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Regulation of histone gene expression in Hela S_3 cells

Regulación de la expresión de genes histona en células Hela S_3

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Throughout the cell cycle of continuously dividing cells as well as after the stimulation of nondiving cells to proliferate, a complex and interdependent series of biochemical events occurs requiring modifications in expression of information encoded in the genome. Hence, the cell cycle provides an effective biological system for studying the regulation of gene readout. For the past several years our laboratory has been focusing on the cell cycle stage-specific regulation of the genes that code for histones. In the present paper, several lines of evidence are presented which suggest that (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 involved in the regulation of histone gene transcription.

Histone mRNAs

In HeLa cells there exist five classes of histones coded by at least five mRNA species. Histone mRNAs lack poly A at their 3' -OH termini (Adesnik and Darnell, 1972) and contain capped 5' termini of the types m^7GppX^mYp and $m^7GppX^mpY^mpZp$ (Stein *et al.*, 1977; Moss *et al.* 1977).

Recently we have observed that two distinct mRNA species in S phase HeLa cells code for histone H_4 (Lichtler *et al.* 1977). When ^{32}P-labeled 4-18 S RNA from S phase HeLa cells was fractionated electrophoretically on a 6% polyacrylamide gel according to the method of Grunstein *et al.* (1973), the profile shown in Fig. 1A was obtained. The individual bands were excised and the RNAs were eluted electrophoretically. The RNAs were then translated in a wheat germ protein-synthesizing system containing ^{3}H-lysine, and the translation products were electrophoresed with unlabeled marker histones on acetic acid-urea polyacrylamide gels (Fig. 1B). No preliminary purification to separate the histones from other translation products was carried out prior to electrophoresis. The difference in electrophoretic mobility between the two H_4 histone

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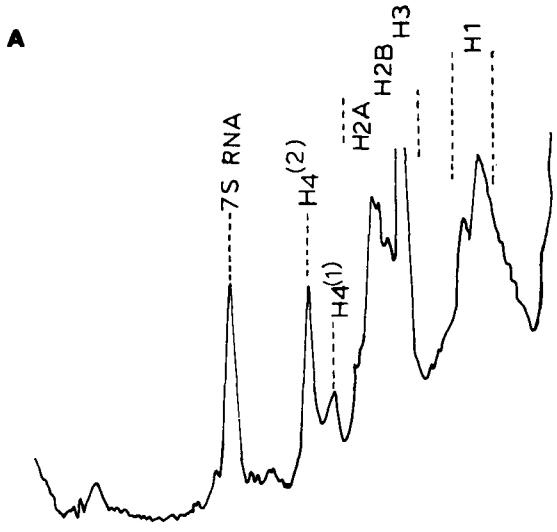
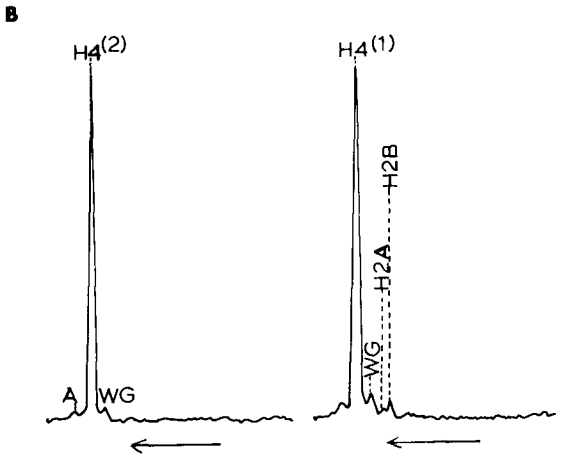
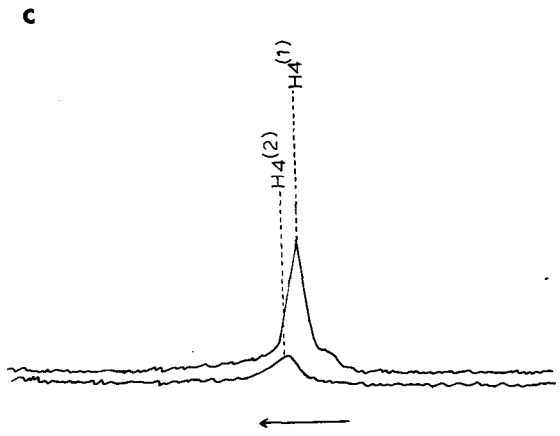


Fig. 1. A) Acrylamide gel electrophoretic fractionation of 4-28 S polysomal RNA from S phase HeLa S_3 cells. 75 μg of unlabeled RNA were combined with 7 × 10^5 cpm of ^{32}P-labeled, 4-18 S RNA from S phase HeLa cells, loaded on 0.3 × 0.4 cm wells of a 6% acrylamide gel and electrophoresed as described by Grunstein *et al.* (1973). The gel was analyzed autoradiographically and a densitometric tracing of one of the wells is shown. Details of the procedures have been reported (Lichtler *et al.* (1977). **B) Acetic acid-urea acrylamide gel electrophoretic** analysis of *in vitro* translation products of RNA extracted from bands H4 (1) and H4 (2) shown in Fig. 1A. 15 μl of ^{3}H-lysine-labeled wheat germ translation products were electrophoresed in the presence of marker histones, and fluorography was performed as described by Bonner and Laskey (1974) and Laskey and Mills (1975). C) Electrophoretic analysis under denaturing conditions of RNAs codings for histone H4, ^{32}P-labeled RNAs extracted from an acrylamide gel similar to that shown in Fig. 1A were electrophoresed in adjacent wells on an 8% acrylamide-98% formamide gel as described by Maniatis *et al.* (1975). Densitometric scans were superimposed to facilitate comparison.





