Rad53-dependent phosphorylation of Swi6 and down-regulation of *CLN1* and *CLN2* transcription occur in response to DNA damage in *Saccharomyces cerevisiae*

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Budding yeast possesses a checkpoint-dependent mechanism of delaying G_1 progression in response to UV and ionizing radiation DNA damage. We have shown that after a pulse of DNA damage in G_1 with the alkylating agent MMS, there is also a *MEC1*-, *RAD53*-, and *RAD9*-dependent delay in G_1. This delay occurs at or before Start, as the MMS-treated cells do not bud, remain sensitive to α-factor, and have low *CLN1* and *CLN2* transcript levels for a longer time than untreated cells. We further show that MMS directly and reversibly down-regulates *CLN1* and *CLN2* transcript levels. The initial drop in *CLN* transcript levels in MMS is not *RAD53* dependent, but the kinetics of reaccumulation of *CLN* messages as cells recover from the damage is faster in *rad53-11* cells than in wild type cells. This is not an indirect effect of faster progression through G_1, because *CLN* transcripts reaccumulate faster in *rad53-11* mutants arrested in G_1 as well. In addition, the recovery of *CLN* mRNA levels can be also hastened by a *SWI6* deletion or by overexpression of the truncated Swi4 (Swi4-t) that lacks the carboxy-terminal domain through which Swi4 associates with Swi6. This indicates that both Rad53 and Swi6 are negative regulators of *CLN* expression after DNA damage. Finally, Swi6 undergoes an MMS-inducible, *RAD53*-dependent phosphorylation in G_1 cells, and Rad53, immunoprecipitated from MMS-treated cells, phosphorylates Swi6 in vitro. On the basis of these observations, we suggest that the Rad53-dependent phosphorylation of Swi6 may delay the transition to S phase by inhibiting *CLN* transcription.

[*Key Words:* MMS; *CLN1; CLN2; RAD53; SWI6;* cell cycle; checkpoint]

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DNA damage checkpoints ensure that a cell with lesions in its DNA does not divide before the damage is eradicated. In the yeast *Saccharomyces cerevisiae*, three DNA damage-inducible checkpoints have been identified that operate in G_1, S, and G_2 phases of the cell cycle (Weinert and Hartwell 1989; Siede et al. 1993, 1994; Weinert et al. 1994; Paulovich and Hartwell 1995; Paulovich et al. 1997a). Two genes, *MEC1/ESR1/SAD3* and *RAD53/MEC2/SPK1/SAD1*, appear important for the performance of all three checkpoints (Allen et al. 1994; Weinert et al. 1994; Paulovich and Hartwell 1995; Siede et al. 1996). In addition, *RAD9, RAD17, RAD24*, and *MEC3* are involved in G_1 and G_2 checkpoints (Siede et al. 1993, 1994; Weinert et al. 1994). These checkpoint gene products delay the cell cycle when DNA damage is present, which allows time for repair and improves survival in the presence of damaging agents. *MEC1* encodes a member of a PI kinase family and has homologs in fission yeast, *Drosophila*, mice, and humans (Al-Khodairy and Carr 1992; Hari et al. 1995; Morrow et al. 1995; Savitsky et al. 1995; Pecker et al. 1996). The kinase activity of Mec1 has not been demonstrated, although it is thought that Mec1, its close yeast homolog Tel1, and similar kinases, like ATM of humans, are protein, rather than lipid, kinases (Hunter 1995). *RAD53* encodes a dual specificity kinase, whose catalytic activity has been demonstrated, but its substrate specificity remains unidentified (Zheng et al. 1993; Sun et al. 1996).

Rad9, Rad17, Rad24, and Mec3 are thought to be involved in recognition of damage and initiating a signal transduction cascade that activates Mec1 and Rad53 (Lydall and Weinert 1995; Navas et al. 1996; Sanchez et al. 1996; Sun et al. 1996). In turn, Mec1 and Rad53 transmit the signal to critical targets including cell cycle machinery and DNA repair enzymes. Two biochemical events have been detected upon DNA damage in cells. First,

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Rad53 undergoes phosphorylation that is dependent on Mec1, Rad9, Rad17, Rad24, and Mec3 (Navas et al. 1996; Sanchez et al. 1996; Sun et al. 1996). Second, there is an induction of transcription of a large group of genes that are involved in DNA replication and repair (Aboussekhra et al. 1996; Kiser and Weinert 1996; Navas et al. 1996). However, not much is known with regard to the mechanisms of the G_1, S, or G_2 cell cycle arrests in response to the DNA damage.

Recently it has been shown that yeast cells irradiated by UV or X rays in early G_1 can delay the onset of Start in a checkpoint-dependent manner (Siede et al. 1993, 1994). Start, in this context, is operationally defined as the point at which cells have committed to the mitotic cell cycle and are resistant to arrest by α-factor. This commitment process involves accumulation of threshold levels of G_1 cyclin proteins, Cln1 and Cln2, which in turn triggers destruction of B-type cyclin inhibitor Sic1 and thereby irreversibly commits cells to S phase and mitosis (for review, see Cross 1995; King et al. 1996; Nasmyth 1996). The rate of Cln1 and Cln2 accumulation is regulated primarily at the transcript level, which can vary depending on growth conditions and affect the length of the G_1 phase (Baroni et al. 1994; Tokiwa et al. 1994; Willems et al. 1996).

In this work we show that MMS, a DNA-damaging agent, can induce a Mec1-, Rad53-, and Rad9-dependent delay before Start that is similar to the one observed by Siede and coworkers after UV or X-ray irradiation (Siede et al. 1993, 1994). The MMS-induced delay is characterized by a prolonged inhibition of *CLN1* and *CLN2* transcription, followed by a gradual reaccumulation of these transcripts and resumption of the cell cycle. The reaccumulation of *CLN* mRNA is faster in a *rad53-11* checkpoint mutant and in a *swi6* mutant strain than in the wild type. This is not an indirect consequence of the fact that these mutants progress through G_1 faster, because it can also be observed in G_1-arrested cells. Overproduction of a truncated form of Swi4, which activates *CLN1* and *CLN2* transcription but is independent of Swi6 in its activity, can also reduce the delay of S phase upon MMS treatment. This suggests the possibility that Rad53 might act on Swi6 to inhibit *CLN* transcription in damaged G_1 cells. We have found that Swi6 undergoes a Rad53-dependent phosphorylation that is induced in the presence of MMS in vivo. The kinase responsible is likely to be Rad53 or an associated kinase because Swi6 can be phosphorylated by immunoprecipitated Rad53 in vitro in a pattern resembling the damage-induced pattern in vivo. On the basis of these observations, we suggest that reduced transcription of *CLN1* and *CLN2* owing to an inhibitory phosphorylation of Swi6 contributes to the checkpoint-mediated delay of Start.

Results

MMS damage in early G_1 delays the onset of CLN *transcription*

MMS is a DNA-alkylating agent that methylates bases and can induce a variety of lesions including strand breaks (Dhillon and Hoekstra 1994). We have used this agent to study the G_1 checkpoint that delays the onset of S phase in the presence of DNA lesions. Figure 1 shows the behavior of wild-type cells that were arrested in early G_1 by α-factor, treated or not treated with 0.1% MMS for 30 min, and then allowed to progress through the cell cycle by removal of MMS and α-factor. Samples were taken to determine budding index and DNA content. As can be seen from Figure 1A, the MMS-treated cells remain unbudded for at least 45 min, whereas the untreated cells bud between 15 and 30 min after release from the α-factor. FACS profiles of these cultures are consistent with the budding indexes and show (Fig. 1B) that the MMS-treated cells maintain a 1N DNA content and do not enter S phase for ~45 min. In contrast, the untreated cells are already well into S phase 30 min after the release from α-factor. In addition to the slow entry into S phase, a considerable fraction of the MMS-treated cells are still in S phase 105 min after release. This very slow S phase is consistent with previous observations that MMS can induce the S-phase checkpoint (Paulovich and Hartwell 1995).

UV irradiation in early G_1 can transiently arrest cells before Start (Siede et al. 1993, 1994), as judged by the fact that the arrested cells are sensitive to α-factor. Cells arrested in G_1 by MMS treatment are also α-factor sensitive (see below). Moreover, this pre-Start arrest is reflected in *CLN* transcript levels. As seen in Figure 1, C and D, whereas untreated cells rapidly induce a normal burst of *CLN1* transcript 10 min after the α-factor release, MMS-treated cells are greatly delayed in their accumulation of *CLN1* transcript. Two simple possibilities could account for this. First, MMS treatment could inhibit some early event and prevent progression to the time at which *CLN* transcription commences. Alternatively, MMS could directly inhibit *CLN* transcription, and that could be responsible for delaying Start.

