End-joining of reconstituted histone H2AX-containing chromatin in vitro by soluble nuclear proteins from human cells

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Abstract Non-homologous end-joining is an important pathway for the repair of DNA double-strand breaks. This type of DNA break is followed by the rapid phosphorylation of Ser-139 in the histone variant H2AX to form γ-H2AX. Here we report efficient in vitro end-joining of reconstituted chromatin containing nucleosomes made with either H2A or H2AX. This reaction is catalyzed by nuclear extracts from human cells and this end-joining is not suppressed by the PI-3 kinase inhibitor wortmannin. During the end-joining reaction H2AX is phosphorylated at Ser-139 as detected by immunoblot with specific antibodies and this phosphorylation is inhibited by wortmannin. Therefore, in vitro the DNA end-joining reaction appears to be independent of H2AX phosphorylation.

Key words: Reconstituted chromatin; DNA end-joining; Histone H2AX; Phosphorylation

1. Introduction

The correct repair of DNA double-strand breaks (DSBs) is an important mechanism for maintaining the stability of mammalian genomes. Two major pathways of DSB repair are known: non-homologous end-joining (NHEJ) which is controlled by a DNA-dependent protein kinase, the protein XRCC4 and DNA ligase IV [1–3], and homologous recombination (HR) which is controlled by the mammalian homologs and paralogs of the yeast RAD50 and RAD51 proteins [4,5]. It was shown that the formation of DSBs in mammalian cells induces the rapid phosphorylation of Ser-139 in the histone H2AX [6,7]. This histone modification is important for DSB repair in vivo, since inactivation of the H2AX gene in mice results in radiosensitivity and chromosome instability [8]. It has been suggested that chromatin containing γ-H2AX promotes assembly of DSB repair complexes [8,9] but the molecular basis of this process remains to be established.

Here we describe an in vitro end-joining assay to analyze the interactions of chromatin with DSB repair proteins. Human histones H2A, H2AX, H2B, H3 and H4 were used to reconstitute chromatin of defined content on a 3.8 kb DNA substrate. Incubation of chromatin with nuclear extract led to end-joining with a much higher efficiency than that of naked DNA. This nuclear extract was found to catalyze the formation of γ-H2AX, however, the end-joining reaction was independent of the presence of H2AX in the chromatin.

2. Materials and methods

2.1. Human cell lines and isolation of nuclear extracts

Cell lines A549 and IMR-90 were obtained from the ATCC, cell line CRL-7201 from Coriell Cell Repositories. A549 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Primary fibroblasts of the cell lines CRL-7201 and IMR-90 were maintained in DMEM with 10% FCS. Cells were washed in PBS and then incubated in two volumes (packed cells) of hypotonic buffer (20 mM Tris–HCl pH 8, 1 mM dithiothreitol (DTT)) for 30 min on ice. The cells were then disrupted by passing five times through a 24-gauge needle. Protease inhibitor mix (Roche) was added and the nuclei pelleted by centrifugation. Nuclei were extracted by incubation for 30 min on ice in 0.5 M KCl, 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM DTT and the soluble nuclear extract was separated from cell debris by centrifugation for 1 min in an Eppendorf centrifuge.

2.2. Expression and purification of recombinant human histone H2AX (rhH2AX)

A rhH2AX gene was constructed from a *Xenopus* histone H2A pET3a kindly provided by K. Luger [10]. First, part of the *Xenopus* H2A gene was replaced with bacterially optimized codons for the carboxy-terminal 13 amino acids of human H2AX using a 'touchdown' PCR method [11] along with Thermozyme™ DNA Polymerase (Invitrogen), and a combination of the following overlapping primers (Gibco-BRL): T7 promoter CCCGCGAAATTAATACGACTCA; H2AXC1 TTCGGACCAACGGTAGCGGAGGTTTTCTTGGGCAGCAGCACGGA; H2AXC2 GGGTAGCTTTTTTACCACCGGACGGAGCTTTCGGACCAACGGTAGCGGAGGTT; H2AXC3 GGATCCTTAGTATTCCTGGGAAGCCTGGGTAGCTTTTTTACCACCGGACGGAG.

