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Default cycle phases determined after modifying discrete DNA sequences in plant cells

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Abstract. After bromosubstituting DNA sequences replicated in the first, second, or third part of the S phase, in *Allium cepa* L. meristematic cells, radiation at 313 nm wavelength under anoxia allowed ascription of different sequences to both the positive and negative regulation of some cycle phase transitions. The present report shows that the radiation forced cells in late G_1 phase to advance into S, while those in G_2 remained in G_2 and cells in prophase returned to G_2 when both sets of sequences involved in the positive and negative controls were bromosubstituted and later irradiated. In this way, not only G_2 but also the S phase behaved as cycle phases where cells accumulated by default when signals of different sign functionally cancelled out. The treatment did not halt the rates of replication or transcription of plant bromosubstituted DNA. The irradiation under hypoxia apparently prevents the binding of regulatory proteins to Br-DNA.

The transcription of bromodeoxyuridine-labelled DNA (Br-DNA) is selectively inhibited in bacteria and phages when irradiated with radiation at 313 nm wavelength in anoxic conditions (Jones & Dove 1972). In plant cells, the treatment apparently prevents the binding of regulatory proteins to Br-DNA. Hence, anoxic irradiation in prophase, where non-nucleolar transcription is negligible, reverses the on-going process of chromosome condensation (Sans *et al.* 1991) to the same extent as the inhibition of protein synthesis does for cells with native DNA which are already in prophase (De la Torre *et al.* 1989). Similar responses to both radiation of cells with Br-DNA and the inhibition of protein synthesis in cells with native DNA are also observed in other small interphase regions where other crucial cycle transitions are made (De la Torre & González-Fernández 1979, González-Fernández *et al.* 1992).

This paper reports results obtained from experiments carried out by simultaneous inactivation of DNA sequences involved in the positive and negative control of progression through late G_1, G_2, and prophase.

MATERIALS AND METHODS

Root meristems of *Allium cepa* L. bulbs were used, when such roots had reached at least 2 cm in length (2–3 days after the water uptake was initiated). Root growth was produced by

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placing the bulbs so that only their bases remained submerged in 90 ml of filtered tap water at a constant temperature of 15 ± 0.5°C and in darkness. Oxygen was provided continuously by bubbling air at the rate of 10 to 20 ml min. The tap water was renewed at 24-h intervals.

Treatments

Roots growing from bulbs were immersed for different treatments for the time specified in each case, without altering the other environmental conditions.

In order to label as binucleate a synchronous cell population, the roots were immersed in a 5 mM caffeine solution for 1 h. This drug inhibits cytokinesis in the cells which were synchronously progressing throughout their telophase. The binucleate cell populations formed then enters into interphase and goes through the whole cell cycle synchronously (Giménez-Martín *et al.* 1965).

In order to allow the *in vivo* incorporation of bromodeoxyuridine into DNA of the nuclei DNA in replication, a 0.1 mM 5-bromo-2′-deoxyuridine (Sigma, St Louis, MO) solution containing 0.1 μM 5-fluoro-2′-deoxyuridine (Sigma, St Louis, MO) solution containing 0.1 μm 5-fluoro-2′-deoxyuridine (Serva, Heidelberg, Germany) and 5 μM uridine (Sigma) was used to feed the root meristems. Given the enhanced photosensitivity of Br-DNA, special care was taken to prevent the exposure of roots to light during and after that feeding. Bromosubstitution under these experimental conditions leads to a replacement of 17% of the thymidine bases, as assessed after CsCl gradient centrifugation. Under these conditions, the density of the onion native DNA (1.6975 g/cm^{-3}) increased to 1.7153 g/cm^{-3} (Quinzani-Jordão 1987).

For UVA irradiation, the bulbs were placed with their attached roots upwards. They were completely immersed in filtered tap water in a large perpex container. Nitrogen was bubbled through the water for 1 h before and during irradiation, in order to deplete oxygen and prevent unwanted damage to Br-DNA. The roots were shielded by a 1.5-cm thick thymidine solution filter, in order to absorb any possible contaminating ultraviolet irradiation from the light source. Twenty minutes of UVA irradiation was provided from above the filter by an Ultravitalux Osram bulb whose emission spectrum was enriched in the 313 nm wavelength. The light source was placed 30 cm above the root tips. Light energy reaching the roots was in ther 6–10 J/cm^2 range, as measured by a long-wave ultraviolet metre (J-221, Ultraviolet Products Inc., San Gabriel, CA).

