A novel UV-damaged DNA binding protein emerges during the chromatin-eliminating cleavage period in *Ascaris suum*

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**ABSTRACT**

During the early cleavage period of *Ascaris suum*, chromatin diminution takes place in the somatic founder cells. In the process of chromatin diminution numerous heterochromatic blocks, consisting predominantly of highly repeated DNA, are discarded during mitotic anaphase and are later on digested in the cytoplasm. Very little is known about proteins that are involved in chromatin diminution. We have detected a nuclear protein and purified it to near homogeneity by its preferential binding to UV-damaged DNA. We termed this protein chromatin diminution associated factor 1 (CDAF1), because maximum binding activity per nucleus was observed to develop in 4–8-cell stages, when chromatin diminution occurs for the first time. CDAF1 recognizes cyclobutane pyrimidine dimers in UV-damaged double-stranded DNA. Its binding properties identify CDAF1 as a novel kind of damaged-DNA binding protein. CDAF1 activity is almost not detectable in 1-celled embryos. It increases dramatically during formation of somatic founder cells and persists up to the first larval stage. However, CDAF1 is absent in tissues of adults. These findings led us to suggest that CDAF1 plays a dual role: during the early segregative cleavage period it might be involved in chromatin diminution as a transfactor and act in nucleotide excision repair as an accessory factor throughout embryogenesis.

**INTRODUCTION**

During the early cleavage period the fertilized egg of nematodes undergoes an invariant series of asymmetrical cell divisions which define the embryonic germline and four somatic founder cells. The lineage tree of the free living nematode *Caenorhabditis elegans* has been fully unravelled (1). In the parasitic nematode *Ascaris* both this tree and the topogenesis of blastomeres are very similar to those of *C.elegans*.

In *Ascaris* and several other species of parasitic nematodes, a substantial amount of the germline genome is discarded in somatic founder blastomeres (2). As early as 1887, Boveri (3) discovered the founder blastomeres (2). As early as 1887, Boveri (3) discovered the process of selective chromatin elimination in *Parascaris*, which has been later referred to as chromatin diminution (4). In *Parascaris univalens* the two large compound chromosomes disintegrate into numerous somatic chromosomes and large blocks of terminal heterochromatin. The latter fail to move at anaphase and are thus excluded from the daughter nuclei. Recent cytological studies have revealed that the chromosomes undergo differential mitotic condensation; chromosome disintegration results from the excision of non-condensed chromatin segments adjacent to the presumptive somatic chromosomes (5). In the germline lineage the chromosomes do not undergo diminution, thus maintaining the zygotic karyotype (6).

In *Ascaris suum* the germline genome is contained in 19 autosomes and 5 X-chromosomes. The female karyotype consists of 48 chromosomes, the male karyotype of 43 chromosomes. Almost all chromosomes are involved in the process of chromatin diminution. The eliminated DNA comprises ~23% of the haploid germline genome. It predominantly consists of highly repeated satellite DNA (7), termed gll-sat-DNA (germline limited satellite DNA). The gll-sat-DNA consists entirely of one single 123 bp repeat unit (8,9). A small number of single copy genes may also be part of the germline limited DNA (10,11).

A large body of data, derived from manipulated *Parascaris* embryos, demonstrates a considerable regulative potential in the decision-making process of segregative cell divisions which give rise to soma and germline sister cells (4). Just as in normal embryos, the alternative nuclear behaviour (diminution or non-diminution) in experimental embryos is strictly correlated with the fate of blastomeres, i.e. their commitment to either soma or germline. These data have been integrated into a ‘differential activation model’ which assigns a crucial role in cell-fate specification to the cell-cycle oscillatory system. Cellular bifurcations are triggered by a signal produced at the very end of the cell cycle. Polarization of the parental germline cell generates signals of critical difference, which direct the nascent daughter cells to adopt mutually exclusive fates (12,13).

According to this concept, the challenge of chromatin diminution is to define the components of the cascade involved in the process of cellular binary decision-making, the factors acting in the site-directed chromosome fragmentation during diminution, and the mechanisms of their (trans-) activation. Very little is known about those factors. Jansen and Moritz (14) have described an endonuclease activity, derived from early cleavage stages of *A.suum*,...
that acts exclusively on supercoiled plasmid DNA introducing either one single- or double-strand break per molecule. Because open-closed DNA circles as reporter molecules are inefficient substrates, the endonuclease appears to act close to local secondary structures, which form at certain interrupted inverted repeats. This observation corresponds to the results of Müller et al. (15). According to their analyses, DNA disintegration upon diminution appears to occur at several sites within a certain ‘chromosomal breakage region’, which have no obvious sequence homology.

Therefore, we searched for DNA binding proteins in early embryos which recognize structures rather than sequences, that might occur during the period of diminution events as predicted from the ‘differential activation model’. Here we characterize a protein. Binding activity is developmentally regulated, it is maximal per nucleus at the third cleavage step when diminution occurs for the first time. The putative role of CDAF1 in the context of chromatin reorganization during chromatin diminution is discussed.