CLN1 *and* CLN2 *transcripts are reversibly down-regulated upon MMS treatment in G_1*

To address in more detail whether *CLN1* and *CLN2* transcripts are directly affected by MMS addition, wild-type yeast cultures were arrested in G_1 with α-factor and released into fresh media containing increasing doses of MMS. These cells were incubated for 20 min and harvested, and then *CLN1* and *CLN2* transcript levels were measured. We found that the *CLN* transcripts were specifically down-regulated in response to MMS addition in a dose-dependent manner (data not shown). To see if cell cycle progression is required to see this down-regulation, we used a *cdc4* strain, which arrests at the G_1/S transition upon incubation at nonpermissive temperature (37°C). This strain was first incubated at 37°C for 3 hr to establish a uniform arrest. Then MMS was added for 30 min to an aliquot of these arrested cells, and samples were harvested for mRNA measurements. *CLN1* levels were reduced in the MMS-treated cells by 5- to 10-fold compared with untreated controls (Fig. 2, lanes 1,2). The same result was obtained with *cdc28* cells arrested in G_1

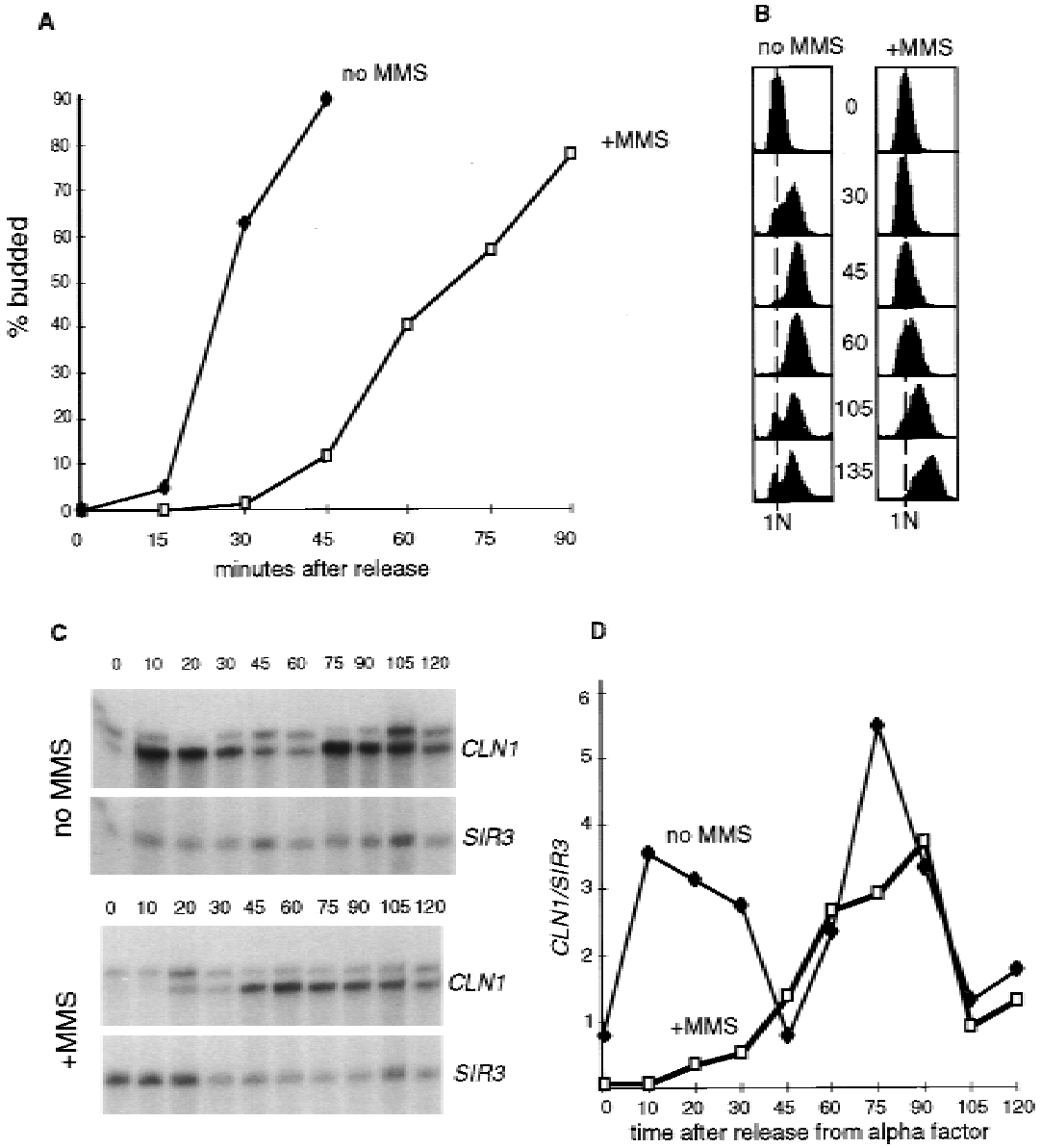

Figure 1. Exposure of G_1 cells to MMS causes a delay of S phase. Wild-type (BY2235) cells were arrested by α-factor in G_1, and one-half of the culture was incubated with 0.1% MMS for 30 min. Then MMS was inactivated as described in Materials and Methods, and both cultures were filtered out of α-factor, resuspended in the fresh media, and allowed to progress into the cell cycle. These cells are 25%–50% viable after such treatment (data not shown). Aliquots of cultures were taken before the release (time point 0) and every 15 min after to determine the percentage of budded cells (*A*) and DNA content (*B*). For the latter, samples were fixed, stained with propidium iodide as described in Materials and Methods, and subjected to FACS analysis. (*C*) A separate culture was treated as described for *A* and *B*, and aliquots for RNA isolation were taken before the release from α-factor and every 10–15 min after. These RNAs were analyzed by S1 protection with radiolabeled probes against *CLN1* and *SIR3* RNAs. The protected fragments corresponding to these RNAs are marked. (*D*) The S1 protection data presented in *C* were quantitated using PhosphorImager software. *CLN1* mRNA levels were normalized to the internal control mRNA (*SIR3*) levels and plotted.

(data not shown). This indicates that MMS treatment significantly inhibits *CLN* transcription or decreases mRNA stability. We then followed *CLN* transcript levels for 2 hr after MMS was removed and found that these transcripts remain low for up to 60 min, after which they start to reaccumulate (Fig. 2B). Therefore, down-regulation of *CLN* transcription by MMS is prolonged but reversible and can occur independently of cell cycle progression.

Importantly, other cell cycle-dependent transcripts that peak during G_1 phase did not show the same response to MMS addition that was observed for *CLN1* and *CLN2*. For example, the *CDC9* transcript, which is known to be DNA damage-inducible (McClanahan and

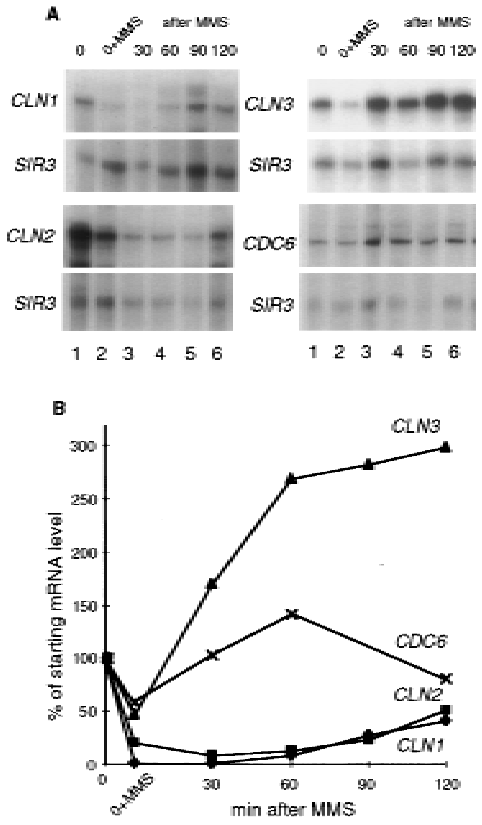

Figure 2. MMS causes reversible down-regulation of *CLN1* and *CLN2* mRNAs. (*A*) The *cdc4-1* (BY665) strain was arrested as described above and divided in two cultures, and 0.1% MMS was added to one part for 30 min, whereas the other part was incubated without MMS for 30 min and harvested (0). An aliquot was harvested out of the MMS-containing culture (0+MMS), and in the rest of the culture, MMS was inactivated and the culture was filtered into the fresh 37°C media and allowed to recover from MMS at this temperature. Aliquots were taken throughout the time of recovery (30, 60, 90, and 120 min after the release from MMS). *CLN1, CLN2, CLN3, CDC6,* and *SIR3* mRNA levels were measured by S1 protection in these aliquots. (*B*) mRNA levels shown in *A* were quantitated, normalized, and plotted. The mRNA levels seen in MMS and after MMS removal are expressed as the percentage of the starting normalized mRNA level seen at 37°C in the untreated culture. Note that the 90-min time point for *CDC6* mRNA could not be reliably normalized to its internal control, because the latter was apparently lost from this sample on the gel.

