The Ratio of Retinoblastoma (RB) to *fos* and RB to *myc* Expression during the Cell Cycle (43940)

ANDREW YEN[1] AND SUSI VARVAYANIS

Cancer Biology Laboratory, Department of Pathology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Abstract. **The putative transregulatory activity of the RB (retinoblastoma tumor suppressor) gene product on the expression of the c-*myc* and c-*fos* proteins during the cell cycle was assessed in HL-60 promyelocytic leukemia cells. Multiparameter flow cytometry was used to simultaneously measure nuclear DNA content, RB protein, and MYC or FOS protein per cell. The amount of RB protein per cell increased with progression through the cell cycle. As the amount of RB protein increased, the ratio of RB to MYC or to FOS protein could be determined per cell as a function of cell cycle phase. Although the amount of RB protein per cell increased with progression through successive cell cycle phases, during S phase the relative rate of increase was not as rapid as that of nuclear DNA. The amount of MYC and FOS per cell also increased throughout the cell cycle, but also more slowly than DNA during S. The ratio of the amount of RB protein to MYC protein remained constant throughout the cell cycle, consistent with putative co-regulation suggested by previous studies of promoter structure. In contrast, the ratio of RB protein to FOS protein increased with progression through the phases of the cell cycle, consistent with a putative negative effect of RB on FOS which was found in previous studies with transgenes and reporters. There was no significant change in these ratios with myelo-monocytic differentiation. Although MYC and FOS have both been implicated as growth-promoting oncogenes putatively transregulated by RB, their behavior during the cell cycle relative to RB is thus distinguishable. Interestingly, in the case of all three of these putative cell cycle regulatory proteins, their cell cycle phase–specific expression levels are consistent with a minimum amount per cell that is necessary but not sufficient for progression to the next cell cycle phase.** [P.S.E.B.M. 1995, Vol 210]

The product of the RB (retinoblastoma) tumor suppressor gene has been implicated in transregulation of two growth promoting genes, c-*myc* and c-*fos* (reviewed in Ref. 1). This premise is motivated in part by studies of motifs occurring in the promoter regions of these genes. The promoters of *myc* and *fos* both contain a retinoblastoma control element (RCE), which allows RB to regulate these promoters in assays using RB transgenes and reporter constructs (2, 3). The RCE is a 30–base pair sequence which binds several proteins, not RB itself. One of them is the Sp1 transcription factor, and confers positive regulation by RB. The RB promoter also contains an Sp1 recognition sequence (4, 5) and can positively autoregulate itself (6). The promoters of *myc* and RB also both contain E2F binding motifs through which overexpression of RB exerts negative regulation (7). Presumably, this may occur through the functional deprivation of E2F by complexing with hypophosphorylated RB protein (1 for review), a mechanism that may also be involved in E2F-dependent cyclin-activated *myc* transcription which can be suppressed by RB over expression (8). Such findings point to the likely

[1] To whom requests for reprints should be addressed at Cancer Biology Laboratory, Department of Pathology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.

Received January 17, 1995. [P.S.E.B.M. 1995, Vol 210]
Accepted July 10, 1995.

0037-9727/95/2103-0205$10.50/0

complexity of the interdigitated regulatory effects on genes such as *myc*. RB overexpression also suppresses transcription from the *fos* promoter in assays using *fos* promoter-reporter constructs (9). In contrast, overexpression of c-*fos* induces overexpression of RB (10). These regulatory relationships between RB, *myc*, and *fos* based on such *in vitro* studies appear complex. For example in different contexts RB might either positively or negatively regulate the *myc* promoter. Furthermore, in differentiating cells expression of the RB and MYC proteins may be co-regulated (11). It would thus be of interest to examine the ratio of RB to MYC and RB to FOS for the endogenous protein levels as RB expression changes naturally during the cell cycle.

When examining the ratio of RB to MYC or to FOS expression as RB varies, assumption of different regulatory relationships such as those above will have different predictable outcomes. For example, if the dominant regulatory modality relating RB and MYC results from common regulatory elements in their promoters causing co-regulation, then the ratio of RB to MYC should remain constant as cells progress through the cell cycle. In contrast, if the dominant regulatory relationship between RB and FOS is RB induced suppression of FOS, then the ratio of RB to FOS should increase as cells progress through the cell cycle. In HL-60 human leukemia cells, for example, it has been shown that the amounts of RB (11–13), *myc* (11, 14), and FOS (15) per cell all increase with progression through the G1, S, and G2 + M phases; however the quantitative ratios of expression are not known.

The actual regulatory relationships are likely even more complicated than the above. One source of additional complication is other proteins which interact at the promoters of these genes. For example, in addition to the E2F and Sp1 binding sequences, the RB promoter also binds ATF (5) as well as p53 (16). The occurrence of mutations in the Sp1 and ATF binding sequences in the RB promoter detected in retinoblastoma patients points to their potential regulatory significance (5). The *myc* promoter likewise responds to other factors in addition to E2F and Sp1. The *myc* promoter also responds to ectopic MYB (17), although the *fos* promoter does not (18). In addition, the *myc* (19) and *fos* (20) promoters both bind the zinc finger transcription factor YY1. Another source of complication is the ability of these transcription factors to undergo protein-protein interactions which might influence their availability to bind DNA. For example, YY1 also binds the *myc* protein (21), as well as complexing with Sp1 (22), suggesting a means by which the amount or avidity of such factors available to bind DNA might be regulated. Furthermore, RB can complex with MYC (23), as can the RB-related p107 protein which binds MYC at the transcriptional activation domain (24). Thus, it is of potential interest to know what relationship between RB and MYC or FOS emerges as the ultimate consequence of a variety of regulatory possibilities.

