Regulation of Human Histone Gene Expression: Transcriptional and Posttranscriptional Control in the Coupling of Histone Messenger RNA Stability with DNA Replication†

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ABSTRACT: The extent to which transcriptional and posttranscriptional regulation contributes to the coupling of histone gene expression and DNA replication was examined during the cell cycle in synchronized HeLa S3 cells. Rates of transcription were determined in vitro in isolated nuclei. A 3–5-fold increase in cell cycle dependent histone gene transcription was observed in early S phase, prior to the peak of DNA synthesis. This result is consistent with a previous determination of histone mRNA synthesis in intact cells [Plumb, M., Stein, J., & Stein, G. (1983) *Nucleic Acids Res. 11*, 2391]. The transcription of these genes did not change appreciably after inhibition of DNA replication by hydroxyurea treatment, although Northern blot analysis indicated that cellular levels of histone mRNA decreased rapidly in the presence of the drug. Total cellular levels of histone mRNA closely parallel the rate of DNA synthesis as a function of cell cycle progression, reaching a maximal 20-fold increase as compared with non S phase levels. This DNA synthesis dependent accumulation of histone mRNA occurs predominantly in the cytoplasm and appears to be mediated primarily by control of histone mRNA stability. Changes in nuclear histone mRNA levels were less pronounced. These combined observations suggest that both transcriptional regulation and posttranscriptional regulation contribute toward control of the cell cycle dependent accumulation of histone mRNA during S phase, while the stability of histone mRNA throughout S phase and the selective turnover of histone mRNAs, either at the natural termination of S phase or following inhibition of DNA synthesis, are posttranscriptionally regulated. The requirement of protein synthesis for histone mRNA turnover is consistent with the involvement of histone protein or a putative short-lived regulatory molecule in the stability of histone mRNAs during S phase and/or in the selective turnover of histone mRNAs in the absence of DNA synthesis.

Histone proteins play a key role in the structural and transcriptional properties of chromatin, the protein–DNA complex that constitutes the eukaryotic genome (Isenberg, 1979; Kornberg, 1979; McGhee & Felsenfeld, 1980; Weisbrod, 1982). The biosynthesis of histones takes place predominantly during the S phase of the cell cycle and is tightly coupled to DNA replication (Spaulding et al., 1966; Robbins & Borun, 1967; Stein & Borun, 1972; Wu & Bonner, 1981; Marashi et al., 1982). It has been suggested that the temporal and functional coupling between histone gene expression and DNA synthesis involves multiple levels of regulation including histone protein synthesis (Jacobs-Lorena et al., 1972; Marashi et al., 1982; Wu & Bonner, 1981; Delegeane & Lee, 1982), histone mRNA turnover (Hereford et al., 1981; Heintz et al., 1983; Plumb et al., 1983b; Stimac et al., 1983; Baumbach et al., 1984; Helms et al., 1984), and histone gene transcription (Stein et al., 1975; Hereford et al., 1981, 1982; Heintz et al., 1983; Sittman et al., 1983; Plumb et al., 1983a,b). Although the interrelationships between these levels of control have been extensively examined, the precise mechanisms regulating the coordinate and stoichiometric coupling between DNA replication and histone gene expression remain unresolved.

Using various metabolic inhibitors, we have previously shown that cellular levels of core and H1 histone mRNAs are temporally and functionally coupled with rates of DNA synthesis in HeLa S3 cells (Baumbach et al., 1984). Moreover, the turnover of histone mRNA that parallels reduced rates of DNA synthesis is not dependent on transcription but requires protein synthesis (Baumbach et al., 1984; Helms et al., 1984). Results from experiments in which RNA synthesis was inhibited by treatment of cells with actinomycin D (Baumbach et al., 1984) indicated that histone gene transcription is not required for the turnover of histone mRNA that occurs in conjunction with DNA synthesis inhibition and that a transcriptional event is not involved in the selective destabilization of histone messages. When HeLa cells were treated with protein synthesis inhibitors (Baumbach et al., 1984; Helms et al., 1984), histone mRNA levels were stabilized and accumulated with time, although DNA synthesis was inhibited. Addition of protein synthesis inhibitors, prior to inhibition of DNA replication by treatment with hydroxyurea, prevented the rapid decline in cellular histone mRNA levels that occurred when HeLa cells were treated with hydroxyurea alone. These results suggest that protein synthesis is an important component of the posttranscriptional control of histone mRNA metabolism in HeLa cells. Our observations are in agreement with results from several laboratories, where similar studies were conducted in exponentially growing (Stimac et al., 1983; Graves & Marzluff, 1984) or synchronized mammalian cells (Butler & Mueller, 1973; Stimac et al., 1983; Sive et al., 1984).

In this paper we present results from studies in which the contribution of transcription to human histone gene expression was examined. Transcriptional rates of histone genes were determined in vitro in isolated nuclei, followed by hybridization of the radiolabeled RNAs to cloned human histone DNA. Levels of total cellular, cytoplasmic, and nuclear histone mRNAs were also determined during the cell cycle. The results of these experiments support the cell cycle periodicity in levels of human histone gene transcription. These genes are transcribed throughout the cell cycle and exhibit an en-

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hanced level of transcription in early S phase prior to the maximal accumulation of histone mRNAs. Our results also demonstrate the predominant contribution of posttranscriptionally mediated control of the coupling of cellular histone mRNA levels with rates of DNA synthesis in HeLa cells. The implications of these findings for several possible mechanisms of histone gene regulation, including autogenous control, are discussed.

Materials and Methods

Materials. [*methyl*-^{3}H]Thymidine (20–32 Ci/mmol) was purchased from New England Nuclear; [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]UTP (3000 Ci/mmol) were purchased from ICN; X-ray film (XAR 5) was from Eastman Kodak Co.; proteinase K was obtained from Boehringer Mannheim; hydroxyurea and puromycin were from Sigma Chemical Co.; cycloheximide (Acti-dione) was provided by Upjohn Co.

Cell Culture, Synchronization, and Pulse Labeling. HeLa S3 cells were grown in suspension culture at 37 °C in Joklik-modified Eagle's minimal essential medium supplemented with 7% newborn calf serum and were synchronized by two successive treatments with 2 mM thymidine (Stein & Borun, 1972). Rates of DNA synthesis, in the absence and presence of metabolic inhibitors, were determined by pulse labeling cells with [^{3}H]thymidine for 30 min as previously reported (Baumbach et al., 1984).

Human Gene Probes. The isolation and characterization of the λCh4A recombinant phages containing human histone genes that were used in these studies have been described (Sierra et al., 1982). Genomic restriction fragments were subcloned into pBR322 and contained the following human histone genes: H1 (pFNC16), H2A + H2B (pFF435B or pFF435D), H3 (pFF435C), and H4 (pFO108A) (Carozzi et al., 1984; Stein et al., 1984).

Preparation of Human Histone Gene Plasmids. Plasmid-bearing bacteria (*Escherichia coli* strain HB101) were grown in Luria-Bertani medium (Maniatis et al., 1982) containing 0.2% D-glucose, 10 mM $MgSO_4$, and 50 μg/mL ampicillin. All experiments using viable recombinant bacteria were performed under the specifications of the NIH Guidelines for Research Involving Recombinant DNA Molecules. Plasmid DNA was isolated by the alkaline lysis method as described by Birnboim and Doly (1979) and modified by Ish-Horowitz and Burke (Maniatis et al., 1982).

