NUCLEOLAR AGING IN _TETRAHYMENA_ DURING
THE CULTURAL GROWTH CYCLE

BIRGIT SATIR and ELLEN ROTER DIRKSEN

From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720, and the Cancer Research Institute, University of California, San Francisco, California 94122

**ABSTRACT**

Nucleolar morphology was studied by electron microscopy in control and actinomycin D-treated populations of _Tetrahymena pyriformis_ (W) during the cultural growth cycle. Nucleoli exhibit an “aging” cycle concomitant with the cultural growth cycle, but independent of the individual cell cycle. Four different stages in the course of this aging process have been defined. Stage I occurs upon inoculation (low number of cells per milliliter) and lasts through lag and accelerating growth phases. In this stage, many small nucleoli are found at the nuclear periphery. In stages 2 and 3, nucleolar fusion begins. Stage 2 dominates the first half of logarithmic growth, and stage 3 dominates the second half. In late decelerating growth phase, the nucleoli enter stage 4. In this stage, only a few large nucleoli are present and these are apparently inactive in ribosome production. In stationary phase, where total RNA remains constant, only stage 4 nucleoli are present. The relative preponderance of granular vs. fibrillar components in the nucleoli changes during this cycle, the granular component dominating stage 1 nucleoli and the fibrillar, stage 4 nucleoli. There is a shortening of the intermediate nucleolar stages in the treated cultures; fusion occurs early and is now pronounced. Not enough ribosomes accumulate to carry the treated cultures through the number of generations equivalent to those of the control, which produces a premature stationary phase.

**INTRODUCTION**

In a previous study of the growth of populations of an amicronucleate strain of _Tetrahymena pyriformis_ (Satir, B., 1967) it was shown that cultures exposed to actinomycin D during the entire growth cycle exhibit a prolonged lag phase and a “premature” stationary phase compared to control cultures. These phenomena were shown to coincide with changes in the pattern of total RNA accumulation. It is known that RNase activity is low in _Tetrahymena_ populations during initial growth and log phases (Lazarus and Scherbaum, 1968), so that these results probably reflect RNA synthesis, which, in turn, is assumed to reflect variations in ribosomal RNA, since about 80% of the cell’s RNA is ribosomal. Numerous studies (Stevens, 1964; Rodriguez, 1967; Stenram, 1968; and Goldblatt et al., 1969) have shown that one of the organelles affected by actinomycin D is the nucleolus. In eucaryotes the centers for the synthesis of ribosomal RNA were shown, by Brown and Gurdon (1964), McConkey and Hopkins (1964), and later by Ritossa and Spiegelman (1965), to be the nucleoli. Actinomycin D causes changes in nucleolar morphology by altering the relationship between the granular vs. fibrillar components, in extreme cases separating the granular component from the main body of the nucleolus. In these cases rRNA (ribosomal RNA)
synthesis is also interfered with or stopped completely.

For a micronucleate strain of *Tetrahymena*, Cameron and Guile (1965) and Cameron et al. (1966) showed that nucleoli were flexible structures capable of reflecting nutritional changes and undergoing fusion and disaggregation. As cultures grew, Cameron and Guile found that numerous, small, peripheral nucleoli progressively aggregated into a few huge masses, and that upon inoculation the process was reversed. Using the same strain, Flickinger (1965) was unable to demonstrate consistent changes during the cell cycle, although some variation in nucleolar number and distribution was present. In this paper we have undertaken a detailed study of nucleolar morphology during the cultural growth cycle, and an examination of the effects of actinomycin D on this morphology. We will show that it is possible to stage nucleoli, independent of the cell cycle, with regard to the phase of cultural growth. Our conclusions suggest that nucleoli age during population growth and that this aging can be accelerated by treating the cultures with actinomycin D.

MATERIALS AND METHODS

Cultural Growth

Cultures of *Tetrahymena pyriformis* (W) were grown axenically on 2% proteose peptone at 26°C in 250 ml Erlenmeyer flasks in the dark. All cultures were inoculated with cells from stationary phase (65-75 hr old) to give a final cell concentration of 10,000-15,000 cells per ml in a total volume of 50 ml. Increase in cell number was routinely followed by counting with a Coulter counter, model B. Actinomycin D was added to cultures prior to inoculation, to give a final concentration of 10 μg per ml. The drug, when added, remained present throughout the experiment. A detailed analysis of the cultural growth cycle has been presented previously (Satir, B., 1967) and the major results are summarized in Fig. 1. The growth curves obtained here confirm previous work in all respects.

