Mitomycin C-induced pairing of heterochromatin reflects initiation of DNA repair and chromatid exchange formation

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Summary

Chromatid interchanges induced by the DNA cross-linking agent mitomycin C (MMC) are over-represented in human chromosomes containing large heterochromatic regions. We found that nearly all exchange breakpoints of chromosome 9 are located within the paracentromeric heterochromatin and over 70% of exchanges involving chromosome 9 are between its homologues. We provide evidence that the required pairing of chromosome 9 heterochromatic regions occurs in G_0/G_1 and S-phase cells as a result of an active cellular process initiated upon MMC treatment. By contrast, no pairing was observed for a euchromatic paracentromeric region of the equal-sized chromosome 8. The MMC-induced pairing of chromosome 9 heterochromatin is observed in a subset of cells; its percentage closely mimics the frequency of homologous interchanges found at metaphase. Moreover, the absence of pairing in cells derived from XPF patients correlates with an altered spectrum of MMC-induced exchanges. Together, the data suggest that the heterochromatin-specific pairing following MMC treatment reflects the initiation of DNA cross-link repair and the formation of exchanges.

Key words: Chromosome pairing, Chromosome positioning, Cross-link repair, Heterochromatin, MMC, XPF

Introduction

Mitomycin C (MMC) is a natural antitumor antibiotic and cytotoxic drug used in clinical chemotherapy regimens for the treatment of the various carcinomas (for a review, see Verweij and Pinedo, 1990). The cytotoxicity of MMC is due primarily to the formation of the DNA adducts – in particular, DNA inter-strand cross-links (ICL) (Iyer and Szybalski, 1963; Dorr et al., 1985; Keyes et al., 1991).

Cytogenetical studies have revealed that MMC frequently produces chromatid exchanges in human cells and that these exchanges do not occur randomly within the genome. Particularly, human chromosomes 1, 9 and 16 are frequently involved in so-called quadriradials, with the exchange breakpoints predominantly observed within the C-bands of the involved chromosomes. Moreover, exchanges between homologous chromosomes occur far in excess of what might be expected by chance (Shaw and Cohen, 1965; Morad et al., 1973). Although the frequency of MMC-induced exchange events in these three chromosomes tends to increase with the increase of C-band size, on average more exchanges are induced in chromosome 9 than in 1, in spite of a similar C-band size (Joseph et al., 1982a). Two mechanisms have been proposed to explain the chromosome bias of MMC-induced exchanges. First, pairing in interphase might provoke the formation of exchanges between the paired chromosomes (Shaw and Cohen, 1965; Morad et al., 1973; Haaf et al., 1986). Second, it has been suggested that the excess of homologous exchanges might result from recombination-associated repair between matching repetitive DNA sequences (Brogger and Johansen, 1972).

Pairing of homologous chromosomes has been observed in somatic cells in a variety of organisms but most information has been gained from studies with *Drosophila melanogaster* (Henikoff, 1997; Fung et al., 1998). However, transient pairing has also been reported in human somatic cells. Pairing of the homologous heterochromatic regions of chromosome 1, 9 and 16 has been observed in metaphases of human lymphocytes after 5-azacytidine incorporation (Haaf et al., 1986). Also, pairing of homologous band 1q12 in normal human brain cells (Arnoldus et al., 1989) and centromeres of chromosome 15 in human lymphocytes (Lewis et al., 1993; Lasalle and Lalande, 1996) was observed. Moreover, homologous association of the centromeres of chromosome 9 and 17 was evident in prostate cancer cells (Williams et al., 1995). Although pairing of homologous chromosomes outside of the meiotic program has been suggested to be of functional importance in relation to nuclear organization, gene expression, malignant- or disease-state, or DNA repair (Henikoff, 1997; Stout et al., 1999; Abdel-Halim et al., 2004), the biological relevance of pairing is poorly understood.

MMC induces ICLs in the DNA, which, when left unrepaired, form impenetrable blocks for DNA replication and are highly cytotoxic. The repair of ICL is a complex process involving proteins belonging to nucleotide excision repair (NER), homologous recombination and translesion synthesis pathways (for a review, see Dronkert and Kanaar, 2001). A

central role was suggested for the NER heterodimeric endonuclease XPF-ERCC1, as mammalian cell lines defective in ERCC1 or XPF are extremely sensitive to DNA cross-linking agents (De Silva et al., 2000; De Silva et al., 2002). Also, studies in yeast and Drosophila showed that the homologues of XPF and ERCC1 proteins are implicated in recombination (Carr et al., 1994; Sekelsky et al., 1995). For S-dependent chemicals like MMC, the formation of structural aberrations requires that the DNA bearing ICLs should pass through a round of replication. However, whether exchanges are the consequences of the removal of ICLs before or during replication is a matter of debate, and whether pairing is preceding the formation of exchanges between homologous chromosomes is not firmly established.

In this study we assessed the localization of defined heterochromatic and euchromatic chromosomal regions in interphase nuclei of human cells exposed to MMC and made correlations with frequencies of chromatid exchange observed in metaphase spreads. We show that chromosome 9 homologues are most frequently involved in MMC-induced exchanges and almost exclusively in the paracentromeric heterochromatic regions. These regions associate and colocalize in G_0 and G_1 phases immediately after MMC treatment, indicating that pairing is not confined to S-phase. Xeroderma pigmentosum (XP) cells belonging to complementation group F (XPF) appeared to be deficient in MMC-induced pairing of chromosome 9 heterochromatin. Furthermore, we found that treatment of XPF-deficient cells results in a strongly reduced frequency of chromatid interchanges compared with repair-proficient cells. On the basis of these results, we propose that pairing of homologous heterochromatin in G_0/G_1 cells upon MMC treatment reflects the initiation of a recombination-dependent ICL repair pathway, ultimately leading to chromatid interchanges between homologous chromosomes in case of misrepair.

Materials and Methods

Cell culture and MMC treatment

In the present studies, three different types of human cells were used. The culture conditions, MMC treatment regimes and fixation times are described for each cell type in the following section.

Isolated human blood lymphocytes from a healthy donor were cultured in RPMI 1640 medium supplemented by 15% fetal bovine serum (FBS), 20 μg/ml phytoheamagglutinin, 1% glutamine and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. Seven hours after culture initiation, the cells were treated with 4 μM MMC (Kyowa) for 1 hour. Parallel cultures treated with 0.1% methanol were included as solvent controls. Subsequently, cells were washed three times with RPMI 1640 medium and cultured in fresh culture medium containing 5 μM BrdU to allow cell-cycle analysis. Following a 2 hour colcemid treatment (0.1 μg/ml), MMC exposed and control cells were harvested at 54 hours and 46 hours after culture initiation, respectively. After hypotonic treatment with 75 mM potassium chloride, the cells were fixed with methanol:acetic acid (3:1) and metaphase preparations were made. Before processing for fluorescence in situ hybridization, the preparations were aged for about a week at room temperature.

