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p56^{chk1} protein kinase is required for the DNA replication checkpoint at 37°C in fission yeast

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Fission yeast p56^{chk1} kinase is known to be involved in the DNA damage checkpoint but not to be required for cell cycle arrest following exposure to the DNA replication inhibitor hydroxyurea (HU). For this reason, p56^{chk1} is considered not to be necessary for the DNA replication checkpoint which acts through the inhibitory phosphorylation of p34^{cdc2} kinase activity. In a search for *Schizosaccharomyces pombe* mutants that abolish the S phase cell cycle arrest of a thermosensitive DNA polymerase δ strain at 37°C, we isolated two *chk1* alleles. These alleles are proficient for the DNA damage checkpoint, but induce mitotic catastrophe in several S phase thermosensitive mutants. We show that the mitotic catastrophe correlates with a decreased level of tyrosine phosphorylation of p34^{cdc2}. In addition, we found that the deletion of *chk1* and the *chk1* alleles abolish the cell cycle arrest and induce mitotic catastrophe in cells exposed to HU, if the cells are grown at 37°C. These findings suggest that *chk1* is important for the maintenance of the DNA replication checkpoint in S phase thermosensitive mutants and that the p56^{chk1} kinase must possess a novel function that prevents premature activation of p34^{cdc2} kinase under conditions of impaired DNA replication at 37°C.

Keywords: cell cycle/chk1/DNA replication/heat shock/*S.pombe*

Introduction

The integrity of the genome is ensured by accurate DNA replication and repair and correct ordering of cell cycle events by control pathways known as checkpoints (Hartwell and Weinert, 1989). Studies in fission yeast have provided a great deal of insight into the mechanism that couples mitosis to completion of DNA replication and DNA repair in eukaryotes (Nurse, 1994).

The temporal order of S phase and mitosis is dependent on the state of the p34^{cdc2}– cyclin B complex whose active form brings about mitosis (Hayles *et al*., 1994). The complex is rendered inactive after Start by the wee1 and mik1 kinases which phosphorylate p34^{cdc2} on tyrosine 15 (Y15) (Gould and Nurse, 1989; Lundgren *et al*., 1991; Smythe and Newport, 1992; Hayles and Nurse, 1995). Subsequent dephosphorylation by the cdc25 phosphatase activates the p34^{cdc2}–cyclin B complex allowing passage into mitosis (Moreno *et al*., 1989). Thus, in cycling cells, the timing of mitosis is determined by a gradual change in the wee1/cdc25 ratio. This ratio influences the cell size at division and maintains the dependence of mitosis on the completion of DNA replication (Enoch and Nurse, 1990; Moreno *et al*., 1990). Recently, it has been shown that during the S phase cell cycle block at the restrictive temperature of the thermosensitive *cdc22-M45* mutant, the cdc25 phosphatase accumulates to high levels although its basal activity remains low. This indicates that the DNA replication checkpoint operates despite the accumulation of cdc25 protein (Kovelman and Russell, 1996).

Fission yeast mutants deficient in the DNA replication checkpoint have been isolated which fail to arrest the cell cycle in the presence of the DNA replication inhibitor hydroxyurea (HU) and thereby undergo mitotic catastrophe (*hus* mutants). Analysis of *hus* mutants provided evidence for a partial overlap between DNA replication and DNA damage checkpoints since many of these mutants were also affected in the ability to arrest the cell cycle in G_2 phase after DNA damage, resulting in the radiation-sensitive phenotype (*rad* mutants). However, in fission yeast, the radiation checkpoint seems not to involve the Y15 phosphorylation of p34^{cdc2}. Indeed, mutations affecting elements involved in Y15 regulation do not abolish the G_2 delay when cells are irradiated (Al-Khodairy and Carr, 1992; Enoch *et al*., 1992; Barbet and Carr, 1993; Sheldrick and Carr, 1993; Al-Khodairy *et al*., 1994).

The fission yeast checkpoint gene *chk1*$^+$/*rad27*$^+$ has been proposed to be solely involved in the DNA damage checkpoint, as deletion of this gene abolishes cell cycle arrest after irradiation (rad phenotype) but does not abolish cell cycle arrest when cells are exposed to the DNA replication inhibitor HU at 30°C (absence of hus phenotype) (Walworth *et al*., 1993; Al-Khodairy *et al*., 1994; Carr, 1995). Recently, it has been shown that p56^{chk1} is phosphorylated when cells are treated with different DNA-damaging agents (Walworth and Bernards, 1996). We reported that deletion of the *chk1* gene in S phase thermosensitive mutants abolishes cell cycle arrest and leads to mitosis in the absence of complete DNA replication under non-permissive conditions (Francesconi *et al*., 1995). These findings were intriguing since deletion of *chk1* does not result in the hus phenotype when the cells are treated with HU under normal growth conditions (30°C).

Here we show that the response to HU treatment is dependent on the growth temperature, as cells deleted for the *chk1* gene undergo mitotic catastrophe if exposed to HU at 37°C. Furthermore, we isolated *chk1* mutant alleles that do not abolish cell cycle arrest after UV irradiation and that are not sensitive to the alkylating agent methylmethane sulfonate (MMS). These alleles induce mitotic catastrophe either in the presence of HU at 37°C or when combined with S phase thermosensitive mutants. Thus, we demonstrate that chk1 protein kinase has a function in the DNA

replication checkpoint that is unrelated to its function in the DNA damage checkpoint. The role of chk1 in the DNA replication checkpoint is apparent at 37°C, suggesting a possible link between this checkpoint and the heat-shock response.

Results

Genetic screening for mutants inducing lethal mitosis in a DNA polymerase δ thermosensitive strain

We performed a screening in order to identify mutations that abolish the S phase cell cycle arrest of strain *polδts3* at the non-permissive temperature. This strain carries a thermosensitive mutation in the gene encoding DNA polymerase δ (*pol3*$^+$) and, when shifted to 37°C, cells arrest in S phase with a cdc (cell division cycle) terminal phenotype (Pignede *et al*., 1991; Francesconi *et al*., 1993). A total of 16 000 clones derived from the mutagenized *polδts3* strain were grown at 37°C and scored under the microscope for the absence of cdc terminal phenotype (Figure 1A). Nine clones were isolated which lacked the cdc terminal phenotype but were still thermosensitive for growth. We first analysed these clones for genetic linkage to some of the known checkpoint genes. In order to perform this analysis, we crossed the *polδts3* thermosensitive strain with the following checkpoint mutant strains: *cdc2-3w*, *hus1-14*, *rad1-1* and *dchk1* (Table I) (Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994). When possible, double mutants were isolated and their phenotypes analysed at the restrictive temperature (Table II). We found that the *polδts3* allele is synthetic lethal (SL) at the permissive temperature with both *hus1-14* and *rad1-1* alleles (Table II). The double mutants that we could isolate (*polδts3 cdc2-3w* and *polδts3 dchk1*) were then crossed with the nine clones derived from the screening in order to determine genetic linkage. We found that two out of nine clones from the screening which showed the cut (cell untimely torn) phenotype at 37°C (Figure 1B) were harbouring the *polδts3* allele and a mutation in the *chk1* gene (we will refer to the *chk1* mutations as *chk1-1* and *chk1-2* alleles). Furthermore, none of the nine clones regained the cdc terminal phenotype at 37°C when transformed with a plasmid carrying the wild-type checkpoint gene *cut5*$^+$ (Saka and Yanagida, 1993; Saka *et al.*, 1994).

