DNA double-strand breaks induce formation of RP-A/Ku foci on in vitro reconstituted *Xenopus* sperm nuclei

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SUMMARY

Replication protein A (RP-A) is involved in DNA replication, repair and recombination. It has been demonstrated that RP-A clusters in foci prior to DNA replication and redistributes over chromatin during S-phase. Here, we show that RP-A foci also form in response to DNA double-strand (ds) breaks produced on *Xenopus laevis* sperm nuclei by restriction enzymes and then reconstituted with *Xenopus* egg high-speed extracts. Ku86 co-localizes with RP-A in the same foci. An unscheduled RP-A-dependent DNA synthesis takes place overlapping with RP-A and Ku86 foci. Immunoelectron-microscopy analysis reveals that these foci correspond to spherical bodies up to 300 nm in diameter, which contain RP-A, Ku86 and DNA. In an independent in vitro assay, we incubated linear dsDNA bound to magnetic beads with *Xenopus* egg extracts. Here, also RP-A and Ku cluster in foci as seen through immunofluorescence. Both proteins appear to enrich themselves in sequences near the ends of the DNA molecules and influence ligation efficiency of ds linear DNA to these ends. Thus, the *Xenopus* in vitro system allows for the generation of specific DNA ds breaks, RP-A and Ku can be used as markers for these lesions and the repair of this type of DNA damage can be studied under conditions of a normal nuclear environment.

Key words: Reconstituted *Xenopus* sperm nuclei, DNA double-strand breaks, Replication protein A/Ku protein foci, Unscheduled DNA synthesis

INTRODUCTION

Replication protein A (RP-A, also known as SSB) is an ubiquitous protein complex, which plays essential roles in DNA replication, repair and recombination. In eukaryotes, it is composed of three subunits of 70 kDa, 32-34 kDa and 11-14 kDa (Wold, 1997). RP-A binds with high affinity (10⁹-10¹¹ M⁻¹) to single-stranded DNA (ssDNA) and with lower affinity (10⁶-10⁷ M⁻¹) to double-stranded DNA (dsDNA). During DNA replication, RP-A binds and stabilizes the ssDNA generated at the replication fork, it directly interacts with pol³-primase complex and stimulates the activity of polδ during the elongation phase (Fairman and Stillman, 1988; Kenny et al., 1989; Longhese et al., 1994). More recently, in an in vitro assay for rolling-circle DNA replication, RP-A has been shown to be directly required for efficient elongation of the DNA strand (Walther et al., 1999).

By using *Xenopus laevis* egg extracts to assemble nuclei around *Xenopus* sperm heads, RP-A has been localized to foci on chromatin prior to DNA replication (Adachi and Laemmli, 1992). Similar focal RP-A distribution has been found on somatic cells at the G1-S phase boundary (Murty, 1996). These RP-A pre-replication foci coincide with the sites of initiation of DNA replication as shown by the co-labeling of RP-A70 and incorporated dNTPs at the beginning of S phase (Adachi and Laemmli, 1992). Although the exact function of RP-A pre-replication foci is not known, it has been speculated that they might facilitate the assembly of the replication machinery perhaps through molecular crowding (Adachi and Laemmli, 1994; Newport and Yan, 1996).

As far as DNA repair reactions are concerned, it has been shown that RP-A participates in the nucleotide excision pathway (Sancar, 1996) and is required for mismatch repair (Lin et al., 1998). RP-A has a conserved stimulatory effect on in vitro DNA strand exchange reactions catalyzed by RAD51, the eukaryotic homologue of RecA (Baumann et al., 1997; Baumann and West, 1997; Thacker, 1999; Shinohara and Ogawa, 1998; New et al., 1998). Phenotypic analysis of mutant alleles of yeast RP-A70 has revealed a role for normal mitotic and meiotic recombination rates in vivo (Smith and Rothstein, 1995; Sugiyama et al., 1997). Interestingly, strains harboring RP-A mutations are very sensitive to DNA-damaging agents like γ-irradiation, methyl methane sulfonate (MMS) or strong UV doses, which provoke DNA double-strand breaks (dsb), indicating that RP-A plays an essential role in the repair of this type of DNA lesions (Longhese et al., 1994; Firmenich et al., 1995).
Among the factors involved in the repair of DNA dsb through the end-joining pathway (Kanaar et al., 1998; Karran, 2000), the DNA-PK/Ku protein complex plays an important role. It consists of the Ku80/Ku70 heterodimer (Taccioli et al., 1994; Smider et al., 1994; Boubnov et al., 1995) and the DNA-dependent protein kinase (DNA-PK) (Finnie et al., 1995; Smith and Jackson, 1999). The Ku heterodimer binds tightly to dsDNA ends and facilitates the loading of DNA-PK onto DNA (Gottlieb and Jackson, 1993; Dynan and Yoo, 1998); once bound to DNA, DNA-PK phosphorylates a number of proteins involved in the repair of DNA dsb, including RP-A middle subunit and p53 (Brush et al., 1994; Boubnov and Weaver, 1995). Yeast Ku mutants are strongly impaired in the repair of DNA dsb in the presence of mutated RAD52 (Bouton and Jackson, 1996).

Although mammalian cell-free systems have been developed to study the repair of DNA dsb on plasmid DNA substrates (Fairman et al., 1992; Mason et al., 1996; Baumann and West, 1998), there is as yet no in vitro system available in higher eukaryotes that would enable us to study DNA dsb repair in a nuclear environment. In this respect, in vitro reconstitution of functional nuclei with Xenopus egg extracts represents a suitable system for studying the role of factors involved in DNA dsb repair directly in such nuclei. This system is amenable to biochemical and functional studies of the factors involved in nuclear processes as DNA replication and repair (Almouzni and Wolffe, 1993). Interphase nuclei capable of replicating their DNA can be assembled around naked DNA or demembranated Xenopus sperm heads using crude interphase Xenopus egg extracts (LSS) (Lohka and Masui, 1983; Blow and Laskey, 1986; Newport, 1987). A high-speed supernatant (HSS) of the crude egg extracts supports rapid chromatin assembly (Philpott and Leno, 1992; Dimitrov and Wolffe, 1996) but not nuclear envelope formation and DNA replication (Sheehan et al., 1988). These HSS-treated nuclei, however, are prompted for DNA replication, which occurs only if membranes are added (Newport, 1987; Sheehan et al., 1988), or if the nuclei are incubated with nuclear extracts prior to the addition of HSS (Walter et al., 1998).

