The cell cycle and its relationship to development in *Acanthamoeba castellanii*

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Summary

The cell cycle and the relationship between particular cell cycle phases and the differentiation of trophozoites into cysts were reinvestigated in *Acanthamoeba castellanii* using flow fluorometric measurements of nuclear DNA content and synthesis, and synchronization of cells by release from the stationary phase. The investigation was performed with cultures growing in non-defined medium (ND cells) showing a high degree of encystation in response to starvation, and with subcultures growing in chemically defined nutrient medium (D cells) exhibiting a very low encystation competence. In both cultures the cell cycle starts with a short S phase taking place simultaneously with cytokinesis, followed by a long G_2 phase. A G_1 phase seems to be either absent or very short. Synchronization experiments reveal that in ND cells encystation is initiated from a particular position of late G_2. The high encystation competence of stationary phase ND cells seems to be due to arrest of cells at this particular cell cycle position. The lack of encystation competence of stationary phase D cells correlates with the loss of accumulation of cells at this particular stage of the cell cycle. This change of the property of cells is related to the growth condition and not to an irreversible loss of encystation competence of D cells.

Key words: *Acanthamoeba castellanii*, cell cycle, encystation, anti-BrdUrd labelling, flow cytometry.

Introduction

During the growth phase of *Acanthamoeba castellanii* little spontaneous encystment of trophozoites takes place. However, encystation can be induced by starvation, desiccation, high salt concentration and several drugs, especially inhibitors of mitochondrial macromolecule synthesis. It has been proposed that encystment is initiated from the G_1 phase of the cell cycle or from early or late S phase. More recently it has been suggested that development might occur from the G_2 phase (reviewed by Byers, 1979). Thus, until now it was not clear whether development in *Acanthamoeba* is related to a specific phase of the cell cycle.

Estimates of cell cycle phase duration in *Acanthamoeba* differ considerably. Early studies indicated cell cycles with M phases of 2%, G_1 phases of 10%–20%, S phases of 3% and G_2 phases of 75%–85%. Recent results indicated a G_1 phase of nearly 50% of the cell cycle (Edwards & Lloyd, 1980) or gave no evidence of a G_1 phase (reviewed by Byers, 1979, 1986).

In an effort to re-examine the question of whether there is any correlation between a specific phase of the cell cycle and the competence to differentiate, cell cycle phases were measured in *Acanthamoeba* cells, growing in a non-defined nutrient medium (ND cells), which show synchronous encystment after transfer of stationary phase cells into non-nutrient medium, as well as in a subculture, growing in chemically defined medium (D cells), which does not show starvation-induced encystment.

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Flow cytometric determination of DNA content in nuclei and of DNA synthesis by incorporated bromodeoxyuridine stained with fluorescein-conjugated anti-bromodeoxyuridine reveals that under both culture conditions G_1 phase nuclei seem to be absent and that the cell cycle starts with a short S phase (about 0·5 h of cell doubling times of 6–8 h in ND cells and 8–10 h in D cells).

Whereas only 5 to 8 % of asynchronously growing ND cells show starvation-induced encystment, 50–70 % of stationary phase ND cells encyst within 20–30 h. Synchronization experiments reveal that development in *Acanthamoeba* is initiated from a particular position of G_2 phase of the cell cycle. The high competence of encystation of stationary phase ND cells seems to be due to an arrestment of cells at this particular stage of the cell cycle whereas non-encysting stationary phase D cells are not being blocked at this position. These findings place the development of *Acanthamoeba* among those of other primitive eukaryotic organisms like *Dictyostelium* (Weijer *et al.* 1984*b*; Sharpe & Watts, 1985), yeast (Nurse & Bissett, 1981; Beach *et al.* 1982; Nurse, 1985) and *Hydra* (Hoffmeister & Schaller, 1987) in the sense that they also show a relationship between particular cell cycle phases and cell differentiation.

Materials and methods

Conditions of growth and development

Cultivation of *Acanthamoeba castellanii* (Neff strain) in the non-defined yeast extract–proteose peptone medium (ND cells) was carried out as described previously (Jantzen, 1974; Jantzen & Schulze, 1987). Cells grew with a doubling time of 6–8 h until a density of $1{\cdot}5\times10^7$ cells ml^{-1} was reached. Adaptation of these cells to the chemically defined growth medium DGM-21B containing eleven amino acids (D cells), was performed according to Byers *et al.* (1980) except as described (Jantzen & Schulze, 1987). These D cell cultures have been maintained in this laboratory for two years, and grew with a doubling time of 8–10 h until a density of 1×10^7 cells ml^{-1} was reached.

Standard stationary phase ND and D cells were taken three days after the termination of growth when the growth medium exhibited a pH of 7·3–7·4. To induce encystation stationary phase cells were transferred into non-nutrient medium (Weisman & Moore, 1969) to a final cell density of 2×10^6 ml^{-1}. Encystation-induced cultures of 25 ml volume were agitated by gentle shaking in 100 ml Erlenmeyer flasks. The degree of encystation was estimated from differential counts of amoebae and cysts. In order to obtain synchronously growing cultures, stationary phase cells were diluted out into fresh non-defined nutrient medium (2×10^6 cells ml^{-1}). The effectiveness of synchronizing procedures was assessed using the synchrony index $F=(N/N_0)-2^{t/g}$ (Blumenthal & Zahler, 1962), in which N is the number of amoeba after division, N_0 is the initial number of amoeba, t is the time taken to divide and g is the generation time. Cell numbers were determined in a Fuchs-Rosenthal haemocytometer or in an electronic cell counter (Coulter).

Preparation of nuclei

Nuclei were prepared by the Triton lysis method (Weijer *et al.* 1984*a*). 2×10^7 cells were pelleted by centrifugation for 3 min at 1700 ***g***. Cells in growth phase were resuspended in a solution of 0·6 ml H_2O and 2 ml 2·5 % Ficoll buffer (0·5 M-sorbitol, 0·5 mM-$CaCl_2$, 20 mM-Tris·HCl, pH 7·5). The cell suspension and 0·2 ml of 10 % Triton X-100 in 2·5 % Ficoll buffer were carefully mixed. Stationary phase ND or D cells were resuspended in a solution of 0·6 ml H_2O and 1·7 ml 2·5 % Ficoll buffer and lysed by addition of 0·5 ml 10 % Triton X-100 in 2·5 % Ficoll buffer. As soon as the majority of the cells were lysed (after about 5 min at 30 °C), lysates were layered onto a 1 ml cushion of 10 % Ficoll buffer and centrifuged at 1000 ***g*** for 20 min. Pelleted nuclei were resuspended in 1 ml 30 % glycerol in 2·5 % Ficoll buffer and stored at −15 °C. Counts of nuclei before and after cell lysis indicated that less than 10 % of the nuclei were lysed.

Determination of nuclear DNA content and size by flow cytometry

Samples of nucleus suspensions corresponding to 1×10^6 nuclei were centrifuged for 20 min at 2500 ***g***, and pelleted nuclei were resuspended in 1 ml diluted 2·5 % Ficoll buffer (2 vol. 2·5 % Ficoll buffer: 1 vol. H_2O). Without fixation of nuclei, the DNA was stained by addition of either 5 μl 10^{-3} M-4,6-diamidino 2-phenylindole (DAPI, AT-dye) or 40 μl 10^{-3} M-chromomycin A_3 (CA3, GC-dye). The distribution of DNA content of nuclei was determined by flow cytometry.

