Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair

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In eukaryotes a cell-cycle control termed a checkpoint causes arrest in the S or G_2 phases when chromosomes are incompletely replicated or damaged. Previously, we showed in budding yeast that *RAD9* and *RAD17* are checkpoint genes required for arrest in the G_2 phase after DNA damage. Here, we describe a genetic strategy that identified four additional checkpoint genes that act in two pathways. Both classes of genes are required for arrest in the G_2 phase after DNA damage, and one class of genes is also required for arrest in S phase when DNA replication is incomplete. The G_2-specific genes include *MEC3* (for mitosis entry checkpoint), *RAD9*, *RAD17*, and *RAD24*. The genes common to both S phase and G_2 phase pathways are *MEC1* and *MEC2*. The *MEC2* gene proves to be identical to the *RAD53* gene. Checkpoint mutants were identified by their interactions with a temperature-sensitive allele of the cell division cycle gene *CDC13*; *cdc13* mutants arrested in G_2 and survived at the restrictive temperature, whereas all *cdc13* checkpoint double mutants failed to arrest in G_2 and died rapidly at the restrictive temperature. The cell-cycle roles of the *RAD* and *MEC* genes were examined by combination of *rad* and *mec* mutant alleles with 10 *cdc* mutant alleles that arrest in different stages of the cell cycle at the restrictive temperature and by the response of *rad* and *mec* mutant alleles to DNA damaging agents and to hydroxyurea, a drug that inhibits DNA replication. We conclude that the checkpoint in budding yeast consists of overlapping S-phase and G_2-phase pathways that respond to incomplete DNA replication and/or DNA damage and cause arrest of cells before mitosis.

[*Key Words*: Checkpoints; DNA replication and repair; budding yeast; cell-cycle control]

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The eukaryotic mitotic cell cycle proceeds by a series of events essentially invariant in their order; DNA replication is completed before mitosis, chromosome condensation before chromosome segregation, and mitosis before cell abscission (for review, see Hartwell and Weinert 1989; Murray 1993). The order is maintained by mechanisms that ensure the dependence of events, where late events require the completion of early events. A checkpoint is one type of mechanism that ensures the dependence of events, and studies from a variety of organisms demonstrate that a checkpoint ensures the dependence of mitosis on DNA replication (for review, see Hartwell and Weinert 1989; Murray 1993). Checkpoints appear to govern the dependence of other cell-cycle events as well [e.g., initiation of DNA replication (Kastan et al. 1991; Kuerbitz et al. 1992); chromosome segregation (Hoyt et al. 1991; Li and Murray 1991)], though some dependencies may be governed by controls other than checkpoints.

The mitotic checkpoint addressed here provides a cell the time to complete DNA replication and repair DNA damage before mitosis. This function has two related consequences for the cell. First, the checkpoint ensures that cells maintain genome integrity (e.g., remain euploid) despite the low but continuous levels of DNA damage that occur in normal cell division. Second, the checkpoint allows cells to withstand episodic DNA damage or delays in DNA replication These two consequences of checkpoint function are inferred from genetic studies of yeast mutants; the checkpoint null mutant *rad9Δ* is viable but mutant cells do lose chromosomes spontaneously when unperturbed (without radiation) at a higher rate than do wild-type cells, and *rad9Δ* cells are sensitive to radiation (Weinert and Hartwell 1990).

Other cell-cycle checkpoints apparently play similar but more complex roles than that of the *RAD9* gene. Yeast cells defective for the checkpoint gene *MAD2* are unresponsive to a defect in microtubule structure or function: *mad2* mutant cells with disrupted microtubules continue the cell cycle and die, whereas wild-type cells arrest and remain viable (Li and Murray 1991). *MAD2* plays a role in genomic stability because *mad2* mutants (in unperturbed cells) lose chromosomes at a higher rate than wild-type cells. *MAD2* is an essential

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gene; therefore, the checkpoint is either essential for normal cell division or it is nonessential and *MAD2* has a second essential cellular function. [Recent studies show that Mad2p can act as a prenyltransferase that effects protein trafficking (Jiang et al. 1993; Li et al. 1993)] The p53 gene in mammalian cells also acts in a cell-cycle checkpoint early in cell division (Kastan et al. 1991; Kuerbitz et al. 1992). Like *RAD9*, the p53 gene is not essential for cell viability (Donehower et al. 1992) but does provide for genomic stability because p53 mutants suffer gene amplification and chromosome loss (Livingston et al. 1992; Yin et al. 1992). p53 mutant cells, however, are not radiation sensitive (Lee and Bernstein 1993; Slichenmeyer et al. 1993), perhaps because they do have an intact G_2 checkpoint and can still complete DNA repair before mitosis.

Studies of the p53 gene also reveal a relationship among cell-cycle checkpoints, genomic stability, and cancer (see Hartwell 1992; Weinert and Lydall 1993). The p53 gene is found mutated in most types of human cancers. Cancer may arise more rapidly in p53 mutant cells than in wild-type cells because the checkpoint defect in mutant cells results in genomic instability and accumulation of mutations that contribute to abnormal growth.

We began dissecting the mitotic checkpoint that orders DNA replication and mitosis in the budding yeast *Saccharomyces cerevisiae*. By examining available radiation-sensitive mutants, we showed that the *RAD9* and *RAD17* genes are essential for cell-cycle arrest in the G_2 phase after DNA damage (Weinert and Hartwell 1988, 1990, 1993; Hartwell and Weinert 1989). The roles of the *RAD9* and *RAD17* genes were evaluated by comparing the cell-cycle responses of mutant and wild-type cells to radiation and to a drug that inhibits DNA replication (hydroxyurea); *rad9* and *rad17* mutants failed to arrest after irradiation (X- or UV-), but did arrest when treated with hydroxyurea. In addition the *RAD9* and *RAD17* genes also showed a striking genetic interaction with a class of *CDC* genes that encode structural genes for DNA replication enzymes, including DNA ligase (*CDC9*; Johnston and Nasmyth 1978); DNA polymerase α (*CDC2*; Sitney et al. 1989), DNA polymerase γ (*CDC17*; Johnson et al. 1985; Carson 1987), and *CDC13* [whose function in DNA replication is inferred from genetic analysis (Hartwell and Smith 1985)]. For example, DNA ligase mutants (*cdc9*) arrested in the G_2 phase (determined by flow cytometry) and remained viable after shift to the restrictive temperature, whereas *cdc9 rad9* and *cdc9 rad17* double mutants failed to cell-cycle arrest and lost cell viability more rapidly. The inactivation of the *cdc* mutant gene product apparently leads to DNA lesions that trigger arrest at the checkpoint. Cell death is probably attributable in part to cell division with unrepaired DNA breaks, although additional causes of death may include defects in DNA repair and/or defects in direct gene product interactions (e.g., defective interaction between the mutant DNA ligase and the mutant checkpoint gene protein). Failure to arrest in the G_2 phase after radiation and after inactivation of DNA replication enzymes argues that the checkpoint genes are essential for arrest in the G_2 phase after DNA damage. [We cannot distinguish whether the four mutants *cdc2*, *cdc9*, *cdc13*, and *cdc17* arrest in the late S phase or in the G_2 phase after shift to the restrictive temperature. Flow cytometry used to measure DNA content is not sensitive enough to distinguish the two possibilities. For simplicity, we will refer to arrest in the G_2 phase in these *cdc* mutants but acknowledge the ambiguity (for further discussion, see Weinert and Hartwell 1993)].

The striking interaction of *RAD9* and *RAD17* with *CDC* genes suggested the genetic strategy used here to identify additional checkpoint genes. We have used the observation that after shift to the restrictive temperature, *cdc13* mutant cells arrest and remain viable, whereas *cdc13*—checkpoint double mutants fail to arrest and die more rapidly. We identify alleles in four checkpoint genes, *MEC1*, *MEC2* (*RAD53*), *MEC3*, and *RAD9*, and also describe studies on a sixth checkpoint gene, *RAD24*, found by screening *RAD* mutants. Our studies show that all four genes are essential for arrest in the G_2 phase after DNA damage and that two of these genes have an additional role in arrest in early S phase when DNA replication is blocked. The mitotic checkpoint has discrete S-phase and G_2-phase pathways.

