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Reassessment of histone gene expression during cell cycle in human cells by using homologous H4 histone cDNA

(mRNA/cell synchronization/hybridization)

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ABSTRACT The representation of H4 histone mRNA sequences in RNAs isolated from G_1 and S phase HeLa cells was assessed by use of a homologous H4 histone cDNA. S phase cells were obtained by double thymidine block, and G_1 cells were obtained by double thymidine block or mitotic selective detachment. Nuclear and cytoplasmic RNAs from S phase cells hybridized with H4 histone cDNA as did nuclear and cytoplasmic RNAs from G_1 cells synchronized by double thymidine block. In contrast, significant levels of hybridization were not observed between H4 histone cDNA and nuclear, polysomal, or postpolysomal cytoplasmic RNAs of G_1 cells synchronized by mitotic selective detachment. Double thymidine block yields a G_1 cell population containing 20–25% S phase cells whereas the G_1 population obtained by mitotic detachment contains <0.1% S phase cells. The ability of H4 histone cDNA to hybridize with the RNAs from G_1 cells obtained after release from double thymidine block can therefore be explained by the presence of S phase cells in such a G_1 population—an artifact of the synchronization procedure. We interpret these results to be consistent with the presence of H4 histone mRNA sequences during the S but not G_1 phase of the cell cycle in continuously dividing HeLa S_3 cells.

Several lines of evidence suggest that, in continuously dividing HeLa S_3 cells, expression of histone genes is restricted to the S phase of the cell cycle. It has been known for some time that histone synthesis and DNA replication occur concomitantly in this as well as in several other mammalian cell lines (1–3). Additionally, *in vitro* translation (4–8) and nucleic acid hybridization data (9) indicate that translatable histone mRNAs are associated with cytoplasmic polysomes only during S phase. A functional relationship between histone synthesis and DNA replication is suggested by the rapid shutdown of histone synthesis and the loss of histone mRNA from the polysomes after treatment of HeLa cells with inhibitors of DNA synthesis such as hydroxyurea, 1-β-D-arabinofuranosylcytosine (cytosine arabinoside), or amethopterin (1–3).

To assess the level(s) at which regulation of histone gene expression resides, we previously analyzed *in vivo* synthesized RNAs isolated from various intracellular compartments of G_1 and S phase HeLa cells for the presence of histone mRNA sequences. In those experiments, G_1 cells were obtained by mitotic selective detachment—a synchronization procedure that yields a population containing <0.1% S phase cells. Using RNA excess hybridization to a homologous ^{3}H-labeled cDNA probe complementary to HeLa cell H2A, H2B, H3, and H4 histone mRNAs, we observed histone mRNA sequences in the nucleus, on the polysomes, and in the postpolysomal cytoplasmic fractions of S phase HeLa cells but not of G_1 cells (9–11). These results are consistent with control of histone gene expression being mediated at least in part at the transcriptional level. Other results from *in vitro* nuclear (11) and chromatin transcription (12–14) experiments, despite their technical limitations, are also consistent with S phase-specific transcription of histone sequences.

However, Melli *et al.* (15, 16) recently reported that a high molecular weight precursor of histone mRNAs is present in G_1 as well as S phase HeLa cells. These results are from studies in which G_1 and S phase HeLa cells, synchronized by double thymidine block, were pulse-labeled with [^{3}H]uridine and hybridized with cloned sea urchin (*Psammechinus miliaris*) histone DNA.

To resolve this apparent conflict in results, we have reinvestigated the levels of histone mRNA sequences during the cell cycle of HeLa cells by using a ^{3}H-labeled DNA complementary to a single histone mRNA species—one of the HeLa cell H4 histone mRNAs. The representation of H4 histone mRNA sequences in S phase cells and in G_1 cells synchronized by mitotic selective detachment or by double thymidine block was measured. In agreement with our previous results, H4 histone mRNA sequences were observed in the nucleus and cytoplasm of S phase cells but not in the nucleus or cytoplasm of G_1 cells obtained by mitotic selective detachment. However, when cells were synchronized by double thymidine block, a procedure that yields a G_1 population containing 20–25% S phase cells, we were able to detect hybridization of H4 histone cDNA to "G_1" nuclear and cytoplasmic RNAs. Our results suggest that significant levels of histone H4 mRNA sequences are not present during the prereplicative period of the cell cycle in continuously dividing HeLa cells. The apparent presence of histone mRNA sequences in G_1 cells obtained by double thymidine block can be explained as an artifact of the cell synchronization procedure.

