Normal and Perturbed Chinese Hamster Ovary Cells: Correlation of DNA, RNA, and Protein Content By Flow Cytometry

H. A. CRISMAN, Z. DARZYNKIEWICZ,* R. A. TOBEY, and J. A. STEINKAMP
Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and *Memorial Sloan-Kettering Cancer Center, New York City, New York 10021

ABSTRACT Quantitative, correlated determinations of DNA, RNA, and protein, as well as RNA to DNA and RNA to protein ratios, were performed on three-color stained cells using a multiwavelength-excitation flow cytometer. DNA-bound Hoechst 33342 (blue), protein-fluorescein isothiocyanate (green), and RNA-bound pyronin Y (red) fluorescence measurements were correlated as each stained cell intersected three spatially separated laser beams. The analytical scheme provided sensitive and accurate fluorescence determinations by minimizing the effects of overlap in the spectral characteristics of the three dyes. Computer analysis was used to generate two-parameter contour density profiles as well as to obtain numerical data for subpopulations delineated on the basis of cellular DNA content. Such determinations allowed for analysis of RNA to DNA and RNA to protein ratios for cells within particular regions of the cell cycle. The technique was used to study the interrelationship of DNA, RNA, and protein contents in exponentially growing Chinese hamster ovary cells as well as in cell populations progressing the cell cycle after release from arrest in G1 phase. The sensitivity of the method for early detection of conditions of unbalanced growth is demonstrated in the comparison of the differential effects of the cycle-perturbing agent, adriamycin, on cells treated either during exponential growth or while reversibly arrested in G1 phase.

DNA, RNA, and protein comprise the bulk of macromolecules in cells. Interrelationships in synthesis and accumulation of these moieties appear to play an important role in regulating cell cycle traverse capacity, cell division, growth, and size. Consistency in the size range of the population volume distribution and cycle generation time is controlled by transcriptional and translational processes that rigidly couple temporal metabolism of DNA, RNA, and protein. With few rare exceptions (e.g., histone proteins), rates of RNA and protein synthesis in cells are constant, and increases in cellular content are linear and proportional across the cell cycle of exponentially growing mammalian populations (1, 2). However, a variety of cycle-perturbing agents, including drugs, induce a differential uncoupling of normal synthetic patterns, causing a disproportionate accumulation of these cellular constituents. These conditions may lead to states of unbalanced growth, loss of long-term viability, and eventual cell death. A technique which could directly measure and correlate cellular DNA, RNA, and protein content in single cells would be useful in detecting alterations in normal metabolic patterns and assessing the functional state of cells under a variety of experimental conditions.

Flow cytometry (FCM) provides a rapid and precise method of performing multiple biochemical measurements on single cells, thereby allowing for subsequent correlation of the various metabolic parameters. Simultaneous FCM analysis of cellular DNA and RNA (3, 4) and DNA and protein (5, 6) contents, for example, permits distinction between quiescent and cycling cells (7, 8), cells in various stages of differentiation (4), and determination of the relationship between cell growth and the cell division cycle (9). To date, no method has been described for rapid, simultaneous measurement of DNA, RNA, and protein in cells. Such measure-

Abbreviations used in this paper: AdR, adriamycin; CHO, Chinese hamster ovary; FCM, flow cytometry; FITC, HO, and PY fluorescence, fluorescein-isothiocyanate (green), Hoechst 33342 (blue), and pyronin Y (red) fluorescence, respectively.
gauge on metabolism of cells located at particular stages of
(PY) (11–14), and protein with fluorescein isothiocyanate
incorporates modifications of procedures for fluorochroming
DNA and RNA to protein per cell, can provide a sensitive
DNA with Hoechst 33342 (HO) (10), RNA with pyronin Y
for direct determination and correlation of DNA, RNA, and
ments, when combined with assessment of ratios of RNA to
DNA and RNA to protein per cell, can provide a sensitive
gauge on metabolism of cells located at particular stages of
the cell cycle.

