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Syntheses and Modulations in the Chromatin Contents of Histones H1° and H1 during G_1 and S Phases in Chinese Hamster Cells†

Joseph A. D'Anna,* Lawrence R. Gurley, and Robert A. Tobey

ABSTRACT: Flow cytometry, conventional autoradiography, and autoradiography employing high concentrations of high specific activity [^{3}H]thymidine indicate that (1) treatment of Chinese hamster ovary (line CHO) cells with butyrate truly blocks cells in G_1 and (2) cells blocked in G_1 by isoleucine deprivation remain blocked in G_1 when they are released into complete medium containing butyrate. Measurements of H1° content relative to core histones and H1°:H1 ratios indicate that H1° is enhanced somewhat in G_1 cells arrested by isoleucine deprivation; however, (1) treatment with butyrate greatly increases the H1° content in G_1-blocked cells, and (2) the enhancement is very sensitive to butyrate concentration. Measurements of relative histone contents in the isolated chromatin of synchronized cultures also suggest that the acid-soluble content of histone H1 (relative to core histones) becomes greatly depleted in the isolated chromatin when synchronized cells are blocked in early S phase by sequential use of isoleucine deprivation and hydroxyurea blockade. We also have measured [^{3}H]lysine incorporation, various protein ratios, and relative rates of deposition of newly synthesized H1°, H1, and H4 onto chromatin during G_1 and S in the absence of butyrate. These measurements show that (1) H1° is synthesized and deposited onto chromatin during traverse of G_1 and S phases so that its specific activity during G_1 is 50–60% of its maximum value in S phase enriched (60–70%) cultures, (2) the ratio between rates of deposition of new histones H1° and H1 onto chromatin reaches a maximum during G_1 at 1.5–2.0 h after cells are released from the G_1 block, and it declines about 6-fold as cells enter S phase, and (3) the H1°:H1 molar ratio is modulated in the isolated chromatin of synchronized cultures so that it reaches a maximum near the G_1–S boundary. These results suggest a dynamic picture of chromatin organization in which (1) newly synthesized histone H1° binds to chromatin during traverse of G_1 and S phases and (2) histone H1 dissociates from (or becomes loosely bound to) chromatin during prolonged early S-phase block with hydroxyurea.

In 1969, Panyim & Chalkley (1969a) reported the isolation and amino acid analysis of a minor histone which has become known as histone H1°. The original observations of Panyim & Chalkley (1969a) and those of subsequent investigators [see D'Anna et al. (1981b) for a review] have led to the generalization that the cellular content of H1° is inversely proportional to the rate of DNA synthesis (Marsh & Fitzgerald, 1973). Although the inverse relationship between the cellular content of H1° and DNA synthesis indicates that the synthesis of H1° can be uncoupled from that of DNA, only a few studies of H1° synthesis or details of H1° enhancement have been reported. Gurley et al. (1972) observed that the rate of isotope incorporation into the H1° region of electrophoretic gels relative to that of the nucleosome core histones was greater in G_1-arrested and G_1-traversing cells than in exponentially growing cultures. More recently, Zlatanova (1980, 1981) has reported that (1) brief treatment of Friend erythroleukemia cells with hydroxyurea reduces synthesis of histone H1 and

† From the Toxicology Group, University of California, Los Alamos National Laboratory, Los Alamos, New Mexico 87545. *Received November 18, 1981; revised manuscript received April 21, 1982.* This work was supported by National Institutes of Health Grant GM24564 and by the U.S. Department of Energy.

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the core histones, but it does not inhibit synthesis of H1° (usually called IP25[1]), and (2) the rate of synthesis of H1° in Friend erythroleukemia cells blocked in G_1 by isoleucine deprivation is greater than that measured in exponentially growing cultures.

Other recent studies indicate that treatment of cells with butyrate increases the proportion of G_1 cells in culture (Rastl & Swetly, 1978; Fallon & Cox, 1979; D'Anna et al., 1980a; Long et al., 1980; Pragnell et al., 1980; Darzynkiewicz et al., 1981) and causes an increase in the cellular content of H1° (Candido et al., 1978;[1] D'Anna et al., 1980a; Pieler et al., 1981). While these studies suggest that H1° can become enhanced during the G_1 phase of the cell cycle in butyrate-treated cultures, this has not been proven. All of the studies of butyrate block (including our own) have employed flow cytometry (FCM[2]) and conventional autoradiography to show that butyrate blocks cells in G_1. Although FCM and conventional autoradiography are powerful tools for cell-cycle analysis, they cannot distinguish very early S-phase from G_1 cells (Walters et al., 1976a; Gurley & Jett, 1981; Jett & Gurley, 1981). Furthermore, it has not been shown that H1° enhancement actually is occurring during the period of G_1 block. Here we report that (1) butyrate truly blocks CHO cells in G_1, (2) cells blocked in G_1 by isoleucine deprivation remain blocked in G_1 when they are released into the presence of butyrate, and (3) H1° really does become enhanced during G_1 block in butyrate-treated cultures. We also report (1) measurement of specific activities of lysine incorporation and relative rates of synthesis for histones H1°, H1, and H4, (2) modulations in the H1°:H1 molar ratio during traverse of G_1 and S, and (3) large variations in the quantity of histone H1 (relative to core histones) which is extracted from the chromatin of synchronized cells. These results suggest that in proliferating cultures (1) H1° is not just a packaging protein for new DNA during the S phase and (2) histone H1 may dissociate from (or become loosely bound to) chromatin during the early stages of DNA replication.

Experimental Procedures

Cell Growth and Cell Synchrony. Suspension cultures of Chinese hamster (line CHO) cells were grown in F-10 medium supplemented with 15% newborn calf serum, streptomycin, and penicillin (Tobey et al., 1966). Cells were examined periodically to ensure that they were free of *Mycoplasma* (Walters et al., 1974). Cell concentrations were measured with a Coulter Model Z_B particle counter.

Cells were synchronized in G_1 by the isoleucine deprivation method of Tobey & Ley (1971). In some experiments, the G_1-blocked cells were treated with butyrate as previously described (D'Anna et al., 1980a). To determine if G_1-arrested cells (not treated with butyrate) would resume cell-cycle progression when they were released from the G_1 block into the presence of complete F-10 medium (includes isoleucine) containing butyrate, we centrifuged and resuspended cells in 3 × 1 L of complete F-10 medium to which had been added 0, 5.0, or 15 mM butyrate. At 4–6.5-h intervals, aliquots of 5 and 40 mL were taken for measurement of cell concentration and for cell-cycle analysis by FCM.

Cultures were synchronized near the G_1–S boundary (early S phase) by use of isoleucine deprivation (30–36 h) followed by resuspension in complete F-10 containing 1.0 mM hydroxyurea (Tobey & Crissman, 1972). To determine if CHO cells released from hydroxyurea blockade would resume cell-cycle traverse in the presence of butyrate, we divided 3 L of cells into three portions which were resuspended (in sterile 12-mL Corex tubes) in fresh F-10 containing 0, 5, and 15 mM butyrate. The cells were quickly centrifuged at low speed to remove residual hydroxyurea from the supernatant and resuspended in 1.0 L of warm F-10 containing the respective concentration of butyrate. Two aliquots of 5 mL were taken at 1.0-h intervals for measurement of cell concentration and autoradiography.

For measurement of the H1°:H1 ratios following release of cells from hydroxyurea blockade, 4.0 L of cells was synchronized by sequential use of isoleucine deprivation (31 h) and 1 mM hydroxyurea (10 h). The culture was then released into 4.0 L of F-10 (the control) or F-10 containing 15 mM butyrate. Prior to and at 1.5–2.0-h intervals after release, aliquots of 2, 5, and 300 mL were taken for measurement of cell concentration, autoradiography, and isolation of histones. Histones H1 and H1° were isolated by perchloric acid (PCA) extraction from the chromatin of blended cells (see below).

Cell-Cycle Analysis. The distribution of cellular DNA content in a culture was determined by flow cytometry, using the fluorescent dye mithromycin C as previously described (D'Anna et al., 1980a). Fractions of cells in G_1, S, and G_2 plus M were computed by the method of Dean & Jett (1974) or the method of Jett & Gurley (1981).

The fraction of cells synthesizing DNA at intermediate to high rates was determined by "conventional autoradiography" employing 15-min pulses of [*methyl*-^{3}H]thymidine (6 Ci/mM) at 2.0 μCi/mL (Tobey & Ley, 1970).

