The involvement of discrete genome regions in post-mitotic chromosome decondensation and in G₁ timing in *Allium cepa* L. meristematic cells

A. GONZALEZ-FERNANDEZ, J. SANS*, P. ALLER and C. DE LA TORRE†

Centro de Investigaciones Biológicas, CSIC, Velázquez, 144, E-28006-Madrid, Spain

*Present address: Departamento de Biología Celular y Genética, Facultad de Medicina, Universidad de Chile, Casilla 70061, Correo 7, Santiago, Chile
†Author for correspondence

Summary

The role of DNA regions replicated at different times during the S period in the process of chromatin decondensation that takes place in the next G₁ period, as well as in the timing of the G₁ to S transition, was analyzed in synchronous populations of cells in *Allium cepa* L. root meristems. For this analysis, DNA bromosubstitution (10⁻⁷ M 5-bromo-2'-deoxyuridine feeding) was carried out at similar times corresponding to the first, middle and last thirds of the S period prior to telophase when anoxic 313 nm irradiation was carried out.

Evaluation, after Feulgen staining, of the chromatin pattern corresponding to G₀ and G₁ phenotypes in these synchronous cells showed that chromatin decondensation after mitosis appeared to be under the control of a double negative/positive regulatory mechanism related, respectively, to early and late replicating sequences. Thus, such decondensation was favoured when bromosubstitution took place during the first part of the S period, but delayed when bromosubstitution took place during the third part or the whole of the S period. Nevertheless, bromosubstitution at both the early and late stages of the S period delayed the timing of the G₁ to S transition. In this way, the initiation of replication appears to be positively controlled by the function of early replicating DNA. Finally, the DNA sequences replicated in the middle third of the S period did not modify the decondensing capacity of the chromatin in G₁ or the timing of the G₁ to S transition.

Key words: genome regions, G₁ regulation, chromosome decondensation, DNA bromosubstitution, UVA irradiation.

Introduction

The incorporation of base analogues into replicating DNA is a way of permanently modifying such molecules. For instance, 5-azacytidine incorporation, which was previously seen to favour de-differentiation, prevents the binding of a negative regulatory protein to the resulting de-methylated DNA (Boyes and Bird, 1991).

Bromosubstitution of DNA - namely, incorporation of bromouracil instead of thymine - does not affect cycle kinetics *per se* in onion meristems. However, anoxic 313 nm irradiation of cells possessing bromosubstituted DNA (Br-DNA) halts their cycle progression in G₂ (De la Torre and González-Fernández, 1979). Indirect evidence also suggests that this combined treatment prevents the binding of regulatory proteins to the resulting bromosubstituted DNA (Sans et al., 1991).

The involvement of specific DNA segments in any cycle step can be worked out by allowing bromosubstitution of DNA for restricted intervals only during the whole S period. By selecting the irradiation time other regulatory intermediate steps between the bromosubstitution period and the checkpoint in the study will remain unaffected.

In this work the bromosubstitution plus irradiation method was used to analyze the replication time of DNA sequences involved in chromatin decondensation and in the timing of the G₁ to S transition in onion meristematic cells. Our results show that early and late replicating sequences are differentially involved in both processes while those replicating in mid S phase are apparently irrelevant.

Materials and methods

The materials used were root meristems of *Allium cepa* L. bulbs. Bulbs weighing 15 to 30 g were of the flat violet variety obtainable locally. They were grown in the dark, at a constant temperature of 15(±0.5)°C, in cylindrical glass receptacles of approximately 80 ml capacity. The bulbs were grown in filtered tap water, which was renewed at 24 hour intervals and aerated by continuous bubbling at a rate of 10 to 20 ml air per min. Only the bases of the bulbs remained immersed in the water. After 2 to 3 days most of the sprouted roots ranged from 1 to 3 cm in length.

**Bromosubstitution**

A 0.1 mM 5-bromo-2'-deoxyuridine (B UdR) (Sigma, St. Louis, MO, USA), 0.1 μM 5-fluoro-2'-deoxyuridine (Serva, Heidelberg,
F.R. Germany) and 5 µM uridine (Sigma, St. Louis, MO, USA) solution (in filtered tap water) was used to feed root meristems, as tested for Allium cepa L. in our laboratory. The roots, still attached to the bulbs, were immersed in the solution for the time specified in each case. The culture conditions were maintained throughout the treatment period.

