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Sequestration of mRNAs Modulates the Timing of Translation during Meiosis in Budding Yeast

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Starvation of diploid cells of the budding yeast *Saccharomyces cerevisiae* induces them to enter meiosis and differentiate into haploid spores. During meiosis, the precise timing of gene expression is controlled at the level of transcription, and also translation. If cells are returned to rich medium after they have committed to meiosis, the transcript levels of most meiotically upregulated genes decrease rapidly. However, for a subset of transcripts whose translation is delayed until the end of meiosis II, termed protected transcripts, the transcript levels remain stable even after nutrients are reintroduced. The Ime2-Rim4 regulatory circuit controls both the delayed translation and the stability of protected transcripts. These protected mRNAs localize in discrete foci, which are not seen for transcripts of genes with different translational timing and are regulated by Ime2. These results suggest that Ime2 and Rim4 broadly regulate translational delay but that additional factors, such as mRNA localization, modulate this delay to tune the timing of gene expression to developmental transitions during sporulation.

Formation of haploid gametes from diploid cells through the specialized cell division of meiosis is central to the life cycle of sexually reproducing organisms. Gametogenesis involves exit from the mitotic cell cycle, progression through the meiotic divisions, and differentiation into specialized gametes that can later undergo fertilization to restore diploidy. In *Saccharomyces cerevisiae* gametogenesis, haploid genomes are packaged into gametes called spores, and the process is referred to as sporulation. Shared characteristics of sporulation and gametogenesis in metazoans include the dynamics of chromosome behavior in the meiotic prophase, postmeiotic hypercondensation of chromatin, and generation of specialized gametes (1–3).

Sporulation is triggered by nitrogen starvation in the presence of a poor carbon source (1). These starvation signals lead to the transcription of *IME1*, which encodes a transcription factor that controls entry into meiosis (4). Ime1 induces expression of a set of genes that are required for premeiotic DNA synthesis, as well as the initial steps of meiosis, particularly those involved in recombination during the meiotic prophase (5, 6). A key target of Ime1 is the gene encoding the Ime2 protein kinase (4, 7). The combined action of Ime1 and Ime2 leads to the induction of a second transcription factor, encoded by *NDT80* (8). Ndt80 upregulates its own expression, as well as that of ~300 additional genes termed the *NDT80* regulon (8, 9). This regulon includes genes required for entry into the meiotic divisions, and thus, deletion of *NDT80* results in the arrest of cells in the meiotic prophase (8, 10). *NDT80* also governs the induction of genes whose products are required for late meiosis events, such as the packaging of daughter nuclei into spores, and postmeiotic events, such as spore wall development (9).

After induction of the *NDT80* regulon, there are two other temporally regulated sets of transcriptionally induced genes, termed the mid-late genes and late genes (11). However, after meiotic prophase, the fine control of the timing of gene expression appears to be performed predominantly at the level of translational regulation rather than transcription (12). To obtain the high degree of synchrony necessary to distinguish differences in timing of translation during the meiotic divisions, an inducible *NDT80* system (*NDT80-IN*) was used for ribosome-profiling studies (13–15). *NDT80-IN* results from the combination of *NDT80* fused to the *GAL1* promoter and the presence of a *GAL4*-estradiol receptor gene (*ER*) fusion gene (13). Transcription of *NDT80* can therefore be controlled by addition of estradiol to the sporulation medium (SPM). Ribosome profiling has demonstrated that genes within the *NDT80* regulon may be coordinately transcribed, but the translational efficiency of these messages is differentially regulated so that protein production is coordinated with development (12).

A regulatory pathway has been defined that controls a set of transcripts, including *CLB3* and *SPO20*, that are delayed in translation until the onset of meiosis II (15). This pathway involves the Ime2 kinase and the RNA binding protein Rim4 (16). Binding of Rim4 to the 5′ untranslated region (UTR) of the *CLB3* transcript represses *CLB3* translation (15). Ime2 activity increases as cells progress through the meiotic divisions (13). Phosphorylation of Rim4 by Ime2 destabilizes Rim4, allowing the translation of Clb3 and other messages translated at the onset of meiosis II, when Ime2 kinase becomes active (15). Many additional Ndt80-regulated transcripts, with translational timing distinct from that of *CLB3*, bind Rim4 (15). How the translation of these other transcripts is controlled and whether the Ime2/Rim4 regulatory system contributes to their regulation have not been described.

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At some point during meiosis, cells become committed to the process of sporulation. Commitment was discovered by the use of "return-to-growth" experiments that involve inducing cells to undergo meiosis and then transferring them at different times into rich medium. Committed cells are defined as those that complete the process of sporulation even when the inducing signal (starvation) is removed (17). While the precise moment of commitment has not been defined, it occurs after induction of *NDT80*, as cells up to that point return to growth when transferred to rich medium (18). Surprisingly, comparison of transcript levels between committed cells before and after transfer to rich medium shows that committed cells returned to rich medium in fact display an extensive response to the change in environment (19). In particular, transcript levels for ribosomal genes and other genes associated with cell cycle entry increase, while transcript levels for genes in the *NDT80* regulon (as well as *NDT80* itself) decrease significantly within 40 min. Despite these changes in transcript abundance, the cells complete sporulation.

Within the *NDT80* regulon, a subset of genes was seen whose transcript levels remained high after transfer to rich medium (19). These genes were referred to as "insulated," since they were refractory to the change in environmental cues (19). Because the term "insulation" has been used in other contexts to describe a different regulatory phenomenon in both yeast and higher cells (20, 21), we refer to these genes instead as "protected" genes. This work shows that protection of genes from nutrient signals correlates with a delay in their translation until the end of meiosis II. Thus, the return-to-growth assay reveals an aspect of the regulation of these transcripts that may account for their extended translational delay. Our results indicate that the Ime2/Rim4 pathway may provide a general mechanism to delay translation during meiosis and that sequestration of specific messages can further tune the timing of the start of translation.

