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The telomeric region is excluded from nucleosomal fragmentation during apoptosis, but the bulk nuclear chromatin is randomly degraded

T Schliephacke[1], A Meinl[1], M Kratzmeier[1], D Doenecke[1] and W Albig*,[1]

[1] Institut für Biochemie und Molekulare Zellbiologie, Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, Germany
* Correspondence: W Albig, Institut für Biochemie und Molekulare Zellbiologie, Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, Germany. Tel: +49-551-395971; Fax: +49-551-395960; E-mail: walbig@gwdg.de

Abstract

Characteristic steps during cellular apoptosis are the induction of chromatin condensation and subsequent DNA fragmentation, finally leading to the formation of oligomers of nucleosomes. We have examined the kinetics and local distribution of this nucleosomal fragmentation within different genomic regions. For the induction of apoptosis, HL60 cells were treated with the water-soluble camptothecin derivative topotecan (a topoisomerase I inhibitor). The genomic origin of the fragments was analysed by Southern blot hybridisation of the cleaved DNA. In these experiments we observed similar hybridisation patterns of the fragmented DNA, indicating a random and synchronous cleavage of the nuclear chromatin. However, hybridisation with a telomeric probe revealed that, in contrast to the other analysed genomic regions, the telomeric chromatin was not cleaved into nucleosomal fragments despite our observation that the telomeric DNA in HL60 cells is organised in nucleosomes. We determined just a minor shortening of the telomeric repeats early during apoptosis. These observations suggest that telomeric chromatin is excluded from internucleosomal cleavage during apoptosis.
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Introduction

Programmed cell death known as apoptosis plays an important role in the development of an organism and in the homeostasis of adult tissues. The process of apoptosis is characterised by multiple intracellular alterations, which finally lead to the breakdown of the cell into apoptotic bodies, subcellular particles that are removed by neighbouring cells.[1] One feature of this disintegration process is the fragmentation of the chromosomal DNA. The cleavage of chromosomal DNA into nucleosome size fragments of 180–200 bp was first demonstrated in glucocorticoid-induced cell death in thymocytes[2] and has rapidly become a biochemical hallmark of apoptosis. Visualisation of the characteristic DNA 'ladder' is now widely used for monitoring apoptosis in many experimental cell systems. Besides the nucleosomal fragments DNA-fragments of 50–300 kb are also detectable.[3] The size of these large DNA fragments is in the same range as the size of chromatin loop domains, implying that the first cleavage sites are the matrix attachment regions (MAR) of the nuclear scaffold. The appearance of these larger fragments precedes the generation of nucleosomal fragments during the execution of cellular apoptosis. It is, however, as yet unknown whether the nucleosomal fragments are generated from the larger fragments or whether these two fragmentation processes are independent from each other.

Eukaryotic chromatin is organised as euchromatin and heterochromatin. Heterochromatin comprises mainly the telomeric and centromeric regions of the chromosomes but also the inactivated regions of the sex chromosomes.[4] It is more condensed than euchromatin that represents the transcriptionally active regions of the genome. The first level of DNA-compaction is the nucleosome. The nucleosome consists of the nucleosome core particle and the linker DNA, which connects two adjacent core particles.[5] Limited digestion of chromatin with micrococcal nuclease, which cleaves at the linker DNA, generates nucleosomes and oligomeric nucleosomal units.[6] Analysis of the nucleosomal DNA reveals that bulk chromatin is composed of nucleosomes with a DNA repetition unit of about 200 bp, that is, the same size as observed during apoptotic fragmentation. Liu *et al.*[7] identified a DNAase that is activated during apoptosis by caspase-mediated degradation of an inhibitor. Due to this mode of regulation, the nuclease has been named 'caspase-activated DNAase (CAD) and its inhibitor is termed ICAD. CAD appears to be responsible for the internucleosomal DNA cleavage, but also other DNAases are discussed to be involved in apototic DNA fragmentation (reviewed in Nagata *et al.*[8]).

The activity of CAD is modulated by chromosomal proteins like high mobility group protein (HMG 17)[9] and histone H1.[10] In mammals seven different subtypes of H1 histones have been identified.[11] In our previous work we have observed that during apoptosis the H1 histones are rapidly dephosphorylated.[12,13] Therefore, the local distribution of the different subtypes and the extent of phosphorylation of linker histones may influence the genomic sites of cleavage during apoptosis. But, whether the different subtypes or the phosphorylation

status of these linker histones influences the activity or specificity of CAD remains to be clarified.

Since there are only limited and controversial data about the spatial and temporal fragmentation of the nuclear genome and the specificity of these DNAases for specialized regions of the genome, we first analysed the kinetics of fragmentation of different regions of the genome. Hybridisation of the fragmented DNA with probes corresponding to different regions of the genome revealed that the telomeric region was not cleaved into nucleosome sized fragments, whereas the bulk nuclear chromatin including the centromeric chromatin, representing another heterochromatic region, was accessible to cleavage at the level of nucleosomes.

Experimental

Cell culture and induction of apoptosis

HL60 human promyelocytic leukemia cells were cultured in RPMI-1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom KG, Berlin, Germany) in a humidified 5% CO_2 atmosphere. Prior to apoptosis induction, cells were blocked at the G1–S transition of the cell cycle with 15 μM aphidicolin (Alexis Corporation, San Diego, CA, USA) for 24 h. To release the cells from the block, they were washed two times with PBS and transferred in new culture flasks to remove aphidicolin. Aliquots of cells at this stage were used as synchronisation control cells and are described as 0 in the kinetics. For induction of apoptosis, cells were exposed for 60, 75, 90, 105, 120, 135, 150 and 180 min with 150 ng/ml of the topoisomerase I inhibitor topotecan (SmithKline Beecham Pharma GmbH, München, Germany). For analysis they were harvested by centrifugation (300 g, 7 min) and washed with PBS.

DNA fragmentation assay

After induction of apoptosis, 5×10^7 cells were harvested by centrifugation at 300 g and washed with PBS. To isolate the low molecular weight (LMW) DNA, cells were lysed in 450 μl buffer containing 10 mM Tris, 10 mM EDTA, 0.2% Triton X-100, pH 7.5 for 30 min at 4°C, centrifuged and the DNA of the supernatant was precipitated with 450 μl of propan-2-ol and 100 μl of 10 M LiCl for 2 h at −20°C. The collected DNA was washed twice with 70% (v/v) ice-cold ethanol, vacuum dried and resolubilised in 50 μl TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Samples were extracted twice with chloroform/3-methylbutane-1-ol (24 : 1) and the purified DNA was again precipitated with 15 μl of 3 M sodium acetate (pH 4.8) and two volumes of ethanol at −20°C for at least 2 h. After centrifugation, pellets were dried and solubilised in 30 μl TE buffer. Extracted DNA was then analyzed on a 1.5% agarose gel using a constant voltage of 50 mV, stained in ethidium bromide (10 μg/ml) and visualised on a UV transilluminator.

