Coupling of *Saccharomyces cerevisiae* Early Meiotic Gene Expression to DNA Replication Depends Upon *RPD3* and *SIN3*

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ABSTRACT

It has been established that meiotic recombination and chromosome segregation are inhibited when meiotic DNA replication is blocked. Here we demonstrate that early meiotic gene (EMG) expression is also inhibited by a block in replication. Since early meiotic genes are required to promote meiotic recombination and DNA division, the low expression of these genes may contribute to the block in meiotic progression. We have identified three Hur$^-$ (*HU* reduced *r*ecombination) mutants that fail to couple meiotic recombination and gene expression with replication. One of these mutations is in *RPD3*, a gene required to maintain meiotic gene repression in mitotic cells. Complete deletions of *RPD3* and the repression adapter *SIN3* permitted recombination and early meiotic gene expression when replication was inhibited with hydroxyurea (HU). Biochemical analysis showed that the Rpd3p-Sin3p-Ume6p repression complex does exist in meiotic cells. These observations suggest that repression of early meiotic genes by *SIN3* and *RPD3* is critical for the normal response to inhibited replication. A second response to inhibited replication has also been discovered. HU-inhibited replication reduced the accumulation of phospho-Ume6p in meiotic cells. Phosphorylation of Ume6p normally promotes interaction with the meiotic activator Ime1p, thereby activating EMG expression. Thus, inhibited replication may also reduce the Ume6p-dependent activation of EMGs. Taken together, our data suggest that both active repression and reduced activation combine to inhibit EMG expression when replication is inhibited.

MEIOSIS and sporulation comprise a complex developmental pathway that the diploid yeast *Saccharomyces cerevisiae* undergoes when starved for nitrogen and a fermentable carbon source (reviewed in Mitchell 1994; Kupiec *et al.* 1997). The physical events that make up this pathway occur in an orderly fashion starting with DNA replication and recombination and ending with spore packaging and maturation. In some cases, downstream events are contingent upon completion of the prior event. For example, when replication is inhibited with hydroxyurea (HU), the downstream recombination and meiotic division events do not occur (Silvia-Lopez *et al.* 1975; Simchen *et al.* 1976). This dependency relationship is similar to the replication checkpoint that occurs in mitosis, and indeed both meiotic and mitotic cells depend upon *MEC1* to delay cell division when replication is compromised (Weinert *et al.* 1994; Navas *et al.* 1995; Sanchez *et al.* 1996). However, an important mitotic Mec1p target, Cdc5p (Sanchez *et al.* 1999), is not a likely meiotic target since a *cdc5* mutation does not inhibit meiotic replication or commitment to meiotic recombination (Simchen *et al.* 1981). Combined with the observation that the meiotic checkpoint inhibits recombination, this result suggests that the meiotic replication checkpoint does not function through *CDC5*. Since meiotic division is controlled by meiosis-specific regulatory genes, it is likely that the replication checkpoint may have unique meiotic targets.

Progression through the meiotic pathway is controlled by the activation of temporally distinct classes of meiosis-specific genes (Holaway *et al.* 1985; Kupiec *et al.* 1997; Chu *et al.* 1998). *IME1* encodes the master activator that is rapidly induced after a shift to sporulation conditions (Kassir *et al.* 1988). *IME1* promotes meiotic replication (Kassir *et al.* 1988) and induces the early meiotic genes (EMG; Mitchell *et al.* 1990). Some early meiotic genes promote recombination (Klapholz *et al.* 1985; Hollingsworth and Byers 1989; Menees and Roeder 1989) and induce the expression of middle meiotic genes that promote cell division. Finally, late genes that promote spore packaging and maturation are expressed. How the meiotic replication checkpoint leads to a block in meiosis is not known, but work on a *spo7* mutant (Esposito *et al.* 1975) correlated impaired replication with reduced expression of *SPR3*, a middle meiotic gene (Kao *et al.* 1989). Although the specific replication defect of the *spo7* mutant is not known, this result suggested that inhibited replication might control downstream events by controlling meiotic gene expression.

Several early meiotic genes are required to promote middle and late gene expression (Sia and Mitchell 1995; Chu and Herskowitz 1998; Hepworth *et al.*

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1998; Soushko and Mitchell 2000). Thus, early meiotic genes are possible targets for regulation by the meiotic DNA replication checkpoint. Surprisingly, early meiotic genes were properly activated in the meiotic replication-defective *clb5 clb6* double mutant (Dirick *et al.* 1998; Stuart and Wittenberg 1998). This result suggested that early meiotic gene expression is not a target for replication checkpoint regulation. However, it is unclear if a *clb5 clb6* mutant is capable of generating a blocked replication signal. An alternate way of generating the meiotic replication checkpoint is to inhibit replication with HU. Here, we show that the HU-activated replication checkpoint does inhibit early meiotic gene expression.

There are two regulatory mechanisms that govern early meiotic gene expression. Both mechanisms rely on Ume6p that binds to early meiotic promoters at the URS1 site (Bowdish and Mitchell 1993; Anderson *et al.* 1995; Rubin-Bejerano *et al.* 1996). In mitotic cells, Ume6p interacts with the Sin3p-Rpd3p deacetylase complex to repress early meiotic promoters (Strich *et al.* 1994; Steber and Esposito 1995; Kadosh and Struhl 1997). In meiotic cells, Ume6p becomes phosphorylated and interacts with Ime1p, leading to the activation of early meiotic promoters (Malathi *et al.* 1997; Xiao and Mitchell 2000). Thus, the replication checkpoint could inhibit early meiotic gene expression by active repression or by inhibited activation. Our genetic and biochemical studies suggest that both regulatory mechanisms are involved in the proper response to inhibited replication.

MATERIALS AND METHODS

Strains and media: *S. cerevisiae* strains (see Table 1) derived from the SK-1 genetic background were used for all Northern blots, protein analysis, and for some β-gal assays. The 1241 strain background was used for recombination testing and for some β-gal assays. *rpd3Δ::URA3* strains were constructed by transforming the *Xba*I cut plasmid pMV130 (Vidal and Gaber 1991). *sin3Δ::LEU2* strains were obtained by cross or by integration of a *Bam*HI-*Xho*I fragment of plasmid pCS117 (Wang *et al.* 1990). Proper formation of deletions was confirmed by Southern analysis or PCR and phenotypic analysis. A hemagglutinin (HA)-tagged version of *UME6* was integrated at the *ura3* locus by transforming the *Nco*I cut plasmid pYX 148 (Xiao and Mitchell 2000) into strain TLY446 to create strain TLY487.

