Inducers of Leukemic Cell Differentiation Cause Down-Regulation of RB Gene Expression (43359)

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Abstract. **Expression of the retinoblastoma (RB) tumor suppressor gene during cell differentiation induced by dimethyl sulfoxide or sodium butyrate was studied in HL-60 human promyelocytic leukemia cells. As cells progressed through the cell cycle, the amount of RB protein per cell increased with homeostasis maintained, so that the amount of RB protein relative to the total cell mass remained almost constant. Dimethyl sulfoxide was used to induce these promyelocytic leukemia cells to undergo terminal differentiation into mature myeloid cells. There was an early reduction in the RB protein expressed per cell. The reduction in expression was similar for cells in all cell cycle phases. There was also progressively reduced expression at later times as cells terminally differentiated. This was compared to the case in which sodium butyrate was used to induce the differentiation of HL-60 cells into mature monocytic cells. An early reduction in RB protein expression per cell also occurred. It occurred for cells in all cell cycle phases as well. Thus, the induced differentiation of HL-60 cells along either the myeloid or the monocytic differentiation lineage involves an early reduction in RB expression, which is common to both pathways. The reduction anteceded proliferative arrest or differentiation. In both cases, the final, resulting G_0-differentiated cells had less RB protein per cell than the proliferating, immature, leukemic precursor cells.**
[P.S.E.B.M. 1992, Vol 199]

The retinoblastoma (RB) tumor suppressor gene is the recessive gene whose loss of function confers susceptibility to development of retinoblastoma (1–3) and other tumors (4–7). Its function in normal cell regulation is largely obscure. It might be speculated that it is involved in proliferation and differentiation control since loss of the appropriate RB protein apparently allows tumor growth. The potential role of the RB protein in cell proliferation is also suggested by certain findings about properties of its phosphorylation state. The RB protein is differentially phosphorylated with respect to the cell cycle. The unphosphorylated form predominates in early G_1 period, while the phosphorylated form occurs more frequently with progression through the cell cycle phases (8–10). Phosphorylation of the protein is also reduced with terminal ($G_{1/0}$) differentiation (10) of HL-60 cells. The RB protein has been found to bind *in vitro* to the viral transforming agents papilloma virus-16 E7, adenovirus E1A, and SV-40 T antigen (11–13). The binding of RB protein to T antigen depends on its phosphorylation state where the hypophosphorylated protein preferentially binds (13). Thus, it has been suggested that the RB protein has an antiproliferative action which may be neutralized when it complexes with viral transforming agents, or, alternatively, that it may inhibit the proliferation promoting activity of the viral transforming agents or some cellular homolog. Finally, the amount of RB protein per cell is regulated when normal lymphocytes are transformed by Epstein-Barr virus, as well as during the mitogenesis of normal lymphocytes (14). Given its potential growth regulatory role, it might be expected that the amount of the RB protein per cell may be regulated during terminal cell differentiation, when $G_{1/0}$ cells arrest and assume a mature differentiated phenotype. The HL-60 human promylocytic leukemia cell line will undergo terminal differentiation along either the myeloid or

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Received May 15, 1991. [P.S.E.B.M. 1992, Vol 199]
Accepted September 24, 1991.

0037-9727/92/1993-0291$3.00/0
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monocytic pathways when exposed to different inducers (15, 16). Dimethyl sulfoxide (DMSO) induces myeloid differentiation, whereas sodium butyrate induces monocytic differentiation. The two inducers, DMSO, a membrane-reactive compound, and sodium butyrate, a phosphodiesterase inhibitor, are disparate in biochemical character, but both evoke cell differentiation. If RB is an antiproliferative tumor suppressor gene, then this context provides a test of whether it is regulated during terminal cell differentiation.

Previous studies have shown that the induced onset of terminal differentiation of HL-60 cells is anteceded by an induced metabolic cascade, which occurs over a period corresponding to two cell-division cycles. This metabolic cascade segregates into two basic steps, early and late events, with an intermediate "pre-commitment" regulatory state (15–17). The precommitment state represents a memory state in which cells are primed to differentiate. Thus, consummation of early events results in a differentiation lineage-independent priming of cells to differentiate. Previous studies showed that retinoic acid, another inducer of myeloid differentiation, and 1,25-dihydroxy vitamin D_3, another monocytic inducer, elicit the same essential early events leading to precommitment, although they induce different ultimate phenotypes (15, 16).