mRNAs does not appear to be due to the presence of AMP residues at the 3' -OH termini of one of the RNA species, since both mRNAs were recovered in the unbound fraction during oligo dT-cellulose chromatography. When the two H_4 histone mRNAs were eluted from a 6% acrylamide-0.2% SDS gel and re-run under denaturing conditions in parallel wells of an 8% acrylamide-95% formamide gel, both RNA species retained their distinct electrophoretic mobilities (Fig. 1c). The latter result indicates that these H_4 histone mRNA species are of different molecular weights; therefore, separation in the aqueous gel system was not simply because of differences in secondary structure or because of aggregation with smaller RNA species.

There are several possible explanations for the apparent differences in molecular weight between the two RNA species which code for histone H_4. One is that the smaller molecular weight RNA represents a cleavage product of the higher molecular weight species; another possibility is that since histone genes are reiterated in human cells, the different RNAs could represent transcripts from different copies of the gene. Studies are presently underway to further characterize the two H_4 mRNA species and the proteins for which they code.

Evidence for transcriptional control of histone gene expression

It has been established in many systems that histone synthesis and the deposition of these proteins on DNA is restricted to the S phase of the cell cycle (Spalding *et al.* 1966; Robbins and Borum 1967; Stein and Borun 1972), both in continuously dividing populations of cells and after stimulation of nondividing cells to proliferate. It has also been observed that inhibition of DNA replication results in a rapid and complete shutdown of histone synthesis (Spalding *et al.* 1966; Robbins and Borun 1967; Borun *et al.* 1967; Gallwitz and Mueller 1969; Stein and Borun 1972; Stein and Thrall 1973). These findings suggest that expression of histone genes is confined to the S phase of the cell cycle, and the coupling of histone and DNA synthesis is consistent with a functional relationships between these two events. We have been examining the regulation of histone gene expression and the level at which control is mediated. The presence of histone mRNA sequences on HeLa S_3 cell polyribosomes, in the post-polysomal cytoplasmic fraction and in the nucleus during the G_1, S and G_2 phases of the cell cycle has been examined. Additionally, *in vitro* transcription of histone mRNA sequences from nuclei and chromatin isolated from HeLa cells at various times during the cell cycle has been assayed.

Since these studies require a high resolution probe for identification of histone mRNA sequences, we have synthesized a ^{3}H-labeled, single-stranded DNA complementary to histone mRNAs. Histone mRNAs were isolated from polyribosomes of S phase HeLa cells and chromatographed on oligo dT-cellulose to remove poly A-containing material. Poly A was then added to the 3'-OH ends of the histone, mRNAs with an ATP: polynucleotidylexotransferase isolated from maize seedlings (Mans and Huff 1975), and the polydenylated mRNAs were transcribed with RNA-dependent DNA polymerase isolated from avian myeloblastosis virus, using dT_{10} as a primer in the presence of ^{3}H-dCTP and ^{3}H-dGTP. Transcription was carried out in the presence of actinomycin D to insure that the DNA copy was single-stranded. Isolation, purification, and characterization of histone mRNAs as well as synthesis and properties of the histone cDNA probe have been reported (Thrall *et al.* 1974; Stein *et al.* 1975a, 1975b; Thrall *et al.* 1977). Identification and quantitation of histone mRNA sequences synthesized *in vivo* or transcribed *in vitro* from nuclei or chromatin were based on the kinetics of hybridization to ^{3}H histone cDNA. Hybridization was carried out in RNA excess in the presence of 50% formamide and 0.5 M NaCl, and hybrid formation was assayed by resistance to single-strand specific S_1 nuclease.

Histone mRNA Sequences in Cellular Fractions

Our initial attempts to assess the level(s) at which regulation of histone gene expression resides involved determination of the representation of histone mRNA sequences on polysomes during the cell cycle of synchronized HeLa S_3 cells (Stein *et al.* 1975b). Two methods were employed to achieve cell synchrony. S and G_2 phase cells were obtained by two cycles of 2 mM thymidine block. As shown in Fig. 2, 3 hours after release from the second thymidine block, 98% of cells are in S phase. 7.5 hours after release from thymidine

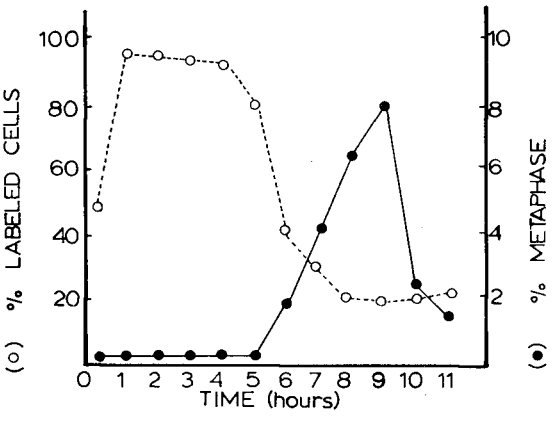


Fig. 2. Percentage of HeLa S_3 cells synthesizing DNA and mitotic index at various times after release of HeLa S_3 cells from two cycles of 2 mM thymidine block. Cells were pulse labeled for 15 min with 5 μCi of ^{3}H-thymidine/ml and the percentage of cells synthesizing DNA was determined autoradiographically (o).