This report describes the development of an FCN method
for direct determination and correlation of DNA, RNA, and
protein in individual cells. The fluorescent labeling protocol
incorporates modifications of procedures for fluorochroming
DNA with Hoechst 33342 (HO) (10), RNA with pyronin Y
(PY) (11–14), and protein with fluorescein isothiocyanate
(FITC) (5, 15). Analysis is performed in a three-laser excitation
system that has been described recently (16).

Using this technique, patterns of DNA, RNA, and protein
contents, and ratios of RNA to DNA and RNA to protein
were analyzed in exponentially growing Chinese hamster
ovary (CHO) cells and in populations reinitiating cycle trans-
verse following reversible arrest in the G1 phase (17). The
sensitivity of the method for early detection of potential
metabolic impairment is demonstrated in analysis of the
differential effect of the antineoplastic drug, adriamycin
(AdR), on these two cell populations.

MATERIALS AND METHODS

Cell Culture: Suspension (spinner) cultures of CHO cells were main-
tained in exponential growth phase in Ham’s F-10 medium containing 15%
newborn calf serum, streptomycin, and penicillin. Populations of CHO cells
were reversibly arrested in the G1 phase by growth in isoleucine-deficient
medium for 36 h as previously described by Tobey and Ley (17). The synchro-
nized G1 populations were subsequently cultured in complete (isoleucine-
containing) medium to initiate cell cycle traverse.

Drug Treatment: AdR (Adria Laboratories, Columbus, OH) was
added for a single 2-h interval at a final concentration of 6 µg/ml to both
exponentially growing and G1-arrested populations. Cells were then centrifuged,
washed, resuspended in fresh medium, and cultured in drug-free complete medium.

Cell Survival Studies: At 15 h after drug treatment and subsequent
culture in drug-free, complete medium, cells were collected from the AdR-
treated and control populations for determination of colony-forming ability.
Cells were placed in dishes, allowed to attach, and cultured for 7 d prior to
staining and scoring of colonies. Each colony contained at least 50 cells. Cell
membrane integrity was also assayed by trypan blue staining and microscopic
examination; under such conditions, only damaged cells are stained.

Cell Harvest and Fixation: At various time intervals cells from
untreated (control) and drug-treated cultures were harvested by centrifugation.
After removal of culture medium, the cell pellet was resuspended in one part
cold saline GM (g/l; glucose 1.1, NaCl 8.0, KCl 0.4, Na2HPO4 12 H2O 0.39,
KH2PO4 0.15) containing 0.5 mM EDTA, and then three parts of ice cold 95%
ethanol was added, with mixing of the cell suspension. The final ethanol
concentration was ~70%. Sample tubes were chilled on ice for at least 1 h and
then stored at 4°C for at least 12 h prior to staining. The volume of fixative
that was nearly identical to distributions obtained for cells
within the G1 region of the cell cycle. Since data are collected and stored in list
mode fashion, data for any of the individual measurement can be retrieved in
a similar manner for cells in any phase of the cell cycle. Potentially, gated
analysis can be performed on any of the distributions, (i.e., protein or RNA
content) as well, and data can be correlated as described above.

RESULTS

Fluorescence Analysis and Spectral Resolution

Quantitative analysis of DNA, RNA, and protein were
derived from measurements of HO (blue), PY (red), and FITC
(green) fluorescence, respectively. The spectral characteristics
of the three dyes (Fig. 1, A and B) required sequential excita-
tion of each dye at the wavelengths indicated (Fig. 1A) fol-
lowed by emission analysis over preselected fluorescence mea-
surement (wavelength) regions (Fig. 1C). This sequential
analysis scheme minimized potential problems in spectral resolu-
tion due to the overlap in fluorescence emission of dyes
(Fig. 1B).