Determination of 5-bromodeoxyuridine incorporation into nuclear DNA.

Incorporation of 5-bromodeoxyuridine (BrdUrd) into DNA was determined according to Dolbeare *et al.* (1983). To exponentially growing cells at a density of 2×10^6 ml^{-1}, 5-fluorodeoxyuridine (FdUrd) and BrdUrd was added for 30 min to a final concentration of 3 μM and 400 μM, respectively. Simultaneous addition of FdUrd and BrdUrd was necessary in order to increase the incorporation of BrdUrd into DNA. Control experiments (e.g. see Fig. 5), and the results of other authors (Roti Roti & Stevens, 1974) indicated that concomitant addition of 3 μM-FdUrd and 400 μM-BrdUrd did not influence growth rates during an 8 h period. Samples corresponding to 2×10^6 cells were centrifuged and washed in 6 ml wash buffer (0·1 M-NaCl, 1 mM-EDTA, 10 mM-Tris·HCl, pH 7·4). For fixation of cells, cell pellets were resuspended in 100 μl wash buffer, mixed with 2 ml 70 % (v/v) ethanol (precooled at −20 °C) and stored at −20 °C for at least 2 h. In an effort to avoid clumping of cells during centrifugation, ethanol suspensions were underlayered with 500 μl 10 % glycerol in PBS (0·15 M-NaCl, 0·05 M-Na_3PO_4 (pH 7·2)). After centrifugation of cells at 1700 ***g*** for 4 min, cells were resuspended in 100 μl PBS and added to 5 ml 2 M-HCl for 30 min at 25 °C. The HCl-treated cell suspension was underlayered with 1 ml 10 % glycerol in 0·1 M $Na_2B_4O_7$ (pH 8·5) and cells were pelleted by centrifugation at 1700 ***g*** for 4 min, washed with 2 ml PBS and resuspended in 100 μl 0·5 % Tween 20 in PBS. Incorporation of BrdUrd was

determined by addition of 15 μl fluorescein (FITC)-conjugated anti-BrdUrd (Becton Dickinson, Catalog No. 7583) for 60 min. The suspension was underlayered with 60 μl 10 % bovine serum albumin in PBS and centrifuged at 1700 ***g*** for 4 min. Cells were suspended in 200 μl PBS and stored at +5 °C. Aliquots corresponding to 1×10^6 cells were suspended in 1 ml PBS, and the DNA was stained by addition of propidium iodide (PI) to a final concentration of 0·07 $\mu g\,ml^{-1}$. Analysis of stained cells by fluorescence microscopy (Zeiss, IM 35) revealed that the FITC label was exclusively located within the nuclei. Cells not pulsed with FdUrd/BrdUrd, but stained with anti-BrdUrd/PI showed no anti-BrdUrd staining. Bivariate DNA/BrdUrd distributions were analysed by flow cytometry.

Flow cytometry

Analysis of distribution of nuclear DNA content was performed with a computerized Ortho Cytofluorograph 30 L (Ortho Diagnostic Instruments) using the 360 nm or the 457 nm line of the argon laser as the excitation wave length for DAPI or CA3 stained nuclei, respectively.

Flow cytometry also permits simultaneous determinations of laser light scattering by nuclei (dependent on nuclear diameter) and DNA content. The results are displayed as two-dimensional contour plots of light scattering *versus* DAPI fluorescence.

Bivariate DNA/BrdUrd distributions were measured by using the 488 nm line of the same laser at 1 Watt for simultaneous excitation of FITC (BrdUrd incorporation) and PI (DNA content). The scattered laser line was blocked by using two low-pass filters at 500 nm. FITC and PI fluorescence were separated by a dichroic mirror (560 nm). Fluorescence was registered through a green bandpass filter (520 ± 10 nm) for FITC and a high-pass filter in the red (620 nm) for PI. The results are displayed on two-dimensional contour plots of FITC/PI fluorescence.

Results

Flow fluorometric measurements of nuclear DNA content and size

The distribution of nuclear DNA content of cells cultivated in a non-defined medium (ND cells) and in a chemically defined medium (D cells) was determined. Several results indicate that in *Acanthamoeba castellanii* a substantial amount of total DNA is represented by mitochondrial DNA (Byers, 1979, 1986). In an effort to avoid misinterpretations of the distribution of cellular DNA content, isolated nuclei instead of whole cells were stained with the fluorochromes DAPI or CA3. From flow fluorometric analysis of nuclear DNA content of exponentially growing cells one would expect a distribution indicating G_1, S and G_2 phase nuclei. However, the DNA distribution profiles show one prominent peak, indicating that the majority of exponentially growing cells must be present in one particular phase of the cell cycle (Fig. 1). Since the DNA content of the majority of ND and D cell nuclei is identical, it follows that growing ND and D cells are in the same phase of the cell cycle. However, whereas in log phase ND nuclei a symmetrical DNA distribution is obtained (Fig. 1A and C), this is not the case in log phase D cell nuclei (Fig. 1B and D). Irrespective of whether log phase nuclei are stained with the AT-dye DAPI or the GC-dye CA3 about 20 % of D cell nuclei exhibit a higher DNA content than the majority of D and ND nuclei. Furthermore, the distributions of light scattering suggest that the higher DNA contents are associated with larger nuclei than are found in ND cells (Fig. 1C and D). Fig. 2 shows the distribution of DNA content and size of stationary phase nuclei. In relation to the pattern obtained with nuclei of growing cells, the DNA and size distribution of stationary phase ND nuclei does not change. However, during the stationary phase of D cells, changes of nuclear DNA and nuclear size distribution take place. The DNA content seems to be somewhat lower. Mixing of stationary phase ND cell nuclei with stationary phase D cell nuclei reveals two DNA peaks after staining and flow cytometry (Fig. 3). This finding could suggest that during the stationary phase of D cells a small reduction of the nuclear DNA content takes place. Bivariate light scattering/DNA content distributions clearly indicate a twofold reduction of nuclear size (Figs 1D and 2D). In contrast to nuclei from growing D cells (Fig. 1B and D), stationary phase D cell nuclei (Fig. 2B and D) do not show the assymetrical distribution of DNA content. Thus, the population of log phase nuclei exhibiting higher than average DNA content could point to S phase nuclei. Consequently, this might suggest that the majority of growing D cells and of growing ND cells as well are G_1 cells. The absence of a comparable asymmetry in DNA plots obtained with log phase ND nuclei could indicate a very short S phase in these cells. Alternatively, the assymetry of distribution of DNA content suggests an increased nuclear DNA content during growth of D cells.

