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High mobility group proteins stimulate DNA cleavage by apoptotic endonuclease DFF40/CAD due to HMG-box interactions with DNA

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The DFF40/CAD endonuclease is primarily responsible for internucleosomal DNA cleavage during the terminal stages of apoptosis. It has been previously demonstrated that the major HMG-box-containing chromatin proteins HMGB1 and HMGB2 stimulate naked DNA cleavage by DFF40/CAD. Here we investigate the mechanism of this stimulation and show that HMGB1 neither binds to DFF40/CAD nor enhances its ability for stable binding to DNA. Comparison of the stimulatory activities of different truncated forms of HMGB1 protein indicates that a structural array of two HMG-boxes is required for such stimulation. HMG-boxes are known to confer specific local distortions of DNA structure upon binding. Interestingly, the presence of DNA strand cross-links formed by cisplatin or transplatin, which may somehow mimic distortions induced by HMG-boxes, also affects DNA cleavage by the nuclease. The data presented suggest that changes induced in DNA conformation upon HMG-box binding makes the substrate more accessible to cleavage by DFF40/CAD nuclease and thus may contribute to preferential linker DNA cleavage during apoptosis.

Keywords: nuclease, DFF, CAD, chromatin, HMG-box, HMGB1 protein, cisplatin

INTRODUCTION

Apoptosis, or programmed cell death, is an essential process that participates in development and the maintenance of tissue homeostasis. The process depends on actively controlled degradation of intracellular structures and allows the removal of unwanted, incorrect or damaged cells from multicellular organisms (reviewed in Wyllie *et al*., 1980; Hengartner, 2000; Kaufman & Hengartner, 2001). The sequential generation of large chromatin fragments followed by internucleosomal fragmentation is a biochemical hallmark of apoptosis. This genomic DNA fragmentation is correlated with chromatin condensation and nuclear breakdown (reviewed in Zhang & Xu, 2002; Samejima & Earnshaw, 2005). The major nuclease primarily responsible for internucleosomal chromatin fragmentation is DNA fragmentation factor (DFF), also termed caspase-activated DNase (CAD) (reviewed in Widłak & Garrard, 2005).

In its inactive form, DFF is a heterodimer composed of a 40-kDa latent endonuclease subunit (DFF40/CAD, hereafter termed DFF40) and a 45-kDa chaperone and inhibitory subunit (DFF45/ICAD, hereafter termed DFF45) (Liu *et al*., 1997; 1998; Enari *et al*., 1998; Halenbeck *et al*., 1998). DFF45 carries two caspase-3 recognition sites. Upon caspase-3 cleavage of DFF, DFF45 is cut and released from DFF40, which forms homo-dimers (Woo *et al*., 2004) and higher homo-oligomers (Liu

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Abbreviations: Ac-DEVD-Cho, acetyl-Asp-Glu-Val-Asp-aldehyde; CAD, caspase-activated DNase (also termed DFF40); DFF40, DNA fragmentation factor 40 kDa subunit; DFF45, DNA fragmentation factor 45 kDa subunit; DTT, dithiothreitol; GST, glutathione sulfotransferase; HMG, high mobility group (protein); ICAD, inhibitor of CAD (also termed DFF45); mAb, monoclonal antibody; MAPKK, mitogen-activated protein kinases' kinase; PAGE, polyacrylamide gel electrophoresis; TFAM, mitochondrial transcription factor A.

et al., 1999; Widłak *et al*., 2003) that are forms of the enzymatically active nuclease. Several lines of evidence show that homo-oligomers of activated DFF40 can further bind additional regulatory proteins, both activators and inhibitors. The C-terminal domain of histone H1 acts as an activator and binds to caspase-activated DFF40, which increases its ability to bind to DNA (Widłak *et al*., 2005). Topoisomerase II, another chromatin activator of DFF40 (Widłak, 2000), also binds to the enzyme (Durrieu *et al*., 2000); this interaction may contribute to the involvement of DFF in the large-scale apoptotic DNA fragmentation. Several protein inhibitors that bind to DFF40 homo-oligomers after removal of DFF45 have also been identified. Among such DFF inhibitors are nucleophosmin/B23, the nuclear receptor of $PI(3,4,5)P_3$ (Ahn *et al*., 2005), and CIIA protein, which also regulates apoptosis signal-regulating kinase 1 of the MAPKK kinase family (Cho *et al*., 2003).