MATERIALS AND METHODS

Isolation of H4 Histone mRNA. Growth and synchronization of HeLa S_3 cells have been reported (3). Total polysomal RNA from 100 liters of S phase HeLa cells was fractionated on 5–30% sucrose gradients containing 0.1% sodium dodecyl sulfate/0.1 M NaCl/10 mM Na acetate/1 mM EDTA, pH 5.4, in a Beckman Ti 14 zonal centrifuge rotor. For identification of histone mRNA species by autoradiography, ^{32}P-labeled RNA was prepared by incubating 5×10^8 S phase HeLa cells at a concentration of 2×10^6 cells/ml in phosphate-free growth medium containing 2% dialyzed calf serum and 50 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{32}P]PO_4. The 7–12S fraction of the polysomal RNAs was electrophoresed on a 0.3 × 16 × 30 cm slab gel by a modification of the gel and buffer system of Grunstein *et al.* (17). RNA bands were located and excised with a scalpel (18) and were eluted from gel slices electrophoretically (17).

***In Vitro* Translation.** *In vitro* translation was carried out in the presence of [^{3}H]lysine (200 μCi/ml; 38 Ci/mmol) by using a cell-free protein synthesizing system derived from wheat germ according to a modification of the method of Roberts and

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Paterson (19). Details of this procedure have been reported (20).

Preparation of H4 Histone cDNA. The reaction mixture for preparation of H4 histone cDNA from polyadenylylated H4 histone mRNA contained the following in 135 μl: 5 μg of polyadenylylated H4 histone mRNA, 50 mM Tris (pH 8.0), 30 mM KCl, 0.1 mM EDTA, 20 mM dithiothreitol, 9 mM $MgCl_2$, 1 mM dATP, 1 mM dTTP, 0.19 mM dGTP (19 Ci/mmol), 0.20 mM dCTP (18 Ci/mmol), 20 μg of dT_{10} per ml, 210 units of reverse transcriptase per ml, and 30 μg of actinomycin D per ml.

RNA Extraction. Subcellular fractionation of G_1 and S phase HeLa S_3 cells yielding nuclear, polysomal, and postpolysomal cytoplasmic fractions and RNA extractions were carried out as reported (9).

Nucleic Acid Hybridization. H4 histone [^{3}H]cDNA and RNA fractions were hybridized in RNA excess at 58°C as described (12), and the amount of nuclease S1-resistant, trichloroacetic acid-precipitable material was determined (12).

RESULTS

To identify and quantitate histone mRNA sequences in the nucleus and cytoplasm of G_1 and S phase HeLa cells, we synthesized a single-stranded DNA that was ^{3}H-labeled and complementary to one of the HeLa cell H4 histone mRNAs. We have reported elsewhere (ref. 21) the presence of multiple forms of H4 histone mRNAs in S phase HeLa cells, which translate identical H4 histone polypeptides *in vitro*. The cDNA used primarily in the present studies was synthesized by using the lower molecular weight H4 histone mRNA (386 nucleotides and designated H4-1) as a template.

H4-1 Histone mRNA. H4-1 histone mRNA was isolated by sucrose gradient fractionation followed by polyacrylamide gel electrophoresis. When the H4-1 histone mRNA was electrophoresed under denaturing conditions in two different systems, it migrated as a single band. Fig. 1*A* shows the electrophoretic profile of HeLa cell H4-1 histone mRNA in a 98% formamide gel (22). In Fig. 1*B* the electrophoretic profile of H4-1 histone mRNA in a 6% acrylamide/10 mM glyoxal gel is shown along with *Hin*dIII fragments of ^{32}P-labeled simian virus 40 DNA present in an adjacent well of the slab gel during electrophoresis and used as molecular weight markers. Because the molecular weights of DNA and RNA are directly comparable in glyoxal gels (23), it can be calculated that HeLa cell H4-1 histone mRNA contains 386 nucleotides—approximately 80 nucleotides (20%) more than required to code for H4 histone.

