DTD 5
ARTICLE IN PRESS

Available online at www.sciencedirect.com
SCIENCE DIRECT®

ELSEVIER

Mutation Research xxx (2005) xxx–xxx

Fundamental and Molecular Mechanisms of Mutagenesis

www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

The DNA demethylating 5-azaC induces endoreduplication in cultured Chinese hamster cells

Santiago Mateos, Inmaculada Domínguez, Nuria Pastor, Gloria Cantero, Felipe Cortés *

Department of Cell Biology, Faculty of Biology, University of Seville, Avda Reina Mercedes 6, E-41012 Seville, Spain

Received 20 September 2004; received in revised form 9 February 2005; accepted 18 February 2005

Abstract

We have investigated the possible influence of 5-azacytidine (5-azaC) substitution for cytidine into DNA on topoisomerase II (topo II) function in chromosome segregation. The endpoint chosen has been the induction of endoreduplicated cells at mitosis showing diplochromosomes. Experiments were performed in the presence and absence of the cytidine analogue to assess the degree of 5-azaC-induced DNA hypomethylation, using differential cutting by restriction endonucleases *Hpa* II and *Msp* I. Using the pulsed-field gel electrophoresis (PFGE) technique, we have also observed a protective effect provided by 5-azaC treatment against DNA breakage induced by the topo II poison *m*-AMSA. Concentrations of 5-azaC shown as able to induce extensive DNA hypomethylation and capable to protect DNA from double-strand breaks induced by *m*-AMSA were used for our cytogenetic experiments to analyze chromosome segregation. Our results seem to indicate that the presence of 5-azaC in DNA induces a dose-dependent increase in the yield of endoreduplicated cells that parallels the levels of hypomethylation observed.

Keywords: DNA hypomethylation; DNA segregation; Endoreduplication; DNA topoisomerases

1. Introduction

The essential nuclear enzymes DNA topoisomerases (topo I and topo II) regulate DNA topology during many cellular processes, such as replication, transcription, recombination and segregation of daughter molecules through transient cleavage of the molecule, strand passing and religation (for a review, see [1]). There are two classes of topos according to their catalytic mechanisms. While class I enzyme (topo I) breaks and rejoins one DNA strand at a time, allowing the DNA to swivel and release torsional strain, class II enzyme (topo II) is able to do so with the two strands that make up duplex DNA, allowing the passage of another intact DNA duplex through the gap. Since topoisomerase-induced breaks in DNA are transient intermediates in the strand passage reaction, they are normally present at low steady-state levels and hence, well

* Corresponding author. Tel.: +34 954 557039; fax: +34 954 610261.
E-mail address: cortes@us.es (F. Cortés).

0027-5107/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.mrfmmm.2005.02.001

tolerated by the cell as a necessary, though otherwise potentially dangerous, process that proves mutagenic and even lethal when the enzyme is poisoned [2,3].

A key feature that establishes a difference between both types of enzyme is that while both type I and type II are proficient in relaxing supercoiled DNA in order to relieve torsional tension that generates during replication and transcription, only topo II is able to resolve intertwined DNA molecules. This unique activity of DNA topo II in decatenating and unknotting is essential for segregating fully replicated daughter chromosomes for G2/M to proceed [4–10]. Besides its essential functional roles in chromosome condensation and segregation, topo II is a basic structural protein particularly abundant in the chromosome scaffold and nuclear matrix [11].

The beginning of the enzyme's catalytic cycle of topoisomerization of DNA consists of the binding of the homodimer to its double-stranded substrate. This binding does not seem to require any cofactor, but the presence of divalent cations has been reported to stimulate it [12]. Concerning the double helix properties influencing such an interaction topo II–DNA, on the other hand, both nucleotide sequence and topology seem to play a role. Within its recognition/binding sites, it seems that topo II cleaves DNA at preferred sequences, while there is no report on high specificity [13–16]. In general, the principles that govern the nucleic acid specificity of topo II are as yet rather obscure and further investigation is needed.

As to the importance of the topological form of DNA for binding and cleavage by topo II as a whole, on the other hand, it has been reported that the enzyme has a preferential interaction with supercoiled DNA as compared with relaxed forms of the molecule [3]. This behaviour of topo II provides a plausible explanation to its strong interaction with supercoiled DNA as well as to why it readily releases its reaction product when relaxation is accomplished.

Endoreduplication is a variety of endomitosis that consists of two successive rounds of DNA replication without intervening mitosis, i.e., segregation of daughter chromatids [10,17]. Diplochromosomes, made up of four chromatids held together instead of the normal two, are the visible mitotic manifestation of this rare, although sometimes spontaneous, phenomenon.

In spite of being a normally rare event in animal cells, a variety of agents have been reported to induce endoreduplication to different degrees, either through interference with cytoskeleton assembly [18,19] or as a result of DNA damage [20–23]. More recently, focusing on topo II agents that interact with the enzyme have been used to provide further evidence that it is essential for separation of daughter chromosomes. Topoisomerase "poisons", i.e., chemicals that cause DNA strand breaks through stabilization of topo II covalently bound to DNA in the intermediate form so-called cleavable-complex [2] as well as those considered as true catalytic inhibitors [24] are able to induce endoreduplication [10,25,26] due to prevention of decatenation of replicated chromosomes by topo II with the subsequent failure to complete anaphasic segregation.