Isolation of Total HeLa Cell RNA. A total of 5×10^7 cells were lysed in 4.5 mL of solution containing 1.3 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.4), 0.7 mM ethylenediaminetetraacetic acid (EDTA), 1.3 μg/mL poly(vinyl sulfate), 2.4% (w/v) sodium dodecyl sulfate (SDS), and 0.9 mg/mL proteinase K. After a 15-min incubation at room temperature, and the addition of 0.3 mL of 5 M NaCl, the aqueous phase was extracted twice with 2 volumes of buffered phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), and once with 1 volume of chloroform/isoamyl alcohol (24:1 v/v). Nucleic acids were precipitated with 3 volumes of ethanol at −20 °C (overnight) after addition of potassium acetate to a final concentration of 53 mM. Total cellular RNA was further purified from the nucleic acid preparation as previously described (Baumbach et al., 1984).

Total cellular RNA preparations were monitored initially for intactness by electrophoresis in 1.5% (w/v) agarose gels containing 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 8.0), 5 mM sodium acetate, 37% (w/v) formaldehyde, and 1 mM EDTA (Lehrach et al., 1977). RNA was dissolved in the above buffer containing 50% (v/v) formamide, 5% (v/v) glycerol, and 0.04% bromphenol blue by heating at 65 °C for 10 min. After electrophoresis, gels were stained for 1 h with 0.5 μg/mL ethidium bromide in 0.1 M ammonium acetate and destained in water (prior to photography). The relative abundance of 28*S* and 18*S* RNA bands, as shown by ethidium bromide staining, was used as a reflection of intactness of the RNA preparations.

Isolation of Nuclear and Cytoplasmic HeLa Cell RNAs. Synchronized HeLa cells (5×10^8) were resuspended in 12 mL of RSB (10 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1.5 mM $MgCl_2$) and swollen on ice for 15 min. Cells were then placed in a 15-mL Wheaton homogenizer and lysed with 15–20 strokes of a type "A" tight pestle. Nuclei were pelleted by centrifugation at 1500*g* for 3 min at 4 °C and washed 3 times with RSB containing 0.5% Triton X-100. The pooled supernatants from nuclear pelleting constituted the cytoplasmic fraction. Cytoplasmic RNA was isolated as described above for total cellular RNA.

Nuclear RNA was isolated in the following manner. Nuclei were first treated with electrophoretically purified deoxyribonuclease I (DNase I) (100 μg/mL) in the presence of 0.6 M NaCl, 50 mM Tris-HCl (pH 7.5), and 20 mM $MgCl_2$ for 20 min at room temperature. The DNase had been pretreated with proteinase K for 2 h [as described by Tullis and Rubin (1980)] to remove contaminating ribonucleases. The mixture was then incubated with proteinase K (200 μg/mL) for 30 min at 37 °C in the presence of 150 mM NaCl, 12.5 mM EDTA, 100 mM Tris-HCl (pH 7.5), and 20 mM $MgCl_2$. The reaction was ended by addition of sodium acetate (pH 5.5) to 0.2 M. At this point the majority of nuclear DNA–protein complexes had been disrupted. Nucleic acids were then extracted several times by hot phenol extractions (Clayton & Darnell, 1983) using phenol equilibrated with 200 mM sodium acetate (pH 5.0) and 10 mM EDTA, followed by a room temperature extraction as for total cellular RNA. One hundred micrograms of yeast tRNA and potassium acetate (adjusted to a final concentration of 250 mM) were added to the aqueous phase containing nuclear RNAs, and 2.5 volumes of ice-cold 95% ethanol were added to this mixture to precipitate the nucleic acids. An additional precipitation in the presence of 3 M sodium acetate was added to remove small nuclear RNAs. RNA concentrations were estimated by absorbance at 260 nm (1 OD = 40 μg/mL).

Northern Blot Analysis of HeLa Histone mRNA Levels. After electrophoretic separation, total cellular, nuclear, and cytoplasmic RNAs were transferred to 0.45-μm nitrocellulose (Schleicher and Schuell) in 20 × SSC [1 × SSC = 150 mM NaCl, 15 mM sodium citrate (pH 7.0)] as recommended (Thomas, 1980). The filters were then baked in vacuo for 2 h at 80 °C.

For detection of core histone mRNAs, the nitrocellulose blots were prehybridized at 50 °C for 6 h in 20 mL of 50% (v/v) formamide, 5 × SSC, 5 × Denhardt solution minus bovine serum albumin [1 × Denhardt solution = 0.02% (w/v) poly(vinylpyrrolidone), 0.02% (w/v) Ficoll 400, 0.02% BSA], 0.1% (w/v) glycine, and 100 μg/mL *E. coli* total nucleic acids. Hybridization with a ^{32}P-labeled histone gene probe was performed in prehybridization buffer without glycine for 40 h at 47 °C by using 1×10^6 cpm/mL and 10 mL per 11 × 14 cm blot. For detection of H1 histone mRNA, prehybridization and hybridization were carried out at 42 °C. After hybridization, filters were washed once in 250 mL of each of the following: 5 × SSC and 1 × Denhardt solution (minus BSA) for 35 min at 60 °C; 2 × SSC and 0.1% (w/v) SDS for 35 min at 60 °C; 1 × SSC and 0.1% (w/v) SDS for 35 min at 60 °C. For blots containing subcellular RNA prepa-

rations, the stringency of the wash procedure was increased by raising the temperature to 62 °C and adding a wash with 0.1 × SSC and 0.1% (w/v) SDS. Filters were briefly air dried on Whatman 3MM paper and exposed to XAR-5 film that had been preexposed to light (preflashed) in order to bring the film to a threshold level (Laskey & Mills, 1977). Kodak X-OMAT Regular or Du Pont Lightning-Plus intensifying screens were used at −70 °C, which increases the sensitivity of detection of ^{32}P by 8–10-fold (Laskey & Mills, 1977).

Hybridization was quantitated by both liquid scintillation spectrometry of the RNA blots and densitometric analysis of autoradiograms. Liquid scintillation spectrometry was performed by excision of the appropriate bands from Northern blots (aligned with the corresponding autoradiogram). Densitometric analysis was performed by using a scanning densitometer from Helena or Zeineh laboratories.

For quantitation of histone mRNA levels on Northern blots that contained RNAs isolated from subcellular fractionation experiments, hybridization signals detected for histone mRNAs were adjusted for minor differences in the amounts of RNA loaded on the gels. This was accomplished by normalization of the hybridization data to the amount of 28S and 18S RNAs on gels. The quantitative analysis of 28S and 18S RNA was performed by densitometry of photographs of the ultraviolet shadowed gel corresponding to a given autoradiogram.

Transcription in Isolated Nuclei. Nuclei were isolated from HeLa cells as described by Flint et al. (1984). The nuclei were used immediately in transcription assays which contained the following components in a total volume of 0.17 mL: 10^7 nuclei, 30 mM Tris-HCl (pH 7.9), 50 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM β-mercaptoethanol, 25% glycerol, 1 mM ATP, 0.25 mM GTP, 0.25 mM CTP, and 100 μCi [α-^{32}P]UTP. The mixture was incubated for 30 min at 30 °C. The isolation of RNA was performed with hot phenol/chloroform extractions as described for nuclear RNA except that final isolation of RNA was by ethanol precipitation.

DNA Filter Hybridization of Nuclear Transcripts. Restriction enzyme digested plasmid DNAs were fractionated electrophoretically in 0.8% agarose gels and transferred to nitrocellulose by diffusion blotting with 20 × SSC (Southern, 1975). The resulting Southern blots were prehybridized as described (Flint et al., 1984). Prior to hybridization, radiolabeled RNA samples were precipitated with trichloroacetic acid and quantitated by liquid scintillation spectrometry. Radioactive RNAs were denatured by boiling for 10 min and then added to ice-cold hybridization mixtures as described (Flint et al., 1984). Prehybridizations were conducted at 65 °C for at least 6 h, while hybridizations were at the same temperature for 72 h. For a blot of 4 × 13 cm, the volume of prehybridization solution was 15 mL, while that of the hybridization solution was 10 mL. After hybridization, filters were treated to remove nonspecifically adhered radioactivity (Flint et al., 1984), without an RNase treatment, air dried briefly, and autoradiographed by using XAR-5 film and Du Pont Lightning-Plus screens. Quantitation of relative transcription rates was by densitometric analysis of autoradiograms. Amounts of plasmid DNAs on Southern blots were confirmed by rehybridization to homologous nick-translated gene probes, and the resulting autoradiograms were quantitated. Blots contained duplicate samples of at least one histone gene plasmid.

