Preferential repair of DNA double-strand break at the active gene in vivo.

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Key words: DNA repair, DSB, active gene, ADH1, PHO5, and MATα.

Running Title: Preferential DSB repair at the active gene.

Background: To determine whether DNA double-strand break (DSB) repair is coupled to transcription, we analyzed DSB repair at the active and inactive genes.

Results: Our results reveal that DSB repair at the active gene is faster than that at the inactive gene.

Conclusion: Present results demonstrate a preferential DSB repair at the active gene.

Significance: This study supports the existence of transcription-coupled DSB repair.

Summary: Previous studies have demonstrated transcription-coupled nucleotide/base excision repair. We report here for the first time that DNA double-strand break (DSB) repair is also coupled to transcription. We generated a yeast strain by introducing a homing (Ho) endonuclease cut site followed by a nucleotide sequence for multiple Myc epitopes at the 3’ end of the coding sequence of a highly active gene, ADH1. This yeast strain also contains the Ho cut site at the nearly silent or poorly active mating type α (MATα) locus, and expresses Ho endonuclease under the galactose-inducible GALI promoter. Using this strain, DSBs were generated at the ADH1 and MATα loci in galactose-containing growth medium that induced HO expression. Subsequently, yeast cells were transferred to dextrose-containing growth medium to stop HO expression, and the DSB repair was monitored at the ADH1 and MATα loci by PCR, using the primer pairs flanking the Ho cut sites. Our results revealed a faster DSB repair at the highly active ADH1 than that at the nearly silent MATα locus, hence implicating a transcription-coupled DSB repair at the active gene in vivo. Subsequently, we extended this study to another gene, PHO5 (carrying the Ho cut site at its coding sequence), under transcriptionally active and inactive growth conditions. We find a fast DSB repair at the active PHO5 gene in comparison to its inactive state. Collectively, our results demonstrate a preferential DSB repair at the active gene, thus supporting transcription-coupled DSB repair in living cells.

Introduction: Cellular DNA is continuously attacked by both endo- and exogenous factors causing DNA damage (1-3). The most versatile cellular pathway for dealing with a large variety of structurally-unrelated DNA lesions is nucleotide excision repair (NER) which mainly removes helix-distorting lesions, including UV-induced cyclobutane pyrimidine dimers, 6-4 photoproducts and 4NQO (4-nitroquinoline-1-oxide)-induced bulky chemical adducts. The other frequent damages like oxidative lesions and small base alterations are processed by base excision repair (BER) (4). The very toxic DNA DSBs induced by ionizing radiation are repaired via homologous recombination (HR) or non-homologous endjoining (NHEJ) (5-8). Further, the occurrence of DNA lesions triggers checkpoints at the key stages in the cell cycle. Checkpoints monitor the progression of cell cycle post DNA damage, and maintain the proper order of events (3, 9-11). The up-regulation activity of checkpoint proteins in response to DNA damage will
impose a temporary arrest of cell-cycle progression to allow DNA repair or to induce apoptosis (cell death).

Although DSB is a threat to the genomic integrity of a cell, yet it occurs during normal DNA metabolism such as replication, meiosis, and immune system development. Programmed DSBs in meiosis are found to promote several major events beyond recombination and synaptonemal complex formation (5, 12). Cells can take advantage of DSB-induced recombination in order to generate genetic diversity in physiological processes such as meiosis and generation of antibodies by V(D)J recombination in lymphocytes.

An extremely cytotoxic ramification of DNA damage is when lesions in the actively transcribed coding sequence cause stalling of the transcription machinery (13, 14). Persistent transcriptional arrest interferes with cellular function or triggers apoptosis (15, 16), and thus the efficient removal of lesions from the coding regions of active genes is essential for proper cellular function. A specific repair mode, referred to as transcription-coupled repair (TCR), removes lesions from the coding sequences of active genes in both prokaryotes and eukaryotes (17-24). In prokaryotes, transcription repair coupling factor displaces DNA damage-stalled RNA polymerase, which facilitates the recruitment of the DNA repair machinery to the lesion in the active gene. Eukaryotic TCR is considerably more complex and is not well understood, although the phenomenon of TCR in eukaryotes was reported more than 20 years ago (19, 21, 22).