Flow fluorometric determination of the S phase by incorporation of bromodeoxyuridine into DNA

In an effort to identify nuclei from S phase amoeba, exponential cells were grown for a 0·5 h period in the presence of FdUrd and BrdUrd. The simultaneous addition of FdUrd and BrdUrd was necessary in order to enhance the incorporation of BrdUrd into DNA. Control experiments show that this procedure does not alter the growth rates of cells during an 8 h period (e.g. Fig. 5). The amount of incorporated BrdUrd *versus* the total DNA amount of nuclei was determined by staining of fixed cells with FITC-conjugated anti-BrdUrd and PI. If the assumption is correct that the majority of log phase D cell nuclei represents G_1 nuclei and the smaller population, exhibiting a higher DNA content, represents S phase nuclei, contour plots of

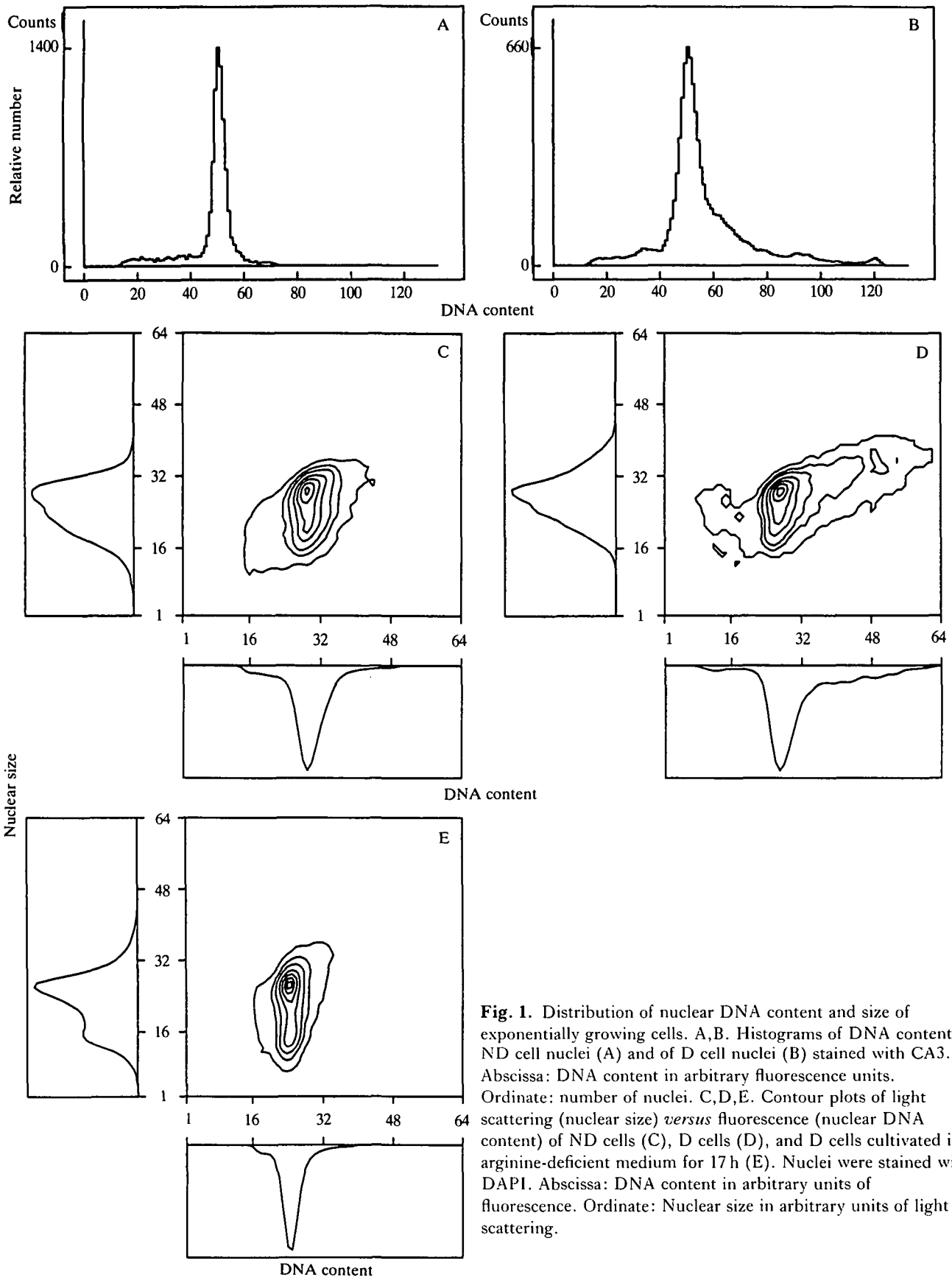

Fig. 1. Distribution of nuclear DNA content and size of exponentially growing cells. A,B. Histograms of DNA content of ND cell nuclei (A) and of D cell nuclei (B) stained with CA3. Abscissa: DNA content in arbitrary fluorescence units. Ordinate: number of nuclei. C,D,E. Contour plots of light scattering (nuclear size) *versus* fluorescence (nuclear DNA content) of ND cells (C), D cells (D), and D cells cultivated in arginine-deficient medium for 17 h (E). Nuclei were stained with DAPI. Abscissa: DNA content in arbitrary units of fluorescence. Ordinate: Nuclear size in arbitrary units of light scattering.

bivariate DNA/BrdUrd distributions would indicate that the FITC/PI-stained cell population shows higher DNA content than the majority of cells, which would stain with PI only. Fig. 4 shows that this obviously is not the case. After a 0·5 h FdUrd/BrdUrd pulses in D (Fig. 4A) as well as in ND cells (Fig. 4B), substantial FITC fluorescence is found in the left area of the PI fluorescence distribution, indicating that the FITC/PI labelled cohort has a lower DNA content than the major PI-stained cell population. Since the small

