Retinol Inhibition of Ornithine Decarboxylase Induction and G1 Progression in Chinese Hamster Ovary Cells

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ABSTRACT

Vitamin A inhibits ornithine decarboxylase (ODC) induction in G1 phase of the cell cycle of synchronous cultures of Chinese hamster ovary (CHO) cells. The retinol-promoted inhibition of ODC was not the result of an effect on general protein synthesis, was effective only in G1, prior to the time an increase in ODC activity had begun, did not involve disruption of the G1-phase increase in cyclic adenosine 3',5'-monophosphate-dependent protein kinase activity, and required transcription-dependent events for reversal. The inhibition of ODC induction was associated with a block of cell cycle progression in G1 phase. In the presence of the vitamin, there was no incorporation of [3H]thymidine, no induction of S-phase-dependent S-adenosyl-L-methionine decarboxylase, and no ultimate cell doubling. The inhibition of ODC and the inhibition of S-phase transition displayed a similar concentration dependence, with 60% inhibition occurring in the presence of 80 μM retinol, a similar dependence on cell locus in early G1 phase for efficacy, and a similar reversal after removal of the vitamin, with the increase in ODC preceding the increase in S-phase transition in a parallel fashion. Arrest of the cells at the G1-retinol-sensitive restriction point prior to ODC induction resulted in the inhibition of the G1-phase-dependent increase in RNA synthesis.

Other naturally occurring retinoids also inhibit CHO cell growth (retinal > retinol > retinyl acetate > retinoic acid). Retinal, the most potent, displayed a paradoxical effect on CHO cells. At very low concentrations (1 to 5 μM), retinal stimulated CHO growth parameters; ODC activity was enhanced in a parallel fashion. At higher concentrations, retinal inhibited in a manner similar to that described for retinol.

INTRODUCTION

Vitamin A and several of its analogs have prophylactic and therapeutic effects on the chemical induction and growth of benign and malignant tumors in whole animals (4, 5, 66). In general, there is a direct, inverse relationship between the level of retinoid in the diet and the incidence of neoplasms after a chemical carcinogen (65). Carcinogenesis is a prolonged, multistage process, and the vitamin apparently acts to inhibit the promotion phase and does not affect initiation. Whether the action of vitamin A is directed to an event specific to some aspect of the transforming process or results from inhibition of an essential aspect of cell growth is unknown.

Synchronous cells in culture provide a model to study the sequential events of the growth response under controlled conditions without the influence of a transforming or promoting stimulus. We have previously demonstrated in mitotically synchronized CHO cells that vitamin A treatment arrested cells in mid-G1 phase (16, 17). The inhibitory effect of vitamin A was a function of cell cycle position; cell cycle progression was blocked only when retinol was added to the cells prior to mid-G1 progression. The arrest was reversible and resulted in a 2-hr delay in the onset of DNA synthesis in cells exposed to a G1 pulse of retinol, suggesting that, in the presence of the vitamin, the cells progressed to a point in mid-G1 prior to some key event required for G1-S-phase transition. An increase in ODC activity is a ubiquitous event marking G1 progression of proliferating cells (28, 57). The excursion of ODC activity in retinol-treated cells was used, therefore, as a marker of the extent of G1 progression to localize the temporal site of vitamin action. It was found that the vitamin-induced arrest blocked the cells at a site prior to the induction of ODC. These results suggested the presence of a retinol-sensitive restriction (50) or commitment point in CHO cells during mid-G1 phase that ODC induction was associated with passage through or beyond this point. In the present studies, we have examined this relationship between the inhibition of ODC and the inhibition of growth in order to establish any obligatory correlations between the 2 vitamin-inhibited processes.

MATERIALS AND METHODS

Cell Culture Techniques. CHO cells were grown in monolayer in McCoy's Medium 5A containing 20% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cell cultures were incubated at 37° in a humidified 95% air-5% CO2 atmosphere. Cell-doubling times were 13 to 15 hr under these conditions. Cell numbers were determined by an electronic particle counter (Coulter Electronics, Hialeah, Fla) on samples collected by scraping with a rubber policeman into Puck's Saline A. Cells were synchronized by selective detachment of cells blocked in metaphase by a 2-hr incubation in the presence of 0.06 μg colcemid per ml (67). The mitotic cells (mitotic index, >90%) were washed free of the drug by centrifugation, resuspended in fresh, complete medium (first wash, 4°; second wash, 37°), plated (0.5 to 1 x 106 cells per 5 ml media) in plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37°, and allowed to progress through the cell cycle. Within 60 min, greater than 90% of the cells had exited mitosis. Flow microfluorometric measurements of cellular DNA content were performed with a Coulter Model FPS-1 cell sorter on Puck's