In a recent investigation, we analyzed the possible influence of DNA substitution by halogenated nucleoside analogues of thymidine on chromosome segregation. Our observation was that all the thymidine analogues tested are able to induce endoreduplication to different degrees as a result of segregation failure being the yield of endoreduplication parallel to the relative level of halogenated nucleoside substitution for thymidine in DNA achieved [27].

In order to continue our studies on the specificity of topo II for DNA sequence in mammalian cells, we have carried out an investigation on the possible influence of the methylated state of DNA on chromosome segregation. The drug 5-azacytidine (5-azaC), which acts as a strong hypomethylating agent at the C5 position of cytidine after its incorporation into DNA, was used to induce extensive modification of the DNA sequence. Our results seem to indicate that severe hypomethylation of DNA leads to reduced chromatid decatenation that ends up in endoreduplication, most likely due to a failure in topo II function.

2. Materials and methods

2.1. Chemicals and enzymes

A stock solution of 5-azaC (Sigma) was prepared in distilled water (H_2O_D) and kept in 200 ml vials at −20 °C until use. Just before an experiment, a vial was thawed and then further diluted in medium in order to obtain the final concentration of 5-azaC desired. Restriction endonucleases *Hpa* II and *Msp* I (Promega)

were used according to the suppliers' recommended protocol in the activity buffer provided.

2.2. Cell culture

Chinese hamster ovary AA8 cells were grown as monolayers in McCoy's 5A medium with 10% fetal calf serum, 2 mM L-glutamine and the antibiotics, penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were cultured at 37 °C in 5% CO_2 in air.

2.3. Genomic DNA preparations

Untreated control cells or cells treated for 24 h with 0.1, 0.5 and 50 μM 5-azaC were processed to obtain an extract of genomic DNA. Briefly, about 15×10^6 cells was collected in 10 ml PBS using a scraper and then centrifuged at 1200 rpm for 6 min. After centrifugation, the supernatant was discarded and the pellet was resuspended with 500 μl of lysis solution (10 mM Tris–HCl at pH 6, 25 mM EDTA, 100 mM NaCl, 0.5% SDS). Lysate of cells was obtained by passing the mixture at least 10 times through a sterile insulin syringe. Then, 500 μl of lysate per sample was incubated at 50 °C for 60 min in a water bath in the presence of proteinase K (100 μg/ml) and RNase (10 μg/ml). At the end of the incubation, DNA was extracted twice with phenol–chloroform–isoamyl alcohol mixture (25:24:1). The genomic DNA was then precipitated with 7.5 M ammonium acetate and with 100% ethanol. After centrifugation at 14,000 rpm for 15 min at 4 °C, the supernatant was carefully removed and the pellet rinsed with cold 70% ethanol. Subsequently, the DNA pellet was dried and dissolved in 50 μl TE buffer at pH 7.4. DNA concentration was measured by spectrophotometry at 260 nm. Purity of DNA was assessed using the ratio of OD 260/280 with a ratio of 1.8–2.0 being considered of high purity [28]. Quantification of all DNA samples was performed using a Beckman DU-64 Spectrophotometer.

2.4. Genomic DNA digestion and electrophoresis

A total of 2 μg of genomic DNA per sample was digested overnight with approximately 10-fold excess of either *Hpa* II or *Msp* I endonucleases according to the manufacturer's protocol (Promega). Also, a DNA aliquot per sample was incubated without restriction enzyme addition and served as background control. Briefly, the DNA digestion was performed in 20 μl reaction mixtures containing 2 μg of DNA, 10× Buffer A (in the case of digestion with *Hpa* II) containing 60 mM Tris–HCl (pH 7.5), 60 mM NaCl, 60 mM $MgCl_2$ and 10 mM DTT or 10× Buffer B (when *Msp* I was used for the enzyme digestion) containing 60 mM Tris–HCl (pH 7.5), 500 mM NaCl, 60 mM $MgCl_2$ and 10 mM DTT, 0.1 mg/ml BSA. In all cases, 15 units of the restriction enzyme were used and H_2O_D was added to complete the final reaction volume. The reaction mixtures were incubated overnight at 37 °C and terminated by adding 2 μl of loading buffer consisting of 5% (v/v) Sarkosyl, 0.0025% Bromophenol Blue and 25% (v/v) glycerol. The mixtures were subjected to 1% (w/v) agarose-gel electrophoresis in TAE (Tris/acetate/EDTA) running buffer. The agarose gels were stained with ethidium bromide and DNA was revealed with a UV transilluminator. The relative changes in methylation status were compared between samples by a densitometric analysis of the gel using the software program PCBAS 2.0.

2.5. Pulsed-field gel electrophoresis (PFGE) of DNA

Cells were treated for 24 h with either 35 or 50 μM 5-azaC and then, treated for 3 h with the topo II poison *m*-AMSA at a dose of 2 μM to induce DNA double-strand breaks through stabilization of topo II–DNA "cleavable complexes". Cells untreated, treated with 5-azaC alone, or *m*-AMSA alone served as controls. At the end treatments, cells were immediately embedded in agarose and DNA double-strand breaks were analyzed by clamped homogeneous field (CHEF) gel electrophoresis.