To extend the analysis of the genetic interaction between the different checkpoint mutants and S phase *cdc* mutants, we crossed the thermosensitive strains *polδts1*, *polδts2*, *polαts1* (DNA polymerase α *cdc* mutant, our unpublished result), *cdc17* (DNA ligase), *cdc20* and *cdc24* with the checkpoint mutant strains listed in the previous paragraph (Nasim and Smith, 1975; Nasmyth and Nurse, 1981; Damagnez *et al*., 1990; Francesconi *et al*., 1993). The phenotype of double mutants was then analysed and results are summarized in Table II. Synthetic lethality at the permissive temperature of 25°C was observed only in the combination of *polδ* and *polα* mutants with either *rad1-1* or *hus1-14* checkpoint mutations. All the others combinations were viable at 25°C and showed the cut phenotype at 37°C (Table II).

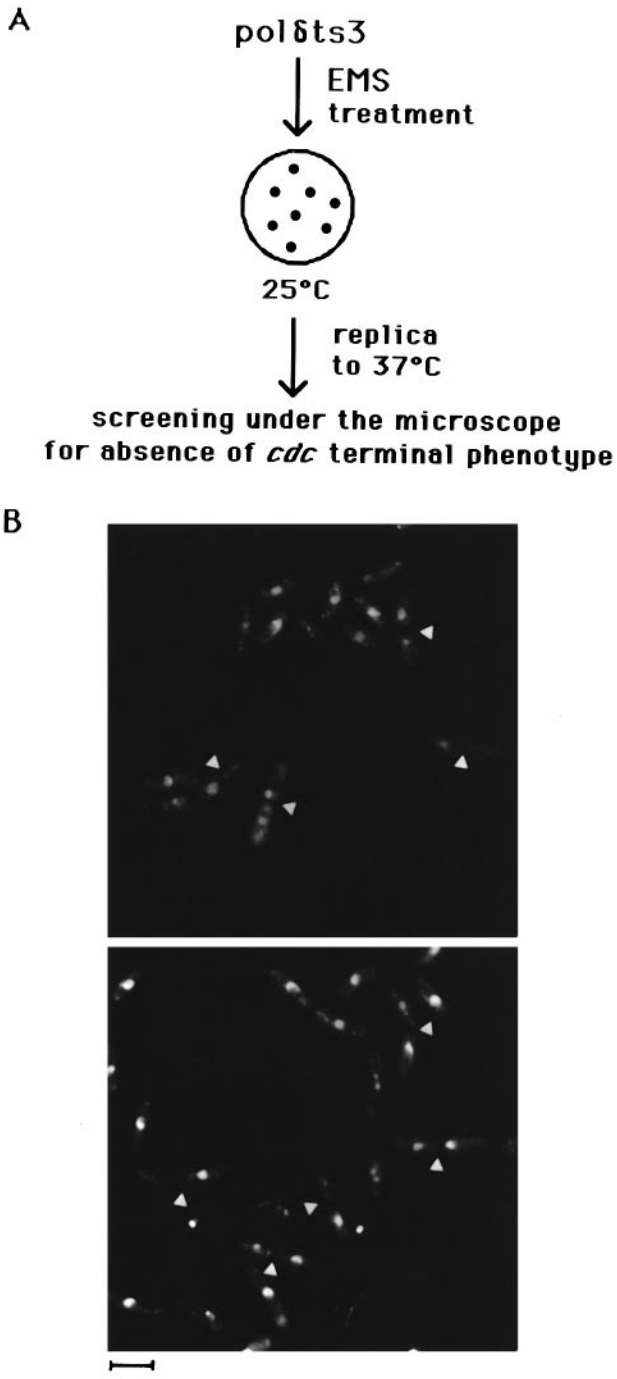

Fig. 1. (**A**) Screening strategy to identify mutants that abolish the cell cycle arrest in strain *polδts3*. (**B**) Nuclear morphology of *polδts3 chk1-1* (upper panel) and *polδts3 chk1-2* cells (lower panel) shifted to 37°C for 7 h; arrows indicate aberrant mitosis; bar represents 10 μm.

Primary characterization of polδts3 chk1-1 and polδts3 chk1-2 double mutants

The double mutants *polδts3 chk1-1* and *polδts3 chk1-2* isolated from the screening described above were transformed with a plasmid carrying the wild-type *chk1*$^+$ gene. Transformants regained the cdc terminal phenotype at 37°C. Double mutants were then back-crossed to the *polδts3* strain and finally crossed with wild-type, allowing isolation of *chk1-1* and *chk1-2* single mutants. Combination of either of the two *chk1* alleles with the *polδts1*,

Table I. Strains used in this study

Strain	Genotype	Source
wt (972)	h^{-S} wild-type	a
dchk1 (SP1182)	h^{-S} *chk1::ura4*$^+$ *ura4-D18*	b
chk1-1	h^{-S} *ura4-D18 leu1-32 ade6 chk1-1*	c
chk1-2	h^{-S} *ura4-D18 leu1-32 ade6 chk1-2*	c
polδ ts3 chk1-1	h^{+N} *ura4-D18 leu1-32 ade6-M216 polδ ts3 chk1-1*	c
polδ ts3 chk1-2	h^{+N} *ura4-D18 leu1-32 ade6-M216 polδ ts3 chk1-2*	c
polδ ts1	h^{+N} *ura4-D18 leu1-32 ade6-M216 polδ ts1*	d
polδ ts2	h^{+N} *ura4-D18 leu1-32 ade6-M216 polδ ts2*	d
polδ ts3	h^{+N} *ura4-D18 leu1-32 ade6-M216 polδ ts3*	d
polα ts1	h^{+N} *ura4-D18 leu1–32 ade6-M216 polα ts1*	g
polδ ts3 dchk1 (SPC3)	h^{+N} *chk1::ura4*$^+$ *ura4-D18 leu1-32 polδ ts3*	h
cdc17	h^{+N} *cdc17-K42*	a
cdc20 (ED087)	h^{+N} *cdc20-M10*	e
cdc24 (ED092)	h^{+N} *cdc24-M38*	e
cdc25	h^{+N} *cdc25-22*	a
cdc2-3w	h^{-S} *cdc2-3w*	f
hus1-14	h^{-S} *hus1-14*	f
rad1-1	h^{-S} *rad1-1*	f

[a]From the National Collection of Yeast Cultures, Norwich, UK.
[b]From David Beach, Cold Spring Harbor Laboratory, NY, USA.
[c]This study.
[d]Francesconi *et al.* (1993).
[e]From Peter Fantes, University of Edinburgh, UK.
[f]From Paul Nurse, Imperial Cancer Research Fund, London, UK.
[g]S phase thermosensitive *cdc* mutant in DNA polymerase α encoding gene, our unpublished result.
[h]Francesconi *et al.* (1995).