**MATERIALS AND METHODS**

### Preparation of peeled eggs

The proximal segments of uteri, prepared from gravid females, were collected on ice and minced in distilled water by an Ultra-Turrax. From the crude egg suspension ‘peeled eggs’, which are still surrounded by the peritelline membrane and the ascaroside layer, were prepared as described (5). These eggs, still at the pronuclear stage, were allowed to develop in distilled water at 30°C in the dark.

### Preparation of 123 bp restriction fragments of the gll-sat-DNA

Gll-sat-DNA was isolated from testes, as described previously (8). The DNA was digested to completion with BstI or AsnI. After agarose gel electrophoresis, the respective monomer variants of the 123 bp repeat unit, termed BstI and AsnI monomers, were isolated with ‘agarose spin columns’ (Supelco) according to the instructions of the manufacturer.

### 3'-Endlabelling of the 123 bp monomers with digoxigenin

The reactions were carried out in 50 µl 10 mM Tris, pH 7.5, 10 mM MgCl2, 50 mM NaCl, 1 mM DTE containing 250 ng 123 bp monomers, 50 µM digoxigenin-d-UTP (Boehringer Mannheim), 2 U DNA polymerase I and 50 µM dATP for AsnI monomer labelling, additional dCTP and dGTP (50 µM each) in case of BstI monomer labelling. After 30 min at room temperature, the reactions were terminated with 2 µl 0.2 M EDTA, pH 8.0. The DNA was recovered by ammoniumacetate/ethanol precipitation.

### UV-irradiation and cisplatin treatment of DNA

DNA (400–800 ng) was irradiated in a small volume of H2O (8 µl) with a 100 W germicidal lamp (Bioblock Scientific) emitting at 254 nm. By means of the nicking activity of T4 endonuclease V a dosage of 2000 KJ/m² was determined to be sufficient to introduce at least one damaged site per Ascaris monomer molecule. For cisplatin treatment DNA (240 ng) was incubated in 50 µl 3 mM NaCl, 1 mM sodiumphosphate, pH 7.4, in the presence of 24 µM cisplatin for 20 h. After precipitation, the DNA was redissolved in H2O at 40 ng/µl.

### Preparation of cell extracts from embryos and tissues

Protein fractions were prepared from selected stages as follows: 200 µl packed embryos (2 × 10⁷ embryos) were homogenized at 4°C in 1 ml buffer L (10 mM HEPES, pH 7.8, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol) and 100 µg/ml leupeptin. The homogenate was fractionated at 15 000 g for 15 min. The supernatant was clarified at 100 000 g for 20 min to yield the LS- (low salt) supernatant. The 15 000 g pellet was washed three times in buffer L and resuspended in 200 µl buffer H (buffer L + 350 mM NaCl). Nuclear proteins were extracted for 40 min at 4°C. Finally, the suspension was clarified at 100 000 g for 20 min. The resulting supernatant, termed HSP- (high salt) pellet, extract, was stored at −80°C. LS-supernatants and HSP-extracts from body muscle and gut of *Ascaris suum* females were prepared essentially in the same way.

### Standard DNA mobility-shift assay (DMSA)

LS-supernatants (20 µg protein) or HSP-extracts (5 µg protein), respectively, were preincubated with poly [d(I-C)] (1.25–2.5 µg) as a general competitor in 25 mM HEPES, pH 7.8, 75 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol for 5 min at room temperature. After adding 1 ng digoxigenin-labelled probe and, in several cases, additional competitor DNA of various sources, the binding reaction (10 µl) was allowed to continue for 20 min and terminated with 5 µl of DTT-solution (5 mM DTT, 0.023% bromphenolblue). The samples were immediately electrophoresed on a 5% polyacrylamide gel in 1/3 TBE-buffer (30 mM Tris, 30 mM boric acid, 0.8 mM EDTA) for 70 min at 14 V/cm in a water-jacketed electrophoresis unit. The DNA was blotted on a nylon membrane (Biodyne B) in 1/2 TBE-buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA) overnight at 100 mA and 4°C. Probe DNA was visualized by chemiluminescence (CSPD, AMPPD) and/or colour reaction (NBT, BCIP) mediated by alkaline phosphatase, conjugated with anti-digoxigenin FAB fragments (Boehringer Mannheim), according to the Bohringer protocol.

### Photoreactivation of UV-irradiated DNA

Digoxigenin-labelled AsnI monomers (15 ng) were UV-irradiated, as described above. The DNA solution was adjusted to 100 mM NaCl; 10 mM K/Na-phosphate, pH 7.0, 5 mM 2-mercaptoethanol, 0.1% BSA. The DNA was photoreactivated with the light of a lamp used for room illumination at 25°C for 30 min in the presence of 50 ng *Anacytis nidulans* photolyase (gift of A.P.M.Eker). After proteinase K treatment, the DNA was recovered by phenol/ chloroform extraction and precipitation.