TCR is one of the two sub-pathways of NER. The other one is the global genome repair (GGR or GG-NER) that is responsible for the removal of DNA lesions throughout the genome. The basic steps of NER are:- (a) recognition of DNA lesion, (b) dual incisions of the DNA strand carrying the lesion to form a 24–32-nucleotide oligomer, (c) release of the excised oligomer bracketing the lesion, (d) repair synthesis to fill in the resulting gap, using the undamaged strand as template, and finally, (e) ligation. DNA damage recognition differs between GG-NER and TC-NER, but the subsequent steps are shared. The damage recognition step makes TC-NER a faster process than GG-NER. In TC-NER, recognition of the lesion is tied to RNA polymerase stalling at the DNA damage site and involves Rad26p in yeast or Cockayne syndrome group B protein (CSB) in humans (25-34). Like TC-NER, BER is also coupled to transcription (35-37). However, it is yet to be determined whether DSB repair is coupled to transcription. Here, we report a fast DSB repair at the transcriptionally active gene as compared to the inactive gene, thus implying transcription-coupled DSB repair.

Experimental Procedures:

Plasmids: The plasmid, pFA6a-13Myc-KanMX6 (38), was used for genomic myc epitope tagging of Ino80p at the C-terminal. The same plasmid was also used to insert Ho cut site just before multiple myc epitope tags at the ADH1 and PHO5 coding sequences. The plasmid, pFA6a-3HA-His3MX6 (38), was used for genomic HA epitope tagging of Rad50p and Ku70p at their C-terminals. The plasmid, pRS406 (39), was used to knock out RAD52 and DNL4.

Strains: The yeast (Saccharomyces cerevisiae) strain, JKM179, contains a Ho cut site at the MATα locus, and expresses HO under the GAL1 promoter in galactose-containing growth medium. JKM179 was obtained from the Haber laboratory (40). The genotype of JKM179 is hoΔ MATα hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO. The Ho cut site followed by a nucleotide sequence encoding multiple myc epitope tags was added before the stop codons at the original chromosomal loci of ADH1 and PHO5 in JKM179 to generate the PCY23 and RSY33 strains, respectively. Multiple myc epitope tags were added to the chromosomal locus of INO80 in JKM179 to generate the ASY32 strain. Likewise, multiple HA epitope tags were added to the chromosomal loci of RAD50 and KU70 in PCY23 to generate the RSY30 and RSY31 strains, respectively. The RAD52 and DNL4 genes were knocked out in
PCY23 to generate the PCY36 and PCY37a strains, respectively.

**Growth Media:** For induction of the *GAL1* promoter to express *HO*, yeast cells were grown in galactose-containing medium (YPG; yeast extract, peptone plus 2% galactose). To stop the expression of *HO*, yeast cells were grown in raffinose (YPR; yeast extract, peptone plus 2% raffinose) or dextrose (YPD; yeast extract, peptone plus 2% dextrose)-containing medium. To study the DSB repair at the Ho cut sites at *ADH1* and *MATα* loci, yeast cells were initially grown in YPG, and subsequently switched to YPD as described in the Figure 3B legend. To study DSB repair at the Ho cut site at *PHO5*, yeast cells (RSY33) were initially grown in YPG without inorganic phosphate (Pi), and then switched to YPD (transcriptionally inactive condition) or YPD-Pi (transcriptionally active condition) as described in the legends of Figure 6B and 6C.

**Genomic DNA preparation:** The genomic DNA was extracted from 5 ml of yeast culture. Briefly, the harvested cells were suspended in 200 µl lysis buffer (50 mM HEPES, pH 7.5; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; and 0.1% Na-deoxycholate) with 200 µl volume-equivalent of glass beads, and then vortexed for 30 min at 4 °C using a Tomy vortexer (MT-360). The whole-cell extract was collected by punching a hole at the bottom of the eppendorf tube, and then partitioned with 200 µl of phenol:chloroform:isoamylalcohol. The aqueous phase following phenol:chloroform extraction was treated with ethanol to precipitate genomic DNA.

**Analysis of DSB and its repair:** The genomic DNA was analyzed for Ho-induced DSB and repair at the *ADH1, PHO5* and *MATα* loci, using the primer pairs flanking the Ho cut sites at the *ADH1, PHO5* and *MATα* loci. The primer pair used to analyze DSB at the Ho cut site at *PHO5* was:

5'-ACCTCTAATTCTAGAGATGTCATGAC-3', and 5'-GAATTCTAGAGCTCGTTAAAC-3'.

The primer pair used to analyze the DSB at the Ho cut site at the *MATα* locus was:

5'-AGTTGCTCGATTAAACTCATCTGTG ATTTGTGG-3', and 5'-GATGCTAAGATTGATTGTGGCTTGAG3'.