B-lymphoblastoid cell lines derived from a healthy individual (JVM) and a xeroderma pigmentosum patient (complementation group F, XP7NE) were grown in RPMI 1640 medium containing 10% FBS, 2 mM glutamax (Gibco), 20 mM sodium pyruvate and antibiotics. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. Exponentially growing cells were treated with 4 μM MMC for 1 hour, washed and further incubated at 37°C. The cultures were fixed at various time intervals post-treatment. Metaphase preparations were made and processed for analysis using Giemsa staining and fluorescence in situ hybridization (FISH).

Primary human skin fibroblasts derived from a healthy individual (VH25) and XP patients (XP25RO, complementation group A; XP24KY and XP7NE, group F) were grown in Ham's F10 medium, supplemented with 15% FBS and antibiotics. The cells were incubated in a humidified 2.5% CO_2 atmosphere at 37°C. The VH25 cells were treated with MMC for two different types of experiments. In the first type, confluent (G_0) cells grown on glass slides were treated with various doses of MMC for different time periods. Then the cells were washed with Ham's F10 medium and fixed either immediately or allowed to recover by incubation up to 20 hours in fresh culture medium containing 5 μM BrdU to restrict the analysis to noncycling cells. In the second type of experiments, confluent cells were trypsinized and seeded on slides at a threefold reduced cell density to allow cell-cycle progression. After 24 hours the cells were treated with 4 μM MMC in the presence of 5 μM BrdU for 1 hour and fixed immediately. The fibroblasts from XP patients were grown to confluency on glass slides, treated with 4 μM MMC for 1 hour and fixed. In each experiment, cultures treated with appropriate solvent concentrations were included. In all situations, the fibroblasts were washed twice with ice-cold PBS before a 5 minute fixation step with 2% paraformaldehyde in PBS at 4°C. To permeabilize the nuclear membrane the cells were washed with ice-cold PBS and treated with methanol (–20°C) for 10 minutes, after which the slides were air-dried.

Premature chromosome condensation

The technique of premature chromosome condensation (PCC) was applied on isolated human lymphocytes as described previously (Darroudi et al., 1998). Briefly, 7 hours after culture initiation the lymphocytes were treated with 4 μM MMC for 1 hour, washed with Ham's F10 medium and fused with mitotic Chinese hamster ovary (CHO) cells in the presence of 40% polyethylene glycol. After washing with Ham's F10 medium the cells were incubated for 1 hour at 37°C in culture medium containing 0.3 μg/ml colcemid. The cell suspension was fixed and the preparations were made as described above for human lymphocytes.

Fluorescence in situ hybridization

On metaphase and PCC preparations, FISH was performed using a combination of human whole chromosome specific (Cambio) and band specific (Research Genetics) DNA probes. The probes were labeled with FITC-12-dUTP or biotin-16-dUTP. Three different combinations of probes were used: chromosome 1 and 2 with their respective bands 1q11-12 and 2p11.1q11.2; chromosome 8 and 9 with the respective bands 8p11.2 and 9q12-13; and chromosome 1 and 9 with the respective bands 1q11-12 and 9q12-13. To discriminate between the painted chromosomes and chromosome bands, dual colour FISH was performed using reversed colors for the whole chromosome and band-specific DNA probes (Figs 1, 6). For the analysis of interphase fibroblasts, only the band specific probes 8p11.2 and 9q12-13 were applied.

Before in situ hybridization, slides were treated with RNAse and pepsin, post-fixed with 1% formaldehyde and denaturated in 70% formamide as previously described in detail (Boei et al., 1996). The band-specific probes were labeled during a PCR reaction using a protocol supplied by the manufacturers and purified using a QIAquick kit (Qiagen). The band specific probes were precipitated together with Cot1 DNA, dissolved in hybridization buffer (Cambio) and, if required, mixed with whole chromosome-specific probes.

Denaturation of probes was performed by incubation at 67°C for 10 minutes followed by incubation at 37°C for 1 hour to allow probe competition. The probe mixture was then applied to the denaturated slides and hybridized overnight at 37°C.

Post-hybridization, slides were washed with 50% formamide in 2×SSC (pH 7.0) at 45°C and treated with 10% blocking protein (Cambio). For immuno-fluorescence detection of FITC-labeled probes, rabbit anti-FITC and FITC conjugated goat anti-rabbit IgG were used (Cambio), whereas Texas-Red conjugated avidin and biotinylated goat anti-avidin (Cambio) were used to detect biotin labeled probes. In several experiments FISH was combined with the visualization of incorporated BrdU. This was achieved using mouse IgG monoclonal anti-BrdU antibody (Boehringer Mannheim) followed by a second antibody, Cy3 conjugated anti-mouse antibody (Jackson ImmunoResearch). After the immunological detection, the cells were counter-stained in 10 ng/ml DAPI/PBS solution for 10 minutes and embedded with Citifluor mounting medium (Agar Scientific).

Microscopy and scoring criteria

FISH analysis was performed using a Zeiss Axioplan microscope equipped with filters suited for the observation of DAPI, FITC, Texas red and Cy3. Furthermore, a triple filter for simultaneous observation of DAPI, FITC and Texas red was applied.

Well-spread metaphases were analyzed for the presence of chromosomal aberrations involving the painted chromosomes. The analysis was restricted to chromatid interchanges, i.e. exchanges involving a painted chromosome and other unpainted chromosomes, or exchanges involving two painted chromosomes, including two homologous chromosomes. In both classes we distinguished whether or not the exchange breakpoints were located in the band of the painted chromosome (Fig. 1).

Interphase cells hybridized with band-specific probes were scored manually or by means of a computerized image analysis system consisting of a CH250 photometrics CCD camera, an Apple Macintosh PowerPC and IPlab software (Scanalytics). In the manual scoring, a distinction was made between nuclei with two entirely separated hybridization signals for a particular chromosome (class 1), and nuclei in which signals were so close together that only a single (usually larger) hybridization signal or two touching signals were observed (class 2, Fig. 3B). For the computerized analysis, single 2-D images of 200 nuclei were captured from randomly selected control and MMC-treated cells. Image analysis was performed as described by Abdel-Halim et al. (Abdel-Halim et al., 2004). Briefly, for each cell the lengths of both major and minor axes were determined and taken as a measure of nuclear size. The relative position and the radial distance of both homologous hybridization signals for each chromosome band (8p11.2 and 9q12-13) were determined and used to plot the hybridization signals of all nuclei in one quadrant of the nucleus to assess their nuclear distribution. Finally, the distances between the two intensity weighted centers of the hybridization signals of the homologous chromosome bands were determined for each image, sorted and plotted against ascending nucleus numbers (Fig. 5B).