Among the first identified activators of DNA cleavage by DFF40 were the major non-histone chromatin proteins HMGB1 and HMGB2 (Liu *et al*., 1998; Toh *et al*., 1998; Widłak *et al*., 2000), but the molecular mechanism of such activation remained to be addressed. HMGB1 is the most abundant HMG-box containing protein, which interacts with internucleosomal linker DNA (about 1 molecule per 10–15 nucleosomes). HMGB1 and its close homologue HMGB2 (both proteins share > 80% identity) have a tripartite structure. They possess N-terminal and central domains called HMG-boxes that are largely homologous and consist of about 70 highly basic residues forming three α-helices. The HMG-boxes constitute non-specific DNA-binding regions. An additional C-terminal segment contains about 40 highly acidic residues and is involved in interactions with other proteins. The HMG-box is found in other proteins, including mitochondrial TFAM, which possesses two boxes, and several known or putative transcription factors that contain a single HMG-box embedded in a larger protein. All HMG-box-containing proteins interact with DNA primarily through contacts with the minor groove, which is responsible for their low sequence-specificity for DNA binding (reviewed in Landsman & Bustin, 1993; Bustin & Reeves, 1996; Thomas & Travers, 2001).

Here we aimed to clarify the mechanism of DFF40-mediated DNA cleavage in the presence of HMGB1 protein. We have found that HMGB1 does not stably interact with the nuclease. This finding leads us to postulate that changes induced in DNA conformation upon HMG-box binding make the substrate more susceptible to cleavage by DFF40 nuclease.

MATERIALS AND METHODS

Protein preparation. His_6-tagged recombinant human DFF40 was co-expressed in bacteria with DFF45 using the pRSFDuet1 vector (Novagen) and purified on Ni-NTA agarose (synthetic genes were engineered to optimize codon usage and efficiency of expression in *E. coli* (Xiao *et al*., 2007)). Alternatively, mouse GST-tagged DFF40 was co-expressed in *Escherichia coli* with human DFF45 using a two-vector system described previously (Korn *et al*., 2002), and purified on GSH-Sepharose. Hamster recombinant caspase-3 was expressed in *E. coli* and purified as described previously (Liu *et al*., 1998). The cDNAs encoding full-length or truncated forms of human HMGB1 were amplified by RT-PCR by using specific primers. Selected primers allowed us to amplify nucleotides 164–811 (intact HMGB1), 164–655 (HMG/A-B), 185–403 (HMG/A) and 443–655 (HMG/B), according to the numbering in GenBank (accession No. NM_002128). The PCR products were cloned into pET21a expression vector (Novagen) and the resulting His_6-tagged recombinant proteins were expressed in bacteria and purified on Ni-NTA agarose. Contaminating proteins were precipitated with 2% trichloracetic acid and then preparations were dialyzed and concentrated. Alternatively, HMGB1 purified from calf thymus was purchased (Wako Chemicals). His_6-tagged recombinant rat TFAM was expressed in *E. coli* and purified on Ni-NTA agarose (the mature form of TFAM without the mitochondria localization signal — residues 43–246 — was cloned into an expression vector, as described in Dong *et al.* (2002). Total histone H1 was purified from HeLa cell chromatin using hydroxyapatite as described elsewhere (Widłak *et al*., 2000).

Nuclease assay. DFF40/45 heterodimer was activated by incubation with caspase-3 (in an approximate molar ratio of 2 : 1) for 15 min at room temperature and then caspase-3 was inhibited with 10 μM Ac-DEVD-Cho. One microgram of pWLTR11 plasmid DNA (4.2 kb in length) was incubated with 20 ng of caspase-activated DFF40 in buffer consisting of 4 mM $MgCl_2$, 10 mM KCl, 50 mM NaCl, 1 mM EGTA, 1 mM DTT and 20 mM Tris/HCl, pH 7.5, in the presence of different amounts of HMG-box-containing proteins or histone H1 (or bovine serum albumin as a control). After incubation for 30 min at 33°C reactions were terminated by adding EDTA, SDS and proteinase K, and then DNA samples were separated electrophoretically on 1.5% agarose gels and stained with ethidium bromide. For some experiments plasmid DNA was incubated for 20 h at 37°C with 0.1, 0.3, 1.0, 3.0 or 10 μM cisplatin (*cis*-diamine-dichloro-platinum, *cis*-DDP) or transplatin (*trans*-diamine-dichloro-platinum, *trans*-DDP) to introduce strand cross-links, then DNA was purified

by phenol/chloroform extraction and ethanol precipitation.