The disappearance of the PCR signal would indicate the presence of DSB. A specific region of *SMC2* was amplified as a control. *SMC2* is not damaged by Ho. The primer pair for amplification of a specific region of *SMC2* was:

5'-GACGCACCCTTGTAACAGTCAG-3', and 5'-GGCGAATTCCATCACATTATACTAAC GCG-3'.

**Chromatin immunoprecipitation (ChIP) assay:** The ChIP assay was performed as described previously (41-44). Briefly, yeast cells were treated with 1% formaldehyde, collected and resuspended in lysis buffer. Following sonication, cell lysate (400 µl lysate from 50 ml of yeast culture) was precleared by centrifugation, and then 100 µl lysate was used for each immunoprecipitation. Immunoprecipitated protein–DNA complexes were treated with proteinase K, the cross-links were reversed, and DNA was purified. Immunoprecipitated DNA was dissolved in 20 µl TE 8.0 (10 mM Tris HCl pH 8.0 and 1 mM EDTA), and 1 µl of immunoprecipitated DNA was analyzed by PCR. PCR reactions contained [α-32P]dATP (2.5 µCi for 25 µl reaction), and the PCR products were detected by autoradiography after separation on a 6% polyacrylamide gel. As a control, ‘input’ DNA was isolated from 5 µl lysate without going through the immunoprecipitation step, and dissolved in 100 µl TE 8.0. To compare PCR signal arising from the immunoprecipitated
DNA with the input DNA, 1 µl of input DNA was used in the PCR analysis.

For analysis of recruitment of Ino80p, Ku70p, and Rad50p, the above ChIP protocol was modified as described previously (25). Briefly, a total of 800 µl lysate was prepared from 100 ml of yeast culture. Following sonication, 400 µl lysate was used for each immunoprecipitation (using 10 µl of anti-HA or anti-myc antibody and 100 µl of protein A/G plus agarose beads from Santa Cruz Biotechnology, Inc.), and immunoprecipitated DNA sample was dissolved in 10 µl TE 8.0 of which 1 µl was used for the PCR analysis. In parallel, the PCR analysis for ‘input’ DNA was performed using 1 µl DNA that was prepared by dissolving purified DNA from 5 µl lysate in 100 µl TE 8.0. The primer pairs used for PCR analysis were as follows:

MAT\(\alpha\)-Ho:
5'-GGTTTTGTAGAGTGTTGGACGAAT-3', and
5' - GCTATACTGACAACATTCACTACTCG-3';

ADH1-ORF:
5'-CGGTAACAGAGCTGACACCAGAGA-3', and
5'-ACGTATCTACCAACACGATTGACCC-3';

ADH1-UAS:
5'-GAGTTTCCGGGTGATAATAGG-3', and
5'-CTATTGTATACTCCTCCCTCCGC-3';

ADH1-Core:
5'-GGTATACGGCCTTCCTTCCAGTTAC-3', and
5' - GAACGAGAACAATGACGAGGAAACAA AAG-3';

PHO5-ORF:
5'- ACCTCTAATTCTAAGAGATGTCATGAC-3', and
5' - ACAATGTCATCATGCGCATGAGTC-3'; and
Chr-V:
5'- GGCTGTCAGAATATGGGGCCGTAGTA-3', and
5'-CACCCCGAAGCTGCTTTCACAATAC-3'.

Autoradiograms were scanned and quantitated by the National Institutes of Health image 1.62 program. Immunoprecipitated (IP) DNAs were quantitated as the ratio of IP to input in the autoradiogram. ORF, open reading frame; UAS, upstream activating sequence; core, core promoter; and Chr-V, Chromosome-V.

**Total RNA preparation:** Total RNA was prepared from yeast cell culture as described by Peterson et al. (45). Briefly, 10 ml yeast culture was harvested, and then suspended in 100 µl RNA preparation buffer (500 mM NaCl, 200 mM Tris-HCl, 100 mM Na2EDTA, and 1% SDS) along with 100 µl phenol/chloroform/isoamyl alcohol and 100 µl volume-equivalent of glass beads (acid washed; Sigma). Subsequently, yeast cell suspension was vortexed with a maximum speed (10 in VWR mini-vortexer; Cat. No. 58816-121) for 5 times (30 seconds each). Cells suspension was put in ice for 30 seconds between pulses. After vortexing, 150 µl RNA preparation buffer and 150 µl phenol/chloroform/isoamyl alcohol were added to yeast cell suspension followed by vortexing for 15 seconds with a maximum speed on VWR mini-vortexer. The aqueous phase was collected following 5 min centrifugation at a maximum speed in microcentrifuge machine. The total RNA was isolated from aqueous phase by ethanol precipitation.