Preparation and separation of HMW DNA fragments

Cells (1×10^7) were harvested by centrifugation, washed with PBS and resuspended in 50 μl buffer I (150 mM NaCl, 2 mM phosphate buffer pH 6.4, 1 mM EGTA and 5 mM $MgCl_2$). The suspension was heated to 45°C and mixed with 50 μl of a 1.5% solution of low melting point agarose in buffer I (45°C). This suspension was transferred to a plug former device and cooled on ice. Plugs were incubated at 50°C for 18 h in 3 ml of digestion buffer containing 10 mM NaCl, 25 mM EDTA, 10 mM Tris/HCl pH 9.5, 10% (w/v) lauroyl sarcosine and 35 μg/ml proteinase K. After washing in sterile TE the plugs were used immediately or stored at 4°C. The HMW DNA fragments were separated on rotating field agarose gel electrophoresis (ROFAGE) in 0.9% agarose gels in 0.25 × TBE running buffer (1 × TBE: 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) using a Rotaphor R23 (Biometra, Goettingen, Germany) with a ramped voltage of 180–120 V for 19 h, a ramped switch time from 60 to 10 s and a ramped angle from 120° to 110°. The separated HMW DNA fragments were transferred to nylon membranes (Hybond N, Amersham) under standard conditions with the exception of extended denaturation (1 h) in 0.5 N HCl. The filter was hybridised with the radioactively labelled probes. The size of the fragments was determined by comparing with the size pattern of the chromosomes from the yeast ENY-WA-4D.[14]

Preparation of nuclei

Cells were harvested by centrifugation, washed twice with PBS and lysed in buffer I containing 0.3 M sucrose, pH 8.0, 2 mM Mg acetate, 3 mM $CaCl_2$, 10 mM Tris and supplemented with 1% Triton X-100 and 0.5 μM DTT. Cells were further disrupted by 16 strokes in a Dounce homogeniser (pestle S). This suspension was mixed with the same volume of buffer II containing 2 M sucrose, pH 8.0, 5 mM Mg acetate, 10 mM Tris and 0.5 μM DTT, and layered on a cushion of the same buffer (Sorvall HB4 Rotor, 45 min, 10 000 rpm). The nuclei were resuspended in 10 ml Hewish buffer (60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris and 15 mM β-mercaptoethanol, pH 7.4).[15]

Digestion of nuclei with micrococcal nuclease

Nuclei were preincubated for 2 min at 37°C in Hewish buffer complemented with 1 mM $CaCl_2$ and digested for 10 s, 1 min or 3 min with 10 U Micrococcus Nuclease (Roche Diagnostics, Germany). Digestion was stopped by adding 200 μl 0.1 M EDTA and chilling on ice. After centrifugation at 5000 rpm for 5 min, digested nuclei were resuspended in 10 ml phosphate–EDTA–buffer (5 mM $NaPO_4$, 0.2 mM EDTA and 250 μM PMSF at 1 l). To extract nucleosomes, nuclei were further disrupted by 20 strokes in a Dounce homogeniser (pestle S), and sheared by five passages through a 0.90 × 40 mm hypodermic needle (20G × 11/2, Braun Melsungen AG, Melsungen, Germany) and five passages through a 0.40 × 20 mm^2 hypodermic needle (27G × 4/5″) fitted to a 5 ml plastic syringe. The extracted nuclei were sedimented by centrifugation for 5 min at 15 000 rpm and the supernatant containing nucleosomes was extracted twice with chloroform-3-methylbutane-1-ol (24 : 1). The purified nucleosomal DNA was again precipitated with 250 μl/ml of 5 M NaCl and two volumes of absolute ethanol at −20°C for at least 2 h. After centrifugation, pellets were washed twice with 70% (v/v)

ice-cold ethanol, vacuum dried and solubilised in 200 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Extracted DNA was then analyzed on a 1.4% agarose gel using a constant voltage of 50 mV, stained in ethidium bromide (10 μg/ml) and visualised on a UV transilluminator.

Probes and southern blot hybridisations

The telomere repeat probe (TTAGGG)$_n$ was generated by PCR as described by Ijdo et al.[16] The Sau3A-repeat probe is a 171 bp monomer of the human centromeric α-satellite DNA and was prepared as published by Dunham et al.[17] Oligonucleotide probes: heterochromatin probe het266: 5'-(CCCTAA)$_6$-3'; heterochromatin probe het405: 5'-GAA-GAAGCTTTCTGAGAAACTGCTTAGTG-3'[18] and the long interspersed repetitive elements (LINE) probe was: 5'-CATGGCACATGTATACATATGTAACWAACC-3'.[19]

After electrophoresis, DNA in the gel was acid depurinated, denatured, neutralised and blotted onto a GeneScreen membrane (NEN™ Life Science Products, Inc., Boston, USA) as recommended by the supplier. DNA was immobilised by UV crosslinking. Probes were ^{32}P-labeled by the random priming method using a Rediprime kit (Amersham Life Science, England) following the manufacturer's recommmended procedure. For Southern blot hybridisation, blots were prehybridised for at least 2 h in 0.125 ml/cm^2 hybridisation buffer (tablets, Amersham Life Science, England) at 65°C. Hybridisation was carried out over night at 65°C. The stringent washes consisted of 2 × SSC (1 × SSC: 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS for two times at RT (15 min each) and two washes with 0.2 × SSC and 0.1% SDS for 30 min at 65°C. Radioactive bands were detected by using a PhosphoImager system (Molecular Dynamics GmbH, Krefeld, Germany).

Determination of telomere length

Telomere length was determined by hybridisation of terminal restriction fragments (TRF) with the telomere specific probe.[20] Chromosomal DNA was prepared using the Qiagen Blood & Cell Culture DNA Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instruction. In all, 10 μg of chromosomal DNA was digested with the combination of two frequently cutting restriction enzymes (HinfI and Csp6I), the cleavage sites of these endonucleases are not present within the telomere repeat sequence. The resulting fragments were separated by agarose gel electrophoresis, transferred onto nylon membranes and hybridised with the radioactively labelled telomere repeat probe. The size distribution of the TRFs was determined by comparison with the DNA size standard.

Quantitative dot blot analysis of chromosomal DNA

Nonfragmented chromosomal DNA was isolated by treatment of the cells with 0.2% Triton-X100 in TE-buffer for 30 min on ice. After centrifugation at 14 000 rpm for 10 min unfragmented DNA was isolated from the pellet by the DNA Maxi Kit from Qiagen. The isolated DNA was dissolved in 0.6 M NaCl to a concentration of 10 μg/ml. The DNA was heat denatured at 95°C for 10 min and cooled on ice for 5 min. From this stock solution a series of three dilutions in 10 × SSC were prepared. In all, 200 μl aliquots of each dilution were spotted on a nylon membrane in a vacuum dot blot device (BioRad). After additional denaturation and neutralisation, the membrane was hybridised with a radioactively labelled telomere repeat probe. For subsequent hybridisation with the different probes the labelled membranes were stripped with 0.5% SDS (100°C) for 10 min and subsequent gradual cooling to room temperature. Hybridisation was analysed by a PhospoImager and quantified with the ImageQuant software from MolecularDynamics. The relative hybridisation intensity was calculated by comparing the mean value of signal intensities of each time point with the intensity at 0 h (uninduced). In order to normalise for variations in DNA content per spot the filters were reprobed with labelled total chromosomal DNA. The calculated relative intensities at 0 h of the different probes were set to 1.