### Nicking of UV-damaged DNA with T4 endonuclease

Photoreactivation, catalysed by the photolyase, was monitored as follows: 1.5 ng samples of UV-irradiated digoxigenin-labelled AsnI was incubated with T4 endonuclease.
Figure 1. Lineage tree and topogenesis of blastomeres in early embryogenesis of *A. suum*. A series of segregative cell divisions of the germline represented by P₀, P₁, P₂, and P₃ generates four somatic founder cells; AB, EMSt, C and D, according to Boveri’s nomenclature (6). The lifetimes of cells display a considerable variation. A, B, EMSt, C and D undergo chromatin diminution. The somatic lineages participate in the formation of the embryonic blastemas, as indicated. Right: living specimens (anterior is to the right) representative of the embryo batches from which nuclear proteins were prepared.

monomers, photolyase-treated or mock-treated, were incubated with an excess of 12 ng T₄ endonuclease V (gift of R.S.Lloyd) in 10 µl 25 mM NaH₂PO₄, pH 6.8, 1 mM NaCl, 0.01 mM EDTA, 100 µg/ml BSA for 45 min at 37°C. After DNA denaturation, the samples were mixed immediately with 1/3 vol 15 mM DTT, 0.023% bromo-mephenolblue, 20% glycerol and electrophoresed on a 6% polyacrylamid gel in 1/3 TBE-buffer.

Purification of CDAF1 by DNA-affinity chromatography

A 10 ml HSP-extract, prepared from 4–8-cell stages (18.5 mg protein), was diluted with 3 vol buffer L to precipitate egg-shell proteins. The precipitated proteins were discarded by centrifugation. The supernatant, concentrated to 10 ml by ultrafiltration (UF 15, Millipore), was loaded onto a DNA–cellulose column (4 ml), equilibrated with buffer L + 75 mM NaCl. Proteins were fractionated stepwise by increasing the NaCl concentration in buffer L, i.e. at 75, 200 and 500 mM NaCl. The 500 mM NaCl fractions containing CDAF1 were pooled and applied to an UV-irradiated DNA–cellulose column (0.5 ml), equilibrated with buffer L + 500 mM NaCl after saturation with BSA. Proteins were eluted at 500 and 2000 mM NaCl. The peak fractions were dialysed in buffer L + 160 mM NaCl and assayed for CDAF1 activity and protein patterns. Active fractions were stored in aliquots at −80°C.

RESULTS

Aspects of cellular and nuclear differentiation

Embryogenesis of *A. suum* is still perceived as the classical paradigm of the mosaic-type of development. The egg cell, P₀, already undergoes an asymmetrical division. The larger blastomere, which incorporates the animal hemisphere of the egg, is the first order somatic founder cell, AB, the smaller vegetal sister is the germline blastomere P₁ (Fig. 1). At the second cleavage step AB divides by a meridional cleavage into A and B, and P₁ by a subequatorial division into the second order presomatic cell, EMSt, and the smaller P₂. The resulting T stage transforms into the planar rhomb stage through the movement of P₂ around EMSt. At that stage the embryonic axes are fixed: A and B specify dorsal, EMSt and P₂
ventral. P₂ defines the posterior end of the embryo (Fig. 1). Cleavage of the embryo to the 8-cell stage occurs via three steps: first, the dorsal cells, A and B, both divide into left and right daughter blastomeres. Second, P₂ cleaves into the anterior smaller P₃, and the posterior third order presomatic cell, C. Third, EMSt gives rise to the anterior, larger MSt and the posterior E by an unequal division. P₃ divides into the smaller P₄ and the posterior D, the final presomatic cell. Then P₄ cleaves equally into the progenitors of the germ cells, UG₁ and UG₂ (Fig. 1). The constancy of early cell genealogy and blastomere topogenesis contrasts with the variation in developmental timing. Three features contribute to this variation: (i) embryos differ in cleavage tempo. The high degree of asynchrony becomes obvious already at the first cleavage step. (ii) Somatic founder cells display a considerable variation in cell-cycle length. Hence, embryos with identical cell numbers may differ in cell qualities. A 7-cell embryo, for example, may consist of four AB descendants and either MSt, E and P₂ or EMSt, P₃ and C. (iii) Cells of the same somatic lineage differ in their cleavage rhythm (Fig. 2B). Cell proliferation drops drastically at the late comma stage, where the embryo consists of ~480 cells. The comma stage transforms into the first larvae by morphogenetic processes which include organogenesis and cytodifferentiation.

In the presomatic cells A, B, EMSt, C and D (Fig. 1) the process of chromatin diminution takes place. Thus, during the third
chromatin diminution (Fig. 2). Because of the variable length of cleavage step three blastomeres, A, B and EMSt, undergo age 0 h) which represent the germline state; (ii) 1–2-cell stages (42 h) embryonic stages for DMSAs: (i) pronuclear stages (developmental proliferation and cytodifferentiation, we selected the following nuclear differentiation (non-diminution versus diminution), cell the embryo consists of at least 50 cells. final diminution event, upon cell division of D, takes place when the cell cycle in somatic founder cells (see above), diminution in