We used the Xenopus nucleus reconstitution system to analyze the dynamics of RP-A when only DNA dsb are produced in the sperm nuclei by addition of restriction enzymes. We have found through indirect immunofluorescence and immunoelectron microscopy (immunoEM) that RP-A clusters in distinct foci together with the Xenopus homolog of Ku80 (XKu80) and that a nuclear-envelope-independent DNA synthesis occurs in these foci. Moreover, our results have indicated that RP-A and Ku preferentially bind at the ends of linear dsDNA linked to magnetic beads and that both factors are required for a more efficient ligation of DNA ends.

**MATERIALS AND METHODS**

**Preparation of Xenopus egg extracts**

*X. laevis* females were primed and induced to lay eggs as described by Murray (Murray, 1991). Interphase extracts were prepared essentially as described by Adachi and Laemmli (Adachi and Laemmli, 1992; Adachi and Laemmli, 1994) with some minor modifications. After being washed with extraction buffer XB (10 mM HEPES/KOH pH 7.4, 250 mM sucrose, 50 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, 10 μg ml⁻¹ cytochalasin B, 1× protease inhibitor cocktail), the eggs were crushed by means of a 12 minute centrifugation in a Beckman HB4 rotor at 10,000 rpm (15,000 g) at 2-4°C. The turbid cytoplasmic layer was retrieved by syringe suction with a 20/21 gauge needle and, for the preparation of low speed supernatants (LSS), the 15,000 g spin was repeated once. In order to obtain high-speed supernatants (HSS), the turbid cytoplasmic layer was transferred into 1 ml ultraclear Beckman tubes, overlayed with paraflin oil and spun at 55,000 rpm (200,000 g) in a TLS55 swing-out rotor for 80 minutes at 2-4°C. The clear supernatant was retrieved by syringe aspiration with a 20/21 gauge needle. Both LSS and HSS were divided into 20 μl or 50 μl aliquots, frozen in liquid nitrogen and stored at −70°C. Mitotic extracts were prepared as described by Murray (Murray, 1991).

**Induction of DNA dsb on Xenopus sperm heads**

Demembranated Xenopus sperm heads were prepared according to Lohka and Masui (Lohka and Masui, 1983). In order to produce dsb on demembranated sperm heads, the following standard mix was prepared (RE mix): 40,000 demembranated sperm heads (~200 ng of DNA, diluted in XB buffer), 0.2 units of the chosen restriction enzyme (RE) in a final volume of 20 μl with XB buffer and incubated for 20 minutes at 23°C. For inactivation, the RE was put at 95°C for 5 minutes. Control DNA digestion was performed by adding a similar amount of plasmid DNA (e.g. puC19) per unit enzyme. Prior to using the RE digested sperm in the immunofluorescence assay, the RE mix was cooled on ice for 5 minutes. Alternatively, the RE digested sperm were centrifuged for 10 minutes at 6,000 rpm (3300 g) in a Hereus minifuge, the supernatant was removed and the sperm pellet was carefully resuspended in a 20 μl XB buffer.

**Immunofluorescence assays**

The standard nuclei assembly mix for immunofluorescence consisted of: 5 μl of freshly thawed egg extract (HSS or LSS), 5000 intact or RE-digested sperm heads, an ATP regenerating system (2 mM ATP and 20 mM creatin phosphate, 0.5 μg creatin kinase) in a final volume of 10 μl with XB buffer and incubated for 20 minutes at 23°C. For temperature-dependence control, the mix was put at 4°C for 1 hour; for energy-dependence control, the ATP-regenerating system was omitted and 5 μl final 5′-adenyllylimidodiphosphate (AMP-PNP) was added instead. The ss/dsDNA competition experiments were performed as described (Adachi and Laemmli, 1994). In order to label the sites of DNA synthesis, biotinylated dUTP/dATP were added at 0.2 mM (final concentration) to the assembly mix.

After incubation, the assembly mix was placed on ice for 5 minutes and then fixed for 5 minutes with freshly dissolved paraformaldehyde (2.5% final concentration in XB). The fixed mix was spun onto coverslips through a 0.7 ml of freshly thawed egg extract (HSS or LSS), 5000 intact or RE-digested sperm heads, an ATP regenerating system (2 mM ATP and 20 mM creatin phosphate, 0.5 μg creatin kinase) in a final volume of 10 μl with XB buffer. After two quick washes with PBS, the coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 20 minutes, incubated for 1 hour with the primary antibodies diluted in 3% BSA/PBS (affinity-purified rabbit anti-RPA70/34/14 and anti-XKu86 antibodies: 1:100; affinity purified chicken anti RPA70 antibodies 1:50; Streptavidin-FITC (Molecular Probes) 1:200) washed three times (5-10 minutes each) with PBS and PBS, the coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes at 23°C. For temperature-dependence control, the mix was put at 4°C for 1 hour; for energy-dependence control, the ATP-regenerating system was omitted and 5 μl final 5′-adenyllylimidodiphosphate (AMP-PNP) was added instead. The ss/dsDNA competition experiments were performed as described (Adachi and Laemmli, 1994). In order to label the sites of DNA synthesis, biotinylated dUTP/dATP were added at 0.2 mM (final concentration) to the assembly mix.