Analysis of populations of CHO cells stained with all three dyes
provided distribution profiles for DNA, protein, and RNA
that were nearly identical to distributions obtained for cells
stained with only one of each respective dye (Fig. 2). Cells
stained with all three dyes (Fig. 2D) showed a slight decrease
in (FITC) green fluorescence and a slight increase in (PY) red
fluorescence as compared with respective single dye stained
cells (Fig. 2, B and C, respectively). This, in all probability, is
due in part to energy transfer from FITC to PY. The DNA
profiles in Figs. 2 A and 2 D are similar, indicating no change
in HO-DNA fluorescence intensity in the three-color stained
cells. Data in Fig. 2 were all obtained using the same electronic
gain and laser power settings for all three lasers. Cells
pre-
treated with RNase prior to PY staining and analysis showed
an 85–90% decrease in red fluorescence compared with distrib-
tions for non-RNase-treated cultures (Fig. 2 C). Collectively
the data above demonstrate the sensitivity and reliability
of multicolor staining and fluorescence analysis for accurate
assessment of DNA, RNA, and protein in single cells under
the staining and analysis conditions described.

142 THE JOURNAL OF CELL BIOLOGY • VOLUME 101, 1985
Analysis of Asynchronous and G1-arrested CHO Cells

The DNA, RNA, and protein staining and analysis techniques were used to characterize and compare populations of exponentially growing CHO cells and the noncycling CHO cells in cultures deprived of isoleucine. Isoleucine deprivation is a method routinely used for synchronization of rodent cells in cultures deprived of isoleucine. Isoleucine deprivation is a method routinely used for synchronization of rodent cells in cultures deprived of isoleucine. The cell distribution patterns with respect to RNA and protein contents are surprisingly similar both in exponentially growing (Expon.) as well as noncycling (Ileu-) cell populations (Fig. 3, A and B). However, the populations show greater heterogeneity in protein rather than RNA content. This is evident from comparisons of the width of the RNA and protein distributions in the G1 or G2 + M clusters.

One characteristic feature of the G1 population is the apparent presence of a threshold RNA or protein content (Fig. 3A, arrows). The data indicate that cells with a subthreshold content of either RNA or protein do not immediately enter the S phase. As described before, this threshold discriminates two different compartments of G1 phase, G1A and G1B, which are believed to have different functions (7). The exponentially growing population shows about half of the G1 cells in the G1A compartment (i.e., cells characterized by the subthreshold RNA or protein content) (Fig. 3A). A remarkably constant relationship was observed between RNA and protein content of individual cells in the cycling population (i.e., RNA vs. protein) (data not shown).

Analysis of the RNA to DNA ratio (Fig. 3A) in relation to cell cycle position, as based on DNA content, reveals a characteristic pattern of changing rates of RNA replication vs. transcription. The data show that during G1, when DNA content is constant, cells accumulate increasing quantities of RNA which is reflected in high heterogeneity of the RNA to DNA ratio. During progression through S phase, the rate of DNA replication exceeds the rate of accumulation of RNA giving rise to a nonvertical, negative slope of the RNA to DNA ratio for the S phase cell cluster. It can be noted that cells in G2 + M have, on average, an RNA to DNA ratio that is similar to that of the majority of G1 cells.

The ratio of RNA to protein is a novel parameter. Simultaneous measurements of DNA, RNA, and protein made it possible not only to estimate this ratio, but also to assess the RNA to protein relationship for cells at various positions in the cell cycle. The RNA/protein ratio thus provides a useful parameter which would detect unbalanced growth when the rates of RNA and protein accumulation (reflecting DNA transcription and RNA turnover as well as protein synthesis and degradation) vary with respect to each other. These studies detect no such variability during the cell cycle; in fact, the RNA to protein ratio remains strikingly constant and uniform for all cells regardless of their DNA content (Fig. 3A). Such a pattern is expected because of the very good correlation between RNA and protein content in individual cells mentioned previously.

Noncycling cells such as those shown in Fig. 3B represent CHO cells arrested in G1 by isoleucine deprivation. Cells from these cultures show a much higher heterogeneity in RNA and protein content than G1 cells in the cycling population. The threshold RNA or protein content of the G1 population (similar to that observed in the cycling population) is also apparent in the respective profiles of cells deprived of isoleucine (Fig. 3B, arrows). Based on gating analysis, >90% of isoleucine-deprived cells have the subthreshold RNA content and may be characterized as G1A cells (7). A good correlation was also observed between RNA and protein content of individual cells (data not shown) although the distribution is more asymmetrical in comparison with cycling cells.