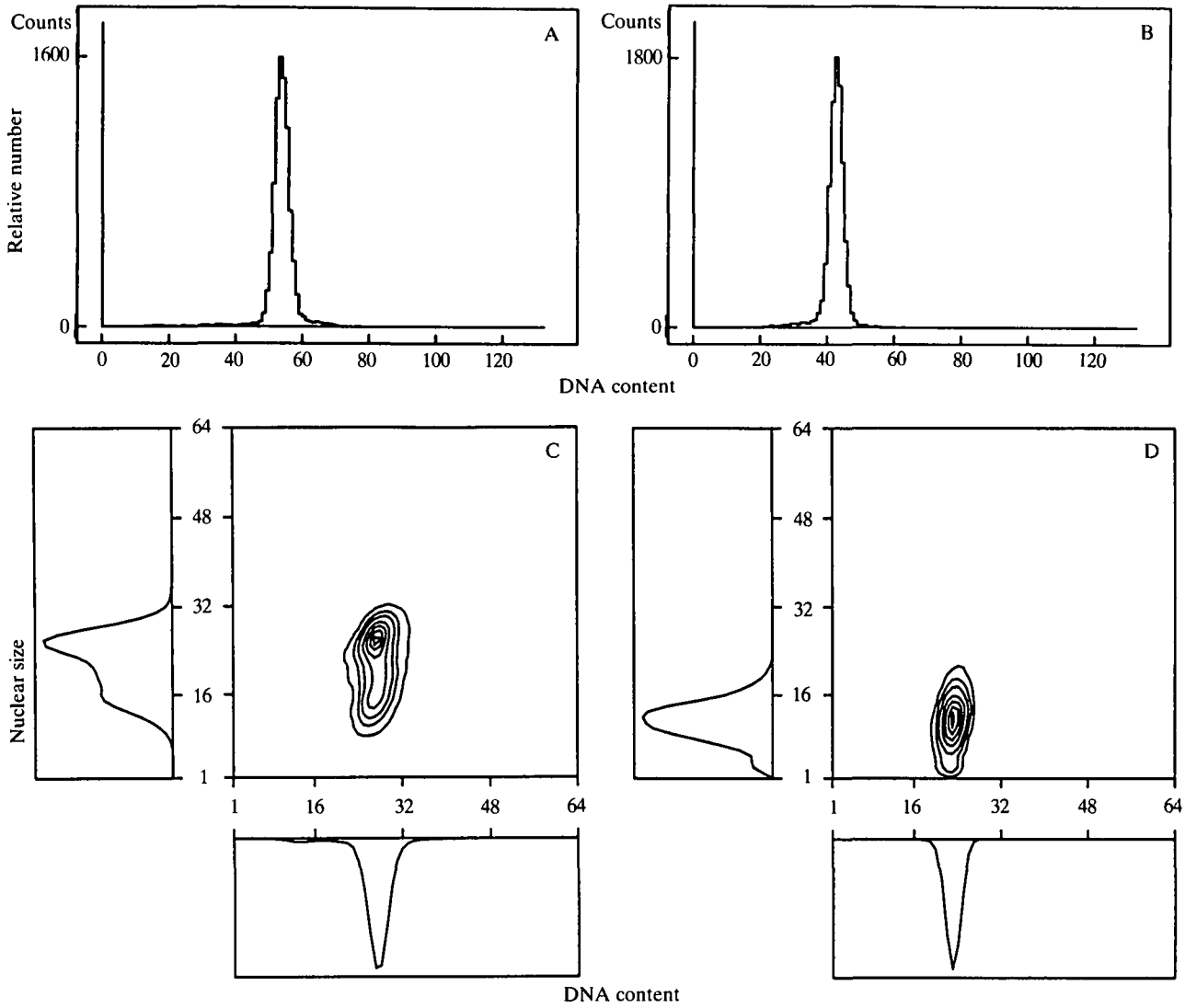

Fig. 2. Distribution of nuclear DNA content and size of stationary phase cells. A,B. Histograms of DNA content of ND cell nuclei (A), and of D cell nuclei (B) stained with CA3. C,D. Contour plots of nuclear size *versus* DNA content of ND cell nuclei (C) and D cell nuclei (D) stained with DAPI.

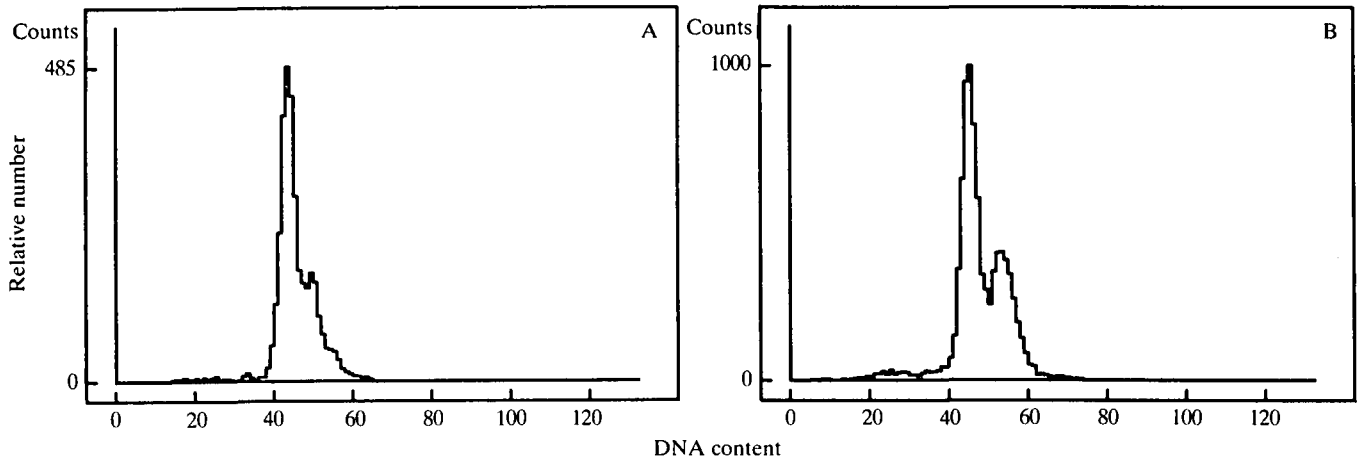

Fig. 3. Distribution of nuclear DNA content of a mixture of stationary phase ND cell nuclei with stationary phase D cell nuclei. Nuclei were prepared from stationary phase ND cells and D cells, then mixed and stained with either CA3 (A) or DAPI (B).