Results

Identification of four additional checkpoint genes

We devised a genetic screen for checkpoint mutants based on the observation that *cdc13*–checkpoint double mutants failed to arrest in the G_2 phase and died rapidly after shift to the restrictive temperature (Fig. 1) (Weinert and Hartwell 1993). *cdc13* mutants are defective for the metabolism of telomere-associated DNA; they accumulate single-stranded DNA in regions near the ends of chromosomes at the restrictive temperature (B. Garvik and L.H. Hartwell, pers. comm.). We inferred that these DNA lesions cause arrest at the checkpoint and that the low cell viability of *cdc13*–checkpoint double mutant cells may be attributable to cell division with unrepaired DNA lesions (though other explanations are possible—discussed below). *cdc13* mutants were chosen for the genetic screen, instead of other *cdc* mutants (e.g., *cdc9*; DNA ligase), because *cdc13*–checkpoint double mutants have the most dramatic arrest–defect phenotype (Fig. 1A,B) (see Hartwell and Weinert 1989; Weinert and Hartwell 1993).

We mutagenized *cdc13* cells (strain TWY146, see Table 1) and first screened for strains that died rapidly (the rapid death phenotype) and then identified mutants that failed to cell-cycle arrest after shift to the restrictive temperature (the *cdc13* arrest–defect phenotype; see Materials and methods). Of ~12,000 ethylmethane sulfonate (EMS)-mutagenized cells screened, ~500 strains died more rapidly than a *cdc13* strain and 21 of these strains also failed to cell-cycle arrest after shift to the restrictive temperature.

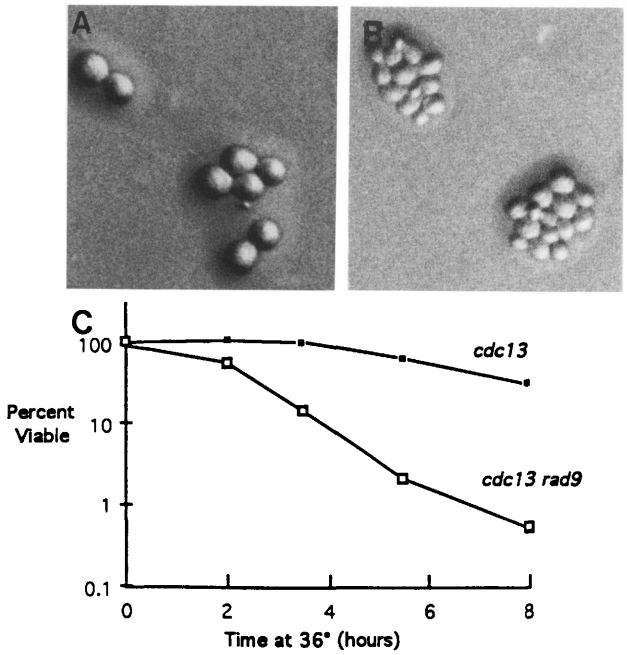

Figure 1. *cdc13* cells arrest in the first cell cycle and retain cell viability, whereas *cdc13 rad9* cells fail to arrest and lose viability more rapidly. *cdc13* (*A*) and *cdc13 rad9* (*B*) cells grown at the permissive temperature were placed on agar plates and shifted to the restrictive temperature of 36°C for 10 hr. Each microcolony arose from a single cell. Cells were photographed at the same magnification. (*C*) *cdc13* and *cdc13 rad9* cells grown at the permissive temperature were shifted to the restrictive temperature of 36°C in liquid culture, at various times cells were plated at the permissive temperature (23°C), and cell viability was determined by colony formation after incubation for 2–3 days. The average of duplicate cultures is shown.

Genetic characterization and mapping of checkpoint mutants

In each mutant analyzed the *cdc13* arrest–defect and rapid death phenotypes cosegregated and were attributed to single mutations (see Materials and methods). All 21 mutants were recessive to wild type and formed four complementation groups, with multiple alleles of *mec1*, *mec2*, and *rad9* and a single allele of *mec3* (Table 2). In a separate screen of radiation-sensitive mutants, we found that *rad24-1* is also a checkpoint mutant and included it in further analyses. In our genetic screen we did not identify any alleles of the checkpoint mutant *rad17* or that of *rad24*. We have now screened *rad6, 8, 9, 10, 11, 12, 17, 18, 24, 52, 53* (identical to *mec2*), and *rad54* (this study, Weinert and Hartwell 1988; unpubl.).

mec1 identifies a previously undescribed gene and maps to the long arm of CHRII between *lys2* and *cdc28* (Table 3). The genetic mapping was confirmed by results of DNA sequence analysis; *MEC1* is immediately adjacent to but distinct from *CKS1*, which was mapped to the same chromosomal region (Hadwiger et al. 1989; unpubl.). *mec2* maps to CHRXV and proved to be allelic with *rad53-1*; both *mec2-1* and *rad53-1* alleles confer sensitivity to hydroxyurea (HU sensitivity) and fail to complement each other for HU sensitivity, and meiotic analysis shows that the mutations are tightly linked (Table 3). *MEC3* maps physically to CHRIV and is neither *RAD55* nor *RAD9*, but we have not yet identified its chromosomal location (see Materials and methods).

Checkpoint mutants are sensitive to DNA-damaging agents and fail to cell-cycle arrest after X-irradiation

We tested the newly isolated checkpoint mutant alleles for their responses to DNA-damaging agents and found that at least some alleles of each checkpoint gene were methylmethane sulfonate (MMS)-, UV-, and X-ray sensitive (Table 2; data not shown). Some alleles of *mec1* and *mec2* showed a complex spectrum of phenotypes (see Discussion).

On the basis of their radiation and drug sensitivities, the strongest alleles from each complementation group, *mec1-1*, *mec2-1*, and *mec3-1*, were selected for further studies. We also tested the strongest alleles for each gene for cell-cycle arrest after X-irradiation and found that each failed to arrest in the G_2 phase after DNA damage (Table 4).

The strongest allele for each checkpoint gene appears to be a nonconditional mutant for checkpoint function. Although the *cdc13*-arrest defect phenotype was necessarily determined at 36°C, we found that the same mutants tested at 23°C were arrest-defective after X-irradiation and were DNA damage sensitive (X-ray, UV, and MMS). Whether alleles other than *mec1-1*, *mec2-1*, and *mec3-1* are temperature sensitive for function has not been examined. *mec1-1*, *mec3-1*, and *rad24-1* all grow well at 23°C and at 36°C (in CDC^+ strains), and *mec2-1* grows well at lower temperatures but not at 36°C (see Table 5).

Interaction of checkpoint and CDC *genes*

The roles of the checkpoint genes were examined by interactions with *CDC* genes that act at specific stages of cell division. We introduced each checkpoint mutation into *cdc* mutants that arrest after shift to the restrictive temperature in either the G_1 phase (*cdc28*); in G_1–S (*cdc7*); in S phase (*cdc8* and *cdc17* at 38°C); in G_2 phase (*cdc2, cdc9, cdc17* at 34°C, and *cdc13*); in G_2–M phase (*cdc16*); or in postanaphase (*cdc15*). We classify the arrest of *cdc16* mutants as in G_2–M although we have no formal proof that the arrest point is distinct from the class of *cdc* mutants that arrests in the G_2 phase. The *cdc16* mutant is distinctive in that it does not generate DNA damage at the restrictive temperature as does the other class of *cdc* mutants that arrest in G_2 (for discussion, see Weinert and Hartwell 1993). *cdc6* mutants were also analyzed and arrest apparently early in nuclear division (see Discussion). The stages of arrest are based on previous observations, including genetic, morphological, and flow cytometric analyses (see Pringle and Hartwell 1981; Carson 1986; Weinert and Hartwell 1993). The interactions of *CDC* and checkpoint genes were assessed by comparing the *cdc* single mutants with the corre-

