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Interrelationships of Protein and DNA Syntheses During Replication of Mammalian Cells

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During the replication of chromatin, the syntheses of the histone protein and DNA components are closely coordinated but not totally linked. The interrelationships of total protein synthesis, histone protein synthesis, DNA synthesis, and mRNA levels have been investigated in Chinese hamster ovary cells subjected to several different types of inhibitors in several different temporal combinations. The results from these studies and results reported elsewhere can be brought together into a consistent framework which combines the idea of autoregulation of histone biosynthesis as originally proposed by W. B. Butler and G. C. Mueller (Biochim. Biophys. Acta 294:481–496, 1973] with the presence of basal histone synthesis and the effects of protein synthesis on DNA synthesis. The proposed framework obviates the difficulties of Butler and Mueller's model and may have wider application in understanding the control of cell growth.

Most histone synthesis in eucaryotic cells occurs coordinated with DNA synthesis during the S-phase period of the cell cycle and is inhibited when DNA synthesis is inhibited (27, 32); however, an appreciable amount of histone synthesis has been found to occur in the G_1 portion of the cell cycle and the G_0 state when there is no DNA replication (49, 51, 52). Butler and Mueller (8) showed that the coordination between histone and DNA synthesis resulted from alterations in the level of histone mRNA. The level of translatable histone mRNA in exponentially growing cells was greatly decreased when they were treated with inhibitors of DNA synthesis, and this in turn resulted in the inhibition of histone protein synthesis. However, when total protein synthesis was inhibited by cycloheximide or puromycin at the same time that DNA synthesis was inhibited with hydroxyurea, translatable histone mRNA levels did not decrease. Subsequently, investigators in several laboratories, measuring the levels of histone mRNAs by using cloned histone genes (5, 12, 17, 20, 21, 30, 38, 39, 42, 43), have substantiated and extended those results to show that the histone mRNA is rapidly degraded during the inhibition of DNA synthesis but protected from degradation when protein synthesis is also inhibited.

To explain their own results, Butler and Mueller (8) proposed a model in which the histone not bound to chromatin inhibited the translation of its own mRNAs, and presented evidence that the level of free histone in the cytoplasm did increase after treatment of cells with hydroxyurea. Others have also found evidence for a small but measurable pool (25).

The general idea of autoregulation of histone synthesis has persisted (17, 40, 43), but several researchers have explained their more recent data in other ways. For example, Stimac et al. (42, 43) have reported that histone mRNA levels rose when cells were treated with translation inhibitors alone and suggested that inhibition of protein synthesis may uncouple DNA synthesis from histone mRNA levels. They proposed that a protein with a short half-life coupled the two processes; thus, it rapidly disappeared when protein synthesis was inhibited. Alternatively, Graves and Marzluff (17) proposed that changes in deoxynucleotide metabolism may be involved in the regulation of histone mRNA levels.

All these reports have concentrated on studies of histone mRNA levels and metabolism. In attempting to gain further insight into the relationship between histone and DNA synthesis, we have used several different types of inhibitors in several different temporal combinations and have measured the rate of DNA synthesis, the rate of total protein synthesis, the rate of histone synthesis, and the pattern of histone variant synthesis as well as the level of histone mRNA. We found that the results of these experiments can be interrelated by a four-component model, which combines the idea of autoregulation of histone biosynthesis with basal histone synthesis.

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells were grown at 37°C in complete HAM F10 medium supplemented with 10% heat-inactivated fetal calf serum, streptomycin, and penicillin. Hydroxyurea, cytosine arabinoside, aphidicolin, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1,3-bis(cyclohexyl)-1-nitrosourea (BCyNU) were obtained from the Drug Development Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md. Novobiocin, cycloheximide, and puromycin were purchased from Sigma Chemical Co., St. Louis, Mo. Before use, hydroxyurea, cytosine arabinoside, novobiocin, cycloheximide, and puromycin were dissolved in sterile water, aphidicolin was dissolved in dimethyl sulfoxide, and CCNU and BCyNU were dissolved in 95% ethanol. For measurements of the inhibition of DNA and protein synthesis, cells were pretreated with the inhibitor for 30 min and then labeled in its continued presence for 120 min with [^{3}H]thymidine (specific activity, 84 Ci/mmol; final activity, 1 μCi/ml) or [^{14}C]lysine (specific activity, 275 mCi/mmol; final activity, 10 μCi/ml) in lysine-free medium containing 10% heat-inactivated fetal calf serum. The extent of inhibition of DNA synthesis was

