

Note

## The Yeast Checkpoint Kinase Dun1 Downregulates *DIN7* in the Absence of DNA Damage

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**Yeast *DIN7* is a DNA damage-inducible gene. Its expression is increased in the absence of Dun1, a DNA damage checkpoint kinase. We identified a *DIN7* promoter region responsible for Dun1-mediated downregulation and found that *DIN7* expression was not further increased in response to hydroxyurea in  $\Delta$ *dun1* cells. Thus *DIN7* repression by Dun1 can be released upon DNA damage.**

**Key words:** mitochondria; transcriptional regulation; DNA damaging checkpoint

Mitochondria contain multiple copies of mitochondrial DNA (mtDNA) that encode components essential to supplying cellular energy through oxidative phosphorylation respiration. The accumulation of mtDNA mutations is the pathogenesis of aging and several disorders, *e.g.*, mitochondrial disease and neurodegenerative disease.<sup>1,2</sup> So far, two mtDNA repair genes, *NTG1* and *DIN7*, have been found to be induced by DNA damage in *Saccharomyces cerevisiae*.<sup>3,4</sup> Although Ntg1 is localized to both the nucleus and mitochondria,<sup>3</sup> Din7 locates only to mitochondria.<sup>5</sup> Overexpression of Din7, a mitochondrial nuclease, causes an elevated incidence of petites, in which mitochondrial respiratory function is defective due to the deletion of mtDNA.<sup>6</sup> Thus the expression of *DIN7* should be kept low under unstressed conditions. Gene expression of *DIN7* is induced when yeast cells are exposed to DNA damaging agents, *e.g.*, hydroxyurea (HU), methanesulfonate (MMS), and ultraviolet (UV) light<sup>4,7</sup> but little is known about the mechanism by which DNA damage induces *DIN7* expression. We have identified a novel *cis*-acting element in the *DIN7* promoter that is responsible for DNA-damage inducible expression of *DIN7*.<sup>7</sup> In order to understand the transcriptional regulation of *DIN7*, identification of both the positive and negative factors for *DIN7* transcription is necessary.

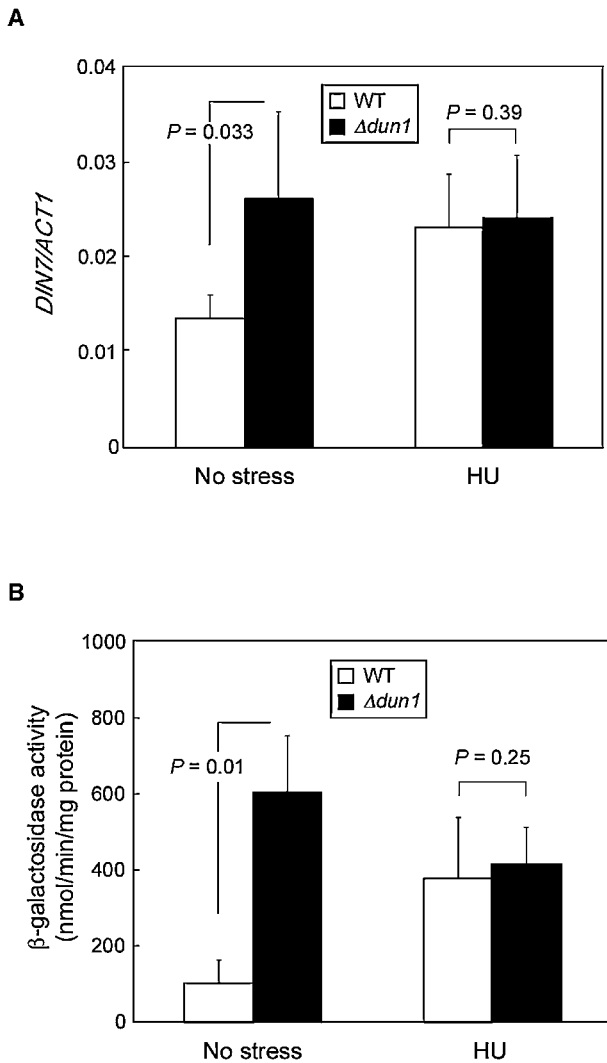
Dun1 is an effector kinase at the Mec1/Rad53 DNA damage checkpoint.<sup>8,9</sup> It upregulates several DNA damage-inducible genes (*e.g.*, *RNR* genes encoding ribonucleotide reductase) by phosphorylating Crt1, a transcriptional repressor in the presence of DNA-damaging agents.<sup>10–12</sup> It has also been reported that *DIN7* expression is increased in  $\Delta$ *dun1* cells,<sup>4</sup> suggesting that Dun1 negatively regulates the expression of *DIN7*. We investigated to determine whether Dun1 is involved in this DNA damage-inducible *DIN7* expression by comparing the *DIN7* mRNA level in  $\Delta$ *dun1* cells with that in wild-type *DUN1* (WT) cells in the presence and the absence of HU. Yeast strains were cultured as previously described.<sup>13</sup>

