Published in final edited form as:

Methods. 2009 May ; 48(1): 19–22. doi:10.1016/j.ymeth.2009.03.017.

Assays for chromatin remodeling during nucleotide excision repair in *Saccharomyces cerevisiae*

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Abstract

How DNA repair proteins interact with the dynamic structure of chromatin is an emerging question. Chromatin structure impedes the access of repair proteins to sites of DNA damage. Several recent studies have implicated chromatin remodeling complexes in DNA repair. In this report we summarize the methods we used to investigate chromatin remodeling during nucleotide excision repair (NER) *in vivo*. We describe a procedure to analyze UV-induced chromatin remodeling at the silent mating-type locus *HML* using isolated nuclei from UV treated yeast cells. In addition, a method to capture transient protein-protein associations in chromatin is outlined. We have used the methods described here to demonstrate that the SWI/SNF chromatin remodeling complex is involved in chromatin rearrangement during NER.

Keywords

Chromatin; Nucleotide excision repair; Methods; Yeast; SWI/SNF

Introduction

It has been known for three decades that nucleosome structure is disrupted during nucleotide excision repair (1). Recent work has suggested that nucleosomes are also disrupted during repair of DNA double strand breaks (2,3). Despite these progresses, the mechanisms of chromatin dynamics during DNA repair in eukaryotes are not well understood (4–6). Latest studies have demonstrated that the ATP-dependent chromatin remodeling complexes are involved in base excision repair (7,8), nucleotide excision repair (NER) (9,10) and the repair of DNA double strand breaks (2,11–13). NER has been an active area of research to study how repair proteins function in the context of chromatin. NER is a key mechanism repairing a variety of bulky DNA lesions, including UV damage and chemical adducts (14,15). Inactivation of NER causes a disease known as xeroderma pigmentosum (XP), an autosomal recessive disease that strongly predisposes affected individuals to skin cancer (16).

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Chromatin structure impedes the access of repair proteins to sites of DNA damage (17). Soon after chromatin remodeling complexes were identified they were hypothesized to be involved in chromatin rearrangement during NER (1,17). Indeed, the SWI/SNF chromatin remodeling complex stimulates NER of 6-4photoproducts in reconstituted positioned mononucleosomes *in vitro* (10). Physiological relevance of these *in vitro* observations has been suggested by our finding that the NER damage recognition heterodimer Rad4-Rad23 associates with SWI/SNF in yeast cells (9). These findings shed light on the molecular mechanisms of nucleosome rearrangement during NER.

Here we describe the method we used to examine UV-induced chromatin remodeling and the involvement of SWI/SNF in this process *in vivo*. Our method addresses two major events necessary to justify further investigation of the direct role of SWI/SNF in NER: 1) To look directly at chromatin remodeling following UV irradiation; 2) To investigate a potential interaction of the SWI/SNF complex with the NER damage recognition proteins. This procedure involves isolation of nuclei from UV irradiated yeast cells followed by analysis of nucleosome remodeling using a restriction enzyme accessibility assay. We also detail a protocol to examine SWI/SNF associations with NER recognition proteins in chromatin that combines chemical crosslinking with His-tag pull-down assay to study *in vivo* protein associations/interactions.

1. Materials

Dark room equipped with gold fluorescent light (Philips F40T12/GOLD) to prevent repair of UV lesions by photoreactivation. Ultraviolet Crosslinker (UVP, with peak emission at 254 nm). Yeast strains and expression constructs: Yeast BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), *snf6* (BY4741; *snf6::kanMX4*) and cells expressing TAP-tagged Rad4 (Open Biosystems). DNA fragments encoding C-terminal V5-His6-tagged Snf6 (Snf6 is a SWI/SNF subunit) and LacZ (used as control) fusion proteins were cloned into the *CEN URA3* plasmid (pYC2/CT, Invitrogen). Expression of fusion proteins is induced by galactose. Plasmids were introduced into yeast cells and expression of tagged-proteins was confirmed by Western blot using anti-V5 antibody (Invitrogen).

2. UV irradiation and isolation of yeast Nuclei

In budding yeast, UV-induced pyrimidine dimers can be repaired by either NER or photoreactivation catalyzed by photolyase in the presence of light ranging from 350 nm to 450 nm. To study chromatin remodeling associated with NER, it is important to prevent repair of UV damage by photoreactivation. All steps described below are conducted in a darkroom with gold fluorescent light.