The procedure was as follows: exponential cells were collected using a cell scraper, washed twice in cold phosphate-buffered saline (PBS) and counted in PBS. They were mixed with low-melting temperature agarose (LMP-agarose, Sigma) at 4×10^6 cells/ml. The suspension was pippeted into plug moulds (250 μl, Bio-Rad) and kept at 4 °C for 30 min to allow the agarose to set. Subsequently, plugs were transferred to three volumes of lysis solution (0.5 mol dm^{-3} EDTA, 2% sarkosyl and 0.5 mg/ml proteinase K), maintained on ice for 1 h to prevent any repair occurring during diffusion of the lysis solution and then, incubated at

37 °C for 24 h. Before electrophoresis, the plugs were washed twice in PBS and cut into halves corresponding to about 2×10^4cells.These samples were inserted into 0.6 cm × 0.5 cm × 0.1 cm wells of a precast 0.8% agarose gel in 0.5× TBE buffer (0.05 mol dm^{-3} Tris, 0.05 mol dm^{-3} borate, 0.1 mmol dm^{-3} EDTA; pH 8.4). *Saccharomyces cerevisiae* yeast chromosomes were used as DNA size standards in each gel. The wells were sealed with 0.8% agarose.

Electrophoresis was carried out using a CHEF-DRII system (Bio-Rad). The gels were electrophoresed at 45 V (1.3 V/cm) for 96 h with a switch time of 60 min. Electrophoresis buffer was 0.5× TBE. Buffer temperature was maintained at 14 °C by circulation through a cooling bath. Following electrophoresis, the gels were placed in 200 ml of electrophoresis buffer with 0.5 μg/ml of ethidium bromide to stain the DNA. Finally, gels were photographed under UV illumination and densitometrically analyzed using the software programme PCBAS version 2.08. DNA damage was measured as the percent of DNA migration from the well: % DNA migration = [optic density in lane/total optic density on the lane and well] × 100. Statistical analysis for significance was used (Student's *t*-test).

2.6. Induction of endoreduplication

Actively growing AA8 cells were cultured for 24 h in the presence of a wide range of different concentrations of the cytidine analogue 5-azaC (0.05; 0.1; 0.5; 15; 35 and 50 μM) shown as efficiently inducing hypomethylation at the C5 position of cytidine after its incorporation into DNA. After treatment, the cultures were thoroughly washed and maintained in fresh medium for 18 h to allow them to recover. Cultures that did not receive any treatment served as control. Colcemid (2×10^{-7} M) was finally added for 2 h 30 min to all the cultures for metaphase arrest. The flasks were gently shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 2 min (hypotonic treatment), fixed in methanol:acetic acid (3:1) and dropped onto clean glass microscope slides. The slides were stained with 3% Giemsa in phosphate buffer pH 6.8 and mounted in DPX. Two thousand metaphases per culture were counted and classified as normal or as having diplochromosomes. All the experiments were carried out in triplicate.

3. Results

3.1. DNA hypomethylation after treatment with 5-azacytidine

The cytidine analogue 5-azaC, containing an N atom at the 5′-position instead of a carbon, has been reported as efficiently able to induce hypomethylation at the C5 position of cytidine after its incorporation into DNA [29]. With the ultimate purpose of assessing the level of DNA demethylation following 5-azaC treatment in AA8 Chinese hamster ovary cell line, the cells were given a 24 h treatment with different doses of the cytidine analogue and DNA was subsequently extracted for restriction enzyme digestion and gel electrophoresis. Two restriction endonucleases, namely *Hpa* II and *Msp* I were selected for our study.

While methylation of cytosine in 5′-CpG-3′ sequence renders DNA resistant to cutting by restriction enzyme *Hpa* II, *Msp* I cuts CpG islands regardless of its methylation status. Accordingly, we carried out a comparative restriction enzyme digestion to see whether a treatment with 5-azaC was effective in changing the methylation pattern of DNA in AA8 cells. As can be seen in Fig. 1, incubation with *Msp* I enzyme led to extensive DNA fragmentation, yielding a smear of similar characteristics regardless of the dose of 5-azaC given to the cells. Contrasting with this observation, on the other hand, the pattern of *Hpa* II cutting was in all cases that corresponding to a much more limited fragmentation of DNA, as expected from the enzyme limited ability to cut DNA CpG sequences, only when they are demethylated for methylation protects from cutting by *Hpa* II.

The above notwithstanding, treatment of the cells with doses of 5-azaC ranging from 0.1 to 50 μM yielded a dose-dependent increase in the amount of DNA migrated from the wells, clearly detectable densitometrically (Fig. 1, lanes 5, 8 and 11).

