

Mitochondrial transcription factor A is the major protein in rodent hepatocytes that recognizes DNA lesions induced by N-acetoxy-acetylaminofluorene

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Extracts from rodent liver cells contain an abundant protein that recognizes DNA adducts induced by the chemical carcinogen N-acetoxy-acetylaminofluorene (AAAF). This protein also has a strong affinity for DNA damaged by cisplatin (DDP), but not by benzo(a)pyrene diolepoxide or UV-radiation, and has been termed AAAF/DDP-DDB. Here we purified this protein from rat tissue and analyzed it by mass spectrometry and identified it as mitochondrial transcription factor A (TFAM). Experiments with bacterially expressed recombinant TFAM confirmed its high affinity for DNA damaged by AAAF. Assuming its abundance and specificity for AAAF induced lesions, TFAM may significantly impede recognition and repair of DNA adducts induced by AAAF and other derivatives of 2-aminofluorene.

Keywords: AAAF, cisplatin, TFAM, UV-radiation

The first step of each DNA repair mechanism is damage recognition carried out by different protein detectors, which depend on the type of damage and repair pathway. Among detector complexes involved in NER, an important and universal pathway which removes a broad spectrum of DNA damage, are XPC/HR23B, UV-DDB (XPE) and XPA/RPA (reviewed in Wood, 1999; Sancar *et al.*, 2004). Many other proteins that bind DNA lesions repaired by NER have been detected in different organisms, but their relevance to DNA repair remains elusive. Among these damaged DNA-binding (DDB) proteins is chromatin protein HMGB1 (formerly termed HMG-1) and other HMG-box-containing proteins, which were found to have a high affinity for 1,2-intrastrand crosslinks induced in DNA by *cis*-diaminedichloroplatinum (*cis*-DDP, cisplatin) (Pil & Lippard, 1992) and other bulky DNA damage (reviewed in Widłak *et al.*, 2006). It has been proposed that HMG-box-containing proteins may compete

with DNA repair proteins, making repair less efficient and facilitating apoptosis induced by *cis*-DDP and other DNA-damaging factors; this was called the damage-shielding model (reviewed in Widłak *et al.*, 2006; Reeves & Adair, 2006).

Derivatives of the aromatic amine 2-aminofluorene (2-AF) are known hepatocarcinogens and the formation of covalent DNA adducts seems to be an essential step in carcinogenesis induced by these compounds (Kroese *et al.*, 1988; Poirier *et al.*, 1991). N-acetoxy-acetylaminofluorene (AAAF), which induces mostly the dG-C8-AAF form of adduct, is among the best studied of such compounds (reviewed in Heflich & Neft, 1994). Although DNA adducts induced by derivatives of 2-AF are removed by NER, some aspects of their repair differ from that of other bulky DNA adducts. Adducts induced by AAAF were not preferentially removed from transcriptionally active genes (Tang *et al.*, 1989), and their removal was not biased toward the tran-

Abbreviations: AAAF, N-acetoxy-acetylaminofluorene; BPDE, benzo(a)pyrene diolepoxide; *cis*-DDP, *cis*-diamine-dichloroplatinum, cisplatin; DDB, damaged-DNA binding (protein); EMSA, electrophoretic mobility shift assay; HMG, high mobility group (protein); MNase, micrococcal nuclease; mtDNA, mitochondrial DNA; mtTFA, mitochondrial transcription factor A; NER, nucleotide excision repair; PAGE, polyacrylamide gel electrophoresis; TFAM, mitochondrial transcription factor A; 2-AF, 2-aminofluorene.

scribed strand (McGregor *et al.*, 1997). In contrast to DNA lesions induced by either UV-radiation or benzo(a)pyrene, adducts induced by 2-AF derivatives were not preferentially removed from matrix attached DNA (Widłak & Rzeszowska-Wolny, 1999). It seems probable that structural properties of AAAF-induced damage and/or specific mechanisms of such damage recognition contribute to low efficiency of repair of DNA adducts induced by AAAF.