*DIN7* mRNA was upregulated in  $\Delta$ *dun1* cells not stressed with HU (Fig. 1A). In contrast to WT cells, HU treatment did not induce further increases in *DIN7* mRNA levels in  $\Delta$ *dun1* cells. To determine the transcriptional activity of the *DIN7* promoter, we analyzed *DIN7* promoter activity using  $\beta$ -galactosidase as a reporter. In the absence of HU,  $\beta$ -galactosidase activity in  $\Delta$ *dun1* cells was 6-fold higher than in WT cells (Fig. 1B). In contrast,  $\beta$ -galactosidase activity in the presence of HU was almost the same as in HU-treated WT cells, suggesting that Dun1 downregulates *DIN7* expression in the absence of HU, but fails to do so in the presence of HU.

To identify the repression region responsible for Dun1-mediated downregulation in the *DIN7* promoter, we used a single-copy plasmid in which the *DIN7* promoter region from –654 (bp) to –1 (bp) was placed upstream of the *lacZ* reporter (pYC2-654).<sup>7</sup> In addition, a series of deletion mutants of the *DIN7* promoter was constructed. We have found that a 19-bp and a 7-bp region of the promoter are *cis*-acting elements required for induction of *DIN7* expression in response to HU.<sup>7</sup> We measured  $\beta$ -galactosidase activity in WT and  $\Delta$ *dun1* cells harboring each of the reporter plasmids (Fig. 2).

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Abbreviations: HU, hydroxyurea; mtDNA, mitochondrial DNA