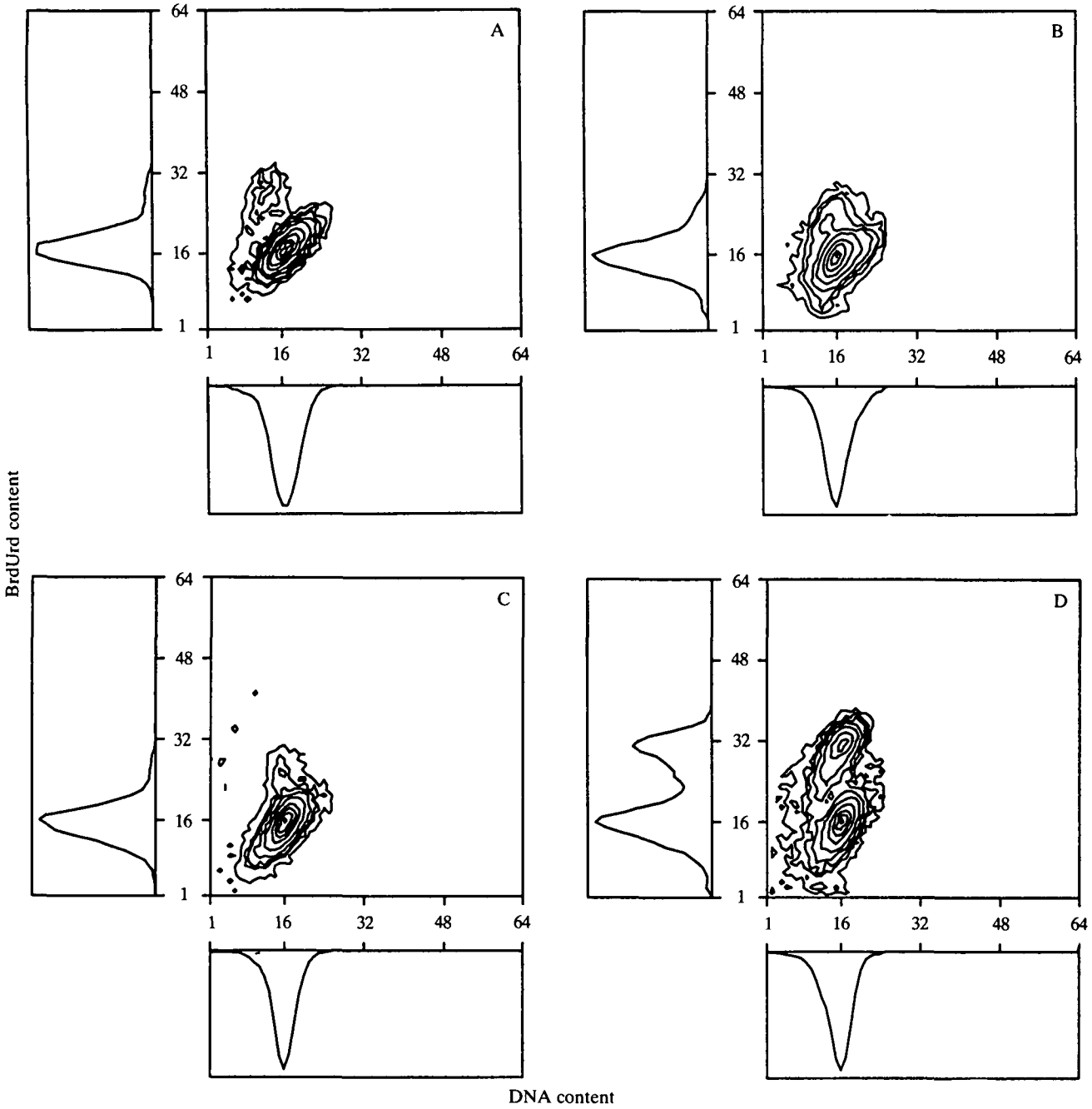

Fig. 4. Contour plots of bivariate DNA/BrdUrd distributions measured for exponentially growing cells. A,B. Exponential D cells (A) and ND cells (B) were grown in the presence of FdUrd/BrdUrd for 0·5 h and then fixed and stained with FITC-conjugated anti-BrdUrd and PI. C. Exponential D cells labelled for 0·5 h with FdUrd/BrdUrd and then transferred into fresh growth medium for 1 h. D. Exponential ND cells labelled for 1·8 h with FdUrd/BrdUrd. Abscissa: content of DNA in arbitrary fluorescence units (PI). Ordinate: content of incorporated BrdUrd in arbitrary fluorescence units (FITC).

population of FITC-labelled cells represents S phase cells, the major PI-labelled cell population represents G_2 phase cells. These results do not support the above suggestion, in contrast, they reveal that in D as well as in ND cultures the majority of cells represent G_2 phase cells.

In relation to the cell cycle duration of 6–8 h in ND cells and 8–10 h of D cells, the S phase is short. The evidence for this comes from the three following observations. (1) According to the FdUrd/BrdUrd pulse experiment shown in Fig. 4, the population of S phase nuclei should appear at the left-most side of the fluorescence distribution if the nuclear DNA content of exponentially growing cells is analysed by flow cytometry. However, as shown in Fig. 1, the distribution of nuclear DNA content in growing ND and D cells does

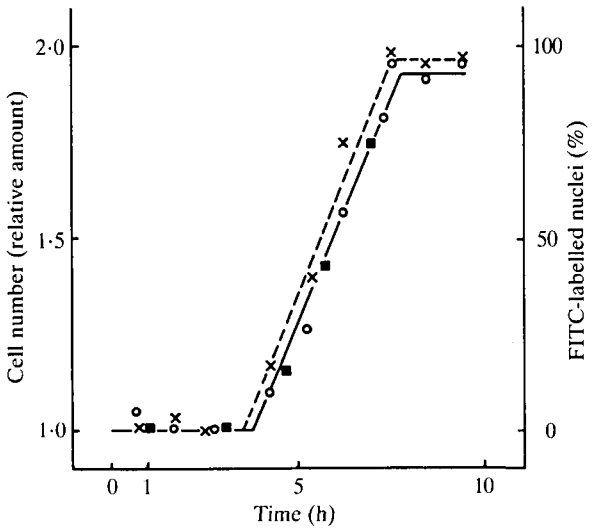

Fig. 5. Cell proliferation and DNA synthesis in a synchronous ND cell culture. Synchronization of cell growth was performed by release of standard stationary phase ND cells into fresh nutrient medium. Cell number was measured in a Fuchs-Rosenthal haemocytometer. DNA synthesis was determined by cultivation of cells in the presence of FdUrd/BrdUrd and staining of fixed cells with FITC-conjugated anti-BrdUrd. ○, cell number of a culture containing FdUrd/BrdUrd. ■, percentage of FITC-labelled nuclei in this culture. ×, cell number of a control culture.

not indicate a substantial amount of nuclei showing distributions of S phase DNA content. (2) During the 0·5 h FdUrd/BrdUrd pulse, only 7–8 % of ND nuclei and 5–6 % of D cell nuclei in exponentially growing cells become labelled. (3) Neither the distributions of nuclear DNA content shown in Fig. 1 nor the contour plots of bivariate DNA/BrdUrd distribution shown in Fig. 4 indicate the existence of a G_1 phase cell population in growing ND or D cell cultures. Thus, in *Acanthamoeba* this cell cycle phase seems to be either absent or very short. Assuming for simplicity the absence of a G_1 phase and a constant rate of DNA synthesis, the distribution of the FITC fluorescence should coincide with the distribution of the PI fluorescence if the duration of FdUrd/BrdUrd pulse equals or exceeds the length of the S phase. As shown in Fig. 4, a 0·5 h FdUrd/BrdUrd pulse in growing ND and D cells does not lead to an exact coincidence of the FITC and PI fluorescence distribution. The average DNA content of the small FITC/PI-labelled cohort is slightly smaller than that of the major PI-labelled cell population, indicating that the duration of the S phase exceeds (about 10 %) the 0·5 h duration of the BrdUrd/FdUrd pulse. If D cells are pulsed for 0·5 h with FdUrd/BrdUrd and then transferred into fresh growth medium for 1 h, and if ND cells are pulsed for 1·8 h instead of for 0·5 h, the FITC-labelled cohort and the PI-labelled population exhibit identical DNA distributions (Fig. 4C and D), indicating that the S phase in D as well as in ND cells can not be longer than 1·8 h. Since the 0·5 h FdUrd/BrdUrd pulse leads to nearly identical FITC/PI distributions, a duration of the S phase of about 0·5 h in ND as well as in D cells can be estimated.

The results indicate that independent of growth conditions, the cell cycle of *Acanthamoeba* starts with a short S phase followed by a long G_2 period and mitosis. However, whereas in ND cultures the majority of log phase as well as of stationary phase cells contain G_2 phase nuclei, this is not the case in D cultures. Nuclei of stationary phase D cells exhibit a lower than G_2 phase DNA content and nuclear size. In contrast, in about 20 % of the nuclei of growing D cells the G_2 phase DNA content seems to be exceeded.