We have previously shown that HMGB1 protein has elevated affinity to DNA damaged by AAAF (Łanuszewska & Widłak, 2000). Two additional proteins that specifically bind DNA damaged by AAAF have also been detected in nuclear extracts from rat liver cells (Rzeszowska-Wolny & Widłak, 1999; Pietrowska *et al.*, 2000). Major protein detected in extracts from rat and mouse liver cells that preferentially binds to DNA damaged by AAAF has an apparent molecular mass of about 25 kDa. This abundant protein (at least 10^5 molecules per cell) also has a strong affinity for DNA damaged by cisplatin, but not by BPDE or UV-radiation, and was termed AAAF/DDP-DDB (Pietrowska & Widłak, 2005). Here we aimed to isolate and identify this putatively novel DDB protein. The AAAF/DDP-DDB protein has been chromatographically purified from rat liver cells and identified by mass spectrometry as mitochondrial transcription factor A (TFAM, mtTFA), which was verified using bacterially expressed recombinant protein for *in vitro* DNA-binding assays.

MATERIALS AND METHODS

Preparation of fractions from rat liver cells.

Liver tissue of adult male Wistar rats was mechanically homogenized on ice in buffer consisting of 10 mM Hepes/NaOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 2.2 M sucrose, 1% Nonidet P40 and a protease inhibitor mixture (Complete™, Boehringer). The homogenate was centrifuged for 10 min at $400 \times g$ and washed in the same buffer without detergent to obtain the fraction of cell nuclei. To obtain nuclear extracts nuclei were incubated for 30 min on ice with a buffer consisting of 10 mM Hepes/NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 10 mM KCl and 0.4 M NaCl, and then insoluble remnants were pelleted by centrifugation for 30 min at $16000 \times g$ at 4°C. The chromatin fraction was purified from rat liver nuclei treated with micrococcal nuclease (MNase). Briefly, nuclei suspended in 80 mM phosphate buffer (pH 6.8) containing 5 mM $CaCl_2$, 1 mM DTT and 350 mM NaCl were incubated with MNase at 37°C until about 50% of chromatin was cleaved into mononucleosomal fragments. After the nuclease digestion, the reaction mixture was diluted twofold

with 350 mM NaCl in 80 mM phosphate buffer (pH 6.8) and EDTA (final conc. 2 mM), and incubated on ice for 10 min, then insoluble remnants were pelleted by centrifugation for 10 min at $16000 \times g$ at 4°C. Protein concentration was assayed with the Protein Assay Kit (BioRad). Proteins were resolved on 13% polyacrylamide/SDS gels and stained with silver or Coomassie Brilliant Blue.

Purification of AAAF/DDP-DDB protein. A chromatin fraction isolated from liver cells was subjected to chromatography on hydroxyapatite (type I, BioRad). Chromatin was applied onto the column equilibrated with buffer consisting of 160 mM phosphate buffer (pH 6.8), 1 mM DTT and 350 mM NaCl, and then the column was washed with the same buffer. Protein fractions were eluted with a linear 0.4–2 M NaCl gradient in 160 mM phosphate buffer (pH 6.8), 1 mM DTT. Selected fractions were diluted with 160 mM phosphate buffer and 1 mM DTT until 150 mM NaCl was reached, and then applied onto a CM-Sephadex column pre-equilibrated with the same buffer. Column-bound proteins were eluted with 0.2, 0.6 and 1.0 M NaCl in phosphate buffer. The activity of AAAF/DDP-DDB protein was analyzed in chromatographic fractions using EMSA and Southwestern-blot assays (see below).

Purification of recombinant TFAM protein.

Six-His-tagged rat recombinant TFAM was expressed in an *Escherichia coli* bacterial expression system and purified using chromatography on Ni-NTA agarose. cDNA for the mature form of rat hepatoma TFAM (residues 43–246; without MLR domain) was cloned into pQE30 (Qiagen) expression vector, as described elsewhere (Dong *et al.*, 2002).

Identification of purified protein. Proteins from selected chromatographic fractions were resolved on 13% polyacrylamide/SDS gel and stained with Coomassie Brilliant Blue. Bands corresponding to the DDB activity in a Southwestern blot assay were excised from the gel and analyzed by tandem mass spectrometry (ESI-QUAD-TOF) in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics (Warsaw, Poland). Resulting peptides were annotated to proteomic data bases using the Mascot software (www.matrixscience.com).

