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Regulation of Human Histone Gene Expression During the HeLa Cell Cycle Requires Protein Synthesis

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We have examined the effects of protein synthesis inhibition on histone gene expression during the HeLa cell cycle. Histone mRNAs, which normally are rapidly degraded in the absence of DNA synthesis, persist and increase in concentration when translation is inhibited before DNA replication is halted. This is not a function of polysomal shielding of these mRNAs from active degradation mechanisms since inhibitors of translation initiation alone effect stabilization and induction. The superinduction of histone mRNAs by protein synthesis inhibition is effective at the G1/S border, and in the S-phase and non-S-phase periods of the cell cycle. However, the relative increase in histone mRNA is greater when cells not synthesizing DNA are treated with a protein synthesis inhibitor than when S-phase cells are so treated. Non-histone mRNAs examined are not superinduced by translation inhibition. Transcription rates from both histone and non-histone genes increase after protein synthesis inhibition. Although the decrease in histone gene transcription associated with DNA synthesis inhibition is prevented and reversed by protein synthesis inhibition, we have no evidence that histone gene-specific transcriptional regulation is dependent on protein synthesis. Transcriptional increases may contribute to the superinduction effect but cannot explain its differential extent during the cell cycle, since these increases are similar when replicating or nonreplicating cells are treated with a protein synthesis inhibitor. We believe that changes in histone mRNA stability can account for much of the differential superinduction effect. Our results indicate a requirement for continuing protein synthesis in the cell cycle regulation of histone mRNAs.

The concentrations of most histone proteins and their mRNAs change with the progression of the cell cycle (11, 13, 17, 20). During the S phase of HeLa cells, for example, core histone mRNAs accumulate to a level ca. 15-fold above that of their non-S-phase levels (11). This is mediated both by an approximate threefold increase in transcription rate and by a fivefold increase in histone mRNA stability (11).

Upon treatment of S-phase cells with inhibitors of DNA synthesis, histone mRNAs rapidly disappear from the cytoplasm and nucleus (9, 11, 20). Non-histone RNA species (11), and probably histone mRNAs from genes not subject to S-phase regulation (22), are not so affected. The decline in histone RNA levels is accompanied by a decrease in transcription rate from these genes (11), but the extent and rate of the decline cannot be accounted for by a change in the rate of histone RNA synthesis alone, and must involve a specific destabilization of these RNAs.

Evidence has accrued that translatable histone RNAs can persist in the absence of DNA replication, if cells are treated with protein synthesis inhibitors before DNA synthesis is stopped (3, 22, 23). Early studies used cycloheximide, an inhibitor of polypeptide chain initiation and elongation that immobilizes polysomes on mRNAs (3, 23). It was not clear therefore whether polysomal persistence shielded histone mRNAs from active degradation mechanisms or whether replication and histone RNA stabilization could really be uncoupled by some other means. Subsequently, Stahl and Gallwitz (23) blocked the initiation of protein synthesis by hypertonic shock during the HeLa cell S phase. In vitro translation assays led these authors to conclude that clearance of these RNAs requires continued protein synthesis. Very recent studies in which a variety of protein synthesis inhibitors and cloned histone gene probes were used (10, 12) support early reports.

We decided to reinvestigate, by direct nucleic acid analysis, the coupling between histone gene expression and DNA synthesis, and the role of protein synthesis in this process. We therefore have used various protein synthesis inhibition regimes to examine in synchronized HeLa cells the following. (i) The requirement for protein synthesis in regulating histone RNA concentration at several points during the cell cycle. These include the S phase, the G1/S-phase boundary, and the non-S-phase period (comprising G2, M, and a very short G1) between two sequential S phases. (ii) The dependence of histone gene transcription on continued protein synthesis. We hoped to dissect the protein synthesis-dependent mechanisms normally controlling histone gene expression and to understand whether synthesis of these mRNAs, as well as their persistence, is obligatorily linked to the DNA replicative process.

We believe that a more detailed understanding of those mechanisms responsible for regulating S-phase-associated histone gene expression will facilitate and compliment their reproduction in vitro.

MATERIALS AND METHODS

Suppliers. Aphidicolin was supplied by the Natural Products Branch Div. of Cancer Treatment, National Cancer Institute. Cycloheximide was obtained from Sigma Chemical Co., and pactamycin was kindly given to us by C. Baglioni, State University of New York, Albany. Radioactive precursors were supplied by New England Nuclear Corp. CsCl was obtained from Varlakoid Chemical Co. Nitrocellulose (BA 85) was purchased from Schleicher & Shuell, Inc.

Plasmids. Genomic clones used for histone H4 and H3 mRNA and transcription quantitation were pHu4A and pHh5B, respectively, and are characterized in references 12

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and 23. Isolation and characterization of pHe27, a cDNA corresponding to an abundant HeLa cell polyadenylated RNA is described in reference 1. phsG is a cDNA clone corresponding to the 70-kilodalton heat-shock protein mRNA (14), and pA1 is a chicken β-actin cDNA clone (5).

Cell culture and synchronization. HeLa cell culture, synchronization, and DNA synthesis assays were exactly as described previously (11).

Polysome analysis. A 50-ml volume of cells (at 6×10^5/ml) were concentrated and resuspended on ice in 0.75 ml of reticulocyte standard buffer (8) (10 mm KCl, 10 mm Tris [pH 7.4], 1.5 mm $MgCl_2$). After the cells swelled for 10 min, they were homogenized by 15 to 20 strokes in a hand-held Dounce homogenizer. The homogenate was spun at 12,000 × *g* for 10 min at 4°C and subsequently loaded onto a 13-ml 15 to 40% (wt/vol) sucrose gradient (made in reticulocyte standard buffer). This gradient was centrifuged for 9 or 18 h at 42,000 × *g* at 4°C in a Beckman SW40 rotor. Gradients were poured and fractionated with a Buchler AutoDensiflow IIC pump. Absorbance of gradient fractions was analyzed in an LKB Bromma 2238 Uvicord SII continuously recording spectrophotometer.

RNA isolation and analysis. RNA was isolated from nuclei or cytoplasmic supernatants as described previously (11). Polysome fractions to be analyzed for RNA content were made 0.2% sodium dodecyl sulfate, phenol and chloroform extracted, and ethanol precipitated. Formaldehyde-agarose gel electrophoresis and Northern blot hybridization were as described previously (11). Densitometric quantitation was obtained with a Beckman Du-8 recording spectrophotometer.

Nuclei preparation. Nuclei were prepared by methods described previously (4). Briefly, cells were washed in cold phosphate-buffered saline and then lysed by incubation in 0.5% Nonidet P-40–160 mM NaCl–10 mM Tris-hydrochloride (pH 8.4)–1.5 mM $MgCl_2$ on ice for 5 min. Nuclei were separated from cytoplasmic debris by centrifugation at 1,000 *g* for 5 min and then washed twice in 1× cold wash buffer (20 mM Tris-hydrochloride [pH 8.0], 20% glycerol, 140 mM KCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 14.3 mM 2-mercaptoethanol) at 4°C. Samples of ca. 5×10^7 nuclei were either labeled immediately (see below) or frozen in lipid N_2 in 1.5× cold wash buffer, and then stored at −80°C.

Transcription reactions. Transcription reactions were done essentially as described previously (4). Reaction volumes were 300 μl (consisting of 200 μl of nuclei [ca. 5×10^7] and 100 μl of other components). Final concentrations in the reaction were 1× cold wash buffer (see above), 0.67 mM each ATP, CTP, and GTP, 6.7 mM phosphocreatine (Sigma Chemical Co.), 100 μg of creatine phosphokinase (Sigma Chemical Co.) per ml, and 700 μCi of high-specific-activity [α-^{32}P]UTP (New England Nuclear Corp.) per ml. Where appropriate, α-amanitin was added to 2 or 200 μl/ml. Reactions were for 15 min at 30°C and were terminated by pelleting the nuclei at 4°C.