3.2. 5-Azacytidine treatment protects against DNA double-strand breaks induced by the topo II poison m-AMSA

The incorporation of 5-azaC instead of cytidine has a widespread effect in that methyltransferases proceed

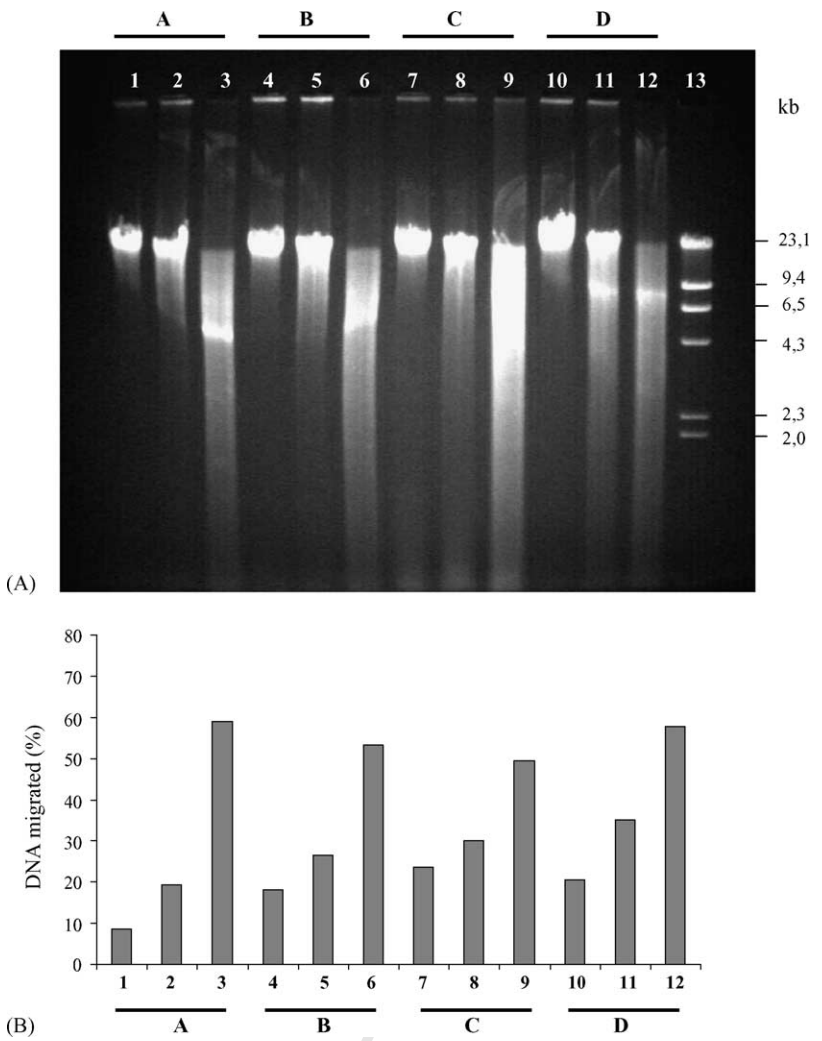

Fig. 1. Pattern of DNA digestion by restriction endonucleases *Hpa* II and *Msp* I depending upon the methylation of cytosine in 5′-CpG-3′ sequences. While methylation renders DNA resistant to cutting by *Hpa* II, restriction enzyme *Msp* I cuts CpG islands, regardless of their methylation status. (A) Electrophoretic image of DNA fragmentation pattern. Lanes 1, 4, 7 and 10, uncut control genomic DNA; lanes 2 and 3, DNA from cells untreated with 5-azaC digested with *Hpa* II and *Msp* I, respectively. Lanes 5, 8 and 11, *Hpa* II digestion pattern for cells grown in the presence of 0.1, 0.5 and 50 μM 5-azaC. Lanes 6, 9 and 12, the same for *Msp* I. Molecular weight marker (*Hind* III cut phage lambda DNA) is included (lane 13). (B) Densitometric pattern obtained from (A). Note that while for *Msp* I the pattern is roughly similar regardless of 5-azaC treatment, given that it cleaves DNA independently of the methylation status of CpG islands, a clear dose-dependent effect is observed for cleavage by *Hpa* II (DNA migrated) depending upon demethylation.

to hypomethylate DNA not only at the site of 5-azaC misincorporation, but far beyond that location in DNA. Topo II cleavage for its function during essential processes, such as segregation of fully replicated DNA, depends on recognition/binding of the enzyme homodimer to preferred nucleotide sequences, though there is no report on a high specificity [13–16].

In order so see whether DNA demethylation induced by 5-azaC had any effect on topo II catalytic activity, the effectiveness of the topo II poison *m*-AMSA to induce DNA double-strand breaks through stabilization of covalent “cleavable complexes” topo II–DNA was analyzed. Fig. 2 shows that AA8 Chinese hamster ovary cells pre-treated with 5-azaC for