Table 1. *Strains used in this study*

Strain	Genotype
TWY397[a]	*MATa ura3 his7 leu2 trp1*
TWY398[a]	*MATa rad9Δ::LEU2 his7 ura3 leu2 trp1*
TWY308	*MATα mec1-1 ura3 trp1*
TWY312	*MATα mec2-1 ura3 his7 trp1*
TWY316	*MATa mec3-1 ura3 his3 trp1*
TWY399	*MATa rad24-1 ura3 his7 leu2 trp1*
TWY123[a]	*MATα/MATa ura3/ura3 his3/+ his7/+ leu2/leu2 trp1/trp1*
TWY162	*MATα/MATa mec1-1/mec1-1 ura3/ura3 his3/+ his7/+ leu2/+ cdc13/+*
TWY409	*MATα cdc2-2 his7*
TWY410	*MATα cdc2-2 mec1-1 ura3 ura1*
TWY411	*MATα cdc2-2 mec2-1 ura3 his3 his7*
TWY412	*MATα cdc2-2 mec3-1 his7*
TWY413	*MATα cdc2-2 rad24-1 ura3 ura1 his7 leu2*
TWY414	*MATα cdc6-1 ura3 ura1*
TWY415	*MATα cdc6-1 mec1-1 ura1 trp1*
TWY416	*MATα cdc6-1 mec2-1*
TWY417	*MATα cdc6-1 mec3-1 ura3 ura1 his7 trp1*
TWY418	*MATa cdc6-1 rad24-1 ura3 ura1 his7*
TWY419	*MATα cdc7-4 ura1*
TWY420	*MATα cdc7-4 mec1-1 ura1 his7*
TWY421	*MATα cdc7-4 mec2-1 ura3 his7*
TWY422	*MATα cdc7-4 mec3-1 ura1 his7*
TWY423	*MATα cdc7-4 rad24-1 ura3 his 7 leu2*
TWY424	*MATa cdc8-1*
TWY425	*MATa cdc8-1 mec3-1 ura1 his7 leu2 trp1*
TWY426	*MATα cdc8-1 rad24-1 ura1 his7 leu2*
TWY54[b]	*MATα cdc9-8 ura3 ade3 ade2 trp1 leu1 can1 cyh2 sap3 trp1 SCE::URA3*
TWY285	*MATa cdc9-8 mec1-1 ade2 ade3 ura3 trp1 leu⁻*
TWY283	*MATα cdc9-8 mec2-1 ura3 his3 trp1 leu⁻*
TWY429	*MATα cdc9-8 mec3-1 his7 trp1*
TWY430	*MATa cdc9-8 rad24-1 his7 leu2 trp1 ade3*
TWY431	*MATa cdc13-1 ura3 his3*
TWY432	*MATα cdc13-1 mec1-1 ura3 his3 his7 leu2*
TWY433	*MATα cdc13-1 mec2-1 ura3*
TWY434	*MATa cdc13-1 mec3-1 ura3 his7 leu2 trp1*
TWY435	*MATα cdc13-1 rad24-1 mec3-1 ura3 his3 trp1*
TWY436	*MATa cdc15-1 ura1 ura3 his3 his7*
TWY437	*MATa cdc15-1 mec1-1 ura3 his7*
TWY438	*MATα cdc15-1 mec2-1 his7 trp1*
TWY439	*MATα cdc15-1 mec3-1 ura1 his7*
TWY440	*MATa cdc15-1 rad24-1 ura3 his7 trp1*
TWY441	*MATa cdc16-1 ura1 ura3 his7*
TWY442	*MATα cdc16-1 mec1-1*
TWY443	*MATa cdc16-1 mec2-1 ura3 trp1*
TWY444	*MATα cdc16-1 mec3-1 his7 trp1*
TWY445	*MATa cdc16-1 rad24-1 ura1 ura3 his7*
TWY446	*MATa cdc17-1 his7*
TWY447	*MATα cdc17-1 mec1-1 trp1*
TWY448	*MATa cdc17-1 mec2-1 his7*
TWY449	*MATα cdc17-1 mec3-1 ura1 ura3 his7 trp1*
TWY450	*MATa cdc17-1 rad24-1 his7 leu2 trp1*
TWY451	*MATa cdc28-1 ura1 ura3 his3 his7*
TWY452	*MATa cdc28-1 mec1-1 his7*
TWY453	*MATa cdc28-1 mec2-1 ura3 his7*
TWY454	*MATa cdc28-1 mec3-1 ura1 his7*
TWY455	*MATa cdc28-1 rad24-1 ura1 ura3 his7*
TWY146	*MATα cdc13-1 ura3 his7*
TWY148	*MATα cdc13-1 mec2-1 ura3 his7*
TWY158	*MATα cdc13-1 mec1-1 ura3 his7*
TWY159	*MATα cdc13-1 mec3-1 ura3 his7*
KSS255[c]	*MATα rad24-1 ura3 trp1 his3 leu2 lys2*
g151-12c[c]	*MATα rad53-1 ade2-1 ade1 arg4-17 lys1-1 his5-2*

Strains were generated in this study, except for the following:
[a]Weinert and Hartwell (1990).
[b]Hartwell and Weinert (1989).
[c]KSS255 from K. Sitney and g151-12a from the Berkeley Stock Center are not congenic with A364a.

Table 2. *Phenotypes of checkpoint mutants*

Strain[a]	Allele	Phenotypes			
		cdc13 arrest[b]	MMS[c]	HU[c]	X-ray[c]
MEC$^+$		+	+	+	+
mec1	−1	−	−	−	−
	−3, −4, −6, −11	−	−	−	+
	−7	−	−	±	+
	−8, −10	−	−	±	−
	−2, −5, −9, −12	−	+	+	+
mec2	−1, −2, −3	−	−	−	−
	−4	−	+	−	−
mec3	−1	−	−	±	−
rad24	−1	−	−	±	−
rad9	−10, −11, −12	−	−	+	−
	−13	−	±	+	−

[a]Strains were derived from *TWY146 MATa cdc13 ura3 his7*, except for the *rad24* mutant (TWY435) (see Table 1). Genes and alleles were assigned by complementation and allelism tests (see Materials and methods).

[b]The *cdc13*-arrest phenotype measures arrest in G_2 after DNA damage. Cells were grown to midlog in liquid culture at the permissive temperature, plated on agar plates, and shifted to the restrictive temperature of 36°C. After 10–16 hr, plates were examined microscopically. Strains that had an intact checkpoint produced microcolonies that primarily contained either two or four buds, and produced strains with a defective checkpoint, mostly microcolonies that contained five buds or more.

[c]Sensitivity to MMS, HU, and X-rays was determined by comparing patches of cells replicated onto selective medium (0.01% MMS, 0.1 M HU), or complete medium (without drug), or after x-irradiation. Alternatively, we determined the ability of single cells to form visible colonies when streaked on plates containing selective medium. *mec3* and *rad24* were consistently more HU sensitive than *rad9* mutants but less sensitive than *mec1* and *mec2* mutants. *mec1-1*, *mec2-1*, and *mec3-1* are each UV-sensitive (data not shown), as is *rad24* (Eckardt-Schupp et al. 1987).

Table 3. *Genetic mapping of* mec1 *and* mec2

Mutant	Cross[a]	PD	NPD	T
mec1	*cdc28 mec1* / + +	38	0	4
	cdc28 + / + *lys2*	25	0	23
	mec1 + / + *lys2*	22	0	18
mec2	*mec2-1* / *rad53-1*	16	0	0

Conclusion: 1. *cdc28* → *mec1* → *lys2*

4.8 cM 22.5 cM

2. *mec2-1* and *rad53-1* are allelic

[a]To map *mec1*, diploids were analyzed that were either *cdc28 mec1* +/+ + *lys2* or *cdc28 mec1* +/+ +::*URA3 lys2* (contains a partial duplication of the *MEC1* gene with the *URA3* gene integrated at the *MEC1* locus; T. Weinert, unpubl.) To map *mec2-1*, a diploid formed from *mec2-1* and *rad53-1* haploid parents was analyzed.

