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Differential regulation of gene expression by RNA polymerase II in response to DNA damage

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Abstract

Cells change their gene expression profile dynamically in various conditions. By taking the advantage of ChIP, we examined the transcription profile of *Saccharomyces cerevisiae* genes in response to DNA damaging agents such as MMS or 4NQO. Gene expression profiles of different groups of genes roughly correlated with that revealed by Northern blot assay or microarray method. Damage-inducible genes showed increased cross-linking signals of RNA polymerase II, TFIIH, and TFIIF, meanwhile damage repressible genes decreased them, which means that gene expression is mainly regulated at the level of transcription. Interestingly, the characteristic occupancy pattern of TFIIH and polymerase with phosphorylated carboxy-terminal domain (CTD) in promoter or in coding regions was not changed by the presence of DNA damaging agents in both non-inducible and inducible genes. ChIP data showed that the extent of phosphorylation of CTD per elongating polymerase complex was still maintained. These findings suggest that overall increase in CTD phosphorylation in response to DNA damage is attributed to the global shift of gene expression profile rather than modification of specific polymerase function.
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Keywords: DNA damage; RNA polymerase carboxy-terminal domain; Transcription factor IIH; Chromatin immunoprecipitation

Eukaryotic RNA polymerase II (pol II) is a multisubunit complex. The largest subunit contains a unique C-terminal domain (CTD), which consists of multiple heptapeptide repeats with the consensus of YSPTSPS [1,2]. Pol II with an unphosphorylated CTD participates in the formation of the preinitiation complex, whereas pol II, in the process of elongation, has a highly phosphorylated CTD. Therefore, transcription cycle involving phosphorylation and dephosphorylation of the CTD has been proposed which includes many kinases and phosphatases [3]. CTD phosphorylation occurs largely at serine 2 and serine 5 within a heptapeptide repeat. Furthermore, phosphorylation of different serines predominates during different phases of transcription [4]. Serine 5 is preferentially phosphorylated at initiation/early elongation phase, whereas serine 2 is phosphorylated during the elongation phase. CTDK-I of budding yeast, the functional homolog of human pTEFb, is necessary for serine 2 phosphorylation and proper transcription regulation in vivo [5,6]. The overall level of serine 2 phosphorylation in elongating pol II is balanced by Fcp1 CTD phosphatase [7–9]. Interestingly, the mutations of CTDK-I subunit genes cause yeasts sensitive to DNA damage [10]. This indicates that proper regulation of serine 2 phosphorylation might be essential for survival in the genotoxic environment. In this regard, genes that are undergoing transcription show a faster rate of repair relative to the other inactive DNA region. This preferential repair of the transcribed region is called transcription-coupled repair (TCR) [11]. Although the mechanism(s) enabling TCR remains

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elusive, the fact that many of transcription elongation factors have affected TCR indicates that elongating pol II plays a critical role in TCR.

Immediate response on DNA damage and maintenance of DNA integrity is critical for the viability of living organisms. Cells respond by adjusting their transcription programs. The DNA microarray methods for genomewide analysis of gene expression provided a way to determine the overall functional state of the cell [12,13]. Many biochemical and genetic approaches as well as DNA microarray have focused on the global response to DNA-damaging agents that induce and repress a variety of genes.

Here we examined the transcriptional response of *Saccharomyces cerevisiae* to chemical DNA damaging agents such as MMS or 4NQO. Although the microarray method has been used to monitor global gene expression in *S. cerevisiae* [12,13], this method depends on the steady state level of RNA, which is a function of both RNA synthesis and turnover, and actually many parameters have been accounted for to determine the RNA levels in vivo.

In this study, chromatin immunoprecipitation was used to monitor the regulation of gene expression based on the occupancy of various transcription factors. It enabled us to dissect the regulation steps of various genes and observe it specifically at the level of transcription. We also analyzed different phosphorylation of pol II CTD during the transcription cycle. Our results led us to conclude that many genes are dynamically regulated in response to DNA damaging agents. Their expression is either induced or repressed by enhancing or reducing the recruitment of the transcription factors. In addition, there might be more elaborated regulatory mechanisms targeting steps beyond the simple recruitment of the transcription initiation factors. Although the CTD phosphorylation at serine 2 analyzed in bulk protein preparation was induced in response to DNA damage, polymerase engaged in productive transcription of each gene did not represent the higher density of phosphorylation per molecule. Our data indicate that changes in CTD phosphorylation reflect the global reprogramming of gene expression rather than changes in specific polymerase function.

