# EVIDENCE THAT THE COUPLING OF HISTONE GENE EXPRESSION AND DNA SYNTHESIS IN HeLa $S_3$ CELLS IS NOT MEDIATED AT THE TRANSCRIPTIONAL LEVEL

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SUMMARY: Representation of histone mRNA sequences in various intracellular fractions and in vitro transcripts of chromatin was examined after inhibition of DNA synthesis in S phase HeLa $S_3$ cells by cytosine arabinoside or hydroxyurea. Histone mRNA sequences were assayed by hybridization to a $^3H$-labeled, single-stranded DNA complementary to histone mRNAs. Both inhibitors bring about a drastic reduction (greater than 99%) in the level of histone mRNA sequences on polysomes. The representation of histone mRNA sequences in nuclei and in chromatin transcripts is not affected by treatment for 30 min with cytosine arabinoside or hydroxyurea. Cytosine arabinoside or hydroxyurea treatment results in an elevated level of histone mRNA sequences in the post-polysomal cytoplasmic fraction. Taken together these results provide evidence that in vivo as well as in vitro coupling of histone gene expression and DNA synthesis is not mediated at the transcriptional level. The specific post-transcriptional process at which the coupling mechanism is operative remains to be identified.

Several lines of evidence suggest that histone gene expression and DNA synthesis are biological processes which are tightly coupled. It has been known for some time that in continuously dividing cells, as well as after stimulation of nondividing cells to proliferate, the synthesis of histones is restricted to the period of the cell cycle when DNA replication occurs (S phase) (1-4). In vitro translation (5-11) and nucleic acid hybridization studies (12) suggest that histone mRNA sequences are associated with polyribosomes exclusively during the S phase of the cell cycle. Previous studies from this laboratory have shown that histone mRNA sequences are transcribed in vitro from chromatin of S phase cells but not from chromatin of $G_1$ phase cells (13-15). A functional relationship between histone

Abbreviation : SDS=sodium dodecyl sulfate





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gene expression and DNA replication is further indicated by the complete and rapid shutdown of histone synthesis when DNA replication is inhibited by drugs such as cytosine arabinoside or hydroxyurea (1-4,6,9). However, the mechanism by which such a coupling occurs remains to be resolved.

We have examined the levels of histone mRNA sequences present in various intracellular compartments after inhibition of DNA synthesis in S phase HeLa $S_3$ cells by cytosine arabinoside or hydroxyurea. Additionally, the influence of inhibition of DNA synthesis on transcription of histone mRNA sequences in vitro from chromatin was assayed. Histone mRNA sequences were analyzed by hybridization to a $^3H$-labeled, single-stranded DNA complementary to histone mRNA sequences (13,16). Results are presented which suggest that the coupling of histone gene expression and DNA replication is not mediated at the transcriptional level.

## MATERIALS AND METHODS

Cell Culture and Synchronization: HeLa $S_3$ cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum and were synchronized by two cycles of 2 mM thymidine block, as previously reported (3). S phase cells were harvested two hours after release from the second thymidine block at which time greater than 98% of the cells are undergoing DNA replication (3).

Histone Synthesis: $10^8$ S phase cells were harvested and resuspended at a concentration of $10^7$ cells/ml in Earl's balanced salt solution containing 2% fetal calf serum and 5 µCi/ml of $^3H$-leucine (45 Ci/mmole). Following incubation for 30 min at 37°C cells were harvested and chromatin was isolated as described previously (17). Chromatin was extracted twice with 0.4 N $H_2SO_4$, and histones were precipitated at -20°C from the combined acid extracts by addition of two volumes of ethanol. The histones were fractionated electrophoretically in acetic acid-urea polyacrylamide gels (18). Details of the procedure have been reported (19). Samples in which DNA synthesis was inhibited were treated with cytosine arabinoside (40 µg/ml) or hydroxyurea (10 mM) for 30 min prior to labeling with $^3H$-leucine and during the labeling period.

Subcellular Fractionation: All procedures were carried out at 4°C. Cells were washed three times with Earl's balanced salt solution, resuspended at a concentration of $4 \times 10^7$ cells/ml in 10 mM KCO-1.5 mM $MgCl_2$-10 mM Tris (pH 7.4), and allowed to swell at 4°C for 20 min. Cells were then lysed in a Dounce homogenizer by 15 strokes with a loose-fitting (type A) pestle. Nuclei were pelleted by centrifugation at 1000 xg for 5 min, sucrose was added to the supernatant to a final concentration of 10% (w/v), and mitochondria and microsomes were pelleted by centrifugation at 8000 xg for 10 min. The post-microsomal supernatant was then centrifuged at 100,000 xg for 90 min to pellet polyribosomes. The resulting supernatant will be referred to as the post-polysomal-cytoplasmic fraction.

