ON THE MECHANISM OF 5-BROMODEOXYURIDINE INDUCTION OF PROLACTIN SYNTHESIS IN RAT PITUITARY TUMOR CELLS

DEBAJIT K. BISWAS, KARIM T. ABDULLAH, and BARBARA A. BRENNESSEL

From the Laboratory of Pharmacology, Harvard School of Dental Medicine, Boston, Massachusetts 02115

ABSTRACT

GH_12C_1, a clonal strain of rat pituitary tumor cells in culture (GH cells), does not produce detectable amounts of prolactin. 5-Bromodeoxyuridine (BrdUrd), the thymidine analogue, at sublethal concentrations (3–5 μg/ml) induces prolactin synthesis in these cells. BrdUrd also induces prolactin synthesis in $F_1BGH_12C_1$ cells, a BrdUrd resistant ($BrdUrd^r$) substrain isolated from GH_12C_1 cells. The $F_1BGH_12C_1$ strain is not drug dependent, but its resistance to BrdUrd is a stable phenotype. The significant features of the induction of prolactin synthesis in the $BrdUrd^r$ strain are the increased net synthesis of prolactin and the shortening of the lag period of prolactin induction. As BrdUrd concentration in the growth medium is increased, the rise in prolactin synthesis parallels the increased incorporation of BrdUrd into DNA. Prolactin synthesis is first detected when BrdUrd replaces 20–25% of the thymidine in DNA. BrdUrd can replace up to 75–80% of the thymidine within 2 d of treatment. Partial starvation of these cells under specified growth conditions does not affect the general growth pattern of the cells, general protein synthesis, and thymidine uptake, but does affect DNA synthesis. When cells are cultured under conditions in which DNA synthesis is preferentially inhibited, BrdUrd does not induce prolactin synthesis, suggestive of a DNA-mediated mechanism of action for the drug.

KEY WORDS 5-bromodeoxyuridine · prolactin · gene expression · rat pituitary tumor cells

The drug 5-bromodeoxyuridine (BrdUrd), a thymidine analogue, seems to have diverse effects on the various processes of differentiation in eukaryotic cells. These may be classified into two distinct types. BrdUrd suppresses the synthesis of specific proteins in certain cells, and in other cell types the drug induces the production of certain cell-specific proteins. Rutter et al. (12) described in detail the role of BrdUrd on different eukaryotic cell systems. On the basis of the different effects of this drug, these authors discussed the possible DNA-linked and non-DNA-linked mechanisms of action of BrdUrd.

Holtzer and Abbott (7) postulated that BrdUrd selectively inhibits the synthesis of luxury molecules without grossly depressing the synthesis of essential molecules of the cells. Priesler et al. (11) claimed that inhibition of differentiated function

is dependent upon incorporation of BrdUrd into DNA whereas induction of cell-specific protein synthesis does not require such incorporation. Davidson and Kaufman (6), in their studies with Syrian hamster melanoma cells, demonstrate that BrdUrd suppresses pigment formation in these cells and that this suppression can be reversed by addition of deoxycytidine (dCyd) to the culture medium. The effect of dCyd on the suppression of pigmentation does not depend on the incorporation of BrdUrd into DNA. In contrast, Stellwagen and Tomkins (14) and O'Brien and Stellwagen (10) postulate that incorporation of BrdUrd into DNA is obligatory for its effect on the inhibition of synthesis of tyrosine aminotransferase in hepatoma cells.

We have reported previously (2) that BrdUrd induces prolactin synthesis in a clonal strain of rat pituitary tumor cells (GH_12C_1), and in a substrain of GH_12C_1 cells isolated by exposure of these cells to 30 μg/ml BrdUrd. Prolactin synthesis in the BrdUrd resistant ($BrdUrd^r$) cells can be detected after 8–10 d of treatment with the drug (30 μg/ml). Previous results (2) demonstrate that uptake and incorporation of BrdUrd and thymidine into DNA is identical in the BrdUrd sensitive ($BrdUrd^s$) parent strain and in the $BrdUrd^r$ strain. These results would suggest that the resistant phenotype of $F_1BGH_12C_1$ is not caused by a lesion in any of these steps leading to the incorporation of the drug into DNA. In this investigation, we deal with the mechanism of induction of prolactin synthesis by BrdUrd in $F_1BGH_12C_1$ cells, and present experimental evidence which is consistent with a DNA-mediated mechanism of BrdUrd action on cellular differentiation.

MATERIALS AND METHODS

Materials

Culture media and sera were obtained from Grand Island Biological Co. (Grand Island, N. Y.), and culture dishes and flasks from Falcon Plastics (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.). BrdUrd, thymidine, dAMP, dGMP, dTMP, dCMP, and BrdUMP were purchased from Sigma Chemical Co. (St. Louis, Mo.). [Methyl-^{3}H]thymidine (20 Ci/mmol), [6-^{3}H]BrdUrd (23.6 Ci/mmol), L-[U-^{14}C]leucine (297 mCi/mmol), ^{32}P as carrier-free orthophosphoric acid, and Omnifluor were obtained from New England Nuclear (Boston, Mass.). Rat prolactin was obtained from the Rat Pituitary Hormone Distribution Program (National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health) and polyethyleneimine (PEI) cellulose sheets from Brinkmann Instruments, Inc. (Westbury, N. Y.).