**RT-PCR (Reverse transcriptase-PCR) analysis:** RT-PCR analysis was performed according to the standard protocols (46). Briefly, total RNA was prepared from 10 ml yeast culture. Ten micrograms of total RNA was used in the reverse transcription assay. RNA was treated with RNase-free DNase (M610A, Promega) and then reverse-transcribed into cDNA using oligo(dT) as described in the protocol supplied by Promega (A3800, Promega). PCR was performed using synthesized first strand as template and the primer pairs targeted to the \(ADH1\) and \(MAT\alpha\)
ORFs. RT-PCR products were separated by 2.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The primer pairs used in the PCR analysis were as follows:

**ADH1:**
5’-CGGTAACAGAGCTGACACCAGAGA-3’, and 5’-ACGTATCTACCAACGATTTGACCC-3’;

**MATα:**
5’-GGTTTTGTAGAGTGGTTGACGAAT-3’, and 5’-GCTATACTGACAACATTCAGTACTCG-3’.

**Results and Discussion:**

**Induction of DSBs at the highly active ADH1 and nearly silent MATα genes.**

Cells in S-phase have a fast kinetics of DNA DSB repair post irradiation as compared to cells in G1- or G2-phase (47). The fast repair kinetics of DSBs in S-phase is attributed to be due to functionally active DNA-polymerases. To determine whether RNA-polymerases have any influence on the repair of DNA DSB, we analyzed here the effect of RNA polymerase II-dependent transcription on DSB repair by introducing the Ho cut site at the ADH1 coding sequence and MATα locus, as described below. Our RT-PCR analysis revealed that ADH1 is a highly active gene as compared to the MATα locus (Figure 1A). MATα appeared to be a poorly active or nearly silent gene, consistent with previous studies (48). These observations were further substantiated by the analysis of the association of RNA polymerase II with these two loci. We find a robust association of RNA polymerase II with ADH1 as compared to the MATα locus (Figure 1B). A primer pair targeted to the transcriptionally inactive region of chromosome V was also used in the PCR analysis as a non-specific DNA control (49). Collectively, these results demonstrate that ADH1 is a highly active gene, consistent with previous studies (48, 50), and MATα is a nearly silent gene as compared to ADH1.

However, the coding sequences of both genes have similar nucleosomal density (51).

To determine whether DSB repair is coupled to transcription, we first analyzed the DSB at the nearly silent MATα locus, using the yeast strain that has a Ho cut site at the MATα locus and expresses HO under the control of the GAL1 promoter in galactose-containing growth medium (Figure 2A). The yeast strain was initially grown in raffinose-containing medium up to an OD₆₀₀ of 0.9, and then switched to galactose-containing growth medium for 30 and 60 min. In raffinose-containing growth medium, HO was not expressed as the GAL1 promoter is not induced in the presence of raffinose (44, 52). Therefore, Ho cut site at the MATα locus was intact in raffinose-containing growth medium (Figure 2B). Upon switching to galactose-containing growth medium, HO was expressed as the GAL1 promoter is induced in the presence of galactose (44, 52). Such expression of HO would induce DSB at the Ho cut site at the MATα locus. The generation of DSB at the MATα locus was monitored by PCR, using the primer pair flanking the Ho cut site (Figure 2A). The disappearance of the PCR signal would indicate the DSB at the MATα locus. Our PCR analysis revealed that PCR signal was significantly reduced upon switching the growth media from raffinose to galactose for 30 and 60 min (Figure 2B). As a control, we amplified a specific region of SMC2 that is not damaged by Ho. We find that like the MATα locus, the PCR signal at SMC2 did not decrease upon switching the growth media from raffinose to galactose for 30 and 60 min (Figure 2B). These results support that the expression of HO in galactose-containing growth medium induced DSB at the MATα locus.

To further support the induction of DSB at the MATα locus, we analyzed the recruitment of the INO80 complex at the Ho cut site, since previous studies (53, 54) have demonstrated the recruitment of INO80 to DSB. INO80 is an ATP-dependent chromatin remodeling complex, and is required to promote DSB repair (53-55). To analyze the recruitment of the INO80 complex at the
MATα-Ho locus, we tagged the Ino80p component of INO80 by myc epitope in the JKM179 strain that has a Ho cut site at the MATα locus and expresses HO under the GAL1 promoter. Subsequently, the ChIP assay was performed following the switch of the growth media from raffinose to galactose for 60 min. Immunoprecipitated chromatin was analyzed by PCR at the site proximal (represented as “ChIP region” in Figure 2A) to DSB. Consistent with previous studies (53, 54), we observed an enhanced recruitment of INO80 to the ChIP region in galactose-containing growth medium (Figure 2C), thus supporting the presence of DSB at the MATα locus.