RNA isolation and hybridization. RNA isolation and hybridization were conducted essentially as described previously (4). Briefly, nuclei were incubated with 50 μg of DNase I (RNase free; Boehringer Mannheim Biochemicals) per ml in 500 mM NaCl–10 mM Tris (pH 7.4)–50 mM $MgCl_2$–2 mM $CaCl_2$ for 5 min at 37°C. This was followed by digestion with proteinase K (25 μg/ml) in 10 mM Tris (pH 8.0)–10 mM EDTA–10 mM NaCl–0.5% sodium dodecyl sulfate for 60 min at 37°C. After phenol-chloroform extraction and ethanol-ammonium acetate precipitation, a second round of DNase I (in 10 mM Tris [pH 7.4], 10 mM $MgCl_2$) and proteinase K digestions (60 min each) was carried out. RNA was ethanol precipitated twice before hybridization.

Hybridizations to excess plasmid DNAs immobilized on nitrocellulose filters were done exactly as previously described (4), except that 8 μg of DNA was bound to each filter.

RESULTS

Protein synthesis inhibition stabilizes and induces histone mRNAs. In initial experiments, we examined the effects of protein synthesis inhibitors alone and in conjunction with a DNA synthesis inhibitor on S-phase histone RNA concentrations. Since the predominant changes in histone protein and RNA concentrations occur during the S phase, we carried out our study on highly synchronous HeLa cell populations. Cells were synchronized at the G1/S border by sequential thymidine and aphidicolin treatments (see above). Aphidicolin is a fully reversible inhibitor of DNA polymerase-α (18). After release from the aphidicolin block, cells progress in close coordination through the S phase, which in these HeLa cells is ca. 9 h long (11).

The inhibitor protocols in the initial and subsequent experiments are displayed in Fig. 1. Briefly, they entailed a 10-min treatment of cells at 3 h into the S phase with a protein synthesis inhibitor (cycloheximide or pactamycin), before interrupting replication with aphidicolin. Histone RNA levels were examined 30 min after aphidicolin application (40 min after translation inhibition) by filter hybridization analysis. The clone used in these studies for histone H4 mRNA

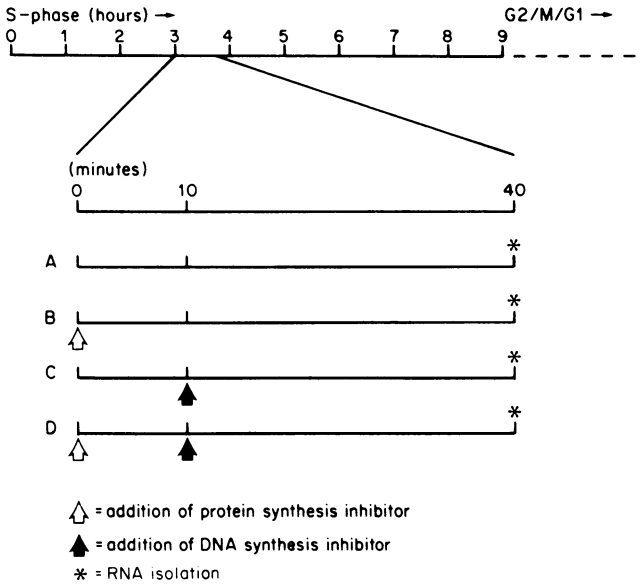

FIG. 1. Inhibitor treatment protocols. The various phases of the cell cycle, including the approximate duration of the S phase (through which HeLa cells progress after synchronization by sequential thymidine and aphidicolin blocks [12]), are represented. The 40-min period beginning 3 h into the S phase, during which these experiments were conducted, is expanded. In treatment A (the control), no inhibitors were applied. In treatment B, either cycloheximide or pactamycin was added to a concentration of 20 μg/ml or 10^{-7} M, respectively, at the start of the 40-min period. In treatment C, aphidicolin was added to a final concentration of 5 μg/ml at 10 min after the start of the 40-min period. In treatment D, either cycloheximide or pactamycin was added at the start of the period (as in B), followed 10 min later by aphidicolin application (as in C).

quantitation has been shown to encode a cell cycle-regulated histone mRNA (11, 25). Results essentially identical to those presented here also have been obtained with an H3 gene probe (data not shown).

Independent 40-min treatments of S-phase cells with cycloheximide and pactamycin resulted, respectively, in ca. 1.4- and 1.2-fold increases in cytoplasmic H4 mRNA concentrations (compared with untreated cells) (Fig. 2A, lanes 1 to 3). In contrast, a 30-min aphidicolin treatment of S-phase cells reduced the H4 mRNA level to 14% of that observed in untreated cells (Fig. 2A, lane 4). This dramatic loss of histone mRNA, however, was totally prevented by arresting translation with either pactamycin (lane 5) or cycloheximide (lane 6) before DNA replication is blocked (Fig. 2A). In fact, the levels of histone mRNAs present after such dual treatments approximated the slightly elevated levels observed after treatment with pactamycin or cycloheximide alone.

When nuclear RNA concentrations are examined, the histone H4 mRNA level in aphidicolin-treated S-phase cells again decreased to ca. 14% of its untreated level (Fig. 2B, lanes 1 and 2). However, after administration of pactamycin (lane 3) or pactamycin plus aphidicolin (lane 4), nuclear RNA concentrations were maintained at ca. 90% of their corresponding untreated values (Fig. 2B).

Quantitation of histone mRNA levels and DNA synthesis rates after various inhibition procedures is presented in Table 1. Aphidicolin treatment alone decreased the DNA synthesis rate to 5% of its peak S-phase value when administered alone or in conjunction with pactamycin or cycloheximide. However, the protein synthesis inhibitors, when administered alone, also decreased DNA synthesis rates to ca. 40% of their uninhibited value. Under conditions used here pactamycin and cycloheximide reduced protein synthesis by at least 95% (data not shown).

It is apparent, therefore, that translation inhibitors maintain and increase histone mRNA concentrations during periods of decreased DNA synthesis. Furthermore, both nuclear and cytoplasmic histone mRNA populations are resistant to degradation after appropriate inhibitor treatments.

We pursued three aspects of these results. First, we wished to confirm rigorously that protein synthesis initiation only is interrupted by pactamycin, and is sufficient to prevent histone mRNA degradation. Second, we examined

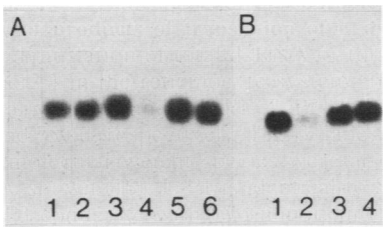

FIG. 2. Histone RNA concentrations after inhibitor treatments. Cytoplasmic or nuclear RNA was extracted upon completion of the inhibitor treatments detailed in Fig. 1 and separated on a 1.5% formaldehyde–agarose gel. H4 sequences were assayed by Northern blot hybridization as described previously (1). (A) Cytoplasmic RNA (20 μg) from cells treated with the various inhibitors was loaded in each lane as follows: 1, no inhibitor; 2, pactamycin; 3, cycloheximide; 4, aphidicolin; 5, pactamycin plus aphidicolin; 6, cycloheximide plus aphidicolin. (B) Nuclear RNA (20 μg) from cells treated with the various inhibitors was loaded in each lane as follows: 1, no inhibitor; 2, aphidicolin; 3, pactamycin; 4, pactamycin plus aphidicolin.

TABLE 1. Relative rates of DNA synthesis and levels of histone mRNA

Inhibitor treatment[a]	Relative amount of:		
	DNA synthesis	Histone mRNA	
		Cytoplasmic	Nuclear[b]
None	1	1	1
Aphidicolin	0.06	0.14	0.14
Pactamycin	0.38	1.24	0.85
Cycloheximide	0.38	1.40	ND
Pactamycin plus aphidicolin	0.07	1.47	0.93
Cycloheximide plus aphidicolin	0.05	1.37	ND

[a] Inhibitor treatment protocols are displayed in Fig. 1.
[b] ND, Experiment was not done.

the extent of histone mRNA concentration increase after more prolonged protein synthesis inhibition. Third, we measured transcription rate changes upon translation interruption to determine whether these could account for the induction and stabilization phenomena.