MATERIALS AND METHODS

Yeast media, strains, and plasmids. Unless otherwise noted, standard media and growth conditions were used (22). All the yeast strains used are listed in Table 1. Diploid strains carrying P_{SPS4}-5′ UTR$_{SPO20}$-*SPO20*, P_{SPS4}-5′ UTR$_{SPS4}$-*SPO20*, or P_{SPO20}-5′ UTR$_{SPS4}$-*SPO20* at the *SPO20* locus; *SPS4-3×HA*, *SPS4-GFP*, P_{SPO20}-5′ UTR$_{SPS4}$-*SPS4*, or P_{SPO20}-5′ UTR$_{SPO20}$-*SPS4* at the *SPS4* locus; or *IME2ΔC241* were constructed by PCR-based integration in the haploid parents A14154 and A14155 and subsequent mating of the transformants (14, 23). To introduce the *SPO20* or *SPS4* upstream region, the plasmids pFA6a-HIS3MX6-P_{SPS4}, pFA6a-HIS3MX6-P_{SPO20}, and pFA6a-KanMX6-P_{SPO20} were constructed for use as PCR templates by replacing the *GAL* promoter in the pFA6a-HIS3MX6-PGAL1 and pFA6a-KanMX6-PGAL1 plasmids (24) with 1 kb of sequence upstream of the translational start site from *SPO20* and *SPS4*, respectively. The positions of the transcriptional start sites used for *SPO20* and *SPS4* were based on an earlier study defining the 5′ ends of transcripts in sporulating cells (25). Strains carrying bacteriophage MS2 loop-tagged mRNAs were generated using CRISPR/Cas reagents provided by B. Futcher. Detailed descriptions of their construction will be presented elsewhere (G. Zhao and B. Futcher, unpublished data). We first generated plasmid pRS425-Cas9-*Sk*HIS3-381. This plasmid expresses both the *Streptococcus pyogenes cas9* gene under the control of the yeast *TEF2* promoter and a noncoding guide RNA with a sequence that targets Cas9 for cleavage of the *Saccharomyces kluyveri HIS3* sequence. MS2 loop sequences were amplified from the plasmid pLOXHIS5MS2L (26) with oligonucleotides that contained 5′ sequences homologous to the fusion junctions created by insertion of the green fluorescent protein (GFP) gene and *S. kluyveri HIS3* in construction of the GFP-tagged strain collection (27). When pRS425-Cas9-*Sk*HIS3-381 and the MS2 loop PCR product are cotransformed into strains from the GFP collection, Cas9 generates double-stranded breaks within the *S. kluyveri HIS3* gene adjacent to the GFP sequence, and these breaks can be repaired by recombination integrating the MS2 PCR fragment. The resulting strains have lost the coding regions of both GFP and *S. kluyveri HIS3*, and in their place at the 3′ end of the open reading frame (ORF) are (in order) 30 nucleotides (nt) encoding the first 10 residues of GFP, a stop codon, the MS2 loops, 48 nucleotides from the original GFP tagging vector, and, finally, the genomic 3′ UTR. Details of both the Cas9 plasmid construction and this technique will be described elsewhere (Zhao et al., unpublished). The final integrations in all strains were verified by PCR and sequencing. These MS2 loop-tagged strains in the BY4741 background were then mated with the SK-1 background strain AN117-4B carrying plasmids expressing a GFP-tagged MS2 coat protein (MS2CP) (pMS2-CP-GFP [26]) and a red fluorescent protein (RFP) prospore membrane marker (pRS426-SPO20-mCherry [28]) and sporulated to observe mRNA localization. To make yLJ92, the *ime2-as* (analog-sensitive) allele was first PCR amplified from KBY516 (13), using an oligonucleotide 500 bp upstream of the translational start site and a downstream oligonucleotide that introduced a stop codon, followed by 30 nt of the wild-type *IME2* 3′ UTR immediately after codon 241. The PCR product was then cotransformed with the plasmid pRS425-Cas9-skHIS3-381 into the *IME2ΔC241*::*HIS3MX6* strains. After screening for transformants that had lost the *HIS3* marker, correct integrations in all the strains mentioned above were verified by PCR and sequencing. To make yLJ159, PCR-mediated integration was first used to replace codon 349 of *RIM4* with a kanamycin resistance cassette in both yLJ97 and AN117-4B. The *rim4 F349L* allele was then amplified by PCR from strain A31421, and this product was cotransformed with the plasmid pRS425-Cas9-Kan-280 into the strains with the kanamycin insertions in *RIM4*. Correct integrations were identified by loss of G418 resistance and then verified by PCR and sequencing. Finally, the two resulting haploids were mated.

Return-to-growth conditions. For return-to-growth experiments with *GAL-NDT80* strains, cells were grown in yeast extract-peptone-dextrose (YPD) overnight at 30°C and then transferred to yeast extract-peptone-acetate (YPA) at an optical density at 660 nm (OD_{660}) of 0.3. After incubation at 30°C for 16 to 18 h, the cells were washed and resuspended in SPM at a final OD_{660} of 1.6. The cells were incubated at 30°C for 6 h to allow the population to accumulate in meiotic prophase, and then 1 mM β-estradiol was added to the cell culture to induce meiotic entry. At each time point, the cells were washed once with distilled water (dH_2O), resuspended in a 2× volume of YPD, and incubated at 30°C. For strains carrying *ime2-as1*, the inhibitor 3-methylbenzyl-PP1 (EMD Millipore) was add to the YPD at a final concentration of 50 μM at the time of transfer from SPM. The experiments examining commitment were performed three times (for a representative example, see Fig. 6).

mRNA localization. To observe the localization of MS2 loop-tagged mRNAs, diploids heterozygous for the tagged mRNAs and carrying the MS2CP-GFP and Spo20$^{51–91}$-mCherry plasmids were grown in YPD overnight at 30°C and then diluted in YPA to an OD_{660} of 0.2. After incubation at 30°C overnight, the cells were washed and resuspended in SPM at an OD_{660} of 1.2 and incubated at 30°C. After 6 h of incubation, samples were placed on microscope slides, and images were collected on a Zeiss Axioplan2 microscope with a Zeiss mRM digital camera. The images were processed using Axiovision 4.0 software.

Western blot assays. For Western blots, the *NDT80-IN* strains were sporulated as described above. At intervals, 5 ml of sporulating culture was collected, pelleted, resuspended in 5 ml of 5% trichloroacetic acid, and incubated at 4°C for 10 min. The cells were then pelleted and washed with 1 ml acetone, and the pellet was allowed to air dry for 2.5 h. The pellets were then resuspended in 100 μl freshly made lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 27.5 mM dithiothreitol, 11 mM phenylmethylsulfonyl fluoride, 2-fold-concentrated EDTA-free cOmplete protease inhibitor cocktail tablets [Roche]). The cells were broken by addition of 50

TABLE 1 Strains used in this study

Strain name	Genotype	Reference
AN120	*MAT***a**/*MAT*α *ura3/ura3 his3ΔSK/his3ΔSK trp1::hisG/trp1::hisG arg4-NSP1/ARG1 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1Δ::LEU2/RME1 leu2/leu2*	36
AN117-4B	*MAT*α *ura3 his3 trp1::hisG leu2 arg4-NSP1 lys2 hoΔ::LYS2 rme1Δ::LEU2*	36
A14201	*MAT***a**/*MAT*α *hoΔ::LYS2/hoΔ::LYS2 lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG GAL-NDT80::TRP1/GAL-NDT80::TRP1 ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3*	14
A31421	*MAT***a**/*MAT*α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG GAL-NDT80::TRP1/GAL-NDT80::TRP1 ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 CLB3-3HA::Kan/CLB3-3HA::Kan rim4F349L-3V5/rim4F349L-3V5*	15
yLJ28	As A14201 plus *HIS3MX6-*P_{SPS4}*-5′ UTR*$_{SPS4}$*-SPO20/HIS3MX6-*P_{SPS4}*-5′ UTR*$_{SPS4}$*-SPO20*	This study
yLJ29	As A14201 plus *HIS3MX6-*P_{SPS4}*-5′ UTR*$_{SPO20}$*-SPO20/HIS3MX6-*P_{SPS4}*-5′ UTR*$_{SPO20}$*-SPO20*	This study
yLJ40	As A14201 plus *HIS3MX6-*P_{SPO20}*-5′ UTR*$_{SPS4}$*-SPS4/HIS3MX6-*P_{SPO20}*-5′ UTR*$_{SPS4}$*-SPS4*	This study
yLJ41	As A14201 plus *HIS3MX6-*P_{SPO20}*-5′ UTR*$_{SPO20}$*-SPS4/HIS3MX6-*P_{SPO20}*-5′ UTR*$_{SPO20}$*-SPS4*	This study
yLJ44	As A14201 plus *KanMX6-*P_{SPO20}*-5′ UTR*$_{SPS4}$*-SPO20/KanMX6-*P_{SPO20}*-5′ UTR*$_{SPS4}$*-SPO20*	This study
yLJ50	As A14201 plus *IME2ΔC241::HIS3MX6/IME2ΔC241::HIS3MX6*	This study
yLJ80	As A14201 plus *SPS4-3HA-HIS3MX6/SPS4-3HA-HIS3MX6*	This study
yLJ89	As A14201 plus *IME2ΔC241::HIS3MX6/IM2ΔC241::HIS3MX6 SPS4-3HA-KanMX/SPS4-3HA-KanMX*	This study
yLJ92	As A14201 plus *IME2ΔC241(M146G)-as/IME2ΔC241(M146G)-as*	This study
yJL97	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SPS4-12×MS2L*	This study
yLJ99	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPS4-12×MS2L/SPS4*	This study
yLJ111	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPR6-12×MS2L/SPR6*	This study
yLJ112	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 LDS2-12×MS2L/LDS2*	This study
yLJ113	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPS1-12×MS2L/SPS1*	This study
yLJ119	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SGA1-12×MS2L/SGA1*	This study
yLJ120	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 CTS2-12×MS2L/CTS2*	This study
yLJ121	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPO20-12×MS2L/SPO20*	This study
yLJ122	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPR28-12×MS2L/SPR28*	This study
yLJ123	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPS4-12×MS2L/SPS4 IME2ΔC241::KanMX6/IME2ΔC241::KanMX6*	This study
yLJ137	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPO77-12×MS2L/SPO77*	This study
yLJ139	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SSP2-12×MS2L/SSP2*	This study
yLJ141	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 CDA1-12×MS2L/CDA1*	This study
yLJ159	*MAT***a**/*MAT*α *his3Δ1/his3 leu2Δ0/leu2 met15Δ0/MET15 ura3Δ0/ura3 trp1::hisG/TRP1 arg4-NSP1/ARG4 lys2/LYS2 ho::LYS2/ho rme1::LEU2/RME1 SPS4-12×MS2L/SPS4 rim4-F349L/rim4-F349L*	This study
yLJ167	As A31421 plus *SPS4-GFP-HIS3MX6/SPS4-GFP-HIS3MX6*	This study