Expression of recombinant proteins

For expression of the human DFF45/DFF40 complex in E. coli the strategy of polycistronic expression was used. The coding regions of DFF45 and DFF40 were amplified by PCR of cDNA from HeLa cells. The DFF45 ORF was cloned into the NdeI/NcoI sites of pRSET-B (Invitrogene, San Diego, USA) resulting in pWA377. The DFF40 ORF was fused with a His-tag by insertion into the BglII/HindIII sites also of pRSET-B, resulting in pWA378. The coding region of the fusion protein together with the upstream ribosome-binding site (RBS) was amplified from pWA378 by PCR with primers containing an NcoI and HindIII restriction site, respectively. This fragment was then inserted downstream of the coding region of DFF45 into the NcoI/HindIII sites of pWA377. From the resulting expression plasmid (pWA379) a polycistronic mRNA that codes for DFF45 and DFF40 with a N-terminal penta-His-tag was transcribed. The DFF45/DFF40 complex was expressed in E. coli JM109 and purified by affinity chromatography on Ni-NTA beads (Qiagen, Hilden, Germany). For storage the purified complex was dialyzed against Buffer S (10 mM HEPES-KOH pH 7.0, 50 mM NaCl, 2 mM MgCl$_2$, 20% glycerol, 5 mM DTT) and kept at −20°C.

Human caspase 3 was expressed as fusion protein with a N-terminal His-tag. The coding region of this fusion protein was isolated by amplification from plasmid DNA (gift from Thomas Meergans, Konstanz, Germany),[21] by PCR. The isolated fragment was cloned into the SalI/BamHI sites of YEp51.[22] The His-tag protein was expressed in Saccharomyces cerevisiae ENY.WA-4D[14] and purified by Ni-NTA chromatography.

DFF in vitro assay

The inactive DFF45/DFF40 complex (5 μl) was incubated with 14 μU of recombinant caspase 3 in 50 μl plasmid assay buffer (20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) at 37°C for activation of DFF40. Different volumes of this incubation mixture, with the active DFF40, was

then incubated with a mixture of two different DNA fragments containing the 170 bp *Sau*3A-repeat and a 350 bp telomere repeat, respectively, in a total volume of 50 μl plasmid assay buffer. After 10 min incubation at 30°C the assay was stopped by adding phenol/chloroform to the incubation mixture. After repeated chloroform extraction the DNA was precipitated with ethanol, resolved in TE and analysed by agarose gel electrophoresis.

Results

Site-specific DNA fragmentation

During apoptosis DNA fragments of two different sizes can be detected. Early in apoptosis fragments in the range of 50–300 kb can be observed, whereas later in apoptosis smaller fragments of the size of nucleosomal DNA and multiples thereof are generated. To analyse the sites of cleavage within the genome we separated the DNA fragments by ROFAGE (high molecular weight (HMW) fragments) and conventional agarose gel electrophoresis (LMW fragments) and analysed the DNA fragments by Southern hybridisation with probes specific for different regions of the mammalian genome. The *Sau*3A-repeat probe is specific for constitutive heterochromatin (β-heterochromatin) of the centromeric region. It belongs to the family of α-satellite-DNA and consists of 170 bp that are tandemly repeated in the centromeric region. The telomere probe is specific for the telomeric region of the chromosomes and consists of fragments in the range of 1–5 kb of repetitive telomere-repeat sequences 5'-TTAGGG-3' amplified by PCR.[16] The heterochromatin probes het266 and het404 are specific for different regions of the centromere region. het266 corresponds to satellite-III-sequences and het405 represents repetitive alphoid sequences. The LINE probe hybridises to the LINE, which are interspersed in euchromatic regions but are excluded from coding sequences.

HMW DNA fragmentation

The genomic sites of early cleavage were analysed by Southern hybridisation of HMW fragments separated by ROFAGE. Since DNA fragments of this size were observed at the onset of apoptosis, DNA was prepared from cells harvested shortly after induction of apoptosis (from 1 to 3 h in 15 min intervals). For each time point total DNA from equal amounts of cells were applied to the gel. The parameters for electrophoresis were selected for separation of fragments between 10 and 1000 kb. After blotting to nylon filter the DNA was hybridised either with end-labelled oligonucleotides or random primed DNA fragments corresponding to various regions of the mammalian genome. The filters were stripped and rehybridised several times with different probes. Thus, the results for each probe were directly comparable.

The hybridisation signals obtained with all probes used in this experiment showed similar hybridisation patterns (Figure 1) indicating that the fragments were released simultaneously from all sites of the genome. Fragmentation to HMW fragments starts about 100 min after induction of apoptosis. The hybridisation intensity of the HMW fragments

peaks at 135 min after induction. Cells that further proceeded in the apoptotic execution process reveal less and shorter HMW fragments. This may be due to further cleavage of the HMW fragments, perhaps into nucleosomal fragments. Fragments shorter than 20 kb cannot be detected with the used ROFAGE system.

These results show that for those regions of the chromatin that we have analysed, there are no local preferential cleavage sites within the chromatin. Initial apoptotic chromatin cleavage appears to be random.

LMW DNA fragmentation

During apoptosis chromatin is cleaved into nucleosomal units; therefore, we can isolate DNA fragments with a size of 200 bp or a multiple thereof from apoptotic cells. These fragments were separated by conventional agarose gel electrophoresis. After transfer of the DNA onto nylon filters the DNA was hybridised with the same probes as used for hybridisation of the HMW-DNA filter. Nucleosomal DNA fragments appeared about 2 h after induction, whereas HMW fragments were detectable already 1.5 h postinduction (see DNA stained agarose gel of Figure 1 *versus* Figure 2).

The hybridisation patterns generated by the different probes were nearly identical. These results indicate that also the cleavage of the chromatin in nucleosomal units occurs randomly and is not site specific.

The only difference in the signals between the different probes was the observation that the telomere-repeat probe did not hybridise with the nucleosomal fragments. We could only detect a smear in the size range between 2 and 10 kb. The size of the hybridising fragments persisted with ongoing apoptosis. This indicates that these fragments were not further cleaved during apoptosis. The size of these hybridising fragments corresponds to the length of the telomeric repeat region. Therefore, we suggest that the telomere region was not cleaved during the execution of apoptosis.

