Chapter 4

Pairing of heterochromatin in response to cellular stress

Research Article

Pairing of heterochromatin in response to cellular stress

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ABSTRACT

We previously reported that exposure of human cells to DNA-damaging agents (X-rays and mitomycin C (MMC)) induces pairing of the homologous paracentromeric heterochromatin of chromosome 9 (9q12–13). Here, we show that UV irradiation and also heat shock treatment of human cells lead to similar effects. Since the various agents induce very different types and frequencies of damage to cellular constituents, the data suggest a general stress response as the underlying mechanism. Moreover, local UV irradiation experiments revealed that pairing of heterochromatin is an event that can be triggered without induction of DNA damage in the heterochromatic sequences. The repair deficient xeroderma pigmentosum cells (group F) previously shown to fail pairing after MMC displayed elevated pairing after heat shock treatment but not after UV exposure. Taken together, the present results indicate that pairing of heterochromatin following exposure to DNA-damaging agents is initiated by a general stress response and that the sensing of stress or the maintenance of the paired status of the heterochromatin might be dependent on DNA repair.

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Introduction

In interphase, chromosomes are folded into subnuclear regions called chromosome territories (reviewed in [1]). The different chromosome territories display a non-random radial organization [2–5] that is mainly directed by the gene density and the size of chromosomes. Although the large variation of the inter-homologue distances suggests a large degree of freedom in the spatial arrangement of homologous chromosome territories in mammalian interphase nuclei [6–8], pairing of centromeres of homologous chromosomes has been observed in brain cells [9,10] and in normal and neoplastic cells of hemopoietic and lymphoid origin [11]. Moreover, there is growing evidence for alterations in the positioning of homologous chromosomes as a result of exposure to DNA-damaging agents. Ionizing radiation [7,12,13], the cross-linking agent MMC [8] and H2O2 in combination with L-histidine [14] reduce the average distances between homologues of some chromosomes and lead to pairing of homologous chromosomal regions. The positional changes after infliction of DNA damage have been suggested to be prerequisites or consequences of recombinational repair of DNA damage [7,8,12,15].

Strikingly, almost similar frequencies of pairing were observed for the paracentromeric heterochromatic regions (9q12–13) of chromosome 9 homologues in human fibroblasts exposed to ionizing radiation and MMC [7,8], although these two agents induce different types and frequencies of DNA lesions [16,17]. In the present study, we addressed the question whether heterochromatin pairing is the result of...
general response to cellular stress rather than the consequence of DNA damage. We found that both UV radiation and the non-genotoxic heat shock treatment are able to induce pairing of the heterochromatic regions (9q12–13) in a subset of confluent human fibroblasts. In contrast, pairing of the euchromatic regions (8p11.2) was virtually absent. Interestingly, pairing of 9q12–13 was not only induced in UV-exposed cells but also occurred in neighboring unexposed nuclei indicative for a bystander effect. Moreover, XPF cells responded differently to different types of cellular stress. Based on these results, we propose that pairing of heterochromatin is a genetically controlled response to cellular stress that can be initiated by genotoxic and non-genotoxic stressors.

Materials and methods

Cell culture

Primary human skin fibroblasts derived from a healthy individual (VH25) and xeroderma pigmentosum (XP) patients (XP25R0, complementation group A; XP7NE, group F) were grown in Ham’s F10 medium, supplemented with 15% FBS and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). The cells were grown to confluency on glass slides in a humidified 2.5% CO2 atmosphere at 37°C before UV exposure or heat shock treatment.

UV irradiation

Prior to irradiation, cells were rinsed in PBS and exposed to UV light using a Philips TUV lamp (predominantly 254 nm) at a dose rate of ∼0.41 J/m2/s. Subsequently, the cells were incubated in the preserved medium for the described time intervals or fixed immediately. For local UV exposure, the cells were transferred to the dishes containing the irradiated cells and were incubated for different time intervals before fixation. The dishes containing the slides with confluent cells were submerged in a water bath at 45°C for different time intervals. The cells were fixed immediately at the end of the heating period. For studying the effect of recovery after heat exposure, cells were treated with heat for 1 h then incubated at 37°C for different time periods before fixation.

