A Comparison of Cell Cycle-related Changes in Postmitotic and Quiescent AF8 Cells as Measured by Cytofluorometry after Acridine Orange Staining

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ABSTRACT

AF8 cells were collected by mitotic detachment or made quiescent by serum restriction. Replated mitotic cells or serum-stimulated quiescent cells were then compared by flow cytofluorometry, with the use of acridine orange staining. Red fluorescence intensity ($F_{\geq 600}$) was the same in quiescent cells and in cells immediately after mitosis. However, $F_{\geq 600}$ increased very rapidly in postmitotic cells, while there was a delay in serum-stimulated quiescent cells. $F_{\geq 600}$ reached a peak at 4 hr in postmitotic cells and between 16 and 19 hr in serum-stimulated quiescent cells. A similar delay in the time of entry into S phase occurred after serum stimulation of resting cell populations. The results are compatible with the hypothesis that cells after mitosis may enter a state that is different from the state of cells made quiescent by serum restriction.

INTRODUCTION

Smith and Martin (16) have postulated that following mitosis all cells enter an indeterminate (A) phase, from which they leave asynchronously after an indefinite period of time, as a result of the random occurrence of some hypothetical critical event. The cells then enter a deterministic (B) phase of relatively constant duration which is terminated by mitosis. According to the transition probability theory of Smith and Martin (16), all cells enter the A state after mitosis, including cells that are made quiescent by a variety of conditions restrictive for growth (for a review, see Refs. 2 and 13).

Darzynkiewicz et al. (4, 8) have developed a simple cytofluorometric assay that distinguishes stimulated from nonstimulated lymphocytes. The assay consists of staining fixed cells with AO and of determining with a flow microfluorometer the intensity of the green and red fluorescences caused by the interaction of the dye with the cellular nucleic acids (18). Under appropriate conditions, green fluorescence ($F_{\text{glo}}$) is proportional to the amount of DNA. Red fluorescence ($F_{\geq 600}$) is largely related to RNA content, and it is its intensity that progressively increases when quiescent lymphocytes are stimulated by phytohemagglutinin, even before they enter DNA synthesis (8).

In this paper we have asked 2 simple questions: (a) is $F_{\geq 600}$ the same in cells collected by mitotic detachment (postmitotic cells) and in cells made quiescent by serum deprivation; and (b) do changes in $F_{\geq 600}$ also occur in mitotically collected cells as they progress toward S? To answer these questions we have used a temperature-sensitive (ts) mutant of baby hamster kidney cells called AF8, which arrests at the nonpermissive temperature in G1 (1, 5).

MATERIALS AND METHODS

Cell Cultures. The culture of AF8 cells, the methodology for collection of mitotic cells (without the use of colchicine or trypsin), the preparation and stimulation of quiescent cultures, the labeling with [3H]thymidine, and the autoradiographic technique have all been described in detail by Ashihara et al. (1).

Cell Staining. The cells collected from cultures were rinsed once with PBS and then fixed in suspension in 70% ethanol, for at least 16 hr at 4°. Further steps of staining, in essence, were similar as described in detail before (18), except that in the present experiments the method was modified because the cells had been fixed. Thus, after fixation the cells were centrifuged, rinsed again with PBS, and resuspended in PBS at an approximate concentration of 1 to 2 x 10⁶ cells/ml. Aliquots (0.2 ml) of cell suspensions (2 to 4 x 10⁶ cells) were then mixed with 0.4 ml of a solution containing 0.05 N HCl and 0.15 N NaCl, and 30 sec later 1.2 ml of a solution containing AO (8 μg/ml) dissolved in 0.2 M Na₂HPO₄, 0.1 M citric acid buffer (pH 6.0), 1 mM sodium EDTA, and 0.15 N NaCl were added. Chromatographically purified AO, obtained from Polysciences Inc. (Warrington, Pa.) was used. In this method pretreatment of cells at low pH increases stainability of nuclear DNA (6), while at that low pH nucleic acids remain insoluble. Subsequent staining with AO in the presence of chelating agents (EDTA, citrate) results in denaturation of cellular RNA (7) which then stains metachromatically (3), while the native DNA intercalates the dye and stains orthochromatically (12). The specificity of staining was controlled by incubation of fixed cells suspended in PBS with 10⁶ units of RNase A per ml for 20 min at 35° (chromatographically purified RNase A; Worthington Biochemical Corp., Freehold, N. J.) or with DNase (1 mg/ml) (Worthington) for 30 min at 35°. The cells treated with nucleases were then stained as described above.