Table II. Relevant phenotype of double mutants

h^+ \ h^-	*cdc2-3w*	*hus 1-14*	*rad 1-1*	*dchk1*
polδ ts1	cut 37°C	SL 25°C	SL 25°C	cut 37°C
polδ ts2	cut 37°C	SL 25°C	SL 25°C	cut 37°C
polδ ts3	cut 37°C	SL 25°C	SL 25°C	cut 37°C
polα ts1	cut 37°C	SL 25°C	SL 25°C	cut 37°C
cdc17-K42	cut 37°C	cut 37°C	cut 37°C	cut 37°C
cdc20-M10	cut 37°C	cut 37°C	cut 37°C	cut 37°C
cdc24-M38	cut 37°C	cut 37°C	cut 37°C	cut 37°C

polδts2 polδts3 mutant alleles or with other known S phase *cdc* mutants (*polαts1*, *cdc17*, *cdc20* and *cdc24*) results in cells with the cut phenotype at 37°C (not shown) (Nasmyth and Nurse, 1981; Francesconi *et al*., 1993, 1995).

We further analysed the phenotype of *polδts3 chk1* double mutants at 37°C in synchronized cultures. Unlike strain *polδts3* (Figure 2B), synchronizations by centrifugal elutriation of double mutant strains *polδts3 chk1-1* and *polδts3 chk1-2* at 37°C showed that the cells enter mitosis despite incomplete DNA replication (Figure 2C and D), leading to accumulation of cells with the cut phenotype (not shown). This behaviour is similar to that observed for the *polδts3 chk1::ura4*$^+$ double mutant (Francesconi *et al*., 1995). Thus, we conclude that in double mutant strains at the non-permissive temperature, mitosis is uncoupled from DNA replication.

We then obtained and sequenced *chk1* alleles from both mutants by PCR amplification. Allele *chk1-1* has a single point mutation resulting in the substitution of Glu92 by aspartic acid. This amino acid is conserved in the catalytic domain of many serine/threonine protein kinases, suggesting that this mutant could be altered in the kinase activity. Allele *chk1-2* also has a single point mutation changing Ile484 to threonine. This mutation, at the very carboxy-terminus of the protein, is not located in the catalytic domain of protein kinases.

Characterization of the DNA damage checkpoint in chk1-1 and chk1-2 mutants

We analysed the radiation sensitivity of *chk1-1* and *chk1-2* single mutants at different temperatures. Surprisingly, we found that, unlike the *dchk1* strain (*chk1* deletion), *chk1-1* and *chk1-2* mutants are not radiation sensitive (data not shown). We then investigated whether these mutants were able to arrest the cell cycle after UV irradiation at the same temperature at which they had been isolated, 37°C. Like the wild-type strain, strains *chk1-1* and *chk1-2* show a decrease in septation index after irradiation with 200 J/m^2 at 37°C, indicating that the DNA repair checkpoint is intact (Figure 3A). In contrast, control strain *dchk1* had completely lost the DNA damage checkpoint as previously shown (Walworth *et al*., 1993; Al-Khodairy *et al*., 1994). We further analysed the radiation checkpoint in the synchronized double mutant *polδts3 chk1-1* at 37°C. Similarly to the wild-type strain, the double mutant delays mitosis after irradiation in a dose-dependent manner, confirming that the radiation checkpoint is active despite mutations in *polδ* and *chk1* genes (Figure 3B). The same

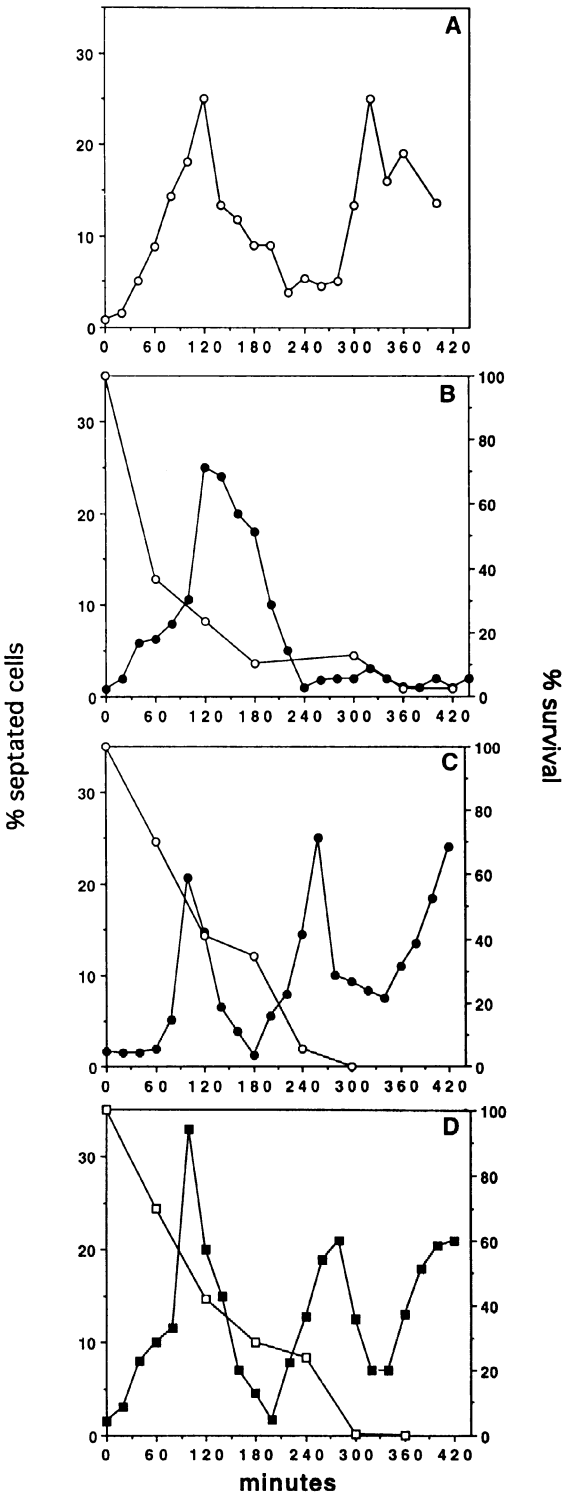

Fig. 2. Synchronized *polδts3 chk1-1* and *polδts3 chk1-2* double mutants do not arrest the cell cycle at 37°C despite incomplete DNA replication. (**A**) Synchronized *polδts3* at 25°C, (○) % septated cells; (**B**) synchronized *polδts3* at 37°C, (●) % septated cells, (○) % surviving cells; (**C**) synchronized *polδts3 chk1-1* at 37°C, (●) % septated cells, (○) % surviving cells; (**D**) synchronized *polδts3 chk1-2* at 37°C, (■) % septated cells, (□) % surviving cells. Synchronized double mutants at the permissive temperature of 25°C behave as a *polδts3* single mutant (A).

