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A novel *cis*-acting element required for DNA damage-inducible expression of yeast *DIN7*

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Abstract

Din7 is a DNA damage-inducible mitochondrial nuclease that modulates the stability of mitochondrial DNA (mtDNA) in *Saccharomyces cerevisiae*. How *DIN7* gene expression is regulated, however, has remained largely unclear. Using promoter sequence alignment, we found a highly conserved 19-bp sequence in the promoter regions of *DIN7* and *NTG1*, which encodes an oxidative stress-inducible base-excision-repair enzyme. Deletion of the 19-bp sequence markedly reduced the hydroxyurea (HU)-enhanced *DIN7* promoter activity. In addition, nuclear fractions prepared from HU-treated cells were used in *in vitro* band shift assays to reveal the presence of currently unidentified *trans*-acting factor(s) that preferentially bound to the 19-bp region. These results suggest that the 19-bp sequence is a novel *cis*-acting element that is required for the regulation of *DIN7* expression in response to HU-induced DNA damage.

Keywords: *Saccharomyces cerevisiae*; Mitochondria; DNA-damage response; Transcriptional regulation

Damage to the DNA of nuclear chromosomes and mitochondrial genomes occurs during the normal life cycles of eukaryotic cells. Repair of this inevitable DNA damage is required for the preservation of various cellular functions. Multiple nuclear genes that are inducible by DNA damage have been identified in yeast and human cells [1,2]. Nuclear DNA as well as mitochondrial DNA (mtDNA) encodes proteins that are essential for supplying cellular energy through oxidative respiration and mutations in mtDNA caused by various damaging agents are associated with aging and mitochondrial diseases [3,4]. Thus, it is important to understand how the expression of mtDNA repair-related genes is regulated at the transcriptional level in response to DNA damage.

Two genes from the budding yeast *Saccharomyces cerevisiae* that are expressed in response to DNA-damage have been reported to be involved in mtDNA repair. The nuclear gene *DIN7* encodes a nuclease, which is exclusively localized in mitochondria [5]. The expression of *DIN7* is markedly increased in response to DNA-damaging agents such as hydroxyurea (HU), methyl methanesulfonate (MMS), and ultraviolet (UV) light [6,7]. HU inactivates ribonucleoside reductase resulting in a block of deoxynucleotide synthesis, whereas MMS treatment and UV irradiation cause the formation of alkylated DNA and cyclobutane pyrimidine dimers, respectively. The second gene is *NTG1*, which encodes an oxidative DNA damage-inducible base-excision repair enzyme that is localized in both nuclei and mitochondria [8,9]. Because the expression of *NTG1* should be influenced by the Ntg1-mediated base excision repair of nuclear chromosomes, however, *DIN7* is likely to be a simpler model gene for investigations of how the expression of mtDNA repair-related genes is regulated at the transcriptional level.

In this study, we identified a novel highly conserved 19-bp sequence in the promoters of *DIN7* and *NTG1*, which serves as a required *cis*-acting element for the induction of *DIN7* expression in response to HU. We also demon-

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strated the presence of protein(s) that specifically bound to the 19-bp sequence in cells.

Materials and methods

Strains, media, and genetic techniques. Yeast *Saccharomyces cerevisiae* W303 (MATa, *ade2*, *leu2*, *his3*, *ura3*, *trp1*) [10] was used as the parental strain in this study. The *din7*-null mutant was constructed by displacing the gene with the *URA3* gene. The 19-bp sequence in the *DIN7* promoter was replaced with the *TRP1* gene via PCR-mediated gene disruption to yield the Δ19-bp mutant. The conditions for yeast cell cultivation and the media used in this study have been previously described [11]. Yeast transformations were performed as described [12]. Standard procedures were used for all DNA manipulations and *Escherichia coli* transformations [13].

Promoter alignment. Promoter regions were aligned using the ClustalW version 1.83 sequence alignment program [14] and the results were displayed using BOXSHADE.