Results and discussion

Experimental design

In an effort to determine the cellular response to the DNA damaging agents, we used chromatin immunoprecipitation (ChIP) method in which proteins are cross-linked in vivo to DNA using formaldehyde [4]. Yeast cells were treated with methyl methanesulfonate (MMS) or 4-nitroquinoline *n*-oxide (4NQO). MMS is a base alkylating agent, whereas 4NQO forms UV mimic cyclobutan pyrimidine dimers. Damage treated cells were harvested, washed, and resuspended in PBS buffer. Cross-linking agent, formaldehyde, was then added to minimize any interfering effect of drugs on cross-linking efficiency. The presence of individual transcription factors around specific locus was monitored by quantitative PCR using appropriate primers.

Genes for study were chosen and categorized based on DNA microarray results as follows (www.hsph.harvard.edu/geneexpression) [12,13]. Group 'MC' (minimally changed): their expression is not much changed by an hour treatment of 0.02–0.1% MMS. This includes *ADH1*, *PMA1*, *PYK1*, *TDH3*, and *SED1*, each encoding for alcohol dehydrogenase (glucose fermentation), cytoplasmic H^+-ATPase (proton transport), pyruvate kinase (glycolysis), glyceraldehyde 3-phosphate dehydrogenase (gluconeogenesis), and cell wall component (structural elements). Their expression has been reported changed barely or if any, reduced slightly. Group 'In' (induced): this category includes genes *GTT2*, *UBI4*, *SSU1*, *FLR1*, *HIS5*, *RNR1*, and *YFL061w*. They represent various cellular functions such as amino acid metabolism (*GTT2*, *HIS5*), protein degradation (*UBI4*), sulfur metabolism (*SSU1*), stress response (*FLR1*), DNA replication (*RNR1*), and include one whose function is unknown (*YFL061w*). They are known to be induced by an hour treatment of 0.1% MMS to a various degree from at least 3.7- (*RNR1*) to 156.9-fold (*YFL061w*). Group 'Re' (reduced): the genes repressed in the same condition are included; *DBP2*, *GAR1*, and *RKI1*. Each plays a role in cellular metabolism such as mRNA, rRNA, and pentose-phosphate cycle, respectively. Their expression is reduced by 7.3- (*GAR1*) or up to 31.3-fold (*DBP2*). The genes above were chosen as they were transcribed strong enough to detect by ChIP and also well separated from neighboring genes.

Transcription programs of various genes are dynamically changed in response to DNA damaging agents

Yeast cells were grown to an exponential phase (OD_{600} = 0.8–1.0) and treated with MMS at final concentrations of 0.02% and 0.1% or with 0.7 μg/ml of 4NQO for an hour at 30 °C. The cells were subsequently harvested and the chromatin fractions were prepared as described under Materials and methods. Occupancy of transcription factors at specific locus was monitored as a parameter of cross-linking signals amplified from the target genes. Recruitment of pol II was probed by ChIP using 8WG16 monoclonal antibody that recognizes non-phosphorylated CTD. One of the typical results is shown in Fig. 1A. The cross-linking of pol II with non-phosphorylated CTD coincided roughly with the grouping of genes that was rationalized based on

