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Incorporation of DUF/FACT into chromatin enhances the accessibility of nucleosomal DNA

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Abstract

DNA unwinding factor (DUF) was discovered as an essential DNA replication factor in *Xenopus* egg extracts. DUF consists of an HMG protein and a homolog of Cdc68p/Spt16p, and has the capability of unwinding dsDNA. Here we have examined the interaction of DUF with chromatin. DUF was incorporated into chromatin assembled from sperm heads and from plasmid DNA in egg extracts. It was revealed that the chromatin assembled in egg extracts immunodepleted of DUF is less sensitive to micrococcal nuclease (NNase) digestion than that assembled in control extracts, indicating that chromatin containing DUF has more decompact structure than that without DUF. Also we found that DUF has a high affinity for core histones in vitro. We suggest that the function of DUF may be to make the chromatin structure accessible to replication factors.

Keywords: DNA replication; DNA unwinding factor; Chromatin; FACT; CP complex; *Xenopus* egg

In eukaryotic cells, DNA dynamics such as transcription, replication, and repair are regulated by chromatin structure. Thus, alteration of the nucleosome structure is one of the key steps in eukaryotic DNA dynamics. Recent studies have revealed the mechanism of chromatin alteration in the initiation and elongation steps of transcription [1–5]. Remodeling factors such as SWI/SNF complex, NURF, and ACF alter the chromatin structure, resulting in an increase in the accessibility of nucleosomal DNA [1–4]. Another type of chromatin modification is histone acetylation catalyzed by histone acetyltransferases (HATs), such as yeast Gcn5p, Esa1p/mof/Tip60, and mammalian GCN5/PCAF [1,2,4]. Recent progress in studies of remodeling factors and HATs has revealed that they cooperate in decompacting chromatin [1,2]. In transcription elongation, it has been suggested that facilitates chromatin transcription (FACT) facilitates the movement of RNA polymerase by disassembling nucleosomes [5–10]. As for DNA replication, HMG17 or chromatin accessibility complexes (CHRAC) were reported to enhance the SV40 DNA replication in vitro [11,12]. These reports indicating that the chromatin factors positively regulate replication of viral DNA in the form of chromatin strongly suggest that alterations in chromatin structure are also prerequisite for eukaryotic DNA replication.

In our previous report, we purified DNA unwinding factor (DUF), which introduces negative supercoiling into duplex DNA, from *Xenopus* egg extracts [13]. Immunodepletion of DUF from *Xenopus* egg extracts drastically reduced the DNA replication activity of the egg extracts, indicating that this factor is required for DNA replication. DUF consists of two polypeptides with molecular masses of 140 kDa (DUF140) and 87 kDa (DUF87). DUF140 shows homology to *Saccharomyces cerevisiae* Cdc68p/Spt16p, which was originally identified as a regulator of G1/S transition [14].

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Various genetic studies have indicated that Cdc68p/Spt16p is involved in the regulation of the transcription of a wide variety of genes [14–21]. The phenotypic similarities between mutants of *CDC68/SPT16* gene and those of histone genes (*HTA1* and *HTB1*), and the identification of *SAN1*, a putative chromatin factor, as a suppressor of *cdc68/spt16* mutants have indicated that *CDC68/SPT16* is involved in the alteration of chromatin structure [16,17]. DUF87 is an HMG protein with high sequence homology to human structure-specific recognition protein-1 (SSRP1) and murine T160. SSRP1 interacts with cisplatin-damaged DNA [22] and has been implicated in the regulation of transcription [23]. T160 was first identified as a protein that binds to the signal sequence of V(D)J recombination [24,25]. Interestingly, Wittmeyer and Formosa [26] and Wittmeyer et al. [27] have found that a complex of Cdc68p/Spt16p and Pob3p (CP complex), which show homology to DUF140 and DUF87, respectively, interacts with DNA polymerase α. Hertel et al. [25] have found that T160 colocalizes with replication foci. These data suggest that DUF is involved in a wide variety of DNA dynamics including transcription, recombination, repair, and replication. Recently, the cloning of FACT showed it to be a human homolog of DUF [6–8]. This suggests that DUF is involved in DNA replication by modulating chromatin structure.