Next, we analyzed DSB at the highly active ADH1 gene. In this direction, we introduced a Ho cut site followed by a nucleotide sequence for multiple myc epitope tags just before the translational stop codon of ADH1 in the JKM179 strain. To confirm the presence of Ho cut site at the ADH1 locus in the generated strain (PCY23), DSB was analyzed in raffinose, dextrose, or galactose-containing growth media. In the raffinose or dextrose-containing growth media, the GAL1 promoter is not induced (44, 52), and thus, HO would not be expressed. Hence, DSB would not be observed at the ADH1 locus in raffinose or dextrose-containing growth media. The DSB at the ADH1 locus was analyzed by PCR, using the primer pair flanking the Ho cut site. The PCR signal at the ADH1 locus disappeared in galactose-containing growth medium, but not raffinose or dextrose-containing growth media (Figure 2D). As a control, we amplified a specific region at the SMC2 locus, and found that PCR signal at SMC2 did not disappear in galactose-containing growth medium (Figure 2D). These results demonstrate the presence of DSB (and hence Ho cut site) at the ADH1 locus in galactose-containing growth medium.

We next analyzed the induction of DSB at the ADH1 locus following the switch of the growth medium from raffinose to galactose for 30 and 60 min. In this direction, we inoculated the yeast strain in raffinose-containing medium, and grown up to an OD600 of 0.9 at 30°C. Subsequently, yeast cells were transferred to galactose-containing growth medium for 30 and 60 min, and DSB was analyzed at the ADH1 locus by PCR. We find a modest decrease in the PCR signals after 30 and 60 min in galactose-containing growth medium (Figure 2E). The PCR signal at the control SMC2 gene did not decrease after 30 and 60 min in galactose-containing growth medium (Figure 2E). Thus, there is a modest level of DSB at ADH1 following the switch of the growth media from raffinose to galactose for 30 and 60 min (Figure 2E). On the other hand, we observed a dramatic decrease in the PCR signal at the Ho cut site at the MATα locus (Figure 2B). These observations indicate that nearly silent MATα locus has significantly more DNA DSB than the highly active ADH1 gene.

Fast DSB repair at ADH1 in comparison to MATα.

Why is the extent of DSB at ADH1 less than that at the MATα locus? When HO is expressed in galactose-containing growth medium, both the generation and repair of DSB occur simultaneously, as Ho is constantly present in galactose-containing growth medium. Therefore, we do not observe 100% DSB. However, less DSB would be observed if DSB repair occurs faster than its generation in galactose-containing growth medium. Intriguingly, we observed less DSB at the highly active ADH1 gene than the nearly silent MATα locus. Thus, these observations indicate that there might be a fast DSB repair at ADH1 as compared to the MATα locus. To confirm that the DSB at the highly active ADH1 gene is repaired fast in comparison to the nearly silent MATα locus, we analyzed DSB repair at the ADH1 and MATα loci. In this direction, we grew the yeast cells in galactose-containing growth medium up to an OD600 of 0.2 to induce maximal DSB at ADH1 (as done in Figure 2D), and then switched to dextrose-containing growth medium for 0.5, 2 and 3 hrs to allow the cells to repair DSB as schematically shown in Figure 3A. We isolated genomic DNAs from these yeast cultures, and analyzed DSB repair by PCR, using the primer pairs...
flanking the Ho cut sites at the ADH1 and MATα loci. The increase in PCR signal would indicate the progression of DSB repair. Intriguingly, we find that the PCR signal at ADH1 increased more than that at the MATα locus (Figure 3B). These observations support that the DSB at the highly active ADH1 is repaired fast as compared to the nearly silent MATα locus. Therefore, we detected less DSB at ADH1 than the MATα locus (Figures 2B and 2E).

HR-dependent DSB repair is absent at the MATα locus due to the deletion of the donor in the parent JKM179 strain (40). Thus, DSB repair at the MATα locus occurs via NHEJ. An enhanced DSB repair at ADH1 as compared to MATα could be due to the presence of HR at ADH1. To test this possibility, we analyzed DSB repair at ADH1 in the presence and absence of Rad52p that is essential for HR-dependent DSB repair (56). We find that the absence of Rad52p did not significantly alter the DSB repair at ADH1 (Figure 4). Thus, DSB repair at ADH1 does not appear to be promoted via HR, but rather NHEJ. Such DSB repair is impaired in the absence of DNA ligase Dnl4p (Figures 5A and 5B).