Results

Nonrandom induction of chromatid interchanges by MMC

MMC-induced chromatid interchanges were analyzed in isolated human lymphocytes. G_1 human lymphocytes were treated with 4 μM MMC for 1 hour, washed thoroughly and propagated to mitosis. BrdU-based harlequin staining was used to select fixation times in which the percentage of cells in the second mitosis post-treatment did not exceed 8%. The genomic frequency of chromatid interchanges obtained by analysis of Giemsa-stained preparations was found to be 0.38 per cell (Fig. 2A). FISH analysis of chromatid interchanges involving chromosomes 1 and 9, and their similar sized chromosomes 2 and 8, respectively, revealed that MMC-induced exchanges were not homogeneously distributed over the genome. About 50% of all exchanges induced in the genome involve chromosomes 1 or 9, although the DNA content of these two chromosomes only amounts to about 13% of the genome (Morton, 1991). In sharp contrast, chromosomes 2 and 8, also with a DNA content of about 13%, participated in only about

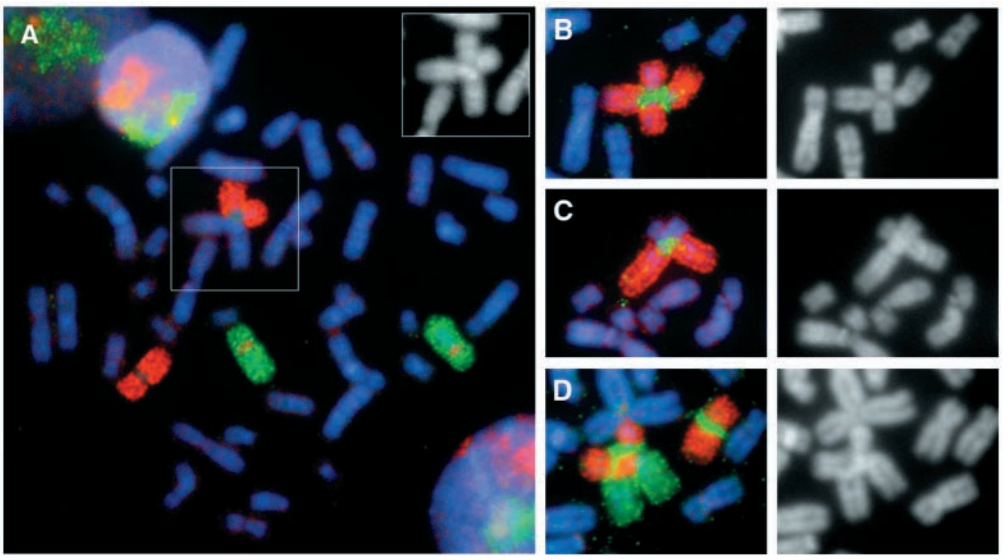

Fig. 1. MMC-induced chromatid interchanges. Examples of MMC-induced chromatid interchanges as observed in human lymphocytes using FISH combining whole chromosome and band-specific probes. (A) Metaphase spread containing a quadriradial chromatid exchange between chromosome 9 (red) and an unpainted chromosome (blue) with the exchange breakpoint at the paracentromeric heterochromatic band of chromosome 9 (green). Two unaffected chromosomes 8 (green, with red paracentromeric euchromatic band) are also present. (B) The most commonly observed quadriradial between the homologues of chromosome 9 (red) with both exchange breakpoints within the heterochromatic bands (green). (C) An exchange involving the heterochromatic band (green) of chromosome 1 (red) and an unpainted chromosome. (D) The heterochromatic bands of chromosomes 1 (green) and 9 (red) are involved in a chromatid exchange. The black and white images of the DAPI counterstained chromosomes were included to visualize the exchanges at higher contrast.

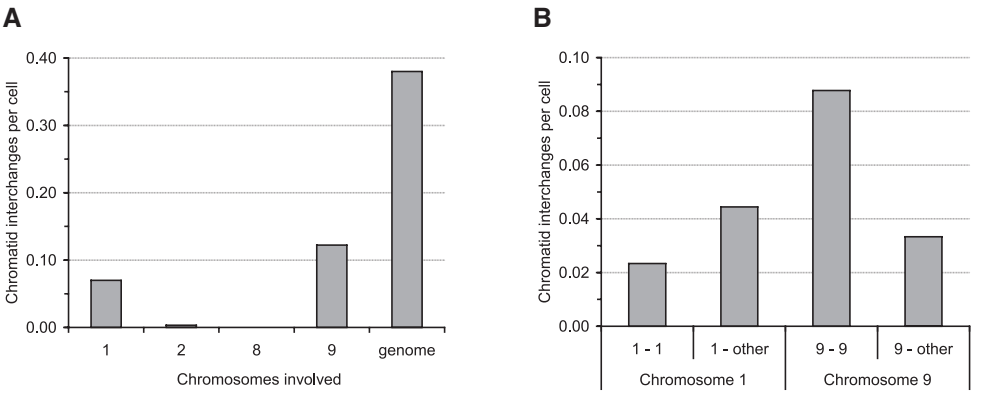

Fig. 2. Distribution of MMC-induced chromatid interchanges over the genome. Frequencies of chromatid interchanges observed in human lymphocytes after treatment in G_1 with 4 μM MMC for 1 hour. (A) The genomic frequency (estimated by analysis of 200 Giemsa-stained metaphase cells) as well as the specific frequencies for chromosomes 1, 2, 8 and 9 (analysis of 900 cells after FISH painting) are plotted. Chi-square statistics showed a highly significant (P<0.001) difference among the four chromosomes used taking into account their DNA contents. No chromatid interchanges were observed in 900 mock-treated cells. (B) The frequencies of chromatid interchanges observed for chromosome 1 and 9 are subdivided into two categories – namely, those between the painted homologues (1-1 or 9-9) and between two non-homologous chromosomes (1-other or 9-other).

1% of exchanges (Fig. 2A). From the chromatid interchanges involving chromosome 9, about 70% occurred between the two homologues and 30% between chromosome 9 and another chromosome. In the case of chromosome 1, the picture was reversed: about 30% of exchanges occurred between the homologues and 70% with other chromosomes (Fig. 2B). The exchanges between nonhomologous chromosomes frequently involved chromosome 1 and 9: about 50% of the non-homologous exchanges of chromosome 9 were with chromosome 1 (Fig. 1D). In the majority (>97%) of exchanges involving chromosome 1 or 9, the exchange breakpoints were observed in the paracentromeric heterochromatic bands of these chromosomes (Fig. 1), showing the high potency of MMC to induce exchanges in these regions.