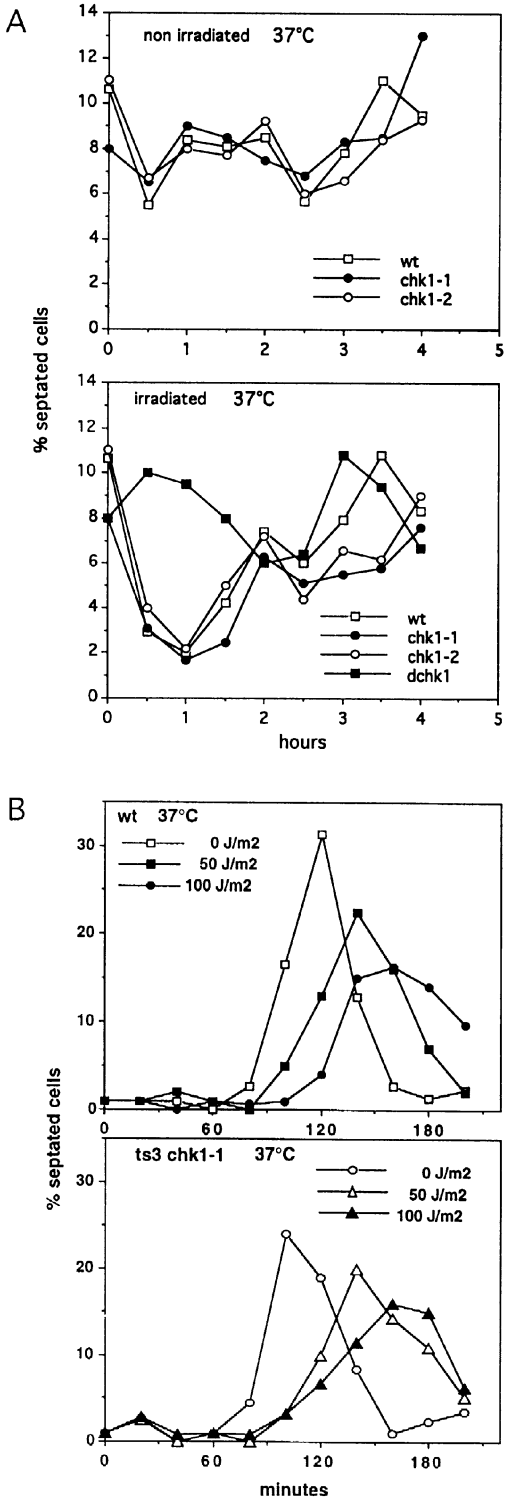

Fig. 3. Mutant alleles *chk1-1* and *chk1-2* are proficient for the DNA damage checkpoint after UV irradiation at 37°C (the same results were obtained at 30°C). (**A**) Asynchronous cultures of *chk1-1* and *chk1-2* single mutants arrest the cell cycle after UV irradiation at 200 J/m^2 similarly to wild-type (wt) (irradiation at 100 J/m^2 gave similar results). (**B**) Synchronous culture of the *polδts3 chk1-1* double mutant (lower panel) delays mitosis after irradiation at 37°C in a dose-dependent manner similarly to wild-type strain (upper panel) (the same results were obtained for the *polδts3 chk1-2* double mutant).

results were obtained for the *polδts3 chk1-2* double mutant (not shown). Furthermore, *chk1-1* and *chk1-2* mutants are not sensitive to the alkylating agent MMS which induces DNA strand breaks. Mutant strains behave similarly to the wild-type when exposed to MMS at 37°C (a behaviour similar to the wild-type strain was also observed at 30°C). In contrast, the control strain *dchk1* is highly sensitive to exposure to MMS (Figure 4A). Unlike the *dchk1* strain, the partial sensibility to MMS of the wild-type and *chk1* alleles does not result from illegitimate passage into mitosis as shown by 4′,6′-diamidino-2-phenylindole (DAPI) staining of the cells after 4 h of MMS treatment at 37°C (Figure 4B). These experiments clearly demonstrate that *chk1-1* and *chk1-2* alleles are proficient for the DNA repair checkpoint.

HU sensitivity of chk1 mutants

Our results, suggesting a role for $p56^{chk1}$ kinase in the DNA replication checkpoint, are in conflict with previous reports that the *chk1*-deleted strain is insensitive to the DNA replication inhibitor HU (Walworth *et al*., 1993; Al-Khodairy *et al*., 1994). We re-examined this phenotype at 30 and 37°C. Cells were grown in YEA medium and 12 mM HU was added at time 0 and after 3 h of incubation at 30 and 37°C. The *dchk1* and *chk1-1* mutants behaved similarly to the wild-type strain in the presence of HU at 30°C, as demonstrated by survival, septation index and cut index of the different strains (Figure 5A, panels a, b and c). The control strain *hus1-14* rapidly lost viability at both 30 and 37°C and cells showed the cut phenotype as previously described (Enoch *et al.*, 1992). The *dchk1* strain is slightly more sensitive to exposure to 12 mM HU than are wild-type cells at 30°C. This sensitivity does not result from the absence of the DNA replication checkpoint as shown by the septation and cut index and in agreement with a previous report (Figure 5A) (Al-Khodairy *et al*., 1994). In addition, the *dchk1* strain showed a slight increase of cells with the cut phenotype after 10 h of incubation when compared with wild-type and *chk1-1* strains (Figure 5A, panel c). Since FACS analysis showed that DNA replication was completed at 30°C after 9 h of incubation in the presence of HU (not shown), the percentage of *dchk1* cells with the cut phenotype after 10 h of incubation could depend on the lack of the G_2 DNA repair checkpoint. In marked contrast, *dchk1* and *chk1-1* mutants lost viability when exposed to 12 mM HU at 37°C (Figure 5B, panel a). Mutant cells showed first an attempt to block the cell cycle, as shown by the decrease in septation index during the first 3 h of treatment, followed by an abnormal increase in the percentage of septated cells (Figure 5B, panel b). DAPI stainings of mutant cells exposed to HU at 37°C clearly showed an accumulation of cells with the cut phenotype (Figure 5B, panel c and e). The septation and cut index of *chk1* mutants increased after 4 h of incubation while the wild-type strain started to septate after 6 h of incubation in the same experimental conditions and did not accumulate cells with the cut phenotype (Figure 5B, panels b and c). FACS analysis showed that after 4 h of incubation at 37°C, cells exit the G_1 block imposed by HU and enter S phase (Figure 5B, panel d). In the wild-type strain, the S phase in the presence of HU took place in ~3 h and it is during this time period that *chk1* mutants start to lose