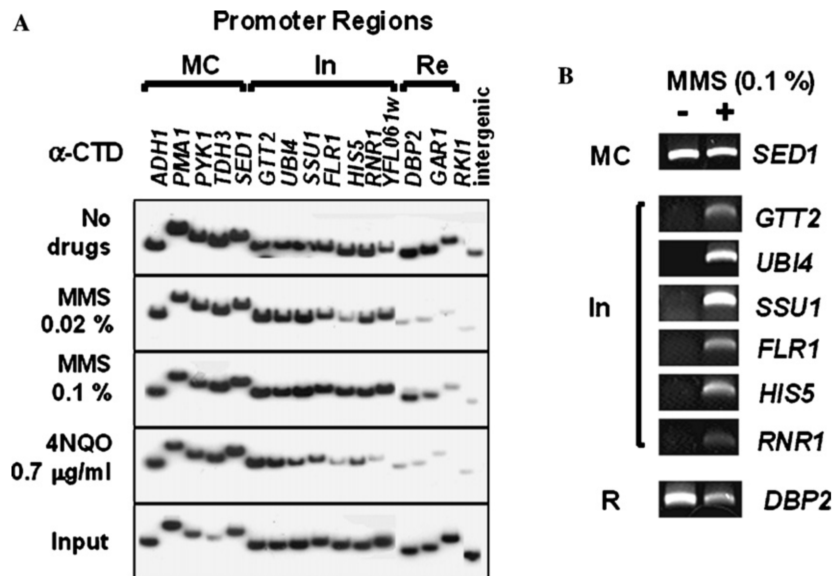

Fig. 1. Differential gene expression in the presence of MMS or 4NQO. (A) Yeast cells were grown at 30 °C in the YPD media containing 2% glucose. When growth reached 0.8 U of OD_{600}, the culture was divided into appropriate number and further incubated in the presence or absence of MMS (0.02%, 0.1%) or 4NQO (0.7 μg/ml). A ChIP assay with 8WG16 was used to monitor the occupancy of the different genes by RNA polymerase II. PCR was performed with appropriate primers to detect the promoter sequences of indicated genes. 'MC,' minimally changed genes, includes *ADH1*, *PMA1*, *PYK1*, *TDH3*, and *SED1*; 'In,' inducible genes, includes *GTT2*, *UBI4*, *SSU1*, *FLR1*, *HIS5*, *RNR1*, and *YFL061w*; and 'Re,' reduced genes, includes *DBP2*, *GAR1*, and *RKI1*. As an internal background control, a region of chromosome V devoid of ORFs was amplified and indicated as an intergenic region. The top panel with 'no drugs' indicates the ChIP with the chromatin solution prepared from the culture without any drug treatment. Input (bottom panel) shows the signal from the chromatin before immunoprecipitation. (B) The steady state level of RNA was monitored by RT-PCR. Yeast cells were grown and treated with MMS (0.1%) as in (A). RNA was purified from each sample and the induction of various genes was analyzed by RT-PCR. The experiments were repeated three times and the representative is shown.

DNA microarray results (Fig. 1A). The genes in Group 'MC' were barely changed or slightly reduced while those in 'Re' group were greatly reduced by MMS or 4NQO. Interestingly, genes in Group 'Re' responded more sensitively in the low concentration of damaging agents (Fig. 1A compare MMS 0.02% and 0.1% panels). Their expression was reduced at the transcriptional level due to the restriction of the formation of transcription complexes in the damage-induced condition. In case of Group 'In,' transcription was induced (~2-fold; *GTT2*, *UBI4*, *FLR1*, *HIS5*, and *RNR1*: ~3.6-fold; YFL061w) by 0.1% MMS, yet their fold difference in terms of pol II crosslinking was not as much as expected. The treatment of cells with 4NQO resulted in several genes grouped in 'In' unchanged or rather repressed if any. Two different agents seemed to induce substantially different subset of genes in a various range of drug concentrations. The microarray method has observed an increase at higher concentration (2 or more than 8 μg/ml of 4NQO was required to detect strong induction signals which were comparable with those of MMS 0.1%) [13].

To further elucidate dynamic change of gene expression, it was examined by the steady state level of RNA using RT-PCR (Fig. 1B). The result displayed the overall correlation of gene expression profile between two methods. However, the fold difference, especially induction fold estimated by RT-PCR was much greater than that by ChIP. The fold difference might be attributed to overestimation by RT-PCR or underestimation by ChIP, or both [13]. For example, if the basal transcript level is below the detection limit, as the case shown in Group 'In,' RT-PCR often overestimates the extent of induction. The antibody used to monitor pol II was 8WG16 that recognizes any of non-phosphorylated serines 2 out of 26 heptapeptide (YSPTSPS) repeats. Note that the epitopes might be presented over the IP limit of antibody in an induced condition where single molecule carries multiple targets. If that is the case, antibody saturation is one of the possible ways to explain that ChIP with 8WG16 underestimates.