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TABLE 1. Effect of various inhibitors on DNA, total protein, total histone, and histone variant synthesis

Compound	Concn	Relative synthesis			H3.1 + H3.2 / H3.3	H2A.1 + H2A.2 / H2A.X + H2A.Z	Synthesis pattern
		DNA	Protein	Histone			
Control		100	100	100	2.5	4.2	S
DNA synthesis inhibitors							
Hydroxyurea	1 mM	7	155	17	0.2	0.4	G_1
Cytosine arabinoside	10 μM	3	121	ND[a]	0.3	0.7	G_1
Hydroxyurea/cytosine arabinoside	1mM/10 μM	<1	116	16	0.2	0.5	G_1
Aphidicolin	50 μM	<1	108	17	0.4	0.8	G_1
Protein synthesis inhibitors							
Cycloheximide	50 μg/ml	10	2	3	6.0	7.5	S+
Puromycin	100 μg/ml	8	7	ND	1.9	3.7	S
Pleiotropic inhibitors							
Novobiocin	2 mM	4	25	ND	2.2	2.9	S
CCNU[b]	100 μM	<1	5	ND	2.9	2.3	S
BCyNU[c]	100 μM	<1	4	ND	1.6	3.1	S

[a] ND, Not determined.
[b] CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.
[c] BCyNU, 1,3-bis(cyclohexyl)-1-nitrosourea.

measured by the method of Schmidt and Thannhauser (34) as modified by Wu and Wilt (53). The extent of inhibition of protein synthesis was measured by determining the amount of [^{14}C]lysine incorporated into trichloroacetic acid-precipitable material. The ratios of histone variant synthesis were determined from two-dimensional polyacrylamide gels (7) of the 0.5 N HCl extractable material from whole cells as described by Wu and Bonner (49).

The relevant gel islands were excised from the dried and fluorographed (6, 23) gel, solubilized overnight at 37°C in H_2O_2–NH_4OH (95:5) in tightly capped scintillation vials for scintillation counting. Ratios of counts per minute for different histone variants were calculated as indicated in Table 1.

RESULTS

Inhibitors of DNA synthesis. For a number of different mammalian cell lines, it has been observed that when replication is inhibited by compounds which interfere directly with DNA synthesis, the level of histone mRNA rapidly decreases (5, 8, 12, 15, 17, 20, 21, 30, 38, 39, 42, 43). The synthesis of histone protein is also inhibited even though total protein synthesis is not (8). More recently, the inhibition of histone protein synthesis was found to be specific for particular variants (49, 51).

The inhibitors of DNA synthesis used in this study effectively depressed the rate of DNA synthesis and histone synthesis but did not inhibit the rate of total protein synthesis (Table 1); in fact, there were small but reproducible increases in the incorporation of labeled amino acid into total protein. A comparison of Fig. 1B and C demonstrates the effect of hydroxyurea on the pattern of histone variant synthesis. The synthesis of the S-phase H2A variants .1 and .2 and the H3 variants .1 and .2 was greatly inhibited relative to the synthesis of the basal H2A variants .X and .Z and H3 variant .3. Quantitation of the effect of several inhibitors of DNA synthesis on the pattern of H3 and H2A synthesis is shown in Table 1.

These several inhibitors exerted the same effect on histone synthesis even though their mechanisms of action on DNA synthesis are quite different. Hydroxyurea is an inhibitor of the ribonucleotide reductase, an enzyme necessary for the synthesis of TTP (41). Cytosine arabinoside is a competitive inhibitor of deoxycytidine and is a chain terminator when it is incorporated into DNA in place of deoxycytidine (14). Aphidicolin, on the other hand, inhibits DNA polymerase α, the polymerase involved in DNA replication (28). These inhibitors have all been found to depress the level of histone mRNA (5, 17, 29, 39). In addition, three other inhibitors of DNA synthesis—fluorodeoxyuridine, mycophenolic acid, and methotrexate—have also been found to depress histone mRNA levels (17). Figure 2 shows the mRNA levels of one histone, H4, after various treatments of CHO cells. A comparison of columns 1 and 2 shows the depression of the H4 mRNA level after treatment of cells with hydroxyurea, results which are in agreement with others in the literature (5, 17, 29).

Inhibitors of protein synthesis. It is notable that in concentrations of cycloheximide which inhibit total protein synthesis by 98%, the pattern of the residual protein synthesis is the same as that present in untreated cells (Fig. 1D). The labeled histones from inhibited and uninhibited cells show the same mobilities when analyzed by gel electrophoresis. Since acetic acid–urea–Triton X-100 first-dimension gels can be sensitive to a single amino acid substitution in a protein (47, 54), this indicates that the histones synthesized under these conditions have the correct protein sequence and should be able to function as histones.