Histone mRNA stabilization does not result from polysomal shielding of these mRNAs. In the first case, we examined whether histone mRNAs remained associated with immobilized polysomes in the absence of translation, such that they might be protected from degradation when DNA synthesis was subsequently halted. To this end, we used an inhibitor of both translation initiation and elongation (cycloheximide), as well as an inhibitor of initiation alone (pactamycin). Pactamycin, at appropriately low concentrations, allows polysome runoff from mRNAs but prevents initiation of new polypeptide chains (11). In the presence of this inhibitor, therefore, all or most of a mRNA species should be free from polysomes and accessible to soluble factors.

Figure 3 shows a series of polysome profiles from cells subjected to the inhibitor regimens detailed in Fig. 1. Untreated, aphidicolin-treated, and cycloheximide-treated (with or without aphidicolin present) cells show profiles with prominent polysome and monosome peaks, the proportions of each which are virtually identical among these treatments (Fig. 3A to D). Conversely, cells subjected to treatment with pactamycin or pactamycin plus aphidicolin yielded polysome profiles with enormously increased monosome concentrations and negligible polysome peaks (Fig. 3E and F).

Despite the general effect of pactamycin on translation, it appeared possible that some small number of polysomes remained intact, and that histone mRNA might be sequestered in these polysomes and potentially shielded from degradative mechanisms. We therefore examined the sedimentation position of histone H4 mRNA in polysome profiles from cells subjected to the various inhibitor treatments. Parallel profiles (to those shown in Fig. 3) were spun for twice the usual length of time to resolve clearly small polysomes (in which histone mRNAs sediment [3, 7]) from the monosome peak. RNA was isolated from pooled fractions across these profiles (see above) and examined for H4 sequences by Northern hybridization analysis (Fig. 4). In the absence of any drug treatment, 85% of total cytoplasmic histone H4 mRNA sedimented in polysome fractions (Fig. 4A). After 30 min of aphidicolin treatment, at least 80% of the remaining histone mRNA was found in polysome fractions (Fig. 4B), although the amount of H4 mRNA was grossly reduced. This mRNA (persisting after replication inhibition) was distributed among the different-size polysomes resolved in these gradients in the same proportions as was histone mRNA from untreated cells, suggesting that

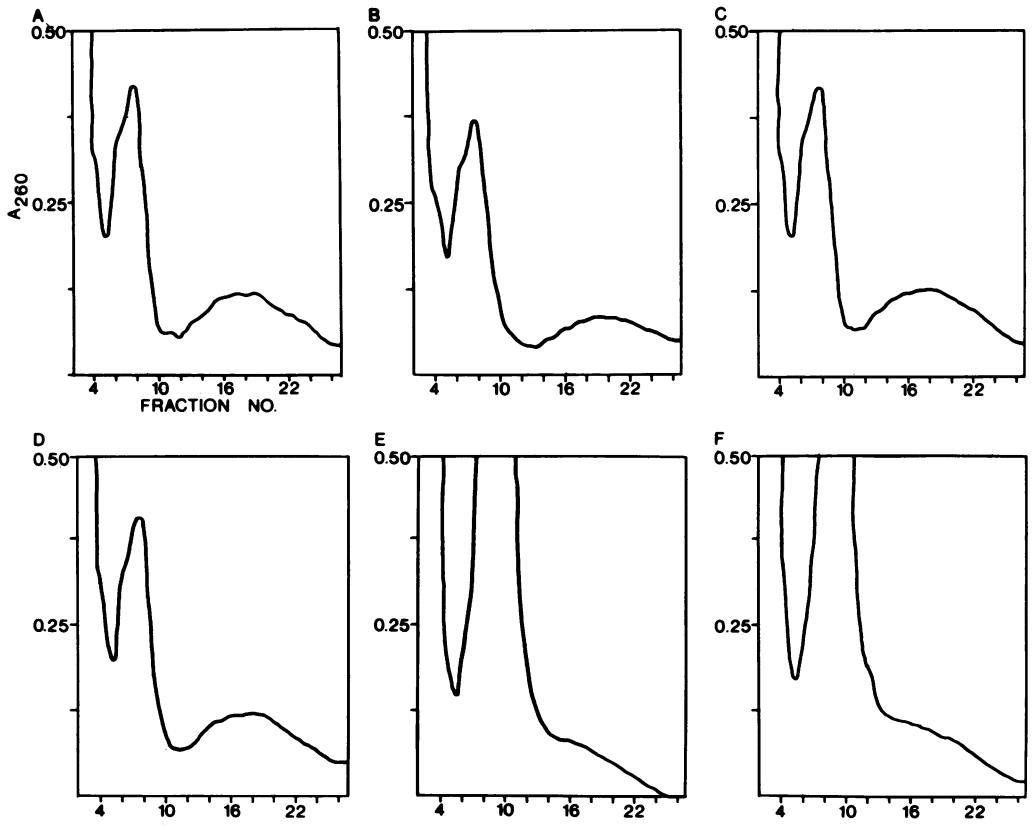

FIG. 3. Polysome profiles after inhibitor treatments. Profiles were prepared on completion of the protocols detailed in Fig. 1. Postmitochondrial supernatants were loaded onto 15 to 40% sucrose gradients and centrifuged at 42,000 × *g* for 9 h at 4°C. Gradient fractions were monitored for absorbance at 260 nm as described in the text. Fraction 1 was obtained from the top of the gradient. Profiles are from cells subjected to inhibitors as follows: A, none; B, aphidicolin; C, cycloheximide; D, cycloheximide plus aphidicolin; E, pactamycin; and F, pactamycin plus aphidicolin.

aphidicolin-resistant histone mRNA is being translated at the same unit rate as S-phase mRNA. In cells treated with cycloheximide (Fig. 4C) or with cycloheximide and aphidicolin (Fig. 4D), 89 and 85%, respectively, of H4 mRNA sedimented in the polysome fractions, with fractional distribution between gradient fractions that was similar to each other and those of untreated or aphidicolin-treated mRNA distributions.

A dramatic contrast to these results is provided by H4 mRNA sedimentation profiles from cells treated with pactamycin- and cells treated with pactamycin plus aphidicolin. At least 75 and 79%, respectively, of H4 mRNA in these gradient sedimented in pre-monosome and monosome fractions (Fig. 4E and F). The remaining H4 mRNA sedimented mostly in the small-polysome fraction, suggesting either that polysome runoff after pactamycin application was not complete or that the large monosome peak was not resolved from the adjacent gradient fractions. The significant amount of histone mRNA at the top of the gradients may reflect sequestration of free histone mRNAs into ribonucleoprotein particles.

In summary, histone mRNA stabilization by protein synthesis inhibition does not result from protection of these mRNAs by polysomes. In the absence of translation initiation only, histone mRNAs sediment in post-polysomal fractions. This reflects a real requirement for protein synthesis in the coupling of histone mRNA stability and DNA synthesis.

Histone mRNAs are superinduced throughout the cell cycle by protein synthesis inhibition. We wished to investigate the extent to which histone mRNAs are superinduced by more prolonged protein synthesis inhibition during the S phase and to examine whether histone mRNAs can be induced by translation inhibition during periods of the cell cycle when DNA synthesis is not normally occurring.

Synchronized HeLa cells were subjected to extensive (up to 5-h) periods of protein synthesis inhibition at the G1/S boundary, during the early S phase, after replication inhibition during the S phase, and in the period between S phases (ca. 4 h in our synchronized cells). RNA was isolated and examined for histone and non-histone mRNA sequences as described below.

