Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1549–1552, March 1982
Cell Biology

Growth inhibitory and stimulatory effects of retinoic acid on murine 3T3 cells

(cell cycle/flow cytometry/comitogenesis/DNA replication)

EDWARD W. SCHRODER, ELIEZER RAPAPORT, ALISA KASTAN KABCENELL, AND PAUL H. BLACK

Department of Microbiology, Boston University School of Medicine, and Hubert H. Humphrey Cancer Research Center at Boston University, Boston, Massachusetts 02118

Communicated by Herman M. Kalckar, December 3, 1981

ABSTRACT ***All-trans*-β-retinoic acid (RA) has both comitogenic and antiproliferative effects on murine Swiss 3T3 cells. Treatment of quiescent 3T3 cells for <24 hr with micromolar concentrations of RA potentiates subsequent mitogenic response of those cells to phorbol 12-myristate 13-acetate. Longer exposures of 3T3 cells to RA result in inhibition of DNA replication as measured by [^{3}H]thymidine incorporation and decreased growth rates and saturation densities for cells grown in either 2% or 10% serum. Both the comitogenic and antiproliferative activities of RA for 3T3 cells are RA-dose dependent. RA-induced decreases in the 3T3 cell saturation density are reversible only after resuspension of cells by trypsinization and replating. Treatment of 3T3 cells for 48 hr with RA inhibits the rate of [^{3}H]thymidine incorporation by 35–50%, while autoradiographic data show that labeling indices are similar to control values. Equal percentages of control and 48-hr RA-treated quiescent 3T3 cells respond to subsequent stimulation with 10% serum as determined by autoradiographic and flow cytometric analyses. However, the progression of RA-treated cells through the S phase of the cell cycle is slowed. These data suggest that inhibition of 3T3 cell proliferation by RA is established after a minimum 24-hr treatment and that this inhibition is the result of a decreased rate of DNA replication in S-phase cells.**

Retinoids are derivatives of vitamin A, many of which have been shown to have potential value as cancer-chemopreventive (1) or -therapeutic (2) agents. It has been suggested that these potentialities could relate, in part, to direct effects of retinoids on tumor cells (1–3). There is evidence that retinoids can modify patterns of cell proliferation *in vitro*. A large number of cell lines have been examined, yielding data indicating that retinoids can be inhibitory (4–9), ineffectual (4, 5, 7), or stimulatory (5, 8, 10–12).

The mechanisms by which retinoids modulate cell proliferation of various cell lines in opposite ways are unknown. We, and others, have approached this question by examining the effects of retinoids on the response of untransformed quiescent murine 3T3 cells to mitogens. These experiments have led to reports of retinoid potentiation of the mitogenic response of 3T3 cells to various growth factors and the tumor promoter, phorbol 12-myristate 13-acetate (PMA) (12, 13). We have extended these studies and now report data that clarify the relationship between retinoid potentiation of the 3T3-cell mitogenic response and the long-term effects of retinoids on 3T3-cell proliferation. Our results suggest that continuous treatment of 3T3 cells with *all-trans*-β-retinoic acid (RA) results in inhibition of DNA synthesis in S-phase cells and prevents a proliferative manifestation of the enhanced mitogenic response observed with shorter exposures. The relevance of serum concentration to RA effects on 3T3-cell proliferation is also considered as is altered density inhibition of cellular proliferation and its reversibility.

MATERIALS AND METHODS

Cells and Tissue Culture. The origin and history of the murine Swiss 3T3 cells used in this study have been described (14). Cells were grown at 37°C in Dulbecco's modification of Eagle's minimal essential (DME) medium supplemented with various concentrations of fetal calf serum, penicillin at 250 units/ml, and streptomycin at 250 μg/ml under humidified 90% air/10% CO_2. All medium components were obtained from GIBCO. Cells were passaged every 3 to 4 days by dispersion with trypsin/EDTA (15), replated at $5.5 \times 10^3/cm^2$, and used between passages 8 and 15 in our laboratory. These cells have been demonstrated to be free of *Mycoplasma* contamination (15).