Cell cycle-linked encystation

Transfer of asynchronously growing ND cells into non-nutrient medium leads to encystation of 5 to 8 % of cells within 72 h, whereas 50 to 70 % of standard stationary phase cells encyst within 20 to 30 h. As shown in the preceding sections, the majority of growing as well as of stationary phase ND cells exhibit G_2 phase DNA content. Thus, encystation could be either related to processes occurring specifically during the stationary phase or linked to a particular position of the G_2 phase at which stationary phase cells become arrested and from which encystation is initiated. To follow the encystation property during the cell cycle, cells were synchronized by release from the stationary phase and transferred into encystment medium at various times. Fig. 5 shows the increase in cell number and in BrdUrd-labelled nuclei after cells have been diluted out into fresh growth medium. After a lag of 3–4 h, cell division and concomitant increase of BrdUrd-labelled nuclei proceeds within 3–4 h, indicating a synchrony index of about 0·5. In a parallel culture which does not contain the BrdUrd/FdUrd label, the increase in cell number was also monitored. Comparison of the growth rates of these two cultures shows that the presence of BrdUrd/FdUrd does not influence the growth of cells. The concomitant increase of cell number and BrdUrd-labelled nuclei indicates that in exponentially growing ND cells the S and cytokinesis phase occur simultaneously. The degree of starvation-induced encystation of cells at various stages of the cell cycle is shown in Fig. 6. Standard stationary phase cells show a strong tendency to encyst. The encystment response of cells decreases up to the point at which about 90 % of the cells have been divided. During further progression through the cell cycle, the degree of encystation increases again. Although at no point in the cell cycle do the growing synchronized cultures have the same encystation potential as the stationary phase arrested

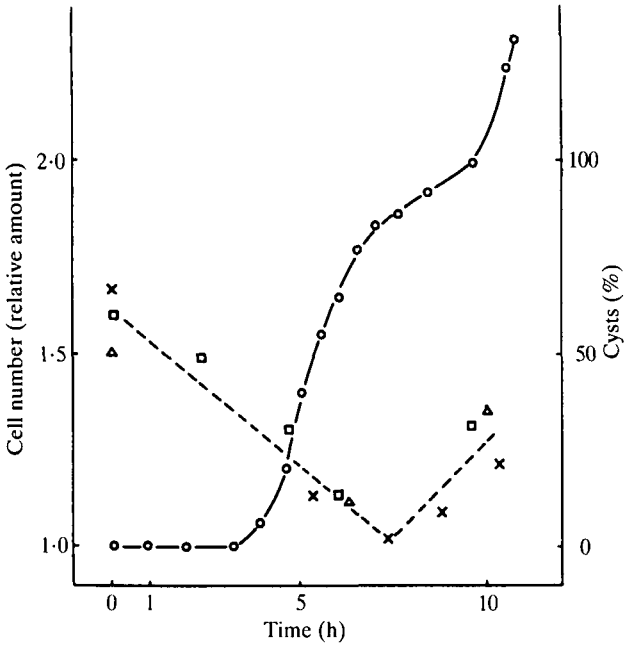

Fig. 6. The variation in the percentage of cysts formed from cells of synchronously growing ND cell cultures. Synchronization of cells was performed as described in the Fig. 5 legend. Cell number was measured with a Coulter counter. At the indicated times, cells were transferred into non-nutrient medium. After 48 h, the degree of encystation was determined from differential counts of amoeba and cysts. The results of three independent experiments are shown. (○) cell number. (×, □, △) percentage of cysts.

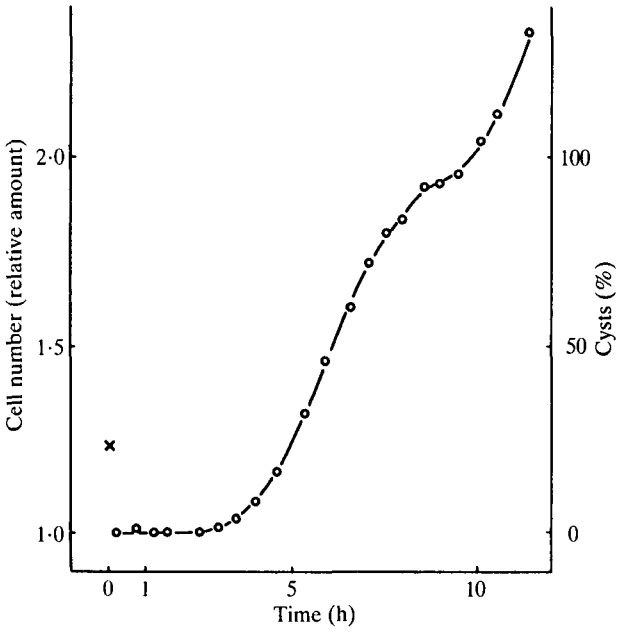

Fig. 7. Induction of encystation and growth in non-standard stationary phase ND cells. Non-standard stationary phase ND cells were taken two days after the termination of growth (instead of three days as in standard stationary phase cells) and transferred either into non-nutrient or fresh growth medium. The cell and cyst numbers were determined as in the Fig. 6 legend. (○) cell number. (×) percentage of cysts.

amoebae, the encystation potential of about 30 % of late G_2 phase cells clearly exceeds that of early G_2 phase cells and of asynchronously growing cells. The encystation potential of stationary phase cells of an earlier stage (two days instead of three days after termination of growth) is low. Only 20 % of these cells form cysts in non-nutrient medium. The low encystation potential correlates with a low synchrony of growth which starts after a 3 h lag when earlier stationary phase cells are transferred into fresh growth medium (Fig. 7). These results indicate a well-defined differentiation decision point in G_2 from which encystation can be initiated.

The encystation property of D cells is very low. Only 3 to 5 % of growing and 2 % of stationary phase D cells encyst after transfer into encystment medium. The above results suggest that the incompetence of stationary phase D cells to develop into cysts is due to an arrest of cells at an inappropriate position of the cell cycle. Differences of nuclear DNA content and size between stationary phase ND and D cells (Fig. 2) support this suggestion. Furthermore, unlike in ND cells, transfer of stationary phase D cells into fresh chemically defined nutrient medium does not lead to synchronous growth of cells. After a lag of about 15 h the cell number doubles within 11 h.

D cells can also be growth-arrested by omission of one of the five essential amino acids from the growth medium (Dolphin, 1976; Jantzen & Schulze, 1987). After deprivation of arginine, exponentially growing cells are arrested in the G_2 phase of the cell cycle (Fig. 1E). Thus, with respect to nuclear DNA content and size and arrest of growth, arginine-starved log phase D cells and stationary phase ND cells are similar. However, arginine-starved D cells neither develop into cysts when transferred into non-nutrient medium, nor grow synchronously when arginine is re-added.

In an effort to test whether the altered property of D cells is related to the growth condition or to an irreversible loss of encystation competence in response to prolonged cultivation of *Acanthamoeba* in the chemically defined nutrient medium, stationary phase D cells were inoculated in non-defined growth medium (DND cells). Like ND cells, DND cells double within 6–8 h, and encyst (50–60 % of cells) or grow synchronously (synchrony index of 0·5) when standard stationary phase cells are transferred into non-nutrient medium or fresh non-defined nutrient medium, respectively. Thus, although the growth rate and the cell cycle of ND and D cells are similar, the correct cell cycle arrest and thus the encystation competence seems to be affected by the altered growth condition of D cells.

Discussion

The cell cycle of ND cells

In exponentially growing *Acanthamoeba*, cultivated in a non-defined medium, the distribution of nuclear DNA content is unimodal as measured by flow cytometry. Contour plots of bivariate DNA/BrdUrd distribution and synchronization experiments show that the S phase occurs at the beginning of the cell cycle simultaneously with cytokinesis. These results imply that the G_1 phase is either absent or very short and that the majority of growing cells represent G_2 phase cells. The duration of the S phase and of the G_2 phase can be estimated to be about 0·5 h, and 6·5 h, respectively, in an average cell cycle of 7 h.