Initially we examined the extent of increase in H4 mRNA levels during the early S phase, in response to longer periods of protein synthesis inhibition. In the inhibitor protocols used (Fig. 5A), cells at 2 h into the S phase were treated with pactamycin or cycloheximide for up to 3 h, with or without the addition of aphidicolin 10 min after the initial protein synthesis inhibition (treatments B and C). Alternatively, cells were treated for 1 h with aphidicolin before the addition of pactamycin or cycloheximide for an additional 3 h (treatments D and E). RNA was isolated at the times indicated and analyzed for H4 and, in some treatments, for He27 sequences by Northern hybridization analysis. He27 is a cDNA clone corresponding to a polyadenylated RNA abundant in HeLa cells (1). H4 mRNA levels were induced two- and fourfold over untreated concentrations by 3-h treat-

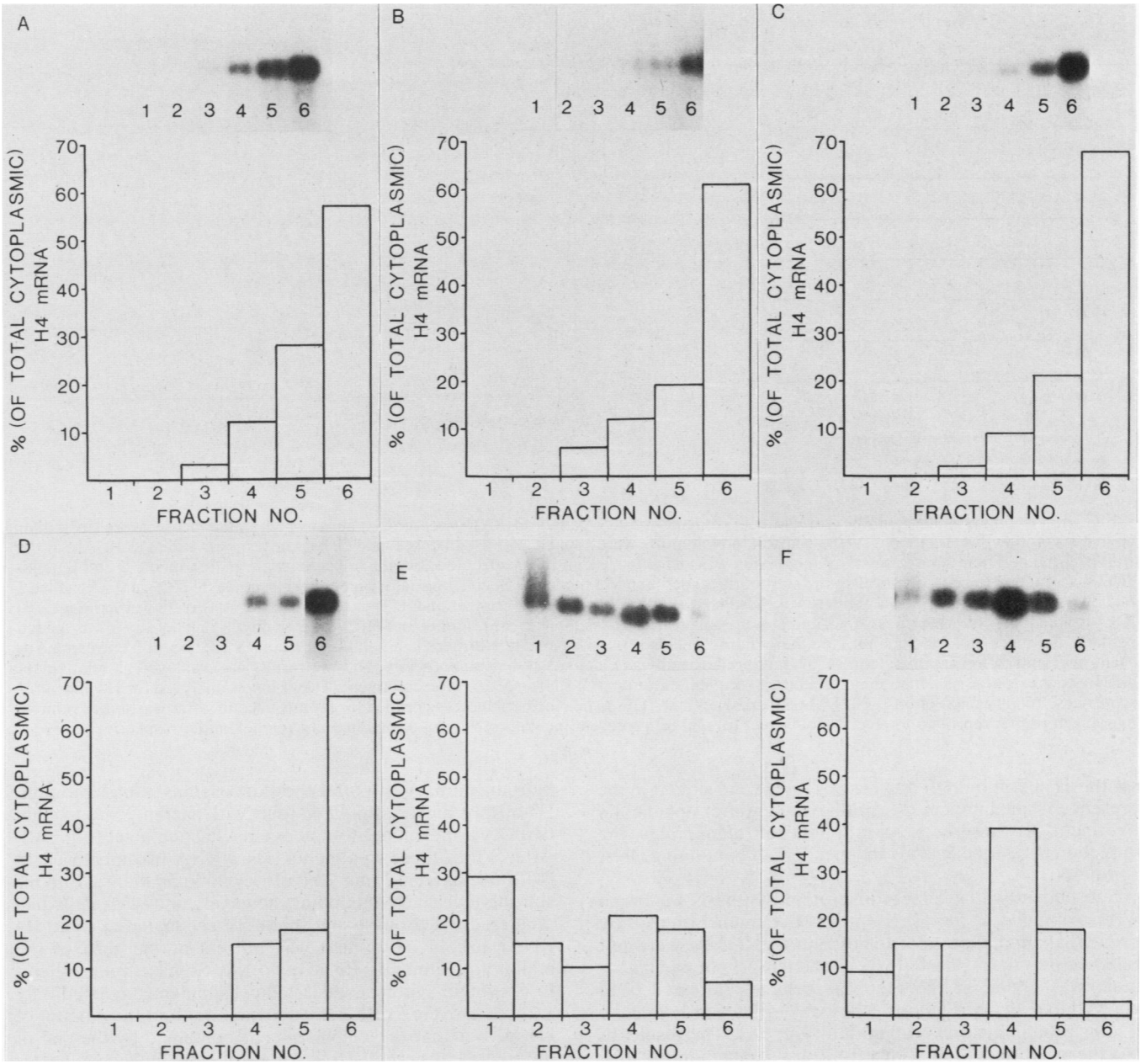

FIG. 4. Distribution of H4 RNA sequences in polysome profiles after inhibitor treatments. Cells were subjected to the protocols outlined in Fig. 1, and polysome profiles were determined as described in the text. Gradients were centrifuged at 42,000 × *g* for 18 h before fractionation. RNA was isolated as described in the text from gradient fractions pooled as follows: lane 1, top of the gradient; lane 2, 40S ribosomal subunits; lane 3, 60S ribosomal subunits; lane 4, monosome peak; lane 5, small polysomes; lane 6, remainder of large polysomes. Three fraction equivalents of RNA from each pooled sample of each gradient were analyzed by Northern hybridization analysis for H4 sequences. Autoradiograms were quantitated densitometrically, and the results are displayed as percentage of total cytoplasmic histone H4 RNA in each fraction. RNA analyzed is from cells treated with the following inhibitors: A, none; B, aphidicolin (to obtain an autoradiogram of sufficient density to allow quantitation, the autoradiogram shown was exposed for seven times the length of each of the other exposures shown here); C, cycloheximide; D, cycloheximide plus aphidicolin; E, pactamycin; F, pactamycin plus aphidicolin.

ments of either pactamycin plus aphidicolin or pactamycin, respectively (Fig. 5B and C). Similar results were obtained for equivalent cycloheximide treatments (data not shown). After aphidicolin treatment for 1 h, H4 mRNA levels were reduced to 10% of their untreated value. DNA synthesis was reduced 50% after 1-h and 75% by 3-h protein synthesis inhibitor treatments. Subsequent to aphidicolin treatment, the effects of translation inhibition on residual H4 mRNA levels were dramatic. Figure 5C diagrams the 8 (with cycloheximide)- and 16 (with pactamycin)-fold increase in H4 mRNA concentrations elicited by 3 h of protein synthesis inhibition, started after a 1-h aphidicolin treatment. H4 mRNA levels in the presence of aphidicolin alone remained low, and He27 mRNA levels were unaltered by protein

