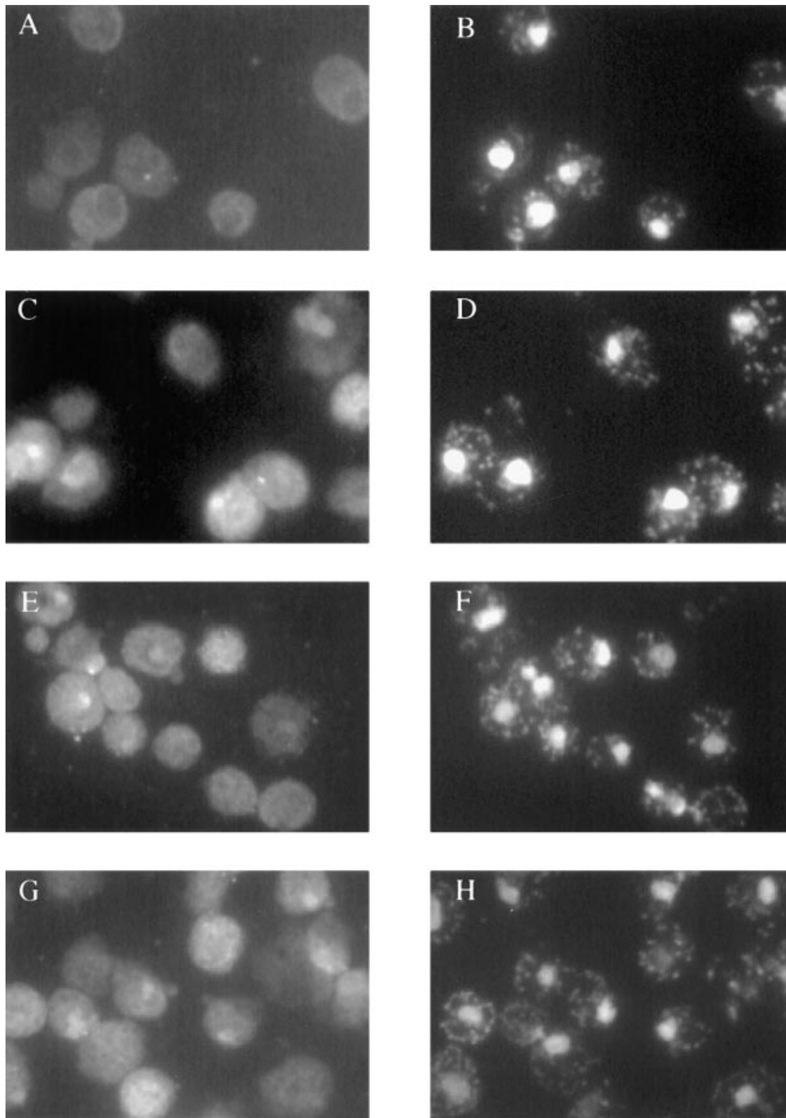

Figure 8.—Effects of NLS addition and NLS truncation upon the location of Mod5p-II. Indirect immunofluorescence of MT-8 yeast cells expressing *MOD5* mutant alleles. A, C, E, and G display the detection of Mod5p with affinity-purified anti-Mod5 primary antibody and FITC-conjugated secondary antibody. B, D, F, and H display DAPI staining of nuclear and mitochondrial DNA. (A and B) Cells containing vector alone. (C and D) Cells expressing pJDBmod5-M1. (E and F) Cells expressing pJDBmod5-M1-SV40. (G and H) Cells expressing pJDBmod5-M1-KRK.

sidase fusion protein is located in the nucleus of yeast cells (Moreland *et al.* 1987; Figures 5, C and D and 9A). We inserted portions of the *MOD5* gene in-frame at the junction of H2B and *lacZ* of plasmid pFB1-67a in an effort to identify sequences in Mod5p that would cause the karyophilic H2B/β-galactosidase to have a cytosolic location.

Initially we assessed the location of fusion proteins possessing nearly the entire Mod5p-II. The fusion protein encoded by pFB1-67aMOD5(12–427) is located primarily in the cytosol (Figures 9B and 10). Although the protein could be located in the cytosol because it is misfolded and the NLS is masked, this is not likely as the fusion protein has similar activity to the protein encoded by pFB1-67a (Table 4). To map the sequences responsible for the cytosolic location of the fusion protein we generated two plasmids each encoding about one-half of Mod5p-II. pFB1-67aMOD5(12–234) contains *MOD5* codons 12–234 whereas pFB1-67aMOD5(230–403) contains *MOD5* codons 230–403. The plasmids were transferred to strain MT-8, and the activities and locations of H2B/Mod5p/β-galactosidase proteins were determined. Surprisingly, both fusion proteins located primarily in the cytosol (Figures 9, C and D and 10). Again, the cytosolic location did not appear to be an artifact of grossly misfolded proteins as the fusion proteins had about the same level of activities as the starting H2B/β-galactosidase protein (Table 4). We considered the possibility that Mod5p-II contains more than one *cis*-acting sequence regulating its nucleus/cytosolic distribution. To further map the putative regulatory sequences, the sequences in pFB1-67MOD5(12–234) and pFB1-67aMOD5(230–403) were each divided into two halves generating plasmids that individually contained Mod5p amino acids 12–109, 102–234, 230–326, and 324–403, respectively (Figures 9, E–H and 10). Each encoded the appropriate β-galactosidase activity (Table 4). Fusion proteins with Mod5p amino acids 12–109 or 324–403 were located primarily in the nucleus (Figure 9, E and H) whereas proteins with Mod5p amino acids 102–234 or 230–326 were located in the cytosol and nucleus (Figure 9, F and G). However, there was hetero-