Differential association of TFIIH in response to DNA damage

To further confirm the reproducibility of DNA damage dependent induction of genes in Group 'In,' ChIP was carried out with anti-Kin28 antibody that recognizes one of the essential subunits of Transcription Factor IIH (TFIIH), Kin28. ChIP assay showed strong and increased cross-linking to the genes in Group 'In' upon MMS treatment, in contrast to the genes in Group 'MC' (Fig. 2). The fold difference was estimated to be greater than those of signals performed by 8WG16. In

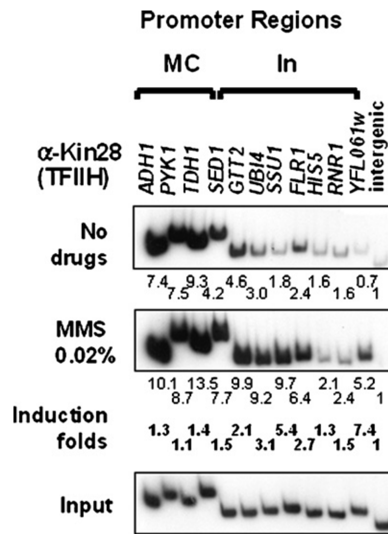

Fig. 2. The occupancy of Kin28 kinase subunit of TFIIH was increased by MMS. ChIP was carried out as described in Fig. 1. Polyclonal rabbit antibody was used to precipitate Kin28. The numbers immediately below each lane are quantitated PCR signals in arbitrary units after normalization for amplification efficiency and subtraction of background. The induction folds were obtained by dividing each number of specific locus from MMS treated condition with the ones from normal condition.

the similar condition (0.05% MMS), microarray assay reported that each gene was induced in a various degree, such as 13.8 (*GTT2*), 7.4 (*UBI4*), 12.0 (*SSU1*), 3.7 (*FLR1*), 4.4 (*HIS5*), 3.9 (*RNR1*), and 180.0 (*YFL061w*) folds. The quantitation of ChIP signal showed that the ratio of induction folds correlates with above within an experimental error range (Fig. 2 compare top and middle panels, and see induction folds). The induction folds estimated by ChIP were 2.1 (*GTT2*), 3.1 (*UBI4*), 5.4 (*SSU1*), 2.7 (*FLR1*), 1.3 (*HIS5*), 1.5 (*RNR1*), and 7.4 (*YLR061w*). This result suggests that the total increase of RNA in response to MMS was primarily due to the increased number of transcription events (or formation of transcription complex). The data also suggest a possibility that there is more elaborated regulatory mechanism to control the RNA level. The TFIIH cross-linking signal in the *HIS5* and *RNR1* was not increased as much as microarray or RT-PCR determined (see Fig. 2, also Figs. 4 and 5). This consideration indicates that subset of genes might be also regulated at the post-transcriptional level. In addition, the RNA message level without DNA damage for *GTT2* calculated by Samson' group was 37.3, the lowest value among genes tested above, for example, 228 for *UBI4*, 145 for *SSU1*, 271 for *FLR1*, 224 for *HIS5* or 76 for *RNR1* [12,13]. However, the TFIIH cross-linking signal showed the highest for *GTT2* without MMS (see Fig. 2 no drug panel), even though the amplification efficiency of each primer set was similar between target loci (see Fig. 2 input panel). This proposes another possibility that preformed initiation complexes could be regulated at the post-transcription initiation level as observed for many heat shock genes.