In this work, we have examined the role of *Xenopus* DUF/FACT in the modulation of chromatin structure. We assembled chromatin from demembranated sperm heads and from exogenous DNA in *Xenopus* egg extracts. In both cases, DUF/FACT was shown to be incorporated into the assembled chromatin. It was also revealed that chromatin assembled in egg extracts that had been immunodepleted of DUF is less sensitive to micrococcal nuclease than that assembled in the presence of DUF. In addition, we found that DUF interacts with core histones. We present our data on the effects of DUF on chromatin structure and discuss the possible function of DUF in DNA replication.

Materials and methods

Sperm chromatin assembly in egg extracts. Interphase *Xenopus* egg extracts and sperm heads were prepared as described [13]. Sperm heads were added to 10 μl egg extract at a concentration of 45 μ^{-1}l and the mixture was incubated at 23 °C for 60 min, diluted with 50 μl NWB buffer (200 mM sucrose, 15 mM Hepes–NaOH (pH 7.4), 50 mM NaCl, 2.5 mM $MgCl_2$, and 1 mM DTT), loaded onto a 0.5 ml glycerol cushion (20% glycerol in NWB), and centrifuged at 5000 rpm (1500*g*) and 4 °C for 10 min to yield reconstituted nuclei. Chromatin was prepared by suspending the nuclei in 50 μl NWB containing 0.2% Triton X-100 and centrifuging the mixture through a glycerol cushion consisting of 20% glycerol and NWB. After washing with 200 μl NWB, the chromatin was suspended in 20 μl NWB and 10 μl SDS–PAGE sample buffer was added. DUF in the samples was analyzed by Western blotting [13].

Minichromatin assembly in egg extracts. Single-stranded circular M13 DNA and [α-^{32}P]dCTP were added to an egg extract at concentrations of 5 ng/μl and 5 μCi/μl, respectively, and the extract was incubated at 23 °C for 100 min. Minichromatin assembled in the egg extract was purified as follows. The egg extract containing minichromatin was supplemented with 0.25% Triton X-100 and filtered through a Sepharose CL4B column equilibrated with minichromatin purification buffer (MPB) (20 mM Hepes (pH 7.5), 100 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.2 μg/ml each of leupeptin, chymostatin, and pepstatin). Fractions containing DNA and DUF were combined, loaded onto a sucrose cushion (20 mM Hepes (pH 7.5), 100 mM KCl, 1 mM EDTA, and 35% sucrose), and centrifuged at 40,000 rpm (130,000*g*) and 4 °C for 2.5 h in a Hitachi RSP50 rotor. The precipitated minichromatin was suspended in 40 μl MPB and further filtered through a Sepharose CL4B column equilibrated with MPB containing 0.1% Triton X-100. The DNA in each fraction was recovered and analyzed by electrophoresis and autoradiography. DUF in each fraction was detected by Western blotting.

MNase digestion of minichromatin. Immunodepletion of DUF from egg extracts was described previously [13]. Minichromatin was assembled from 60 ng of single-stranded M13 DNA in 10 μl DUF-depleted or mock-depleted extract. The extracts were incubated at 23 °C for 90 min, after which aphidicolin and Triton X-100 were added to final concentrations of 30 ng/ml and 0.25%, respectively. Limited digestion of DNA was started by the addition of 0.7 U/μl MNase and 1 mM $CaCl_2$, and the mixtures were incubated at 23 °C. Aliquots were taken at 0, 2, 5, 10, and 20 min and the digestion was stopped by adding lysis buffer (100 mM Tris–HCl (pH 8.0), 50 mM EDTA, 0.5 M NaCl, and 1% SDS). Digested DNA in each sample was purified, electrophoresed, and autoradiographed.

