Replicon clusters may form structurally stable complexes of chromatin and chromosomes

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SUMMARY

Nuclear DNA replication was monitored ‘in situ’ in pea nuclei with the bromodeoxyuridine antibody technique. The labelling appeared to be restricted to a number of finely distinct spots. The labelling was followed through three subsequent cell cycles in meristematic and differentiating pea root cells. The results show that the spots as seen just after the labelling persist distinctly over the mitotic chromosomes as well as in the nuclei of the following cell cycles up to 44 hours after the pulse. Moreover, they are also present in the nuclei of differentiating cells. The spots over the mitotic chromosomes in specific cases give rise to a dynamic banding. Nuclei of the second and third cycle show absence of labelling in specific zones, owing to the segregation of the labelled strands of chromosomal DNA. The maintenance of the spotted appearance of the replication clusters through all stages of the three subsequent cell cycles may be an indication in favour of the hypothesis that such clusters represent structurally stable replicon complexes held together by the nuclear matrix and the chromosome scaffold.

Key words: DNA replication, immunofluorescence, bromodeoxyuridine labelling, cell cycle, chromatin structure

INTRODUCTION

DNA replication in eukaryotic cell nuclei is achieved through the simultaneous activation of a number of groups of adjacent replicons called replicon clusters (Huberman and Riggs, 1968). Analysis of the temporal and spatial order in which such replicon clusters are activated has led to the conclusion that it is not a random process. Early results obtained through autoradiography of [3H]thymidine-labelled nuclei showed that heterochromatin replicated at the end of the S-phase and was localized at the nuclear periphery (Ockey, 1972; Comings and Okada, 1973; Sparvoli et al., 1976). In especially favourable cases it was possible to show that such labelling appeared as a defined number of spots located at the nuclear periphery, which corresponded to specific heterochromatic segments of the chromosomes (Sparvoli et al., 1977).

The limitation imposed by the low resolution level of autoradiographs of [3H]thymidine-labelled DNA was overcome by immunofluorescence detection of incorporated bromodeoxyuridine (BrdUrd) or biotin-labelled dUTP. In fact this technique allowed a much finer detection of the sites of DNA replication (Nakamura et al. 1986; Nakayasu and Berezney, 1989; Jackson, 1990; Hozák et al., 1993).

Nevertheless, since the real presence of a nuclear matrix has often been challenged, other authors have proposed different interpretations (Adachi and Laemmli, 1992). The different ways of interpreting the observed foci have meaningful consequences regarding the model of DNA organization in the nucleus and its method of replication.

On the basis of such contrasting hypotheses, taking advantage of the pattern of plant growth and development, we devised an experimental protocol that could provide some evidence that might help in discriminating between these different interpretations. We showed previously that when pea roots are fed with a pulse of BrdUrd, the incorporated precursor can be detected in nuclei by immunofluorescence (Levi et al., 1987) and, also in this plant material, the labelling appears in the form of distinct spots (Levi et al., 1990). In the root tip, the meristematic cells undergo a series of cell cycles and their derivatives, in a more proximal zone, can stop dividing and start to differentiate. After a brief pulse with BrdUrd, it is then possible to follow the fate of the labelled DNA replication sites in interphase nuclei and mitotic chromosomes in the intact organism throughout subsequent cell cycles and in differentiating cells. The evidence that we obtained shows that the replicon clusters appear to form stable complexes. This finding
is interpreted as being in agreement with the hypothesis that replicon clusters are held together by the nuclear matrix.

**MATERIALS AND METHODS**

Three-day-old seedlings of *Pisum sativum* L. var. Lincoln (Ingegnoli, Italy), grown on Agriperlite (Superlite, Vic Italiana, Italy) at 25°C in the dark were used.

The seedlings were directly pulsed with BrdUrd (asynchronous labelling) or synchronized with hydroxyurea (HU) in order to label the nuclei in specific moments of the S-phase (Fig. 1).

**Synchronization**

Seedlings were transferred with the root immersed in an aerated bath containing 2.5 mM HU for 12 hours in the dark at 25°C, and then washed thoroughly and transferred to aerated distilled water. The cell cycle kinetics of the recovery was monitored by flow cytometry (Fig. 1) (Levi et al., 1992).

**BrdUrd labelling**

The seedlings were transferred with the roots immersed in an aerated solution of BrdUrd (100 µM) for 30 minutes at 25°C in the dark. At the end of the pulse, the roots were rinsed and fixed immediately, or the seedlings were transferred for the chase period in distilled water. In some experiments, after 3 hours of chase with distilled water, the seedlings were transferred in 2 mM 8-quinolinol, a microtubule inhibitor, in order to accumulate more mitotic figures.

**Fixation and slide preparation**

At the proper time, root apices (0-2 mm) or differentiating root segments (2-5 mm from the tip) were fixed in 4% formaldehyde in Tris buffer (Levi et al., 1986) and then rinsed thoroughly in buffer. Nuclei were extracted by crushing the tissue with a glass rod, and nuclear smears were prepared as described previously (Levi et al., 1986). The slides were stored without problems up to several weeks.
in absolute ethanol at −20°C. A sample of the suspension of nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI) and used for flow cytometric analysis (Levi et al., 1992).