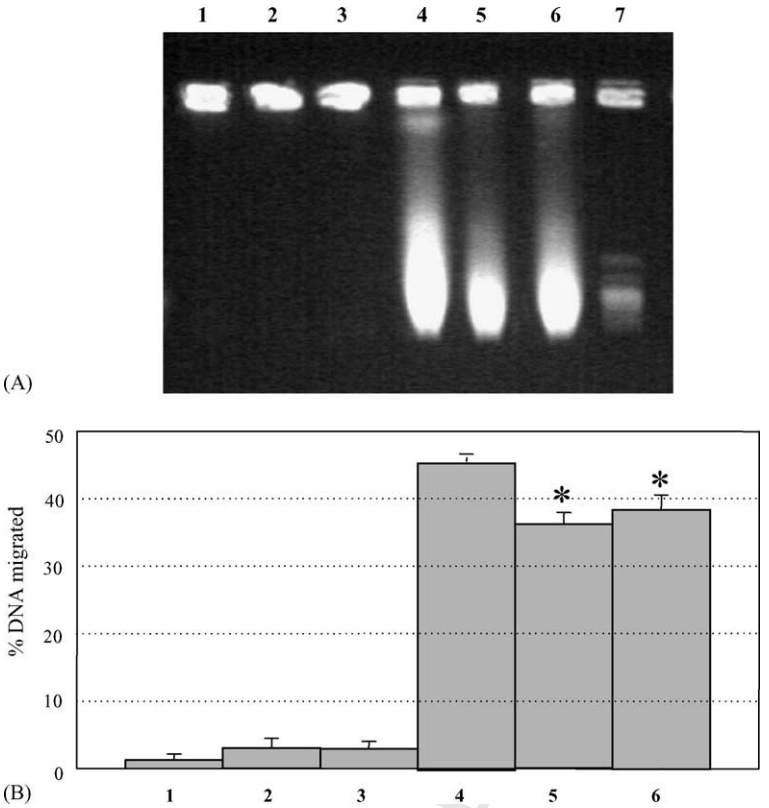

Fig. 2. Protection provided by 5-azaC against the induction of DNA double-strand breaks by the topo II poison *m*-AMSA assessed by PFGE. AA8 Chinese hamster ovary fibroblasts were treated with 5-azaC for two cell cycles and then with the topo II poison *m*-AMSA (see Section 2.5 for details). (A) Representative PFGE gel of three independent experiments is shown. Lane 1: untreated control; lanes 2 and 3: cells exposed to 35 and 50 μM 5-azaC, respectively; lane 4: cells treated with 2 μM *m*-AMSA; lane 5: cells grown in 35 μM 5-azaC before *m*-AMSA treatment; lane 6: the same with a pre-treatment with 50 μM 5-azaC; lane 7: *S. cerevisiae* DNA marker. (B) Densitometric analysis of the percent of DNA migration in PFGE gels as shown above, indicative of DNA double-strand breaks produced. Each bar represents the mean of three independent experiments ± S.D. ($^{*}P < 0.005$, according to Student's *t*-test).

24 h (about two consecutive cell cycles) appear as less sensitive to *m*-AMSA, as compared with control cells treated with the topo II poison alone, as assessed by PFGE measurements of DNA double-strand breaks.

3.3. Endoreduplication induced by 5-azacytidine

Once established that 5-azaC, at the doses tested by us, is capable of inducing extensive DNA demethylation in AA8 Chinese hamster ovary cells, we analyzed the possible influence of 5-azaC substitution for cytidine into DNA on normal chromosome segregation. The endpoint chosen by us to assess missegregation leading to aberrant mitosis was endoreduplication [10,17], typically visible as metaphases made up of diplochromosomes (Fig. 3).

5-Azacytidine substitution into DNA took place for two consecutive S-periods (total 24 h) before allowing the cells to recover for an additional cell cycle during which endoreduplication (if any) might take place. As can be seen in Fig. 4, endoreduplication was effectively induced in a dose-dependent fashion at doses of 5-azaC ranging from 0.05 to 35 μM. At concentrations of the cytidine analogue of 50 μM and higher, while the induction of endoreduplication was still observed, the dose-dependent relationship was more variable (data not shown).

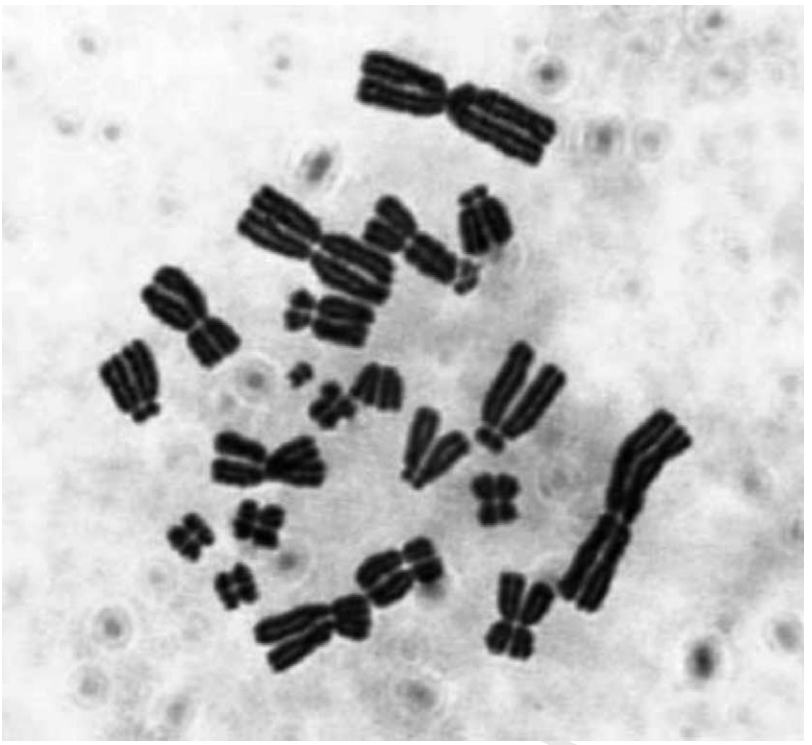

Fig. 3. Endoreduplicated cell at metaphase showing the characteristic diplochromosomes. 5-azaC was present for two consecutive rounds of DNA replication, followed by an additional S-period in absence of the cytidine analogue during which endoreduplication took place.