Apoptotic chromatin fragmentation under different conditions

It is known that the execution of the apoptotic program shows cell type and inducer specificity. To analyse whether the random cleavage of the chromatin is a general principle in apoptotic chromatin fragmentation or a specific apoptotic response of HL60 cells treated with topotecan, we analysed the nucleosomal fragments of apoptotic U937 cells induced by topotecan (Figure 3) and of apoptotic HL60 cells induced by teniposide and staurosporine (Figure 4), respectively. The progress of induced cell death was monitored by flow cytometry analysis and other apoptotic markers (not shown). Since we have observed in previous experiments that the cleavage of chromatin into nucleosomal units in U937 is delayed compared with HL60 cells, we isolated DNA from cells induced for up to 24 h. Execution of apoptosis in HL60 cells induced by teniposide or staurosporine was also slower in comparison with topotecan-induced apoptosis. Therefore, we isolated DNA from cells for up to 16 or 22 h after induction in these experiments, respectively.
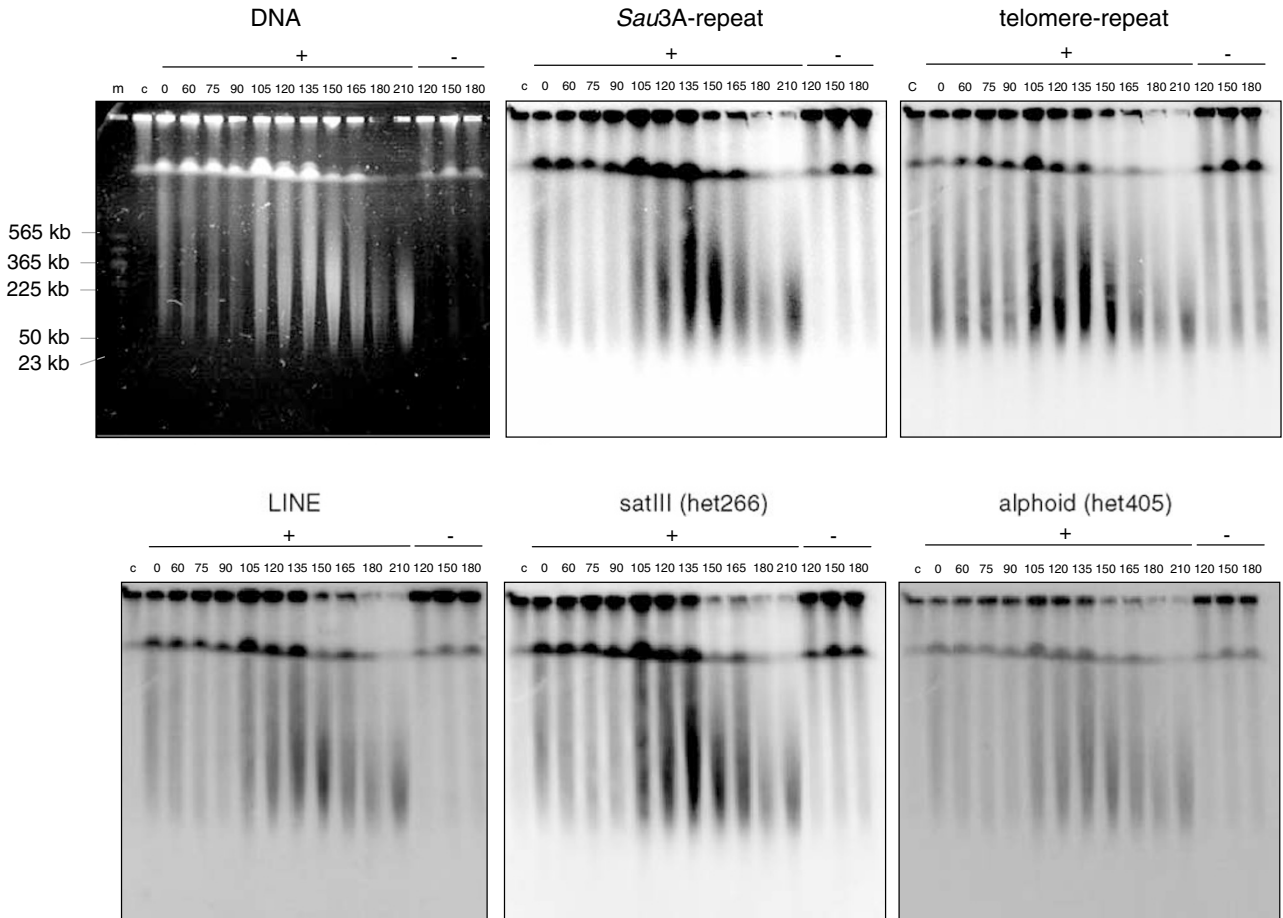
Figure 1 Hybridisation pattern of HMW DNA fragments from topotecan treated HL60 cells. Synchronised HL60 cells were treated with 150 ng/ml topotecan (+). After 60 min cells were harvested in intervals of 15 min until 210 min. HMW DNA of the cells was separated by ROFAGE. After transfer onto nylon filter the fragments were successively hybridised with radioactively labelled probes specific for repetitive genome regions. Hybridisation was detected using a PhosphoImager system. To eliminate growth effects untreated synchronised cells (−) were also harvested 120, 150 and 180 min after release from the cell cycle block. For estimation of the size of the DNA fragments yeast chromosomes from ENY-WA-4D[14] were also applied to the gel (m). DNA from unsynchronised cells served as a control (c). Note: the accumulation of DNA in each lane on the gel of the aberrant size of about 4 Mb represents a compression band of unseparated fragments. This is a typical feature of this gel system; the apparent size of this band represents the resolution limit of the system running with these parameters

Again, all hybridisation probes resulted in similar patterns of signals with the exception of the telomere probe (Figures 3 and 4). In particular, in HL60 cells either induced by teniposide or staurosporine, chromosomal DNA was cleaved in a similar way. In addition, treatment of HL60 or U937 cells with topotecan resulted in similar apoptotic DNA fragmentation. These results indicate that the random chromosomal DNA cleavage and the exclusion of the telomeric region from nucleosomal DNA cleavage is not an exclusive feature of apoptotic HL60 cells treated with topotecan, but it rather seems to be a general feature of apoptotic DNA cleavage.

From these results the question arose whether the telomeric chromatin region of the cells analysed herein is actually organised in nucleosomes. Therefore, we cleaved the chromatin of HL60 cell nuclei with micrococcal nuclease, separated the resulting fragments by agarose gel electrophoresis and hybridised the fragments with the telomere-repeat probe. This hybridisation showed a nucleosomal fragment pattern (Figure 5) generated by the limited nuclease digestion. In contrast to the signals detected with the Sau3A-repeat probe, the repeat size of the telomeric fragments was

slightly shorter (about 160 bp instead of 200 bp, indicated by the horizontal lines in Figure 5). Telomeric mononucleosomes have been found to be hardly detectable in such experiments since they are highly sensitive to overdigestion with micrococcal nuclease.[23] These results clearly show that the telomeric region in HL60 cells is indeed organised in nucleosomes but with a shorter linker region.

The telomere stability during apoptosis

To get information about the question whether the telomeric region is absolutely excluded from chromatin cleavage during apoptosis or whether it is randomly cleaved, we analysed the length of the telomeres in apoptotic cells.