Table 4. *Checkpoint mutants fail to arrest the cell cycle after x-irradiation*

Strain[a]	Percent lethality	Percent arrest	Arrest lethality
MEC$^+$	49	42	0.82
mec1-1	79	19	0.24
mec2-1	68	22	0.32
mec3-1	68	24	0.35
rad24-1	69	11	0.16
rad9Δ	84	16	0.19

[a]The fraction of arrested cells/cell lethality at a low dose of x-rays (2 krads) provides a convenient metric of cell cycle arrest (discussed in Materials and methods). Results are from a single experiment. Strains are *MEC*$^+$, TWY397; *mec1-1*, TWY158; *mec2-1*, TWY148; *mec3-1*, TWY159; *rad24-1*, TWY435; *rad9*, TWY398.

sponding *cdc*–checkpoint double mutants for four phenotypes, including arrest morphology, first cycle arrest, cell viability, and maximum permissive temperature for colony formation (see Materials and methods). This strategy was used previously to characterize *rad9* and *rad17* mutants.

All six checkpoint genes are required for arrest of cdc *mutants in the G_2 phase with DNA damage*

One class of *cdc* mutants arrests in the G_2 phase after shift to the restrictive temperature and encodes gene products that are involved in DNA metabolism (*cdc9*, DNA ligase; *cdc2*, DNA polymerse γ; *cdc17* at 34°C, DNA polymerase α; and *cdc13*, function unknown; see introductory section). Each of the corresponding *cdc*–checkpoint double mutants showed some defect in cell-cycle arrest (although for some mutants the arrest defect is relatively small), and all double mutants died more rapidly at the restrictive temperature compared with the *cdc* single mutants (Table 5). Each *cdc*–checkpoint double mutant also showed an altered maximum permissive temperature compared with the *cdc* strain. For *cdc2*, *cdc17*, and *cdc9* the maximum permissive temperature was lower in the *cdc*–checkpoint double mutant; mutant cells grown at intermediate temperatures are limited for the function of these DNA replication enzymes and likely require a delay at the checkpoint to complete replication before mitosis and to remain viable. The maximum permissive temperature is higher in the *cdc13*–checkpoint double mutants, though most cells formed at the higher temperature were inviable (for discussion, see Weinert and Hartwell 1993). The phenotypes for this DNA replication-defective class of *cdc*–checkpoint double mutants are similar to those of the corresponding *cdc rad9* and *cdc rad17* double mutants. The results indicate that all six checkpoint genes are required for arrest in the G_2 phase after DNA damage resulting from defects in DNA replication enzymes.

Though we consistently observed a qualitative correlation between the increased cell death and a defect in cell-cycle arrest, we do not always see a quantitative

correlation between the extent of cell death and of the defect in cell-cycle arrest (see Table 5; e.g., cf. *cdc13 mec1* and *cdc13 mec2*; or *cdc9 mec1* and *cdc9 rad24*). This suggests that the cause of cell death may not be attributable solely to a defect in cell cycle arrest. Some cell death may be due instead to defects in DNA repair or to defects in gene product interactions between the checkpoint and DNA replication proteins.

MEC1 *and* MEC2 *are also essential for arrest in S phase*

Three results indicate that *mec1* and *mec2* mutants have a defect in S-phase arrest when DNA replication is blocked. First, *cdc8 mec1* and *cdc8 mec2* double mutants were synthetically lethal; after sporulation of appropriate diploids we were unable to recover any viable double mutants (from *cdc8/+ mec1/+* or *cdc8/+ mec2/+* diploids, analyzing 22 and 25 tetrads with 66% and 70% spore viability, respectively). We infer that the double mutants were formed during meiosis but were inviable because we did recover the three other expected genotypes (*cdc8*, *mec1*, and *CDC$^+$ MEC$^+$*) at frequencies expected for unlinked genes (*cdc8*, *mec1*, and *mec2* are on CHRX, CHRII, and CHR XV, respectively). Because the double mutants were apparently inviable under conditions where both single mutants are viable, *cdc8 mec1* and *cdc8 mec2* mutants are synthetically lethal. *cdc8* mutants at 23°C are partially defective for DNA replication (see Weinert and Hartwell 1993 and references therein), so *cdc8* spores germinated at 23°C might be expected to experience an S-phase delay and therefore require *MEC1* and *MEC2* for that delay and for cell viability.

Second, *cdc17 mec1* mutants died more rapidly than did *cdc17 MEC$^+$* mutants at the high temperature (38°C) that causes *cdc17* single mutants to arrest in S phase (Table 5). The *cdc17 mec1* mutant cells at 38°C also displayed a higher frequency of abnormal microtubule morphologies than did *cdc17* strains, suggesting that *cdc17 mec1* cells fail to arrest under conditions where *cdc17 MEC$^+$* cells do arrest (data not shown). However, the interpretation of the *cdc17 mec1* phenotypes is complicated because of the ambiguous phenotypes of other *cdc17*–checkpoint double mutants. For example, both *cdc17 mec2* and *cdc17 mec3* strains showed a mild cell-cycle arrest defect at 38°C, yet the cell viability of both strains was comparable with that of the *cdc17 MEC$^+$* strain. By other criteria (e.g., interaction with *cdc8*, response to HU) *mec2* mutants are S-phase arrest defective, whereas *mec3* mutants are not. The analysis of *mec2* may also be complicated by its temperature sensitivity for growth (in a *CDC$^+$* background; see Table 5). The basis for this variability of phenotypes in some of the *cdc17*–checkpoint mutants at 38°C is unknown.

The third and most dramatic result that indicates an S-phase arrest defect in *mec1* and *mec2* mutants was their response to incubation with HU. HU-treated *mec1* and *mec2* mutant cells failed to cell-cycle arrest and died rapidly, whereas wild-type cells arrested and remained viable (Table 6; Fig. 2). From their DNA and tubulin morphologies (Fig. 2; data not shown), *mec1* and *mec2* mutants appeared to attempt and even complete mitosis in the presence of HU. The low viability of HU-treated *mec1* and *mec2* cells may be attributable to entry into mitosis of mutant cells under conditions where *MEC$^+$* cells remain arrested. Again, other contributions to cell inviability (e.g., defects in DNA repair, defective gene product interactions) cannot be ruled out at present.

Interactions of checkpoint mutants with other cdc *mutants*

Checkpoint mutations did not affect the phenotypes of *cdc* mutants that arrest in G_1 (*cdc28*), G_1–S (*cdc7*), G_2–M (*cdc16*), or postanaphase (*cdc15*) after shift to the restrictive temperature (Table 5). By the criteria of interaction with *CDC* genes, the role of the checkpoint genes appears restricted to the S and/or G_2 phase of the cell cycle and to respond to incomplete DNA replication or DNA damage.

The analysis of *cdc6*–checkpoint mutants gave results that were less dramatic and more difficult to interpret. For example, from their nuclear morphology *cdc6 mec1* mutants appeared arrest-defective compared to the *cdc6* strains (most double mutant cells did not arrest as large-budded cells with an undivided nucleus), however cell viabilities were similar in the two strains. Although the strongest interactions appeared between *cdc6* and *mec3*, there may be some interaction with the other checkpoint mutants as well (including *rad9* and *rad17*, Weinert and Hartwell 1993), because all double mutant combinations have a lower maximum permissive temperature than the *cdc6* strain. The interaction of *CDC6* and checkpoint genes requires additional studies.

Discussion

Six checkpoint genes are required for arrest in the G_2 phase after DNA damage

We have now identified and characterized mutants in six checkpoint genes, *RAD9*, *RAD17*, *RAD24*, *MEC1*, *MEC2* (*RAD53*), and *MEC3*. All are essential for the DNA damaged-induced cell-cycle arrest in the G_2 phase. This conclusion is based on several observations. First, all checkpoint mutants were defective for cell-cycle arrest following X-irradiation (Table 4); those tested also failed to cell-cycle arrest after UV-irradiation (*rad9* and *rad17*; Weinert and Hartwell 1993), and all of the mutants are also sensitive to DNA-damaging agents (X-ray, UV, and MMS). The sensitivity to DNA-damaging agents may be attributable solely to a failure to cell- cycle arrest, though we have not ruled out an additional direct role in DNA repair.