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Saline A-washed, ethanol-fixed (70%, 10 min) cells suspended in hypotonic citrate containing 5 mg propidium iodide per 100 ml as the fluorescent DNA stain (33). Retinoid solutions were prepared in dioxane under subdued light and used immediately. Control cell populations received an equivalent amount of dioxane (0.5%), which was found to have no effect on growth parameters. Survival of cells in the presence of retinol or dioxane was determined by the ability of the cells to form colonies. Three dilutions of cell number were plated in triplicate into new plastic Petri dishes containing prewarmed media in growth media alone or containing 0.5% dioxane or retinol and incubated for 10 days. Colonies were fixed with 50% acetic acid, stained with crystal violet, and counted. Cell viability was determined by the capacity to give rise to a colony of 50 or more cells.

Macromolecular Synthesis. Entry into S phase of synchronized cell populations was estimated by pulse measurements of [3H]thymidine incorporation into acid-insoluble material. At the times indicated after mitotic release, 10 x 10⁶ cells in 5 ml full medium were incubated with the isotope (5 μCi/ml) for 15 to 30 min. The incubation was terminated by removing the media and washing the monolayer twice with fresh media prior to harvesting the cells. Cells were pelleted by centrifugation and extracted by sonication (two 30-sec bursts) at 4° in 500 μl 0.5 mM trichloroacetic acid. Insoluble radioactivity was determined by filtration of the extract through cellulose 1<sub>e</sub> filters (0.45-μm pore; Millipore Corp., Bedford, Mass.); the level of cpm/10<sup>⁶</sup> cells/min was taken as an index of DNA-synthetic activity. RNA- and protein-synthetic rates were determined by similar pulse measurements upon incubating the cells with [3H]uridine or [3H]leucine, respectively.

Polyamine Determinations. Aminoguanidine bicarbonate (10⁻⁵ M) was included in the medium of cultures (2 x 10⁶ cells) to be analyzed for polyamine concentrations to inhibit diaminooxidase present in serum. At the times indicated after mitosis, the medium (5 ml) was poured off into 1 ml 50% trichloroacetic acid, and 2.5 ml of chilled 10% trichloroacetic acid was rapidly added to the cell monolayer. The cell precipitate was scraped into solution, the dish was rinsed with an additional 2.5 ml acid, and the extract was sonicated (two 30-sec bursts) to ensure cellular disruption. Five hundred pmol of 3,3'-iminobispropylamine were added to both media and cell extracts to serve as an internal standard for estimation of polyamine recovery, and the samples were stored at -80° until processing for analysis. Extracts were centrifuged for 15 min at 20,000 x g, and the supernatant was removed and extracted 3 times with 3 volumes of water-saturated ether to remove the acid, evaporated to dryness, and finally suspended in 200 μl of 0.1 N HCl. An 80-μl aliquot of the reconstituted extract was analyzed for putrescine, spermidine, and spermotline concentrations utilizing a Durrum D-500 automatic amino acid analyzer (41, 58).

Assay for ODC Activity. ODC activity was measured in cell supernatants as previously described with minor modifications (59). Cells were harvested by scraping, sedimented and sonicated (two 30-sec bursts) in 0.5 ml 50 mM Na₂H₇K₂PO₄, pH 7.2, containing 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride, and 30 μM pyridoxal phosphate. Two 200-μl aliquots of a 10,000 x g supernatant from each sample were incubated for 60 min at 37° in the presence of 0.25 mM L-[1-¹⁴C]orotidine (0.5 μCi) in 15-mli