Studies of Cell Proliferation. Cells were plated at 2×10^4 per 35-mm-diameter tissue culture dish in DME medium/10% serum. After an overnight attachment period, cells were changed to various test media. Cell numbers were determined for duplicate cultures at 24-hr intervals by duplicate cell counts with an electronic particle counter (Coulter Electronics, Hialeah, FL). Cell-doubling times were calculated for the period of exponential growth 24–72 hr after addition of test media and represent the 48-hr period divided by the number of cell doublings ($\log_2$ cell number at 72 hr minus $\log_2$ cell number at 24 hr) (16).

Assay of DNA Synthesis. Incorporation of [^{3}H]thymidine (17 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Schwarz/Mann, Orangeburg, NY) by subconfluent serum-deprived 3T3 cells in response to various stimuli was used as a measure of DNA synthesis. 3T3 cells were plated at 4×10^4 per 35-mm-diameter tissue culture dish in DME medium/10% serum. After a 4-hr attachment period, cells were changed to DME medium/0.25% serum and incubated for 72 hr. This protocol yields $0.8–1.0 \times 10^5$ quiescent cells per 35-mm dish (labeling indices, <4.0).

For mitogenesis experiments, quiescent cells were treated with RA in serum-free DME medium for various times, washed once with DME medium, and stimulated. Synthesis of DNA in response to various stimuli was assessed during the peak of the response by labeling for 30–60 min with [^{3}H]thymidine (2 μCi/ml) in DME medium, 18–22 hr after stimulation. The concentrations (20 or 50 ng/ml) of PMA used as mitogenic stimuli in some experiments were selected to yield <20% of the rate of [^{3}H]thymidine incorporation obtained in response to 10% serum (13). Labeled cells were washed, extracted with 5%

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; RA, *all-trans*-β-retinoic acid; DME medium, Dulbecco's modification of Eagle's minimal essential medium.

Cl_3CCOOH, solubilized, and assayed by liquid scintillation spectrometry as described (17).

Autoradiographic analyses were carried out by exposing Cl_3CCOOH-extracted cells to Kodak NTB-2 nuclear track emulsion for 4 days. After developing, cells were stained with Giemsa, and labeling indices were determined by counting 1000 cells in each of two dishes under ×430 magnification.

Flow Cytometric Analyses. Cells cultivated in 100-mm-diameter dishes were prepared for flow cytometry by washing twice with trypsin/EDTA (15) and leaving a small volume of this solution over the cells until most cells had rounded. Five milliliters of DME medium/10% serum was added to each dish, and the cells were scraped with a rubber policeman. Resuspended cells were pelleted by low-speed centrifugation at 4°C, and suspended again in 1 ml of propidium iodide at 50 μg/ml in 0.1% sodium citrate at 0°C by vigorous mixing. Propidium iodide-stained cells were subjected to flow cytometric analysis using a Bio/Physics 4800 A cytofluorograph with 10-mW excitation at 488 nm from an argon ion laser source. Total fluorescence was measured in the 510- to 700-nm range. Distribution of fluorescence from individual cells was accumulated in a Bio/Physics 2101 distribution analyzer (10^4 cells were analyzed for each sample).

Reagents. RA and PMA were obtained from Sigma. RA was dissolved to a concentration of 10 mM in 100% ethanol and stored at −20°C in small portions. All procedures involving the use of RA were performed in subdued light. Control cultures were treated with equivalent concentrations of ethanol, which never exceeded 0.1%. PMA was dissolved in dimethyl sulfoxide, diluted in DME medium to 10 μg/ml, and stored in small portions at −20°C.

RESULTS

We, and others, have previously reported that micromolar concentrations of RA can enhance the response of quiescent 3T3 cells to mitogenic stimuli (12, 13). However, cultures of asynchronously growing 3T3 cells do not show increased proliferative activity (Fig. 1). Cultivation of 3T3 cells in the presence of 10 μM RA results in continued growth at a decreased rate and to a lower saturation density as compared with controls for cells