In contrast to inhibitors of DNA synthesis, the inhibitors of protein synthesis, cycloheximide and puromycin, did not greatly decrease the variant synthesis ratios of either histone H3 or H2A, even though DNA synthesis was greatly inhibited (Fig. 1D and Table 1). With cycloheximide, the variant synthesis ratios were consistently higher than those of untreated cells (denoted as S+ in Table 1); this will be discussed below. Histone H4 mRNA levels were elevated in cells treated with cycloheximide (Fig. 2, column 1 versus 3), results which again are in agreement with others reported in the literature (5, 21, 39, 43). This increase in the histone mRNA level after cycloheximide treatment suggests that all of the cells are slowly synthesizing protein rather than just a few resistant cells rapidly synthesizing protein. For puromycin, the histone variant synthesis ratios were not significantly higher or lower than the controls. This may simply be due to the less complete inhibition of protein

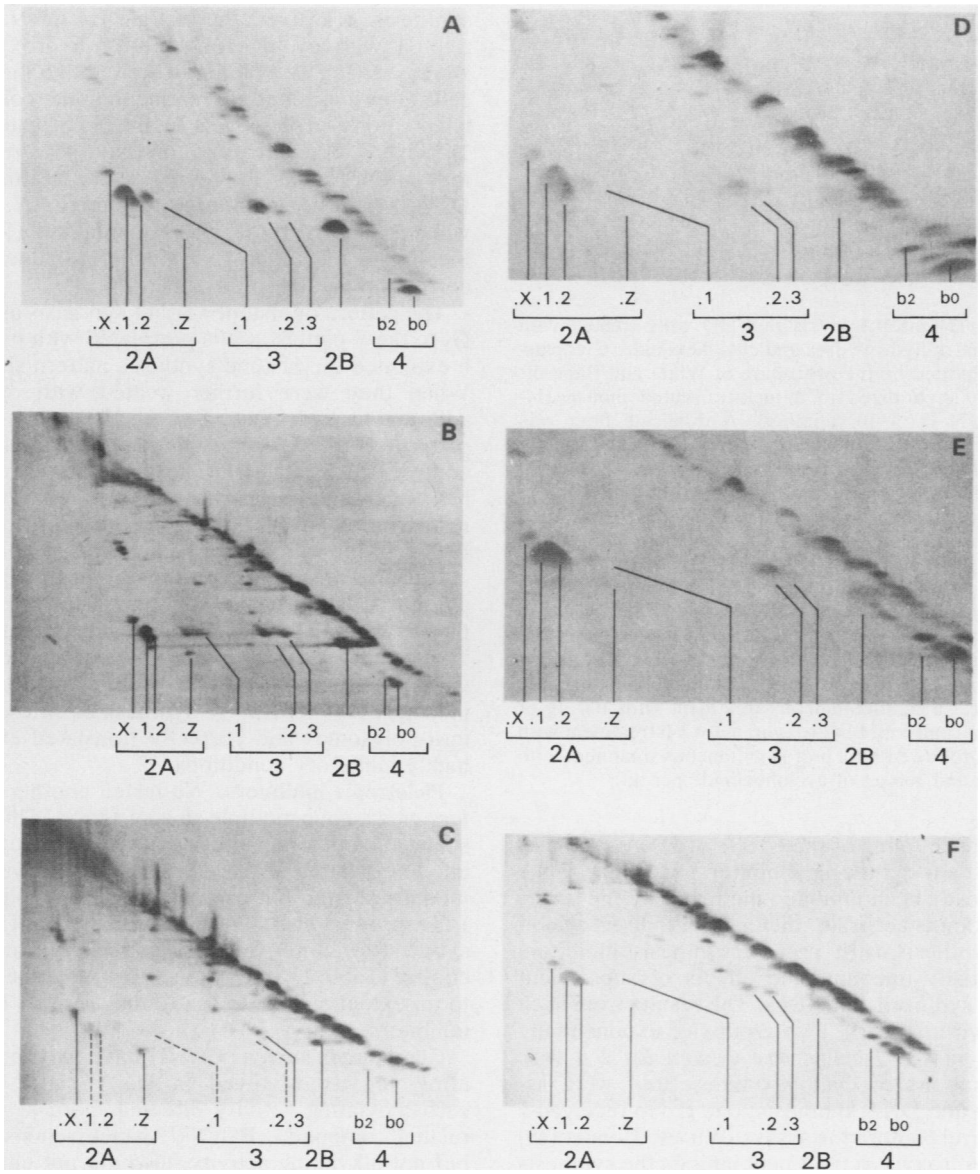

FIG. 1. Histone variant synthesis patterns. (A) Mass pattern (stained with Coomassie brilliant blue R) for CHO cells. Variants are noted, except for b_2 and b_0, which refer to modified forms of H4. Other panels are all synthesis patterns, cells labeled with [^{14}C]lysine for 2 h during the treatment period as described in the text. (B) Untreated control cells; (C) 2-h treatment with 1 mM hydroxyurea; (D) 2-h treatment with 50 μg of cycloheximide per ml; (E) simultaneous 2-h treatment with 1 mM hydroxyurea and 50 μg of cycloheximide per ml; (F) 2-h pretreatment with 1 mM hydroxyurea for 2 h followed by simultaneous 2-h treatment with 1 mM hydroxyurea and 50 μg of cycloheximide per ml.

synthesis that we were able to obtain with puromycin (Table 1). However, this difference may also be related to the very different mechanism of inhibition of these compounds, particularly with respect to the fate of the polysomal mRNAs. Cycloheximide merely slows the rate of translation, but puromycin alters the process, causing premature peptide termination, the release of incomplete proteins, and polysome disaggregation. Puromycin is also not as efficient as cycloheximide when combined with hydroxyurea (Table 2).