Construction of DIN7 promoter–reporter fusion constructs. The PCR primers used for plasmid construction are listed in Table 1 (see supplemental data). All *DIN7* promoter-*lacZ* fusions were constructed using the pYC2/NT/*lacZ* vector (Invitrogen). The *DIN7* promoter fragments from −654, −402, −654 (Δ − 371/−353), −352, −371, −654 (Δ − 119/−113), and −654 (Δ − 371/−353, Δ − 119/−113) to −1 were PCR-amplified and inserted into the SpeI/KpnI site of pYC2/NT/lacZ after the SpeI/KpnI fragment containing the *GAL1* promoter was removed; this resulted in the pYC2-654, pYC2-402, pYC2-Δ19, pYC2-352, pYC2-371, pYC2-Δ7, and pYC2-Δ19Δ7 plasmids, respectively. The *LEU2-lacZ* fusion was constructed by inserting the *LEU2* DNA sequence from position −125 to +41 into the SpeI/KpnI site of pYC2/NT/lacZ. The *LEU2* fragment included both the *LEU2* transcriptional and translational start sites but lacked the sequence that is required for the regulation of its expression [15]. Tandem repeats of the 19-bp or 7-bp sequence (one, four, or seven copies) were inserted next to the 5′ end of the *LEU2* basal promoter to yield the 1 × 19-*LEU2-lacZ*, 4 × 19-*LEU2-lacZ*, 7 × 19-*LEU2-lacZ*, 1 × 7-*LEU2-lacZ*, 4 × 7-*LEU2-lacZ*, and 7 × 7-*LEU2-lacZ* plasmids, respectively.

β-Galactosidase assay. The β-galactosidase assay was performed according to Burke et al. with some modifications [12]. The W303 cells that harbored the reporter constructs were grown at 30 °C for approximately 24 h in 5 ml of SD medium supplemented with amino acids required for growth. SD medium (50 ml) with or without 0.15 M HU (Wako) was inoculated with the preculture to a density of 5×10^5 cells/ml and incubated for 9 h at 30 °C. When the cell number reached 2–4 × 10^7 cells, crude cell-free extracts were prepared and assessed for β-galactosidase activity.

Quantitative RT-PCR. Cells were cultured at 30 °C for 9 h in YPD medium with or without 0.15 M HU. Total RNA was purified using RNeasy (Qiagen) and reverse transcription was performed using a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara). The gene expression level of *DIN7* was measured using qPCR MasterMix Plus for SYBR Green I Low ROX (Eurogentec) and a 7500 Real-Time PCR system (Applied Biosystems). The expression level of *ACT1* was used for normalization. DIN7-realF (5′-TGGAGCAACCGCTAGTGGAT-3′), DIN7realR (5′-GATGC GTTTTTGTTATCAAGAGAATTC-3′), ACT1realF (5′-TGGATTC CGGTGATGGTGTT-3′), and ACT1realR (5′-TCAAAATGGCGT GAGGTAGAGA-3′) were designed using Primer Express (Applied Biosystems) and used as primers to detect these genes. C_t measurements were performed in triplicate.

Preparation of nuclear extracts. Large volumes of YPD medium with or without 0.15 M HU were inoculated with the overnight preculture of W303 cells and cultured for 9 h at 30 °C. Nuclear extracts were prepared as described by the Hahn laboratory (http://www.fhcrc.org/science/labs/hahn/index.html).

Electrophoretic mobility shift assay (EMSA). Each 50-bp oligonucleotide containing a centrally positioned 19-bp or mutated 19-bp sequence from the *DIN7* promoter was biotinylated at its 3′ end using a Biotin 3′ End DNA Labeling Kit (Pierce) and then annealed to form double-stranded DNA, which was used as a probe. The biotin-labeled double-stranded DNA (200 fmol) and 1 μg of nuclear extracts were mixed and incubated for 20 min at room temperature in 20 μl of standard reaction buffer (pH 7.5) containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 1 μg of double-stranded Poly(dI–dC), and 0.05% NP-40. Protein-DNA complexes were separated on a 5% polyacrylamide gel in 0.5× TBE buffer and the band shifts were observed using a LightShift chemiluminescent EMSA kit (Pierce). In the competition assay, a 10- or 50-fold excess of unlabeled double-stranded DNA was added to the reaction mixture.