GST pull-down assay. DFF40-GST fusion protein, either inactive DFF40/45 heterodimer (0.5 μg) or an equivalent amount of caspase-activated DFF40 nuclease, was bound to 50 μl of GSH-Sepharose (Pharmacia) beads. The GSH-Sepharose with bound DFF40-GST was incubated with HMGB1 (1 μg), in the presence or absence of plasmid DNA (1 μg), in 250 μl of binding buffer consisting of 10 mM KCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100 and 20 mM Tris/HCl, pH 7.5, for 15 min at room temp. Sepharose beads were then washed twice with 1 ml of binding buffer. Bound proteins were eluted with 50 mM reduced glutathione, separated electrophoretically on 15% polyacrylamide/SDS gels and electro-transferred onto nitrocellulose membranes (Schleicher & Schuell). Membrane-immobilized proteins were probed with rabbit anti-human DFF40 polyclonal antibodies (Pharmingen) and anti-HMGB1 mAb (Santa Cruz). The antigen–antibody complexes were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia).

Binding to DNA-cellulose. Inactive DFF40/45 heterodimer (0.5 μg) or an equivalent amount of caspase-activated DFF40 was mixed with HMGB1 (1 μg) in binding buffer consisting of 10 mM KCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100 and 20 mM Tris/HCl, pH 7.5, and then the mixture was incubated with a suspension of DNA-cellulose (equivalent to 20 μg of DNA) for 30 min at room temp. in a final volume of 250 μl of binding buffer. Cellulose beads were then washed twice with 1 ml of binding buffer. Bound proteins were eluted with 2% SDS, separated on PAGE/SDS gels and Western-blot detected as described above.

RESULTS AND DISCUSSION

Previous studies have shown that in normal cells, DFF40 apoptotic nuclease exists in a latent form in a heterodimer complex with an inhibitor subunit, DFF45, and that upon exposure of cells to apoptotic stimuli, caspase-3 becomes activated and cleaves the inhibitor, resulting in homo-oligomer formation of DFF40 and enzyme activation (reviewed in Widłak & Garrard, 2005). We also found that HMGB1 and HMGB2 proteins stimulate DFF40 cleavage on naked DNA substrates (Liu *et al*., 1998; Widłak *et al*., 2000). Here we show that this effect is independent of the order of addition of HMGB1, DNA and caspase-3 to the DFF40/45 heterodimer. Because HMGB1 added either before caspase-3 treatment of DFF40/45 heterodimer or after such treatment shows the same stimulatory effect on DNA cleavage (Fig. 1A, lanes 4 and 3, respectively) this means that stimulation occurs by affecting enzyme activity and/or substrate susceptibility and not by a caspase-3-dependent activation process. The effect of HMGB1, however, can be reversed by the post-addition of an excess of intact DFF45 (Fig. 1A, lanes 5 and 6). The activation by HMGB1 is specific for DFF40 because the activities of either DNase I, micrococcal nuclease (MNase) or endonuclease G are unaffected by this protein (not shown). The stimulatory effect of HMGB1 on DNA cleavage was observed at the whole range of protein/DNA ratios tested (one HMGB1 molecule per 1000 to 30 bp of DNA substrate). The highest HMGB1 stimulatory effects were detected at higher protein/DNA ratios. In marked contrast, the same high protein/DNA ratios of histone H1 were inhibitory (Fig. 1B, lanes 12 and 16 represent 1 μg of either HMGB1 or histone H1 added per 1 μg of DNA, respectively).