After incubation, the assembly mix was placed on ice for 5 minutes and then fixed for 5 minutes with freshly dissolved paraformaldehyde (2.5% final concentration in XB). The fixed mix was spun onto coverslips through a 0.7 ml of XB +30% sucrose cushion for 5 minutes at 3000 rpm in a Beckman HB4 rotor. After two quick washes with PBS, the coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 20 minutes, incubated for 1 hour with the primary antibodies diluted in 3% BSA/PBS (affinity-purified rabbit anti-RPA70/34/14 and anti-XKu86 antibodies: 1:100; affinity purified chicken anti RPA70 antibodies 1:50; Streptavidin-FITC (Molecular Probes) 1:200) washed three times (5-10 minutes each) with PBS and PBS, the coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes at 23°C. For temperature-dependence control, the mix was put at 4°C for 1 hour; for energy-dependence control, the ATP-regenerating system was omitted and 5 μl final 5′-adenyllylimidodiphosphate (AMP-PNP) was added instead. The ss/dsDNA competition experiments were performed as described (Adachi and Laemmli, 1994). In order to label the sites of DNA synthesis, biotinylated dUTP/dATP were added at 0.2 mM (final concentration) to the assembly mix.
owing to the lower amount of DNA synthesis. 0.25 mM final 5-bromo-
sample whereas, for the RE digested sperm, 150
measure replicative DNA synthesis, 30
described in Blow and Laskey (Blow and Laskey, 1986). In order to
loaded in a 0.8% agarose gel in 1
K added and incubated at 56°C for 2 hours. The samples were then
8, 1 mM EDTA, 100 mM NaCl, 1% SDS) containing 10
immediately in the different assays.

In order to cut open the plasmid molecules, which were bound to
Heald et al. (Heald et al., 1996) with minor modifications. In detail,
DNA synthesis (replicative and repair) was monitored by adding 1
µCi of α32P-dATP to every 4 µl of HSS or LSS extract used to the
assembly mix. After 1 hour and 2 hours, respectively, at 23°C, aliquotes of 6 µl were taken, 6 µl TENS buffer (10 mM Tris/HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1% SDS) containing 10 µg proteinase K added and incubated at 56°C for 2 hours. The samples were then
loaded in a 0.8% agarose gel in 1× TBE buffer and run for 4-5 hours at 100 V. The gel was then dried and exposed to X-omat photographic film.

cesium chloride density gradients were basically performed as described in Blow and Laskey (Blow and Laskey, 1986). In order to measure replicative DNA synthesis, 30 µl of LSS were used for each
sample whereas, for the RE digested sperm, 150
measure replicative DNA synthesis, 30

Immunoelectron microscopy

After incubation with HSS, intact or damaged sperm nuclei were collected by centrifugation through a 30% sucrose cushion in Xb and immediately fixed with 8% formaldehyde in 0.2 M Hepes-KOH pH 7.4 or with 2% glutaraldehyde in the same buffer for 3 hours and then stored in 8% formaldehyde/0.2 M Hepes-KOH before EM processing for up to 4 days. After several rinsings in cold PBS, the glutaraldehyde-fixed samples were postfixed in 2% osmium tetroxide in 0.2 M phosphate buffer at 4°C for 1 hour, washed thoroughly in 50% ethanol and contrasted in 1% uranylacetate/50% ethanol for 30 minutes. The dehydration was continued by increasing the concentration of ethanol, then propyleneoxide was added and, finally, the samples were embedded in Epon.

Paraformaldehyde-fixed sperm nuclei were embedded in Lowicryl
K4M or processed for cryosectioning according to Tokuyasu
(Tokuyasu, 1989). For Lowicryl embedding, the specimens were washed in PBS, encapsulated in low melting point agarose (Sigma, 2% in PBS), gradually dehydrated in ethanol at decreasing temperatures down to −35°C, infiltrated in Lowicryl K4M with UV-induced polymerization at −35°C, basically according to Armbruster et al. (Armbruster et al., 1983). For cryosectioning, sperm chromatin was embedded in gelatin blocks (10% gelatin in PBS), infused in a polynvinlypyrrolidone-sucrose mixture and frozen on copper pins.

Ultrathin Epon and Lowicryl sections were cut on a Reichert Ultracut ultramicrotome. Cryosections approximately 70 nm thick were obtained on Leica cryoultramicrotome using a Drukker diamond cryoknife and collected according to Tokuyasu (Tokuyasu, 1989).

For immunolabeling, Lowicryl sections and thawed cryosections were blocked in 10% of fetal calf serum (FCS) in PBS. Primary and secondary gold adducts (Jackson Immunoresearch Laboratories) were diluted in 5% FCS. The working dilution for anti-RPA70, 34 and 14 affinity purified antibodies was 1:10. The same dilution was used for anti-XKu86 affinity-purified antibodies. After extensive washing in PBS, the sections were postfixed in 2% glutaraldehyde/PBS for 30 minutes and then washed in water. Lowicryl sections were contrasted in a saturated solution of uranyl acetate for 30 minutes. Cryosections were embedded in a methylcellulose-uranyl acetate mixture (Griffiths et al., 1984).

Immunocytochemical in situ terminal transferase (TdT) reactions on ultrathin Lowicryl K4M sections were performed as described in Raška et al. (1995). Mouse anti-biotin 5 nm gold coupled antibodies were used for the detection of incorporated biotinylated bases. In the case of double labeling of biotinylated DNA and RP-A, 0.5% BSA in PBS was used for blocking and for the antibody dilution.

The ultrathin sections were examined with Philips CM100 or Opton EM 109 Turbo (Opton Feintechnik, Oberkochen, Germany) electron microscopes.

DNA beads

Plasmid DNA attached to magnetic beads was prepared according to Heald et al. (Heald et al., 1996) with minor modifications. In detail, 25-30 µg of plasmid DNA (pSP64 Sat-Sar8, 8258 bp in length (Strick and Laemmli, 1995)) were cut open at a single BamHI site and then filled in by Klenow according to the presence of 50 µM biotinylated (bio) dATP/dUTP for 2 hours at 37°C. In order to eliminate the unincorporated bio-dNTPs, NaCl (final 2 M) and polivinylalcohol (PVA, final 5%) were added. The resulting mix was incubated for 20 minutes on ice and then centrifuged for 20 minutes at 13,000 rpm in an Eppendorf minifuge at 4°C. The supernatant, which mainly contained the dNTPs, was discarded, the pellet enriched in the biotinylated plasmid DNA was resuspended in 100 µl of the bead wash buffer (10 mM Tris pH 8, 0.5 mM EDTA pH 8, 2 M NaCl) and PVA (final 5%) was added, put on ice for 10 minutes and centrifuged as before. This purification step was repeated three times. Finally, the DNA pellet was resuspended in 200 µl of 10 mM Tris pH 8, 1 mM EDTA pH 8 added to 130 µl of streptavidin-coated magnetic beads (Dynal M 280, 10 mg ml−1 solution) equilibrated in beads wash buffer, together with 150 µl of 10% PVA, 150 µl of 5 M NaCl, 37.5 µl of 1 M Tris pH 8, 3 µl of 0.5 M EDTA pH 8 and incubated on a rotating wheel at room temperature overnight. Using the magnet, the supernatant was removed and the beads were then washed two or three times in 500 µl of beads wash buffer and resuspended in 130 µl of the same buffer. The extent of DNA binding to the beads was followed by agarose gel/ethidium bromide staining and was always around 80-90% of the plasmid DNA input.

