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Impaired chromosome segregation in plant anaphase after moderate hypomethylation of DNA

C. Leyton, D. Mergudich, C. de la Torre* and J. Sans

*Departamento de Biología Celular y Genética, Facultad de Medicina, Universidad de Chile, Santiago, Chile and *Centro de Investigaciones Biológicas, CSIC, Madrid, Spain*

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Abstract. 10^{-6} M and 10^{-5} M 5-azacytidine, demethylated around 9% and 17% of the 5-methylcytosine residues found in *Allium cepa* L. native DNA, respectively. Both treatments stimulated RNA synthesis in the cells of root meristems. On the other hand, the 10^{-5} M treatment gave rise to multiple chromosomal anomalies in mitosis before any fall in the mitotic index was detectable, but no chromosomal breaks were ever seen.

Serious lesions involved in chromatids and segregation in anaphase were preferentially found after hypomethylation of DNA sequences replicated in the second half of the previous S period: (i) sister telomeres remained unresolved at the cell equator while kinetochores had reached the poles, (ii) whole unsegregated chromosomes were pulled to one of the poles by obviously disfunctional kinetochores, resulting in an unbalanced distribution of chromatids, (iii) unsegregated chromosomes in other cells remained at the spindle equator as if kinetochores were nonfunctional, while cytoplasmic division took place before their migration to the poles. Frequently, a growing cytokinetic plate randomly cut the unsegregated chromosomes, giving rise to aneuploid nuclei. These anaphase failures are a firm basis to explain why the 10^{-5} M treatment selectively depressed the rate of cell proliferation in these cells in the long run. On the other hand, if hypomethylation occurred at the first half of the previous S period, enlarged chromosomal segments were evident in most metaphases, while chromosome laggards and bridges were recorded in anaphase at rather similar frequencies after the different 5-azacytidine treatments. These data were consistently obtained both in the native mononucleate cells of meristems and in one subpopulation of synchronous cells labelled as binucleate by 5 mM caffeine.

DNA methylation is a natural repressor of gene expression, due to the affinity of proteins which halt transcription for methylated DNA (Boyes & Bird 1991, Tate & Bird 1993). Conversely, hypomethylation induced by the incorporation of 5-azacytidine (AZA) into the DNA increases its expression (Eden & Cedar 1994). Plant DNA is methylated (Gruenbaum *et al.* 1981) and ribosomal genes are indeed activated for at least two subsequent cycles after

Correspondence: Professor J. Sans, Departament de Biología Celular y Genética, Facultad de Medicina, Universidad de Chile, Casilla 70061, Correo 7, Santiago, Chile.

AZA treatment in onion (De la Torre *et al.* 1991, Mergudich *et al.* 1992). However, undetected negative effects related to the transmission of some hypomethylated genes may exist, since reduced methylation is lethal for mouse embryos (Li *et al.* 1992). The recorded diminution in chromosome condensation after AZA (Fucik *et al.* 1970) seems in principle not a challenge to cell viability, but the induction of breaks in fixed positions of the AZA-treated chromosomes might be one valid reason for cell lethality (Schmidt *et al.* 1985).

In this paper, the mitotic behaviour of chromosomes was studied to see whether fragile sites exist in this plant genome after their experimental hypomethylation. Instead, serious disturbances in the balanced distribution of sister chromatids in ana-telophase occurred after the 10^{-5} M AZA treatment. Such altered chromosomal segregation is incompatible with cell viability.

MATERIALS AND METHODS

Material

Root meristems of *Allium cepa* L. bulbs were used. The bulbs weighed 15–20 g. Roots were grown attached to the bulbs, in filtered tap water, which was renewed at 24 h intervals, and aerated by continuous bubbling at a rate of 10–20 ml air per min in cylindrical glass receptacles of about 80 ml capacity in the dark, and at a constant temperature of 15 ± 0.5°C. The bulbs were placed in such a way that only their bases remained submerged in water. After 2–3 days most of the roots ranged from 15 to 30 mm in length. Initiation of treatments were then started, since the roots achieve dynamic equilibrium of growth from 12 mm onwards (Díez *et al.* 1970).

5-azacytidine (AZA) treatments

The roots, still attached to the bulbs, were treated for up to 4 days with two different concentrations (10^{-6} M and 10^{-5} M) of 5-azacytidine (Sigma, St Louis, MO, USA) which were prepared with filtered tap water. The conditions described above were maintained throughout the period of treatment.

Caffeine treatment

In order to label the spontaneous synchronous population of telophase cells as binucleate the roots, still attached to the bulbs, were submerged in a 5 mM caffeine (Merck, Darmstad, Germany) prepared in filtered tap water, for 1 h. This drug inhibits cytokinesis in cells going through telophase during this time (Giménez-Martín *et al.* 1965) and these cells then enter interphase and go synchronously through the whole cell cycle (López-Sáez *et al.* 1966).

Cytological procedures

Roots were fixed in 3:1 (v/v) ethanol-acetic acid mixture at the time specified for each experiment. Their apical meristems were squashed after staining them with acetic orcein.

Preparation of spread metaphase chromosomes

To obtain chromosomes after different AZA treatments, the root attached to the bulbs were treated with 1 mM colchicine (BDL, Poole, UK) for 3 h. After their fixation the apical meristems were treated for 1 h with a 0.5% solution of pectinase (from *Aspergillus niger*, Sigma, St Louis, MO, USA) in citrate buffer pH 4.2, at 37°C. Coverslides were removed by the dry ice method, and preparations were hydrated by passing them through ethanol (100%,

96%, 70%, 50% and 30%) and distilled water. Finally, after washing them several times with phosphate buffer pH 6.8, the slides were stained for 10 min with 3% Giemsa in the same phosphate buffer, washed again, air dried and mounted with Merckaglass resin.