Histone columns. Individual species of core histone (H2A, H2B, H3, and H4) were conjugated with CNBr-activated Sepharose as described [28]. Briefly, purified histones H2A, H2B, and H3 (Roche) were dissolved in distilled water, dialyzed against 0.1 M $NaHCO_3$ (pH 8.5), and immobilized on CNBr-activated Sepharose 4B (Pharmacia). Histone H4 was dialyzed against 0.1 M potassium phosphate (pH 7.5) and $NaHCO_3$ was added to a final concentration of 0.1 M before immobilization. DUF was purified as previously described [13,29]. Purified DUF was dialyzed against binding buffer (20 mM Tris (pH 7.5) and 50 mM NaCl) and loaded onto histone columns equilibrated with binding buffer. The columns were washed with binding buffer and adsorbed materials were eluted with buffer solution (20 mM Tris, pH 7.5) containing increasing concentrations of NaCl (0.15, 0.5, 1, and 2 M). DUF in the eluates was analyzed by Western blotting.

Results

Incorporation of DUF/FACT into chromatin assembled from sperm heads

To determine whether *Xenopus* DUF is incorporated into chromatin assembled in egg extracts, we prepared chromatin by incubating demembranated *Xenopus* sperm heads with interphase egg extracts. Reconstituted nuclei were treated with detergent and the chromatin was isolated by centrifugation through a glycerol cushion (Fig. 1A). Immunoblot analysis of the chromatin fraction indicated that DUF140 and DUF87 were incorporated into chromatin assembled from sperm heads (Fig. 1B; lanes 1 and 2), although intact sperm heads themselves did not contain DUF (Fig. 1B; lane 3).

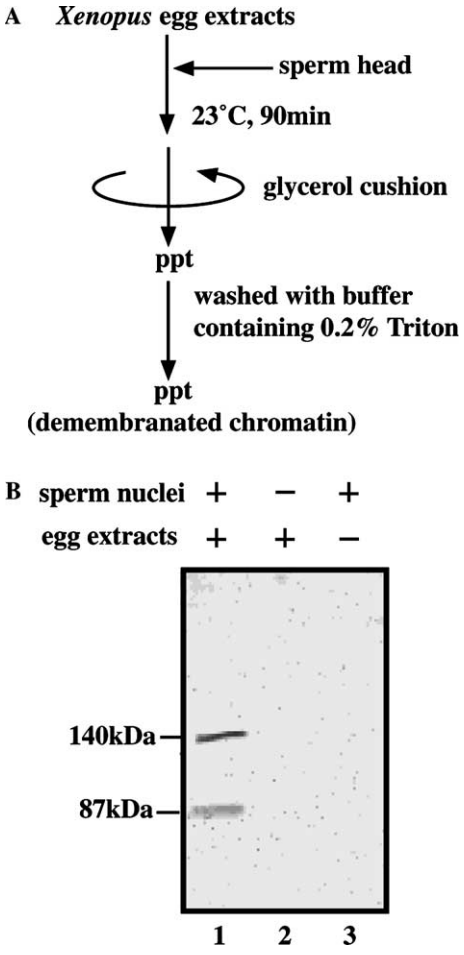

Fig. 1. Incorporation of DUF into sperm chromatin. Procedures for the purification of sperm chromatin (A). *Xenopus* egg extracts were incubated with (lane 1) or without (lane 2) *Xenopus* sperm heads and DUF incorporated into the purified nuclei was detected by Western blotting. Intact sperm nuclei were analyzed in the same round of Western blotting (lane 3) (B).

Incorporation of DUF/FACT into minichromatin

When single-stranded DNA is incubated with *Xenopus* egg extracts, double-stranded DNA is synthesized by DNA polymerase(s) in the extract and histones bind to the synthesized double-stranded DNA to form chromatin [30,31], hereafter called minichromatin. Using this system, we examined the incorporation of DUF into minichromatin assembled in *Xenopus* egg extracts. We incubated single-stranded circular M13 DNA with interphase egg extracts in the presence of [α-^{32}P]dCTP. Minichromatin was purified by gel filtration, centrifugation through a sucrose cushion, and a second gel filtration (Fig. 2A). Western blot analysis revealed that in each step of purification, DUF coeluted with DNA, suggesting that DUF is incorporated into minichromatin (Figs. 2B, C, and D). Note that significant amounts of DUF co-eluted in later fractions in the first gel filtration (Fig. 2B).