Methods

Cells used in this investigation were rat pituitary tumor cells in culture, designated as GH cells. GH_12C_1 is a clonal strain of GH cells. It produces large amounts of growth hormone but does not produce any detectable prolactin (for review, see reference 9). $F_1BGH_12C_1$ cells are surviving populations isolated by exposure of GH_12C_1 cells to 30 μg/ml BrdUrd (2). Cells were maintained in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. Growth conditions are described in greater detail in the legend to individual figures. Growth of cells was measured by determination of total cell protein by the method of Lowry et al. (8).

Prolactin in the growth medium of cell cultures was determined by microcomplement fixation immunoassay (15). The absolute sensitivity of the method for prolactin is 3–5 ng.

DNA synthesis in cells under different growth conditions was determined by measurement of the incorporation of [^{3}H]thymidine (in some experiments [^{3}H]BrdUrd) into trichloroacetic acid (TCA)-insoluble radioactivity after a 3-h pulse. Most of this (90–95%) TCA-insoluble radioactivity was solubilized upon prior treatment of the cell extracts with DNase 1 (2).

Uptake of [^{3}H]thymidine or [^{3}H]BrdUrd by the cells in different stages of DNA synthesis was determined by measuring intracellular TCA-soluble radioactivity. TCA-soluble radioactivity attained maximum levels within 10–15 min of exposure of these cells to the radioactive precursors.

Protein synthesis under specified growth conditions was measured by pulse-labeling the cell with [^{14}C]leucine 3 h before harvesting (see figure legends for details). Hot TCA-precipitable radioactivity was measured in an aliquot of cell-extract prepared as described in the figure legends.

Base Analysis of ^{32}P-Labeled DNA

Cells were exposed to ^{32}P (as carrier-free orthophosphoric acid, 2 μCi/ml) for 48 h. Cells were harvested and washed with Tris-buffered saline (TBS) (4) and stored at −80°C. DNA from these cells was extracted by using the method described by Stellwagen and Tomkins (14). [^{32}P]RNA was removed from the DNA preparations by treatment with heat-treated pancreatic RNase A. The twice-precipitated DNA was pelleted by centrifugation at 10,000 *g* for 15 min at 4°C. The DNA was dissolved and dialyzed against buffers under the conditions described by Bick and Davidson (1).

The dialyzed [^{32}P]DNA solution was then hydrolyzed by sequential treatment with two enzymes, DNase 1 and snake venom phosphodiesterase under the conditions described by Bick and Davidson (1). The 5′-deoxymon-

onucleotides obtained after such enzymatic hydrolysis of DNA were then concentrated by lyophilization and extracted with methanol as described by Couch and Hanawalt (5). 100- to 200-μl samples were then analyzed by two-dimensional PEI cellulose chromatography by using the method described by Couch and Hanawalt (5). For the identification of the different [^{32}P]deoxymononucleotides obtained after such hydrolysis of DNA, authentic samples of dAMP, dGMP, dCMP, dTMP, and BrdUMP were co-chromatographed with the samples to be analyzed and identified under ultraviolet light. The location of the well-separated deoxymononucleotides was circled, and these areas of the cellulose plates were cut and counted in Omnifluor in a Packard Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.). For autoradiographic identification of ^{32}P-labeled deoxymononucleotides, the dried plates were exposed to Kodak x-ray films (X-Omat R-film, XR-5) for 16–18 h and subsequently developed. Authentic deoxymononucleotides, as visualized under UV light, and radioactive spots were superimposable.

RESULTS

Growth Characteristics of and Prolactin Production by BrdUrd-Resistant $F_1BGH_12C_1$ Cells

The results presented in Fig. 1*A* show the growth pattern of the $F_1BGH_12C_1$ cells in the presence or absence of BrdUrd. The growth of these cells was not different when tested after maintaining the cells in the absence of the drug for at least 1 yr. These results suggest that the cells are not drug dependent and also that the resistant phenotype of these cells is a stable one. Though the subclone was isolated by selecting the survivors in the presence of 30 μg/ml BrdUrd, the results presented in Fig. 1*A* demonstrate that these cells could tolerate as much as 300 μg/ml of the drug in the growth medium. Prolactin production by $F_1BGH_12C_1$ cells in the presence of 30 μg/ml and that by GH_12C_1 cells in the presence of 3–5 μg/ml of the drug could be detected after 8–10 d of exposure of the cells to the drug. However, results presented in Fig. 1*B* demonstrate that this long latent period of prolactin induction could be reduced by treatment of the cells with higher concentrations of the drug. In the presence of 300, 120, and 60 μg/ml of BrdUrd, prolactin production could be detected at 1, 4, and 7 d, respectively.

While prolactin synthesis in these cells was induced, there was no detectable increase in the total protein or DNA synthesis after treatment with the drug. Total RNA synthesis in intact cells, and RNA synthesis in a cell-free system with isolated nuclei (3), was the same in both control and treated cells. By comparing RNA synthesis in nuclei from control and BrdUrd-treated cells, it was found that K_m values for all four nucleoside triphosphates were unaltered after drug treatment ($K_m = 3–4 \times 10^{-7}$ M; unpublished data). Growth hormone, which constitutes a significant fraction of the total protein synthesized by $F_1BGH_12C_1$ cells, is also unaffected by treatment with BrdUrd (2).