Localization of homologous chromosomes in interphase cells exposed to MMC

The aforementioned metaphase analysis revealed that chromosome 9 is very frequently involved in MMC-induced exchanges and that about 70% of these exchanges are between the homologues with virtually all exchange breakpoints located within the paracentromeric heterochromatin. Obviously, somewhere between G_1 (the time of treatment) and mitosis (the time of cytogenetic analysis) the homologous chromosomes must have been in physical contact to form the exchanges. The obvious first question is whether the frequent interactions between the homologues of chromosome 9 can be explained by close proximity of the two chromosomes. To investigate the relative position of homologous chromosomes 9 within the interphase cells, we employed FISH using the band-specific probe 9q12-13 (covering the paracentromeric heterochromatic region) and for comparison, we applied the band-specific probe 8p11.2 (paracentromeric euchromatin). The interphase FISH analysis was performed on in situ fixed human fibroblasts, since the flat nuclei of this cell type allow simple two-dimensional analysis (Fig. 3). This was especially beneficial given that large numbers of cells had to be analyzed. Image analysis of confluent fibroblasts revealed that chromosomes 8 and 9 are apparently randomly distributed within the nucleus (Fig. 5A, upper panels) with similar average inter-homologue distances for bands 8p11.2 (8.3 μm) and 9q12-13 (7.6 μm). Hence, a close proximity of chromosome 9 homologues in untreated cells can not account for the frequently observed chromatid interchanges between these two chromosomes.

The pairing of chromosome 9 homologues must therefore be either induced by MMC or should occur naturally when cells progress from G_0 to mitosis. To assess the influence of MMC on the localization of chromosome 8 and 9, confluent fibroblasts were treated for 1 hour with 4 μM MMC and fixed immediately or after recovery periods up to 20 hours in fresh

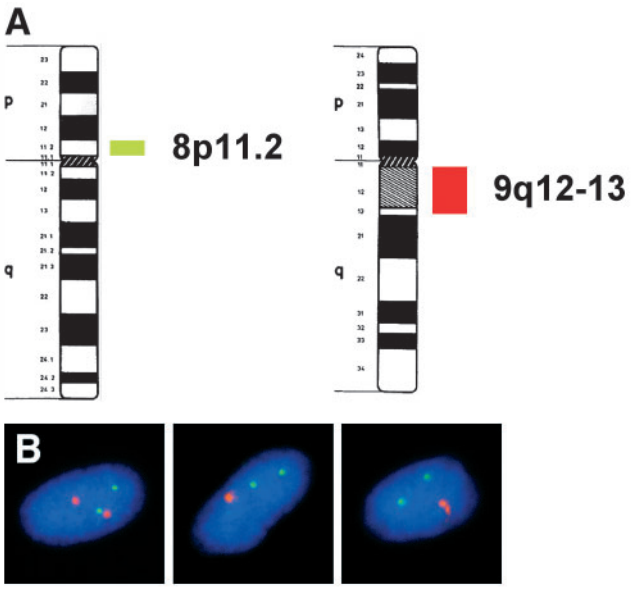

Fig. 3. Interphase FISH with band-specific probes for chromosome 8 and 9 on human fibroblasts. (A) G-banded chromosomes 8 and 9, showing the position and size of the bands covered by the applied chromosome band-specific probes 8p11.2 (green) and 9q12-13 (red). (B) Example of confluent human fibroblasts after hybridization. Criteria for manual analysis were made according to the number of hybridization signals in the nucleus. Discrimination was made between cells with: two separate hybridization signals for each probe (left) and nuclei in which signals of homologous chromosomes were so close together that only one single, usually larger and brighter, hybridization signal (middle) or two touching signals (right) could be observed.

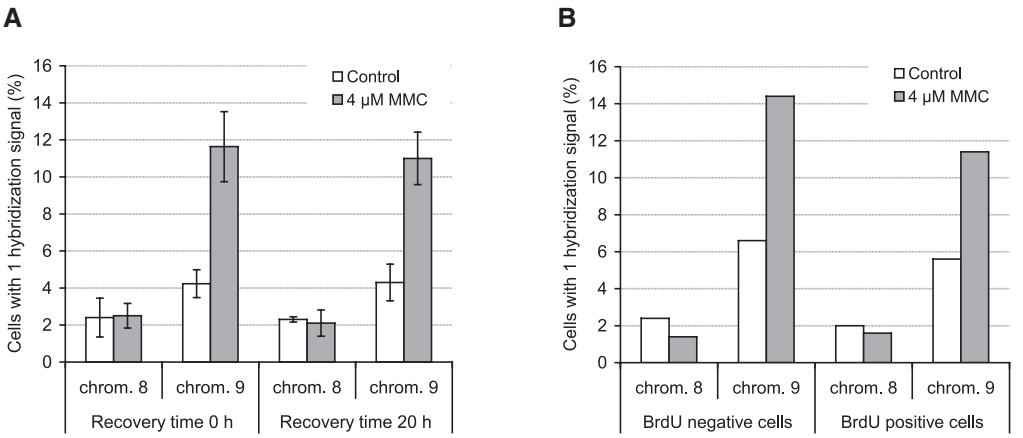

Fig. 4. Colocalization of hybridization signals of homologous chromosome bands in human fibroblasts. The percentage of cells with one hybridization signal for the homologues of either chromosome 8 or 9 was analyzed manually. (A) The analysis was performed on confluent cells treated with 4 µM MMC for 1 hour and fixed either immediately (recovery time 0 hours) or after a recovery time of 20 hours. Solvent controls were included for both time points. Each bar represents the average percentage obtained from at least three independent experiments. In each experiment 500 cells were analyzed. The error bars are standard deviations (s.d.) of the mean values. A significantly increased colocalization was evident for chromosome 9 in the treated cells ($P<0.05$, Student t-test) and no difference was observed between 0 hour and 20 hour recovery. (B) The analysis was performed on cycling cells treated with 4 µM MMC for 1 hour, 24 hours after they were released from confluency by subcultivation to reduced cell density. The cells were fixed directly after the treatment. Since BrdU was present during treatment, BrdU-positive cells must have been treated in the S-phase of their cell cycle. 500 BrdU-positive and -negative cells were analyzed. A significant increase of the number of cells containing one hybridization signal for chromosome 9 was observed in MMC-treated cells ($P<0.05$, Wilson's method for difference in proportions).

medium. In the manual analysis two classes of cells were recognized, i.e. cells displaying two hybridization signals and cells with one (or two adjacent) hybridization signal for the band-specific probes for chromosome 8 or 9 (Fig. 3B). Following MMC-treatment, washings and subsequent incubation in fresh medium for up to 20 hours, a subset of cells was found to progress from G_0 into S-phase. By simultaneous detection of incorporated BrdU and FISH signals we were able to restrict the analysis to G_0/G_1 cells. The outcome of these experiments is presented in Fig. 4A. In untreated cells we noticed a difference in the percentage of cells with one hybridization signal when stained for chromosome 8 and 9. This difference can be explained by the difference in size of the hybridization signal. The larger size of band 9q12-13 (covering about 0.6% of the nuclear surface) compared with band 8p11.2 (covering about 0.4%) (Fig. 3A), resulted in an about two-fold higher probability to colocalize in case of random positioning (Abdel-Halim et al., 2004). MMC treatment of G_0 cells did not influence the percentage of cells with one hybridization signal for chromosome 8. By contrast, for chromosome 9 the percentage of cells with one hybridization signal increased significantly (approximately 2.5-fold) due to the treatment with MMC. Strikingly, the altered distribution of chromosome 9 observed already directly after the 1 hour treatment with no significant changes at later fixation times (only 20 hours is shown in Fig. 4A).