Next, 14 codon changes were made to produce a bacterially optimized rhH2AX gene using the following primers (Gibco-BRL): X1A ATGTCTGGACGTGGCAAAACCGGCGGTAAAGCTCGCGCTAAGGCCAAGTCTCGCTC; X1B GAAGGAGATATACATATGTCTGGACGTGGCAAAACCG; X2 TCTGCATAATGGCCTTTCCGTAACAGCCG; X3 GGCTGTTACGGAAAGGCCATTATGCAGAGCG; X4 ACCTCCGAGCAGTTTGTTCAGTTCCTCATCGTTGCGGATAGCGAGCTGC; X5A CAGGGCGGTGTCCTGCCGAACATCCAGGCCGTGCTGCTGCCGAAGAAAAC; X5B GAACAAACTGCTCGGAGGTGTCACTATCGCTCAGGGCGGTGTCCTGCCGAAC; X5C ATCCGCAACGATGAGGAACTGAACAAACTGCTCGGAGGTGTC; X6 CCAACTCAGCTTCCTTTCGGGCTTTG.

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Abbreviations: NHEJ, non-homologous end-joining; DSB, double-strand DNA break; DTT, dithiothreitol; AFM, atomic force microscopy; rhH2AX, recombinant human H2AX; NCP, nucleosome core particle

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Primer sets X1A/X1B with X2, X3 with X4, and X5A/X5B/X5C with X6 were used for PCR and then 0.25 μl from each reaction was combined in a fourth reaction containing the primer pair X1B and X6 to PCR ligate the overlapping gene fragments into a 488 bp rhH2AX gene cassette. This gene construct was subcloned into pCR2.1 (Topo™TA Cloning®, Invitrogen) and grown in Top10 *Escherichia coli*. Standard methods [12] were used to subclone the *Nde*I/*Bam*HI (New England Biolabs) rhH2AX coding cassette into pET3a. H2AX-pET3a was sequenced and then transformed into BL21(DE3) pLysS for IPTG-inducible protein expression (Novagen).

Inclusion bodies were isolated from the bacterial lysate and dissolved into 7 ml of 7 M guanidinium hydrochloride, 20 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol per liter of cell culture [10]. The dissolved protein was filtered (0.45 micron) and loaded onto a Superose 12 column (2.5×100 cm). FPLC was conducted at a flow rate of 1 ml/min and the entire run was complete after 500 ml of buffer (7 M urea, 1.2 M NaCl, 20 mM sodium acetate pH 5.2, 5 mM β-mercaptoethanol, 1 mM EDTA). The major H2AX fractions were combined and dialyzed in 8 kDa molecular weight cut off (MWCO) dialysis tubing (BioDesign Inc.) against three changes of 4 l distilled water at 4°C. Lyophilized protein was then dissolved in 1 ml of Inclusion Body Solubilization™ buffer (Genotech) and 4 ml of 7 M urea, filtered (0.45 micron), and purified to homogeneity by reverse phase high-performance liquid chromatography (HPLC) on a prep-scale Vydac C4 column (2.2×25 cm). A gradient of 30–60% acetonitrile (0.1% trifluoroacetic acid) in 60 min was used at a flow rate of 9.9 ml/min. The peak H2AX fractions were combined and lyophilized. The H2AX was checked by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Protein Resource Center, The Rockefeller University) and the protein concentration determined by amino acid analysis (Biomolecular Structure Lab, UC Davis).

2.3. DNA and histone purification

A plasmid (pUC208-18) containing substrate DNA was amplified in bacteria, isolated, and restriction-digested with *Cfo*I. The 3.8 kb fragment containing 18 repeats of a nucleosome positioning sequence was purified by MonoQ (Pharmacia) FPLC [13]. Purification of the individual HeLa cell core histones by acid extraction, ion exchange chromatography and C4 reverse phase HPLC was conducted as described previously [14]. Native chicken erythrocyte (C.E.) histone octamers were isolated from nuclei as described earlier [15].