Both the RNA to DNA and RNA to protein ratio distributions of noncycling cells show higher heterogeneity of the G1 population in comparison to respective distributions for exponentially growing G2 phase cells. The distribution with respect to the RNA to protein ratio is less symmetrical than in cycling populations and the skew of the distribution indicates the presence of G1 cells with lower protein content but still relatively high RNA content. The RNA and protein contents, however, are somewhat decreased in the cells deprived of isoleucine. Thus, on the basis of the RNA to protein...
Analysis of Cycle Progress in Synchronized Cultures

When transferred to complete (isoleucine-containing) medium, the G1-arrested cells (Fig. 3 B) will initiate DNA synthesis and progress through the cell cycle (Fig. 3, C–F). By 6 h (Fig. 3 C), cells with elevated RNA and protein contents are the first to enter S phase. The RNA to DNA ratio is increased in this subpopulation but the RNA to protein ratio profile is similar to the subpopulation of cells still residing in G1 phase at that time. At 9 h (Fig. 3 D), cells have reached G2 + M phase, and by 12 h (Fig. 3 E), the single parameter DNA profile for the population (Fig. 3 E, left panels) closely resembles the DNA profile for exponentially growing CHO cells in Fig. 3 A. However, assessment of the RNA and protein contents profiles as well as the RNA to DNA and RNA to protein ratios in Fig. 3, B–E indicate greater heterogeneity in cellular levels of these constituents in cultures initially arrested in G1 phase. By 24 h, the respective distributions patterns (Fig. 3 F) begin to more closely resemble those shown in Fig. 3 A, especially the RNA to DNA ratio profile. At 36 h, patterns of the two cultures are indistinguishable (data not shown).

Analysis of Drug-treated Populations

AdR treatment induced a differential response in metabolism of DNA, RNA, and protein in exponentially growing cells and cells initially in G1 phase at the time of treatment. Analysis of the single parameter DNA profiles at 15 h after drug treatment (Fig. 4 B, left panel) showed a large accumulation of cells from the exponential culture arrested in the G2 + M phase (i.e., 85%). Based on numerical data (Table I), this subpopulation had mean ratio values for RNA to DNA and RNA to protein that were 44% and 31% elevated, respectively, above control ratio values. Few cells are observed in S phase, but the cells remaining in G1 phase (i.e., 15%) had RNA to DNA and RNA to protein ratio values almost identical to control values (Table I). Survival studies showed that, compared to the exponential control CHO cell population, the AdR-treated exponential population had only a 12% surviving fraction. Analysis with trypan blue revealed that, at the time the survival studies were performed (15 h after AdR treatment), >97% of the drug-treated cells excluded the dye.

By comparison, the AdR-treated G1 cells, when placed in drug-free, complete medium (Fig. 4 D), show only a moderate accumulation of cells in G2 + M phase with RNA to DNA and RNA to protein mean ratio values that are only 5% and 11% below respective values for the untreated control (Table I). Comparison of these values and those in Table I for cells...
in all phases of the cell cycle show that, based on these criteria, the G₁ drug-treated population is in a more nearly balanced state of metabolic equilibrium 15 h after drug treatment than the drug-treated exponential culture at 15 h after AdR treatment. Cells in the former population continue to progress through S phase (Fig. 4 D), and based on cell count data, the cell number of the population had doubled by 30 h compared to an increase of only 8% in the drug-treated asynchronous
FIGURE 4  Single parameter DNA distribution (left panels) and two-parameter contour density profiles for DNA (y axis) vs. protein and RNA, as well as the RNA to DNA and RNA to protein ratios (left to right) for control (untreated), exponentially growing CHO cells (A) and control CHO cells at 15 h after release from synchrony in G1 phase (C). Corresponding populations of AdR-treated cells at 15 h after drug treatment (i.e., 6 μg/ml AdR for 2 h) are shown in B and D, respectively.