Figure 3. CDAF1 binding properties. (A) CDAF1 binds UV-damaged repeated sequences of the gll-sat-DNA with high specificity. UV-irradiated, digoxigenin-labelled Bcl I monomers (1 ng) were incubated with HSP-extract (5 µg protein), prepared from 4–8-cell stages, and increasing amounts of poly [d(I-C)] competitor: without competitor (lane 1), with 20 ng (lane 2), 80 ng (lane 3), 310 ng (lane 4) and 1250 ng (lane 5). (B) CDAF1 binds UV-damaged DNA of any complexity. HSP-extracts (5 µg protein), prepared from 4–8-cell stages, were incubated with 1 ng UV-irradiated Asnl monomers as probe and additional competitors: 100 ng pBR 322 DNA (lane 1), and sonicated salmon DNA (lane 3); 50 ng UV-irradiated DNA from pBR 322 (lane 2) and salmon (lane 4). (C) CDAF1 binds exclusively to UV-induced pyrimidine lesions in double-stranded DNA. Standard DMSAs were carried out with the following additional competitors, at 120 ng per 10 µl assay in lanes 1–4 and 40 ng in lanes 5–7; oligo(dT)19–24, UV-irradiated (â) (lane 1); double-stranded oligo(dA)20(dT)20, subsequent to UV-irradiation of oligo(dT)19–24 (lane 2), of oligo(dA)19–24 (lane 3), and untreated (lane 4); Asnl monomers untreated (lane 5), cisplatin-treated (CPT) (lane 6), and UV-irradiated (lane 7). (D) CDAF1 recognizes cyclobutane pyrimidine dimers, but not (6–4) photoproducts. In standard DMSAs the UV-damaged probe (1 ng Asnl monomer) was mock-treated (lane 1), and photolyase-treated (lane 2). Photolyase-mediated repair of UV-damaged DNA dramatically reduces complex formation.

cleavage step three blastomeres, A, B and EMSt, undergo chromatin diminution (Fig. 2). Because of the variable length of the cell cycle in somatic founder cells (see above), diminution in C occurs earliest in 14-celled and latest in 26-celled embryos. The final diminution event, upon cell division of D, takes place when the embryo consists of at least 50 cells.

In order to correlate the appearance of DNA binding proteins with crucial events in embryonic patterning, i.e. early cell commitment, nuclear differentiation (non-diminution versus diminution), cell proliferation and cytodifferentiation, we selected the following embryonic stages for DMSAs: (i) pronuclear stages (developmental age 0 h) which represent the germline state; (ii) 1–2-cell stages (42 h) performing the first segregative cleavage, (iii) 4–8-cell stages (63 h). In these egg batches consisting of 2–3-cell stages (18%), rhomb stages (54%) and 5–8-cell stages (28%), 82% of the embryos prepare for, execute or have completed diminution for the first time; (iv) ~26-cell stages (96 h). In these batches of 6–13-cell stages (17%), 14–32-cell stages (57%) and 33–60-cell stages (25%), ~79% of the embryos have completed diminution in the C lineage; (v) comma stages (168 h) of ~480 cells, which initiate the morphogenetic processes; (vi) first larvae (300 h) which have completed cytodifferentiation.

In A. suum the diploid germline genome (3 × 10^5 kb) is contained in multiple tiny chromosomes, 48 = 2(19A + 5X) in females, 43 = 38A + 5X in males. Almost all chromosomes carry terminal blocks of heterochromatin which differ in size. Several chromosomes show intercalarily located heterochromatic knobs. The gll-sat-DNA is localized in the heterochromatic blocks, as has been shown by in situ hybridization (Niedermaier, unpublished). The Asnl and the Bcl I monomers, that were used in DMSAs, both represent a substantial portion of the gll-sat-DNA.

Since its detection in 1887 (5), the process of diminution has been studied repeatedly. However, several features which are relevant in understanding this programmed DNA-rearrangement at the molecular level are still not well understood. Diminution is accomplished essentially via a three step process: (i) during prophase the heterochromatic blocks and the intercalary chromosome segment undergo normal condensation. However, short interstitially located regions connecting the terminal heterochromatin blocks and the intercalary chromosome segments remain completely uncondensed. (ii) Diminutive fragmentation occurs at the onset of anaphase and results in releasing the heterochromatin termini. (iii) The eliminated akinetic fragments are rapidly degraded in the cytoplasm, because they are unable to assemble nuclear envelopes. To summerize, the site-directed DNA-rearrangement of diminution requires karyokinesis in a presomatic cytoplasm. Chromosome breakage is preceded by differential local condensation. Differential chromosome condensation does not occur in germline mitoses.