SIN3-MYC was constructed as follows: A *SIN3* complete open reading frame fragment was obtained from the AMP109 genome in a PCR with the oligos Sin3-up1 (5′ CAGTCTTGTAAC TACTGTTG) and Sin3-dwn1 (5′ TACAATGTTATATCGTT GAC) and ligated into the plasmid pGEM-T (Promega, Madison, WI) to generate pTL13 plasmid. *Apa*I and *Not*I sites flanking the fragment were used to clone *SIN3* into pRS424 plasmid to generate pTL15 plasmid. The oligos Sin3-PET-up (5′ AATA TAGAAACGACTGGGAATACTGAATCTTCAGACAAGGG GGCTAAGATTCAAAGGGAACAAAAGCTGG) and Sin3 PET-dwn (5′ GAAGAAAGACCCTGTCGTACTAAAGATTTTTGTT CTAAATCTAGTTAAAACTACCTATAGGGCGAAT TGG) were used in a PCR on the plasmid pMPY-MYC (Schneider *et al.* 1995) to generate a fragment containing the following: 60 bp upstream of the *SIN3* stop codon-triple *MYC-URA3*-triple *MYC*-60 bp of *SIN3* downstream of stop codon. An *in vivo* recombinant between this PCR fragment and plasmid pTL15 cut with *Tth*111I (cuts just downstream of stop codon) was obtained, and loop out of the *URA3* gene selected for on 5′-fluoroorotic acid (5-FOA). The resulting plasmid, pTL18 containing *SIN3* with a C-terminal triple *MYC* tag, was recovered from yeast and shown to be functional. A *Pvu*II fragment from pTL18 containing the *SIN3-MYC* allele was cloned into *Pvu*II cut pRS306 to create the integrating pTL26 plasmid. pTL26 was cut inside the *SIN3* gene with *Eco*RI or *Sal*I and transformed into strain TLY2. Proper integration of the fragment at the *SIN3* locus was confirmed by PCR, and Ura− isolates were obtained on 5-FOA. Replacement of *SIN3* with *SIN3-MYC* was confirmed by PCR and Western analysis.

A similar strategy was used to generate *RPD3-HA*, which contains a triple HA tag at the C terminus of the protein.

Yeast and bacterial media, including Luria broth, yeast extract-peptone-dextrose (YPD), yeast extract-peptone-potassium acetate (YPAc), synthetic complete (SC), sporulation medium (SPO), and 5-FOA, were prepared as previously described (Kaiser *et al.* 1994). HU (Sigma, St. Louis) was added to SPO medium at 0.1 M or 0.04 M concentrations.

Plasmid/genome recombination assay: Strains of the 1241 background were used to monitor recombination between a genomic copy of *leu2-c* and a plasmid-borne *leu2-e* allele (pSR1 or pTL5). *leu2-c* and *leu2-e* are frameshift mutations produced by filling in and religating the *LEU2 Cla*I and *Eco*RI sites, respectively, and were provided by G. S. Roeder. Recombination between these two alleles can generate a wild-type *LEU2* gene. The production of Leu^+ descendants is stimulated by sporulation medium in *rme1*, but not *RME1* haploids, suggesting that this assay recapitulates key features of meiotic chromosome metabolism. Independent nonpetite pSR1 (*leu2e* allele in a *URA3* marked *CEN* plasmid) transformants were patched on SC-Ura and grown for 2 days. These were then replicated to SC-Ura, SPO, or SPO + 40 mM HU plates. After 2 days on SC-Ura or 4 days on SPO ± HU they were replicated to SC-Ura-Leu to assess recombination or SC-Ura to assess viability. To quantitate recombination, independent pTL5 (*leu2e* allele in a *TRP1* marked *CEN* plasmid) transformants were patched on SC-Trp and replicated to nylon filters on SC-Trp, SC-Trp + 40 mM HU, SPO, or SPO + 40 mM HU plates. At the times indicated above, three patches of each strain were resuspended in water, diluted to appropriate densities, and plated on SC-Trp and SC-Trp-Leu. Recombination frequencies were calculated as the number of Trp^+Leu^+ colonies divided by the number of Trp^+ colonies.

Mutagenesis, screen, and cloning: TLY 77 cells carrying pSR1 and pREY138, an *IME2-lacZ TRP1* plasmid (Sia and Mitchell 1995), were mutagenized with EMS to ~20% survival and plated on SC-Ura-Trp. Approximately 50,000 colonies were screened for their ability to produce Leu^+ papillae after incubation on SPO + 40 mM HU plates. Potential positives were retested and 43 isolates were found to be hyperrecombinogenic since they produced numerous Leu^+ papillae after mitotic growth; these were discarded. For the remaining 137 isolates, production of Leu^+ papillae depended on incubation in SPO medium and these were secondarily screened for their ability to promote *IME2* expression. Forty-two isolates gave moderate induction of *IME2-lacZ* in the presence of HU and were purified, retested, and crossed to a wild-type strain to determine whether a single gene segregated with the Rec^+ phenotype. Twenty-two isolates showed a clear 2:2 segregation pattern and were examined further. Cross-complementation suggested that these mutations fell into at least three groups, but it also revealed that a *hur-B51/hur-B51* diploid failed to sporulate. Complementation cloning revealed that *hur-B51* is an allele of *RPD3* and recovery of *hur-B51* from the genome

TABLE 1

Yeast strains

Strain	Genotype
SK1 derived[a]	
AMP 109	*MAT***a**/α *RME1/RME1*
AMP 614	*MAT*α *RME1 arg6 his3*Δ*SK*
AMP 734	*MAT***a** *IME2-5-lacZ::URA3 arg6*
TLY 2	*MAT***a** *arg6*
TLY 7	*MAT***a**/α *RME1/RME1 sin3*Δ*::LEU2/sin3*Δ*::LEU2 arg6/arg6 his3*Δ*SK/his3*Δ*SK*
TLY 18	*MAT*α *RME1 rpd3*Δ*::URA3 arg6 his3*Δ*SK*
TLY 397	*MAT***a** *sin3*Δ*::LEU2 arg6*
TLY 401	*MAT***a** *rpd3*Δ*::URA3 arg6 his3*Δ*SK met4*
TLY 446	*MAT***a** *SIN3-MYC arg6*
TLY 449	*MAT*α *rpd3*Δ*::URA3 SIN3-MYC*
TLY 475	*MAT***a** *SIN3-MYC RPD3-HA arg6*
TLY 483	*MAT***a** *RPD3-HA ura3::UME6(N3)HA::URA3 arg6*
TLY 485	*MAT***a** *ura3::UME6(N3)HA::URA3 arg6*
TLY 487	*MAT***a** *SIN3-MYC ura3::UME6-HA-URA3 arg6*
TLY 491	*MAT***a** *rpd3-418* arg6*
TLY 401 × TLY 838	*MAT***a**/α *rpd3*Δ*::URA3/rpd3*Δ*::URA3 arg6/+ his3*Δ*SK/his3*Δ*SK met4/+*
TLY 552 × TLY 585	*MAT***a**/α *+/ura3 arg6/arg6*
TLY 572	*MAT*α *rpd3-418* arg6*
TLY 590	*MAT***a** *RME1 arg6*
TLY 605	*MAT***a** *ime2*Δ*2::LEU2*
1241 derived[b]	
TLY 77	*MAT***a** *rme1 his4-712 cyh*^{r}
TLY 78	*MAT***a** *RME1 his4-712*
TLY 162	*MAT*α *rme1 his3*Δ*SK*
TLY 356	*MAT*α *rme1 hur-B51 cyh*^{r}
TLY 354	*MAT*α *rme1 hur-E3*
TLY 355	*MAT*α *rme1 hur-B42 cyh*^{r}
TLY 405	*MAT***a** *rme1 rpd3*Δ*::URA3 his4-712 cyh*^{r}
TLY 472	*MAT***a** *rme1 sin3*Δ*HIII his4-712 cyh*^{r}