Micrococcal nuclease sensitivity of minichromatin assembled in the presence or absence of DUF

A variety of experimental results on DUF/FACT [6–9] and Cdc68p/Spt16p [17,21,27,32–35] suggest that DUF/FACT is involved in the modulation of chromatin structure. In the present study, we compared the micrococcal nuclease sensitivity of minichromatin assembled in DUF-depleted or mock-depleted egg extracts. As shown in our previous paper, DNA synthesis on single-stranded DNA as a template is not affected by immunodepletion of DUF [13]. The same result was obtained in the present experiments (Fig. 3, lanes, mock 0 min and depleted 0 min). The assembled chromatin preparations were treated with MNase for various lengths of time to achieve limited digestion of DNA. Fig. 3 shows the electrophoretic pattern of DNA after the incubation of minichromatin with MNase for various lengths of

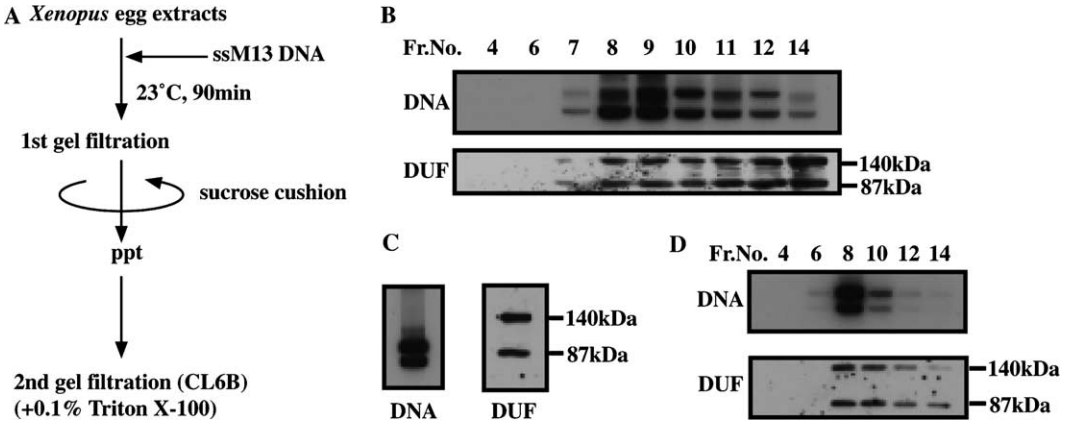

Fig. 2. Incorporation of DUF into minichromatin. Purification scheme for minichromatin assembled in egg extracts (A). Detection of DNA and DUF in the first gel filtration fractions (B). Precipitate from the sucrose cushion was analyzed (C). Detection of DNA and DUF in the second gel filtration fractions (D).