μl of zirconia beads, followed by two pulses at 6 m/s for 40 s in a FastPrep-24 high-speed benchtop homogenizer (MP Biomedicals). Then, 50 μl 3× SDS sample buffer was added, and each lysate was boiled for 5 min before loading on an SDS polyacrylamide gel. Sps4-GFP was detected using monoclonal anti-GFP antibodies (ClonTech) at 1:1,000 dilution. As a loading control, porin was detected by antiporin antibodies (Molecular Probes) at 1:1,000 dilution or Arp7 was detected using polyclonal anti-Arp7 antibodies (Santa Cruz Biotechnology) at 1:5,000 dilution. Quantitation was performed using an ImageQuant 4000 (GE Healthcare).

Microarrays. Total RNA from cells pelleted at each time point was extracted and purified using a RiboPure yeast kit (Ambion). Cy3- and Cy5-labeled cRNAs were produced using an Agilent QuickAmp labeling kit (Agilent) and purified using an RNeasy minikit (Qiagen). Probes were hybridized to an Agilent yeast gene expression 8,000 by 15,000 microarray (Agilent) using an Agilent gene expression hybridization kit and hybridization oven. After hybridization, the arrays were scanned using an Agilent DNA microarray scanner, and the fluorescence was analyzed and normalized using Agilent feature extraction software. Basic analysis was performed using the LIMMA package in R. Clustering was performed using Cluster 3.0, and clustering data were visualized in Java Treeview.

qPCR. Five milliliters of cells was collected at each time point. Total RNA was extracted and purified with a RiboPure yeast kit (Ambion), and then cDNAs were synthesized using the Transcriptor first-strand cDNA synthesis kit (Roche) and treated with RNase A (0.5 μg/μl) for 30 min at 37°C. After synthesis, the cDNA was purified using a PCR purification kit

(Qiagen), and the cDNA concentration was then measured and adjusted to 1 ng/μl. The mRNA level of each gene was determined by quantitative PCR (qPCR) using a Mastercycler EP Realplex (Eppendorf) and LightCycler 480 DNA Sybr green I PCR master mix (Roche). All time points were assayed in triplicate in each experiment, and all experiments were performed at least twice.

RESULTS

Protected transcripts exhibit delayed translation. Friedlander et al. (19) defined a set of 24 protected transcripts within the *NDT80* regulon whose levels were not reduced when cells committed to sporulation were transferred into rich medium. Ribosome profiling throughout a sporulation time course demonstrated that, while the *NDT80* regulon is transcriptionally induced at the same time, the timing of peak translation varies between messages (12). There is a sizable overlap between the protected gene set defined by return-to-growth experiments and a cluster of *NDT80*-induced genes that are translated very late in sporulation (with peak translation around 3 h after *NDT80* induction) (12, 19). Comparisons between these two studies may not provide a complete picture, however, because of differences in synchrony between the strains. The return-to-growth study identifying protected transcripts used a wild-type yeast strain and lacked the degree of synchrony achieved by the ribosome-profiling experiments using the *NDT80-IN* system.

To allow direct comparison between the ribosome-profiling data and protected gene expression, a return-to-growth experiment was performed in the *NDT80-IN* background and transcript levels were analyzed using microarrays. Cells were arrested in meiotic prophase by incubation in sporulation medium for 6 h, and then *NDT80* expression was induced by addition of estradiol. mRNA was isolated from cells taken immediately prior to estradiol addition, labeled with Cy3, and used as the normalizing control. At 1-h intervals after addition of estradiol, cells were taken directly from the sporulation medium or transferred to rich medium for 1 h. mRNA was then isolated from these cells, labeled with Cy5, mixed with the Cy3-labeled control mRNAs, and hybridized to microarrays. This experiment revealed a larger number of genes than in the previous study whose transcripts appeared protected upon return to growth (Fig. 1A) (19). Nonetheless, when the set of protected transcripts seen in this experiment is compared with the protected transcripts from Friedlander et al. and with the very late-translated genes from the ribosome-profiling data of Brar et al. (12), there is extensive overlap (Fig. 1A).

In our microarray data, the mRNA levels for most of the messages, such as *SPO20*, dropped 1 h after return to growth, but for protected transcripts, such as *SPS4*, message levels remained high (Fig. 1B). Clustering of the microarray data from the *NDT80-IN* return-to-growth experiment with the ribosome-profiling data from Brar et al. (12) revealed that the majority of protected transcripts fall primarily into one of two clusters (Fig. 1C). Transcripts in cluster 1 show a broad peak of ribosomal association centered 1.5 to 2 h after the addition of estradiol, corresponding to meiosis I (12). The second, larger cluster contains 19 genes (Fig. 1D) whose translation is delayed until more than 2.5 h after the induction of *NDT80*, a time point that corresponds to the end of the meiosis II division. Importantly, when the extent of protection of the transcripts in each cluster was assessed by measuring the ratio of the transcript levels before and after return to growth, only the transcripts in cluster 2 showed significant protection (Fig. 1E). Further, qPCR analysis of three genes in cluster 1 (*CDA2*, *SMK1*, and *SMA2*) demonstrated that their transcript levels dropped after return to growth (L. Jin, unpublished observation). Thus, cluster 1 transcripts, though they appear red in the heat map, are reduced after the introduction of nutrients, and cluster 2 represents the strongly protected transcripts. Cluster 2 is enriched (8/19) for genes involved in spore wall assembly, consistent with the idea that these protected transcripts encode proteins that function in postmeiotic stages of spore development. The strong correlation between protection and prolonged translational delay suggests they may be related phenomena.