Materials and Methods

Cells and Culture Conditions. HL-60 human promyelocytic leukemia cells, a generous gift of Drs. John Sokolowski and Allen Sartorelli, were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum as described previously (16). The doubling time of the cells is approximately 24 hr. The cells are periodically tested to verify their capability to undergo terminal differentiation, that is, $G_{1/0}$ specific growth arrest and phenotypic differentiation in response to retinoic acid, DMSO, 1,25-dihydroxyvitamin D_3, sodium butyrate, and tetradecanoyl phorbol myristate acetate. Cell cycle distribution is assayed by flow cytometry, as described previously (16). Functional differentiation is assayed by inducible oxidative metabolism detected by reduction of formazan, as described previously (16).

Cells were initiated in culture at a density of 0.25 $\times 10^6$ cells/ml culture medium and incubated at 37°C in a 5% CO_2 humidified atmosphere. Inducer, either DMSO or sodium butyrate, was added, as described before (18, 19), to make a final concentration of 1.25% DMSO or 1×10^{-3} *M* sodium butyrate, which was added from a stock of 1.0 *M* butyric acid in phosphate-buffered saline.

RB Expression per Cell Assay. RB protein expression per cell was assayed simultaneously with genomic DNA content using flow cytometry as previously described (10, 14). Briefly, 10^6 cells were fixed in cold (−80°C) 90% methanol and stored at −20°C. The fixed cells were resuspended in phosphate-buffered saline with heat-inactivated goat serum and Triton X-100. A previously described antibody (10, 20, 21) against a peptide fragment of the RB protein was used as a primary antibody in immunostaining for cellular RB protein content. The antibody was a generous gift from Dr. Y. K. Fung. A secondary goat antirabbit immunoglobulin-fluorescein isothiocyanate reagent (Cappel Laboratories) was used. DNA staining with propidium iodide was done following digestion with RNase. Flow cytometry was done with a multiparameter dual laser flow cytometer (EPICS; Coulter Electronics), as described previously. Computer-implemented programs performed statistical and listmode data analysis. Each analyzed histogram represents 10,000 cells. The histograms and data shown are typical of three repeat experiments in each instance.

Results

DMSO and sodium butyrate induce a reduction in the per cell content of RB protein. The reduced expression is pan-cell cycle in that it occurs similarly for cells in all cell cycle phases. In both cases, the decrease in expression is evident after one division cycle in the presence of the inducer and progresses. HL-60 cells, which were initially exponentially proliferating, were initiated in culture with 1.25% DMSO. At this and periodic subsequent times, cells were harvested for analysis of RB protein content and DNA content by flow cytometry. Cells were immunostained using fluorescein isothiocyanate to detect cellular RB protein, and propidium iodide stained to detect DNA. Thus, for each cell in the harvested sample, the RB protein and DNA content could be simultaneously determined. Figure 1 shows the sequence of isometric plots of number of cells as a function of DNA content and RB protein expression for cells treated with DMSO. The plots are of cells at 0, 24, and 48 hr, when onset of terminal differentiation occurs. By integrating cells with all DNA contents, a frequency histogram of RB protein expression for the entire population was derived. From this, the mean RB protein expression per cell could be calculated. Figure 2 shows the density plots relating RB protein expression per cell and DNA content (cell cycle progression).

From the isometric plots of cells as a function of RB protein versus DNA content, it can be seen that as cells progressed through the cell cycle, there was a progressive increase in RB protein content consistent with ongoing accumulation of the protein throughout G_1, S, and G_2. This relative relationship persisted with the addition of DMSO. However, there was a reduction in RB expression that was similar for cells throughout the cell cycle. This can be seen in the isometric plots (Fig. 1) for DMSO-treated cells. (The plots for sodium butyrate-treated cells are similar.) It is also evident in the dot density plots for cells treated with DMSO (Fig.