^{3}[H]-Thymidine, ^{3}[H]-uridine and ^{3}[H]-leucine incorporation

Single 1 h treatments with (5-^{3}[H]-)thymidine, (5,6-^{3}[H])uridine or L-(4,5-^{3}[H])leucine (Amersham International, Amersham, UK) were used after 0 (control), 48 or 96 h of the different AZA treatments. They were used at 0.4; 1.5 and 0.4 MBq/ml, respectively (with specific activities of 925; 1500 and 24.5 GBq/mmol, respectively). After each of these treatments, 20 to 30 roots were collected and thoroughly washed with 1% calcium hypochlorite in order to eliminate contaminating bacteria. Afterwards, the roots were washed with cold distilled water. The meristematic zone of each root was isolated (0.5 to 1.5 mm from the root apex). In order to precipitate macromolecules, a pool of 10 meristems were treated with cold 5% TCA. Later on, these roots segments were homogenated in a Potter grinder in 10 mM NaOH. The homogenate was incubated for 15 min at 80°C. Samples of the homogenate (0.1 ml) were transferred onto GF/A Whatman glass fibre filters, dried and counted in a scintillation counter in toluene PPO-POPOP scintillation mixture. The protein concentration was measured in other similar aliquots of the homogenate (Bradford, 1976). The incorporated radioactivity was expressed in c.p.m./100 μg of protein.

Oxygen consumption

The oxygen consumption was estimated in root segments, from 1–2 cm from the apex, at 0 (control), 48 and 96 h after the AZA treatments (10^{-6} M and 10^{-5} M) were initiated. Five root segments were selected from each different treatment. Their wet weight was then recorded. Oxygen consumption was measured by polarography by an oxygen electrode (Clark nr. 5331, Yellow Springs Instruments, USA). For this record, the electrode was placed in a 1.8 ml volume glass cell, at 25°C. The electrode signal was amplified by the oxygen monitor ISI (model 53), and registered by a Groerz RE 511 channel recorder, set with 100 mV of entrance potential difference. To calibrate the oxygen electrode at 100% saturation, a 435 ng/ml solution was used, since it is the oxygen solubility in water at 25°C at a pressure of 718 mm of Hg that is required. Correction for the actual atmospheric pressure was carried out. The 0% oxygen saturation level was fixed by adding sodium dithionite crystals at a 0.02 M sodium sulfite solution (pH 7.0–7.2) previously bubbled with nitrogen (Reynafarge *et al.* 1976). The line obtained represents the rate of oxygen consumption, expressed in nmol of oxygen per minute and per gram of fresh tissue.

DNA extraction

Groups of 50 isolated 1 mm long-root segments corresponding to the meristems were frozen in liquid nitrogen, and ground to a fine powder in a mortar. Extraction buffer (600 μl) (10 mM tris-HCl buffer pH 8.0, 10 mM EDTA pH 8.0, 1% sarcosyl) and proteinase K (1 mg/ml) were added to the extract. All components were mixed and kept for 12 h at 37°C, under continuous shaking. Then, an equal volume of chloroform/phenol mixture saturated with 0.1 mM Tris-HCl buffer pH 8.0 was added. The mixture was centrifuged at 14000 r.p.m. in an Eppendorf microcentrifuge for 4 min. The aqueous phase was removed, mixed with 2 volumes of ethanol and kept overnight at −20°C. The precipitated DNA was collected by centrifugation at 10000 r.p.m. for 10 min. The pellet was air dried, and redissolved in 1 ml of 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE buffer). RNase was added to a 10 mg/ml final concentration and the solution was incubated at 37°C for 1 h. After extraction with a chloro-

form/phenol mixture, the aqueous phase was removed and the DNA precipitated with ethanol. The DNA was redissolved in the TE buffer.

Content in 5-methylcytosine (5mC)

In order to separate the DNA bases, DNA extracted from cells treated with different concentrations of AZA during 96 h was hydrolysed in 96% formic acid at 180°C for 60 min. A Bruker LC 211 HPLC system and a Spherisorb ODS II 5 UM column, were used to separate and analyse the bases with a linear gradient from 4.0% methanol/10 mM ammonium phosphate pH 5.3 to 15% methanol/10 mM ammonium phosphate pH 5.1 at rate of 1 ml/min for 20 min. Individual compounds were quantitated by UV absorption peaks corrected for differences in extinction coefficients. HPLC standards for guanine, cytosine, adenine, 5-methyl-cytosine and thymine were identified by their UV spectra. Guanine (G) eluted at 3.1 min, cytosine (C) eluted at 4.3 min, 5-methylcytosine (mC) eluted at 7.2 min, thymine (T) at 8.7 and adenine (A) at 12.7 min. Each DNA sample was subjected to at least three acid hydrolyses. Three analyses were performed for each acid hydrolysate. The 5mC content of DNA was estimated at the 5mC/5mC+C ratio.

RESULTS

Effect of AZA treatment on DNA methylation

In order to investigate whether AZA treatments changed the pattern of cytosine methylation into DNA, DNA extracted from control and AZA-treated meristematic cells was hydrolyzed and its bases were separated by HPLC, as it can be seen in Figure 1*a–c*. The estimated 5mC/5mC+C ratio for control DNA was of 0.6. When the meristems were kept in the presence of AZA for 96 h (a time which roughly corresponds to three cycle times in control conditions) 9.4% and 16.6% of the native 5mC bases were demethylated at 10^{-6} M (Figure 1b) and 10^{-5} M AZA concentrations (Figure 1c), respectively.

Effect of AZA treatment on metabolism

In order to see whether DNA hypomethylation modified gene expression and/or had any general toxic effect, ^{3}[H]-thymidine, ^{3}[H]-uridine, ^{3}[H]-leucine incorporations and oxygen consumption were also measured after 96 h of continuous treatment with 5-azacytidine. *In vivo* incorporation of the radioactive precursors showed that the rate of ^{3}[H]-uridine and ^{3}[H]-leucine incorporations nearly doubled at both AZA concentrations (Table 1). On the contrary, 10^{-5} M AZA (but not 10^{-6} M) reduced ^{3}[H]-thymidine incorporation. None of these AZA treatments modified the oxygen consumption (Table 1).

