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Role of RNA Polymerase II Carboxy Terminal Domain Phosphorylation in DNA Damage Response

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The phosphorylation of C-terminal domain (CTD) of Rpb1p, the largest subunit of RNA polymerase II plays an important role in transcription and the coupling of various cellular events to transcription. In this study, its role in DNA damage response is closely examined in *Saccharomyces cerevisiae*, focusing specifically on several transcription factors that mediate or respond to the phosphorylation of the CTD. CTDK-1, the pol II CTD kinase, *FCP1*, the CTD phosphatase, *ESS1*, the CTD phosphorylation dependent cis-trans isomerase, and *RSP5*, the phosphorylation dependent pol II ubiquitinating enzyme, were chosen for the study. We determined that the CTD phosphorylation of CTD, which occurred predominantly at serine 2 within a heptapeptide repeat, was enhanced in response to a variety of sources of DNA damage. This modification was shown to be mediated by CTDK-1. Although mutations in *ESS1* or *FCP1* caused cells to become quite sensitive to DNA damage, the characteristic pattern of CTD phosphorylation remained unaltered, thereby implying that *ESS1* and *FCP1* play roles downstream of CTD phosphorylation in response to DNA damage. Our data suggest that the location or extent of CTD phosphorylation might be altered in response to DNA damage, and that the modified CTD, *ESS1*, and *FCP1* all contribute to cellular survival in such conditions.

Key words: CTD phosphorylation, DNA damage, DNA repair, RNA polymerase II carboxy terminal domain, *Saccharomyces cerevisiae*

In all eukaryotes, the largest subunit of RNA polymerase II (pol II) harbors a highly conserved C-terminal domain (CTD), which is composed of multiple heptapeptide repeats with the consensus sequence; Tyr_1-Ser_2-Pro_3-Thr_4-Ser_5-Pro_6-Ser_7 (Chambers & Kane, 1996). The CTD is essential for the function of pol II, but its role is not completely understood.

The CTD is reversibly phosphorylated on the serines at the second (Ser2) and fifth positions (Ser5), by a variety of kinases and phosphatases (Dahmus, 1996). Among these, CTDK-1 is known to be the primary kinase involved in the Ser2 phosphorylation of the CTD. CTDK-1 consists of three subunits, encoded by *CTK1*, *CTK2*, and *CTK3* (Prelich, 2002). CTDK-1 stimulates efficient elongation by pol II *in vitro*. The deletion of *CTK1*, which encodes for the kinase subunit, induces a loss of CTD phosphorylation at Ser2, and affects the expression of genes associated with the post-diauxic growth phase (Jona *et al.*, 2001). Although multiple kinases in addition to CTDK-1 target the CTD of pol II, only two phosphatases have identified thus far; Fcp1p and Ssu72p (Krishnamurth *et al.*, 2004). Fcp1p appears to be required for the recycling of pol II, via the dephosphorylation of the CTD. Clearly, understanding the molecular functions of *FCP1* in the dephosphorylation of the CTD during different steps of the transcription cycle is important to obtain an understanding of the manner in which gene expression is regulated (Licciardo *et al.*, 2001).

Among those proteins that bind the CTD and affect pol II function are Ess1p and Rsp5p (Wu *et al.*, 2001). Ess1p, an essential yeast peptidyl prolyl isomerase, has been shown to bind the phosphorylated CTD of pol II via its WW domain (Morris *et al.*, 1999; Myers *et al.*, 2001). While the WW domain mediates the binding between Ess1p and its substrates, the PPIase domain catalyzes the enzymatic activity. Ess1p and its human homologue, Pin1, have been determined, in model peptides, to be specific for phosphorylated Ser-Pro or Thr-Pro bonds (Yaffe *et al.*, 1997; Hani *et al.*, 1999). Prolyl bond isomerization would obviously affect the binding of other proteins to the phosphorylated CTD (Wu *et al.*, 2000). Rsp5p (reversion of Spt phenotype) and its mammalian orthologue, Nedd4, are ubiquitin-protein ligase, which binds to the CTD (Gavva *et al.*, 1997; Huibregtse *et al.*, 1997; Chang *et al.*, 2000). Proteins of this family have HECT domains in