Relationship of Prolactin Induction to DNA Synthesis

The BrdUrd induction of prolactin synthesis was studied in $F_1BGH_12C_1$ in relation to DNA synthesis in these cells. The main emphasis of the experimental approach was to preferentially inhibit DNA synthesis without drastically affecting other cellular biochemical processes. The cellular processes which were monitored under these conditions are (*a*) general growth, (*b*) general protein synthesis, (*c*) [^{3}H]thymidine uptake, (*d*) DNA synthesis, and (*e*) prolactin synthesis. The two inhibitors of DNA synthesis, mitomycin C and cystosine arabinoside, at 0.05 μg/ml and 2–5 μg/ml, respectively, inhibited protein synthesis within 2–3 d of treatment of the cells with the drugs (data not shown). Thus, these inhibitors of DNA synthesis were found to be not suitable for this study. However, experimental conditions which would preferentially block DNA synthesis were established by addition of limited amounts of growth medium to a specified number of cells ($6–8 \times 10^5$ cells/4 ml F-10 medium). Under these conditions (see legend to Fig. 2), DNA synthesis reached a maximum level within 3–4 d, and declined thereafter (Fig. 2*A*). Protein synthesis under these growth conditions was not affected and continued for at least 3–4 d more, though at a slower rate (Fig. 2*A*). RNA synthesis, as determined by incorporation of [^{3}H]adenosine and [^{3}H]guanosine into TCA-precipitable material, was also found to be not affected and continued for 3–4 d more. (Unpublished results, obtained by Dr. T. F. J. Martin, Dept. of Zoology, University of Wisconsin, Madison, Wis., by growing another clonal strain of GH cells under similar growth conditions, i.e., 2×10^5 cells/ml F-10 medium). DNA synthesis resumed in these cells after a short lag period upon replenishment with fresh medium,

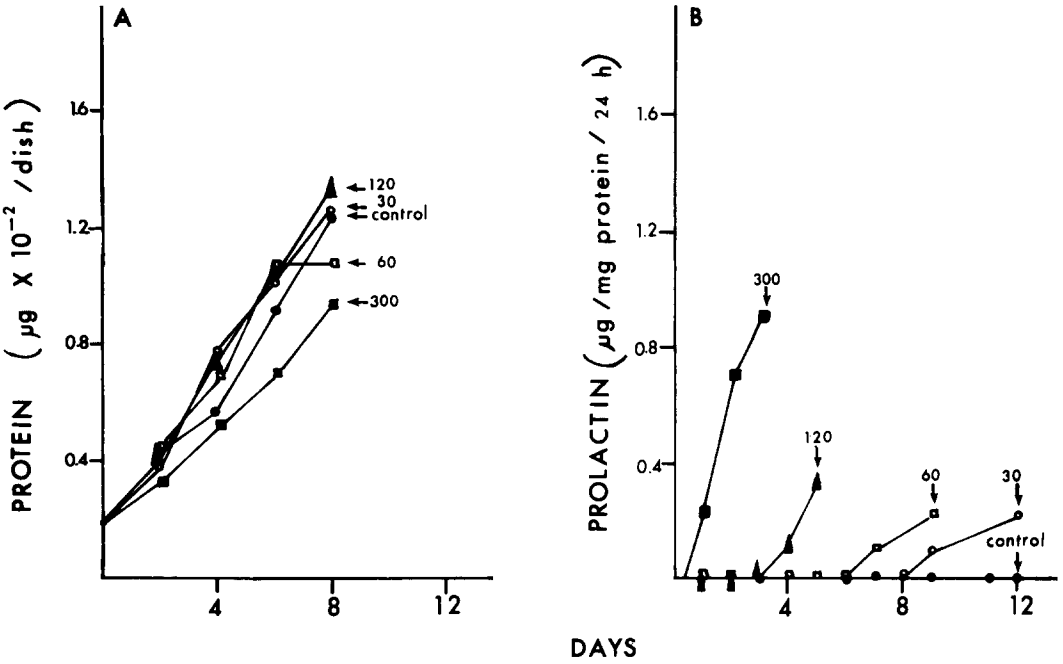


FIGURE 1 Growth and prolactin production by $F_1BGH_12C_1$ cells at different concentrations of BrdUrd. $F_1BGH_12C_1$ cells were maintained in F-10 medium without BrdUrd for 12 mo before initiation of this experiment. Fig. 1*A* shows the growth of these cells in the presence of different amounts of BrdUrd (in μg/ml), as indicated by the numbers with the arrows, and in absence of the drug (control). Cells were grown in 60-mm dishes and scraped from the dish after the indicated days of treatment with the appropriate amounts of the drug. Protein was determined in an aliquot of the cell extracts by the method of Lowry et al. (8). Each point represents the amount of protein averaged from duplicate dishes. Fig. 1*B* shows the prolactin production by $F_1BGH_12C_1$ cells at different concentrations of BrdUrd. Prolactin in the medium of the cells was determined by complement fixation assay. Each point represents the amount of prolactin averaged from duplicate dishes. Numbers indicated by the arrows are concentrations of BrdUrd (in μg/ml) in the growth medium.