Prolonged treatment with 4 µM MMC for up to 6 hours did not increase the level of pairing when compared with 1 hour treatment (data not shown). We also assessed the effect of MMC dose on the intranuclear position of the chromosomal bands. Up to a dose of 8 µM a proximate linear dose-response for the percentage of cells with one hybridization signal was obtained for chromosome 9, with no further increase at higher doses. For chromosome 8, the percentages of class 2 cells did not increase significantly above control levels over the entire dose range tested (data not shown).

To compare the localization of homologous chromosomes between G_0/G_1 and S-phase, cells were released from confluency and treated 24 hours later with 4 µM MMC for 1 hour, directly followed by fixation. During the treatment, BrdU was present in the medium. As a consequence, BrdU-positive cells were treated during the S-phase, whereas BrdU-negative cells were in G_0/G_1. No significant difference in the percentage of cells with one hybridization signal for chromosome 9 was found between BrdU-positive and BrdU-negative cells (Fig. 4B). Moreover, the percentage of cells with colocalizing chromosome 9 bands among these cycling fibroblasts was similar to the percentage observed in confluent cells.

So far, the analysis was performed manually, discriminating between cells with two or one hybridization signals. To gain more detailed insight into the localization of homologous chromosomes we applied interphase image analysis to measure the distance between homologous hybridization signals. Image analysis was performed on 200 cells from confluent cultures treated for 1 hour with 4 µM MMC or with solvent only (control) and fixed directly after treatment. MMC treatment resulted in a slight but not significant increase in nuclear size; the average length of the major axis of the nucleus increased from 11.9 µm to 12.5 µm and the minor axis from 6.7 µm to 6.9 µm (compare outer curves of upper and lower panels of Fig. 5A). There was also a slight change in the intra-nuclear distribution of the individual homologous bands of chromosome 8 and 9. In control cells, 50% of the hybridization signals were found within a radial distance of 5.7 µm (chromosome 8) and 5.3 µm (chromosome 9). After MMC treatment, this radial distance increased nonsignificantly to 6.0 µm and 5.5 µm, respectively (compare inner curves of upper and lower panels of Fig. 5A). The effect of MMC on the relative position of chromosome 8 and 9 was assessed by measurement of the distance between the hybridization signals of homologous chromosomes in each cell. In Fig. 5B, cells with ascending distance between the homologues are plotted against the absolute distance between the homologues. Treatment with MMC did not change significantly the average

distance between the homologues of chromosome 8 (8.3±4.1 μm). For chromosome 9, reduction (P<0.01) in the average inter-homologue distance was observed in MMC treated cells (6.5±4.4 μm) compared with the control (7.6±4.2 μm). In 10% of control cells the distance between the homologues of chromosome 9 was less than 3 μm, whereas after MMC treatment, significantly (P<0.01) more cells (23%) were found in this category. These measurements confirm the manual observations and indicate a specific movement of chromosome 9 homologues towards each other over fairly large distances.

PCC analysis of G_1 chromosomes

We showed an increase in the number of G_0/G_1 human fibroblasts displaying colocalization of paracentromeric heterochromatin of the homologues of chromosome 9 directly following 1 hour treatment with 4 μM MMC. Human lymphocytes treated in G_1 with MMC, and subsequently propagated to reach mitosis, exhibited the formation of exchanges predominantly between the homologues of chromosome 9 at the position of the heterochromatic band. This correlation raises the question whether exchange interactions already take place during the G_1 phase of the cell cycle. To address this question, we utilized the premature chromosome condensation (PCC) technique in combination with FISH. Unfortunately, the morphology of the prematurely condensed chromosomes did not allow unambiguous recognition of exchanges. Therefore, we had to restrict our analysis to the colocalization of homologous chromosomes 8 and 9 within the PCC spread and to distinguish between colocalization at the position of the painted bands and other loci of the painted chromosomes. A significant induction of colocalization of chromosome 9 homologues was observed (Fig. 6F), with roughly half of the homologues colocalizing at the position of the painted heterochromatin band (Fig. 6A). A partial alignment of the homologous chromosomes 9 was