McEntee 1984; Ruby and Szostak 1985), underwent virtually no reduction in MMS and was elevated after MMS removal (data not shown). The *CDC6* transcript level was reduced <50% in MMS and was rapidly restored after MMS was removed (Fig. 2). The *CLN3* transcript also dropped by 50% in the presence of MMS but was actually induced to a reproducibly higher level shortly after MMS removal. We have noted the presence of STRE elements and three short stretches of homology to the DRE (damage response element) of *RNR2* (Elledge and Davis 1989) in the *CLN3* promoter, which could be responsible for this damage-induced increase. In addition, the *HIS3, MAT***a1,** and *SIR3* mRNAs, which were used as internal controls, displayed little or no sensitivity to MMS within the range of MMS concentrations and incubation times applied in our study. Therefore, although high levels of MMS and/or prolonged exposure to it eventually eliminate all transcripts (data not shown), the *CLN1* and *CLN2* transcripts are consistently more sensitive to it than the other transcripts we have monitored.

Start delay in MMS-treated cells can be reduced by deregulation of CLN *transcription*

The experiments shown so far suggest that *CLN* transcript levels may be directly affected by MMS treatment. To see whether this down-regulation of *CLN* levels is sufficient to explain the observed delay of S phase in damaged cells, we asked whether modest increases in *CLN* expression could shorten the delay. To do this, we first tried a strain producing elevated levels of the Swi4 transcription factor, which is a primary activator of *CLN1* and *CLN2* transcription (for review, see Breeden 1996). We found that simply overproducing Swi4 from the *GAL* promoter does not noticeably reduce the G_1 delay after MMS as compared with the wild-type strain (data not shown). However, GAL induction of carboxy-terminally truncated Swi4 (*GAL::SWI4-t*) can reduce the delay of S phase after MMS treatment. The Swi4-t strain deregulates *CLN* transcription such that *CLN1* message is easily detectable in G_1 cells treated with MMS, and then it increases modestly during the recovery period to a peak level that is about twofold higher than that of the wild-type cells (Fig. 3A). Consistent with the deregulated *CLN1* levels, Swi4-t cells bud and become α-factor resistant more rapidly than wild-type cells after MMS treatment (Fig. 3B,C). Thus, it appears that increased *CLN* levels allow a partial bypass of the MMS-induced delay of Start. This is not particularly surprising in view of the observed reduction of the *CLN* transcripts in response to MMS (Fig. 1), but it argues against an alternative model, which is that the G_1 delay mechanism in response to DNA damage operates exclusively via CDK inhibition.

Checkpoint mutants have a shorter G_1 delay after MMS treatment than wild-type cells

Mutations in *MEC1, RAD53,* and *RAD9* make cells defective in G_1 delay upon UV and ionizing radiation damage (Siede et al. 1993, 1994, 1996), and they are sensitive to MMS (Paulovich and Hartwell 1995; Paulovich et al. 1997a). These mutants are also defective in their G_1 delay after MMS treatment. Figure 4 shows the kinetics of budding and DNA synthesis in isogenic wild type, *rad9*Δ, *rad53-11,* and *mec1-1* checkpoint-defective mutants. In the absence of damage, these mutants and the wild-type cells have a similar rate of progression through the G_1/S transition (Fig. 4A). By 30 min after release, all three of the untreated strains have budded and entered S phase. However, after treatment with a pulse of MMS damage, (Fig. 4A,B), the wild-type cells delay entry into S

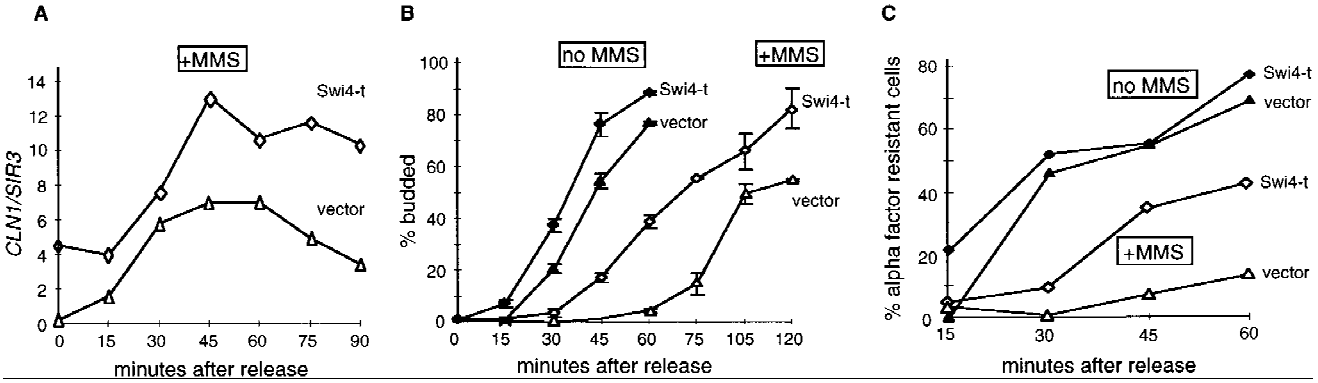

Figure 3. Overproduction of the carboxy-terminally truncated Swi4, Swi4-t, diminishes the MMS sensitivity of *CLN1* mRNA and shortens the delay of budding and of the S-phase entry after MMS treatment. (*A*) The wild-type W303-1a strains transformed with the vector (pBD860) or with the *GAL::SWI4-t* plasmid (pBD1168) were arrested by α-factor in rich media with raffinose. Galactose was added to induce Swi4-t overexpression 1 hr before the release, and in 30 min 0.1% MMS was added. After an additional 30 min, cells were released both from MMS and α-factor and allowed to grow in rich media with galactose. Aliquots were collected before the release and in 15-min intervals after it to perform S1 protection measurements of *CLN1* and *SIR3* RNA levels. These levels were then quantitated, and *CLN1* was normalized to *SIR3* and plotted. (*B*) The same two strains were arrested by α-factor as before, and a half of the culture of each strain was treated with 0.1% MMS for 30 min before the release from α-factor arrest. Aliquots were taken to count the percentage of budded cells. Note that the budding results shown in *B* are the average of two independent experiments. (*C*) The wild-type W303-1a strain with vector (pBD860) or with the *GAL::SWI4-t* plasmid (pBD1168) were arrested and treated with MMS as described in *B*. After the release from α-factor alone or MMS and α-factor, the cultures were allowed to grow in rich media with galactose and aliquots were removed and placed into microtiter plate wells. To these aliquots, α-factor (at 10 μg/ml) was added. α-Factor samples were incubated for 30–45 min, then formaldehyde was added to 4% to fix the cells, and the percentage of budded cells was counted.

phase and are still primarily in G_1 as judged by budding (Fig. 4A) and FACS analysis (Fig. 4B) at the 150-min time point, whereas the *rad9*Δ, *rad53-11*, and *mec1-1* strains go through this transition ~30 min faster. The checkpoint-defective strains also go through Start earlier than wild type, as judged by α-factor resistance (Fig. 4C). In-

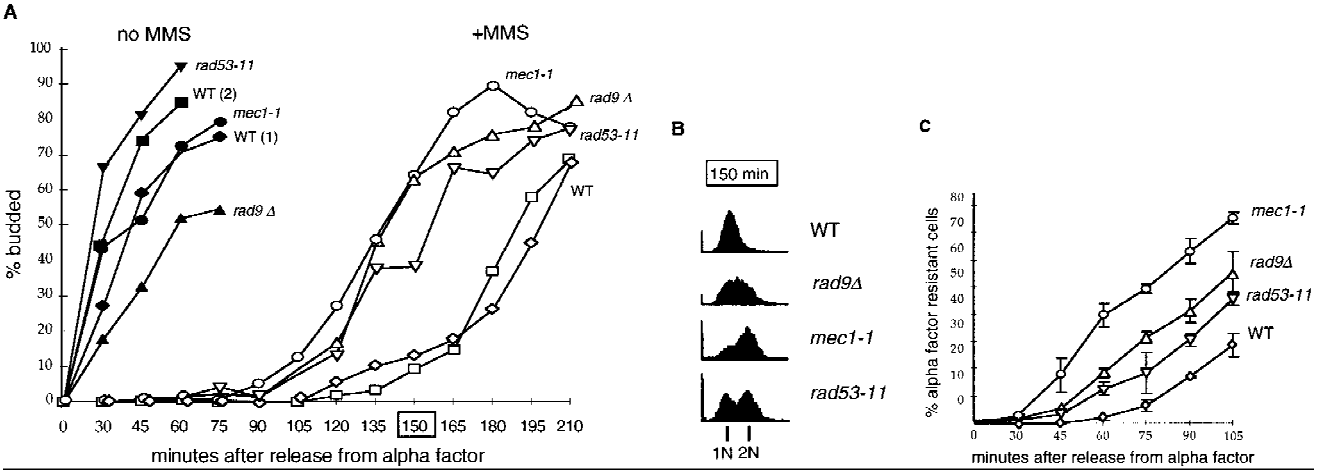

Figure 4. The delay of budding and S-phase entry is shorter in *mec1-1*, *rad53-11*, and *rad9*Δ strains than in the wild type. (*A*,*B*) The isogenic wild type (BY2006), *mec1-1* (BY2226), *rad53-11* (BY2007), and *rad9*Δ (BY2227) strains were arrested by α-factor and released into fresh media with or without 0.2% MMS. The ''no MMS'' cultures were allowed to progress into the cell cycle. In the ''+MMS'' cultures, after incubation for 30 min, MMS was inactivated and the cultures were filtered, resuspended in another change of media, and allowed to grow. (*A*) Aliquots were taken from treated and untreated cultures to count the percentage of budded cells. Note that the 30-min time point for MMS-treated cultures corresponds to the moment of release from MMS. In *B*, the DNA content for the cells shown in *A* was determined by FACS and an example of DNA content distribution for the 150-min time point is presented. The 150-min time point for which FACS data are shown is marked by a box in *A*. (*C*) The same four strains were arrested by α-factor, and 0.2% MMS was added for the last 30 min before the release from the pheromone. After that, MMS was inactivated and the cultures were filtered into the fresh media. Samples were taken in 15-min intervals and transferred to microtiter plates with 10 μg/ml of α-factor, as described in the legend to Fig. 3. Percentage of budded cells was scored after 30 to 45-min incubation in α-factor. Presented are the average values of two independent experiments.