Second, all of the checkpoint genes show a striking interaction with a class of *CDC* genes that function in DNA replication. Each of the *CDC* genes in this class (*CDC2*, *CDC9*, *CDC13*, and *CDC17*) encode proteins involved in DNA metabolism (see introductory section),

Table 5. *Role of checkpoint genes in arrest of* cdc *mutants*

Strain[a]	Cell cycle phase[b]	Arrest: nuclear morphology[c] 23	Arrest: nuclear morphology[c] RT	Arrest: first cycle[d]	Viability[e] 23	Viability[e] RT	Maximum permissive temperature[f] (°C)
cdc13	G_2	15	97	90	89 ± 2.2	83 ± 5.7	25
cdc13 mec1		9	15	3	87 ± 4.2	14 ± 6.3	28
cdc13 mec2		16	42	43	92 ± 0.9	19 ± 1.3	28
cdc13 mec3		4	24	15	87 ± 2.3	39 ± 3.4	28
cdc13 rad24		17	34	10	86 ± 4.2	45 ± 1.0	30
cdc2	G_2	15	94	90	99 ± 0.9	19 ± 2.9	30
cdc2 mec1		13	45	36	52 ± 8.4	0.24 ± .07	25
cdc2 mec2		28	56	45	88 ± 3.1	0.61 ± 0.2	28
cdc2 mec3		12	68	38	86 ± 4.4	0.82 ± 0.4	25
cdc2 rad24		26	64	39	72 ± 2.3	0.22 ± .06	28
cdc9	G_2	21	96	79	93 ± 3.0	11 ± 1.4	32
cdc9 mec1		8	51	49	87 ± 4.2	1.1 ± 0.1	25
cdc9 mec2		15	71	69	89 ± 3.5	1.5 ± 1.0	25
cdc9 mec3		17	67	73	68 ± 1.6	0.5 ± .09	25
cdc9 rad24		18	71	74	82 ± 0.0	0.17 ± .02	25
cdc17 (34°C)	G_2	19	93	71	97 ± 1.4	64 ± 11.1	32
cdc17 mec1 (34°C)		16	59	62	80 ± 9.0	0.27 ± .12	28
cdc17 mec2 (34°C)		25	66	51	82 ± 0.5	0.16 ± .07	28
cdc17 mec3 (34°C)		15	47	24	86 ± 1.7	9 ± 2.5	30
cdc17 rad24 (34°C)		9	32	19	85 ± 3.8	3 ± 2.1	28
cdc28	G_1	44	97	96	97 ± 1.7	59 ± 1.3	32
cdc28 mec1		37	78	90	89 ± 2.9	57 ± 13.6	32
cdc28 mec2		24	89	84	96 ± 1.9	67 ± 3.7	32
cdc28 mec3		24	92	88	88 ± 5.5	57 ± 10.2	32
cdc28 rad24		20	89	87	93 ± 1.6	79 ± 4.3	32
cdc6	see text	20	67	86	92 ± 3.7	26 ± 1.9	32
cdc6 mec1		13	34	53	86 ± 2.5	22 ± 7.6	30
cdc6 mec2		25	67	64	90 ± 4.4	51 ± 6.8	28
cdc6 mec3		12	44	67	88 ± 3.1	7.2 ± 2.5	30
cdc6 rad24		22	74	73	85 ± 0.8	5.6 ± 2.7	28
cdc7	G_1–S	21	71	83	87 ± 5.1	29 ± 4.1	28
cdc7 mec1		35	61	87	81 ± 1.3	17 ± 1.7	25
cdc7 mec2		20	77	75	96 ± 4.0	22 ± 2.9	28
cdc7 mec3		26	71	88	82 ± 7.4	22 ± 1.3	25
cdc7 rad24		18	75	85	78 ± 5.2	22 ± 3.8	28
cdc8	S	20	96	94	95 ± 0.5	24 ± 0.5	28
cdc8 mec1						inviable	
cdc8 mec2						inviable	
cdc8 mec3		15	82	86	82 ± 1.3	14 ± 2.2	28
cdc8 rad24		16	97	93	64 ± 3.1	6.0 ± 2.0	28
cdc17 (38°C)	S	19	77	80	97 ± 1.4	14 ± 4.9	32
cdc17 mec1 (38°C)		16	43	82	80 ± 9.0	1.6 ± 1.2	28
cdc17 mec2 (38°C)		25	49	58	82 ± 0.5	11 ± 5.3	28
cdc17 mec3 (38°C)		15	50	64	86 ± 1.7	28 ± 7.6	30
cdc17 rad24 (38°C)		9	74	72	85 ± 3.0	11 ± 5.2	28
cdc16	G_2/M	3	81	59	99 ± 1.0	66 ± 9.7	30
cdc16 mec1		14	85	51	91 ± 3.0	40 ± 7.3	30
cdc16 mec2		18	86	53	90 ± 2.1	62 ± 4.8	30
cdc16 mec3		22	89	58	88 ± 2.3	52 ± 6.6	30
cdc16 rad24		13	72	83	71 ± 1.0	39 ± 2.1	30
cdc15	postanaphase	38	95	86	94 ± 8.7	45 ± 3.8	32
cdc15 mec1		32	92	75	94 ± 0.8	42 ± 1.1	32

(*Table 5 continued on facing page*)

Table 5. *(Continued)*

Strain[a]	Cell cycle phase[b]	Arrest: nuclear morphology[c] 23	Arrest: nuclear morphology[c] RT	Arrest: first cycle[d]	Viability[e] 23	Viability[e] RT	Maximum permissive temperature[f] (°C)
cdc15 mec2		38	87	87	91 ± 6.6	67 ± 11.5	32
cdc15 mec3		22	92	85	88 ± 6.1	53 ± 6.3	32
cdc15 rad24		11	70	98	85 ± 2.9	40 ± 4.9	32
MEC$^+$	asynchronous	22	28	15	97 ± 1.8	93 ± 2.7	36
mec1	asynchronous	16	19	40	93 ± 2.1	92 ± 4.2	36
mec2	asynchronous	38	33	18	79 ± 5.7	68 ± 5.9	32
mec3	asynchronous	10	12	25	83 ± 4.9	83 ± 4.9	36
rad24	asynchronous	20	22	16	90 ± 5.3	86 ± 1.3	36

[a]Strains are shown in Table 1. Restrictive temperature (RT) for *cdc* mutations was 36°C, except as noted.
[b]Conclusions from these and previously published observations.
[c]Percentage of cells with a large bud and undivided nucleus, except *cdc28* (unbudded) and *cdc15* (large budded with divided nucleus).
[d]Percentage of microcolonies that contain either two or four buds (microcolony assay), except for *cdc28* (one or two buds).
[e]Mean value from three to six cultures; standard deviation shown.
[f]Maximum permissive temperature of colony formation.

and DNA replication in the corresponding temperature-sensitive mutants at the restrictive temperature is either incomplete or complete with errors (causing arrest in late S or G_2 phase, respectively). Each of these four *cdc* DNA replication-defective mutants arrested in the G_2 phase at the restrictive temperature, whereas each of the corresponding *cdc*–checkpoint double mutants failed to arrest and died rapidly. Unrepaired DNA lesions in these *cdc* mutants at the restrictive temperature probably activate the checkpoint, and cell death in the checkpoint mutants occurs either because of cell division with damaged chromosomes, defects in DNA repair, defects in gene product interactions, or a combination of these. We cannot determine the exact cause(s) of cell death in the *cdc*–checkpoint double mutants from these studies.

Our results suggest that any type of DNA lesion (generated by MMS-, UV-, or X-irradiation or by defects in DNA replication) causes an arrest that requires all six checkpoint genes. We have no evidence that some checkpoint genes are required for arrest due to specific types of DNA lesions, although if such mutants do exist, we may not have detected them because our mutant isolation strategies used only radiation- or *cdc13*- induced DNA lesions. Some alleles of *mec1* are more resistant to DNA-damaging agents than are other alleles (Table 2), a result that could suggest lesion-specific detection by different alleles of *MEC1*. However, these different phenotypes may simply reflect different quantitative levels of function and not qualitatively different functions. This question needs to be examined further. In sum, we favor the idea that all of these genes play a role in signal transduction once some form of DNA damage has been generated.