The increase in the H2A variant synthesis ratio indicates that there is a selective elevation in the level of the S-phase histone mRNAs. In an experiment performed with various concentrations of cycloheximide, we found that the histone mRNA level (from quantitating the ^{32}P-labeled H4-pBR322 bound to the dot blots) and the H2A variant synthesis ratio increased together (Fig. 3). The close correspondence of these two curves suggests that the level of the basal variant mRNAs may not have changed at all in the presence of cycloheximide, and that the increase was specific for the S-phase variant mRNAs.

Inhibitors of protein synthesis added before inhibitors of DNA synthesis. When cells were pretreated with cycloheximide before the addition of hydroxyurea (Fig. 2, column 5), histone mRNA levels were elevated to an extent similar to that when cycloheximide was present alone (column 3). The pattern of histone variant synthesis was also indistinguishable under these two conditions (data not shown). For cells in culture, it is known that inhibition of protein synthesis very quickly leads to the inhibition of DNA synthesis (16, 44), and this result suggested that perhaps the explanation

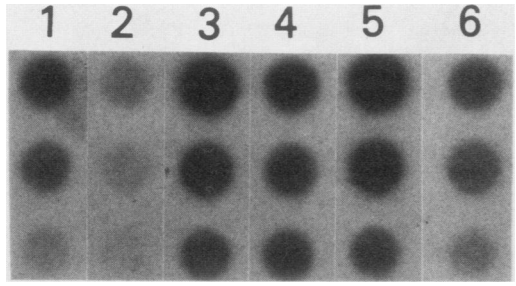

FIG. 2. Histone H4 mRNA levels in CHO cells treated with various combinations of hydroxyurea and cycloheximide. Cytoplasmic RNA was dot blotted by the procedure of White and Bancroft (48). The filter was hybridized to a nick-translated mouse H4-PBR322 plasmid (37) (5×10^8 dpm/μg). A duplicate filter was hybridized to a nick-translated plasmid containing chicken β-actin (9; a gift from B. Paterson) as a control. The control showed no significant differences among samples. The concentrations of inhibitors used in this experiment are somewhat different from those used in the other experiments, but this does not alter the results. From top to bottom there are 4×10^5, 2×10^5, and 1×10^5 cell equivalents per dot. Lanes: 1, untreated control cells; 2, 2-h treatment with 5 mM hydroxyurea; (3) 2-h treatment with 100 μg of cycloheximide per ml; (4) 2-h simultaneous treatment with 5 mM hydroxyurea and 100 μg of cycloheximide per ml (the lowest dot in this case has 2×10^5 cell equivalents); (5) 2-h treatment with 100 μg of cycloheximide per ml followed by 2 h of simultaneous treatment with 100 μg of cycloheximide per ml and 5 mM hydroxyurea; (6) 2-h treatment with 5 mM hydroxyurea followed by 2 h of simultaneous treatment with 5 mM hydroxyurea and 100 μg of cycloheximide per ml.

for the apparent uncoupling of the effects of DNA synthesis inhibitors by protein synthesis inhibitors was that DNA synthesis had already been inhibited indirectly by the latter.