terestingly, the *mec1-1* mutant appears to bud and become α-factor resistant more rapidly than the others. In summary, just as was observed for *GAL::SWI4-t*, in the absence of checkpoint function, the MMS-induced delay of Start is clearly diminished but not completely eliminated.

A RAD53 *checkpoint mutant treated with MMS recovers* CLN1 *transcription faster than wild type*

In the signal transduction cascade from DNA damage to the cell cycle machinery targets, Rad53 is thought to be the most proximal of all the known checkpoint genes to the targets (Navas et al. 1996; Sanchez et al. 1996; Sun et al. 1996). Because *mec1-1*, *rad9*Δ, and *rad53-11* mutants exhibit a similarly shortened G_1 delay after MMS treatment, we chose to characterize the *rad53-11* mutant in more detail. As expected on the basis of Figure 4A, control and *rad53-11* cells express comparable levels of *CLN1* mRNA after release from α-factor arrest in the absence of DNA damage (Fig. 5A, B). When these cells are treated with MMS, the initial down-regulation of *CLN1* mRNA is about the same in the *rad53-11* and wild-type cells (Fig. 5C,D; data not shown). However, the recovery of high *CLN1* mRNA levels is more rapid in the *rad53-11* mutant than in the wild-type cells (Fig. 5C, D).

Because we have shown that *CLN* mRNA down-regulation and recovery are not dependent on cell cycle progression, we were interested to see whether the same is true for the fast recovery of *CLN* transcripts in the *rad53-11* strain. *cdc4 rad53-11* double mutants were constructed (see Materials and Methods), and the *cdc4* arrests and MMS treatments were repeated as described for Figure 2. In this case, the results of two experiments with two independent *cdc4 rad53-11* isolates were quantitated and used to produce the plot shown in Figure 6A. It is clear from this analysis that *cdc4 rad53-11* strains arrested in G_1 recover their *CLN1* message levels faster than the *cdc4 RAD* strain. During the *cdc4* arrest, *CLN1* transcript levels are slightly lower in the *cdc4 rad53-11* strain as compared with the *cdc4* strain (data not shown); so its ability to recover *CLN1* mRNA after MMS treatment cannot be a result of the unusually high *CLN1* levels.

Swi6 mutants treated with MMS also recover CLN1 *transcription faster than wild type*

GAL::SWI4-t is capable of reducing the MMS-induced delay of Start, but *GAL*-induced wild-type Swi4 is not.

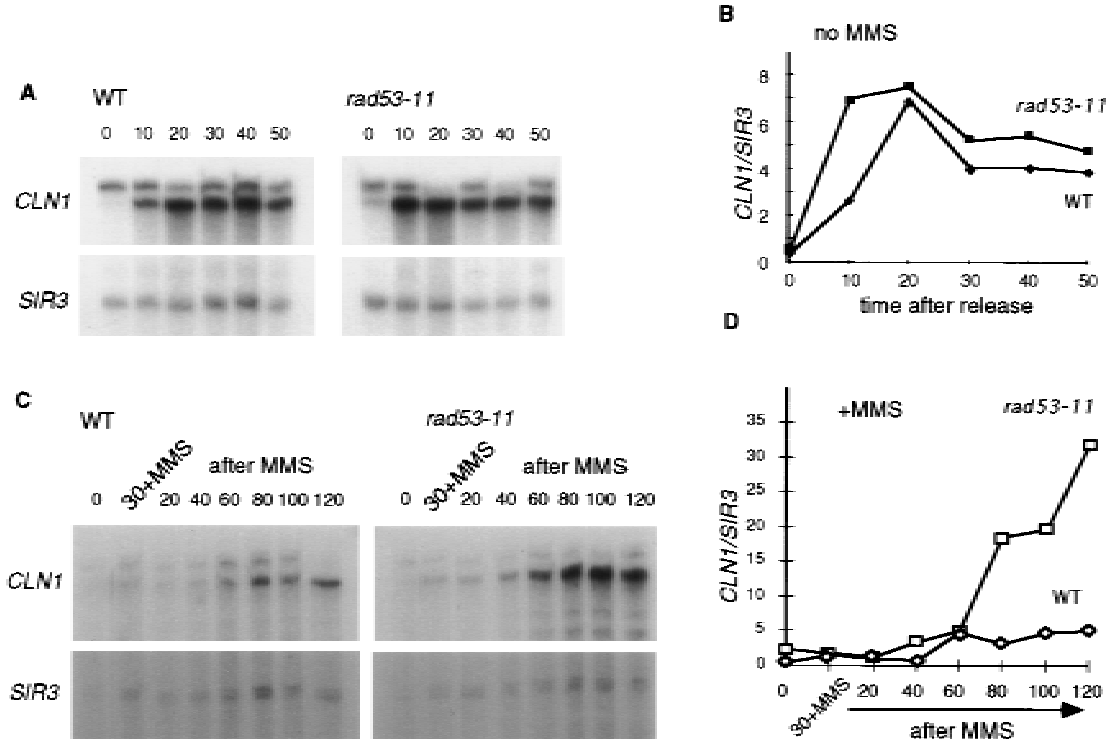

Figure 5. The *rad53-11* strain has a faster rate of *CLN1* transcript recovery after MMS than the wild type. (*A*) The isogenic wild-type (BY2006) and *rad53-11* (BY2007) strains were arrested by α-factor and released as usual. Aliquots were taken in 10-min intervals for 50 min, RNAs were isolated, and the levels of *CLN1* and *SIR3* were measured by S1 protection. (*B*) Data obtained in *A* were quantitated as above. (*C*) The same two strains were treated with MMS in the same way as described in the legend to Fig. 4A. Aliquots were taken before the release from α-factor (0), with 0.2% MMS (30+MMS) and at intervals after MMS was removed. Levels of *CLN1* and *SIR3* were measured by S1 protection. (*D*) The *CLN1* and *SIR3* levels shown in *C* were quantitated, and *CLN1* was normalized to *SIR3* and plotted.

Swi4-t is more stable than wild-type Swi4, but both proteins are expressed at such high levels under these conditions that this difference is unlikely to be the reason for the selective deregulation of *CLN* transcripts by Swi4-t. The other important difference between Swi4 and Swi4-t is that Swi4-t has lost the carboxy-terminal domain necessary for association with Swi6 (Sidorova and Breeden 1993). Thus, we wondered whether Swi6 could be the target of negative regulation by the Rad53 pathway under conditions of DNA damage. *swi6* mutants grow slowly and do not recover synchronously from α-factor arrest; so to avoid these complications, we generated *cdc4 swi6*Δ and *cdc4 SWI6* strains. These cells were arrested at the G_1/S transition, and *CLN1* transcript was monitored after cells were treated with a pulse of MMS. As can be seen in Figure 6B, the absence of Swi6 does not eliminate the drop of *CLN1* transcript levels upon MMS treatment, but, like *rad53-11*, it allows a faster recovery of *CLN1* transcription after MMS (Fig. 6A). Thus, the maintenance of low *CLN1* mRNA levels in response to DNA damage is Rad53 dependent and Swi6 dependent. The requirement for Swi6 to inhibit *CLN* expression is surprising because it was first identified as an activator of transcription. However, this result could be explained if Swi6 is modified in response to DNA damage to a form that causes the Swi4/Swi6 complex to repress rather than activate transcription.