2.4. Chromatin reconstitution and characterization

Stoichiometric amounts of the four core histones (either HeLa H2A, H2B, H3, and H4 or rhH2AX and HeLa H2B, H3, and H4) were combined with 25 μg of substrate DNA in a starting buffer containing 4 M urea and 1.8 M NaCl in a microdialyzer using 8 kDa MWCO membrane (BioDesign Inc.). This mixture was equilibrated with buffer A (7.4 M urea, 2 M NaCl, 20 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol). A linear gradient of buffer A and buffer B (2 M NaCl, 20 mM Tris–HCl pH 7.5, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM PMSF) followed by a gradient of buffer B and buffer C (10 mM NaCl, 10 mM triethanolamine pH 8.0, 0.2 mM EDTA, 5 mM β-mercaptoethanol) was used for chromatin reconstitution [16]. An aliquot of each sample for use in microscopy was crosslinked in buffer C containing 0.15% glutaraldehyde for 15 h and then equilibrated with buffer C. All dialysis was conducted at 4°C and each gradient was pumped over 7 h. Samples of the reconstituted chromatin (1.5 μg) were digested with 0.1 unit of micrococcal nuclease in buffer supplemented with 1 mM calcium at 37°C for 10 min. The reaction was stopped by addition of EDTA. The digested chromatin core particles were then examined on a native polyacrylamide gel (5% acrylamide, 0.5×TBE buffer pH 8.0) confirming proper nucleosome core reconstitution.

2.5. Atomic force microscopy (AFM)

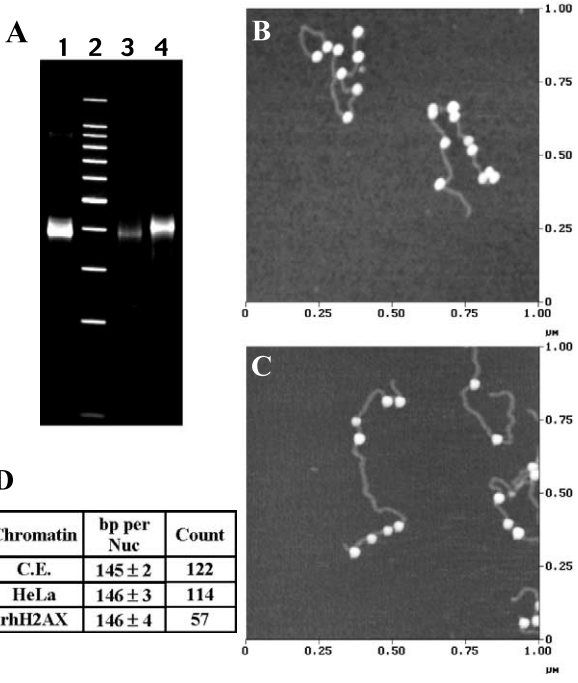
Crosslinked chromatin was diluted to approximately 3 ng/μl in 0.2 mM $MgCl_2$ and immediately deposited (12 μl) onto freshly cleaved mica. After 5 min the mica was rinsed with 2 ml of Nanopure water (Millipore), dried with nitrogen gas and imaged at ambient temperature and humidity using a Nanoscope 3a AFM (Digital Instruments). Three micron images were scanned at a rate of 1.3 Hz with an FESP tip (Digital Instruments) using Tapping mode. Images were processed, rendered, and analyzed using Digital Instruments Nanoscope software and Scion Image (Scion Corp.).