**Fig. 1.** Expression of *DIN7* in W303 (WT) and  $\Delta dun1$  Cells.

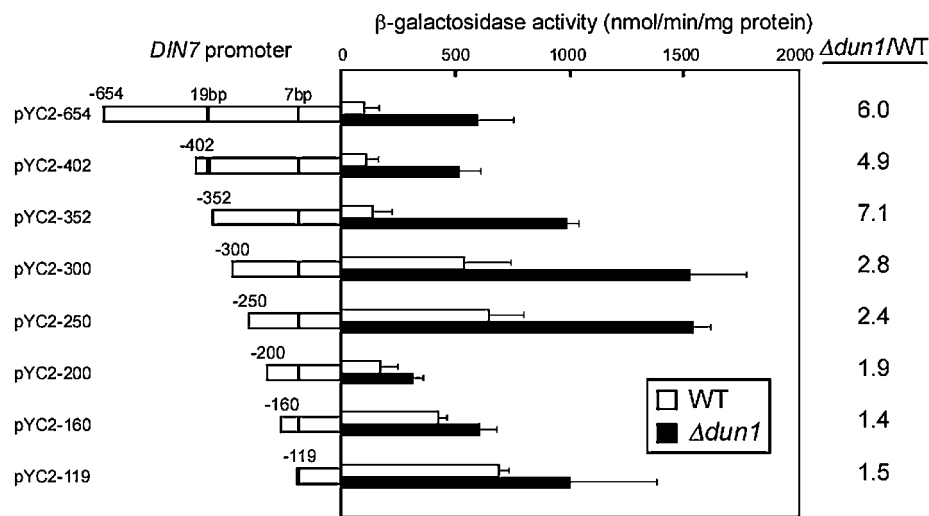
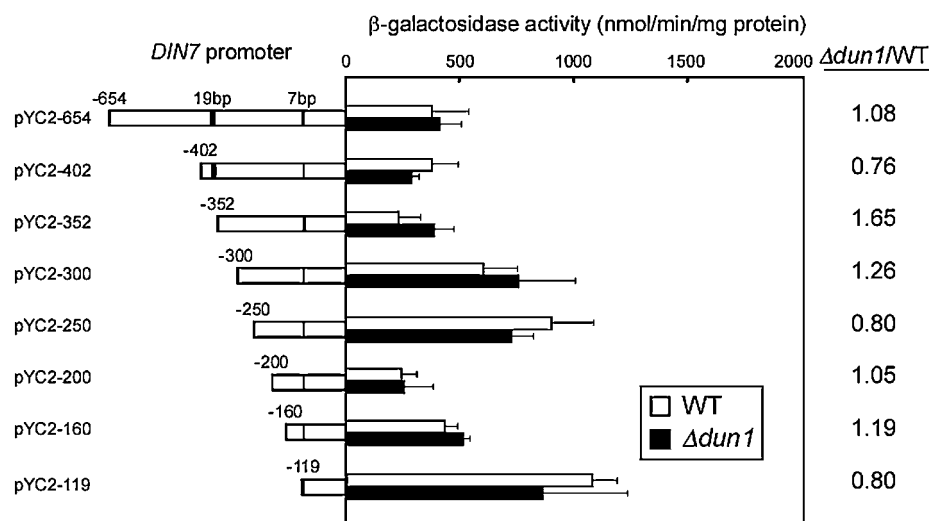
A, Expression levels were determined by quantitative reverse transcriptase-PCR. W303 (MATa, *ade2*, *leu2*, *his3*, *ura3*, *trp1*) (open bars) and  $\Delta dun1$  (closed bars) cells were cultured in SD medium at 30°C for 9 h in the absence and the presence of 0.15 M HU. Quantitative RT-PCR was performed as previously described.<sup>7)</sup> The vertical axis indicates the relative expression level of *DIN7*. B, *DIN7* promoter activity as measured by  $\beta$ -galactosidase assay. W303 (open bars) and  $\Delta dun1$  (closed bars) cells carrying pYC2-654 (*DIN7-lacZ*) were cultured in SD medium at 30°C for 9 h in the absence and the presence of 0.15 M HU.  $\beta$ -Galactosidase activities in the cell extracts were measured as previously described.<sup>7)</sup> Mean values and standard deviations were obtained from at least five independent experiments.

In the absence of HU, the deletion mutants from  $-352$  to  $-201$  (pYC2-352, pYC2-300, pYC2-250, and pYC2-200) showed a gradual decrease in relative  $\beta$ -galactosidase activity in  $\Delta dun1$  cells versus WT cells as the deleted region length increased (Fig. 2A). In contrast, deletions from  $-200$  to  $-120$  (pYC2-200, pYC2-160 and pYC2-119) did not show any significant difference in their activities. These results indicate that the *DIN7* promoter region responsible for Dun1-dependent transcriptional repression is located in a relatively long

region between  $-352$  and  $-201$ . In the presence of HU, the  $\beta$ -galactosidase activities of the various plasmids showed almost no difference between WT and  $\Delta dun1$  cells (Fig. 2B). These results support the thesis that Dun1 is a negative regulator of *DIN7* in the absence of DNA damage.

To further verify that these regions are required for the suppression of *DIN7* expression, we inserted fragments of the *DIN7* promoter, from  $-352$ ,  $-300$ , or  $-200$  to  $-120$ , into the region upstream of the *LEU2* basal promoter. As shown in Fig. 3A, in the case of the fragments from  $-352$  and  $-300$  to  $-120$ ,  $\beta$ -galactosidase activity was repressed in WT cells but was restored in  $\Delta dun1$  cells, suggesting that these fragments contain the region for the Dun1-mediated repression of *DIN7* expression. In the case of the fragment from  $-200$  to  $-120$ , however, the  $\beta$ -galactosidase activity in  $\Delta dun1$  cells was almost the same as in WT cells. These results suggest that the *DIN7* promoter region between  $-352$  and  $-201$  is necessary and sufficient for the repression mediated by Dun1 and that the major repression region is located in the region  $-300$  to  $-201$ . Moreover, the  $\beta$ -galactosidase activity of the promoter containing the fragment from  $-200$  to  $-120$  was greatly reduced regardless of the presence of Dun1 (Fig. 3A), suggesting that this fragment contains another repression region independent of Dun1.