This was done by TRF analysis. Total DNA of apoptotic HL60 cells was digested with high frequent cutter HinfI/Csp6I. These restriction enzymes were used since there is no cleavage site for these within the telomeric region. The resulting fragments were separated by conventional agarose gel electrophoresis and after transfer onto a nylon filter, they were hybridised with the telomere-repeat probe. As control
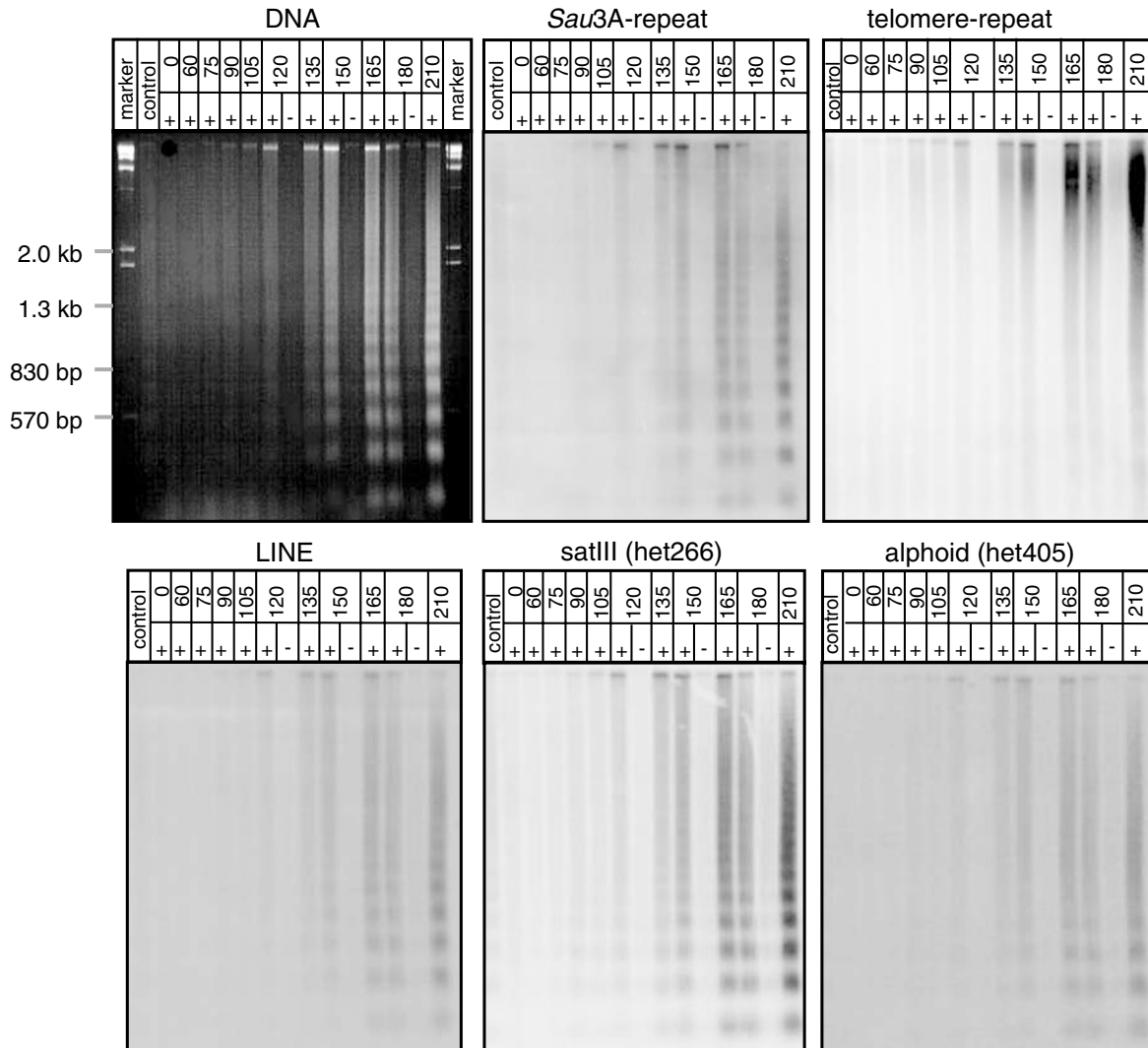
Figure 2 Hybridisation pattern of LMW DNA fragments from topotecan treated HL60 cells. An aliquot of the HL60 cells used for the detection of HMW DNA fragments (Figure 1) was used for isolation of LMW DNA to allow separation of the nucleosomal DNA by conventional agarose gel electrophoresis. After transfer of the DNA onto a nylon filter the fragments were successively hybridised with radioactively labelled probes specific for repetitive genome regions. Hybridisation was detected by using a PhosphoImager system. To eliminate growth effects untreated synchronised cells (−) were also harvested at different time points and analysed the same way. For determining the fragment size λ-phage DNA cleaved by *Hin*dIII/*Eco*RI was used as a size-marker (m). DNA from unsynchronised cells served as a control

the stripped blot was hybridised with the *Sau*3A-repeat probe. The hybridisation pattern resulting from the two different probes was totally different. Hybridisation with the telomere-repeat probe resulted in hybridisation signals corresponding to fragments of the size between 4 and 10 kb (Figure 6). This reflects the size of telomeres of HL60 cells.[24] The maximum of the hybridisation signals decreased from about 8 kb in uninduced and early apoptotic cells to about 6 kb in apoptotic cells (5 h after induction with topotecan). These hybridising fragments were also detected with DNA from apoptotic cells that was not cleaved with restriction enzymes. In addition, the intensities of the signals did not dramatically decrease during apoptosis. The signals of the hybridisation by the *Sau*3A-repeat probe were of defined size when the DNA was cleaved by the restriction endonucleases whereas without additional cleavage we observed the typical nucleosomal ladder.

These results indicate that the telomeric region is only slightly shortened during apoptosis but is not cleaved to nucleosomal units.

Quantification of regional DNA cleavage by dot blot analysis

HMW DNA was isolated from apoptotic cells at 1 h intervals from 4 to 8 h after induction and from nonapoptotic cells (0 h). Equal amounts of DNA were spotted onto nitrocellulose filters and hybridised with the same probes as used for the Southern blot experiments. The resulting hybridisation signals were quantified using a PhosphoImager system. The intensity of each hybridisation signal was normalised to the signal intensity of the corresponding spot obtained by hybridisation with radiolabelled total chromosomal DNA as
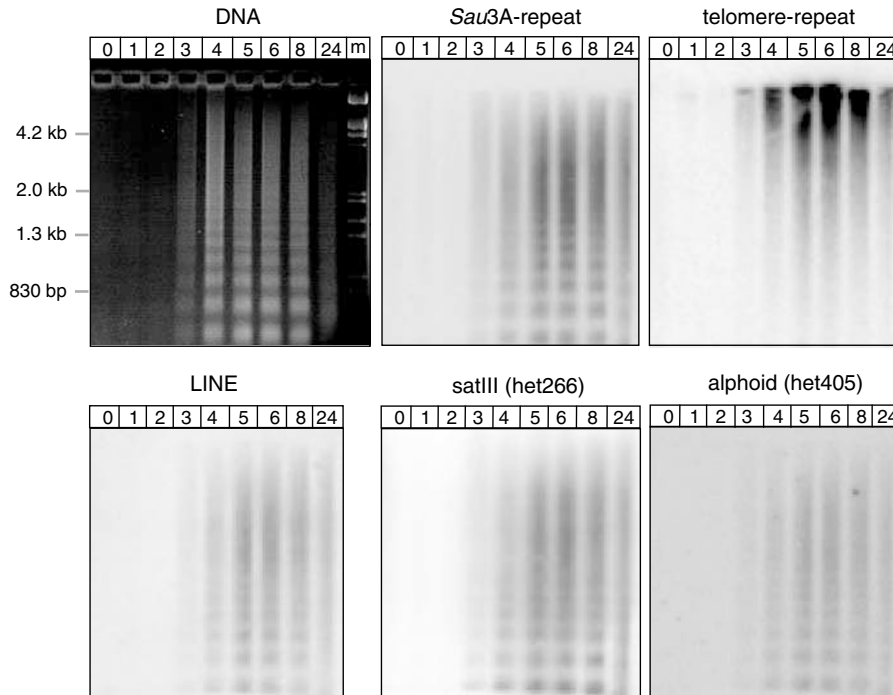
Figure 3 Hybridisation pattern of LMW DNA fragments from topotecan treated U937 cells. U937 cells were treated with 150 ng/ml topotecan. At the indicated time (h) after induction of apoptosis, cells were harvested and the isolated LMW DNA was separated by agarose gel electrophoresis. Transfer and hybridisation was performed as described in Figure 2