To determine whether cells released from isoleucine deprivation G_1 block into complete F-10 medium containing butyrate would remain in G_1 or if they would enter early S phase, we used autoradiography employing high concentrations of high specific activity [*methyl*-^{3}H]thymidine (Walters et al., 1976a). Four hundred milliliters of cells synchronized in G_1 by the isoleucine deprivation method was divided into four 100-mL portions, each of which was centrifuged and resuspended in 90 mL of complete F-10. Two of the 90-mL cultures contained 100 μCi/mL high specific activity [*methyl*-^{3}H]-thymidine (76 Ci/mM) and 0.0 or 15 mM butyrate. The other two cultures contained 0.1 μCi/mL [*methyl*-^{3}H]thymidine (6 Ci/mM) and 0 or 15 mM butyrate. Aliquots of 5 mL were taken for autoradiography at 2-h intervals for the first 14 h, and final aliquots were taken 26 h after release.

Isotopic Labeling for Synthesis Experiments. The relative rates of deposition of newly synthesized histones onto chromatin during traverse of G_1 and S were measured in two similar experiments. In both experiments, 4.0 L of cells was blocked in G_1 by the isoleucine deprivation method for 36 h and then released into 4.0 L of complete F-10. Prior to release from the G_1 block and at 1.0–1.5-h intervals after release, aliquots of 340–470 mL were withdrawn and labeled for 1.0 h with [^{3}H]lysine (60 Ci/mM) at 1 μCi/mL. Fifteen minutes before the harvest of each pulse-labeled aliquot, 5 mL of cells was withdrawn from the main (initially 4 L) culture for conventional autoradiographic analysis. Cells were harvested by centrifugation at 4 °C, and histones were isolated by PCA

[1] IP25 is the name given to the murine erythroleukemia cell protein which becomes enhanced when cells are stimulated to differentiate with butyrate, dimethyl sulfoxide, and a number of other agents (Keppel et al., 1977, 1979). While IP25 and H1° from all species may not be identical [see D'Anna et al. (1981)], other evidence (Pieler et al., 1981; Smith & Johns, 1980; D'Anna et al., 1980b) suggests that IP25 and the H1°'s from different species all belong to the same family of proteins (Smith et al., 1980; Pehrson & Cole, 1981).

[2] Abbreviations: FCM, flow cytometry; $NaDodSO_4$, sodium dodecyl sulfate; NP-40, Nonidet P-40 nonionic detergent; PCA, perchloric acid; TCB, 15 mM Tris-HCl–3 mM $CaCl_2$, pH 7.2; Tris, tris(hydroxymethyl)aminomethane; CHO, Chinese hamster ovary.

extraction from the blended chromatin of whole cells or from isolated nuclei (see below).

Protein Extraction. Nuclei were isolated by a modification of a procedure we used previously (D'Anna et al., 1980a; Wigler & Axel, 1976). Cells [(1.5–2.0) × 10^8] were harvested by low-speed centrifugation. They were resuspended in cold TCB (15 mM Tris-HCl–3 mM $CaCl_2$, pH 7.2) and pelleted by low-speed centrifugation in a clinical centrifuge. The cells were then resuspended in 8 mL of TCB, and 0.65 mL of 10% (v/v) Nonidet P-40 (NP-40) was added, with vigorous stirring on a vortex mixer (30 s), to make the solution 0.75% in NP-40. Cells were homogenized 12–14 strokes with a tight B pestle in a 15-mL glass Dounce homogenizer (Kontes glassware); release of cytoplasm was monitored by phase contrast microscopy. The homogenate and a 2.0-mL wash (0.50% NP-40 in TCB) of the homogenizer were transferred to a 12-mL Corex tube, and the nuclei were pelleted by low-speed centrifugation. The pellet was resuspended in 0.5 mL of 0.5% NP-40 in TCB by vortexing for 15 s; it was then diluted further with 4.5 mL of 0.5% NP-40 in TCB and mixed by vortexing for an additional 20 s. After centrifugation, the nuclei were washed twice more in TCB without NP-40.

Pelleted nuclei were transferred with a plastic-tipped pipet (nuclei stick to glass) to a Sorvall Omni-Mixer microblender cup (Du Pont Instruments) with 2.25 mL of isotonic saline containing 50 mM $NaHSO_3$. The nuclei were blended in an ice–water bath at a motor speed of 1.4 × 10^4 rpm for 1.25 min. The blended chromatin was pelleted by centrifugation and treated with 0.83 M PCA to extract H1 and H1° and with ethanol and HCl to extract nucleosomal core histones (Johns, 1964; Gurley & Hardin, 1968; Gurley et al., 1975).

For extraction of total histones, the pellet from the blended nuclei was extracted 3 times (45 min each) with 0.15 mL of 0.20 M H_2SO_4. The pooled extracts were clarified by centrifugation (30 min at 7700*g*) and precipitated by the addition of 10 volumes of acetone. The next day, the histones were washed twice with acetone, dissolved in water, and lyophilized.

Histones also were extracted from the chromatin of blended cells by the first method of Johns as previously described (Gurley & Hardin, 1968; Gurley et al., 1978).

Electrophoresis. Histones were separated by electrophoresis in two variations of the acid–urea–polyacrylamide gels of Panyim & Chalkley (1969b) and by electrophoresis in the sodium dodecyl sulfate ($NaDodSO_4$) gel system of Laemmli (1970). PCA-extracted proteins and H_2SO_4-extracted proteins were subjected to electrophoresis for 27 and 23 h, respectively, in long, cylindrical (0.5 cm × 25 cm) 2.5 M urea–15% acrylamide–5.2% acetic acid gels as described previously (Gurley et al., 1978). Histones extracted with ethanol in the Johns procedure were subjected to electrophoresis for 26 h in long cylindrical (0.5 cm × 25 cm) 6 M urea–12% acrylamide–5.2% acetic acid gels (D'Anna et al., 1977). All of the acid–urea gels were stained 12–18 h with 0.2% amido black–30% methanol–9% acetic acid and destained by diffusion in the same solvent without amido black.

Histones also were subjected to electrophoresis in $NaDodSO_4$ slab gels (Laemmli, 1970) as described previously (D'Anna et al., 1981a). After electrophoresis, the gels were fixed for 1 h with 20% trichloroacetic acid. They were then stained 4 h in 0.10% Coomassie blue, 7.5% acetic acid, and 50% methanol. Gels were destained by diffusion in 7.5% acetic acid–10% methanol.

Absorbance profiles of the cylindrical gels were measured at 630 nm with a Gilford Model 240 spectrophotometer equipped with a linear transport device. Absorbance profiles of slab gels were measured with a band-pass filter (522 nm) in a Quick Scan R & D electrophoresis/TLC densitometer (Helena Laboratories). A series of calibrated filters was used to determine that the photomultiplier response was linear in the absorbance range of the gels. Histone absorbance ratios were calculated from the weights of appropriate regions of photocopies of the absorbance profiles.

After absorbance profiles were measured, the cylindrical gels were cut into 2.2-mm pieces and dissolved in 1.0 mL of H_2O_2 for 18 h (55–60 °C). Isotopic incorporation was determined by scintillation spectrometry as described previously (D'Anna et al., 1980a).

Specific Activity Computations. Specific activities of H1°, H1, and H4 from the pulse-labeling experiments were computed from the absorbance and radioactivity profiles of the long acid–urea gels: the total counts per minute (cpm) from a given region of the gel were divided by the weight of that region (proportional to the integrated absorbance) cut from a photocopy of the absorbance profile. The weight of the absorbance region from the photocopy was an average of at least two determinations using the same ream of paper in the photocopy machine. (We find that the weights vary by less than 5% from sheet to sheet.) For conversion of counts per minute per unit weight to counts per minute per microgram for histones H1 and H4, counts per minute per unit weight was multiplied by the unit weight per gram which was determined from electrophoresis of known weights of histones H1 and H4. Similar coefficients of unit weight per microgram for H1° were computed from relative specific activity measurements of H1° and H1 (from cells labeled with [^{3}H]lysine for several generations), the number of lysines per molecule, and the calculated coefficient of H1. We used molecular weights of 23 000, 21 500, and 11 300 and lysine compositions of 65, 66, and 11 lysines/molecule for CHO H1 (D'Anna et al., 1980b), H1° (D'Anna et al., 1980b), and bovine H4 (Delange & Smith, 1972), respectively.