RESULTS

Involvement of Transcription in the Coupling of Histone mRNA Stability with DNA Replication. Initially, we addressed the extent to which transcription is involved in regulating cellular levels of histone message when DNA synthesis is inhibited. Cells were treated with hydroxyurea at a concentration that reduced DNA synthesis and histone mRNA levels by greater than 95% and 90%, respectively. Nuclei were isolated from treated cells and transcribed in vitro in order to assay histone gene expression. In parallel studies in which cells were treated with cycloheximide, we assessed the extent of the effect of protein synthesis inhibition on histone gene transcription.

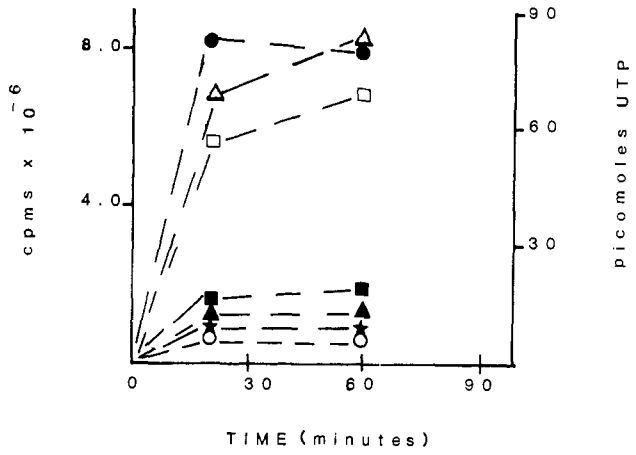

FIGURE 1: Synthesis of radiolabeled RNA by isolated nuclei in the presence of varying amounts of unlabeled ribonucleoside triphosphates. Nuclei were isolated from exponentially growing HeLa cells and transcribed in vitro. The effects of varying amounts of unlabeled UTP on incorporation of radiolabeled UTP were assayed and expressed either as the amount of radioactivity incorporated (closed symbols) or picomoles of total UTP incorporated per 10^7 nuclei (open symbols). The effects of 25 (triangles), 15 (squares), or 0 μM (circles) unlabeled UTP, as well as the effect of α-amanitin in the presence of 15 μM unlabeled UTP (stars), on transcription are shown.

The relative transcription rates of genes transcribed by RNA polymerase II can be determined with isolated nuclei in which previously initiated RNA chains are elongated during an in vitro incubation in the presence of [α-^{32}P]UTP [reviewed in Marzluff and Huang (1985)]. Isolation of nuclei, in vitro transcription reactions, and isolation of radiolabeled RNA were carried out as described under Materials and Methods. An initial characterization of the in vitro transcription system is shown in Figure 1, which demonstrates the results of a reaction using 1 × 10^7 nuclei in the presence of 50 μCi of [α-^{32}P]UTP for 60 min at 37 °C. The addition of ribonucleoside triphosphates (ATP, GTP, CTP) resulted in stimulation of incorporation of radiolabeled UTP. To optimize this incorporation, we assayed transcription in the presence of varying amounts of unlabeled UTP. Conditions were adopted that yielded transcripts with a specific activity of approximately 10^9 cpm/μg, to facilitate use in hybridization analysis. Nuclear transcription in the presence of α-amanitin at a concentration of 1 μg/mL indicated that approximately 45% of the radiolabeled RNAs were transcribed by RNA polymerase II, in agreement with previously reported studies (Detke et al., 1978; Clayton & Darnell, 1983; Sive et al., 1984; Marzluff & Huang, 1985).

Using this nuclear transcription system, we have observed that the decrease in histone mRNA levels which accompanies inhibition of DNA synthesis cannot be accounted for by changes in histone gene transcription alone. For these studies, exponentially growing HeLa cells were exposed to hydroxyurea (1 mM) and nuclei were isolated in duplicate. Duplicate transcription assays were performed for each sample; total radioactivity incorporated ranged from 2.0 × 10^6 to 8.8 × 10^6 cpm. The radiolabeled RNAs were isolated, and equivalent