a probe. The intensity of the hybridisation signal of nonapoptotic DNA (0 h) of each hybridisation probe was set to 1 (equals 100%). The relative signal intensities of the other time points were expressed in relation to the 0 h value of the corresponding probe. As each spot contains the same amount of HMW DNA, a relative intensity of 1 for the individual time points represents a nonpreferential cleavage of the corresponding chromosomal region. Values higher than 1 represent a relative increase of DNA from this specific region within the remaining (not degraded) HMW DNA in apoptotic cells and therefore indicate a cleavage rate below average. Values below 1 indicate a preferred cleavage of the corresponding region.

The results of this experiment are shown in Figure 7. The relative amount of telomeric DNA increases during apoptosis indicating that telomeric DNA is excluded from cleavage in the execution of apoptosis. The relative amount of all other examined regions decreased during apoptosis, due to apoptosis related cleavage in these regions. The uniformity of the decrease of the relative amount of uncleaved DNA confirmed the results obtained from the hybridisation experiments of nucleosomal DNA shown above, which indicate a nonpreferred cleavage of genomic DNA in the execution of apoptosis.

From these results we conclude that the telomeric region is excluded from total cleavage during apoptosis. We only detected a slight shortening of the telomeric region at the onset of apoptosis. This cleavage seems not to be a result of internucleosomal cleavage, since no hybridising nucleosomal fragments can be detected.

In vitro analysis of CAD specificity

One reason for the exclusion of the telomeric region from internucleosomal cleavage may be the sequence of the telomeric repeats. It was shown that CAD preferentially cleaves within the sequence 5′-RRRYRYYY-3′.[25] Since the sequence of the telomeric repeat differs from this preferred sequence we compared the kinetics of the cleavage of DNA fragments of the telomeric region and of the *Sau*3A repeat. This was done in an *in vitro* assay. The DNA fragments were incubated with recombinantly expressed CAD/ICAD complexes and recombinant caspase 3. The kinetics of the cleavage was monitored by measuring the disapearance of the DNA fragments on an agarose gel (Figure 8a). The intensities of the bands were determined with the ImageQuant software. For each time point the relation of the intensities of two different bands (telomere repeat *versus* Sau3A repeat) was calculated. The relation of the input was set to 1. In the diagram the results of four independent experiments are presented (Figure 8b). The results clearly demonstrate that the telomere repeat is more resistant to CAD cleavage than the *Sau*3A repeat. However, after longer exposure or higher concentration (data not shown) of active CAD the telomeric sequence is also cleaved. This indicates that the sequence of the telomeric region contributes only partially to the exclusion of the telomeric region from internucleosomal cleavage.

Discussion

To analyse the temporal and spatial order of cleavage of chromosomal DNA during apoptosis, we analysed the HMW
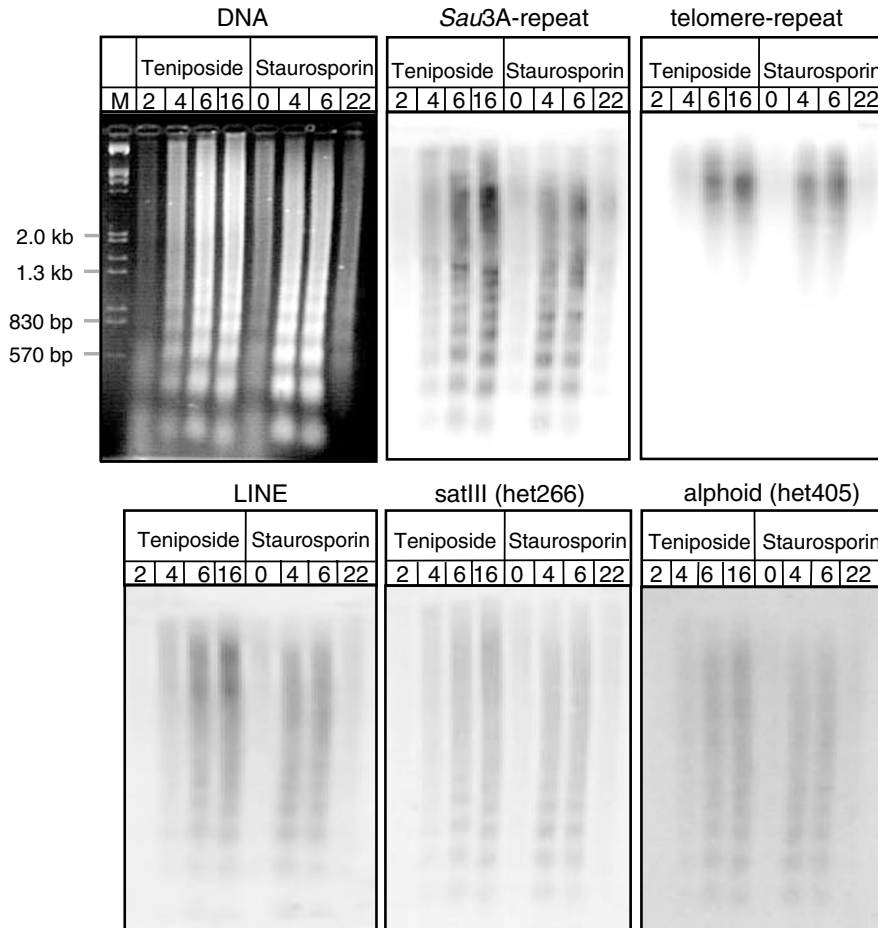
Figure 4 Hybridisation pattern of LMW DNA fragments from HL60 cells treated with teniposide or staurosporine. HL60 cells were grown in medium with 10 μM teniposide or 1 μM staurosporine, respectively. Incubation time (h) is indicated at the top of the figure

DNA fragments and LMW DNA fragments released from the nuclear chromatin, by hybridisation with probes specific for different regions of the human genome. Neither for the HMW fragments nor for the LMW fragments we could identify specific regions that are preferentially cleaved. Therefore, our results point to a random cleavage of the chromatin during apoptosis. This is different from other reports that found preferential sites of apoptotic DNA cleavage,[26,27,28] but there is an equal number of reports supporting our findings.