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their C-terminal regions and a variety of other domains in their N-terminal regions. The binding of Rsp5p to the CTD results in the ubiquitination of Rpb1p, followed by its degradation. Therefore, by ubiquitinating Rpb1p and targeting it for destruction, Rsp5p has been suggested to exert a negative effect on pol II transcription (Wu *et al.*, 2001).

In this study, we have attempted to delineate the role of CTD phosphorylation in DNA damage responses. In a recent model of Transcription Coupled Repair (TCR), a repair pathway in which an active gene is repaired more rapidly than those in the non-transcribed region, pol II arrested at a DNA lesion was suggested to be phosphorylated prior to clearance by ubiquitination (Svejstrup, 2003). The transcription factors controlling phosphorylation of the CTD may link transcription to the repair pathway. Therefore, we evaluated the roles of transcription factors including CTDK-1, *FCP1, ESS1*, and *RSP5,* in the DNA damage response pathway, using a well-characterized yeast system (Kim *et al.* 2004a, 2004b). This study indicated that the phosphorylation of the CTD increased in response to DNA damage occurring specifically at Ser2. In addition, mutations in *CTK1*, *FCP1*, and *ESS1* were found to be sensitive to a variety of DNA damaging agents, including MMS, 4NQO, and UV. In contrast, the *RSP5* mutant was not sensitive to these agents. Ctk1p is responsible for damage dependent increases in CTD phosphorylation at Ser2, but its specific activity was not increased in response to DNA damage. Taken together, our data suggest that pol II responds to DNA damage by increasing the CTD phosphorylation, and factors that mediate or respond to this modification contribute to survival under DNA damage conditions.

Materials and Methods

Yeast extract preparation after treatment with DNA damaging agenst

In order to prepare the yeast whole-cell extracts, cells were grown to an OD_{600} 1.0 - 2.0 in 50 ml of YPD. The cells were incubated for an additional 1 h in either the presence or absence of MMS or 4NQO. The cells were collected, washed with ice-cold Tris-buffered saline, and suspended in 200 µl of lysis buffer [200 mM Tris-Cl (pH 8.0), 320 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 10 mM EDTA, 10 mM EGTA, 20% glycerol, 1 mM dithiothreitol (DTT), protease and phosphatase inhibitors]. The whole cell extracts was then constructed via the vortexing of yeast suspensions with 0.5 g of glass beads. Cell debris was removed by centrifugation at 4°C for 10 min. The yeast strains used in this study are shown in Table 1.

Immunoblot analysis

Whole cell lysate (50 µg) was boiled for 3 min in sample buffer, then separated on 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in TBS containing 0.1% Tween 20 and 5% (w/v) skim milk, then incubated overnight with H5, H14, or 8WG16 primary antibodies (Covance, USA). The membranes were then washed three times and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse immunoglobulin M (IgM) (Sigma, USA). The bound antibodies were visualized using chemiluminescence reagents. The Tfg2p antibody used in this study was a kind gift from Dr. S. Buratowski.

Cell viability assay

For spotting analysis, yeasts were grown in either YPD or synthetic medium (SC). The cells were freshly grown to an OD_{600} 1.0 and serially diluted 10-fold. A 10 µl aliquot of each of the dilutions was then spotted on plates containing MMS (0.01, 0.025, 0.05, or 0.075%), or treated with UV after spotting. The plates were incubated for 3-4 days at 30°C.