A defect in mitochondrial respiration generates petites in yeast, which do not grow on a glycerol (YPGly) plate but form small colonies on a glucose (SD) plate. Either overexpression of *Din7* or disruption of the *DUN1* gene can cause an elevated level of petite formation.<sup>5,6)</sup> Hence we proceeded to examine the role of the repression regions in petite formation by deleting the entire *DIN7* ORF including its promoter (from  $-440$  to  $-1$ ). The  $\Delta din7$  cells harboring *DIN7* expression plasmid lacking the region from  $-300$  to  $-120$  (p $\Delta$ 300/120) or that from  $-200$  to  $-120$  (p $\Delta$ 200/120) in the *DIN7* promoter exhibited approximately 3-fold increases in petite formation as compared to WT cells and  $\Delta din7$  cells carrying the plasmid for the *DIN7* ORF with an intact promoter (p*DIN7*) (Fig. 3B). These results suggest that the increase in petite formation resulted from elevated expression of *Din7*, which was due to deletion of the repression regions in the *DIN7* promoter. In addition, the level of petite formation in  $\Delta din7$  cells increased as compared with that in WT cells, suggesting that the level of *Din7* expression, either higher or lower, causes deleterious effects on mitochondrial functions. Thus *DIN7* expression should be strictly maintained at certain levels in cells. Because mitochondrial dysfunctions induced by excess amounts of *Din7* are toxic, unnecessary expression of *DIN7* should be avoided. Despite the risk associated with increases in petite formation, the fact that *DIN7* expression is induced to a high level in response to DNA damage<sup>4,7)</sup> suggests that a considerable amount of *Din7* is required to overcome DNA damage in cells.

**A****B**

**Fig. 2.** Identification of the Repression Region in the *DIN7* Promoter.

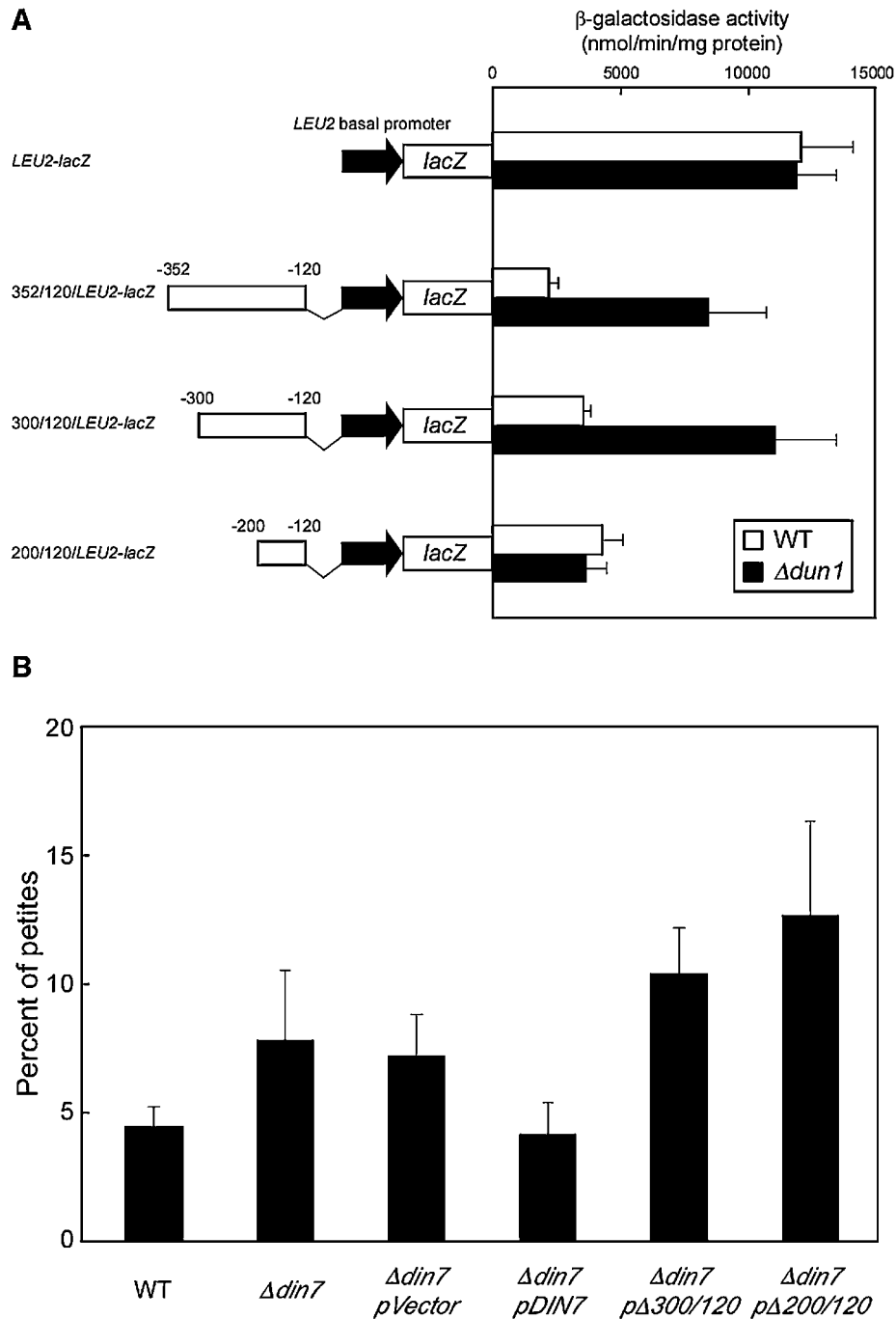
Schematic diagrams of the *DIN7-lacZ* constructs and the resulting  $\beta$ -galactosidase activities are shown. W303 (open bars) and  $\Delta dun1$  (closed bars) cells carrying each plasmid were cultured at 30°C for 9 h in the absence (A) and the presence (B) of 0.15 M HU. Mean values and standard deviations were obtained from at least five independent experiments. Values in the right column of the figure indicate the ratio of  $\Delta dun1$  relative to WT.