Table 6. *Inhibition of DNA replication by hydroxyurea confirms the S-phase arrest defect in* mec1 *and* mec2 *mutants*

Strain[a]	Nuclear morphology[b] (−HU/+HU)	Viability (−HU/+HU)	Spindle[c] (% normal in HU)
MEC$^+$	27/91	96 ± 1.2/91 ± 2.3	97
mec1	20/27	95 ± 3.5/0.2 ± 0.07	3
mec2	30/69	93 ± 3.6/0.5 ± 0.07	38
mec3	16/89	85 ± 2.3/63 ± 2.9	97
rad24	18/74	83 ± 3.8/79 ± 1.0	95

[a]Strains: *MEC*$^+$, TWY397; *mec1-1*, TWY308; *mec2-1*, TWY312; *mec3-1*, TWY316; *rad24-1*, TWY399.
[b]Cells were incubated with 0.2 M hydroxyurea in liquid media at 30°C for 4 hr and analyzed for cell viability and morphology. Arrested cells are large budded and have an undivided nucleus.
[c]The spindle morphology observed specifically in large-budded cells is presented. Normal short spindles seen in HU-arrested wild-type cells were at the neck of the bud. Abnormal spindles were clearly neither short nor elongated, though details of their abnormality were not always easily identified (abnormalities are more easily seen in larger diploid cells; see Fig. 2). The microtubule phenotypes of unbudded cells were normal. Few large-budded cells with an elongated spindle and bipolar nucleus were observed.

MEC1 *and* MEC2 *define distinct S- and* G_2*-phase pathways of the mitotic checkpoint*

Mutants in the *MEC1* and *MEC2* genes were identified by their defects in the G_2 pathway but, when analyzed, proved to be defective for an S-phase pathway as well. This conclusion is based on the following observations: *mec1* and *mec2* mutants treated with HU failed to arrest and were inviable; *mec1* and *mec2* were synthetically lethal with *cdc8*; and *cdc17 mec1* failed to arrest and died more rapidly than *cdc17* mutants at the high temperature (38°C) at which *cdc17* cells arrest in S phase. From the nuclear morphology of HU-treated cells, *mec1* and *mec2* mutant cells with incompletely replicated DNA appeared to enter (and even complete) mitosis. Mi-

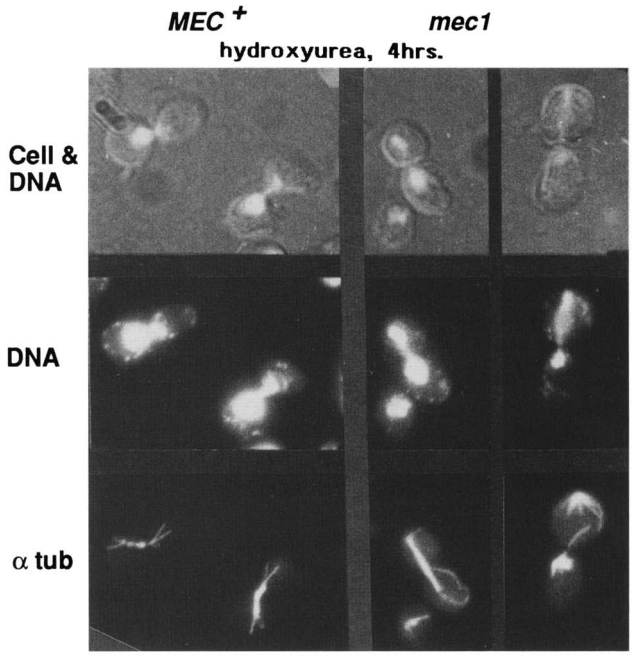

Figure 2. Inhibition of DNA replication by incubation of cells with HU causes cell-cycle arrest of *MEC*$^+$ cells but not *mec1* cells. Diploid cells (TWY123, *MEC*$^+$/*MEC*$^+$; TWY162 *mec1*/*mec1*) were treated for 4 hr with HU at 30°C, fixed, and stained for nuclear and microtubule morphologies. (*Top*) Cell morphology, using Nomarski optics, and nuclear morphology simultaneously; (*middle*) nuclear morphology alone; (*bottom*) microtubule morphology.

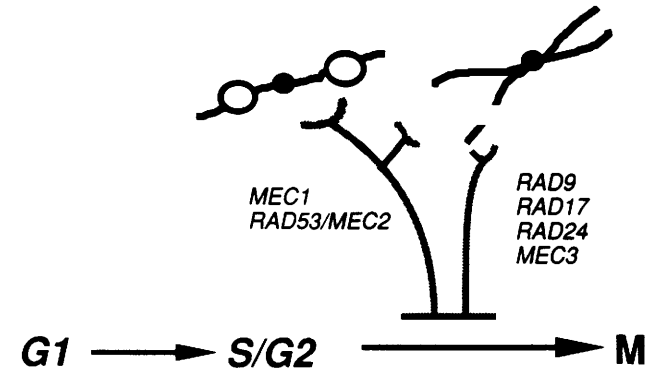

Figure 3. Summary of the roles of the checkpoint genes in the S- and G_2-phase pathways in checkpoint control. (*Top*) Partially replicated chromosomes with two replication bubbles and centromere, and two fully duplicated sister chromatids, one of which has a break.

tosis with unreplicated DNA has been observed in mammalian cells treated with both HU (to block DNA replication) and with caffeine (to override the checkpoint) (described as the MUG phenotype–mitosis with unreplicated genome; Brinkley et al. 1988.). In addition, several fission yeast genes function in an S-phase pathway analogous to that mediated by *MEC1* and *MEC2* (Enoch and Nurse 1990; Al-Khodairy and Carr 1992; Enoch et al. 1992; Jimenez et al. 1992; Rowley et al. 1992b; for review, see Murray 1993). The *RCC1* gene in mammalian cells (Nishimoto et al. 1978) and in fission yeast (Matsumoto and Beach 1991), and the *bimE* gene in filamentous fungi (Osmani et al. 1988; 1991; for review, see Dasso 1993.) are also essential for an S-phase pathway in those organisms. The existence of an S-phase pathway appears to be universal among eukaryotic cells.

The dependence of mitosis on DNA replication is enforced by an S- and a G_2-phase pathway

The model in Figure 3 summarizes one view of the relationship between the six checkpoint genes and the cell cycle in budding yeast. [The EMS-induced checkpoint mutations are uncharacterized as to their molecular lesions, so except for the *rad9* mutation, which is a null, these mutants may retain partial function. We believe that the general conclusions on phase specificity are correct because null mutants of *rad17*, *rad24*, and *mec3* have phenotypes similar to those of the mutants studied here (D. Lydall and T. Weinert, unpubl.)]. We imagine that the checkpoint genes have some role(s) in the signal transduction process. It is unlikely that any of these genes represent the target of checkpoint control for the following reason: We anticipate that the target will be a gene(s) essential for mitosis, and a checkpoint-defective mutant allele in that gene might be dominant. This expectation derives from mutations in fission yeast in the *CDC2* gene (*cdc2-3w*), which are dominant and have a checkpoint defect (Enoch and Nurse 1990). All of the checkpoint mutant alleles that we have characterized in budding yeast are recessive (Table 2; Weinert and Hartwell 1988, 1993).

Possible mechanistic relationships between S and G_2 pathways

Why does the cell employ two DNA-sensitive mechanisms to ensure chromosome integrity before mitosis? Conceptually, the two may play fundamentally different roles (Enoch and Nurse 1991; Li and Deschaies 1993; see Murray 1993). We imagine the G_2 pathway has a conceptually simpler function where DNA lesions trigger arrest in G_2, and the pathway constitutes an extrinsic control to mitosis normally not essential for viability. The G_2 pathway ensures that damage generated either spontaneously during replication or by specific environmental insults is repaired before mitosis. This control is analogous to that of the DNA damage-sensitive *recA*-dependent response in bacteria.