If this explanation is accurate, then partial indirect inhibition of DNA synthesis with protein synthesis inhibitors should only partially uncouple the effects of subsequent addition of DNA synthesis inhibitors. The results from such a study are shown in Fig. 3. Two groups of exponentially growing cultures of CHO cells were treated for 2 h with various concentrations of cycloheximide. Both were assayed for histone and DNA synthesis; one group received 1 mM hydroxyurea just before the assays. Wu and Bonner (49) had shown that hydroxyurea treatment inhibits the synthesis of H2A variants .1 and .2 with very little effect on the synthesis of H2A.Z and H2A.X; thus, the differences in the H2A synthesis ratios at any particular cycloheximide concentration primarily reflect differences in the rate of H2A.1 +.2 synthesis. Hydroxyurea treatment inhibited the synthesis of the S-phase H2A variants to the same extent as in the untreated control up to a cycloheximide concentration of 0.1 μg/ml (protein and DNA synthesis inhibited to 40% of the control) (Fig. 3). Upon more extensive inhibition, the ability of hydroxyurea to inhibit the synthesis of H2A.1/2 decreased, disappearing at 3.0 μg/ml. It is obvious from Fig. 3 that this apparent uncoupling could be attributed to the prior inhibition of DNA synthesis rather than to the inhibition of protein synthesis per se.

Inhibitors of DNA synthesis added before inhibitors of protein synthesis. In their model, Butler and Mueller (8) predicted that once histone mRNA levels were depressed due to the inhibition of DNA synthesis, histone mRNA synthesis could resume only after resumption of DNA synthesis, because without DNA synthesis, free histone concentration could not decrease. Thus, histone mRNA levels could be increased only after resumption of DNA synthesis. However, the histone H4 mRNA levels of cells treated with cycloheximide after hydroxyurea treatment increased (Fig. 2, column 6) well above the levels found in cells after hydroxyurea treatment alone (column 2) and may have surpassed the levels found in untreated cells (column 1). Several other investigators have also reported that histone mRNA levels, depressed due to treatment of cells with DNA synthesis inhibitors, recovered to various extents when protein synthesis was also inhibited (17, 39, 43). Thus, the model of Butler and Mueller (8) does not make the correct prediction in this case.

The pattern of histone variant synthesis also changes from G_1 to the S pattern. Cells pretreated with hydroxyurea for 2 h exhibited the histone synthesis pattern shown in Fig. 1C. When they were further treated with cycloheximide in addition to hydroxyurea (Fig. 1F), the histone synthesis pattern returned to one qualitatively similar to that of the control cells (Fig. 1B). The quantitative results for cycloheximide and for puromycin (Table 2) show that both restored the histone synthesis pattern to the S-phase one, but again the ratios were higher with cycloheximide.

It is also notable that in these cells in which both protein and DNA synthesis are suppressed to a small pecentage of their uninhibited rates, transcription continues, leading to an increase in the histone mRNA level and to changes in the variant pattern of histone protein synthesis. This result indicates that the newly synthesized mRNA is complexed into polysomes and correctly translated even under these highly inhibitory conditions.

Pleiotropic inhibitors. We tested another group of inhibitors which have multiple sites of action. This group included some chemotherapeutic agents such as cyclohexylchloroethylnitrosourea (Table 1), which reacts with DNA to form interstrand and intrastrand DNA cross-links as well as DNA-protein cross-links. The pattern of histone variant synthesis was not altered significantly compared with the control (Table 1), even though DNA synthesis was inhibited to an extent similar to that obtained with DNA replication inhibitors.

Compounds such as CCNU have two activities, an alkylating activity involved in DNA-related lesions, and a carbamoylating activity directed more at proteins (22). A related compound, BCyNU, which retains carbamoylating but not alkylating activity, also did not change the histone variant synthesis pattern, suggesting that the DNA-related lesions are not so important in this case. Novobiocin, acompound that competes with ATP on multiple enzymes (24), interfering with deoxynucleotide metabolism but also inhibiting protein synthesis, did not significantly alter the

TABLE 2. Recovery of histone variant synthesis ratios in log-phase CHO cells after pretreatment with an inhibitor of DNA synthesis[a]

Pre-treatment (0–2 h)	Treatment (2–4 h)	H3.1 + H3.2 / H3.3	H2A.1 + H2A.2 / H2A.X + H2A.Z	Synthesis pattern
None	None	2.5	4.2	S
None	HU	0.6	1.0	G_1
HU	HU	0.5	0.7	G_1
HU	HU + CH	4.5	4.9	S
HU	HU + PU	2.0	2.6	S

[a] CHO cells in exponential growth were treated as indicated with 1 mM hydroxyurea (HU) and 50 μg of cycloheximide (CH) per ml or 100 μg of puromycin (PU) per ml. All samples were labeled with [^{14}C]lysine during the treatment period. Patterns of histone variant synthesis were determined as described in the text.