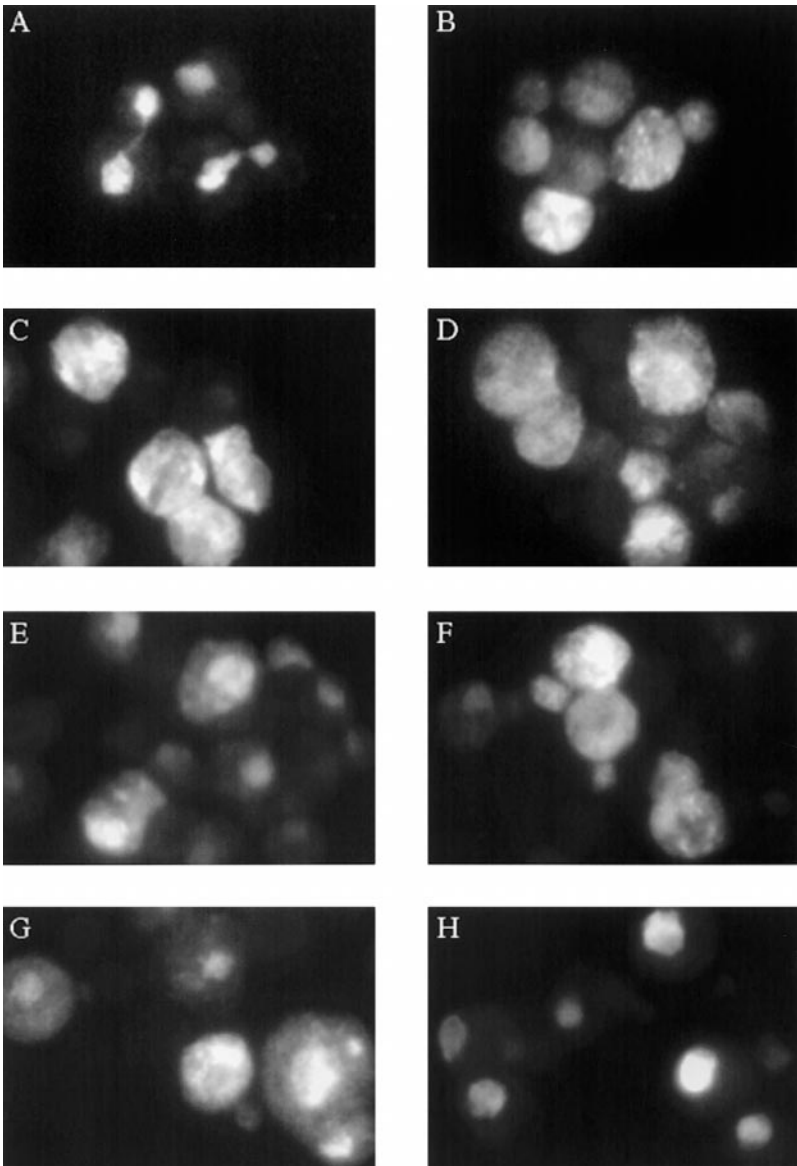

Figure 9.—Localization of karyophilic β-galactosidase by various fragments of Mod5p-II in MT-8. (A) MT-8 cells expressing fusion proteins consisting of histone H2B NLS in-frame with β-galactosidase alone or with (B) Mod5(12–427), (C) Mod5(12–234), (D) Mod5(230–403), (E) Mod5-(12–109), (F) Mod5(102–234), (G) Mod5(230–326), or (H) Mod5(324–403) from multicopy plasmids were subjected to immunofluorescence with rabbit anti-β-galactosidase and FITC-conjugated anti-rabbit IgG.

geneity among cells with respect to location of these latter two fusions. Perhaps the entire region causing a cytosolic pool is not included in either of them.

Mod5p amino acid regions 197–206 and 225–236 are leucine-rich and resemble the NES consensus motif. To investigate the possibility that either/both of these sequences might function in nuclear export, additional fine mapping in this vicinity of Mod5p was performed. The H2B/Mod5p/β-galactosidase proteins encoded by pFB1-67aMOD5(170–243), pFB-67aMOD5(206–243), pFB1-67aMOD5(206–276), pFB1-67aMOD5(230–276), and pFB1-67aMOD5(275–326) were found to have enzymatic activities similar to H2B/β-galactosidase alone (Table 4). Proteins with Mod5p amino acids 206–276 were predominantly cytosolic (Figure 10). Removal of sequence from either the carboxyl terminus (in proteins with amino acids 170–243 and 206–243) or from the amino terminus (in proteins with amino acids 230–276) of this Mod5p region resulted in increased nuclear pools or heterogeneous distribution. Fusion proteins containing amino acids 275–326 were also located in both the nucleus and cytosol. Even though our studies indicate that Mod5p-II possesses sequences in the middle of the protein that are responsible for the cytosolic pool, we have been unable to define them precisely because they appear to be redundant, long, and complicated. (However, see below.)