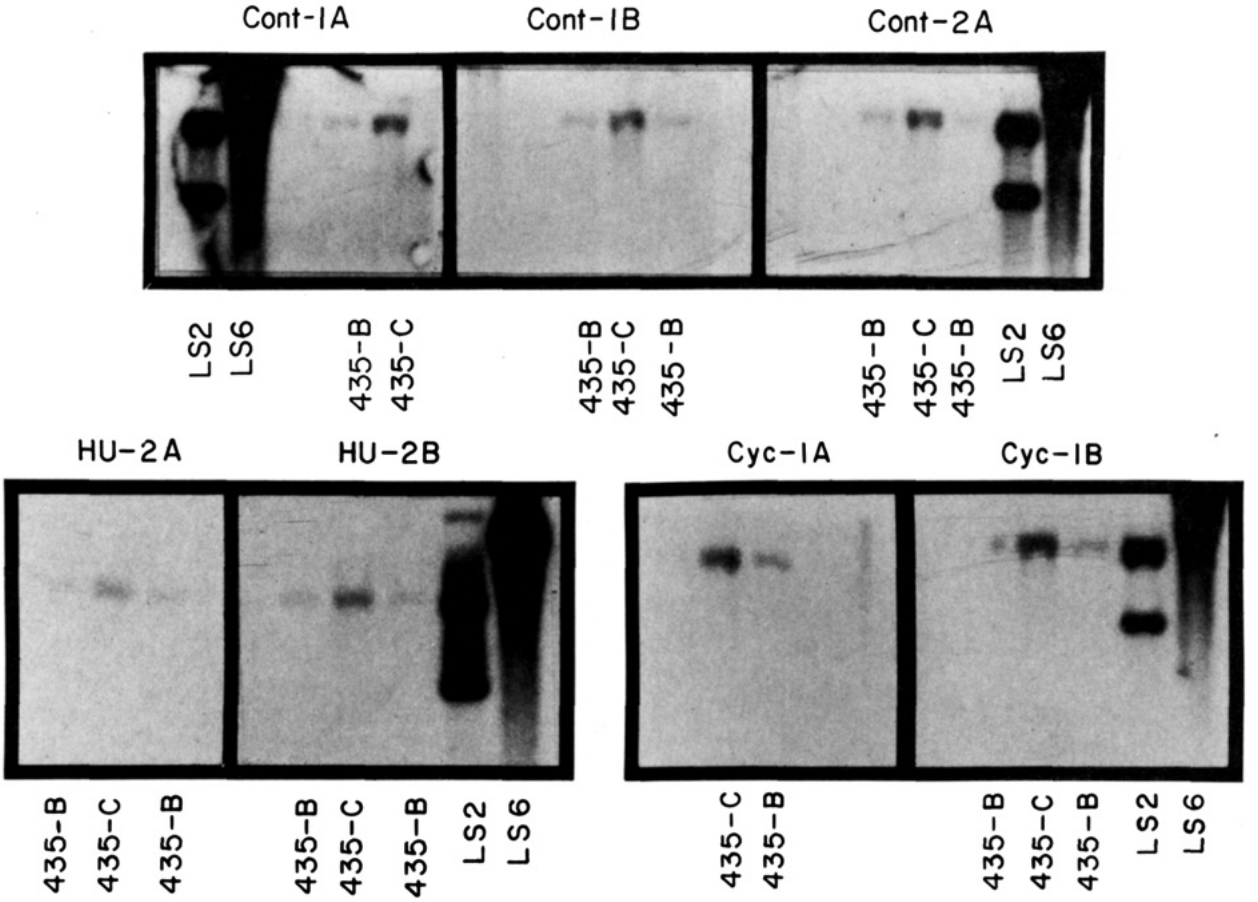

FIGURE 2: Analysis of histone gene transcription in the presence of metabolic inhibitors. Exponentially growing HeLa cells were exposed to cycloheximide or hydroxyurea for 90 min, and nuclei were isolated. Transcription reactions were conducted immediately following isolation of nuclei, and total RNA was isolated. Equivalent amounts of radioactive RNA (dpm) were hybridized to Southern blots containing human histone (435B = H2A + H2B; 435C = H3) and ribosomal (LS2 = 18S; LS6 = 28S) gene plasmids. Quantitation of results was performed as described under Materials and Methods, and a summary of the data is presented in Table I. Representative autoradiograms, corresponding to duplicate or triplicate samples isolated from control (Cont), hydroxyurea-treated (HU), or cycloheximide-treated (Cyc) cells, are shown.

amounts of acid-precipitable radioactivity from each sample (2×10^6 cpm) were hybridized to Southern blots of plasmids containing human H2A and H2B (pFF435B) and H3 (pFF435C) histone genes. The isolation and characterization of these genes have been reported (Sierra et al., 1982), and it has been demonstrated that they encode cell cycle dependent histone mRNAs (Plumb et al., 1983a; Marashi et al., 1986; D. Collart, unpublished results). Prior to electrophoretic fractionation and transfer to nitrocellulose, plasmids were cleaved with appropriate restriction enzymes to permit separation of the human histone gene sequences from the vector. The presence of the vector segment in the Southern blot served as an internal control for nonspecific hybridization. As an additional control, Southern blots included restriction enzyme digested plasmids containing human 18S (LS2) and 28S (LS6) rRNA genes to monitor transcripts for which rates of transcription and RNA stability are not dependent on DNA replication. The human ribosomal genes also permit an assessment of overall levels of transcription. The relative transcription rates are summarized in Table I, and representative autoradiograms are shown in Figure 2.

The minimal influence of DNA synthesis inhibition on histone gene transcription (~20%), together with a greater than 90% reduction in cellular histone mRNA levels, suggests that changes in the rate of histone mRNA turnover are primarily responsible for the coordinate and stoichiometric coupling of histone mRNA levels and rates of DNA synthesis in HeLa cells. Furthermore, because inhibition of RNA synthesis does not directly influence histone mRNA stability (Baumbach et al., 1984), predominant control of histone mRNA turnover at a posttranscriptional level is strongly implicated.

Table I: Histone Gene Transcription in the Presence of Hydroxyurea or Cycloheximide[a]

sample	transcription[b] H2A + H2B	transcription[b] H3	% of control[c]
control	2.4 ± 0.2	2.9 ± 0.3	100
hydroxyurea	2.0 ± 0.4	2.2 ± 0.5	80
cycloheximide	5.0 ± 0.5	5.9 ± 0.5	205[d]

[a] Exponentially growing HeLa cells were treated with hydroxyurea (1 mM) or cycloheximide (40 μM) for 90 min. For each experiment nuclei were isolated in duplicate, and duplicate transcription assays were carried out for each set of nuclei, as described under Materials and Methods. [b] These values represent the relative strengths of autoradiographic signals from hybridization to the histone gene probes (see Figure 2 for examples). Quantitation was by densitometric analysis of autoradiograms; values were normalized for the amount of DNA bound to the blots. [c] These values represent an average for H3 and H2A + H2B. [d] This value is not corrected for the effect of cycloheximide on ribosomal RNA transcription. Inhibition of protein synthesis does not appear to modify histone gene transcription significantly.

We have also observed that the stabilization and accumulation of histone mRNAs in the absence of protein synthesis do not appear to be associated with a substantial increase in histone gene transcription. Exponentially growing HeLa cells were treated with cycloheximide (40 μM), and histone gene transcription was assayed in isolated nuclei as described above. Within 90 min of cycloheximide treatment, there was an apparent increase of approximately 200% in histone gene transcription (Table I), accompanied by a reduction of approxi-

Table II: Histone Gene Transcription during the Cell Cycle

	x-fold increase over control[a]		
	435B/(H2A + H2B)	108A/H4	LS2/ribosomal
mitosis/early G1			
control, 9 h	1.0	1.0	1.0
control, 10 h	1.5 ± 0.6	1.5 ± 0.2	1.0 ± 0.2
control, 11 h	0.8 ± 0.2	1.5 ± 0.2	
cycloheximide, 1 h (postaddition at 9 h)	2.6 ± 0.7	2.0 ± 0.2	1.1 ± 0.2
cycloheximide, 2 h (postaddition at 9 h)	1.1 ± 0.4	2.2 ± 0.3	0.6 ± 0.1
control, 15 h	0.9 ± 0.2	1.2 ± 0.2	1.0 ± 0.1
S phase			
control, 19 h	2.6 ± 0.8	1.0 ± 0.1	0.8 ± 0.2
control, 20 h	3.4 ± 1	1.6 ± 0.2	1.1 ± 0.2
control, 21 h		2.9 ± 0.4	
control, 22 h	1.2 ± 0.4	0.9 ± 0.1	0.7 ± 0.1
control, 24 h	2.4 ± 0.7	1.4 ± 0.2	

[a] The relative transcriptional activity of each of these histone genes was measured throughout the cell cycle as explained for Figure 3 and expressed relative to values obtained from control samples at 9 h after release from thymidine block. Values shown were obtained by using 5 μg of each of the plasmid DNAs.

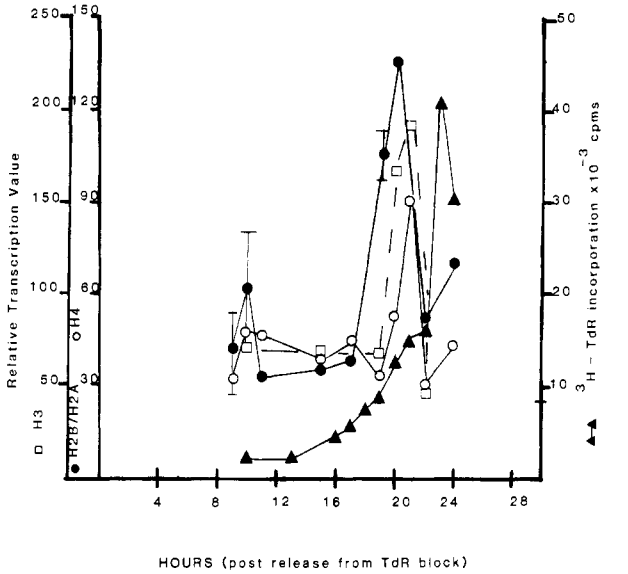

FIGURE 3: Cell cycle dependent transcription of histone genes. HeLa cells were synchronized as described under Materials and Methods, and nuclei were isolated at times indicated and used immediately for transcription reactions in the presence of 100 μCi of [α-^{32}P]UTP. Equal amounts of radioactive RNAs were hybridized to dot blots containing 10, 5, and 2.5 μg of histone gene containing plasmids. Quantitation of relative transcription rates was carried out as described under Materials and Methods. The relative rates of transcription, with 5 μg of plasmid DNA, are shown for H3 (□), H4 (○), and H2A/H2B (●). In parallel cultures, rates of DNA synthesis were assayed by using [^{3}H]thymidine and are expressed as acid-precipitable radioactivity incorporated (▲) as a function of time after release from thymidine block. Standard errors are obtained from analysis of duplicate blots at hours 9, 10, and 15.

mately 50% in ribosomal RNA synthesis (Table II and data not shown). Because ribosomal RNA transcription constitutes approximately 50% of total transcription in vitro, and hybridizations were performed by using equal amounts of radioactivity, the apparent 2-fold increase in histone gene transcription can be largely accounted for by the observed decrease in ribosomal RNA synthesis and, hence, an overrepresentation of histone mRNA sequences in in vitro nuclear transcripts from cycloheximide-treated cells.