***IME2* and *RIM4* regulate the translational delay of *SPS4*.** Transcripts exhibiting translational delays can be divided broadly between those that are delayed until early meiosis II and those that are delayed until the end of meiosis II. Only transcripts in the latter class show protection. A question, then, is whether the mechanisms of translational delay are the same in the two groups. Previous work has demonstrated that several nonprotected *NDT80*-regulated genes, including *SPO20* and the cyclin gene *CLB3*, are delayed in translation until the onset of meiosis II and that this delay requires the RNA binding protein Rim4, the 5′ UTR of the transcript, and the absence of Ime2 kinase activity (15). A carboxy-terminal truncation of Ime2 (*IME2ΔC241*) creates a stable, hyperactive form of the kinase that leads to early translation of *CLB3*, *SPO20*, and several other transcripts with the same delay (15, 29). Ime2 promotes translation by antagonizing the RNA binding protein Rim4 (15). A point mutant in one of the Rim4 RNA binding domains (*rim4-F349L*) reduces RNA binding and leads to early translation of the *CLB3* message, similar to premature activation of Ime2 (15, 25). *rim4-F349L* alters one of two RNA binding domains within Rim4, and in contrast to the *rim4* deletion, which blocks gametogenesis prior to meiosis, a fraction of *rim4-F349L* cells in the *NDT80-IN* background are able to escape this early block and progress through meiosis (16). To test whether the same pathway also regulates the translational delay of protected transcripts, the timing of *SPS4* translation was compared in wild-type, *IME2ΔC241*, and *rim4-F349L* cells. An in-frame fusion of sequences encoding three tandem copies of the hemagglutinin (HA) epitope was created at the 3′ end of *SPS4* in both the wild-type and *IME2ΔC241* strains in the *NDT80-IN* background. To monitor Sps4 protein levels in the *rim4-F349L* mutant strain, an *SPS4*::*GFP* fusion was used. The three cultures were transferred to sporulation medium for 6 h, and then samples were removed at 1 h and then every 15 min after the addition of estradiol. In the wild-type strain, Sps4-HA did not appear until 3 h after estradiol addition, consistent with the ribosome-profiling data (12) (Fig. 2A). In contrast, constitutive activation of Ime2 or mutation of *RIM4* results in detectable Sps4 protein 1 h after *NDT80* induction and accumulation of the protein more than an hour earlier than in the wild type (Fig. 2A and B). This is due to premature translation and not to an effect on meiotic progression or the transcriptional induction of *SPS4*, as these are unaffected by the *IME2ΔC241* allele (Fig. 2C and data not shown). For the *rim4-F349L* strain, only about 20% of the cells progress into meiosis, probably due to defects in the premeiotic role of *RIM4* (16). Nonetheless, in the fraction of cells that progress, meiosis I and meiosis II occur with kinetics similar to those of the wild type (Fig. 2C). These results indicate that at least one mechanism for translational control is shared by both nonprotected and protected transcripts.

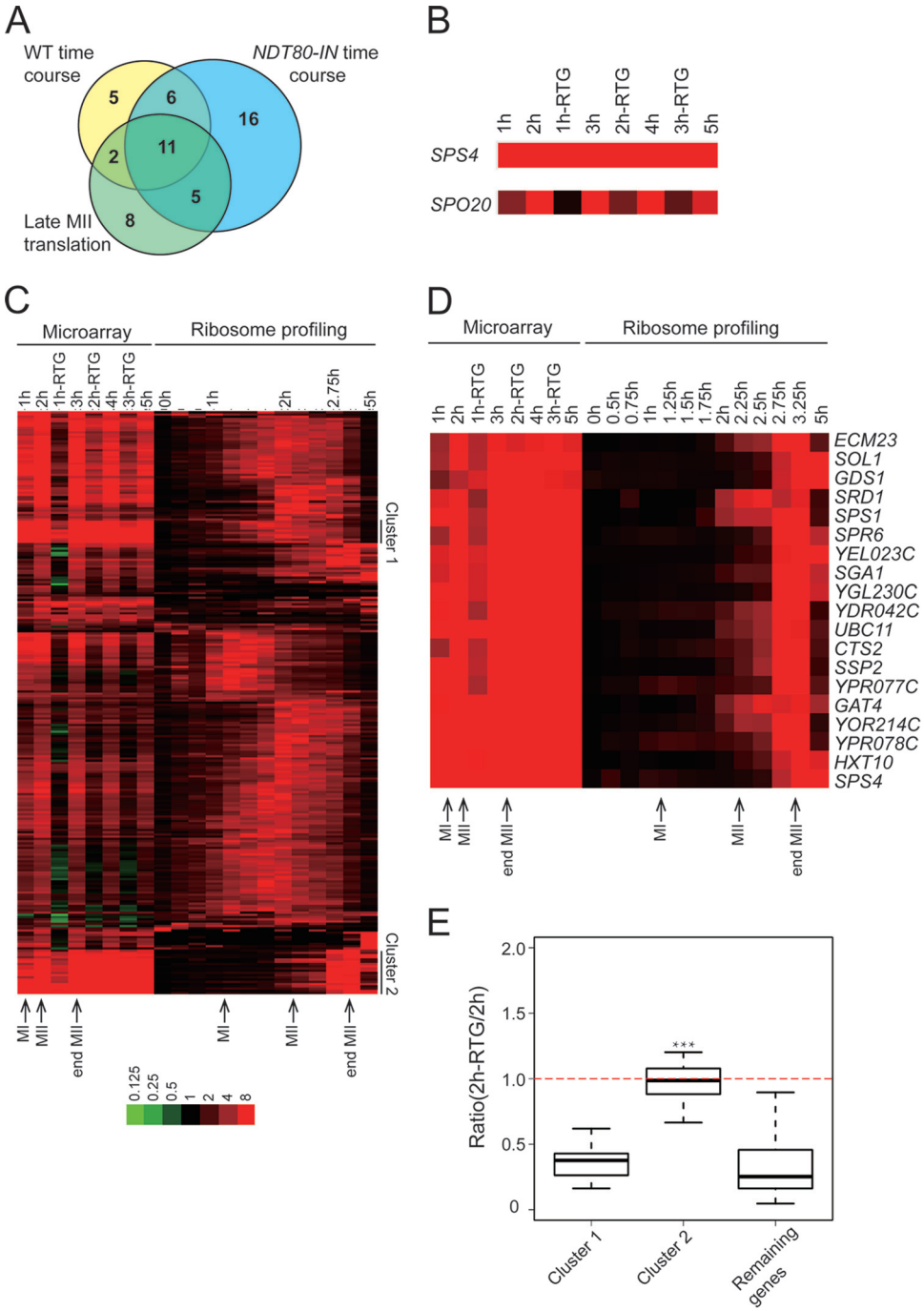

FIG 1 A set of protected transcripts is translated at the end of meiosis II (MII). (A) Venn diagram showing the overlap between the set of protected transcripts identified in return-to-growth experiments using either *NDT80-IN* (A14210) or *NDT80* (WT [wild-type] time course) (19) and the cluster of genes showing peak translation in late MII (Late MII translation) (12). The numbers indicate the number of genes in each subset. (B) Behaviors of a protected gene, *SPS4*, and a nonprotected gene, *SPO20*, in the microarray experiment. At the 1, 2, and 3-hour time points, aliquots were transferred to rich medium for 1 h prior to mRNA extraction. For example, "2 h-RTG" indicates cells that were transferred to YPD 2 h after addition of β-estradiol. RTG, return to growth. (C) Coclustering of the *NDT80-IN* return-to-growth expression data with the ribosome-profiling data for the *NDT80* regulon from reference 12. Most of the protected transcripts fall into one of two translational clusters, indicated on the right. The color intensity scale ($\log_2$) is shown at the bottom. The times of meiosis I, meiosis II, and the end of meiosis II in the two time courses are indicated. The data used to generate the heat map are provided in Table S1 in the supplemental material. (D) Heat map of cluster 2 genes that are both protected and delayed in translation until the end of meiosis II. (E) Box plot of expression ratios (expression at 2 h RTG/expression at 2 h) for genes in cluster 1 and cluster 2 and the remaining genes in the microarray. The thick horizontal lines denote median values, the boxes represent the middle 50%, and the whiskers mark the 95% limits. The asterisks indicate that the distribution in cluster 2 is significantly different than those for both cluster 1 and the remaining genes (Student's *t* test; $P < 0.001$).