In order to cut open the plasmid molecules, which were bound to the beads with both biotinylated sites, the DNA beads were equilibrated with a buffer (NEB) and incubated with the enzyme (1 unit per 100 ng DNA) for 1 hour at 37°C. In this manner, one DNA fragment of ~3 kb and one of ~5 kb were left bound to the beads.
Prior to being used in the egg extracts, the DNA beads were equilibrated in XB buffer. Generally, the equivalent of 5 ng DNA bound to beads per μl egg extract was used. Immunofluorescence assays with DNA beads were done exactly as with the sperm nuclei.

For the isolation of the DNA end bound complexes after incubation with the egg extract, the beads were retrieved onto the magnet and extensively washed in XB buffer + 0.01% NP40, followed by two washes with the relevant restriction enzyme buffer and then incubated for 1 hour at room temperature with the chosen restriction enzyme. Three consecutive digestions were performed with EcoRI, EcoRV and PstI. This resulted in the consecutive liberation of 30 bp (EcoRI) and 230 bp (EcoRV) external fragments on the two DNA chains bound to the beads and one internal fragment of 700 bp (PstI).

For DNA ligation experiments, the beads were incubated with a pUC19 plasmid (Stratagene) end labeled with α32P-dATP in the presence of egg extracts. After 1 hour of incubation, the beads were extensively washed with XB buffer + 0.1% TWEEN 20, TE + 1% SDS buffer, BglII restriction buffer and then BglII enzyme was added (5 U per 100 ng DNA) and incubated for 2 hours at 37°C. The released fragments were retrieved in the supernatant by collecting the beads onto the magnet, loaded in a 1% agarose gel and run at 100 V for 5 hours in 1× TBE buffer.

Miscellaneous
DNA manipulations such as plasmid amplification and labeling were performed according to Sambrook et al. (Sambrook et al., 1989).

RESULTS
RP-A foci are induced on sperm nuclei incubated with restriction enzymes
Genetic analysis in yeast (Longhese et al., 1994; Longhese et al., 1996; Firmenich et al., 1995; Umezu et al., 1998) and in vitro assays using naked DNA and recombinant mammalian proteins (Shinohara and Ogawa, 1998; New et al., 1998) have shown that RP-A is required for dsb repair. However, the timing and mechanism of RP-A function in the process have not yet been determined.

We used in vitro reconstituted nuclei containing solely DSB to follow the dynamics of DNA repair factors such as RP-A. In order to generate DSB, demembranated Xenopus sperm heads were incubated with different restriction enzymes for 20 minutes at 23°C (see Materials and Methods). The successful digestion of the sperm heads was confirmed by the extent of electrophoretic migration in agarose gels compared with intact...
Xenopus nuclei lacking the double nuclear membrane (pronuclei) formed around the damaged sperm heads through the addition of HSSs of Xenopus egg extracts. Like undamaged nuclei, restriction enzyme-treated sperm heads swell gradually in the presence of the extracts (Dimitrov and Wolff, 1996; and data not shown). Affinity-purified anti-RP-A70 antibodies (Adachi and Laemmli, 1992) were used to monitor by indirect immunofluorescence the distribution of RP-A on intact versus restriction-enzyme-digested sperm pronuclei (Fig. 1A). When no restriction enzyme was added, some RP-A dots were seen (Fig. 1A, no addition). The number of dots was variable (Fig. 1A, no addition) but was lower, and had a lower intensity of labeling, than parallel experiments on digested sperm. After the incubation of the sperm chromatin with restriction endonucleases, RP-A70 (as well as RP-A34 and 14, data not shown) always clustered in numerous foci scattered along the sperm pronuclei as seen through confocal microscopy (Fig. 1A). RP-A foci were induced by several restriction enzymes but the number of foci per sperm nucleus varied depending on the specific conditions.

Fig. 2. RP-A foci on restriction-endonuclease-treated sperm chromatin form with different types of egg extracts. Immunofluorescence with purified rabbit anti-RP-A70 antibodies on intact sperm heads (Sperm) or sperm heads pretreated with 0.05 units of EcoRI (Sperm+RE) incubated with interphase HSS, interphase LSS or mitotic HSS for 1 hour at 23°C. A cumulative image of 10-12 confocal sections is shown for each sample. Bars, 5 μm.

Fig. 3. Unscheduled DNA synthesis (UDS) occurs at RP-A foci induced by restriction endonuclease. (A) Double immunofluorescence with affinity-purified rabbit anti-RP-A70 antibodies (RPA) and with streptavidin conjugated to FITC in order to reveal incorporation of biotinylated dNTPs (dNTPs). Demembranated sperm heads (~4000 heads) pretreated with 0.02 units of EcoRI (RE+HSS) or mock (HSS) are incubated for 1 hour at 23°C with HSS containing 20 μM biotinylated dATP and dUTP. Cumulative images representing 10-12 confocal sections for each fluorescence channel are shown. Most fluorescence foci owing to dNTP and RP-A signals co-localize as shown in the merged figure. It should be mentioned that, in some foci, there is an imbalance between the two color intensities, one being much higher than the other. Therefore, the yellow color in the merged figure is not that apparent in some co-localized foci (arrows). With mock pretreated sperm, the signal of the biotinylated dNTPs is barely visible. Bars, 5 μm. (B) Cesium chloride centrifugation gradient. Intact sperm heads were incubated with LSS extracts for 2 hours in the presence of [32P]dATP and with BrdCTP (LSS-HL) or without BrdCTP (LSS-LL). EcoRI-pretreated sperm heads (0.02 U EcoRI per 4000 sperm heads) were incubated with HSS extracts for 2 hours in the presence of [32P]dATP and BrdCTP (HSS RE). Equivalent amounts in sperm heads of LSS-HL and LSS-LL samples and five equivalents of HSS 43 fractions were taken from each gradient and their refractometric index as well as the total radioactivity measured. On the graph, the radioactivity expressed in arbitrary units (AU) is plotted for each fraction. The density of the fraction with the highest radioactivity value is written for each sample above the respective peaks.
endonuclease used (Fig. 1A, compare EcoRI, DraI, PstI). No RP-A foci were seen when damaged sperm nuclei were incubated with the HSS at 4°C or when a non-hydrolyzable ATP analog was added to the HSS (Fig. 1C, 4°C, AMP-PNP), which indicates that physiological temperature and ATP hydrolysis are required for the formation of these RP-A foci.