In vitro CTD Kinase assay

The $(HA)_3$-tagged Ctk1p was partially purified by immunoprecipitation from whole-cell extracts, using 12CA5 antibody. The precipitate was then resuspended in 20 µl kinase reaction buffer containing 20 mM HEPES-NaOH (pH 7.6), 7.5 mM magnesium acetate, 2 mM dithiothreitol, 100 mM potassium acetate, 2% glycerol, 1 µM ATP, 0.3 µl of [γ-^{32}P]ATP (250 µCi; GE Healthcare Bioscience, USA), and 3 g of the CTD peptide. The reactions were

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
W3031A	*MAT*a, *ura3-1*, *leu2-3*, *112 trp1-1*, *his3-11*, *15*, *can1-100*, *ade2-1*, phi+	Wu *et al.*; 2001
H164R1A	Isogenic to W3031A, *ess1*^{H164R}	Wu *et al.*; 2001
A144T1A	Isogenic to W3031A, *ess1*^{H144T}	Wu *et al.*; 2001
YXW29	Isogenic to W3031A, *rsp5*Δ::*LEU2*, pRS416-*RSP5* (*URA*, CEN)	Wu *et al.*; 2001
YMK16	*MAT*a, *ura3-1*, *leu2*-3, *112*, *trp1-1*, *his3-11*, *15*, *can1-100*, *ade2-1*, *fcp1*Δ::*LEU2* pMK86	Cho *et al.*; 2001
YFC26	*MAT*a, *ura3-1*, *leu2-3*, *112*, *trp1-1*, *his3-11*, *15*, *can1-100*, *ade2-1*, *fcp1*Δ::*LEU2* p(*fcp1-1 TRP*)	Cho *et al.*; 2001
YFC28	*MAT*a, *ura3-1*, *leu2-3*, *112*, *trp1-1*, *his3-11*, *15*, *can1-100*, *ade2-1*, *fcp1*Δ::*LEU2* p(*fcp1-2 TRP*)	Cho *et al.*; 2001
YSB772	*MAT*a, *ura3-52*, *leu2*Δ*1*, *trp1*Δ*63*, *his3*Δ*200*, *lys2*Δ*202*, *ctk1*-$(HA)_3$::*TRP*	Cho *et al.*; 2001

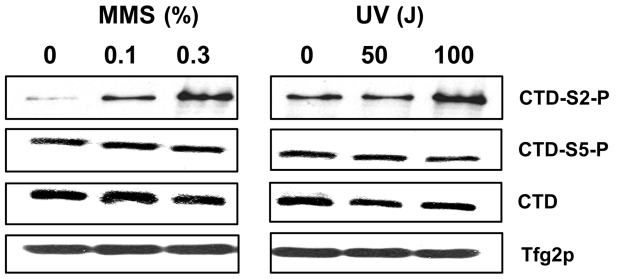

Fig. 1. Phosphorylation of pol II CTD at serine 2 increases in response to DNA damage. *HA-ctk1* cells (YSB772) were treated with MMS or with indicated doses of UV. Whole cell extracts (50 μg) were subjected to 7.5% SDS-PAGE, and immunoblot analyses was carried out using H14 (CTD-S5-P, phosphorylated CTD on Ser5), H5 (CTD-S2-P, phosphorylated CTD on Ser2), 8WG16 (CTD, recognizing the nonphosphorylated CTD), and a subunit of TFIIF (Tfg2).

incubated at 30°C for 1 h, resolved by SDS-7.5% PAGE, dried down, and finally exposed to X-ray film.

Results

Phosphorylation of RNA pol II at Ser2 on the CTD is increased in response to the DNA damage