The recruitment of TFIIH and TFIIF to the promoters of damage activated genes

Nucleotide excision repair (NER) and base excision repair (BER) consist of a general pathway termed global genome repair (GGR) and a specialized pathway termed transcription-coupled repair (TCR). Whereas GGR removes lesions from the entire genome, repair by TCR is confined to DNA lesions in the transcribed strand of transcriptionally active genes and strictly depends on ongoing transcription by RNA pol II. Many laboratories have reported multiple connections between the transcription, especially elongation and repair. Assembly of repair complex involves the recruitment of TFIIH [14]. The helicase subunits of TFIIH, XPB, and XPD play a role in NER and BER [15]. Therefore, TFIIH exerts a dual function in the cell, being an essential factor in transcription initiation and in excision repair [16]. The recruitment of TFIIH during a transcription cycle has been reported previously [4,17]. TFIIH is known to be cross-linked preferentially to the promoter regions. It means that TFIIH dissociates from the initiation complex as it leaves the promoter. In this regard, we asked whether the distribution profile of TFIIH might be changed in the presence of DNA damaging agents. In Fig. 3, TFIIH (Kin28) showed strong cross-linking to the promoter regions of tested genes. In contrast, little

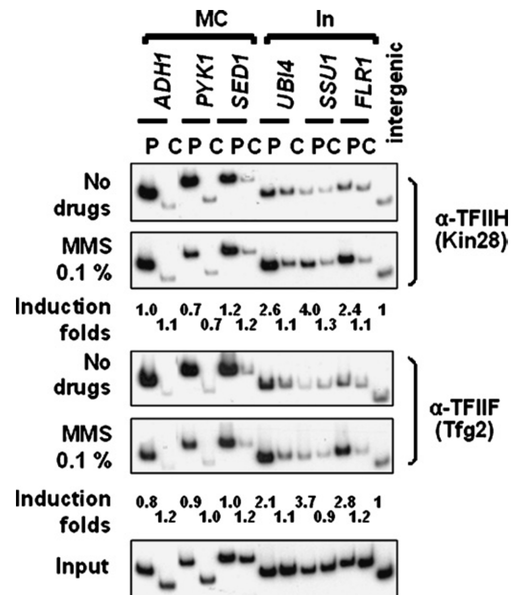

Fig. 3. TFIIH (Kin28) and TFIIF (Tfg2) cross-link preferentially to the promoter region. The occupancy of transcription initiation factors was monitored by ChIP. Immunoprecipitation was performed with antibodies against Kin28 or Tfg2, a subunit of TFIIF. Primers were designed to amplify two separate regions of each gene, promoter (P) or coding region (C).

cross-linking was seen in the coding regions either in the absence or presence of MMS. Biased cross-linking pattern of TFIIH was obvious in strongly transcribed genes and was not affected by MMS. The three inducible genes tested, *UBI4*, *SSU1*, and *FLR1*, also showed higher cross-linking of TFIIH at the promoter region. MMS enhanced the TFIIH recruitment, still maintaining its characteristic occupancy profile. The cross-linking pattern of TFIIH (Kin28) was closely paralleled with that of TFIIF (Tfg2), one of other essential initiation factors, which predominantly located in the promoter region. This result showed that the recruitment of initiation factors was induced in the DNA damage responding genes to turn the transcription on for accumulation of transcripts in time. TFIIH did not necessarily travel with elongating pol II in a damage-induced condition. It indicates that it might be independently recruited to the stalled pol II complex whenever an assembly of repair complex is demanded [14].

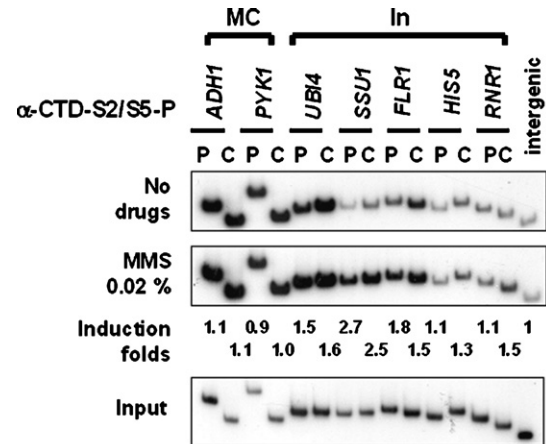

Fig. 4. Cross-linking of phosphorylated CTD was increased as the inducible genes were turned on by MMS. ChIP and PCR were carried out using the B3 monoclonal antibody that recognizes the phosphorylated form of CTD. Primer pairs for promoter (P) or coding sequences (C) of the indicated genes were used as in Fig. 3.