Given the enhanced photosensitivity of Br-DNA, special care was taken to prevent exposure of roots to light during and after the BUdR-treatment. Bromosubstitution under these experimental conditions corresponded to a replacement of 17% of the native thymidine bases, as assessed by CsCl gradient centrifugation (Quinzani-Jordao, 1987). Chromosomal studies at the second mitosis after BUdR feeding previously confirmed such Br incorporation (Gutiérrez et al., 1983).

Synchronous binucleate cell population

The roots, still attached to the bulbs, were immersed in a 5 mM caffeine solution for 1 h. This drug inhibits cytokinesis in cells going through telophase at this time and produces a binucleate cell population, which then enters interphase and goes through the whole cell cycle synchronously.

UVA irradiation

Bulbs with their attached roots in the upright position were immersed in filtered tap water in a large perspex container to irradiate the root tips. Nitrogen was passed through the water 1 h before and during irradiation in order to deplete oxygen and prevent damage to bromosubstituted DNA. The roots were shielded by a 1.5 cm thick 1 mM thymidine solution filter in order to absorb any possible contaminating ultraviolet irradiation from the light source. Twenty minutes of UVA irradiation was provided by an Ultravioletix Osram bulb, the emission spectrum of which was enriched in the 313 nm wavelength. The light source was placed 30 cm above the root tips. The irradiation took place at the time specified for each experiment. Light energy reaching the roots was in the 6-10 joules/cm² range, as measured by a black ray long-wave ultraviolet meter.

Labelling of nuclei in DNA replication

Sequential pulse treatments (20 min) with [3H]thymidine ([3H]TdR: Amersham International, Amersham, Buckinghamshire, Great Britain; 370 KBq/ml, 925 GBq/mmol) were applied to every set of bulbs from the 7th to 15th hour after caffeine treatment, so that the arrival of the synchronous population at the S period could be studied.

Processing of meristems after isotopic labelling

After each [3H]thymidine treatment, a minimum of 20 roots (4 roots from each bulb from a lot of 5) were fixed in a freshly prepared 3:1 (v/v) ethanol/acetic acid mixture.

Squashes were prepared from each of the roots after they were either stained with acetic orcein containing 10% of 1 M hydrochloric acid or stained with Feulgen. Subsequently, the coverslips were removed by placing the slides on solid CO₂.

In order to detect isotope incorporation the slides were covered with Kodak NTB2 autoradiographic emulsion diluted 1:1 (v/v) with water. After drying, they were exposed at −70°C for 9 days. The silver grains were developed with Kodak D-19 and fixed with Kodak ultrarapid acid fixer.

Microdensitometry

After Feulgen staining the absorbance at 550 nm of the nuclei was measured by using an M-85a scanning microdensitometer (Vickers Ltd. York, U.K.). The readings were taken for eight seconds with a ×40 objective, using masks which furnished a visual field 30 µm in diameter, under a light of 550 nm wavelength with a 30 nm band-width, using a reading spot of about 0.3 µm in diameter (size 2). Under these reading conditions, the range of values obtained for the 2C DNA content in these binucleate meristematic cells (33 pg of DNA per nucleus) was located in the central part of the scale, 200 arbitrary units being the maximum possible value that could be recorded in the microdensitometer used. One hundred nuclei of fifty synchronous binucleate cells were scored in each experiment. The projected areas of the nuclei were measured over different density thresholds. In this way, the areas of chromatin at different degrees of condensation were estimated. An area of chromatin was considered to be dense if it absorbed more than 80% of the light. The binucleate cells possessing chromatin areas with density above such absorption were considered to be cells with a G₀ chromatin phenotype (see below).

Results

Assessment of G₀ and G₁ nuclear phenotypes in cells with 2C DNA content

The G₀ nuclear phenotype was evaluated in the same A. cepa bulbs by dissecting from two to four ungerminated primordia from the onion bulbs before they were stimulated to proliferate. Mean DNA content was assessed in the interphase cells forming these primordia. These dormant meristems exclusively contained interphase cells with DNA contents corresponding to the pre- (2C) or postreplicative (4C) phases of the cycle. The pattern of chromatin condensation was evaluated in the nuclei of the ungerminated primordia with a 2C DNA content (G₀ nuclear phenotype).

The G₁ nuclear phenotype was also evaluated in the active meristems from roots growing under steady-state kinetics. Autoradiography after a short treatment with [3H]thymidine permitted the discarding of the replicating interphase cells. The pattern of chromatin condensation was then evaluated in the 2C nuclei corresponding to cells in early interphase (G₁ nuclear phenotype).