Fluorescence Measurements. Fluorescence of individual cells was measured in the FC 300 cytofluorograph (Ortho Diagnostic Instruments, Bio/Physics Systems, Westwood, Mass.) interfaced to a Nova 1220 minicomputer (Data General Corporation, Southboro, Mass.). The cells sus-
pended in AO solution are transported through the instrument at rates of about 200 cells/sec. Fluorescence and light-scattering signals are generated by each cell as it passes through the focus of a 488 nm argon-ion laser beam. The red fluorescence (\(F_{>600}\), measured in a band from 600 to 650 nm) and green fluorescence (\(F_{530}\), measured in a band from 515 to 575 nm) emissions for each cell are separated by optical filters measured by separate photomultipliers, and their integrated values are recorded and stored in the computer. The pulse width, i.e., the time taken by the cell to pass through the focus of the laser beam, was also recorded and was used to distinguish single cells from cell doublets, as well as to estimate cell size or nuclear diameter, as described (15). The data presented were based on a total of \(5 \times 10^3\) cells/sample. The experiment was repeated 4 times.

**RESULTS**

**Entry of Cells into DNA Synthesis.** AF8 cells collected by mitotic detachment or made quiescent by serum deprivation (0.5% serum) for 48 hr were incubated at either the permissive (34°) or nonpermissive (40.6°) temperatures. \([^3H]\text{Thymidine}\) was added immediately after plating of the mitotic cells or serum stimulation of the quiescent monolayers, so that the data in Chart 1 represent cumulative labeling indices. At nonpermissive temperature neither postmitotic nor serum-stimulated AF8 cells can enter S. At permissive temperature postmitotic AF8 cells enter the S phase approximately 16 hr before AF8 cells that are serum stimulated from quiescence. The median time of entry into S (1) was 13 hr for postmitotic cells and 29 hr for cells serum stimulated from quiescence.

**Analysis of AO-induced Fluorescence in AF8 cells.** Charts 2 to 4 illustrate how AO-induced fluorescence in postmitotic and serum-stimulated AF8 cells was analyzed.

Postmitotic cells consist of a relatively uniform population characterized by low \(F_{>600}\) and by a mean \(F_{530}\) typical of cells with a 2n DNA content (Chart 2A). During their synchronized growth prior to DNA replication (i.e., before an increase in their \(F_{530}\) could be seen), the cells markedly increase \(F_{>600}\) (see below). Eight hr following mitosis the first cells enter S phase (Chart 2B). Most \(F_{>600}\) is RNase sensitive (Chart 2C).

Chart 3 illustrates populations of mitotic cells immediately after replating. There are 2 main subpopulations among these cells. The subpopulation with \(F_{530}\) characteristic of cells with 4n DNA content represents cells still in mitosis. The other subpopulations with \(F_{530}\) of 2n DNA content most probably represent very early postmitotic cells. Based on the differences in \(F_{530}\), it is possible completely to separate these subpopulations and to analyze their mean \(F_{>600}\) and the intercellular variation (Chart 3, B and C). As predicted the \(F_{>600}\) of mitotic cells is nearly twice that of the early postmitotic cells (Chart 3; see also Table 1).