Conservation of regions implicated for Mod5p subcellular distribution: Genome sequencing has uncovered putative *MOD5* homologs from many organisms. For eukaryotes, there are sequences that would encode the homologs for *C. elegans* and *S. pombe* and there are partial sequences for the putative human, *Drosophila melanogaster*, and Plasmodium homologs. For eubacteria, 33 sequences for the putative *MOD5* homologs (*mia* in *E. coli*) are in the databases. None of the sequenced archaeal genomes contains a *MOD5/miaA* gene. All of the eukaryotic and eubacteria sequences

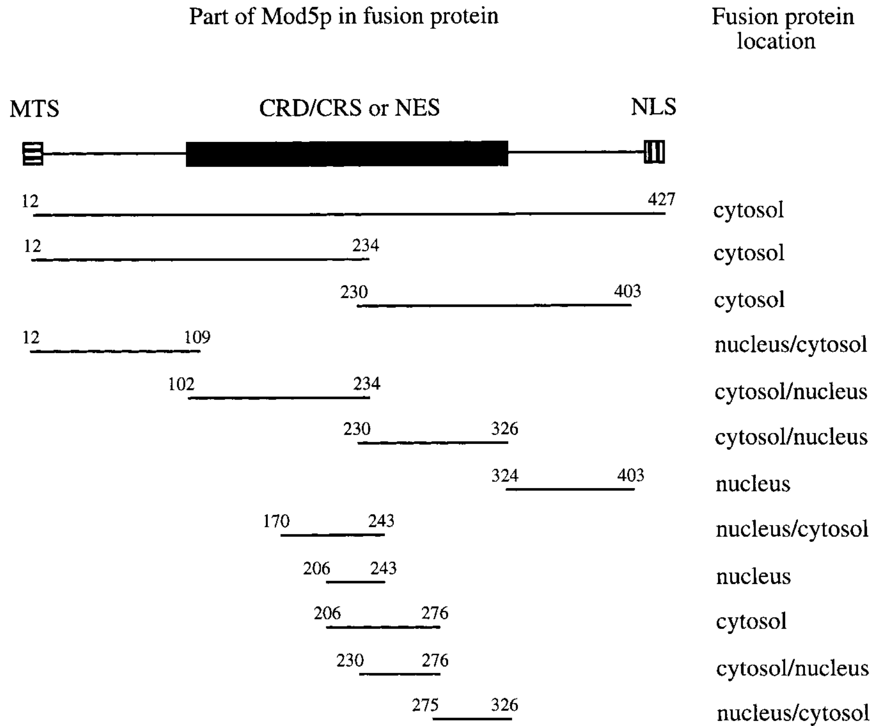

Figure 10.—Summary of results of localization of karyophilic β-galactosidase by various fragments of Mod5p-II in MT-8. Boxes indicate sequences responsible for mitochondrial targeting (horizontal bars), nuclear targeting (vertical bars), and the cytoplasmic pool (solid box) of Mod5p. Lines with numbers indicate the beginning and ending Mod5p amino acids incorporated into the H2B-β-galactosidase fusion proteins that were used to map the CRD/CRS or NES sequences. The locations of the fusion proteins are summarized in this diagram: cytosol, primarily cytosol location; nucleus, primarily nucleus location; nucleus/cytosol, protein in both nucleus and cytosol with bias for a nuclear location; cytosol/nucleus, protein in both nucleus and cytosol with bias for cytosol location.

share striking conservation. However, the eukaryotic sequences have additions. The *S. cerevisiae* and *C. elegans* sequences possess short amino-terminal additions and two in-frame AUGs at codons 1 and 12 or 15, respectively (Figure 11). The GenBank entry for the *C. elegans MOD5* gene (U13642) begins the coding sequence at the second AUG; however, an in-frame upstream AUG is present and the sequence can be translated as: MIFRKFLNFLKPYKM. . . . In yeast, the N-terminal 11 Mod5p amino acids are a necessary part of the mitochondrial targeting sequence (MTS; Boguta *et al.* 1994). Like the yeast sequence and other proteins targeted to the mitochondria (for a review, see Attardi and Schatz 1988), the *C. elegans* aminoterminal sequence is basic, hydrophobic, and predicted to be amphiphilic (von Heijne 1986). Thus, the *C. elegans* aminoterminal sequence resembles the structure of other MTS. Given the two inframe AUGs and the similarity to MTS, we predict that the *C. elegans* putative homolog also encodes sorting isozymes—an amino-extended protein distributed to mitochondria and a form lacking this extension distributed differently. The *S. pombe* Mod5p homolog has no aminoterminal extension, and if there is a mitochondrial Mod5p isoform it must be encoded by a separate gene that has yet to be sequenced. There is insufficient sequence information to make predictions as to whether the human, Drosophila, or Plasmodium Mod5p homologs are sorting isozymes or whether mitochondrial and nucleus/cytosol activities are encoded by separate genes.