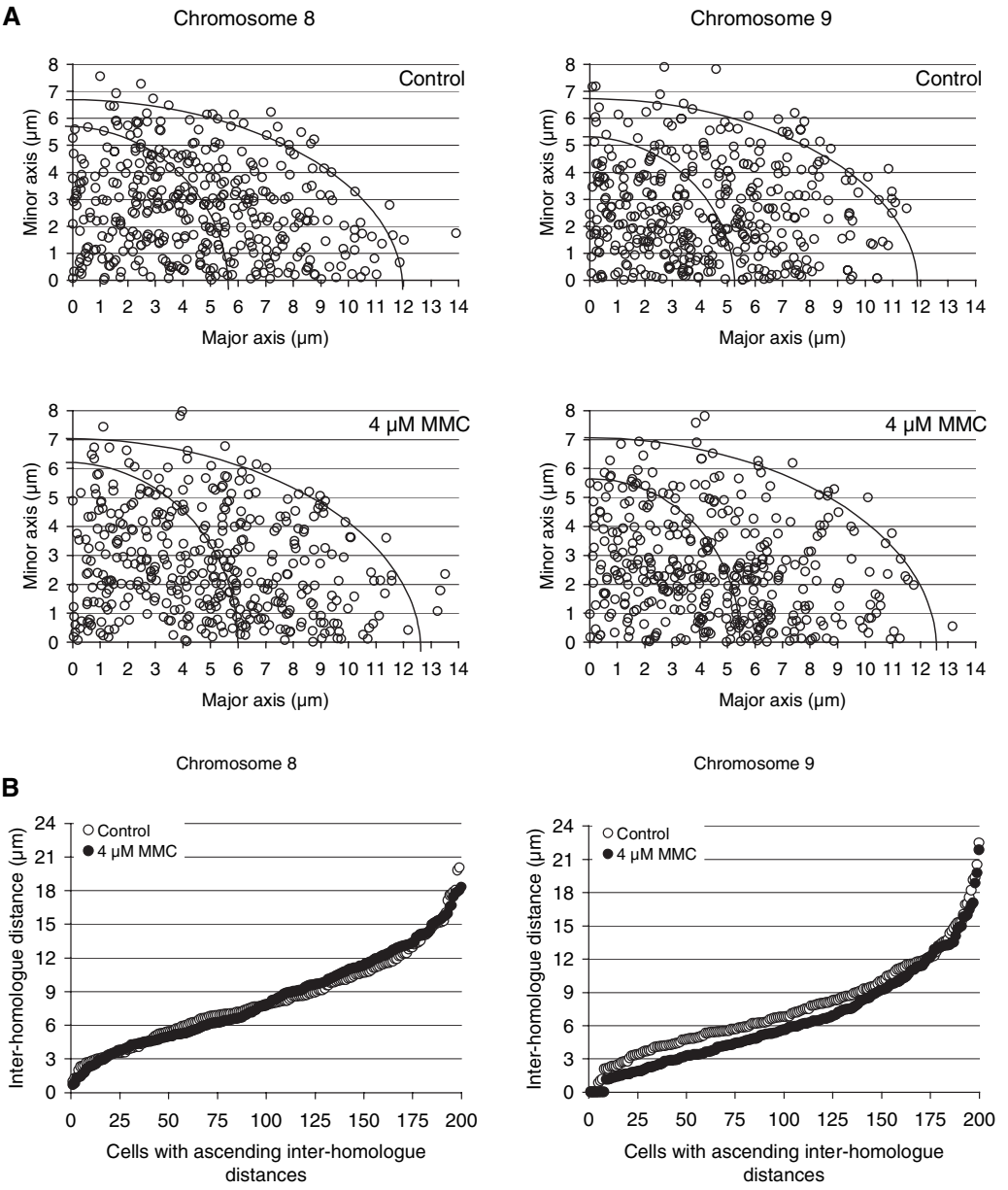

Fig. 5. Position of chromosome bands 8p11.2 and 9q12-13 within confluent fibroblasts. (A) The position of hybridization signals observed in 200 nuclei from control and MMC-treated confluent human fibroblasts are plotted in one quadrant of the nucleus. The outer curve represents the contours of an average sized nucleus. The inner curve corresponds to one quadrant of a circle with a radius equal to the median of the radial distances of the hybridization signals. Therefore, 50% of hybridization signals are located inside this circle. (B) The inter-homologue distance distribution of chromosome bands 8p11.2 and 9q12-13 was determined by analyzing 200 nuclei from control and MMC-treated confluent human fibroblasts. Cells with ascending inter-homologue distances are plotted against the inter-homologue distances.

observed in a small fraction of PCC spreads (Fig. 6B,C). In the same PCC preparations, the frequency of breaks in chromosomes 8 and 9 were analyzed (Fig. 6D-F). The frequency of MMC-induced breaks was about fivefold higher for chromosome 9 than for chromosome 8 (Fig. 6F). For both chromosomes, the majority of breaks were observed in (or near) the painted paracentromeric chromosome regions.

Colocalization of homologous chromosomes in XP fibroblasts

The heterodimeric protein ERCC1-XPF has been implicated in repair of cross-links and homologous recombination, besides its role in the NER pathway (Adair et al., 2000; Mu et al., 2000; Niedernhofer et al., 2001). Exposure of confluent XPF fibroblasts derived from two different patients to 4 μM MMC for 1 hour did not result in a significant change in the colocalization of the 9q11-12 bands of the homologues of chromosome 9 (Fig. 7). XPA cells, which are completely deficient in NER, responded to MMC treatment similarly as normal human fibroblasts indicating that the observed pairing is NER independent (Fig. 7). Other treatment regimes including higher doses of MMC, longer exposure times and different recovery times failed to show an increased colocalization of chromosome 9 in XPF fibroblasts (data not shown). The percentage of cells with colocalizing chromosome bands 8p11.2 in the repair-deficient fibroblasts was similar to the wild-type fibroblasts (data not showm).

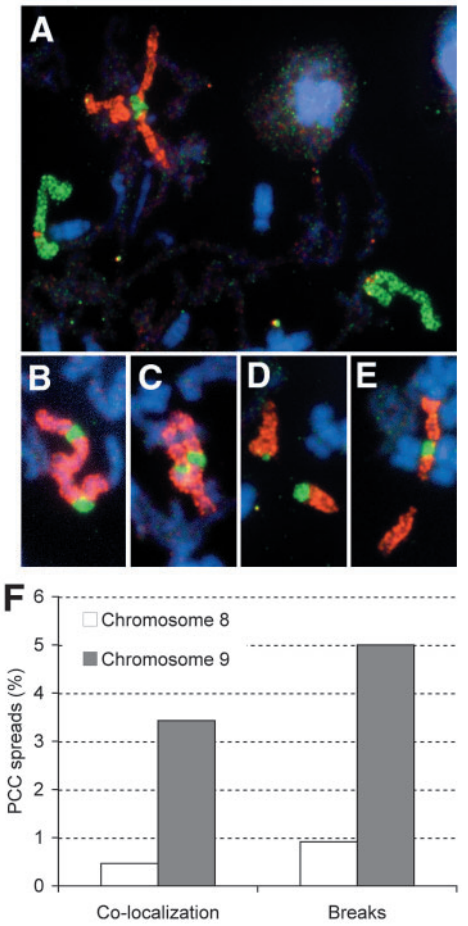

Fig. 6. PCC analysis of MMC-treated human lymphocytes. (A-E) Sections of PCC spreads of MMC-treated G_1 human lymphocytes after in situ hybridization using whole chromosome-specific probes for chromosome 8 (green) and 9 (red) together with band-specific probes 8p11.2 and 9q12-13 in reversed colors. Colocalization of the chromosome 9 homologues was observed at the position of the heterochromatic bands (A) or at other part of chromosome 9 sometimes leading to a partial alignment (B,C). Also, chromosome breakage within the green-painted heterochromatic band (D) or in an arm of chromosome 9 (E) was frequently observed. (F) The induced colocalization of homologous chromosomes 8 or 9, or breaks in these chromosomes in G_1 human lymphocytes after treatment with 4 μM MMC for 1 hour (background frequencies are deduced). The induced colocalization and breaks were significantly different (P<0.01, Wilson's method) between chromosomes 8 and 9.

Interphase chromosome repositioning correlates with chromatid exchanges observed at metaphase

Normal human cells are proficient in the gathering of chromosomes 9 upon MMC treatment and these cells frequently display chromatid interchanges between the homologous chromosomes 9 at metaphase. XPF cells, however, are deficient in pairing of chromosomes 9 in interphase cells. To assess the role of XPF in the formation of chromatid exchanges and to correlate MMC-induced chromatin pairing at interphase and chromatid exchanges at metaphase, we analyzed the frequency of chromatid exchanges induced by 4 μM MMC in lymphoblastoid cell lines derived from a healthy individual and from a XPF patient. The genomic frequency of chromatid interchanges in the wild-type lymphoblastoid cells was about 0.45 per cell at both 28 hour and 40 hour intervals after treatment (Fig. 8A). This frequency is comparable with that observed using lymphocytes (0.38, Fig.

