Observations on rRNA gene replication in *Chara vulgaris* evaluated by *in situ* $[^{3}H]$rRNA–DNA hybridization

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Summary

*In situ* $[^{3}H]$rRNA–DNA hybridization showed that: (1) during the cell cycle in antheridial filaments of *Chara vulgaris* the rDNA gene content doubles during a 2h period in mid-S-phase, which corresponds to about 13% of the duration of this phase; (2) doubling of the number of grains occurs during mid-S-phase irrespective of the developmental stage of the antheridial filaments; (3) the numbers of autoradiographic grains in comparable phases of successive cell cycles preceding spermatid formation are the same. Thus the gradual reduction of total nucleolar volume (estimated per nucleus) occurring in successive cell cycles preceding spermatid formation is not associated with a decrease in the number of rDNA genes.

Key words: *Chara vulgaris*, ribosomal gene replication, cell cycle, antheridial filaments, $[^{3}H]$rRNA–DNA hybridization.

Introduction

Several authors have shown that *in situ* $[^{3}H]$rRNA–DNA hybridization can be used as a quantitative method for determining the relative rDNA content in a cell population (Côté et al. 1980; Miller et al. 1979; Olszewski et al. 1981; Wolgemuth-Jarashow et al. 1976). In the present study this method has been used to compare the alterations in rDNA levels during the cell cycle in *Chara vulgaris* antheridial filaments. Antheridial filaments are very convenient for performing cytochemical analyses because of their naturally synchronized cell divisions and the correlation of cell length with specific stages of interphase (Kwiatkowska & Maszewski, 1979a, 1986; Olszewski & Godlewski, 1972, 1973).

The cell cycle of *Ch. vulgaris* antheridial filaments is characterized by the absence of $G_{1}$ phase (type $S+G_{2}+M$) (Kwiatkowska & Maszewski, 1979b; Olszewski & Godlewski, 1972). Furthermore, the following gradual changes during the successive mitotic cycles of the 2-, 4-, 8-, 16- and 32-celled antheridial filaments were found: (1) shortening of the length of interphase (Godlewski & Olszewski, 1973); (2) diminution in cell size as well as in the dry mass of cytoplasm and nucleus (Kuran, 1975); (3) decrease in number and total volume of mitochondria (Kwiatkowska, 1981), and in the number of Golgi structures counted per cell (Kwiatkowska & Maszewski, 1979a); (4) decrease in the transcriptional activity of the nucleus and nucleoli during consecutive interphases (Kwiatkowska & Maszewski, 1979b). Recent studies have shown that this process also involves a considerable reduction of the maximum nucleolar volume, which was achieved in antheridial filament cells in successive preprophases (Kwiatkowska & Maszewski, 1985a). The present observations were aimed at: (1) the determination of rDNA replication timing during interphase; (2) checking whether the rDNA replication pattern was changing during successive interphases; (3) the investigation of whether the reduction in nucleolar volume in the course of spermatogenesis was associated with changes in rDNA gene contents.

Materials and methods

*Chara vulgaris* plants were obtained from the Botanical Garden, Lodz.

1-, 2-, 4-, 8-, 16- and 32-celled antheridial filaments in various phases of the cell cycle were used (see Fig. 1). The stage of interphase was recognized on the basis of cell length; this is characteristic for each developmental stage of the antheridial filaments (Olszewski & Godlewski, 1972). Apical fragments of thallus with antheridia were fixed in ethanol:
glacial acetic acid mixture (3:1, v/v). Squash preparations were made from isolated antheridia.

In situ [3H]rRNA/DNA hybridization was carried out according to the procedure described by Avanzi et al. (1973). [3H]rRNA (6-8 x 10^4 cts mm^-1 per mg RNA) was used, isolated according to the method of Cherry et al. (1965) from Spriodela oligorrhiza cultured for 3 days with [3H]uridine. Preparations coated with Ilford K2 emulsion were exposed for 6 months and, after development and fixing, the cells were stained with Toluidine Blue according to Smetana et al. (1969).

Results

Cellular localization of [3H]rRNA

In Ch. vulgaris antheridial filaments, 3–12 nucleoli (6-5 on average) can be discerned within a nucleus. Most frequently encountered are cells with six or seven nucleoli. Their diameter ranges from 0-2 to 3-5 μm (Maszewski & Kwiatkowska, 1984).