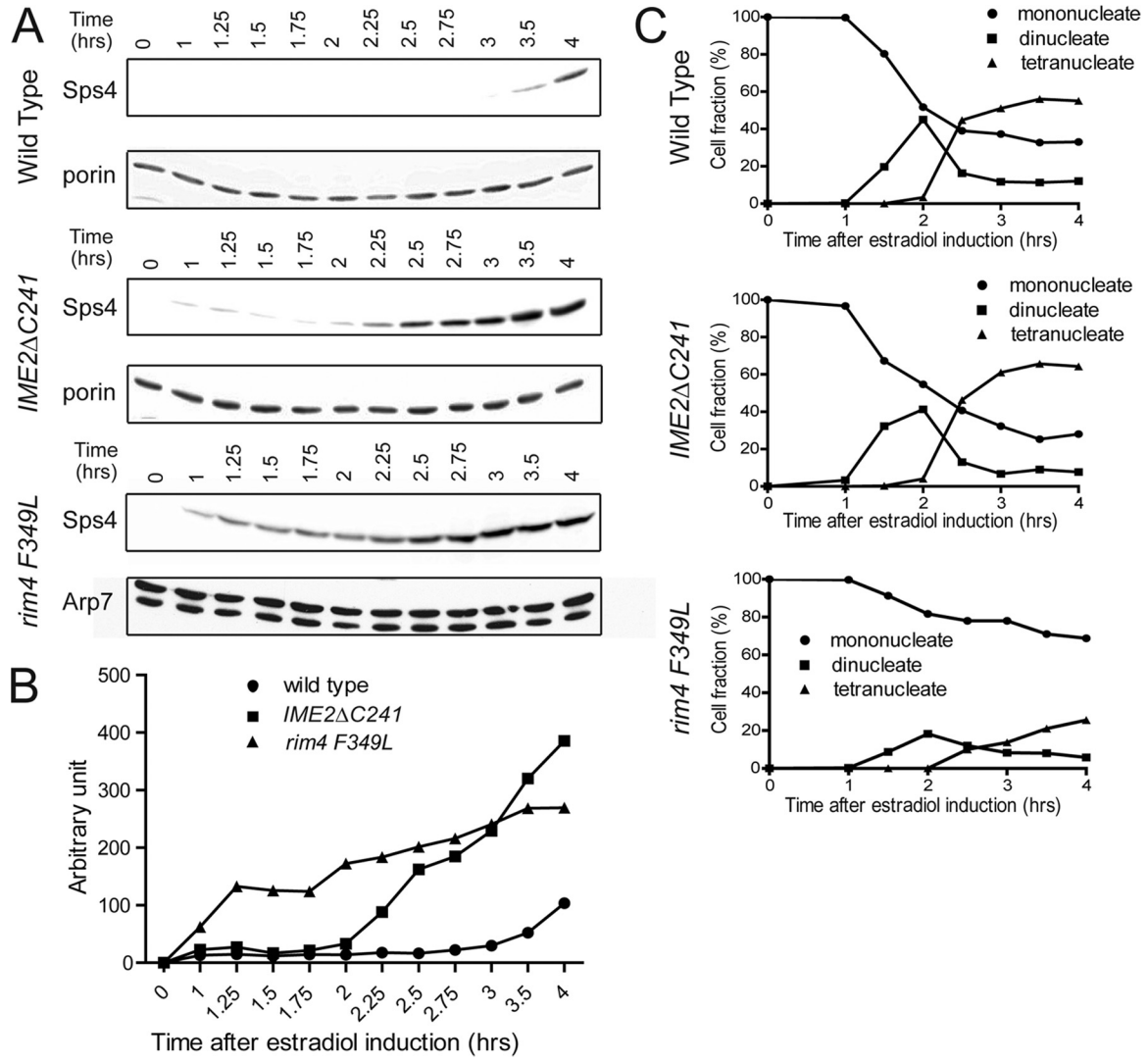

FIG 2 Timing of Sps4 translation in wild-type and *IME2ΔC241* cells. (A) The levels of Sps4-3HA (strains yLJ80 and yLJ89) or Sps4-GFP (strain yLJ167) were monitored by Western blotting. Cells were incubated in SPM for 6 h prior to the addition of β-estradiol (time zero), and aliquots were removed at the indicated times after induction. As a loading control, the same samples were probed with antibodies to the mitochondrial Por1 protein. (B) Quantitation of the levels of Sps4 in panel A. The values are normalized to the amount of Por1 protein in each lane for yLJ80 and yLJ89 and the amount of Arp7 protein for yLJ167. (C) DAPI (4′,6-diamidino-2-phenylindole) staining was performed to monitor the progression of cells through the meiotic divisions in the same time courses. The percentages of cells containing one nucleus (before MI), two nuclei (MI), or four nuclei (MII or later) are indicated.

***IME2* and *RIM4* regulate protection.** If translational delay is linked to protection, then premature activation of Ime2 should cause not only early translation but also loss of protection. This idea was tested by comparing mRNA protection in wild-type and *IME2ΔC241* diploids by qPCR using primers specific for the open reading frames of various genes. Two hours after *NDT80* induction, cells were transferred to rich medium for 1 h. Transcript levels were then compared before and after return to growth (Fig. 3). In wild-type cells, for the protected transcripts *SPS4* and *GAT4*, the transcript levels did not drop after return to growth, consistent with the microarray data (Fig. 3). In contrast, the *GAT4* and *SPS4* transcripts were no longer protected in the *IME2ΔC241* mutant, as transcript levels decreased after transfer to rich medium, similar to unprotected *SPO20* (Fig. 3). Further support for the idea that translational delay is a requirement for protection comes from the observation that protection of the *GAT4* and *SPS4* transcripts is lost in *rim4-F349L* diploids (Fig. 3). These results demonstrate that the Ime2/Rim4-mediated mechanism for translational delay is necessary for protection.

Protection of *SPS4* requires the 5′ UTR. For the *CLB3* gene, control of translational timing by the Ime2/Rim4 pathway is exerted through binding of Rim4 to the 5′ UTR of the *CLB3* mRNA. Moreover, fusion of 1,000 bp upstream of the translational start site of *SPS4* to the *NDT80* open reading frame is sufficient to protect *NDT80* transcripts, suggesting that protection is provided either by the promoter or by the 5′ UTR of the *SPS4* transcript (19). To map the *cis* elements involved in protection more precisely, a series of chimeras between the protected gene *SPS4* and the nonprotected gene *SPO20* were constructed in the *NDT80-IN* background and assayed for mRNA protection using qPCR.