To assess further the purity of the H4-1 histone mRNA, translation was carried out in a wheat germ cell-free protein synthesizing system containing [^{3}H]lysine. The amount of [^{3}H]lysine incorporated into hot acid-resistant, trichloroacetic acid-precipitable material was proportional to the amount of RNA added to the translation assay system. The products of translation were electrophoresed according to charge and molecular weight on acetic acid/urea polyacrylamide gels in the presence of unlabeled HeLa cell histones as markers. No preliminary purification to separate the histones from other translation products was carried out prior to electrophoresis. The gel was prepared for fluorography according to the method of Laskey and Mills (27) and the resulting fluorogram was scanned with a Joyce–Loebl densitometer. The labeled translation product comigrates with marker HeLa H4 histone (Fig. 1*C*). Peak WG is a wheat germ protein (based on its translation in the absence of added mRNA), and the fast migrating material in band A apparently represents incomplete polypeptides. On the basis of the assumption that each of the protein products has a similar specific activity and that the area of each peak is proportional to the amount of radioactivity in the gel band, planimetric integration indicates that >98% of the material coded for by the H4-1 histone mRNA preparation is H4 histone. Purity of the HeLa cell H4-1 translation product was confirmed by two-dimensional tryptic peptide mapping (data not shown). Further characterization of the H4-1 histone mRNA has included two-dimensional T1 oligonucleotide mapping (unpublished data). We have also determined that H4-1 histone mRNA contains 5′-terminal capped structures of the types $m^7GpppXmY$ and $m^7GpppX_mY_mZ$, lacks poly(A) at the 3′ terminus, and does not contain internal methylated bases (unpublished data).