Several reports show that in *Acanthamoeba* G_2 is the predominant phase of the cell cycle, and that the S phase is relatively short (3–10% of the cell cycle duration). However, the estimated percentages of time in G_1 differ considerably. On the basis of [^{3}H]thymidine incorporation into nuclei, evidence for the absence of G_1 and initiation of nuclear DNA synthesis in late telophase was obtained for *A. castellanii* HR (*A.* rhysodes; Band & Mohrlok, 1973). However, other results indicate that the G_1 phase may occupy 10 and even 50% of a total generation time (Neff, 1971; Edwards & Lloyd, 1980). These varying results may be due to differences in the methodology. The results of Neff (1971) are based on [^{3}H]thymidine incorporation into cellular DNA, and those of Edwards & Lloyd (1980) on DNA content of synchronously growing cells obtained by centrifugation of exponential cultures and cultivation of the most slowly sedimenting population. Thus, these results do not rule out the possibility that also non-nuclear DNA synthesis has been determined or that the selection method isolated an atypical subpopulation of cells. Variations in the results on durations of the G_1 phase are also reported for other primitive eukaryotes. In *Dictyostelium discoideum*, for example, a G_1 phase seems to be absent and the reported variabilities of G_1 phase duration are proposed to be due to differences in the methodology (Weijer *et al.* 1984*a*). Furthermore, do cells from other primitive eukaryotic organisms like *Amoeba* (Makhlin *et al.* 1979), *Physarum* (Mohberg & Rusch, 1971) and *Hydra* (David & Campbell, 1972; Campbell & David, 1974) not go through a G_1 period of significant length.

The cell cycle of D cells

Acanthamoeba cultivated in a chemically defined medium for two years, double over a 8–10 h period. As in ND cells, the cell cycle starts with a short S phase (about 0·5 h) followed by a long G_2 phase. Thus, in relation to ND cells, the lengthening of the cell cycle duration seems to be due primarily to a lengthening of the G_2 phase. However, several observations also indicate important differences between ND and D cells. (1) The distribution pattern of nuclear DNA content in growing D cells shows an asymmetry not observed in ND cells. In view of the contour plots of bivariate DNA/BrdUrd distribution, this asymmetry indicates that a population of growing D cells exhibits an increased G_2 phase DNA content.

(2) During the stationary phase ND cells become blocked in the G_2 phase of the cell cycle. D cells do not seem to be halted at an equivalent position during the stationary phase, since these cells exhibit a smaller nuclear size and presumably a lower nuclear DNA content. The small degree of the reduction of nuclear DNA content exclude the simple explanation that stationary phase D cells are arrested in G_1. Other possible explanations could be: (a) arrest of cells during the S phase. (b) Reduction of the overall ploidy level of a polyploid genome (Byers, 1986), or selective sub-genomic reductions. (c) In view of the twofold reduced nuclear size, a changed structure of chromatin could be assumed. This could alter the binding of the fluorochromes. On the basis of agarose gel electrophoresis of nuclear DNA, preliminary results suggest chromosomal fragmentation in growing but not in stationary phase D cells. In an effort to obtain information about numbers, sizes and linkage groups of *Acanthamoeba* chromosomes the method of orthogonal-field-alternation gel electrophoresis will be used (Altschuler & Yao, 1985). Using this method we hope to answer the question whether selective sub-genomic reductions occur in stationary phase cells cultivated in the chemically defined medium.

(3) Transfer of stationary phase ND cells into non-nutrient medium or fresh growth medium leads to synchronous encystation or synchronous growth, respectively. Whereas, in stationary phase D cells neither synchronous encystation nor synchronous growth can be achieved by these procedures.

Cell cycle-linked encystation

The results presented show that the majority of exponentially growing cells as well as stationary phase cells of ND cultures exhibit G_2 phase DNA content. However, the data also indicate that these G_2 phase cells differ in their competence to develop into cysts after transfer into non-nutrient medium. Whereas 50 to 70% of standard stationary phase G_2 cells develop into cysts, only 5 to 8% of cells of an asynchronous exponential culture differentiate. The high competence of standard stationary phase ND cells to differentiate into cysts seems to be due to arrest of cells at a particular cell cycle position of late G_2 phase from which encystation can be initiated. This suggestion is supported by several observations. (1) Dilution of standard stationary phase cells into growth medium rather than starvation medium leads to synchronous

growth ($F = 0{\cdot}45$) after a 3 to 4 h lag. (2) The encystation potential of earlier stationary phase cells is low (20% cysts), and after transfer of these cells into nutrient medium cells grow with low synchrony ($F = 0{\cdot}27$) after a 3 h lag. (3) Considerable encystation of cells is also achieved when late G_2 phase cells of a synchronously growing culture are transferred into non-nutrient medium. However, relative to standard stationary phase cells the encystation potential is 2-fold lower. One possible hypothesis which could explain this relative low level of encystation is that although initiation of encystation is related to a specific position of the G_2 phase of the cell cycle, the formation of cysts is promoted by processes occurring specifically during the stationary phase. Another suggestion could be that the degree of synchrony decreases rapidly after transfer of cell cycle-arrested standard stationary phase cells into growth medium. The calculated synchrony index of 0·45 to 0·5 in these cultures could support this suggestion. (4) Stationary phase D cells which exhibit altered nuclear states are not committed to encystment. (5) Log phase D cells, growth arrested by amino acid starvation, neither encyst nor grow synchronously after transfer into complete medium although these cells exhibit the G_2 phase DNA content and nuclear size. This result would suggest that amino acid starvation does not arrest cells at the differentiation decision point of G_2 phase. However, secondary effects of depletion of an essential amino acid which lead to inhibition of encystment and synchronous growth can not be excluded.

A relationship between the cell cycle phase and the competence of encystment in *Acanthamoeba* has been proposed by earlier studies. From some reports the conclusion can also be drawn that encystation is initiated from the G_2 phase of the cell cycle. These studies were based either on microspectrophotometric analysis of nuclear DNA distributions (Byers, 1979) or on initiation of encystment in synchronous cultures obtained by a centrifugation selection method (Chagla & Griffiths, 1978). However, other studies suggest initiation of encystment from S phase (Neff & Neff, 1969; Neff, 1971; Rudick, 1971). These results are based on inhibitor studies, possibly a problematic methodology.