Swi6 undergoes MMS-inducible Rad53-dependent phosphorylation in vivo

Rad53 is a dual-specificity protein kinase whose consensus phosphorylation site is not known (Zheng et al. 1993). Upon treatment with DNA-damaging agents such as hydroxyurea and MMS, Rad53 undergoes phosphorylation, which alters its mobility in SDS-PAGE (Sanchez et al. 1996; Sun et al. 1996). This phosphorylation is performed by an upstream kinase, possibly Mec1, rather than by Rad53 itself (Sanchez et al. 1996; Sun et al. 1996). In vitro Rad53 can phosphorylate itself (Zheng et al. 1993; Fay et al. 1997) and histone H1 (Sun et al. 1996), and Rad53 kinase activity is increased upon treatment with a checkpoint-activating agent, hydroxyurea (Sun et al. 1996). One potential target of Rad53 in living cells is the Dun1 kinase, which is phosphorylated in a Rad53-dependent, MMS-inducible manner (Allen et al. 1994). To see whether MMS also affects the Swi6 phosphorylation pattern, we monitored Swi6 phosphorylation by peptide mapping using the same MMS treatment conditions we used to follow *CLN* transcription. Wild-type cells were arrested in G_1 and released into radioactive orthophosphate-containing media, and MMS was added to one-half of the culture for 40 min. Extracts were made, Swi6 was immunoprecipitated from these cells, and its phosphorylation state was determined by peptide mapping. Figure 7A shows that upon MMS treatment Swi6 gains phosphates on at least two new peptides (designated a and b). This phosphorylation can be acquired as early as 20 min after introduction of MMS, and it persists for at least 90 min after MMS is eliminated (data not

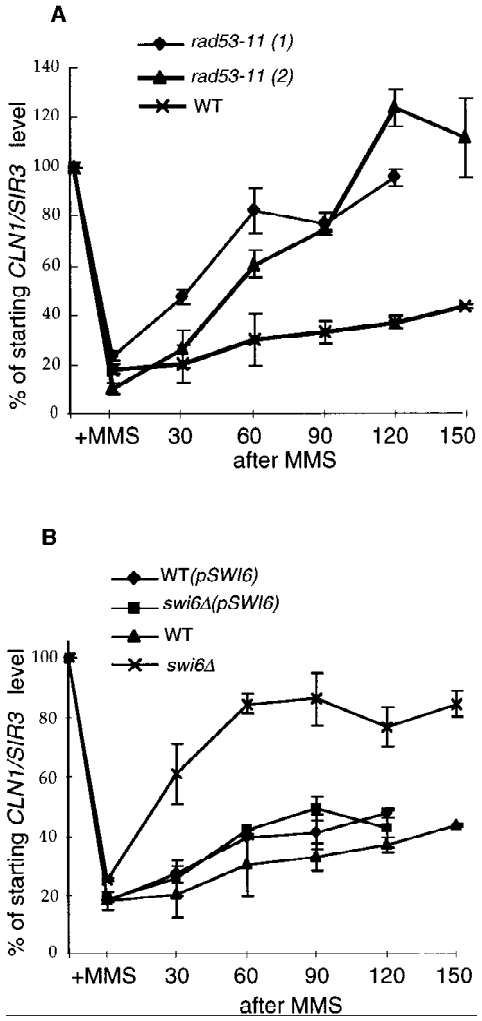

Figure 6. The *CLN1* transcription is recovered faster in the *cdc4 rad53-11* and *cdc4 swi6*Δ strains than in the *cdc4* strain upon MMS treatment during the arrest at nonpermissive temperature. The *cdc4* (BY2240), two isolates of *cdc4 rad53-11* (BY2243 and BY2287) and *cdc4 swi6::TRP1* (BY2241) strains, BY2240 transformed with pBD1265 (with *SWI6* gene), and BY2241 transformed with pBD1265 were treated the same way as described in the legend to Fig. 2. They were first arrested at 37°C for 3 hr, and then an aliquot from both cultures was collected to serve as control (0), and to the rest of the cultures, 0.1% MMS was added for 30 min (+MMS). Then MMS was inactivated and removed by filtration, and the cultures were resuspended in the fresh 37°C media and incubated at this temperature for up to 150 min. Aliquots were taken throughout the time of recovery from MMS. RNAs were isolated, and *CLN1* and *SIR3* transcript levels were measured. In each case, data from two to three experiments were quantitated and their average plotted. The mRNA levels seen in MMS and after MMS removal are expressed as the percentage of the starting normalized mRNA level seen at 37°C in the untreated culture. *wt, cdc4* BY2240, *wt(pSWI6), cdc4* BY2240 transformed with *pSWI6* pBD1265, *rad53-11 (1)* and *rad53-11 (2), cdc4 rad53-11* BY2287 and BY2243, respectively, *swi6*Δ, *cdc4 swi6::TRP1* BY2241, *swi6*Δ*(pSWI6) cdc4 swi6::TRP1* BY2241 transformed with *pSWI6* pBD1265.

shown). Thus, these phosphorylations are temporally correlated with the interval during which *CLN* mRNA levels remain low.

We then asked if this phosphorylation of Swi6 occurs in a checkpoint-defective *RAD53* mutant, *rad53-11*, which is incapable of delaying G_1 or of prolonging the inhibition of *CLN1* transcription upon MMS treatment. The *rad53-11* strain was synchronized and labeled as above, and the Swi6 phosphorylation pattern was analyzed (Fig. 7B). In contrast to the isogenic wild-type cells, there was no MMS-inducible phosphorylation of Swi6 isolated from the MMS-treated *rad53-11* strain. Therefore, this phosphorylation of Swi6 requires the checkpoint-proficient Rad53 kinase.

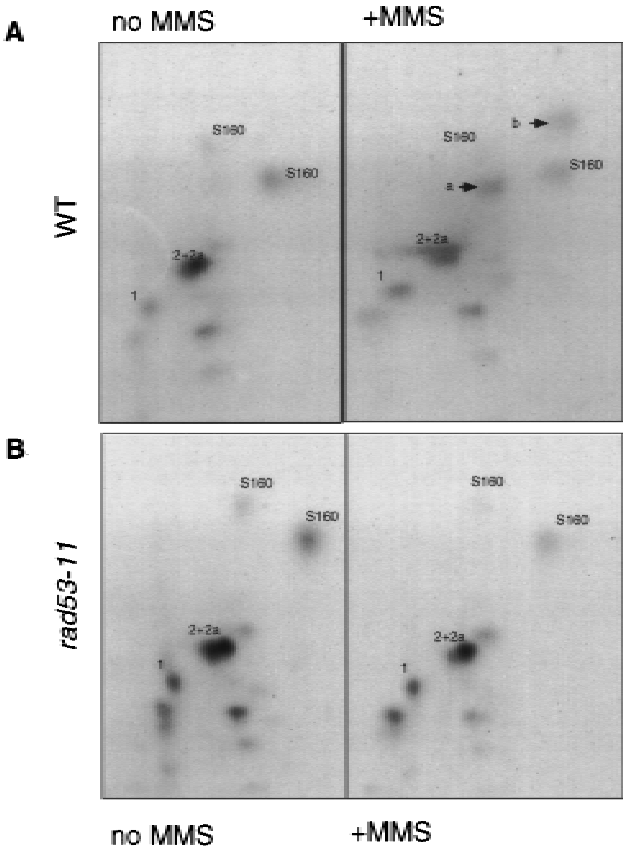

Figure 7. The Swi6 protein undergoes MMS-inducible *RAD53*-dependent phosphorylation. (*A*) The wild-type (BY2006) strain was arrested in G_1 by α-factor and released into radiolabeled orthophosphate-containing media. MMS (0.1%) was added to one-half of this culture for 40 min, after which time both cultures were harvested. Swi6 was immunoprecipitated out of extracts made from harvested cells, digested with trypsin, and resolved on TLC plates as described in Materials and Methods. MMS-inducible peptides *a* and *b* are marked by arrows. (*B*) The *rad53-11* (BY2007) strain was arrested by α-factor and released as in *A*, 0.1% MMS was added to one-half for 40 min, and then both halves were harvested and treated as above. S160 stands for serine-160-containing peptides (Sidorova et al. 1995) that are marked here for the reference. Other major constitutive phosphopeptides of Swi6 are numbered.

Rad53 or an associated kinase phosphorylates Swi6

To begin to address whether this phosphorylation of Swi6 is performed directly by Rad53, we sought to reconstitute the MMS-inducible, Rad53-dependent kinase reaction in vitro. First, Rad53 was immunoprecipitated out of untreated and MMS-treated wild-type (WT) or *rad53-11* cells, and radioactive ATP was added to monitor phosphorylation. As seen in Figure 8A, the wild-type Rad53 immunoprecipitate incorporates phosphate into a single polypeptide of ~90 kD that has been shown previously to be Rad53 itself (Zheng et al. 1993; Sun et al. 1996; Fay et al. 1997). This basal level of Rad53 kinase activity is relatively low but is markedly stimulated by MMS. In contrast, we observe a very low kinase activity in immunoprecipitates of the checkpoint-defective Rad53 isolated from the isogenic *rad53-11* strain. Moreover, this low kinase activity cannot be stimulated by MMS (Fig. 8A, lane 4), despite the fact that Rad53-11 undergoes the mobility shift that is correlated with the Mec1-dependent phosphorylation and activation (data not shown). This could indicate that the *rad53-11* defect is in its catalytic function, though more complicated scenarios could be proposed. It is of interest because *RAD53* is an essential gene (Zheng et al. 1993), and *rad53-11* is a viable, checkpoint-deficient allele (Weinert et al. 1994) that has not been characterized in vitro previously. Here, it serves as the ideal negative control, allowing us to conclude that the kinase activity we observe in this assay is Rad53 dependent.