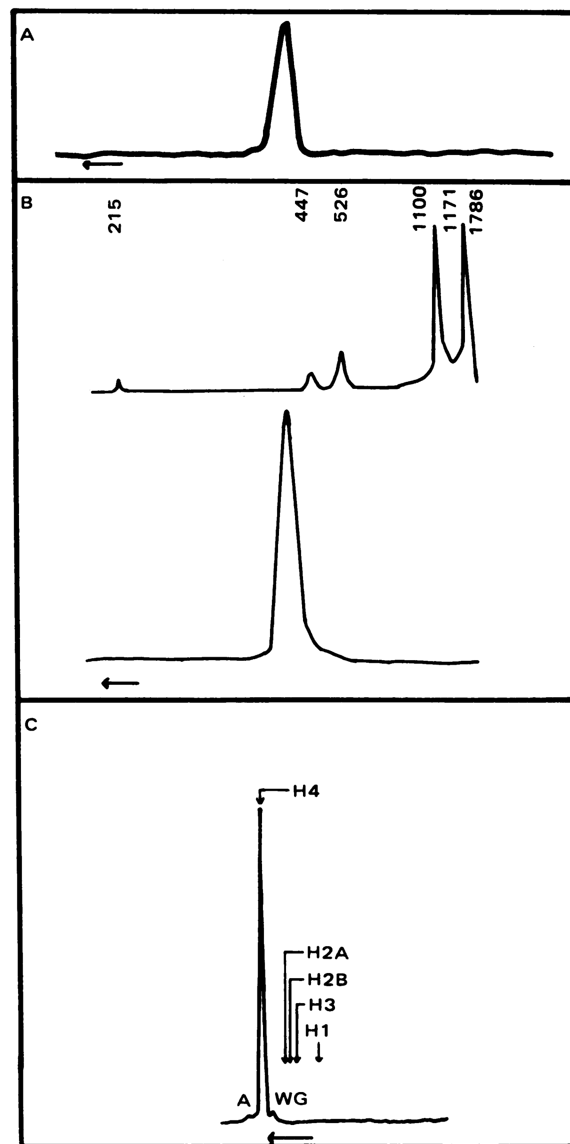

FIG. 1. Electrophoretic analysis of H4-1 histone mRNA and its translation products. (*A*) ^{32}P-Labeled H4-1 mRNA was isolated as described (ref. 21) and electrophoresed in an 8% acrylamide/98% formamide gel (22). The gel was analyzed autoradiographically and the film was scanned with a densitometer. (*B*) ^{32}P-Labeled H4-1 mRNA was electrophoresed on a 6% acrylamide gel in the presence of 10 mM glyoxal (23). Lower curve, H4 mRNA; upper curve, *Hin*dIII digest of simian virus 40 [^{32}P]DNA (sizes of nucleotides are indicated). (*C*) H4 mRNA was translated in the presence of [^{3}H]lysine (200 μCi/ml; 38 Ci/mmol) in a cell-free protein synthesizing system derived from wheat germ. An aliquot (15 μl) of the assay mixture was electrophoresed in an acetic acid/urea gel (24, 25) in the presence of marker HeLa histones, and fluorography was performed as described (26, 27). The direction of migration is indicated by the arrows.

H4-1 Histone cDNA. Because it lacks poly(A) at the 3′ terminus, H4-1 histone mRNA is ineffective as a template for

dT_{10}-primed transcription with RNA-dependent DNA polymerase. AMP residues were therefore added enzymatically to the 3′ termini of H4-1 histone mRNAs with ATP-polynucleotidyl exotransferase as described by Mans and Huff (28). After poly(A) addition to H4-1 histone mRNA in the presence of [^{14}C]ATP, 89% of the incorporated radioactivity bound to oligo(dT)-cellulose. The average size of the poly(A) chain added to various H4-1 histone mRNA preparations was in the range 12–22 AMP molecules per molecule. Using ^{32}P-labeled RNA we have observed that, after polyadenylylation, 73% of the radiolabeled RNA bound to oligo(dT)-cellulose. These results indicate that the majority of the H4-1 histone mRNAs are polyadenylylated to a length sufficient for binding to oligo(dT)-cellulose and argue against selective polyadenylylation of any minor RNA species in the H4-1 histone mRNA preparation.

The size of the ^{3}H-labeled H4-1 histone cDNA was determined by electrophoresis under denaturing conditions in 6% polyacrylamide/10 mM glyoxal gels. Electrophoresis was carried out with ^{3}H-labeled *Hae* III fragments of ϕX174 present in an adjacent well as molecular weight standards. A substantial portion of the cDNA preparation was approximately 400 nucleotides long, indicating that 30–40% of the H4-1 cDNA was full length copy; no significant amounts of cDNA larger than the H4-1 mRNA template were present (Fig. 2). As observed by others (29), several molecular weight classes of less than full-length copy size are present in the cDNA preparation, possibly due to termination by reverse transcriptase at sites of secondary structure in the H4-1 mRNA.

The H4-1 histone cDNA is totally sensitive to micrococcal nuclease but exhibits a somewhat high resistance to nuclease S1. Under standard nuclease S1 digestion conditions, 10–15% of the acid-precipitable H4-1 cDNA is nuclease resistant. This observation is consistent with the high level nuclease S1 resistance of protamine cDNA (20%) which has been shown to have a high secondary structure (30). Alternatively, the high nuclease resistance in cDNAs to small mRNAs such as H4-1, compared with those of larger mRNAs, may be due to the greater relative contribution in the small cDNAs of the double-stranded loop at the 3′ end.

Fig. 3 shows the hybridization of HeLa cell H4-1 cDNA to its template mRNA. Hybridization was carried out in RNA excess in 0.5 M NaCl/50% formamide. It was determined empirically (Fig. 3*A*) that the rate of hybridization was optimal at 58°C (which is 15° below the melting temperature). The H4-1 mRNA·cDNA hybrids melted with a single, sharp transition and a melting temperature of 73°C (Fig. 3*C*), indicating a G+C content of approximately 53% (32). The $Cr_0t_{1/2}$ of the H4-1 mRNA·cDNA hybridization reaction was 2.2×10^{-3} sec × mol per liter, and greater than 90% of the hybridization occurred within the range of log $Cr_0t_{1/2} = \pm 1$.