Yeast and *C. elegans MOD5* genes each encode an ∼100-amino-acid carboxyl motif (yeast Mod5p amino acids 303–344 and 373–428) not found in prokaryotes. Here, we show that the yeast NLS is located near the end of this C-terminal extension (amino acids 408–424). We did not find a perfect match to either the SV40 large T antigen or the bipartite-like NLS consensus by inspection of the *C. elegans* carboxyl-terminal extension. However, in a location close to the Mod5p NLS the *C. elegans* has an imperfect match to the bipartite NLS consensus: **K**HIDGKKHKHHA**K**Q**KK** (Figure 11, underlined sequences). The similarities lead us to predict a nuclear pool of the *C. elegans* Mod5p.

Yeast, *C. elegans*, and Plasmodium Mod5p sequences each possess two short internal stretches of amino acids that are absent or different from the bacterial counterparts (yeast Mod5p amino acids 238–257 and 273–277; Figure 11). These sequences overlap with the location of one of the putative export/retention domains we identified (amino acids ∼230–326) perhaps providing auxiliary evidence that they have a role other than catalysis. Future experiments guided by the information regarding eukaryotic additions may help delineate more precisely those sequences involved in nucleus/cytosol distribution. In sum, it appears that the eukaryotic *S. cerevisiae* and *C. elegans* Mod5p have acquired "accessory" sequences that are important to the cellular distribution of this highly conserved family of proteins. Use of two in-frame AUGs allows for the synthesis of proteins containing or lacking part of the accessory information.

DISCUSSION

Mod5p-II *cis*-acting sequences important for the nuclear pool: Three lines of evidence support the conclusion that the carboxyl terminal motif located between

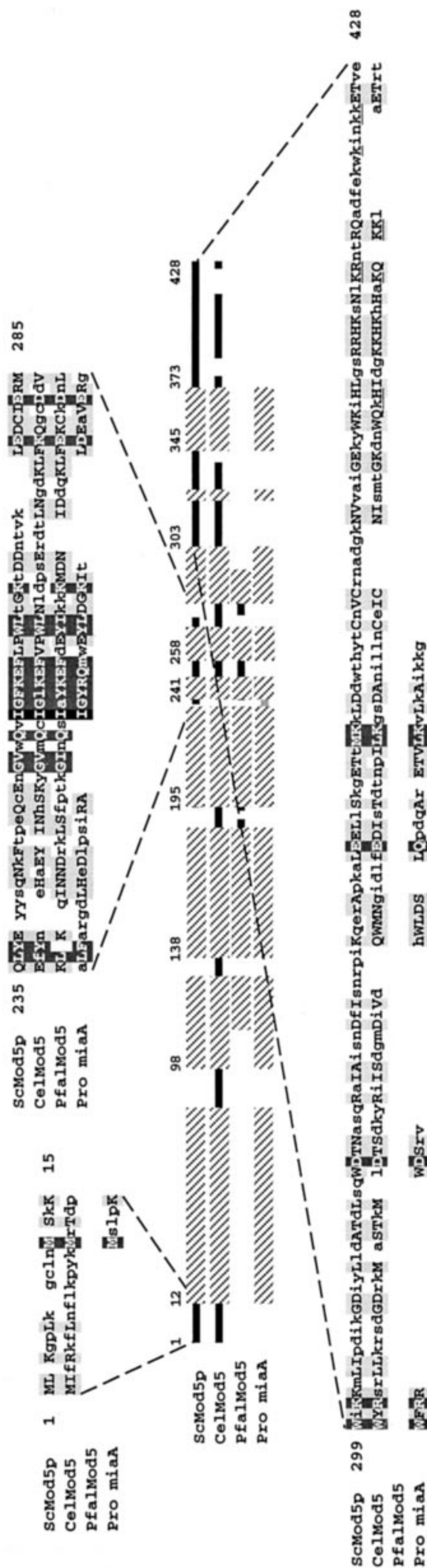

Figure 11.—Alignment of eukaryotic Mod5 proteins and a prokaryotic consensus sequence for miaA proteins. Mod5p homologs from S. cerevisiae (ScMod5p), C. elegans (CelMod5), and P. falciparum (PfalMod5) are shown. Pro miaA is a consensus sequence derived from 33 prokaryotic organisms (see materials and methods). The schematic diagram in the center shows the alignment of the full-length proteins (where the sequence is available). The hatched boxes represent regions of sequence that are conserved in eukaryotes and prokaryotes. The black lines represent regions of sequence that are found only in eukaryotes, while the gray line represents a short stretch of sequence that is dissimilar between eukaryotes and prokaryotes. For three regions the amino acid alignments are shown. Identical residues are presented as white letters on a black background; white on dark gray is 75–99% conserved or similar; black on dark gray is 50–74% conserved or similar; and black on white is not conserved or similar. The basic residues of the S. cerevisiae NLS and the C. elegans putative NLS are indicated by underlining.

amino acids 408 and 424 is the Mod5p-II NLS. First, this sequence conforms exactly to the bipartite NLS consensus sequence [KR (X_{10}) 3 of 5 K or R]. Second, a region of Mod5p-II containing this sequence is sufficient to target a passenger protein to the nucleus and mutations of the consensus amino acids affect the efficiency of nuclear accumulation of the passenger protein. Third, this motif is necessary for the nuclear pool of Mod5p.