For *SPO20*, mRNA levels decrease when cells are transferred to rich medium 2 h after *NDT80* induction compared to cells in

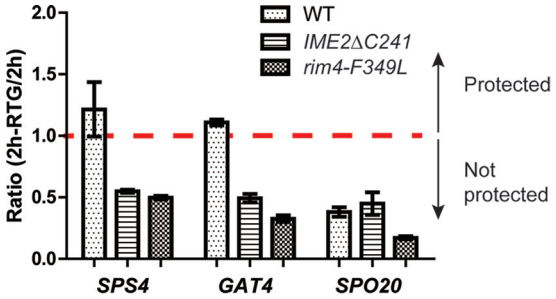

FIG 3 The effect of hyperactive Ime2 or mutation of *RIM4* on protection. The levels of two protected transcripts, *SPS4* and *GAT4*, and one nonprotected transcript, *SPO20*, were examined in a wild-type *NDT80-IN* strain (A14201) or in *IME2ΔC241* (yLJ50) or *rim4-F379L* (A31421) derivatives. Two hours after addition of estradiol, the cultures were split; half was harvested, and half was transferred to rich medium for 1 h. The expression levels at all time points were measured by qPCR and normalized to the expression at time zero for each gene. The graph displays the ratios of the expression levels before and after transfer. Ratios greater than or equal to 1 indicate protection. The error bars represent the ranges of values from two independent experiments.

SPM, whereas for *SPS4*, the levels do not drop after return to growth (Fig. 1B and 3). One set of chimeras replaced 1,000 bp upstream of the AUG translation start codon from *SPS4* with the corresponding sequence from *SPO20* and vice versa. In both cases, transcript behavior was correlated with the upstream region and not the ORF. That is, the *SPO20* transcripts generated using the *SPS4* upstream region are protected, whereas the *SPS4* transcripts fused to *SPO20* upstream are not (Fig. 4C, D, and H). This 1,000-bp region contains both the promoter and the 5′ UTR. Chimeras were therefore constructed which retained the 5′ UTR for each ORF (at bp −133 from the translational start site for *SPO20*; bp −64 for *SPS4*) (25). In this case, protection was correlated with the presence of the 5′ UTR. *SPO20* expressed from the *SPS4* promoter region but carrying its own 5′ UTR was not protected, while transcripts from an *SPS4* gene expressed from the *SPO20* promoter but retaining the *SPS4* 5′ UTR were protected (Fig. 4E, F, and H).

These results suggest that the regulatory element that confers protection is harbored within the *SPS4* 5′ UTR. To determine if the 5′ UTR is sufficient for protection, the 5′ UTR of *SPO20* was replaced with the 5′ UTR of *SPS4*. Indeed, transcripts derived from P_{SPO20}-5′ UTR_{SPS4}-*SPO20* were protected, similar to those from the endogenous *SPS4* gene (Fig. 4G and H). Thus, the 64 nucleotides of the *SPS4* 5′ UTR are necessary and sufficient for protection.

The *SPS4* transcript is localized in *IME2*-regulated foci. Though Ime2 and Rim4 regulate both translational delay and protection, many genes, such as *SPO20*, exhibit translational delay without protection, suggesting that an additional mechanism is required to establish protection. mRNAs whose translation is regulated temporally or spatially often exhibit specific localizations within the cell (30, 31). Therefore, one possible mechanism for protection from nutrient-induced turnover is sequestration of mRNAs from the degradation machinery. To investigate mRNA localization, transcripts were tagged by insertion of multiple copies of a hairpin sequence from the bacteriophage MS2 into their 3′ UTRs. These hairpin loops are specific binding sites for the MS2CP. Localization of transcripts can then be indirectly determined using the localization of an MS2CP-3×GFP fusion ex-

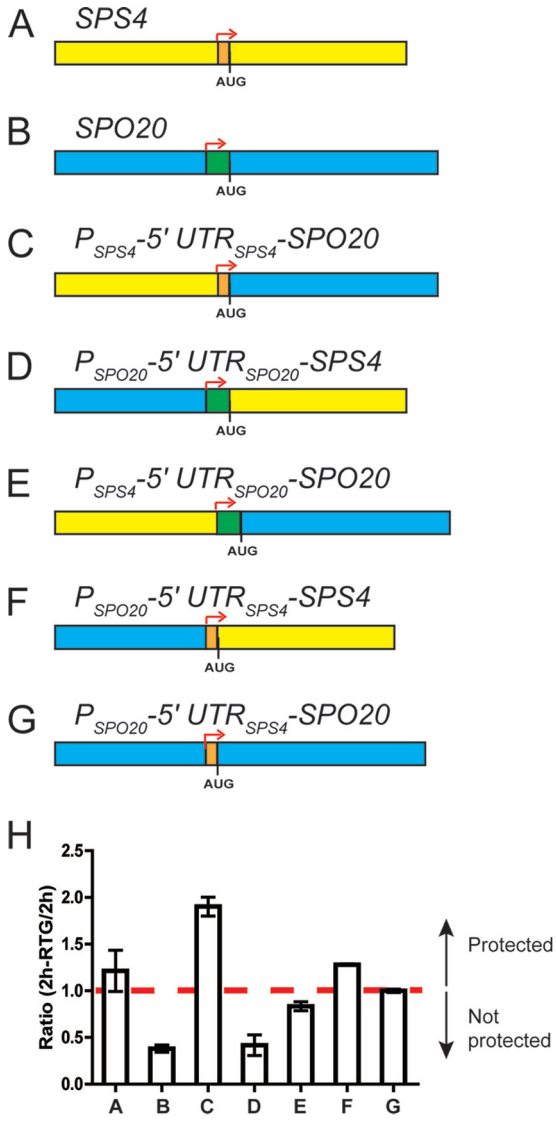

FIG 4 Mapping of the *cis*-acting determinants of protection. A series of *SPS4* (yellow, with the 5′ UTR indicated in orange)-*SPO20* (blue, with the 5′ UTR indicated in green) chimeric genes were constructed in the *NDT80-IN* background (A14201). Sequence swaps were made at the translational start site (AUG) or the reported transcriptional start sites (indicated by the red arrows). (A) *SPS4*. (B) *SPO20*. (C) *SPS4* upstream region fused to the *SPO20* coding region. (D) *SPO20* upstream region fused to the *SPS4* coding region. (E) *SPS4* promoter fused to the *SPO20* 5′ UTR and coding region. (F) *SPO20* promoter fused to the *SPS4* 5′ UTR and coding region. (G) *SPO20* promoter and coding region with the *SPS4* 5′ UTR. (H) Transcript levels before and after return to growth were assayed for each construct. The values for bars A and B are from Fig. 3. The dashed line indicates a ratio of 1. The error bars represent the range of values from the two experiments.

pressed in the same cells (26). Localization of six protected transcripts (*SPS4*, *SSP2*, *SPR6*, *CTS2*, *SGA1*, and *SPS1*) and five nonprotected transcripts (*LDS2*, *SPO20*, *CDA1*, *SPR28*, and *SPO77*) was monitored in strains containing a red fluorescent marker for the prospore membrane ($Spo20^{51–91}$-mCherry) (28). Prospore membranes form and grow during the second meiotic division, so the mCherry reporter allows the identification of cells in meiosis II (1). In mid-meiosis II, all the protected transcripts are present in discrete foci clustered near the open ends of the pros-

pore membranes (Fig. 5A and data not shown). In contrast, all the nonprotected transcripts show dispersed MS2CP-3×GFP fluorescence throughout the cytosol, similar to control cells with no tagged message (Fig. 5A and data not shown). Thus, there is perfect correlation between transcript protection and localization to foci within the cell.

Given that translation delay is required for protection, the question arises whether it is also necessary for transcript localization to foci. In fact, activation of Ime2 using *IME2ΔC241* abolished *SPS4* transcript foci, with the mRNA instead exhibiting some concentration near the prospore membrane with diffuse cytoplasmic localization similar to that of nonprotected transcripts (Fig. 5B). This result indicates that loss of translational delay and protection correlates with loss of focus formation. In contrast, when *SPS4* foci were examined in a *rim4-F349L* background, no changes in the transcript localization were seen (Fig. 5C), despite the fact that this mutation removes both protection and translational delay (Fig. 2 and 3). This result is considered further in Discussion below.