RNA Fractionation: Nuclear RNA was isolated by the following procedure. Nuclei were digested with DNAse I (100 μg/ml) for 60 min at 37°C. The material was brought to a final concentration of 0.1 M NaCl-10 mM Na acetate-1 mM EDTA-1% SDS (pH 5.4) and incubated at 37°C for 15 min. The sample was extracted twice with one volume each of phenol and chloroform-isoamylalcohol (24:1 v/v). The aqueous phase was then extracted twice with chloroform-isoamyl alcohol. RNA was precipitated from the aqueous layer with two volumes of ethanol at -20°C. The sample was then digested again with DNAse I and the series of organic extractions described above was repeated. The RNAs were chromatographed on Sephadex G50 (fine), eluted with 0.1 M NaCl-10 mM Na acetate-1 mM EDTA (pH 5.4) and precipitated with two volumes of ethanol at -20°C.

Polysomal RNA was prepared by resuspending polysomes in 0.1 M NaCl-10 mM Na acetate-1 mM EDTA-1% SDS (pH 5.4) and then executing a series of organic extractions as described above for isolation of nuclear RNAs. RNA was isolated from the post-polysomal-cytoplasmic fraction in a similar manner and then chromatographed on Sephadex G50 (fine), eluting the column with 0.1 M NaCl-10 mM Na acetate-1 mM EDTA (pH 5.4). RNAs were precipitated by addition of two volumes of ethanol at -20°C.

In Vitro Chromatin Transcription: RNA was transcribed using fraction V *E. coli* RNA polymerase prepared according to the method of Burgess and Jendrisak (20). Transcription was carried out for 60 min at 37°C in a Dounce homogenizer fitted with a wide clearance pestle, and the reaction mixture was periodically homogenized to maintain chromatin solubility. The incubation mixture in a final volume of 3.4 ml contained 40 mM Tris (pH 8.3)-4 mM $MgCl_2$-1 mM $MnCl_2$-20 μM EDTA-0.008% β-mercaptoethanol-0.4 mM each of ATP, UTP, CTP and GTP-150 μg of DNA (as chromatin)/ml and 40 units of RNA polymerase. The reaction mixture was brought to a concentration of 1% SDS-0.1 M NaCl-10 mM Na acetate-1 mM EDTA, and was incubated at 37°C for 15 min. Nucleic acid was extracted as described above with phenol-chloroform-isoamylalcohol, with chloroform-isoamyl alcohol and precipitated with two volumes of ethanol. The material was then resuspended in 10 mM Tris-0.1 M NaCl-5 mM $MgCl_2$ (pH 7.4) containing DNAse I (40 μg/ml) and incubated at 37°C for 60 min. The reaction mixture was deproteinized by extraction with phenol-chloroform-isoamylalcohol and extraction with chloroform-isoamylalcohol. The RNA transcripts were chromatographed on Sephadex G50 (fine) as described above, precipitated with two volumes of ethanol and resuspended in distilled water for hybridization analysis.

Hybridization Analysis: Histone mRNA sequences were detected by hybridization to a $^3H$-labeled, single-stranded DNA complementary to histone mRNAs. Preparation and properties of the histone cDNA probe have been reported (12,13,16). 0.04 ng of $^3H$-cDNA (27,000 cpm/ng) were annealed at 52°C in sealed glass capillary tubes in a volume of 15 μl containing 50% formamide-0.5 M NaCl-25 mM HEPES (pH 7.0)-1 mM EDTA with various amounts of RNA. Hybridization was carried out in RNA excess. The reaction mixtures were assayed for hybrid formation using fraction IV single-strand specific $S_1$ nuclease isolated from *Aspergillus oryzae* (21). Each sample was incubated for 20 min in 2 ml of 30 mM Na acetate-0.3 M NaCl-1 mM $ZnSO_4$-5% glycerol (pH 4.6), containing $S_1$ nuclease at a concentration sufficient to degrade at least 95% of the single-stranded nucleic acids present. The amount of radioactive DNA resistant to digestion was determined by trichloroacetic acid precipitation. $Cr_0t$ = moles of ribonucleotides x seconds/l.