[a] SK1 strains contain *rme1*Δ*5::LEU2 ura3 trp1::hisG leu2::hisG lys2 ho::LYS2* unless indicated.
[b] 1241 strains contain *leu2-c ura3 trp1 can1 HML*α *HMR*α unless indicated.

showed that the mutation was a C to T transversion at nucleotide 1255 creating a TAA stop codon. We have also identified *SOK1*, a dosage suppressor of the *tpk1 tpk2 tpk3* triple mutant lethality (Ward and Garrett 1994), as an extragenic high-copy suppressor of the Hur E3 strain (our unpublished results). Because protein kinase A signaling is known to inhibit early meiotic gene expression in normal meiosis (Matsuura *et al.* 1990), suppression the Hur$^-$ phenotype by *SOK1* is probably independent of the checkpoint pathway.

Miscellaneous: For Northern analysis, RNA was isolated and 10 to 20 μg were run on formaldehyde denaturing gels, transferred to nylon membranes, and probed for *IME1*, *IME2*, *SPO13*, *HOP1*, and PC4-2 as described (Smith and Mitchell 1989; Sia and Mitchell 1995). The *RNR2* probe is an internal 0.7-kb *Eco*RI-*Sal*I fragment from pSE310 (Elledge and Davis 1987). Probes were labeled with [α-^{32}P]dCTP using High Prime (Roche) labeling mix, hybridized, and washed according to standard procedure. Quantitation was carried out as described (Figure 3). The plasmids pKB852 and pTL7 contain the *IME2* 5′ region (from −852 to −18 from the AUG) fused to −*CYC1-lacZ*. For β-galactosidase assays, *o*-nitrophenyl-β-d-galactopyranoside color reactions were carried out on permeabilized cells as described (Bowdish and Mitchell 1993).

Immunoprecipitation and Western blotting: Protein extracts were prepared in "extraction buffer" (EB: 100 mm NaCl, 100 mm KCl, 1 mm EDTA, 5% glycerol, 0.05% β-mercaptoethanol, 50 mm Tris-Cl pH 7.4 supplemented with protease inhibitors, 0.15 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml each leupeptin, aprotinin, and pepstatin, and phosphatase inhibitors, 20 mm β-glycero-phosphate, 10 mm *p*-nitrophenyl phosphate, 5 mm NaF, and 1 mm $NaVO_4$), and protein concentration was determined with Bio-Rad (Hercules, CA) reagent as described (Bowdish *et al.* 1994). For immunoprecipitations, 4 mg of protein were resuspended in 0.5 ml of EB, and 15 μl of polyclonal anti-Rpd3p (produced against yeast Rpd3p purified from *Escherichia coli*) antiserum were added and incubated at 4° for 10 min. Fifty microliters of a 50% slurry of EB-equilibrated Protein A Sepharose beads was added and mixed by inversion for 1 hr at 4°. After binding, the beads were washed 4× with 0.5 ml EB. The final wash was removed and the beads were boiled in 40 μl of 3× Laemmli buffer. Thirty microliters of this was loaded on an 8% SDS-PAGE. Immunoblots were probed with anti-cMyc (Ab-1, Calbiochem, San Diego, CA), anti-HA (BAbCo, Richmond, CA), anti-Rpd3p (described above), or anti-Ime2p (Sia and Mitchell 1995) antibodies.

RESULTS

Impaired replication downregulates early meiotic gene expression: We used Northern analysis to test whether the expression of early meiotic genes is responsive to impaired replication. An *rme1*Δ haploid strain

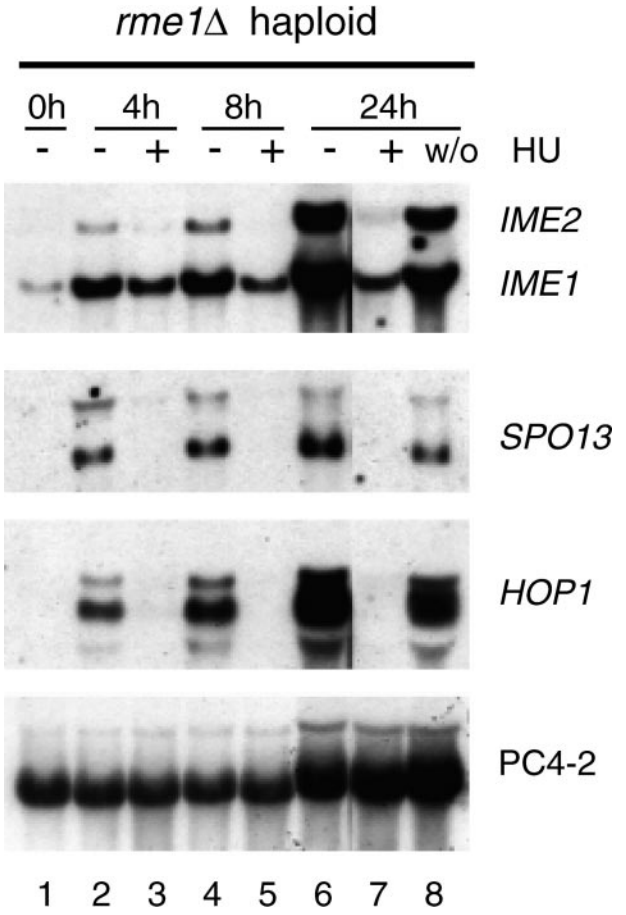

FIGURE 1.—The effect of HU on meiotic gene expression. RNA was prepared from cells (AMP 734) grown to mid-log phase in YPAc (lane 1) or at the indicated duration after a shift to SPO in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of 0.1 M HU. Lane 8 was prepared from cells washed free of HU after 8 hr in SPO + 0.1 M HU and incubated a further 16 hr in SPO. Fifteen micrograms of total RNA per lane was run on a formaldehyde denaturing gel, transferred to nylon, and sequentially probed for the indicated transcripts (see MATERIALS AND METHODS).

that can undergo the early events of meiosis was initially examined (Figure 1) to provide a basis for genetic studies, but diploids showed a similar response (see below). Cells shifted to SPO induced the expression of *IME1*, which in turn activated expression of the early meiotic genes *IME2*, *SPO13*, and *HOP1* (Figure 1, lanes 2, 4, and 6). When DNA synthesis was impaired by the ribonucleotide reductase inhibitor HU, *IME1* was induced at a slightly lower level (Figure 1, lanes 3, 5, and 7). Transcript accumulation of the early genes *IME2*, *SPO13*, and *HOP1*, however, was severely reduced by HU. The reduction of early meiotic transcripts in response to HU was not due to cell death because cells that were washed free of HU after an 8-hr treatment remained competent to express early meiotic genes (Figure 1, lane 8). Thus, HU caused a reversible reduction in early meiotic gene expression.