The amount of nuclear area above the density threshold corresponding to 20% light transmission or less was considered to be a good indicator of the degree of chromatin condensation. Thus, 98% of the 2C nuclei in the ungerminated primordia possessed a high chromatin density (G₀ phenotype). Dense chromatin occupied from 3.1 to 5.7 arbitrary units of the area of nuclear projection, the mean value being 4.4. On the other hand, 97.4% of the nuclei of 2C cells in the proliferating meristems (that is to say in G₁) had areas of projection of dense chromatin between 0.5 and 2.9, with a mean value of 2.15. These data are not shown, since they essentially confirm previously published results.

Chromatin patterns and proliferative fraction in binucleate cells

When nuclei were differentiated on the basis of their chromatin condensation pattern in binucleate cells in control conditions, two distinct subpopulations were discerned at G₁ shortly (4 h) after their formation (Fig. 1A). These two subpopulations corresponded to the G₀ and G₁ nuclear phenotypes defined in both the ungerminated primordia and the proliferating meristems, respectively. Thus, a portion of these cells displayed small nuclei with a large amount of dense chromatin (arrowheads in Fig. 1A), while the other
Role of genome portions on $G_1$

portion showed enlarged nuclei with few regions of dense chromatin (thin arrows in Fig. 1A).

Considering that shortly after telophase the $G_1$ to ($G_0 + G_1$) ratio is a measure of the proliferative or growth fraction, this parameter was calculated in the binucleate cells. The proliferative fraction (PF) estimated at hour 4 after telophase (mid $G_1$) was around 50% in the binucleate population.

Table 1. Proliferative fraction (PF) at hour 4 of interphase in binucleate cells

<table>
<thead>
<tr>
<th></th>
<th>PF, at h 4</th>
<th>LI, at h 13</th>
<th>LI/PF</th>
</tr>
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<tbody>
<tr>
<td>Native DNA</td>
<td>50.7 ± 4.8</td>
<td>59.2 ± 5.6</td>
<td>1.17</td>
</tr>
<tr>
<td>Br all S</td>
<td>48.3 ± 4.6</td>
<td>57.4 ± 5.4</td>
<td>1.19</td>
</tr>
<tr>
<td>Native DNA+UVA</td>
<td>53.1 ± 4.3</td>
<td>63.3 ± 5.8</td>
<td>1.19</td>
</tr>
<tr>
<td>Br1+UVA</td>
<td>66.7 ± 5.9</td>
<td>15.3 ± 3.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Br2+UVA</td>
<td>49.9 ± 4.5</td>
<td>59.2 ± 4.8</td>
<td>1.19</td>
</tr>
<tr>
<td>Br3+UVA</td>
<td>24.4 ± 3.7</td>
<td>3.1 ± 2.8</td>
<td>0.13</td>
</tr>
<tr>
<td>Br all S+UVA</td>
<td>6.7 ± 5.8</td>
<td>4.1 ± 3.2</td>
<td>0.61</td>
</tr>
</tbody>
</table>

The PF is expressed by the frequency of cells with $G_1$ phenotype versus the frequency of cells with $G_0 + G_1$ nuclear phenotypes. Recorded labelling indices (LI) at hour 13 of interphase are also included. Both sets of data (±95% confidence limits) are expressed as %. The last column represents the estimated LI to PF ratios.

The PF detected in the native 2C mononucleate cells (97.4%, see above). This indicates that the binucleate population behaves in a specific way, at least in the short term.

The timing of the cycle phases

Telophase is the cycle stage where caffeine blocks the fusion of the Golgi vesicles which is responsible for the formation of the cytokinesis plate. The evaluation of the duration of the whole $S$ period in relation to telophase was previously accomplished by recording the frequency of labelled telophases at different times after a short treatment with $[^3]H$thymidine (Quastler and Sherman, 1959). The data on $S$ phase duration have been omitted, since they confirmed previous published results (De la Torre et al., 1989; Sans et al., 1991). They placed the $S$ period from 6 to 18 hours before telophase (Fig. 2).

Decondensation of chromatin in relation to the time of DNA bromosubstitution

In order to analyze the role of specific DNA sequences in the process of chromatin decondensation, bromosubstitution was carried out for either the whole $S$ period (Br all S, in Fig. 2) or the three 4-hour non-overlapping segments (Br1, -2, -3) shown in Fig. 2. UV irradiation was always carried out immediately before caffeine treatment (see upper bar in Fig. 2). This time of irradiation corresponded to the early telophase of the synchronous populations used.