4. Discussion

The question as to the relative importance of DNA sequence for its recognition by DNA topoisomerases and their subsequent binding and cutting of the polynucleotide chain (s) for enzyme function is still a matter of scientific investigation. Concerning this, it is generally agreed upon that nucleotide sequence plays a role but the rules that determine the nucleic acid specificity of topo II are as yet far from being fully understood.

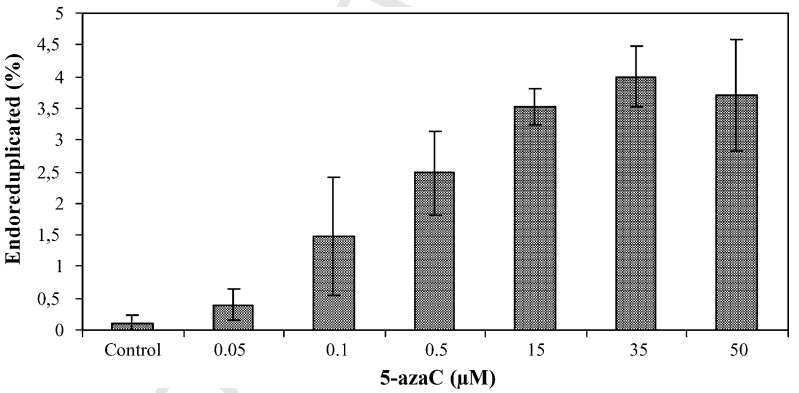

Fig. 4. Induction of endoreduplication by 5-azaC. In good agreement with the degree of hypomethylation observed (see Fig. 1) 5-azaC was shown as capable of inducing endoreduplication in a dose-dependent fashion. Bars indicate standard errors from three experiments.

Reports on topo II cleavage at preferred sequences of DNA within its recognition/binding sites have been presented, while high specificity does not seem to exist [13–16].

The frequencies of the nucleotides and dinucleotides in the region near the site of phosphodiester bond breakage was analyzed in order to study the sequence specificity of double-strand DNA cleavage by *Drosophila* topo II [30] and clearly revealed a non-random distribution. The consensus sequence derived was 5′GT·A/TAY↓ATT·AT··G3′ where a dot means no preferred nucleotide and Y stands for pyrimidine [30]. Furthermore, analysis of DNase I footprint has revealed that *Drosophila* topo II can protect a region in both strands of the duplex DNA, with the cleavage site located near the center of the protected region [31], and it has been proposed that the strong DNA cleavage sites of *Drosophila* topo II [30] likely correspond to specific DNA-binding sites of the enzyme [13,32].

On the other hand, the interaction between calf thymus topo II and DNA was also characterized by means of a transcription assay [33] and it was concluded that topo II binds to a region of DNA located symmetrically around the enzyme-mediated cleavage site.

In a study aimed at the assessment of the possible role played by the nature of DNA in chromosome segregation, we have recently reported the induction of endoreduplication in AA8 Chinese hamster cells treated with different halogenated nucleosides that incorporate into DNA for thymidine [34], namely CldU, IdU and BrdU. The observation was that treatment with any of the halogenated nucleosides for two consecutive cell cycles resulted in endoreduplication, the frequency of endoreduplicated cells depending upon the thymidine analogue and the percent of substitution for thymidine into DNA [27]. We concluded that even though the possible involvement of other proteins cannot be ruled out at present, our observations seem to favor the likely hypothesis that the nature of DNA might play a role for the recognition/binding of topo II and its subsequent cleavage of the fully replicated molecule for chromosome segregation [27]. It has been reported that eukaryotic topo II preferentially cleaves alternating purine–pyrimidine repeats within the consensus sequence, and additionally, GT, AC and AT repeats were better substrates for cleavage than GC repeats [14,33]. Furthermore, the distribution of DNA cleavage sites induced by topo II in the presence or absence of enzyme poisons were mapped in the simian virus 40 genome [16] and the finding was that strong sites tended to occur within A/T runs, such as those that have been associated with binding to the nuclear scaffold [16].

In the present investigation, we have found that DNA substitution of 5-azaC for cytidine that results in a drastic DNA demethylation, provides a protection against DNA double-strand breaks induced by the topo II poison *m*-AMSA. This observation seems to point out to a loss in the capacity of the poison to induce the stabilization of covalent DNA–topo II "cleavable complexes" that, in turn, are responsible for the DNA damage when the replication fork machinery collides with them [35].

Concerning endoreduplication, a dose-dependent increase in the yield of endoreduplicated cells after treatment with 5-azaC was observed. This parallels the corresponding level of DNA hypomethylation induced in DNA by 5-azaC incorporation for cytidine, as assessed using restriction enzyme digestion, and also correlates with the protection of DNA against damage induced by the topo II poison *m*-AMSA.

Taken as a whole, our previous report [27] as well as the present results seem to support the idea that the presence of anomalous bases, such as halogenated pyrimidines or demethylated cytosines in DNA, results in a defective function of topo II in chromosome segregation that eventually leads to aberrant mitosis and the subsequent endoreduplication [26]. Supporting this, it has been reported that the cleavage activity of topo I is affected by both the removal of the CH_3 group from thymidine (by substituting uridine for thymidine) or its addition onto cytosine (replacement of cytosine by 5-methylcytosine) [36–38].