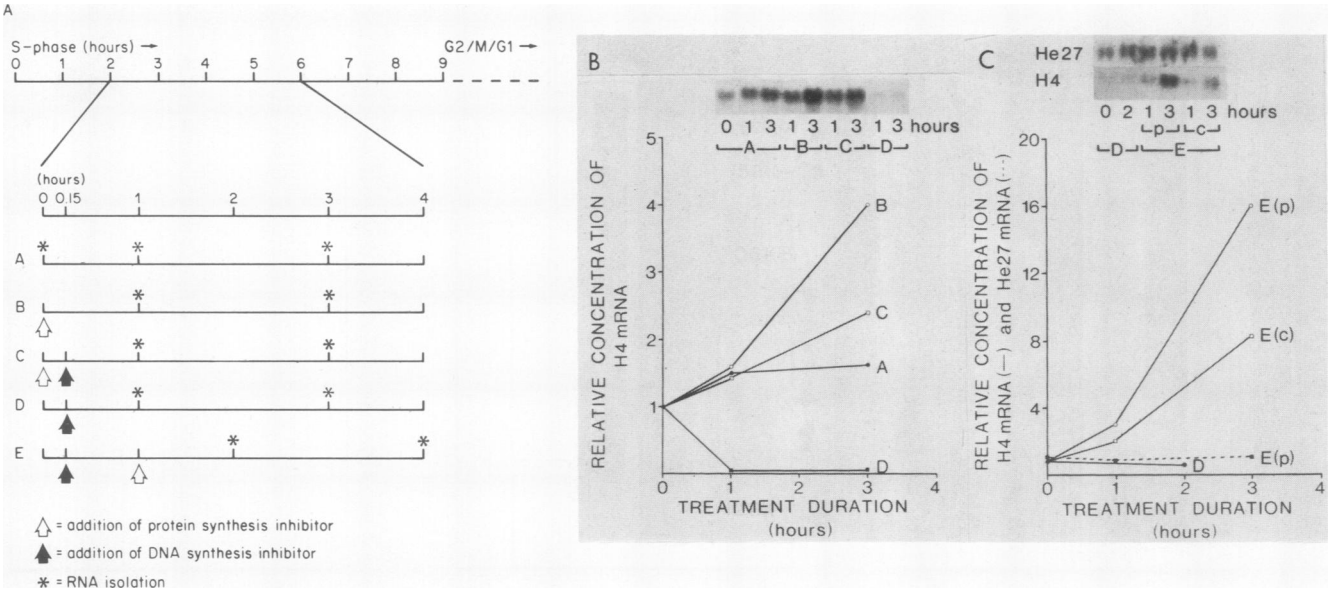

FIG. 5. Prolonged S-phase inhibitor treatment protocols and RNA analyses. (A) Synchronized cells, 2 h into the S phase, were treated for up to 3 h as follows. In treatment A (the control), no inhibitors were applied. For treatments B and C, pactamycin was added to 10^{-7} M at the start of the experiment; in treatment C, aphidicolin was added to 5 μg/ml 10 min after the addition of pactamycin. In treatments D and E, aphidicolin was added to cells 10 min after the start of the experiment; in treatment E, either pactamycin or cycloheximide (to 50 μg/ml) was added 1 h after aphidicolin application. Total RNA was isolated at the start of the experiment and at 1 and 3 h after the start of the experiment. (B) RNA isolated after treatments A, B, C, and D was analyzed for H4 sequences as described in Fig. 2. Twenty micrograms of RNA was loaded per lane. Northern blots and quantitation thereof are displayed. Letters beneath Northern lanes and beside curves on the graph correspond to each other and to the treatments in A. The autoradiogram was quantitated densitometrically, and the results are displayed as relative H4 mRNA concentration as a function of inhibitor treatment duration. (C) RNA isolated after treatments D and E was analyzed for H4 and He27 sequences. Twenty micrograms of RNA was loaded per lane. The same Northern blot was probed for H4 mRNA and, after washing to remove hybridized probe, reprobed for He27 sequences. (p) and (c) represent incubation with pactamycin and cycloheximide, respectively.

synthesis inhibition. DNA synthesis rates were similar in the presence of pactamycin or cycloheximide (after aphidicolin treatment) to those seen with aphidicolin alone. Thus the induction in histone RNA is independent of continuing DNA synthesis.

Subsequently, the effects of protein synthesis inhibition were examined at the G1/S border. Cells synchronized by sequential thymidine and aphidicolin treatments were not released into the S phase. Instead, pactamycin or cycloheximide was added at the time the cells would have been allowed to enter the S phase. RNA was isolated at 1, 3, and 5 h after protein synthesis inhibition (Fig. 6A). The results of Northern hybridization of this RNA are depicted in Fig. 6B. H4 mRNA levels increased essentially linearly 16 (with pactamycin)- to 22 (with cycloheximide)-fold after 5 h of protein synthesis inhibition. Histone H4 mRNA levels from control cells maintained in aphidicolin alone were unchanged. This protein synthesis inhibitor-mediated RNA concentration increase is not general since He27 RNA levels were unaltered. The superinduction of H4 mRNA occurred with no increase in DNA synthesis (Table 2).

We were interested to examine these RNA superinduction phenomena in cells that were free of aphidicolin (or any other DNA synthesis-suppressing agent). Cells synchronized by the thymidine-aphidicolin procedure described proceeded through one S phase and then entered a non-S-phase period (consisting of G2, M, and some G1) of ca. 4 h in length before beginning a second S phase. At 1 h after the start of the non-S-phase period, cells were subjected to pactamycin or cycloheximide treatment. RNA was analyzed at the times indicated (Fig. 7B) for H4 and He27 sequences by Northern hybridization. At 1 h after the start of translation inhibition, H4 mRNA had accumulated four (with pactamycin)- to eight (with cycloheximide)-fold above its starting level (Fig. 7B). After 3 h of these treatments, H4 mRNA had increased 12 (with pactamycin)- and 22 (with cycloheximide)-fold above starting levels. By this point, however, untreated cells had reentered the S phase and therefore accumulated some H4 RNA, 10-fold more than was present at the start of the inhibitor treatments. Relative to this S-phase value, therefore, protein synthesis-inhibited cells had accumulated only 1.2 (pactamycin)- or 2.2 (cycloheximide)-fold the amount of H4 mRNA that these control cells contained by the end of the treatment period. Unlike control cells, however, pactamycin- or cycloheximide-treated cells did not reenter the S phase, as indicated by relative DNA synthesis rates detailed in Table 2.

In summary, these results indicate that histone mRNA levels can be superinduced (that is, above their normal values) at any point during the cell cycle but that the induction is relatively smaller when cells are in the S phase, than when they are not synthesizing DNA, at the time of protein synthesis inhibitor application.

Transcription rates of histone and non-histone genes are increased by protein synthesis inhibition. Since modulation of histone mRNA levels is achieved by an S-phase-associated increase in mRNA stability and transcription rate, we wanted to understand to what extent each of these processes contributes to the superinduction phenomena observed here. In particular we have examined, in isolated nuclei, changes in transcription rates when protein synthesis is halted in the absence of, before or after, DNA replication interruption.

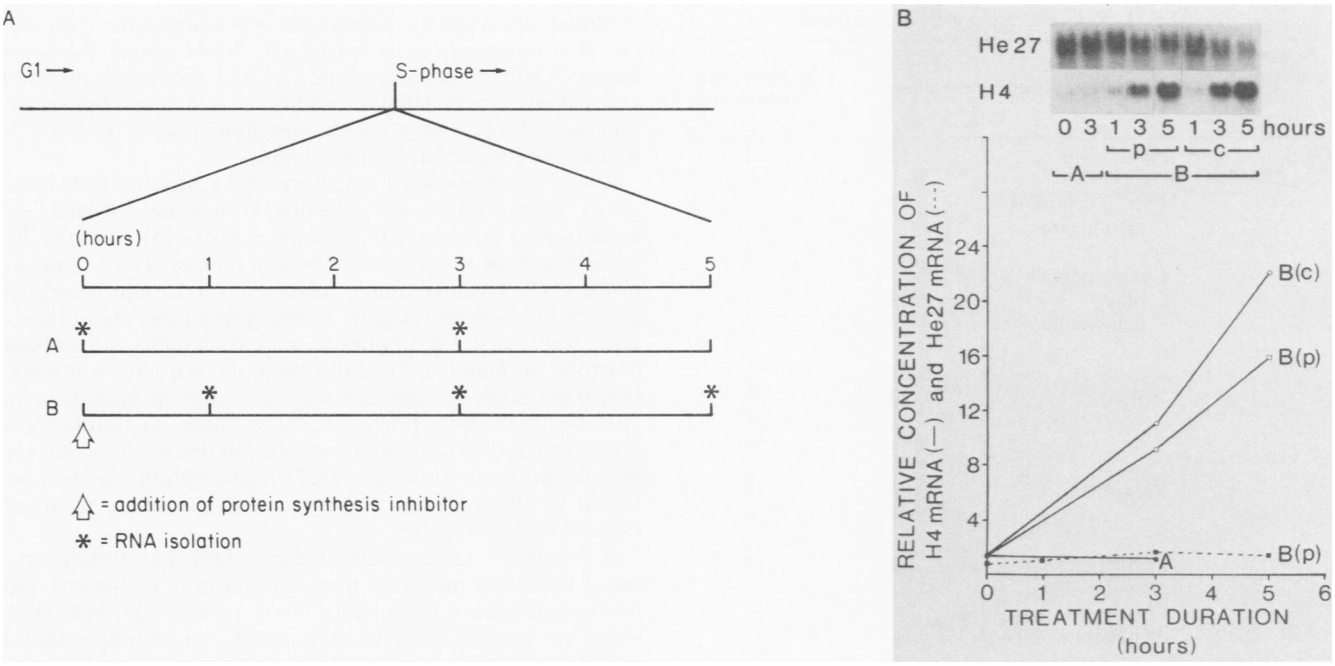

FIG. 6. G1/S-phase inhibitor treatment protocols and RNA analysis. (A) Cells were synchronized to the G1/S boundary as described previously (1) but were not released into the S phase. Samples of these cells were either not subjected to further manipulation (treatment A) or treated with either 10^{-7} M pactamycin (treatment B) or 50 μg of cycloheximide per ml (treatment B) for up to 5 h. Total RNA was extracted at the times indicated. (B) RNA isolated at the times indicated in A was subjected to Northern hybridization analysis. Twenty micrograms of RNA was loaded per lane. The same filter was probed for H4 sequences and, after removal of hybridized probe by washing, for He27 sequences. Letters beneath autoradiographic lanes and beside curves on the graph correspond to each other and to the treatments described in A. (p) and (c) represent incubations with pactamycin and cycloheximide, respectively. Autoradiograms were quantitated densitometrically, and results are expressed as relative concentration of mRNA as a function of treatment duration.