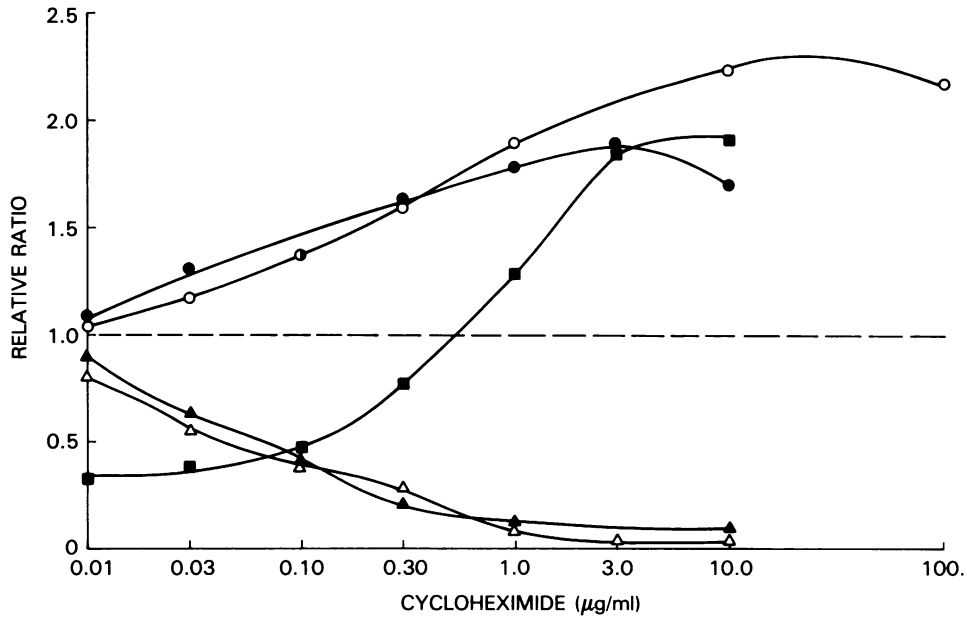

FIG. 3. Uncoupling of the effects of hydroxyurea by prior treatment with cycloheximide. Two groups of exponentially growing CHO cell cultures were treated with the indicated concentration of cycloheximide for 2 h; then 1 mM hydroxyurea was added to one group for 5 min. Media on all cultures were replaced with fresh lysine-free Ham F10 with 10% fetal calf serum, containing the appropriate concentrations of cycloheximide, hydroxyurea, and [^{14}C]lysine, and the cultures were labeled for 2 h. Histones were analyzed as described in the text. The inhibition of total protein synthesis was determined by measuring the incorporation of [^{14}C]lysine into certain nonhistone proteins on the two-dimensional gels. DNA synthesis was measured in parallel cultures as described in the text. Cytoplasmic histone mRNA levels were determined as described in the legend to Fig. 2. All values are expressed relative to the value in the controls without cycloheximide or hydroxyurea. Symbols: ●,■, H2A variant synthesis ratios, relative to a control value of 4.2; ●, no hydroxyurea; ■, 1 mM hydroxyurea; △, rate of protein synthesis (no hydroxyurea); ▲, rate of DNA synthesis (no hydroxyurea; all hydroxyurea-treated samples had rates less than 2% of the control); ○, H4 mRNA level (no hydroxyurea).

histone variant synthesis pattern (Table 1). Treatment of cells with novobiocin does not lead to decreases in histone mRNA levels (17). Because pleiotropic inhibitors act on both protein and DNA synthesis, the effect of combinations of cycloheximide and hydroxyurea added to cells at the same time was studied. The pattern of histone variant synthesis was the same in cells treated with the mixtures of inhibitors (Fig. 1E) as with the pleiotropic inhibitors (data not shown). The same result was obtained when puromycin was substituted for cycloheximide in the mixture (data not shown). The H4 mRNA level in cells treated with mixtures of inhibitors was somewhat elevated above that of untreated cells but below that of cells treated with cycloheximide alone (Fig. 2, column 4), results which agree with those reported for novobiocin (17). Thus, the effects of pleiotropic inhibitors were similar to those of protein and DNA synthesis inhibitors added together. The direct effects of pleiotropic inhibitors on DNA synthesis seem to be partially to completely uncoupled by their concomitant inhibition of protein synthesis. This provides an alternative explanation for the finding reported by Graves and Marzluff (17) that treatment of cells with novobiocin did not destabilize histone mRNA but altered nucleotide pools. Based on that finding, the authors suggested that nucleotide pools were involved in histone mRNA stability. Our results show that treatment of cells with novobiocin also inhibits protein synthesis and thus would not be expected to destabilize histone mRNA.