Correlating parameters measured by population sampling such as Western analysis of the cellular expression of a specific molecular entity in nonhomogeneous cell populations can potentially be misleading. For any given cellular properties, such as the expression level of a specific molecule, correlation of population means may, but does not necessarily, reflect correlation in individual cells. For example, the correlation of induced increases in mean expression for two proteins seen in a cell population may be due to at least two real processes. One is that for each cell, as the expression of one protein increases, so does the other, implying coupling. The other is that there are two distinct subpopulations within the whole population such that the increase in the population means reflects increases that are due to two distinct subpopulations. In contrast to the former case, the latter would imply lack of coupling. To discern rigorously between these possibilities, it is necessary to examine expression of both proteins in each single cell, which can be done by multiparameter flow cytometry.

Materials and Methods

HL-60 cells were maintained in continuous exponential growth in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum in a humidified atmosphere of 5% CO_2 as previously described (11, 15). Stock cultures of 10 ml were initiated at 0.2×10^6 or 0.1×10^6 cells/ml and resuspended in fresh medium every 2 or 3 days, respectively. The doubling time of the cells is approximately 20 hr.

Staining of cells for flow cytometric analysis of DNA, RB, and MYC, or FOS in lieu of MYC, was done essentially as previously described (25). The staining procedure was originally reported for cell cycle phase–specific measurement of RB (25) and was subsequently used to detect MYC (11, 14) and also FOS (15). In the present case, the procedure was modified to simultaneously measure RB plus MYC or FOS by adding the primary antibodies against RB and MYC or RB and FOS simultaneously while reducing the amount of buffer to maintain the 200-μl reaction volume. Secondary staining for the rabbit anti-RB or the murine anti-MYC or FOS was likewise done by simultaneously adding FITC-conjugated goat anti-rabbit immunoglobulin and AMCA-conjugated goat anti-mouse immunoglobulin. Briefly, the cell fixation was done as follows. At indicated times, 1×10^6 cells were harvested from experimental cultures and resuspended in 0.1 ml ice-cold phosphate-buffered saline (PBS), pH 7.2. To fix the cells, 0.9 ml of absolute methanol at −80°C was layered over the PBS cell suspension to

form a discontinuous interface. The suspension was vortexed and stored in sealed microfuge tubes at −20°C until every sample was collected. All samples were then fluorescently stained for DNA, RB, and MYC or FOS protein content as follows. Procedures were carried out on ice. All reagents were ice-cold except as noted. The fixed cells were resuspended and disaggregated by vortexing and centrifuged to a pellet for 45 sec in a microfuge. The pellet was aspirated dry and resuspended in 150 μl PBS. To minimize nonspecific binding, 150 μl of NGS/PBS (50% normal goat serum (heat-inactivated for 30 min at 60°C), 0.002% Triton X-100 in PBS) was then added and the suspension vortexed. The cells were centrifuged to form a pellet which was loosened and resuspended in NGS/PBS so that the final volume was 200 μl when 10 μl of primary anti-RB protein and 40 μl of anti-c-*myc* protein antibody or 50 μl of anti-v-*fos* antibody were added. The suspension was vortexed and incubated for 1.5 hr at 37°C in a water bath with periodic agitation to allow primary antibody binding. The cell suspension was then cooled on ice for 2 min. The cells were centrifuged to a pellet and resuspended in 150 μl PBS to which 150 μl NGS/PBS was then added. To promote dissociation of nonspecific antibody binding, the suspension was stored at 4°C for 30 min on ice with periodic agitation. Then the cells were again centrifuged and resuspended in 150 μl PBS to which 150 μl NGS/PBS was added. For secondary staining, the cells were centrifuged and resuspended in 190 μl NGS/PBS to which 5 μl FITC-GAR (fluorescein-conjugated goat anti-rabbit IgG, heavy and light chain, 12 mg/ml, reconstituted to 2 ml per the manufacturer's instructions and then diluted 1:10 with PBS/NGS [Cappel Division Organon Teknika Corp., West Chester, PA]) plus 5 μl AMCA-GAM (7-amino-4-methylcoumarin-3-acetic acid [AMCA]–conjugated goat anti-mouse IgG, $F(ab)_2$ fragment–specific, affinity-purified [Jackson Immunoresearch Labs, Inc., West Grove, PA], reconstituted per the manufacturer's instructions, and diluted 1:4 with PBS/NGS) were added and mixed. The cells were incubated for 1 hr at 37°C with periodic agitation. The suspension was cooled on ice for 2 min. The cells were centrifuged and resuspended in 150 μl PBS to which 150 μl NGS/PBS and 5 μl RNase (3368 U/mg, 13 mg/ml ribonuclease A; Worthington Biochemicals, Freehold, NJ) was then added. To promote dissociation of nonspecifically bound secondary antibody, the cells were incubated for 0.5 hr at 37°C in a water bath with periodic agitation. The cells were centrifuged and resuspended in 150 μl PBS to which 150 μl NGS/PBS was then added. This wash was repeated. The pellet was then resuspended in 0.75 ml PI/PBS (0.05 mg/ml propidium iodide in PBS). The fixed stained cells were stored on ice until analysis by flow cytometry to derive list mode data files.

