Role of Nonhistone Chromosomal Proteins in the Regulation of Histone Gene Expression


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Summary

Histone gene expression was studied during the cell cycle of continuously dividing HeLa S1-cells and following stimulation of confluent monolayers of WI-38 human diploid fibroblasts to proliferate. The presence of histone messenger RNA (mRNA) sequences was assayed by hybridization to a 3H-labeled single-stranded DNA complementary to histone mRNA's. In HeLa S1-cells, histone mRNA sequences were found in the nucleus and associated with polyribosomes during S phase but not during G1. Transcripts of S-phase chromatin contained histone mRNA sequences but those of G1 chromatin did not. Similarly, in WI-38 cells association of histone mRNA sequences with polyribosomes and transcription of histone mRNA sequences from chromatin parallel DNA replication. Together these results suggest that the regulation of histone gene expression resides, at least in part, at the transcriptional level. Chromatin reconstitution studies provide evidence that nonhistone chromosomal proteins play a key role in activation of histone gene transcription during the period of the cell cycle when DNA is replicated. Phosphate groups associated with the S-phase nonhistone chromosomal proteins appear to be functionally involved in the control of histone gene readout. Although WI-38 human diploid fibroblasts transformed by SV40 exhibit morphological and biochemical modifications characteristic of neoplastic cells, transcription of histone mRNA sequences remains unaltered.

Introduction

Cell proliferation in eukaryotic cells involves a complex and interdependent series of biochemical processes requiring the selective expression of specific genes. An understanding of the mechanisms by which defined genes are rendered available for transcription during the cell cycle is central to understanding the normal course of growth and development as well as aberrations that arise in association with neoplasia and a broad spectrum of disease processes. During the past several years, evidence has been presented that suggests an important role for chromosomal proteins in the regulation of gene expression during the cell cycle. Variations observed in the composition and metabolism of the nonhistone chromosomal proteins during G1, S, G2, and mitosis and their correlation with changes in transcription are consistent with a regulatory function for these proteins (reviewed in Refs. 3, 11, 37, and 44). More direct evidence that nonhistone chromosomal proteins may be responsible for cell cycle stage-specific transcription comes from a series of chromatin reconstitution studies that indicate that nonhistone chromosomal proteins determine the differences in availability of DNA as template for RNA synthesis during the cell cycle of continuously dividing cells (40), as well as following stimulation of nondividing cells to proliferate (39).

Recently, we have been focusing on the regulation of a specific set of genes during the cell cycle, those that code for histones. The histone genes are of particular significance, since their expression appears to be functionally related to DNA replication. Furthermore, the gene products, histones, are of key importance in the regulation of transcription in general and in the maintenance of genome structure. We have synthesized a single-stranded, 3H-labeled DNA complementary to histone mRNA's (48) to serve as a high-resolution probe for identification of histone mRNA sequences in RNA's isolated from intact cells, as well as in transcripts from chromatin. In this report, evidence is presented that suggests that in both continuously dividing cells and nondividing cells stimulated to proliferate: (a) regulation of histone gene expression resides, at least in part, at the transcriptional level; and (b) a subset of the nonhistone chromosomal proteins associated with the genome during the S phase of the cell cycle is responsible for activation of histone gene transcription when DNA replication occurs.

Transcriptional Regulation of Histone Gene Expression during the Cell Cycle in Continuously Dividing Cells (43, 47)

A functional relationship between histone synthesis and DNA replication in many biological systems is suggested by the restricted synthesis of these proteins and their deposition on the DNA during the S phase of the cell cycle (33, 36, 38). Further support for the coupling of histone and DNA synthesis comes from the observation that inhibition of DNA replication results in a rapid and complete shutdown of histone synthesis (6, 14, 33, 36, 38). It has previously been
sequences on G2 polysomes is complex. The kinetics of the hybridization reaction between G2 polysomal RNA and histone cDNA suggests that the amount of histone mRNA sequences present on the polyribosomes of G2 cells is 21% of that present on S-phase polyribosomes. However, thymidine labeling followed by autoradiography indicates that 20% of the G2 cell population consists of cells that are undergoing DNA replication (38, 47). It is therefore reasonable to conclude that the histone mRNA sequences present in the G2 polyribosomal RNA are due to the presence of S-phase cells in the G2 population. This implies that histone mRNA sequences are not associated with polyribosomes during the G2 phase of the cell cycle. Unfortunately, to date no effective methodologies are available for obtaining a pure population of G2-phase HeLa S0-cells to establish this point definitively.

These results demonstrate that in HeLa cells, histone mRNA sequences become associated with polyribosomes during the transition from the G2 to the S phase of the cell cycle. It therefore follows that the activation of histone gene expression in this system is not regulated at the translational level, and transcriptional control is strongly implicated. This interpretation is supported by our recent findings that histone mRNA sequences are present in the mRNA of S-phase cells, but not in the mRNA of G1 cells.4