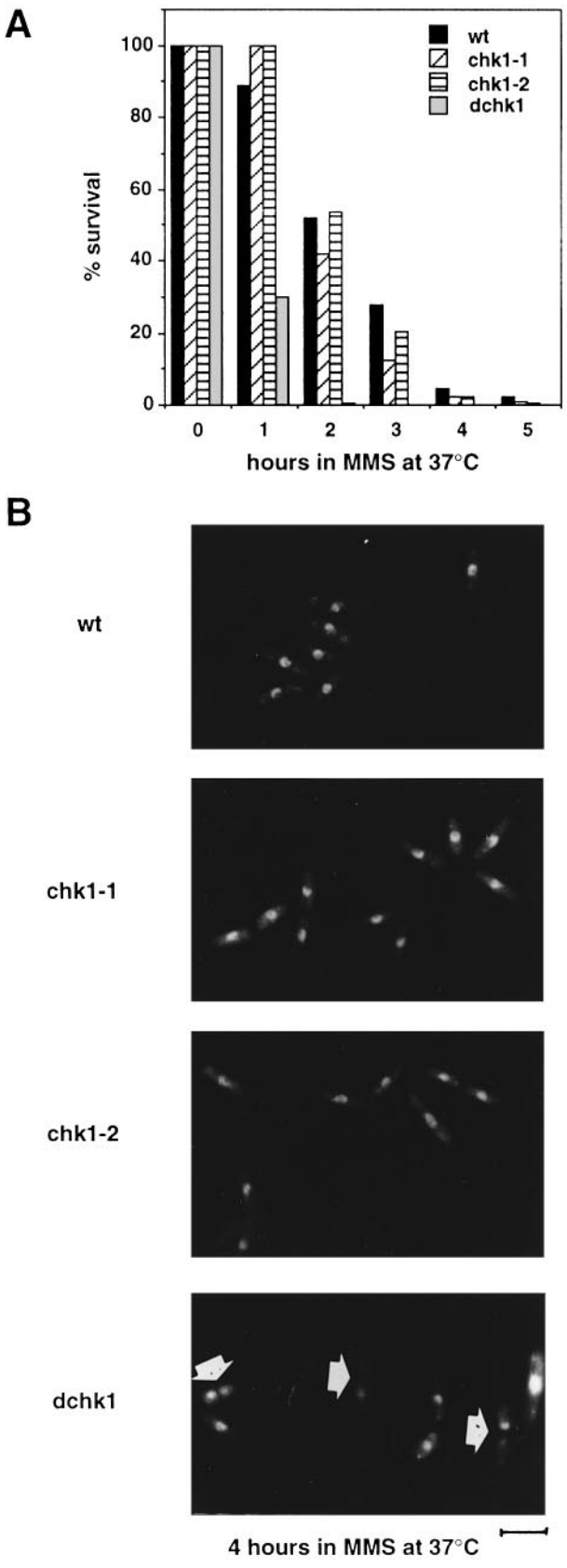

Fig. 4. Mutant strains *chk1-1* and *chk1-2* are not sensitive to MMS. (**A**) Survival after exposure to 0.01% MMS at 37°C for different times. (**B**) Nuclear morphology of cells treated with 0.01% MMS at 37°C for 4 h; arrows indicate aberrant mitosis; bar represents 10 μm.