To determine whether the 5′ regulatory region of an early meiotic gene was the target of replication control, we tested the HU response of an *IME2-CYC1-lacZ* reporter (abbreviated *IME2-lacZ*; Table 2). If the *IME2* 5′-region is a target for regulation, then β-galactosidase activity should respond to HU just like the meiotic transcripts. We verified that *IME2-lacZ* was under meiosis-specific control in these strains by showing that an *RME1* haploid (TLY 78) failed to express *IME2-lacZ* and that a *rme1*Δ haploid (TLY 77) induced *IME2-lacZ* more than 100-fold in response to sporulation medium. In the presence of HU, the *rme1*Δ haploid failed to substantially induce *IME2-lacZ*. These results suggest that the reduction of early meiotic gene expression in response to HU is mediated by 5′ regulatory sequences.

Early meiotic gene activation is dependent on *IME1* (MITCHELL *et al.* 1990), and HU caused a slight reduction in *IME1* transcript levels. Therefore, it seemed possible that the reduced early meiotic gene expression in response to HU could be due to reduced *IME1* expression. If this were the case, then ectopic *IME1* expression from a heterologous promoter should restore early gene expression in the presence of HU. Cells carrying a P_{ACT1}-*IME1* plasmid expressed *IME2-lacZ* during mitotic growth and further activated expression in sporulation medium (Table 2). However, in sporulation medium containing HU, *IME2-lacZ* expression remained at the mitotic level. Thus, ectopic *IME1* expression did not overcome the block in early meiotic gene expression when replication was compromised. This result suggests that the reduced *IME1* levels in the presence of HU cannot account for the severely reduced expression of early meiotic genes.

Screen for genes that couple replication with recombination and EMG expression: To learn how HU-blocked replication caused inhibition of early meiotic gene expression and recombination, we designed a screen to identify genes required for the HU response. To monitor recombination in haploids, we constructed strains carrying *leu2-c* in the genome and *leu2-e* on a plasmid and assessed the production of Leu^+ progeny on plates. This assay maintains three critical features of meiotic recombination: (i) dependence on sporulation medium (Figure 2 and Table 3), (ii) inhibition by *RME1* (not shown), and (iii) inhibition by HU (Figure 2 and Table 3). It is also sufficiently robust to monitor the response of single colonies. Thus, this recombination assay and the *IME2-lacZ* assay described above can be used to monitor meiotic progression in *rme1*Δ haploids.

We used these assays to perform a screen for mutants that maintain the ability to recombine at meiotic levels and permit *IME2* transcription when meiotic replication is impaired. Cells were EMS mutagenized and screened for their ability to give rise to Leu^+ papillae after incubation on sporulation medium containing 40 mM HU. Potential positives were secondarily screened for the ability to express *IME2-lacZ* in the presence of HU. The phenotypes of three Hur^- (*HU* reduced *r*ecombination)

TABLE 2

IME2-lacZ* expression in cells carrying P_{ACT1}-*IME1

Strain	*RME1*	P_{ACT1}-*IME1*	*IME2-lacZ* expression		
			Mitosis	SPO	SPO + HU
TLY 78	+	−	0.02	0.05	0.02
TLY 77	−	−	0.04	67	0.3
TLY 78	+	+	42	70	19
TLY 77	−	+	39	286	33

All strains carry the reporter plasmid pKB852 (P_{IME2}-*CYC1-lacZ-URA3*) and the indicated strains carry pTL5 (P_{ACT1}-*IME1-TRP1*), which can complement the sporulation defect of an *ime1*Δ/*ime1*Δ diploid. Cells were grown for 2 days in selective media containing 0.5% glucose. Mitotic cultures were collected at this time, and the remaining culture shifted to SPO. After 1 hr in SPO, the culture was divided and one-half was brought to 40 mM HU, while the other remained untreated. These samples were cultured an additional 23 hr and then collected. The β-galactosidase activity (Miller units) of three independent cultures were all within 22% of the presented average value.

mutants are shown in Figure 2 and Table 4. Wild-type cells and the Hur⁻ mutants all produced Leu⁺ papillae after incubation on sporulation medium (Figure 2). Addition of 40 mM HU severely inhibited recombination in the wild-type strain, moderately inhibited recombination in Hur B42 and Hur B51 strains, and had very little effect on recombination in the Hur E3 strain. The reduced Leu⁺ papillation of the wild-type strain was not due to reduced viability, and all strains failed to papillate after growth on SC-Trp, indicating that they still required a starvation signal to induce recombination. The *IME2-lacZ* response of these strains was very similar (Table 4). Wild-type cells and the Hur⁻ mutants all promoted *IME2* reporter activity in sporulation medium. Addition of 40 mM HU severely inhibited expression in the wild-type strain (1000-fold reduction), moderately inhibited expression in the Hur B42 strain (30-fold reduction), and only weakly inhibited expression in the Hur B51 and Hur E3 strains (8- and 4-fold reduction, respectively). Thus, the Hur⁻ mutants are defective in reducing recombination and meiotic gene expression when replication is inhibited.

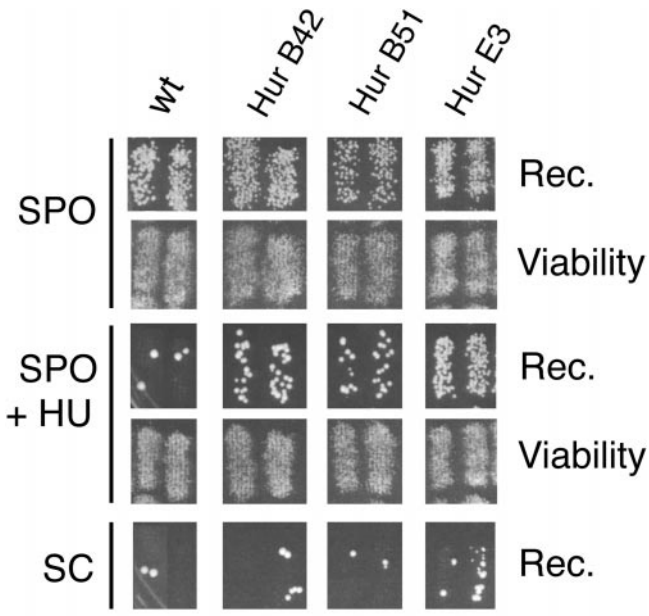

FIGURE 2.—Mutations that uncouple recombination from replication. Recombination phenotype of wild-type and Hur⁻ mutant derivatives. Two patches of each strain were grown on SPO ± 40 mM HU for 4 days and subsequently replicated to SC-Leu to assess recombination and SC to assess viability. Cells grown vegetatively were also replicated to SC-Leu to assess mitotic recombination.