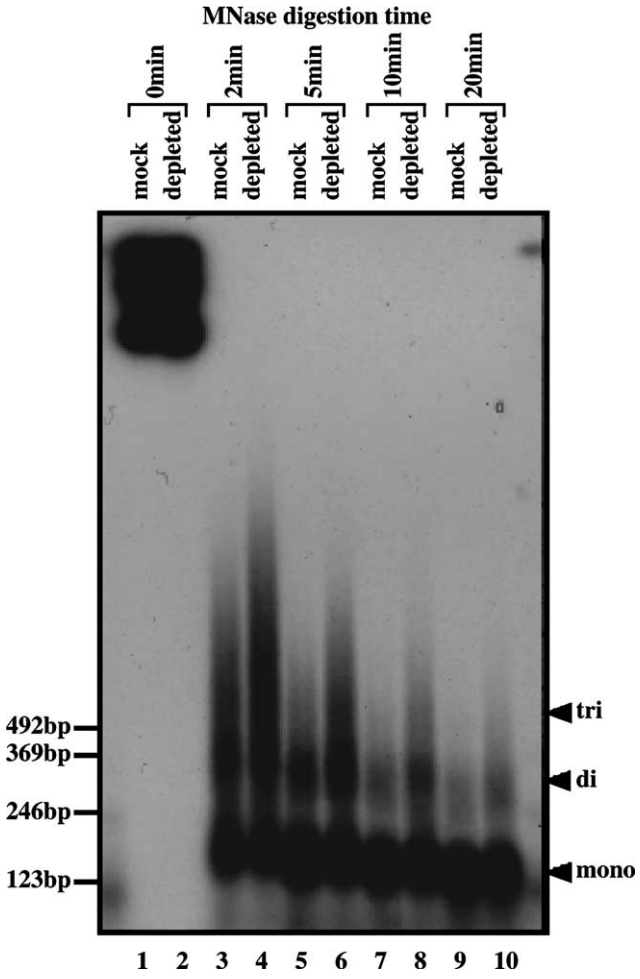

Fig. 3. Sensitivity of minichromatin to MNase. Minichromatin was assembled on the template of ssM13 plasmid in mock-depleted (lanes 1, 3, 5, 7, and 9) and DUF-depleted (lanes 2, 4, 6, 8, and 10) extracts. The assembled minichromatin was then digested with MNase for 0 (lanes 1 and 2), 2 (lanes 3 and 4), 5 (lanes 5 and 6), 10 (lanes 7 and 8), or 20 (lanes 9 and 10) min. The sizes of the123 bp ladder and mono-, di-, and tri-nucleosome(s) are indicated.

time. The sizes of the DNA fragments derived from DUF-depleted and mock-depleted minichromatin preparations corresponded to those of mono-, di-, and tri-nucleosomes (Fig. 3). This result shows that regularly positioned nucleosomes were assembled in both the DUF-depleted and mock-depleted extracts. It was found that minichromatin assembled in the DUF-depleted extracts was digested by MNase to a lesser extent than that assembled in mock-depleted extracts (Fig. 3, lanes 2 and 5 min). Similar results were obtained reproducibly in three rounds of independent experiments. These results suggest that DUF confers minichromatin with a higher sensitivity to MNase.

Interaction of DUF with core histones

Next we tested whether DUF binds to core histones. Purified DUF was applied to CNBr–Sepharose columns

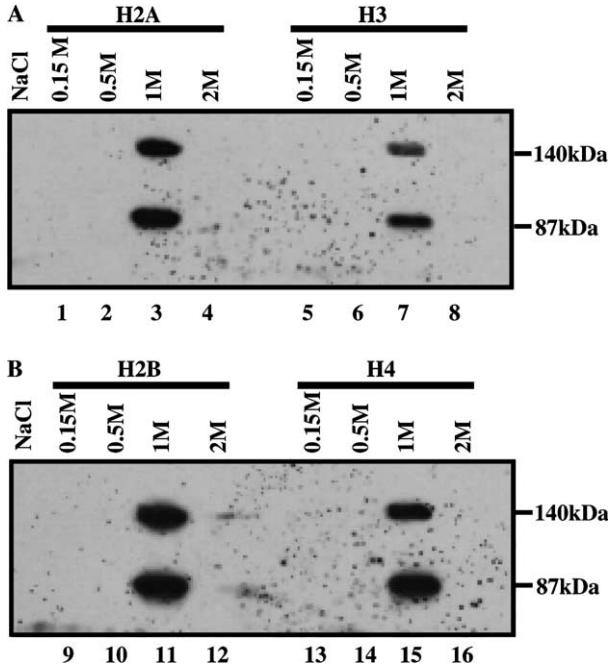

Fig. 4. Interaction of DUF with core histones. Purified DUF was loaded onto CNBr–Sepharose columns conjugated with core histones H2A (A; lanes 1–4), H3 (A; lanes 5–8), H2B (B; lanes 9–12) or H4 (B; lanes 13–16), and eluted with 0.15 M (lanes 1, 5, 9, and 13), 0.5 M (lanes 2, 6, 10, and 14), 1.0 M (lanes 3, 7, 11, and 15), or 2.0 M (lanes 4, 8, 12, and 16) NaCl. The eluates were analyzed by Western blotting to detect DUF.