The mitotic index (•) was also determined from the autoradiographic preparations.

block, when G_2 cells are harvested, approximately 20% of the cells are still undergoing DNA replication as assayed by thymidine labeling followed by autoradiography. This high background of S phase cells in the G_2 population complicates interpretation of G_2 nuclei acid hybridization studies. Unfortunately, better methods are not available for obtaining a pure population of G_2 phase HeLa cells.

Doble thymidine synchronization is even less suitable for obtaining G_1 cells because, as Fig. 2 clearly indicates, when cells synchronized by this procedure reach G_1 (11 hours after release) 25% of the cells are undergoing DNA replication (are in S phase). Therefore, we routinely obtain G_1 cells by mitotic selective detachment- a procedure which yields 97% G_1 cells 2 hours after harvest of mitotic cells, without detectable levels of S phase cells.

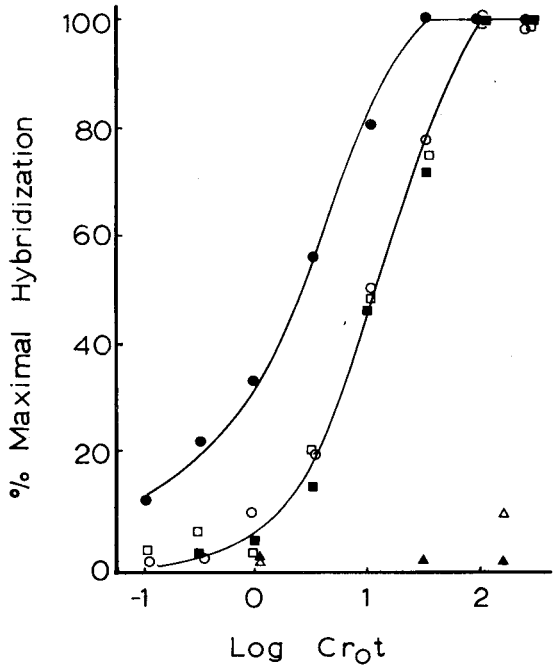


Fig. 3. Kinetics of annealing of histone cDNA to RNA isolated from non-membrane-bound plolyribosomes of G_1, S. and G_2 phase HeLa S_3 cells. ^{3}H-labeled cDNA (27,000 dpm/ng) and unlabeled RNA were hybridized at 52°C in sealed glass capillary tubes containing in a volume of 15 μl: 50% formamide-0.5 M NaCl-25mM Hepes (pH 7.0)-1 mM EDTA-0.04 ng of cDNA and 3.75 or 7.5 μg of polyribosomal RNA from G_1 (□), S (o) or G_2 (●) phase HeLa S_3 cells. Samples were removed at various times and incubated for 20 min in 2.0 ml of 30 ml sodium acetate-3.0 M NaCl-1 mM $ZnSO_4$-5% glycerol (pH 4.6), containing S_1 nuclease at a concentration sufficient to degrade at least 96% of the single-strandend nucleic acids present. Te amount of labeled DNA, resistant to digestion was determined by trichloroacetic acid precipitation. Polyribosomal RNA was isolated as previously reported (Stein *et al.* 1975b) Cr_0t = mole ribonucleotides × sec/liter.

As shown in Fig. 3, formation of hybdrids between S phase polyribosomal RNA and histone cDNA indicates the presence of histone-specific sequences on polyribosomes of S phase cells. In contrast, the absence of G_1 polyribosomal RNA hybridization demonstrates that histone mRNA sequences are not components of G_1 polyribosomes. Comparison of the kinetics of the hybridization reaction between S phase polyribosomal RNA and histone cDNA (Cr_0t = 1.8) with the kinetics of the histone mRNA-cDNA hybridization reaction ($Cr_0t = 1.7 \times 10^{-2}$) indicates that histone mRNA sequences account for 0.9% of the RNA from S phase non-membrane bound polyribosomes (Stein *et al.* 1975b). This value is consistent with the situation *in vivo* where approximately 10-15% of the protein synthesis in S phase HeLa cells is histone synthesis (Stein and Borun 1972). Additionally, the absence of hybrid formation between G_1 polyribosomal RNA and histone cDNA establishes the absence of ribosomal RNA (5S, 18S and 28S) and tRNA complementary sequences in the histone cDNA probe.

Determination of the presence or absence of histone mRNA sequences on G_2 polyribosomes is complex. The kinetics of the hybridization reaction between G_2 polyribosomal RNA and the histone cDNA (Cr_0t = 8.5) suggests that the amount of histone mRNA sequences present on the polyribosomes of G_2 phase cells is 21% of that present on S phase polyribosomes. However, as discussed previously, 20% of the G_2 phase cell population consists of S cells. It is therefore reasonable to conclude that the histone mRNA sequences present in the G_2 polyribosomal RNA are due to the S phase cells in the G_2 population. This implies that histone mRNA sequences are not associated with polyribosomes during the G_2 phase of the cells cycle.