Results and discussion

Identification of a highly conserved sequence in the promoters of DIN7 and NTG1 that is involved in responses to HU-induced DNA damage

Because *DIN7* and *NTG1* are DNA damage-inducible genes that are required for mtDNA repair, we hypothesized that their transcription may be activated by a common mechanism in response to DNA-damaging agents. We first examined whether their promoters contain *cis*-acting elements that are required for the induction of gene expression. The promoter regions of *DIN7* and *NTG1* were aligned with each other as described [14]. We found that the two genes contain a highly conserved 19-bp sequence that is located at approximately nucleotide position −360 in both the *DIN7* and *NTG1* promoters (Fig. 1A). The 19-bp sequence was not found in the promoter regions of other known mtDNA repair-related genes that are not DNA damage-inducible, such as *OGG1* [16–18], *MIP1* [19–21], and *MHR1* [11,22,23].

To investigate the effects of the 19-bp sequence on the expression of *DIN7* in response to HU, we constructed a single copy plasmid in which the *DIN7* promoter region from −654 to −1 was placed upstream of the *lacZ* reporter (pYC2-654). In addition, *DIN7* mutant promoters containing various deletions were constructed. The resulting β-galactosidase activities were then measured (Fig. 1B). The wild-type (WT) promoter resulted in a 4-fold increase in the β-galactosidase activity in response to HU. Deletions upstream of −372 bp did not affect the HU-induced β-galactosidase activity (pYC-402 and pYC2-371). On the other hand, deletion of the 19-bp sequence (pYC2-Δ19 and pYC2-352) resulted in HU-induced β-galactosidase activity levels that were about 50% of the activity observed with the WT promoter. These results indicate that the 19-bp sequence plays a critical role in the induction of *DIN7* gene expression in response to HU. The importance of the 19-bp sequence was further confirmed by measuring the *DIN7* mRNA level. A significant decrease in the *DIN7* mRNA level was detected with real-time PCRs when the 19-bp sequence was deleted (Fig. 2).

A 7-bp sequence is necessary but not sufficient for HU-induced expression of DIN7

Using a decamer consensus sequence from the promoters of genes involved in chromosome DNA repair [24], a