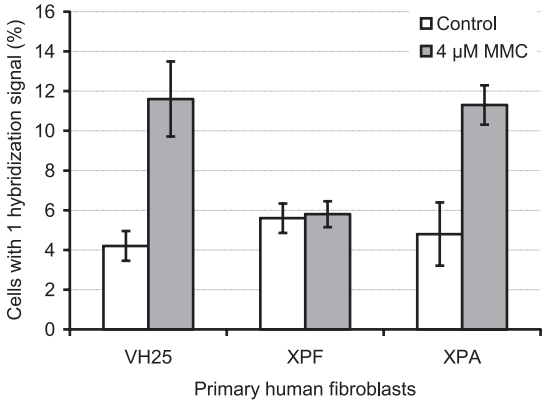

Fig. 7. Colocalization of chromosome 9 homologues in wild-type and DNA repair-deficient fibroblasts. Percentage of cells with one hybridization signal for chromosome 9p12-13 band-specific probe observed in wild-type human fibroblasts (VH-25) and fibroblast cell lines derived from XPA and XPF patients. Confluent cells were treated for 1 hour with 4 μM MMC and fixed directly afterwards. Analysis was preformed manually in 500 cells per experiment. The average percentage of at least three experiments is presented together with the standard deviation of the mean values. A significant increase (P<0.05, Student's t-test) in the number of cells with one hybridization signal was observed for wild-type and XPA fibroblasts. In XPF cells there was no significant change; the data were taken from cells of two different XPF patients.

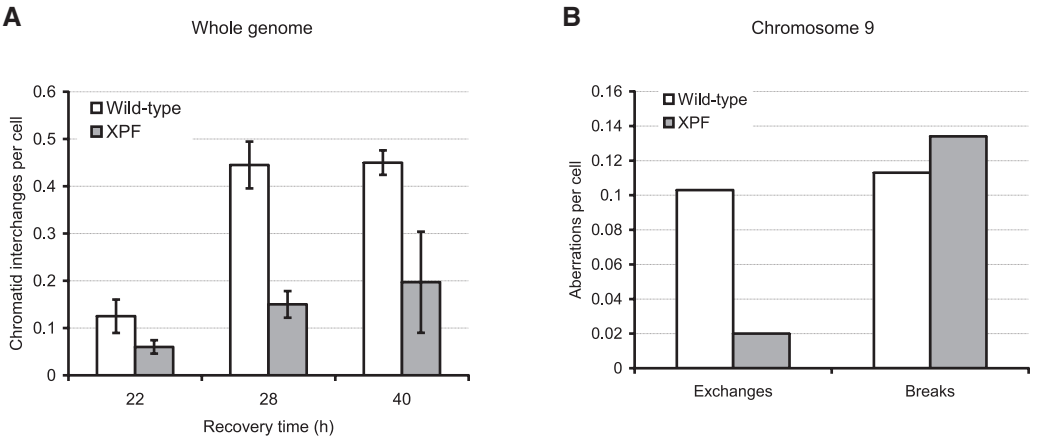

Fig. 8. Chromatid interchanges and breaks induced by MMC in wild-type and XPF lymphoblastoid cells. (A) Genomic frequencies for chromatid interchanges as observed in wild-type and XPF lymphoblastoid cells. Exponentially growing cells were treated with 4 μM MMC for 1 hour at different recovery times. Data from at least two experiments were analyzed. The difference between wild-type and XPF cells is significant at 28 hour and 40 hour time points ($P<0.05$, Student t-test). (B) Frequencies of chromatid exchanges and breaks observed for chromosome 9 after a recovery of 28 hours. A significant difference was observed only for exchanges ($P<0.01$, Wilson's method).

2A). In XPF cells, the yield of chromatid interchanges was significantly lower than that found in wild-type cells at all time periods examined (Fig. 8A). Importantly, XPF and wild-type human lymphoblastoid cells displayed a similar growth rate, mitotic index and cell cycle progression even after exposure to MMC (data not shown). We also examined the frequency of chromatid interchanges and breaks involving chromosome 9 in cells fixed at 28 hours post-treatment (Fig. 8B). In line with the whole genome analysis, chromosome 9 was significantly less involved in the exchanges induced in XPF cells compared with the wild-type cells. But breakage in chromosome 9 (mainly in the paracentromeric heterochromatin) was frequently observed in XPF cells making the ratio between breaks and exchanges about sevenfold higher in XPF cells than in the wild-type cells (Fig. 8B).

Discussion

Classic cytogenetic studies revealed an overrepresentation of human chromosomes 1, 9 and 16 in MMC-induced chromatid exchanges (Shaw and Cohen, 1965; Morad et al., 1973). Here we confirmed these findings by the application of FISH using a combination of whole chromosome and band-specific probes for chromosomes 1, 2, 8 and 9. The preferential involvement of chromosomes 1 and 9 is related to the presence of large paracentromeric heterochromatic bands in these chromosomes. These large C-bands might be particularly prone for cross-linking by MMC. Sequence-specific induction of DNA lesions by MMC is related to a particular order of repeated CpG sequences (Teng et al., 1989; Borowy-Borowski et al., 1990) and is enhanced (two- to fourfold) when the cytosines of these MMC-susceptible sequences are 5-methylated (Johnson et al., 1995; Li et al., 2000; Li et al., 2001). Paracentromeric heterochromatic bands have been reported to contain GC-rich repeats and harbor highly methylated DNA (Miller et al., 1974; Gosden et al., 1975; Meneveri et al., 1993). In line with this, it has been shown recently that an antibody raised against 5-methylcytosine preferentially stained the juxtacentromeric regions of chromosomes 1, 9, 16 and Y (Koch and Strätling, 2004). In addition to a nonuniform distribution of MMC-induced interchanges among human chromosomes, a striking preference for homologous interactions, particularly between chromosome 9 homologues, was observed in this study. This is consistent with results obtained by the analysis of MMC-treated human lymphocytes using solid staining (Shaw and Cohen, 1965) and chromosome banding (Morad et al., 1973; Joseph et al., 1982a).

Homology itself appears to be an important factor in the formation of MMC-induced interchanges. In contrast with the C-bands of chromosome 1, 9 and 16, the noncentric C-band of the Y chromosome is almost unaffected in normal cells on MMC-treatment (Shaw and Cohen, 1965; Morad et al., 1973), despite a comparable size of the C-bands (Joseph et al., 1982b). However, in lymphocytes from a XYY male patient MMC treatment resulted in a frequent involvement of the Y chromosomes in interchanges, almost exclusively in Y-Y configurations (Schmid et al., 1981; Joseph et al., 1982b). Hence it is clear that also the C-band of chromosome Y is susceptible to MMC ICLs, but that sequence homology is required for a subsequent formation of chromosome interchanges.