To label nuclei in replication, roots were treated for 15 min with [5′-^{3}H]thymidine (Amersham, Buckinghamshire, UK) at a concentration of 370 kBq/ml and a specific activity of 925 GBq mmol, at the times specified. To detect isotope incorporation the slides were covered with 1:1 (v/v) diluted NTB-2 Kodak autoradiographic emulsion. After drying, they were left at −70°C for 9 days. Silver grains were developed with Kodak D-19 developer and fixed with Kodak ultrarapid acid fixer.

Incorporation of [5,6-^{3}H]uridine, [^{3}H]cytidine, [^{3}H]adenosine and [^{3}H]guanosine, in separate treatments, was carried out, after 1-h incubation, at 74 kBq/ml concentrations and between 30 and 40 GBq/mmol, after 4 h of recovery from the anoxic UVA irradiation which immediately followed a 36 h feeding with 5-bromo-2′-deoxyuridine. Root apical meristems were cut in 1 mm-long segments and homogenized in a glass homogenizer in 5% TCA at 4°C. Aliquots were placed in GFA glass filters, were extensively washed by 5% TCA and, finally washed with 3:1 ethanol–ether mixture. After drying, the radioactivity incorporated in the TCA-insoluble material was measured in a scintillator counter.

For cytological analysis, roots were fixed in a 3:1 ethanol–acetic acid mixture. Their apical meristems were stained with acetic orcein according to Tjio & Levan (1950). The meri-

stematic region of each root was cut (0.5 to 1.5 mm from the root apex) and then squashed. Mitotic and labelling indexes were estimated in at least 2000 cells from at least four different roots.

RESULTS

Transcription and replication of bromosubstituted DNA after anoxic irradiation

Firstly, the transcription capacity of the onion single strand bromosubstituted genome was assayed in root meristems 4 h after recovery from a treatment of 36 h with 5-bromo-2′-dcoxyuridine, followed by irradiation at 313 nm wavelength in anoxic conditions. Incorporation of [5,6-^{3}H]uridine, [^{3}H]cytidine, [^{3}H]adenosine and [^{3}H]guanosine into the acid-insoluble material was seen to reach vales of 2.1, 1.3, 1.0 and 0.9 times the incorporation recorded in meristems which possessed native DNA when irradiated under the same conditions (control). Thus, no depression in transcription seemed to have taken place in Br-DNA when compared to native DNA. There was only an unbalanced increase in uridine incorporation.

In relation to replication, our previous unpublished data showed that the treatment with 5-bromo-2′-deoxyuridine strongly modified the internal pool of deoxyribonucleosides. Thus, the estimates based on incorporation of exogenous deoxyribonucleosides. Thus, the estimates based on incorporation of exogenous deoxyribonucleosides are unreliable. However, cells with Br-DNA which were irradiated under anoxia at any time from very late G_1 to late S were able to reach their 4C DNA content—which corresponds to G_2—with a maximum 15% delay in relation to control (data not shown). This suggests that replication of Br-DNA is not importantly depressed in relation to that of native DNA.

Strategies used to study the role of DNA sequences replicated at different times of the S period in cycle progression

In order to deal with the study of the role of different DNA sequences in cycle progression, three experimental schemes were used (Figure 1). All of them took into account the recorded parameters of the cell cycle in onion root meristematic cells growing under fixed environmental conditions (De la Torre *et al.* 1989). The values were confirmed for the control cells, as we will see later in the different panels of Figure 2. It was found that G_1 took 8.3 h while the whole interphase took 24.4 h in the third of the binucleate population formed by its fastest cells. The duration of G_2 (5.5 h) was also confirmed. The time schedule for durations and locations of treatments can be followed (lower bar of Figure 1).

Under the first protocol (Figure 1a), bromosubstitution occurred in the S period of the same cycle where the cells were later labelled as binucleate, when passing through their subsequent telophase. In the two other experimental schemes, bromosubstitution occurred in the S period of the binucleate cells, in the cycle immediately after caffeine (Figures 1b and c). The first protocol was used to record the response of cells located in G_1, while the other two were used for studying the response of cells in both G_2 and prophase. In the three protocols, bromosubstitution was allowed to occur in roots from different bulbs for one of the three conventional fragments of the S period, namely S1 (early S phase), S2 (mid S phase) and S3 (late S phase). These experiments tried to confirm our own previous results (De la Torre & González-Fernández 1979, Sans *et al.* 1991, González-Fernández *et al.* 1992), but under strict analogous conditions. Some of the bromosubstitution treatments for a single subperiod of the S phase advanced, while others delayed, cycle progression of the binucleate