Winter *et al.*[29] compared the rate of fragmentation of transcriptionally active genes *versus* nontranscribed genes in B-lymphocyte hybridoma cells by slot blot hybridisation. They observed that all examined genes were degraded at the same rate regardless of their transcriptional state. From these results they concluded that nuclear DNA is degraded in a homogenous manner during apoptosis. Since only 30% of the human genome comprises genes and gene-related sequences this suggestion may be an overinterpretation. Nevertheless, their results in combination with the results reported herein confirm this suggestion. Another approach to analyse the regional cleavage of chromosomal DNA during apoptosis was to clone and sequence the released oligonucleosomal DNA fragments.[27,30,31] Whereas the two former reports detected a relative accumulation of interspersed

sequences (SINE and LINE) in the isolated fragments, Khodarev *et al.*[27] observed a normal distribution of repetitive DNA within the entire cloned material. These authors observed, however, a preferential cleavage site in such repetitive sequences. This preference referred only to the sequence of cleavage, but not to a preferred regional cleavage. In the studies mentioned above only a limited range or special regions of the human genome has been analysed. In our study we compare for the first time the bulk of the human genome in respect to temporal and spatial apoptotic cleavage.

With our analysis system we are not only able to detect cleavage but we also can detect the mode of cleavage (loop sized, nucleosomal sized or unspecific). We observed that under apoptotic conditions the telomeric region is not cleaved into nucleosomal fragments, although telomeric DNA is organised in nucleosomes (herein and Bedoyan *et al.*[32]).

The obvious hybridisation signals of HMW DNA fragments in our analysis (Figure 1) with the telomeric probe may be the result of apoptotic cleavage of the subtelomeric region generating fragments of the size of the telomeres.

The nucleosomal arrays of telomeric chromatin differ from those of bulk chromatin with respect to the length of the spacer, the linker DNA, but not in the structure of the core
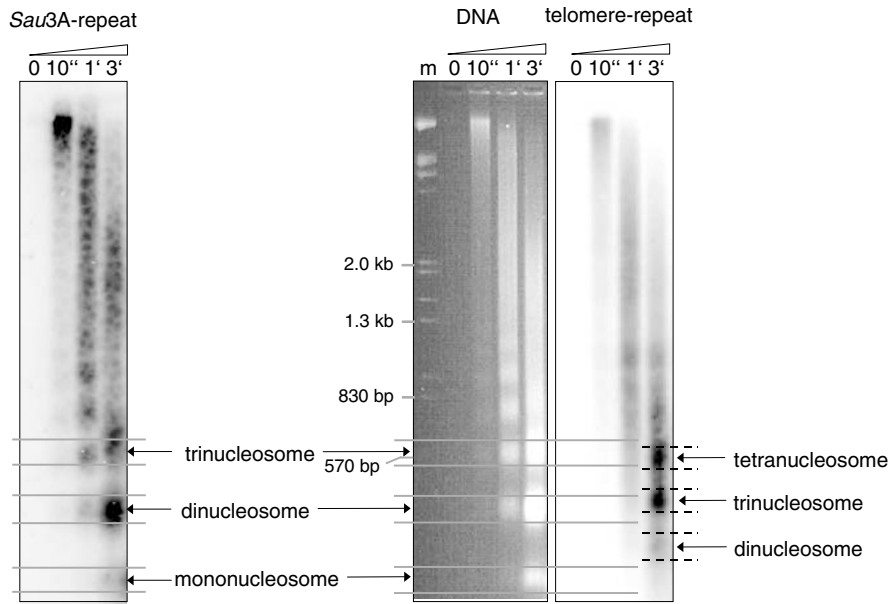
Figure 5 Examination of the nucleosomal organisation of the telomeric region. Nuclei of HL60 cells were prepared and treated with 10 U micrococcal nuclease for the indicated time. After lysis of the treated nuclei nucleosomes were isolated and the nucleosomal DNA was separated by conventional agarose gel electrophoresis. After transfer of the DNA to nylon filter the DNA was hybridised with the probe specific for the telomeric region. As a control the stripped filter was hybridised with the *Sau*3A-repeat specific probe. The size of the respective nucleosomal DNA fragments was determined by comparing with the size marker. To emphasize the different lengths of the nucleosomal repeats horizontal lines mark the hybridising fragments
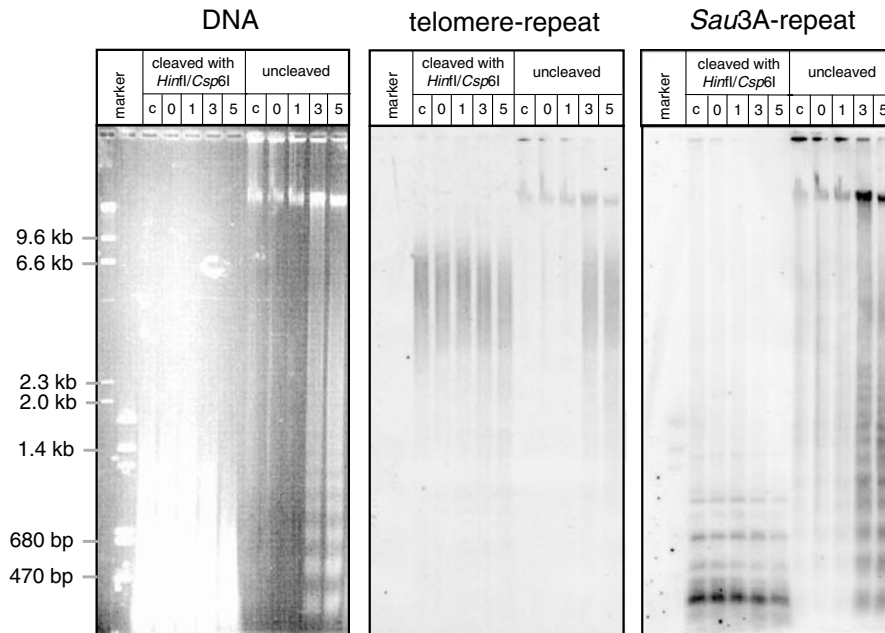