CTD phosphorylation per RNA polymerase II is not changed during transcription cycle in response to DNA damage

The CTD of pol II plays an essential role in gene expression and its role is in part mediated by phosphorylation within the heptapeptide repeats. It has been suggested that phosphorylation status of the CTD determines proteins that interact with pol II. Pol II also becomes ubiquitinated in yeast and mammalian cells after exposure to DNA damaging agents, which might lead pol II to preferential degradation [18,19]. Interestingly, the ubiquitinated pol II is hyperphosphorylated on the CTD. Furthermore, in budding yeast, DNA damaging agents increase phosphorylation of the CTD on serine 2 and its phosphorylation is dependent on Ctk1 kinase subunit of CTDK-I complex [10]. To test whether the typical phosphorylation profile is changed by DNA damage, ChIP was carried out with phosphorylation specific CTD antibodies. And the effect of DNA damage on transcription and localization of phosphorylated pol II within a unit of gene was carefully analyzed. In this study monoclonal B3 and H5 antibodies were used. B3 recognizes phosphorylated serine 2 and 5 without differentiation, while H5 recognizes phosphorylated serine 2 preferentially. We sought to address whether pol II engaged in on-going transcription responds to DNA damage-induced condition by changing its CTD phosphorylation pattern, specifically focusing on serine 2 site. As seen in Fig. 4, MMS increased the cross-linking of phosphorylated CTD (at serine 2 and 5) in both promoter and coding regions of *UBI4*, *SSU1*, and *FLR1* in Group 'In.' However, the cross-linking of the phosphorylated CTD to the *ADH1* and *PYK1* in 'MC' was barely changed by MMS. It means that DNA damage turned on subset of genes by increasing the transcription complex formation. That is, many rounds of transcription cycle increased the cross-linking chance of phosphorylated pol II to the genes in Group 'In,' therefore, it did not necessarily mean that phosphorylation per polymerase engaged in transcription has been increased by MMS. If CTD phosphorylation is a means to change specific function of pol II appropriate to the DNA damage condition, the CTD crosslinking should be increased in both Group 'MC' and 'In' genes. The cross-linking of TFIIH and phosphorylated CTD to *HIS5* and *RNR1* genes was low (Figs. 2 and 4), and the fold increase by MMS was less than 1.5.

We further analyzed serine 2 phosphorylation (Fig. 5). Phosphorylation of serine 2 on CTD was strongly detected in the coding regions of tested genes by MMS. In case of *UBI4*, *SSU1*, and *FLR1*, their cross-linking was also increased somehow in the promoter region even though it still maintained the coding to promoter bias. That is, serine 2 phosphorylation of pol II was increased by MMS, again still maintaining its typical profile during transcription cycle. It was increased in genes in Group 'In' as the cross-linking of Kin28, CTD, and Tfg2 was by MMS. This shows that global changes in gene expression profile led to an overall increase in CTD serine 2 phosphorylation but certain phosphorylation density on CTD or its profile during transcription cycle is maintained.

Environmental signals are able to influence the extent of CTD serine 2 phosphorylation which is mediated by Ctk1 kinase [5]. The diauxic shift when yeast cells enter stationary growth phase or other kind of stressful conditions such as heat shock induces serine 2 phosphorylation too. DNA damage is simply one of stressful conditions. Eukaryotic cells respond to environmental changes by changing the pattern of gene expression. It is noteworthy that Ctk1 is dispensable under the normal

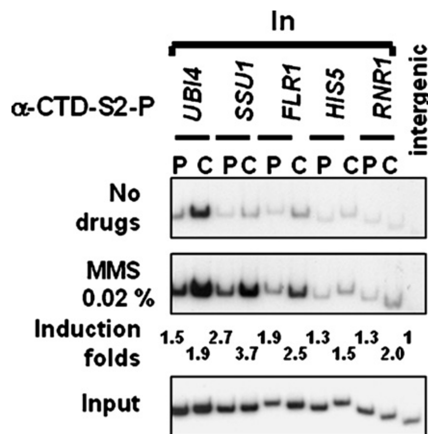

Fig. 5. Phosphorylation of serine 2 in the CTD of polymerase was enriched in the coding region and increased as the inducible genes were turned on by MMS. ChIP and PCR were carried out using the H5 monoclonal antibody that recognizes the phosphorylated serine 2 of CTD. Primer pairs are as in Fig. 4.

