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

T. Ashihara, F. TraganoS, R. Baserga, and Z. Darzynkiewicz

Fels Research Institute and Department of Pathology, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140 [T. A., R. B.], and Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [F. T., Z. D.]

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$_{>600}$) was the same in quiescent cells and in cells immediately after mitosis. However, F$_{>600}$ increased very rapidly in postmitotic cells, while there was a delay in serum-stimulated quiescent cells. F$_{>600}$ 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, the red fluorescence (F$_{>600}$) is proportional to DNA content. Under the same conditions, the green fluorescence (F$_{>500}$) is largely related to RNA content, and it is the 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$_{>600}$ the same in cells collected by mitotic detachment (postmitotic cells) and in cells made quiescent by serum deprivation; and (b) do changes in F$_{>600}$ 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 [H]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 fixing the cells were centrifuged, rinsed again with PBS, and resuspended in PBS at an approximate concentration of 1 to 2 x 10$^6$ cells/ml. Aliquots (0.2 ml) of cell suspensions (2 to $4 \times 10^6$ cells) were then washed 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 /g/ml) dissolved in 0.2 M Na$_2$HPO$_4$-0.1 M citric acid buffer (pH 6.0), 1 mm sodium EDTA, and 0.15 n NaCl were added. Chromato graphically 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 10$^6$ 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-
suspended 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 ($F_{r>600}$, 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]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 $F_{r>600}$ 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 $F_{r>600}$ (see below). Eight hr following mitosis the first cells enter S phase (Chart 2B). Most $F_{r>600}$ 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 $F_{r>600}$ and the intercellular variation (Chart 3, B and C). As predicted the $F_{r>600}$ 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...
the same results, however, were obtained in 2 other sepa-

erver, in cells collected by mitotic detachment, F>li(M) in-

selected for Chart 5, which is from 1 experiment (essentially

quiescence. Only cells with a G, content of DNA were

Serum Stimulation. Chart 5 shows the time curve of F>ti,M1 of

precedes DMA replication (Chart 4).

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>)00). G0 cells were obtained from stationary cultures

maintained in the presence of 0.5% serum for 48 hr at 37°. The

eye G1 population represents the synchronized G1 population 20

min after harvesting and replating of mitotic cells. The value of

F>,00 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>soo characteristics of 4n DNA value.

F>,00 of some of these populations was also measured after incubation of cells with RNase.

<table>
<thead>
<tr>
<th>Phase of the cell cycle</th>
<th>Mean F&gt;,00 Before RNase</th>
<th>Mean F&gt;,00 After RNase</th>
</tr>
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<tbody>
<tr>
<td>G0</td>
<td>35.7</td>
<td>10.0</td>
</tr>
<tr>
<td>G1 (early)</td>
<td>39.7</td>
<td>10.0</td>
</tr>
<tr>
<td>G1 (asynchronous)*</td>
<td>42.8</td>
<td>10.0</td>
</tr>
<tr>
<td>G1 (late)</td>
<td>55.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Early S*</td>
<td>56.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Mid-S*</td>
<td>58.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Late-S*</td>
<td>60.9</td>
<td>10.0</td>
</tr>
<tr>
<td>G0 + M*</td>
<td>69.4</td>
<td>18.8</td>
</tr>
<tr>
<td>M</td>
<td>71.8</td>
<td>19.1</td>
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</tbody>
</table>

arrested in G0 phase (10). The quiescent cells have lower

F>,00 than do their proliferating counterparts in G1. Stimula-
tion of quiescent cells to proliferate by addition of serum is accom-
panied by an increase in cellular F>,00 which precedes DNA replication (Chart 4).

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>soo values represents cells in mitosis, while the subpopulations with mean F>,00 value one-half of that of mitotic cells represent cells in early G1. After simultaneous measurement of F>soo 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>,00 values (bottom panels). Thus, the frequency histograms of F>soo (top panels) represent the intercellular distribution of red fluorescence values in the mixed population (A), in cells in early G1 (B), and in M cell populations (C), respectively.

Changes in red fluorescence of AF8 cells immediately after mitosis or after serum stimulation from quiescence. Only cells with a G1 content of DNA were selected for Chart 5, which is from 1 experiment (essentially the same results, however, were obtained in 2 other separate experiments). Red fluorescence intensity is the same in postmitotic cells (see above) and in quiescent cells. However, in cells collected by mitotic detachment, F>,00 in-

creased very rapidly, about 20% within 30 min, 30% in 1 hr, and reaching a peak of 50% at 4 hr. In serum-stimulated AF8 cells, F>,00 began to increase at 4 hr after serum and reached a peak between 8 and 17 hr (from the results of 2 other experiments, the peak of red fluorescence is between 16 and 19 hr). RNase digestion eliminates from 70 to 90% of the red fluorescence.

In 1 experiment, mitotic cells were replated at a high density, to simulate the conditions of serum-stimulated quiescent cells. This is actually the experiment shown in Chart 5 which clearly shows that the rapidity of progress of postmitotic cells is not due simply to a low cell density. In
were set to circumscribe the G, S, and G, M populations based on F, axis. These values are included in Table 1.

some experiments, but not in others (see Chart 4), a transient decrease in F>_600 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 F>_600 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 F>_600. After longer periods of time (over 20 hr), cells growing at the nonpermissive temperatures always have higher F>_600 than do the proliferating cells at 34°.

Chart 5 deals only with cells with a G, content of DNA. Table 1 shows the same data on F>_600 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 G, and S until in G,M it reaches a value that is roughly twice the value of G, or postmitotic cells.

DISCUSSION

Flow cyt fluorometry was used to measure the fluorescence of cells stained with the metachromatic dye AO under conditions when the orthochromatic green fluorescence (F>330) is proportional to the quantity of DNA per cell, while the red metachromasia (F>_600) 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).

F>_600 after AO staining has about the same intensity in G, 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 F>_600 increases within 30 min and reaches a peak by 4 hr. In serum-stimulated cells F>_600 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 indistinguishable from G, 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 F>_600 similar to that of resting cells. However, an absolute distinction cannot be obtained by this technique with these cells, although G, lymphocytes can be distinguished from postmitotic cells.

F>_600 increases also in cells incubated at the nonpermissive temperature; in fact, in selected cells with a G, 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°C, AF8 cells do not enter S. If F>_600 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 F>_600? 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 F>_600 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 F>_600 in postmitotic and the serum-stimulated cells would become more dramatic. Yet, if F>_600 measures RNA amount, it is difficult to believe that postmitotic cells could double their RNA content in 30 min or so. Perhaps F>_600 may have something to do with RNA processing or the state of polysome aggrega-
There is disaggregation of polysomes in both mitotic and 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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