The number of RP-A foci correlated with the amount of the restriction enzyme used to digest the sperm heads (Fig. 1B, compare 0.02 U, 0.05 U and 1 U of EcoRI). Moreover, when a rare cutting restriction nuclease (NotI) was used in similar unit amounts, many fewer RP-A foci were formed per nucleus (Fig. 1B, compare 1 U EcoRI with 1 U NotI), whereas pUC19 plasmid DNA was linearized equally well by each of the two enzymes in these conditions (data not shown). Thus, the formation of RP-A foci on sperm nuclei can be triggered by DNA dsb.

In order to monitor the strength of the binding of RP-A to the damaged nuclei, competition experiments were performed with ds and ssDNA (Adachi and Laemmli, 1994). Restriction-enzyme-digested sperm nuclei were first incubated with HSS for 1 hour and then an excess of either ds or ssDNA was added (see Materials and Methods). RP-A was completely displaced from restriction-enzyme-induced foci only by ssDNA (Fig. 1C, compare +ssDNA and +dsDNA), suggesting that, like pre-replication centers (preRCs) (Adachi and Laemmli, 1994), restriction-enzyme-induced foci provide mainly low-affinity binding sites for RP-A. Interestingly, lower amounts of ssDNA (4 μg ml⁻¹) did not suffice for the displacement of RP-A (data not shown).

Restriction-enzyme-induced RP-A foci are sites of ‘unscheduled DNA synthesis’

Three types of Xenopus egg extracts can be obtained, which mimic different phases of the embryonic cell cycle: interphase HSS (G1), interphase LSS (S) and mitotic HSS (M) (Murray, 1991; Almouzni, 1998). We analyzed the requirement for a distinct cell cycle stage of the formation of RP-A foci in damaged sperm nuclei. As previously shown (Adachi and Laemmli, 1994), RP-A foci, albeit not as many, were obtained on intact sperm pronuclei only when interphase HSS was used (Fig. 2, interphase HSS). In intact nuclei, which are capable of replication, the RP-A signal was also positive (Fig. 2, interphase LSS). No RP-A labeling could be detected on mitotic condensed chromatin (Fig. 2, mitotic HSS). By contrast, upon being damaged, regardless of the type of extract used to assemble the nuclei, RP-A was always present in numerous foci (Fig. 2, sperm+RE).

RP-A foci formed on intact sperm nuclei co-localized with the sites of initiation of DNA replication only when membranes or LSS were added to the sperm pronuclei. Conversely, when sperm nuclei are assembled in HSS, no fluorescent signal corresponding to the incorporated labeled nucleotides (owing to DNA synthesis) is seen (Adachi and Laemmli, 1992; Fig. 3A, HSS, dNTPs). However, unscheduled DNA synthesis (UDS) caused by repair might occur on the damaged pronuclei even in the absence of a nuclear envelope. In order to test this possibility, biotin-labeled dNTPs were added to the damaged sperm nuclei and their localization was followed by immunofluorescence. The labeled dNTPs were indeed incorporated in the pronuclei and their signal overlapped to a large extent with RP-A foci (Fig. 3A, RE+HSS).

We further analyzed the migration of the newly synthesized DNA labeled with BrdCTP by cesium chloride density gradient centrifugation. Damaged sperm nuclei incubated with HSS were compared with intact sperm nuclei incubated with LSS. The complete substitution of one DNA strand with the heavier dCTP analog (which reflects replicative synthesis) sharply peaked at δ=1.4028 g ml⁻¹ (Fig. 3B, LSS HL), whereas unsubstituted DNA (obtained by the incubation of intact sperm with LSS but leaving out BrdCTP) migrated at density δ=1.4000 g ml⁻¹ (Fig. 3B, HSS RE). The broad peak of newly synthesized DNA in the case of the damaged sperm is found at an intermediate density value of 1.4009 g ml⁻¹ (Fig. 3B, HSS RL). This demonstrates that short fragments of DNA are synthesized, possibly as a result of single strand gap-fill DNA synthesis for the repair of dsb (Shinohara and Ogawa, 1995).

In conclusion, restriction-enzyme-induced RP-A foci clearly differ from preRCs because they are not restricted to a particular cell cycle stage and also because they co-localize with sites of DNA synthesis of the repair type under conditions when replicative DNA synthesis does not take place.

Restriction-enzyme-induced RP-A foci also contain Xenopus Ku86

The fact that RP-A foci form on nuclei in response to DNA dsb and harbor UDS suggest that these foci might represent active sites for DNA repair. For this reason, we checked whether other proteins known to be involved in the repair of DNA dsb localize to these foci. One of these factors, Ku, is a heterodimeric DNA-binding complex that is required for the non-homologous end-joining repair of DNA dsb in both Saccharomyces cerevisiae and mammals (Dyman and Yoo, 1998).