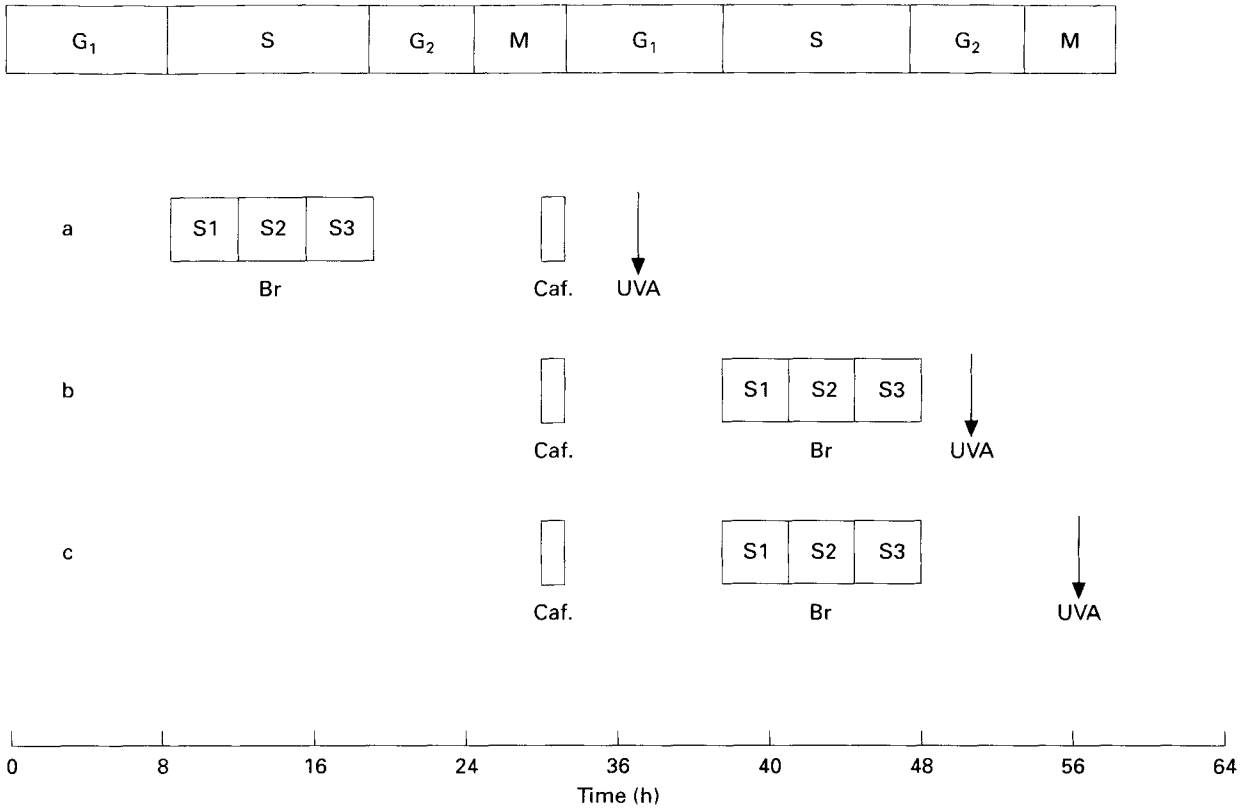

Figure 1. Experimental schemes developed to record the effects of DNA bromosubstitution, followed by anoxic irradiation at 313 nm wavelength, in cycle progression. The upper bar represents the relative time duration of two subsequent cell cycles in these meristems. In the fastest cells in this population, G_1 lasts 7 h, S lasts 10.5 h and G_2 lasts 5.5 h. (a) Scheme for analysing the response to irradiation of cells in mid G_1. Bromosubstitution takes place for only one or two of the three sequential thirds of the whole S duration (S1, S2 or S3). Five mM caffeine labels as binucleate the cells enduring cytokinesis. To see whether the cells are or are not blocked in their progression through G_1, their arrival into S phase is studied. For this, short treatments with [^{3}H]thymidine were given at 7, 9, 11, 13 and 15 h after the UVA irradiation. (b) Protocol to analyse the response of G_2 cells. Feeding with bromodeoxyuridine also takes place for one or two of the different thirds of the S period (S1, S2 or S3). Cells are irradiated in G_2. Entrance into prophase is used as a landmark to check whether the cells are blocked or advance into mitosis. (c) Scheme used to analyse the response of cells in prophase to DNA bromosubstitution followed by anoxic UVA irradiation in mid prophase. Entrance into prophase and metaphase of the portion of the cell population lagging behind the fastest cells were analysed.