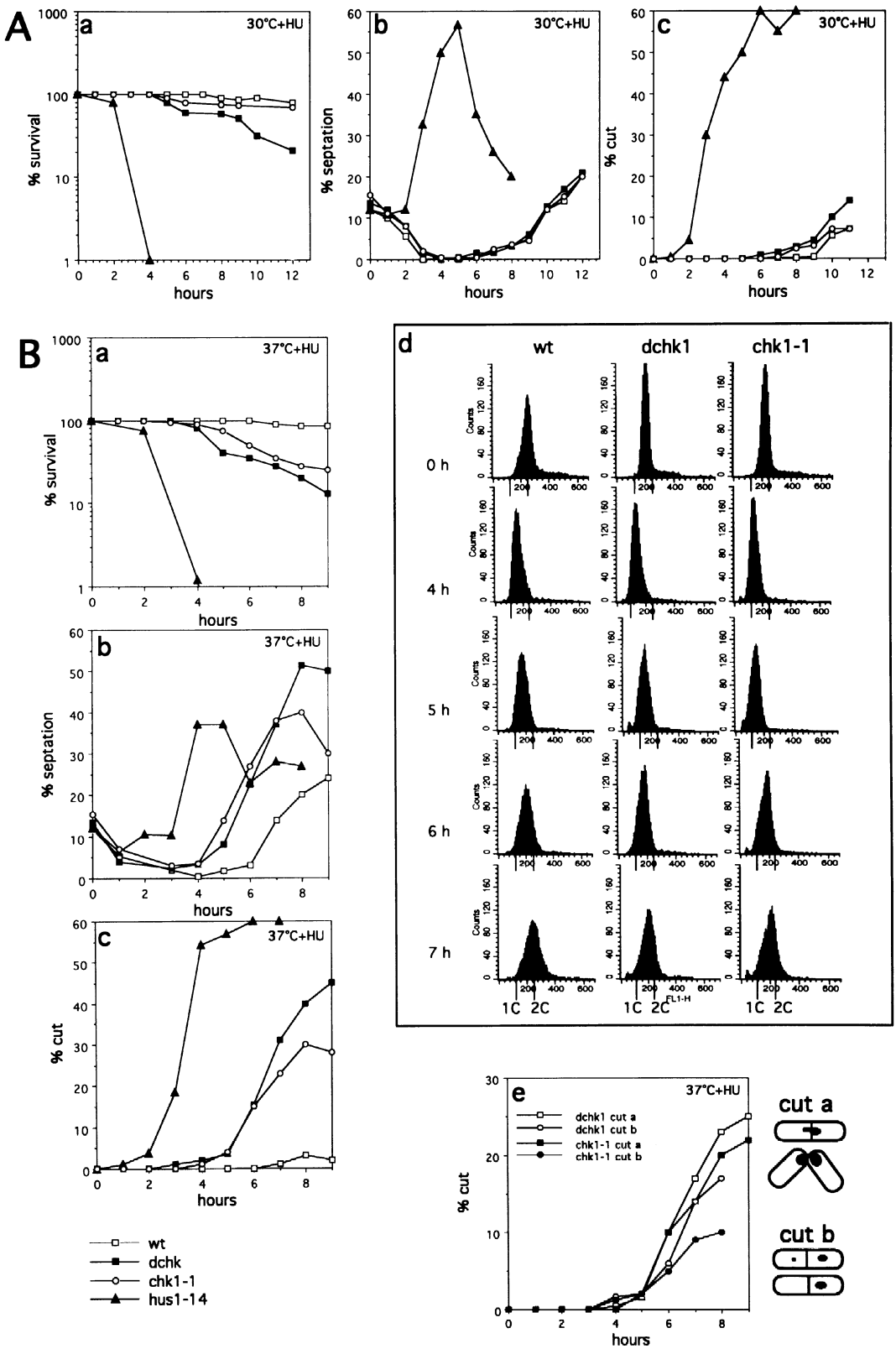

Fig. 5. HU sensitivity of *chk1* mutants. (**A**) *chk1* mutants are not sensitive to transient exposure to HU at 30°C: survival (a), septation index (b) and cut index (c) of wild-type, *hus1-14*, *dchk1* and *chk1-1* strains. (**B**) *chk1* mutants are sensitive to transient exposure to HU at 37°C: survival (a), septation index (b) and cut index (c) of wild-type, *hus1-14*, *dchk1* and *chk1-1* strains. (d) FACS analysis performed on wild-type, *dchk1* and *chk1-1* cells at 0, 4, 5, 6 and 7 h of incubation at 37°C in the presence of HU. (e) Percentage of the different cut phenotypes observed in *dchk1* and *chk1-1* mutants at 37°C in the presence of HU.

viability and to show the cut phenotype. At 6 h, cells are clearly in S phase and both *chk1* mutants have ~25% of septated cells and 15% of cells with the cut phenotype. It is worthwhile underlining that the *chk1-1* mutant, which is proficient for the DNA repair checkpoint (Figures 3 and 4), is sensitive to HU at 37°C similarly to the *chk1*-deleted strain, suggesting that the observed phenotypes do not depend on the absence of a functional DNA damage checkpoint. In addition, the *dchk1* strain, which lacks the DNA repair checkpoint at both 30 and 37°C, does not show sensibility to HU at 30°C.

These experiments demonstrate that, unlike the *hus1-14* mutant that is sensitive to the block in late G_1/early S phase at all the temperatures, the *chk1* mutants are sensitive to HU in middle/late S phase at 37°C. This result is consistent with the observation that the chk1 checkpoint is essential to maintain the S phase cell cycle block of DNA polymerase δ thermosensitive strains whose DNA replication begins but is not completed, leading to cells arrested with a DNA content comprised between 1C and 2C (Francesconi *et al.*, 1993, 1995). Thus, we can conclude that the chk1 checkpoint is essential for coupling mitosis to completion of S phase when DNA replication is impaired at 37°C. Furthermore, since at 37°C in the presence of HU there was an attempt to stop the cell cycle, the chk1 checkpoint might act after inhibitory tyrosyl phosphorylation of $p34^{cdc2}$ has occurred in order to prevent premature activation of the kinase when DNA replication is impaired at 37°C.

Analysis of $p34^{cdc2}$ tyrosine phosphorylation in polδts3 and polδts3 chk1 mutants

We analysed the level of tyrosine phosphorylation of $p34^{cdc2}$ in strains *polδts3* and *polδts3 chk1-1*. Cells in log phase were shifted to 37°C and protein extracts were prepared at 1 h intervals after the shift. An equal amount of total protein was incubated with $p9^{CKS}$ beads (Zhang *et al.*, 1995) that precipitate $p34^{cdc2}$ kinase. Precipitated $p34^{cdc2}$ was probed with anti-phosphotyrosine antibodies and, after stripping, re-probed with anti-cdc2 PSTAIRE antibodies (Figure 6A). Western blot shows that in the *polδts3* single mutant, which arrests the cell cycle at the non-permissive temperature, the level of tyrosine-phosphorylated $p34^{cdc2}$ increased during the shift (Figure 6A, a and b). This result is in agreement with the results obtained for *cdc20* and *cdc22* S phase thermosensitive mutants (Hayles and Nurse, 1995). On the contrary, the double mutant strain, which at 37°C does not display cell cycle arrest, shows a level of phosphorylated $p34^{cdc2}$ which initially increased after 6 h of shift and then decreased considerably (Figure 6A, c and d). The fluctuation of the amount of tyrosine-phosphorylated $p34^{cdc2}$ in the double mutant strain suggests that, as previously discussed for the HU experiment, chk1 acts after the DNA replication checkpoint has been activated and that it is necessary to maintain the inhibitory Y15 phosphorylation of $p34^{cdc2}$–cyclin B complex during the DNA replication block at 37°C.

We further investigated the level of phosphotyrosine by analysing the phosphoamino acid content of $p34^{cdc2}$ precipitated from an equal amount of *polδts3* and *polδts3 dchk1* mutant cells shifted to 37°C. We chose the *polδts3 dchk1* double mutant strain because cells at 37°C enter

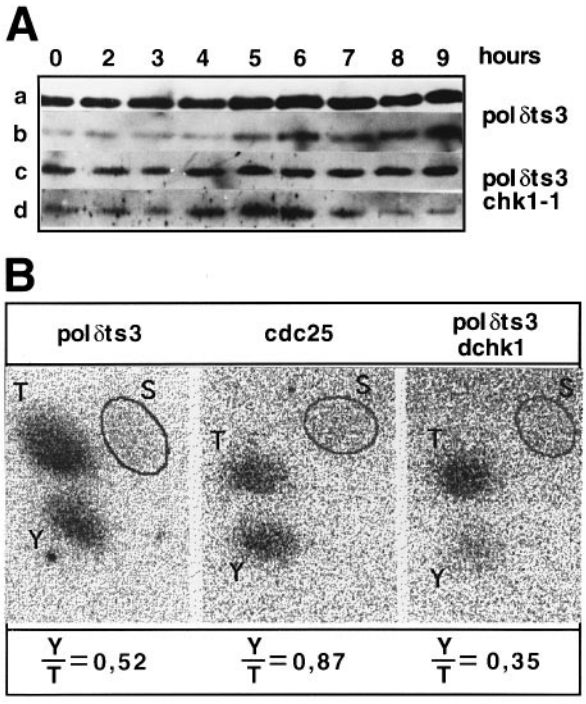

Fig. 6. (**A**) Tyrosine phosphorylation level of $p34^{cdc2}$ precipitated from *polδts3* and *polδts3 chk1-1* strains at time 0 and after 2, 3, 4, 5, 6, 7, 8 and 9 h of shift to 37°C. (a) $p34^{cdc2}$ precipitated from the *polδts3* mutant and revealed with anti-cdc2 PSTAIRE antibodies. (b) $p34^{cdc2}$ precipitated from the *polδts3* mutant and revealed with anti-phosphotyrosine antibodies. (c) $p34^{cdc2}$ precipitated from the *polδts3 chk1-1* double mutant and revealed with anti-cdc2 PSTAIRE antibodies. (d) $p34^{cdc2}$ precipitated from the *polδts3 chk1-1* double mutant and revealed with anti-phosphotyrosine antibodies. (**B**) Phosphoamino acid analysis of *in vivo* labelled $p34^{cdc2}$ precipitated from *polδts3* and *polδts3 dchk1* strains shifted to 37°C for 6 h. The control *cdc25* strain was shifted to 37°C for 4 h. Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine. TLC (thin layer cellulose) plates were exposed to Molecular Dynamics Screen. Screens were scanned using a Molecular Dynamics PhosphoImager. Quantification was done with ImageQuant Software.