**Immunofluorescent staining**

The slides stored in ethanol were immersed for 30 minutes in methanol at room temperature, air dried, rehydrated for a few minutes in Tris buffer and hydrolysed for 1 hour at 25°C in 2 M HCl. The immunofluorescent staining was performed essentially as described previously (Levi et al., 1987, 1990), with an indirect method: monoclonal anti-BrdUrd antibody (Becton Dickinson), biotinylated secondary antibody and Texas Red-streptavidin. Slides were counterstained with DAPI and mounted in Tris buffer for immediate observation and photographing, or dried and conserved unmounted at 4°C in the dark for a few days. The nuclei and stain remained well preserved. Slides were examined with a Zeiss Axioplan fluorescence microscope and photographed with Kodak T-Max 400 professional film.

**RESULTS**

**Patterns of labelling of S-phase nuclei**

After a 30 minute pulse of BrdUrd, the nuclei always show a distribution of labelling in the form of finely distinct spots or foci whose number and localization appear to conform to three basic patterns (Fig. 2), which show some similarities with those found in mammalian cells (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992).

The first pattern (Fig. 2A,B) appears as a very large number of finely distinct small spots (we counted an average of 352 spots per nucleus, with a minimum of 210 and a maximum of 410). Their main characteristic is to appear to be distributed throughout the nucleus except for the nucleolar zone. This kind of pattern is very similar to type I described by Nakayasu and Berezney (1989) and to pattern 1 described by O'Keefe et al. (1992), and should be ascribed to the early S-phase.

The second distinct pattern (Fig. 2D) is characterized by the presence of a ring of spots around the nucleolar zone in addition to a limited number of spots that appear distributed prevalently around the nuclear periphery. This pattern is similar to Nakayasu and Berezney’s (1989) type II and to O’Keefe’s (1992) pattern 3. The pattern shown in Fig. 2C could be intermediate between those in A,B and that in D.

The third pattern represented in Fig. 2E,F shows a moderate

**Fig. 3.** Appearance of BrdUrd immunofluorescence localization in mitotic figures, 3 and 6 hours after the pulse. (A-C) Prophases; (D-G) metaphases; (B,E,G) the same as A,D,F stained with DAPI. The labelling still appears in the form of tiny spots very similar to those observed over the nuclei immediately after labelling. Bar, 5 µm.
number of spots preferentially located at the nuclear periphery and corresponding to the distribution of heterochromatin. This last pattern in our opinion corresponds to Nakayasu and Berezney’s (1989) type III and to O’Keefe’s (1992) patterns 4 and 5 and should be ascribed to the late S-phase. That this is the case is also confirmed by their high frequency in cells labelled at the end of the S-phase after synchronization with HU. In the micrographs of the last two stages a number of spots appear to be larger, with curved or horseshoe shapes as is also observed in mammalian cells.

Patterns of labelling of mitotic chromosomes

If the root tips are fixed 3 to 6 hours after a late S-phase pulse (Fig. 1) and the nuclei extracted and spread as described in Materials and Methods it is possible to find several labelled prophases and a number of group or single metaphasic chromosomes (because of the extraction procedure and of spreading, it is very difficult to obtain complete metaphasic spreads). Fig. 3A,C show the appearance of the labelling of two prophasic nuclei in which the nucleus in Fig. 3C corresponds to a cell labelled at the end of S-phase. As can be seen, the chromosomal portions in the focal plane exhibit a number of finely distinct spots similar to those observed in the S-phase nuclei. The spotted appearance of chromosomes is most evident in metaphasic chromosomes either when they form clusters as in Fig. 3D,F or when they are observed as single chromosomes as shown in Fig. 4. Fig. 5, moreover, shows a gallery of metaphasic chromosomes that correspond to nuclei labelled at the end of the S-phase. They exhibit the clear presence of ‘replication banding’, each band is made up of two or more spots. It can be observed that such bands occur mostly in the pericentromeric regions, and in secondary constrictions and satellites. All the mitotic chromosomes, then, show labelling made up of a number of distinct spots similar to those observed in S-phase nuclei and these spots, in favourable conditions, appear to determine a replication banding.

Nuclei labelling patterns 20 and 44 hours after the pulse

Fig. 6 shows a number of different labelled nuclei as they appear 20 or 44 hours after the BrdUrd pulse. Fig. 6A,B represent a nucleus from the root tip (0-2 mm zone), while the nuclei from 6C to 6H are from the 2-5 mm zone, where a relevant number of nuclei have ceased dividing because their cells have started the differentiation process. As can be seen, all the nuclei, which are representative of the population of labelled nuclei, show a typical spotted appearance similar to that already encountered in the previous stages. Nevertheless, most of them (Fig. 6A,C,E) show that the labelling is restricted to specific well delimited areas. Some of the nuclei, like the one in Fig. 6G, do not exhibit labelling distributed to specific areas but have a uniformly spotted appearance. We believe that these nuclei may come from cells that ceased dividing and started the differentiation process. The elongated appearance of the nucleus in Fig. 6G is in agreement with this interpretation. Whether this interpretation is correct or not, all the labelled nuclei in the 2-5 mm zone show the spots and, according to our cytometric analyses, a relevant number of them should belong to cells that ceased dividing and initiated the differentiation process.