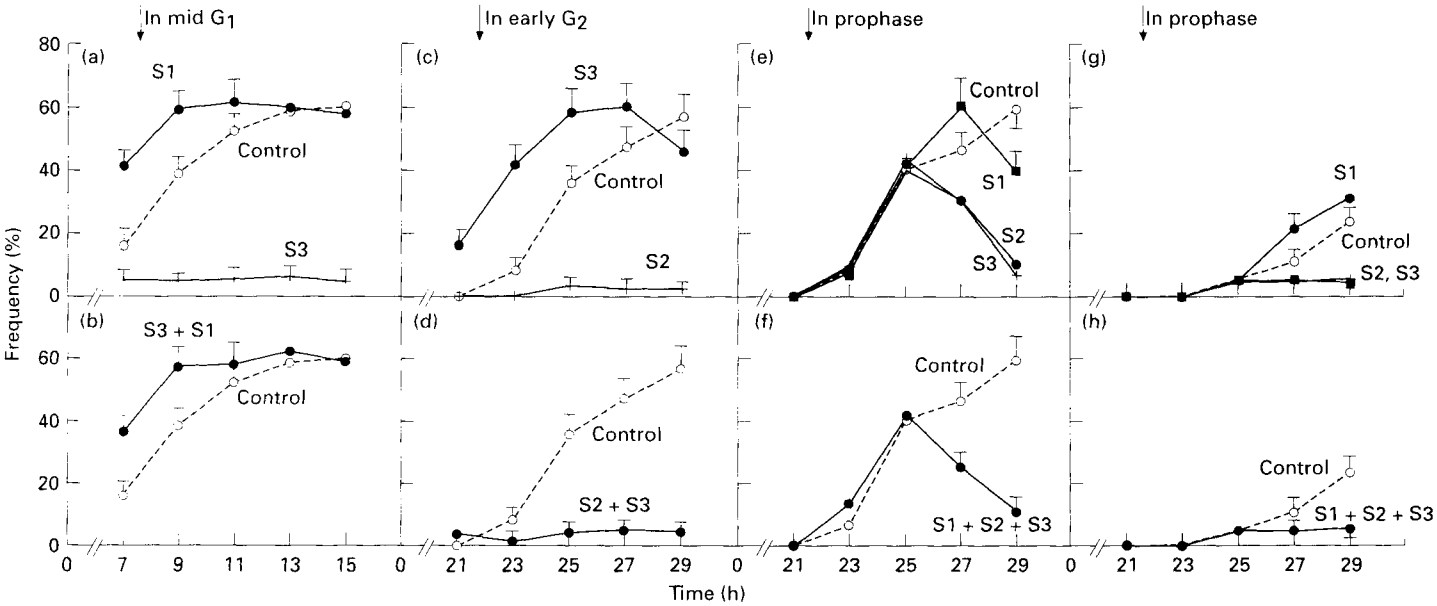

Figure 2. Kinetics of the entrance of the synchronous binucleate population into the different landmarks, after the protocols outlined in Figure 1. Abscissae correspond to time after the caffeine treatment, when the cells were fixed and studied. Ordinates correspond to frequency of the binucleate cells in the different cycle compartments. All the upper panels correspond to experiments in which only S1, S2 or S3 sequences were bromosubstituted, while the lower panels correspond to experiments in which the sequences advancing and blocking progression to the corresponding landmark were simultaneously inactivated by irradiation. The control curves correspond to cells which possessed only native DNA irradiated under anoxic conditions at 313 nm wavelength. (a, b) ordinates=frequency of the binucleate cells with labelled nuclei after [^{3}H]thymidine feeding. The protocol in Figure 1a was followed. The only difference in (b) is that the binucleate cells possessed both S1 and S3 sequences bromosubstituted. (c, d, e, f) ordinates=frequency of binucleate cells in prophase. In (e) and (f), part of the cell population was already in prophase when irradiation took place (experimental scheme in Figure 1c). (g, h) ordinates=frequency of binucleate cells in metaphase. (g) Bromosubstitution of DNA sequences replicated in a single subperiod of the S phase. (h) Simultaneous bromosubstitution of sequences involved in positive and negative regulation of the metaphase triggering. Vertical bars represent the corresponding standard deviations for the means. These SD have been drawn only on one side of the mean values for the sake of simplicity.

cells, after the corresponding radiation. Irradiation alone or bromosubstitution alone hardly modified the control curves (González-Fernández & De la Torre 1979), so that these controls were omitted from this study.