Electron Microscopy

Control or actinomycin D-treated cultures were fixed for electron microscopy at various times during the cultural growth cycle. The cells were either (a) fixed in 3% glutaraldehyde and postfixed with 2% OsO₄ in 0.025 M phosphate buffer, pH 7.4, or (b) treated according to the procedure of Williams and Luft (1968). In some experiments the cells were stained "in block" according to the method of Kellenberger et al. (1958). Material was dehydrated in ethanol or acetone and embedded in Epon. Sections were cut on a Porter-Blum MT2 ultramicrotome and were usually gold-silver in color. They were doubly stained with uranyl acetate and lead citrate (Reynolds). Micrographs were taken with a Siemens 1A electron microscope.

Distribution of Stages during Cultural Growth

In a series of cultures, histograms (Figs. 1 and 2) of the distribution of nucleolar stages at various times...
FIGURE 3 5 hr old control culture (stage 1). Numerous nucleoli (nu) are present, peripherally located along the nuclear membrane, and the nucleolar organizer region (NO) can be seen in several of them. Microtubules (arrows) are evident in several areas of the nucleus (N), apparently not aligned along any particular axis but distributed at random. The chromatin stains intensely and is found evenly distributed throughout the nucleus. The cytoplasm is rich in ribosomes, and several mitochondria (M) with their characteristic tubular cristae can be seen. × 10,000. Insert: Note the predominance of the granular component (g) at this stage, and fibers (arrow) radiating into this mass from the center. × 120,000.
in the cultural growth cycle were constructed by staging, at the electron microscope level, 10-15 different nuclei at each critical age. Care was taken to avoid duplicate counting of serial sections from the same cell. Several different blocks of cells at the same age were used for the count. Since a single stage predominates at each age, additional preparations were examined qualitatively. The results are entirely consistent. To test if a correspondence existed between nucleolar stage and the cell cycle, we staged each of the nuclei shown in Figs. 11-18 of Flickinger (1965) taken at equidistant time points in the cell cycle. We have assumed that a log phase population is a truly random population, in that equal numbers of cells of each age of the cell cycle are present. On this basis we were able to plot the distribution of nucleolar stages predicted for log phase (Fig. 1, striped area).

Several criteria were used in nucleolar staging: (a) the number of nucleoli present in each nucleus, (b) their size and shape, (c) the morphology of each nucleolus in regard to the relative proportion of granular vs. fibrillar material, and (d) the relation of the nucleoli to the nuclear membrane.

RESULTS

Nucleolar Morphology of Control Populations

Here we deal with the characteristic morphology of the nucleoli of control populations from stage to stage.

Stage 1 (Fig. 3) Here is shown a nucleus from a 5 hr old cell. Numerous, small, cup-shaped nucleoli (nu), having an average size of 500 m, are present. They are aligned in groups of approximately six nucleoli, along and in close contact with the nuclear membrane. The major component present in these nucleoli is a granule, 100-150 A in diameter. Electron-opaque areas can be seen in association with several nucleoli: at this stage there is one per nucleolus. Using labeled thymidine, Charret (1969) has convincingly demonstrated that these dense globules resembling chromatin in nucleoli of this appearance are nucleolar organizer regions (NO).

The insert shows a typical example of the appearance of nucleoli at this stage, where granular material is the dominant component. The granules vary in size from 100 to 150 A in diameter and form the entire bowl of the cup. Thin fibers (arrow) extend from the chromatin mass at the center of the cup to the granular region (g).

Stage 2 (Fig. 4) An example of a nucleus from a 24 hr old population. There are fewer nucleoli present and these have an average size of 700 m. These nucleoli are in close contact with the nuclear membrane. Several of them have a cup-shaped appearance, and in longitudinal section it is possible to see the nucleolar organizer region at the center of the cup (NO). This region is now surrounded by a medulla of fibrous material while the granules are organized into a distinct cortex. Fine connections are found between the cortex and the nuclear membrane (see insert, top), and attached ribosomes can also be seen on the cytoplasmic side of the nuclear membrane.