The S-phase pathway may be more complex and may be intrinsic to DNA replication itself, perhaps by ensuring that the time for DNA replication is sufficient. How does the cell sense incomplete DNA replication? The cell may detect some feature of a replication fork. Specific proteins may serve both in DNA replication and in cell-cycle control. In this regard, the role of *CDC6* in cell

division may be of interest. Recently, a *CDC6* homolog in fission yeast, *cdc18*$^+$, was reported to have a checkpoint function, acting as a positive regulator of DNA replication and as a negative regulator of mitosis (Kelly et al. 1993). In budding yeast genetic evidence also suggests *CDC6* may function both in the initiation of DNA replication (Hartwell 1976; Hogan and Koshland 1992) as well as an inhibitor of mitosis (Bueno and Russell 1992). The mitotic arrest caused by overexpression of *CDC6* does not require the *RAD9* gene (Bueno and Russell 1992). We have seen weak interactions between *CDC6* and the checkpoint genes (Table 5), and the meaning of these possible interactions remains to be established.

A model of the mitotic checkpoint must account for why *MEC1* and *MEC2* are required in both the S and G_2 pathways, whereas the other four checkpoint genes function solely in the G_2 pathway. We consider two possibilities. First, the signal in the S and G_2 phases may be processed by distinct pathways that subsequently converge on the *MEC1/MEC2* function and then on the target. This model predicts the existence of genes that act in an as-yet-unidentified and distinct S-phase pathway (see Murray 1993). In a second model, *MEC1/MEC2* function during S phase, and their function in S phase is required for the subsequent function of the G_2 pathway. This model posits that *MEC1* and *MEC2* act before the G_2 pathway genes. If this model is correct, *MEC1/MEC2* must have a constitutive role in the cell cycle because previous results showed that when cells in the G_2 phase were irradiated, the G_2 pathway was activated and cells arrested in G_2 (Weinert and Hartwell 1988, 1993). At present, we have no observations that distinguish between the two possibilities.

Comparison of the mitotic checkpoints in fission and budding yeasts

The general organization of the checkpoint that orders DNA replication and mitosis appears similar in fission and budding yeast (for review, Li and Deschaies 1993; Murray 1993). Both yeasts have genes that function specifically in the G_2 pathway in response to DNA damage: Four genes in budding yeast (see Fig. 3) and at least two genes in fission yeast, *chk1*$^+$ (Walworth et al. 1993) and *wee1*$^+$ (Rowley et al. 1992a; but see Barbet and Carr 1993.). Both yeasts have genes that function in both the S- and G_2-phase pathways: *MEC1* and *MEC2* in budding yeast and at least 11 genes in fission yeast (see Murray 1993). The genetic analyses in both yeasts appear incomplete so yet more checkpoint genes will likely be identified: Whether these genes from the two yeasts share sequence similarities and/or molecular functions awaits their molecular analysis.

Target of the mitotic checkpoint

A possible target of checkpoint regulation is $p34^{CDC2/CDC28}$ (Nurse 1990). Support for this hypothesis comes from experiments in fission yeast showing that mutants in *cdc2*$^+$ and its regulators (*cdc25*$^+$ and *wee1*$^+$) have checkpoint defects. Another possible direct link between the checkpoint genes and *cdc2*$^+$ in fission yeast was reported recently in the genetic suppression of a *cdc2* mutation by the checkpoint gene *chk1*$^+$ (Walworth et al. 1993). In budding yeast it is unclear what interaction, if any, exists between checkpoint genes and *CDC28*, the *cdc2*$^+$ homolog. For example, the same *cdc2* mutants that have a checkpoint defect in fission yeast have no detectable phenotype in budding yeast (Amon et al. 1992; Sorger and Murray 1992; for review, see Murray 1993; Weinert and Lydall 1993). We have also not yet detected genetic interactions between *CDC28* and the budding yeast checkpoint genes (see Table 4; D. Lydall and T.A. Weinert, unpubl.). The identity of the essential mitotic controls and how it is regulated by checkpoint genes remains a central unanswered question.

Limitations of the checkpoint screen

The screen based on the *cdc13* arrest phenotype has limitations indicating that additional checkpoint mutants might be identified using other criteria. The screen was apparently biased in identifying some but not all checkpoint genes; we recovered multiple alleles of *mec1*, *mec2*, and *rad9* but only a single allele of *mec3* and no alleles of *rad17* and *rad24*. Some checkpoint mutants may have been under-represented in our screen because they were not sufficiently lethal in combination with *cdc13*. For example, *cdc13 rad24*, *cdc13 mec3*, as well as *cdc13 rad17*, retain greater viability after 4 hr at the restrictive temperature than do the other three *cdc13*–checkpoint double mutants (Table 5; Weinert and Hartwell 1993). In addition, our screen may be limited to genes involved in recognizing a subset of possible DNA lesions (*cdc13*-defective cells accumulate single-stranded DNA at the restrictive temperature). We note, however, that the lesions in *cdc13*-defective cells must eventually share a common feature with radiation-induced lesions because all checkpoint mutants are radiation sensitive as well. Additional searches for checkpoint mutants might exploit the lethality of other *cdc*–checkpoint double mutants at the restrictive temperature for the *cdc* mutation (Table 5), or the observation that all six *cdc13*–checkpoint double mutants have a higher maximum permissive temperature than do *cdc13* single mutants.

The use of synthetic lethality to identify checkpoints and checkpoint genes

The checkpoint mutant isolation strategy used here relied on the following simple hypothesis: A checkpoint detects cellular defects and arrests cell division to provide the cell time for repair. Cell lethality results in checkpoint-defective cells when cell division occurs without repair of damage. In this study we introduced DNA damage by using a temperature-sensitive mutation (*cdc13*) and identified checkpoint mutants because they died rapidly and continued cell division despite the

damage. A conceptually similar approach was used to isolate mutants in the S-phase pathway in fission yeast because they failed to arrest and died rapidly when treated with HU (*hus* mutants; Enoch et al. 1992). Similarly, mutants that identify a checkpoint responsive to microtubule function or assembly (*MAD* and *BUB* genes) were identified from their failure to arrest and their low viability when treated with the microtubule poison benomyl (Hoyt et al. 1992; Li and Murray 1992). The checkpoint mutants identified here exhibited unselected phenotypes (e.g., radiation sensitivity) that provide further evidence for their role in checkpoint function. Similarly, the *hus*, *mad*, and *bub* mutants exhibit unselected phenotypes consistent with their roles in a checkpoint function (Hoyt et al. 1991; Li and Murray 1991; Enoch et al. 1992; also see Neff and Burke 1992).

Materials and methods

Strains and genetic methods

All strains are congenic with A364a, except as noted in Table 1. The *rad24-1* mutation was introduced into A364a by backcrossing the original *rad24-1* mutation (from KSS255; Table 1) to an A364a parental strain six times. Yeast media and genetic methods were standard (Sherman et al. 1974). Cell-cycle studies were done with cells grown to midlog stage (2×10^6 to 5×10^6/ml) in complete medium, except where noted.

Radiation and drug sensitivities

X-irradiation of cells was performed by use of a Machlett OEG600 tube set at 50 kV, 20 mA, which delivered 106 rad/sec, and UV-irradiation was delivered by use of a Stratalinker 1800. Qualitative UV- and X-ray sensitivities were determined by irradiating replicas of patches of cells two times, once at the time of replica plating and a second time 8–16 hr later. Dose of radiation: X-rays, 8 krads; UV, 80 J/m^2. Strain survival was determined after incubation for 2–3 days at 23°C.

For drug sensitivites, patches of cells were replica plated on medium containing 0.01% of MMS (Sigma M-4016) or 0.1 M HU (Sigma H-8627). To determine MMS sensitivity, patches of cells were replica plated and immediately replica plated a second time (double-replicas) to MMS-containing solid medium. This procedure effectively dilutes the number of cells transferred and improved the discrimination of MMS^s and MMS^r strains. We also monitored the ability of single cells to form visible colonies on solid medium containing either MMS or HU. The results from these qualitative tests of drug sensitivity varied with temperature and cell ploidy; we found that the most reproducible results were obtained using haploid strains at 23°C or diploid strains at 30°C (in *CDC*$^+$ strains).