These results demonstrate that in HeLa cells histone mRNA sequences become asso-

ciated with polyribosomes during the transition from the G_1 to the S phase of the cell cycle. Such findings are in agreement with *in vitro* translation studies from several laboratories wich indicate that RNA isolated from polyribosomes of S phase HeLa cells supports the synthesis of histone, whereas the RNA from polyribosomes of G_1 cells or of S phase cells treated with inhibitors of DNA synthesis does not (Borun *et al.* 1975). The hybridization studies eliminate the possibility that histone mRNAs are components of the polyribosomes during periods of the cell cycle other than S phase, but at such times are in some way rendered non-translatable. These findings suggest that histone gene expression in HeLa cells is not regulated at the translational level and transcriptional control is implied. This interpretation is supported by other data from our laboratory suggesting that histone mRNA sequences are present in the nuclei of S phase cells but not in the nuclear RNA of G_1 phase cells (Fig. 4), and that histone mRNA sequences are not sequestered in the post-polysomal cytoplasmic fraction of G_1 cells.

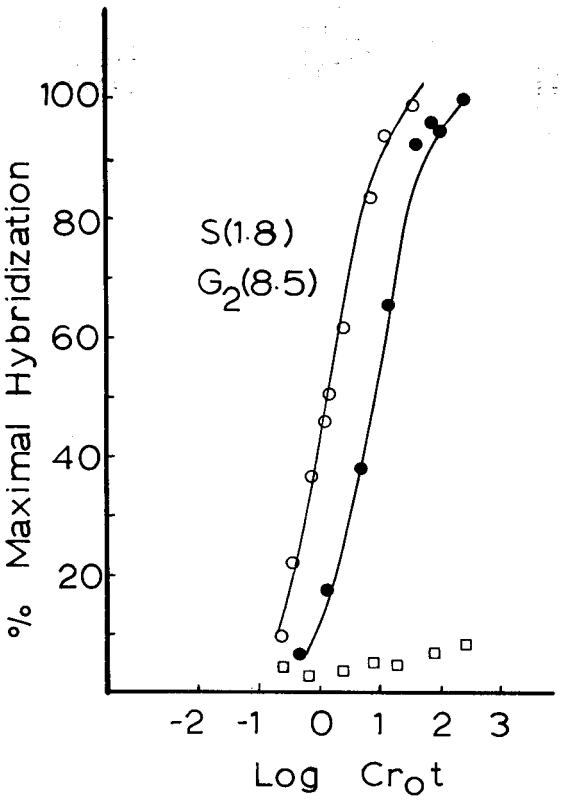


Fig. 4. Kinetics of hybridization with histone ^{3}H cDNA of RNAs transcribed from isolated nuclei of G_1 and S phase HeLa S_3 cells. The transcription reactions contained 0.4 mM GTP, CTP, UTP and ATP-5 mM Mg acetate - 70 mM KCl-25 mM Hepes (pH 7.6)-0.04% 2-mercaptoethanol- 12.5% glycerol-1-5×10^7 nuclei/ml. Incubation was at 25°C for 45 min. RNA transcribed from G_{1S1} (▲) and S phase (●) nuclei. RNA isolated from G_1 (△) and S phase (○) nuclei that were incubated in the absence of ribonucleoside triphosphates. RNA from S phase nuclei transcribed in the presence of α-amanitin at concentration of 1 μg/ml (□) and 100 μg/ml (■).

It should be emphasized that 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, post-transcriptionally (Farquhar and McCarthy 1973; Skoultchi and Gross 1973; Gross *et al.* 1973; Gabrielli and Baglioni 1975). In such circumstances histone mRNA sequences appear to be components of a stored maternal mRNA population which become templates for protein synthesis after fertilization.

In Vitro Transcription of Nuclei

Another line of evidence suggesting that regulation of histone gene expression resides at least in part at the transcriptional level is provided by *in vitro* transcription of isolated nuclei (Detke *et al.* 1977). Nuclei were isolated by a modification of the method of Sarma *et al.* (1976) and transcribed using the endogenous RNA polymerase. In this system incorporation of ^{3}H-UMP into RNA is linear for 45 min and is dependent upon the addition of exogenous ribonucleoside triphosphates. In a 45 min incubation, nuclei from S phase HeLa cells synthesize 0.17 pg of RNA/nucleus. The isolated nuclei retain activity representative of all three classes of DNA-dependent RNA polymerase. If transcription is inhibited by incubating the nuclei with increasing amounts of α-amanitin, a three-component inhibition curve is obtained. Based on known sensitivities of the

solubilized polymerases from HeLa cells to α-amanitin (Hossenlopp *et al.* 1975; Benecke and Siefart 1975; Weil and Blatti 1976), it is apparent that the nuclei possess all three classes of RNA polymerase. Under our conditions, the class I polymerase comprises 35% of the total RNA synthesizing activity, polymerase II, 58% and polymerase III, 7%.

Results of hybridization of ^{3}H-labeled histone cDNA with nuclear RNAs indicate that histone mRNA sequences are being actively synthesized in isolated S phase nuclei. The RNA from transcriptionally active nuclei incubated in the presence of all four ribonucleoside triphosphates hybridizes with histone cDNA with a Cr_ot of 2.7, whereas RNA from nuclei which are not actively transcribing due to the absence of the four ribonucleoside triphosphates hybridizes with a Cr_ot of 10 (Fig. 4). The increase in the representation of histone mRNA sequences which we have observed ranges from 2-4 fold. The histone mRNA sequences comprise 0.38-0.63% of the total RNA of active nuclei and 0.13-0.17% of the endogenous pool of nuclear RNA. Although nuclei isolated by this method have been reported to possess the capability for initiation of transcription (Sarma *et al.* 1976), it is not known whether the observed stimulation is due to the *de novo* initiation of histone mRNA synthesis or whether we are merely detecting the completion of preinitiated histone mRNAs. In contrast, G_1 nuclei did not synthesize detectable amounts of histone mRNA sequences (Fig. 4), although the general transcriptional activity of G_1 and S phase nuclei was similar. Neither RNA of the endogenous pool of G_1 nuclei nor the RNA of active G_1 nuclei was found to hybridize with the histone cDNA probe, even at a Cr_ot of 320. It thus appears that the histone genes are transcribed only during the S phase portion of the cell cycle.