The sensitivity of the *ctk1Δ* cells to the genotoxic agents suggested that the phosphorylation of CTD is required for the DNA damage response (Ostapenko & Solomon, 2003). In order to determine whether or not DNA damage influences pol II CTD phosphorylation, we treated the yeast cells with various doses of MMS (alkylating agent, methyl methanesulfonate) or UV, and the total protein extracts were prepared and subjected to immunoblot analysis. A variety of CTD isoforms were detected using monoclonal antibodies that recognize phosphorylated Ser2 (H5), phosphorylated Ser5 (H14), and unphosphorylated CTD (8WG16). As is shown in Fig. 1, MMS or UV treatment resulted in an increase in CTD phosphorylation, specifically at Ser2, in a dose dependent manner, whereas detected levels of phospho-Ser5 and unphosphorylated CTD were not significantly altered by either type of DNA damage. Tfg2p, an essential subunit of general transcription factor IIG, was visualized as a loading control. This data indicates that cells respond to DNA damage by inducing the CTD Ser2 phosphorylation of pol II. Our findings in this regard suggest that alterations in CTD phosphorylation may play an important role in the activities of pol II, adjusting it to stressed conditions.

Ctk1p is a major DNA damage dependent Ser2 kinase and plays a role in DNA damage response

Ctk1p exhibits kinase activity, and forms a complex with Ctk2p, a cyclin-like protein, and Ctk3p. Genes encoding for CTDK-1 subunits are not essential for viability, but a *ctk1Δ* mutant has been previously determined to exhibit growth-impaired cold-sensitive phenotypes (Sterner *et al.*, 1995). In order to precisely delineate the role of CTDK-1 in DNA damage response, we tested the viability of the *ctk1* deletion mutant under a variety of damage-induced conditions. Fig. 2A indicates that the yeast cells with *ctk1Δ* were sensitive to MMS and 4NQO (4-nitroquinoline-1-oxide). These *ctk1Δ* mutants were also shown to be sensitive to UV irradiation (data not shown), as reported previously (Ostapenko and Solomon, 2003). Therefore, although Ctk1p is dispensable under normal growth conditions, it becomes essential in damaged conditions, thereby indicating its crucial role in DNA damage responses.

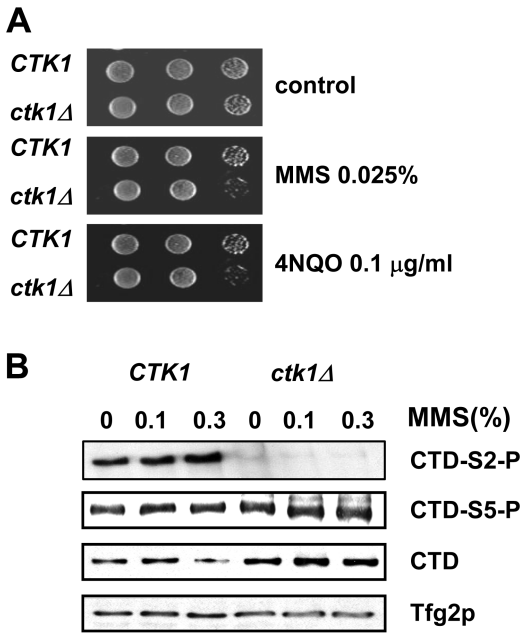

Fig. 2. Ctk1p induces Ser2 phosphorylation in response to DNA damage. A, *ctk1Δ* is sensitive to MMS and 4NQO. *CTK1* wild type and *ctk1Δ* strains were diluted 10-fold, and 10 μl were spotted onto YPD plates containing either MMS or 4NQO. All plates were incubated for 3 days at 30°C. B, Ctk1p is the primary Ser2 kinase in DNA damage response. The *CTK1* and *ctk1Δ* cells were treated with MMS, as described. The whole cell extracts (50 μg) were then subjected to 7.5% SDS-PAGE, and immunoblot analysis was carried out using H14, H5, and 8WG16. An immunoblot against a subunit of TFIIF (Tfg2p) was included as a loading control.

As Ctk1p is known to phosphorylate Ser2, we compared the phosphorylation levels of pol II in MMS-treated wild type and *ctk1Δ* cells in order to determine whether it was also responsible for damage-induced Ser2 phosphorylation. Fig. 2B shows that the deletion of *ctk1* abolished not only normal Ser2 phosphorylation, but also damage-induced Ser2 phosphorylation. Therefore, the Ctk1p of CTDK-1 is clearly the primary Ser2 kinase operating under both normal and DNA-damaged conditions.