For calculation of the ratio of the rates of deposition of newly synthesized molecules of H1° and H1 onto chromatin during each 1-h pulse with [^{3}H]lysine, the counts per minute for each protein (from the same gel) were first corrected for the number of lysines per molecule. This was done by dividing each specific activity by the number of lysines per molecule.

For calculation of the ratio of the rates of deposition of newly synthesized molecules of protein onto chromatin for each mole of that protein extracted with PCA or H_2SO_4, the specific activity of each protein was first corrected for the number of molecules per microgram and the number of lysines per molecule. This was done by multiplying each specific activity by the ratio of the molecular weight and number of lysines per molecule.

Since the specific activities and the ratios of the rates of deposition of new histones onto chromatin were measured from 1-h pulses, they represent specific activities and rates of deposition per hour. Therefore, the specific activities and rates of deposition are plotted at the midpoint of the labeling period.

Results

Verification of G_1 Arrest in Butyrate-Treated Cultures. Three experiments were performed to investigate the position of butyrate block relative to G_1 block induced by isoleucine deprivation (Tobey & Ley, 1971) and early S-phase block induced by hydroxyurea (Walters et al., 1976a). In the first experiment, cells were released from early S-phase (hydroxyurea) block into warm F-10 containing 0, 5.0, or 15 mM butyrate. The purpose of the experiment was to determine if cells would remain blocked in S, or if they would progress

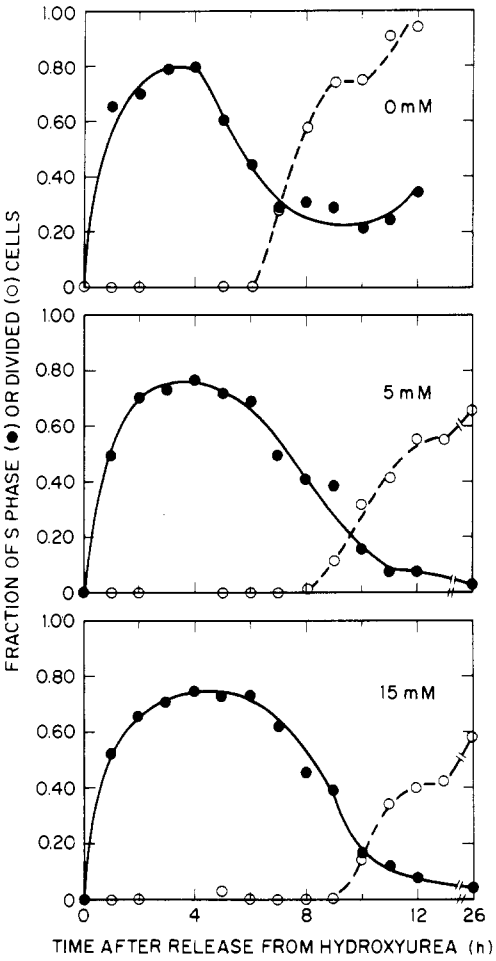

FIGURE 1: Release of CHO cells from hydroxyurea blockade into 0.0, 5.0, and 15 mM butyrate. Cells were synchronized by sequential use of isoleucine deprivation (30 h) and hydroxyurea blockade (10 h). The fraction of S-phase cells (●) and the divided fraction of cells (○) are plotted as functions of time after release from hydroxyurea blockade.

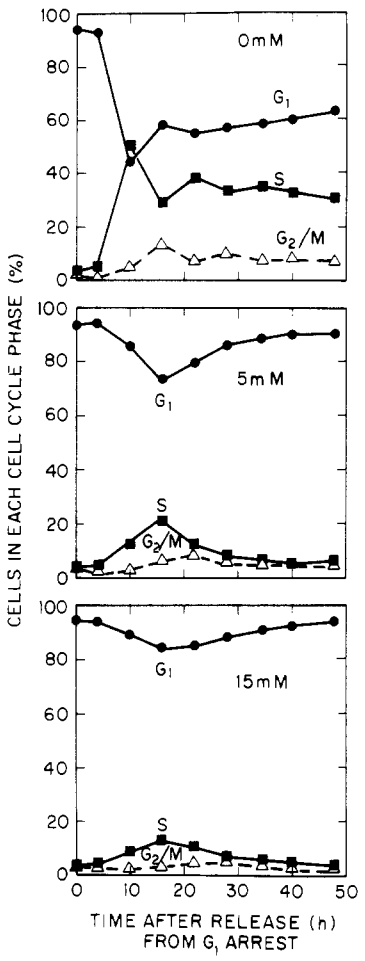

FIGURE 2: Release of CHO cells from G_1 arrest (isoleucine deprivation, 36 h) into 0.0, 5.0, and 15 mM butyrate. The percentages of cells in G_1 (●), S (■), and G_2–M (△) were determined by flow cytometry.

through the cell cycle to become blocked in the subsequent G_1 or early S. Analysis of the cultures by cell concentration measurements and conventional autoradiography (Figure 1) indicates that similar numbers of cells incorporate [^{3}H]thymidine in all of the cultures, but the duration of S phase is longer at increasing concentrations of butyrate. These direct observations that butyrate retards cell progression through the S phase confirm our previous deduction from FCM analyses of asynchronous cultures (D'Anna et al., 1980a). Increased concentrations of butyrate cause a reduction in the divided fraction of cells, besides retarding progression through the S phase. Since FCM analyses of the cultures at 26 h indicate only G_1 cells, a number of cells die prior to or after cell division. We conclude that while release of cells from hydroxyurea into butyrate can kill up to 40% of the cells, it does not prevent cell-cycle progression through the S phase.

In the second experiment, cells were released from isoleucine deprivation G_1 block into complete F-10 medium containing 0, 5, or 15 mM butyrate. The purpose of the experiment was to determine if cells would remain blocked in G_1 (or very early S), or if they would progress through the cell cycle. FCM analyses (Figure 2) show that in the absence of butyrate, 51% of the cells contained increased quantities of DNA by 10 h, indicating entry into the S phase. This is in contrast to 5 and 15 mM butyrate, where maximum percentages of 21% and 13% of the cells, respectively, enter the S phase as parasynchronous waves ~16 h after release. Measurements of cell concentrations at the end of 48 h show increases in the cell concentrations of 480, 34, and 15% at 0, 5, and 15 mM butyrate which are consistent with the FCM analyses. Although the FCM analyses do not distinguish very early S-phase cells from G_1 cells, they do show that (1) cells need not be exposed to butyrate prior to entry into G_1 for butyrate to block cell-cycle progression, (2) butyrate can block cells at the same point or later in G_1 (or in early S) than the block point of isoleucine deprivation, and (3) a portion of the cells can escape G_1 (or early S phase) block to replicate and divide before becoming arrested during the next G_1 (or early S) phase of the cell cycle.

To determine if cells were being blocked in G_1 or very early S phase, we released cells from isoleucine deprivation G_1 block into complete F-10 medium containing 15 mM butyrate and high concentrations of high specific activity [^{3}H]thymidine. At these high concentrations of [^{3}H]thymidine, even very low levels of incorporation should be detectable (Walters et al., 1976a; Gurley & Jett, 1981). Figure 3 shows the fraction of cells which incorporate [^{3}H]thymidine in cultures labeled with 100 μCi/mL (high level label) or 0.1 μCi/mL (low level label) in the absence of butyrate or in similar cultures treated with 15 mM butyrate. In the absence of butyrate, 90% of the cells in the low level labeled culture and 99% of the high level labeled culture incorporate [^{3}H]thymidine by 14 h. In the presence of butyrate, only 12% of the cells in the low level culture and 23% of the cells in the high level culture begin to incorporate [^{3}H]thymidine by 14 h. Even at 26 h, only 18% and 32% of the cells have incorporated label. These results differ greatly from experiments in which cells progress through

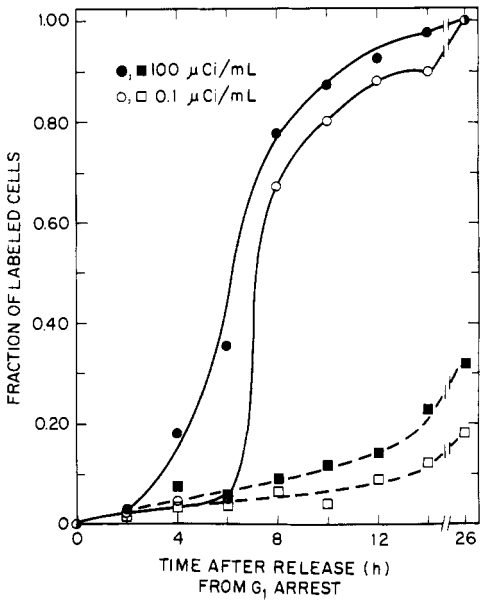

FIGURE 3: Autoradiography of cells following release from G_1 arrest (isoleucine deprivation, 36 h) into 0.0 (●, ○) or 15 mM (■, □) butyrate. Cultures were labeled continuously after release from G_1 arrest with either high specific activity [^{3}H]thymidine at 100 μCi/mL (●, ■) or low specific activity [^{3}H]thymidine at 0.1 μCi/mL (○, □).