Affinity-purified anti-XKu 86 antibodies were used to localize XKu86 by immunofluorescence on reconstituted sperm nuclei (Fig. 4). In restriction-enzyme-damaged sperm, XKu86 localized to numerous foci, which co-localized with RP-A (Fig. 4B), whereas, in intact sperm, the XKu86 signal was different. The number and intensity of fluorescence foci were lower and the overall pattern was smoother (Fig. 4A). Moreover, in restriction-enzyme-treated sperm, the bulk of XKu86 foci co-localized with incorporated dNTPs (Fig. 4C) as was the case with RP-A (Fig. 3A).

In order to examine the roles of RP-A and Ku in the formation of foci and DNA repair synthesis, specific antibodies were used to deplete RP-A or XKu86 from the extracts (>95% removal as judged by western blots, data not shown). The damaged sperm were then incubated with the depleted extract in the presence of labeled dNTPs and immunofluorescence was performed (Fig. 5). The removal of XKu86 did not affect either DNA repair synthesis (Fig. 5B, dNTPs) or the localization of RP-A in the foci (Fig. 5B, RP-A). Conversely, the incorporation of labeled dNTPs in the damaged sperm nuclei decreased dramatically after RP-A depletion (Fig. 5A, dNTPs). Moreover, in the absence of RP-A, XKu86 signal appeared smoother (Fig. 5A, XKu86).

The presence of XKu86 in the restriction-enzyme-induced RP-A foci supports the concept that these structures are involved in the repair of the dsb. The fact that RP-A is required for efficient repair DNA synthesis might indicate that the activity of either polδ or polε, which are known to be
Ultrastructural analysis of restriction-enzyme-induced RP-A foci

In order to analyze restriction-enzyme-induced RP-A foci at the ultrastructural level, immunogold electron microscopy was performed on sections of damaged Xenopus sperm nuclei after incubation with egg extracts. By postembedding labeling and EM analysis (both Lowicryl and cryosections), these foci appeared as spherical bodies up to 300 nm in diameter, specifically labeled by antibodies against either of the three RP-A subunits and antibodies against XKu86 (Fig. 6; and data not shown). The RP-A/Ku bodies appeared to be the only distinguishable structures on the restriction-enzyme-treated sperm chromatin incubated with HSS. Interestingly, the ultrastructure of RPA-bodies assembled in intact Xenopus sperm heads in the presence of HSS (Eltsov et al., 2000) was similar to that of the enzyme-treated heads.

In order to obtain more information on the ultrastructure of the RP-A bodies, the samples were fixed with glutaraldehyde, stained with osmium and embedded in Epon. The RP-A foci exhibited a fine fibrillar ultrastructure that was already discernible in Lowicryl and thin cryo-sections, but better observed in Epon sections (Fig. 6A; and data not shown).

In order to know whether or not the RP-A labeled foci...
contained DNA, we performed a terminal transferase reaction on ultrathin Lowicryl sections by which labeled deoxynucleotides (biotin-dUTP) were covalently added to the exposed ends of DNA generated by the cutting procedure (Rasˇka et al., 1995). As shown in Fig. 6D, the RP-A bodies on damaged sperm nuclei contained DNA even though the level of labeling was lower than in the surrounding chromatin.

In conclusion, restriction-enzyme-induced RP-A foci can be discerned at the ultrastructural level as distinct bodies that contain RP-A, Ku and DNA.

RP-A and Ku bind close to DNA ends

We used DNA molecules bound to a solid phase in order to study at the molecular level the interaction of RP-A and XKu86 with DNA/chromatin at the dsb. Linear plasmid DNA containing biotinylated dNTPs was bound to magnetic beads and then incubated with Xenopus HSS (see Materials and Methods). These DNA beads have already been shown to form regular chromatin (Sandaltzopoulos et al., 1994) and nuclei can be assembled around them in the presence of Xenopus egg extracts (Heald et al., 1996; and data not shown). DNA bound to magnetic beads was added to HSS in amounts corresponding to 1000 sperm nuclei per ml extract and incubated for 1 hour at 23°C. Immunofluorescence was then performed on the DNA beads with anti-RP-A and anti-XKu86 antibodies. Both proteins clustered in distinct dots over the entire surface of the beads (Fig. 7).

The labeling pattern, absent from naked beads incubated with HSS (Fig. 7), consisted of tiny fluorescence dots and was somewhat reminiscent of the RP-A and Ku foci obtained with damaged sperm nuclei. Moreover, by changing the amount of DNA bound to the magnetic beads, the number of foci varied proportionally (data not shown). These results suggested that a significant amount of RP-A and XKu86 was bound to restricted sequences of DNA attached to the beads.

In order to map the location of RP-A and Ku at the molecular level with respect to the DNA dsb, three consecutive digestions by restriction enzymes were performed on the DNA bound to the magnetic beads. The fragments of 30 bp and 230 bp were consecutively cut off by restriction enzymes from the two extremities of the DNA linked to the beads, followed by a liberation of one internal fragment of 700 bp. The released material was examined by SDS-PAGE and western blotting. The 34 kDa subunit of the RP-A complex bound to DNA beads showed a slower migration than the RP-A present in the extract, which is known to be due to phosphorylation upon DNA binding (Blackwell, 1996; Niu et al., 1997) (Fig. 8, RP-A 34/34*, compare lanes 1 and 2). Strikingly, the amount of RP-A bound to the 30 bp close to the DNA ends was proportionally higher than the amount of RP-A bound to an internal DNA fragment of ~700 bp that resulted from the PstI digest (Fig. 8, RP-A compare lanes 4 and 8). Similarly, the amount of RP-A bound to the 230 bp external fragment was higher than that associated with the 700 bp internal fragment (Fig. 8 RP-A, compare lanes 6 and 8). Thus the ratio between the number of RP-A molecules and the...
bp of DNA to which RP-A is bound was larger close to the ends of the DNA. Although our data represent only a semiquantitative measurement, they indicate that RP-A was enriched at the DNA ends. This is in agreement with the presence of shining dots of RP-A by immunofluorescence (Fig. 7).

XKu86 does not seem to be as enriched as RP-A in the small 30 bp DNA fragment, but more XKu bound to DNA was found when a longer fragment (i.e. 230 bp) is released from the DNA ends (Fig. 8, XKu86, lanes 2, 4 and 6). This can be explained by the fact that Ku is known to ‘walk’ rapidly along the DNA and also to alternate quickly between DNA ends (Dynan and Yoo, 1998).