The low encystation competence of stationary phase D cells in relation to stationary phase ND cells is not related to heritable changes but to the growth conditions. Whereas the growth rate and the cell cycle is not affected appreciably by the growth conditions, fluorometric analysis of nuclear DNA content display a subpopulation of log phase D cell nuclei with increased genome size indicating differential DNA amplification. This phenomenon seems to be reversible, since after cultivation of D cells in the non-defined growth medium distributions of nuclear DNA content do not indicate populations showing an increased DNA content. Differential DNA amplification is a pervasive phenomenon in continuous cell lines and in tumours (Stark, 1986) as well as in lower eukaryotes like *Leishmania* (Coderre *et al.* 1983; Kink & Chang, 1987). In about 2% of growing *Amoeba proteus* cells some part of the nuclear DNA also seems to be replicated in interphase more than once (Makhlin *et al.* 1979). It is proposed that perturbations of cells that predominately affect DNA synthesis relative to RNA or protein synthesis will be particularly effective in stimulating over-replication (Schimke *et al.* 1986). Recently, genomic size variations have been found in various strains of the acellular slime mould *Physarum polycephalum* (Kubbies *et al.* 1986). In relation to our findings it is interesting to note that in two of these strains the emergence of mixoploidy parallels the loss of the ability to sporulate. Whether genome size variation in *Acanthamoeba* may affect cell cycle arrest and thus encystation competence deserves further investigation.

References

Altschuler, M. J. & Yao, M.-C. (1985). Macronuclear DNA of *Tetrahymena thermophila* exists as defined subchromosomal-sized molecules. *Nucl. Acids Res.* **13**, 5817–5831.

Band, R. N. & Mohrlok, S. (1973). The cell cycle and induced amitosis in *Acanthamoeba*. *J. Protozool.* **20**, 654–657.

Beach, D., Durkacz, B. & Nurse, P. (1982). Functionally homologous cell cycle control genes in budding and fission yeast. *Nature, Lond.* **300**, 706–709.

Blumenthal, L. K. & Zahler, S. A. (1962). Index for measurement of synchronization of cell populations. *Science* **135**, 724.

Byers, T. J. (1979). Growth, reproduction, and differentiation in *Acanthamoeba*. *Int. Rev. Cytol.* **61**, 283–341.

Byers, T. J. (1986). Molecular biology of DNA in *Acanthamoeba, Amoeba, Entamoeba*, and *Naegleria*. *Int. Rev. Cytol.* **99**, 311–341.

Byers, T. J., Akins, R. A., Maynard, B. J., Lefken, R. A. & Martin, S. M. (1980). Rapid growth of *Acanthamoeba* in defined media; induction of encystment by glucose-acetate starvation. *J. Protozool.* **27**, 216–219.

Campbell, R. D. & David, C. N. (1974). Cell cycle kinetics and development of *Hydra attenuata*. II. Interstitial cells. *J. Cell Sci.* **16**, 349–358.

Chagla, A. H. & Griffiths, A. J. (1978). Synchronous cultures of *Acanthamoeba castellanii* and their use in the study of encystation. *J. gen. Microbiol.* **108**, 39–43.

Coderre, J. A., Beverley, S. M., Schimke, R. T. & Santi, D. V. (1983). Overproduction of a bifunctional thymidylate synthetase-dihydrofolate reductase and DNA amplification in methotrexate-resistant *Leishmania tropica*. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2132–2136.

DAVID, C. N. & CAMPBELL, R. D. (1972). Cell cycle kinetics and development of *Hydra attenuata*. I. Epithelial cells. *J. Cell Sci.* **11**, 557–568.

DOLBEARE, F., GRATZNER, H., PALLAVICINI, M. G. & GRAY, J. W. (1983). Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5573–5577.

DOLPHIN, W. D. (1976). Effect of glucose on glycine requirement of *Acanthamoeba castellanii*. *J. Protozool.* **23**, 455–457.

EDWARDS, S. W. & LLOYD, D. (1980). Oscillations in protein and RNA content during synchronous growth of *Acanthamoeba castellanii*. *FEBS Lett.* **109**, 21–26.

HOFFMEISTER, S. A. H. & SCHALLER, H. C. (1987). Head activator and head inhibitor are signals for nerve cell differentiation in *Hydra*. *Devl Biol.* **122**, 72–77.

JANTZEN, H. (1974). Polyadenylsäure enthaltende RNA und Genaktivitätsmuster während der Entwicklung von *Acanthamoeba castellanii*. *Biochim. Biophys. Acta* **374**, 38–51.

JANTZEN, H. & SCHULZE, J. (1987). Effect of essential amino acids on the phosphorylation of a 40 S ribosomal protein and protein synthesis in *Acanthamoeba castellanii*. *J. cell. Physiol.* **130**, 444–452.

KINK, J. A. & CHANG, K.-P. (1987). Tunicamycin-resistant *Leishmania mexicana amazonensis*: Expression of virulence associated with an increased activity of *N*-acetylglucosaminyltransferase and amplification of its presumptive gene. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1253–1257.

KUBBIES, M., WICK, R., HILDEBRANDT, A. & SAUER, H. W. (1986). Flow cytometry reveals a high degree of genomic size variation and mixoploidy in various strains of the acellular slime mold *Physarum polycephalum*. *Cytometry* **7**, 481–485.

MAKHLIN, E. E., KUDRYAVTSEVA, M. V. & KUDRYAVTSEV, B. N. (1979). Peculiarities of changes in DNA content of *Amoeba proteus* nuclei during interphase. *Expl Cell Res.* **118**, 143–150.

MOHBERG, J. & RUSCH, H. P. (1971). Isolation and DNA content of nuclei of *Physarum polycephalum*. *Expl Cell Res.* **66**, 305–316.

NEFF, R. J. (1971). Synchronous cultures in cytodifferentiation studies. *In Vitro* **6**, 300–308.

NEFF, R. J. & NEFF, R. H. (1969). The biochemistry of amoebic encystment. *Symp. Soc. Exp. Biol.* **23**, 51–81.

NURSE, P. (1985). Cell cycle control genes in yeast. *Trends Genet.* **1**, 51–55.

NURSE, P. & BISSETT, Y. (1981). Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. *Nature, Lond.* **292**, 558–560.

ROTI ROTI, L. W. & STEVENS, A. R. (1974). Effect of 5-bromodeoxyuridine on growth, encystment, and excystment of *Acanthamoeba castellanii*. *J. Cell Biol.* **61**, 233–237.

RUDICK, V. L. (1971). Relationship between nucleic acid synthetic patterns and encystment in aging unagitated cultures of *Acanthamoeba castellanii*. *J. Cell Biol.* **49**, 498–506.

SCHIMKE, R. T., SHERWOOD, S. W., HILL, A. B. & JOHNSTON, R. N. (1986). Overreplication and recombination of DNA in higher eukaryotes: Potential consequences and biological implications. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2157–2161.

SHARPE, P. T. & WATTS, D. J. (1985). The role of the cell cycle in differentiation of the cellular slime mould *Dictyostelium discoideum*. *Molec. Cell Biochem.* **67**, 3–9.

STARK, G. R. (1986). DNA amplification in drug resistant cells and in tumours. *Cancer Surveys* **5**, 1–23.

WEIJER, C. J., DUSCHL, G. & DAVID, C. N. (1984*a*). A revision of the *Dictyostelium discoideum* cell cycle. *J. Cell Sci.* **70**, 111–131.

WEIJER, C. J., DUSCHL, G. & DAVID, C. N. (1984*b*). Dependence of cell-type proportioning and sorting on cell cycle phase in *Dictyostelium discoideum*. *J. Cell Sci.* **70**, 133–145.

WEISMAN, R. A. & MOORE, M. O. (1969). Bead uptake as a tool for studying differentiation in *Acanthamoeba*. *Expl Cell Res.* **54**, 17–22.

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