After this, the set of experiments which fulfils the objectives of the present work was carried out. Bromosubstitution of replicating DNA was successively allowed for the two segments of the S period displaying opposite effects in a specific cycle transition, as shown in the previous experiments. Entering into S phase, prophase or metaphase were used as landmarks to know whether the anoxic irradiation of the cells under any of these treatments prevented or permitted their progression towards later stages of the cycle.

Progression through late G_1

Radiation was carried out in mid G_1 of the cells whose DNA had been previously bromosubstituted in the early, mid or late thirds of the S period of the previous cycle. Thus, the kinetics of entering into S phase were looked at after the S1, S2 and S3 schemes of Figure 1a.

The data obtained are shown in Figure 2a. The entrance into S phase was used as a landmark to know whether any of the treatments prevented or advanced cycle progression in the binucleate cells. The control curve corresponds to cells which only possessed native DNA irradiated under anoxic conditions at 313 nm wavelength. The binucleate cells having their S1 sequences bromosubstituted were advanced into S in relation to control. This means that S1 sequences are normally involved in a safety checkpoint repressing the precocious triggering of replication, i.e. in a negative control. On the other hand, sequences replicated in S3 behaved as if involved in a positive regulatory mechanism of progression throughout the last half of G_1. Thus, their modification by bromosubstitution and anoxic irradiation prevented the start of replication (Figure 2a).

The simultaneous inactivation of the sequences replicated in both S1 and S3 allowed the entrance of those cells which were irradiated at mid G_1 into S phase, as shown by the kinetics of [^{3}H]thymidine incorporation (Figure 2b). In this way, the S period was the default phase for these cells. They accumulated in S when the genome sequences were modified so that they were unable to respond to both positive and negative regulatory signals involved in the G_1 to S transition.

G_2 progression

Prophase was used as a landmark to see how the irradiation in early G_2 interferred with cycle progression in cells with Br-DNA in those sequences replicated at different times of the S period, following the protocol of Figure 1b. The anoxic irradiation of cells in which S3 portions of their DNA were bromosubstituted was seen to advance the entrance of the population into prophase (Figure 2c). It confirmed that S3 segments are involved in the negative regulation of the G_2 to prophase progression. On the other hand, sequences which are replicated in the central third of the S period (S2) were seen to prevent the entrance into prophase (Figure 2c). S2 sequences seem to be involved in the positive regulation of the G_2 to prophase transition.

The simultaneous inactivation of S2 and S3 sequences of this *Allium cepa* L. genome prevented the G_2 to prophase transition (Figure 2d). Thus, the cells in G_2 which were unable to respond to both positive and negative regulatory signals were unable to enter into mitosis. G_2 was the default phase for these particular cells.

Progression through G_2 and prophase

In order to detect changes in the frequency of binucleate cells in prophase, when irradiated in prophase itself, the same scheme displayed in Figure 1c was followed. As can be seen in Figure 2e, the frequency of prophases rose slightly in relation to control when S1 sequences were bromosubstituted. Their inactivation accelerated the entrance into prophase of the binucleate cells which were still in G_2 when the irradiation took place. However, S2 and S3 sequences decreased the frequency of prophases. In this sense, S1 sequences appear to be involved in the negative regulation of prophase triggering in cells located in late G_2, while S2 and S3 sequences seem to be involved in its positive control. The fall in frequency of prophases after the anoxic irradiation of those cells supported a normal kinetics of exit from prophase, as suggested by the parallelism between the right part of the S1, S2 and S3 curves. These data confirm previous results (Sans *et al.* 1991).

When performing an experiment involving the simultaneous inactivation by anoxic UVA irradiation of the sequences replicated in the whole S phase (S1 + S2 + S3) (Figure 2f), as well as the simultaneous inactivation of S1 + S2 sequences or that of S1 + S3 sequences (data not shown) it was found that, in all cases, the frequency of cells in prophase declined after irradiation. Prophase was not a default phase at all for cells whose DNA was missing positive and negative regulatory signals.

The prophase to metaphase transition

In order to see whether the fall in the frequency of prophases observed in the previous experiments when irradiated in prophase itself was due either to an increased rate of entrance into metaphase or to decondensation of the prophase chromosomes—a sort of return to G_2—the frequency of binucleate cells in metaphase was recorded.