2.6. End-joining reactions and immunoblot analysis of γ-H2AX

Reaction mixes (50 μl each) contained 20 mM Tris–HCl pH 7.5, 1 mM ATP, 10 mM DTT, 10 mM $MgCl_2$ (if not otherwise indicated), 2 μl of nuclear extract (2–3 μg of total protein) and 200 ng of the 208-18 DNA substrate or the equivalent amount of 208-18 reconstituted chromatin. After incubation for 90 min at room temperature 2 μl of solution containing 20 μg of proteinase K was added and incubation continued for 60 min at 37°C. The reaction products were separated in a 0.5×TBE 0.6% agarose gel. The gel was stained with ethidium bromide and images captured using a CCD camera (Alpha Inotech) with a UV transluminator (UVP). Western blot analysis of γ-H2AX was conducted as previously described [17].

3. Results

The 3.8 kb DNA substrate (208-18 DNA), HeLa cell core histones, and bacterially expressed rhH2AX used in this study were all purified individually to homogeneity using gel filtration, ion exchange, and reverse phase chromatography. This allowed the reconstitution of chromatin with exactly defined content. Micrococcal nuclease digestion of the H2AX chromatin (Fig. 1A, lane 4) produced nucleosome core particles (NCPs) indistinguishable from control erythrocyte (Fig. 1A, lane 1) and reconstituted HeLa NCPs (lane 3). Fig. 1B shows a typical image of the reconstituted H2AX-containing 208-18 chromatin visualized using AFM. This chromatin appears normal and was determined to contain an average of 10 nucleosomes per fiber ($n = 64$) with a standard deviation of 1.5 and a range of 6–14 nucleosomes. Naked DNA was not found in these samples. Similar results were obtained with chromatin containing normal histone H2A. Fig. 1C shows an AFM im-



D

Chromatin	bp per Nuc	Count
C.E.	145 ± 2	122
HeLa	146 ± 3	114
rhH2AX	146 ± 4	57

Fig. 1. Characterization of reconstituted chromatin. A: Micrococcal nuclease digestion of lane 1 – C.E. chromatin, lane 3 – HeLa chromatin, lane 4 – H2AX chromatin. Lane 2 shows a 100 bp DNA ladder. B, C: Images of reconstituted H2AX chromatin (B) and reconstituted HeLa (H2A) chromatin (C) obtained by AFM. The maximum vertical scale on AFM images is 12 nm (white). D: DNA packaging in reconstituted chromatin ± one standard deviation (bp per nucleosome) calculated from chromatin contour plots containing the given number of chromatin fibers (Count).

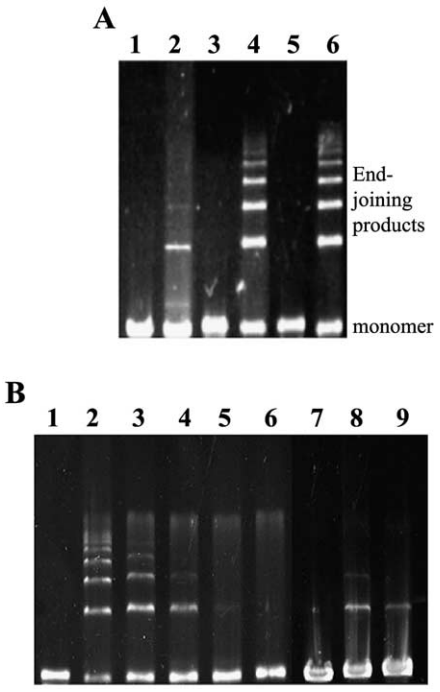


Fig. 2. Reconstituted chromatin and DNA end-joining by A549 human cell nuclear extract. All images show ethidium bromide-stained 0.6% agarose gels. A: Products of end-joining in 10 mM $MgCl_2$ of naked 208-18 DNA (lane 2), H2A chromatin (lane 4) and H2AX chromatin (lane 6); lanes 1, 3 and 5 show corresponding controls without nuclear extract. In (B), lanes 2–6 reactions with H2AX chromatin were performed at 10, 5, 2.5, 1.25 and 0 mM $MgCl_2$, respectively. Lanes 8 and 9 show products of reactions with naked 208-18 DNA incubated at 10 and 1.25 mM $MgCl_2$. Lanes 1 and 7 show controls with H2AX chromatin and DNA without nuclear extract.

age of reconstituted HeLa control chromatin (containing normal histone H2A) also used in our experiments. Analysis of many AFM images of the chromatin allowed chromatin contour plots to be made comparing nucleosome loading to fiber length [23]. From these plots the DNA packaging values of the various chromatin were calculated and found to be 146 bp per nucleosome as expected for normal nucleosome cores (see the table in Fig. 1D).