Stage 3 (Fig. 5) The nucleus of a cell from a 45 hr old population just prior to entering stationary phase. There are fewer nucleoli and these are commonly found in groups of three to five, with the average size and distance between groups becoming larger. The nuclear membrane sometimes forms outpocketings which contain the nucleoli. Occasional nucleoli are observed which seem to have two nucleolar organizer regions associated with them (arrow). In the insert, bottom, another example is shown at higher power. Fine fibers radiating from the nucleolar organizer regions are sometimes seen (arrow). Again at this stage, a thin granular cortex (g) surrounds the fibrous core (f) of each nucleolus, the latter repre-

Figure 4 24 hr old control culture (stage 2). Total number of nucleoli has decreased and their size has slightly increased. In the nucleus, microtubules (arrow) can still be found, and the chromatin appears somewhat thickened. It has been suggested that this thickening represents chromosome duplication (Roth and Minick, 1961). Note that in the posterior end of the nucleus there appears to be a line-up of heterochromatic regions connected by finer, less dense threads. X 12,500. Insert, top: Note many fiber-like connections (black lines) which are present between the inner nuclear membrane and the granular cortex of the nucleolus. Also, note the dense nucleolar organizer region (NO). X 72,000.

Figure 5 45 hr old control culture (stage 3). Still fewer nucleoli are present. In one case, two nucleolar organizer regions are associated with one nucleolus (arrow). X 20,000. Insert, bottom: This shows the predominance of the fibrous core (f) and the now narrow granular cortex (g). Fine fibers (arrow) radiate from the nucleolar organizer region. X 98,000.
FIGURE 6 68 hr old control culture (stage 4). Very few nucleoli are present. Note the extensive loop formation. Arrow indicates where two or three nucleolar organizer regions are present. × 24,000. Insert: Higher magnification shows the thin granular cortex (g) and the extensive fibrillar core (f). A nuclear pore (np) is also seen. × 84,000.

senting the major component now. The presence of two nucleolar organizer regions and the generally smaller number of nucleoli in each group might suggest fusion of at least two of the smaller nucleoli present at earlier cultural ages (i.e., nucleoli of stages 1 and 2). Actual fusion has not been observed, but is inferred from changes in size, shape, and number. In control cultures, no more than two, or rarely three, nucleolar organizer regions are ever observed in each nucleolus.

stage 4 (Fig. 6) The smallest number of nucleoli and the largest in size of any stage in the cultural growth cycle are found in 68 hr old cultures. These nucleoli are up to circa two times larger than stage 1 nucleoli. Arrow indicates where two or three nucleolar organizer regions are present. The close association with the nuclear membrane has decreased and the nucleoli appear to migrate towards the center of the nucleus. The fibrous material has become pronounced while only a thin rim of granular material still persists. The increase in size seems somewhat larger than could be accounted for by fusion of two or three stage 1 nucleoli, and probably also represents accumulation of nuclear products. Around the nucleoli extensive loops and rings have formed which normally consist of granular material, whereas the major nucleolar component is the fibrous core (f). At a higher power (insert) the rim of granular material can be followed along the edge of the nucleolus and into the loops and rings. One nucleolar organizer region is clearly seen, as well as a nuclear pore (np).
Sequence of Nucleolar Stages

Figs. 1 and 2 show the distribution of nucleolar stages in control and actinomycin D–treated cultures during the cultural growth cycle. In 5 hr old control cultures (Fig. 1), 90% of the cells have their nucleoli in stage 1 and the remaining 10% in stage 4. Since stage 4 characterizes all the cells at 68 hr, and these cells are used to inoculate cultures (0 hr), we interpret this profile to mean that the nucleoli disaggregate during the first 5 hr of cultural growth. This is in agreement with the data of Cameron and Guille (1965). The granular component is accumulating at this time, and the nucleoli approach the nuclear membrane. Morphologically, stage 1 must correspond to profiles of nucleoli actively synthesizing ribosomes, since it has been previously demonstrated that RNA per cell is increasing 5 hr after inoculation (Satir, B., 1967). The 24 hr profile shows the majority (75%) of cells in nucleolar stage 3. Stages 2 and 4 are also present (10% and 15% of the population, respectively), but no stage 1 nucleoli are encountered. By decelerating growth phase just prior to stationary phase, all cells appear to possess stage 4 nucleoli. We interpret these nucleoli as being largely inactive in RNA synthesis, insofar as RNA per cell is constant throughout stationary phase (Satir, B., 1967). The bulk of the nucleoli are invariably found in one stage, which then changes sequentially as the culture ages. Nucleolar stage, therefore, does correspond to cultural age.