Flow cytometry was performed with a dual laser flow cytometer (EPICS 750 series, Coulter Electronics, Hialeah, FL). The primary laser delivered 75 mW of UV excitation. The secondary laser delivered 200 mW of 488 nm excitation with a 7.5-μsec delay. AMCA emission was segregated by a 470-nm long pass dichroic mirror and detected by a photomultiplier tube masked with a 440-nm band pass filter. FITC fluorescence was segregated by a subsequent 550-nm long pass dichroic mirror and detected with a photomultiplier tube masked with a 525-nm band pass filter. PI fluorescence was detected in the remaining fluorescence emission by a photomultiplier tube masked with a 630-nm long pass filter. Forward angle light scatter was used as the trigger signal. The AMCA, FITC, and PI photomultiplier signals were processed by gated amplifiers with windows synchronized to the time of primary and secondary excitation. Ten thousand cells were analyzed per distribution. A typical case from three or more repeat experiments is shown in each instance.

c-*myc* and v-*fos* antibodies, used for flow cytometry as previously described (11, 15), were derived from hybridoma cells (ATCC and Microbiological Assoc.) as follows. Supernatant was collected from exponentially growing cultures (at 48 and 72 hr) and filtered through a 0.2-μm membrane to assure acellularity. Gamma globulin was isolated from the culture supernatants by ammonium sulfate precipitation at room temperature, yielding an approximately 40-fold concentration. Briefly, saturated ammonium sulfate was added dropwise to the culture supernatant to a final concentration of 50% (v/v) with constant stirring overnight. The pH was maintained at neutrality. The suspension was then centrifuged at 1400*g* for 30 min. The pelleted precipitate was dissolved in PBS to a final volume of half than that of the original sample and reprecipitated as above, stirring for 2–3 hr. The suspension was again centrifuged as before and the pellet resuspended in a minimum volume of PBS. The ammonium sulfate was then removed by dialysis in a 12,000– to 14,000–molecular weight exclusion Spectropor membrane against large volumes of PBS for 2 days at 4°C, changing the dialyzate mornings and evenings. The antibody solution was filtered through a 0.2-μm membrane and stored at 4°C. Each batch of antibody was titrated to determine optimum binding before use. The stock *myc* and *fos* antibody concentrations as used for presented experiments were typically 9 to 14 mg/ml. Typically the reaction mixture contained 360 to 700 μg of antibody.

Results

In exponentially proliferating HL-60 human promyelocytic leukemia cells, the amounts of RB and MYC proteins per cell increase with progression

through the cell cycle such that their ratio remains constant throughout the cycle. Multiparameter dual laser flow cytometry was exploited to simultaneously analyze cellular DNA content, RB protein expression, and either MYC or FOS protein per cell. Using exponentially proliferating HL-60 cells, nuclear DNA was fluorescently stained with propidium iodide, a DNA intercalating fluorochrome. RB was fluorescently stained using a rabbit primary antibody and fluorescein conjugated secondary antibody. MYC was fluorescently stained using a murine primary antibody and AMCA-conjugated secondary antibody. The AMCA was UV excited by the primary laser while the propidium iodide and fluorescein were excited at 488 nm by a delayed second laser. AMCA fluorescence emission is blue, while fluorescein emission is green, and propidium iodide emission is red. The delayed propidium iodide and fluorescein emission signals were segregated from the AMCA emission by temporally gated amplifiers which intercept the photomultiplier tube analog voltage signal before analog to digital conversion. The propidium iodide and fluorescein emissions were optically segregated chromatically. Thus, nuclear DNA, RB and either MYC or FOS expression could be simultaneously measured per cell.

The correlation plots between DNA (Fig. 1, horizontal) and either RB, MYC, or FOS (Fig. 1, vertical) are shown in Figure 1. Each dot in the plots represents a measured cell. The amount of each of these proteins per cell increases with progression through G1, S, and G2 + M as shown by the course of the plot going from the beginning of G1 at the lower left to the end of G2 + M at the upper right. In G1 all cells have the same 2C DNA, but increasing protein with advancement through G1. During S phase, as cells synthesize DNA, the amount of RB, MYC or FOS per cell increased as did the amount of DNA. In G2 + M the cells had the same 4C DNA content, but increasing protein with advancement through G2 + M. Figure 2 shows the correlation plots between DNA (Fig. 2, horizontal) and the arithmatic quotient of either RB, MYC, or FOS, and DNA per cell. The amount of RB, MYC, or FOS per DNA increased with progression through G1 when DNA content remained constant. However, the amount of these proteins per DNA decreased with progression through S phase when DNA was being replicated. The rate of increase in the amount of RB, MYC or FOS per cell is slower than the rate of increase in DNA. The short duration of G2 in these cells (approximately 2 hr) makes it difficult to be able to discern any further appreciable changes in G2. Similar qualitative relationships for RB and *myc* were seen in the rate of accumulation relative to DNA synthesis. However the rate of increase of FOS during S was somewhat slower than that of RB and MYC. This was evident in the steeper downward slope of FOS per DNA versus DNA compared with the cases of RB or MYC. Thus the amount of RB, MYC and FOS are all increasing with progression through the cell cycle although their quantitative interrelationships are not apparent from the analysis so far.