In summary, modifications of histone mRNA levels that occur with changes in rates of DNA synthesis appear not to be dependent on rates of histone gene transcription. Rather, the changes in cellular histone mRNA levels that are observed when either DNA replication or protein synthesis is inhibited are primarily posttranscriptionally regulated.

Cell Cycle Analysis of Histone Gene Transcription. The contribution of transcriptional control to the temporal coupling of histone gene expression and DNA replication in HeLa cells was examined throughout the cell cycle in synchronized cells. As previously reported (Hereford et al., 1981; Plumb et al., 1983a,b; Heintz et al., 1983, Delisle et al., 1983), total cellular and cytoplasmic histone mRNA levels accumulate in parallel with rates of DNA synthesis upon entry of cells into S phase. However, little is known about the extent to which transcriptional and posttranscriptional processes contribute to the control of this accumulation. Also, histone mRNAs are destabilized at the end of S phase, accompanying completion of DNA replication, and the control of this relationship has yet to be defined. The contribution of transcriptional and posttranscriptional processes to the natural decline in histone mRNAs at the end of S phase was therefore analyzed. The results were compared with those obtained when DNA synthesis was inhibited.

HeLa cells were synchronized by double thymidine block, and aliquots of cells were harvested at intervals beginning at 10 h postrelease from thymidine block through the next S phase. To avoid potential ambiguities from synchronization with excess thymidine, sample isolation began after completion of the first S phase. Transcription reactions were conducted in isolated nuclei by using [α-^{32}P]UTP. Total radiolabeled RNA was isolated, and equal amounts of radioactivity were hybridized to Southern blots containing human histone and 18S ribosomal RNA gene plasmids. The saturability of the hybridization reaction was tested by placing differing amounts (2.5, 5, and 10 μg) of plasmid DNA on dot blots (data not shown). The results indicated that the conditions of DNA excess hybridization were achieved with 5 μg of plasmid DNA; therefore, this amount was used for analysis in subsequent experiments. To test whether any observed differences in histone gene transcription during the cell cycle were due to differences in the amount of plasmid DNA on Southern blots, corresponding blots were washed free of hybridized radiolabeled RNA and rehybridized with nick-translated histone gene plasmids. The amounts of plasmid DNAs on the blots were quantitated by densitometry. Transcription data were then normalized to any detected quantitative differences among the amounts of plasmid DNA.

The results from one such cell cycle experiment are shown in Figure 3 and Table II. Results from four independent experiments indicate that there is an increase in histone gene transcription that immediately precedes or coincides with the onset of S phase. DNA synthesis rates were minimal at 10 h after release from double thymidine block and gradually increased until hour 22, reaching a maximum at approximately hour 23. However, histone gene transcription for each of the core histone genes analyzed was maximal between hours 20 and 21, several hours before maximal DNA synthesis. There was only a 3–4.5-fold increase in the transcription of histone genes, in association with the onset of S phase. The patterns were the same for all histone genes analyzed. For comparison, the relative rate of transcription of 18S ribosomal RNA, as detected by hybridization analysis to a homologous plasmid, LS2, did not change significantly during progression through the cell cycle (Table II).

It is apparent from these results that histone gene transcription occurs throughout the cell cycle. However, significant cellular levels of mRNAs transcribed from cell cycle regulated

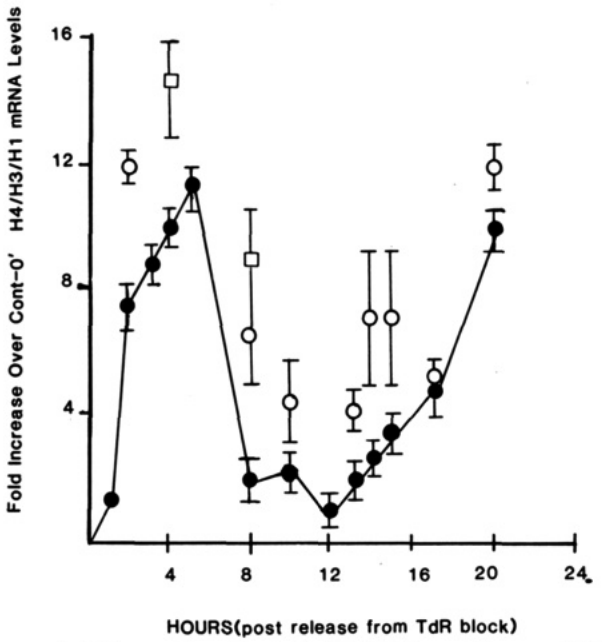

FIGURE 4: Influence of protein synthesis inhibition on histone mRNA steady-state levels during the cell cycle. Total cellular RNA was isolated at times indicated from synchronized HeLa cells, and histone mRNA levels were determined by Northern blot analysis as described under Materials and Methods. The results are expressed as *x*-fold increase over histone mRNA levels in thymidine-blocked cells, and as a function of time after release from thymidine block. The graphs represent a summary of at least three independent experiments and four blots per experiment. Analyses of H4, H3, and H1 mRNA levels in the absence (●) or presence (○) of cycloheximide or puromycin (□) at times after release from thymidine block are shown. Values for drug-treated samples are plotted at times corresponding to the end of the 2-h drug treatment.

histone genes accumulate only during S phase. Therefore, it can be inferred that histone mRNA is destabilized at times outside of S phase. Previous studies have shown that histone mRNAs are stabilized when protein synthesis is inhibited. Therefore, to investigate further the destabilization mechanism(s) involved in cell cycle dependent histone mRNA turnover, cells were treated with cycloheximide at different times during the cell cycle to analyze potential qualitative differences in protein synthesis dependent destabilization of histone mRNAs. Total cellular RNA was isolated from synchronized cells, as well as from cells treated with cycloheximide, and histone mRNA levels were analyzed by Northern blot hybridization using homologous human histone gene probes. Densitometric analysis of at least seven blots representing three experiments was performed, and the data were expressed relative to results from thymidine-blocked cells (Figure 4). H4, H3, and H1 mRNA levels increased approximately 10-fold during the first and second S phases after release from thymidine block. The pattern of histone mRNA accumulation and decline followed that of DNA synthesis (data not shown).

In parallel studies, cycloheximide (10 μg/mL) or puromycin (0.4 mM) was added for a period of 2 h to cells at 0, 2, 6, 8, 10, 11, 12, 13, 15, and 17 h postrelease from thymidine block. Total cellular RNA was isolated, and histone mRNA levels were analyzed as above. The data obtained from cells at the end of the 2-h inhibitor treatment are expressed relative to values obtained from prereleased cells. As evident from Figure 4, a general pattern appears in which there is a greater relative stabilization of histone mRNAs in the presence of protein synthesis inhibitors at times outside of S phase (8–15 h) than at times when DNA synthesis and histone mRNA levels are approaching maximal levels (2, 4, 17, and 19 h). These results further support a protein synthesis dependent turnover event operative on histone mRNA levels during the cell cycle and, most importantly, in controlling availability of histone mRNAs at times outside of S phase.