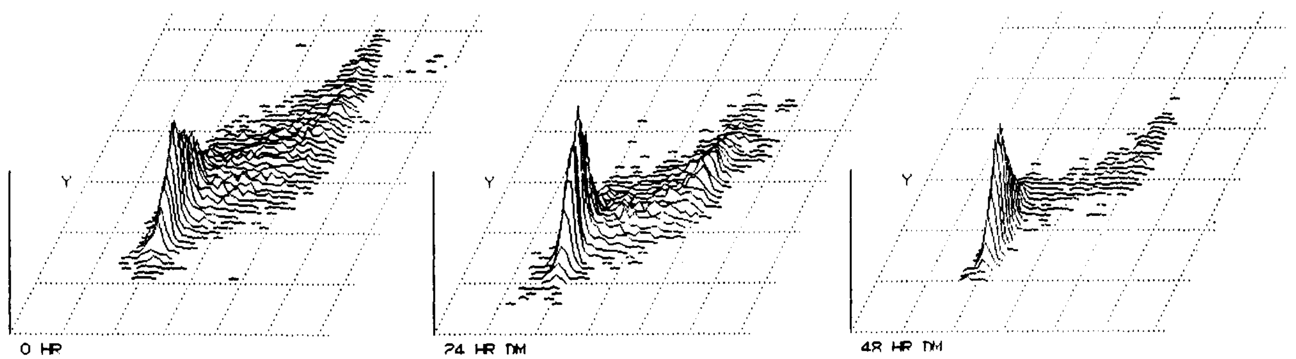

Figure 1. Bivariate isometric plots relating RB protein expression (*y* axis) and DNA content (*x* axis) for HL-60 cells treated with 1.25% DMSO for 0, 24, and 48 hr. (left, middle, and right, respectively). The *z*-axis (vertical) represents the relative number of cells. The peak proximal to the origin is G_1 cells, with S and then, finally, G_2+M cells progressively more distal to the origin. Each distribution contains 10,000 cells.

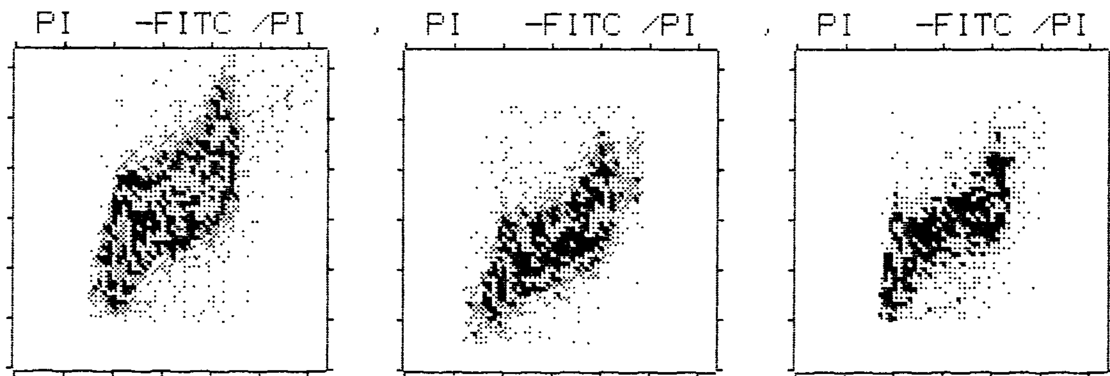

Figure 2. Dot density plots of RB protein (*y* axis) correlated with DNA content (*x* axis) for HL-60 cells treated with 1.25% DMSO for 0, 24, and 48 hr (left, middle, and right, respectively).

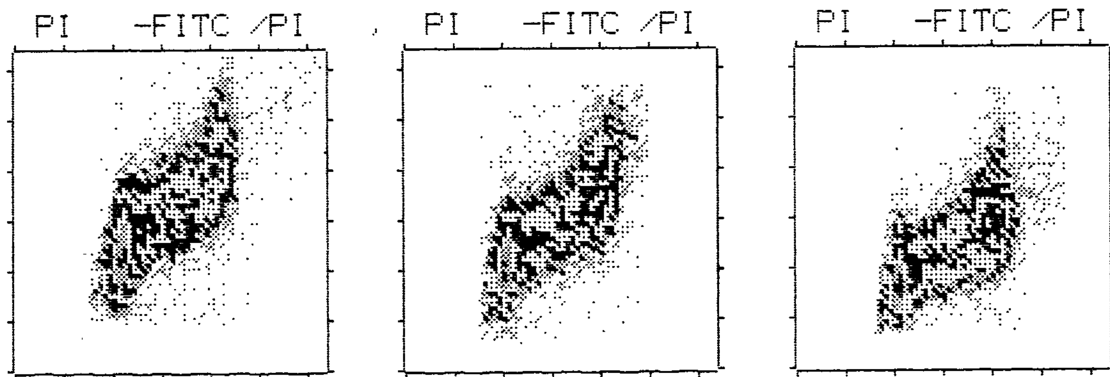

Figure 3. Dot density plots of RB protein (*y* axis) correlated with DNA content (*x* axis) for HL-60 cells treated with 10^{-3} *M* sodium butyrate for 0, 24, and 48 hr.