DNA binding *in vitro* assays. The ability of proteins to bind to damaged DNA *in vitro* was tested using an EMSA and a Southwestern-blot assay, as described in detail elsewhere (Pietrowska & Widłak, 2005). Briefly, a synthetic double-stranded 36 bp-long oligonucleotide was incubated with AAAF, BPDE (Midwest Research Institute, USA) or *cis*-DDP (Ebewe) to generate molecular probes containing specific types of DNA damage. The treatments introduced a single lesion into 10–20% of the DNA molecules, on average (Rzeszowska-Wolny & Widłak, 1999). Oligonucleotides were then ^{32}P -end-labeled and purified

from a 6% polyacrylamide gel. For the EMSA test radioactive oligonucleotides (25 ng) were incubated with proteins (5 μ g of nuclear extracts or corresponding amounts of purified fractions) for 30 min at 4°C in buffer consisting of 20 mM Tris/HCl (pH 7.6), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 5% glycerol, 150 mM NaCl, in the presence of competitor non-radioactive homologous oligonucleotides. Protein-oligonucleotide complexes were resolved by electrophoresis on a 6% polyacrylamide gel in 0.5 × Tris/borate/EDTA buffer, and gels were dried and autoradiographed. For Southwestern-blot analysis proteins (50 μ g of nuclear extracts or corresponding amounts of purified fractions) were separated on 13% polyacrylamide/SDS gels and electrotransferred onto PVDF membranes. Membrane-bound proteins were renatured and complexed with radioactive oligonucleotides (250 ng) at 25°C in a buffer consisting of 25 mM Tris/HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂ and 0.25% bovine serum albumin, in the presence of an 80-fold excess of non-radioactive non-specific competitor (*E. coli* DNA). After washing with the same buffer filters were autoradiographed.

RESULTS AND DISCUSSION

Nuclear extracts from rat liver cells were analysed for the presence of proteins that bind to damaged DNA using an electrophoretic mobility shift assay. The radio-labeled oligonucleotide damaged by AAAF or *cis*-DDP was used as a probe, and complexes with proteins were formed in the presence of excess of non-radioactive oligonucleotide, either non-damaged or damaged with AAAF or *cis*-DDP, as a homologous competitor. The results in Fig. 1 show that AAAF-damaged DNA is specifically bound by AAAF/DDP-DDB protein complex (lane 1), whereas *cis*-DDP-damaged DNA is bound by HMGB1 and AAAF/DDP-DDB protein complexes (lane 4). Accordingly, the presence of AAAF-damaged competitor DNA inhibits the probe binding by AAAF/DDP-DDB protein but not HMGB1 (lanes 3 and 6), while the presence of *cis*-DDP-damaged competitor DNA inhibits the probe binding by both AAAF/DDP-DDB protein and HMGB1 (lanes 2 and 5) (the identity of HMGB1 in appropriate complex was confirmed in our earlier work; Łanuszewska & Widłak, 2000).

Aiming to identify the AAAF/DDP-DDB protein we attempted to purify it to homogeneity from the chromatin fraction of rat liver cells. Proteins were fractionated by column chromatography on hydroxyapatite and CM-Sephadex, and then activity of the AAAF/DDP-DDB protein was detected in such fractions (Fig. 2). When proteins were eluted

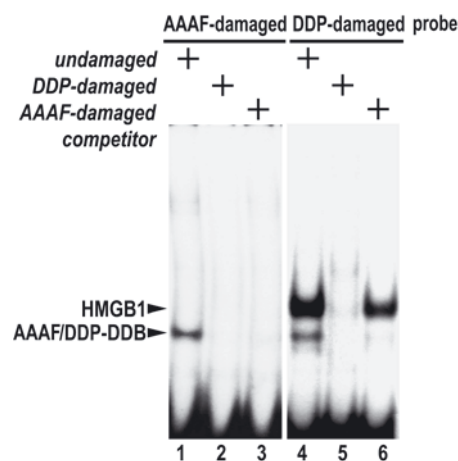


Figure 1. The major rat liver protein that recognizes DNA damaged by AAAF also binds to DNA damaged by *cis*-DDP and has been termed AAAF/DDP-DDB.