CDAF1, a protein of the HSP-extract of 4–8-cell stages, recognizes UV-damaged DNA Following incubation of HSP-extracts (5 µg protein), prepared from 4–8-cell stages, with UV-damaged, digoxigenin-labelled Bcl I or Asnl monomers as probes (1 ng) in the presence of poly [d(I-C)] competitor, a protein was detected to bind specifically to the probes (Fig. 3A). We termed this protein CDAF1. Formation of CDAF1–DNA complexes depends on the amount of poly [d(I-C)] competitor: in assays without this general competitor, the probe DNA is completely retained on top of the 5% polyacrylamide gel, indicative of the relative abundance of DNA binding proteins in the HSP-extract. If 20 ng competitor is present in the binding reaction a faint CDAF1 retardation band is formed. At competitor amounts higher than 100 ng all non-specific complexes disappear (Fig. 3A). We termed this protein CDAF1. Formation of CDAF1–DNA complexes depends on the amount of poly [d(I-C)] competitor: in assays without this general competitor, the probe DNA is completely retained on top of the 5% polyacrylamide gel, indicative of the relative abundance of DNA binding proteins in the HSP-extract. If 20 ng competitor is present in the binding reaction a faint CDAF1 retardation band is formed. At competitor amounts higher than 100 ng all non-specific complexes disappear (Fig. 3A). Even at a 5000-fold excess of competitor, the retardation band does not diminish, indicating that CDAF1 binds to the probe with high specificity. The intensity of the CDAF1 retardation band does not depend on the specific Ascaris probe, i.e. Bcl I or Asnl monomers. The LS-supernatant of 4–8-cell stages (20 µg protein), representing the cytoplasmic fraction, contains trace amounts of CDAF1. Therefore, CDAF1 at least in its DNA binding form must be located in the nuclei at earliest developmental stages. Extracting
the 15 000 g pellet with 1000 mM NaCl, instead of routinely used 350 mM, did not increase the yield of CDAF1 (not shown). One extraction is sufficient to recover the factor quantitatively.

In a second series of experiments, UV-damaged S. nidulans fragments of pBR 322 (75, 78 and 105 bp) were used as probes: CDAF1 also binds effectively to these digoxigenin-labelled probes (not shown). Therefore, binding specificity does not depend on the source (sequence) of DNA. This was confirmed by applying DNAs of quite different complexity as additional competitors to the binding reactions. Salmon DNA and pBR 322 DNA, each at 100 ng, did not affect the formation of CDAF1–UV-irradiated DNA complexes. However, 50 ng of the respective DNAs, subsequent to UV-irradiation, effectively competed for CDAF1 binding to the cognate sites in the Ascaris monomer probes (Fig. 3B).

**CDAF1 recognition of UV-lesions requires double-stranded DNA**

The results presented above indicate that CDAF1 displays properties of DDB-proteins. To further characterize the cognate sites in UV-damaged DNA by CDAF1, defined simple-sequences were used as additional competitors: first, oligo (dA)_{19-24} and oligo (dT)_{19-24} were UV-irradiated and subsequently annealed to the respective complementary strands, but undamaged. Unaffected competition for CDAF1-binding was observed to occur, if the oligo dT had been UV-irradiated (Fig. 3C). In contrast, the double-stranded oligonucleotide with irradiated oligo (dA) as additional competitor did not affect complex formation. Therefore, CDAF1 does not recognize UV-induced adducts in oligo (dA). Second, oligo (dT)_{19-24} and oligo (dT)_{174}, both UV-damaged, were used separately as single-stranded additional competitors in DMSAs. Neither nucleotide did affect complex formation (Fig. 3C). Thus, single-stranded DNA, even if UV-damaged, is no substrate for CDAF1. Third, Asnl monomers of the Ascaris gll-sat-DNA were treated with cisplatin and tested for competitor property. Cisplatin-damaged DNA could not compete for CDAF1–probe DNA complex formation either (Fig. 3C). CDAF1 apparently does not recognize lesions which are different from those introduced by UV-irradiation.

**CDAF1 recognizes CPDs**

The observations described above indicate that CDAF1 binds to lesions in double-stranded DNA caused by UV-irradiation of pyrimidine nucleotides. CPDs and (6–4) photoproducts are the major UV-induced adducts (16). To find out which adduct is recognized by CDAF1, defined simple-sequences were used as additional competitors to the binding reactions. Salmon DNA and pBR 322 DNA, each at 100 ng, did not affect the formation of CDAF1–UV-irradiated DNA complexes. However, 50 ng of the respective DNAs, subsequent to UV-irradiation, effectively competed for CDAF1 binding to the cognate sites in the Ascaris monomer probes (Fig. 3B). CDAF1 binding activity is resistant to proteinase K and tolerates high salt