How is the DSB repair facilitated at the active gene? We hypothesize that RNA polymerase II promotes the recruitment of the repair factors to the DSB site at the active gene (and hence stimulates DSB repair), analogous to the fact that RNA polymerase II facilitates the recruitment of TC-NER specific factor, Rad26p, to the DNA lesion at the active gene to promote NER (25). In support of this hypothesis, a recent study (57) implicated the interaction of RNA polymerase II with RPA (Replication protein A complex) which is involved in DSB repair. Further, INO80 chromatin remodeling complex that promotes DSB repair is recruited to the gene in a transcription-dependent manner (58, 59). Similarly, the recruitment of the MRX (Mre11p-Rad50p-Xrs2p) complex and Ku proteins to the DSB site might be promoted by RNA polymerase II or transcription machinery to stimulate DSB repair. To test this, we generated yeast strains carrying HA epitope tags at the C-terminals of Rad50p (MRX) and Ku70p (Ku proteins), and then performed the ChIP experiments to analyze their association with the active ADH1 gene. Our ChIP analysis revealed that Rad50p and Ku70p did not associate with ADH1 (Supplementary Figures S1A and S1B). Thus, RNA polymerase II or active transcription machinery does not appear to promote the targeting of MRX or Ku70p to DSB. However, like the association of RPA and INO80 with active gene (57-59), we have recently demonstrated that TC-NER factor, Rad26p, associates with active genes (25, 60) and facilitates chromatin disassembly (60, 61). Such chromatin regulatory function of Rad26p might be enhancing DSB repair at the active gene. Further, DSB repair at the active coding sequence is likely to be promoted by transcription-coupled open chromatin structure or chromatin remodeling/modifying factors. These possibilities remain to be investigated. Nonetheless, this study demonstrates for the first time that DSB repair at the active gene occurs faster than that at the inactive gene, thus supporting transcription-coupled DSB repair.

Fast DSB repair at PHO5 under transcriptionally active condition in comparison to inactive state.

To extend our results to another set of transcriptionally active and inactive genes, we introduced a Ho cut site followed by a nucleotide sequence for multiple myc epitope tags just before the translational stop codon of an inducible PHO5 gene in the aforesaid yeast (JKM179) strain that had a Ho cut site at the MATα locus and expressed HO under the GAL1 promoter. The PHO5 gene is transcriptionally induced in the absence of Pi and repressed in the presence of Pi (62-65). Thus, transcription of PHO5 would be repressed in YPD (that contains Pi) and induced in the YPD-Pi (YPD without Pi) medium. Using this generated strain, we induced DSB at the PHO5 coding sequence in YPG-Pi (Supplementary Figure S2), and then analyzed DSB repair under transcriptionally active (YPD-Pi) and inactive (YPD) conditions as schematically shown in Figure
6A. We find that DSB repair at the PHO5 coding sequence occurs fast under transcriptionally active condition as compared to inactive state (Figures 6B and 6C). However, at the later time point (4 hr.), DSB repairs at the active and inactive PHO5 were the same (Figure 6B). Therefore, our data support a fast DSB repair at the transcriptionally active PHO5 gene as compared to inactive PHO5, hence implying transcription-coupled DSB repair.

Conclusion:

Previous studies (17-24, 35-37) have demonstrated transcription-coupled nucleotide/base excision repairs. However, it remained unknown whether transcription-coupled DSB repair exists. Here, we have developed experimental systems to analyze the induction and repair of DSBs at the active and inactive genes. Using such systems, we demonstrate that DSB repair at the active gene occurs faster than that at the inactive genomic locus. Therefore, our results support for the first time the existence of transcription-coupled DSB repair, hence providing a new regulatory process of genome repair in vivo.

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Figure Legends:

Figure 1: MATα is a poorly active gene in comparison to a highly active ADH1 gene. (A) RT-PCR analysis of ADH1 and MATα mRNAs. Yeast cells were grown in YPR as well as YPG. Total RNA was prepared from harvested culture and analyzed for ADH1 and MATα mRNAs. The level of ADH1 mRNA was set to 100, and MATα mRNA was normalized with respect to 100. Normalized mRNA levels are plotted in the form of a histogram. (B) Analysis of RNA polymerase II association with the ADH1 and MATα loci. Yeast cells were grown in YPR as well as YPG, and crosslinked by formaldehyde. Immunoprecipitation was performed as described previously (41-44), using a mouse monoclonal antibody 8WG16 (Covance) against the carboxy terminal domain of the largest subunit (Rpb1p) of RNA polymerase II. Immunoprecipitated DNAs were analyzed by PCR using the primer pairs targeted to ADH1 and MATα. The ratio of the PCR signal of immunoprecipitated DNA to that of input DNA was determined, and referred to as the ChIP signal. The ChIP signal at ADH1 was set to 100, and the ChIP signals at MATα and Chr-V were normalized with respect to 100. The normalized ChIP signals (represented as normalized occupancy) are plotted in the form of a histogram.