The frequent involvement of homologous chromosomes in MMC-induced interchanges have been taken as cytological evidence for pairing of at least some of the human chromosomes (Shaw and Cohen, 1965; Brogger and Johansen, 1972). Obviously, somewhere between the moment of treatment (G_1) and analysis (mitosis), the homologous chromosomes must have been in physical contact to form the exchange. We used primary human fibroblasts to investigate the relative positioning of the homologues of chromosomes 8 and 9 in various stages of the cell cycle. Interphase analysis of confluent fibroblasts showed an apparently random positioning of chromosomes 8 and 9 centromeres, with a large range of inter-homologue distances. This result is in concordance with previous observations made in confluent human fibroblasts indicating that similar sized chromosomes occupy similar radial positions with a large range of inter-homologue distances (Sun and Yokota, 1999; Sun et al., 2000). Hence, close proximity of homologous chromosomes 9 cannot account for the frequently observed exchanges between the homologues. Instead, pairing of homologues 9 could occur at a later stage during cell cycle progression or can be triggered

by MMC treatment. We provide evidence that in untreated S-phase cells, pairing is not elevated when compared with G_0/G_1 fibroblasts and that treatment with MMC is able to induce pairing of homologous chromosomes 9 in a subset of cells independently of the cell cycle. Interestingly, the pairing frequency observed in fibroblasts correlates with the interchange frequency observed in the MMC-treated lymphocytes. Thus, the induced pairing might underlie the formation of interchanges between the homologues observed at metaphase. Unfortunately, direct comparison could not be made since insufficient numbers of human fibroblasts reach mitosis after treatment with MMC.

The above-described pairing of chromosome 9 heterochromatin was confirmed by image analysis revealing a decrease in the average inter-homologue distances. This indicates a repositioning of at least one of the homologues. Two distinct models have been put forth for how this pairing might take place. One of the models emphasizes active movement of chromatin to bring the homologous regions into contact. In the other model, contact formation depends on random movement of chromatin by diffusion, referred to as random walk motion (for a review, see Loidl, 1990). Studies in yeast and *Drosophila* showed that simple random contacts through diffusion are sufficient to allow homologous pairing (Marshall et al., 1997; Fung et al., 1998). Constrained diffusion motion confined to a subregion of the nucleus might explain the observation that pairing is restricted to a subset of cells. Most probably, pairing is only possible in those cells carrying the homologous chromosomes in sufficient close proximity.

Our observations suggest the involvement of active cellular processes to induce and/or maintain pairing. First, we show that MMC-induced pairing is absent in XPF cells with a defect in DNA cross-link repair. Second, in our previous study (Abdel-Halim et al., 2004) pairing of homologous chromosomes 9 was observed within minutes after exposure to X-rays. At low temperature this extremely fast response was not observed. Both observations argue against pairing being based on random diffusion. Particularly, diffusion-controlled processes are known to be rather independent of temperature (Hoogstraten et al., 2002).

Not only repositioning of the heterochromatin is required for pairing but also the stability of the paired chromatin might be critical. We cannot rule out that the extent of pairing reflects equilibrium between association and dissociation of homologous chromosome regions. The fact that pairing frequencies were not reduced during recovery periods up to 20 hours might favor a stable association of the paired homologues. Additional support for a stable association comes from PCC analysis of G_1 lymphocytes. Despite the destructive nature of this technique, MMC-induced association of the heterochromatic regions of chromosome 9 was evident. Apparently, once the physical association is established, the homologous chromosomes are firmly held together. The association might last till late S-phase when the heterochromatin is replicated and ICLs are repaired. In case of misrepair the former association is converted into a permanent interaction between the homologues, i.e. the formation of an exchange aberration. To sustain a possible correlation between interphase pairing and the formation of metaphase exchanges, misrepair should be a common event in the paired heterochromatin regions. Indeed, we found that XPF fibroblasts were deficient in pairing of homologues 9, and that XPF lymphoblastoid cells exhibited a reduced frequency of chromatid interchanges when compared with wild-type cells. This reduction was even more pronounced when only exchange aberrations involving chromosome 9 were considered.

The heterodimeric ERCC1-XPF appears to have a key role in the repair of ICLs (De Silva et al., 2000; De Silva et al., 2002). It has been proposed that the ERCC1-XPF complex is involved in the removal of non-homologous ssDNA tails during recombinational repair of ICL by single-strand annealing (Adair et al., 2000; Sargent et al., 2000). Pairing of certain homologous chromosome regions as observed in the present study might reflect an initial step in cross-link repair. The fact that XPA cells are not deficient in pairing proves that the pairing is not dependent on NER. This gives a clue that the XPF protein is either required to trigger pairing or alternatively, might be required to prevent dissociation once homologous regions have paired. The reduced yield of MMC-induced chromatid interchanges in XPF lymphoblastoid cells supports this hypothesis. Incomplete ICL repair, leading to chromosome breaks as intermediate repair product, seems not to be affected by the absence of the XPF protein. Alterations in chromosome exchange to break ratio were also observed in ERCC1/XPF-deficient mouse and hamster cells after exposure to UV light (Melton et al., 1998; Chipchase and Melton, 2002).

Most models propose that recognition and repair of ICL in human cells occur primarily during the S-phase. However, recently a refined model was presented in which ICLs are recognized and incised by ERCC1/XPF independently of DNA replication (Rothfuss and Grompe, 2004). The incised ICLs are then processed further in S-phase where misrepair leads to chromosome exchanges and breaks. Present data are consistent with the initiation of ICL repair prior to S-phase, however, our PCC analysis suggests additional processing in G1 phase leading to the formation of chromosome breakage.

In summary, the frequent involvement of heterochromatic regions of chromosome 9 homologues in MMC-induced chromatid-type exchange aberrations appears to correlate with pairing of these regions directly on treatment. Probably owing to constrained chromatin mobility the pairing is observed in only a subset of cells. We propose a model in which paired homologous regions are firmly held together and are subsequently converted into chromatid interchanges during S-phase. The absence of pairing in XPF cells point towards the involvement of an active cellular mechanism in the pairing process. Alternatively, XPF might be required to maintain the association of homologous chromosomes. Pairing might be initiated by a general cellular stress response as treatment with different genotoxic agents such as X-rays (Abdel-Halim et al., 2004) and MMC resulted in a similar level of pairing of heterochromatin. In this respect, the recent observation of accumulation of heat shock transcription factor 1 (HSF1) at the paracentromeric region of chromosome 9 in heat-shocked human cells (Jolly et al., 2002) could represent a link between stress and pairing. Future investigations aimed to elucidate the underlying mechanism of pairing should consider both homology-related DNA repair pathways and stress-related alteration in chromatin organization.

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