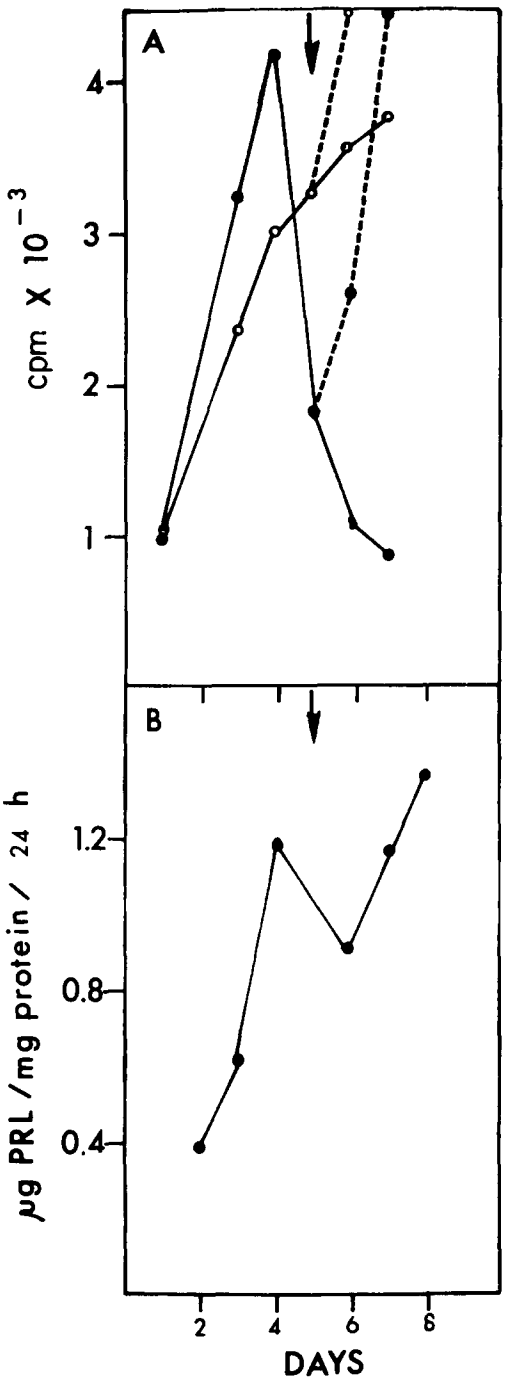
and protein synthesis resumed its normal rate after the medium change (Fig. 2*A*).

The incorporation of [^{3}H]BrdUrd into DNA in $F_1BGH_12C_1$ follows the same pattern as the incorporation of thymidine (2). Furthermore, the incorporation of [^{3}H]BrdUrd into TCA-soluble radioactivity was not affected by limiting the growth medium, indicating that the uptake of BrdUrd by these cells remains unaltered. Thus, aside from the inhibition of DNA synthesis, no other gross effects on the macromolecular synthesis and on the physiology of the cells could be detected under the conditions of cell culture.

Under the defined growth conditions, prolactin synthesis in $F_1BGH_12C_1$ cells followed the same pattern as DNA synthesis (Fig. 2*B*). Prolactin synthesis was detectable within 1–2 d of growth of these cells in the presence of 300 μg/ml BrdUrd, reached a maximum in 3–4 d, and declined thereafter in parallel with the decline of DNA synthesis. Prolactin synthesis resumed in conjunction with DNA synthesis after addition of fresh medium to the cells (Fig. 2*B*).

The results presented in Fig. 3*A* show the level of DNA synthesis, and Fig. 3*B* shows the BrdUrd induction of prolactin synthesis in $F_1BGH_12C_1$ cells. $F_1BGH_12C_1$ cells were grown (under the same conditions as described in the legend to Fig. 2) initially in the absence of BrdUrd. Addition of BrdUrd (300 μg/ml) on the 4th d of growth (as indicated by the arrow in Fig. 3) induced prolactin synthesis only in those cells (Fig. 3*B*, ○—○) in which DNA synthesis (Fig. 3*A*, ○—○) was permitted to continue. The cells that did not receive any fresh medium, i.e., cells in which DNA synthesis could not continue (Fig. 3*A*, ●—●),

did not produce any detectable prolactin (Fig. 3 *B*, ●—●) even in the presence of BrdUrd (added on the 4th d as indicated by the arrow). The cells that received fresh medium on the 4th d of growth, in the absence of BrdUrd, also did not produce any detectable prolactin (Fig. 3 *B*, ▲—▲). These results demonstrate that there is a parallel relationship between DNA synthesis and BrdUrd induction of prolactin synthesis in these cells, under these defined growth conditions.