Localization of autoradiographic grains after hybridization with [3H]rRNA was analysed in two- or four-celled antheridial filaments in which the nucleoli were most readily visible due to their relatively large size (Kwiatkowska & Maszewski, 1985a). In cells stained with Toluidine Blue after extraction of RNA and proteins the nucleoli were seen as pale blue bodies on a darker background (Fig. 1B,C).

The distribution of autoradiographic silver grains in preparations on which [3H]rRNA–DNA hybridization has been performed is specific: they are localized over the nucleoli or on its periphery (Fig. 1B,C). Therefore, it can be concluded that [3H]rRNA isolated from Spriodela oligorrhiza hybridizes with rDNA of Ch. vulgaris. Analogous results indicating that considerable similarity rDNA nucleotide composition exists between systematically distant groups have been obtained in several other studies (Johnson & Strehler, 1972; Olszewskas et al. 1981; Schubert & Wobus, 1985). The average number of autoradiographic grains formed as a result of hybridization amounts to 1-6 per nucleolus. Relatively low labelling of the nucleoli results from the dispersion of rDNA genes in several nucleolus organizing regions. The total number of grains per nucleus is similar to that in other cell types on which in situ [3H]rRNA hybridization has been performed (e.g. see Cote et al. 1980; Olszewskas et al. 1981). No preferential labelling of large nucleoli over the smaller ones has been found: on the contrary, the largest nucleoli sometimes show an absence of labelling (Fig. 1B). This phenomenon is connected with the specificity of this method, which consists of several preliminary treatments. These may not result in every nucleolus in the formation of single-stranded rDNA fragments able to hybridize with exogenous [3H]rRNA (Coté et al. 1980; Henderson, 1982). Our studies suggest that in the largest nucleoli – those richest in RNA and proteins – the preliminary treatment was more likely to result in incomplete extraction than in others. This prevented effective demonstration of single-stranded rDNA.

Cell labelling at different stages of interphase

Nucleolar labelling has been analysed at 12 interphase points in four-celled antheridial filaments. For each point from 30–60 cells were counted, derived from about six antheridia. As in the previous studies, only the generation in which four-celled filaments had been most numerous was chosen for analysis. The investigations carried out by Olszewskas & Godlewski (1972) showed that in most antheridial filaments there was a strong correlation between the cell length and the specific stage of interphase.

From early telophase to mid-S-phase the average number of autoradiographic grains over all nucleoli within a nucleus is constant, amounting to about five (Fig. 2A). The average number of grains in the cells from 15 up to 17 μm long increases rapidly and reaches a level twice that found over cells from earlier stages of S phase. This high level of labelling of the nucleus is maintained up to the end of interphase.

S phase in the four-cell stage of antheridial filaments lasts about 15 h; it has been initiated in cells 13 μm long and is completed in cells attaining a length of 19 μm (Kwiatkowska & Maszewski, 1979b). The average growth rate of cell length during S phase is thus:

\[
\frac{19 \text{ μm} - 13 \text{ μm}}{15 \text{ h}} = 0.4 \text{ μm h}^{-1}.
\]
It can be assumed that the doubling of rDNA genes during the cell cycle at the four-cell stage started 7 h after the initiation of S phase in telophase and terminated about 5 h before the late replication of heterochromatin and 6 h before the termination of S phase (cf. Kwiatkowska & Maszewski, 1979b). Therefore, the replication of rDNA takes about 2 h, i.e. 13% of the duration of S phase.

rDNA replication in relation to the developmental stage of antheridal filaments

The course of rDNA replication during interphase has been compared in four- and 32-celled filaments. The difference between 32-celled and four-celled filaments is that the former have mitotic cells half the length of the latter due to a slower rate of cell growth and a shorter interphase that results from a reduction in the duration of G2 phase. The duration of S phase is, however, the same in both stages and lasts about 15 h (Godlewski & Olszewska, 1973).

From curves illustrating variations of the relative rDNA gene content, which was estimated from the number of autoradiographic grains, it may be concluded that the stage of development does not influence the rDNA replication pattern (compare Fig. 3 with Fig. 2A).

Former studies indicated that the rate of increase of cell length in 32-celled filaments in S-phase was about 0.1 μm h⁻¹ (Kwiatkowska & Maszewski, 1978). From the data presented in Fig. 3, it can be calculated that...

Fig. 3. The number of autoradiographic silver grains over interphase nuclei of different stages in 32-cell antheridal filaments after [³H]rRNA–DNA hybridization. Arrows indicate the sizes of cells at the end of S phase; T, telophase cells initiating S phase.