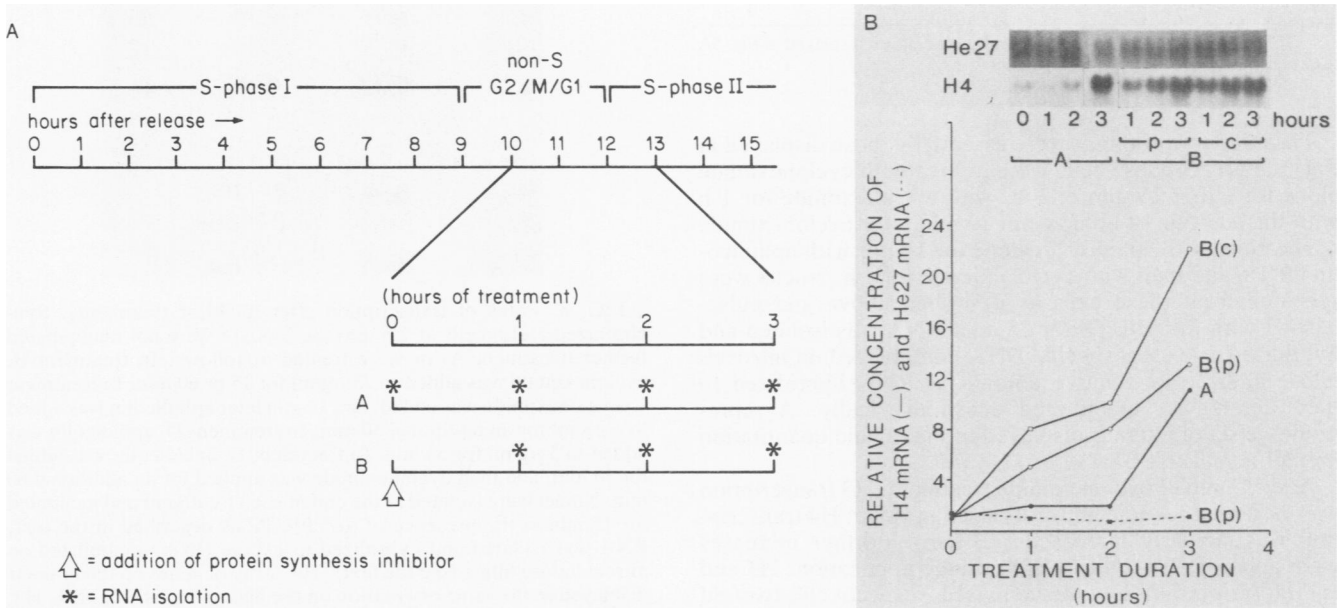

FIG. 7. Non-S-phase inhibitor treatment protocols and RNA analysis. (A) Synchronized cells were allowed to progress through one S phase. Cells remained untreated (treatment A) or at 1 h after completion of the S phase were treated with either 10^{-7} M pactamycin or 50 μg of cycloheximide per ml (treatment B) for up to 3 h. Total RNA was isolated at the times indicated. (B) RNA isolated at the times indicated in A was subjected to Northern hybridization analysis. Twenty micrograms of RNA was loaded per lane. The same filter was probed for H4 sequences and, after washing to remove hybridized probe, for He27 sequences. Letters beneath autoradiographic lanes and beside curves on the graph correspond to each other and to the treatments in A. (p) and (c) represent incubations with pactamycin and cyloheximide, respectively. Autoradiograms were quantitated densitometrically, and results are expressed as relative concentration of mRNA as a function of treatment duration.

TABLE 2. Relative rates of DNA synthesis

Position in cell cycle at start of treatment[a]	Inhibitor treatment	Treatment duration (h)	Relative DNA synthesis rate
G1/S	Aphidicolin	12	1
	Pactamycin plus aphidicolin	3	1
	Cycloheximide plus aphidicolin	3	1
S	None	0	29
		1	36
		3	38
	Pactamycin	1	11
		3	6
	Pactamycin plus aphidicolin	1	1
	Aphidicolin	1	1
Non-S	None	0	5
		1	3
		2	3
		3	8
	Pactamycin	1	1
		2	1
		3	2
	Cycloheximide	1	1
		2	1
		3	1

[a] G1/S, S, and non-S inhibitor treatment protocols are displayed in Fig. 5A, 6A, and 7A.

The inhibitor protocols were essentially those displayed in Fig. 5. Early S-phase cells were treated with cycloheximide alone for either 15 min or 1 h, with cycloheximide for 1 h with the addition of aphidicolin 10 min after cycloheximide application, with aphidicolin alone for 1 h, or with aphidicolin for 1 h and then with cycloheximide for 1 h. Nuclei were prepared from these cells as described above and pulse-labeled with [α-^{32}P]UTP for 15 min. RNA was isolated and hybridized to excess specific DNA immobilized on nitrocellulose filters. The relative amount of RNA hybridized to these filters was quantitated densitometrically. A representative set of filters is displayed in Fig. 8, and quantitation thereof is in Table 3.

After 15 min of cycloheximide treatment, H3 transcription was increased 1.4-fold above its starting value; H4 transcription was similarly increased (1.3-fold). Further increases were apparent 1 h after cycloheximide application; H3 and H4 transcription rates were increased, respectively, twofold and about threefold above starting levels. The H3 clone used for transcription rate measurements has been shown to encode a cell cycle-regulated histone mRNA (11, 26). In the presence of aphidicolin alone, H3 and H4 transcription rates decreased twofold. This decrease was prevented by the addition of cycloheximide before aphidicolin, when H3 and H4 transcription rates were sustained two- and threefold higher, respectively, than their starting early S-phase values. Additionally, when cycloheximide was added after aphidicolin, H3 transcription increased ca. 3-fold above the level achieved with aphidicolin alone (1.5 fold above the untreated cell value). Under similar conditions, H4 transcription increased 3.5-fold above aphidicolin-alone levels, or about 2-fold above untreated, S-phase levels.

These increases were not, however, restricted to histone genes. Transcription was measured from genes for He27, β-actin (with a chicken cDNA clone as probe [5]), and the 70-kilodalton heat-shock protein (with a human cDNA clone as probe [14]). Cycloheximide treatment for 15 min increased β-actin and heat-shock gene transcription rates about threefold, whereas a 1-h treatment increased these rates about fourfold. Although aphidicolin alone did not alter transcription from actin or heat-shock genes, after incubation with cycloheximide for 1 h approximately three- to sixfold transcriptional increases were observed relative to untreated (or aphidicolin-treated) values. He27 transcription was less affected by these treatments, increasing maximally to about twofold its initial value.