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RESULTS
Concentration-dependent Inhibition of ODC and Thymidine Incorporation by Retinol Addition to Mitotically Synchronized CHO Cells. In a previous report, we described the coincident inhibition of ODC induction and cell cycle progression after the addition of retinol (120 μM) to mitotically synchronized CHO cells (16, 17). Chart 1 depicts the concentration dependence of these 2 effects of vitamin A. ODC varied in
activity in the control cultures as a function of the CHO cell cycle (Chart 1, inset). The enzyme activity was maximal in mitotic cells and was rapidly turned over after mitotic exit to achieve a minimal expressed activity at 1 hr, which is maintained during the first 2 hr of G1 phase. An induction of the enzyme was first apparent at Hr 3, after which a progressive increase occurred in a biphasic manner, with the first peak of activity at 6 hr, a time corresponding to an increase in [3H]thymidine incorporation. The inhibitory effect of retinol on the cell cycle-dependent increase in ODC activity was concentration dependent. Enzyme activity was depressed in cultures allowed to progress in the presence of 50 μM retinol. A 60% inhibition was apparent after the addition of 80 μM, while in cultures exposed to 160 μM the ODC activity was completely inhibited and remained at the basal 2-hr control cell level. This was not due to a direct inhibitory effect of retinol on ODC since in vitro addition of retinol (120 μM) to cell supernatants had no effect on the enzyme activity. There was no effect of the vitamin on the time course of ODC expression; the submaximal increases in ODC which did occur in response to lower concentrations of retinol displayed a biphasic nature similar to that of the control cultures.

The inhibition of cell cycle progression by retinol displayed a similar concentration dependence to the inhibition of ODC induction. G1-S phase transition, as indicated by [3H]thymidine incorporation, occurred in the control cells at 5 to 6 hr after mitotic release (Chart 1, inset). In cultures exposed to increasing concentrations of retinol, the rate of DNA synthesis was progressively decreased at all times tested from 5 to 13 hr after mitosis. A 65% inhibition was observed with 80 μM retinol, while in cultures exposed to 160 μM the cells failed to enter S phase, as indicated by the lack of label incorporated at any time tested. Cell counts conducted after 15 hr of incubation indicated that, while the control cells had doubled in number, those cultures plated in the presence of 120 or 160 μM retinol remained at the original number plated. These results indicated a block of cell progression prior to entrance into S phase in accordance with previous findings that exposure of asynchronous cells to the vitamin arrested the cells at some point in G1 prior to an increase in cellular DNA content as assessed by flow microfluorimetry (17). Furthermore, the concentration dependence for the inhibition of both ODC induction and S-phase entry is similar to that previously established for inhibition of cell number increase in logarithmically growing asynchronous CHO cells. Although some variation in the maximal concentration of retinol required to totally inhibit ODC and S-phase entry was seen in different experiments (i.e., from 100 to 160 μM), the parallelism between the effect on ODC and S-phase entry was always apparent. The variation may relate to differences in the composition of either the different lots of retinol or the different batches of media used.

To ensure that the inhibitory effects of retinol on these growth events were not due to a nonspecific detergent-like action of the vitamin, the ability of the cells to form colonies when incubated for 10 days in the presence of 80 or 160 μM retinol was assessed. It was possible to obtain plating efficiencies in this manner without removing the vitamin, because the cells are apparently able to metabolize it to an inactive state. We have shown previously that CHO cells plated in the presence of retinol, after a 24- to 40-hr period of incubation in which no increase in cell number occurs, begin to increase in number at the same rate as the control cultures (17). The escape from inhibition apparently results from metabolic removal, since a second addition of retinol after 24 hr preserves the proliferation block for at least an additional 30 hr. Therefore, the 10-day incubation period allowed those cells which remained viable during the initial period of growth inhibition by the vitamin to recover and grow to form visible colonies. The antiproliferative effect of the vitamin was not the result of a general toxicity, since the plating efficiency of the dioxane and the 80 μM retinol-treated cells was the same as that of the control (80%), while that of the 160 μM retinol-treated cells was only decreased by 24%.