We found that a *hur-B51* diploid failed to sporulate, suggesting that besides preventing meiotic gene expression and recombination in the presence of HU, it also functions in normal meiosis (data not shown). A YCp50 genomic clone (ROSE *et al.* 1987) that complemented the sporulation defect of the *hur-B51* mutant was identified. Sequencing of the ends showed that it contained ~14.2 kb of chromosome XIV including *RPD3*, *PEX6*, *YNL328c*, and *EGT2*. A plasmid containing only *RPD3* also rescued the sporulation defect. A cross of TLY 356 (*hur-B51*) and an *RPD3-URA3* strain indicated tight *hur-B51-RPD3* linkage because the Hur⁻ phenotype always segregated away from Ura⁺ (16 tetrads). Also, a *hur-B51*/*rpd3*Δ strain was sporulation defective. Therefore, linkage and complementation indicated that the Hur B51 strain carried a mutation in *RPD3*. Recovery of *RPD3* from the genome of a Hur B51 strain and subsequent sequencing showed that it encoded a protein truncated by 15 amino acids at the C terminus, Rpd3-418*p. These results indicate that *RPD3* is required to couple early meiotic gene activation and recombination with replication.

Role of mitotic repressor genes in the response to impaired replication: The isolation of an *RPD3* allele that reduced the response to HU suggested that the Rpd3p-Sin3p repression complex might be critical for a normal response to impaired replication. Therefore, we examined the effects of HU on recombination (Table 3) and gene expression (Table 4) in *rpd3*Δ and *sin3*Δ

TABLE 3

Recombination frequencies

Strain	Genotype	Frequency of Leu$^+$ recombinants ($\times 10^{-6}$)				Fold reduction by HU (SPO medium)
		SC	SC + HU	SPO	SPO + HU	
TLY 77	*wt*	3	40	500	30	16
TLY 405	*rpd3*Δ	4	50	800	150	5
TLY 472	*sin3*Δ	4	30	500	200	2.5

Strains were grown on the media indicated for 2 days (SC, SC + HU) or 5 days (SPO, SPO + HU) and plated on SC-Leu (identifies Leu+ recombinants) and SC-Trp (identifies pTL5 plasmid-bearing cells). Recombination frequencies were calculated as the number of SC-Leu colonies divided by the number of SC-Trp colonies.

mutants. Wild-type, *rpd3*Δ, and *sin3*Δ strains have a low frequency of recombination ($\sim 4 \times 10^{-6}$) in mitotic culture (SC). HU stimulated recombination to the same extent in these three strains during mitotic growth in SC medium (SC + HU). A shift to sporulation medium promoted recombination ~100-fold over the mitotic (SC) values. HU inhibited the production of meiotic recombinants (SPO + HU) in a wild-type strain by 16-fold; however, *rpd3*Δ and *sin3*Δ strains were reduced only 5- to 2.5-fold, respectively. Similarly, HU inhibited *IME2-lacZ* expression in a wild-type strain by 100-fold, while *rpd3*Δ and *sin3*Δ strains were reduced only 2.5- to 3-fold, respectively. Furthermore, when *rpd3-418** was introduced into this strain background, *IME2-lacZ* expression was reduced only 5-fold by HU. Thus, *RPD3* and *SIN3* are required for the full recombination and gene expression responses to impaired replication.

To determine the effect of HU on expression of early meiotic genes in diploids, we performed Northern analysis (Figure 3). As expected, *IME1* transcript levels were only slightly reduced by HU (quantitated in Figure 3, C and D). *IME2* and *HOP1* were poorly expressed in the wild-type diploid treated with HU. At the 6-hr timepoint, *IME2* levels were reduced 6-fold and *HOP1* levels were reduced nearly 10-fold by HU in the wild-type strain. In *rpd3*Δ and *sin3*Δ diploids there was a low but detectable level of *IME2* and *HOP1* in mitotically growing cells because of the lack of mitotic repression (Figure 3A, lane 8 and 3B, lane 1). When the mutants were shifted to sporulation medium containing HU, there was greater expression of early genes than in similarly treated wild-type cells, and this expression increased with time. For example at 6 hr, the *rpd3*Δ mutant had less than a 2-fold reduction in *IME2* and little reduction of *HOP1* expression in response to HU (Figure 3C). Thus, mutation of *RPD3* or *SIN3* permits induction of early genes in the presence of HU, suggesting that the Rpd3p/Sin3p complex represses meiotic gene expression when replication is inhibited.

One possible explanation for the reduced meiotic checkpoint response of *rpd3*Δ and *sin3*Δ mutants is that they have general defects in HU uptake or response. Two lines of evidence argue against this explanation. First, FACS analysis of cells taken from this experiment

TABLE 4

Effect of HU on *IME2-lacZ* expression

Strain (bkgd)	Genotype	*IME2-lacZ* expression			Fold reduction by HU
		SC	SPO	SPO + HU	
TLY 162(1241)	Wild type	<0.1	102	0.1	1000
TLY 355(1241)	*hur-B42*	<0.1	97	3.3	30
TLY 356(1241)	*hur-B51*	0.1	45	5	8
TLY 354(1241)	*hur-E3*	<0.1	65	16	4
TLY 590(SK1)	wt *RME1*	<0.1	<0.1	<0.1	NA
TLY 2(SK1)	wt *rme1*	<0.1	63	0.6	105
TLY 491(SK1)	*rpd3-418* rme1*	4	76	16	4.8
TLY 401(SK1)	*rpd3*Δ *rme1*	5	130	42	3.1
TLY 397(SK1)	*sin3*Δ *rme1*	8	200	84	2.4

Transformants of each strain carrying the reporter pTL7 (P_{IME2}-*CYC1-lacZ-TRP1*) were grown in SC-TRP 0.5% glucose overnight. Cultures were divided into three samples and either collected for SC or shifted to SPO ± 40 mM HU. For the 1241 strains, SPO cultures were collected after 26 hr; for the SK1 strains, they were collected after 6 hr. The values presented are average Miller units of three independent samples with each determination within 30% of the average value.