Procedure

1. Yeast strains (BY4741 and *snf6* in our study) are grown overnight then diluted into fresh 200 ml YPD to a starting A_{600} of ~0.1. Inoculated cultures are grown at 30 °C with shaking to an A_{600} of 1.0.
2. Centrifuge cells at $4000 \times g$ for 5 min at 4 °C, then wash once with 40 ml ice-cold PBS, and spin as before. Suspend yeast cells in 200 ml cold $1 \times$ PBS.
3. Aliquot cells into a container such that the layer of cells is ~2 mm thick. We used 50 ml aliquots into plastic trays (23 cm $\times$ 15 cm; Rubbermaid).
4. Irradiate cells with 100 J/m^2 of UV using a UV crosslinker with predominantly 254 nm UV light. Prior to irradiation remove an aliquot of cells as the untreated control.

5. Pour irradiated yeast cells into sterile flasks and add an equal volume of warm $2 \times$ YPD. Incubate cells at 30°C with shaking. Collect repair time course samples after 0, 1, 2, 3 and 4 hrs and store samples on ice.
6. Centrifuge cells at $4000 \times g$ for 5 min at 4°C to collect cells. For convenience, pellets can be frozen at -80°C at this step.
7. Suspend each pellet in 1 ml of sorbitol buffer (1.4 M Sorbitol, 20 mM Tris-HCL, pH 6.8, 5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)).
8. Centrifuge cells $4000 \times g$ for 5 min at 4°C and discard supernatant.
9. Suspend each pellet in 1 ml of sorbitol buffer and add Zymolyase 100T (MP Biomedicals) to a final enzyme concentration of 0.5 mg/ml. Incubate at 30°C with gentle shaking for 20 minutes.
10. Place $2\mu\text{l}$ of cell suspension on a glass slide and mix with one drop of 1% SDS, placing a cover slip over the sample and examining it under a light microscope. Spheroblasts should pop open in 1% SDS while yeast cells stay intact. A good zymolyase treatment should convert ~80% of the cells into spheroblasts.
11. Centrifuge the spheroplasted cells at $4000 g$ for 5 min.
12. Wash spheroplast pellet in 1.4 M sorbitol with 1 mM PMSF twice.
13. Suspend each pellet in 1 ml Ficoll buffer (18% Ficoll 400, 20 mM Tris-HCl, pH 6.8, 1 mM MgCl_2 , 5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride).
14. Lyse spheroblasts with 10 strokes in a 1-ml type A Dounce homogenizer to release nuclei. Nuclei will be stabilized by low concentration of Ficoll.
15. Spin samples at $3000 \times g$ for 5 min to pellet and remove cell debris and intact cells (nuclei will stay in the supernatant).
16. Transfer supernatant into a new tube and pellet nuclei at $20,000 \times g$ for 30 min at 4°C .
17. Carefully discard supernatant. Nuclei are ready for enzyme accessibility assay. Nuclei can be stored at -80°C .

3. Restriction Enzyme accessibility assay to examine UV-induced chromatin remodeling

Nucleosome is an effective barrier to restriction enzymes and prevents the digestion of enzyme recognition sites within the nucleosomal DNA. A restriction enzyme analysis provides a relatively easy and reliable method of quantifying the nucleosomal DNA accessibility. This method has been widely used to study nucleosome dynamics in the promoter region during transcription activation (18,19). We adapted this method to monitor UV-induced chromatin rearrangement at the silent *HML* locus by measuring the accessibility of *EcoRV* to its recognition site at the *HML* locus. An *EcoRV* recognition site is located in the silent *HML* locus within a positioned nucleosome as illustrated by the nucleotide resolution of the nucleosome position (Fig. 1). The strategy used is outlined in Fig. 1.

Procedure

1. Nuclei from approximately 50 ml starting yeast culture with an A_{600} of 1.0 are used for each restriction enzyme digestion. Suspend nuclei in $200\mu\text{l}$ $1 \times$ *EcoRV* digestion buffer (50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM MgCl_2 , 1 mM Dithiothreitol)

by pipeting. Centrifuge at 13,000 g for 10 min to wash the nuclei and resuspend the pellet in 200 μ l 1 $\times$ *EcoRV* buffer.

2. Add 20 Units of *EcoRV* (1 μ l of 20 U/ μ l stock, NEB) to each tube with nuclei isolated from UV- and mock-treated yeast cells. Incubate at 37 °C for 15–30 min.
3. Stop *EcoRV* digestion by adding 50 μ l solution (5% SDS and 5 mg/ml proteinase K). Vortex to mix and incubate at 37 °C for 2 hr.
4. Extract with phenol/chloroform. Collect the aqueous phase (~250 μ l) and precipitate DNA by adding 25 μ l of 3 M sodium acetate and 600 μ l of ethanol. Resuspend the precipitated DNA in 100 μ l H₂O.
5. Digest the purified DNA by *HpaI* to release the *HML* locus (Fig. 1) and separate digested DNA on a 1% agarose gel followed by Southern blot analysis. The probe design is such that only α 2 gene and the 5'-end of α 1 gene can hybridize (Fig. 1).
6. Quantify accessibility by measuring the cut and uncut fragments hybridized to the radio-labeled probe using a phosphorImager for quantitative autoradiography.