DISCUSSION

During the course of this work, we found that the idea of autoregulation of histone synthesis as originally proposed by Butler and Mueller (8), combined with two other sets of results, provided a plausible framework for the reciprocal and complex relationships between protein and DNA syntheses in replicating cells. The two other sets of results were the immediate effect of inhibitors of protein synthesis on the rate of DNA synthesis, and the synthesis and incorporation of histone into chromatin in the absence of replication.

Chromatin as a structural entity can be considered to be composed of two major macromolecules, DNA and histone. DNA is synthesized at the site of chromatin replication, but histone is synthesized on cytoplasmic polyribosomes and must migrate through the cytoplasm to the site of chromatin replication. We include four elements in this model. (i) The rate of chromatin replication can be limited by either the rate of DNA synthesis or the rate of protein synthesis as mediated by the concentration of free cytoplasmic histone. (ii) The concentration of free cytoplasmic histone also regulates the level of S-phase histone mRNA through uncharacterized mechanisms. (iii) Histones are synthesized and incorporated into chromatin at all times, very rapidly during replication, more slowly at other times. Thus, the level of free histone is always in a dynamic steady state even when DNA synthesis is inhibited or absent. (iv) The set point of the regulatory system in uninhibited cells is with DNA synthesis limiting, so that only a fraction of the potential maximum histone mRNA level is needed.

The effects of DNA synthesis inhibitors alone can be explained as originally described by Butler and Mueller (8). Limiting DNA synthesis limits histone utilization, but continued protein synthesis causes the concentration of free histone to rise. This increased concentration of free histone leads to a decrease in the level of S-phase histone mRNA,

resulting in a decrease in the rate of S-phase histone synthesis.

The effects of protein synthesis inhibitors alone can be explained as being mediated by the concentration of free histone. Limiting protein synthesis limits histone synthesis. The decreased production of histone leads to decreased concentration of free histone, which limits chromatin production and leads to elevated S-phase histone mRNA levels. The limitation on chromatin production inhibits DNA synthesis. Because the histone mRNA level is maintained at a fraction of its potential maximum in uninhibited cells (Fig. 2), it could also be adjusted up as well as down in inverse proportion to the concentration of free histone.

Two findings favor the idea that the concentration of free histone mediates the effects of protein synthesis on DNA synthesis. First, in early sea urchin embryos, in which there is a known pool of excess histone (33), DNA synthesis is totally unaffected by the complete inhibition of translation (45). Second, in mammalian cells, a small amount of DNA synthesis does continue for a while after translation is completely inhibited (2–4, 31, 35, 36, 46). This nascent chromatin is partially deficient in histones (35), suggesting that the cell attempts to continue DNA synthesis even when too little histone is available to make mature chromatin. These findings together argue against a purely regulatory linkage between protein and DNA synthesis, and suggest simply that the rate of DNA synthesis may be limited by the availability of free histone in the pool.

Inhibition of protein synthesis immediately leads to inhibition of DNA synthesis; thus, the subsequent addition of an inhibitor of DNA synthesis might not be expected to exert any effect on the pattern of histone synthesis or the level of histone mRNA. Therefore, the histone mRNA level was similar whether or not cultures were treated with inhibitors of DNA synthesis after protein synthesis (and thus DNA synthesis) had been inhibited (Fig. 2).

When inhibitors of DNA and protein synthesis are added at the same time, or when a pleitropic inhibitor with both of these inhibitory effects is added, both the production and the utilization of free histone are inhibited. Thus, the concentration of free histone may not be immediately limiting DNA synthesis, but its level would still decrease as histone is incorporated into existing chromatin by replacement and turnover (50). However, the level of histone mRNA might be expected to rise more slowly when cells are treated with pleiotropic inhibitors or inhibitors of protein and DNA synthesis together than when treated with inhibitors of protein synthesis alone.