Heat shock treatment

The dishes containing the slides with confluent cells were washed twice with ice-cold phosphate-buffered saline (PBS) prior to a 5-min in situ fixation step with ice-cold PBS containing 2% paraformaldehyde. To permeabilize the nuclear membrane, the cells were treated with methanol (−20°C) for 10 min after which the slides were air-dried and aged for 1–3 days prior to fluorescence in situ hybridization (FISH).

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) was performed using human DNA probes specific for the bands 8p11.2 (paracentromeric euchromatic band of chromosome 8) and 9q12–13 (paracentromeric heterochromatic region of chromosome 9) as previously described [7]. Briefly, the probes were labeled with FITC-12-dUTP or biotin-16-dUTP by PCR using a protocol supplied by the manufacturers (Research Genetics). The probes were mixed and precipitated together with human Cot1 DNA (Roche) and dissolved in hybridization mixture (50% denatured formamide, 2× SSC, 50 mM phosphate buffer (pH 7.0) and 10% dextran sulfate). Probe denaturation was performed by incubation for 7 min at 80°C followed by competition for 1 h at 37°C. Prior to in situ hybridization, slides were treated with RNAase, pepsin, MgCl2 and formaldehyde as previously described [20] followed by denaturation in 60% denatured formamide, 2× SSC and 50 mM phosphate buffer (pH 7.0) for 2.5 min at 80°C. Finally, the probes were added to the slides and hybridized overnight at 37°C. Post-hybridization, slides were washed with 50% formamide in 2× SSC (pH 7.0) at 42°C and treated with 10% blocking protein (Cambio). For immunofluorescent detection of FITC-labeled probes, rabbit anti-FITC and fluorescein-conjugated goat anti-rabbit IgG were used (Cambio), whereas Texas-Red avidin and biotinylated goat anti-avidin (Cambio) were used to detect biotin-labeled probes. To detect the UV damage in locally irradiated cells, a mouse IgG monoclonal antibody against 6-4PP and a secondary goat anti-mouse antibody conjugated goat anti-rabbit IgG were used (Cambio), whereas Texas-Red avidin and biotinylated goat anti-avidin (Cambio) were used to detect biotin-labeled probes. To detect the UV damage in locally irradiated cells, a mouse IgG monoclonal antibody against 6-4PP and a secondary goat anti-mouse antibody conjugated with Alexa Fluor 488 were used. Subsequently, the slides were counter-stained with 10 ng/ml DAPI/PBS solution for 10 min and embedded with Citifluor mounting medium (Agar Scientific).

Microscopy and scoring criteria

Fluorescence microscopy was performed with a Zeiss Axioplan microscope equipped with filters for observation of DAPI, FITC and TRITC. Interphase nuclei hybridized with band specific probes were analyzed manually to assess the colocalization of homologous chromosome regions. For each chromosome pair, a distinction was made between cells which displayed entirely separated hybridization signals and cells in which signals were so close together that only a single (usually larger) hybridization signal or two touching signals were observed (both are called paired or colocalized signals) (Fig. 1A). Representative images were captured using a computerized imaging system consisting of an Nu200 CCD camera, an Apple Macintosh PowerPC and IPLab software (Scanalytics). In control cells, the difference in the percentage of cells containing colocalized signals when stained for chromosome 8 (ranging from 2.6 to 3.6%) and 9 (ranging from 6.8 to 10.2%) relates to the size of the hybridization signals [7,8]. Colocalization of chromosome 9 or 8 homologues in control cells...
covered with the filter (mock control) was not different from the colocalization in uncovered control cells. In all experiments, the induced colocalization was calculated by subtracting the percentage of colocalization in controls from the corresponding value of colocalization in UV-irradiated or heat-treated samples. For each data point, 500 cells were analyzed and Student’s t test was used to check for the statistical significance of difference between groups. When a significant difference was mentioned, the $P$ value was at least <0.01.