Loss of protection does not cause loss of meiotic commitment. The identification of mutants that lose protection allowed us to test whether the maintenance of certain transcripts is important for commitment to differentiation in the *NDT80-IN* background. At half-hour intervals after the addition of estradiol, cells were transferred to rich medium, and after 3 h in rich medium, the budding index of each culture was determined by light microscopy. As cells enter meiosis from the G_1 phase of the cell cycle, sporulating cells are unbudded, and committed cells, which finish the process of sporulation before reentering the cell cycle, do not produce buds within 3 h. However, uncommitted cells return more quickly to the mitotic cycle and can produce buds in this time window. In the wild-type strain, cells transferred to rich medium before the addition of estradiol, or within 1 h of estradiol addition, showed ~70% budding (Fig. 6A). Between 60 and 90 min after estradiol addition, this response dropped to below 20%. indicating that the bulk of the population had undergone commitment. This timing correlates with the appearance of binucleate cells in the culture. Thus, in the wild type, commitment occurs at the time the population passes through the first meiotic division. This timing of commitment is consistent with that found in a recent study examining commitment in single cells rather than populations (32).

Return-to-growth experiments cannot be performed using *IME2ΔC241* cells, because active Ime2 blocks bud emergence, and therefore, no budding is seen in cultures shifted back to rich medium even from premeiotic (and therefore precommitment) time points (33; L. Jin, unpublished observation). To assay commitment in the presence of constitutively active Ime2, an analog-sensitive (*as*) version of *IME2CΔ241* was used. This mutation creates a conditional form of Ime2 that can be inhibited by addition of the purine analog, 3-methylbenzyl-PP1 (13). Thus, the constitutive activity of Ime2ΔC241-as can be used to prevent protection, but its activity can be inhibited when cells are returned to growth by addition of 3-methylbenzyl-PP1 to the rich medium. Cells carrying *ime2ΔC241-as* were analyzed for commitment in the budding assay described above, except that 3-methylbenzyl-PP1 was added at the time of transfer to YPD to inactivate Ime2. Addition of the inhibitor at the same time as return to growth did not restore protection in these cells; thus, commitment can be monitored in the absence of protection (data not shown). Under this

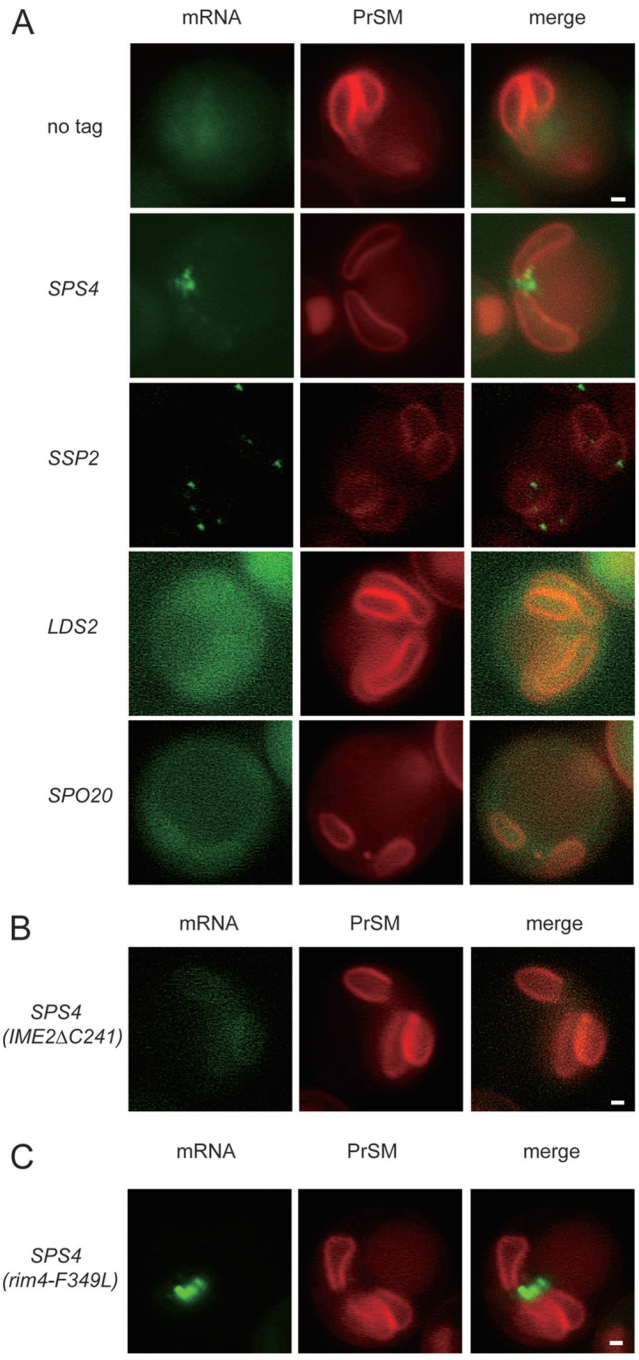

FIG 5 Localization of mRNAs during meiosis. (A) Untagged (AN120), *SPS4* tag (yLJ99), *SSP2* tag (yJL139), *LDS2* tag (yLJ112), or *SPO20* tag (yLJ121) diploids containing *MS2CP-GFP* and the prospore membrane marker $SPO20^{51-91}$*-mCherry* were sporulated at 30°C. Cells in mid- to late meiosis II were identified by the prospore membrane morphology and the specific mRNA localization revealed by GFP fluorescence. *SPS4* and *SSP2* are protected transcripts. *LDS2* and *SPO20* are nonprotected members of the *NDT80* regulon. (B) Effect of *IME2ΔC241* on *SPS4* mRNA foci. Strain yJL123, carrying the *SPS4*-MS2 loop transcriptional fusion and homozygous for *IME2ΔC241*, was sporulated and examined for localization of the *SPS4*-MS2 mRNA. (C) Effect of *rim4-F349L* on *SPS4* mRNA foci. Strain yJL159, carrying the *SPS4*-MS2 loop transcriptional fusion and homozygous for *rim4-F349L*, was sporulated and examined for localization of the *SPS4*-MS2 mRNA. For all strains, the image shown for each tagged mRNA is representative of the pattern seen in >95% of the meiosis II cells, with >20 cells scored in each strain. Scale bar, 1 μm.