It is reasonable to conclude that as cells terminate DNA replication and enter the G2 phase of the cell cycle, the expression of histone genes is “turned off.” However, the mechanism by which histone genes cease to be expressed at this point in time is an enigma. It remains to be established whether exit from S phase is accompanied by: (a) cessation of histone mRNA synthesis and processing; (b) degradation of existing histone mRNA’s; or (c) inhibition of histone synthesis at the translational level. A combination of these possibilities may be operative. Control at the nuclear level, perhaps mediated transcriptionally, is suggested as a component of the mechanism by studies of mRNA in G2-phase cells. When the presence of histone mRNA sequences in G2 mRNA was assayed by hybridization with histone cDNA, a limited representation (14%) of histone mRNA sequences in G2 compared with S-phase mRNA was observed, most likely attributable to S-phase cells in the G2 population.4 This result suggests that in HeLa cells histone mRNA sequences are not present in nuclei of cells that have completed DNA replication. Transcription of histone genes may be repressed with the shutdown of DNA synthesis or a rapid degradation of histone mRNA sequences may occur when DNA replication is terminated. The mechanism by which histone synthesis is terminated at the end of S phase may be somewhat analogous to the inhibition of histone synthesis when DNA replication is blocked by drugs such as hydroxyurea or cytosine arabinoside. In such situations there is a rapid loss of histone mRNA’s from polyribosomes (5, 7, 8, 43). It is not clear whether inhibition of DNA synthesis brings about a degradation of histone mRNA’s or results in dissociation of histone mRNA’s from the polyribosomes. Furthermore, these findings do not preclude involvement at the transcriptional level. The type of regulation of histone gene expression observed during the cell cycle of HeLa cells may not be universal. For example, there is evidence that during early stages of embryonic development, control of histone synthesis may be mediated, at least in part, posttranscriptionally (12, 35).

To ascertain directly whether the genes that contain the information for histone synthesis are transcribed during a restricted period of the cell cycle in continuously dividing HeLa S0-cells, the following approach was pursued. Chromatin from G2- and S-phase cells was transcribed with Escherichia coli RNA polymerase in a cell-free system, the
RNA’s were isolated, and their ability to form S₁ nuclease-resistant, acid-precipitable hybrids with histone [³H]cDNA was determined (43). The kinetics of the hybridization of histone cDNA and RNA transcripts from G₁, as well as S phase chromatin are shown in Chart 1. While transcripts from S-phase chromatin hybridize with histone cDNA at a C₅,₊, of 2 × 10⁻¹ compared with a C₅,₊, of 1.7 × 10⁻² for the histone mRNA-cDNA hybridization reaction, there is no evidence of hybrid formation between histone cDNA and G₁ transcripts even at a C₅,₊, of 100. The maximal level of hybrid formation (65%) between histone cDNA and S-phase transcripts was the same as that observed between histone cDNA and histone mRNA. Fidelity of the hybrids formed between histone cDNA and transcripts from S-phase chromatin is suggested by the fact that the melting point Tₘ of these hybrids is identical to the Tₘ of histone mRNA-cDNA hybrids [65° in 50% formamide:0.5 M NaCl:25 mM N-2-hydroxyethylpipperazine-N’-2-ethanesulfonic acid (pH 7.0):1 mM EDTA]. It should be noted that the Tₘ obtained under these conditions is consistent with a RNA-DNA hybrid having a GC content of 54%, which is the nucleotide composition of histone mRNA reported by Adesnik and Darnell (1) and Thrall et al. (48).

RNA synthesized in intact cells may remain associated with chromatin during isolation and in part account for hybrid formation between in vitro RNA transcripts and complementary DNA’s for specific genes. It is possible that the extent to which this phenomenon occurs varies with the tissue or cell and the method of chromatin preparation. To determine whether such endogenous RNA sequences account for histone-specific sequences, which are detected in transcripts from S-phase chromatin, the following control was executed. S-phase chromatin was placed in the in vitro transcription mixture without RNA polymerase and an amount of E. coli RNA equivalent to the amount of RNA transcribed from S-phase chromatin was added. RNA was immediately extracted by the same procedure utilized for the isolation of in vitro RNA transcripts. When this control RNA was annealed with histone cDNA, no significant level of hybridization was observed (Chart 1). Additionally, RNA isolated from S-phase chromatin in the absence of carrier RNA showed no hybrid formation with the histone cDNA. These results established that endogenous histone-specific sequences associated with S-phase chromatin are not contributing significantly to the hybridization observed with S-phase in vitro transcripts. It is therefore reasonable to conclude that the histone sequences present in S-phase transcripts can be accounted for by in vitro synthesis.

When G₁ chromatin is transcribed in the presence of an amount of histone mRNA equivalent to that transcribed from S-phase chromatin, and the mixture of G₁ transcripts and added histone mRNA is subsequently isolated, hybridization of histone cDNA occurs at the expected C₅,₊, (2 × 10⁻¹) (27). This result suggests that the absence of histone mRNA sequences among RNA transcripts from G₁ chromatin is not attributable to a specific nuclease associated with chromatin during the G₁ phase of the cell cycle. The possibility that histone sequences are present in G₁ transcripts but are not detected because they are in a double-stranded form due to symmetrical transcription is unlikely, since heating the hybridization mixture to 100° for 10 min before incubation has no effect on the hybridization of [³H]cDNA to the transcripts (27).

The results from these studies clearly indicate that histone sequences are available for transcription during S phase and not during G₁. Such findings are consistent with the restriction of histone synthesis to the S phase of the cell cycle and the association of histone mRNA’s with poly-somes only during S phase. Together, this evidence suggests that in continuously dividing HeLa S₁-cells, the expression of histone genes is regulated, at least in part, at the transcriptional level and that the readout of these genetic sequences occurs only during the period of DNA replication. It is also reasonable to conclude that chromatin is a valid model for studying the regulation of cell cycle stage-specific transcription of histone genes.