The specific kinase activity of Ctk1p toward the CTD remains unchanged in response to DNA damage

Damage-dependent increases of Ser2 phosphorylation by

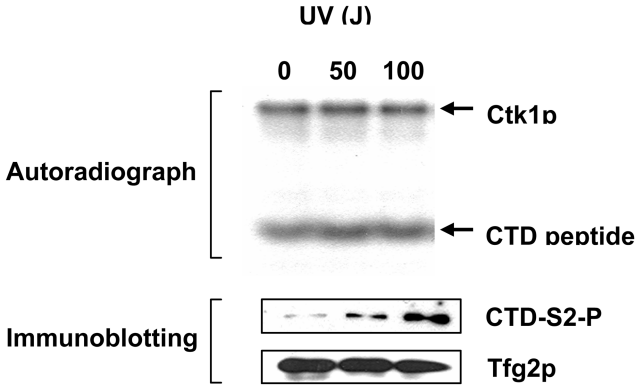

Fig. 3. The specific kinase activity of Ctk1p is unaltered by DNA damage. Yeasts expressing $(HA)_3$-Ctk1p were treated with the indicated doses of UV, after which the whole cell extracts were prepared. $(HA)_3$-Ctk1p was partially purified by immunoprecipitation. The CTD (three repeats of heptapeptides) was then phosphorylated with [γ-^{32}P]-ATP by incubation with the immunoprecipitated $(HA)_3$-Ctk1p. The products were resolved by 15% SDS-PAGE and visualized by autoradiography. Lower panel; immunoblot analysis shows the induction of the CTD Ser2 phosphorylation in the extracts used for the immunoprecipitations. Immunoblot analysis was carried out as described in the previous figures.

Ctk1p led us to attempt to determine whether its specific kinase activity is upregulated by DNA damage. To this end, a yeast strain with C-terminal HA tag at the genomic locus of *CTK1* was constructed. This strain behaves identically to the wild type strain, and responds to a variety of DNA damage signals. Using protein extracts identical to those used to verify the UV-dependent increase of Ser2 phosphorylation, Ctk1p-HA was partially purified via immunoprecipitation. In order to monitor the kinase activities of Ctk1p, we incubated the immuno-precipitates with the CTD peptides harboring three copies of the heptapeptide repeat. As is shown in Fig. 3, although total CTD phosphorylation at Ser2 was increased (lower panel), the specific kinase activity of Ctk1p toward the CTD peptides remained unaltered (upper panel). This result indicates that the kinase activity of Ctk1p is not the regulatory target induced by DNA damage, although we were not able to rule out the possibility that its kinase activity might be altered in the context of the entire transcription complex.

FCP1 mutants are sensitive to DNA-damaging agents

Fcp1p is the major CTD phosphatase that is conserved among eukaryotic organisms, and appears to play a significant role in pol II recycling. Mutations in *FCP1* result in the increased phosphorylation of Ser2, suggesting that *FCP1* functions *in vivo* via the dephosphorylation of Ser2 (Cho *et al.*, 2001). In order to determine the role of *FCP1* in DNA damage response, we evaluated the viability of *fcp1* temperature-sensitive mutants under a variety of damaging conditions at the permissive temperature of 30°C. We determined that the *FCP1* mutants, *fcp1-1* and