The minimum RB, MYC, or FOS protein per cell for G1, S, or G2 + M cells increases, but the maximum is similar for all phases. The frequency distributions of RB per cell for cells restricted to G1, S, or G2 + M derived by list mode analysis of exponential HL-60 cells stained for DNA, RB, and MYC is shown in Figure 3. As can be seen, the mean of the distributions increases with the progression of G1, S and G2 + M distributions. There is also a progressive increase in the apparent minimum RB protein allowed per cell with progression from G1 to S and to G2 + M. However, the maximally occurring RB per cell, evident in the coincident right sides of the distributions, is similar for G1, S, or G2 + M. The distributions are consistent with a minimum RB per cell needed to progress out of

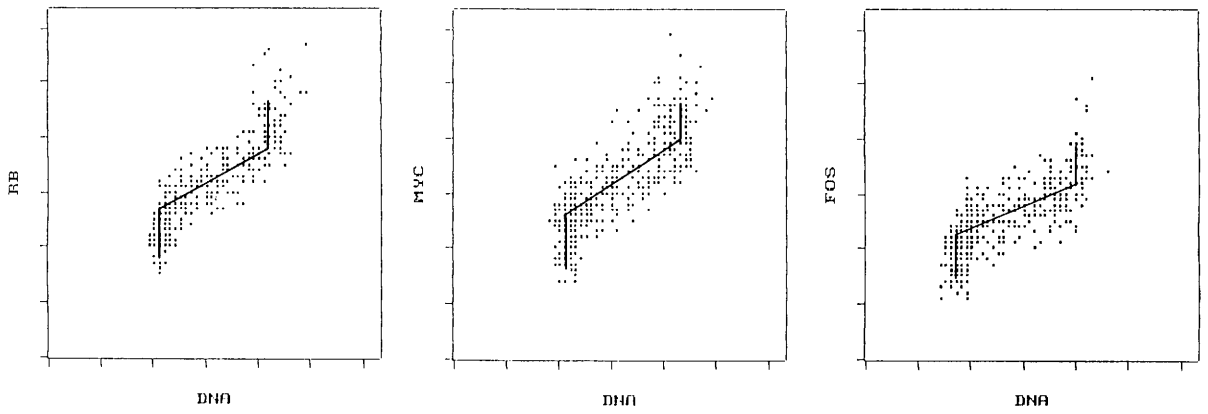

Figure 1. Bivariate correlation plots showing (vertical) RB (left), MYC (middle), or FOS (right) protein per cell versus (horizontal) DNA content. The ascending sigmoid shape shows the increasing RB, MYC, or FOS protein per cell as DNA content increases from G1 (left) through S to G2 + M (right). The amount of protein is represented in arbitrary relative units of fluorescence.

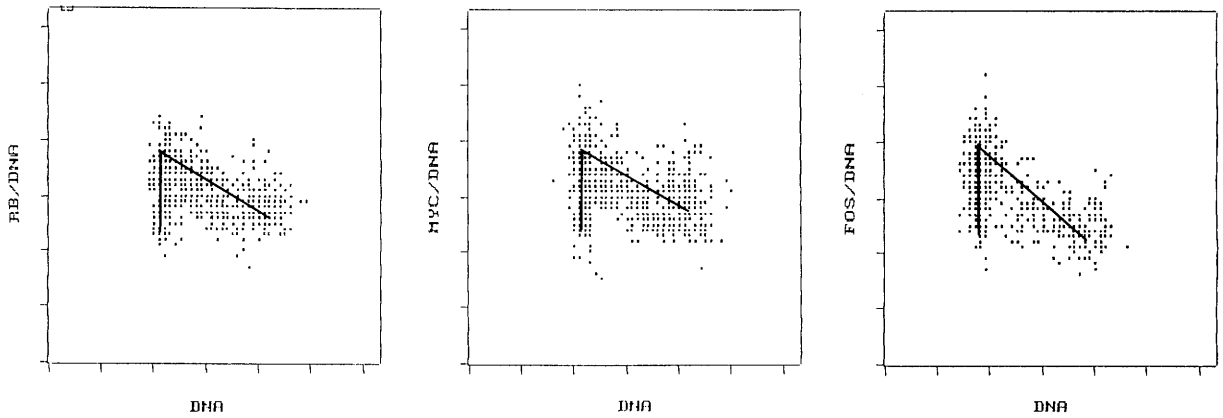

Figure 2. Bivariate correlation plots showing the (vertical) quotient of RB (left), MYC (middle), or FOS (right) to DNA per cell versus (horizontal) DNA content. The quotient increases vertically with advancement through G1 and then decreases progressively with progression through S as DNA is replicated.