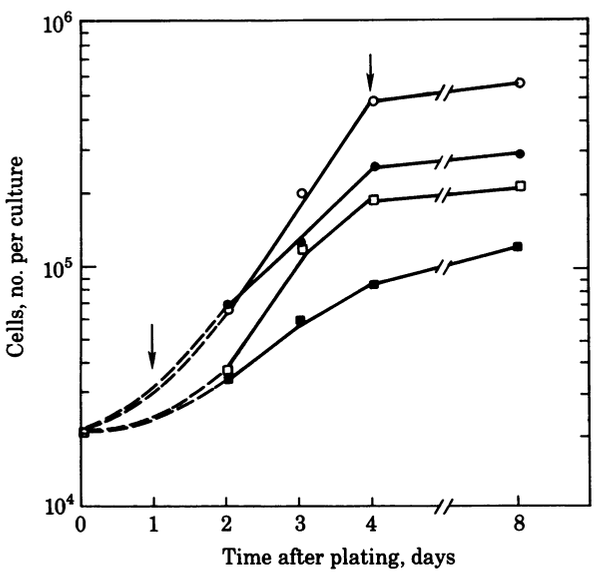

FIG. 1. Growth curves of 3T3 cells. Cells were grown in DME medium/10% (○ and ●) or 2% (□ and ■) serum in the absence (○ and □) or presence (● and ■) of 10 μM RA. ↓, Days on which media were changed.

Table 1. Reversibility of RA-induced decrease in 3T3 cell saturation density

Treatment with 10 μM RA		
Previous two passages	Final passage	Final density, cells × 10^{-4}/cm^2
−	−	14.6
−	+	5.7
+	−	13.7
+	+	5.5

grown in DME medium supplemented with either 2% or 10% serum. Decreased growth rates are not apparent until cells have been treated with RA for longer than 24 hr. Refeeding of density-inhibited cultures (days 4–8) does not significantly increase the saturation density of RA-treated cultures. In other experiments, we have found that refeeding of RA-treated 3T3 cells with fresh control medium does not reverse the effect of RA on saturation density, even after 4 days in control medium (data not shown). However, RA-treated cells that are trypsinized and replated in control medium reacquire a normal saturation density (Table 1). The decreased growth rates of logarithmically growing RA-treated 3T3 cells are RA-dose dependent (Table 2) for cells grown in medium supplemented with either 10% or 2% serum. Cells grown in 2% serum are more sensitive to the inhibitory effects of RA than are cells grown in 10% serum.

In view of the disparity between the effects of continuous treatment of asynchronously growing 3T3 cells with RA and the enhanced mitogenic response of quiescent 3T3 cells treated with RA (12, 13), we have further examined the effects of RA on 3T3-cell mitogenesis. We found that treatment of quiescent 3T3 cells with 10 μM RA for increasing lengths of time followed by addition of a suboptimal concentration of PMA results in RA enhancement of the mitogenic response to PMA only in cultures treated with RA for 24 hr or less (Table 3). Cultures treated with RA for 30 or 48 hr incorporate less [^{3}H]thymidine than controls. The RA stimulation of the mitogenic response to PMA is optimal with 4–7 hr treatments. The RA enhancement of the mitogenic response of quiescent 3T3 cells to PMA is also RA-dose dependent (Table 4). Stimulation of the response to PMA is maximal at 10 μM RA. Sustained treatment of 3T3 cells with higher concentrations of RA results in cell detachment and death. Stimulation of the response to PMA is still detected at 1 μM RA. Treatment with RA without subsequent stimulation results in a small increase in control levels of [^{3}H]thymidine incorporation at 10 μM only. In other experiments, we have shown that RA enhances the mitogenic response of quiescent 3T3 cells to other suboptimal stimuli, including supranormal calcium concentrations (13) and low concentrations of serum (unpublished observations). Control experiments have shown that RA treatment alone does not increase [^{3}H]thymidine uptake into acid-soluble pools of quiescent 3T3 cells (data not shown). We have shown previously that potentiation of the mitogenic response of 3T3

Table 2. RA-dose dependence of 3T3 cell-doubling times

	Doubling time, hr	
RA, μM	10% serum	2% serum
0 (Control)	17.1 (0)	20.3 (0)
10	25.0 (100)	37.2 (100)
1	21.4 (54)	35.8 (92)
0.1	21.0 (49)	28.0 (46)
0.01	18.5 (18)	28.4 (48)
0.001	17.9	23.8 (21)
0.0001	17.1	18.5

Values in parentheses are percent of maximal increase.