Mutant isolation

A *cdc13 RAD* strain (TWY146; *MATα cdc13 ura3 his7 CAN1*) was mutagenized with EMS to ~50% viability, a dose that increased the frequency of point mutations ~230-fold (the frequency of canavanine resistance increased from 6×10^{-6} to 1.4×10^{-3}). Mutagenized cells were grown to single colonies on solid medium at 23°C, the permissive temperature for *cdc13*. Each colony represented a potential independent mutant.

The screen for checkpoint mutants involved two steps. First we screened individual colonies for cell viability after incubation at the restrictive temperature (rapid death screen). Colonies were double replica plated (to reduce the number of cells transferred; see above) and subjected to a temperature upshift–downshift regime; 6 hr at 36°C, then 23°C overnight, followed by the same regime the following day. This procedure effectively squares the difference in cell survival between rapidly dying strains and the *cdc13* control strains. After a subsequent 2- to 3-day incubation at 23°C, growth of each colony was compared with that of a *cdc13* strain, a *cdc13 rad9* strain, as well as with the mutant colony incubated only at 23°C. We showed that *cdc13* cells survive this temperature shift regime, whereas *cdc13 rad9* cells do not. Strains that died rapidly were retested for their rapid death phenotype, and candidates were then tested for their cell-cycle arrest phenotype. All strains with the rapid death phenotype were also screened qualitatively for X-ray sensitivity, although this was not a necessary criterium for a checkpoint mutant.

Strains that exhibited a rapid death phenotype were screened individually for microcolony formation at the restrictive temperature. Cells from each strain were resuspended in liquid culture, grown for several generations at the permissive temperature, sonicated, plated on agar plates, and incubated at the restrictive temperature (36°C). After 8–16 hr, the morphology of microcolonies arising from single cells was examined microscopically, and strains were characterized as arrested (forming microcolonies containing mostly two and four buds) or not-arrested (forming microcolonies containing greater than four buds; see Fig. 1A,B).

Genetic analysis of checkpoint mutants

The checkpoint mutants were crossed to *MAT***a** *cdc13* and sporulated, and haploid segregants were tested for single gene segregation of mutant phenotypes. The *cdc13* arrest phenotype and the rapid death phenotypes cosegregated where tested. The *cdc13* arrest phenotype was used initially to follow all checkpoint mutations in crosses and to test for complementation because some alleles were not sensitive to DNA-damaging agents (see Table 2). We isolated appropriate *MAT***a** *cdc13*– checkpoint double mutants for complementation and allelism tests. Initial gene assignments were made from complementation tests and were verified by allelism tests; all mutants showed tight linkage to the prototype allele from its complementation group (though spore viability was low in some strains). The strongest mutant alleles were selected for further study (*mec1-1*, *mec2-1*, and *mec3-1*, as well as *rad24-1*; obtained from K. Sitney and B. Mortimer, University of California, Berkeley).

Cell-cycle arrest after X-irradiation

Cells were grown in rich liquid medium to midlog phase, sonicated, plated on solid medium, and treated with a low dose of X-rays (2 krad) that generates a few double-strand breaks per cell (see Weinert and Hartwell 1988). After incubation of irradiated cells for 8–10 hr, cell-cycle arrest was determined from the morphology of microcolonies and from the cell inviability (determined 24 hr after irradiation). The ratio of percent arrested cells (microcolonies with either a large-budded cell or with two adjacent large-budded cells) divided by the percent of inviable cells provides a metric of the efficiency of cell cycle arrest. For wild-type cells this metric approaches 1.0 because essentially all cells with unrepairable DNA breaks die and arrest in the G_2 phase;

haploid cells in the G_1 or postanaphase stages when X-irradiated cannot repair the DNA double-strand breaks, arrest in the next G_2 phase, and die as large-budded and two adjacent large-budded cells, respectively. Wild-type cells that can repair the double-strand break (S- and G_2-phase cells) form large microcolonies that are not counted. In contrast, checkpoint mutant cells that die after X-irradiation do not usually arrest immediately, rather they continue to divide for a few generations. Therefore, in checkpoint mutants, the ratio of arrested to inviable cells is $<<1.0$ and typically <0.3. Cell death of irradiated cells is likely attributable to unrepaired DNA damage though may not be due solely to the defect in cell-cycle arrest, as discussed in the text.

Cell-cycle response to HU

HU inhibits DNA replication by depleting cells of dNTP precursors, presumably by inhibiting ribonucleotide reductase, and causes wild-type cells to arrest cell division in S phase. Cells were grown to midlog in liquid medium and then incubated for 4 hr with 0.2 M HU. HU was removed by washing cells twice with water and plated to determine cell viability, or fixed with 70% ethanol for 1 hr and stained with 4,6-diamino-2-phenyl indole (DAPI) for analysis of cell and nuclear morphology. To determine microtubule morphology, cells were fixed with freshly prepared paraformaldehyde and stained with DAPI and anti-tubulin antibodies (Pringle et al. 1989). For each strain, at least 100 cells was examined by fluorescence microscopy.

We found that with HU a first cycle arrest assay was not a particularly informative phenotype. HU-resistant cells plated on medium containing HU arrested in the first cycle (forming microcolonies of large-budded and two adjacent large-budded cells) and eventually recovered to resume cell division. HU-sensitive cells (*mec1* and *mec2*) plated on medium containing HU also appeared to arrest in the first cycle as large-budded cells but never resumed cell division. Analysis of nuclear morphology provides an explanation for why *mec1* and *mec2* mutants fail to recover: HU-sensitive cells appear to attempt mitosis before completion of DNA replication, die, and therefore do not resume cell division like HU-resistant cells.

Analysis of cdc–*checkpoint double mutants*

cdc mutants that arrest in specific stages of the cell cycle were crossed to checkpoint mutants and *cdc*–checkpoint double mutants were isolated (Table 1). The interactions of checkpoint and *CDC* genes were assessed by comparing four phenotypes of the *cdc* and respective *cdc*–checkpoint double mutants. After shift to the restrictive temperature for 4 hr, we determined the cell and nuclear morphology, cell viability, and whether cells arrested in the first cell cycle. Cell and nuclear morphology was determined from cells fixed in 70% ethanol, stained with DAPI, and viewed by fluorescence and light microscopy. Cell viability was determined microscopically from colony formation 24 hr after plating. First cycle arrest was determined by plating cells on solid medium and scoring the percent of cells that arrest in the first cycle (as either large-budded or as two adjacent large-budded cells; see Fig. 1). For each phenotype, at least 100 cells or microcolonies were analyzed.

The fourth criterion is the ability of cells to form colonies when grown at intermediate temperatures (the maximum permissive temperature). This test measures the cumulative effect, over ~20 cell divisions, of a checkpoint defect in cells limited for a *cdc* function. The maximum permissive temperature is the temperature (23, 25, 28, 30, 32 or 36°C) at which cells form macroscopically visible colonies similar to those formed at 23°C after 2–4 days of incubation.

Genetic mapping

In crosses to generate *cdc28 mec1* double mutants we noted their genetic linkage. *MEC2* (on CHRXV) and *MEC3* (on CHRIV) were each mapped physically by use of DNA fragments from the cloned genes as probes of a Southern transfer of chromosomes separated by pulse-field gel electrophoresis (S. Kim and T. Weinert, unpubl.). We have not yet determined the map position of *mec3* on CHRIV; preliminary data indicate that *mec3* is not tightly linked to *cdc2*, *cdc13*, *trp1*, *cdc34*, *rad9*, *aro8*, or *sac2* (T. Weinert et al., unpubl.). Hybridization of a *MEC3*-containing DNA probe to collections of phage and cosmid recombinant clones, provided by M. Olson, has proven inconclusive thus far in physical mapping (T. Weinert et al., unpubl.).

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