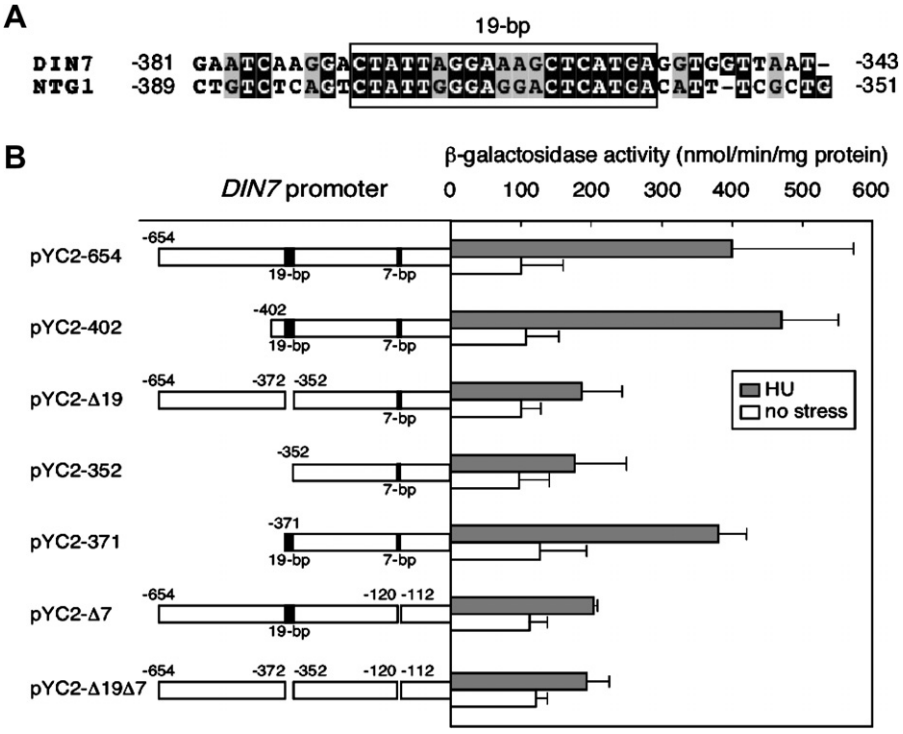

Fig. 1. Identification of a *cis*-acting element in the *DIN7* promoter. (A) Alignment of the promoter sequences of *DIN7* and *NTG1*. The box indicates the highly conserved 19-bp sequences in the *DIN7* and *NTG1* promoters. (B) Schematic diagrams of the *DIN7-lacZ* constructs and the resulting β-galactosidase activities. W303 cells carrying each plasmid were cultured at 30 °C for 9 h in the presence (gray bars) or absence (open bars) of 0.15 M HU. The mean values and standard deviations were obtained from at least five independent experiments.

7-bp sequence located 120-bp upstream of the *DIN7* transcriptional start site was predicted as a potential *cis*-acting element, the 7-bp sequence that matched the first seven nucleotides of the decamer consensus sequence [7]. We therefore deleted the 7-bp sequence (pYC2-Δ7) in a promoter-reporter construct, and tested the effect on the β-galactosidase activity. As shown in Fig. 1B, the 7-bp deletion caused a marked decrease in the β-galactosidase activity, as was observed with the deletion of the 19-bp sequence. These results suggested that both the 19-bp and 7-bp sequences are required for the induction of *DIN7* expression in response to HU.

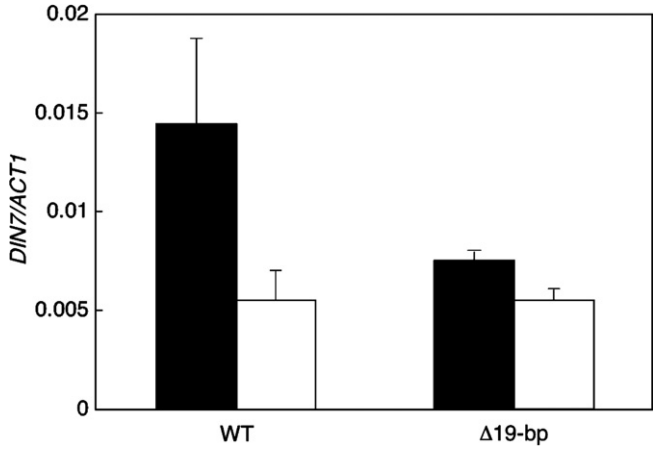

Fig. 2. Expression levels of *DIN7* mRNA in W303 (WT) and mutant (Δ19-bp) cells bearing the 19-bp deletion. Expression levels were obtained using quantitative reverse transcriptase-PCR. Cells treated with 0.15 M HU (closed bars) or untreated (open bars) were incubated in YPD medium at 30 °C for 9 h. The vertical axis indicates the relative expression level of *DIN7*.

To test whether or not additional copies of the 19-bp and 7-bp motifs enhanced *DIN7* transcription, we inserted several tandem repeats of the 19-bp or the 7-bp sequence into the region upstream of *LEU2* basal promoter. The insertion of four tandem repeats of the 19-bp sequence increased the β-galactosidase activity, and seven tandem repeats of the 19-bp sequence produced more than double the activity in the presence of HU (Fig. 3A). Even seven tandem repeats of the 7-bp sequence, however, failed to increase the β-galactosidase activity regardless of the presence of HU (Fig. 3B). These results suggested that the 19-bp sequence acts as an upstream activating sequence (UAS), whereas the 7-bp sequence alone is insufficient for HU-induced *DIN7* expression.