Figure 2: Analysis of DSB at the MATα and ADH1 loci. (A) Schematic diagram showing the generation of DSB at the MATα locus following the induction of HO expression. The primers flanking the Ho cut site are marked by two arrow-headed lines, and were used in PCR for analysis of DSB. (B) PCR analysis of DSB at the MATα locus. Yeast strain that contains a Ho cut site at the MATα locus and expresses HO under the GAL1 promoter was initially grown in YPR up to an OD600 of 0.9 (log phase), and then switched to YPG for 30 and 60 min (an OD600 of 1.0 is approximately 3 X 107 yeast cells/ml; 46). Genomic DNA was analyzed by PCR to determine DSB, using the primer pair flanking the DSB site. The PCR signal at 0 min was set to 100. The PCR signals at 30 or 60 min in YPG were normalized with respect to 0 min time point in YPG.
(C) The ChIP assay for analyzing the recruitment of Ino80p to the DSB site. The yeast strain (ASY32) was grown as in panel B prior to crosslinking. Immunoprecipitation was performed using an anti-myc antibody (9E10, Santa Cruz Biotechnology, Inc.) against myc-tagged Ino80p. An anti-HA was used as a non-specific antibody control. The ratio of immunoprecipitate over the input in the autoradiogram is indicated below the immunoprecipitated band. (D) Analysis of DSB at ADH1 in YPR, YPG and YPD media. The yeast strain, PCY23, was grown in YPR, YPG, or YPD up to an OD600 of 0.2 at 30 °C, and harvested. Genomic DNA was analyzed for DSB by PCR, using the primer pair flanking the HO cut site at ADH1. (E) Induction of DSB at the ADH1 locus. The yeast strain PCY23 was grown as in panel B.

**Figure 3:** Analysis of DSB repair at the ADH1 and MATα loci. (A) Schematic diagram for the experimental strategy. (B) The yeast strain, PCY23, was initially grown in YPG up to an OD600 of 0.2, and then switched to YPD for 0.5, 2 and 3 hrs. Genomic DNAs from these yeast cultures were analyzed by PCR for DSBs at the ADH1 and MATα loci. The PCR signals at 0 hr time point for both the ADH1 and MATα loci were set to 100, and the signals at other time points were normalized with respect to 100. Normalized PCR signals are plotted in the form of a histogram.

**Figure 4:** Analysis of DSB repair at the ADH1 and MATα loci in the presence and absence of Rad52p. Both the wild type (PCY23) and Δrad52 (PCY36) strains were grown as in Figure 3B. Genomic DNAs from these yeast cultures were prepared, and analyzed by PCR for DSBs at the ADH1 and MATα loci. A specific region within SMC2 was amplified as control, using the same genomic DNAs. The PCR signals at 0 hr time point for the ADH1, MATα and SMC2 loci were set to 100, and the PCR signals at 3 hr were normalized with respect to 100. The ratios of normalized PCR signals at 3 hr at the ADH1 and MATα Ho cut sites to that at SMC2 are plotted in the form of a histogram. A ratio that is greater than 1 would indicate the DSB repair.

**Figure 5:** Analysis of DSB repair at ADH1 in the presence and absence of Dnl4p. Both the wild type (PCY23) and Δdnl4 (PCY37a) strains were grown as in Figure 3B. The DSB repairs at the ADH1 (A) and MATα (B) loci were analyzed as in Figure 4.