Nonhistone Chromosomal Proteins and Regulation of Histone Gene Transcription in Continuously Dividing HeLa S₁-Cells (27, 43, 46)

Evidence has been presented that strongly suggests that among the nonhistone chromosomal proteins are macromolecules that are responsible for the regulation of chromatin template activity during the cell cycle (39, 40). To examine directly the involvement of nonhistone chromosomal proteins in the control of the cell cycle stage-specific transcription of a defined set of genetic sequences, histone genes, we have pursued the following approach. Chromatin isolated from G₁- and S-phase cells was dissociated in high salt-urea, and each chromatin preparation was fractionated...
into DNA, histones, and nonhistone chromosomal proteins. Chromatin preparations were then reconstituted by the gradient dialysis method of Bekhor et al. (4) utilizing DNA and histones pooled from G1- and S-phase cells and either G1- or S-phase nonhistone chromosomal proteins (Chart 2). Essentially DNA, histones, and nonhistone chromosomal proteins are combined in high salt-urea, and the salt is progressively removed by stepwise dialysis, followed by removal of the urea. Details of the procedure for reconstitution of HeLa cell chromatin have been reported, as well as several lines of evidence for fidelity of chromatin reconstitution by this method (42). In vitro RNA transcripts from chromatin reconstituted with G1 nonhistone chromosomal protein and from chromatin reconstituted with S-phase nonhistone chromosomal proteins were annealed with histone [3H]cDNA. Chart 1 indicates that RNA transcripts from chromatin reconstituted with G1 nonhistone chromosomal protein and from chromatin reconstituted with S-phase nonhistone chromosomal proteins were annealed with histone [3H]cDNA. Chart 1 indicates that RNA transcripts from chromatin reconstituted with S-phase nonhistone chromosomal proteins hybridized with histone cDNA, while those from chromatin reconstituted with G1 nonhistone chromosomal proteins do not exhibit a significant degree of hybrid formation (43). It should be emphasized that the kinetics and extent of hybridization with the cDNA are the same for transcripts of native S-phase chromatin and transcripts of chromatin reconstituted with S-phase nonhistone chromosomal proteins. Furthermore, the amounts of RNA transcribed and the recovery during isolation of these transcripts from native and reconstituted chromatin preparations are essentially identical. These results clearly implicate a functional role for nonhistone chromosomal proteins in regulating the availability of histone sequences for transcription during the cell cycle. Such a regulatory role for nonhistone chromosomal proteins is in agreement with results from several laboratories which have indicated that these proteins are responsible for the tissue-specific transcription of globin gene (2, 9, 28) and the hormone-induced transcription of ovalbumin genes (B. W. O'Malley, personal communication). However, these results represent the first demonstration that nonhistone chromosomal proteins regulate the transcription of genes that are transiently expressed.

An important question that then arises is whether the difference in the in vitro transcription of histone genes from G1- and S-phase chromatin is due to an activator of histone gene transcription present in the S-phase nonhistone chromosomal proteins or, alternatively, to a specific repressor of histone gene transcription present among the G1 nonhistone chromosomal proteins. If the difference in histone gene activity of G1- and S-phase chromatin were due to an activator that is present or operative only in S phase, one would anticipate that dissociation of G1 chromatin with high-salt and urea, followed by reconstitution in the presence of S-phase nonhistone chromosomal proteins, would result in an increase in the availability of histone genes for transcription. One would not anticipate any major effect on histone gene transcription if S-phase chromatin were reconstituted in the presence of G1 nonhistone chromosomal protein. In contrast, if the difference in histone gene expression in G1- and S-phase chromatin can be accounted for by a repressor of histone gene expression that is associated with chromatin during the G1 phase of the cell cycle, one would anticipate that dissociation of S-phase chromatin followed by reconstitution in the presence of increasing amounts of G1 nonhistone chromosomal proteins would result in a progressive decrease in the availability of histone genes for transcription. If the latter alternative prevails, the presence of S-phase chromosomal proteins during reconstitution would not be expected to effect significantly the expression of histone genes from G1 chromatin. If the regulation of histone genes involves repressors and activators acting in an antagonistic manner, one would anticipate a more complex intermediate result.