Retinol Inhibition of SAMD Induction. Another index of a G1 phase progression block would be the lack of an induction of an S-phase-dependent enzyme. SAMD, the second enzyme of the polyamine biosynthetic pathway, increases in activity during S phase of the CHO cell cycle (13). As shown in Table 1, the enzyme failed to increase in retinol-treated cells. Both putrescine-dependent and spermidine-dependent SAMD have approximately doubled in the control cultures by mid-S phase (10 hr). The activity of both forms of the enzyme in the vitamin-exposed cells, however, remained at the level expressed in the G1 phase control cultures.
Effect of Retinol on RNA and Protein Synthesis in Mitotically Synchronized CHO Cells. All of the effects of the vitamin on CHO cells examined thus far could result from a general effect to inhibit protein synthesis. Translation is required for the induction of both the polyamine-biosynthetic enzymes and for the enzymes involved in DNA replication. Furthermore, protein synthesis inhibitors have previously been shown to inhibit G1 progression (20, 43, 63, 69). Therefore, the rate of protein synthesis was examined after retinol addition. Chart 2, left, shows the effects of 120 µM retinol on protein synthesis during synchronous CHO cell cycle progression. The rate of [3H]-leucine incorporation remained fairly constant during the initial 8 hr of CHO cell progression, with a maximum occurring between Hr 4 and 6 at a rate 20% higher than demonstrated at the other times tested. At all times in the presence of the vitamin, the rate of amino acid incorporation was inhibited 20 to 30%. Therefore, it seems unlikely that the 90% inhibition of ODC expression observed at this vitamin concentration was the result of a general inhibition of protein synthesis.

In contrast, the effect of vitamin addition on RNA synthesis was marked. During G1 progression, the rate of [3H]uridine incorporation increased 2-fold in the control cultures peaking at 6 hr (Chart 2, right). In the retinol-treated cells, there was no increase in RNA synthesis. The rate was 70% of the control at 2 hr and remained constant so that uridine incorporation at 8 hr was only 30% that of the control.

Ability of Retinol to Inhibit ODC and Block Cell Cycle Progression Restricted to G1 Phase. As shown in Chart 3, the effect of retinol to block the cell cycle-dependent increase in ODC activity was only apparent when the vitamin was added prior to Hr 4 of the cell cycle. If retinol was added at the time of mitotic exit or after 2 hr of G1 progression, the increase in ODC detectable in the control cells was inhibited. If retinol was added after 4 or 6 hr of progression, the vitamin was without effect, and ODC activity continued to increase as in the control cells. This lack of effect was not due to a time dependence for vitamin transport or action, since only 2 hr were required for the inhibitory effect to become apparent after the addition at Hr 2, while even 4 hr after the addition at Hr 4 the control level of activity was maintained. A similar G1-phase dependence of the effect of retinol on cell cycle progression was noted in the previous report (17). Addition of retinol at 0 or 2 hr blocked any increase in cell number, while cultures to which the vitamin was added after 4 or 6 hr of progression doubled as did the controls.

G1-Phase-specific Expression of Cyclic AMP-dependent Protein Kinase Not Altered by Retinol. The transcription-dependent events required for ODC induction, which are sensitive to enhancement by cyclic AMP, occur during the initial hours of G1 progression. Cytoplasmic cyclic AMP-dependent protein kinase is activated during G1 transition prior to the increase in ODC activity (10). If exogenous cyclic AMP is added at the time of kinase activation, the increase in ODC is potentiated (9). Wertz and Mueller (74) have recently shown that retinoic acid can inhibit TPA-stimulated membrane events, suggesting that the action of the vitamin in CHO cells could be to disrupt the serum factor(s) membrane growth stimulus. Thus, the action of the vitamin could be to interfere with the transmission of the cyclic AMP-mediated trophic stimulus responsible for ODC

Table 1

<table>
<thead>
<tr>
<th>Time after mitosis (hr)</th>
<th>Putrescine-dependent SAMD</th>
<th>Spermidine-dependent SAMD</th>
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<tbody>
<tr>
<td>2</td>
<td>200</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>530</td>
<td>80</td>
</tr>
<tr>
<td>10 + retinol</td>
<td>249</td>
<td>26</td>
</tr>
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Chart 2. Retinol inhibition of leucine and uridine incorporation. Control (●) or 120 µM retinol-treated (○) mitotically synchronized cells were incubated at various times after mitosis for 30 min in the presence of [3H]-leucine or [3H]uridine. The cells were washed and harvested, and the incorporation of radiolabel into insoluble material was determined. Estimations of total [3H] precursor in the cells showed no effect of the vitamin on leucine uptake and a 30% inhibition of uridine uptake at the 2 later times tested. Bars, S.D.
induction. Table 2, however, shows that retinol addition did not affect the increase in cyclic AMP-dependent protein kinase activity which occurred 2 hr after mitotic exit. The ratio of active to total kinase increased 2-fold in both control and retinol-treated cultures.