When AF8 cells are incubated in low serum (0.5%) concentration, proliferation gradually ceases and they become

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**Chart 1.** Entry into S phase of postmitotic or serum-stimulated AF8 cells. Cells collected by mitotic detachment (●, △) or quiescent cultures stimulated by 10% serum (○, ▲) were continuously exposed to \([^3H]\text{thymidine}\), and the percentage of labeled cells was determined by autoradiography. ○, ●, cells at 34°; ▲, △, cells at 40.6°.

**Chart 2A.** Changes in fluorescence of AO-stained AF8 cells during the initial 8 hr of synchronized growth following mitosis. Cells in mitosis were selectively collected as described in “Materials and Methods.” These cells were replated and then collected either after 30 min (A) or 8 hr (B) of incubation at 34°. The results are presented as a computer-drawn scattergram in which each point represents an individual cell; its distance from the abscissa and ordinate is a measure of green (\(F_{530}\)) and red (\(F_{>600}\)) fluorescence of the cell, respectively. \(B(\text{RNase})\) scattergram, cells collected 8 hr after mitosis (B), and treated with RNase prior to staining.
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Chart 3. Red and green fluorescence of AF8 cells immediately after mitosis. In these samples of mitotic cells, within 5 min after collection there were 2 main subpopulations represented by separate peaks on frequency histograms. A (bottom), abscissa, green fluorescence in arbitrary units; ordinate, fraction of cells with a given green intensity. The subpopulation of cells with high \( F_{>600} \) values represents cells in mitosis, while the subpopulations with mean \( F_{>600} \) value one-half of that of mitotic cells represent cells in early G1. After simultaneous measurement of \( F_{>600} \) and pulse width for 5000 cells and excluding cell doublets based on their pulse width values; see the paper of Sharpless et al. (15), the main cell subpopulations were separated on the basis of their different \( F_{>600} \) values (bottom panels). Thus, the frequency histograms of \( F_{>600} \) (top panels) represent the intercellular distribution of fluorescence values in the mixed population (A), in cells in early G1 (B), and in M cell populations (C), respectively.

Table 1

<table>
<thead>
<tr>
<th>Phase of the cell cycle</th>
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<td>G0</td>
<td>35.7</td>
<td>10.0</td>
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<tr>
<td>G1 (early)</td>
<td>39.7</td>
<td>10.8</td>
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<tr>
<td>G1 (asynchronous)*</td>
<td>42.8</td>
<td>10.9</td>
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<tr>
<td>G1 (late)</td>
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<tr>
<td>Early S*</td>
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<tr>
<td>Mid-S*</td>
<td>56.2</td>
<td>18.4</td>
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<tr>
<td>Late-S*</td>
<td>60.9</td>
<td>18.4</td>
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<tr>
<td>G0 + M*</td>
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<td>M</td>
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Table 2

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Changes in red fluorescence of AO-stained AF8 cells during the cell cycle

Subpopulations of cells were selected on the basis of green fluorescence \( (F_{>600}) \). G0 cells were obtained from stationary cultures maintained in the presence of 0.5% serum for 48 hr at 37°. The early G1 population represents the synchronized G1 population 20 min after harvesting and replating of mitotic cells. The value of \( F_{>600} \) for the asynchronous G1 population, as well as for other populations marked by asterisks, were obtained by selection of cells from serum-stimulated cultures as illustrated in Chart 6. Cells in late G1 were obtained from synchronized cultures 8 hr after mitotically detached cells were plated, as shown in Chart 2B. Mitotic cells were selected from the sample of the mitotically detached cells as having \( F_{>600} \) characteristics of 4n DNA value.

\( F_{>600} \) of some of these populations was also measured after incubation of cells with RNase.

Changes in green fluorescence after mitosis or after serum stimulation.

S*Mid-S*Late-S*G2+

After RNase35.739.742.855.056.558.260.969.471.8

Mean \( F_{>600} \) of some of these populations was also measured after incubation of cells with RNase.