Chart 2. Flow diagram of experimental protocol for chromatin reconstitution.
As shown in Chart 3, when G\textsubscript{1} chromatin is dissociated and then reconstituted in the presence of increasing amounts of S-phase nonhistone chromosomal proteins, hybrid formation between transcripts from these chromatin and histone cDNA is seen at progressively lower $C_{\text{m}}$ values, indicating a dose-dependent activation of histone genes of the G\textsubscript{1} chromatin by the S-phase nonhistone chromosomal proteins. It can be seen that histone genes from G\textsubscript{1} chromatin can be activated to approximately the same degree as in native S-phase chromatin by comparing the kinetics of the hybridization of histone cDNA with transcripts from S-phase chromatin ($C_{\text{m,112}} = 2 \times 10^{-11}$) and the kinetics of the hybridization of histone cDNA with transcripts from G\textsubscript{1} chromatin reconstituted with a 1:1 ratio of S-phase nonhistone chromosomal proteins to DNA ($C_{\text{m,112}} = 3 \times 10^{-11}$) (27). The fidelity of the hybrids formed between the transcripts and histone cDNA as well as the validity of comparing $C_{\text{m,112}}$ values is suggested by the fact that the $T_m$ of the hybrid in all cases is identical to the $T_m$ of the hybrids formed between histone mRNA and histone $[^{3}H]$cDNA. Also, the maximal hybridization, as estimated by a double reciprocal plot, is equal in all cases to that of the histone mRNA-cDNA hybridization reaction (65%). In contrast, when G\textsubscript{1} chromatin is dissociated and then reconstituted in the presence of S-phase histones, even at a 1:1 ratio of S-phase histone to DNA, a significant stimulation of the transcription of histone genes is not observed (Chart 3). There were no significant differences among the various chromatin preparations in the yield or recovery of RNA during isolation, even though the presence of S-phase nonhistone chromosomal proteins during reconstitution could cause a greater than 1000-fold stimulation in the amount of histone sequences transcribed from G\textsubscript{1} chromatin. Therefore, the observed increase in representation of histone mRNA sequences cannot be attributed to nonspecific alteration of template activity. Stimulation of histone gene transcription is not observed when G\textsubscript{1} chromatin is dissociated and then reconstituted in the presence of additional G\textsubscript{1} chromosomal proteins, even at a 1:1 ratio of additional G\textsubscript{1} protein to DNA (Chart 3). The latter result suggests that specific chromosomal proteins are required to elicit activation of histone gene readout.

To eliminate the possibility that the small amount of nucleic acid present in the S-phase chromosomal proteins is responsible for the observed hybridization with histone $[^{3}H]$cDNA, either by containing histone sequences or by having the ability to render histone genes transcribable, the residual nucleic acid was removed from the S-phase chromosomal proteins by buoyant density centrifugation in cesium chloride-urea. As shown in Chart 4, there is no significant difference in the kinetics of hybridization with histone cDNA of transcripts from G\textsubscript{1} chromatin reconstituted with equal amounts of either cesium chloride-treated S-phase chromosomal proteins or untreated S-phase chromosomal proteins (27).

In order to determine whether G\textsubscript{1} chromatin contains an inhibitor of histone gene transcription, which is degraded or inactivated as the cells progress from the G\textsubscript{1} to the S phase of the cell cycle, chromatin from S-phase cells was dissociated and then reconstituted in the presence of total chromosomal proteins from G\textsubscript{1}-phase cells. The ability of transcripts from this reconstituted chromatin preparation to hybridize with histone cDNA was determined. As shown in Chart 5, the presence of G\textsubscript{1} total chromosomal proteins,
are the same as those seen with the histone mRNA-cDNA
ribosome chromosomal proteins is responsible for the observed
depending fashion. These results
der the histone genes of G1-phase chromatin available
for transcription in a dose-dependent fashion. These results
do not indicate which component(s) of the S-phase nonhis-
tone chromosomal proteins that has the ability
difference can be accounted for by a component(s) of the 5-
these results provide support for the contention that the
dicator fidelity of the RNA transcripts.
transcription from S-phase chromatin is due to the nonhistone
chro-mosomal proteins can be overridden by S-phase chromo-
somal proteins. This would suggest that any additional spe-
cific repressor of histone gene expression is lost during
isolation, dissociation, fractionation, or reconstitution or
that any inhibition of histone gene transcription by G1 chro-
mosomal proteins can be accounted for by a component(s) of
the G1, chromosomal proteins that has the ability to render the
histone genes of G1, phase chromatin available for transcription in
a dose-dependent fashion. These results do not indicate which component(s) of the S-phase nonhis-
tone chromosomal proteins is responsible for the observed
activation or by what mechanism the activation is achieved,
but they do provide an assay by which this histone gene activator can be purified and characterized.

To date most chromatin transcription studies have been
executed utilizing bacterial RNA polymerase. While these
studies have demonstrated a role for nonhistone chromo-
somal proteins in dictating the availability of histone (27, 43,
46), globin (2, 9, 28), and ovalbumin (B. W. O'Malley, per-
sonal communication) genes for transcription in chromatin,
it is quite possible that there is an additional level of regula-
tion existing in the intact cell that can be recognized only by
the appropriate homologous eukaryotic RNA polymerase.

Regulation of Histone Gene Transcription following Stimu-
lation of Nondividing Human Diploid Cells to Proliferate

To determine whether the mode of histone gene regula-
tion observed in continuously dividing HeLa S, cells is of
broader biological relevance, we examined the control of
histone gene expression following stimulation of nondivid-
ing WI-38 human diploid fibroblasts to proliferate. Con-
fluent monolayers of WI-38 human diploid fibroblasts can
be induced to proliferate by replacing exhausted growth
medium with fresh medium containing 20% fetal calf serum
(32, 34). The addition of serum to such cells triggers a
complex and interdependent series of biochemical events
(reviewed in Ref. 3). Activation of DNA synthesis as mea-
sured by incorporation of [3H]thymidine into DNA is evident
at 10 hr following stimulation of WI-38 cells and reaches a
maximum at 12 hr (Chart 6A). The activation of DNA synthe-
sis in WI-38 cells is supported by a similar (600-fold) in-
crease in the percentage of nuclei labeled with [3H]thymidine as determined autoradiographically (Chart
6B). An increase in mitotic activity is observed beginning at
20 hr (Chart 6C). Concomitant with the activation of DNA
synthesis, there is a stimulation of histone synthesis. The
tight coupling between histone synthesis and DNA replica-
cation in WI-38 cells is suggested by the rapid and complete
shutdown of histone synthesis by inhibition of DNA replica-
cation (45).