mitosis more quickly when compared with the *polδts3 chk1-1* double mutant, probably because of the different nature of the *chk1* mutation (deletion of the gene versus point mutation). The phosphoamino acid detection and quantification showed that the phosphotyrosine content of $p34^{cdc2}$ precipitated from the *polδts3 dchk1* double mutant after 6 h of temperature shift is significantly reduced (Figure 6B). Interestingly, the *polδts3* single mutant has a phosphotyrosine/phosphothreonine ratio of 0.5 while the *cdc25* mutant strain did show, as expected from previous reports, a phosphothreonine/phosphotyrosine ratio close to 1. A possible explanation for the phosphotyrosine/phosphothreonine ratio obtained for the *polδts3* strain could be that the time employed to harvest cells mimics a short temperature release leading to phosphorylation of T14 as previously shown for the S phase *cdc17* and *cdc21* thermosensitive mutants (Den Haese *et al.*, 1995). If this is the case, each molecule of $p34^{cdc2}$ would contain two residues of phosphothreonine (T167 and T14) and one of phosphotyrosine (Y15), justifying the ratio of 0.5.

Discussion

The $chk1^{+}/rad27^{+}$ gene was identified previously by two different screenings: the first screening was designed in

order to look for multicopy suppressors of the cold-sensitive allele *cdc2-r4* (Walworth *et al.*, 1993), and the second screening was aimed at finding mutants that were sensitive both to radiation and to a transient temperature shift in a *cdc17-k42* background (ts DNA ligase) (Al-Khodairy *et al.*, 1994). It was considered that *chk1*$^+$/*rad27*$^+$ is necessary for the DNA damage checkpoint but is not required for the DNA replication checkpoint. We previously showed that *chk1* deletion induces rapid death and passage into mitosis despite incomplete DNA replication in several S phase thermosensitive mutants (Francesconi *et al.*, 1995). Similarly, combination of the *cdc2-3w* mutation, which abolishes the checkpoint for DNA replication but not that for DNA repair, with S phase *cdc* mutants results in mitotic catastrophe under non-permissive conditions (Table I). The *chk1* requirement for the cell cycle block of S phase *cdc* mutants was difficult to reconcile with the observation that *chk1*-deleted cells are still able to block the cell cycle in the presence of the DNA replication inhibitor HU (Al-Khodairy *et al.*, 1994).

Here we report the identification of two *chk1/rad27* alleles that induce mitotic catastrophe in the DNA polymerase δ thermosensitive strain and in several S phase *cdc* mutants. We demonstrate that the identified *chk1* alleles are proficient for the DNA damage checkpoint, implying that the mitotic catastrophe observed in the S phase *cdc* mutant background does not result from loss of this checkpoint. Thus, these *chk1* alleles reveal a new function for the p56^{chk1} protein kinase in the DNA replication checkpoint. We re-examined the behaviour of a *chk1*-deleted strain and of the identified *chk1-1* mutant in the presence of HU. We found that at 37°C mutant cells are unable to maintain the cell cycle block imposed by HU treatment, suggesting that chk1 kinase is not required for activating the DNA replication checkpoint but for keeping it active when DNA replication is impaired at 37°C. Thus, the *chk1*$^+$ gene product would appear to be a necessary element of the DNA replication checkpoint under heat shock conditions. We previously suggested that in the *polδts3 chk1::ura4*$^+$ double mutant both the DNA replication and the repair checkpoints were inactive, leading to uncontrolled entry into mitosis at 37°C. This interpretation implied that DNA polymerase δ is required for the replication feedback control (Francesconi *et al.*, 1995). In the light of the results presented here, it is clear that the absence of the replication checkpoint in double mutants at 37°C is due to mutations in the *chk1* gene rather than in *polδ*. However, we report (Table I) that *hus1-14* and *rad1-1* checkpoint mutants, which are deficient in both DNA replication and repair checkpoints, are synthetic lethal at permissive temperature with either DNA polymerase α or DNA polymerase δ thermosensitive *cdc* mutants. The synthetic lethality is also not observed with the *cdc2-3w* mutant, which is only deficient in the replication checkpoint, nor with the *chk1* null allele mutant, which is affected at 25°C only in the repair checkpoint. Altogether these results suggest that the *polα* and *polδ* mutants have a defect in DNA replication at the permissive temperature requiring an additional function of hus1 and rad1 essential to allow cell survival. Indeed, an additional function necessary for recovery from S phase arrest has been proposed for the *hus1*$^+$ and *rad1*$^+$ genes since the mutants lose viability dramatically in the presence of HU even before cells undergo mitotic catastrophe (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992; Al-Khodairy *et al.*, 1994). The observation that the recovery function of hus1 and rad1 could be essential in DNA polymerase α and δ mutants at the permissive temperature suggests that these replicative enzymes are important targets for monitoring a correct progression through S phase.

The *chk1* alleles described in this report are proficient for the DNA damage checkpoint, but induce mitotic catastrophe in a *cdc17* background (ts DNA ligase). This suggests that monitoring unligated DNA does not require the DNA damage checkpoint. This observation is in agreement with the recent report of mutational analysis of checkpoint *rad1*$^+$ (Kanter-Smoler *et al.*, 1995). Mutant *rad1-S3* retains the DNA damage checkpoint but not the DNA replication checkpoint and induces a phenotype of rapid death and lethal passage into mitosis in the *cdc17* background. Interestingly, it has been reported that p56^{chk1} kinase is constitutively phosphorylated in a *cdc17* strain at the permissive temperature of 25°C (Walworth and Bernards, 1996).