conjugated with histones H2A, H2B, H3, and H4. The adsorbed DUF was eluted with increasing concentrations of NaCl. Immunoblot analysis of the eluates showed that DUF remained bound to the columns during elution with 0.15 and 0.5 M NaCl but was eluted with 1 M NaCl, indicating that DUF was tightly bound to the four species of core histones (Figs. 4A and B). We observed no interaction of DUF with control CNBr–Sepharose column blocked by glycine (data not shown).

Discussion

In our previous paper, we showed that DUF is incorporated into nuclei reconstituted from demembranated sperm heads incubated in interphase egg extracts. In the present study, we show that DUF is bound to chromatin in the reconstituted nuclei. We also show that DUF is incorporated into minichromatin assembled in the extract on the template of single-stranded M13 DNA. This is consistent with the findings that human FACT interacts with reconstituted nucleosomes in vitro [8] and that maize SSRP1 protein associates with chromatin in vivo [36].

We previously reported that *Xenopus* DUF binds to double-stranded DNA and introduces a negative twist into the DNA [13]. In the present study, DUF was

shown to bind to core histones. This is consistent with the in vitro results for human FACT [8]. However, in contrast to FACT, which interacts only with histones H2A and H2B [8], *Xenopus* egg DUF shows strong affinity for histones H2A, H2B, H3, and H4. This contradiction might arise from the differences in the species or type of cells from which DUF and FACT are purified (DUF from *Xenopus*, FACT from HeLa; DUF from eggs, FACT from somatic cells). It is also possible that some unknown modification of DUF/FACT regulates the specificity of its interaction with histones.

In our experiments, minichromatin assembled in the presence of DUF showed a higher sensitivity to MNase than that assembled in the absence of DUF. Recently, Formosa et al. [32] reported that an SPN complex consisting of Cdc68p/Spt16p (homolog of DUF140), Pob3p (with homology to the N-terminal 2/3 of DUF87/SSRP1 but without an HMG domain), and Nhp6p (an HMG protein) interacts with the reconstituted nucleosomes, and that the binding of the SPN complex to nucleosomes enhances DNase I sensitivity to nucleosomal DNA. These data are consistent with our results of the higher MNase sensitivity of minichromatin assembled in the presence of DUF. It is believed that the capabilities of DUF to bind to core histones and introduce a negative twist into double-stranded DNA contribute to the alteration in the structure of chromatin into one that is more sensitive to MNase. Interestingly, the yeast Spt16–Pob3p complex associates with histone acetyltransferase complex NuA3 [33]. It is possible that DUF/FACT, a higher eukaryotic homolog of Spt16–Pob3p, in association with HAT, could function in the alteration of chromatin structure.

What is the function of DUF/FACT in DNA replication? FACT was first identified as a factor that activates the elongation step in the transcription of the chromatin template. Kang et al. [21] have found that Cdc68p/Spt16p, a subunit of DUF/FACT, interacts with initiation factor TFIIE, which suggests that it also participates in the initiation step of transcription. By analogy with the functions of FACT in transcription, a similar role of DUF in DNA replication can be hypothesized. The activity of DUF to modulate chromatin structure could facilitate replication initiation by recruiting initiation factors to replication origins in the form of nucleosomes. DUF/FACT could also function in stimulating the elongation step of DNA replication, as it has been shown for FACT in the elongation of transcription [5–10]. In the case of DNA replication, a structural change in chromatin caused by the binding of DUF to duplex DNA and core histones may enable the replication machinery to proceed on template DNA in the form of nucleosomes. It is also considered that DUF might facilitate the elongation step of DNA replication by recruiting DNA polymerase α to the replication fork, since an interaction between the Cdc68p/Spt16p–Pob3p complex and DNA polymerase α has been observed in *S. cerevisiae* [26,27]. Further studies on the function of DUF/FACT will provide novel insights into the mechanism of replication of chromatin DNA.

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