**CDAF1 activity per nucleus is maximal at the 4–8-cell stage**

To address the important question whether CDAF1 activity can be correlated with developmental stages of chromatin diminution, we assayed binding activity at various embryonic stages and in tissues of adult females. CDAF1 activity is not detectable in gut and body muscle of adult animals (Fig. 5E). The HSP-extracts of these tissues, nevertheless, contain at least as many DNA binding proteins as those prepared from embryos. In 1-celled embryos, developed for 0, 10 and 21 h, respectively, CDAF1 activity was detected at a comparatively low level. This small amount of activity might have developed during the incubation required to prepare ‘peeled eggs’. Subsequent to the initial lag phase, activity increases dramatically in the late 2 cell stage. The linear rate of increase drops down after the cleavage period of diminution events has been terminated. From the comma stage the increase remains at almost zero during the final 120 h of morphogenesis into the first larval stage (Fig. 5A). Thus, the time-course of CDAF1 binding activity per embryo appears to parallel the period of cellular propagation, but not the number of exponentially increasing cells (nuclei). The duration of cell cycles becomes progressively shorter during the early cleavage period. The accelerating cleavage tempo does not seem to trigger the rate of CDAF1 production. Rather, the production is set in motion after the first cleavage division, and the binding potential would not change during the period of early cellular segmentation of the egg cytoplasm. Because CDAF1 activity increases continuously up to the first larvae, it seems most probable that the factor is bound to the somatic segment of the genome. If CDAF1 would reside preferentially in the gll-sat-DNA, its amount should decrease during the cleavage period of diminution events. If CDAF1 emerges in all (somatic) cells and does not segregate preferentially upon mitotic proliferation, the activity per nucleus is maximal at 4–8-cell stages, as indicated by the graphical representation in Figure 5A. This is visualized in Figure 5B–D, where dilutions of HSP-extracts of four developmental stages were assayed.
Figure 4. CDAF1 binding activity is resistant to proteinase K and tolerates high salt. (A) Exhaustive proteolysis of HSP-extracts with trypsin (Tryp) (100 µg/ml) results in a defined pattern of fragments (lane 2), with proteinase K (Prot K) (100 µg/ml) mostly in a smear of peptides (lane 3), as shown by silver stained aliquots (4.5 µg) after SDS–PAGE (12.6%). Lane 1 untreated HSP-extract (Co), left positions of marker proteins. (B) DMSAs reveal that the specific binding property of CDAF1 is destroyed with trypsin (lanes 2) and conserved upon digestion with proteinase K (lanes 3). The higher mobility of the complex, compared to that of control (lane 1), indicates a limited digestion of CDAF1. The binding reactions were performed with HSP-extract from 4–8-cell stages, 1 ng UV-irradiated AsnI monomers and 1.25 µg poly [d(I-C)] in lanes 1, 2 and 4. In the absence of competitor (lanes 3 and 5) the complex had been formed in the proteinase K-treated nuclear extract, apparently because the DNA binding potential is mostly destroyed. (C) CDAF1 specific binding occurs at a wide range of salt concentration. Standard DMSAs were performed with UV-irradiated Ascaris AsnI monomers (1 ng) in the absence of poly [d(I-C)] (lanes 1–4), and in the presence of 1.25 µg poly [d(I-C)] (lanes 5–8) at NaCl-concentrations as follows: 75 mM (lanes 1 and 5), 150 mM (lanes 2 and 6), 300 mM (lanes 3 and 7) and 500 mM (lanes 4 and 8).

Figure 5. CDAF1 activity per nucleus is maximal in 4–8-cell stages. HSP-extracts were prepared in exactly the same way from 1-cell (developmental age 0, 10 and 21 h), 1–2-cell (42 h), 4–8-cell (63 h), 26-cell (96 h), comma 480-cell stages (168 h), first larvae (300 h), and gut and muscle of adult females, respectively, and assayed for CDAF1 activity. The PCBAS densitometry program was used for quantitation of colour reaction in retardation bands. (A) (●) relative CDAF1 activity per embryo, (■) relative CDAF1 activity per nucleus, considering the mean cell number at the respective stages. The dashed curve represents the presumptive increase of CDAF1 activity per embryo according to the ‘differential activation model’ (see Discussion). (B–D) DMSAs were performed with the following dilutions of the HSP-extracts. (B) 1/1 of the 1-cell stage (5 µg protein) (lane 1), 1/4 of 4–8-cell stages (1.25 µg protein) (lane 2); (C) 1/1 of 4–8-cell stages (5 µg protein) (lane 3), 1/4 of 26-cell stages (1.25 µg protein) (lane 4); (D) 1/1 of 26-cell stages (5 µg protein) (lane 5), and 1/8 of comma stages (0.63 µg protein) (lane 6). UV-irradiated BclI monomers (1 ng) were used as probe. Poly [d (I-C)] was 0.63 µg in B and C and 1.25 µg in D. Note that in each pair (B, C, D) approximately equal numbers of nuclei were assayed. (E) CDAF1 activity is absent in HSP-extracts of gut (10 µg protein) (lane 7) and body muscle (10 µg protein) (lane 8) of adult females. UV-irradiated AsnI monomers (1 ng) were used as probe, poly [d (I-C)] was 1.25 µg.

CDAF1 without destroying its active center. Proteinase K treatment did not generate an active CDAF1 fragment from the cytoplasmic fraction of 1-cell stages (not shown). (ii) HSP-extracts prepared from 1-cell and 4–8-cell stages were incubated for 10 min at 30 °C in the presence of MgCl2 (25 mM) and ATP (0.5 mM), a procedure which has been shown to effectively stimulate endogenous phosphorylation of proteins (18). As verified by
UV-irradiation (20). Second, several proteins have been described that both recognize damaged DNA and are involved in DNA-rearrangements (21). We detected CDAF1 that preferentially binds to UV-damaged DNA, and discuss why it is probably involved in chromatin diminution. CDAF1, a novel damaged-DNA binding protein

In eukaryotes, several DDB proteins and enzymes operating on UV-damaged DNA have been described (22–24). The human 106 kDa XPC protein, which copurifies with 43 kDa HHR23B, binds DNA with a preference for single-stranded or damaged DNA (25,26). Patients suffering from xeroderma pigmentosum (XP) complementation group E frequently lack a dimer protein of 48 and 127 kDa, which selectively binds to (6–4) photoproducts, even at 1.5 M NaCl (27–30). A homolog of this protein has been purified from monkey kidney cells (31). In cell-free extracts the XPE protein appears to act as an accessory factor in nucleotide...