In summary, these results indicate first that protein synthesis inhibition increases transcription from all histone and non-histone genes examined. These include H3, H4, H2A (data not shown), β-actin, heat-shock, an abundant HeLa cell RNA (He27), and α-tubulin (data not shown) genes. Second, this increase is rapid. Both histone and non-histone transcription rates rose within 15 min of cycloheximide application, although not to the values measured 1 h after protein synthesis inhibition. Third, the decrease in histone transcription normally associated with DNA synthesis inhibition is prevented by protein synthesis interruption. Fourth,

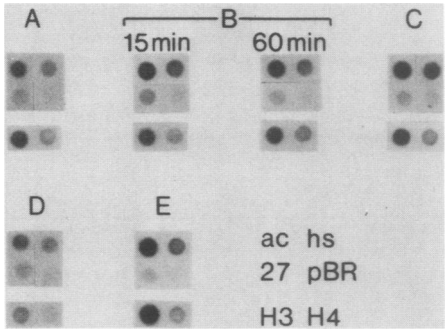

FIG. 8. Rates of transcription after inhibitor treatments. Synchronized HeLa cells at 2 h into the S phase were not manipulated further (treatment A) or were treated as follows. In treatment B, cycloheximide was added to 75 μg/ml for 15 or 60 min. In treatment C, cycloheximide was added, and 10 min later aphidicolin was added to 5 μg/ml for an additional 50 min. In treatment D, aphidicolin was added to 5 μg/ml for 50 min. In treatment E, aphidicolin was added for 50 min, and then cycloheximide was applied for an additional 60 min. Nuclei were isolated at the end of each treatment and incubated for 15 min in the presence of [α-^{32}P]UTP as described in the text. RNA was isolated and hybridized to excess DNA immobilized on nitrocellulose filters (see the text). The array of genes on the filters is displayed in the same orientation on the figure: ac, hs, 27, pBR, H3, and H4 represent, respectively, clones of β-actin, heat shock, He27, pBR322, histone H3, and histone H4 (see the text). Input counts per minute were ca. 4×10^5 for H3 and H4 and 8×10^6 for β-actin, heat shock, He27, and pBR (see Table 3 for the actual input of counts per minute and the results of quantitation). Autoradiographs were quantitated densitometrically. Different exposures were used such that they were in the linear range of the film and densitometer; however, the same exposure was used to compare the response of one gene to inhibitor treatments.

TABLE 3. Transcription rates

Treatment[a] (duration)	Input cpm[b] ($\times 10^6$)	Densitometric area/10^6 input cpm[c]						Relative change[d]				
		pBR	H3	H4	Actin	Heat shock	He27	H3	H4	Actin	Heat shock	He27
None	0.57		6.79	1.99				1	1			
	14.17	0.07			0.87	0.49	0.23			1	1	1
Cycloheximide	0.35		9.14	2.66				1.4	1.3			
(15 min)	10.21	0.11			2.42	1.26	0.48			2.9	2.7	2.3
Cycloheximide	0.28		13.32	6.36				2.0	3.3			
(1 h)	8.82	0.15			3.08	1.78	0.34			3.7	3.9	1.2
Cycloheximide plus	0.34		13.53	6.15				2.0	3.2			
aphidicolin	9.63	0.11			2.75	2.13	0.38			2.9	5.8	1.7
								1	1			
Aphidicolin	0.40		3.18	1.20				0.5	0.6			
	10.20	0.10			0.89	0.42	0.28			1	1	1
										1.0	0.8	1.1
								3.2	3.3			
Aphidicolin plus	0.30		10.17	4.13				1.5	2.1			
cycloheximide	8.35	0.11			3.92	1.77	0.33			4.2	4.0	1.4
										4.8	5.2	1.2

[a] Inhibitor protocols used are displayed in Fig. 5A and described in the text. This experiment has been repeated at least three times for all genes shown, with similar results.

[b] Input counts per minute reflect conditions required for DNA excess in filter hybridizations and are actual counts per minute for the experiment shown in Fig. 8.

[c] Area is expressed as millimeters squared. Gels were scanned such that exposures were in the linear range of the film and densitometer. Areas have been normalized between genes for similar exposure times.

[d] pBR background has been subtracted.

the increase in histone transcription observed when protein synthesis was inhibited after replication had been halted (relative to levels with the replication inhibitor alone) is similar to that observed when protein synthesis was inhibited in S-phase cells.

One potential problem in the interpretation of these transcription rate results arises in the following way. Nuclei isolated from cells that had been subjected to protein synthesis inhibition (at concentrations of inhibitors described here) incorporated about twofold less [α-^{32}P]UTP than did untreated cells. This may have resulted from decreases in synthesis of only one or a few particular class(es) of RNA (e.g., rRNA and tRNA). In this event transcription of RNA classes unaffected by translation inhibitors would apparently increase if transcription rate data are expressed (as in Table 3) relative to a constant amount of radioactivity incorporated. If, however, all classes of RNA synthesized are equally depressed by protein synthesis inhibition, then normalization of transcription rate data in this fashion will truly reflect relative numbers of transcription complexes present on the genes examined.

For these reasons, we examined the relative proportions of transcripts produced by RNA polymerases I, II, and III in nuclei isolated from cells that had been treated with a protein synthesis inhibitor, with or without a DNA replication inhibitor. Cells 2 h into the S phase were treated (Fig. 5) with cycloheximide for 1 h, with or without aphidicolin added 10 min after the addition of cycloheximide, or with aphidicolin for 1 h and then with cycloheximide for 1 h. Nuclei were prepared as for transcription rate measurements. Pulse-labeling was with [α-^{32}P]GTP for 15 min in the presence of 0-, 2-, or 200 μg of α-amanitin per ml to distinguish RNA polymerases I, II, and III activities (see the legend to Table 4). RNA was isolated as for transcription rate assays, and total incorporated radioactivity was determined. Table 4 details the results of these experiments. Although total incorporation was reproducibly decreased about twofold in cycloheximide-treated nuclei, the relative contributions of RNA polymerases I, II, and III transcripts to total transcription were very similar in all cases, ca. 30, 50, and 15%, respectively. These results indicate that on average all classes of transcription are equally depressed by protein synthesis inhibition, by a mechanism(s) we do not understand. The results (Table 3) therefore reflect accurately that the numbers of transcription complexes per gene increase, for some genes, in the absence of protein synthesis.

TABLE 4. Relative contribution of RNA polymerase I, II, and III transcripts to total transcription

Inhibitor treatment[a]	Relative total ^{32}P incorporation[b]	% of total transcription contributed by RNA polymerase[c]		
		I	II	III
None	1	38	53	15
Cycloheximide	0.44	36	52	13
Cycloheximide plus aphidicolin	0.52	26	58	16
Apidicolin plus cycloheximide	0.40	23	61	16

[a] Inhibitor treatment protocols are displayed in Fig. 5A and described in the text.

[b] Total radioactivity incorporated for the untreated sample was 3×10^6 cpm.

[c] RNA polymerase I transcription was estimated to be the amount of transcription resistant to 200 μg of α-amanitin per ml. RNA polymerase II transcription was estimated to be the amount of transcription sensitive to 2 μg of α-amanition per ml. RNA polymerase III transcription was calculated as the difference between transcription resistant to 2 and 200 μg of α-amanitin per ml.

DISCUSSION

We have rigorously demonstrated by direct nucleic acid analysis that, in the absence of protein synthesis, histone mRNA degradation does not occur when replication is inhibited. This stabilization is not a function of polysomal shielding of these RNAs from active degradative mechanisms since histone mRNA not associated with polysomes, whether monosomal, post-monosomal, or nuclear, was not degraded when translation initiation was blocked by pactamycin treatment. Our results thus reflect a requirement for continued protein synthesis in replication-linked histone RNA destabilization and demonstrate that DNA synthesis is not inextricably linked to histone gene expression. The results obtained here are in agreement with earlier studies (3, 23), including those of Stahl and Gallwitz (23). These authors demonstrated that the decline in translatable histone mRNAs effected by hydroxyurea treatment could be prevented by the simultaneous inhibition of translation initiation by hypertonic shock. Very recently (10, 12), similar results have been obtained by using cloned histone gene probes as a means of mRNA quantitation.