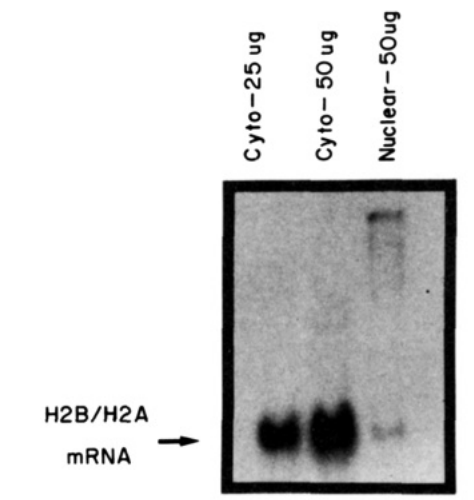

FIGURE 5: Subcellular representation of histone mRNAs. Total cytoplasmic and nuclear RNAs were isolated from exponentially growing HeLa cells as described under Materials and Methods. Histone mRNA levels were determined by Northern blot analysis using pFF435B (H2A + H2B histone genes) as a probe. The autoradiogram reflects H2A/H2B mRNA levels in 25 and 50 μg of cytoplasmic RNA and 50 μg of nuclear RNA.

In summary, the results presented in Figures 3 and 4 and Table II demonstrate that there is a transcriptional component to the control of coupled histone gene expression and DNA replication. However, the increase in histone gene transcription at the onset of S phase is minimal (3–5-fold) compared with the increase in rates of DNA synthesis and histone mRNA steady-state levels (10–30-fold). In agreement with earlier results (Plumb et al., 1983a,b), the peak of transcription precedes the peak of DNA synthesis. These results also indicate that there is a strong posttranscriptional component to the regulation of cellular histone mRNA levels, which involves selective degradation of histone mRNAs and may include variations in the stability of histone mRNAs during the cell cycle.

Subcellular Localization of Histone mRNA. To elucidate further the cellular mechanisms involved in coupling of histone mRNA stability and rates of DNA synthesis, we analyzed the subcellular localization of histone mRNA as a function of cell cycle progression. In addition, we studied the effects of protein synthesis inhibition on subcellular levels of histone mRNA. Total cellular, cytoplasmic, and nuclear RNAs were isolated as described under Materials and Methods. Histone mRNAs were detected by Northern blot analysis, and quantitation was performed by densitometric analysis. Normalization of the densitometric values to actual amounts of RNA on the blots was performed with 18S and 28S RNAs as quantitation standards as described under Materials and Methods. A representative autoradiogram of nuclear and cytoplasmic histone mRNA levels is shown in Figure 5. Of note is the limited representation of histone mRNA sequences in nuclear RNA, which is less than 3% of that detected in an equal amount of cytoplasmic RNA. A more exact quantitation was not possible due to the limit of sensitivity of the densitometer used in these studies.

In the following experiments, HeLa cells were synchronized by double thymidine block and total cellular, cytoplasmic, and nuclear RNAs were isolated at various times after release from

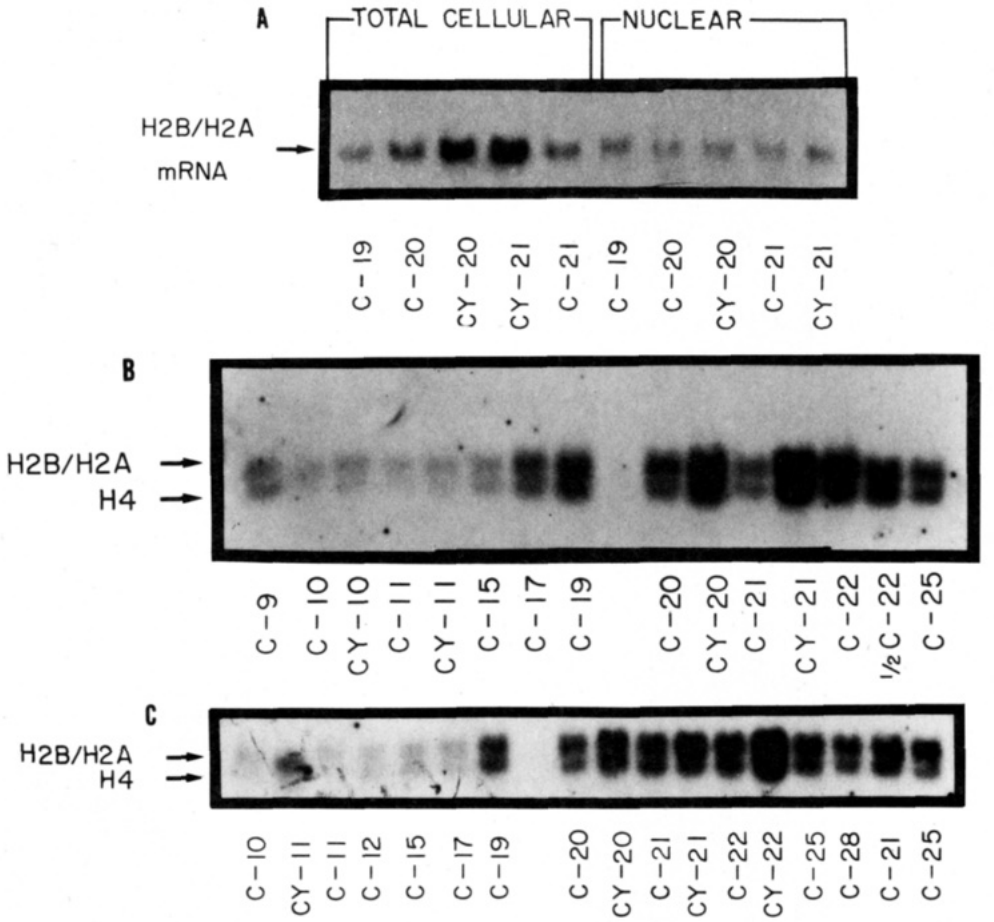

FIGURE 6: Subcellular analysis of histone mRNA levels during the cell cycle. HeLa cells were isolated at various times after release from thymidine block, and total cellular, cytoplasmic, and nuclear RNAs were isolated as described under Materials and Methods. Equal amounts of the RNA preparations (50 μg unless indicated) were electrophoretically fractionated and transferred to nitrocellulose. The RNAs were then hydridized to radiolabeled human histone gene probes. Representative autoradiograms are shown. In parallel cultures, cycloheximide (40 μM) was added at hours 9 and 19 for 1 and 2 h of treatment. (A) Cell cycle dependent changes in total cellular and nuclear H2B + H2A histone mRNAs in the absence (C) or presence of cycloheximide (CY). (B) Periodic accumulation of H4, H2A, and H2B total cellular mRNAs after release from double thymidine block. (C) Cell cycle dependent alterations in H4, H2A, and H2B histone mRNA levels in the cytoplasm of synchronized cells.

thymidine block. In parallel cultures, cycloheximide was added at 9 and 19 h (G2-M and early S phase, respectively), and cells were harvested after 1 or 2 h in the presence of the drug. The RNA preparations were electrophoretically separated and transferred to nitrocellulose paper, and histone mRNA levels were detected by hybridization to radiolabeled human histone gene plasmids. Representative autoradiograms are shown in Figure 6, and quantitative analysis of histone mRNA levels is displayed in Figure 7.