Changes in red fluorescence of AF8 cells immediately after mitosis. In these samples of mitotic cells, within 5 min after collection there were 2 main subpopulations represented by separate peaks on frequency histograms. A (bottom), abscissa, green fluorescence in arbitrary units; ordinate, fraction of cells with a given green intensity. The subpopulation of cells with high \( F_{>600} \) values represents cells in mitosis, while the subpopulations with mean \( F_{>600} \) value one-half of that of mitotic cells represent cells in early G1. After simultaneous measurement of \( F_{>600} \) and pulse width for 5000 cells and excluding cell doublets based on their pulse width values; see the paper of Sharpless et al. (15), the main cell subpopulations were separated on the basis of their different \( F_{>600} \) values (bottom panels). Thus, the frequency histograms of \( F_{>600} \) (top panels) represent the intercellular distribution of fluorescence values in the mixed population (A), in cells in early G1 (B), and in M cell populations (C), respectively.

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Changes in red fluorescence of AF8 cells growing at permissive and nonpermissive temperatures following mitosis or after stimulation of quiescent cultures by addition of fresh medium. Two sets of cultures were investigated. In the first, cells in mitosis were collected (see "Materials and Methods") and replated in cultures which were then maintained either at 34° (B) or 40.6° (C). In the second set, the cells were preincubated at 34° in the presence of 0.5% serum for 48 hr. During that time cell proliferation ceases, and the cells become arrested predominantly in G0. These cells were stimulated by addition of fresh serum, and the cultures were incubated at 34° (C) and 40.6° (B). In both sets of cultures, cells were harvested at various time intervals after replating or serum stimulation (abscissa). The results represent the mean value of \( F_{>600} \) for the selected populations of cells with a 2n content of DNA (G0 and G1). Repeated incubation with RNase prior to cell staining of various parallel samples made it possible to establish the base line, indicating the level of RNase-resistant \( F_{>600} \) of the G0 or G1 subpopulations (A). The value of \( F_{>600} \) for the mitotically detached cells at 0 time was established from the sample collected as described in Chart 3.
were set to circumscribe the G₁, S, and G₂/M populations based on F₅₆₆₄ axis. These values are included in Table 1.

which was sensitive to DNase The S subpopulation was further subdivided for 28 hr. With the use of an interactive computer program, the thresholds by serum. The cells, prior to harvest, were stimulated with 10% fresh medium decrease was followed by a gradual increase occurring between 4 and 16 hr. The reason for this variation in the F₆₀₀ decrease between experiments is unclear; the phenomenon perhaps is related to the actual “depth” of cell quiescence at the onset of the experiment which could vary slightly from experiment to experiment.

Cell growth at the nonpermissive temperatures is paralleled by an increase in F₆₀₀. After longer periods of time (over 20 hr), cells growing at the nonpermissive temperatures always have higher F₆₀₀ than do the proliferating cells at 34°C.

Chart 5 deals only with cells with a G₁ content of DNA. Table 1 shows the same data on F₆₀₀ from populations of cells with different DNA contents. The data were collected from cell cultures as illustrated in Chart 6 and also from synchronized cells as shown in Chart 2. Red fluorescent intensity obviously continues to increase during G₁ and S until in G₂M it reaches a value that is roughly twice the value of G₀ or postmitotic cells.

DISCUSSION

Flow cytometry was used to measure the fluorescence of cells stained with the metachromatic dye AO under conditions when the orthochromatic green fluorescence (F₅₁₀) is proportional to the quantity of DNA per cell, while the red metachromasia (F₆₀₀) is largely related to RNA content (7, 18). This technique has already been applied to distinguish stimulated from quiescent lymphocytes and to obtain multiparameter analysis of lymphocyte stimulation (8, 9).

some experiments, but not in others (see Chart 4), a transient decrease in F₆₀₀ was seen in serum-stimulated cells during the first 2 hr following addition of serum; the decrease was followed by a gradual increase occurring between 4 and 16 hr. The reason for this variation in the F₆₀₀ decrease between experiments is unclear; the phenomenon perhaps is related to the actual “depth” of cell quiescence at the onset of the experiment which could vary slightly from experiment to experiment.