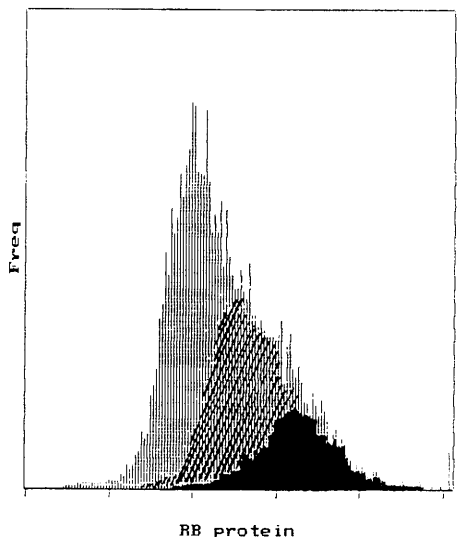

Figure 3. Frequency (vertical) histogram for relative RB protein per cell (horizontal) for G1 (vertical lined), S (cross hatched), and G2 + M (solid) cells. The minimum RB per cell increases with progression from G1 to S and then G2 + M, but the maximum RB expressed in each phase does not substantially change. The relative size (area) of the G1, S, and G2 + M histograms represents the relative number of cells in each phase.

G1 to S and then G2 + M, but the amount of RB alone is not sufficient to cause progression. The frequency distributions for *myc* of G1, S, and G2 + M cells are qualitatively similar, as are those for FOS, and the same analysis applies (not shown). Thus, in all cases the G1, S, and G2 + M frequency distributions are consistent with a condition for a necessary, but not sufficient, amount of RB, MYC, or FOS for G1 cells to go into S and S into G2.

The mean RB, MYC, or FOS protein per cell for G1, S, or G2 + M cells increases, but the quotient of RB to MYC remains constant while the quotient of RB to FOS increases. For cells in G1, S, or G2 + M, Figure 4 shows the mean RB and MYC protein per cell and the mean quotient of RB and MYC per cell. Mean RB and MYC each increase with progression from G1 to S and then G2 + M, but the quotient of RB and MYC remains constant throughout. This is in contrast to the case of FOS. For exponentially proliferating HL-60 cells simultaneously stained for DNA, RB, and FOS, Figure 5 shows the same analysis as performed for RB and MYC. As in Figure 4, protein is shown in relative arbitrary units, though not in the same units as in Figure 4. Likewise, the quotient is normalized to arbitrary units. Mean FOS increases with progression from G1 to S and then G2 + M, but unlike the case of

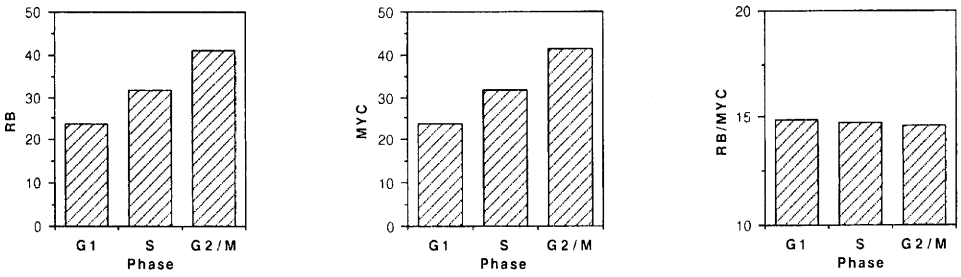

Figure 4. Relative change per cell of RB to MYC with progression through the G1, S, and G2 + M cell cycle phases. Mean RB protein per cell for G1, S, and G2 + M (left panel). Mean MYC protein per cell for G1, S, and G2 + M (middle panel). Mean ratio of RB to *myc* protein per cell for G1, S, and G2 + M (right). (vertical) Arbitrary units which physically represent relative fluorescence (left and middle panels) or the normalized ratio of fluorescence signal intensities (right panel). (horizontal) G1, S, and G2 + M phases.

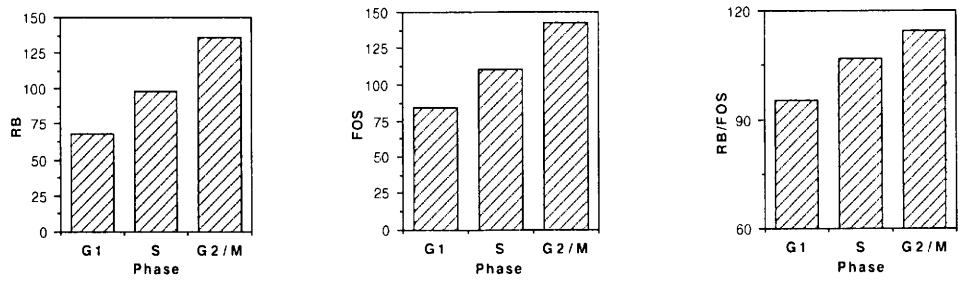

Figure 5. Relative change per cell of RB to FOS with progression through the G1, S, and G2 + M cell cycle phases. Mean RB protein per cell for G1, S, and G2 + M (left panel). Mean FOS protein per cell for G1, S, and G2 + M (middle panel). Mean ratio of RB to *fos* protein per cell for G1, S, and G2 + M (right). (vertical) arbitrary units of fluorescence (left and middle panels) or the normalized ratio of fluorescence signal intensities (right panel). (horizontal) G1, S, and G2 + M phases.