growth condition, but becomes essential in stressed condition. It means that CTD serine 2 phosphorylation is indispensable for survival in this condition. We assume that subset of genes that are expressed in stressed condition must depend critically on CTD serine 2 phosphorylation. Further studies on the differential phosphorylation and dephosphorylation of CTD and the interacting proteins that read out phosphorylation code should provide an insight into understanding the exact role of CTD in gene expression either in normal or stressed condition.

Materials and methods

Yeast, growth conditions, and analysis. Yeast strain used in this study was YC43 [MATα, *ura3-52 or ura3-1*, *leu2-3*, *trp1-1*, *his3Δ200* or *his3-11*, *rpb3-*(*HA*)$_3$*::leu2::TRP1*, *rpb2Δ297::HIS3*, *ade2-1*, pRP214(*RPB2*, *LEU2*)]. It is a kind gift from Dr. S. Buratowski (Harvard Medical School, USA). The yeasts were grown in YPD containing 2% glucose. For MMS or 4NQO treatment, cell culture grown to the exponential phase was divided and DNA damaging agent was added into appropriate samples (MMS; 0.02% or 0.1%, 4NQO; 0.7 μg/ml). Usually, cells were treated for an hour. The media preparation, yeast transformation, and other yeast manipulations were performed using the standard methods as described previously.

Chromatin immunoprecipitation. Chromatin immunoprecipitations (ChIPs) were performed essentially as described in Kormanitsky et al. [4]. Briefly, all yeast strains were grown to $OD_{600} = 0.8$–1.0 in YPD media containing 2% glucose. Subsequently after drug treatment, cells were harvested, quickly washed with water, and resuspended in the same volume of PBS. Formaldehyde was then added to a final concentration of 1% and incubated at room temperature for 20 min. Cross-linking was quenched by addition of glycine to 240 mM. Cells were collected and lysed with glass beads in FA lysis buffer (50 mM Hepes–KOH at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, and 1 mM PMSF). Chromatin fraction was prepared by sonication method. Anti-Tfg2 (subunit of TFIIF) serum was a kind gift of Dr. S. Buratowski. Anti-Kin28 polyclonal serum was from Santa Cruz and monoclonal antibodies 8WG16, H5, and B3 were purchased from Covance. For IPs, all antibodies except H5 and B3 were preincubated with protein A–Sepharose CL-4B beads (Amersham/Pharmacia) for an hour at the room temperature. The beads pre-linked with antibodies were incubated with chromatin solution overnight at 4 °C. For H5 and B3 IPs, anti-mouse IgM agarose (Santa Cruz Biotechnologies) was incubated with both antibodies and chromatin solution overnight at 4 °C. Conditions for PCRs were described previously. Where noted, PCR signals were quantitated by Phosphoimager (Fujix BAS 2040) scanning and normalized to the input DNA reaction and the intergenic control. The sequence information of primers used in this study is provided as Supplementary data.

RNA analysis. Yeasts grown to the exponential phase ($OD_{600} =$ 0.8–1.0) were used for the RNA isolation. Total RNA was prepared using the Trizol method according to the manufacturer's instructions (Invitrogen). Before RT-PCR, the RNA was usually treated with DNase I (Promega) to remove any residual chromosomal DNA remaining in the sample. Double-stranded cDNA was made from 1 μg of total RNA using the reverse transcriptase (Promega). Semi-quantitative PCR amplification of the target regions of various genes was performed in a 25 μl reaction mixture by using a following program: 5 min at 94 °C followed by 16–20 cycles with 40 s at 94 °C, 40 s at 50 °C, and 50 s at 72 °C. The reaction was finished by an extra extension step at 72 °C for 10 min. Signals were quantified by Chemi-digital image analysis system using Labworks software (UVP, USA). Oligonucleotide primers used in RT-PCR were same with the ones used for amplification of coding regions in ChIP assay.

Acknowledgments

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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.2004.10.101.

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