Table I: Effects of Butyrate Concentration upon the H1°:H1 Absorbance Ratio[c] in Cells Blocked in G_1 by Isoleucine Deprivation

total time in Ile⁻ medium (h)	[butyrate] (mM)	time in butyrate (h)	% G_1 cells[b]	$A_{H1°}{}^c/A_{H1}$
0[a]	0.0	0	58	0.08
36	0.0	0	92	0.11
60	0.0	0	92	0.15
60	2.5	24	95	0.19
60	5.0	24	94	0.29
60	15.0	24	92	0.37

[a] Exponentially growing control. [b] Determined by FCM.
[c] This is the ratio of the integrated absorbances of H1° and H1 obtained from absorbance profiles of stained acid-urea-polyacrylamide gels. It closely approximates the H1°:H1 molecular ratio.

G_1 into the early S phase in the presence of hydroxyurea (Walters et al., 1976a) or picolinic acid (Gurley & Jett, 1981). With those agents, the percentage of labeled cells (high level label only) increases at the same rate as that of untreated controls. We conclude, therefore, that (1) butyrate blocks cells in G_1, rather than early S, and (2) the position of G_1 block can be the same or later in G_1 than that induced by isoleucine deprivation.

Modulations of the H1°:H1 Ratio in Synchronized Cultures Treated with Butyrate. Since butyrate blocks cells in G_1, we wanted to determine whether butyrate caused H1° enhancement during G_1 block and, perhaps, other phases of the cell cycle. Because (1) we can measure precisely the H1°:H1 absorbance ratio (which closely approximates the molar ratio) from PCA-extracted proteins (D'Anna et al., 1980a) and (2) we previously observed no measurable difference in the chromatin content of histone H1 (relative to core histones) between exponentially growing and butyrate-treated (10 mM sodium butyrate, 24 h) cultures (D'Anna et al., 1980a), our initial experiments were designed to monitor the H1°:H1 ratio and, presumably, modulations of only the H1° content. We shall see, however, that modulations in the chromatin content of histones H1 and H1° are more complex than we initially assumed.

Table II: Effects of Butyrate Concentration upon the H1°:H1 Absorbance Ratio[c] in Cells Released from Isoleucine Deprivation Induced G_1 Block (36 h)

[butyrate] (mM)	time after release (h)	time in butyrate (h)	% G_1 cells[b]	$A_{H1°}{}^c/A_{H1}$
0.0[a]	0	0	92	0.11
0.0	24	0	54	0.09
5.0	24	24	92	0.44
15.0	24	24	87	0.47

[a] Exponentially growing control. [b] Determined by FCM.
[c] This is the ratio of the integrated absorbances of H1° and H1 obtained from absorbance profiles of stained acid-urea-polyacrylamide gels. It closely approximates the H1°:H1 molar ratio.

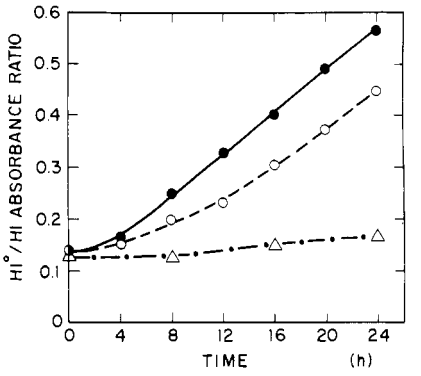

FIGURE 4: Measurements of H1°:H1 enhancement as a function of time in G_1-blocked cultures. Cultures were synchronized in G_1 arrest by the isoleucine deprivation method for 36 h; one culture was maintained in isoleucine-deficient medium (△); another culture was maintained in isoleucine-deficient medium, but it was made 15 mM in butyrate (○); the third was released from isoleucine deprivation into complete F-10 medium containing 15 mM butyrate (●). Aliquots were removed from each culture during the next 24 h for the isolation of histones and eventual measurement of H1°:H1 absorbance ratios.

Table I shows the effects of prolonged G_1 block and the effects of butyrate concentration upon the H1°:H1 absorbance ratio in cultures arrested in G_1 by isoleucine deprivation. Table II shows the effects of butyrate concentration upon the H1°:H1 absorbance ratio in cultures released from G_1 block (isoleucine-deficient medium) into 5 and 15 mM butyrate for 24 h (where most cells remain blocked in G_1). Figure 4 shows the time dependence of H1°:H1 absorbance enhancement in (1) cultures blocked in G_1 by isoleucine deprivation, (2) cultures blocked in G_1 by isoleucine deprivation, but containing 15 mM butyrate, and (3) cultures released from isoleucine deprivation G_1 blockade into 15 mM butyrate. These data clearly show that (1) the H1°:H1 ratio is increased somewhat in cells blocked in G_1 (by isoleucine deprivation) in the absence of butyrate (Table I), (2) butyrate greatly elevates the H1°:H1 enhancement observed in G_1-blocked cells (Table I and Figure 4), (3) the enhancement caused by butyrate is even larger in cultures released from isoleucine deprivation into complete medium containing butyrate (Table II and Figure 4), and (4) the initial time dependence of the H1°:H1 ratio (Figure 4) exhibits a time lag which suggests a delay in the entry of butyrate into the cells or a delay in the enhancement process itself. We note that while the H1°:H1 ratio reflects changes in the chromatin content of both H1 and H1°, experiments in the next section verify that H1° does exhibit a net enhancement during G_1 in the butyrate-treated cultures.

We next investigated the possibility that butyrate would modulate the H1°:H1 ratio during the S phase in butyrate-treated cultures. In the first experiment, cultures were blocked in early S phase by sequential use of isoleucine deprivation

Table III: Various Histone Absorbance Ratios Computed from Absorbance Profiles of Long Acid-Urea-Polyacrylamide Gels[f]

source[a]	no. of independent samples	H1°:H1[c,d]	H1°:H4[c,e]	H1:H4	(H1° + H1):H4[c,e]
exponential	5	0.075 ± 0.010	0.035 ± 0.007	0.50 ± 0.03	0.54 ± 0.04
exponential + 15 mM NaBu (24–28 h)	3[b]	0.41	0.19 ± 0.01	0.46 ± 0.01	0.65 ± 0.01
Ile⁻ (total of 60 h) + 15 mM NaBu (last 24 h)	3[b]	0.33 ± 0.06	0.15 ± 0.03	0.45 ± 0.01	0.60 ± 0.04
Ile⁻ (36 h) → F-10 + 15 mM NaBu (24 h)	3[b]	0.46 ± 0.07	0.18 ± 0.03	0.39	0.57 ± 0.03
Ile⁻ (36 h)	2	0.12	0.064 ± 0.002	0.52 ± 0.02	0.59 ± 0.02
Ile⁻ (60 h)	2	0.12	0.054 ± 0.003	0.46 ± 0.02	0.51 ± 0.02
Ile⁻ (36 h) → hydroxyurea (10 h)	3	0.13 ± 0.02	0.046 ± 0.010	0.36 ± 0.02	0.40 ± 0.03
Ile⁻ (36 h) → hydroxyurea (24 h)	2	0.22 ± 0.02	0.031 ± 0.003	0.14 ± 0.02	0.17 ± 0.01
Ile⁻ (36 h) → hydroxyurea (24 h), last 14 h in 15 mM NaBu	2	0.30 ± 0.09	0.075 ± 0.30	0.25	0.33 ± 0.04

[a] Ile⁻ refers to growth in isoleucine-deficient medium to induce G_1 arrest; NaBu = sodium butyrate. [b] The H1°:H1 ratio for one sample was measured from a $NaDodSO_4$ gel. [c] Relative to a coefficient of 1.0 for H1, there are 1.01 ± 0.07 molecules of H1° per unit absorbance on the gel stained with amido black. [d] H1°:H1 ratios computed from PCA-extracted proteins. [e] Computed from the H1°:H1 and H1:H4 ratios. [f] The histones were extracted with PCA and H_2SO_4 from isolated nuclei.