Complexes were formed between labeled oligonucleotide, damaged either with AAAF or *cis*-DDP, and nuclear extracts from rat liver cells, in the presence of a 50-fold excess of homologous non-radioactive competitor (either undamaged or damaged with AAAF or *cis*-DDP), and then separated by native polyacrylamide gel electrophoresis.

from hydroxyapatite-bound chromatin material by increasing NaCl concentrations, the AAAF/DDP-DDB activity could be detected mostly in a fraction eluted with 0.4–0.45 M NaCl (lane 3). This fraction was then applied onto a CM-Sephadex column and proteins were eluted with 0.2, 0.6 and 1 M NaCl. The AAAF/DDP-DDB activity was detected in the fraction eluted with 0.6 M NaCl (lane 9). This protein fraction contained two major protein species of about 25 and 18 kDa (Fig. 2A). Southwestern-blot analysis (Fig. 2C) revealed that both proteins form *in vitro* complexes with an AAAF-damaged DNA probe but not with a control non-damaged probe. However, only the 25 kDa protein form *in vitro* complexes with a *cis*-DDP-damaged probe, and we assumed that this protein band corresponds to the AAAF/DDP-DDB protein. This protein was excised from a Coomassie-stained gel and subjected to mass spectrometry analysis. The protein was identified as mitochondrial transcription factor A (TFAM, mtTFA) (identified peptides covered 39% of the mature form of rat TFAM). Similarly, the AAAF/DDP-DDB protein was purified from chromatin fraction of mouse liver cells using the same procedure and also identified as TFAM (identified peptides covered 53% of the mature form of mouse TFAM), which fully confirmed its identity. We also tried to identify the 18 kDa protein. However, the obtained peptides did not match any protein in proteomic data bases. In addition, the 18 kDa protein was eluted from a gel and renatured but it did not form any complexes with either AAAF-damaged or *cis*-DDP-damaged DNA (not shown).

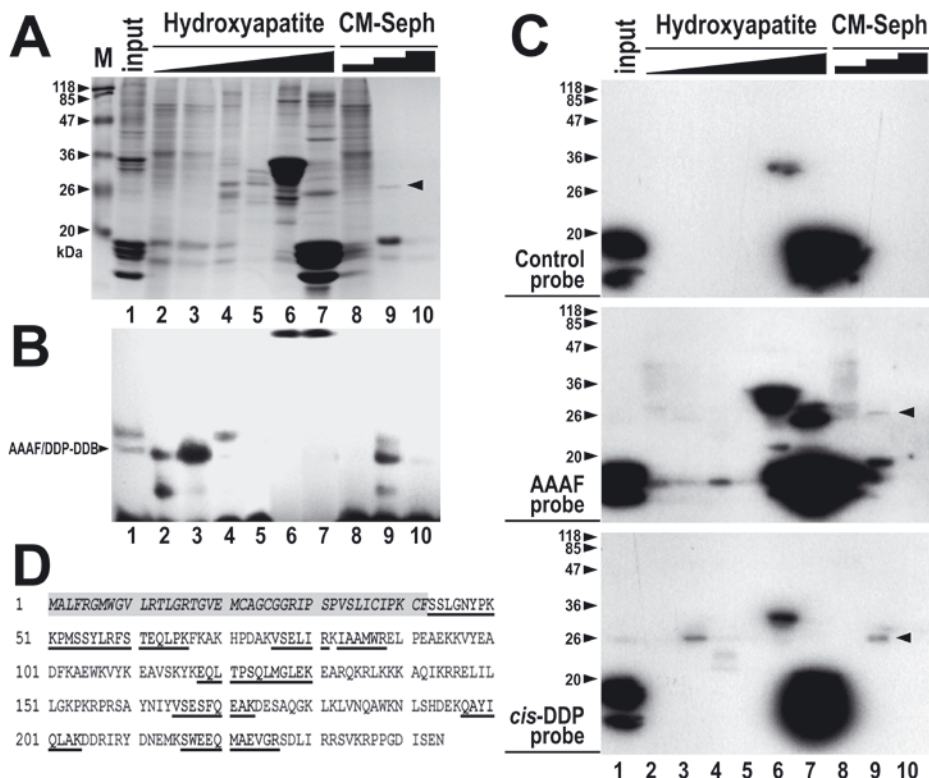


Figure 2. The AAAF/DDP-DDB protein was purified and identified as TFAM.