In our opinion, however, an alternative hypothesis based upon the degree of chromatin condensation must be considered. It is well known that the dynamics of chromatin compaction can be altered by incorporation of the deoxycytidine analogue 5-azaC into DNA during the second-half of S-phase, as a consequence of drastic DNA demethylation. This prevents the normal compaction of late-replicating structures, i.e., G-bands and constitutive heterochromatin [39–41]. Interestingly, also BrdU incorporation into DNA has been reported to induce alterations in chromatin compaction, although more limited than those induced by 5-azaC [42]. The mechanisms are different; while BrdU plays a direct role by steric hindrance, 5-azaC is responsible for

an epigenetic effect leading to drastic DNA demethylation [41].

It has been reported that topo II has a much higher affinity for supercoiled DNA than for the relaxed forms of the molecule [3], which explains the strong interaction of the enzyme with the former, while the relaxed products of its reaction are readily released. Taking into account this behaviour of topo II depending upon the supercoiled state of DNA, the results reported in the present investigation could be explained as due to a possible loss of affinity of the enzyme for decondensed demethylated DNA. In turn, unresolved intertwined daughter DNA molecules, unable to segregate at G2/M, should lead to the triggering of an endoreduplication cycle, most likely as an emergency mechanism mediated by checkpoint signalling.

At present, discriminating between both hypotheses, i.e., DNA sequence versus DNA supercoiling/relaxed status appears as technically difficult and further investigation is needed.

Acknowledgements

This work has been partly funded by Grants from Spanish Ministry of Education and Science (DGI; BFU-2004-00884/BMC) and Junta de Andalucía (CVI 120), Spain.

References

[1] J.C. Wang, DNA topoisomerases, Annu. Rev. Biochem. 65 (1996) 635–692.
[2] L.F. Liu, DNA topoisomerase poisons as antitumor drugs, Annu. Rev. Biochem. 58 (1989) 351–375.
[3] D.A. Burden, N. Osheroff, Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme, Biochim. Biophys. Acta 1400 (1998) 139–154.
[4] T. Uemura, H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, M. Yanagida, DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. Pombe*, Cell 50 (1987) 817–925.
[5] C.S. Downes, A.M. Mullinger, R.T. Johnson, Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis, Proc. Natl. Acad. Sci. USA 88 (1991) 2616–2620.
[6] C.E. Shamu, A.W. Murray, Sister chromatid separation in frog extracts requires topoisomerase II activity during anaphase, J. Cell Biol. 117 (1992) 921–934.
[7] P. Buchenau, H. Saumweber, D.L. Arndt-Jovin, Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by in vivo confocal laser scanning microscopy, J. Cell Sci. 104 (1993) 1175–1185.
[8] J.F. Giménez-Abián, D.J. Clarke, A.M. Mullinger, C.S. Downes, R.T. Johnson, A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes, J. Cell Biol. 131 (1995) 7–17.
[9] A.T. Sumner, Inhibitors of topoisomerase II delay progress through mitosis and induce doubling of the DNA content in CHO cells, Exp. Cell. Res. 217 (1995) 440–447.
[10] A.T. Sumner, Induction of diplochromosomes in mammalian cells by inhibitors of topoisomerase II, Chromosoma 107 (1998) 486–490.
[11] U.K. Laemli, E. Käs, L. Poljak, Y. Adachi, Scaffold-associated regions: *cis*-acting determinants of chromatin structural loops and functional domains, Curr. Opin. Genet. Dev. 2 (1992) 275–285.
[12] N. Osheroff, Role of the divalent cation in topoisomerase II mediated reactions, Biochemistry 26 (1987) 6402–6406.
[13] M. Sander, T. Hsieh, A. Udvardy, P. Schedl, Sequence dependence of *Drosophila* topoisomerase II in plasmid relaxation and DNA binding, J. Mol. Biol. 194 (1987) 219229.
[14] J.R. Spitzner, M.T. Muller, A consensus sequence for cleavage by vertebrate DNA topoisomerase II, Nucleic Acids Res. 16 (1988) 5533–5556.
[15] M.P. Lee, M. Sander, T. Hsieh, Nuclease protection by *Drosophila* DNA topoisomerase II. Enzyme/DNA contacts at the strong topoisomerase II cleavage sites, J. Biol. Chem. 264 (1989) 21779–21787.
[16] Y. Pommier, G. Capranico, A. Orr, K.W. Kohn, Distribution of topoisomerase II cleavage sites in simian virus 40 DNA and the effects of drugs, J. Mol. Biol. 222 (1991) 909–924.
[17] F. Cortés, S. Mateos, P. Escalza, Acetaldehyde induces mature endoreduplicated *Allium cepa* root tip cells to divide, Experientia 43 (1987) 205–206.
[18] M. Rizzoni, F. Palitti, Regulatory mechanism of cell division. I. Colchicine-induced endoreduplication, Exp. Cell Res. 77 (1973) 450–458.
[19] S. Sutou, Spontaneous sister chromatid exchanges in Chinese hamster cells in vivo and in vitro, Mutat. Res. 82 (1981) 331–341.
[20] S. Sutou, F. Tokuyama, Induction of endoreduplication in cultured mammalian cells by some chemical mutagens, Cancer Res. 34 (1974) 2615–2623.
[21] C.J. Kusyk, T.C. Hsu, Induction of high frequencies of endoreduplication in mammalian cell cultures with 33258 Hoechst and rubidazone, Cytogenet. Cell Genet. 23 (1979) 39–43.
[22] Y. Huang, C. Chang, J.E. Trosko, Aphidicolin-induced endoreduplication in Chinese hamster cells, Cancer Res. 43 (1983) 1361–1364.
[23] C. Lüke-Huhle, Endoreduplication in Chinese hamster cells during α-irradiation induced G2 arrest, Mutat. Res. 119 (1983) 407–413.
[24] T. Andoh, R. Ishida, Catalytic inhibitors of DNA topoisomerase II, Biochim. Biophys. Acta 1400 (1998) 155–171.