Transcription of Histone Genes following Stimulation of
WI-38 Cells to Proliferate. To determine the availability
of histone genes for transcription as a function of time follow-
ing stimulation of WI-38 cells to proliferate, we examined in
vitro transcripts of chromatin from confluent WI-38 cells,
from WI-38 cells during the prereplicative phase (1, 4, and 7
hr after stimulation) and from cells at 10 and 12 hr following
stimulation (S phase). The presence of histone mRNA se-
quences was assayed by hybrid formation with 3H-labeled
cDNA complementary to HeLa S, cell histone mRNA's. Utili-
ization of a histone cDNA probe templated by HeLa cell
histone mRNA's is justifiable for detection of histone mRNA
sequences isolated from WI-38 cells or transcribed from WI-
38 cell chromatin. One would not expect significant dif-
ferences in the genetic sequences of HeLa and WI-38 cells
since these cells are both of human origin. The identity of
the histone genes in HeLa and WI-38 cells is substantiated

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R. L. Jansing, J. L. Stein, and G. S. Stein. Activation of Histone Gene
Transcription by Nonhistone Chromosomal Proteins in WI-38 Human Diploid
Fibroblasts, submitted for publication.
stimulation of WI-38 cells to proliferate. A comparison of the $C_{r_{t/2}}$ values of the hybridization reactions between histone cDNA and RNA transcripts from chromatin as a function of time following stimulation to proliferate clearly demonstrates that activation of histone gene transcription parallels the onset of DNA synthesis in WI-38 cells (Chart 6). A similar time course for the appearance of histone mRNA on polyribosomes is observed (Chart 8). The low level of hybridization between histone cDNA and RNA transcripts from chromatin in G1 and unstimulated cells is most likely attributable to the few proliferating cells which escape "contact inhibition" and hence continue to synthesize DNA and histones. This interpretation is supported by the observation that stimulation of semiconfluent WI-38 cells results in a time course and maximal level for activation of histone genes similar to that observed when confluent cells are stimulated. However, in these semiconfluent cells, an elevated level of histone gene transcription from chromatin is detected prior to stimulation and during the prereplicative period ($C_{r_{t/2}} = 14$). Control experiments were carried out to eliminate the possibility that the endogenous RNA's associated with chromatin from S-phase (12 hr) cells account for hybrid formation of RNA transcripts with histone cDNA.

Transcription of Histone Genes in Normal and SV40-transformed WI-38 Cells. Transformation of mammalian cells by DNA and RNA tumor viruses results in pronounced modifications in cell structure and function that are evident at both morphological and biochemical levels. Such changes may be due, in part, to the expression of the viral genetic information, the interaction of viral gene products with the host-cell genome or other cell components, and/or the insertion of the viral genome into the host-cell DNA.

by indistinguishable $T_m$ values for hybrids formed between HeLa histone cDNA and HeLa histone mRNA's, HeLa histone cDNA and HeLa S-phase chromatin transcripts, and HeLa histone cDNA and WI-38 cell S-phase chromatin transcripts. There is 70% sequence homology in the histone mRNA sequences of divergent organisms such as humans and sea urchins (49).

The kinetics of hybridization of histone cDNA with RNA transcripts from chromatin of WI-38 cells at various times following serum stimulation are shown in Chart 7. There is a significant increase in the rate of hybridization of histone cDNA to RNA transcripts 10 hr following stimulation ($C_{r_{t/2}} = 1.0$), with a maximal rate of hybridization observed at 12 hr ($C_{r_{t/2}} = 4.0 \times 10^{-4}$). In contrast to the limited extent of hybrid formation between histone cDNA and RNA transcripts from chromatin of confluent cells and cells 1, 4, and 7 hr following stimulation ($C_{r_{t/2}} = 180$), the kinetics of the hybridization reaction of histone cDNA and RNA transcripts from S-phase (12 hr) chromatin revealed a 500-fold activation of histone mRNA sequence transcription following

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**Chart 6.** DNA synthesis at various times following serum stimulation of WI-38 human diploid fibroblasts. Cells were labeled with [3H]thymidine. To determine the rate of DNA synthesis, cells were harvested and nuclei were isolated. Nuclei were washed twice with cold (4°C) 0.3 M perchloric acid, and nucleic acids were extracted with hot (90°C) 1 M perchloric acid. The amount of DNA present in nucleic acid extracts was assayed by the diphenylamine reaction. A, an average of at least 4 determinations; the range of values did not exceed 7%. B, labeled nuclei per 1000 cells at various times following serum stimulation of WI-38 human diploid fibroblasts. Cells were labeled with [3H]thymidine. To determine the percentage of cells with [3H]thymidine-labeled nuclei, cells were harvested, smeared on acid-washed microscope slides, and prepared for autoradiography. Autoradiographs were exposed for 14 days and stained with hematoxylin following development. The values were obtained by counting 2000 cells. Each value represents an average of 4 determinations and the range of values did not exceed 7%. C, mitotic figures/1000 cells at various times following serum stimulation of WI-38 human diploid fibroblasts. Colcemid was added 12 hr following serum stimulation and, at the indicated times, cells were harvested, smeared on acid-washed microscope slides, fixed in alcohol:acetic acid (3:1), and stained with hematoxylin. The values for mitotic figures per 1000 cells were obtained by counting 2000 cells. D, represents an average of at least 4 determinations and the range of values did not exceed 7%.