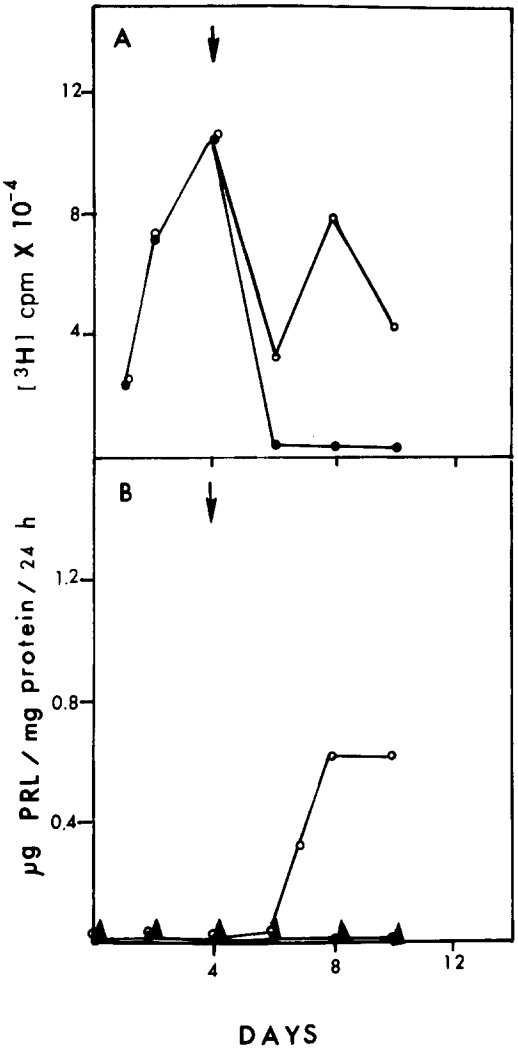


Base Composition of DNA Isolated from Control and BrdUrd-Treated Cells

Base composition of the DNA isolated from control and BrdUrd-treated cells was determined, and the results are presented in Table I. It is evident from these results that BrdUMP, designated by letter *B*, was absent in DNA isolated from control cells. BrdUMP in DNA isolated from BrdUrd-treated cells (300 μg/ml for 2 d) constituted as much as 26% of the total bases with concomitant reduction in the amount of dTMP (designated by the letter *T*). The amount of

FIGURE 2 Effect of growth of cells in limited medium on DNA, protein, and prolactin synthesis in $F_1BGH_12C_1$ cells. 6–8 × 10^5 cells were plated in 100-mm dishes containing only 4 ml of F 10 medium. BrdUrd was added to the final concentration of 300 μg/ml. Cells were pulsed with [^{3}H]thymidine (5 μCi/ml) and with [^{14}C]leucine (20 μCi/ml) 3 h before harvesting on the specified day and incubated in the humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were then harvested after this period, washed with TBS three times, and resuspended in 1 ml of TBS. Cells were lysed by addition of 0.05 ml of 2% SDS, and the radioactivity in an aliquot was measured after precipitation with 2 ml of 10% TCA. Both ^{3}H- and ^{14}C-radioactivity were determined in each sample. About 90–95% of the TCA-insoluble ^{3}H-radioactivity became TCA soluble upon prior treatment of the cell extracts with DNase 1 under conditions described previously. Prolactin, in the medium withdrawn on the specified day, was determined by microcomplement fixation assay. At the arrow, medium was removed and fresh medium (4 ml) was added. Fig. 2 *A* shows the [^{3}H]thymidine incorporation into DNA (●—●) and [^{14}C]leucine incorporation into protein (○—○) as determined by TCA-insoluble ^{3}H- and ^{14}C-radioactivity in aliquots of extracts of cells harvested on the specified day. Dashed lines represent [^{3}H]thymidine and [^{14}C]leucine incorporations after replenishment with the fresh medium on the day indicated by the arrow. Each point represents average radioactivity in extracts of cells prepared from duplicate dishes. Fig. 2 *B* shows the amount of prolactin produced by cells on the indicated day. Each point represents the amount of prolactin in the medium averaged from duplicate dishes. The arrow indicates the day of addition of fresh medium (4 ml).

dGMP, dCMP, and dAMP, designated by the letters G, C, and A, respectively, seemed to be unaltered in the DNA isolated from control and BrdUrd-treated cells. These results demonstrate that treatment of the cells with increasing amounts of the drug for the periods (days) indicated in parentheses caused increased replacement of thymidine. Treatment of the cells with the drug at any concentration did not alter the G, C, or A content of the DNA. The total B and T content was equivalent to the A content of the DNA isolated from treated cells with each concentration of BrdUrd. These results show that B replaces only T of the DNA in the treated cells.



Relationship between BrdUrd Replacement of Thymidine in DNA and Prolactin Synthesis

The results presented in Fig. 4*A* show the percent replacement of thymidine in DNA by BrdUrd and prolactin production by $F_1BGH_12C_1$ cells at different concentrations of the drug. Treatment of the cells with increasing concentrations of BrdUrd (ranging from 30 to 300 μg/ml) led to a concentration-dependent increased replacement (○—○) of thymidine in the DNA by the drug. As BrdUrd concentration was raised, the increase in thymidine replacement of the drug paralleled the increase in prolactin found in the culture medium (●—●). Similarly, the results presented in Fig. 4*B* demonstrate that when the drug was withdrawn, prolactin production declined in parallel with the reduction of the level of thymidine substitution by BrdUrd.

Fig. 5 shows the early and late kinetics of induction of prolactin synthesis (○—○) in relation to altered base composition of the DNA (●—●). BrdUrd (300 μg/ml), when added to $F_1BGH_12C_1$ cells grown under the conditions which permit continued DNA synthesis, replaced thymidine in the DNA to the extent of 5% after 6 h. However, no prolactin could be detected in the medium of these cells (Fig. 5). BrdUrd substitution for thymidine in the DNA of treated cells continued to increase with time and reached ~75% after 2 d.

FIGURE 3 Relationship between BrdUrd induction of prolactin synthesis and DNA synthesis. $F_1BGH_12C_1$ cells were plated at 6–8 × 10^5 cells/100-mm dish with 4 ml of F-10 medium in the absence of BrdUrd. Three sets of such dishes in duplicates were used in this experiment. Fig. 3*A* shows the levels of DNA synthesis in cells as determined by pulsing the cells with [^{3}H]thymidine 3 h before withdrawal of the medium and harvesting of the cells on the specified day. TCA-insoluble radioactivity was determined as described in the legend to Fig. 2. Fig. 3*B* shows the prolactin production by these cells as determined after assay of cell medium by microcomplement fixation. From one set of dishes (○—○) medium was removed and saved for prolactin determination and, to this set of dishes, fresh medium (4 ml) and BrdUrd (300 μg/ml) were added on the 4th d of growth as indicated by the arrow. The second set of dishes (●—●) received only BrdUrd (300 μg/ml), and the cells were maintained without medium replacement. From the third set of dishes (▲—▲, Fig. 3*B* only) medium was removed and 4 ml of fresh medium was added. The third set of dishes did not receive any BrdUrd.