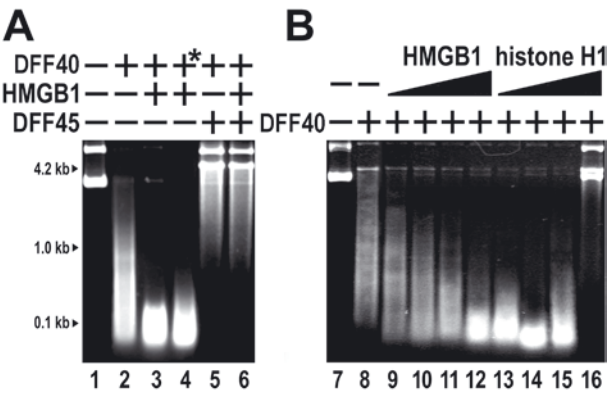

Figure 1. HMGB1 protein stimulates naked DNA cleavage by DFF40 endonuclease.
Panel A. Plasmid DNA was incubated with caspase-3-activated recombinant DFF40 nuclease in the presence of HMGB1. HMGB1 was added to reaction mixtures either before (asterisk, lane 4) or after (lanes 3 and 6) DFF40 activation with caspase-3, or an excess of DFF45 was added after caspase-3 inhibition (lanes 5 and 6). **Panel B.** Plasmid DNA was incubated with activated DFF40 in the presence of increasing amounts (30, 100, 300 or 1000 ng) of either HMGB1 or histone H1.

Knowing that histone H1 and topoisomerase II bind to DFF40, we aimed to use a DFF40-GST fusion protein in a GST-pull down experiment to determine whether the nuclease interacts with HMGB1 *in vitro*. Either DFF40/45 heterodimer or caspase-activated DFF40 were immobilized on GSH-Sepharose and then incubated with comparable amounts of HMGB1. Unbound proteins were removed from beads by intensive washing and then proteins bound to GSH-Sepharose were eluted by an excess of reduced glutathione and analyzed by Western-blotting. The results revealed that HMGB1 bound neither DFF40/45 heterodimer nor caspase-activated

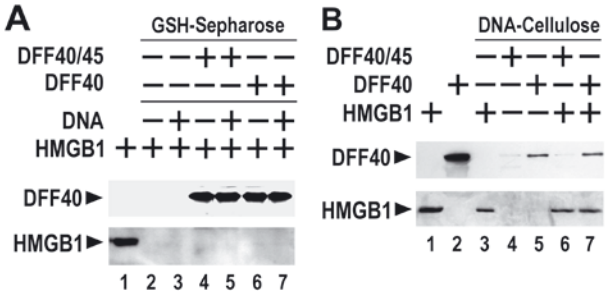

Figure 2. HMGB1 neither binds DFF40 endonuclease nor enhances its stable binding to DNA.
Panel A. DFF40–GST, in the form of either DFF40/45 heterodimer (lanes 4 and 5) or caspase-activated DFF40 nuclease (lanes 6 and 7), was immobilized on GSH-Sepharose. Sepharose beads were incubated with HMGB1 in the presence or absence of plasmid DNA, and then washed. Bound proteins were eluted with free GSH, separated by SDS/PAGE and detected by Western blotting with anti-DFF40 or anti-HMGB1 antibodies. Lane 1, amount of input HMGB1 used for reactions. **Panel B.** DFF40/45 heterodimer (lanes 4 and 6) or caspase-activated DFF40 (lanes 5 and 7) was incubated with DNA-cellulose in the presence (lanes 6 and 7) or absence (lanes 4 and 5) of HMGB1 (or HMGB1 was incubated with DNA-cellulose itself — lane 3). Cellulose beads were washed and bound proteins eluted with SDS, separated by SDS/PAGE and detected by Western blotting with anti-DFF40 or anti-HMGB1 antibodies. Lane 1 and 2, amounts of input HMGB1 and DFF40 used for reactions, respectively.