TABLE I.  Mean Values of the RNA to DNA and RNA to Protein Ratios*  

|                  | RNA to DNA | RNA to Protein |
|------------------|------------|----------------|
|                  | G1  | S   | G2+M | G1  | S   | G2+M |
| Exponential control | 64.0 | 62.6 | 58.0 | 46.1 | 48.2 | 47.3 |
| AdR treated      | 65.4 | —   | 83.5 | 45.9 | —   | 62.2 |
| Synchronized (G1 phase control) | 112.4 | 101.5 | 91.0 | 71.6 | 72.3 | 65.0 |
| AdR-treated      | 101.9 | 94.6 | 86.7 | 57.2 | 58.3 | 57.4 |

*Obtained for cells at various stages of the cell cycle after a 2-h treatment with AdR (6 μg/ml) and resuspension in drug-free medium for 15 h prior to analysis. 

Mean values of ratios are in arbitrary units (i.e., mean channel number) derived from the same ratio data used in Fig. 4. Numerical values were calculated using gated analysis (5, 18) which allows for analysis of correlated measurements made within preselected regions of the DNA histograms.

DISCUSSION

Simultaneous analysis of DNA, RNA, and protein by FCM provided for direct correlation of cellular levels of these macromolecules in exponential and G1-arrested populations of CHO cells. Ratios of RNA to DNA and RNA to protein on a cell-to-cell basis were useful in assessing both graphically and numerically the metabolic condition of cells at distinct phases of the cell cycle. The technique was effective in demonstrating the accumulation of the respective cell constituents during the progression of synchronized cells from G1 to S and G2 + M phases of the cell cycle. Although the DNA to RNA and the DNA to protein profiles in this initially synchronized population appeared quite similar, the dispersion in the RNA to protein ratio indicated a considerable heterogeneity in cells arrested than for actively cycling cells. In contrast to the nearly two log reduction in survival of exponentially growing cells treated with AdR and then released into drug-free medium for 15 h prior to plating, the isoleucine deficient, drug-treated cells released into drug-free medium for 15 h prior to plating exhibited a survival value of 84%. Trypan blue measurements indicated that virtually all of the cells from the cohort yielding an 84% survival fraction maintained an intact plasma membrane.
entering S phase. The RNA to protein ratio is a new parameter not previously demonstrated on a per cell basis. The analysis correlates transcriptional and translational activity and can also be useful for detecting conditions of unbalanced cell growth. For example, G₁-arrested cells were initially in a moderate state of unbalanced growth after 36 h of isoleucine deprivation; however, 24 h after growth in complete medium, the population regained equilibrium and metabolic patterns resembled those of exponentially growing cells. At 36 h, the profiles for the two populations were virtually identical (data not shown).

These FCM analyses further revealed conditions of gross imbalance of cellular levels of DNA, RNA, and protein induced by the cycle-perturbing agent, AdR. As early as 15 h after drug treatment, metabolic patterns showed uncoupling of DNA synthesis and cell division associated with continued but disproportionate accumulation of RNA and protein, particularly in the exponential drug-treated population. Based upon the relatively greater degree of imbalance induced in exponentially growing populations in Fig. 4, we would predict that the toxicity of AdR would be greater for cells of this type than cells that were initially arrested in G₁ at the time of drug addition. Survival measurements indicated that this is indeed the case. In the present study, cells assayed for colony-forming ability at 15 h after adriamycin treatment showed an 84% surviving fraction for cells exposed to the drug while in G₁-arrest compared to 12% for the drug-treated exponentially growing cells. At the time of plating, both drug-treated populations exhibited >97% exclusion of trypan blue, indicating that samples processed for and analyzed by FCM were composed predominately of intact cells. Thus, in this instance at least, there is excellent correlation between cell survival and early (i.e., 15 h after drug treatment) indications of cellular metabolic impairment and unbalanced growth (reflected in the abnormal ratios of RNA to DNA and RNA to protein, and drug toxicity).

The multiparameter analysis procedure outlined in this report should be useful in yielding information on the metabolic state of cell populations treated with a variety of experimental agents. In addition, the technique should provide new insight into normal metabolic processes associated with cell cycle progression–related events.

The work was performed under the auspices of the Los Alamos National Flow Cytometry and Sorting Research Resource, funded by the Division of Research Resources of the National Institutes of Health (grant P41-RR01315-02), the Department of Energy, and the United States Public Health Service (grants 1ROCA23296 and CA 28704).