Fig. 2. Antheridal filaments in the four-cell stage. Changes of autoradiographic grain number after [³H]rRNA–DNA hybridization (heavy lines, means ± S.E.) in comparison with changes of: A, mean number of cells labelled with [³H]thymidine (after Kwiatkowska & Maszewski, 1979b); B, mean total volume of nucleoli during interphase (after Maszewski & Kwiatkowska, 1984); C, activity of [³H]uridine incorporation into nucleoli (after Kwiatkowska & Maszewski, 1979b). T, telophase, beginning of interphase; an arrow indicates the size of cells when they terminate S phase.
rRNA replication in 32-celled filaments begins about 7 h after initiation of S phase in telophase and lasts about 2 h, the same values as were obtained for four-celled filaments.

**Content of rDNA, transcriptional activity and volume of nucleoli**

Two periods of increased transcriptional activity of nucleoli have been found in antheridial filaments of Ch. vulgaris: the first in mid-S-phase and the second in mid-G2-phase (Kwiatkowska & Maszewski, 1976). On the other hand, nucleolar volume increases throughout the whole of interphase, attaining the highest values in late G2 (Maszewski & Kwiatkowska, 1984). Lack of correlation between the transcriptional activity of nucleoli and their volume results from the restriction of rRNA transport from the nucleoli into the cytoplasm during the periods of reduced ribosome synthesis and intensification of their transport in the same cell cycle phases in which the intensified synthesis of nucleolar RNA occurred (Kwiatkowska & Maszewski, 1985c).

As can be seen from comparing the data presented in Fig. 2B and C, the number of rDNA genes correlates with their transcriptional activity and nucleolar volume only in the first half of S phase. In the remaining interphase periods the correlation does not exist. It is characteristic, however, that two equally high peaks of nucleolar transcriptional activity (in mid-S-phase and mid-G2-phase) occur in the period of doubled rDNA level.

**Content of rDNA genes at different stages of spermatogenesis**

The content of rDNA genes does not undergo any change during morphogenesis, in spite of the drastic reduction in nucleolar volumes at successive developmental stages of the antheridial filaments (Figs 4, 5).

Independently of the number of cells in the antheridial filament, the autoradiographic grains occurring over telophase and post-telophase nuclei average ≈5 (Fig. 6A–C and Fig. 7A,B,F). The mean number of grains over cells of a size characteristic of late S phase, as well as in prophase, metaphase and G2 phase, is ≈10 per cell (Fig. 6A,D,E; and Fig. 7C,D,E).

**Fig. 4.** The number of autoradiographic silver grains over G2 phase nuclei after [3H]rRNA–DNA hybridization (black bars) and the volume of nucleoli within preprophase nuclei (white bars) in the 2-, 4-, 8-, 16- and 32-cell antheridial filaments.

**Fig. 5.** Antheridial filaments at different stages of development after silver staining. A, 1cf, 2cf, 4cf (filaments in the one-, two- and four-celled stages, respectively); B, a fragment of filament in the 32-cell stage, G2 phase. Bar, 10 μm.
Fig. 6. Antheridial filaments after [3H]rRNA–DNA hybridization. A. A fragment of the eight-celled filament in which desynchronization of mitotic phases between adjacent cells can be seen (p, prophase; t, telophase); B–F, fragments of 16-cell filaments: B,C, early S phase; D,E, G2 phase; F, telophase. Bar, 10 μm.

Fig. 7. Fragments of 32-cell filaments. A. Early S phase; B, mid-S-phase; C, G2 phase; D, early prophase; E, late prophase/metaphase; F, telophase. Bar, 10 μm.

Discussion

Timing of ribosomal gene replication during the cell cycle

Earlier studies on rDNA replication during the cell cycle were based either on autoradiographic methods using [3H]thymidine (Gosh et al. 1970; Kasten & Strasser, 1966; Tourte, 1975) or on the [3H]rRNA hybridization technique with isolated DNA (Amaldi et al. 1969; Balazs & Schildkraut, 1971; Howell, 1972; Zellweger et al. 1972). In experiments using in vitro hybridization of nucleic acids, Andersen & Engberg (1975) found that replication of rDNA in Tetrahymena was limited to a short period during S phase. On the other hand, results obtained with electron-microscope autoradiography indicated that rDNA replication lasts throughout interphase but is more intense during S phase (Nilsson & Zeuthen, 1980, cit. after Engberg, 1985). This divergence of results may, according to Engberg (1985), be due to difference in the sensitivity of the techniques used. Accuracy in establishing the timing of rDNA replication depends, to a large extent, on the possibility of precisely specifying the stage of interphase in the cell population in question, as well as on the method of synchronizing the cell cycles.