Recovery of ODC after Retinol Removal is Blocked by Actinomycin D. To determine if transcription of mRNA for ODC was occurring in the presence of retinol, cells were exposed to the vitamin for the first 4 hr of G1 progression, and then the monolayers were washed and supplemented with fresh media containing additional retinol, actinomycin D, or dioxane (control). Cells resupplemented with retinol retained the inhibition of ODC expression (Chart 4A). Those cells removed from the presence of the vitamin and placed in control media displayed increased ODC activity within 30 min which continued to progressively increase, lagging behind the activity of control cells by about 2 hr. This recovery paralleled and preceded the recovery of S-phase transition after vitamin removal (Chart 4B). However, cultures to which actinomycin D was added after removal of the vitamin showed no increase in enzyme activity when tested from 30 min to 4 hr after media replacement.

Increased Putrescine Concentrations in Retinol-blocked Cells. Several studies examining the effects of addition of direct inhibitors of ODC expression (i.e., substrate analogs) to cells in culture have shown concomitant inhibition of cell proliferation (6, 26, 37, 38, 68). In some cases, this effect has been reversible by the addition of exogenous putrescine (26, 37). However, putrescine addition (1 mM) to CHO cells had no effect on preventing or relieving the retinol-induced cell cycle block as measured by either [3H]thymidine incorporation or ultimate cell doubling. Measurements of intracellular polyamines showed putrescine not to be a limiting factor. As shown in Table 3, the diamine level was actually higher in the vitamin-treated cells after 4 or 6 hr of progression. Although the mechanism in these cells for such an increase in putrescine in the absence of any induction of ODC is unknown, a similar phenomenon has been shown to occur in several other growing tissues (23, 25, 64). In those studies, the biosynthetic pathway was shown to be reversed, and the increased putrescine was synthesized from spermidine and spermine. Similarly, the concentrations of the polyamines were significantly decreased in the retinol-treated cells. No significant amounts of any of the polyamines were detectable in the growth media from either the control or retinol-treated cells.

Effects of Other Naturally Occurring Retinoids on Growth Parameters. The efficacy of the other naturally occurring retinoids to affect asynchronous CHO cell logarithmic growth was examined. Chart 5 shows that retinol was the most potent retinoid, completely inhibiting cell doubling at a concentration of 25 μM. Retinol was the second most effective, while 200 μM retinal acetate and 1000 μM retinoid acid were required to fully inhibit growth. Although retinal was the most potent, it was not the main object of study in these experiments because of its paradoxical effect on CHO cell growth parameters. As shown in Table 4, very low doses of retinal (1 to 5 μM) often stimulated CHO cell proliferation as measured by an increase in the extent of ODC induction throughout G1, by a stimulation of the rate of [3H]thymidine incorporation, and by an enhancement of cell growth rate. Higher doses of retinal (greater than 5 μM) inhibited all aspects of growth in a manner similar to the effects of retinol previously discussed. Similar contrasting effects of retinoids on DNA synthesis have been demonstrated in lymphocytes (32) and may reflect different functional receptors for different forms of the vitamin (49).

DISCUSSION

Pardee (50) has suggested that restriction points occur during the cell cycle which involve events on which all others thereafter depend. The present study provides further support to the hypothesis that vitamin A acts to limit CHO cell growth at such a target event in G1 phase (16, 17). (a) Cells incubated in the presence of retinol do not enter S phase as shown by flow
Mitotically synchronized cells were incubated in the presence of 0.5% dioxane (control) or 120 μM retinol. At the time indicated, the cells were harvested, and TCA-extracted for analysis of polyamine concentrations, which are expressed as pmol/10^6 cells.

<table>
<thead>
<tr>
<th>Time after mitosis (hr)</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
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<tbody>
<tr>
<td>Control Retinol</td>
<td>Control Retinol</td>
<td>Control Retinol</td>
<td>Control Retinol</td>
</tr>
<tr>
<td>2</td>
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<td>0.64 ± 0.20</td>
<td>3.64 ± 0.21</td>
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<tr>
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<td>0.83 ± 0.07</td>
<td>2.96 ± 0.33</td>
</tr>
<tr>
<td>6</td>
<td>0.78 ± 0.01</td>
<td>1.18 ± 0.02</td>
<td>2.44 ± 0.08</td>
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* Mean ± S.D.