TABLE I

Base Composition of DNA Isolated from Control and BrdUrd-Treated Cells

BrdUrd	G	C	A	T	B	$\left[\frac{B}{B+T}\right]$
μg/ml			%			
None	18.1	19.8	32.0	30.1	–	–
30 (9)	19.1	19.8	31.0	24.0	6.2	20
60 (8)	18.6	19.3	31.3	22.8	8.0	26
120 (5)	18.2	20.6	31.3	17.6	12.0	40
300 (3)	18.5	18.8	31.1	5.8	26.1	81

$F_1BGH_12C_1$ cells were grown in F-10 medium in 100-mm dishes in the presence of BrdUrd at the indicated concentrations. Medium was changed every 3 d. Cells were pulsed with ^{32}P for 48 h and harvested on the days indicated in parentheses. [^{32}P]DNA was isolated from these cells and hydrolyzed enzymatically to ^{32}P-labeled deoxymononucleotides. These were subsequently separated by two-dimensional PEI-cellulose chromatography. The radioactivity in each spot was determined as described in Materials and Methods.

Substitution of thymidine by BrdUrd in DNA did not reach >82% even after continued treatment of the cells with 300 μg/ml of the drug for as long as 8–10 d. Prolactin (Fig. 5, ○—○) in the medium of the cells could not be detected at 6, 12, and 16 h of treatment, times at which thymidine replacement was 5, 12, and 18%, respectively (Fig. 5, ●—●). However, prolactin could be detected (Fig. 5, ○—○) after treatment for 24 h when thymidine replacement was ~25%. In some experiments, prolactin production could be detected at the base substitution level as low as 20%.

The results presented in Fig. 4*A* and *B* and Fig. 5 suggest that BrdUrd induction of prolactin synthesis in $F_1BGH_12C_1$ cells is mediated via incorporation of the drug into DNA, and also demonstrates a parallel relationship between prolactin production and the extent of thymidine substitution by BrdUrd. The results presented in Table II show that detectable prolactin synthesis occurred in GH-cell strain GH_12C_1 after treatment with BrdUrd for 9 d at 5 μg/ml, in $F_1BGH_12C_1$ cells after 9 d at 30 μg/ml, and after 1 d at 300 μg/ml. In all cases, when prolactin synthesis was first detected, replacement of thymidine by BrdUrd was comparable and found to be in the order of 22–25%.

DISCUSSION

We have reported previously that BrdUrd induces prolactin synthesis in GH_12C_1, a clonal strain of rat pituitary tumor cells, and in $F_1BGH_12C_1$, a subclone of GH_12C_1 (2). While prolactin synthesis in these cells was induced, there was no detectable increase in the total protein or DNA synthesis after treatment with the drug. The induction of prolactin synthesis in $F_1BGH_12C_1$ cells occurs only in the presence of BrdUrd with a concentration dependent lag period (Fig. 1*B*). An important property of these cells is that they can be grown in the presence of higher concentrations of the drug (300 μg/ml), which reduces the lag period for the induction of detectable prolactin synthesis to 24 h. This permits us to study the induction phenomenon in relation to DNA synthesis in greater detail. Culturing cells in a limited amount of medium (2×10^5 cells/ml of medium) does not seem to affect their growth, protein synthesis, RNA synthesis, or thymidine uptake until after 6–7 d. However, $F_1BGH_12C_1$ cells cannot reenter the "S" phase (DNA synthesis phase) of the cell cycle after growth in limited medium for 3–4 d (Fig. 2*A*), whereas total protein synthesis, in the absence of DNA synthesis, continues for at least 3–4 additional d. Thus, studies on the induction of prolactin synthesis have been undertaken during the period (6–7 d in limited medium) when none of the major cellular processes, except DNA synthesis, are affected grossly. It is still possible, though unlikely, that there are minor non-DNA effects on cellular metabolism which do not affect general protein synthesis but affect synthesis of a specific protein, e.g., prolactin. However, our results suggest that prolactin synthesis can be induced only when BrdUrd is added to cells during the "S" phase of the cell's growth cycle or when the cells are permitted to enter the "S" phase (Figs. 2*B* and 3*B*), but not when DNA synthesis is arrested (Fig. 3*B*).