MYC, the ratio of RB to FOS increases. Thus, the FOS per cell increases more slowly than the RB per cell with progression through the cell cycle. This is consistent with the faster decline of FOS per DNA compared with RB per DNA that was apparent in Figure 2. Thus, with progression through the cell cycle, increases in MYC per cell are closely coupled to increases in RB, whereas increases in FOS are relatively inhibited. In the case of FOS, it should be noted that the inhibition was not great and did not prevent FOS from increasing with progression through the cell cycle, although it did so at a slower rate than RB or MYC. Similar quantitative results were observed in five repeat analyses of cells at 0, 24, and 48 hr since time of re-initiation in culture, indicating that the results are independent of time in culture of the cells, which is otherwise a possibility since these genes can react to culture and nutritional conditions.

The nonspecific background and crossover staining signals for FITC in the AMCA channel and AMCA in the FITC channel for the flow cytometric measurement are shown in Figure 6. Cells were stained for DNA and either of the primary antibodies against RB or MYC with either of the secondary antibodies conjugated to FITC or AMCA to test all combinations of background staining and cross-reactivity. As shown in Figure 6, the nonspecific background and crossover signals were minimal relative to the specific signal. Similar results occurred in the case of staining for FOS in lieu of MYC (data not shown).

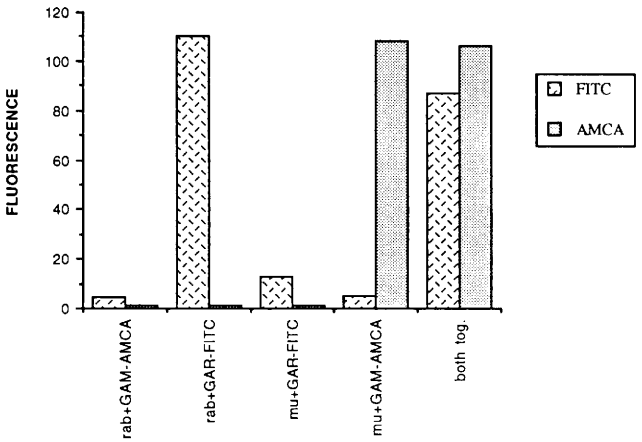

Figure 6. Nonspecific background and crossover signal for cells stained with (left to right) the rabbit anti-RB primary and either the GAM-AMCA or GAR-FITC secondary stained with the murine anti-MYC primary and either secondary and stained with both primary antibodies and both secondary antibodies. The FITC and AMCA signals in each case are shown.

Discussion

In summary, the present results describe the expression of RB, MYC, and FOS per cell for G1, S, and G2 + M cells, measuring RB and MYC or RB and FOS simultaneously with DNA for each cell in exponentially proliferating HL-60 cells. The amount of RB, MYC, or FOS increased with progression through the cell cycle. Frequency distributions of RB, MYC, or FOS per cell for G1, S, or G2 + M cells indicate progressively increasing minimum levels expressed for each successive phase but comparable maximum levels for all phases, consistent with a threshold level for each phase transition that is necessary but not sufficient. As the amount of RB per cell increased with progression through the G1, S, and G2 + M phases, the amount of MYC increased commensurately. The ratio of the RB to MYC protein per cell thus remained constant with progression through the cell cycle. In contrast, the ratio of the RB to FOS protein increased comparing G1 with G2 cells. This reflected the slower increase in FOS relative to RB during cell cycle progression. The data indicate that putative transregulation mechanisms where RB actively enhances or inhibits MYC expression are not the dominant mode of regulation during the cell cycle, since the relative amounts RB and MYC appear to be co-regulated to sustain a constant balance. In contrast, the data for RB and FOS show an increase in the ratio of RB to FOS as RB increased, consistent with the previously reported negative regulation of FOS promoter-reporter constructs by ectopic RB expression. However, the relative inhibition of increases in FOS was much more modest than the extent of inhibition observed with ectopic expression of RB and FOS promoter constructs, indicating the potential presence of compensatory influences.

In some cells, it has been shown that the RB protein exists in two states, unphosphorylated and phosphorylated (1), which may reflect different regulatory capabilities. These states are distinguishable as two bands on a Western analysis, corresponding to a faster migrating unphosphorylated protein distinct from a slower migrating phosphorylated protein. However, in exponentially proliferating HL-60 cells, there is essentially no unphosphorylated RB protein, and a Western analysis shows only a band corresponding to phosphorylated protein (26–28). This simplifies the present analysis by removing the heterogeneity in RB protein resulting from distinguishing between the unphosphorylated and phosphorylated RB protein. Thus, all of the RB protein detected in the present study is in the phosphorylated form.