The polymerase responsible for the synthesis of histone mRNA sequences in S phase nuclei can be determined by incubating the nuclei in the presence of varying concentrations of α-amanitin. The complete inhibition of the synthesis of histone mRNA sequences at as little as 1 μg of α-amanitin/ml (Fig. 4) indicates that the class II RNA polymerase is responsible for the transcription of histone genes. It is the only class of polymerase which is inhibited completely at this concentration, whereas the other two polymerase classes are inhibited only slightly or not at all. Since the $Cr_ot_{1/2}$ of the hybridization reaction between histone cDNA and RNA isolated from nuclei transcribed in the presence of either 1 μg of α-amanitin/ml is equal to 10, a value identical to that obtained with endogenous S phase nuclear RNAl no new histone RNA sequences are synthesized in the presence of α-amanitin at a concentration of 1 μg/ml or more. Thus the synthesis of histone mRNA sequences appears to be performed by the class II RNA polymerase.

Chromatin Transcription

Chromatin from G_1 and S phase cells was transcribed with *E. coli* RNA polymerase in a cell-free system, the RNA molecules were isolated and their ability to form hybrids with histone cDNA was determined (Stein *et al.* 1975a). The kinetics of the hybridization of histone cDNA and RNA transcripts from G_1 as well as S phase chromatin are shown in Fig. 5. Although transcripts from S phase chromatin hybridize with histone cDNA with a $Cr_ot_{1/2}$ value of 2×10^{-1} compared with a value of 1.7×10^{-2} for the histone mRNA-histone cDNA hybridization reaction, there is no evidence of hybrid formation between histone cDNA and G_1 phase transcripts, even at a $Cr_ot_{1/2}$ value of 100, indicating at least a 1000-fold increase in availability of histone genes for transcription. Since the overall template activity *in vitro* for RNA synthesis of G_1 and S phase chromatin is similar, it is unlikely that the failure to detect histone mRNA sequences in G_1 chromatin transcripts results from a dilution effect. The maximal hybrid formation (65%) between histone cDNA and S phase transcripts is 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 T_m (melting temperature) of these hybrids is identical to the T_m of histone mRNA-cDNA hybrids (65°C in 50% formamide-0.5 M NaCl-25 mM HEPES (pH 7.0)-1 mM EDTA). It should be noted that the T_m value obtained under these conditions is con-

sistent with an RNA-DNA hybrid having a GC content of 54% which is the nucleotide composition of histone mRNA reported by Adeskik and Darnell (1972) and Thrall *et al.* (1972).

RNAs synthesized in intact cells may remain associated with chromatin during isolation and in part account for hybrid formation between RNA transcripts formed *in vitro* and cDNA for specific genes. Undoubtedly, the extent to which this phenomenon occurs varies with the tissue or cell and the method of chromatin preparation. To determine if such endogenous RNAs account for the histone-specific sequences which are detected in transcripts from S phase chromatin, the following controls were carried out. S phase chromatin was placed in the 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. When RNA was extracted and annealed with histone cDNA, no significant hybridization was observed (Fig. 5). Additionally, RNA isolated from S phase chromain in the absence of carrier RNA showed no hybrid formation with histone cDNA. These results indicate that endogenous histone-specific sequences associated with S phase chromatin are not contributing significantly to the hybridization observed with S phase transcripts. It is therefore reasonable to conclude that histone sequences in S phase transcripts can be accounted for by synthesis *in vitro*.

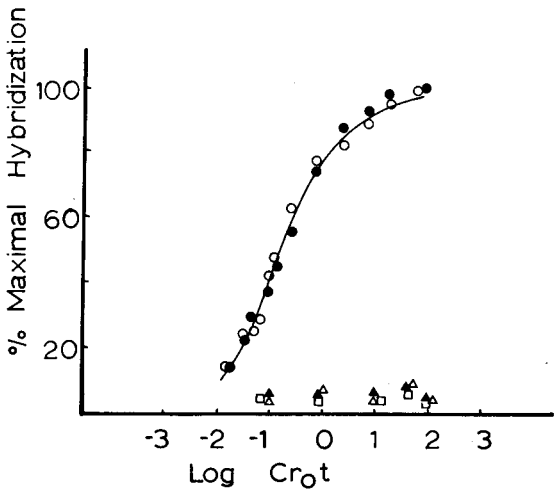


Fig. 5. Hybridization of histone cDNA to *in vitro* transcripts from native and reconstituted HeLa S_3 cell chromatin. ^{3}H-labeled cDNA and 0.15 or 1.5 μg of RNA were hybridized as described in the legend of Fig. 3. RNA transcripts from native S phase chromatin (●), native G_1 phase chromatin (▲), chromatin reconstituted with S phase nonhistone chromosomal proteins (○) and chromatin reconstituted with G_1 phase nonhistone chromosomal proteins (△). Histone ^{3}H-cDNA was also annealed to RNA isolated from native S phase chromatin in the presence of E. coli RNA as carrier (□).