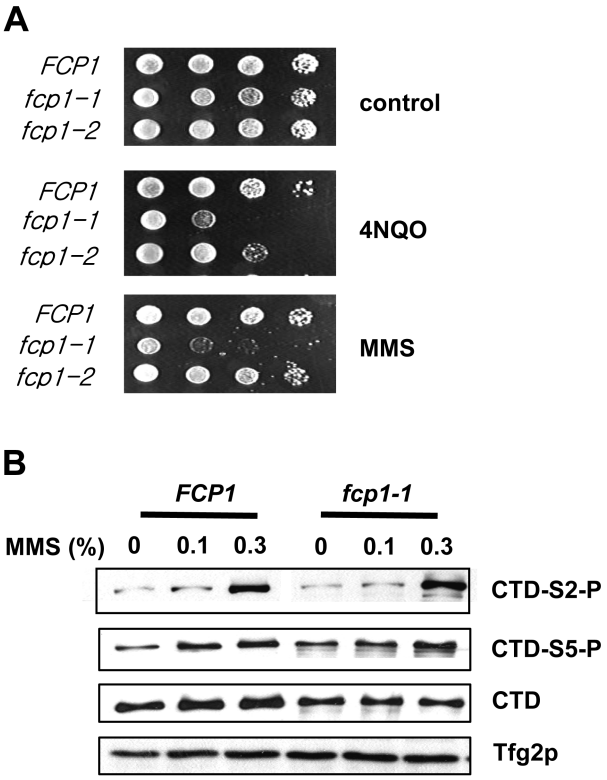

Fig. 4. The *fcp1 mutant* is sensitive to DNA-damaging agents. A, *FCP1*, *fcp1-1*, and *fcp1-2* were grown to an OD_{600} = 1.0, diluted 10-fold, and spotted onto YPD plates containing MMS (0.01%) or 4NQO (0.075 g/ml). All plates were incubated for 3 days at 30°C. B, CTD Ser2 phosphorylation is induced by DNA damage in a background of *fcp1* mutation. Whole cell extracts (50 μg) of indicated yeasts were subjected to 7.5% SDS-PAGE, and immunoblot analyses were carried out as described above.

fcp1-2, were sensitive to both MMS and 4NQO, with varying sensitivities as was reported previously. This indicates that *FCP1* plays an important role in DNA damage response (Fig. 4A). However, although *FCP1* down-regulates CTD Ser2 phosphorylation during the normal transcription cycle, damage-dependent Ser2 phosphorylation was not affected by *FCP1* mutations. This demonstrates that damage-dependent Ser2 phosphorylation is not increased by the simple inhibition of the CTD phosphatase activities of Fcp1p (Fig. 4B).

ESS1 mutants are sensitive to DNA-damaging agents

Ess1p is an essential prolyl isomerase. in order to determine Ess1p's role in DNA damage response, the temperature sensitive yeast cells, $ess1^{A144T}$ and $ess1^{H164R}$, were chosen as each of these strains harbors a mutation in either the WW or catalytic domains. We then assessed the viability of the *ess1* mutants under a variety of damage conditions at the permissive temperature, 30°C. Fig. 5 shows that both $ess1^{A144T}$ and $ess1^{H164R}$ were sensitive to MMS, 4NQO, and UV (Fig. 5A). Interestingly, however,