The decamer consensus sequence in the promoter of *RAD2*, which encodes a single-strand DNA endonuclease that belongs to the same XPG family as Din7, is required as a UAS for both the constitutive and DNA damage-induced expression of this gene [25,26]. In addition, the decamer consensus sequence in the promoters of *MAG1* and *MGT1*, which encode alkylated-DNA damage repair enzymes, and in that of *RAD51*, which encodes a homologous DNA pairing enzyme, have been shown to function as upstream repressing sequences (URSs) [24]. This decamer

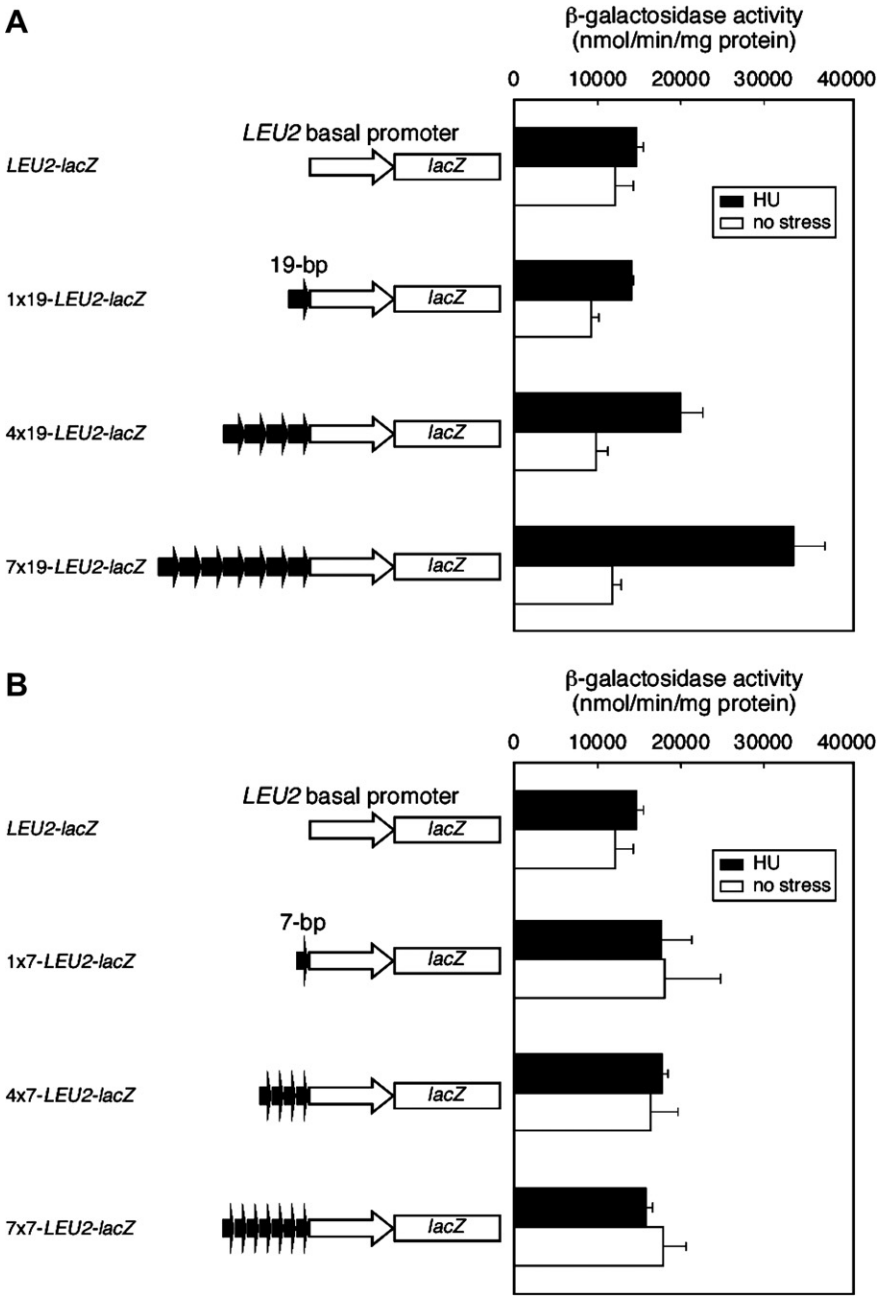

Fig. 3. Elevated levels of β-galactosidase activity due to increased copies of the 19-bp sequence in the presence of HU. The schematic illustrations show plasmids containing several tandem repeats of the 19-bp (A) or 7-bp (B) sequence. W303 cells carrying each plasmid derived from *LEU2-lacZ* fusion construct were treated with 0.15 M HU (closed bars) or were untreated (open bars) at 30 °C for 9 h. β-Galactosidase activities in the cell extracts were measured. The mean values and standard deviations were obtained from at least three independent experiments.

consensus sequence is likely able to function as either a UAS or URS, depending on the other regions of the gene promoter. Here, the decamer consensus sequence appears to function as a UAS for the transcriptional regulation of *DIN7* as it does for the expression of *RAD2*.

The presence of proteins that bind to the 19-bp sequence

In general, *trans*-acting factors that recognize *cis*-elements are required for the regulation of gene transcription. To examine whether there are proteins that specifically bind to the 19-bp sequence, we performed EMSAs using the 19-bp sequence or mutated 19-bp sequences (M1, M2, and M3) as biotin-labeled DNA probes. The proteins in the nuclear fractions extracted from HU-untreated or HU-treated cells were subjected to the EMSAs.

The mutated 19-bp sequences were designed by exchanging purine and pyrimidine bases in the regions of the sequence that were completely identical to the same regions from *NTG1* (Fig. 4A). As shown in Fig. 4B, a strong signal for the shifted band was detected only when the 50-bp double-stranded DNA probe, harboring the