In Ch. vulgaris, antheridial filament cells the cell cycles are naturally synchronized. Furthermore, it is possible to identify the time of onset of the different phases of the cell cycle due to their correlation with specific cell lengths.

The results presented in this paper provide support for the validity of the in situ [3H]rRNA–DNA hybridization technique for precisely establishing the rDNA replication period. A doubling of the number of autoradiographic grains was detected in cells of mid-S-phase, between 7 and 9 h after the initiation of DNA synthesis in telophase. Consequently, rDNA replication occurs during the final 13% of S phase, which

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lasts a total of 15 h. These and other results seem to prove that the rDNA replication pattern is not dependent on the type of cell cycle and is characteristic of the species. In organisms possessing the same type of cell cycle as Chara (S + G2 + M, but lacking G1), i.e. in Physarum polycephalum (Zellweger et al. 1972) and Microasterias fimbriata (Touret, 1975), rDNA replication proceeds differently from the way it does in Chara since it occurs throughout S as well as G2 phase. A rDNA replication pattern resembling that of Chara has been described in Chinese hamster cells with a G1 + S + G2 + M type of cell cycle (Amaldi et al. 1969).

Comparison of alterations of rDNA gene content in the Chara cell cycle with total nucleolar volume and transcriptional activity (Kwiatkowska & Maszewski, 1979; Maszewski & Kwiatkowska, 1984) reveals the lack of a simple relation between these parameters, except during the first half of S phase. Nevertheless, it can be presumed that maximum transcriptional activity of the nucleoli is limited, due to rDNA gene number. Two equally high peaks of rRNA synthesis have been observed in Chara, namely in mid-S-phase and mid-G2-phase, both occurring after rDNA has been replicated. However, in P. polycephalum the first peak of rRNA synthesis occurring in S phase when rDNA replication has not been completed is much lower than the second peak taking place in G2 phase, in which rDNA content has reached its maximum value (Grant, 1972; Mittermayer et al. 1964; Zellweger et al. 1972).

Number of rDNA genes in relation to reduction of nucleolar size during spermatogenesis

Several studies proved that either an increase (Avanzi et al. 1973; Gall, 1968; Gambarini & Meneghini, 1972; Miller et al. 1979) or a decrease (Engberg et al. 1972; Johnson & Strehler, 1972) in the number of rDNA genes in a cell is one of the mechanisms regulating the number of ribosomes synthesized. On the other hand, during dedifferentiation of carrot and Jerusalem artichoke explants, which entails a rapid increase in rRNA synthesis, a constant rDNA gene level is maintained (Hase et al. 1982).

The present studies have proved that in the successive cell cycles preceding spermatid formation in Ch. vulgaris, no change occurred in the number of autoradiographic grains obtained by [3H]rRNA-DNA hybridization. There is a gradual reduction in transcriptional activity of nucleoli associated with morphogenesis (in the subsequent interphases), which is expressed as a decrease in both [3H]uridine incorporation and RNA polymerase I activity (Kwiatkowska & Maszewski, 1979b), as well as a decrease in total nucleolar volume and the growth rate of interphase cells (Kwiatkowska & Maszewski, 1985a); these features are not the result of the elimination of some rDNA genes, neither is there any alteration in rDNA replication pattern during the successive cell cycles.

Nucleolar sizes in antheridial filaments indicate that the decrease in the total nucleolar volume in the successive preprophases is associated with an increase of the percentage of micronucleoli of 0.2-0.5 μm diameter. At the two-cell stage micronucleoli constitute about 23% of the nucleolar population; at the 32-cell stage, 45% (Kwiatkowska & Maszewski, 1985a). It has been assumed that these micronucleoli did not resume active rRNA synthesis (Kwiatkowska & Maszewski, 1985b). They might maintain their micronuclear state throughout interphase, since their number does not diminish during this period (Kwiatkowska & Maszewski, 1985a). The increase in the number of inactive micronucleoli seems to be related to the progressive decrease in rRNA synthesis in the successive developmental stages of antheridial filaments. It has been shown that no relationship exists between the intensity of nucleolar labelling after [3H]rRNA-DNA hybridization and the number of cells in a filament. This suggests that the gradual inactivation of some nucleolar organizer regions after the successive mitoses, manifest in the increase in the number of micronucleoli, is not associated with a reduction in their rDNA genes.

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(Received 11 February 1987 – Accepted, in revised form, 11 May 1987)