Effect of AZA treatment on metabolism

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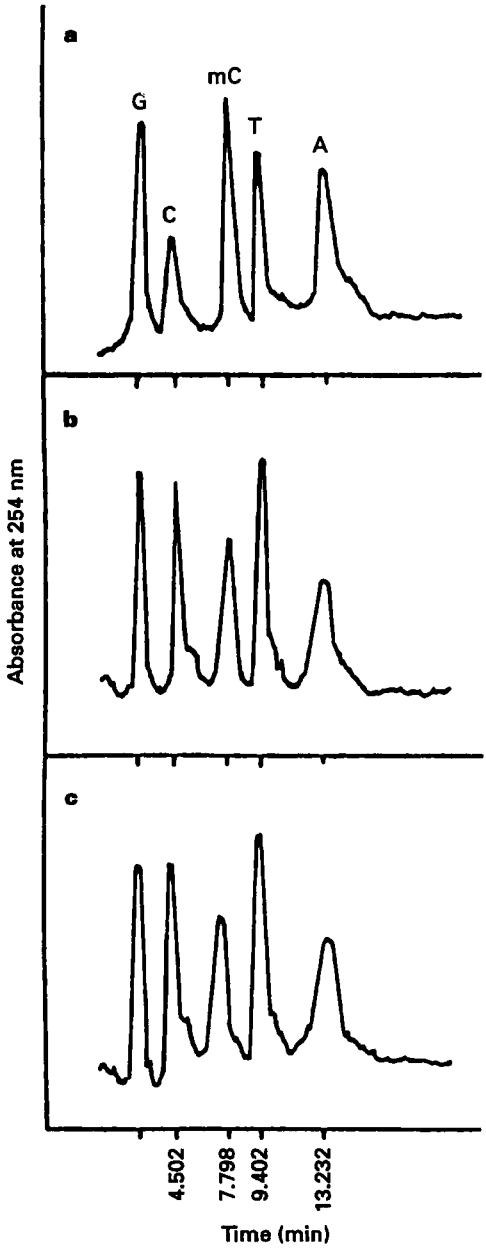

Figure 1. Effect of 5-azacytidine treatment on the content of both 5-methylcytocine (mC) and other DNA bases. Bases from control meristems are shown in **a**. Those from meristems proliferating in either 10^{-6} M or 10^{-5} M AZA for the last 96 h are shown in **b** and **c**, respectively. It could be estimated that 60% of the cytosine bases were methylated in the control. Incorporation into DNA of 10^{-6} M and 10^{-5} M AZA produced the demethylation of roughly 9% and 17% of the mC residues found in the control, respectively, as measured by electronic integration of the areas below such peaks.

Effect of AZA treatment on cell proliferation

Since 10^{-5} M AZA produced a decrease in ^{3}[H]-thymidine incorporation, any effects AZA presented on the proliferative kinetics, as measured by changes in the mitotic index were investigated. Under 10^{-6} M AZA treatment, mitotic index was not modified (Figure 2). On the other hand, the 10^{-5} M treated roots displayed a progressive decrease in mitotic index from the 12th to the 48th hour. It remained very low from then onwards (Figure 2).

Table 1. Incorporation of ^{3}[H]-thymidine, ^{3}[H]-uridine and ^{3}[H]-leucine into 1 mm long root meristems and oxygen consumption in the 2 cm long root apex, both 48 and 96 h after initiation of the different AZA treatments. Values expressed as % of the control

		10^{-6} M AZA	10^{-5} M AZA
^{3}[H]-thymidine	48 h	123	70
	96 h	109	21
^{3}[H]-uridine	48 h	179	183
	96 h	179	271
^{3}[H]-leucine	48 h	201	201
	96 h	218	195
Oxygen consumption	48 h	109	107
	96 h	101	102

The ^{3}[H]-thymidine, ^{3}[H]-uridine and ^{3}[H]-leucine incorporations in control meristems were of 2129 ± 193; 5648 ± 473 and 4980 ± 396 c.p.m./100 μg protein, respectively. The oxygen consumption in the control roots amounted 850 ± 100 nmoles O_2/min × g of wet weight.

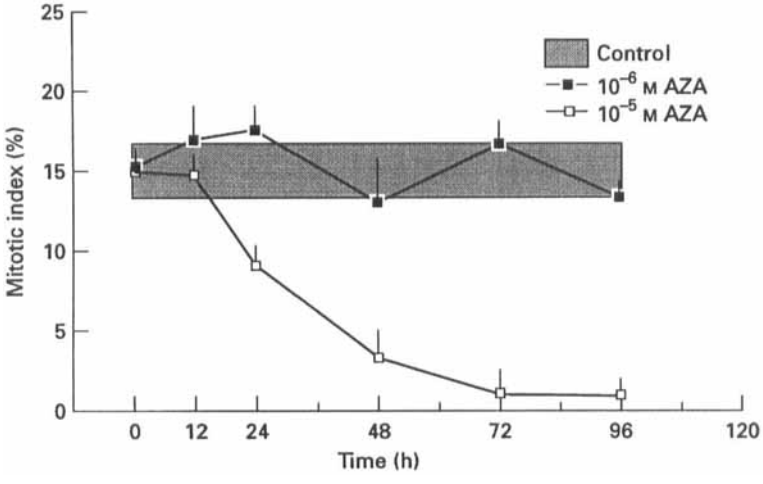

Figure 2. Effect of AZA treatment in mitotic activity in meristems. Control meristems (dotted bar), and meristems continuously treated with 10^{-6} M and 10^{-5} M AZA at times from 0–96 h of their initiation. The mitotic index fell down from 12 h onwards at the higher but not at the lower concentration. Vertical bars are standard deviations.

Effect of AZA treatment on mitotic chromosomal segregation

Anaphases with very long chromosomes (Figure 3b,c) were frequently found in both 10^{-6} M and 10^{-5} M AZA-treated roots. The frequency of enlarged chromosomes was higher at the highest AZA concentration.