When G_1 chromatin was transcribed in the presence of an amount of histone mRNA equivalent to that transcribed from S phase chromatin, and the mixture of G_1 transcripts and added histone mRNA was subsequently isolated, hybridization with histone cDNA occured at the expected Cr_0t value (2×10^{-1}) (Park *et al.* 1976). This result suggests that the absence of histone mRNA sequences amongst RNA transcripts from G_1 chromatin is not attributable to a specific nuclease associated with chromatin during the G_1 phase of the cell cycle. The possibility that histone mRNA sequences were present in G_1 transcripts but were not detected because they were in a double-stranded form due to symmetric transcription is unlikely, since heating the hybridization mixture to 100°C for 10 min before incubation had no effect on the hybridization of histone cDNA with the transcripts (Park *et al.* 1976).

The results from these studies indicate that histone sequences are available for transcription from chromatin during S phase but not during G_1. Such findings are consistent with the restriction of histone synthesis to the S phase of the cell cycle and the presence of histone mRNAs on polyribosomes, in the post-polysomal cytoplasmic fraction and in the nucleus only during S phase. Taken together with results from the *in vitro* nuclear transcriptions studies, this evidence suggests that in continuously dividing HeLa S_3 cells expression of histone genes is regulated, at least in part, at the transcriptional level and that readout of these genetic sequences occurs only during the period of DNA replication. It is also reasonable to conclude that chromatin is a valid and effective model for studying the regulation of cell cycle stage-specific transcription of histone genes.

Coupling of histone gene expression and DNA replication

Inhibition of DNA synthesis is associated with a rapid and complete inhibition of histone synthesis (Spalding *et al.* 1966; Robbins and Borun 1967; Borun *et al.* 1967; Gallwitz and Mueller 1969; Stein and Borun 1972; Stein and Thrall 1973). Although a definitive explanation for the coupling of histone synthesis and DNA synthesis cannot be provided at this time, it is reasonable to speculate that histones are required to complex with newly replicated DNA. Neither nucleoplasmic nor cytoplasmic pools of histones are present and histones are needed for repression of DNA sequences which are not to be immediately transcribed and for imposition of the appropriate structure to the genome, i.e., packaging of the newly replicated DNA.

To examine the level at which the coupling of histone gene expression and DNA replication resides, we have pursued the following approach (Stein *et al.* 1977a). S phase 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 semi-conservative DNA synthesis. Both inhibitors also effectively block histone synthesis. We then assayed the influence of these inhibitors on the levels of histone mRNA sequences present in the various intracellular RNA fractions by hybridization to histone cDNA. Consistent with *in vitro* translation data from several laboratories (Butler and Mueller 1973; Breindl and Gallwitz 1974; Borun *et al.* 1975) cytosine arabinoside and hydroxyurea bring about a drastic reduction (> 99%) in the representation of histone mRNA sequences on polyribosomes (Table I). In contrast, neither inhibitor reduces *in vitro* transcription of histone mRNA sequences from chromatin (Table I), and only a 10% reduction is observed in the level of histone mRNA sequences in nuclei of cells treated with hydroxyurea or cytosine arabinoside.

These results suggest that coupling of histone gene expression and DNA replication is not mediated at the transcriptional level, and post-transcriptional or translational control is strongly implied. This interpretation is further supported by a 10-fold increase in the representation of histone mRNA sequences in the post-polysomal cytoplasmic fraction (Table I). Accumulation of histone mRNA sequences in the cytoplasm after inhibition of DNA synthesis may be the results 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, an elevated level of histone mRNA sequences in the cytoplasm was not observed (Stahl and Gallwitz 1977). However, *in vitro* translation does not eliminate the possibility that histone mRNAs are present in nontranslatable states.

TABLE I

Effect of Hydroxyurea and Cytosine Arabinoside on Representation of Histone mRNA Sequences in Chromatin Transcripts and in Various Subcellular Fractions of S Phase HeLa Cells

	Percent of Untreated S Phase Control	
	Hydroxyurea	*Cytosine Arabinoside*
Chromatin Transcripts	100	100
Nuclear RNA	90	90
Polysomal RNA	< 0.5	0.5
Post-Polysomal Cytoplasmic RNA	1100	1100

S phase HeLa cells were treated with hydroxyurea (10 mM) or cytosine arabinoside (40 μg/ml) for 30 min. RNAs were then isolated from nuclei, polysomes and the post-polysomal cytoplasmic fraction. Chromatin was prepared and transcribed with *E. coli* RNA polymerase, and transcrips were isolated. The kinetics of hybridization of the RNAs with ^{3}H-labeled histone cDNA were measured.

Nonhistone chromosomal proteins in the regulation of histone gene expression

A role for nonhistone chromosomal proteins in the regulation of histone gene expression during the cell cycle has been suggested by several lines of evidence. Variations observed in the composition and metabolism of the nonhistone chromosomal proteins during G_1, S, G_2 and mitosis, and their correlation with changes in transcription are consistent with a regulatory function for these proteins (reviewed by Stein and Baserga 1972; Baserga 1974; Stein *et al.* 1974b; Elgin and Weintraub 1975). Further evidence that nonhistone chromosomal pro-

teins may be responsible for specific transcription at various stages of the cell cycle comes from a series of chromatin reconstitution studies which indicate that nonhistone chromosomal proteins determine the quantitative differences in availability of DNA as template for RNA synthesis during the cell cycle of continuously dividing cells (Stein and Farber 1972), as well as after stimulation of nondividing cells to proliferate (Stein *et al.* 1974a). 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, the histone genes, we initially pursued the following approach.