As Fig. 1 illustrates, the population is not entirely synchronized with respect to nucleolar stage. However, the sequential progression of stages 1–4 in the population suggests that each individual cell goes through these stages in succession. The small, stage 1 nucleoli apparently fuse and become progressively less granular as cultural aging proceeds. A comparison between the log phase profile and the one obtained by staging Flickinger’s results on the single cell cycle (striped area) is also shown in Fig. 1. The two profiles are clearly different, for, according to our calculations, based on Flickinger’s micrographs, 42% of the cells in logarithmic growth should have their nucleoli in stage 1. Since this is clearly not the case, we must conclude that the change in nucleolar staging that we have observed does not correspond to aging during the cell cycle.

Histograms illustrating the distribution of nucleolar stages in actinomycin D–treated cultures are shown in Fig. 2. The exact distribution of stages differs somewhat from that of stages in control populations. RNA synthesis is slower, and possibly stops earlier in these cultures (Fig. 11 of Satir, 1967). In mid log phase, actinomycin D–treated cultures show less incorporation of tritiated uridine into their nuclei than do control cultures. Correspondingly, there is a shortening of the intermediate nucleolar stages in actinomycin D–treated cultures through the cultural growth cycle, compared to that of controls. In a 5 hr old treated culture all cells have been scored as being in stage 1, as in the controls. However, there are slight differences in ultrastructural morphology between treated and control cultures. In particular, the nucleoli appear somewhat more amorphous and less granular in treated cultures. After 24 hr, although the cells are in logarithmic growth phase, the profile of nucleolar stage has shifted drastically so that 70% of the cells appear in stage 4 and 30% in stage 3. The distribution of nucleoli at this age is indistinguishable from that of nucleoli of controls 1 day older (after 45 hr). In treated cultures 45 hr old, all cells have been scored as stage 4*. As in control stage 4 nucleoli, these nucleoli are also fused, large, mainly fibrous, and probably inactive. We distinguish these nucleoli from true stage 4 nucleoli however, because nucleolar fusion in these cases has progressed much further here than is ever found in control cultures.

Nucleolar Morphology of Actinomycin D–Treated Cultures

Fig. 7 shows the nucleus from a cell of a 5 hr old, actinomycin D–treated culture scored as stage 1. These cells contain nuclei in which the nucleoli are spread out like flocculent patches along the nuclear membrane. However, it should be noted that the size of the nucleoli is about twice that of the control, stage 1, and that they are more irregular in shape. It is evident that part of this size difference could be due to the presence of the fibrous material (f) in these nucleoli. The sizes of the granules at this stage are within the same range as those found in the controls (100–150 Å). Typical nuclei from cultures exposed to actinomycin D for 24 hr can be seen in Figs. 8 and 9; Fig. 8 is especially illustrative. The number of nucleoli has decreased while their individual size has increased. Nucleoli can now be found in outpocketings of the nuclear membrane. The nucleoli of these cells are practically identical to stage 4 nucleoli of the controls (Fig. 6). Loop and ring appendages con-
FIGURE 7  5 hr old actinomycin D-treated culture (stage 1). The nucleoli are here increased in size. At this stage the fibrous material (f) is present to a greater extent than in the controls. X 65,000.

FIGURE 8  24 hr old actinomycin D-treated culture. In these cultures, the nucleoli are found in pockets of the nuclear membrane. Note the rows of nuclear pores (arrow). X 12,000.