(36 h) and hydroxyurea blockade (10 h). The cultures were then released into 0 or 15 mM butyrate. The H1°:H1 absorbance ratio, the [^{3}H]thymidine incorporation (low level), and the divided fraction were monitored as functions of time (Figure 5). Both the butyrate-treated culture and, to our surprise, the control culture exhibited substantial modulations in the H1°:H1 ratio. It appears that butyrate has little effect upon the H1°:H1 ratio until about 4 h, when the H1°:H1 ratio begins to increase as compared with the control in which the H1°:H1 ratio begins to decrease. By 8 h, the ratio in the butyrate-treated culture has increased to 0.27. Since (1) at least 75% of the cells enter the S phase, (2) the H1°:H1 ratio at 8 h (with less than 25% G_1 cells) is similar to the value for a butyrate-treated G_1 (>90% G_1 cells) culture at that time (Figure 4), and (3) there is an inflection in the H1°:H1 data near the end of the S phase, it appears that butyrate is capable of modulating the H1°:H1 ratio during S and/or G_2, as well as during G_1. The increase in the H1°:H1 ratio beyond 8 h can be attributed to enhancement occurring during G_1 of the next cycle.

With regard to the control (no butyrate) in Figure 5a, it can be seen that the H1°:H1 ratio declines as cells progress through the S phase, reaching a minimum near the time of cell division. These modulations of the H1°:H1 ratio in the control strongly suggest differential synthesis, turnover, or differential association of histones H1 and H1° with chromatin during the cell cycle.

A second experiment was performed to support the observation that butyrate might be causing H1°:H1 enhancement in S-phase cells. A culture was synchronized in early S phase by sequential use of isoleucine deprivation (31 h) and hydroxyurea blockade (10 h). The culture was then divided into four parts which were treated with 0, 2.5, 5.0, or 15 mM butyrate (without releasing them from hydroxyurea). Fourteen hours later, the cells were harvested, and histones were extracted by the first method of Johns (1964). The measured H1°:H1 ratios of 0.42, 0.37, 0.42, and 0.38 in the PCA-extracted proteins indicated that H1°:H1 enhancement could occur in hydroxyurea-blocked cultures in the presence or absence of butyrate; however, the amount of H1 present in the gels was much less than we normally measure for corresponding weights of the ethanol-extracted proteins (H2A + H3 + H4) in the first method of Johns. Thus, our data also suggested that H1 was being lost from the chromatin and the hydroxyurea-blocked cultures and that this loss was affecting the H1°:H1 ratio.

Modulations in H1° and H1 Contents in the Chromatin of Synchronized Cultures. To determine whether the net contents of both H1° and H1 were modulated in the isolated chromatin of synchronized cultures, we compared the contents of H1° and H1 with the contents of the nucleosomal core histone H4: (1) histones were extracted from the sedimented chromatin of blended nuclei of each culture by use of H_2SO_4 and PCA; (2) they were subjected to electrophoresis in 2.5 M urea–15% acrylamide–5.2% acetic acid gels; (3) ratios of H1°:H1 and H1:H4 were measured from absorbance profiles of the stained gels, and they were used to compute H1°:H4 and (H1° + H1):H4 ratios. This approach was used for the following reasons: (1) most nuclear DNA is associated with nucleosomes (Chambon, 1977); (2) histone H4, along with other core histones or their variants, appears to be a fundamental constituent of all nucleosomes (McGhee & Felsenfeld, 1980), but nucleosomes may or may not contain H1 (Bakayev et al., 1978; Levy W. et al., 1979; Jackson et al., 1979; Jackson & Rill, 1981); (3) except for a minor impurity which migrates to the trailing side of the $H4_4$ band [determined from two-dimensional electrophoresis employing a $NaDodSO_4$ slab in the second dimension (results not shown)] and whose presence may be accounted for in the analyses, the H1 and H4 regions of H_2SO_4-extracted proteins are free of contaminant proteins in the gels; (4) the results are reproducible.

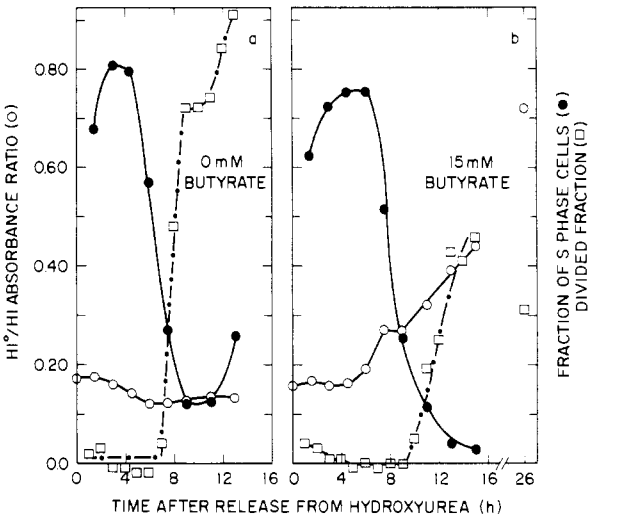

FIGURE 5: Measurements of the H1°:H1 absorbance ratio (○), fraction of S-phase cells (●), and the divided fraction of cells (□) following release from hydroxyurea blockade (10 h) into the presence of (a) 0.0 or (b) 15 mM butyrate.

Table III presents H1°:H1, H1°:H4, H1:H4, and (H1° + H1):H4 absorbance ratios for a variety of synchronized populations. Those ratios show that the chromatin contents of

both H1° and H1 in the isolated chromatin are sensitive to cell-cycle perturbations induced by exposure to butyrate and other synchronizing procedures. In the absence of hydroxyurea, all butyrate-treated cultures exhibit a 4.2–5.2-fold increase in the content of H1° (relative to H4) compared with the exponentially growing control; therefore, butyrate causes a net H1° enhancement in the butyrate-treated G_1 cells of Tables I and II and Figure 4. Even when butyrate is omitted from cultures arrested in G_1 by isoleucine deprivation, there is a 1.5–1.8-fold enhancement in the H1° content; however, we do not know (a) whether the H1° enhancement occurs before or after cells enter G_1 block or (b) why H1° and H1 become somewhat reduced (compared to 36 h in isoleucine-deficient medium) when the cells are left in isoleucine-deficient medium for a total of 60 h.

In general, as the H1° content goes up in the butyrate-treated cultures, the H1 content goes down; however, the sum of H1 plus H1° appears to be somewhat larger in the butyrate-treated cultures than in the controls.

Hydroxyurea blockade causes even more striking changes in the quantity of extracted H1 relative to histone H4 (Table III). When cells are released from isoleucine deprivation (36 h) into hydroxyurea for 10 h, there is a 30% reduction in H1 content relative to the value at the time of release. If the cells remain in the presence of hydroxyurea for an additional 14 h, the H1 content declines even further (a total reduction of 70%). In this regard, we note that 1.0 mM hydroxyurea does not hold cells near the G_1–S boundary; after 24 h in hydroxyurea, cells move 20–25% through S (FCM data not shown). It is possible, therefore, that the slow progression through the S phase in the presence of the drug may be instrumental to the reduced H1 content (see Discussion).

Besides a reduction in the content of H1 in the isolated chromatin from the hydroxyurea-treated cultures (Table III), there is a reduction in the H1° content as well. It appears, therefore, that those factors which reduce H1 content also reduce H1° content. When cultures are exposed to both hydroxyurea and butyrate, there is an enhancement in the H1°:H4 ratio above that measured before butyrate was added and greater than the control which did not contain butyrate. Thus, it appears that butyrate can cause H1° enhancement in the hydroxyurea "blocked" S-phase cultures; furthermore, while H1 is depleted in the hydroxyurea-treated cultures, its depletion is not so great as when butyrate is absent.