2) or with sodium butyrate (Fig. 3) at 0, 24, and 48 hr. Thus, both DMSO and sodium butyrate caused an early reduction in RB protein expression per cell for cells in all cell cycle phases.

The progressive decrease in RB expression induced by either DMSO or sodium butyrate over the first 48 hr (approximately two division cycles) was not sustained at later times. Figure 4 shows the fractional change in RB expression levels for cells treated with DMSO. The progressive decrease in RB protein content per cell was largely restricted to the initial 48 hr of exposure. Thereafter, no further decrease was evident,

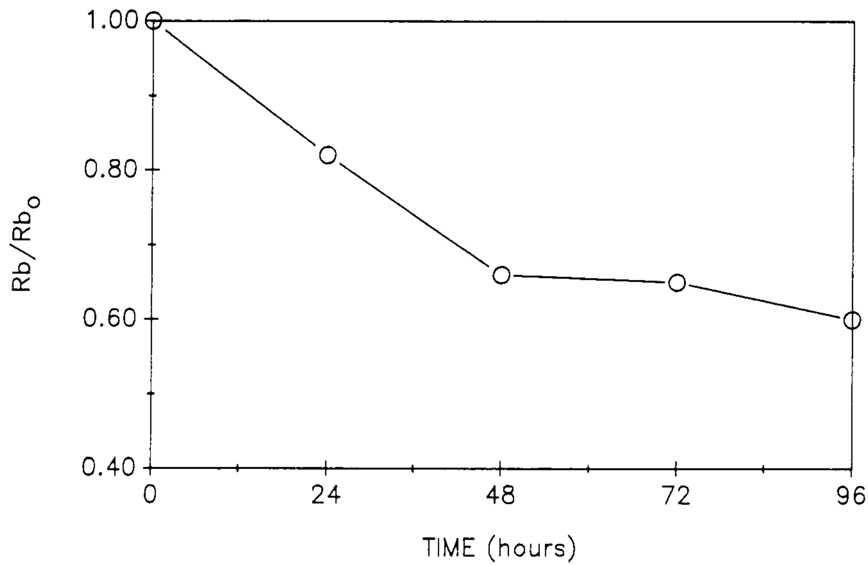

Figure 4. Fractional change in mean RB protein per cell as a function of time during treatment with DMSO. Fractional change is expressed with respect to untreated control cells. Means were derived from analysis of 10,000 cells at each time.

suggesting that the regulatory significance of RB expression levels is exercised relatively early in the induced metabolic cascade. The fractional change in RB expression for sodium butyrate-treated cells is shown in Figure 5. As in the case of DMSO, the induced progressive decrease in RB protein per cell was restricted largely to the initial 48 hr of exposure, except for a decline at 96 hr. The reduction, although obviously consistent, was significantly less than that observed for DMSO. Thus, both the myeloid inducer, DMSO, and the monocytic inducer, sodium butyrate, caused an early decrease in RB expression levels. But at later times, these inducers had different effects on RB expression, where the decrease was either arrested or enhanced. During this period, exposure to these inducers initiated a metabolic cascade, where onset of significant growth arrest or phenotypic differentiation was not apparent until after 48 hr. The distribution of the cells treated with either