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**Chart 7.** Kinetics of annealing of histone [3H]cDNA to in vitro transcripts of chromatin from unstimulated (×) WI-38 cells, and WI-38 cells at 1 (○), 4 (●), 7 (△), 10 (◇), and 12 hr (●) following serum stimulation. Histone cDNA was also annealed to endogenous RNA isolated from S-phase chromatin (△).

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**Chart 8.** Time course of the appearance of histone mRNA sequences on polyribosomes following serum stimulation of confluent WI-38 cells.

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Nonhistone Chromosomal Proteins in Gene Expression

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Direct interaction of a viral gene product with host-cell genetic sequences represents a particularly attractive mechanism for achieving cellular modifications associated with transformation.

Several reports have indicated that transformation by SV40 of WI-38 human diploid fibroblasts is accompanied by modifications in the composition and metabolism of genome-associated proteins, histone (22, 23) and nonhistone chromosomal proteins (10, 20, 21, 25, 30). Since chromosomal proteins play an important role in the regulation of gene expression, the transformation-associated changes in these proteins may be functionally related to the alterations in gene expression that are observed.

To examine the effect of SV40 transformation on the transcription of a specific set of genetic sequences, we have compared the transcription of histone genes from chromatin of normal and SV40-transformed WI-38 cells. The involvement of histones in the regulation of gene expression and their central role in cell proliferation make understanding their control in transformed cells particularly important. Chromatin from exponentially growing normal and SV40-transformed WI-38 cells was transcribed, and the RNA transcripts were hybridized with histone \(^{3}H\) cDNA. Chart 9 shows that no significant differences were observed in the percentage of RNA transcripts that are histone mRNA sequences. The kinetics of the hybridization reactions between histone \(^{3}H\) cDNA and RNA transcripts from chromatin of normal and SV40-transformed cells are indistinguishable. The cell cycle parameters (20) and the percentage of proliferating cells (20) are similar in the normal and SV40-transformed WI-38 fibroblasts utilized in these studies. Although the template activity (the availability of RNA sequences for transcription) does not differ significantly between normal and SV40-transformed cells, it is possible that differences exist in the specific sequences that are transcribed. Within this context, it has been reported that infection and transformation of chick embryo fibroblasts by Rous sarcoma virus result in the expression of globin genes (15).

**Nonhistone Chromosomal Proteins and Regulation of Histone Gene Transcription in WI-38 Cells.** The role of chromosomal proteins in regulating the transcription of histone genes was directly examined by a series of chromatin reconstitution experiments. To assay the involvement of nonhistone chromosomal proteins in rendering histone genes transcribable, chromatin from confluent WI-38 cells was dissociated and reconstituted in the presence of S-phase (12 hr) nonhistone chromosomal proteins. RNA transcripts from the reconstituted chromatin were tested for ability to hybridize with histone \(^{3}H\) cDNA. These data in Chart 10 indicate that the \(C_{R_{0t}}\) of the hybridization reaction between histone cDNA and RNA transcripts from this reconstituted chromatin preparation (\(C_{R_{0t}} \approx 4.0 \times 10^{-1}\)) is indistinguishable from that of the hybridization reaction between histone cDNA and S-phase chromatin RNA transcripts. Transcription of histone mRNA sequences from chromatin of confluent WI-38 cells is unchanged following dissociation and reconstitution in the presence of the histone fraction of S-phase (12 hr) chromatin. These results suggest that nonhistone chromosomal proteins are responsible for determining the availability of histone genes for transcription in chromatin of WI-38 cells and that a component of the S-phase nonhistone chromosomal proteins serves to activate the transcription of histone mRNA sequences. To examine the possibility that a component of the chromosomal proteins of confluent cells specifically restricts availability of histone genes for transcription, S-phase (12 hr) chromatin was dissociated and then reconstituted in the presence of total chromosomal proteins from confluent cells. Transcripts from such reconstituted chromatin preparations exhibit kinetics of hybridization with histone cDNA identical to those of native S-phase chromatin transcripts (Chart 10). A specific repressor of histone genes associated with chromatin of confluent WI-38 cells is therefore unlikely.

**Nonhistone Chromosomal Phosphoproteins and Activation of Histone Gene Transcription**

Results from the studies described above suggest that in

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continuously dividing HeLa S<sub>0</sub>-cells, as well as in WI-38 human diploid fibroblasts following stimulation to proliferate, the cell cycle stage-specific transcription of histone genes is regulated by a component of the S-phase nonhistone chromosomal proteins. One aspect of a possible mechanism by which histone gene transcription is regulated may involve the phosphate groups of the nonhistone chromosomal proteins. Modifications in the phosphorylation of nonhistone chromosomal proteins have been observed throughout the cell cycle of continuously dividing cells and following stimulation of nondividing cells to proliferate (17, 29, 31). Such changes in the metabolism of phosphate groups provide correlative evidence for a functional role of phosphorylation in gene regulation during the cell cycle. More direct evidence that phosphorylation of nonhistone chromosomal proteins is important in determining the availability of defined genes (histone genes) for transcription can be gleaned from previous results of 2 recent studies.