Panel A. SDS/PAGE analysis of chromatographically fractionated chromatin preparation from rat liver. Proteins were first eluted from a hydroxyapatite column with 0.4, 0.45, 0.5, 0.6, 0.8 and 2 M NaCl (lanes 2–7), then the 0.45 M fraction (lane 3) was loaded onto a CM-Sephadex column and proteins eluted with 0.2, 0.6 and 1 M NaCl (lanes 8–10). **Panel B.** Detection of the AAAF/DDP-DDB activity in chromatographic fractions. Complexes were formed with radio-labeled *cis*-DDP-damaged probe in the presence of genomic *E. coli* DNA competitor. **Panel C.** Southwestern-blot analysis of chromatographic fractions. A protein band corresponding to the AAAF/DDP-DDB activity is marked with an arrowhead. **Panel D.** The sequence of amino-acid residues of rat TFAM precursor. The mitochondrial localization region (MLR) removed from the mature protein is highlighted in gray. Peptides identified by mass spectrometry analysis of the AAAF/DDP-DDB protein are underlined.

We have observed that complexes formed by recombinant rat TFAM with damaged DNA have the same electrophoretic mobility as complexes formed by the AAAF/DDP-DDB protein isolated from rat liver extracts (Fig. 3A). We have also aimed to study the affinity of bacterially expressed recombinant TFAM to DNA containing different types of bulky adducts. Recombinant rat TFAM was incubated with a *cis*-DDP-damaged radio-labelled probe in the presence of increasing amounts of different homologous competitors (Fig. 3B). The affinity of recombinant rat TFAM for AAAF-damaged or *cis*-DDP-damaged non-radioactive competitor was at least 33-fold greater than that for undamaged DNA (i.e. 0.06 μ g of AAAF-damaged oligonucleotide was as good competitor as 2 μ g of undamaged one). The affinity of recombinant TFAM for BPDE-damaged competitor was only 2- to 4-fold higher than that for the undamaged one, which was in full agreement with previously reported characteristics of the AAAF/DDP-DDB protein (Pietrowska & Widlak, 2005). Similarly, purified recombinant human TFAM has strong affinity

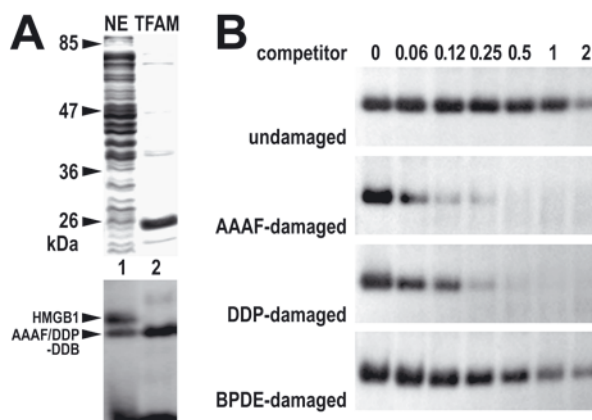


Figure 3. Bacterially expressed recombinant rat TFAM binds with high affinity to DNA damaged by AAAF.

Panel A. Nuclear extract (NE) from rat liver cells (lane 1) and recombinant rat TFAM protein (lane 2) were analyzed by SDS/PAGE (top) and EMSA with radioactive *cis*-DDP-damaged probe (bottom). **Panel B.** Complexes were formed between recombinant rat TFAM and radio-labeled *cis*-DDP-damaged probe (25 ng), in the presence of increasing amounts of homologous non-radioactive competitor (0.06 to 2 μ g, as indicated), either undamaged or damaged with AAAF, *cis*-DDP or BPDE.

for AAAF-damaged or *cis*-DDP-damaged, but not for BPDE-damaged DNA.