Received for publication 28 November 1985 and in revised form 25 March 1985.

REFERENCES

1. Mitchison, J. M. 1971. The Biology of the Cell Cycle. University Press, Cambridge. 29–33.
2. Pardee, A. B., R. Debrow, J. L. Hamlin, and R. A. Kleitzen. 1978. Animal cell cycle. Annu. Rev. Biochem. 47:715–750.
3. Darzynkiewicz, Z., F. Traganos, T. Sharpless, and M. R. Melamed. 1975. Conformation of RNA in situ as studied by acridine orange staining and automated cytofluorometry. Exp. Cell Res. 95:143–153.
4. Darzynkiewicz, Z., F. Traganos, T. Sharpless, and M. R. Melamed. 1976. Lymphocyte stimulation: a rapid, multiparameter analysis. Proc. Natl. Acad. Sci. USA. 76:358–362.
5. Crippman, H. A., and J. A. Steenkamp. 1973. Rapid simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. J. Cell Biol. 59:766–771.
6. Crippman, H. A., J. V. Egmood, R. G. Holdren, A. Pennings, and C. Haenen. 1981. Simplified method for DNA and protein staining of human hematopoietic cell samples. Cytometry. 2:59–62.
7. Darzynkiewicz, Z., T. Sharpless, L. Siaiano-Coico, and M. R. Melamed. 1980. Subcompartments of the G₁ phase of cell cycle detected by flow cytometry. Proc. Natl. Acad. Sci. USA. 77:6686–6699.
8. Darzynkiewicz, Z., F. Traganos, and M. R. Melamed. 1980. New cell cycle compartments identified by multiparameter flow cytometry. Cytometry. 1:98–108.
9. Darzynkiewicz, Z., H. Crippman, F. Traganos, and J. Steenkamp. 1982. Cell heterogeneity during the cell cycle. J. Cell. Physiol. 113:465–474.
10. Arap-Dovin, D., and T. M. Dovin. 1977. Analyses and sorting of living cells according to deoxyribonucleic acid content. J. Histochem. Cytochem. 25:555–589.
11. Barchet, J. 1940. La detection histoehimique des acids pentose nucleiques Comptes Rendus Des Seances De La Societe De Biologie. 133:88–90.
12. Tacke, H. J., A. B. Nieuwenhuis, G. J. M. Koper, J. C. M. Slats, and J. S. Ploem. 1981. Flow cytometry of human reticulocytes based on RNA fluorescence. Cytometry. 1:313–320.
13. Shapiro, H. M. 1981. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and pyronin Y. Cytometry. 2:143–150.
14. Pollack, A., D. L. Proudhonne, D. B. Greenstein, G. L. Irwin III, A. J. Claffin, and N. L. Block. 1982. Flow cytometric analysis of RNA content in different cell populations using pyronin Y and methyl green. Cytometry. 3:28–35.
15. Göösde, W., I. Spies, J. Schumann, T. Buchner, and G. Klein-Dopke. 1976. Two parameter analysis of DNA and protein content of tumor cells. In Pulse-Cytophotometry. T. Buchner, W. Göösde, and J. Schumann, editors, European Press, Ghent, Belgium. 27–29.
16. Crippman, J. A., C. C. Stewart, and H. A. Crippman. 1982. Three-color fluorescence measurement on single cells excited at three laser wavelengths. Cytometry. 2:226–231.
17. Tobey, R. A., and K. D. Ley. 1971. Isoleucine-mediated regulation of genome replication in various mammalian cell lines. Cancer Res. 31:40–51.
18. Saltman, G. C., S. F. Wilkins, and J. A. Whitfield. 1981. Modular computer programs for flow cytometry and sorting: the LACEL system. Cytometry. 1:325–336.
19. Tobey, R. A., H. A. Crippman, and M. S. Oka. 1976. Arrested and cycling CHO cells as a kinetic model: studies with adriamycin. Cancer Treat. Rep. 60:1829–1837.

CRISSMAN ET AL. Normal and Perturbed Chinese Hamster Ovary Cells 147