Some anaphases displayed adherence and non-disjunction of some sister telomeres, which still remained in the equator while sister kinetochores were already at the spindle poles (Figure 3c,d).

Chromosomal laggards and bridges were also frequently observed under 10^{-5} M AZA concentration in stages which corresponded to very late anaphase or telophase (Figure 3e,f).

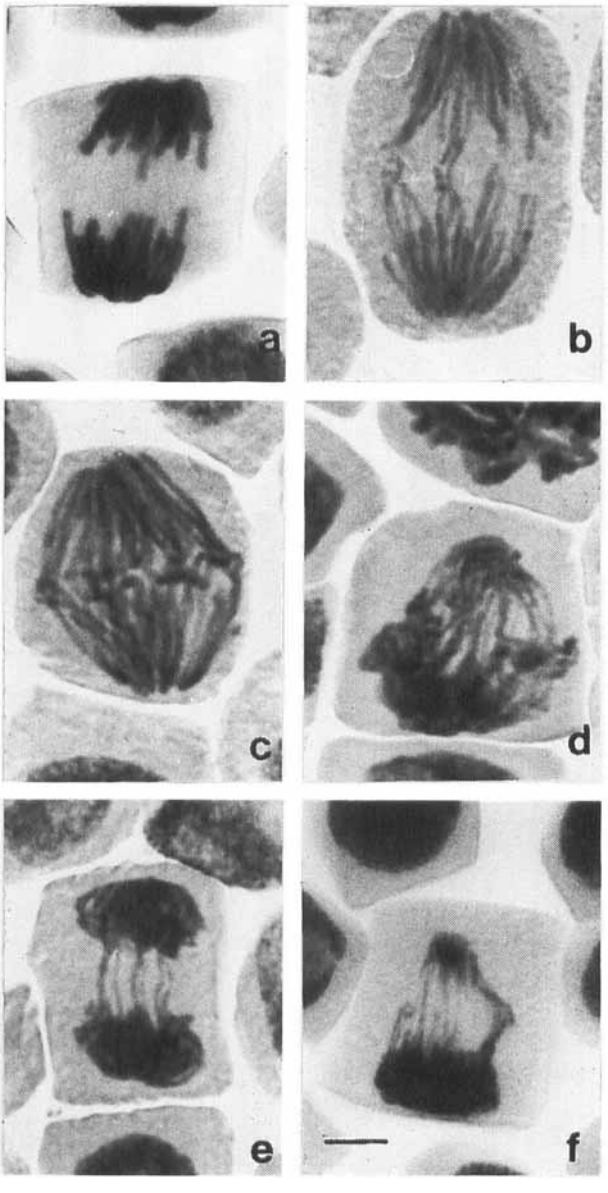

Figure 3. Abnormal ana-telophases after 10^{-6} M AZA treatment. **a** Anaphase in control meristems. **b, f** Ana-telophases in meristems treated for 24 h with 10^{-5} M AZA. Enlarged chromosomes are apparent in **b, c**. The presence of apparently catenated telomeres in the spindle equator are evident in **c, d**. The presence of chromosomal bridges in apparent in **e, f**. The uneven distribution of chromatids is quite evident in **f**, where most chromosomal material stays at the spindle pole situated at the bottom. Bar, 10 μm.

Moreover, serious unbalance of chromosomal distribution was often observed (Figure 3d,f). This was the consequence of chromosomes preferentially going to one of the two poles (Figure 3d,f). This unequal chromosome distribution did not prevent, however, the occurrence of some events normally taking place in late mitosis. Hence, chromosome decondensation and nuclear envelope reformation gave rise to interphase restitution nuclei which were unequivocally aneuploid (Figure 4b–f). Moreover, most of the restitution nuclei remained with a non-spherical shape (Figure 4a,c,d and upper cell in e). This irregular shape represented the disposition of chromosomes in the previous anaphase, as a consequence of

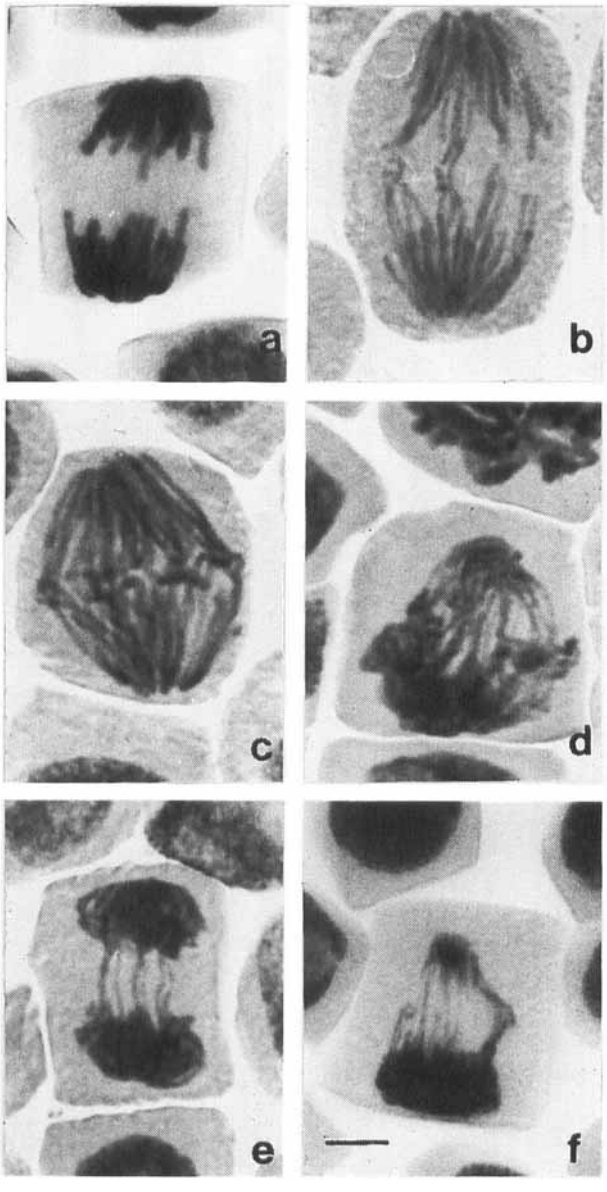

Figure 4. Restitution nuclei formed after disturbed chromosomal segregation in AZA-treated cells. Sister cells often remained connected by undivided chromatin, **b**, **d**, **e**. Strongly unbalanced chromosome segregation was evident in most cells in this figure, even when cytokinesis was complete, **b**, **e** lower cell, **f** lower and upper cells. Partial cytokinetic plate allowed restitution nuclei to remain in partially undivided cells, **a**, **c**, upper cell in **e**. Bar, 10 μm.