Results

Positioning of homologous chromosomes in interphase cells exposed to UV irradiation

To assess the influence of UV irradiation on the positioning of homologous chromosomes in human cells, a combined FISH protocol was used to detect both UV damage and the heterochromatin of chromosome 9q12–13 as shown in Fig.
immediately after UV exposure (Fig. 2). However, post-UV deficient in NER) did not display significant increase of mage. In contrast to normal human cells, XPA cells (completely known to be deficient in the repair of UV-induced DNA da-

pholessions, we investigated colocalization in XP fibroblasts, some 9 homologues is related to the repair of UV-induced DNA da-

photollesions. Moreover, exposure of cells to 30 J/m² UV light resulted in an instant increase in colocalization of chromosome 9 homologues (Fig. 1C) that persisted over a period of up to 24-

h post-UV incubation. Similarly as in the previous reports [7,8], measurement of the nuclear size by image analysis did not reveal significant changes in the size of the nuclei in UV-irradiated cells (data not shown). Therefore, the increased colocalization of the heterochromatin of chromosome 9 sug-

gests a UV-induced positional change of at least one of the homologues. In contrast to chromosome 9, UV irradiation did not affect the percentage of colocalization for chromosome 8 (Figs. 1B, C).

To assess whether the increased colocalization of chromosome 9 homologues is related to the repair of UV-induced photolessions, we investigated colocalization in XP fibroblasts, known to be deficient in the repair of UV-induced DNA da-

mage. In contrast to normal human cells, XPA cells (completely deficient in NER) did not display significant increase of colocalization of chromosome 9 when the cells were fixed immediately after UV exposure (Fig. 2). However, post-UV incubation of cells resulted in increased numbers of XPA cells exhibiting colocalized signals, reaching a maximum of colo-

calization (5%) after 4 h of recovery. In contrast, exposure of confluent XPF cells to a dose of 30 J/m² did not result in a significant change in the percentage of cells with colocalized signals for chromosome 9 at any examined time point (Fig. 2). The frequencies of colocalization of chromosome 8 bands in XPA and XPF cells were similar to that of the normal human cells (data not shown).

Colocalization of homologous chromosomes in cells exposed to local UV irradiation

Local UV irradiation has been successfully applied in the past to study various processes including chromatin organization in interphase nuclei [21]. In the present study, local UV exposure was applied to address whether the colocalization of the heterochromatin of chromosome 9 is dependent on the presence of DNA damage in the heterochromatic region. Confluent normal human cells on slides were covered with a Millipore filter with pores of 8 μm diameter, UV-irradiated with a dose of 30 J/m² and fixed immediately after exposure. To monitor DNA damage and chromosomal bands, we employed a FISH protocol in combination with the immunolog-

detection of UV-induced 6-4PP (Fig. 3A). The percentage of colocalization (6.9%) in locally irradiated cells for chromosome 9 (Fig. 3B) was not significantly different from that in cells exposed to global irradiation (Fig. 1). Since in most of these cells the heterochromatic regions were not in the exposed area, the results indicate that pairing is also induced when exposure occurs outside the target regions. Interestingly, in these experiments, the percentage of unexposed nuclei that displayed colocalized heterochromatin did not differ signifi-

cantly from that in locally or globally irradiated cells (Fig. 3B), i.e., 5.4% after subtraction of control value in mock-treated cells.

To assess whether the exposed cells secrete factors in the culture medium that could induce pairing of the heterochro-

matin of chromosome 9, unexposed confluent cells on slides were co-cultured in Petri dishes containing UV-irradiated cells. As shown in Fig. 3C, the percentage of the induced colocalization of chromosome 9 heterochromatin in unex-

posed cells increased rapidly and significantly to elevated levels similar to that observed in locally or globally exposed cells. The positioning of chromosome 8 homologues was not affected neither by local UV exposure nor conditioned medium (data not shown).

Colocalization of homologous chromosomes in interphase cells after exposure to heat shock

The almost identical results obtained after exposure to X-rays [7], MMC [8] and UV irradiation (this study) suggest that cellular stress might underlie pairing or colocalization of homologous heterochromatin. Moreover, the similar response of unex-

posed (bystander cells) cells neighboring locally UV-exposed cells or unexposed cells incubated with medium conditioned by irradiated cells supports that hypothesis. To get further insights in the putative stress response, human fibroblasts were exposed to heat shock by incubation at 45°C for different

![Graph](image-url)
The induction of colocalization of homologous chromosome 9 after exposure of interphase cells to heat increased with increasing time of heat exposure at 45°C (Fig. 4A), reaching the highest response (10.2%) after 1 h of heat treatment. Heat treatment longer than 1 h changed the cell morphology and did not allow proper analysis of colocalization. Exposure to heat did not change significantly the localization of chromosome 8 bands as shown in Fig. 4A.
Within the first hour after heat shock treatment, a fast reduction of pairing was observed then slowly approaching the control level after 24 h (Fig. 4B).