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F₆₀₀ after AO staining has about the same intensity in G₁ AF8 cells immediately after mitosis (within 15 min after plating) and in cells made quiescent by serum deprivation for 48 hr. Smith and Martin (16) have proposed that after mitosis all cells enter an indeterminate A state from which they exit with different probabilities. By the technique used in these experiments, postmitotic and quiescent cells are indistinguishable. Very rapidly, however, postmitotic cells become distinguishable from serum-stimulated cells. In mitotically collected cells F₆₀₀ increases within 30 min and reaches a peak by 4 hr. In serum-stimulated cells F₆₀₀ does not increase until 4 hr and reaches a peak between 16 and 19 hr. Postmitotic cells also enter S about 16 hr earlier than serum-stimulated cells. It seems therefore that by AO staining and red fluorescence, postmitotic cells exit from the A state much more rapidly than serum-stimulated cells. Since the culture conditions were exactly the same (in 1 experiment, cell densities were also similar), it is difficult to conceive why postmitotic cells exit from the A state much faster than do serum-stimulated cells, unless the latter are different from the former in some other way that is not detectable by the present technique, a finding that militates against the Smith and Martin (16) model. Our results show that this technique can be used to differentiate cycling from noncycling cells, at least within certain limits of error. Since postmitotic cells remain in a state undistinguishable from G₀ for a very brief period of time (less than 30 min), it can be calculated that only about 5% of a population of cycling cells (assuming a cell cycle of 20 hr) will have a F₆₀₀ similar to that of resting cells. However, an absolute distinction cannot be obtained by this technique with these cells, although G₀ lymphocytes can be distinguished from postmitotic cells.

F₆₀₀ increases also in cells incubated at the nonpermissive temperature; in fact, in selected cells with a G₁ DNA content it remains elevated even when it starts to decrease at the permissive temperature. This is due to the fact that, at 40.6°, AF8 cells do not enter S. If F₆₀₀ has something to do with RNA (see below), it would be in agreement with previous results by Rossini and Baserga (14) that RNA synthesis is increased in serum-stimulated cells for several hr even at the nonpermissive temperature. Rossini and Baserga used isolated nuclei and nucleoli, but their results can also be confirmed by in vivo data (unpublished results). The ts block, the execution point of which is 8 to 9 hr before (1) apparently does not interfere with the stimulation of RNA synthesis and/or accumulation.

What is the meaning of F₆₀₀? There is good evidence from previous papers by Darzynkiewicz et al. (8, 18) that red fluorescence after AO staining is correlated to RNA content of the cell. Again in the present experiments, digestion with RNase eliminates 70 to 90% of the red fluorescence (Chart 5). Interestingly enough, after RNase, the residual F₆₀₀ is the same in all groups of cells regardless of the degree of stimulation. If this residual red fluorescence is subtracted from the actual values, the increase in F₆₀₀ in postmitotic and the serum-stimulated cells would become more dramatic. Yet, if F₆₀₀ measures RNA amount, it is difficult to believe that postmitotic cells could double their RNA content in 30 min or so. Perhaps F₆₀₀ may have something to do with RNA processing or the state of polysome aggrega-
There is disaggregation of polysomes in both mitotic and in resting cells, with subsequent reaggregation as cells proceed toward S (11, 17, 19).

Finally, we would like to comment on the stringency of an increased red fluorescence for the entry of cells into S. As Chart 6 shows, no cell enters S (increased green fluorescence) unless $F_{\text{red}}$ is also increased. Whatever the meaning of $F_{\text{red}}$ (RNA synthesis, RNA amount, aggregation of polysomes, or others), it seems to correlate very closely in AF8 cells with their entrance into S.

REFERENCES

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