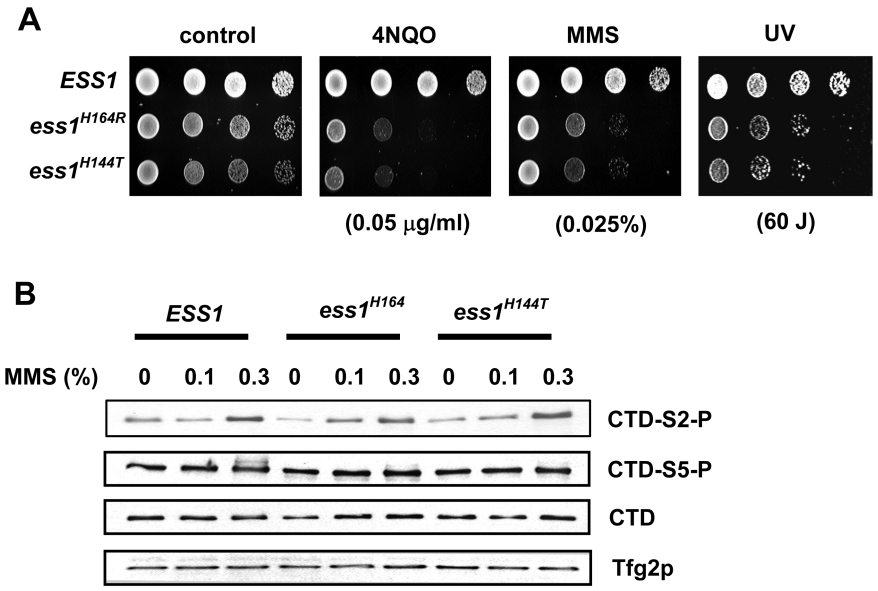

Fig. 5. The *ess1*^{ts} is sensitive to DNA-damaging agents. A, *ESS1*, *ess1*^{H164R}, and *ess1*^{H144T} strains were diluted 10-fold and spotted onto YPD plates containing MMS or 4NQO, or were irradiated with UV. All plates were subsequently incubated for 3 days at 30°C. B, CTD phosphorylation on Ser2 is induced by DNA damage in *ess1* mutation backgrounds. The whole cell extracts (50 μg) of the indicated yeasts were subjected to 7.5% SDS-PAGE, and immunoblot analysis was carried out as described above.

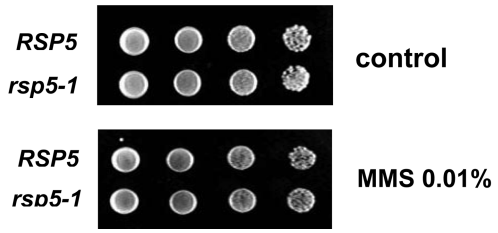

Fig. 6. *RSP5* is not required for survival in the presence of MMS. *RSP5* and *rsp5-1* strains were grown as described above, and cell growth was evaluated by spotting on plates containing MMS (0.01%). The YXW29 strain was transformed with pRS414-*RSP5* or pRS414-*rsp5-1* (Wu *et al.*, 2001). The original *RSP5 plasmid* (pRS416-*RSP5*) was then shuffled out. The resultant strains were employed in this study.

we also observed that the *ess1ts* mutants retained the ability to increase CTD Ser2 phosphorylation upon DNA damage (Fig. 5B). Our findings demonstrate that, although Ess1p plays a role in the DNA damage response, it does not affect Ser2 phosphorylation directly. As it binds preferentially to the phosphorylated CTD, it appears likely that Ser2 is phosphorylated first, and then Ess1p subsequently responds to this.

The RSP5 mutant is not sensitive to the DNA damaging agents.

RSP5 is an essential gene in both yeasts and humans. This gene was originally isolated in a screen for suppressors of *SPT3* mutation. Rsp5p was shown to form a physical and functional complex with pol II (Wang *et al.*, 1999). In addition to its interaction with pol II, Rsp5p contains multiple and diverse substrates, which suggests that Rsp5p exerts effects on a wide range of cellular processes. In order to delineate the role of *RSP5* in DNA damage response, we evaluated the viability of the *rsp5-1* mutant under a variety of damage-induced conditions. We observed that the yeast strain carrying *rsp5-1* allele was sensitive to neither MMS nor 4NQO (Fig. 6 and data not shown). In fact, Rsp5p appears to be responsible for the ubiquitination and degradation of Rpb1p upon UV irradiation (Wu *et al.*, 2001). However, the destruction of pol II may not be essential for survival in the presence of the DNA damage or a redundant pathway may exist, which also regulates the removal of pol II.

Discussion

The largest subunit of RNA pol II contains a conserved CTD, composed of a heptapeptide motif, YSPTSPS, which is repeated between 26 and 52 times. CTD phosphorylation regulates the interaction between pol II and the components of the preinitiation complex, transcription elongation factors, and RNA processing factors. Depending on the temporal and spatial phosphorylation of the CTD, the activity of RNA polymerase can be affected significantly.