**Impact of XPF deficiency on heterochromatin pairing after exposure to heat shock**

XPF cells lack the induction of heterochromatin pairing after UV irradiation (this study) and after MMC [8]. In contrast, heat shock treatment enhanced the induction of the heterochromatin pairing in XPF cells as efficiently as in wild type cells. After 1-h heat shock treatment, the average percentage of XPF cells displaying colocalized signals of the heterochromatin of chromosome 9 was 10.7% (Fig. 4A). No significant change in colocalization frequency was observed for chromosome 8 bands in XPF cells (Fig. 4A).

**Discussion**

We assessed the impact of two different cellular stressors i.e., UV irradiation and heat shock on pairing of heterochromatin in human cells. UVC irradiation primarily induces dimeric photolesions at dipyrimidine sites, whereas heat shock is believed to be principally non-genotoxic and to induce denaturation of proteins. However, recently, Kaneko et al. [22] presented evidence that heat shock induces DSBs in mammalian cells and hence should not be considered as an entirely non-genotoxic treatment. Both types of stress induced colocalization of heterochromatin of chromosome 9 homologues in a subset of cells. In contrast, no changes in the relative positions of the euchromatic regions of chromosome 8 were induced. Previously, we have shown that other DNA-damaging agents i.e., X-rays and MMC, induce similar levels of heterochromatin pairing independent of the cell cycle, that is, the same percentage of pairing of the heterochromatin of chromosome 9 was observed in G0/G1 and S-phase cells after MMC treatment [7,8]. We note here that also the heterochromatic regions of chromosome 1 (1q12) display pairing after UV exposure and heat shock treatment but to a less extent than the heterochromatic regions of chromosome 9. Moreover, MMC treatment leads to 1q11–12 homologous pairing correlating with exchanges observed for chromosome 1 homologues at metaphase (unpublished data). The various treatments are known to induce structurally different DNA lesions at very different frequencies [16,17,23–25]. Taken together, data suggest that pairing of heterochromatin after exposure to various cellular stressors reflects a general stress response.

Pairing of heterochromatin of chromosome 9 is not entirely dependent on DNA damage in the target sequence itself as shown by two types of experiments: (i) local UV damage induced distal from heterochromatin of chromosome 9 provokes pairing; moreover, (ii) pairing was also induced in the unexposed neighboring cells and cells treated with the culture medium obtained from the irradiated cultures. These findings show that the stress signal induced by UV exposure could be transmitted to unexposed cells as a non-targeted bystander effect. Substantial evidence exists for the bystander response by exposure to ionizing radiation both in vitro and in vivo, and several mechanisms involving secreted soluble factors, gap-junction intercellular communication and oxidative metabolism have been proposed to underlie the radiation-induced bystander effect (reviewed in [26–29]). Similar mechanisms could be responsible for the non-targeted effect observed after UV irradiation.

The results obtained with human cell lines deficient in NER revealed a complex role of NER in heterochromatin pairing after UV exposure. The fact that XPA cells (completely deficient in the incision step of NER) clearly exhibited heterochromatin pairing after UV exposure demonstrates that NER is not required for pairing and that transient single-stranded regions generated in normal human cells during NER do not underlie the effect. Yet, heterochromatin pairing is markedly delayed in XPA cells, suggesting that NER has its impact on the process...