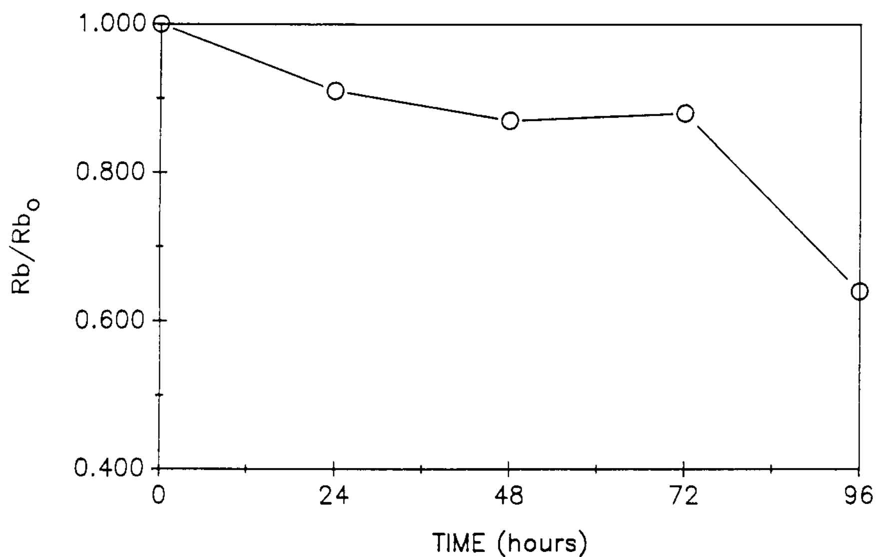

Figure 5. Fractional change in mean RB protein per cell as a function of time during treatment with sodium butyrate.

inducer in the $G_{1/0}$, S, G_2+M cell cycle phases, and the percentage of cells undergoing myelomonocytic differentiation, detected by their increasing ability to produce super oxide and to express the Mol differentiation specific marker, are given in Table I. Thus, the observed early decreases in RB expression preceded overt changes in proliferation or differentiation.

The reduction in RB expression at late times is not due to just the induced arrest of cells in $G_{1/0}$. Both DMSO and sodium butyrate are known (18, 19) to induce the $G_{1/0}$ arrest of HL-60 cells at late times with terminal differentiation. Since the G_1 cells have less RB than cells in other cell cycle phases, the observed decrease in RB expression may reflect, in part, this cell cycle effect. However, a comparison of the bivariate RB protein versus DNA isometric plots shows that the $G_{1/0}$ arrested cells have a reduced RB content compared with G_1 cells in the original cell population. Figure 6 shows the isometric RB protein versus DNA maps for the original cells and for those after 96 hr of DMSO treatment. The maps for sodium butyrate-treated cells are similar. The peak proximal to the origin is the G_1 subpopulation, which can be seen to have a reduced RB protein content at 96 hr.

The dependence of the amount of the RB protein per cell on cell cycle progression observed in all cases above represents an increase in the amount of RB protein with progression through the cell cycle, which is approximately commensurate with the increase in cell size. In both inducer-treated and untreated cells, there was a similar relative increase in the amount of RB protein per cell. If the RB protein per cell is normalized to the size of the cell, then the relative abundance of the RB protein per cell mass as cells advance through the cell cycle is approximately constant. Figure 7 shows the dot density plot of RB protein per cell versus DNA compared with the plot of RB protein per cell size versus DNA for cells that had been exposed to DMSO for 48 hr. The RB protein per cell size was approximated by the quotient of the RB fluorescence signal and the forward angle scattered light intensity, a measure of cell size by flow cytometry (14). Although the total amount of RB protein per cell increased, the RB protein normalized to total cell size was approximately constant[2] as cells went from G_1 to G_2+M. This was true for cells treated with DMSO or sodium butyrate at all the measured times, as well as for the untreated cells. Thus, it appears that cells going through the cell cycle regulate RB protein expression to preserve the amount of RB protein relative to the total cell mass set at the onset of the cell cycle.

Table I. Response to DMSO or Sodium Butyrate[a]

Inducer	Time (hr)	G_1 (%)	S (%)	G_{2+M} (%)	SO(+) (%)	Mol (%)
Control	0	56	33	11	0	0
	24	44	43	13	6	0
	48	50	36	14	3	0
	72	53	33	13	11	0
	96	58	14	14	7	0
DMSO	24	46	36	18	11	0
	48	68	17	15	35	6
	72	67	21	11	60	22
	96	82	11	7	74	48
Sodium butyrate	24	42	36	22	11	0
	48	46	34	20	35	1
	72	63	22	15	78	6
	96	69	19	12	81	14

[a] For untreated (control), DMSO-, or sodium butyrate-treated cultures, the values shown are the percentage of cells in each cell cycle phase (G_1, S, G_{2+M}), as determined by flow cytometry, as well as the percentage of functionally differentiated cells capable of oxidative metabolism (superoxide production [%SO(+)]) and the percentage of cells expressing the Mol myelomonocytic differentiation-specific surface marker.