**XKu86 and RP-A are required for ligation of a dsDNA fragment to the DNA beads**

The ultimate goal in the repair of a DNA dsb is the joining of the two cut ends. In order to see whether or not RP-A/XKu86 are required for ligation of the DNA ends, DNA beads were used in which RP-A and XKu86 apparently concentrate at the DNA tips and form foci. A $^{32}$P-labeled linear DNA fragment (linearized pUC19, 2.7 kbp) was added to the DNA beads and mixed with HSS control or HSS depleted with anti-XKu86 or anti-RP-A antibodies. After 3 hours of incubation at 23°C, the beads were retrieved, extensively washed and incubated with the restriction enzyme Bg/II, whose cutting site was only present in the biotinylated DNA bound to beads (at ~5 kbp or 3 kbp from the ends). Two labeled bands ~7.7 kbp and 5.7 kbp were specifically released by Bg/II from the DNA beads after incubation with the control extract (Fig. 9, autoradiography; compare lanes 1 and 4) witnessing to the ligation of the $^{32}$P-labeled pUC19 to the ends of the DNA bound to the beads. When XKu86 was removed from the extract, neither of the two DNA bands were released in detectable amounts (Fig. 9, lane 3). Even though this experiment did not allow a straightforward quantitative evaluation, the RP-A depletion from the HSS also seemed to decrease the ligation efficiency to some extent (Fig. 9, lane 2).
In conclusion, XKu86 and, possibly to a lesser extent, RP-A, which appear to cluster at the free tips of the DNA molecules, seem to be required to ligate dsDNA ends in the egg extracts.

**DISCUSSION**

DNA dsb represent a severe type of damage that can be provoked in cell nuclei by exposure to ionizing radiation and also to chemicals such as etoposide, methylmethane sulphonate and bleomycin. Both radiation and chemical agents induce a variety of other DNA lesions such as base and sugar modifications, and single-strand breaks mostly mediated by the formation of highly reactive oxygen radicals (Iliakis, 1991). Although dsb are perhaps the lesions that most strongly threaten cell survival, it is likely that damaged cells seek to repair all DNA lesions produced by the oxygen radicals, thus activating several DNA repair pathways (Wood, 1996). Therefore, by using these DNA damaging agents, it might be difficult to analyze exclusively the repair of DNA dsb. On the other hand, it is desirable to have an in vitro system to study the repair of dsb produced in an environment as close as possible to the cell nucleus.

We used prokaryotic restriction enzymes (REs) in order to introduce solely dsb in the eukaryotic genome. As a substrate, we used *Xenopus* sperm heads subsequently assembled into nuclei in the presence of *Xenopus* egg extracts. This in vitro nuclear reconstitution system allows us to manipulate the DNA separately with specific damaging agents without inducing aberrations on most of the nuclear and chromatin proteins.

Here, we show that a known factor involved in the repair of different types of DNA lesions, RP-A, clusters in focal structures on *Xenopus* sperm nuclei after the induction of DNA dsb. The fact that the number of RP-A foci increases when increasing amounts of RE are added to the sperm nuclei (Fig. 1) and that RP-A concentrates near the ends of *Xenopus* chromatin assembled on magnetic beads (Fig. 8) suggests that clusters of RP-A molecules form in proximity to DNA dsb in the nuclei. As seen by immuno EM (Fig. 6), all three RP-A subunits, as well as DNA and Ku, map to RP-A/Ku bodies.

![Fig. 7](image1.png)

**Fig. 7.** RP-A and XKu86 foci form on linear duplex DNA linked to magnetic beads after incubation with HSS. Immunofluorescence with affinity purified anti-RP-A70 (RP-A) and anti-XKu86 antibodies (XKu86) on DNA bound to magnetic beads and incubated with HSS (in DNA amounts equivalent to 5000 sperm heads) for 1 hour at 23°C. The beads were extensively washed and fixed with 2% paraformaldehyde. The DNA bound to the beads was stained with DAPI. Both RP-A and XKu86 form foci all around the beads, whereas the DNA staining appears as a smoother rim. A control immunocytochemical experiment (IC) of naked beads incubated just with the extract and labeled with anti-RP-A70 antibody is shown together with the phase contrast (PC) image. An identical control image was obtained with anti-XKu86 antibodies. Bars, 5 μm.

![Fig. 8](image2.png)

**Fig. 8.** RP-A and XKu are preferentially associated with the DNA sequences proximal to the ends of linear DNA linked to the beads. Western blot analysis with rabbit serum against XKu86 and rabbit anti-RP-A serum, which recognizes all three RP-A subunits (RP-A70, RP-A34 and RP-A14). The slower migrating band might correspond to the phosphorylated form of RP-A34 (RP-A34*). DNA linked to magnetic beads was incubated with HSS for 1 hour at 23°C, extensively washed and then incubated consecutively with the appropriate restriction endonuclease in order to cut off specific DNA fragments. The beads were removed with the aid of the magnet and the excised DNA fragments with the associated proteins were recovered in the supernatant, run on a 7-15% SDS-PAGE gradient gel and transferred onto a nitrocellulose membrane. Lane 1, HSS; lane 2, total proteins bound to the beads; lane 3, wash with restriction enzyme buffer; lane 4, proteins bound to end-proximal 30 bp DNA fragment; lane 6, proteins bound to end-proximal 230 bp fragment; lane 8, proteins bound to 700 bp internal DNA fragment. Lanes 5, 7 and 9, proteins remaining associated with the DNA beads after a consecutive removal of the 30 bp (lane 5), the 230 bp (lane 7) and the 700 bp fragments (lane 9). Lanes 2-9 were loaded with equivalent volumes of sample. Owing to the digestions, there is necessarily less material bound to the beads in lane 7 than in lane 5 and less material in lane 9 than in lane 7.
These are rounded structures up to 300 nm in diameter that, with respect to the surrounding chromatin, exhibit a fine fibrillar ultrastructure. Interestingly, the ultrastructure of the dsb-induced RP-A/Ku bodies is similar to the pre-replication RP-A bodies present on intact sperm chromatin (Eltsov et al., 2000), but dsb-induced RP-A/Ku bodies are smaller. RE-induced RP-A foci also incorporate dNTPs but differently from preRCs; DNA synthesis stimulated by dsb occurs under conditions when replicative DNA synthesis cannot proceed (in the presence of HSS only). Indeed, this type of DNA synthesis is distinct from DNA replication in that only a part of the DNA fiber is replicated (Fig. 3B), which could be explained if single-stranded regions have been created at the site of the dsb by a nuclease and short DNA fragments are synthesized to fill the gaps (Shinohara and Ogawa, 1995). It should be mentioned in this respect that only larger amounts of added ssDNA are sufficient for the displacement of RP-A in RE-treated nuclei (Fig. 1C). This might indicate that, after RE treatment, ssDNA regions are consecutively generated and repaired by filling in the gaps with short DNA fragments. RE-induced RP-A foci might represent active repair factories that assemble and act on clustered damaged DNA sites for repair. Interestingly, the formation of preRCs and repair RP-A foci is an energy-dependent process, which might involve the action of one (or more) helicases. Indeed, the Xenopus homolog of the WRN helicase is required for the formation of preRC RP-A foci (Yan et al., 1998).