The present data describe the net effect of all of the potential RB transregulatory mechanisms relating RB and *myc* or *fos* during the cell cycle. Although they do not explicitly identify the mechanism(s) that might actually dominate the regulation, they eliminate certain of those that have been suggested on the basis of structural motifs as potentially dominant regulatory modes. For example, the suppression of MYC expression by RB which is potentially suggested by E2F motifs in the c-*myc* promoter is eliminated as a dominating mode of regulation during cell cycle progression since the ratio of RB to MYC would increase in this case instead of remaining stable. This is in contrast to the ratio of RB to FOS which increased. Likewise co-regulation of MYC and FOS suggested by common motifs in their promoters is also eliminated. Although the relationship of RB to FOS consistent with a potential inhibitory transregulation is not surprising, the parallel regulation of RB and MYC expression is interesting in view of the traditional perception of these genes as having diametric growth inhibitory and promoting functions. The potential co-regulation of RB with MYC, but not FOS, is consistent with their behavior during the retinoic acid or 1,25-dihydroxyvitamin D_3 induced differentiation of HL-60 cells where there was an apparently coupled early decrease in RB and MYC expression (11). In contrast, there was a later decrease of FOS and MYB expression compared with untreated cells (15), consistent with a lack of co-regulation for RB and FOS.

The above considerations on the potential cell cycle regulatory role of the intracellular ratio of RB to MYC and RB to FOS motivate questions about their potential regulatory role during induced cell differentiation. HL-60 cells undergo G0 arrest and differentiation along either the myeloid or the monocytic lineages. Retinoic acid, for example, induces the G0 arrest and myeloid differentiation of the cells; whereas 1,25-dihydroxyvitamin D_3 causes monocytic differentiation. In either case, the induced metabolic cascade occurs over approximately two division cycles with the onset of phenotypic conversion and G0 arrest at 48 hr of treatment (28). By 72 hr, most of the cells are differentiated. During this time, RB, MYC, and FOS protein levels all undergo downregulation (11, 14, 15). If the stoichiometry of RB to MYC or to FOS were of regulatory significance in effecting cell differentiation, then one might anticipate significant changes in these ratios for the inducer treated cells during this period. We were unable to detect any such changes attributable to treatment with retinoic acid or 1,25-dihydroxyvitamin D_3 (Yen A, Varvayanis S, unpublished, for cells treated as in Ref. 11). For each cell cycle phase, G1, S, or G2 + M, the ratio of RB to MYC per cell observed in retinoic acid treated cells were all within 20% of that of untreated controls. The same was true for 1,25-dihydroxyvitamin D_3 treated cells. Likewise for each cell cycle phase the ratio of RB to *fos* per cell was also not greatly affected by retinoic acid. Thus we found no evidence to indicate that these ratios are of obvious regulatory significance during the induced G0 arrest and differentiation of HL-60 cells along either the myeloid or the monocytic pathways since they fail to appreciably change with treatment.

We are grateful to Amy Pellegrino for skillful secretarial assistance in the preparation of the manuscript. This work was supported in part by grants from the National Institutes of Health (USPHS) and the American Institute for Cancer Research.

1. Zacksenhaus E, Bremner R, Jiang Z, Gill RM, Muncaster M, Sopta M, Phillips RA, Gallie BL. Unraveling the function of the retinoblastoma gene. Adv Cancer Res **61**:115–141, 1993.
2. Udvadia AJ, Rogers KT, Horowitz JM. A common set of nuclear factors bind to promoter elements regulated by the retinoblastoma protein. Cell Growth Dif **3**:597–608, 1992.
3. Udvadia AJ, Rogers KT, Higgins PDR, Murata Y, Martin KH, Humphrey PA, Horowitz JM. Sp-1 binds promoter elements regulated by the RB protein and Sp-1–mediated transcription is stimulated by RB coexpression. Proc Natl Acad Sci USA **90**:3265–3269, 1993.
4. T'Ang A, Wu K-J, Hashimoto T, Liu W-Y, Takahashi R, Shi X-H, Mihara K, Zhang F-H, Chen YY, Du C, Qian J, Lin Y-G, Murphree AL, Qiu W-R, Thompson T, Benedict WF, Fung Y-KT. Genomic organization of the human retinoblastoma gene. Oncogene **4**:401–407, 1989.
5. Sakai T, Ohtani N, McGee TL, Robbins PD, Dryja TP. Oncogenic germ-line mutations in Sp1 and ATF sites in the human retinoblastoma gene. Nature **353**:83–86, 1991.
6. Park K, Choe J, Osifchin NE, Templeton DJ, Robbins PD, Kim S-J. The human retinoblastoma susceptibility gene promoter is positively autoregulated by its own product. J Biol Chem **269**:6083–6088, 1994.
7. Hames PA, Gill RM, Phillips RA, Gallie BL. Transcriptional repression of the E_2-containing promoters EIIaE c-*myc* and RB1 by the product of the RB1 gene. Mol Cell Biol **12**:3431–3438, 1992.
8. Oswald F, Lovec H, Moroy T, Lipp M. E2F-dependent regulation of human MYC: Trans-activation by cyclins D1 and A