Chromatin isolated from G_1 and S phase cells was dissociated in 3 M NaCl-5 M 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.* (1969) using DNA and histones pooled from G_1 and S phase cells, and either G_1 or S phase nonhistone chromosomal proteins. Fig. 5 indicates that RNA transcripts from chromatin reconstituted with S phase nonhistone chromosomal proteins hybridize with histone cDNA ($Cr_0t = 2 \times 10^{-1}$), whereas those from chromatin reconstituted with G_1 nonhistone chromosomal proteins do not exhibit a significant degree of hybrid formation (Stein *et al.* 1975a). It should be emphasized that the kinetics and extent of hybridization with the cDNA are same for transcripts of native S phase chromatin and chromatin reconstituted with S phase nonhistone chromosomal proteins. Furthermore, the amount of RNA transcribed and the recovery during isolation of transcripts from native and reconstituted chromatin preparations were essentially identical. These results suggest a functional role for nonhistone chromosomal proteins in regulating the availability of histone sequences of *in vitro* transcription during the cell cycle. Such a regulatory role for the nonhistone chromosomal proteins is in agreement with results from other laboratories which have indicated that these proteins are responsible for the tissue-specific transcription of globin genes (Paul *et al.* 1973; Barrett *et al.* 1974; Chiu *et al.* 1975, and the hormone-induced transcription of ovalbumin genes (Tsai *et al.* 1976). However, the present results indicate that nonhistone chromosomal proteins are involved in the regulation of genes which are transiently expressed.

We then addressed the question of whether the difference in the transcription of histone genes *in vitro* from G_1 and S phase chromatin is due to a component(s) of the S phase nonhistone chromosomal proteins which renders histone genes transcribable or to a specific inhibitor of histone gene transcription present among the G_1 nonhistone chromosomal proteins (Park *et al.* 1976). As shown in Fig. 6, when G_1 chromatin is dissociated and then reconstituted in the presence of increasing amount of S phase nonhistone chromosomal

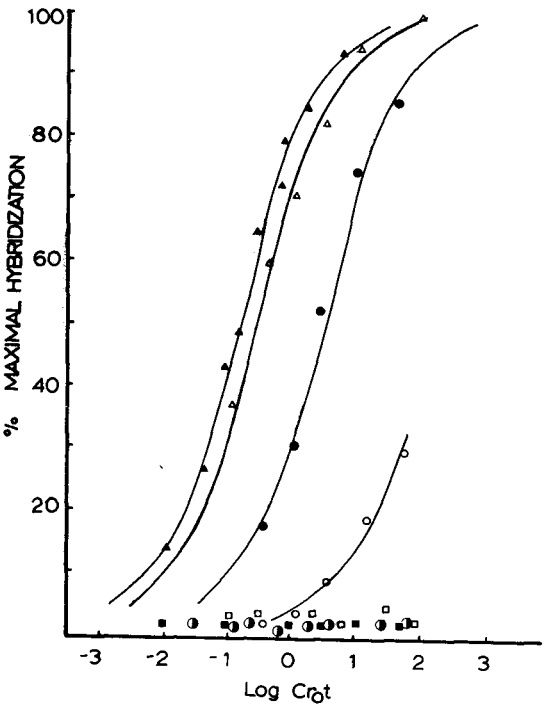


Fig. 6. Hybridization of histone cDNA to *in vitro* transcripts from G_1 phase HeLa chromatin reconstituted in the presence of various amounts of S phase HeLa nonhistone chromosomal proteins. cDNA was annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 0.01 (○), 0.10 (●), or 1.00 (△) mg of S phase nonhistone chromosomal protein or 1.0 mg of S phase histones (□)/mg of G_1 DNA as chromatin. cDNA was also annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 1.0 mg of G_1 total chromosomal protein/mg of G_1 DNA as chromatin (■) and to RNA transcripts from native chromatin of G_1 (◐) and S (▲) phase cells.

proteins, hybrid formation between transcripts from these chromatins and histone cDNA is seen at progressively lower Cr_0t values, indicating a dose-dependent increase in the transcription of histone genes from the G_1 chromatin. By comparing the kinetics of the hybridization of histone cDNA with transcripts from S phase chromatin ($Cr_0t = 2 \times 10^{-1}$), and the kinetics of the hybridization of histone cDNA with transcripts from G_1 chromatin reconstituted with a 1:1 ratio of S phase nonhistone chromosomal proteins to DNA ($Cr_0t_{1/2} = 3 \times 10^{-1}$), it can be seen that the histone genes are transcribed from the reconstituted chromatin to approximately the same extent as from native S phase chromatin. This level of histone gene transcription is the maximum that can be achieved, even if the added S phase nonhistone chromosomal protein to DNA ratio is increased above 1:1. The T_m of the hybrids and the maximal level of hybridization (65%) in all cases are identical to those of the hybrids formed between histone mRNA and histone cDNA. When G_1 chromatin was dissociated and then reconstituted in the presence of S phase histones, even at a 1:1 ratio of S phase histone to DNA, no stimulation of transcription of histone genes was observed (Fig. 6). It should be noted that there were no significant differences among the various chromatin preparations in the yield or recovery of RNA 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_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 was not observed when G_1 chromatin was dissociated and then reconstituted in the presence of additional G_1 chromosomal proteins, even at a 1:1 ratio of additional G_1 protein to DNA (Fig. 6). This result suggest that specific chromosomal proteins are required to elicit histone gene readout.