**Microfluorometric analysis of the G_1-like DNA distribution in asynchronous cells and by the failure of synchronous cells to incorporate thymidine into DNA or to induce the S-phase dependent enzyme, SAMD.** (b) The removal of retinol from synchronous cells incubated in its presence during G_1 phase results in a 2- to 3-hr lag before the initiation of DNA synthesis assessed by thymidine incorporation kinetics. This lag cannot be solely accounted for by a time requirement to clear the vitamin from the cell, since the synthesis of ODC resumes within 30 min after removal. (c) Retinol is effective in inhibiting cell doubling only when added prior to the midpoint of G_1 phase. These results suggest that the site of action of the vitamin, representing a restriction point for cell cycle progression, occurs several hr prior to the onset of S phase.

The effect of the vitamin to inhibit G_1-dependent ODC induction displays characteristics very similar to its effects on the proposed G_1 restriction point. (a) The concentration-dependence of retinol inhibition of the increase in ODC is similar to that for the inhibition of thymidine incorporation and cell doubling. (b) Addition of the vitamin is only effective to inhibit the rise in ODC activity when added during the first 2- to 3 hr of G_1 progression. (c) The recovery of ODC activity after retinol removal precedes the entry of the cells into S phase, suggesting induction of the enzyme constitutes part of the delayed G_1 progression events whose requirement for occurrence imposes the lag in S-phase entry. Vitamin A action to limit growth is believed to represent a physiological function in certain cell types (65). For example, increased epithelial mitotic indices can be shown in states of retinoid dietary deficiency (18, 19). It is consonant, therefore, that the block demonstrated in this report occurs in G_1 prior to ODC expression. The control of cell reproduction *in vivo* usually occurs by arrest at some site in this cell cycle phase (52). Nongrowing cells contain virtually no ODC, and the induction of the enzyme is a ubiquitous initial response to growth stimulants (57). Thus, ODC induction represents a "landmark" event by which the extent of G_1 progression can be charted (51) and may represent the regulatory event on which the antiproliferative action of vitamin A in CHO cells is imposed.

The mechanism by which vitamin A acts to inhibit ODC...
specifically limit translation of the mRNA for the enzyme. Since ODC has an extremely short half-life of 11 to 20 min (22, 31, 60), message must be continually translated during cell cycle progression to maintain the induction of increasing enzyme levels. An inhibitory effect of retinol on ODC synthesis, therefore, would be apparent after vitamin addition at any time. (c) The addition of actinomycin D to cells at the time of retinol removal completely blocked the increase in enzyme activity which was detectable in the control cells within 30 min. If the mRNA for ODC were being transcribed during the time of retinol exposure and the inhibition resulted from vitamin action at another locus, some increase in enzyme activity might be apparent upon release in the presence of the actinomycin D from the translation of this preexisting mRNA. This protocol, of course, presumes that any ODC mRNA synthesized during the retinol incubation would be stable enough to survive and be translated during the 30 min after retinol removal and actinomycin D addition. Although there is no information available on the t1/2 of ODC mRNA, this type of experimental protocol has been used successfully to show in a G1-phase temperature-sensitive mutant of Chinese hamster cells that the translation and not the transcription of ODC mRNA was blocked at the nonpermissive temperature (34). In those studies, an increase in ODC was observed 2 hr after shift to the permissive temperature in the presence of a transcriptional inhibitor. In contrast, in the present study the increase in enzyme activity upon retinol removal was totally blocked by the transcription inhibitor. It thus appears that no mRNA for ODC is being transcribed in the presence of the vitamin. (d) The G1 time dependence of the retinol action in limiting ODC induction coincides with the time of G1 progression previously established for the events constituting ODC gene activation (9).

Retinoids have been postulated to act like steroid hormones (2, 7, 35), and under certain circumstances the vitamin or its binding protein has been localized to the cell nucleus (53, 61, 62). Therefore, the vitamin may act to affect transcription directly without affecting cytoplasmic events. However, several other sites of vitamin A action have been reported in other cell types which could limit nuclear function. For example, Wertz and Mueller (74) have shown that retinol inhibits TPA acceleration of the incorporation of choline into lymphocyte phospholipids and have postulated that such an event may represent the site of action of vitamin A to inhibit subsequent DNA replication. The inhibition of choline incorporation, however, is apparently not involved in the retinol inhibition of TPA induction of ODC in lymphocytes, since preincubation is required to inhibit ODC (32) but not to inhibit phospholipid metabolism (74). This suggests there are at least 2 sites at which retinoids can act to inhibit growth-related events, which may be differentially expressed in different cell types. ODC induction in TPA-stimulated epidermis has been shown to be dependent on prostaglandin synthesis (72), another event catalyzed by a membrane enzyme, which has been suggested as a possible site of retinoid action (71). However, it appears unlikely that this is the mechanism in CHO cells, since the addition of known inhibitors of prostaglandin synthesis (indomethacin, aspirin, and 5,8,11,14-eicosatetraenoic acid) have been found to have no effect on CHO growth parameters.5