In this study, we attempted to characterize the correlation between transcription and DNA damage response, by examining several transcription factors that control the phosphorylation of the CTD. We evaluated the roles of several transcription factors, including *CTDK-1*, the pol II CTD kinase, *FCP1*, the CTD phosphatase, *ESS1*, the

CTD phosphorylation dependent cis-trans isomerase, and *RSP5*, the phosphorylation dependent pol II ubiquitinating enzyme during DNA damage. The specific increase in phosphorylation at the Ser2 of the CTD was a specific focus of the study, as this also increases when yeast cells approach stationary phase, or when they encounter heat shock (Murray *et al.*, 2001). This strongly implies that the CTD responds to environmental stresses via increase in Ser2 phosphorylation. The results of this study also suggest that Ser2 phosphorylation may also play a role in DNA damage response.

Yeast with *ctk1Δ* exhibited a dramatic reduction in Ser2 phosphorylation *in vivo*, thereby indicating that Ctk1p is the primary protein kinase responsible for both ordinary and DNA damage inducible Ser2 phosphorylation (Patturajan *et al.*, 1999, Ostapenko and Solomon, 2003). Yeast cells carrying mutations in the *CTK1* gene have been reported grow in a manner indistinguishable from those of the wild-type strain, but are inviable at reduced temperatures, and also manifest a marked delay during their exit from the stationary phase (Bork *et al.*, 1997). In addition to this, we have obtained evidence that *ctk1*Δ mutant cells are sensitive to the DNA-damaging agents. Interestingly, the specific kinase activity of Ctk1p toward the CTD remained unchanged under DNA damage conditions, even though the total amounts of Ser2 phosphorylation had clearly increased. This finding is consistent with the previous observation that the total amount of phosphorylation of the pol II population is increased under such conditions, rather than the phosphorylation density per pol II molecule (Heo *et al.* 2004).

Next, we examined the role of *FCP1* in DNA damage response. In the transcription cycle, the phosphatase activity of Fcp1p determines the level of CTD phosphorylation occurring, in combination with Ctk1p. We have demonstrated that yeast strains carrying *fcp1* ts alleles were sensitive to DNA-damaging agents, but did not affect damage-inducible Ser2 phosphorylation. Further study will be required in order to determine precisely the role of Fcp1p, in addition to its role in the regulation of Ser2 upon DNA damage.

Among the proteins that bind to the CTD and affect the function of pol II are Ess1p and Rsp5p. Ess1p has been reported to bind to CTD in a phosphorylation-dependent manner and to affect a reconfiguration of the structure of the CTD. According to Hanes's group, Ess1p affects Fcp1p activity, probably via modification of the conformation of the CTD. Both the *ESS1* and *FCP1* mutants proved to be quite sensitive to a variety of DNA damaging agents, but did not exhibit significant differences in the total levels of CTD Ser2 phosphorylation. It appears probable that both *ESS1* and *FCP1* are involved in the step(s) taking place after the phosphorylation of CTD at Ser2. Finally, we attempted to determine whether *RSP5* is involved in DNA damage responses. Although Rsp5p is known to be an ubiquitin conjugating enzyme, crucial to the destruction of pol II, *RSP5* may not be linked directly with cellular survival under DNA damage conditions.

In conclusion, the DNA damage-dependent induction of phosphorylation on the Ser2 of the CTD is mediated by Ctk1p, but is affected by neither Fcp1p nor Ess1p. The average level of Ser2 phosphorylation in the bulk pol II population appeared to increase in response to DNA damage. Ctk1p was determined to be responsible for this change, but Fcp1p and Ess1p may also play roles in this after Ser2 phosphorylation has been induced. We reported previously that overall gene expression profiles shift to adjust to DNA-damaged conditions (Heo *et al.*, 2004). Presumably, the regulation of a subset of those damage-responsive genes is a critical factor in this process and must be dependent on Ser2 phosphorylation.

Acknowledgments

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