## RESULTS AND DISCUSSION

To examine the level at which the coupling of histone gene expression and DNA replication resides, we pursued the following approach. S phase

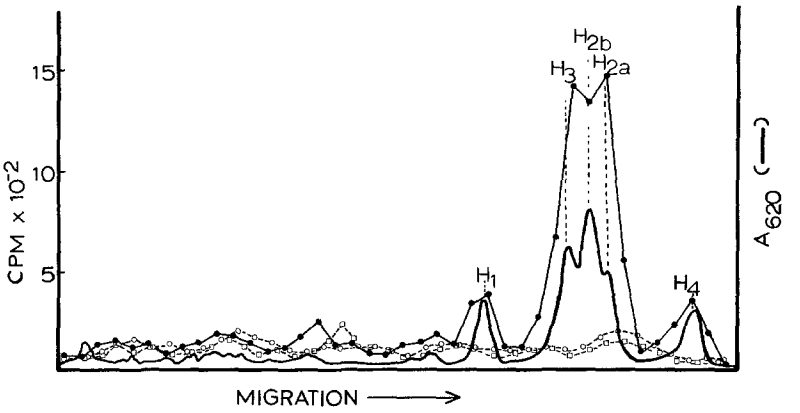


Figure 1 Electrophoretic fractionation of histones from S phase HeLa $S_3$ cells treated with cytosine arabinoside or hydroxyurea. Cells were treated with cytosine arabinoside (40 µg/ml) ( o ) or hydroxyurea (10 mM) ( □ ) for 30 min and then pulse labeled with $^3$H-leucine ( 5 µCi/ml:45 Ci/mmole) for 30 min in the presence of inhibitor. Controls ( ● ) were not exposed to inhibitor. Histones were isolated as described in Materials and Methods, and fractionated electrophoretically in acetic acid-urea polyacrylamide gels (18). The gels were fractionated into 1 mm sections, solubilized in 30% $H_2O_2$ and counted in liquid scintillation cocktail containing triton X100-toluene-liquifluor (1:2:0.126).

TABLE I

Effects of Cytosine Arabinoside and Hydroxyurea

On Incorporation of $^{14}$C Thymidine Into DNA

| | cpm $^{14}$C-Thymidine/mg DNA | % Inhibition |
|---|---|---|
| Control | 25,695 | - |
| Cytosine Arabinoside | 545 | 97.8 |
| Hydroxyurea | 530 | 98.0 |

S phase cells were treated with cytosine arabinoside (40 µg/ml) or hydroxyurea (10 mM). 30 min after treatment with the inhibitor, the rate of DNA synthesis was determined by labeling 2 ml of cells (5 x $10^5$ cells/ml) for 30 min with 0.2 µCi of $^{14}$C-thymidine and determining incorporation of radioactivity into 10% trichloroacetic acid precipitable material. Control samples were S phase cells not treated with inhibitor.

HeLa $S_3$ cells were treated for 30 min with cytosine arabinoside (40 µg/ml) or hydroxyurea (10 mM) - conditions which result in greater than 98% inhibition of DNA synthesis (Table I). As shown in Fig. 1, both inhibitors