In 1 series of experiments chromatin-associated phosphoproteins were isolated from HeLa S<sub>0</sub>-cells, and this subset of the nonhistone chromosomal proteins was compared with other nonhistone chromosomal protein fractions for ability to activate histone mRNA sequence transcription from chromatin. Phosphoproteins were isolated from HeLa S<sub>0</sub> cell chromatin as schematically illustrated in Chart 11.

Concomitantly, protein fractions were isolated in an identical manner from cells that were pulse-labeled with <sup>32</sup>P for 1 hr. The histone gene activating ability of each fraction was correlated with the degree of phosphorylation, and the fractions were also examined by means of polyacrylamide gel electrophoresis. The phosphoprotein fractionation scheme used in these studies subdivided chromosomal proteins into 3 electrophoretically distinguishable fractions as demonstrated in Chart 12. These fractions also differ as to their specific activities with respect to <sup>32</sup>P, with the proteins bound to CaPO<sub>4</sub> gel exhibiting a 10-fold enhancement in phosphorylation (3.2 × 10<sup>6</sup> cpm/mg) compared with the "CaPO<sub>4</sub> nonbinding proteins" (2.3 × 10<sup>5</sup> cpm/mg). Each of the 4 protein fractions was analyzed in the following manner for its ability to activate in vitro transcription of histone mRNA sequences from G<sub>1</sub> chromatin, which is ineffective as a template for histone gene transcription. G<sub>1</sub> chromatin was dissociated in 5 M urea:3 M NaCl and then reconstituted in the presence of 1 of the 4 chromosomal protein fractions. The reconstituted chromatins were transcribed with E. coli RNA polymerase, and the RNA transcripts were assayed for their abilities to form S<sub>1</sub> nuclease-resistant trichloroacetic acid-precipitable hybrids with histone [H]<sub>1</sub>cDNA. As shown in Chart 13, dissociated G<sub>1</sub> chromatin reconstituted alone in the presence of "80,000 × g gel pellet proteins" or CaPO<sub>4</sub> nonbinding proteins, does not serve as a template for the in vitro transcription of RNA sequences that hybridize with histone cDNA. However, dissociated G<sub>1</sub> chromatin reconstituted in the presence of "80,000 × g supernatant proteins" or "phosphoproteins" was capable of transcribing RNA that

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**Chart 11.** Schematic diagram for the fractionation of chromosomal proteins from HeLa cells. Chromatin was prepared as described previously (40) and chromosomal proteins were fractionated according to the procedures of Langan (24) and Kish and Kleinsmith (18). The chromatin was suspended in a Dounce homogenizer in 1.0 M NaCl:50 mM Tris (pH 7.5) at a concentration of 2 mg/ml. Twenty mM Tris (1.5 volumes, pH 7.5) was added dropwise and the mixture was briefly homogenized and centrifuged at 80,000 × g for 1 hr. The 80,000 × g pellet was dispersed in 5 M urea:3 M NaCl:10 mM Tris (pH 8.3) and the mixture was centrifuged at 250,000 × g for 24 hr. The proteins in the supernatant are referred to as the "80,000 × g pellet proteins." BioRex 70, previously equilibrated with 0.4 M NaCl:20 mM Tris-HCl (pH 7.5), was added to the 80,000 × g supernatant proteins at a ratio of 20 mg of BioRex per mg of protein. After stirring for 5 to 10 min, the suspension was centrifuged at 6,000 × g. Calcium phosphate gel was added to the resulting supernatant in a ratio of 0.46 mg of gel per mg of protein, stirred for 5 to 10 min, and then centrifuged at 6,000 × g. The proteins remaining in the supernatant are referred to as "CaPO<sub>4</sub> nonbinding proteins." The pellet of calcium phosphate gel was washed in 40 ml of 1.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>:50 mM Tris (pH 7.5) and solubilized in 0.3 M EDTA (pH 7.5):0.33 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in a ratio of 0.2 ml of solution per mg of gel. The insoluble residue was removed by centrifugation for 15 min at 33,000 × g and the supernatant constituted the protein fraction referred to as the "phosphoproteins."

**Chart 12.** Electrophoretic profiles of the total nuclear proteins (top), the 80,000 × g pellet proteins (A), the CaPO<sub>4</sub> nonbinding proteins (B), and the phosphoproteins (C) isolated by the methods described in Chart 11 from exponentially growing HeLa cells. Aliquots from each sample were dialyzed against 0.1% sodium dodecy] sulfate:10 mM sodium phosphate (pH 7.0):0.1% β-mercaptoethanol and, subsequently, fractionated electrophoretically according to molecular weight on 7.5% acrylamide-0.28% bisacrylamide gels.
hybridizes to histone cDNA. The kinetics of the hybridization reaction between histone cDNA and RNA transcripts from native S-phase chromatin \((Cr_{0.5} = 2 \times 10^{-1})\) are similar to those of the hybridization reaction between histone cDNA and RNA transcripts from G, chromatin reconstituted with 80,000 x g supernatant protein or the phosphoproteins \((Cr_{0.5} = 2.5 \times 10^{-1})\). When RNA polymerase is omitted from the transcription reaction, and RNA is isolated (with an amount of carrier E. coli RNA equivalent to the amount of RNA transcribed in the presence of polymerase) from G, chromatin reconstituted with phosphoproteins, the isolated RNA does not show any significant extent of hybridization with histone cDNA. This experiment indicates that endogenous histone-specific sequences associated with the phosphoprotein fraction do not contribute significantly to the hybridization observed between histone cDNA and transcripts of G, chromatin reconstituted in the presence of phosphoproteins. These results clearly suggest that the ability to activate the histone mRNA sequence transcription resides in a component of the nonhistone chromosomal proteins that is soluble in 0.4 M NaCl and that has a high affinity for CaPO₄ gel.