**Figure 6:** Analysis of DSB repair at the PHO5 gene under transcriptionally active and inactive conditions. (A) Schematic diagram for the experimental strategy. “Inactive” and “active” refer to DSB repairs under transcriptionally active and inactive conditions, respectively. (B and C) The yeast strain, RSY33, was initially grown in YPG-Pi up to an OD600 of 0.4, and then switched to YPD (denoted as +Pi) or YPD-Pi (denoted as –Pi) for 1, 2, 3 and 4 hrs. Genomic DNAs from these yeast cultures were prepared, and analyzed by PCR for DSBs at the PHO5 locus using the primer pair flanking the Ho cut site. A specific region within SMC2 was amplified as control, using the same genomic DNAs. The PCR signals at 0 hr time point for the PHO5 and SMC2 loci were set to 100, and the PCR signals at 1, 2, 3 and 4 hrs were normalized with respect to 100. The ratios of normalized PCR signals at 1, 2, 3 and 4 hrs at the PHO5 Ho cut site to those at SMC2 are plotted in the form of a histogram. A ratio that is greater than 1 would indicate the DSB repair.
References:


Figure 1:

(A) Normalized mRNA level

-20
0
20
40
60
80
100

Normalized mRNA level

ADH1
MATα
Raffinose

ADH1
MATα
Galactose

(B) Normalized association of Rpb1p

0
20
40
60
80
100

Normalized association of Rpb1p

Raffinose
Galactose

ADH1
MATα
Chr-V
Figure 2:

(A) 

(B) 

Galactose 

0 30 60 min

MATα-Ho

100% 23% 30%

SMC2

100% 162% 161%

(C) 

IP

Input Ino80p-Mye HA

Galactose

0 min

0.41 0.06

60 min

1.46 0.10

(D) 

(E) 

Galactose

0 30 60 min

ADH1-Ho

100% 78% 72%

SMC2

100% 162% 161%
Figure 3:

(A) Yeast strain PCY23 (ADH1-Ho & MATα-Ho) → YPG → Induction of DSB at ADH1 and MATα → YPD → DSB repair at ADH1 and MATα

(B) 

<table>
<thead>
<tr>
<th>Galactose → Dextrose</th>
<th>Normalized PCR signal</th>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ADH1-Ho</td>
<td></td>
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<td>MATα-Ho</td>
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<td>MATα-Ho</td>
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Dextrose → 0 hr

0.5 hr

2 hr

3 hr
Figure 4:

![Graph showing the ratio of normalized PCR signal at Ho cut site to that at SmC2](image-url)
Figure 5:

(A) Ratio of normalized PCR signal at ADH1 Ho cut site to that at SMC2

(B) Ratio of normalized PCR signal at MATα Ho cut site to that at SMC2
Figure 6:

(A) Yeast strain RSY33 (PHO5-Ho) → YPG-Pi → Induction of DSB at PHO5 → DSB repair at PHO5 (inactive) → YPD → DSB repair at PHO5 (active)

(B) Bar graph showing the ratio of normalized PCR signal at PHO5 Ho cut site to that at SMC2 under different conditions:
- Galactose → Dextrose
- Dextrose → 0 hr, 3 hr, 4 hr

(C) Bar graph showing the ratio of normalized PCR signal at PHO5 Ho cut site to that at SMC2 under different conditions:
- Galactose → Dextrose
- Dextrose → 0 hr, 1 hr, 2 hr
Supplementary Information:

Supplementary Figures:

Supplementary Figure S1: Analysis of association of Rad50p (A) and Ku70p (B) with the active ADH1 gene. Yeast strains expressing HA epitope-tagged Rad50p and Ku70p (RSY30 and RSY31, respectively) were grown in YPD up to an OD_{600} of 1.0 prior to crosslinking, and then modified ChIP assay was performed as described in experimental procedures. Immunoprecipitation was performed using an anti-HA antibody against HA-tagged Rad50p and Ku70p. An anti-Myc was used as a non-specific antibody control. Immunoprecipitated DNAs were analyzed by PCR using primer pairs targeted to the UAS, core promoter, and ORF of ADH1. The primer pair targeted to an inactive region of chromosome-V (Chr.-V) was used as a non-specific DNA control. The ratio of immunoprecipitate over the input in the autoradiogram is indicated below the immunoprecipitated band.

Supplementary Figure S2: PCR analysis of DSB at PHO5. The yeast strain (RSY33) was grown in dextrose or galactose-containing growth medium in the absence of Pi up to an OD_{600} of 0.5 prior to harvesting. Genomic DNA was analyzed by PCR for DSB at PHO5, using the primer pair flanking the Ho cut site.
Supplementary Figure S1:

(A)

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<td>ADH1 ORF</td>
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<td>Chr.-V</td>
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(B)

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<td>Chr.-V</td>
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Supplementary Figure S2:
Preferential repair of DNA double-strand break at the active gene in vivo.
Priyasri Chaurasia, Rwik Sen, Tej K. Pandita and Sukesh R. Bhaumik

J. Biol. Chem. published online August 21, 2012

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