Figure 6A displays the cell cycle dependent accumulation of total cellular and nuclear histone mRNA levels in control cells as a function of progression into S phase. In the presence of cycloheximide, nuclear and total cellular histone mRNAs are stabilized. The average increase in histone mRNA levels over the corresponding untreated controls after the addition of the inhibitor is approximately the same for nuclear and total cellular histone mRNA levels at hours 20 and 21 (1.5-fold and 2.5-fold at hours 20 and 21, respectively), despite the apparent difference in the relative representation of histone mRNA sequences in these two RNA preparations. Total cellular histone mRNAs accumulate in coordination with an increase in the rate of DNA synthesis (see Figure 7A), whereas nuclear levels demonstrate only a maximal 4-fold accumulation compared with non S phase levels, and this increase occurs 1–2 h before the peak in DNA synthesis (Table III). This pattern is further emphasized in another representative autoradiogram (Figure 6B), which displays the cell cycle dependent accumulation of H4, H2A, and H2B total cellular mRNAs. Quantitation of these autoradiograms is displayed in Figure 7. Figure 6C is an autoradiogram that demonstrates the cell cycle changes in cytoplasmic histone mRNAs from a second independent experiment. Cytoplasmic histone mRNA levels followed the previous pattern of total cellular histone mRNA, increasing as much as 25-fold (as compared to non S phase levels) coordinate with maximal DNA synthesis (Figure 7A).

Table III: Cellular Levels of Histone mRNA during the Cell Cycle

sample[a]	total cell[b]	cytoplasmic[b]	nuclear[c]
C, 10 h[d]	1.0	1.0	1.0
CY, 10 h[e]	6.0 ± 2		
C, 11 h	1.2 ± 0.2	1.4 ± 0.4	1.0
CY, 11 h[f]	7.4 ± 2	5.7 ± 2	
C, 19 h	12 ± 2	10 ± 2	2.6 ± 0.6
C, 20 h	11 ± 4	12 ± 4	5.0 ± 0.5
CY, 20 h[e]	14 ± 3	22 ± 5	8.5 ± 0.8
C, 21 h	9.6 ± 4	20 ± 5	4.0 ± 1
CY, 21 h[f]	25 ± 7	32 ± 15	9.4 ± 3

[a] C = control; CY = cycloheximide. [b] Values are presented as the x-fold increase over histone mRNA levels determined in control samples isolated at 10 h after release from thymidine block and represent an average of two independent subcellular fractionations, corresponding to values shown in Figure 7. [c] Average of values obtained from two separate samples in one subcellular fractionation experiment; see Figure 7. [d] Sample designation is the same as that used in Figure 6. [e] Samples were isolated 1 h after addition of cycloheximide. [f] Samples were isolated 2 h after addition of cycloheximide.

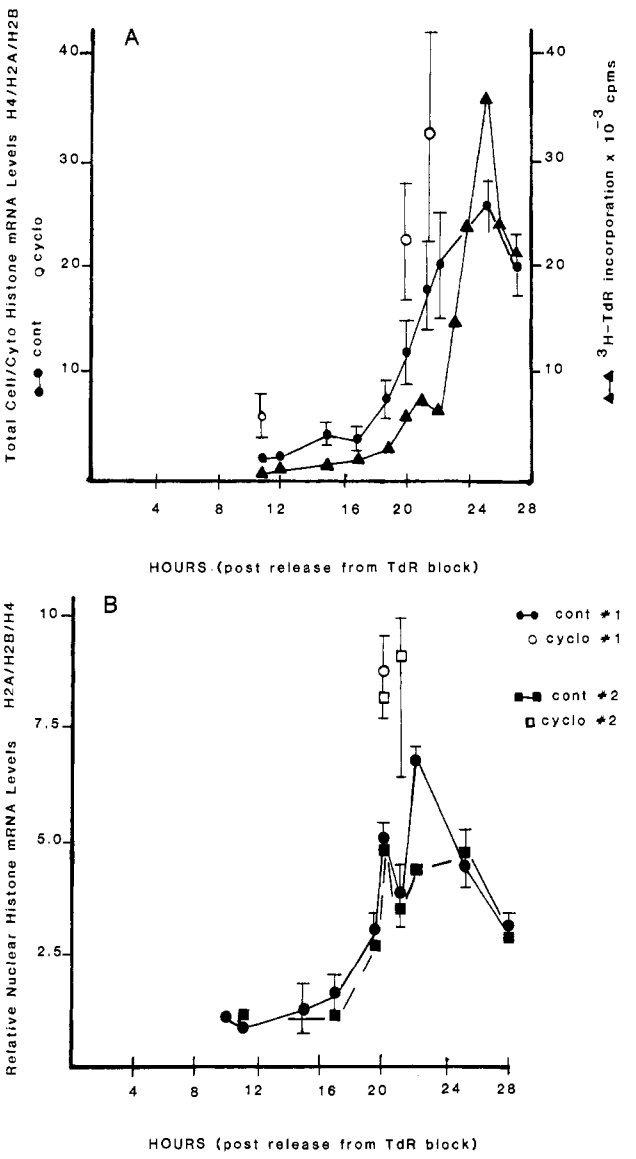

FIGURE 7: Summary of accumulation of total cellular, cytoplasmic, and nuclear histone mRNAs during the cell cycle. HeLa cells were synchronized by double thymidine block, and total cellular, cytoplasmic, and nuclear RNAs were isolated as described under Materials and Methods. Histone mRNA levels were detected by Northern blot analysis using radiolabeled human histone gene plasmids. Autoradiographic data were adjusted for minor differences in the amounts of RNA samples by normalization of the hybridization data to the amount of 28S and 18S RNAs in the RNA samples as described under Materials and Methods. Steady-state levels of histone mRNA are expressed relative to levels at 10 h after release from thymidine block (G1). (A) Cytoplasmic and total cellular mRNAs (●) increase coordinately with rates of DNA synthesis (▲) upon entry into S phase. Also presented are histone mRNA levels in the presence of cycloheximide (○). (B) Nuclear histone mRNA levels were detected and quantitated as described under Materials and Methods. Analyses of two blots are presented in the panel. Control, closed symbols; cycloheximide-treated, open symbols.

Table III indicates that cytoplasmic histone mRNAs are also stabilized in the presence of cycloheximide, demonstrating a slightly greater accumulation after treatment in G1 (approximately 3-fold at hour 11) than in the beginning of the next S phase (approximately 1.5-fold at hours 20 and 21), when compared with untreated control histone mRNA levels at the same times.

In summary, total cellular and cytoplasmic (Figure 7A) histone mRNA levels follow the pattern of DNA synthesis during the cell cycle, demonstrating approximately 20–25-fold accumulation coordinate with peak DNA synthesis. However, nuclear histone mRNA levels (Figure 7B) only increase approximately 4–5-fold compared with non S phase levels, and this accumulation precedes the peak of DNA synthesis. Also apparent from these experiments is the stabilization of cytoplasmic and total cellular histone mRNAs in the absence of protein synthesis (Figure 7A and Table III), with a slightly greater increase over untreated control histone mRNA levels in G1 (hour 10–11) than in early S phase (hour 19–20). Nuclear histone RNA levels also accumulate in the presence of cycloheximide (Figure 7B and Table III).

Discussion

The results reported here further clarify the transcriptional component of the coupled relationship between histone mRNA metabolism and rates of DNA synthesis in mammalian cells (DeLisle et al., 1983; Plumb et al., 1983a,b; Baumbach et al., 1984; Heintz et al., 1983). The objectives of these studies were twofold: first, to analyze the contribution of histone gene transcription to the observed decrease in histone mRNA levels when DNA synthesis is decreased, and to the stabilization of histone mRNA levels in the absence of protein synthesis; and second, to determine the temporal and quantitative changes in histone gene transcription as a function of progression through the cell cycle. In doing so, we measured the relative transcription rates of cell cycle dependent histone genes in isolated nuclei under a variety of metabolic conditions.