**Fig. 4 – Colocalization of homologous chromosomes in interphase cells exposed to heat shock (45°C).** (A) The influence of the duration of heat shock treatment. Data were taken from 3 independent experiments; in every experiment, 500 cells were analyzed for each data point. The error bars represent the standard deviation of the mean values. Data for XPF cells exposed to 1 h heat shock are included. (B) Effect of recovery time on the colocalization of chromosome 9 bands after exposure of human fibroblasts to heat for 1 h. Data obtained from three independent experiments are presented.
that leads to pairing. No induction of heterochromatin pairing was observed in XPF cells exposed to UV irradiation or MMC, although heat shock treatment and X-ray exposure (data not shown) revealed a normal capability of XPF cells to undergo pairing of heterochromatin. So far, the XPF protein has not been implicated in the repair of X-ray-induced DNA damage nor in heat-induced cell injury. This suggests that the sensing of stress factors (most probably mediated by XPF protein) is different in case of different agents. The proficient and deficient induction of heterochromatin pairing after UV irradiation in XPA and XPF cells, respectively, mimic the response of these cells to MMC [8] and again demonstrate that functional NER is not required to generate heterochromatin pairing. Instead, the response of XPF cells suggests that XPF protein is required to either trigger pairing by sensing stress or to maintain the paired status.

Stress events (including heat shock, UV-B and -C irradiation, heavy metals, H$_2$O$_2$, arsenate and alcohols) induce the expression of a set of highly conserved proteins called heat shock proteins (HSPs) or generally termed ‘stress proteins’ [30–35]. Transcriptional regulation of heat shock gene expression is mediated by heat shock transcription factor (HSF1) that binds to specific sequences in the promoter region of heat shock genes [36]. UVB irradiation is known to activate HSF1 in human keratinocytes as evidenced by HSF phosphorylation [37]. Interestingly, in human cells exposed to heat shock, HSF1 granules localize to the pericentromeric heterochromatin regions of specific human chromosomes among which is the 9q11–12 [38,39], the region that displays homologous pairing after exposure to various DNA-damaging agents and cellular stress ([7,8] and this study). This might represent a link between pairing of the heterochromatic regions of chromosome 9 after cellular stress and the formation of HSF1 granules on these regions. However, in contrast to heterochromatin pairing, HSF1 granules are observed in the very majority of heat-treated cells [19]. Recently, Jolly et al. [40] and Rizzi et al. [41] found that the satellite III repeats characteristic of the pericentromeric heterochromatin of chromosome 9 are transcribed into large and stable RNA molecules that remain associated with 9q12 region after heat shock. Based on these findings, we speculate that DNA-damage-mediated stress response leads to transcriptional activation of heterochromatin of chromosome 9 by binding of HSF1 and that this process brings somehow homologous heterochromatin together. Due to restricted movement of interphase chromatin, pairing only occurs in a subset of treated cells [7,8]. Possibly, the delayed pairing of heterochromatin in UV-irradiated XPA cells is due to interference of UV photosensins with RNA Polymerase II [42] accumulating in granules.

In conclusion, clear evidence for non-targeted effects emerged from this study, however, experiments with MMC [8] revealed that this cross-linking agent strongly stimulates pairing and direct interaction of heterochromatin in interphase and that MMC-induced exchange aberrations are also primarily formed between homologous chromosomes in these regions [8,43,44]. Furthermore, UV-irradiated photosensins and possibly other types of damage might provoke interactions between homologous chromosomes as indicated by the persistence of pairing during a 24-h time period post-UV irradiation. Once the heterochromatin comes together, it may provoke recombinational repair via stalled transcripts in the presence of DNA damage as recently shown in yeast [45]. Moreover, the observation that XPF, known to be essential for recombinational repair [46–48], is required for pairing after MMC or UV exposure suggests that recombination-dependent pathways play a role in the maintenance of heterochromatin pairing which could lead to chromosome exchange formation. In line with this, lower frequencies of chromosome exchanges were observed in ERCC1/XPF-deficient mouse and hamster cells after exposure to UV light [49,50] and also in XPF-deficient human cells treated with MMC [8]. In contrast, recovery of pairing occurred within hours in cells exposed to X-rays or heat shock. Moreover, normal induction of heterochromatin pairing was observed in XPF-deficient cells exposed to these agents most likely due to the nature of DNA lesions induced by X-rays or stress caused by heat. Taken together, we propose that stress in general can cause pairing of homologous heterochromatin and that XPF protein could act as a sensor of the stress signal which leads to the maintenance of the paired status when the recombination repair is required.

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