Discussion

Both DMSO and sodium butyrate, inducers of myeloid or monocytic differentiation of HL-60 cells (18, 19), induce an early down-regulation of RB expression per cell. The reduction antecedes onset of differentiation along either the myeloid or monocytic pathways. In both instances, the leukemic, immature, proliferating cells have more RB protein than the final, G_0-differentiated cells. If phosphorylation of the RB protein does not occur until S and subsequently in the cell cycle (8–10), then since G_1 cells have less RB protein after treatment with inducer as shown here by flow cytometry, the untreated leukemic cells also have more of the hypophosphorylated protein than differentiating cells. Similar results have been found in the cases of retinoic acid-induced myeloid differentiation or 1,25-dihydroxyvitamin D_3-induced monocytic differentiation (22). This is in striking contrast to the anticipation of the paradigm where the RB protein, particularly the hypophosphorylated form, functions to inhibit proliferation.

In all cases of myeloid or monocytic differentiation, the reduction in expression occurred for cells in all cell cycle phases. Thus, the observed down-regulation was superimposed on another level of regulation that governed the amount of protein as a function of cell cycle phase, where RB protein content increases parallel to cell size as cells progress through the cell cycle. Therefore, there may be two layers of regulation; one with respect to maintaining the appropriate relative amount of protein as cells go through the cell cycle and another

[2] This is an approximation, since the forward angle light scatter (FALS) intensity is proportional to the cross-sectional area of the scattering body, allowing there are no changes in other optical parameters, such as the index of refraction. The cross-section, S, is proportional to the 2/3 power of the volume, $V^{2/3}$. Because of this, if a given cellular protein and volume double from the beginning to the end of the cell cycle, the amount of protein normalized to FALS will increase by approximately 25%. This was observed when comparing RB normalized to FALS for G_1 and for G_2+M cells using list mode analysis.

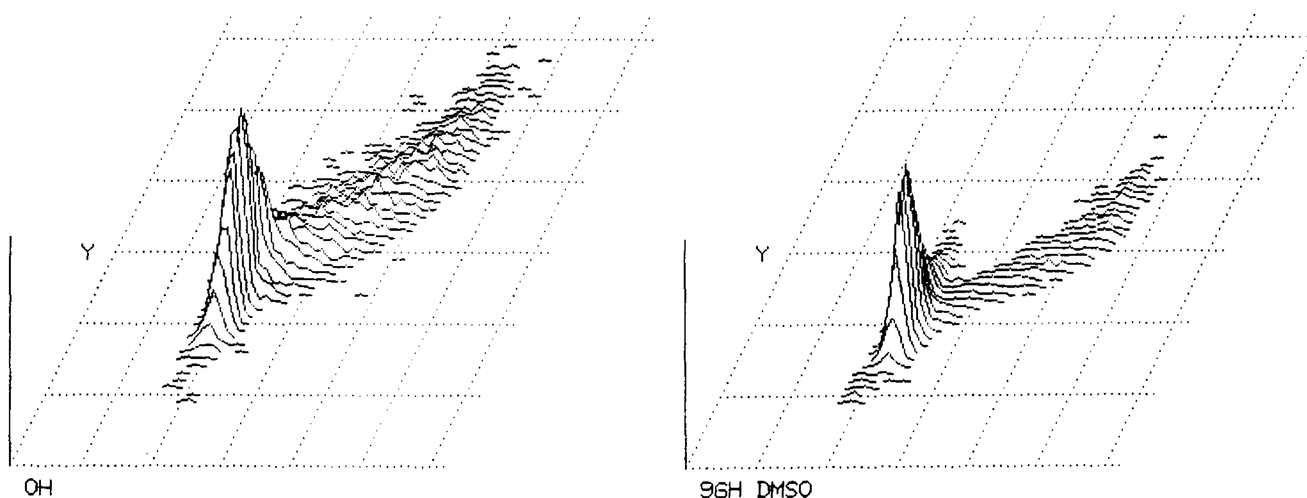

Figure 6. Isometric plots relating RB protein per cell to DNA content for untreated cells (left) and cells treated for 96 hr with DMSO (right). The $G_{1/0}$ peak of the DMSO-treated cells is at a reduced RB protein level (*y* axis) compared with the untreated cells.