DISCUSSION

The first result of our study on the spatial and temporal distribution of DNA replication in plant cell nuclei is that it occurs in a number of distinct foci that show a close similarity to those observed in mammalian cells. Moreover, they show three distinct patterns of distribution of foci that have some basic features similar to those described for mammalian cells (Nakayasu and Berezney, 1989; O’Keefe et al., 1992). This similarity between completely unrelated organisms may
underlie some basic feature of the regulation of the temporal and spatial order of DNA replication in eukaryotes. Concerning this, we previously reported that the auxiliary protein of DNA polymerase δ, PCNA (proliferating cell nuclear antigen), is also distributed in distinct foci in pea nuclei when revealed by immunofluorescence with PC10 monoclonal antibody (Citterio et al., 1992). A speckled distribution was also found with an antibody against a sequence of the human topoisomerase II (Levi et al., 1994).

The appearance of BrdUrd labelling in S-phase nuclei in the form of distinct foci has been interpreted by various authors as reflecting the simultaneous localized activation of different clusters of replisomes. Although there is a general agreement on the interpretation of such foci as representing the simultaneous activation of the various clusters of replisomes, there is no agreement about the mechanism underlying this localized replication. As mentioned above, some authors (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Jackson, 1991) suggest that the observed foci are the consequence of permanent anchoring of the clusters of replisomes to a nuclear matrix. This interpretation would be in agreement with the hypothesis that replication complexes are fixed on the nuclear matrix and DNA is spooled through it while it is being replicated (Cook, 1991). That this may be the case is firmly supported by the evidence presented by Hozák et al. (1993), who were able to visualize what they call ‘replication factories’ attached to the nucleoskeleton, which appear to be the sites where DNA replication occurs.

Our studies, addressed to follow the fate of the replicon clusters from the S-phase to the following stages of mitosis and the subsequent cell cycles up to 44 hours after the pulse, are in agreement with this hypothesis. In fact from our results it is possible to infer that the replicon clusters may form permanent complexes of chromatin and chromosomes.

The first indication comes from the chromosomes as they are observed during the first mitosis immediately following the BrdUrd pulse (Figs 4, 5). When the labelled chromosomes are submitted to close analysis they show that the label is distributed in a number of tiny spots very similar to the foci observed in S-phase nuclei and in G2 nuclei, in apparent continuity with them. The possibility that the spots present in the mitotic chromosomes may correspond to the foci observed after the pulse is reinforced by the clearly spotted appearance of the replication bands that correspond to the late replicating DNA.

To ascertain whether the spots are a permanent feature of chromatin and chromosomes, we examined the nuclei 20 and 44 hours after labelling. In both cases most of the cycling cells should have gone through at least one or two cycles after the labelling, since the duration of the cell cycle in peas lasts about 14 hours (Van’t Hof, 1974). As shown in Fig. 6, all nuclei show a distribution of label in the form of tiny spots, even if most of them show the labelling restricted to specific distinct areas.

All these findings, which are in agreement with the preliminary results obtained by Meng and Berezney (1991), can be explained assuming that the foci observed immediately after labelling and those present in the chromosomes and nuclei in the subsequent stages are the same. Therefore, if the foci represent the replicon clusters, these are structurally stable complexes.

This general picture shows several aspects that strengthen its significance and add particular points of interest.

The first element to be considered concerns the spots present in the mitotic chromosomes. In fact, if they correspond to the clusters of replisomes, we have to consider that they are most probably held together by the chromosome scaffold. If this is the case, it seems plausible that the anchoring scaffold should be preceded, in the interphasic nucleus, by some similar structure, which could be the postulated nuclear matrix.
A further point of interest is the replication banding of chromosomes, which we were able to see clearly in relation to late-replicating DNA. Such bands are made up of contributions from two or more spots. This adds further support to the idea that the banding of chromosomes is related to specific and defined areas, while definite territories appear without labelling. As we have shown, very often this is restricted to specific and defined areas, which can be explained by the segregation of the labelled strands in different nuclei as a consequence of semi-conservative replication of DNA and is in agreement with the findings that show that interphasic chromosomes occupy specific areas (Heslop-Harrison and Bennet, 1990).

We find it difficult to explain all these different findings concerning the labelling patterns without assuming the presence of specific structures that hold the replication clusters in place and cause them to appear as permanent structural complexes. In conclusion, we believe that the evidence presented here may be explained if the presence of an anchoring structure that could consist of the nuclear matrix and a chromosome scaffold is assumed.

This research was supported by the National Research Council of Italy, Special Project RAISA, Subproject no.2, Paper 1918, and by Italian MURST (60%).

REFERENCES


(Received 21 January 1994 - Accepted, in revised form, 27 June 1994)