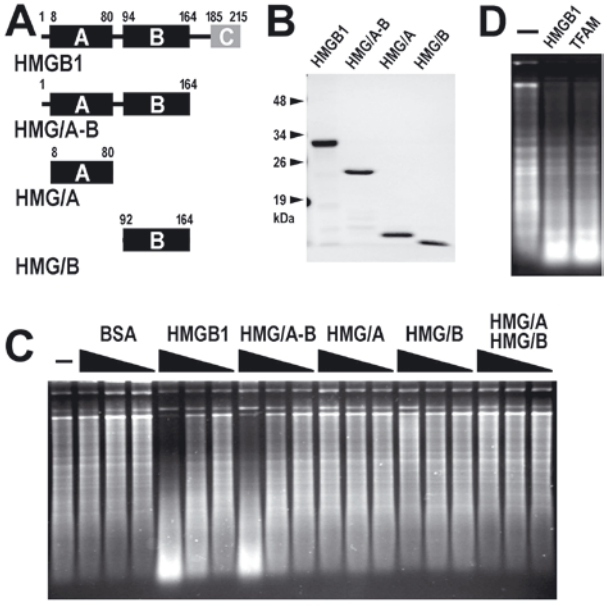

Figure 3. A structural array of two HMG-boxes is responsible for stimulation of DNA cleavage by DFF40 endonuclease.
Panel A. The structure of different truncated forms of HMGB1 protein. Two HMG-boxes (A and B) and the C-terminal acidic domain (C) are shown as rectangles. **Panel B.** SDS/PAGE of forms of HMGB1. **Panel C.** Plasmid DNA was incubated with activated DFF40 in the presence of the indicated forms of HMGB1 or control BSA (1000, 300 or 100 ng each). **Panel D.** Plasmid DNA was incubated with activated DFF40 in the presence of either intact HMGB1 or TFAM protein (300 ng each).

DFF40 (Fig. 2A). Interestingly, the presence of plasmid DNA (when its cleavage was prevented by EDTA) did not induce binding of HMGB1 to GSH-Sepharose-immobilized DFF40-GST, suggesting a lack of stable tertiary complexes involving HMGB1, DFF40 and DNA.

To verify whether the presence of HMGB1 enhances stable binding of DFF species to DNA, we utilized a DNA-cellulose bead assay. Protein species bound to DNA were identified in eluates using Western blotting from SDS/PAGE gels. As shown in Fig. 2B, HMGB1 was recovered from DNA-cellulose whenever it was present in input protein mixtures. Caspase-activated DFF40 also showed significant binding to DNA-cellulose. In addition, some weak binding of DFF40/45 heterodimer to DNA-cellulose could be also detected. Importantly, however, the presence of HMGB1 did not enhance the binding of DFF, caspase-activated or intact heterodimer species, to DNA-cellulose.

To determine the segments within the HMGB1 protein that are responsible for DFF40 stimulation we tested the response of the enzyme activity to different truncated forms of human HMGB1 protein (Fig. 3A and B). We observed that a truncated HMGB1 mutant lacking the C-terminal acidic domain (HMG/A-B) had the same activity as the intact protein, indicating that this domain was redundant for stimulation of DNA cleavage by DFF40 (Fig. 3C). On the other hand, isolated HMG-boxes, either box A or box B (or a mixture of both), did not stimulate DNA cleavage (Fig. 3C). This indicated that an array of both HMG-boxes juxtaposed with a short linker sequence was indispensable for stimulation of DNA cleavage by DFF40. This observation was in full agreement with previously published data that an array of two HMG-boxes joined by a basic 12 amino-acid-long linker is crucial for stable binding of HMGB1 with DNA (Saito *et al*., 1999). To check whether the ability of HMGB1 to stimulate DNA cleavage by DFF40 was due to structural properties of the array of its two HMG boxes but not to their primary sequence of amino-acids another HMG-box containing protein was tested. TFAM is a transcription factor containing two HMG-box structural domains, yet with only moderate amino-acid similarity to HMGB1 (Larsson *et al*., 1996). Here we show (Fig. 3D) that both HMGB1 and TFAM similarly affected DNA cleavage by DFF40, indicating that the structural properties rather than amino-acid sequence of HMG-boxes were indeed crucial for stimulating the nuclease action.