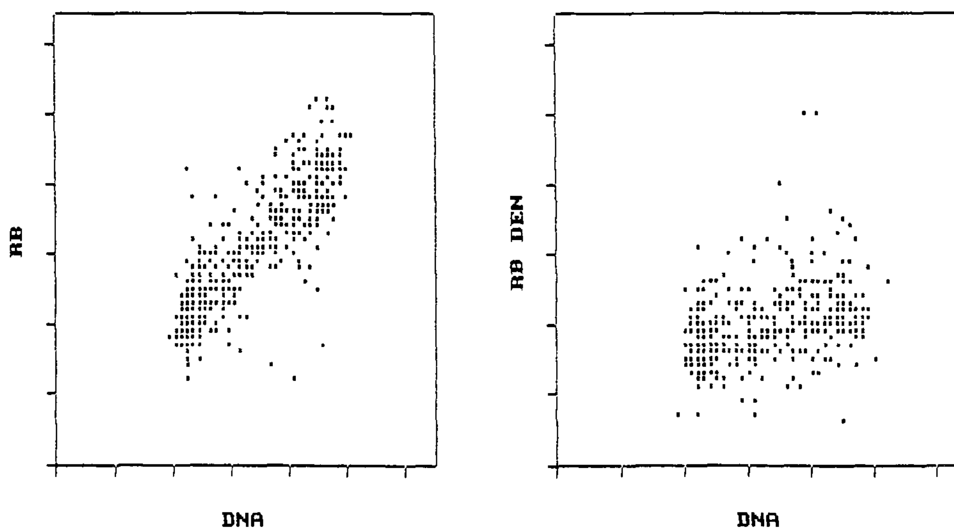

Figure 7. Dot density plots of (left) RB protein (*y* axis) correlated with DNA content (*x*-axis), and (right) RB protein normalized to cell size (RB density) (*y* axis) correlated with DNA content (*x* axis) for cells exposed to DMSO for 48 hr. The RB protein normalized to cell size was approximated from a list mode analysis of the flow cytometric data by forming the quotient of the RB protein-specific fluorescence signal and the forward angle light scatter signal for each cell (14). This quotient was then correlated to the DNA content of the cell and represented in the dot density plot. At other times and for untreated cells, the RB protein per total cell mass was also constant with respect to cell cycle phase.

superimposed on that which sets the overall expression levels.

The current perspective of the RB gene is that it is a tumor suppressor gene whose loss of function renders susceptibility to tumorigenesis. More recent data (10, 14) on its regulation in promyelocytic leukemia cells and lymphocytes show, however, that it may have a role in regulating terminal cell differentiation. Although that role is obscure yet, one possibility suggested by these and our previous studies is that it does not appear to function simply as an inhibitor of proliferation. Significantly, it has also been found that Epstein-Barr virus-transformed, proliferatively active lymphocytes have more RB protein than do normal, proliferatively quiescent lymphocytes (14). A potential reconciliation of the historical and present data might be made by proposing that RB functions normally as a cellular brake against change, that is, as a "status quo" gene. It is hypothesized in this case that elevated levels of RB expression are required to sustain the developmentally ordained differentiation state, whereas reduction in expression allows a change from that state. Consistent with this potential interpretation of its function is the finding that in normal peripheral blood lymphocytes, mitogenesis of the G0 cells by pokeweed mitogen results in a transient down-regulation of RB expression that antecedes recruitment of cells into S phase (14). This is followed by significantly elevated expression levels in

the proliferating lymphocytes. Pokeweed mitogen induces proliferative amplification and the differentiation of B lymphocytes into plasmacytes, and, thus, has been considered as an *in vitro* model for aspects of the humoral immune response. If RB functions as a status quo gene, then it can be rationalized that its transient down-regulation was needed in order for the lymphocytes to undertake a new program of proliferation and differentiation. Finally, the finding that Epstein-Barr viral transformation of normal lymphocytes results in proliferating cells with higher levels of RB expression indicates that viral transformation may affect RB expression (14). This perspective of the possible status quo function of RB thus provides a possible rationalization for the historical data, as well as for these findings.

Supported in part by grants from the United States Public Health Service (National Institutes of Health), American Institute for Cancer Research, Council for Tobacco Research, and Cornell Consolidated and Biotechnology Programs. In particular, this work was supported (in part) by a grant from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the US Army Research Office, and the National Science Foundation.

We are grateful to Amy Pellegrino for skillful secretarial assistance in the preparation of the manuscript. We thank Dr. Y. K. Fung for generously providing the antibody against the RB protein and for many conversations. We thank Mary Forbes for performing the analysis of cell cycle and differentiation.

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