We note that measurements of the ratio of H4 to inner histones from $NaDodSO_4$ slab gels show no difference for all of the samples in Table III (0.217 ± 0.005); therefore, it is unlikely that variations in the H1:H4 ratio arise from discriminatory extraction of H4.

In Figure 5a, we observed changes in the H1°:H1 ratio as cells were released from early S-phase hydroxyurea blockade; in a similar experiment, we have measured the H1:H4 ratios. We find that the H1:H4 absorbance ratio increases from 0.72 of the exponential value in the hydroxyurea-blocked cells to 0.88 near 7 h, when most cells have progressed through the S phase. Therefore, it appears that modulation of the H1 content in the isolated chromatin is a major contributor to the measured changes in the H1°:H1 ratio following release from hydroxyurea blockade.

Deposition of Newly Synthesized Histones H1° and H1 onto Chromatin during Traverse of G_1 and S. Since (1) the chromatin content of H1° becomes greatly enhanced during G_1 in butyrate-treated cultures and (2) the chromatin contents of H1° and H1 are modulated in cultures blocked in G_1, in early S, and following release from hydroxyurea blockade

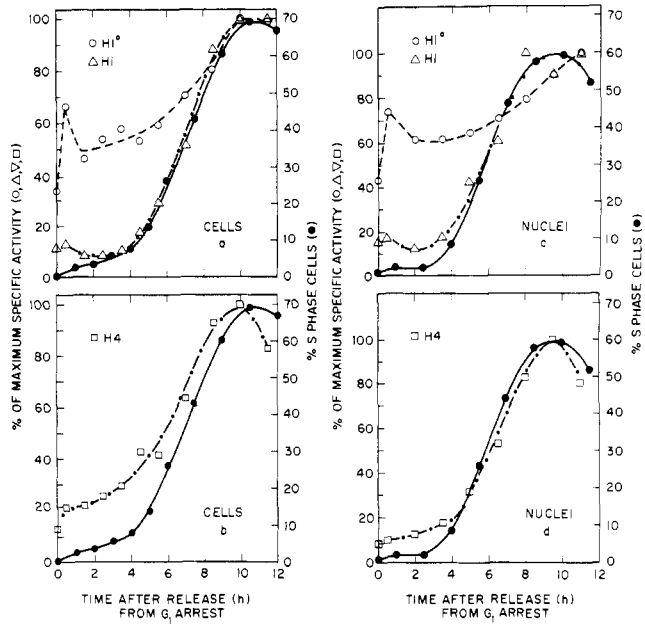

FIGURE 6: Percentages of maximum specific activities of histones H1° (○), H1 (△), and H4 (□) from the chromatin of blended cells (a and b) and blended nuclei (c and d). The percentages of maximum specific activities and the percentages of S-phase cells (●) are plotted as functions of time after release from G_1 arrest (isoleucine deprivation). The maximum specific activities of H1°, H1, and H4 from blended cells are 2.1×10^2, 4.5×10^2, and 1.8×10^2 cpm/μg, respectively. The maximum specific activities of H1°, H1, and H4 from blended nuclei are 2.4×10^2, 4.4×10^2, and 1.5×10^2 cpm/μg, respectively.

(where the H1 content in chromatin is reduced), we wanted to determine if H1° synthesis also occurs during traverse of G_1 and S in the absence of butyrate and hydroxyurea.

Figure 6 shows results of measurements of specific activities (pulse labeling for 1 h with [^{3}H]lysine) of histones H1, H1°, and H4 following release from G_1 block (isoleucine deprivation, 36 h). In each part of the figure, (1) the percentage of the maximum specific activity of each protein during G_1 and S and (2) the percentage of cells in the S phase are plotted as functions of time after release. The proteins were isolated by the first method of Johns from the chromatin of blended cells (Figure 6a,b) or from the blended chromatin of isolated nuclei (Figure 6c,d).

The specific activities of H1 isolated from the chromatin of blended cells and blended nuclei (Figure 6a,c) are similar to one another. In the G_1-blocked cultures ($t = 0$), their specific activities are 12–15% of their maximum values, and by 2–4 h after release, their functional dependencies are similar to the percentages of S-phase cells (which approximate relative rates of DNA synthesis). In contrast to H1, the specific activities of H1° in the G_1-blocked cells are 34–43% of their maximum values, they exhibit sharp increases during the first hour after release from G_1 block, and they remain at >50–60% of their maximum values throughout G_1 traverse. Since the specific activities of H1 and H1° from blended nuclei and blended cells are the same, it is unlikely that H1° is deposited artifactually from the cytoplasm during the isolation procedures. We conclude, therefore, that H1° is extensively synthesized and deposited onto chromatin throughout the traverse of cells from G_1 into S, as well as in G_1-blocked cells.

To compare the syntheses of H1° and H1 in greater detail, we have plotted (1) the H1°:H1 absorbance ratio (Figure 7a), (2) the ratio of rates of deposition of newly synthesized H1° and H1 onto chromatin (Figure 7b), and (3) the ratio of rates of deposition onto chromatin of newly synthesized H1 and H1° per mole of the respective histone isolated from the chromatin

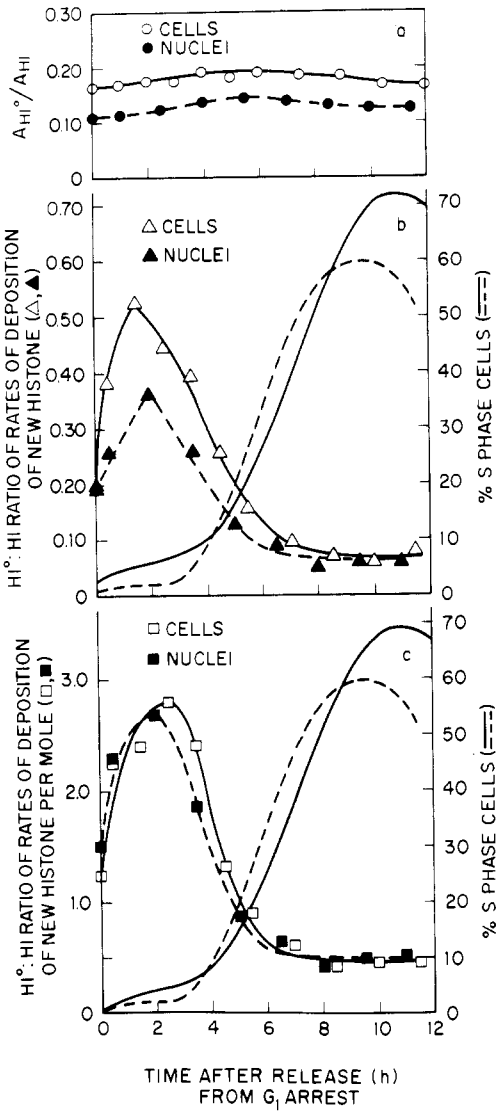

FIGURE 7: Comparisons between H1° and H1. The H1°:H1 absorbance ratio (a), ratio of rates of deposition of new H1° onto chromatin (b), and ratio of rates of deposition of new H1° and H1 per mole of each of those histones obtained by PCA extraction (c) are plotted as functions of time after release from G_1 arrest (isoleucine deprivation, 36 h). The percentages of S-phase cells (—, ---) during the experiments are also plotted in (b) and (c). The open symbols and solid lines represent the quantities from experiments employing blended cells; the closed symbols and dashed lines represent the quantities from blended nuclei.

by PCA extraction (Figure 7c). These variables are plotted as functions of time after release from G_1 block. The computed relationships (from the raw data and data of Figure 6a,c) in Figure 7 reveal a number of important quantitative features. (1) Both sets of data (Figure 7a) exhibit subtle modulations in the H1°:H1 absorbance ratio during traverse of G_1 and S, so that the ratio is maximal near the G_1–S boundary. (2) There is only 0.1–0.2 molecule of H1° for every molecule of H1 in chromatin throughout the G_1 and S periods (Figure 7a); however, on an equimolar basis (Figure 7c), the rate of deposition of new H1° per mole of H1° onto chromatin is 2.8 times greater than the rate of deposition of new H1 per mole of H1 at 2.0–2.5 h, when most cells are in G_1. As cells enter S, the rate of deposition of new H1° per mole declines to only half that of H1, which is consistent with the decline in the H1°:H1 ratio in Figure 7a. (3) While the rate of deposition of new H1° per mole of H1° is much higher than that of H1 during G_1, only 0.4–0.5 molecule of new H1° is actually deposited on chromatin for each molecule of H1 (Figure 7b). When 60–70% of the cells have entered the S phase, only 0.06 molecule of H1° is deposited for each molecule of H1 (Figure 7b).