BrdUrd is incorporated into DNA, substituting for its structural analogue thymidine to the extent of 80% without affecting the content of other bases (Table I). This raises the question: Is the altered DNA structure which results from base

substitution (BrdUrd for thymidine) related to the induction of prolactin synthesis in these cells? Prolactin synthesis increases linearly in parallel with the increasing thymidine substitution by BrdUrd in the DNA of these drug-treated cells. Similarly, when the drug is withdrawn from the cells after maximum base substitution (80%), prolactin synthesis declines in parallel with the reduction in the substituted base (Fig. 4). This parallel relationship between base substitution and prolactin synthesis suggests that the incorporation of the drug into DNA may be involved in the induction process. Initiation of prolactin synthesis appears to be dependent on a minimum amount (20–25%) of thymidine substitution by BrdUrd in DNA (Fig. 5 and Table II), which suggests that the event that triggers BrdUrd induction of prolactin synthesis is also related to a critical alteration in the DNA structure. However, these results do not completely rule out the possibility that the assay for prolactin used in this investigation is not sensitive enough to detect a very small amount of

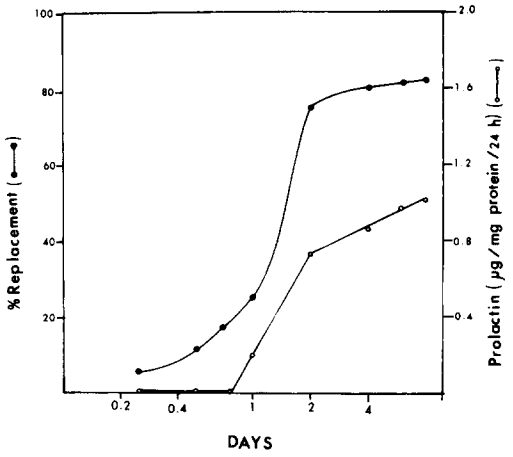


FIGURE 5 Kinetics of prolactin induction. $F_1BGH_12C_1$ cells were grown under the conditions as described in the legend to Fig. 2. Medium was replaced every 3 d. Cells were pulsed with ^{32}P 48 h before harvesting on the specified days of drug treatment (300 μg/ml). Prolactin in the medium and percent replacement of thymidine by BrdUrd in the DNA of these cells were determined as described earlier.

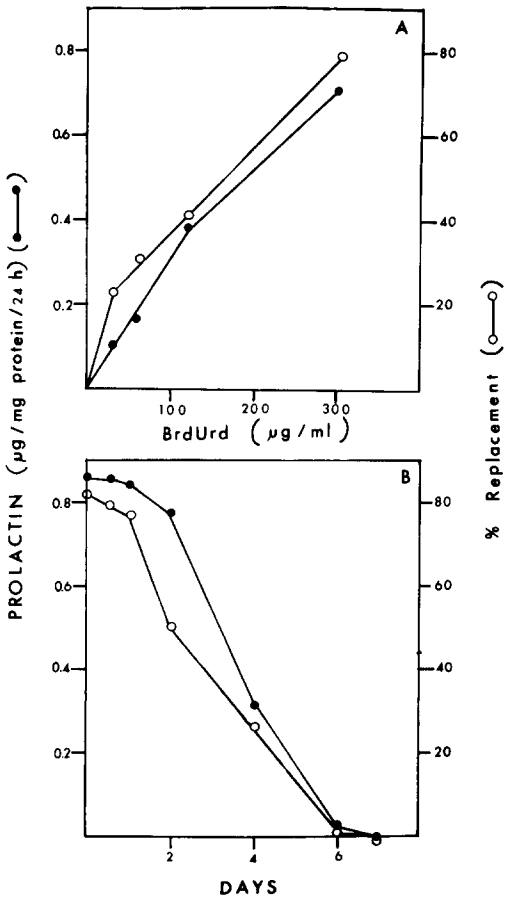


FIGURE 4 Relationship between base substitution and prolactin synthesis. $F_1BGH_12C_1$ cells were grown under the conditions described in the legend to Fig. 2. Fig. 4*A* shows prolactin production (●—●) and percent thymidine replacement of BrdUrd (○—○) in the DNA of cells grown in the presence of different concentrations of the drug. The cells were grown in the presence of 30, 60, 120, and 300 μg/ml of BrdUrd for 9, 7, 5, and 3 d, respectively. Cells were pulsed with ^{32}P (5 μCi/ml) 48 h before harvesting on the specified day. Prolactin in the medium was measured by microcomplement fixation assay, and ^{32}P-labeled DNA was isolated and analyzed for determination of percent replacement of thymidine by BrdUrd as described in Materials and Methods. Fig. 4*B* shows the effect of withdrawal of BrdUrd on prolactin production (●—●) and percent replacement of thymidine by BrdUrd in the DNA (○—○) of cells after prior drug treatment. $F_1BGH_12C_1$ cells were grown in F-10 medium under the conditions described above in the presence of BrdUrd (300 μg/ml) for 6 d. Medium was removed on this day for the determination of prolactin, ^{32}P-labeled DNA was isolated from cells, and the percent replacement of thymidine by BrdUrd was determined. Separate sets of dishes containing drug-treated cells received fresh medium. The cells were then grown in the absence of the drug and harvested on the indicated days after withdrawal of the drug. Prolactin in the medium and percent replacement of thymidine by BrdUrd in the DNA of the cells were determined in each sample as described above.

TABLE II

Replacement of Thymidine by BrdUrd in DNA, and Initiation of Prolactin Synthesis

BrdUrd	Strain	Exposure to drug	% replacement*	Prolactin production
μg/ml		d		
5	GH_12C_1	9	25	+
30	$F_1BGH_12C_1$	9	23	+
300	$F_1BGH_12C_1$	1	22	+

GH_12C_1 and $F_1BGH_12C_1$ cells were grown in the presence of different concentrations of BrdUrd for the indicated period of time under the conditions described in Table I. Cells were pulsed with ^{32}P for 48 h. [^{32}P]DNA was isolated, and the base composition of the DNA was determined by using the procedure described in Materials and Methods. Prolactin in the medium withdrawn on the specified day was determined by microcomplement fixation assay.