A striking consequence of the retinol block of cell cycle

5 M. K. Haddox and D. H. Russell, unpublished observations.
progression is the inhibition of uridine incorporation into RNA. The G1-phase increase in RNA synthetic rate has been shown in several cell types to reflect an increased synthesis of mRNA rather than heterogeneous RNA (1, 29, 42, 54, 70). Inhibitor studies in which low concentrations of actinomycin D were used to specifically block rRNA synthesis have shown that this G1-phase event is essential for entry into S phase (1, 11, 12). Polyamine-biosynthetic events have been shown in many types of growth systems to be intricately tied to RNA synthesis (8, 56). Furthermore, intact nucleolar function has been shown to be essential to ODC expression in at least one cell type, the anucleolar mutants of Xenopus laevis (55). The present demonstration, therefore, of the correlation between the block in ODC and the block in rRNA synthesis produced by retinol adds to the list of examples of this coupling. Since putrescine concentrations were not found to be limiting during retinol exposure, the putative interaction between the 2 systems demonstrated here may involve the direct action of ODC on RNA polymerase I function (39, 40).

The differences that exist between our findings and those in other cell types in which ODC induction is inhibited by retinoids probably reflect differences in the nature of the systems being studied. In epidermis, the inhibitory effect of retinoic acid on TPA-promoted epidermal papilloma formation is associated with a similar inhibition of TPA-induced epidermal ODC (71, 73). Further, the efficacy of different natural and synthetic retinoid analogs to inhibit papilloma formation directly paralleled their inhibition of ODC induction (73). However, in the skin, retinoids had no effect on the TPA-stimulated increase in SAMD, while in CHO cells, the vitamin inhibited the increase in the enzyme. Possibly the SAMD increase in skin is the direct result of a TPA-promoted event independent of the retinoid-sensitive event responsible for ODC induction, while in CHO cells the SAMD increase is a function of cell cycle progression into S phase. Since the retinol prevents entrance into S phase by acting at a point in G1, the lack of SAMD increase occurs as an indirect result of vitamin action. The relative efficacy of the retinoids to inhibit growth in CHO cells and epidermis also differs. This may reflect variations in the cellular distribution of the different retinoid-binding proteins (48, 49, 62) or in the metabolism of the vitamin. The effect of retinoic acid, the most potent inhibitor of TPA induction of ODC in both skin (71) and lymphocytes (32), displays a broad-sloped type of concentration dependence, which suggests that it may not be the active agent and that metabolic activation is required. In contrast, the concentration dependence in CHO cells is fairly precipitous, and therefore retinol may be the actual effector molecule.

Pardee (50) has speculated that the restriction points essential to cell cycle progression will represent sites of fundamental difference in regulation between normal and malignant cells. Therefore, it is interesting to note, in conjunction with the consideration that ODC induction may represent a G1 restriction point for retinol action, the accumulating examples of aberrant, less restricted control of ODC expression during cell cycle progression of transformed cells. In general, the characteristics of polyamine biosynthesis in malignant cells reflect a diminished response to normal control mechanisms (3, 14, 15, 27, 36, 45). Such a loss of regulation of ODC expression has been postulated by Boutwell and O’Brien (44, 46, 47) to be essential to the TPA-promoted transformation process in epidermis, which retinoids block. Although the mechanism of promotion is unclear, Boutwell et al. have pointed out the similarity between the intracellular events after TPA stimulus and those occurring during the normal growth process. ODC induction is such a common event. We show here that ODC induction is a common target site for retinoid inhibition of both TPA-promoted epidermis and CHO cell cycle. Therefore, those cells which possess a receptor for retinoid antiproliferative action may be inhibited in the sequence of growth events at a common point involving polyamine biosynthesis.

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