The molar concentration of nucleotide sequences in solution determines the rate of hybridization. Therefore, we compared the kinetics of the H4-1 histone mRNA·cDNA hybridization reaction with that of rabbit globin mRNA·cDNA hybridization under the same conditions, but at the optimal temperature for each hybridization reaction (58°C for H4-1 histone and 43°C for globin). The similarity of the kinetics of hybridization reaction (Fig. 3*B*) indicates that the calculated sequence complexity is 600 nucleotides for the H4-1 histone mRNA and its cDNA. Considering the variation found for rates of RNA·DNA hybridization reactions and the widely different base compositions of the two mRNAs, this value is in reasonable agreement with the number of nucleotides (386) in the H4-1 histone

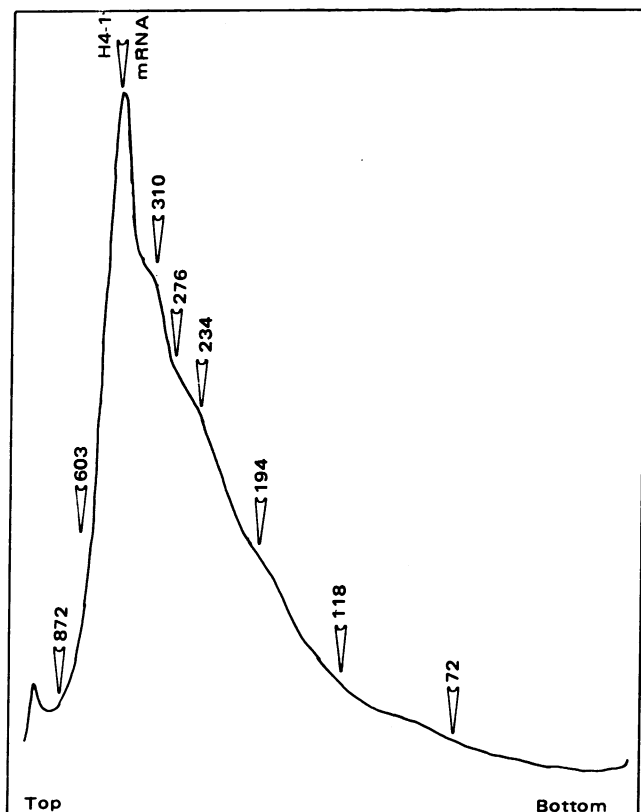

FIG. 2. Electrophoresis of H4-1 cDNA under denaturing conditions. H4-1 cDNA was synthesized and 4.5 ng was electrophoresed in a 6% acrylamide/10 mM glyoxal gel (23). The gel was fixed overnight with 10% (wt/vol) trichloroacetic acid, prepared for fluorography and exposed for 55 hr at −20°C. H4-1 [^{32}P]mRNA and *Hae*III digests of ^{3}H-labeled ϕX174 (6 μg; 9000 dpm/μg) were electrophoresed in adjacent wells. The positions of the small to intermediate fragments of ϕX174 and their sizes in nucleotides are indicated. The 276-nucleotide marker was an average of the 281 and 271 nucleotide fragments which were not adequately resolved.