Assuming that HMGB1 neither binds DFF40 nuclease nor enhances its stable binding to DNA, we hypothesized that the presence of HMG-box containing proteins affected DNA cleavage by DFF40 due to changes induced in DNA conformation upon HMG-box binding. DFF40 is a nuclease specific for double-stranded but not single-stranded DNA, which suggests that the structure of DNA affects its cleavage by this nuclease. In fact, DNA cleavage by DFF40 is inhibited by several drugs that target the DNA minor groove, including ethidium bromide (not shown). Binding of HMG-boxes to DNA induces characteristic bending and unwinding. In addition, HMG-box-containing proteins (as well as isolated HMG-boxes) preferentially bind to DNA that is intrinsically bent and underwound or organized as cruciforms or four-way junctions; this feature of HMG-boxes causes their preferential binding to DNA damaged by cisplatin (*cis*-DDP) (reviewed in Landsman & Bustin, 1993; Thomas & Travers, 2001). Cisplatin is an anticancer drug that induces intra- and inter-strand crosslinks, which cause severe local distortions in the DNA double helix. The 1,2-intrastrand crosslinks, which are the major lesions induced by cisplatin, are known to bend the helix by 34° towards its major groove and unwind it by 13°, and are preferentially recognized by HMG-box-containing proteins (Bellon *et al*., 1991). Transplatin (*trans*-DDP), the geometric isomer of cisplatin, cannot form 1,2-intrastrand crosslinks and DNA damaged by this compound is not preferably recognized by HMGB1. However, transplatin can induce 1,3-intrastrand crosslinks (in addition to interstrand crosslinks), and also bend and unwind the DNA double helix (although contradictory data exist in the literature describing the extent of such DNA helix disturbances) (reviewed in Lippert, 1996). Here we used a plasmid DNA substrate modified with either cisplatin or transplatin to determine whether the presence of drug-induced DNA distortions would affect its cleavage by DFF40. We assumed that incubation of plasmid DNA with 0.1 to 10 μM cisplatin might induce about one crosslink per 5 000 to 50 bp of DNA (as calculated according to formula given by Ushay *et al*. (1981)), and supposed a similar frequency of transplatin-induced crosslinks. Data presented in Fig. 4 show that at a low frequency of damage (about one crosslink per 1000 bp) transplatin-induced disturbances resulted in a higher efficiency of DNA cleavage by DFF40 (lanes 8 and 9). At the same frequency of damage cisplatin barely affected DNA cleavage by the nuclease (lanes 3 and 4). At higher frequencies of damage (about one crosslink per 100 bp and less) both cisplatin and transplatin induced a marked inhibition of DNA cleavage by the nuclease. These data confirm that local disturbances in the DNA double helix affect its cleavage by DFF40 and may help, although not conclusively, to understand the mechanism of action of HMG-boxes upon DNA cleavage by the nuclease.

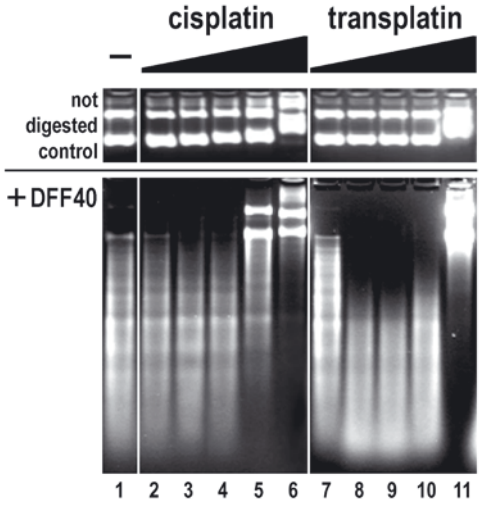

Figure 4. DNA-binding drugs that disturb the conformation of the double helix affect DNA cleavage by DFF40 endonuclease.
Plasmid DNA was incubated for 24 h with either cisplatin or transplatin at 0.1, 0.3, 1.0, 3.0 or 10 μM, purified, and then used as a substrate for caspase-activated DFF40. Corresponding plasmid DNA not treated with the nuclease is shown as a control.

In conclusion, we report here that a tandem array of HMG-boxes, DNA-binding protein domains present in abundant chromatin proteins and transcription factors, is potent to stimulate DNA cleavage by the major apoptotic nuclease DFF40. However, HMG-box-containing proteins neither bind to DFF40 nor enhance the ability of the nuclease for stable binding to DNA. Instead, the data suggest that dynamic changes induced in DNA conformation upon HMG-box binding make the substrate more accessible to cleavage by DFF40 nuclease. In chromatin, HMG-box-containing proteins bind preferentially to internucleosomal linker DNA (reviewed in Bustin & Reeves, 1996). Thus we postulate that the mechanism described here may contribute to the preferential linker DNA cleavage during apoptosis.

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