Table 3. Effect of duration of RA treatment on RA enhancement of the 3T3 cell mitogenic response to PMA

Duration, hr	[^{3}H]Thymidine incorporation,* % of response to 10% serum
0	11.6 ± 3.6
4	44.9 ± 0.3
7	55.8 ± 2.6
18	20.3 ± 2.9
24	15.4 ± 0.8
30	7.7 ± 1.8
48	0.8 ± 0.3

Cells were treated with 10 μM RA for various time durations and then tested for response to PMA at 20 ng/ml. Data are mean ± SD of duplicate determinations for each of two cultures.
* Serum response: 52,153 cpm per culture; unstimulated control, 2.0 ± 0.3%; RA treatment alone, 5.2 ± 3.3%.

cells by RA, as detected by pulse labeling, is also reflected in labeling indices determined by autoradiography of cultures continuously labeled for 24 hr with [^{3}H]thymidine (13).

Despite the failure of 48-hr RA-treated quiescent 3T3 cells to respond to suboptimal doses of PMA (Table 3), these cells do respond to 10% serum in the same numbers as control cells, as indicated by labeling indices (Table 5) and flow cytometry data (Fig. 2 *A* and *B*). The percentage of cells that incorporate [^{3}H]thymidine in response to serum is comparable for control and RA-treated cultures at 18 hr after serum addition. Preliminary experiments have shown that the rate of [^{3}H]thymidine incorporation is maximal at this time for both control and RA-treated cells (data not shown). However, the rate of [^{3}H]thymidine incorporation at this time is 35–50% lower for RA-treated cultures, as determined by liquid scintillation counting procedures (18). We have shown that uptake of [^{3}H]thymidine into dTTP pools is comparable for control and RA-treated cultures under these conditions. This suggests that, although the same percentage of control and RA-treated cells traverse the G_1 phase and enter the S phase of the cell cycle in response to serum, 48-hr treatment of 3T3 cells with RA results in a decreased rate of DNA replication in S-phase cells. This interpretation is further supported by the kinetics of parasynchronous progression of control vs. RA-treated cells through the cell cycle in response to serum (Table 5 and Fig. 2). Labeling indices (Table 5) show that, at 20 and 22 hr after serum addition, higher percentages of RA-treated cells remain in the S phase of the cell cycle as compared with controls. RA-treated cells do complete progression through the S phase, as indicated by reduced labeling indices 24 and 26 hr after serum addition (Table 5). Flow cytometric analyses of relative DNA content are in agreement with these data (Fig. 2). At 18 hr after addition of serum (Fig. 2 *A* and *B*), strong S-phase shoulders are apparent for both control and RA-treated cells. At 22 hr after serum addition (Fig. 2 *C* and *D*), both control and RA-treated cells display a pronounced G_2 + M peak; however, control cultures have fewer S-phase cells, as indicated by the more distinct separation of the G_1 and G_2 + M peaks. By 26 hr after serum addition (Fig. 2 *E* and *F*), control cultures show a more pronounced G_1 peak than do RA-treated cultures while the latter continue to display a more prominent G_2 + M peak. These data suggest that RA-

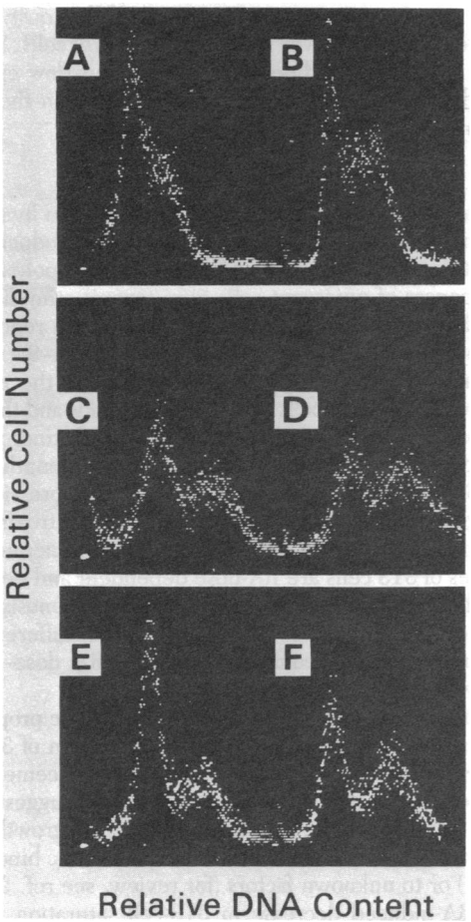

FIG. 2. Flow cytometric analyses of serum-stimulated 3T3 cells. Quiescent 3T3 cells were treated with RA and serum stimulated as described in Table 5. Cells were harvested and prepared for flow cytometry at 18 hr (*A*, control; *B*, RA treated), 22 hr (*C*, control; *D*, RA treated), and 26 hr (*E*, control; *F*, RA treated) after serum addition.