Application of the method—We have used this method to study UV-induced chromatin remodeling (9,20) (Fig. 1). Without UV treatment, only a small fraction of the *EcoRV* sites was accessible in isolated nuclei (Fig. 1, B, lane 1). Limited cutting was also observed immediately after UV irradiation, with no repair incubation (lane 2). In contrast, one hour after UV treatment, ~50% of the *EcoRV* sites were cut in BY4741 cells, compared to less than 10% in *snf6* cells after the same period of repair incubation (lane 3 vs lane 8). In both strains, the accessibility of the recognition site to *EcoRV* decreased with time after reaching a maximum by 1–2 hours after UV irradiation (Fig. 1), suggesting transient remodeling to provide access for repair proteins. We observed attenuation of UV-induced chromatin remodeling at the *HML* locus in *snf6* cells, suggesting that SWI/SNF contributes to UV-induced chromatin remodeling during NER.

4. Chemical crosslinking to capture transient SWI/SNF associations with DNA damage recognition protein

Extensive networks of protein-protein interactions have been demonstrated in the cell. Transient protein interactions are defined by their temporal interaction with other proteins. Due to temporally limited interactions, transiently interacting proteins are more difficult to identify using physical methods like pull-down or co-precipitation assay, an invaluable tool to study protein-protein interactions. Fortunately, chemical cross-linking provides the means for capturing these transient interactions. We have adapted an approach similar to the widely used chromatin immunoprecipitation method to study NER in chromatin. This method was first described in Nature protocol NETWORK (http://www.natureprotocols.com/2006/10/02/comboination_of_chemical_crossl.php).

Procedure

1. 200 ml yeast cells expressing V5-His6 tagged protein are grown in YPG (yeast extract/peptone/galactose) to A₆₀₀ ~0.6–1.0. After UV (100 J/m²) or mock treatment, cells are incubated at 30 °C in the darkroom (to prevent photoreactivation repair of UV lesions by photolyase (21) and time course samples are collected.
2. Add formaldehyde (37% stock solution) directly to the collected yeast culture to 1% (final concentration). After incubation for 20 min at room temperature, cross-linking is quenched by addition of 0.125 M glycine (final concentration).

3. Centrifuge at 4000g for 5 min to harvest cells, wash with cold Tris-buffered saline followed by cold lysis buffer (50 mM HEPES-KOH, pH 7.5, 10 mM MgCl₂, 200 mM KCl, 10 mM EDTA, 10% glycerol and 1% Triton X-100). Resuspend yeast cells (~0.5 ml wet pellet) in 1 ml of cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche).
4. Disrupt cells by vortexing in the presence of 0.5 ml glass beads (vortexing for 1 min at high speed, repeat 6 times with 1 min intervals on ice). Centrifuge at 4000g for 5 min and collect the lysates. Sonicate the lysates to break chromatin (the average size of DNA fragments should be less than 1 kb). Centrifuge for 20 min at 13,000 g to clear the extracts.
5. Mix 0.1 ml Ni-NTA-Agarose beads (Qiagen) with 0.5 ml cell-free extracts (~20 mg/ml protein) and incubate overnight at 4 °C on a rotation platform.
6. Spin down the agarose beads (1000g for 2 min) and wash three times with 1.5 ml lysis buffer, three times with 1.5 ml of the same buffer with 20 mM imidazole and twice with 1.5 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
7. Spin down the beads and add 100 µl 2 × SDS-PAGE sample buffer. Boil for 20 min to reverse formaldehyde cross-links. Separate proteins precipitated with the agarose beads using SDS-PAGE. Samples were analyzed by Western blots using antibodies specific for DNA repair proteins Rad4 and Rad23 (22). The PVDF membrane was stripped and reprobed with V5 antibody (Invitrogen) and the detected Snf6-V5His6 and/or LacZ-V5His6 were used as loading controls.
8. Protein associations in chromatin can be confirmed by a reciprocal experiment. For example, to verify the UV-stimulated SWI/SNF associations with Rad4, yeast cells expressing tandem affinity purification (TAP)-tagged Rad4 from its native promoter can be used to co-purify Rad4-TAP and SWI/SNF. Putative associations of SWI/SNF subunits with Rad4-TAP can then be examined. Yeast culture, crosslinking and extracts preparation are performed as described in step 1 to step 4. To purify TAP-tagged Rad4, Human IgG-Agarose beads (0.1 ml, sigma) are incubated with 0.5 ml cell-free extracts overnight at 4 °C. The beads are transferred to a 2 ml tube and washed three times with 1.5 ml lysis buffer and twice with 1.5 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Spin down the beads and add 100 µl 2 × SDS-PAGE sample buffer. Boil for 20 min to reverse formaldehyde cross-links. Separate proteins precipitated with the IgG-agarose beads using SDS-PAGE. Samples are analyzed by Western blots using antibodies specific for SWI/SNF subunits, e.g. Snf5 (9).