[25] R. Ishida, M. Sato, T. Narita, K.R. Utsumi, T. Nishimoto, T. Morita, H. Nagata, T. Andoh, Inhibition of DNA topoisomerase II by ICRF-193 induces polyploidization by uncoupling chromosome dynamics from other cell cycle events, J. Cell Biol. 126 (1994) 1341–1351.
[26] N. Pastor, M.J. Flores, I. Domínguez, S. Mateos, F. Cortés, High yield of endoreduplication induced by ICRF-193, a topoisomerase II catalytic inhibitor, Mutat. Res. 516 (2002) 113–120.
[27] F. Cortés, N. Pastor, S. Mateos, I. Domínguez, The nature of DNA plays a role in chromosome segregation: endoreduplication in halogen-substituted chromosomes, DNA Repair 2 (2003) 719–726.
[28] J. Sambrook, E.F. Fritsch, T. Maniatis, Gel electrophoresis of DNA, in: N. Irwin, N. Ford, C. Nolan, M. Ferguson (Eds.), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 2003.
[29] P.A. Jones, S.M. Taylor, Cellular differentiation, cytidine analogs and DNA methylation, Cell 20 (1980) 85–93.
[30] M. Sander, T.S. Hsieh, *Drosophila* topoisomerase II double-strand DNA cleavage: analysis of DNA sequence homology at the cleavage site, Nucleic Acids Res. 13 (1985) 1057–1072.
[31] M.P. Lee, M. Sander, T. Hsieh, Nuclease protection by *Drosophila* DNA topoisomerase II. Enzyme/DNA contacts at the strong topoisomerase II cleavage sites, J. Biol. Chem. 264 (1989) 21779–21787.
[32] J.R. Spitzner, I.K. Chung, M.T. Muller, Eukaryotic topoisomerase II preferentially cleaves alternating purine–pyrimidine repeats, Nucleic Acids Res. 18 (1990) 1–11.
[33] B. Thomsen, C. Bendixen, K. Lund, A.H. Andersson, B.S. Sorensen, O. Westergaard, Characterization of the interaction between topoisomerase II and DNA by transcriptional footprinting, J. Mol. Biol. 215 (1990) 237–244.
[34] F. Cortés, W.F. Morgan, S. Wolff, Effect of exogenous thymidine on sister-chromatid exchange frequency in Chinese hamster ovary cells with bromodeoxyuridine- and chlorodeoxyuridine-substituted chromosomes, Mutat. Res. 192 (1987) 277–282.
[35] F. Cortés, T. Ortiz, J. Piñero, Importante of replication fork progression for the induction of chromosome damage and SCE by inhibitors of DNA topoisomerases, Mutat. Res. 303 (1993) 71–76.
[36] F. Leteurtre, G. Kohlagen, M. Fesen, A. Tanizawa, K.W. Kohn, Y. Pommier, Effects of DNA methylation on topoisomerase I and II cleavage activities, J. Biol. Chem. 269 (1994) 7893–7900.
[37] P. Pourquier, L.-M. Ueng, G. Kohlagen, A. Mazumder, M. Gupta, K.W. Kohn, Y. Pommier, Effects of uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I, J. Biol. Chem. 272 (1997) 7792–7796.
[38] C. Carrasco, M.J. Waring, C. Bailly, Substitution of 5-methylcytosines for cytosines enhances the stability of topoisomerase I-DNA complexes and modulated the sequence selectivity of camptothecin-induced DNA cleavage, FEBS Lett. 425 (1998) 337–340.
[39] E. Viegas-Pequignot, B. Dutrillaux, Segmentation of human chromosomes induced by 5-ACR85-azacytidine), Hum. Genet. 34 (1976) 247–254.
[40] M. López-Baena, S. Mateos, J. Piñero, T. Ortiz, F. Cortés, Enhanced sensitivity to topoisomerase inhibitors in synchronous CHO cells pre-treated with 5-azacytidine, Mutat. Res. 421 (1998) 109–116.
[41] D. Flagiello, J. Bernardino-Sgherri, B. Dutrillaux, Complex relationships between 5-aza-dC induced DNA demethylation and chromosome compaction at mitosis, Chromosoma 111 (2002) 37–44.
[42] A.F. Zakharov, N.A. Egolina, Differential spiralization along mammalian mitotic chromosomes. I. BUdR-revealed differentiation in Chinese hamster chromosomes, Chromosoma 38 (1972) 341–365.