Our laboratory (Stein & Borun, 1972; Plumb et al., 1983a,b; Baumbach et al., 1984; Helms et al., 1984) and others (Hereford et al., 1981, 1982; Heintz et al., 1983; Sittman et al., 1983; Stimac et al., 1983; Graves & Marzluff, 1984) have previously reported that cellular levels of histone mRNA are regulated at both transcriptional and posttranscriptional levels. The experiments presented here suggest different roles for these levels of control. Transcriptional control appears to be of primary importance in the initial accumulation of histone mRNAs as the cell enters DNA synthesis. The synthesis of histone mRNAs for the eventual synthesis of histone proteins is essential for packaging of newly replicated DNA into chromatin during the S phase. Conversely, posttranscriptional control appears to be of major importance in regulating the stability and availability of histone mRNAs for translation into histone proteins during the cell cycle.

It has been demonstrated that hydroxyurea (HU) (1 mM) inhibited net transcription by approximately 20%, whereas it produced a dramatic reduction in the rate of DNA synthesis and histone mRNA levels, to approximately 2% and 8% of control levels, respectively (Baumbach et al., 1984). However, in the presence of HU at this concentration for as long as 90 min, an equal level of inhibition of histone gene transcription was not detected. These results are in agreement with earlier studies using indirect assays (Stein et al., 1978), in addition to initial observations that, in the presence of actinomycin D (Act-D) (a transcription inhibitor) and HU, histone mRNA levels decrease with similar kinetics as in HU treatment alone (Baumbach et al., 1984). These results indicate that transcription of histone genes, as well as transcription in general, is not required for the rapid turnover of histone mRNAs in the presence of hydroxyurea. The combined results are consistent with posttranscriptional control of histone mRNA degradation coordinate with reduced rates of DNA synthesis.

In contrast to these results are those obtained from other in vitro transcription experiments (Sittman et al., 1983; Sive et al., 1984; DeLisle et al., 1983), which used high concentrations of DNA synthesis inhibitors and indicated a significant inhibitory effect on histone gene transcription. However, the decrease in histone gene transcription could only partially

account for the more dramatic decrease in vivo of histone mRNA steady-state levels, leaving unresolved the extent to which transcriptional changes mediate this reduction in histone mRNA levels.

Experiments in which cells were exposed to cycloheximide, a protein synthesis inhibitor, showed an apparent relative increase in histone gene transcription, assayed by using isolated nuclei. These results have also been observed by other laboratories (Graves & Marzluff, 1984; Sive et al., 1984). However, we have shown that the relative rate of ribosomal RNA transcription decreased by approximately 50%. Therefore, in the presence of cycloheximide there is an overrepresentation of histone and other nonribosomal transcripts in the hybridization reactions, since equal amounts of radioactivity were used. Ribosomal gene transcription per se was not measured in other studies, although the relative rates of transcription of other polymerase II (Pol II) genes did increase in the presence of cycloheximide. The inhibitory effect that cycloheximide exerts on ribosomal gene transcription has been independently reported (Gokal et al., 1986).

To further assess the control of histone mRNA metabolism during the cell cycle in HeLa cells, the in vitro transcription rates of histone genes in synchronized cells were analyzed. Earlier in vivo experiments (Hereford et al., 1981, 1982; Heintz et al., 1983; Plumb et al., 1983a,b) suggested that histone gene transcription was periodic, occurring at the beginning of S phase. However, these measurements were complicated by the use of indirect assays to measure transcription rates of histone genes. Pulse-labeling studies conducted in our laboratory indicated that the peak of histone gene transcription occurred in the first 2 h of S phase (Plumb et al., 1983a). The results presented here from several cell synchronization experiments suggest that histone gene transcription increases only 3–5-fold at the onset of DNA synthesis and occurs to a substantial degree throughout the cell cycle. Assessment of the temporal coupling of histone gene transcription and DNA replication indicated that the peak of transcription may precede the peak of DNA replication. However, an absolute time was difficult to determine due to the decay of synchrony of the cell population.

In contrast to the minimal increase in histone gene transcription at the onset of S phase, total cellular histone mRNA levels increased in parallel with rates of DNA synthesis, reaching a maximal 25-fold increase over non S phase levels. Therefore, cell cycle dependent changes in total cellular histone mRNA levels appear not to be explained by changes in histone gene transcription alone.

To determine at which subcellular location posttranscriptional control of histone mRNAs might be operative, histone mRNA levels in the total cell, nucleus, and cytoplasm were analyzed as a function of progression through the cell cycle. The results suggested that changes in total cellular RNA were best reflected in the cytoplasmic levels of histone mRNA. Cytoplasmic and total cellular histone mRNAs were stabilized in the presence of cycloheximide, and the degree of histone message accumulation was slightly greater in non S phase cells. Changes in nuclear histone mRNA levels appear not to be as cell cycle dependent as are changes in cytoplasmic levels. Nuclear histone mRNA levels increased only approximately 4-fold during S phase and apparently reflect transcriptional changes of cell cycle dependent histone genes. Cytoplasmic and total cellular levels more accurately reflect the posttranscriptional control of histone mRNA stability and availability.

In summary, this study attempts to delineate the multiple levels of control of histone RNA metabolism. Transcriptional control can be interpreted as serving an important role in the activation of histone gene expression as a cell progresses into S phase. The observed enhancement of histone gene transcription occurs in conjunction with the onset of S phase. The return of histone gene transcription to constitutive levels occurs when the genes replicate in early/mid S phase (Iqbal et al., 1984), and this rate of transcription persists throughout the cell cycle. Posttranscriptional control may serve a different role in controlling the availability of histone mRNAs for protein synthesis. A protein synthesis dependent turnover of cytoplasmic histone mRNAs is suggested in non S phase cells and may mediate the abundance of total cellular histone mRNAs coordinate with rates of DNA synthesis during the cell cycle.

The concept of posttranscriptional control of eukaryotic mRNA stability is not novel. Much evidence has accumulated in the past few years for control that requires protein synthesis as an important regulatory mechanism for eukaryotic gene expression. Eukaryotic message levels that appear to be controlled at a posttranscriptional level include heat shock mRNAs in *Drosophila* (Storti et al., 1980) and mRNAs that encode ribosomal proteins during development (Al-Atia et al., 1985; Pierandrei-Amaldi et al., 1985a,b). Autoregulation of mRNA stability at a level requiring protein synthesis has also been demonstrated for several messages including a major heat shock message, HSP 70, in *Drosophila* cells (DiDomenico et al., 1982) and tubulin mRNAs from several species (Cleveland et al., 1981; Baker et al., 1986).

Evidence has also accumulated for similar patterns of transcriptional activation and posttranscriptional control of mRNA stability for other genes involved in cellular proliferation, including dihydrofolate reductase (Leys & Kellems, 1981), thymidine kinase (Groudine & Casimir, 1984), and the *c-myc* protooncogene (Dani et al., 1984; Thompson et al., 1986). However, the regulation of these cell cycle dependent genes, as well as thymidylate synthase (Jenh et al., 1985), is slightly different in that their S phase mRNA levels remain elevated in the presence of DNA synthesis inhibitors.

The results presented in this paper are consistent with an autoregulatory model for histone gene expression. Such a model was initially proposed by Butler and Mueller (1973), invoking feedback to histone gene transcription. However, our earlier results (Baumbach et al., 1984) and findings presented in this paper support posttranscriptionally mediated regulation of histone mRNA stability as a key element in determining cellular histone mRNA levels. The specific mechanisms by which histone mRNAs are selectively destabilized in a cell cycle dependent manner remain to be established and will provide insight into the coupling of histone gene expression and DNA replication.

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