After incubation of the reconstituted H2A- or H2AX-containing chromatin with nuclear extract from human A549 cells followed by digestion of proteins with proteinase K, the DNA end-joining products were separated and analyzed in a 0.6% agarose gel (Fig. 2A, lanes 4 and 6). A control reaction performed with the 208-18 substrate DNA showed much less efficient ligation (Fig. 2A, lane 2) as compared to chromatin containing either H2A (lane 4) or H2AX (lane 6). This indicates that chromatin is a preferred substrate for the in vitro end-joining.

Sedimentation studies have shown that subsaturated nucleosome arrays do not condense efficiently even in 10 mM Mg^{2+}, however oligomerization does occur [24–26] which could promote end-joining. As shown in Fig. 2B (lanes 2–5), decreasing the $MgCl_2$ concentration in the reaction mix with H2AX chromatin from 10 to 1.25 mM strongly decreases end-joining efficiency but only slightly decreases end-joining of the 208-18 DNA substrate (Fig. 2B, lanes 8 and 9). It is obvious, however, that the naked DNA substrate end-joins poorly in

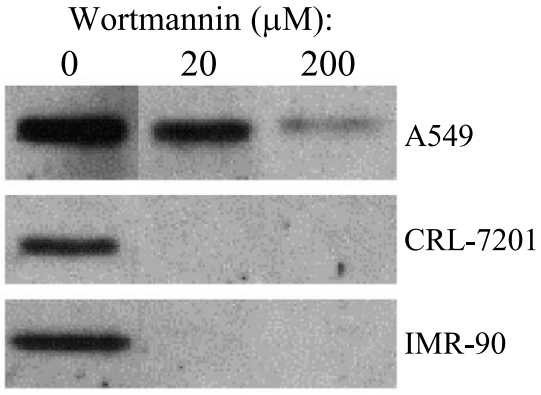


Fig. 3. Phosphorylation of histone H2AX during end-joining of H2AX chromatin by human nuclear extracts prepared from different cell lines. A 10 μl aliquot of the standard end-joining reactions (see Table 1 for results) was separated by SDS–PAGE and blotted using antibodies against γ-H2AX. Nuclear extracts were pre-treated at the indicated concentrations of wortmannin for 30 min on ice.

these reactions regardless of the Mg^{2+} concentration. At 1.25 mM $MgCl_2$ the 1 mM ATP in the reaction should chelate most of the Mg^{2+}, leaving only about 0.25 mM free which is too low to promote chromatin oligomerization [24] and possibly low enough to beginning affecting the rate of the ligation reaction. These results are consistent with the view that Mg^{2+}-mediated chromatin association facilitates in vitro end-joining possibly by juxtaposing the DNA ends for ligation.

To determine whether H2AX is phosphorylated at Ser-139 during the in vitro chromatin ligation we immunoblotted the reaction mixes with antibodies against a Ser-139 phosphorylated H2AX peptide. In vivo DSB-induced phosphorylation of H2AX in chromatin is known to be inhibited by pre-treatment of cells with wortmannin, an inhibitor of PI-3 kinases [9,18]. We pre-incubated the nuclear extracts from three human cell lines with different concentrations of wortmannin. Fig. 3A shows that γ-H2AX is formed during the in vitro end-joining and that this phosphorylation is inhibited by wortmannin. However, in these reactions chromatin end-joining is not inhibited (Table 1) by the same concentrations of wortmannin (20 and 200 μM) which inhibited γ-H2AX formation. This indicates that in vitro H2AX phosphorylation is decoupled from DNA end-joining. This conclusion is supported by the observation of efficient end-joining of the control chromatin (Fig. 2A, lane 3), containing histone H2A which lacks a C-terminal SQEY recognition sequence for PI-3 kinase phosphorylation.