Irradiation in mid prophase of cells with bromosubstituted S2 and S3 sequences did not advance the entrance of these cells into metaphase (Figure 2g). In fact, there was no increase in the appearance of metaphases. This indicated that the cells had returned to interphase. On the other hand, cells with bromosubstituted S1 sequences moderately increased the frequency of cells in metaphase, as if S1 sequences were involved in the negative control of prophase to metaphase progression.

Finally, when all the genome was bromosubstituted, the anoxic irradiation in prophase kept the frequency of the metaphases very low (Figure 2h), in spite of the frequency of prophases decreasing from the 25th hour onwards (previous Figure 2f). These data support the idea that the anoxic irradiation of these cells is merely preventing the chromosome condensation cycle, and reversing it to a G_2 stage. Thus, G_2 also behaved as a default phase for those cells which were in prophase when all their DNA was inactive, for both positive and negative regulatory signals for prophase progression.

DISCUSSION

Bromosubstitution of a single strand of DNA (without further irradiation) was seen to modify, mostly by increasing, DNA affinity for a set of the usual chromosomal proteins, preferentially non histone ones (Gordon *et al.* 1976, Bick & Devine 1977). Interest in this problem is related to the reported inhibition of cytodifferentiation after the change in gene expression which bromosubstitution brings about (see Rutter *et al.* 1973).

In plant cells, the incorporation of RNA precursors was not depressed after bromosubstitution of DNA and its anoxic irradiation, as it is in bacteria (Jones & Dove 1972). Although the molecular effects of this treatment are unknown in higher eukaryotic cells, the

whole set of experiments performed on onions suggests that Br-DNA mostly differs from native DNA after irradiated in their incompetence to bind regulatory proteins. Thus, anoxic UVA irradiation in prophase of cells possessing Br-DNA (Sans *et al.* 1991) produced the same response that the inhibition of protein synthesis induced in the prophase of cells with native DNA (García-Herdugo *et al.* 1974).

The present report shows that S phase is the default phase for cells which were irradiated in late G_1, while G_2 was the equivalent default phase for cells which were in G_2 and in the prophase. When cells were irradiated in prophase, their chromatin decondensed and the nuclear envelope did not break down. These nuclei were indistinguishable from those in control G_2 cells.

The present experiments show that the G_1 to S phase entrance may be retarded or anticipated. Moreover, they also showed that the meristematic cells are already committed to replicate from mid G_1 onwards and that they are prevented for replicating by an inhibitory checkpoint mechanism. These cells probably have the replication machinery already bound to the corresponding early replicating origins which provide the nucleus the competence to initiate replication and that binding is not affected by the irradiation. Thus, cells which were located in mid G_1 initiated replication when both S1 and S3 sequences were unable to interact with the corresponding proteins which prevented and induced replication, respectively. These sequences should be a subset of all the replicon families replicated in that segment of DNA. In fact, they are concentrated in very few chromosomes of this huge onion genome (Panzera *et al.* 1997). The lack of binding of proteins to fully replicated S3 sequences might be the signal responsible for the unbinding of proteins preventing the replication of early origins in control conditions. Such a safety checkpoint would detect missegregation of telomeres unreplicated in the previous mitosis, and thereby prevent the initiation of replication. This process may be redundant to that controlled by the *num* 1^+ gene of fission yeast which conditions the initiation of S phase upon completion of mitosis (Moreno & Nurse 1994).

Finally, G_2 is known to be one of the phases of the cycle where cells become quiescent in plants when the environmental conditions are not good for proliferation progress, as in dry roots (Sans & De la Torre 1979). Here, it is not surprising that cells in G_2 remain in G_2 itself if the binding of the usual set of proteins to their DNA does not take place. These proteins are positively regulating such cycle transition. More surprising is that cells already in prophase lose their chromosome condensation and return to interphase (García-Herdugo *et al.* 1974, Sans *et al.* 1991). Chromosome condensation appears as a reversible and unstable dynamic stage. The completion of chromosome condensation in prophase seems to rely on a control mechanism which depends on protein synthesis taking place in prophase itself. The knowledge of the nature of the proteins which are synthesized in prophase and which are involved in the completion of chromosomal condensation will certainly give a strong push to the discernment of the chromosomal packing mechanisms which operate during mitosis. This paper mostly stresses the need for focusing on DNA as a receptor of signals related to the safe induction of crucial cycle transitions which ensure the accuracy of the DNA replication and segregation cycle in eukaryotic cells.

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