FIGURE 9  Higher magnification of a 24 hr old actinomycin D-treated culture (stage 4). Extensive loop formation, comparable to that of stationary phase control culture (see Fig. 6), is already present in 30% of the cells at this age. X 55,000.
Figure 10  45 hr old treated culture (stage 4*). At this age these cultures contain giant nucleoli (arrow), due to extensive fusion of nucleoli. A large autophagic vacuole (V) can be seen in the cytoplasm; these vacuoles are found more frequently in treated cultures. × 12,600. Insert: High-power micrograph of the giant nucleolus. Note the presence of many nucleolar organizer regions (NO) associated with this nucleolar mass. × 36,000.
sisting of granular material can be seen here. The fibrous core again comprises the majority of the nucleolus.

Fig. 10 represents a cell from a 45 hr old actinomycin D-treated culture, which, at this stage, can possess enormous nucleoli. An example, at low power, of a giant nucleolus, five times the size of a normal stationary phase control nucleolus, can be seen (arrow). Such nucleoli are never seen during the normal cultural growth cycle. For convenience, we have scored these as stage 4* nucleoli, although they are clearly abnormal. Apparently, nucleolar fusion progresses to a far greater extent after drug treatment than in the controls. The cytoplasm is relatively undamaged, although the chromatin appears very dense here. Vacuoles are also frequently present in treated cells, but it should be kept in mind that, if these cells are used for inoculation of new cultures, their growth pattern returns to normal. Therefore, no permanent damage has been done to these cells. In experiments with tritiated actinomycin D, the label is found over the nucleus and many grains are associated with the large cytoplasmic vacuoles. In the insert, at least 10 nucleolar organizer regions can be seen in one nucleolus, which suggests that fusion of 10 or more smaller nucleoli may have formed this giant mass. Further, the fibrous center in such nucleoli is very extensive, but a thin rim of granules can still be seen.

A summary of the nucleolar events is shown in Fig. 11.

**Discussion**

We have demonstrated that, in control and treated cultures of *Tetrahymena*, nucleoli undergo an "aging" cycle concomitant with the cultural growth cycle. This aging cycle has been shown in our system to be independent of the individual cell cycle since (a) the majority of cells at any one age are in one well-defined nucleolar stage and (b) this stage changes sequentially in an easily observed fashion. The cycle is completed, at a new inoculation, with the "old," large, and probably fused nucleoli of stage 4 reverting to the small granular numerous nucleoli which characterize stage 1. This interpretation is in agreement with the results of Cameron and Guile (1965).

The aging cycle can be taken as a direct expression of the cell's accumulation of ribosomal RNA and the activity of the ribosomal genes. It should be recalled that, unlike the nuclear membrane of most metazoan cells, that of *Tetrahymena* does not break down at cell division. The results obtained on total RNA content for control cultures indicate that RNA per cell, which presumably reflects mainly rRNA, reaches a peak after 10-15 hr of growth and then levels off gradually until stationary phase (50 hr), when it remains constant. In this study we find the greatest number of nucleoli per cell at inoculation (stage 1). This number gradually decreases in stages 2 and 3 until, in stationary phase, only a small number of residual nucleoli are found corresponding to stage 4. It is tempting to assume that stage 1 nucleoli are so active in rRNA synthesis that they outpace the cell cycle, thus allowing RNA to accumulate. In late nucleolar stages, synthesis presumably does not keep pace with cell division, which usually manifests itself in these cells by the presence of fewer and fewer nucleoli.

Nucleolar fusion must occur during cultural growth since: (a) stage 4 nucleoli are much larger than stage 1 nucleoli, (b) stage 4 nucleoli often contain two nucleolar organizer regions, and (c) fewer nucleoli are present at stage 4 than at stage 1 in random sections. Fusion must be accelerated in the actinomycin D-treated cells. It seems likely that, in this case at least, fusion represents a mistake in the coordinated release of ribosomal precursors.

In the actinomycin D-treated cultures, RNA piles up during the prolonged lag. There is then a sharper decline in RNA content per cell. This faster decline is mirrored in the earlier and more pronounced nucleolar fusion which has already taken place in mid-log phase. The morphology of the nucleoli of actinomycin D-treated cells at 24 hr corresponds almost exactly to that of controls at 45 hr. Thus, the fused stage 4 nucleoli wherever found, are probably relatively inactive in rRNA synthesis. Since rRNA synthesis is inhibited by actinomycin D, not enough ribosomes accumulate to carry the treated cultures through the numbers of generations equivalent to those of the controls. This is presumably one of the factors producing the premature stationary phase.

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