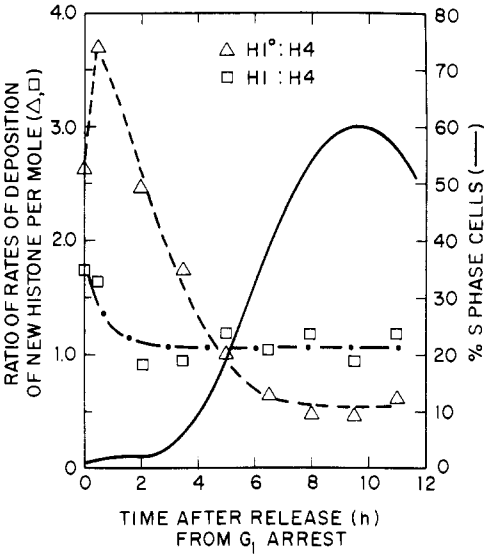

FIGURE 8: Comparison of the rates of deposition of new H1 and new H1° with that of H4. The ratios of rates of deposition of new histones per mole of each histone obtained by PCA extraction from blended nuclei [H1°:H4 (△); H1:H4 (□)] and the percentage of S-phase cells (—) are plotted as functions of time after release from G_1 arrest (isoleucine deprivation, 36 h).

Comparison of Rates of Deposition of New H1 and H1° with That of H4. Since H1 (Varshavsky et al., 1976; Whitlock & Simpson, 1976; Noll & Kornberg, 1977) and H1° (Smith & Johns, 1980) are nucleosomal linker histones, we wanted to compare their specific activities with those of the nucleosomal core histone H4. The relative specific activities of H4 from cells and from nuclei are plotted as functions of time after release from G_1 block in Figure 6b,d. In contrast to H1° and H1, the specific activities of H4 from blended cells and from nuclei are different: the specific activity of H4 from blended cells is approximately twice as large as that from nuclei during the first 4 h after release. Because the H4 from the blended cells contains substantial nonhistone impurities which comigrate with H4 in acid–urea–polyacrylamide gels (D'Anna et al., 1980a), we do not know whether the increased specific activity arises from greater incorporation of newly synthesized H4 or from impurities. We will thus deal only with the specific activity of nuclear H4 which is essentially free of nonhistone impurities (D'Anna et al., 1980a) and whose functional dependence is similar to that of the percentage of S-phase cells (and, thus, DNA synthesis).

Figure 8 shows ratios of the rates of deposition of new histone per mole of that histone in chromatin; these H1:H4 and H1°:H4 rate ratios are plotted as functions of time after release from G_1 block. Although plots of specific activities of H1 (Figure 6c) and H4 (Figure 6d) appear similar, the ratios in Figure 8 show that 1.75 times as much new H1 per mole of H1 is deposited onto chromatin, compared with H4, in the G_1-blocked cells (t = 0 h). This excess incorporation of H1 quickly declines, so that by 2 h (slightly before cells enter S), the rates of H1 and H4 deposition per mole of each histone are essentially the same. These results are in general agreement with those of Tarnowka et al. (1978); however, our data indicate that the excess synthesis of H1 occurs primarily during isoleucine deprivation induced G_1 arrest and that it is readily reversible as cells resume cell-cycle traverse.

Plots of the ratios of H1°:H4 rates of incorporation of new histone per mole (Figure 8) are clearly different from those of H1:H4. They reemphasize the excess synthesis and deposition onto chromatin of H1° which precede DNA synthesis and the deposition of newly synthesized nucleosomal core histones. They also suggest that H1° is not deposited at a sufficient rate to maintain a constant H1°:H4 ratio during the S phase (i.e., the ratio of rates of deposition of new molecules per mole is only 0.5).

Discussion

G_1 Block in Butyrate-Treated Cultures. Cell synchrony experiments and autoradiography employing high concentrations of high specific activity [^{3}H]thymidine indicate (1) butyrate truly blocks cells in G_1 and (2) the position of the G_1 block can be the same or later in G_1 than that induced by isoleucine deprivation. This does not imply that the biochemical state of a butyrate-blocked G_1 culture is the same as that of a cell blocked by isoleucine deprivation or a cell traversing G_1 following recovery from G_1 arrest; it merely indicates that butyrate can retard cell-cycle progression when cells are located within those positions in G_1.

While this manuscript was submitted, Darzynkiewicz et al. (1981) and Xue & Rao (1981) reported investigations of butyrate block in murine leukemic and HeLa cells by other methods. Darzynkiewicz et al. (1981) have employed flow cytometry and the fluorescent dye acridine orange to investigate butyrate block in mouse L1210 cells. Their data indicate that butyrate arrests cells in the G_{1A} part of G_1 (early G_1) characterized by a low RNA content and a condensed state of chromatin. Xue and Rao also have concluded from premature chromosome condensation (PCC) measurements with HeLa cells that butyrate blocks cells in early G_1. Since isoleucine deprivation appears to arrest rodent cells in late G_1 as determined from PCC measurements (Moser et al., 1981), but CHO cells released from isoleucine deprivation G_1 block into complete medium containing butyrate remain in G_1, it is possible that butyrate can block cells at multiple points in G_1 or that butyrate induces a unique biochemical state that is accessible from several points in G_1. Detailed investigations employing several methods and a single cell line will be required to test these possibilities.

Our cell-cycle experiments also (1) confirm our previous deduction from asynchronous cultures that butyrate retards cell-cycle progress through the S phase and (2) support the conclusion of Fallon & Cox (1979) that addition of butyrate to synchronized S-phase cultures causes subsequent loss of G_2 cells from the culture. Therefore, while the addition of butyrate does not appear to cause excessive cell death in exponentially growing cultures, it does appear to cause substantial cell death when it is added to S-phase cells released from hydroxyurea blockade or S-phase cells released from double-thymidine block (Fallon & Cox, 1979). (In this regard, it is possible that synchronizing the cells in early S maximizes the number of cells which will be blocked in or die during G_2; cell death in the exponentially growing culture might only affect the generation time.) Since it has been shown that butyrate induces DNA strand breaks in erythroleukemia cells (Scher & Friend, 1978; Terada et al., 1978), strand breaks may play a role in the apparent toxicity of butyrate to synchronized S-phase cells.

H1° Enhancement in Butyrate-Treated Cultures. Measurements of H1°:H1 ratios and the chromatin contents of H1° and H1 (relative to nucleosome core histones) show that H1° is synthesized and that the chromatin content of H1° becomes greatly enhanced during G_1 in butyrate-treated cultures; therefore, the synthesis and deposition of newly synthesized H1° onto chromatin during G_1 are clearly uncoupled from DNA synthesis in butyrate-treated cultures.

Measurements of H1°:H1 ratios in butyrate-treated cultures following release from hydroxyurea blockade and in butyrate-treated cultures blocked with hydroxyurea suggest tht H1° also can become enhanced during S and, perhaps, G_2. Since some cells always remain in G_1 or near the G_1–S boundary when cells are released from hydroxyurea blockade, we regard the results as tentative. Nevertheless, the data raise the possibility that when exponentially growing cultures are treated with butyrate, H1° may become enhanced in the S and G_2 cells prior to their entry into G_1.

Previously, we observed no measurable differences in the ratio of H1 to the core histone H4 between exponentially growing cultures which had been exposed to 10 mM butyrate for 24 h (D'Anna et al., 1980a). Since that time, (1) Smith & Johns (1980) have reported that the ratio of H1° + H1 to the nucleosomal core histones is nearly constant in various mouse tissues which differ in their H1°:H1 ratios and (2) Pieler et al. (1981) have reported that the ratio of H1° + H1 to total histones in untreated and in butyate-treated murine neuroblastoma cultures is the same. Following our measurements of loss of H1 in hydroxyurea-blocked cells, we reinvestigated the constancy of H1 and of H1 + H1° relative to histone H4 in butyrate-treated cultures. Our new data indicate that the chromatin content of both H1 and H1° can vary in butyrate-treated cultures and in other cultures synchronized in various phases of the cell cycle. In general, we see a decrease in H1 content as H1° increases in the butyrate-treated cultures (in the absence of hydroxyurea); however, our measurements of (H1° + H1):H4 ratios still suggest that the ratios in butyrate-treated cells may exceed those in exponentially growing cultures.