Figure 6 Determination of the telomeric length during apoptosis. Genomic DNA was isolated from HL60 cells treated for the indicated time in hours with topotecan. An aliquot of the chromosomal DNA was cleaved with the frequent cutters *Hin*fI and *Csp*6I in combination, the other aliquot was applied without additional cleavage to agarose gel electrophoresis. Hybridisation with the telomeric repeat probe generated a smear of hybridisation signals in the range of 4–10 kb with both the cleaved DNA and the uncleaved DNA, whereas the *Sau*3A-repeat probe hybridised to discrete fragments in the size range of 0.2–1 kb with the cleaved DNA and in the range of nucleosomal DNA with uncleaved DNA. This indicates that the telomeric region is excluded from apoptotic cleavage

particle. Vertebrate telomeres consist of an array of TTAGGG repeats, comprising 0.01–0.2% of the genome.[33] We determined by TRF analysis an average length of about 8 kb for the telomeres in HL60 cells. This value is in the range of the general length of telomeres in human cells of approximately 2–20 kb, depending on the cell type and the state of differentiation.[24] Recently, Ramirez *et al*.[34] reported a telomere shortening as an early event of DNA damage-induced apoptosis. They induced apoptosis in peripheral blood lymphocytes (PBL) and HL60 cells by camptothecin,
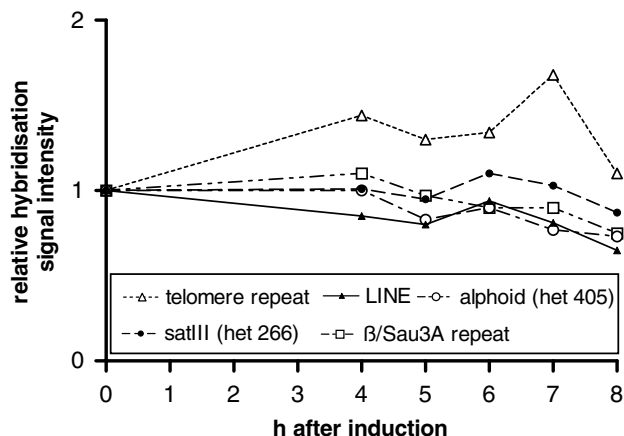
Figure 7 Determination of regional apoptotic cleavage by dot-blot hybridisation of uncleaved DNA. Unfragmented DNA (remaining chromosomal DNA) was isolated from topotecan treated synchronised HL60 cells. Duration of treatment is indicated in hours. Equal amounts of isolated HMW DNA were spotted onto a nylon filter and hybridised with the probes used for southern blot hybridisation. The intensities of hybridisation signals were determined using the ImageQuant software of the PhosphoImager system. The intensities of the individual probes are expressed in relation to the 0 h value (uninduced situation) and are equilibrated to potential deviations of the amounts of the DNA loaded on each spot. The telomeric region is clearly over represented in the remaining chromatin during apoptosis, this means that this region is excluded from apoptotic cleavage.

Figure 8 Analysis of the cleavage preference of CAD. (**a**) A 350 bp fragment of the telomeric region and a fragment comprising one Sau3A repeat (170 bp) were incubated with active CAD. CAD was activated by incubation of the CAD/ICAD complex with caspase 3. The DNA was cleaved with different aliquot of the CAD/ICAD complex for 10 min at 30°C. After agarose gel electrophoresis and ethidium bromide staining the intensities of the bands were determined with the ImageQuant software (MolecularDynamics). The ratio of the intensities (telomere/Sau3A) was calculated from each lane. The ratio from the incubation with no CAD (line 1) was set to 1. (**b**) The results of four independent experiments were evaluated and the relative intensity ratios are shown as bar chart

etoposide and UV irradiation. During the first 4 h telomere shortening was about 10%. From our TRF analysis in apoptotic HL60 cells we observed a decrease in telomere length to a similar extent. However, our results and the results reported by Ramirez *et al.*[34] revealed no continuous cleavage of telomeric DNA. After this initial shortening of 10% of the length of the telomeres we could not observe further cleavage. In our dot blot experiments where we determined the relative content of specific DNA in the uncleaved fraction of chromosomal DNA, we also observed a relative increase of telomeric DNA in this fraction in comparison to the other regions. This again confirms the interpretation that the telomeric DNA is excluded from nucleosomal cleavage during apoptosis. It may be possible that the observed initial shortening of the telomeres is a primary effect of the drugs used for induction of apoptosis in these experiments.

The key nuclease for apoptotic DNA fragmentation is the caspase-activated nuclease (CAD).[35] In nonapoptotic cells this CAD is associated with an inhibitor (ICAD). After induction of apoptosis the inhibitor is cleaved by caspase 3 and CAD becomes active. CAD is a Ca^{2+}-dependent nuclease with a preferred cleavage sequence 5′-RRRYRYYY-3′.[25] Since this sequence does not resemble the telomeric repeat sequence this may be one possible explanation for the exclusion of the telomeric region from internucleosomal cleavage during apoptosis. We checked this possibility in an *in vitro* assay by cleavage of DNA fragments with the telomere repeat sequence and the Sau3A repeat sequence, respectively, using activated CAD. We observed a reduced cleavage of the telomere DNA compared with the Sau3A DNA. However, this reduction cannot be the only reason for the exclusion of the telomeric region from internucleosomal cleavage since with higher CAD concentrations and longer incubation time we
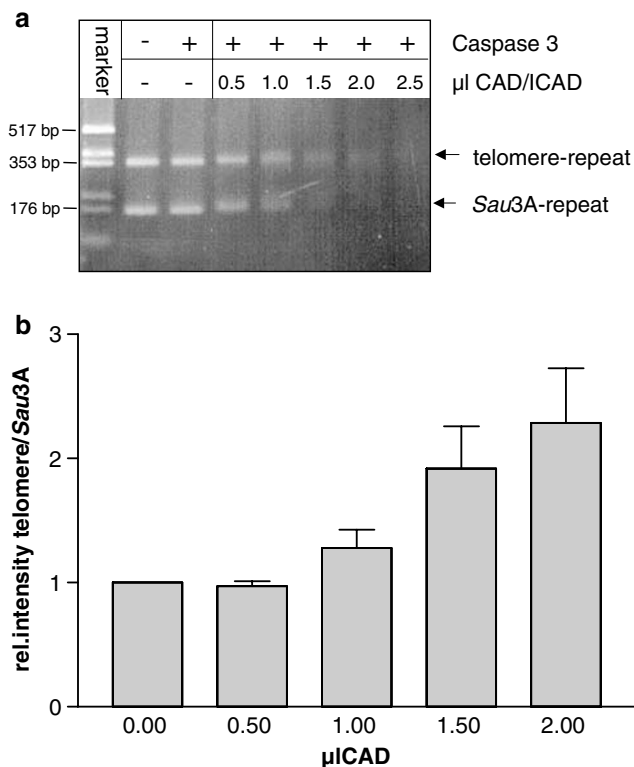
also observed a complete cleavage of the telomeric DNA. An additional reason for the resistance to CAD cleavage may be the chromatin structure of the telomeric region. Besides their nucleosomal organisation the telomeric regions are associated with specific structural proteins that function as structural determinants of this region. It is discussed that the nucleosomes in the telomeric region are organised in a columnar structure[36,37] in contrast to the solenoidal organisation of the other regions. This may also explain the differences of internucleosomal cleavage. It was observed that digestion of the telomeric region by micrococcus nuclease is preferred in comparison to other genomic regions.[23] This behaviour varies from CAD cleavage as outlined herein.

Since execution of apoptosis differs from cell type to cell type and in addition depends on the inducer used for inducing apoptosis, we also analysed the integrity of the telomeric DNA in another cell line and with an inducer using another principle of apoptosis induction. Even in these systems telomeric DNA was excluded from nucleosomal cleavage. This clearly demonstrates that this is a more general effect and is not restricted to a special cell line nor depends on an apoptotic stimulation induced by DNA damage. Further experiments should reveal whether this exclusion is just an effect of

telomeric chromatin organisation or is a prerequisite for proper apoptotic cell elimination.

Acknowledgements

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