Two other Mod5p-II regions resemble the bipartite NLS consensus. Amino acids 122–136 and 170–185 each contain the requisite number of basic amino acids, but the two basic regions are separated by 8 and 9 amino acids, rather than the conserved 10-amino-acid spacer (Robbins *et al.* 1991; for a review, see Dingwall and Laskey 1991). These sequences did not act to deliver the β-galactosidase passenger protein to the nucleus. Nor under normal lab growth conditions do these two motifs play significant roles in Mod5p-II subcellular distribution since mutation of the endogenous Mod5p sequence located at 408–424 alone causes the vast majority of Mod5p-II to be located in the cytosol. Moreover, amino acids 170–185 are conserved in prokaryotes, indicating a role different from organelle targeting for this region of Mod5p.

Although the Mod5p-II motif located between amino acids 408 and 424 appears to be a bonafide NLS, it differs in some respects from other bipartite NLS (Robbins *et al.* 1991). For nucleoplasmin and N1 Xenopus proteins, neither basic half of the bipartite motif is sufficient for nuclear accumulation; that is, the two basic regions are interdependent. In contrast, the basic amino acids of the Mod5p-II motif appear to have a cumulative effect upon nuclear accumulation as mutation of two or three basic amino acids of the carboxyl half of the motif to asparagines does not destroy NLS activity. Only if all five basic consensus amino acids are altered is there a dramatic effect upon nuclear accumulation of the passenger protein. For endogenous Mod5p-II, again only if all five amino acids are altered do we see a significant cellular redistribution of the protein.

Remarkably, alteration of all consensus basic amino acids to asparagines does not completely destroy NLS activity for β-galactosidase. It is possible that, as others have recently shown (for a review, see Makkerh *et al.* 1996), neutral and acidic amino acids in the vicinity of remaining basic amino acids play a role in nuclear import. It is also possible that there is a cryptic bipartite NLS in this region. There are two basic amino acids at positions 401 and 402 upstream of the consensus KR amino acids at positions 408 and 409 and two basic amino acids in the spacer region of the NLS motif (see underlined amino acids in Figure 4). Mutation of the five consensus amino acids generates a sequence that has two basic amino acids separated from a downstream basic amino acid by 9 or 16 residues, respectively. As our results show that alteration of two of the three down-

stream amino acids does not destroy NLS activity and others have shown flexibility in the length of the spacer (Robbins *et al.* 1991), it is possible that mutation of the five consensus amino acids uncovers the activity of a weak/overlapping NLS. The existence of the putative overlapping NLS could explain why mutations of the Mod5p consensus amino acids show cumulative effects, rather than interdependent effects upon NLS activity. Interestingly, other bipartite NLS sequences have similar basic amino acids upstream of the amino half and in the spacer regions (Robbins *et al.* 1991), so there may be other similar cases of putative overlapping signals.

Regulation of the nucleus/cytosol pools of Mod5p-II: Mod5p-II possesses a bipartite NLS that promotes efficient nuclear accumulation of β-galactosidase. However, subcellular fractionation of cells expressing endogenous *MOD5* or Mod5p from multicopy vectors (Boguta *et al.* 1994) and immunofluorescence of cells containing *MOD5* on a multicopy vector (Boguta *et al.* 1994 and these studies) show that much, perhaps the majority, of Mod5p-II is cytosolic. How is a large portion of the Mod5p-II pool in the cytosol despite the presence of a "strong" bipartite NLS? To test for NLS masking, we positioned an additional SV40 large T antigen NLS in Mod5p at a position normally occupied by part of the mitochondrial targeting sequence (Boguta *et al.* 1994), as this region of Mod5p should be "exposed." Despite the additional NLS, substantial Mod5p remained in the cytosol. Thus, it seems unlikely that the Mod5p cytosolic pool is a result of NLS occlusion. Interestingly, the Mod5p-II nucleus/cytosol distribution is regulated differently than Cca1p, which is sorted to the same subcellular compartments. Addition of the same SV40 large T antigen NLS at the analogous Cca1p location resulted in the vast majority of the protein being located in the nucleus (Wolfe *et al.* 1996). Thus, NLS masking or a "weak" NLS can account for the cytosolic pool of Cca1p, but does not appear to account for the Mod5p-II cytosolic pool.