DISCUSSION

Chromatin diminution was the first example violating the rule of genome constancy (3). In A. suum the bulk of the germline specific DNA is almost entirely composed of one 123 bp unit repeat. The copies of the multiple sequence variants are strictly arranged in tandem clusters (8). Thus, we are confronted with two major questions addressing the problem of genome dynamics: (i) what mechanisms manage the ‘segmental amplification’ of the 123 bp sequences in the germline? (ii) What factors establish the apparatus to accomplish the ordered process of their elimination in all somatic lineages? Here we address the latter problem. The results of Müller et al. (15) and of Jansen and Moritz (14,19), mentioned in the Introduction, coherently indicate that structural reorganization of cisacting nucleotide sequences is significant in positioning the enzyme which cuts the DNA backbone during diminution. Therefore, we searched for proteins in early embryos, which recognize unusual DNA structures that might be present during chromatin diminution. The rationale for detecting those factors, by means of DMSAs, is based on two unrelated previous observations. First, diminution in germline cells can be experimentally induced by UV-irradiation (20). Second, several proteins have been described that both recognize damaged DNA and are involved in DNA-rearrangements (21). We detected CDAF1 that preferentially binds to UV-damaged DNA, and discuss why it is probably involved in chromatin diminution.

Purification of CDAF1

CDAF1 was purified from HSP-extract of 4–8-cell stages (10 ml), as described under Materials and Methods. After fractionation of the extract via DNA–cellulose CDAF1 was shown to be contained exclusively in the 500 mM NaCl fraction, comprising 1% of total protein applied. CDAF1 was purified from the enriched fraction by a second chromatography using UV-irradiated DNA–cellulose. CDAF1 elutes from the matrix at 2000 mM NaCl, together with two other proteins that do not bind DNA (Fig. 6A and B). CDAF1 was identified by comparing its activity and the ratios of protein bands in consecutive fractions (not shown). From SDS–PAGE the Mr of CDAF1 was calculated to be 56, and that of the other proteins 64 and 18, respectively. Because in earlier gel filtration experiments native CDAF1 was shown to range between 34 and 87 kDa, and the elution profiles of the two other proteins differ from that of CDAF1 upon fractionation at 500 mM NaCl in the first chromatography, copurification is obviously not due to protein interactions.

Figure 6. Purification of CDAF1. (A) Protein patterns after SDS–PAGE (11%) visualized by silver staining at various stages of purification. HSP-extract prepared from 4–8-cell stages (lane 2) was fractionated via DNA–cellulose. The 500 mM NaCl eluate was applied to a UV-irradiated DNA–cellulose column. Proteins were eluted at 500 mM NaCl and 2000 mM NaCl. From the second column 2 µl of the 500 mM eluate and, in order to visualize CDAF1, 100 µl of the 2000 mM eluate were applied in lanes 3 and 4, respectively. Lane 1 marker proteins. (B) The 2000 mM NaCl eluate (A, lane 4), after dialysis against buffer L + 160 mM NaCl, was assayed for CDAF1 activity in the presence of BSA (2 µg/ml) without (lane 1) and with poly (dI-C) competitor (310 ng) (lane 2), with pBR 322 competitor (50 ng), untreated (lane 3) and UV-irradiated (* (lane 4), using 1 ng UV-irradiated Axi monomers as probe. Because the retardation band forms in the absence of competitor, and does not form in the presence of UV-irradiated competitor, the 2000 mM NaCl eluate must contain CDAF1 as the only DNA-binding protein.
excision repair (NER) (32). In contrast, the 31 kDa XPA factor is indispensable for NER, and thus plays an important role in tumor prevention (33,34). XPA which is homologous to the RAD14 protein of Saccharomyces cerevisiae recognizes (6–4) photoproducts in both single-stranded and double-stranded DNAs (35,36). Complex formation effectively occurs at NaCl concentrations lower than 200 mM. Another protein essential for NER, and also viability, is the RAD3 gene product of S.cerevisiae. This 80 kDa DNA helicase recognizes (6–4) photoproducts in UV-damaged supercoiled DNA in the presence of ATP (37). Kai et al. (38) have described three new DDB proteins from Drosophila embryos, termed D-DBB 1, 2 and 3. D-DBB 1 of 30 kDa preferentially binds to (6–4) photoproducts; ATP and Mg2+ increase binding affinity. D-DBB 2 of 14 kDa probably prefers binding to CPDs, which is enhanced by Mg2+. Hela cells contain an ~40 kDa protein, termed NF 10, that specifically binds to (6–4) photoproducts (39). Because NF 10 is expressed in all XP complementation groups, it might be a novel type of DDB protein. Another human DDB activity recognizes (6–4) photoproducts in both single- and double-stranded DNAs, but not CPDs in double-stranded DNA (40).