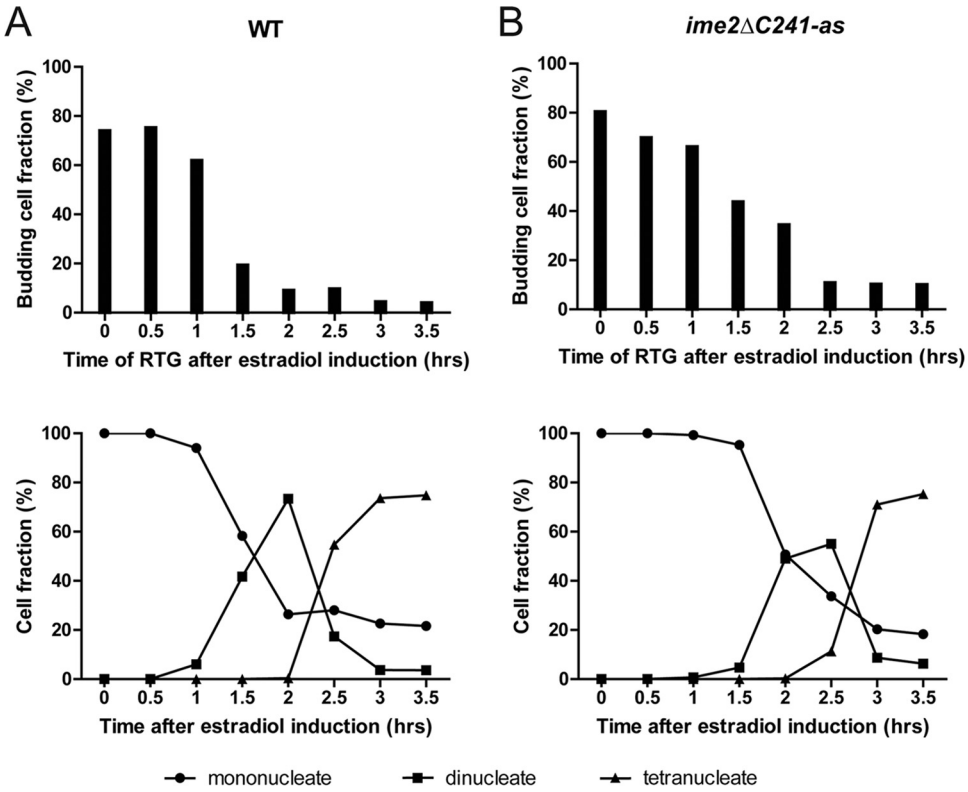

FIG 6 Commitment to meiosis in wild-type and *ime2*Δ*C241-as* cells. (A) (Top) At the indicated times after estradiol addition, commitment was analyzed by transfer of cells to rich medium and determination of the budding index using light microscopy. For *NDT80-IN* cells (A14201), the percentage of budding cells was determined after 3 h of incubation in rich medium. (Bottom) Progression of the population through the meiotic divisions in the same time course. The percentages of cells containing one nucleus (before MI), two nuclei (MI), or four nuclei (MII or later) were determined by DAPI staining. (B) Commitment monitored in *NDT80-IN ime2*Δ*C241-as* cells (yLJ92). The cells were treated as for panel A, except that 50 μM 3-MB-PP1 was added to the rich medium upon return to growth in order to inactivate Ime2-as.

protocol, the *ime2*Δ*C241-as* cells became committed to meiosis between 1 h and 2.5 h after addition of estradiol (Fig. 6B). This delay in commitment relative to the wild type mirrors a delay in the appearance of binucleate cells in the *ime2*Δ*C241-as* cells. Thus, commitment occurs in these cells and does so at the same time with respect to meiotic progression as in the wild type. These results indicate that protection and late translation of transcripts are not required for cells to commit to differentiation.

DISCUSSION

As yeast cells progress from the meiotic prophase into the meiotic divisions, control of translation becomes the critical process controlling the timing of gene expression (12). The results presented here demonstrate that the Ime2/Rim4 regulatory circuit controls the expression timing not only of mRNAs whose translation is delayed until the onset of meiosis II but also of mRNAs such as *SPS4*, whose translation is delayed until the end of the meiotic divisions.

Unlike most genes in the *NDT80* regulon, the levels of these very late-translated mRNAs are stable when meiotic cells are returned to rich medium. The earlier observation that creating a protected form of *NDT80* (by fusion to the *SPS4* upstream region) does not alter the disappearance of other mRNAs in the *NDT80* regulon when cells are transferred to rich medium (19) suggests that the mechanism of protection likely represents, not continued transcription, but rather resistance of specific messages to degradation. Thus, protected messages are sequestered away from the degradation machinery.

Earlier studies that provide the absolute level of mRNAs in sporulating cells suggest that the genes encoding protected transcripts, as a set, tend to be more highly expressed than those encoding nonprotected transcripts; however, there is significant overlap in expression levels between the two sets of genes (12, 25). In particular, *SPO20* is expressed at a higher level than *GAT4*. Therefore, the differing responses of these mRNAs to introduction of nutrients is not simply a matter of the transcript levels.

The Ime2/Rim4 circuit is required for protection. As protected mRNAs are enriched in Rim4 precipitates (15), these results suggest that binding of Rim4 and establishing a translational delay are prerequisites for protection. Binding of Rim4 is not sufficient for protection, however, as translationally delayed Rim4-bound mRNAs, such as *SPO20*, are not protected. A strong correlation was observed between protection and localization of transcripts into discrete intracellular foci, suggesting that localization into these foci may be important for protection. The only exception to this correlation is that the *rim4-F349L* allele loses protection and translation delay but does not alter the localization of the *SPS4* transcript into foci. The *rim4-F349L* allele is a partial loss-of-function allele (16). It may be that protection and translation are more sensitive assays of Rim4 activity than focus formation and that this accounts for the separation of these phenotypes in this strain.

Alternatively, it may be that Rim4 regulates the translation and stability of the transcripts only after they are released from foci, similar to *RIM4*-mediated translational delay of *SPO20*.

These results suggest a model in which the Ime2/Rim4 circuit is the primary regulator of translational delay during meiosis, but additional layers of regulation must be necessary to "tune" the timing of translational onset. In the case of the protected transcripts, we propose that sequestration of the transcripts into foci extends the delay in translation until the end of meiosis II. Rim4 might act in conjunction with additional factors to organize the protected transcripts into foci, and upon Ime2-stimulated destruction of Rim4, the retention of the transcripts in foci would maintain their translational repression. The model predicts that loss of these additional factors would lead to loss of protection and of foci but that these transcripts might still exhibit a translational delay similar to that of *SPO20*. There are a number of genes in the *NDT80* regulon that contain RNA binding motifs. These gene products are strong candidates to play a role in protection and modulation of translational timing.

Protection and differentiation. Loss of protection does not block the commitment of cells to differentiation. Thus, maintaining the levels of protected transcripts is not required to stop cells from returning to mitotic growth. Similarly, though expression of Ime2 can interfere with mitotic growth (33), our data suggest that the activity of the Ime2 kinase is not required to stop cells from reentering mitosis because commitment occurs even in the presence of inhibited Ime2-as. As many of the proteins encoded by protected transcripts are known to be important for spore wall assembly, it is possible that protection, though unnecessary for commitment, is important for successful completion of sporulation under return-to-growth conditions. In this case, loss of protection would lead to cells that complete meiosis but fail to form proper spores. Unfortunately, though meiotic progression is efficient, completion of spore formation is highly variable in the *NDT80-IN* background, making it difficult to establish whether loss of protection affects spore formation under return-to-growth conditions.

Translational regulation during gametogenesis. In midsporulation, the yeast cell switches from fine-grained control of gene expression based primarily on differential transcription to differential translation (12). One possible reason for this change is that the nuclear chromatin undergoes compaction, mediated by both modification of core histones and increased levels of histone H1, as cells progress through meiosis (3, 34). It may be that transcriptional regulation becomes more problematic in this more compacted chromatin so that translational control is preferred. By sequestering certain transcripts from the translational machinery, the cell may also ensure sufficient expression of those gene products to complete sporulation even if nutritional conditions change. Hypercompaction is also seen during spermatogenesis in many metazoans, where the chromatin is marked by histone modifications similar to those seen in sporulation in yeast, and at late stages, many of the histones are replaced by protamines (3, 35). It is unlikely that active transcription is occurring in these hypercompact nuclei. It will be interesting to learn if regulated translation is used to control proper timing of gene expression in the later stages of spermatogenesis, as well.

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