We were intrigued to observe, in our initial experiments, an increase in total histone mRNA levels after S-phase cells had been treated for 40 min with a protein synthesis inhibitor. We extended this observation by examining changes in histone mRNA levels after more prolonged (up to 5-h) translation inhibition treatments begun at different points during the cell cycle. Histone mRNA superinduction was observed when cycloheximide or pactamycin was applied to cells at the G1/S boundary, during the S phase in the presence or absence of the DNA synthesis inhibitor aphidicolin, and in the non-S-phase period at the end of the first S phase in our synchronized cells, before entry into a second S phase. The superinduction was not general for all mRNA species since RNA hybridizing to HeLa cell cDNA clone He27, as well as β-actin and heat-shock (data not shown) mRNAs, was not superinduced by protein synthesis inhibition. The relative induction of histone mRNAs above their levels at the time of translation inhibition varied markedly. S-phase cells achieved, maximally, a 4-fold increase in H4 mRNA after 3 h of protein synthesis inhibition, whereas cells not synthesizing DNA at the time of protein synthesis inhibition achieved up to 22-fold superinduction of H4 mRNA 3 h after the inhibitor was applied. This latter class included cells at the G1/S border, S-phase aphidicolin-treated cells, and non-S-phase cells.

The result is understandable upon consideration of normal histone mRNA regulatory mechanisms. Histone mRNA levels rise during the S phase as a result of increases in histone gene transcription rates and mRNA stability. One or both of these processes must contribute to increase histone mRNA levels during protein synthesis inhibitor-mediated superinduction. The increments by which histone mRNA stabilities or transcription rates, or both, increase relative to their values at the time of inhibitor application will determine the relative increases in histone mRNA concentrations. S-phase cells that are actively synthesizing DNA already have greater histone gene transcription rates and histone mRNA stabilities than do their non-DNA-replicating counterparts. If one or both of these processes increase(s) to the same absolute value(s) after protein synthesis inhibition, the increase(s) will be smaller [and the relative change(s) in histone mRNA concentration accordingly less] in the S-phase cells than in nonreplicating cells.

We examined histone and non-histone gene transcription rates before and after translation inhibition in the presence or absence of DNA synthesis. We hoped to dissect, by inference from these results, the relative importance of transcriptional and mRNA stability changes in the superinduction phenomenon and relative differences in its extent during the cell cycle.

Both histone (H3 and H4) and non-histone (β-actin, heat-shock, and He27) transcription rates were increased to some extent by protein synthesis inhibition (in the presence or absence of DNA synthesis). The induction was rapid since some increase was observable 15 min after translation was blocked. The magnitude of the increase, after 1 h of protein synthesis inhibition, was smaller for He27 (maximally 2-fold) than for H3 or H4 (1.5- to 3-fold) or actin and heat shock (3- to 6-fold). The histone-specific decrease in H3 and H4 transcription observed upon DNA synthesis blockage (about twofold) was prevented by cycloheximide application before aphidicolin treatment was begun. In this event, histone gene transcription rates were similar to those seen with cycloheximide alone. Additionally, if protein synthesis was inhibited after DNA synthesis was stopped, histone transcription rates increased about threefold above their aphidicolin-inhibited values. That is, the relative transcription rate increases observed were similar when S-phase and aphidicolin-blocked cells were treated with a protein synthesis inhibitor. Since the magnitude of histone mRNA superinduction was similar when either non-S-phase or aphidicolin-blocked S-phase cells were subjected to a protein synthesis inhibitor, we expect the subsequent transcriptional increases to be quantitatively similar in these two cell populations. It remains a formal possibility, however, that the relative increases in transcription rates differ in nonreplicating S-phase and non-S-phase cells, and the equivalence of histone mRNA concentration increase was fortuitous. We are confident that these transcription rates reflect the numbers of transcription complexes per gene, since although protein synthesis inhibition decreased total transcription, the proportions of transcripts produced by RNA polymerases I, II, and III did not appear to change.

It is clear that histone mRNA synthesis occurs during the induction phenomenon. However, it seems unlikely that changes in histone gene transcription rates are the major cause of histone RNA superinduction. Both replicating and nonreplicating cells were induced transcriptionally to similar extents by translation inhibition, whereas the relative increases in histone mRNA concentrations were not similar under these conditions. Additionally, the mRNA superinduction was (among the genes examined) restricted to histone mRNAs, whereas the transcriptional potentiation was not. We conclude that stabilization of histone mRNAs is the most important process contributing to their induction in the absence of protein synthesis. The half-lives of histone mRNAs in the absence of protein synthesis are not known, but they are probably at least as long (ca. 40 min) as they are during the S phase. We can therefore account for the differential superinduction at different points in the cell cycle by postulating that a relatively greater increase in stability of histone mRNA occurs when translation inhibitors are applied in the absence of DNA synthesis (where the histone mRNA half-life is ca. 10 min) than when S-phase cells are similarly treated. This conclusion is supported by examining the distribution of histone mRNA across polysome gradients after 40 min of cycloheximide treatment. About 15% of total H4 mRNA was present in monosome and post-monosome fractions (Fig. 4C and D). This may reflect newly synthesized mRNA, because cycloheximide does not effect poly-

some disaggregation (2; Fig. 3C and D). Since the major portion of histone mRNA was sequestered in polysomes, most of the effect we observed must have been due (at least after this period of protein synthesis inhibition) to mRNA stabilization. In agreement with this study, Stimac et al. (24) have recently reported an induction of histone mRNA by translation arrest in a partially purified G1 HeLa cell population.

What is the significance of the transcriptional increases observed and of the histone-specific mRNA superinduction in the metabolism of histone and other RNA species? First, the transcriptional induction observed after protein synthesis interruption is certainly not restricted to histone genes, at least in HeLa cells. Although translation inhibition prevents and reverses the decreases in histone gene transcription associated with DNA replication inhibition, we do not know whether it does so by way of the same mechanisms operative in translationally active cells. Our results are contradictory to those of Graves and Marzluff (10), who reported an increase in histone H3 but not in β-actin transcription when mouse myeloma cells were treated with cycloheximide after 5-fluorodeoxyuridine-mediated DNA synthesis inhibition. These authors suggested that a labile protein represses specifically histone gene transcription. We do not understand the discrepancy in our results, although the cell types, nuclei preparation, and pulse-labeling techniques used in the two studies differ. There is no implication from the study presented here that cell cycle-linked transcriptional regulation of histone gene expression is mediated by a factor sensitive to protein synthesis inhibition.

Transcriptional potentiation by protein synthesis inhibition has been implicated in the superinduction of interferon mRNAs (21). Additionally, in the presence of a functional immediate early gene product, adenovirus early gene transcription is enhanced by cycloheximide (6). The rapidity of transcriptional increase, detectable 15 min after translation is halted, and the broad spectrum of genes for which it has been observed may imply that a labile protein repressor controls some cellular transcription, as has been proposed (16). There are, nevertheless, many other plausible interpretations of these results.

This study indicates clearly, however, that histone mRNA levels are controlled by a labile or rapidly sequestered protein(s) which probably alters mRNA stability. The destabilization of histone mRNAs resulting from DNA synthesis interruption must be effected indirectly since the two processes can be completely unlinked. Our results support a proposal, made nearly 15 years ago (3), that included the suggestion that concentrations of histone mRNAs are controlled in an autoregulatory fashion. In this scheme, a free histone protein may bind to its cognate mRNA and either direct nucleases to the RNA or allow nuclease action after preventing ribosome loading on the RNA. In the absence of, or after a block in, DNA synthesis, cytoplasmic histone protein levels may increase sufficiently (3) to effect rapid destruction of most histone RNAs. We note that such mechanisms may control levels of only those histone mRNAs expressed in a cell cycle-dependent fashion and that histone genes whose expression does not increase in the S phase (25) may be subject to quite different regulatory processes. Regulation of mRNA concentrations by processes requiring continued protein synthesis have been documented for several other eucaryotic mRNA species including *c-myc* (15), interferon (19, 21), and interleukin-2 (7) mRNAs.

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