Application of the method—The Rad4-Rad23 heterodimer plays a crucial role in NER damage binding and recognition in yeast (22). To probe the mechanism by which SWI/SNF stimulates NER, we used this method to examine the possible association of SWI/SNF with the Rad4-Rad23 heterodimer in yeast cells.

We used yeast cells expressing a V5His6-tagged version of Snf6 for easy protein pulldown. Proteins associated with Snf6-V5His6 were cross-linked and captured using nickel–nitrilotriacetic acid (Ni-NTA) agarose beads and identified by Western blotting. We found that both Rad4 and Rad23 copurify with Snf6-V5His6 and Snf5 (Fig. 2), suggesting a potential physiological mechanism for the *in vitro* reports of SWI/SNF stimulation of UV damage repair on nucleosomal templates (10).

Conclusion

Rearrangement of nucleosome structure following excision repair of UV-induced photoproducts in human chromatin was observed three decades ago. These findings led to the “unfolding–refolding” model to explain the transitions in chromatin structure associated with repair. Understanding chromatin rearrangement during NER has been fueled in recent years after the impressive discoveries of ATP-dependent chromatin remodeling complexes and histone modifying enzymes. It is now apparent that chromatin modifications/remodeling is an integrated part of DNA damage repair and signaling (23–26). However, mechanistic studies to understand how DNA repair occurs in the context of chromatin are still lacking and clearly needed. Thus there is a need to develop tools to monitor changes in chromatin structure during DNA repair *in vivo*. The techniques describe here provide researchers tools to study the potential interactions of DNA repair proteins with chromatin modifying factors and examine the role of various histone modifications in regulating chromatin structure during repair.

While our techniques described here will be very useful in addressing the role of chromatin modifications in DNA repair, care must be taken when evaluating the data obtained from these types of experiments because pull-down assays do not show direct protein-protein interactions, but rather a co-localization of two complexes. Therefore it is necessary to couple the *in vivo* methodologies that show physiological relevance with *in vitro* methodologies that address the mechanism of the system. Another potential caveat of the herein described methods is the cross-linking. Care must be taken to ensure the observed co-precipitation of the complexes of interest is not an artifact due to the cross-linking. This can be addressed by conducting the pull-down assay using less stringent conditions under native conditions. Alternatively, consistent results from the *in vitro* assays also address this issue.

The methodologies described here have been useful in starting to unravel the mechanism of a three decade old observation: chromatin remodeling following UV damage (1). The continued use and improvements of these methods will further this area of investigation. More importantly, simple adaptations of the techniques described here will prove useful in a wide range of protein-protein interactions and protein-chromatin interactions studies and coupled with *in vitro* assays will produce a plethora of information regarding the mechanisms of chromatin dynamics under various conditions.

Acknowledgments

This work was supported, in part, by funds from the CONCERN Foundation, the American Cancer Society and the Sylvester Comprehensive Cancer Center (to F.G.), and NIH grants ES04106 and ES002614 (to M.J.S.).

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Figure 1.

A. Schematic diagram of the nucleosome-loaded *HML* locus. Ovals represent nucleosomes whose positions have been mapped at nucleotide resolution (27). **B.** Accessibility of the *EcoRV* site at the *HML* locus in chromatin of isolated nuclei after UV irradiation. These figures were first published in Gong, F., Fahy, D., and Smerdon, M. J. (2006) *Nat Struct Mol Biol* **13**, 902–7.

Figure 2.

A. UV stimulates associations between Rad23 and SWI/SNF. Western blots showing association of Snf5 and Snf6-V5His6 with Rad23. Captured proteins associated with V5His-tagged Snf6 were probed with Rad23 and V5 antibodies. Cell extracts before His-tag pulldown were used as input. **B.** Western blot showing a time course of Rad4 association with Snf6-V5His6. Cells were cross-linked immediately after UV irradiation (lane 2) or after 1 h (lane 3) or 2 h (lane 4) of repair incubation (triangle denotes increasing repair time). His-tag pulldown materials were probed for the presence of Rad4. Corresponding Protein extracts (input) were also probed as controls. The UVC dose used was 100 J/m². These figures were first published in Gong, F., Fahy, D., and Smerdon, M. J. (2006) *Nat Struct Mol Biol* **13**, 902–7.