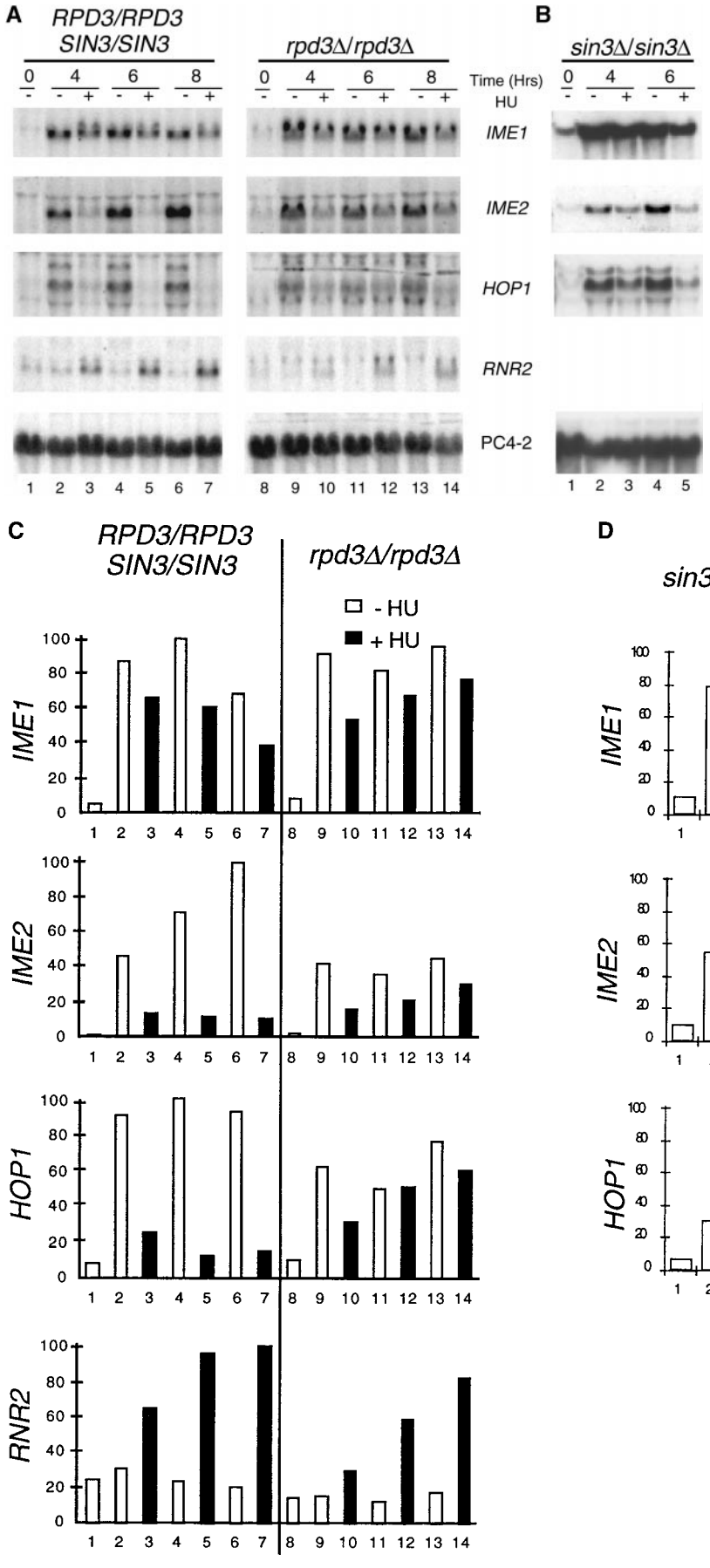

FIGURE 3.—The effect of HU on meiotic gene expression in wild-type, *rpd3*Δ, and *sin3*Δ diploids. (A) RNA was prepared from wild-type (TLY 552 × TLY 585) and *rpd3*Δ/*rpd3*Δ (TLY 401 × TLY 838) diploids grown to mid-log in YPAc (lanes 1 and 8) or shifted to SPO in the absence (lanes 2, 4, 6, 9, 11, and 13) or presence of 0.04 M HU (lanes 3, 5, 7, 10, 12, and 14), collected at various times, and analyzed by Northern blot (see MATERIALS AND METHODS for probes and conditions). (B) RNA was prepared from *sin3*Δ/*sin3*Δ (TLY 7) diploids treated and analyzed in the same way as above, but in a separate experiment. This experiment included a wild-type strain that showed essentially the same expression pattern as the wild-type strain in A. (C) Relative gene expression of the samples in A was determined using phosphor-imaging and Image Quant software. The signal for each transcript was normalized for loading by dividing by the PC4-2 signal, and then relative gene expression was determined by setting the maximal wild-type signal for that transcript to 100. (D) Relative gene expression of the samples in B was calculated as described above, with the wild-type signals from this experiment serving as the relative standard.

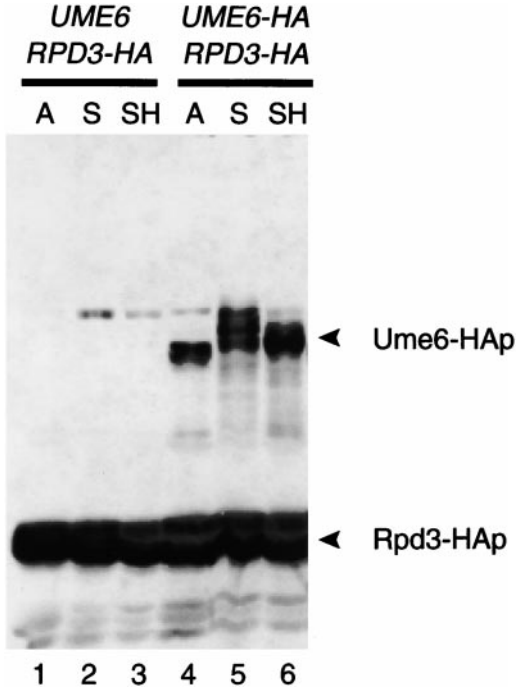

FIGURE 4.—The effect of HU on Ume6p modification. TLY 475 (lanes 1–3) and TLY 483 (lanes 4–6) cultures were grown in YPAc to mid-log and divided in thirds. One-third was harvested for the YPAc (A) protein extract. The remaining cells were shifted to sporulation medium in the absence (S) or presence (SH) of 0.1 M HU, cultured 4 more hours, and then harvested for extracts. A total of 100 μg of crude protein extracts were run on a 10% SDS-PAGE and Western blotted for the HA epitope.

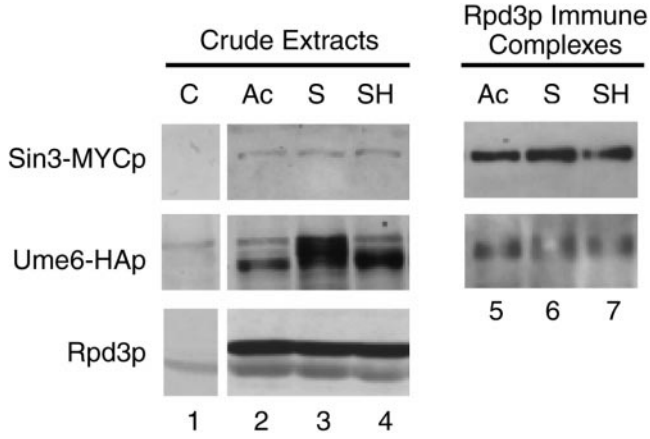

FIGURE 5.—Interaction of Rpd3p with Sin3-MYCp and Ume6-HAp during sporulation. Protein extracts were obtained from cells (TLY 487) grown to mid-log phase in YPAc (Ac, lanes 2 and 5) or from cells incubated in SPO medium (S, lanes 3 and 6) or SPO + 0.04 M HU (SH, lanes 4 and 7) for 4 hr. Lanes 2–4 contain 100 μg total protein extract while lanes 4–6 contain Rpd3p-immune complexes obtained from 4 mg total protein extract. Lane 1 contains 100 μg of total protein extracts from various control strains. For Sin3-MYCp detection, the control is a *SIN3* untagged strain (TLY 485); for Ume6-HAp detection, the control is a *UME6* untagged strain (TLY 446); for Rpd3p detection, the control is an *rpd3*Δ strain (TLY 449). Western blots were probed for MYC-epitope, HA-epitope, and Rpd3p.

showed that HU blocked DNA synthesis in both wild-type and *rpd3*Δ diploids (data not shown). Second, HU treatment induced *RNR2* expression in *rpd3*Δ and *sin3*Δ mutants (Figure 3A, and data not shown). Therefore, deletion of these genes did not simply bypass the normal DNA damage transcriptional response, nor did it permit HU-resistant DNA synthesis. Taken together, our data suggest that Rpd3p and Sin3p cooperate to repress early meiotic gene expression when replication is inhibited.