the altered chromosomal segregation. This feature made these cells distinguishable from the rest of the meristematic cell population, even when they remained as undivided 2n 4C cells (Figure 4a,c and upper cell in e). Many of these sister cells with restitution nuclei still retained chromatin connections which unambiguously proved the cytokinetic plate had randomly separated chromosomes which were not previously segregated. That is to say, the 10^{-5} M AZA treatment allowed the process of cytoplasm division to occur while the sister chromosomes had not completed their segregation (Figure 4b,d). Partial cytokinetic plates were sometimes observed (Figure 4a,c and upper cell in e).

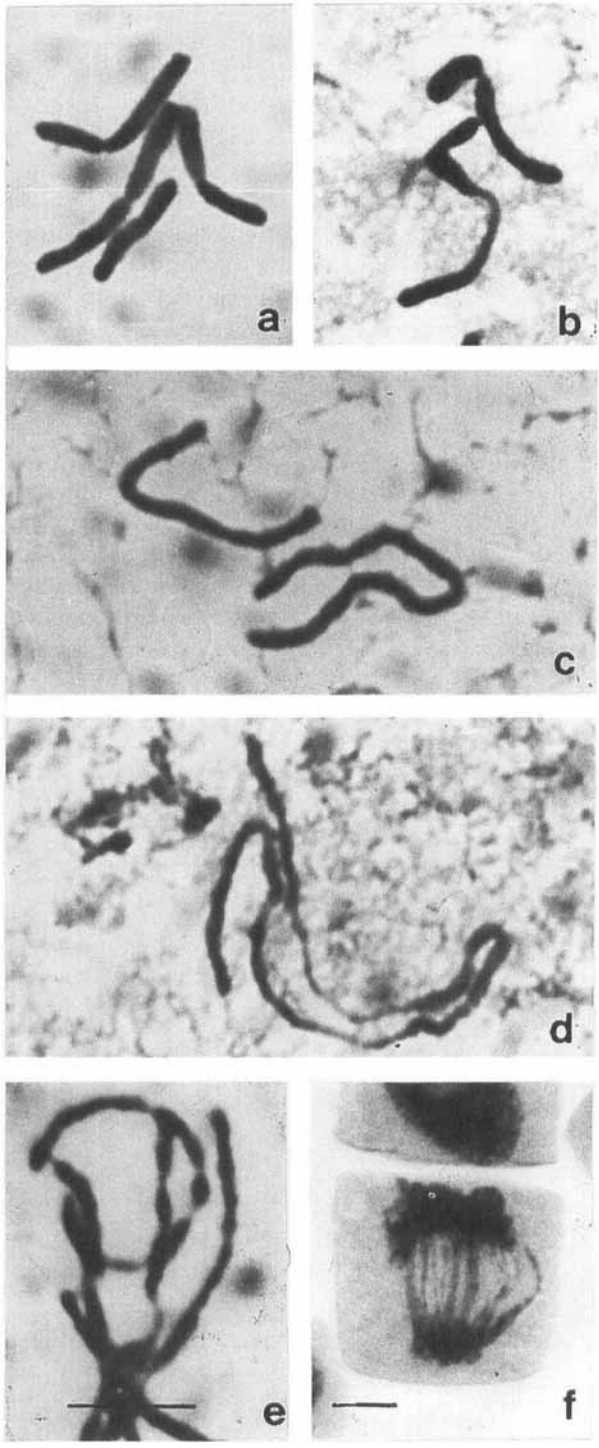

Figure 5. Effect of AZA treatment in chromosomal length. Chromosomes were accumulated by a 3 h-long colchicine treatment, **a**, control meristems. **b**, **c**, **d** and **e** meristems treated for one cycle (30 h) with 10^{-6} M AZA and for a second cycle (30 h) with 10^{-5} M AZA. It can be observed that the treatment seems to preferentially enlarge some chromosomal regions, **b**, **c** and **d**. **e** A pattern of discontinuous domains are apparent. **f** cell in ana-telophase treated with 10^{-5} M AZA for 24 h. It is obvious the unequal distribution of chromosomes into both poles. Numerous chromosomal laggards and bridges are seen. Chromatin in some of these bridges also has continuous differential domains. Bars, 10 μm (enlargement of **a**–**d** are equal to that in **e**).

Effect of the AZA treatment on chromosomal length

In order to see whether the AZA treatment could affect the length of the chromosomes in plant mitosis, an experimental protocol which involved the use of 10^{-6} M AZA treatment for a cycle time (30 h) followed by a 10^{-5} M AZA treatment for a subsequent cycle time (another 30 h) was used. In this way, the DNA was slightly demethylated in the first cycle without preventing cycle progression, while the 10^{-5} M AZA treatment for the second cycle depressed the frequency of cells in mitosis at the end of the whole treatment (60 h). Chromosomes were dispersed by the extreme squashing of the meristems after a three hour-long colchicine treatment (Figure 5a–e). Chromosomal lengths were recorded in isolated control chromosomes and in those belonging to meristems treated with AZA. The values showed a shift towards greater chromosomal lengths when the cells were treated with 10^{-6} M AZA during one cycle (30 h) followed by a 10^{-5} AZA for another cycle time (30 h) (Figure 6). When incubations with 10^{-6} M AZA covered the last two cycles instead (data not shown) there was also a shift but not as large as that recorded in the combined treatment. Chromosomes of control meristems showed regular thicknesses, as seen in Figure 5a. On the other hand, 40% of the chromosomes from the AZA-treated cells were larger than the control ones (Figure 5c), and 21.8% presented either pericentromeric regions or other intercalar regions which were more extended and thinner than most of the chromosome arms (Figure 5b, d). It should be stressed that a new pattern of extended thin domains dispersed between normal domains of regular thickness was displayed in some chromosomes (Figure 5e). A similar pattern could also be observed in some chromosomal bridges in ana-telophases (Figure 5f). Chromosomal breaks were never observed in either c-mitoses or anaphases.