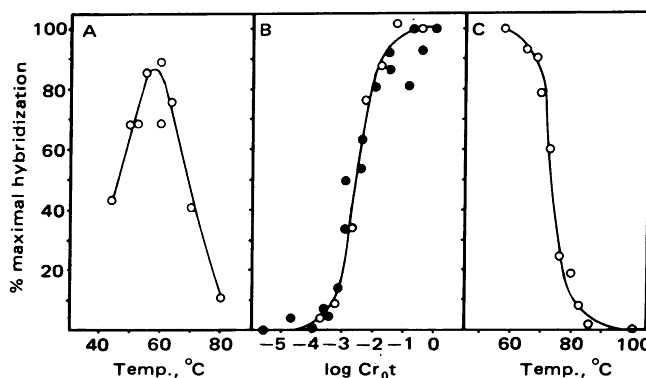

FIG. 3. Analysis of the H4-1 mRNA-to-H4-1 cDNA hybridization conditions. (*A*) Determination of optimal hybridization temperature. S phase polysomal RNA (3.7 ng) was hybridized with 5 pg of H4 [^{3}H]cDNA to a Cr_0t of 4.2 sec × mol of nucleotide per liter at the indicated temperatures. Nuclease S1-resistant material was determined and is plotted here relative to the resistant material present at the completion of hybridization at optimal temperature. (*B*) Hybridization of H4 and globin mRNAs to their respective cDNA. H4 mRNA (3.7 ng) and 2.2 μg of *Escherichia coli* tRNA were hybridized with 5 pg of H4-1 [^{3}H]cDNA at 58°C. Globin mRNA (4.5 ng) was hybridized with 2.9 pg of globin cDNA (7×10^{7} dpm/μg) at 43°C [optimal temperature for globin hybridization under the conditions used for H4 hybridization (31)]. Nuclease-resistant material was determined and plotted as percentage of maximal hybridization. ○, H4 mRNA·cDNA; ●, globin mRNA·cDNA. (*C*) Thermal denaturation profile of H4 mRNA·cDNA hybrids incubated for 10 min at the indicated temperature and processed for nuclease S1 resistance.

mRNA as determined by polyacrylamide gel electrophoresis in denaturing conditions (Fig. 1*B*).

H4 Histone mRNA Sequences During the Cell Cycle. To assess the representation of *in vivo* synthesized histone mRNA sequences in G_1 and S phase HeLa cells, nuclear and cytoplasmic (polysomal and postpolysomal) RNAs were isolated and assayed for their abilities to hybridize with HeLa cell H4-1 histone cDNA. Significant levels of hybridization between histone H4-1 cDNA and G_1 (1.5 hr after mitotic detachment) nuclear, polysomal, or postpolysomal cytoplasmic RNAs were not observed when cells were synchronized by mitotic selective detachment (Fig. 4)—a technique that yields a G_1 population containing <0.1% S phase cells (see Fig. 6). Although in the experiment shown in Fig. 4 there appeared to be some hybridization between H4-1 cDNA and G_1 postpolysomal RNA, the amount was not significant or reproducible. The $Cr_0t_{1/2}$ of the hybridization reaction between H4-1 cDNA and S phase nuclear RNA is 3.2×10^{-1} sec × mol/liter. By comparison with the $Cr_0t_{1/2}$ of the H4-1 mRNA·cDNA hybridization reaction, it can be calculated that 0.7% of the S phase nuclear RNA consists of H4 histone mRNA sequences. From the kinetics of hybridization of H4-1 cDNA with polysomal ($Cr_0t_{1/2} = 3.2 \times 10^{-1}$) and postpolysomal ($Cr_0t_{1/2} = 1.26$) RNAs of S phase HeLa cells, it can be calculated that H4 histone mRNA sequences account for 0.7 and 0.17% of the polysomal and postpolysomal cytoplasmic RNA populations, respectively.

When "G_1" cells were obtained by double thymidine block synchronization, histone H4 mRNA sequences were present in the RNAs isolated from the nucleus and cytoplasm of these cells. The kinetics of hybridization of H4-1 histone cDNA with nuclear, polysomal, and postpolysomal cytoplasmic RNAs of HeLa cells 12 hr after release from thymidine block indicate that significant amounts of H4 histone mRNA sequences were present in these cells (Fig. 5). Ideally, one would expect a slight difference in the kinetics of hybridization of H4 histone cDNA with G_1 and S phase RNAs isolated from double thymidine synchronized cells. However, the absence of such a difference in hybridization kinetics can be explained by the range of variation generally observed and the possibility that there may be significant differences in the amount of H4 histone mRNA per cell in these two cell populations. The results obtained with G_1 cells synchronized by double thymidine block are in sharp contrast to those obtained with G_1 cells synchronized by mitotic selective detachment but are not surprising because [^{3}H]thymidine labeling followed by autoradiography showed that, by 11.5 hr after release from thymidine block, 20–25% of the "G_1" cell population consisted of S phase cells (Fig. 6).