Impaired replication inhibits modification of Ume6p: In mitosis the Rpd3p-Sin3p complex promotes repression of early meiotic genes through their interaction with the DNA-binding protein Ume6p (KADOSH and STRUHL 1997). Recent studies have shown that Ume6p is hyper-phosphorylated in sporulation medium and that this modification is critical for expression of meiotic genes (XIAO and MITCHELL 2000). Thus, it was possible that HU-blocked replication could signal to inhibit Ume6p modification. To test this idea, we examined the mobility of epitope-tagged Ume6-HAp (expressed from its own promoter) in the presence and absence of HU (Figure 4). When cells were grown mitotically in YPAc, Ume6-HAp ran as a tightly migrating ~140-kD band. Incubation in sporulation medium shifted the Ume6-HAp band up, but the presence of HU reduced that shift. Our observations support the idea that a blocked replication signal may reduce early meiotic gene expression by inhibiting Ume6p phosphorylation.

The repression complex exists in sporulating cells: Prior studies of the repression complex were carried out in mitotic cells, so it was unknown whether it existed in meiotic cells. The existence of a meiotic repression complex seemed tenuous because *SIN3* transcripts were not detected in stationary phase cultures (WANG *et al.* 1990). Given that *rpd3*Δ and *sin3*Δ mutants have a reduced response to impaired meiotic replication, we suspected that the repression complex did exist and that it played a role in meiosis. To detect these proteins, we generated an anti-Rpd3p antibody and a functional MYC-tagged version of Sin3p. Expression of *SIN3-MYC* was controlled by the endogenous *SIN3* promoter. Total protein extracts were obtained from a mitotic culture (Figure 5, lane 2) or 4 hr after a shift to sporulation medium in the absence (lane 3) or presence (lane 4) of 40 mM HU. Immunodetection of Sin3-MYCp and Rpd3p showed that their levels were relatively insensitive to sporulation medium or HU. We confirmed that HU inhibited Ime2p expression in this experiment by Western analysis (data not shown). Thus, Rpd3p and Sin3p are present in early meiotic cells, and HU does not cause an increase in the concentration of these repression proteins.

Although protein levels were maintained in sporulation medium, it seemed possible that association of the

repression components would be altered. This idea was especially attractive because of the sporulation-induced Ume6p modification. To determine whether the association of the repression complex subunits was affected by a shift to sporulation medium or sensitive to impaired replication, Rpd3p immune complexes were obtained and the components analyzed by Western blot (Figure 5, lanes 5–7). Sin3-MYCp and Ume6-HAp were detected in Rpd3p-immune complexes isolated from both mitotic and sporulating cultures. Furthermore, complex formation was not grossly affected by HU. These data suggest that modification of Ume6p does not affect association and that inhibited replication does not increase the amount of the repression complex.

DISCUSSION

Previous studies had shown that inhibition of meiotic replication blocked the progression of meiosis (Silvia-Lopez *et al.* 1975; Simchen *et al.* 1976) and hinted that the coupling of replication with cell division might be via control of meiotic gene expression (Kao *et al.* 1989). Our findings directly demonstrate that inhibition of meiotic replication reduces expression of the early class of meiotic genes. Since early meiotic genes are required to promote recombination, middle meiotic gene expression and, ultimately, sporulation, the reduction in early gene expression may contribute to the downstream defects when replication is inhibited.

The coupling of meiotic gene expression and recombination to replication comprises a *bona fide* checkpoint, as defined by Hartwell and Weinert (1989), since loss-of-function Hur$^-$ mutants that are defective in this coupling were isolated. The Hur$^-$ mutations do not completely abolish the response to HU, suggesting that they do not eliminate the checkpoint. Similarly, the first characterized checkpoint mutation, *rad9*, did not completely abolish the response to DNA damage (Weinert and Hartwell 1990). Although it is known that a *mec1-1* mutant uncouples meiotic division from replication (Stuart and Wittenberg 1998), we did not expect to obtain mutations in *MEC1* or in any of the known mitotic replication checkpoint genes because our screen demanded viability on HU-containing medium. However, it is possible that the *HUR* genes work downstream of *MEC1* to inhibit meiotic progression.

One of the Hur$^-$ mutations lies in *RPD3*, and deletions of *SIN3* and *RPD3* partially uncoupled meiotic gene expression and recombination from replication. The model that cell cycle arrest and accumulation of derepressed meiotic transcripts in *sin3*Δ and *rpd3*Δ strains accounts for their meiotic recombination rate in the presence of HU seems unlikely, because cells arrested mitotically with HU fail to achieve SPO + HU recombination levels. However, *SIN3* and *RPD3* are not classical checkpoint genes since deletants still partially respond to inhibited replication. Furthermore, because of their natural positive roles in promoting middle meiotic gene expression and nuclear divisions (Vidal and Gaber 1991; Vidal *et al.* 1991; Hepworth *et al.* 1998), *sin3*Δ and *rpd3*Δ strains would not divide meiotically in the presence of HU. Thus, other checkpoint targets must exist that can account for the full response. In this regard, we showed that the phosphorylation of Ume6p, which is required for early meiotic gene expression, is inhibited when replication is blocked. Thus, the response to inhibited replication relies upon both reduced activation and active repression to inhibit early meiotic gene expression. A model that summarizes our findings on the coupling of meiotic gene expression with replication and incorporates speculation about the natural roles of *SIN3* and *RPD3* in meiosis is outlined in Figure 6.

The coupling of EMG expression to replication depends on SIN3 and RPD3: In mitosis, Rpd3p interacts with Sin3p, which interacts with the DNA-binding protein Ume6p to repress early meiotic genes (Kadosh and Struhl 1997; Kasten *et al.* 1997; Rundlett *et al.* 1998). In meiosis, *IME1* expression is induced, and Ime1p interacts with Ume6p to promote early meiotic gene activation. One simple model to explain the transition between mitotic repression and meiotic activation of early meiotic genes is that Ime1p displaces the repressor proteins from Ume6p. However, we have found that the Sin3p/Rpd3p/Ume6p complex is stable in meiosis, arguing against this model and suggesting that histone deacetylase activity may be an important meiotic function. Thus, one role for the complex in meiosis may be to ensure early meiotic gene repression when DNA synthesis is inhibited.

SIN3 and *RPD3* are normally positive regulators of meiosis because *sin3* and *rpd3* mutants are defective in sporulation, fail to express middle meiotic genes and undergo nuclear divisions, and have reduced expression of early meiotic genes (Vidal and Gaber 1991; Vidal *et al.* 1991; Hepworth *et al.* 1998). Since Sin3p and Rpd3p help to repress early meiotic genes in the presence of HU, it is possible that meiotic DNA synthesis alters the activity of the complex. If this is the case, Sin3p and Rpd3p may normally promote early and middle meiotic gene expression only when an appropriate DNA synthesis signal is received.