Table 4. RA-dose dependence of enhancement of mitogenic response of 3T3 cells to PMA

	[^{3}H]Thymidine incorporation,* % of response to 10% serum			
	Experiment 1		Experiment 2	
RA, μM	Without PMA	With PMA	Without PMA	With PMA
0 (Control)	1.5	13.7	2.4	17.9
10.0	4.4	57.7	9.6	69.2
3.2	2.3	38.7	ND	ND
1.0	1.8	23.0	2.9	26.0
0.3	1.1	15.0	ND	ND
0.1	1.3	17.1	2.2	18.7

Cells were treated for 6 hr with various concentrations of RA and then tested for response to PMA at 50 ng/ml. Data are mean of duplicate determinations for each of two cultures. ND, not determined.
* Serum response: 53,227 cpm per culture, experiment 1; 33,813 cpm per culture, experiment 2.

Table 5. Kinetics of RA-treated 3T3 cell progression through the cell cycle in response to serum

Time after serum addition, hr	Labeling index	
	Control	RA treated
0	8.2 ± 4.4	8.9 ± 0.1
18	68.5 ± 1.1	69.8 ± 3.0
20	51.5 ± 2.2	62.2 ± 0.0
22	31.6 ± 0.8	49.7 ± 2.1
24	32.6 ± 0.5	34.3 ± 3.0
26	37.9 ± 2.5	29.0 ± 1.8

Quiescent 3T3 cells were treated for 48 hr with DME medium/0.25% serum. Cells were stimulated with DME medium/10% serum beginning at time zero. RA (5 μM) or solvent control were present throughout. Data are mean ± SD.

treated cells continue to progress parasynchronously through their cycle but at a slower rate than do control cells. Taken together, the [^{3}H]thymidine incorporation and flow cytometric data indicate that RA-treated cells are slowed in the S or G_2 (or both) phases of the cell cycle.

DISCUSSION

There have been several reports of cell activation by retinoids, including enhanced plasminogen activator production (19), stimulation of prostaglandin synthesis (20), and potentiation of the responses of quiescent cells to mitogenic stimuli (12, 13). These data have been difficult to reconcile with reports of inhibition of cellular proliferation by retinoids (4–9). Our data show that, for Swiss 3T3 cells, potentiation of the mitogenic response of quiescent cells to PMA by retinoids and the growth inhibitory properties of retinoids represent distinct phases of the response to retinoids that depend on the length of treatment. Shorter ($\leq$24 hr) treatments result in expression of the comitogenic properties of retinoids while longer treatments result in inhibition of DNA replication and cell proliferation. Both responses of 3T3 cells are RA-dose dependent and demonstrable in the micromolar dose range. Since mitogenesis and cell growth experiments must be performed with different serum concentrations, exact coincidence of the RA dose–response curves would not be expected.

It is of interest that, although the comitogenic properties of retinoids do not result in enhanced proliferation of 3T3 cells, other reports of retinoid-induced growth enhancement of different cell lines have appeared (5, 8, 10, 11), suggesting cell-specific variation in the effects of retinoids on growth regulation. Such differences could relate to cytoplasmic binding proteins (21) or to unknown factors (for review, see ref. 22).

The RA-induced decrease in 3T3-cell saturation densities reported here is consistent with reports for other cell lines (4–9). The failure of cultures to achieve the same final density as controls on simple replacement of RA-containing media could represent a continued presence of retinoid in an active form, perhaps associated with lipophilic cellular components. Alternatively, the persistence of lower cell densities after removal of RA may relate to previous elaboration of increased amounts of extracellular matrix by RA-treated cells, as has been demonstrated for some cell types (23, 24). The reversal of RA-induced reduction of 3T3-cell saturation density on trypsinization and replating in the absence of RA may reflect a removal of extracellular matrix.