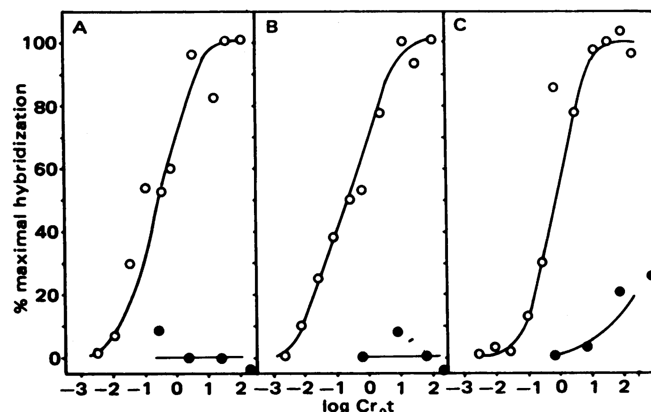

FIG. 4. Hybridization of RNAs from S phase cells (○) and from G_1 phase cells synchronized by mitotic selective detachment (●). HeLa cells were fractionated and the RNA from each fraction was isolated. S phase RNA (55 ng or 1.1 μg) was hybridized with 15 pg of H4-1 [^{3}H]cDNA. For G_1 RNA fractions, 0.68 μg of nuclear (*A*), 3 μg of polysomal (*B*), or 2.2 μg of postpolysomal (*C*) RNA was hybridized with 15 pg of H4 cDNA. Average maximal hybridization was 91%.

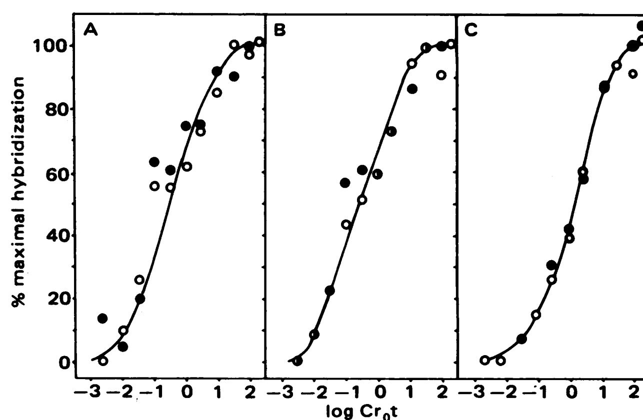

FIG. 5. Hybridization of RNAs from S phase cells (○) and from G_1 phase cells isolated 12 hr after release from the second of two 2 mM thymidine blocks (●). HeLa cells were fractionated and the RNA from each fraction was isolated. RNA (44 ng or 1.1 μg) was hybridized with 15 pg of cDNA. (*A*) Nuclear. (*B*) Polysomal. (*C*) Postpolysomal.

DISCUSSION

The synthesis of a DNA complementary to a single purified histone mRNA species, one of the HeLa cell H4 histone mRNAs (H4-1), has permitted us to reevaluate the representation of H4 histone mRNA sequences in G_1 and S phase HeLa cells. Utilizing the H4-1 cDNA probe and RNA-excess hybridization, we detected the presence of H4 histone mRNA sequences in the nucleus and cytoplasm of S phase cells. However, we were unable to detect significant levels of H4 histone mRNA sequences in nuclear, polysomal, or postpolysomal cytoplasmic RNAs isolated from G_1 phase HeLa cells obtained by mitotic selective detachment. It should be emphasized that this synchronization procedure yields a G_1 cell population containing <0.1% S phase cells. This observation is consistent with previous results from our laboratory which showed that a cDNA containing sequences complementary to H4, H3, H2A, and H2B histone mRNAs did not hybridize with nuclear or cytoplasmic RNAs of G_1 HeLa cells similarly synchronized (9–11).