We then performed a Rad53 kinase reaction with exogenously added recombinant Swi6 (Sidorova and Breeden 1993). Because Swi6 and the phosphorylated form of Rad53 migrate very closely in SDS-PAGE, Swi6 was immunoprecipitated from each reaction before electrophoresis (see Materials and Methods for details). As seen with Rad53, we observed highly efficient phosphorylation of Swi6 that was MMS induced and Rad53 dependent (Fig. 8B). The checkpoint-defective Rad53-11, as well as wild-type Rad53 from untreated cells, incorporated about an order of magnitude less phosphate into Swi6 than the wild-type kinase precipitated from MMS-treated cells. Two possibilities could account for this effect. The kinase activity of the Rad53 from undamaged cells could be too low to incorporate substantial radioactivity into Swi6. Alternatively, MMS could change the specificity of Rad53. We believe that the former possibility is more likely the case, as Rad53 isolated from undamaged cells that overexpresses Rad53 by ~10-fold (Zheng et al. 1993; data not shown) is capable of incorporating substantial amounts of phosphate into Swi6 (Fig. 8C). Thus, the ability to phosphorylate Swi6 is not restricted to the MMS-activated form of Rad53.

To see whether the pattern of Rad53-dependent phosphorylation of Swi6 was similar in vivo and in vitro, we used the higher resolution method of peptide mapping. Figure 9, A and B, shows peptide maps of in vivo labeled Swi6, and in this experiment three Rad53-dependent, MMS-inducible phosphopeptides can be readily detected (marked a, b, c). Figure 9C shows the peptide map of

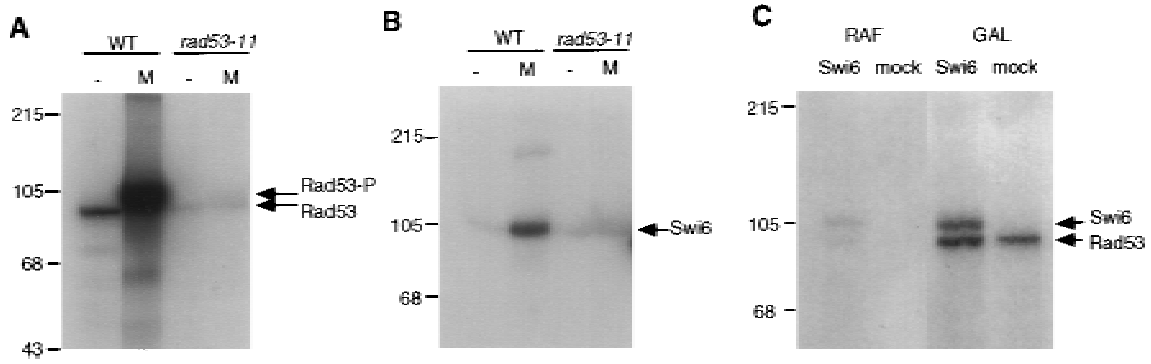

Figure 8. The MMS-inducible, Rad53-dependent phosphorylation of Swi6 can be reconstituted in vitro. (*A*) Rad53 was immunoprecipitated out of lysates of the wild-type BY2006 and *rad53-11* BY2007 cells that were treated (M) or not treated (−) with 0.1% MMS for 40 min. Radiolabeled ATP was added to immunoprecipitates to allow phosphorylation, and reactions were stopped with SDS loading buffer, boiled, and resolved on SDS-PAGE. The arrows mark the position of Rad53, both the steady-state form (Rad53) and the damage-modified form (Rad53-P), and positions of protein molecular weight markers are shown at *left*. (*B*) Rad53 was immunoprecipitated as in *A*, and recombinant Swi6 was added along with radiolabeled ATP. Reactions were stopped by addition of AB buffer (see Materials and Methods), and Swi6 was released by incubation at 4°C for 30 min and immunoprecipitated with Swi6 antibodies. These immunoprecipitates were resolved on SDS-PAGE. Swi6 position is marked by an arrow. (*C*) Wild-type W303 1A strain transformed with *pGAL::RAD53* pBD2146 was grown either in raffinose (RAF) or galactose (GAL) to induce overexpression of *RAD53* (see Materials and Methods). Protein extracts were prepared out of these cells, Rad53 was immunoprecipitated, and kinase assays with recombinant Swi6 (Swi6) or mock preparation (mock) (see Materials and Methods) were performed as described above. Products of kinase reactions were directly loaded onto SDS PAGE. Swi6 and Rad53 positions are marked by arrows.

Swi6 phosphorylated in vitro by Rad53 that has been immunoprecipitated from the cells overproducing Rad53 from a *GAL* promoter. Among the products of this reaction are three peptides whose positions of migration are indistinguishable from those of the inducible, Rad53-dependent peptides a, b, and c seen in vivo (Fig. 9B). Figure 9D confirms that these prominent peptides are derived from Swi6, as they are not detectable among the products of the kinase reaction between Rad53 and a mock substrate, which has been prepared from bacteria harboring vector instead of the *SWI6*-expressing plasmid. In summary, Swi6 undergoes MMS-induced and Rad53-dependent phosphorylations in vivo, and these phosphorylations appear to be reproducible in vitro with immunoprecipitated Rad53.

Discussion

MMS-induced G_1 delay

Previous studies have established that a yeast cell that receives treatment with UV or ionizing irradiation in G_1

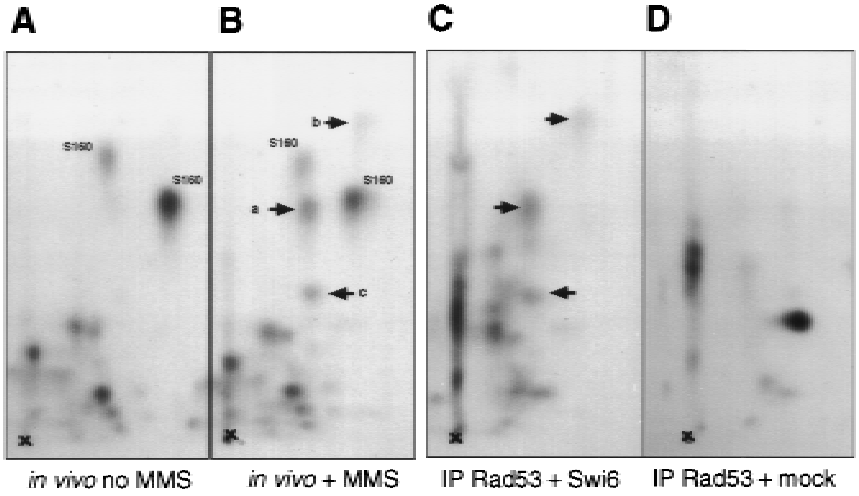

Figure 9. Rad53-dependent phosphopeptides of Swi6 generated in vivo and in vitro migrate to similar positions on phosphopeptide maps. (*A*,*B*) Wild-type (BY2286) strain was synchronized in G_1 by α-factor and released into radiolabeled orthophosphate-containing media, and 0.1% MMS was added to one-half of this culture for 40 min, after which time both halves were harvested. Phosphopeptide maps of Swi6 isolated out of these two cultures were generated as described for Fig. 7 (see Materials and Methods for details). MMS-inducible peptides are marked by arrows. Three of such peptides could be detected (a, b, c). Serine 160-containing peptides (S160) are marked for the reference. (*C*,*D*) Kinase assays were performed as in Fig. 8C with Rad53 isolated out of the wild-type strain overexpressing *RAD53* from the *GAL* promoter (BY2292) with Swi6 (*C*) or mock preparation (*D*) as a substrate. Radioactively labeled products were resolved on SDS-PAGE as in Fig. 8C. Swi6 and a corresponding region out of the SDS-PAGE lane where mock preparation was run were excised, and proteins were eluted and subjected to phosphopeptide analysis as in Figs. 7 and 9A,B. Rad53-dependent in vitro peptides that migrate similar to the MMS-inducible Rad53-dependent peptides generated in vivo are marked by arrows. (**x**) Map origins.

can undergo a transient and checkpoint-dependent arrest in G_1 (Siede et al. 1993, 1994). In this paper we show that a short pulse of MMS, administered either during an α-factor-induced G_1 arrest or immediately after release from the pheromone treatment, can produce a similar effect. With MMS doses of 0.1%–0.2%, these cells remain in G_1 and do not enter S phase for up to 90 min after withdrawal of the genotoxic agent. The MMS-damaged G_1 cells show a prolonged sensitivity to α-factor that indicates that they pause at or before Start. As with the UV studies, this G_1 delay is also dependent on the *RAD53, MEC1,* and *RAD9* genes (Siede et al. 1993, 1994, 1996; Weinert et al. 1994). However, in our studies, we have found that these checkpoint mutants only shorten but do not eliminate the delay of Start, suggesting that there may be other contributing factors. In addition, we observe that this G_1 delay is correlated with a delay in the accumulation of *CLN1* and *CLN2* transcripts.