Table 1
Wortmannin does not inhibit in vitro end-joining of chromatin

Cell line	[Wortmannin] (μM)		
	0	20	200
A549	44.3[a]	42.9	44.3
CRL-7201	41.0	39.6	40.6
IMR-90	41.5	39.8	41.1

[a]Percent end-joining products produced by extracts pre-treated for 30 min on ice with the indicated concentration of wortmannin (μM).

4. Discussion

We demonstrated here a nuclear extract-mediated end-joining reaction that preferentially and efficiently utilizes chromatin as a substrate instead of DNA. The reaction shows $MgCl_2$ dependence. A decrease of $MgCl_2$ concentration below levels known to promote chromatin self-association resulted in the same low efficiency of end-joining of both naked DNA and chromatin. This may indicate that chromatin oligomerization (or higher order organization) mediated by magnesium (and possibly other nuclear proteins) helps to promote the end-joining reaction and may be important for DSB repair in vivo. Surprisingly, chromatin composed of H2A-containing nucleosomes was end-joined just as efficiently as chromatin composed with H2AX. When H2AX was present, the reaction resulted in the phosphorylation of Ser-139 in histone H2AX and γ-H2AX formation was wortmannin-sensitive. However, the end-joining of chromatin was not inhibited by pre-incubation of nuclear extracts with wortmannin. This indicates that the in vitro phosphorylation of H2AX and chromatin end-joining may be uncoupled. Wortmannin-insensitive DNA end-joining reactions by mammalian cell extracts were described in many other studies, reviewed in [27]. Some workers have isolated extracts that catalyze wortmannin-sensitive DNA end-joining reactions [28], but it remains to be established whether these extracts are able to perform wortmannin-sensitive end-joining of chromatin.

Free histone H2AX in known to be phosphorylated in vitro at Ser-139 by a kinase present in mammalian nuclear extract [6]. In our experiments some free histones were present in the reconstituted chromatin preparations, so it remains unclear whether the observed H2AX phosphorylation occurs at chromatin bound H2AX (within nucleosomes) or only upon free histone. Although this question is of interest for future experiments it has no bearing on the present study since the in vitro end-joining of chromatin observed was not dependent on the presence of H2AX.

A strong correlation between DSBs and γ-H2AX formation was observed in vivo [6,7,9] suggesting the existence of mechanisms coupling H2AX phosphorylation and its elimination to DSB repair. It was shown that at least two wortmannin-sensitive protein kinases are involved in H2AX phosphorylation: ATM kinase [18] and ATR kinase [19], but the mechanism of γ-H2AX elimination from chromatin after DSB repair is unknown. This elimination should be linked to chromatin re-assembly after DSB repair [20]. ATM kinase is defective in the chromosome instability syndrome ataxia teleangiectasia (AT) and both AT cells and cells from ATM-deficient mice show strongly decreased H2AX phosphorylation after treatment with ionizing radiation [18]. However, human AT and mouse ATM (−/−) cells have no major defect in NHEJ repair of radiation-induced DSBs [21] indicating that the catalytic steps of end-joining in vivo may also be uncoupled from H2AX phosphorylation. Alternatively, other protein kinases may be involved in the formation of γ-H2AX.

DSB repair in vivo includes not simply DNA end-joining but also the prevention of rejoining incorrect DNA ends (misjoining) and the control of unequal HR between repeats (misrecombination). The frequency of DNA misjoining is increased in human AT cells [22] and the cells from H2AX knockout mice show remarkable chromosome instability [8]. These observations suggest that while H2AX is not necessary for chromatin end-joining, the formation and removal of γ-H2AX may be critical in the control of misjoining.

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