A crucial prediction of the model is that inhibitors of protein synthesis, when added after inhibitors of DNA synthesis have exerted their effects, should still be able to reverse those effects on the histone mRNA levels and on the pattern of histone variant synthesis, even in the continued absence of DNA synthesis. When the inhibitor of DNA synthesis is added first, the concentration of free histone would be elevated, causing the level of S-phase mRNA and the rate of S-phase histone synthesis to be depressed. When the inhibitor of protein synthesis is added later, histone protein synthesis would be further depressed due to the greatly lowered efficiency of translation, and the concentration of free histone would decrease as the histones still become incorporated into chromatin by replacement or turnover (50). The lowered free histone concentration soon becomes limiting for replication, effectively "uncoupling" the effects of the inhibitor of DNA synthesis. The level of S-phase histone mRNA would increase, and as it was translated the pattern of histone variant synthesis would return to the S-phase pattern, even though DNA synthesis would have been almost totally inhibited throughout the whole process (Table 2). Thus, the model suggests that the apparent uncoupling of histone mRNA levels from the rate of DNA synthesis by inhibitors of protein synthesis may be primarily due not to any direct effect on protein synthesis, but to the secondary effect of such inhibitors on DNA synthesis, as mediated by the concentration of free histone.

Although most histone synthesis in proliferating cells is coordinate with DNA synthesis, Wu and Bonner (49) showed that some histone species were synthesized constitutively; thus, in cells treated with inhibitors of DNA synthesis, the pattern of synthesis of histone variants changed (Fig. 1B). These results are not contradictory to the results of studies of mRNA levels mentioned previously, because the different variant mRNAs hydridize with the same histone gene probe and therefore are not distinguishable. When S1 nuclease was used to digest mismatched hybrids, the level of mRNA coding for H3.3, a basal variant, was also found to be independent of the rate of DNA synthesis (38).

Wu et al. (51) further showed that in G_1 and G_0 cells in which DNA replication is absent, a significant amount of histone synthesis with distinguishable variant patterns was present; furthermore, these histones became incorporated into nucleosomes in the absence of replication (50). Thus, the free histone pool would be in a dynamic steady state, its level depending on the rate of histone synthesis on the one hand and the rate of utilization into chromatin in the other, whether or not DNA is being replicated. Even in the absence of DNA synthesis, the level of free histone in the pool could still fall in response to inhibitors of protein synthesis in much the same way that the level of a short-lived protein might be expected to (42, 43). Thus, this model suggests an alternative explanation for the effects of protein synthesis inhibitors.

Histone incorporation into chromatin in the absence of DNA replication implies histone turnover and degradation to provide a long-term balance. It has been shown that in quiescent cells of several species, H3.3 is the only H3 variant synthesized (26, 51, 52) and non-proliferating tissues do gradually change their relative contents of H3 variants (18, 55). All histones, not just the distinguishable protein variants, are gradually replaced, and several investigators have measured half-lives for the turnover of various histone species (11, 13, 19).

This framework does not incorporate an exact mechanism for modulating histone mRNA levels, but evidence from several laboratories indicates that both mRNA transcription and stability can be altered (1, 15, 17, 20, 29, 38) and that these alterations happen in unison for the various major histone mRNAs (9, 12, 14). It is not necessary for the functioning of the proposed mechanism that free histones themselves bind to the mRNA or to the genes, even though they may, but only that those processes which regulate mRNA transcription and stability are controlled by the level of free histone through as yet undiscovered mechanisms. Our results do show, however, that those mechanisms altering S-phase histone mRNA synthesis and stability function even when DNA and protein synthesis are both almost totally inhibited. There is almost a complete lack of knowledge concerning the nature and kinetics of the free histone pool, what sort of carriers might be involved, and what sort of other pathways into (i.e., dissociation from chromatin) or out of (i.e., degradation) the pool may exist. We are currently developing techniques to analyze the histone pool to test some of these ideas. The regulation of chromatin syn-

thesis may be an example of a more general phenomenon in which structural components of cellular complexes or organelles regulate their own synthesis, as analogous situations have been demonstrated in other cases (for example, see reference 10).

In concentrations of cycloheximide which slow but do not stop the growth of tissue culture cells, the immediate inhibition of replication is moderated as histone mRNA levels rise. Histone protein synthesis then substantially increases, as does DNA synthesis (Wu and Bonner, manuscript in preparation), under some conditions recovering to the rates seen in uninhibited cells. Thus, in addition to the short-term balancing of histone synthesis and utilization, the interrelationships between protein and DNA synthesis discussed here may provide for a longer-term adaptive response of cells in S-phase to varying nutritional or environmental conditions.

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