It was observed that the decondensation of chromatin after mitosis was favoured by irradiation only in those binucleate cells in which early replicating DNA had been bromosubstituted. On the other hand, when the DNA segments replicating in the last third of the $S$ period were bromosubstituted, chromatin decondensation was prevented. Finally, bromosubstitution of the sequences replicating in the middle third of the $S$ period apparently did not modify the chromatin condensation pattern (1st column of Table 1).

Initiation of DNA replication in relation to the time of DNA bromosubstitution

Entrance into $S$ phase of synchronous binucleate cell populations was monitored by providing sequential pulses of $[^3]H$thymidine at different times after addition of caffeine,

Fig. 1. (A) Cell preparation obtained after squashing the apical 1.5 mm of onion roots, and acetic orcein staining. The meristems received 1 hour of treatment with 5 mM caffeine before fixation. The arrow indicates a binucleate cell with enlarged nuclei, while the arrowhead indicates a binucleate cell with small nuclei, displaying compact chromatin. Similar Feulgen-stained preparations permitted us to discern two distinct nuclear phenotypes (arrowheads for $G_0$ and thin arrows for $G_1$) for nuclei with 2C DNA content in this binucleate population. Observe that both nuclei of the binucleate cells are usually located in a central position. (B) Binucleate cell having received a $[^3]H$thymidine pulse 7 hours after the 1 hour of caffeine treatment. Both nuclei possess an early interphase phenotype and are actively incorporating the isotope. (C) Binucleate cell displaying a $G_0$ phenotype, after treatment similar to that described for B. The nuclear labelling was not greater than the background.
following the schedule of Fig. 2. Most labelled cells in the samples taken between 7 and 15 hours after caffeine addition still possessed decondensed chromatin (Fig. 1B), while binucleate cells with G₀ phenotype remained unlabelled (Fig. 1C).

As can be seen in Fig. 3, the kinetics of entry into the S period of the total synchronous population of binucleate cells varied with the time of DNA bromosubstitution. Thus, the entrance into S phase was delayed when sequences replicating in the first third, the last third or the whole S period were bromosubstituted. On the other hand, bromosubstitution of the sequences of DNA replicating in the middle third of the S period did not affect G₁ duration.

**Chromatin decondensation and G₁ duration**

The labelling indices (LI) at hour 13 of interphase were recorded and compared with the proliferative fraction (PF) measured at hour 4. Labelling indices were usually larger (about 1.19 times) than proliferative fractions in control cells with either native or bromosubstituted genomes (1st and 2nd rows of Table 1). The same applies after irradiation of cells that had either native DNA (3rd row) or bromosubstituted DNA segments replicating in the middle third of the S period (5th row). The LI to PF ratio was greatly diminished when irradiation took place in cells with early or late replicating bromosubstituted DNA (Br1 and Br3 in Table 1).

**Discussion**

The modification of discrete DNA sequences of a genome by incorporation of base analogues during subperiods of the S phase has the potential to reveal the involvement of those DNA sequences in cycle progression. Previous results obtained on the induction and reversal of chromosome condensation in prophase (Sans et al., 1991) suggested that bromosubstitution plus anoxic UVA irradiation prevented DNA from interacting with the set of proteins controlling such physiological processes (García-Herdugo et al., 1974).

Advancements in the start of rDNA transcription after mitosis are accompanied by anticipated chromosome decondensation (Morcillo and De la Torre, 1979a,b; Aller et al., 1983). However, the anticipated chromatin decondensation observed in cells with bromosubstituted early replicating DNA was not followed by an advancement in the time at which they initiated replication. There was an important delay instead. The results presented here suggest a model for the involvement of discrete DNA sequences in G₁. Chromosome decondensation after mitosis is only achieved after both the removal of some proteins from early replicating sequences and the binding of proteins to some of the late replicating regions. On the other hand, the initiation of replication also requires the binding of new proteins to some sequences co-replicating in early S phase. Early replicating origins are the most likely candidates responsible for this positive control.
It would be interesting to check whether the involvement of late replicating sequences in the positive control of the triggering of replication is but a consequence of their role in chromosome decondensation.

It would be surprising if the same family of late replicons controlled chromatin condensation (Sans et al., 1991) and decondensation (this work) in a positive manner, while early replicons exerted a negative control on both processes. However, it should be noted that both the initiation of replication after a period of chromosome decondensation and the initiation of chromatin condensation at the G₂ to M transition are activated by Cdc2 and NimA protein kinases (Gould and Nurse, 1989; Blow and Nurse, 1990; Fang and Newport, 1991; Osmani et al., 1991).

The present work essentially stresses the role of discrete DNA elements in the control of cycle progression in proliferating plant cells.

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References