* $\left[\frac{B}{B+T}\right]$

prolactin synthesis initiated at base substitution <20%. Though thymidine substitution by BrdUrd reaches a maximum of 80% within 2 d and remains at this level beyond this period of treatment (Fig. 5), prolactin synthesis continues to increase even after 2 d but at a much slower rate. It is possible that once the maximum alteration in the DNA structure caused by base substitution is achieved and maintained, prolactin gene expression is turned on and continues to be at this stage of expression even when no further base substitution occurs.

These results demonstrate that BrdUrd induction of prolactin synthesis maintains a strict parallel relationship with (*a*) DNA synthesis, and (*b*) BrdUrd incorporation and base substitution in the DNA. Thus, these results are in agreement with DNA-linked mechanisms of action of BrdUrd on the control of differentiated function proposed by Rutter et al. (12). It may be postulated that BrdUrd substitution of thymidine in the DNA causes sufficient alteration in the structure of the genome leading to altered binding affinity for the cellular regulatory molecules to DNA. Thus, an increase in the affinity of binding to DNA of an "activator" or decrease in the same for a "repressor" type of molecule will lead to the expression of a specific gene which is normally suppressed. However, such a model does not explain the results obtained by other investigators (6, 13) in different systems in which incorporation of BrdUrd into DNA does not seem to be obligatory for its action. In these systems, it is possible that BrdUrd acts via a mechanism that is different from the one described above, or it is also possible that, in these systems, BrdUrd acts via a mechanism which demands only very low levels of incorporation of the drug and thus undetectable substitution for thymidine in the DNA. The present study provides means to identify the regulatory molecules that are involved in the suppression of a specific gene such as the prolactin gene in GH_12C_1 cells and also helps in understanding the mechanism involved in the effect of BrdUrd on the control of gene expression.

The authors are thankful to Dr. Armen H. Tashjian, Jr. for his encouragement and interest in this work.

This investigation was supported by the National Science Foundation, the National Institute of General Medical Sciences, and the National Institute of Arthritis, Metabolism, and Digestive Diseases.

Received for publication 2 August 1978, and in revised form 5 October 1978.

REFERENCES

1. BICK, M. D., and R. L. DAVIDSON. 1974. Total substitution of bromodeoxyuridine for thymidine in the DNA of a bromodeoxyuridine-dependent cell line. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2082–2086.
2. BISWAS, D. K., J. LYONS, and A. H. TASHJIAN, JR. 1977. Induction of prolactin synthesis in rat pituitary tumor cells by 5-bromodeoxyuridine. *Cell.* **11**:431–439.
3. BISWAS, D. K., T. F. J. MARTIN, and A. H. TASHJIAN, JR. 1976. Extended RNA synthesis in isolated nuclei from rat pituitary tumor cells. *Biochemistry.* **15**:3270–3280.
4. BISWAS, D. K., and A. H. TASHJIAN, JR. 1974. Intracellular site of prolactin synthesis in rat pituitary cells in culture. *Biochem. Biophys. Res. Commun.* **60**:241–248.
5. COUCH, J., and P. HANAWALT. 1971. Analysis of 5-bromouracil distribution in partially substituted deoxyribonucleic acids. *Anal. Biochem.* **41**:51–56.
6. DAVIDSON, R. L., and E. R. KAUFMAN. 1978. Deoxycytidine reverses the suppression of pigmentation caused by 5-bromodeoxyuridine without changing the amount of 5-bromodeoxyuridine in DNA. *Cell.* **12**: 923–929.
7. HOLTZER, H., and J. ABBOTT. 1968. Oscillation of the chondriogenic phenotype in vitro. *In* The Stability of the Differentiated State. H. Ursprung, editor. Springer-Verlag New York, Inc., New York. 1–16.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
9. MARTIN, T. F. J., and A. H. TASHJIAN, JR. 1977. Cell culture studies of thyrotropin-relearning hormone action. *In* Biochemical Actions of Hormones, Vol. 4. G. Litwack, editor. Academic Press, Inc., New York. 269–312.
10. O'BRIEN, J. C., and R. H. STELLWAGEN. 1977. The effect of controlled substitution of 5-bromodeoxyuridine for thymidine in hepatoma cell DNA. *Exp. Cell Res.* **107**:119–125.
11. PREISLER, H. D., D. HOUSMAN, W. SCHER, and C. FRIEND. 1973. Effects of 5-bromo-2-deoxyuridine on production of globin mRNA in dimethylsulfoxide-stimulated Friend leukemia cells. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2956–2959.
12. RUTTER, W. J., R. L. PICTET, and P. W. MORRIS. 1973. Toward molecular mechanisms of developmental processes. *Annu. Rev. Biochem.* **42**:601–645.
13. SCHUBERT, D., and F. JACOB. 1970. 5-Bromodeoxyuridine-induced differentiation of a neuroblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 246–254.
14. STELLWAGEN, R. H., and G. M. TOMKINS. 1971. Preferential inhibition by 5-bromodeoxyuridine of the synthesis of tyrosine aminotransferase in hepatoma cell cultures. *J. Mol. Biol.* **56**:167–182.
15. TASHJIAN, A. H., JR., L. LEVINE, and A. E. WELHELMI. 1968. Studies on growth hormone by microcomplement fixation. *Ann. N. Y. Acad. Sci.* **148**:352–371.