It seems likely that the ability of *sin3* and *rpd3* mutants to permit some meiotic recombination in the presence of HU is a consequence of early meiotic gene expression. However, *rpd3* was recently found to be an allele of *rec3*, a mutant with reduced mitotic recombination rates (Dora *et al.* 1999). This result implies that the derepressed meiotic gene expression in these mitotic cells is not sufficient to activate meiotic recombination. This finding also suggests that Rpd3p and perhaps histone deacetylation modify DNA to make it more accessible for the mitotic recombination apparatus. Our data

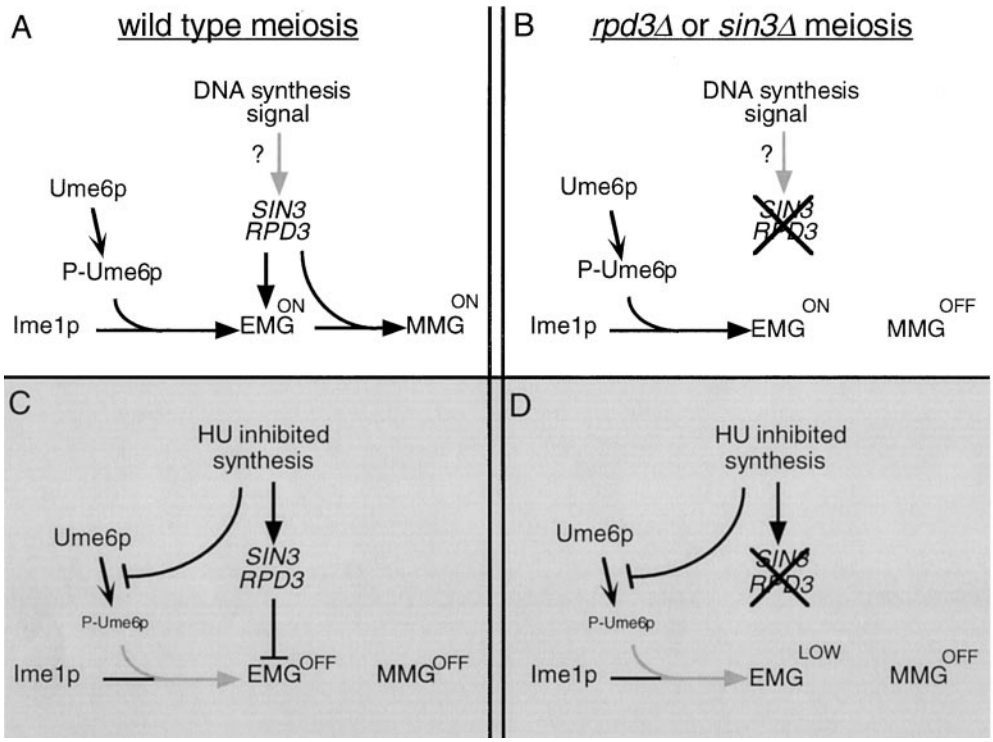

FIGURE 6.—Models for the meiotic gene expression response to replication signals in wild-type (left) and *sin3*Δ or *rpd3*Δ mutants (right). (A) Ume6p becomes phosphorylated in sporulation medium, which permits interaction with Ime1p and activation of EMGs. Middle meiotic genes (MMGs) are expressed only when *both* EMGs and *SIN3* and *RPD3* are present. We propose that the normal role of *SIN3* and *RPD3* is to interpret a DNA synthesis signal. (B) In *sin3*Δ or *rpd3*Δ mutants, Ume6p modification is intact (data not shown), and EMGs are induced. MMGs fail to be induced because the *SIN3-RPD3* positive signal is absent. (C) When replication is inhibited, less phospho-Ume6p is available to interact with Ime1p, thus reducing EMG activation. Additionally, *SIN3* and *RPD3* mediate an impaired replication signal to repress EMGs. Without EMG expression, MMGs are not induced and sporulation cannot proceed. (D) When replication is inhibited in *sin3*Δ or *rpd3*Δ mutants, the low levels of phospho-Ume6p are sufficient to activate a low level of EMG expression (and recombination) because repression by *SIN3* and *RPD3* is lost. With only weak EMG expression and no *SIN3-RPD3* positive signal, MMGs are not expressed.

show that Rpd3p can play an inhibitory role in meiotic recombination when DNA synthesis is impaired. Taken together, these observations suggest that Rpd3p may function to regulate recombination by gene regulation and perhaps by more direct effects on DNA structure.

Control of Ume6p phosphorylation by inhibited replication: Upon a shift to sporulation medium, Ume6p becomes hyper-phosphorylated *in vivo* (XIAO and MITCHELL 2000; Figure 6A). Phosphorylation of Ume6p correlates with its ability to associate with Ime1p and activate early meiotic gene expression (MALATHI *et al.* 1997; XIAO and MITCHELL 2000). Since HU inhibits full levels of Ume6p phosphorylation, we propose that early meiotic genes will be activated only when a DNA synthesis signal promotes Ume6p phosphorylation and subsequent Ume6p-Ime1p complex formation (Figure 6B). The low levels of phospho-Ume6p in the presence of HU can explain why ectopic *IME1* expression could not overcome the HU-mediated defect in early meiotic gene expression. HU-reduced phospho-Ume6p levels may be due to inhibition of kinases or activation of phosphatases that target Ume6p. The protein kinases Rim11p and Rim15p both promote meiotic gene activation and Ume6p phosphorylation (BOWDISH *et al.* 1995; MALATHI *et al.* 1997; VIDAN and MITCHELL 1997; XIAO and MITCHELL 2000). We assessed the kinase activity of Rim11p and Rim15p when isolated from untreated *vs.* HU-treated sporulating cultures and found no *in vitro* regulation by HU (our unpublished results). However, their *in vivo* activity on Ume6p might be inhibited by HU-blocked replication. Alternatively, blocked replication could inhibit the activity of other kinases or activate phosphatases to reduce Ume6p phosphorylation. Although the regulatory mechanism is uncertain, the defective phosphorylation of Ume6p provides one explanation of how impaired replication inhibits early meiotic gene expression.

Relationship between EMG regulators and replication checkpoint control: Combined with the work of STUART and WITTENBERG (1998) our data suggest a multi-factor response that controls early meiotic gene expression and recombination when meiotic replication is inhibited. Since a *mec1-1* mutation permits meiotic nuclear division in the presence of HU, *MEC1* is clearly a critical upstream checkpoint response gene; however, no meiotic targets of *MEC1* are known. HU inhibits Ume6p phosphorylation, which is normally required to promote early meiotic gene expression and sporulation. The molecular target of this effect is unknown, but it could be downstream or independent of the *MEC1* pathway. Finally, Rpd3p and Sin3p repress early meiotic genes in the presence of HU. We do not know if this function is a specific response to inhibited replication or if it simply represents an extension of their known mitotic roles. However, Rpd3p and Sin3p do become positive regulators of meiotic gene expression in meio-

sis, so it is possible that meiotic DNA synthesis controls the transition between their mitotic and meiotic roles.

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