The presence of nuclear export or cytosol retention sequences in Mod5p-II could generate the cytosolic pool. By inspection, Mod5p does not contain any closely matched sequences to the previously characterized NES, CRS, or CRD motifs (Pines and Hunter 1994; Fischer *et al.* 1995; Michael *et al.* 1995; Wen *et al.* 1995; Shou *et al.* 1996). However, only a few examples of these motifs have been studied in detail. Even those with similar activity do not necessarily show sequence similarity. Therefore, we conducted a functional search for NES and/or CRS/CRD *cis*-acting sequences. We found that portions of Mod5p inserted in-frame with an NLS-containing β-galactosidase can cause β-galactosidase to accumulate in the cytosol. However, without a positive test for function, it is difficult to precisely define the boundaries of these sequences. Our studies show that Mod5p-II may contain more than one Mod5p-II retention/export sequence. We conclude that the distribution of Mod5p-II between the nucleus and cytosol results from antagonistic interactions of a "strong" NLS and other *cis*-acting sequences that maintain the isozyme in the cytosol or cause it to exit from the nucleus.

Role of the nuclear pool of Mod5p-II and nucleolar location: The initial discovery of nuclear Mod5p-II was surprising in view of the studies of the processing of yeast pre-$tRNA^{Tyr}$ in Xenopus oocytes that showed i^6A moiety addition to tRNA only after intron removal, which occurs just prior to nuclear export (for a review, see Hopper and Martin 1992) and i^6A-containing $tRNA^{Tyr}$ was detected solely in the cytosol (Nishikura and De Robertis 1981). By characterizing the *cis*-acting sequences responsible for each subcellular pool and then altering these sequences to obtain *MOD5* variants that coded for primarily nuclear or primarily cytosolic Mod5p-II, consequences of mislocalization can be determined. We have altered the *MOD5* NLS so that the majority of Mod5p-II is located in the cytosol. As judged by the efficiency of $tRNA^{Tyr}_{UAA}$ in nonsense suppression, mislocating Mod5p-II to the cytosol does not affect the i^6A modification of $tRNA^{Tyr}$. We have not assessed the modification of other tRNA families. However, since i^6A can be added to otherwise mature tRNAs *in vitro* (Dihanich *et al.* 1987), we would expect that a cytosolic reaction would suffice to modify all tRNA families. As we have not completed characterizing the sequences responsible for the cytosol pool, we have not yet generated variant Mod5p-II located primarily in the nucleus. We would predict, however, that tRNAs encoded by genes lacking an intervening sequence would be modified in this case, whereas intervening sequence-containing pre-tRNAs would not.

If the nuclear pool is not essential for tRNA modification, why should there be a nucleus-located pool of this enzyme? Here we show that nuclear Mod5p-II appears to be concentrated in the nucleolus. Overexpression is necessary to view the subnuclear location of Mod5p-II, so it is possible that the nucleolar location is an artifact of overproduction. However, as the location of other tRNA processing activities does not change upon overproduction (for example, compare Li *et al.* 1989 and Rose *et al.* 1995), endogenous Mod5p-II may be nucleolar. If so, this subnuclear location would be different from pre-tRNA splicing endonuclease (Peebles *et al.* 1983), tRNA ligase (Clark and Abelson 1987), and m^2_2G dimethyltransferase (Li *et al.* 1989; Rose *et al.* 1995) that are associated with the nucleoplasmic surface of the nuclear membrane. Other pre-tRNA processing activities do appear to be nucleolar and studies have shown that rRNA and tRNA biosynthetic pathways can share enzyme activities. For example, RNaseP that processes the 5′ ends of pre-tRNA also affects pre-rRNA processing (Chamberlain *et al.* 1996) and the RNA subunits of mitochondrial RNA processing enzyme (MRP) involved in rRNA biosynthesis and RNaseP are associated with the same protein complex (Stolc and Altman 1997)

and are located in the nucleolus (Lee *et al.* 1996). Moreover, *in situ* hybridization studies have shown particular pre-tRNAs to be localized in the nucleolus (Bertrand *et al.* 1998). It is possible that the nucleolar pool of Mod5p-II modifies tRNAs that are located in the nucleolus during particular processing steps. Another possibility is that Mod5p-II might modify RNAs other than tRNAs. Although it is unlikely that rRNA itself contains i^6A modifications, it is possible that the snoRNAs involved in rRNA modification (for a review, see Tollervey and Kiss 1997) may be so modified.

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LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

Aris, J. P., and G. Blobel, 1988 Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. J. Cell Biol. **107:** 17–31.

Attardi, G., and G. Schatz, 1988 Biogenesis of mitochondria. Annu. Rev. Cell Biol. **4:** 289–333.

Beggs, J. D., 1991 Multiplecopy yeast plasmid vectors, pp. 383–390 in *Molecular Genetics in Yeast, Alfred Benzon Symposium*, Vol. 16, edited by C. Von Wettstein, J. Friis, M. Kielland-Brandt and A. Stenderup. Munksgaard, Copenhagen.

Benton, B. M., W.-K. Eng, J. J. Dunn, F. W. Studier, R. Sternglanz *et al.*, 1990 Signal-mediated import of bacteriophage T7 RNA polymerase into the *Saccharomyces cerevisiae* nucleus and specific transcription of target genes. Mol. Cell. Biol. **10:** 353–360.

Bertrand, E., F. Houser-Scott, A. Kendall, R. H. Singer and D. R. Engelke, 1998 Nucleolar localization of early tRNA processing. Genes Dev. **12:** 2463–2468.