We found p56^{chk1} to be required for the cell cycle arrest in middle/late S phase at 37°C. This indicates that chk1 protein kinase couples cell cycle control to the heat shock response and suggests that functions and interactions of checkpoint proteins in either the DNA repair or DNA replication checkpoint pathways are modulated in response to different growth conditions. It is known that in *S.pombe* the heat shock response and cell cycle control are connected (Polanshek, 1977). The fission yeast homologue of Hsp90, a member of a family of proteins involved in stress responses, has been found to bind to and to be required for wee1 kinase activity at all temperatures (Aligue *et al.*, 1994). More recently, a heat shock-inducible cyclophilin-like protein was found to suppress the cell cycle defect of the *wee1-50 cdc25-22 win1-1* triple mutant (Weisman *et al.*, 1996).

We found that the level of the tyrosine-phosphorylated inactive form of p34^{cdc2} is reduced in *polδts3 chk1* double mutants at 37°C when compared with the *polδts3* single mutant, suggesting that the p56^{chk1} protein kinase could act in the DNA replication checkpoint under conditions of temperature stress by modulating the activities of the wee1 and/or cdc25 p34^{cdc2} regulators. Alternatively, p56^{chk1} could modify the p34^{cdc2}–cyclin B complex, allowing maintenance of its phosphorylated inactive form at 37°C.

Materials and methods

Yeast methods and media

The *S.pombe* strains used in this study are listed in Table I. Standard genetic procedures and media were as described (Gutz *et al.*, 1974; Moreno *et al.*, 1991). We refer to YEA medium, in which cells have been grown, as the conditioned medium.

Mutagenesis was performed by collecting 1×10^8 mid-log phase cells grown in YEA at 25°C and resuspending them in 1 ml of 0.2 M $NaPO_4$ pH 7, 2.5% EMS (methane-sulfonic acid ethyl ester, Sigma). At this EMS concentration cell survival was 50%. The cell suspension was incubated for 1 h at room temperature, 5 ml of 6% Na-thiosulfate were added and cells were washed five times in YEA before plating. Plates were incubated at 25°C until colony formation. A total of 16 000 clones were replicated on YEA plates and incubated at 37°C overnight.

Replicated clones were scored under the microscope in a search for those which did not display the cdc terminal phenotype.

Preparation of synchronous cultures was performed by centrifugal elutriation as previously described (Francesconi *et al.*, 1995). Synchronization was followed every 20 min from time 0 by estimation of the percentage of septated cells under a white light phase contrast microscope. Every hour from time 0 cells from the cultures at 37°C were collected, diluted and plated on YEA at 25°C in order to quantify cell survival.

Measurements of the radiation checkpoint in asynchronous cultures were performed by growing cells in YEA at 37°C to 2×10^6 cells/ml; cells were collected, resuspended in water at 5×10^7 cells/ml and split into two samples one of which was irradiated with 200 J/m^2 in a Stratagene Stratalinker UV source while the other sample served as non-irradiated control. Cells were then re-inoculated in conditioned medium at 37°C at 2×10^6 cells/ml and percentages of septated cells were followed every 30 min.

Measurements of the radiation checkpoint in synchronized cultures were performed by selecting early G_2 populations from cultures grown in YEA to 1×10^7 cells/ml at 25°C. Early G_2 cells were resuspended in water at 5×10^7 cells/ml and each sample was split into three aliquots, two of which were irradiated with 50 and 100 J/m^2, respectively, while the third one served as a non-irradiated control. Non-irradiated and irradiated cells were re-inoculated in conditioned medium at 2×10^6 cells/ml at 37°C; the septation index was followed every 20 min under a white light phase contrast microscope.

Determination of MMS (Fluka) sensibility was performed by growing cells in YEA to mid-log phase at 37°C; at time 0, MMS was added to 0.01% final concentration; from time 0 cell samples were collected every hour, MMS was inactivated with 5% Na thiosulfate, cells were diluted and plated on YEA at 37°C in order to quantify cell survival.

For HU (Sigma) experiments, cultures were grown to 2×10^6 cells/ml and then split into two samples, one of which was re-incubated at 30°C while the other was shifted to 37°C. After 1 h of incubation, HU was added to 12 mM final concentration (time 0) and again was added after 3 h (time 3). From time 0, cell samples were collected every hour, diluted and plated for survival estimation. The septation index was followed under a white light phase contrast microscope. An aliquot of each cell sample was treated for DAPI staining in order to determine the percentage of cells with the cut phenotype, and an aliquot was treated to estimate the cellular DNA content as previously described using a Becton-Dickinson FACScan (Costello *et al.*, 1986).

DAPI staining was performed as described (Moreno *et al.*, 1991). Samples were photographed under an epifluorescence microscope (Zeiss).

PCR genomic amplification and DNA sequencing

Schizosaccharomyces pombe genomic DNA of *chk1-1* and *chk1-2* mutant strains was prepared as previously described (Moreno *et al.*, 1991), further purified using Nucleotrap Kit (Macherey-Nagel) and finally resuspended in water. PCR amplification was carried out using the appropriate oligonucleotides designed according to the published *chk1*$^+$ sequence (Walworth *et al*., 1993; Al-Khodairy *et al*., 1994). Conditions for PCR amplification were determined by testing different DNA and $MgCl_2$ concentrations using final concentrations of 200 mM for each dNTP and 500 nM for each oligonucleotide. Replitherm™ Thermostable DNA polymerase from Epicentre Technologies Corporation was used. Cycling parameters were: 5 min at 94°C, 30 s at 37°C and 30 s at 72°C for one cycle; 30 s at 94°C, 30 s at 37°C and 30 s at 72°C for 35 cycles; 5 min at 72°C for one cycle. Amplified DNA fragments were gel-purified, cloned into pUC19 vector and four independent clones were sequenced using the standard Sanger method (Sanger *et al.*, 1977).

Protein manipulation

Native total protein extracts from *S.pombe* were prepared as previously described (Moreno *et al.*, 1991). One mg of protein extract was incubated with 25 µl of p9^{CKS} Sepharose beads at 4°C for 2 h, washed four times with 1 ml of HB buffer and finally resuspended in 30 µl of HB buffer plus an equal volume of 2× sample buffer. After boiling, samples were electrophoresed on an SDS–polyacrylamide gel (Laemmli, 1970). Western blotting was carried out using Immobilon-P (Millipore). Membranes were probed with anti-phosphotyrosine antibodies PY20 (Transduction Laboratories) and, after stripping, with anti-cdc2 (PSTAIRE) antibodies (Santa Cruz Biotechnology). Immunoblots were revealed by ECL (Amersham).

Ortho [^{32}P]phosphate labelling of cells was performed as previously described (Moreno *et al.*, 1991) using 2.5 mCi of radioactivity. Labelled p34^{cdc2} was precipitated with p9^{CKS} Sepharose beads as described above, electrophoresed on an SDS–polyacrylamide gel and transferred onto Immobilon-P (Millipore). The radioactive band was confirmed to be the p34^{cdc2} protein by probing with anti-cdc2 (PSTAIRE) antibodies and was used for the two-dimensional phosphoamino acid analysis as previously described (Cooper *et al.*, 1983).

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