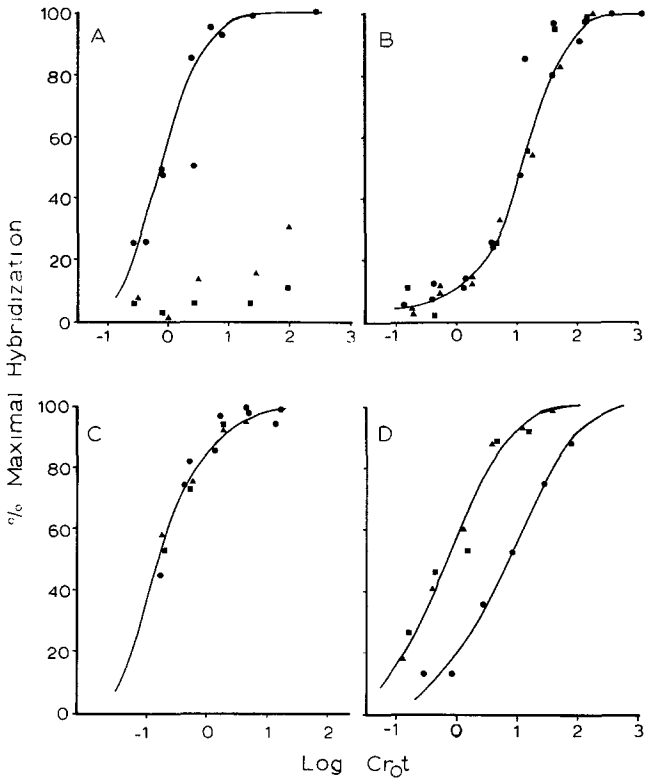


Figure 2 Hybridization of polysomal RNA (A), nuclear RNA (B), in vitro chromatin transcripts (C), and post-polysomal-cytoplasmic RNA (D) from S phase cells ( ● ) and S phase cells treated with cytosine arabinoside ( ▲ ) or hydroxyurea ( ■ ). $Cr_0t$ = moles of ribonucleotides x seconds x $l^{-1}$.

effectively block histone synthesis. We then assayed the influence of these inhibitors on the level of histone mRNA sequences present in various intracellular fractions. A comparison of the kinetics of the hybridization reactions between histone cDNA and polysomal RNA from control, cytosine arabinoside-treated and hydroxyurea-treated cells indicates that cytosine arabinoside and hydroxyurea bring about a drastic reduction (>99%) in the representation of histone mRNA sequences on polysomes (Fig. 2A ). This reduction is consistent with in vitro translation data from several laboratories (5,7-10) which indicate that the amount of translatable histone mRNAs on polysomes is decreased when DNA synthesis is inhibited.

In contrast, the representation of histone mRNA sequences is similar in nuclear RNAs of control cells and cells treated with cytosine arabinoside or hydroxyurea (Fig. 2B). However, it should be noted that the type of nucleic acid hybridization analysis utilized in these experiments does not permit us to distinguish between amounts of pre-existing and newly synthesized histone mRNAs, nor does it allow us to assess the rates of histone mRNA synthesis and/or turnover. These results suggest that the coupling of DNA replication may be at a post-transcriptional level rather than at the transcriptional level.

Results from *in vitro* chromatin transcription experiments support the hypothesis that coupling of histone gene expression and DNA synthesis is not mediated transcriptionally. Chromatin from control cells and from cells treated for 30 min with cytosine arabinoside or hydroxyurea were transcribed *in vitro* with *E. coli* RNA polymerase. The RNA transcripts were then assayed for their ability to hybridize with histone cDNA. The data in Fig. 2C indicate that the kinetics of the hybridization reactions between histone $^{3}$H-cDNA and RNA transcripts from chromatin of control and drug-treated cells are indistinguishable. These data suggest that transcription of histone mRNA sequences is unaffected by inhibitors of DNA synthesis, consistent with results from *in vivo* nuclear RNA studies described above.

Hybridization analysis of RNAs present in the post-polysomal-cytoplasmic fraction of control cells or cells treated with cytosine arabinoside of hydroxyurea (Fig. 2D) suggests that the inhibition of DNA synthesis for 30 min does not reduce the representation of histone mRNA sequences. In fact, a ten-fold increase in the level of histone mRNA sequences is observed in the post-polysomal-cytoplasmic fraction in either cytosine arabinoside or hydroxyurea treated cells compared with control cells. This accumulation of histone mRNA sequences in the post-polysomal-cytoplasmic fraction after inhibition of DNA synthesis may be the result of release of

histone mRNAs from polysomes or may reflect processing of histone mRNAs from the nucleus. In previous studies in which histone mRNAs were assayed by in vitro translation the elevated level of histone mRNA sequences in the post-polysomal-cytoplasmic fraction was not observed (22). However, in vitro translation does not eliminate the possibility that histone mRNAs are present in non-translatable states. Histone mRNAs isolated from the polysomes of S phase HeLa cells have been shown to have capped 5' termini of the types $m^7GpppX^mpYp$ and $m^7GpppX^mpY^mpZp$ (23,24). Release of histone mRNAs from polysomes may be accompanied by removal of the 5' caps, or the RNAs may be otherwise partially degraded. Such alterations in histone mRNAs may not be detected by nucleic acid hybridization analysis but would be expected to influence in vitro translation.

The present results provide in vivo as well as in vitro evidence that the coupling of histone gene expression and DNA synthesis is not mediated at the transcriptional level. However, the specific post-transcriptional process where the coupling mechanism is operative remains to be identified. Elucidating the mechanism underlying this coupling phenomenon may have important implications for understanding the control of histone synthesis, DNA replication, and in a broad sense, the control of cell proliferation.

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