We found that the Xenopus homolog of the Ku80 protein, an essential factor in the repair of dsb in metazoa, co-localizes with RP-A in the RE-induced foci (Fig. 4B). The presence of XKu86 indicates that the dsb might be repaired via the non-homologous end-joining pathway (Kanaar et al., 1998; Karran, 2000). Indeed, the dsb are produced in a haploid nucleus where no homologous sister chromatid is present. Alternatively, XKu86 might simply protect the DNA ends from excessive nuclease attack by nucleases (Gravel et al., 1998; Polotnianka et al., 1998; Laroche et al., 1998).

Several proteins involved in dsb repair have been found to change their localization within the cell nucleus after exposure to DNA-damaging agents. In particular, Mre11, Rad50 and Rad51 form many foci on the nuclei of tissue culture cells exposed to ionizing radiations (Haaf et al., 1995; Maser et al., 1997; Mirzoeva and Petrini, 2001) or X-rays (Nelms et al., 1998). hMre and the Rad50 proteins and, recently, the p95-Nimbrin have been found to form a complex with homologies to the prokaryotic SbCD nuclease (Dolganov et al., 1996; Carney et al., 1998) and were found at sites close to DNA ends in the irradiated nuclei (Nelms et al., 1998). Interestingly, Mre11/Rad50 foci and Rad51 foci do not overlap with each other (Maser et al., 1997; Nelms et al., 1998; Mirzoeva and Petrini, 2001). Although it has been argued that they might represent two distinct repair pathways (Maser et al., 1997), these data could also be explained by a temporal succession of events in which the various proteins participate in the same repair pathway at different times. It will be interesting to see if RP-A and Ku foci are temporally and spatially related with these other focus-forming proteins. It is becoming more and more widely accepted that many factors involved in the repair of DNA dsb will assemble into a large ‘repairosome’ complex, as shown by genetic and biochemical assays (Hay et al., 1995; Dolganov et al., 1996; Clever et al., 1997; Wang et al., 2000). Through immunoprecipitation with anti-RP-A or anti-Ku antibodies in egg extracts, however, we were unable to show any direct interaction between the two proteins (data not shown) although we cannot exclude the possibility that they might interact directly at the sites of DNA damage. Recent data have shown that, in mammalian cells, Ku replaces RP-A in the binding to the DNA-PK upon induction of dsb (Sha et al., 1999), which supports the absence of physical interaction between the two factors. Accordingly, our depletion

Fig. 9. XKu86 and RP-A are required for ligation of dsDNA fragments on DNA beads incubated with HSS. Analysis of duplex DNA ligation by agarose gel electrophoresis and autoradiography. DNA bound to magnetic beads was incubated with 32P-end labeled pUC19 in the presence of mock depleted, RP-A-depleted, XKu86-depleted HSS extracts or with a buffer for 1 hour at 23°C. After incubation, the DNA beads were extensively washed with increasing concentrations of salt (up to 2 M NaCl) and detergent (up to 1% SDS). DNA beads were then equilibrated with BglII restriction enzyme buffer and digested with BglII (5 units per 100 ng DNA) for 2 hours at 37°C. The DNA fragments released from the beads were run on a 0.8% agarose gel. (A) Autoradiography: the labeled DNA fragments released by BglII digest and the linearized pUC19 are indicated on the side. (B) Ethidium bromide staining of the gel. Lane 1, mock depleted extracts; lane 2, RP-A-depleted extracts; lane 3, XKu86-depleted extracts; lane 4, buffer. Molecular weight standards are indicated on the left-hand side. The depletion of XKu86 and, to a lesser extent, RP-A from the extract inhibits end to end ligation of linear DNA fragments (arrows indicate the 7.7 kbp DNA fragments, arrowheads indicate the 5.7 kbp fragment). The asterisks indicate the presence of a contaminating DNA band migrating at ~6.8 kb. Ethidium bromide staining of the gel served as a control measure of the loading. In lane 2 in (B), the presence of the band migrating more rapidly than the 3 kbp fragment might be due to the star activity of BglII.
experiments showed no interdependence between RP-A and Ku in the formation of foci induced by dsb (Fig. 5). The fact that, after RP-A depletion, Ku foci appear smoother supports the argument that RP-A might contribute to the clustering of the broken DNA ends to which Ku is bound, the latter event being independent of the presence of RP-A. Moreover, it has been shown that DNA-PK phosphorylates RP-A in vitro (Henricksen et al., 1996) and that, in mutant scid cells (which harbor an inactive DNA-PK), RP-A is no longer phosphorylated (Boubnov and Weaver, 1995). Indeed, upon Ku depletion in our system, the RP-A 34 kDa subunit bound to DNA is no longer upshifted during SDS-PAGE migration (data not shown). Further biochemical and functional tests are needed to elucidate the interactions of RP-A and Ku and their role in the recognition and repair of DNA dsb. The introduced in vitro system represents a convenient approach for the implementation of such functional tests.

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