Mitochondrial transcription factor A is a protein that contains two HMG-boxes, and its primary function is to regulate replication and transcription of mitochondrial DNA (Larsson *et al.*, 1998). This protein has been previously reported to bind with enhanced affinity to DNA containing either lesions induced by *cis*-DDP or 8-oxo-guanine (Ohndorf *et al.*, 1997; Yoshida *et al.*, 2002), yet its binding to other types of DNA damage was not reported. Though mitochondria are the primary location of TFAM, this protein is detected in other cellular compartments as well. In fact, high levels of TFAM have been detected in nuclei of rat hepatoma cells (Dong *et al.*, 2002). We have compared levels of the AAAF/DDP-DDB activity in the nuclear fraction of liver cells homogenized in the presence of Nonidet P40 and in fractions of liver cells homogenized and lysed in the absence of a detergent (this latter procedure is more likely to protect the integrity of mitochondrial membranes). In fact, we have observed that the level of the AAAF/DDP-DDB protein was 3–4-fold lower in nuclear extracts from liver cells lysed in the absence of detergent as compared to extracts from detergent-treated cells, and that the AAAF/DDP-DDB protein could be detected in mitochondrial extracts (not shown). This indicates that during cell lysis a fraction of TFAM released from mitochondria disrupted with detergent apparently binds to chromatin and could be recovered from nuclear extracts and chromatin fractions. However, it seems possible that a fraction of TFAM that recognizes damaged DNA is also located in intact nuclei of rat hepatocytes. Although TFAM is ubiquitously expressed in rat tissues, the activity of AAAF/DDP-DDB protein was markedly lower in extracts from testes as compared to liver and other somatic tissues (Pietrowska & Widłak, 2005). An additional nuclear isoform of TFAM (ts-HMG), produced from transcripts that have an alternative first exon, was detected in testes of mice (Larsson *et al.*, 1996), but not testes of rats (Rantanen *et al.*, 2001). However, the level of “primary” TFAM was markedly downregulated in both mouse and rat testes, reflecting the progress of spermatogenesis. Thus the observed reduction of the AAAF/DDP-DDB activity in testicular extracts (Pietrowska & Widłak, 2005) apparently reflects downregulation of TFAM protein during spermatogenesis.

TFAM, like other HMG-box containing proteins, can recognize distortions in DNA structure that facilitate its physiological binding to the D-loop region in mtDNA. This feature apparently enables binding of TFAM to unwound or bent sequences at a site of DNA damage. Similar to other HMG-box proteins (e.g. HMGB1), TFAM binds with high affinity to DNA damaged by cisplatin (Ohndorf *et al.*, 1997;

Yoshida *et al.*, 2002). In marked contrast, however, we have shown here that TFAM binds to DNA damaged by AAAF with a much higher affinity as compared to HMGB1. The presence of Pt-DNA 1,2-intrastrand crosslinks, the major type of cisplatin-induced adducts, or dG-C8-AAF, the major type of AAAF-induced adducts, somehow differently distorts the structure of double-stranded DNA (reviewed in Łanuszewska & Widłak, 2000). Although recognition of DNA distorted at a site of cisplatin-induced crosslinks is the general feature of the HMG-box, recognition of DNA containing other bulky damage differently changing the structure of DNA may be characteristic for a particular HMG-box protein.

The physiological consequence of TFAM binding to damaged DNA remains elusive at the moment. The protein is essential for mtDNA maintenance and possibly involved in its repair (Larsson *et al.*, 1998). Mitochondrial DNA is highly susceptible to DNA damage, especially oxidative damage caused by byproducts of the electron transfer reactions in mitochondria. Interestingly, TFAM but not HMGB1 shows high affinity for mismatches containing 8-oxo-guanine, the major type of oxidative base modification, that suggested its involvement in recognition and repair of mtDNA damage (Yoshida *et al.*, 2002). Homozygous disruption of the *Tfam* gene resulted in increased apoptosis *in vivo*, and most probably observed cell deaths resulted from lack of mtDNA expression and respiratory chain dysfunctions, yet more direct effects of TFAM should not be excluded (Wang *et al.*, 2001). Interestingly, TFAM interacts directly with p53 protein, whose fraction relocated to mitochondria at the onset of DNA damage-induced apoptosis, and the presence of p53 enhanced TFAM binding to cisplatin-damaged DNA yet inhibited its binding to 8-oxo-guanine-containing DNA (Yoshida *et al.*, 2003). Hypothetically, TFAM that bound to damaged DNA might either facilitate or delay DNA repair (by recruiting repair complexes or shielding a lesion, respectively). Interactions with p53 in cells exposed to genotoxic agents might affect binding of TFAM to particular types of DNA damage to modulate their repair and induction of apoptosis, as proposed by Kohno and coworkers (Yoshida *et al.*, 2003). According to our earlier data the AAAF/DDP-DDB protein, identified here as TFAM, is the major protein present in somatic tissues of rodents that recognizes and binds to DNA containing damage induced by AAAF (Pietrowska & Widłak, 2005). The data presented here indicate the possibility that a fraction of TFAM protein that has an activity of AAAF/DDP-DDB protein, exists in nuclei of rat somatic cells. Considering the abundance of this protein and its high affinity for DNA alterations induced by AAAF, TFAM might essentially impede recognition and repair of DNA adducts induced by

2-AF derivatives and contribute to their carcinogenic potency.

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