Photolyases repair CPDs or (6–4) photoproducts at visible light (41–44). Photolyases can be detected in DMSAs by comparing complex formation in the dark and under visible light, i.e. photoreactivating conditions: binding to UV-damaged DNA is strong in the dark, but weak or absent under visible light (17,42).

Here we show that A.suum embryos contain a nuclear DDB protein of Mr 56, named CDAF1. CDAF1 recognizes CPDs in double-stranded DNA. Binding does not depend on Mg2+, ATP or light/dark conditions. Concentrations higher than 500 mM NaCl do not impede complex formation. This set of properties defines CDAF1 as a novel kind of DBB protein.

Proteins that recognize UV-damaged DNA take part in three different repair pathways (21); (i) photorepair, (ii) base excision repair and (iii) NER. Because CDAF1 binds to CPDs in the presence of visible light and properly at high salt, it is certainly not involved in photorepair. Since CDAF1 possesses no enzymatic (DNA glycosylase/AP endonuclease) activity, it is not involved in base excision repair. In NER Rad3, XPA and XPE are thought to direct the repair machinery to sites of damage (45). Accordingly, CDAF1 could act as an accessory factor directing the NER machinery to CPD sites during embryogenesis, the only period in the life cycle of Ascaris where nuclear replications occur unprotected from solar UV radiation.

**CDAF1 and chromatin diminution**

CDAF1 cannot be claimed to be a constitutive component of the germline specific heterochromatin, because the binding activity is low in the nuclear fractions of 1-celled embryos. The results of several DMSAs, by combining various probes and competitor DNAs, make it unlikely that the repeat variants of the authentic gll-sat-DNA contain any additional sequence information to establish the CDAF1–DNA complexes in vitro. CDAF1 activity is developmentally regulated. It is maximal per nucleus at the third cleavage step and decreases during further development. It is not observed in tissues of adults. CDAF1 activity is absent in the cytoplasm (LS-supernatants) of any cleavage stage. Therefore, activation of the binding property appears to occur in the course of nuclear localization. If we accept this notion, it may explain why we failed to stimulate binding by combining nuclear HSP-extracts, prepared from pre- and post-diminution stages. The binding property, once established, seems to be resistant to endogenous activities of kinases and phosphatases, and surprisingly, exhaustive protease K treatment. Because of the low degree of cleavage synchrony, the 4–8-celled embryo batches contain cells at different mitotic stages, i.e. germline chromosomes and nuclei, chromosomes undergoing diminution, and somatic chromosomes and nuclei. Nevertheless, CDAF1 binding activity was recovered only from nuclear fractions. Therefore, the bulk of active molecules appears to be irreversibly bound to chromatin throughout the cell-cycles. However, the *in vivo* binding sites appear to differ from the CPDs in double-stranded DNA. This is concluded from the fact that, on the one hand, CDAF1 is routinely extracted from nuclei at 350 mM NaCl, and on the other, high salt, i.e. 500 mM NaCl, in the binding assay does not diminish complex formation *in vitro*.

According to the ‘differential activation model’ of asymmetrical cell division (13), in presomatic cells those factors become activated which mediate chromatin diminution. In the model the cytoplasmic mass establishing the presomatic blastomeres is irrelevant for the amount or degree of activity of putative diminution factors emerging in the course of asymmetrical P cell divisions. If so, the extent of factor activation should decrease geometrically corresponding to the dashed curve in Figure 5A. The curve is based on four suppositions: (i) activation occurs in the second half of the cell cycle, (ii) factor exhaustion is completed during the life span of each somatic founder cell, (iii) exhaustion depends on the cell mass (60% of the respective P mother cell) and (iv) the maximum is achieved upon cleavage of D. Such a concept of factor exhaustion is inconsistent with several observations. For example, diminution does not occur in the largest presomatic cell, AB. Instead, two initial cell (activation) cycles are required to pave the way for diminution in the half-sized AB daughter cells. Cells undergo diminution on schedule, irrespective of chromosome number and cleavage geometry at the first cleavage step, as has been shown in several experimental egg types (5,12,20). There is no evidence that CDAF1 is activated merely by modification(s) of an inactive profactor stored in the egg-cell (see Results). Rather, after an initial lag phase, CDAF1 seems to be synthesized *de novo* at a constant rate in the developing embryo. The factor would be present in all presomatic cells, including the late AB, in abundant quantities, and hence, not sufficient for diminution to occur. Thus, additional events would be necessary to cause diminution. Mainly from our cytological data, derived from A.suum (see Results) and *Punicalens*, we propose that diminution is initiated by transactions which mediate the formation of diminution complexes at sites bordering the ends of the prospective somatic chromosomes. Resolution of the diminution complexes under the stress of anaphasic segregative force would liberate the sister chromatids from the germline specific chromatin. In this scenario, CDAF1 could act as a helper factor in positioning the excision machinery at sites susceptible for breakage.

We failed to detect CDAF1 by means of oligonucleotide probes in whole mounts of early embryos. Next, antibodies to be raised against CDAF1 will allow tracing its nuclear localization and continuing our efforts to assay whether the factor affects the endonuclease-mediated cleavage-sites in supercoiled plasmid DNA.

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