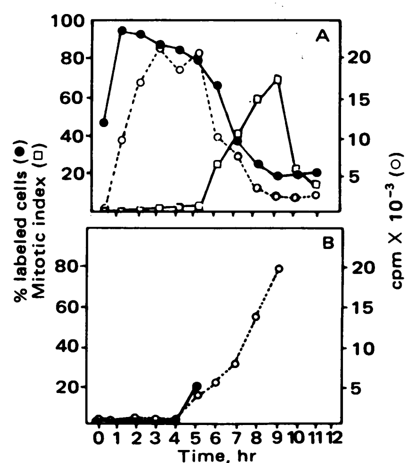

FIG. 6. Incorporation of [^{3}H]thymidine into DNA, percentage of cells in DNA synthesis, and mitotic index (no. of cells in mitosis per 1000 cells) at various times after release of HeLa cells from the second of two 2 mM thymidine blocks (*A*) and at various times after mitotic selective detachment (*B*). Cells were pulsed with [^{3}H]thymidine (5 μCi/ml) for 15 min. The rate of DNA synthesis was determined by the amount of radioactivity incorporated into 5% trichloroacetic acid-precipitable material. The percentage of cells in DNA synthesis and the mitotic index were determined autoradiographically.

Our results appear to be in direct conflict with those of Melli *et al.* (15, 16) who reported the synthesis of a high molecular weight precursor of histone mRNAs throughout the cell cycle in HeLa cells. In their experiments, radiolabeled HeLa cell RNA was hybridized with cloned sea urchin histone DNA. However, the possiblity must be considered that the histone mRNA sequences that they observed in G_1 HeLa cells may in fact have been due to S phase cells in the G_1 population. Melli *et al.* obtained G_1 cells by double thymidine block synchronization, a procedure that yields a G_1 population containing 20–25% S phase cells when assayed by [^{3}H]thymidine labeling and autoradiography. This interpretation is supported by our results showing that "G_1" cells obtained by double thymidine block synchronization contain RNA sequences hybridizable to HeLa cell H4-1 histone cDNA.

Our results indicating the presence of H4 histone mRNA sequences in S but not G_1 phase HeLa cells are consistent with regulation of histone gene expression during the cell cycle of these continuously dividing cells being mediated at least in part at the transcriptional level. Our experimental results do not preclude the existence of a rapidly turning over histone transcript in G_1 cells, which would not be detected by RNA excess hybridization. However, data from *in vitro* chromatin transcription studies, despite their limitations, support the possibility that H4 histone mRNA sequences are transcribed during the S but not G_1 periods of the cell cycle (data not shown). More direct evidence for or against transcriptional control could be obtained by DNA-excess hybridization using unlabeled homologous histone DNA and *in vivo* pulse-labeled RNA.

Recently, Marzluff and coworkers[‡] assayed pulse-labeled RNAs isolated from G_1 and S phase mouse myeloma cells, synchronized by the isoleucine deprivation method, for the presence of histone mRNA sequences by hybridization to cloned sea urchin histone DNA. Consistent with transcriptional level control of histone gene expression during the cell cycle of continuously dividing cells, they found that the synthesis of H3 and H4 histone mRNAs were prominent only during S phase. Parker and Fitscher (I. Parker, personal communication) have also observed a difference (>500-fold) in the representation of histone mRNA sequences in G_1 and S phase mouse 3T6 cells. In the latter experiments, histone mRNA sequences were identified by hybridization of nuclear and cytoplasmic RNAs to homologous histone cDNA.

Although there is evidence to suggest transcriptional level control of histone gene expression in several eukaryotic cell lines, this mechanism does not appear to be operative in all biological situations. During early stages of sea urchin development (33–35) and during *Xenopus laevis* oogenesis (36, 37), histone gene expression appears to be regulated posttranscriptionally.

‡ Chiu, I.-M., Cooper, D. & Marzluff, W. F. (1979) *Abstracts of the Second Annual American Cancer Society (Florida Division) Cancer Research Seminar*, Abstr. 38.

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