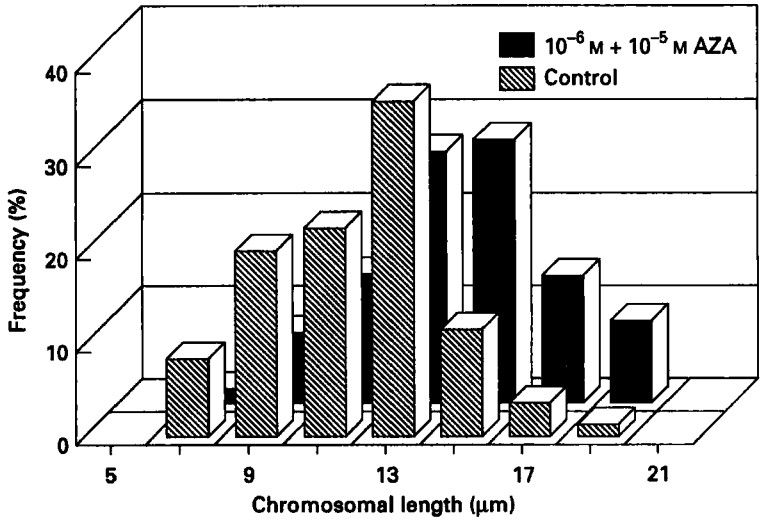

Figure 6. Length (μm) of chromosomes accumulated by colchicine recorded in samples of at least 2000 chromosomes spreads from control meristems (empty bars) and from meristems treated with 10^{-6} M AZA for one cycle (30 h) and with 10^{-5} M AZA for a second one (30 h) (stripped bars). There is a shift towards larger sizes in chromosomes after the AZA treatment. The cumulative frequency for chromosomes that were 15 μm long or larger were of 16.0% and 50.1% for control and AZA-treated meristems, respectively.

The kinetics of appearance of abnormal ana-telophases (AbAs)

In order to detect whether 10^{-5} M AZA preferentially induced chromosomal anomalies in a specific phase of the cell cycle, mitotic index and the frequency of AbAs were recorded during and after treatments with 10^{-5} M AZA for 6, 12, 24 and 36 h (Figure 7a–d). When looking to the frequency of AbAs, it was evident that, independently of the length of the AZA treatments, 24 h after the initiation of any treatment nearly 70% of the ana-telophases presented some of the previously described anomalies. Since the whole cycle lasts 30 h, this suggests that most of the anomalies taking place in ana-telophase should be due to AZA treatment restricted to early S period (see specifically Figure 7a). However, the treatment also produced a high level of AbAs only 12 h (but not 6 h) after the initiation of the treatment (nearly 50% in Figure 7c,d). This suggested that hypomethylation of sequences replicated in late S also produced abnormalities in ana-telophase. It is interesting to note that the peak of AbAs was always observed before any important fall in mitotic index.

Segments of the interphase preferentially sensitive to AZA in synchronous cells

In order to confirm whether the appearance of AbAs was associated with treatments covering different portions of the previous interphase, the experimental protocol displayed in Figure 8B was followed. Caffeine (5 mM) was given to permanently label as binucleate those synchronous cells enduring cytokinesis (Giménez-Martín *et al.* 1965, López-Sáez *et al.* 1966). The fastest cells of this synchronous population (comprising 15%) reached S period about 6 h after caffeine, and finished DNA replication around 18 h, as detected after different sequential treatments with ^{3}[H]-thymidine. It confirmed previous published data (De la Torre *et al.* 1989). The recorded wave of mitosis for this binucleate cell population started at the 23 h and was hardly modified by the different AZA treatments (data not shown). A common time of root fixation was used (30 h after caffeine, when a peak in bimitosis was recorded in control meristem). Synchronous binucleate cells reached anaphase with abnormalities (AbAs) similar to those seen in mononucleate cells (Figure 8A). Hence, there were bi-ana-telophases with disturbed chromosomal segregation (Figure 8A,e), chromosomal laggards and bridges in telophase (Figure 8A,f) and unequal chromatid segregation in restitution nuclei already in interphase (Figure 8A,g,h). Incubations with AZA during either G_1, or G_2+M only gave a small fraction of abnormal ana-telophases. The slight effects recorded when treatments took place outside the S period could be explained by asynchrony in the population. By contrast, the largest frequency of AbAs was recorded when the AZA treatment covered either the first half of the S period (84.5%), or the second part (51.1%).

The relationship between the abnormalities found in ana-telophases and the kind of DNA sequences hypomethylated

To confirm whether the abnormalities found in ana-telophases were related to the time when hypomethylation took place in the previous S period, AZA feeding in the binucleate cells was restricted to four independent but contiguous segments, which corresponded to quarters of the whole S period. They were named S1, S2, S3 and S4 (from early to late S, respectively). The kinetics of entrance into mitosis after AZA (data not shown) were similar in all the treatments, and were no different from those displayed by the binucleate cells with native DNA. The recorded frequencies of abnormal ana-telophases are shown in Table 2. Lengthened chromosomes were preferentially recorded when hypomethylation took place in the first half of the previous S period (S1 and S2 subperiods). On the other hand, the preferential migration of kinetochores to a single pole mostly took place when AZA feeding occurred at S4 (late S), when centromeres and telomeres replicate in these cells