Recently, Paulovich and Hartwell (1995) showed that continual treatment of G_1 cells with low levels of MMS (0.03%) induces a profound slowing of S phase, which is dependent on *RAD53* and *MEC1* and to a lesser degree, on *RAD9, RAD17,* and *RAD24* (Paulovich et al. 1997a). We have reproduced this finding and observed a dosage dependence to the cell's response. For example, administering 0.05% MMS to cells that were released from α-factor-induced G_1 arrest causes a <50% decrease in *CLN* mRNA levels (data not shown) and no significant G_1 delay because these cells will traverse to S phase almost as fast as untreated controls (J. Sidorova, unpubl.). MMS (0.1%) is sufficient to induce a G_1 delay if cells are treated during the α-factor arrest (Figs. 1 and 3), but 0.2% MMS is required if it is administered immediately after release (Fig. 4; J. Sidorova, unpubl.). These observations may be explained by the fact that cells continue to grow during an α-factor arrest and they attain the critical size required to go through to S phase very rapidly upon release. In this context, a low dose of MMS may not cause enough damage to trigger a response before cells exit G_1. This is consistent with the possibility that there is a threshold of damage needed to trigger a slowdown in G_1 or that the delay can only be triggered at an early step in G_1 progression. Alternatively, MMS-induced lesions may be generated too slowly to occur in this time frame or they may be less readily recognized in G_1 than in S and G_2 cells (Paulovich et al. 1997b).

Another striking feature of the checkpoint-dependent response to MMS damage in G_1 is that G_1 cells delay only briefly and then progress into a slow S phase. This slow S is most likely owing to incomplete repair of the damage because it is also checkpoint dependent (Fig. 4; Paulovich and Hartwell 1995). Thus, it appears that the G_1 delay is quite transient and not tightly coupled to the presence of damage. This is in contrast to the G_2 arrest, which can last for many hours after a single double-strand break introduced by HO endonuclease (Sandell and Zakian 1993). Although the extent of the delay may certainly be affected by the type of DNA damage, one can also hypothesize that a tighter G_2 arrest has evolved to protect the cell from an irreversible loss of genetic information that would occur during mitosis of unrepaired chromosomes. As such, the G_2 checkpoint arrest may be fundamentally different from the transient delay that occurs in G_1 cells, despite the fact that the same checkpoint genes are used to detect the damage.

MMS causes the loss of CLN1 *and* CLN2 *mRNAs*

In principle, pausing or slowing down during a given phase of the cycle could be achieved by reducing the amount of the cyclin/Cdk kinase that is active during that phase and/or by preventing the switch from one Cdk form to the other, for example, from Cln/Cdk to Clb/Cdk in the case of the G_1/S transition. These effects could be achieved by direct inhibition of kinase activity, depression of cyclin or CDK protein levels, or inhibition of their transcription. In the case of DNA damage-induced delay in G_1, we have found that *CLN1* and *CLN2* message levels are down-regulated by MMS in a dosage-dependent manner and are kept low for some time after the damage. Subsequently, the levels of these messages are restored slowly. The loss of *CLN* transcripts that occurs after brief exposure to 0.1% MMS cannot be attributed to a global interference with the cell's transcriptional or post-transcriptional activity. Although this nonspecific interference certainly takes place after prolonged incubation with MMS (J. Sidorova, unpubl.), the *CLN1* and *CLN2* messages are more sensitive to the short exposures and low dose of MMS used in this study than are several other messages. Moreover, *CLN1* sensitivity to MMS can be reduced by overexpression of a truncated form of its transcriptional activator, Swi4-t.

The MMS-induced loss of *CLN1* and *CLN2* messages is reversible, and the rate of recovery of these messages is dependent on Rad53 function. Rad53 slows the recovery of *CLN* transcript levels both in cycling cells and in G_1-arrested cells. Because this effect is observed in arrested cells, it cannot be an artifact of the differences in the rate of G_1 progression. Loss of Rad53 function speeds the recovery of *CLN* transcript levels and reduces the delay of Start. Deregulated expression of *CLN1* and *CLN2* can also reduce the G_1 delay in response to DNA damage. Thus, the simplest interpretation of these findings is that Rad53-dependent inhibition of *CLN* transcription contributes to the delay of Start in MMS-treated cells. Rad53 is known to play a role in transcriptional activation of genes required for DNA repair (Aboussekhra et al. 1996; Kiser and Weinert 1996; Navas et al. 1996), and our data indicate that this kinase may have an additional role in repressing transcription of genes that promote progression through the cell cycle.

The role of Swi4 and Swi6 in MMS-induced down-regulation of CLNs

The Swi4/Swi6 complex induces G_1/S-specific transcription of *CLN1* and *CLN2*. Swi4 is the DNA-binding subunit of the complex, and in the absence of Swi4, *CLN* transcription is greatly reduced (Nasmyth and Dirick 1991; Ogas et al. 1991; Cross et al. 1994; Stuart and Wit-

tenberg 1994; Partridge et al. 1997). Lack of Swi6 leads to a constitutive intermediate level of *CLN* transcription (Dirick et al. 1992; Lowndes et al. 1992). These data have led to the view that Swi4 is the primary activator of *CLN* transcription and Swi6 plays a regulatory role, both enhancing and repressing *CLN* transcription depending on the phase of the cell cycle. It is therefore possible that Swi6 may also regulate the activity of the Swi4/Swi6 complex in response to DNA damage. Swi6 could be modified by a DNA damage-dependent mechanism and shift from an activating to a repressing component of the Swi4/Swi6 complex. This would repress *CLN* transcription and delay the G_1/S transition. In agreement with this idea, the absence of Swi6, but not Swi4, causes cells to lose viability rapidly in the presence of MMS (Johnston and Johnson 1995). In addition, we find that the lack of Swi6 can actually increase the rate of *CLN1* transcript recovery after MMS, similar to the effect observed in cells with defective Rad53 (Fig. 6). Finally, we have observed that Swi6 undergoes an MMS-dependent change in phosphorylation in wild-type but not *rad53-11* cells. In vivo, we observe two to three new phosphorylations on Swi6, which are induced by MMS treatment, and these phosphorylations are present throughout the period when *CLN* transcription is low. In vitro Rad53, obtained from MMS-treated wild-type yeast cells or from cells overexpressing this kinase from the *GAL* promoter, can phosphorylate exogenous Swi6, and three phosphopeptides, which appear to comigrate with the MMS-inducible in vivo phosphopeptides, are observed. The *rad53-11* strain is unable to promote MMS-inducible phosphorylation of Swi6 in vivo or in vitro. This mutant kinase has a dramatically reduced activity as judged by its decreased ability to phosphorylate itself, and it does not support phosphorylation of the exogenous Swi6. Taken together, all these observations strongly indicate that Swi6 is either a direct substrate of a damage-activated Rad53 in living cells or it is phosphorylated by a Rad53-activated kinase that is associated with Rad53.

These results give rise to the following model for the checkpoint induced by DNA damage (Fig. 10). When G_1 cells are subjected to DNA damage by MMS, there is an immediate drop in *CLN1* and *CLN2* messages that is Rad53-independent. This drop could be mediated by specific promoter elements or by alterations in mRNA stability, and this would be expected to rapidly reduce the rate of G_1 progression. This delay is extended by a Rad53- and Swi6-dependent inhibition of further *CLN* transcription. Temporally correlated with this inhibition, there is a Rad53-dependent change in the phosphorylation state of Swi6, which we speculate may inhibit Swi6 function and provide the mechanism for repressing *CLN* transcription.

The inhibition of *CLN* expression that we observe in response to MMS may not be universal for every kind of damage but is likely to be one of the mechanisms that delay Start in response to DNA damage. Moreover, the recent finding that ectopic overproduction of *CLN1* can increase genomic instability in wild-type cells and leads to even more instability and cell death in checkpoint-deficient *mec1-1* cells (Vallen and Cross 1995) suggests that the tight control over *CLN* levels may be crucial not only for cells with damaged DNA but also for undamaged cells. At the same time it is clear that the loss of *CLN* transcripts is certainly not the only mechanism involved in delaying Start, because ectopic expression of *CLN*s does not eliminate the delay completely. It is ultimately possible that there are several levels of regulation within G_1, both cyclin-mediated and independent, that help to protect cells from DNA damage.