Evidence presented here suggests that the mechanism of RA-induced inhibition of 3T3-cell proliferation relates to a decreased rate of DNA replication in S-phase cells and is not the result of a block during the G_1 phase of the cell cycle. These data suggest that 3T3 cells respond differently to retinoids than do Chinese hamster ovary cells. The latter are completely blocked in the G_1 phase of their cell cycle by retinol, due to inhibition of polyamine biosynthesis (8, 25). Relevance of polyamine biosynthesis to the 3T3-cell growth inhibition by retinoids is possible nonetheless, since inhibitors of the polyamine pathway have recently been shown to lengthen both the G_1 and the S phases of the Chinese hamster ovary cell cycle (26). Our data do not rule out an RA effect on the G_2 phase of the 3T3 cycle. Further studies will be required to clarify this point. It is of interest that induction of a reversible growth arrest of transformed human cells by substitution of homocysteine for methionine in growth media has recently been shown to result from lengthening of the S and G_2 phases of the cell cycle (27). Additional evidence (18) suggests that the molecular mechanisms of RA-induced potentiation of mitogenesis and subsequent inhibition of DNA replication may both be due to expansion of cellular acid-soluble nucleotide pools.

We thank Drs. R. McCaffrey and M. Osband for providing flow cytometer facilities and E. Cohen for performing the analyses. We also thank Dr. I.-N. Chou for many helpful discussions. This work was supported by National Institutes of Health Grants CA 28107 (to P.H.B.) and CA 28803 (to E.R.). This is publication no. 93 of the Hubert H. Humphrey Cancer Research Center at Boston University.

1. Sporn, M. B. & Newton, D. L. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2528–2534.
2. Bollag, W. (1979) *Cancer Chemother. Pharmacol.* **3**, 207–215.
3. Lotan, R. (1980) *Biochim. Biophys. Acta* **605**, 33–91.
4. Lotan, R. & Nicolson, G. L. (1977) *J. Natl. Cancer Inst.* **59**, 1717–1722.
5. Lotan, R. (1979) *Cancer Res.* **39**, 1014–1019.
6. Lotan, R. & Nicolson, G. L. (1979) *Cancer Res.* **39**, 4767–4771.
7. Jetten, A. M., Jetten, M. E. W., Shapiro, S. S. & Poon, J. P. (1979) *Exp. Cell Res.* **119**, 289–299.
8. Haddox, M. K., Scott, K. F. & Russell, D. H. (1979) *Cancer Res.* **39**, 4930–4938.
9. Patt, L. M., Itaya, K. & Hakomori, S. (1978) *Nature (London)* **273**, 379–381.
10. Christophers, E. (1974) *J. Invest. Dermatol.* **63**, 450–455.
11. Tchao, R. & Leighton, J. (1979) *Invest. Urol.* **16**, 476–482.
12. Dicker, P. & Rozengurt, E. (1979) *Biochem. Biophys. Res. Commun.* **91**, 1203–1210.
13. Schroder, E. W., Chou, I. N. & Black, P. H. (1980) *Cancer Res.* **40**, 3089–3094.
14. Black, P. H. (1966) *Virology* **28**, 760–763.
15. Chou, I. N., O'Donnell, S. P., Black, P. H. & Roblin, R. O. (1977) *J. Cell. Physiol.* **91**, 31–38.
16. Patterson, M. K., Jr. (1979) *Methods Enzymol.* **58**, 141–152.
17. Chou, I. N., Prezyna, C. & Black, P. H. (1979) *J. Biol. Chem.* **254**, 10588–10591.
18. Rapaport, E., Schroder, E. W. & Black, P. H. (1982) *J. Cell. Physiol.* **111**, in press.
19. Wilson, E. L. & Reich, E. (1978) *Cell* **15**, 385–392.
20. Levine, L. & Ohuchi, K. (1978) *Nature (London)* **276**, 274–275.
21. Chytil, F. & Ong, D. E. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2510–2514.
22. Schroder, E. W. & Black, P. H. (1980) *J. Natl. Cancer Inst.* **65**, 671–674.
23. Adamo, S., DeLuca, L. M., Akalousky, I. & Bhat, P. V. (1979) *J. Natl. Cancer Inst.* **62**, 1473–1477.
24. Sasak, W., DeLuca, L. M., Dion, L. D. & Silverman-Jones, C. S. (1980) *Cancer Res.* **40**, 1944–1949.
25. Haddox, M. K. & Russell, D. H. (1979) *Cancer Res.* **39**, 2476–2480.
26. Harada, J. J. & Morris, D. R. (1981) *Mol. Cell. Biol.* **1**, 594–599.
27. Hoffmann, R. M. & Jacobsen, S. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7306–7310.