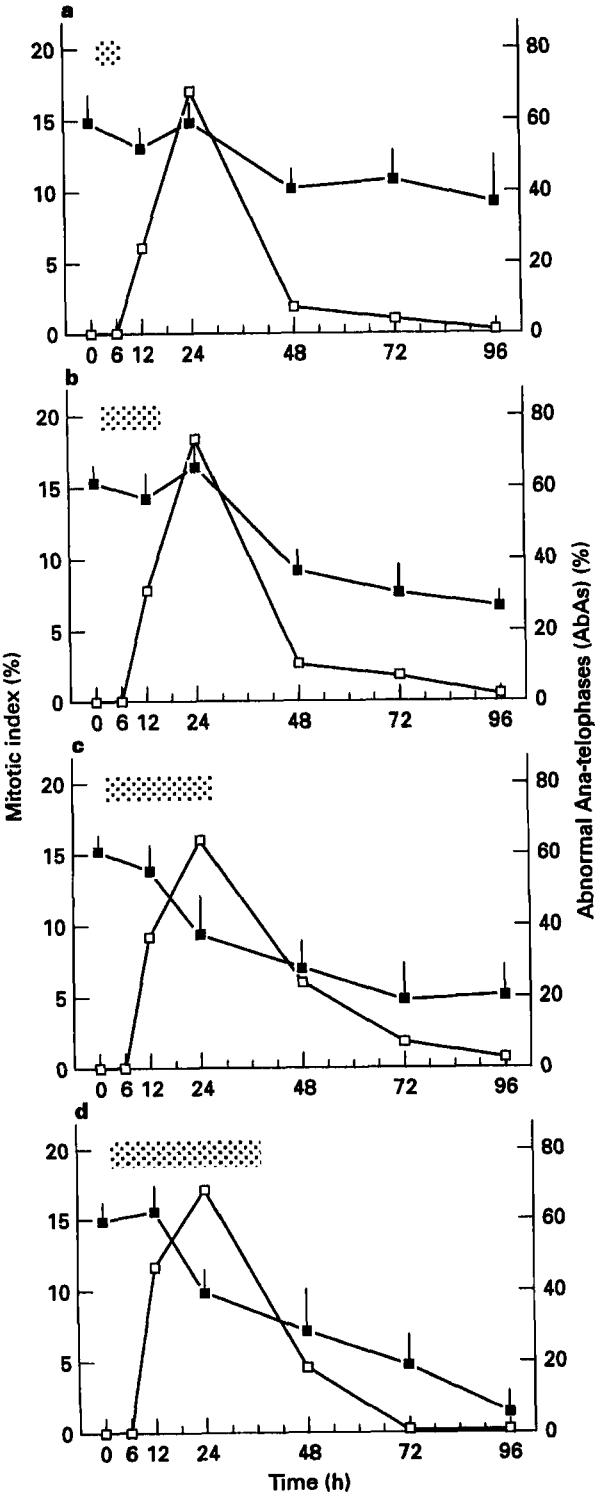

Figure 7. The effect of different AZA treatments in the appearance of abnormal ana-telophases (AbAs). Evolution of mitotic index (dark squares) and kinetics of the appearance of AbAs (empty squares). The length of each AZA treatment is represented by a dotted bar at the upper left part of each panel. Observe the appearance of a peak of AbAs 24 h after any treatment was initiated.