In another series of experiments, we examined the effects on histone gene transcription of dephosphorylating nonhistone chromosomal proteins (19). With a calf thymus protease-free nuclear phosphatase that was covalently linked to agarose, S-phase nonhistone chromosomal proteins from HeLa S₃-cells were partially dephosphorylated. This procedure is effective in removing up to 60% of the phosphate groups from S-phase nonhistone chromosomal proteins. Dephosphorylation is carried out in the presence of 5 M urea, thus maintaining complete solubility, and the procedure yields proteins that, although partially dephosphorylated, are quantitatively and qualitatively identical to native S-phase nonhistone chromosomal proteins. To assay the influence of phosphate groups associated with nonhistone chromosomal proteins on histone gene transcription, chromatin was reconstituted utilizing DNA, S-phase histones, and either native S-phase nonhistone chromosomal proteins or partially dephosphorylated S-phase nonhistone chromosomal proteins. The data in Chart 14 clearly indicate that dephosphorylation results in a 75 to 80% decrease in the transcription of histone mRNA sequences. Such enzymatic dephosphorylation of S-phase nonhistone chromosomal proteins brings about less than 50% reduction in overall template activity and binding sites for E. coli RNA polymerase. Therefore it appears that not all genes are affected randomly and that histone genes are among those that are selectively inhibited.

These 2 lines of evidence provide support for a direct and functional involvement of nonhistone chromosomal protein phosphorylation in the regulation of histone gene transcription. Further elucidation of the involvement of phosphorylation in the regulation of histone gene transcription requires: (a) fractionation of the genome-associated phosphoprotein which constitute a complex and heterogeneous class of macromolecules; (b) determination of whether histone gene transcription is activated by G, protein that is modified at the onset of S phase or a protein that is synthesized and phosphorylated concomitant with the initiation of DNA synthesis; and (c) resolution of whether control of phosphorylation resides with the nonhistone chromosomal protein substrate or phosphorylating enzyme system.

**Conclusions**

While the specific regulatory elements that dictate the availability of histone genes for transcription have to date not been identified, predicated on several observations presented here and elsewhere, it is possible to speculate as to how these genes may be rendered effective templates for transcription of mRNA sequences. DNA is an effective template for the transcription of histone mRNA sequences, and histones by themselves inhibit histone gene transcription from DNA in a dose-dependent, nonspecific manner (46). When complexed with DNA alone, nonhistone chromo-
somal proteins (G, or S phase) do not affect the transcription of histone mRNA sequences (46). However when associated with DNA in the presence of histones, the nonhistone chromosomal proteins are capable of selectively rendering histone genes transcribable (43).

Chromatin reconstituted with S-phase nonhistone chromosomal proteins is an effective template for transcription of histone mRNA sequences, while chromatin reconstituted with nonhistone chromosomal proteins from G, cells is not. Hence, it appears that the cell cycle stage-specific transcription of histone genes depends on the source of nonhistone chromosomal proteins. Also, histone gene transcription during S phase appears to be "activated" by a component of the S-phase nonhistone chromosomal proteins rather than to be "repressed" during the G, phase of the cell cycle by a component of the G, nonhistone chromosomal proteins (27). Together these results suggest that a component of the S-phase nonhistone chromosomal proteins modifies the interaction of histones with DNA in a specific manner to render histone genes transcribable.

It is not clear how such modifications in the association of histones with DNA are achieved. Partial displacement of histones from DNA may be brought about by competition of nonhistone chromosomal proteins with specific sites on the DNA molecule. Alternatively, interaction of nonhistone chromosomal proteins with specific DNA sites may result in conformational modifications in adjacent DNA sequences where histone binding may be altered. Previous data that suggest that nonhistone chromosomal proteins are responsible for cell cycle stage-specific variations in the binding of histones to DNA in chromatin are consistent with such reasoning (41). One may envision “regulatory” proteins being complexed with regions of chromatin that are packaged as "nu-bodies" or with regions of the genome between the "beads." In the specific situation of histone gene activation during S phase, it remains to be established whether the regulatory protein or proteins is: (a) newly synthesized and associated with the genome at the time of DNA replication; (b) recruited from the cytoplasm or nucleoplasm during S phase; or (c) a preexisting chromosomal protein that is enzymatically modified at the onset of S phase to alter its structural and functional properties. Within this context, evidence has been discussed that suggests that nonhistone chromosomal protein phosphorylation influences the transcription of histone genes.

Another important concept that should be considered is that a single regulatory protein may control the transcription of several genes. Such a mechanism may indeed be operative under circumstances where cellular events such as histone synthesis, DNA replication, and possibly numerous other S-phase-specific processes are functionally interrelated or coupled and hence may be coordinately controlled. As fractionation and characterization of the S-phase nonhistone chromosomal proteins progress and additional genes that are selectively transcribed during S phase are examined, properties of regulators of histone gene read-out should become more apparent.

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