Boguta, M., L. A. Hunter, W.-C. Shen, E. C. Gillman, N. C. Martin *et al.*, 1994 Subcellular localization of *MOD5* proteins: mapping of sequences sufficient for targeting to mitochondria and demonstration that mitochondria and nuclear isoforms commingle in the cytosol. Mol. Cell. Biol. **14:** 2298–2306.

Chamberlain, J. R., E. Pagán-Ramos, D. W. Kindelberger and D. R. Engelke, 1996 An RNase P RNA subunit mutation affects ribosomal RNA processing. Nucleic Acids Res. **24:** 3158–3166.

Chen, D.-C., B.-C. Yang and T.-T. Kuo, 1992 One-step transformation of yeast in stationary phase. Curr. Genet. **21:** 83–84.

Chen, S. P., J. S. Brockenbrough, J. E. Dove and J. P. Aris, 1997 Homocitrate synthase is located in the nucleus in the yeast *Saccharomyces cerevisiae.* J. Biol. Chem. **272:** 10839–10846.

Christianson, T. W., R. S. Sikorski, M. Dante and P. Hieter, 1992 Multifunctional yeast high-copy number shuttle vectors. Gene **110:** 119–122.

Clark, M. W., and J. Abelson, 1987 The subnuclear localization of tRNA ligase in yeast. J. Cell Biol. **105:** 1515–1526.

Danpure, C. J., 1995 How can the products of a single gene be localized to more than one intracellular compartment? Trends Cell Biol. **5:** 230–238.

de Beus, E., J. S. Brockenbrough, B. Hong and J. P. Aris, 1994 Yeast *NOP2* encodes an essential nucleolar protein with homology to a human proliferation marker. J. Cell Biol. **127:** 1799–1813.

Dihanich, M. E., D. Najarian, R. Clark, E. C. Gillman, N. C. Martin *et al.*, 1987 Isolation and characterization of *MOD5*, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae.* Mol. Cell. Biol. **7:** 177–184.

Dingwall, C., and R. A. Laskey, 1991 Nuclear targeting sequences—a consensus? Trends Biochem. Sci. **16:** 478–481.

Dove, J. E., J. S. Brockenbrough and J. P. Aris, 1998 Isolation of nuclei and nucleoli from the yeast *Saccharomyces cerevisiae*, pp. 33–46 in *Nuclear Structure and Function,* Vol. 53, edited by M. Berrios. Academic Press, San Diego.

Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj and R. Lührmann, 1995 The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:** 475–483.

Gerace, L., 1995 Nuclear export signals and the fast track to the cytoplasm. Cell **82:** 341–344.

Gietz, D., A. St. Jean, R. A. Woods and R. H. Schiestl, 1992 Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:** 1425.

Gillman, E. C., L. B. Slusher, N. C. Martin and A. K. Hopper, 1991 *MOD5* translation initiation sites determine N^6-isopentenyladenosine modification of mitochondrial and cytoplasmic tRNA. Mol. Cell. Biol. **11:** 2382–2390.

Hanahan, D., J. Jessee and F. R. Bloom, 1991 Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol. **204:** 63–113.

Henriquez, R., G. Blobel and J. P. Aris, 1990 Isolation and sequencing of NOP1. A yeast gene encoding a nucleolar protein homologous to a human autoimmune antigen. J. Biol. Chem. **265:** 2209–2215.

Higgins, D. G., J. D. Thompson and T. J. Gibson, 1996 Using CLUSTAL for multiple sequence alignments. Methods Enzymol. **266:** 383–402.

Hopper, A. K., and N. C. Martin, 1992 Processing of yeast cytoplasmic and mitochondrial precursors to yeast cytoplasmic transfer RNAs, pp. 99–141 in *The Molecular Biology of the Yeast Saccharomyces: Gene Expression*, Vol. II, edited by E. W. Jones, J. R. Pringle and J. R. Broach. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hopper, A. K., A. H. Furukawa, H. D. Pham and N. C. Martin, 1982 Defects in modification of cytoplasmic and mitochondrial transfer RNAs are caused by single nuclear mutations. Cell **28:** 543–550.

Hopper, A. K., H. M. Traglia and R. W. Dunst, 1990 The yeast *RNA1* gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. J. Cell Biol. **111:** 309–321.

Hurt, E. C., 1988 A novel nucleoskeletal-like protein located at the nuclear periphery is required for the life cycle of *Saccharomyces cerevisiae.* EMBO J. **7:** 4323–4334.

Jans, D. A., and S. Hübner, 1996 Regulation of protein transport to the nucleus: central role of phosphorylation. Physiol. Rev. **76:** 651–685.

Kalderon, D., W. D. Richardson, A. F. Markham and A. E. Smith, 1984 Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature **311:** 33–38.

Kilmartin, J. V., and A. E. M. Adams, 1984 Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces.* J. Cell Biol. **98:** 922–933.

Kunkel, T. A., J. D. Roberts and R. A. Zakour, 1987 Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:** 367–382.

Lee, B., A. G. Matera, D. C. Ward and J. Craft, 1996 Association of RNase mitochondrial RNA processing enzyme with ribonuclease P in higher ordered structures in the nucleolus: a possible coordinate role in ribosome biogenesis. Proc. Natl. Acad. Sci. USA **93:** 11471–11476.