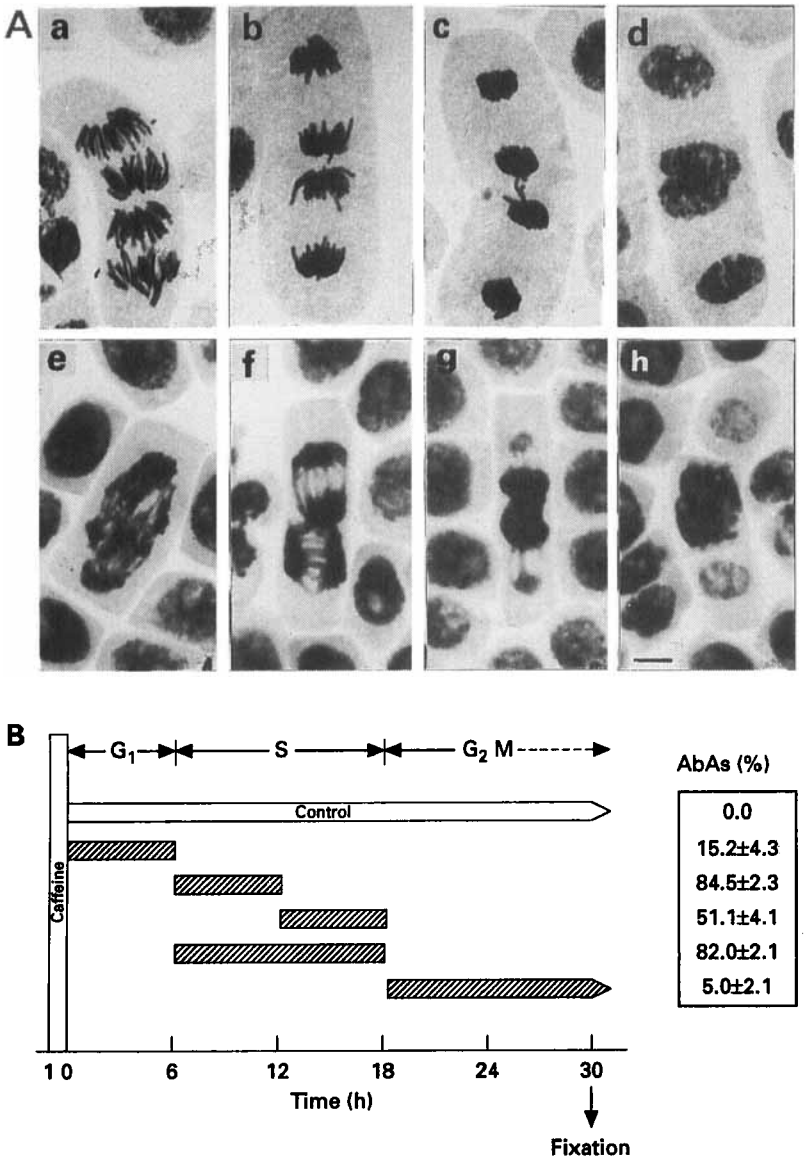

Figure 8. Effect of AZA treatments given during different times of the interphase in a synchronous cell population labelled as binucleate cells by caffeine treatment. **A**: Normal binucleate cells in anaphase (**a**, **b**), and telophase (**c**, **d**). Abnormal binucleate cells in anaphase (**e**, **f**) and in telophase (**g**, **h**) after a 10^{-5} M AZA treatment covering from 6–18 h after caffeine. They were fixed at 30 h. Bar, 10 μm. **B**: Experimental protocol to study the preferential sensitivity to AZA of different interphase stages, as studied in a naturally synchronous cell population labelled as binucleate by 5 mM caffeine treatment (vertical bar). The empty horizontal bars show control binucleate cells. The dark horizontal bars show the different 10^{-5} M AZA treatments. The frequency of recorded AbAs (% of abnormal ana-telophases) recorded at 30 h after the cells were labelled with caffeine treatment are shown in the right portion of this experimental protocol.

Table 2. Frequency of the different abnormalities recorded in ana-telophase, in relation to the fraction of the S period in which DNA hypomethylation was induced, expressed in %

	Enlarged chromosomes	Chromosomal laggards or bridges	Unbalanced distribution
S1	58.2±4.2	25.2±2.7	16.6±1.5
S2	68.9±6.3	16.2±2.0	14.9±1.3
S3	30.5±2.9	21.1±2.3	48.4±4.4
S4	17.2±1.5	19.2±2.1	63.6±6.3

(Schvarztman *et al.* 1977). Finally, chromosomal laggards and bridges appeared at similar frequencies after all four AZA-treatments.

DISCUSSION

A serious malsegregation of chromatids in anaphase was detected before any drop in the mitotic index was recorded and before cell proliferation was depressed or blocked by the 10^{-5} M AZA treatment. These effects were positively correlated to incorporation of AZA into DNA during previous replication. Incomplete DNA replication does not explain the recorded failure in chromosomal segregation, since cells with AZA-substituted DNA entered into mitosis with kinetics similar to that of control cells, even when different times for recovery were allowed (S3 and S4 treatments).

The detected mitotic anomalies were not a consequence of a general cytotoxic effect of the AZA treatment, for no changes in oxygen consumption took place after 96 h of treatment. Moreover, the 12 h-long treatment (covering from early G_2 onwards, from 18–30 h) was less efficient in provoking abnormal ana-telophases than the 6 h-long treatment restricted to the first or second halves of the S period.

The most plausible hypothesis to explain the recorded chromosome disfunctions in mitosis, as well as the rise in the transcription rate, is that the modification of the DNA stereology by the incorporation of the analogue of cytosine prevents the binding of proteins involved in motitic chromosome condensation and in chromatid segregation, as proved to be the case for proteins which usually repress transcription of down-regulated genes (Boyes & Bird 1991). Furthermore, (i) inhibitors of topoisomerase II give rise to segregation anomalies similar to those here described (manuscript in preparation) and to those observed in animal cells (Downes *et al.* 1991, Shamu & Murray 1992, Clark *et al.* 1993, Downes *et al.* 1994), and (ii) ethidium bromide, an intercalating drug which modifies DNA stereology and binding affinities, both prevents chromatid segregation and also uncouples cytoplasmic from chromosomal divisions (González-Fernández *et al.* 1970a,b).

Topoisomerase II is only one of the DNA-binding proteins whose affinity for hypomethylated DNA may have been lost. It is a major component of the chromosomal scaffold required for both chromosome condensation (Adachi *et al.* 1991) and chromosome segregation (Uemura *et al.* 1986).

Faults in the resolution of sister centromeres are obvious mechanisms for blocking the normal chromatid segregation in mitosis. Accordingly, it is not surprising to find an increase in unbalanced chromosomal segregation after AZA feeding in S4, since centromeres and

telomeres are late replicating in *Allium cepa* L. (Fussell 1975, Schvartzman & Díez 1977). The lack of attachment of INCENPs, Clips and other linking proteins to AZA-substituted centromeric DNA could be preventing the formation of a properly functional kinetochore (Tomkiel *et al.* 1994, Holm 1994). The clear preferential segregation of chromatids to one of the two mitotic poles after AZA (Figure 3f, for instance) may depend on two concurrent circumstances: (i) that one of the mitotic poles is always advanced in forming the kinetochore microtubules in relation to the other pole. In the case of the binucleate cells, we ignore mitotic poles in central position and why they would be the most efficient in the early production of kinetochore microtubules (Figure 8A,g), and (ii) that the interface between kinetochore and centromeric DNA may occur in one chromatid with the native DNA strand while in the other one would take place with the AZA-substituted strand (AZA-substitution having taken place for a single S period or fractions).

In relation to the failure in segregation of telomeres, it should be taken into account that they contain satellite DNA, amounting to 4% of the whole onion genome, which is enriched in C-G bases (45.8%) if compared with total DNA (34.6%) (Barnes *et al.* 1985).

Why do specific DNA sequences replicated in the first interphase partly participate in chromatin condensation? One possibility is that sequences rich in mC-G (analogous to the *Alu* family repeats of the human genome: Wagner *et al.* 1993) were regularly distributed throughout the onion complement and were responsible in normal conditions for the binding of proteins inducing chromosome condensation. The beaded appearance of some chromosomes after the induction of DNA hypomethylation favors this view.

Finally, it should be stressed that through DNA segregation and/or migration of chromatids to the mitotic poles was blocked after AZA incorporation, a cytokinetic plate was formed. Its growth led to direct random division of those chromosomes delayed in their mitotic cycle, transforming the resulting sister cells into inviable ones.

The potential of methodologies which selectively modify families of replicons by incorporation of analogues should not be forgotten in the discernment of *cis* regulatory functions of DNA sequences involved in the regulation of cycle progression.

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