Deposition of New H1° and H1 onto Chromatin in the Absence of Butyrate. Our measurements of H1°:H1 ratios, chromatin contents of H1° and H1 in blended chromatin, and the deposition of newly synthesized histones H1° and H1 onto chromatin show that (1) H1° is extensively synthesized and deposited onto chromatin during both G_1 and S, (2) the relative rates of deposition of new histones H1° and H1 onto chromatin vary greatly during traverse of G_1 and S, and (3) the H1°:H1 absorbance (molar) ratio is modulated during traverse of G_1 and S so that it reaches a maximum near the G_1–S boundary.

The synthesis and deposition of new H1° onto chromatin during G_1 are particularly significant because they suggest that (1) H1° is not simply a packaging protein for newly synthesized DNA during the S phase and (2) newly synthesized H1° may play other roles such as modulating chromatin structure, orchestrating cell-cycle progression (Naha & Sorrentino, 1980), preparing chromatin for DNA synthesis, or altering gene expression during traverse of G_1 in proliferating cells. Smith & Johns (1980) have shown that H1° is a nucleosomal linker protein, like H1 and H5, and they have suggested that H1° replaces H1 in chromatin. We do not know if H1° is replacing H1 or other proteins or if it is binding to regions devoid of spacer proteins during cell-cycle progression. In any case, the deposition of new H1° during G_1 provides a mechanism for altering chromatin structure and function. [We note that if H1° is replacing H1 in chromatin during G_1, then it would provide a mechanism for the H1 turnover measured by Gurley & Hardin (1970) in exponentially growing cultures.]

Although the relative rate of deposition of new H1 onto chromatin per mole of H1 is much less than that of H1° during G_1, a substantial number of H1 molecules are deposited onto

chromatin during that period. In G_1-arrested cells, the synthesis (this report; Tarnowka et al., 1978; Zlatanova, 1981) and the turnover of H1 (Gurley et al., 1972) are greater than those of the core histones, but we find that by 2 h after release from G_1 arrest, the rates of deposition of H1 and H4 per mole of each histone are the same. Even then, however, the actual ratio of H1:H1° molecules deposited onto chromatin during G_1 is greater than 1.8:1 in favor of H1. Therefore, it appears that a small portion of the total cellular content of H1 is deposited onto chromatin following release from G_1 block. Hence, as suggested by others (Tarnowka et al., 1978; Zlatanova, 1981), the synthesis and deposition of H1 onto chromatin following release from G_1 block may suggest some specific biological function for newly synthesized H1, as well as new H1°, during that period.

Does H1 Dissociate from Chromatin during the Early Stages of DNA Replication? One of our more surprising results was the large fluctuations in the H1°:H1 ratio observed when cells were released from hydroxyurea blockade (10 h). Subsequent experiments indicated that (1) the modulations in the H1°:H1 ratio arose primarily from a 30% reduction in the quantity of H1 (relative to core histones) that was acid soluble from the chromatin in the hydroxyurea-blocked cells (as cells progressed through S, H1 became replenished in the isolated chromatin) and (2) the acid-soluble H1 content could be reduced even further (a total of 70%) when cells were left in the presence of hydroxyurea for an additional 14 h. [Because of the nature of our extraction procedures (isolation of nuclei, blending, and transfers), the random error in the weight of total recoverable histones per unit of cellular DNA is ~20%; however, we definitely do not see a 300% increase in the weight of core histones (relative to DNA) from the hydroxyurea-blocked culture that would be required to give an apparent 70% loss of histone H1 relative to the core histones.] These results suggest that histone H1 is being lost from chromatin intracellularly or that histone H1 becomes particularly labile to dissociation during the isolation of nuclei and chromatin.

Previous studies also have suggested that histone H1 dissociates from DNA into a "chromatin pool" during the early stages of DNA replication (Jackson & Chalkley, 1981a,b) or during undetermined phases of the cell cycle (Gurley et al., 1973). Since 1 mM hydroxyurea blockade does not prevent the entrance of G_1 cells into the S phase [i.e., they initiate DNA replication as monitored by high levels of [^{3}H]thymidine autoradiography (Walters et al., 1976a)] but does appear to inhibit DNA elongation (Walters et al., 1976b), the sequential use of isoleucine deprivation G_1 block and hydroxyurea blockade may accentuate H1 dissociation or H1 lability (during the early stages of replication) which normally goes unnoticed.

In conclusion, our data suggest a dynamic picture of chromatin structure in which (1) newly synthesized histone H1° is deposited onto chromatin during much of G_1 and S (we do not yet know about G_2) and (2) histone H1 (and perhaps H1°) may be lost or becomes labile to dissociation from chromatin during the early stages of DNA replication. These processes, along with postsynthetic modifications of histones, provide specific molecular mechanisms to alter chromatin structure and function during the cell cycle.

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Variation in the Membrane-Insertion and "Stalk" Sequences in Eight Subtypes of Influenza Type A Virus Neuraminidase†

Janet Blok*,‡ and Gillian M. Air§

ABSTRACT: The two membrane-bound surface antigens of influenza virus, hemagglutinin (HA) and neuraminidase (NA), are known to vary considerably in amino acid sequence. From immunological studies nine serologically distinct subtypes of neuraminidase have been characterized, yet these all exhibit the same enzyme activity. Many studies have been designed to investigate variation in the antigenic properties of the hemagglutinin and/or the neuraminidase. Here we have investigated the sequence variation of regions of the influenza neuraminidase which are not involved in antigenicity. These regions are the hydrophobic transmembrane segment and the "stalk". The NA gene of at least one strain from each of eight of the nine NA subtypes was sequenced from the 3′ end by using the dideoxy method in order to examine these regions. The results reveal that the predicted protein sequence at the N terminus is identical for the first six amino acids in all subtypes while the next six are the same in most subtypes. Following the first 12 amino acids, there is virtually no conservation of particular amino acid side chains in the transmembrane sequence and in the stalk region of the neuraminidase, although the character of the polypeptide is maintained. The transmembrane segment contains a high proportion of hydrophobic amino acids while the stalk region is rich in potential glycosylation sites, and it also contains at least one Cys residue which may form intermolecular disulfide bonds in the neuraminidase tetramer (at variable positions in the different subtypes). The different predicted protein sequences of the eight NA subtypes can therefore be accommodated into four regions of the tetrameric structure of the neuraminidase, which is compatible with the electron micrographs: an N-terminal conserved hexapeptide, the hydrophobic transmembrane segment, a thin stalk which is stabilized by carbohydrate and intermolecular disulfide bonds, and the enzymatically and antigenically active "head". Genetic variation of the influenza neuraminidase is not confined to antigenic properties.

The enzyme neuraminidase (NA)[1] (EC 3.2.1.18) is one of the two projecting surface antigens on particles of influenza viruses, the other being the hemagglutinin. Neuraminidase hydrolyzes terminal *N*-acetylneuraminic acid (NANA) from receptors for the virus located on the surface of cells. Its function in the viral replicative cycle is still not fully understood, but it may be involved with the hemagglutinin in promoting fusion of the viral and host cell membranes in the early stages of the infectious cycle (Huang et al., 1980) or prevent aggregation of virus particles during their release from host cells (Palese et al., 1974).

Influenza neuraminidase is a tetramer of four identical glycosylated polypeptides which is coded by one of the eight negative-stranded RNA molecules of the virus. Electron micrographs show that the NA is composed of a square boxlike "head" containing four coplanar subunits and a long thin "stalk" with a small knob at the end (Laver & Valentine, 1969;

† From the Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2600 Australia. *Received January 18, 1982.* This work was supported in part by Grant AI 15343 from the National Institute of Allergy and Infectious Diseases.

‡ Present address: Virus Ecology Research Group, Research School of Biological Sciences, Australian National University, Canberra, A.C.T. 2600 Australia.

§ Present address: Department of Microbiology, University of Alabama in Birmingham, Birmingham, AL 35294.

[1] Abbreviations: NA, neuraminidase; NANA, *N*-acetylneuraminic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid.

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