Li, J.-M., A. K. Hopper and N. C. Martin, 1989 N^2,N^2-dimethylguanosine-specific tRNA methyltransferase contains both nuclear and mitochondrial targeting signals in *Saccharomyces cerevisiae.* J. Cell Biol. **109:** 1411–1419.

Makkerh, J. P. S., C. Dingwall and R. A. Laskey, 1996 Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. Curr. Biol. **6:** 1025–1027.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Martin, N. C., and A. K. Hopper, 1994 How single genes provide

tRNA processing enzymes to mitochondria, nuclei and the cytosol. Biochimie **76:** 1161–1167.

Michael, W. M., M. Choi and G. Dreyfuss, 1995 A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell **83:** 415–422.

Michael, W. M., P. S. Eder and G. Dreyfuss, 1997 The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. **16:** 3587–3589.

Moreland, R. B., G. L. Langevin, R. H. Singer, R. L. Garcea and L. M. Hereford, 1987 Amino acid sequences that determine the nuclear localization of yeast Histone 2B. Mol. Cell. Biol. **7:** 4048–4057.

Murphy, R., and S. R. Wente, 1996 An RNA-export mediator with an essential nuclear export signal. Nature **383:** 357–360.

Najarian, D., M. E. Dihanich, N. C. Martin and A. K. Hopper, 1987 DNA sequence and transcript mapping of *MOD5*: features of the 5′ region which suggest two translation starts. Mol. Cell. Biol. **7:** 185–191.

Nishikura, K., and E. M. De Robertis, 1981 RNA processing in microinjected *Xenopus* oocytes. J. Mol. Biol. **145:** 405–420.

Peebles, C. L., P. Gegenheimer and J. Abelson, 1983 Precise excision of intervening sequences from precursor tRNAs by a membrane-associated yeast endonuclease. Cell. **32:** 525–536.

Pines, J., and T. Hunter, 1994 The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. EMBO J. **13:** 3772–3781.

Pringle, J. R., A. E. M. Adams, D. G. Drubin and B. K. Haarer, 1991 Immunofluorescence methods for yeast, pp. 589–594 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. Guthrie and G. R. Fink. Academic Press, San Diego.

Reynolds, A., V. Lundblad, D. Dorris and M. Keaveney, 1997 Yeast vectors and assays for expression of cloned genes, pp. 13.6.1–13.6.3 in *Current Protocols in Molecular Biology*, edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. John Wiley & Sons, New York.

Robbins, J., S. M. Dilworth, R. A. Laskey and C. Dingwall, 1991 Two interdependent basic domains in nucleoplasmin nuclear targeting sequences: identification of a class of bipartite nuclear targeting sequences. Cell **64:** 615–623.

Rose, A. M., H. Belford, W.-C. Shen, C. Greer, A. K. Hopper *et al.*, 1995 Location of N^2,N^2-dimethylguanosine-specific tRNA methyltransferase. Biochimie **77:** 45–53.

Sanger, F., S. Nicklen and A. R. Coulson, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

Shou, W., X. Li, C. Wu, T. Cao, J. Kuang *et al.*, 1996 Finely tuned regulation of cytoplasmic retention of *Xenopus* nuclear factor 7 by phosphorylation of individual threonine residues. Mol. Cell. Biol. **16:** 990–997.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics **122:** 19–27.

Stolc, V., and S. Altman, 1997 Rpp1, an essential protein subunit of nuclear RNase P required for processing of precursor tRNA and 35S precursor rRNA in *Saccharomyces cerevisiae.* Genes Dev. **11:** 2414–2425.

Tollervey, D., and T. Kiss, 1997 Function and synthesis of small nucleolar RNAs. Curr. Opin. Cell Biol. **9:** 337–342.

von Heijne, G., 1986 Mitochondrial targeting sequences may form amphiphilic helices. EMBO J. **5:** 1335–1342.

Wen, W., J. L. Meinkoth, R. Y. Tsien and S. S. Taylor, 1995 Identification of a signal for rapid export of proteins from the nucleus. Cell **82:** 463–473.

Wolfe, C. L., Y.-C. Lou, A. K. Hopper and N. C. Martin, 1994 Interplay of heterogeneous transcription start sites and translational selection of AUGs dictate the production of mitochondrial and cytosolic/nuclear tRNA nucleotidyltransferase from the same gene in yeast. J. Biol. Chem. **269:** 13361–13366.

Wolfe, C. L., A. K. Hopper and N. C. Martin, 1996 Mechanisms leading to and the consequences of altering the normal distribution of ATP(CTP):tRNA nucleotidyltransferase from the same gene in yeast. J. Biol. Chem. **271:** 4679–4686.

Zoladek, T., G. Vaduva, L. A. Hunter, M. Boguta, B. D. Go *et al.*, 1995 Mutations altering the mitochondrial-cytoplasmic distribution of Mod5p implicate the actin cytoskeleton and mRNA 3′ ends and/or protein synthesis in mitochondrial delivery. Mol. Cell. Biol. **15:** 6884–6894.

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