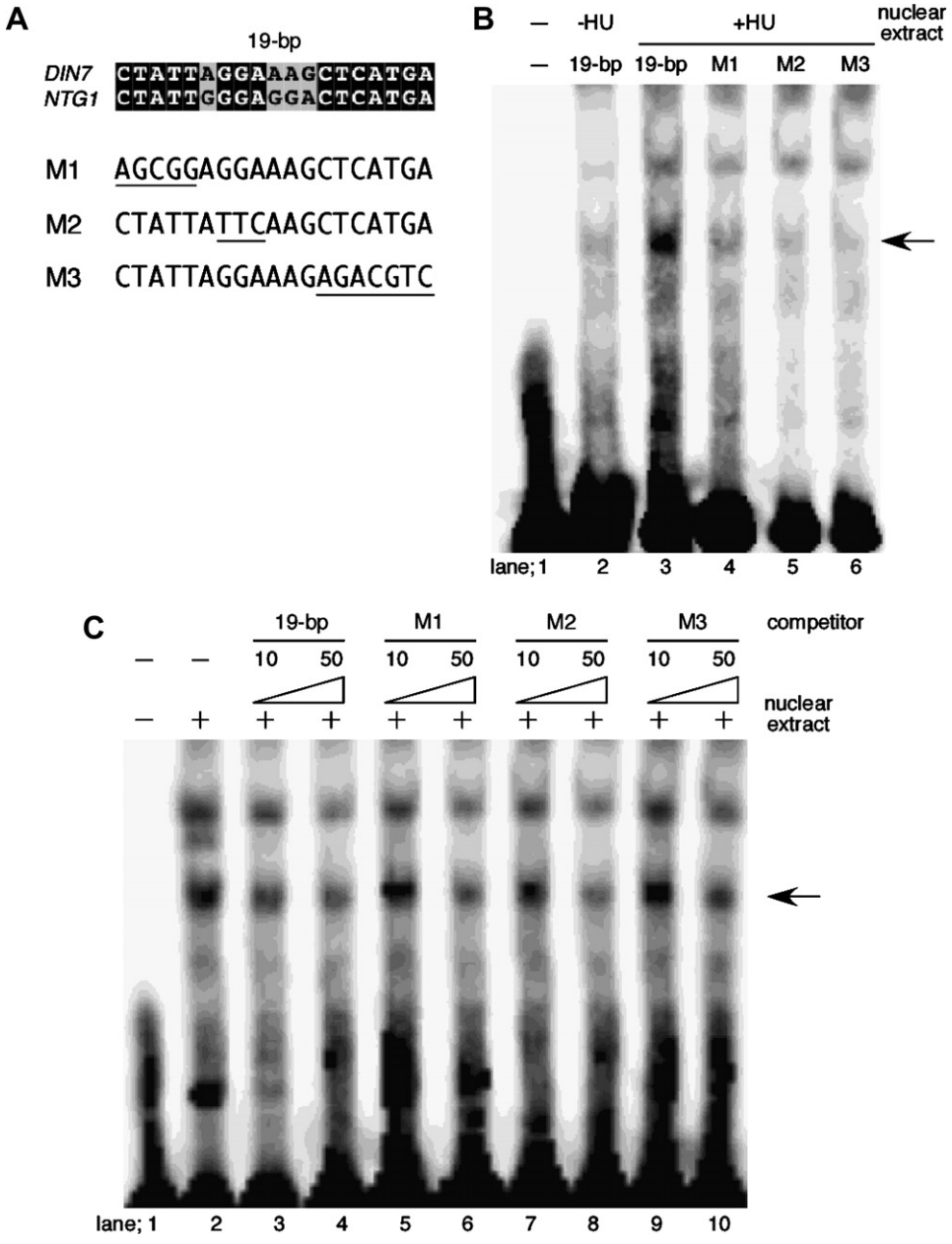

Fig. 4. Band shift assays to search for proteins that bound to the 19-bp sequence. (A) Schematic illustrations of the mutant 19-bp sequences. M1, M2, and M3 were constructed by exchanging purine and pyrimidine bases in the sequence fragments that were identical in the promoters of *DIN7* and *NTG1*. (B) Biotin-labeled double-stranded DNA with the 19-bp sequence (lane 3) or the mutated 19-bp sequences (M1, lane 4; M2, lane 5; and M3, lane 6) was incubated with nuclear extracts from HU-treated cells (lanes 3–6) or untreated cells (lane 2). Nuclear extracts were not added to control samples (lane 1). (C) A 10- or 50-fold excess of unlabeled 19-bp (lanes 3 and 4) or mutant 19-bp sequence (M1, lanes 5 and 6; M2, lanes 7 and 8; and M3, lanes 9 and 10) was incubated with a mixture of biotin-labeled 19-bp DNA fragment and nuclear extracts from HU-treated cells. Arrows indicate major band shifts caused by complexes between the 19-bp DNA sequence and protein(s).

WT 19-bp sequence, was mixed with the nuclear extracts from HU-treated cells (Fig. 4B, lane 3). A weak signal was also detected when the same probe was applied to the nuclear extracts from HU-untreated cells (Fig. 4B, lane 2). When the mutated 19-bp sequences (M1, M2, and M3) were used as DNA probes, markedly weaker signals from the shifted band were detected even from the nuclear extracts from HU-treated cells (Fig. 4B, lanes 4–6). Identification of the *trans*-acting protein(s) that specifically bind to the 19-bp sequence will help to elucidate the mechanism by which *DIN7* gene expression is specifically activated in response to HU-induced DNA damage.

In order to investigate the specificity of the protein(s) that bound to the biotin-labeled 19-bp sequence, a competition assay was carried out using the unlabeled WT or mutated 19-bp DNA fragments as competitors (Fig. 4C). Compared to the amount of DNA probe, addition of a 10-fold excess of the unlabeled 19-bp fragment markedly reduced the signal of the shifted band (Fig. 4, lane 3). In contrast, a 10-fold excess of the unlabeled mutated 19-bp

fragment did not noticeably compete with the probe for binding (Fig. 4, lanes 5, 7, and 9).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.177.

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