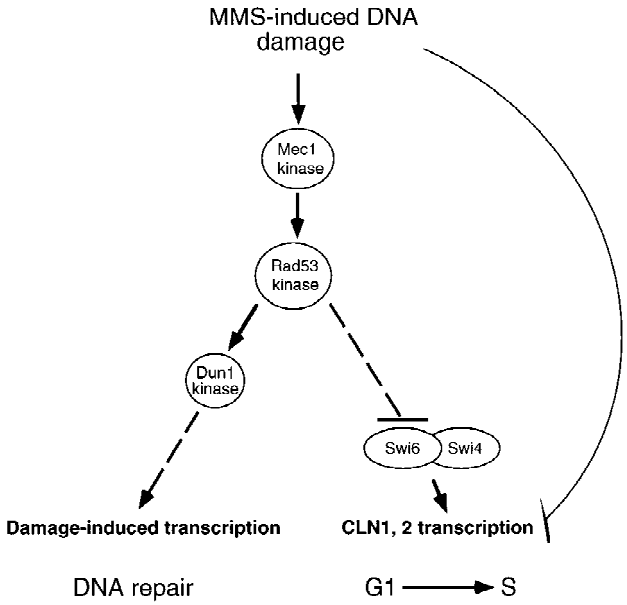

Figure 10. A model for the MMS-induced changes in transcription. As observed previously, MMS-induced DNA damage activates transcription of many genes involved in DNA repair and replication. In addition, it inhibits transcription of the G_1/S-specific cyclins *CLN1* and *CLN2*. MMS causes a rapid and prolonged repression of *CLN1* and *CLN2* transcription, owing to at least two pathways of regulation. The immediate drop in *CLN1* and *CLN2* transcription is Rad53 and Swi6 independent. Subsequent maintenance of this low level requires Rad53 and may involve Rad53-mediated inactivation of the Swi4/Swi6 complex by phosphorylation of Swi6. In this way, Rad53 plays a central role in both inducing repair of DNA damage and in delaying entry into S phase.

Materials and methods

Strains and plasmids

The yeast strains used in this study are listed in Table 1. The plasmid pBD1168 is a YCp50 vector with *GAL::SWI4-t* and was described previously (Sidorova and Breeden 1993). The pBD860 is YCp50 with no insert. The plasmid pBD1265 has been described previously (Sidorova and Breeden 1993) and is a 2μ vector with the *SWI6* gene. The plasmid pBD2146 is a kind gift of David Stern and has been described by Zheng et al. (1993) as pNB187–SPK1.

Growth conditions

All rich (YEP) and minimal (YC) media and growth conditions were as described before (Breeden and Mikesell 1991). Cultures

Table 1. *Strains used in this study*

Strain	Genotype	Source
W303-1a	*MAT***a** *ade2 his3 leu2-3,112 trp1-1 ura3*	Sidorova and Breeden (1993)
BY600	*MAT***a** *swi6::TRP1 ade2 ho::lacZ ura3 his3 leu2-3,112 trp1-1 can1-100 met2*	Sidorova and Breeden (1993)
BY665	*MAT*α *cdc4-1 ade1 ade2 leu2 lys2 ura1*	L. Hartwell (FHCRC, Seattle, WA)
BY1365	*MAT***a** *cdc28-13 ade2 ade3 leu2 trp1-1 ura3*	J. Roberts (FHCRC, Seattle, WA)
BY1699	*MAT*α *cdc28-13 ade2 ura3 trp1 leu2 ho::lacZ*	BY1365 × BY600
BY1956	*MAT***a** *swi6::TRP1 swi6-38::LEU2 ade2 ho::lacZ ura3 his3 leu2-3,112 trp1-1 can1-100 met2*	Breeden collection
BY2006	*MAT***a** *ura3 leu2 trp1 his3*	Paulovich and Hartwell (1995)
BY2007	*MAT***a** *ura3 leu2 trp1 his3 rad53-11::URA3*	Paulovich and Hartwell (1995)
BY2181	*MAT*α *cdc4-1 leu2 rad53-11::URA3*	BY665 × BY2007
BY2226	*MAT***a** *ura3 leu2 trp1 his3 mec1-1::HIS3*	Paulovich and Hartwell (1995)
BY2227	*MAT***a** *ura3 leu2 trp1 his3 rad9Δ::LEU2*	Paulovich et al. (1997a)
BY2235	*MAT***a** *ura3 leu2-3,112 trp1 his3*	Breeden collection
BY2240	*MAT*α *cdc4-1 leu2*	BY1956 × BY2181
BY2241	*MAT*α *cdc4-1 leu2 trp1 swi6::TRP1*	BY1956 × BY2181
BY2243	*MAT*α *cdc4-1 leu2 rad53-11::URA3*	BY1956 × BY2181
BY2286	*MAT***a** *ura3 leu2-3,112 trp1 his3*	Breeden collection
BY2287	*MAT*α *cdc4-1 leu2 trp1 rad53-11::URA3*	BY1956 × BY2181
BY2292	*MAT***a** *leu2-3,112 ura3-52* (pNB187-SPK1)	Zheng et al. (1993)

used for labeling with $^{32}PO_4$ were allowed to double at least once in low phosphate YEP media with 2% glucose (Rubin 1975) before the experiment. For synchrony experiments, cultures were grown to an OD_{660} of 0.2 and arrested by addition of α-factor or by shifting to high temperature. Arrest with α-factor was performed with 5 mg/liter of this pheromone in low phosphate or YEP media with appropriate carbon source typically for 90–120 min. Cells were released from the arrest by filtration. *cdc* strains were arrested at 37°C for 3 hr. MMS was added from a 100% or a 10% solution directly to the cultures and the cultures were shaken vigorously to ensure resuspension of MMS and incubated for times indicated in the figure legends (typically 30 min). As a rule, immediately before washing away by filtration, MMS was inactivated by the addition of an equal volume of freshly made 10% sodium thiosulfate solution to the culture. Thiosulfate addition does not affect the delay of S phase or *CLN* levels (J. Sidorova, unpubl.). In experiments involving induction of the *GAL* promoter, cultures were grown in selective media with 2% raffinose for 12–20 hr, switched to the rich media with raffinose, and synchronized by α-factor. Galactose was added 30–60 min before the release from α-factor. GAL induction of *RAD53* expression was performed in selective media for 3–4 hr.

RNA isolation and S1 protection

These procedures were performed as described previously (Breeden and Mikesell 1991), except for the annealing of radiolabeled probes to RNAs, which was done in formamide buffer (80% formamide, 40 mM PIPES at pH 6.4, 400 mM NaCl, 1 mM EDTA at pH 8.0) at 30°C. The probes used for detection of *CLN1* and *CLN2, CLN3, CDC6, MAT***a***1, HIS3*, and *SIR3* transcripts are described (Breeden and Mikesell 1991; Foster et al. 1993; McInerny et al. 1997). mRNA levels were measured with a PhosphorImager 400A (Molecular Dynamics, Sunnyvale, CA), and the signals were quantitated using ImageQuant software.

FACS analysis

For FACS analysis of DNA content, cells were fixed in 70% ethanol for at least 1 hr, washed twice in 50 mM Tris-HCl (pH 7.8), resuspended in the same buffer, and digested with RNase (20 μg/ml) for 4 hr at 37°C. Cells were pelleted and resuspended in 125 mM Tris-HCl (pH 7.8), 105 mM NaCl, 39 mM $MgCl_2$, and 20 μg/ml of propidium iodide. Cells were analyzed on Becton Dickinson FACScan, and the obtained information was presented using CellQuest software.

Protein analysis

The $^{32}PO_4$ labeling conditions, extract preparation, Swi6 immunoprecipitation, and phosphopeptide mapping procedures were performed exactly as described (Sidorova et al. 1995). Immunoprecipitation of Rad53 for kinase assays was performed as described (Zheng et al. 1993; Sun et al. 1996) with previously characterized polyclonal antibodies, which were kindly provided by David Stern and Steve Elledge (Zheng et al. 1993; Allen et al. 1994), with the following modifications: The extract preparation and immunoprecipitation buffer [20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 0.3 M $(NH_4)_2SO_4$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin A, 1 μg/ml of sodium orthovanadate] was supplemented with 0.1% NP-40. Immunoprecipitates were washed three times with the original washing buffer (PBS, 1% Triton X-100, 10% glycerol, 100 μM sodium orthovanadate), three times with washing buffer with 0.8 M NaCl, and once with the same buffer with 1.6 M NaCl. Rad53 kinase reaction was performed as described (Zheng et al. 1993; Sun et al. 1996). Recombinant Swi6 (100–200 ng) (Sidorova and Breeden 1993) was used per reaction. In some cases Swi6 was substituted with the mock preparation, which is a protein extract fraction obtained using Swi6 purification protocol from *Escherichia coli* harboring an empty vector instead of the Swi6-expressing plasmid. Products of the kinase reaction were either loaded directly onto SDS-PAGE or Swi6 was reprecipitated out of reaction mixture. In the latter case, 24 volumes of AB buffer (20 mM Tris-HCl at pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.5% DOC, 0.3% SDS) were added to the reactions and Swi6 was eluted for 30 min at 4°C. This step was necessitated by the fact that after a kinase reaction, Swi6 is found predominantly associated with beads. After elution was performed as described,

Rad53 remained associated with agarose beads, whereas Swi6 was released (data not shown). These eluates were then separated from the protein–A agarose beads (GIBCO BRL) and incubated with anti-Swi6 polyclonal antibodies (Sidorova and Breeden 1993) and a fresh portion of protein A–agarose beads (Sidorova and Breeden 1993). Precipitates were washed with RIPA and high salt buffers (10 mM Tris-HCl at pH 7.5, 2 M NaCl, 1% NP-40, 0.5% DOC) and resolved on SDS-PAGE.

Acknowledgments

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