overrides tumor suppressor protein functions. Oncogene **9**:2029–2036, 1994.
9. Robbins PD, Horowitz JM, Mulligan RC. Negative regulation of human c-*fos* expression by the retinoblastoma gene product. Nature **346**:668–671, 1991. Erratum: Nature **351**:419, 1991.
10. Pai SR, Bird RC. Overexpression of c-*fos* under control of the SV40 promoter complex induces overexpression of RB in transfected cells. Mol Biol Cell **3**:25a, 1992.
11. Yen A, Chandler S, Forbes ME, Fung Y-K, T'Ang A, Pearson R. Coupled down-regulation of the RB retinoblastoma and c-*myc* genes antecedes cell differentiation: Possible role of RB as a "status quo" gene. Eur J Cell Biol **57**:210–221, 1992.
12. Mihara K, Cao X-R, Yen A, Chandler S, Driscoll B, Murphree AL, T'Ang A, Fung Y-KT. Cell cycle dependent regulation of phosphorylation of the human retinoblastoma gene product. Science **246**:1300–1303, 1989.
13. Stokke T, Erikstein BK, Smedshammer L, Boye E, Steen H. The retinoblastoma gene product is bound in the nucleus in early G1 phase. Exp Cell Res **204**:147–155, 1993.
14. Yen A. Cell cycle-specific control of terminal cell differentiation. In: Campisi J, Cunningham DD, Inouye M, Riley M, Eds. Perspectives on Cellular Regulation: From Bacteria to Cancer. MBL Lectures in Biology. New York: Wiley-Liss, Vol. **11**:pp279–296, 1991.
15. Yen A, Samuel V, Forbes M. Regulation of cell proliferation: Late down-regulation of c-*myb* preceding myelo-monocytic cell differentiation. J Cell Physiol **153**:147–156, 1992.
16. Osifchin NE, Jiang D, Ohtani-Fujita N, Fujita T, Carroza M, Kim SJ, Sakai T, Robbins PD. Identification of a p53 binding site in the human retinoblastoma susceptibility gene promoter. J Biol Chem **269**:6383–6389, 1994.
17. Cogswell JP, Cogswell PC, Kuehl WM, Cuddihy AM, Bender TM, Engelke U, Marcu KB, Ting JP-Y. Mechanism of c-*myc* regulation by c-*myb* in different cell lineages. Mol Cell Biol **13**:2858–2869, 1993.
18. Evans JL, Moore TL, Kuehl WM, Bender T, Ting JP-Y. Functional analysis of c-*myb* protein in T-lymphocytic cell lines shows that it trans-activates the c-*myc* promoter. Mol Cell Biol **10**:5747–5752, 1990.
19. Riggs KJ, Saleque S, Wong KK, Merrell KT, Lee JS, Shi V, Calame K. Yin-Yang 1 activates the c-*myc* promoter. Mol Cell Biol **13**:7487–7495, 1993.
20. Natesan S, Gilman MZ. DNA bending and orientation-dependent function of YY1 in the c-*fos* promoter. Gene Dev **7**:2497–2509, 1993.
21. Shirvastava A, Saleque S, Kalpana GV, Artandi S, Goff SP, Calame K. Inhibition of transcriptional regulator Yin-Yang-1 by association with c-*myc*. Science **262**:1889–1892, 1993.
22. Lee JS, Galvin KM, Shi V. Evidence for physical interaction between the zinc-finger transcription factors YY1 and SP1. Proc Natl Acad Sci USA **98**:6145–6149, 1993.
23. Hateboer G, Timmers HT, Rustgi AK, Billaud M, van't Veer LJ, Bernards R. TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on the c-MYC and adenovirus E1A protein. Proc Natl Acad Sci USA **90**:8489–8493, 1993.
24. Gu W, Bhatia K, Magrath IT, Dang CV, Dalla Favera R. Binding and suppression of the MYC transcriptional activation domain by p107. Science **264**:251–254, 1994.
25. Yen A, Chandler S, Sturzenegger-Varvayanis S. Regulated expression of the RB "Tumor Suppressor Gene" in normal lymphocyte mitogenesis: Elevated expression in transformed leukocytes and role as a "Status Quo" gene. Exp Cell Res **192**:289–297, 1991.
26. Yen A, Forbes ME, Varvayanis S, Tykocinski ML, Groger RK, Platko JD. C-FMS dependent HL-60 cell differentiation and regulation of RB gene expression. J Cell Physiol **157**:379–391, 1993.
27. Yen A, Soong S, Dwon HJ, Yoshida M, Beppu T, Varvayanis S. Enhanced cell differentiation when RB is hypophosphorylated and down regulated by Radicicol a SRC-kinase inhibitor. Exp Cell Res **214**:163–171, 1994.
28. Yen A, Varvayanis S. Late dephosphorylation of the RB protein in G2 during the process of induced cell differentiation. Exp Cell Res **214**:250–257, 1994.