The results obtained in our previous study and in this one provide evidence that at least two types of trans-factors are required for the transcriptional regulation of *DIN7*. One is a factor(s) acting on a 19-bp *cis*-acting element in the *DIN7* promoter to induce *DIN7* expression in response to DNA damage.<sup>7)</sup> The other is a factor(s) binding to the region from -352 to -201 in the promoter of *DIN7* that represses transcriptional activity in the absence of DNA-damaging agents. Crt1 or Crt1-like proteins regulated by Dun1 are candidates for the

factors recognizing this region. The identification of these factors is apparently important to an understanding of the *DIN7* regulatory mechanism in response to DNA damage.

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**Fig. 3.** Biological Activity of the Repression Region in the *DIN7* Promoter in the Absence of HU.

A, Effects of the repression region in the *DIN7* promoter on the transcriptional activity of the *LEU2* basal promoter. W303 (open bars) and  $\Delta dun1$  (closed bars) cells carrying the various plasmids derived from the *LEU2-lacZ* fusion construct<sup>7)</sup> were incubated at 30 °C for 9 h. The *LEU2* basal promoter contained the *LEU2* transcriptional and translational start sites but lacked the sequence required for the regulation of its expression.  $\beta$ -Galactosidase activities in the cell extracts were measured. Mean values and standard deviations were obtained from at least five independent experiments. B, Effects of deletion of the repression region in the *DIN7* promoter on the frequency of petite formation. Strains W303 (WT, *DIN7*),  $\Delta din7$ , and  $\Delta din7$  harboring the vector plasmid pYC2/NT/*lacZ* (Invitrogen), pDIN7, p $\Delta 300/120$ , or p $\Delta 200/120$  were grown in glycerol medium (3 ml), containing 0.67% yeast nitrogen base without amino acids, 3% glycerol and the required amino acids, and incubated at 30 °C for 1 d. The pre-culture (0.1 ml) was inoculated into SD medium (3 ml) supplemented with amino acids required for growth and were cultivated at 30 °C for 1 d. Cells at the appropriate dilution were spread on SD plates and incubated at 30 °C for 3 d. The colonies on each plate were replica-plated onto a YPGly plate and incubated at 30 °C for 3 d. The percentages of colonies that failed to grow on the YPGly plates vs. all colonies on the SD plates were calculated. Mean values and standard deviations were calculated from eight independent cultures of each strain.

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