To determine whether G_1 chromatin contains an inhibitor of histone gene transcription that is degraded or inactivated as the cells progress from the G_1 to the S phase of the cell cycle. chromatin from S phase cells was dissociated and reconstituted in the presence of total chromosomal proteins from G_1 cells. Even et a 1:1 ratio of total G_1 chromosomal proteins to DNA, histone gene transcription from S phase chromatin was not significantly inhibited (Park *et al.* 1976). This result would suggest that any specific inhibitor of histone gene expression is lost during isolation, dissociation, fractionation or reconstitution of chromatin, or that any inhibition of histone gene transcription by G_1 chromosomal proteins can be overridden by S phase nonhistone chromosomal proteins. Results from chromatin reconstitution experiments described above are consistent with a direct role for nonhistone chromosomal proteins in dictating availability of histone genes for transcription from chromatin of continuosly dividing HeLa S_3 cells. Similar experiments indicate that transcription of histone mRNA sequences from chromatin of human diploid fibroblasts after stimulation of these cells to proliferate is also mediated by a component of the S phase nonhistone chromosomal proteins (Jansing *et al.* 1977). Other results suggest that phosphorylation of nonhistone chromosomal proteins may be an important component of the mechanism by which histone gene readout is regulated (Kleinsmith *et al.* 1976; Thomson *et al.* 1976).

Fractionation of nonhistone chromosomal proteins

In order to purify the molecules responsible for the regulation of specific genes, it is necessary to determine not only whether a given fraction has activity but also how much activity is present. We have been using the techniques of chromatin reconstitution and *in vitro* transcription to assay and quantitate the activity of nonhistone chromosomal protein fractions for their involvement in the control of histone gene transcription from chromatin. G_1 chromatin, which does not serve as a template for histone gene transcription, is dissociated in 5 M urea-3 M NaCl-10 mM Tris (pH 8.3) and then reconstituted in the presence of added S phase nonhistone chromosomal protein fractions. The reconstituted chromatin preparations are transcribed in a cell-free system with *E. coli* RNA polymerase, and the transcript are assayed

for their ability to hybridize with histone cDNA Since, as discussed above, this system responds to added nonhistone chromosomal proteins with a dose-dependent, but saturable, increase in the transcription of histone genes, we have a viable method for monitoring nonhistone chromosal protein fractionation.

We have recently been able to achieve a substantial purification of the S phase nonhistone chromosomal protein(s) which exhibit the ability to render histone mRNA sequences transcribable from chromatin. The fractionation of S phase HeLa nonhistone chromosomal proteins was accomplished by ion-exchange chromatography on QAE-Sephadex followed by SP-Sephadex ion-exchange chromatography and then gel filtration chromatography on Sephadex G-100 (Park *et al.* 1977b).

Ion-exchange chromatography of S phase HeLa chromosomal proteins on QAE-Sephadex A-25 was carried out as follows. Chromosomal proteins from which nucleic acids had been removed by ultracentrifugation were dialyzed against 5 M urea-10 mM Tris (pH 8.3), and were loaded on a column of QAE-Sephadex A-25 previously equilibrated with the same buffer. The proteins were then eluted with two column volumes each of 5 M urea-10 mM Tris (pH 8.3), containing 0, 0.1, 0.25, 0.5 and 3 M NaCl. The histones and approximately 10% of the nonhistone chromosomal proteins were not bound and were eluted in the void volume (Fig. 7), whereas a complex but electrophoretically distinct class of nonhistone chromosomal proteins was eluted by each salt concentration (Park *et al.* 1977a). Total recovery of proteins from the column was approximately 85%. To determine the ability of each of the QAE fractions to render histone genes available for transcription, 3 mg of G_1 chromatin (containing approximately 1 mg of DNA) were dissociated and then reconstituted in the presence of 100 μg of each of the QAE fractions. As shown in Fig. 8a, transcripts from G_1 chromatin reconstituted in the presence of the unbound fraction or of the material eluted with 0.1, 0.25, or 3.0 M NaCl did not show significant hybrid formation with histone ^{3}H-cDNA. In contrast, even though the total amount of RNA transcribed was similar, transcripts of G_1 chromatin reconstituted

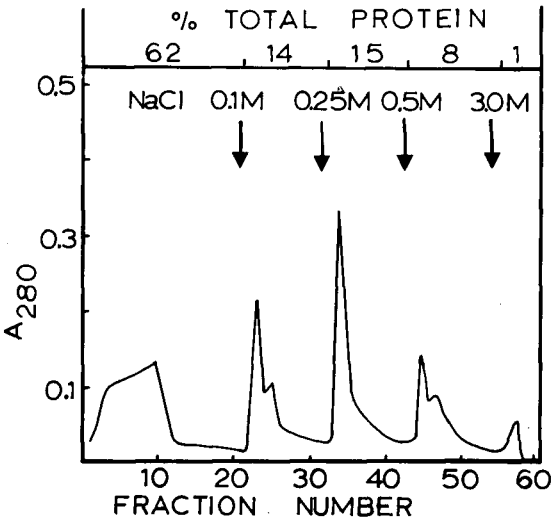


Fig. 7. Elution profile of S phase HeLa chromosomal proteins from QAE-Sephadex. Proteins were loaded in 5 M urea -10 mM Tris (pH 8.3), and were eluted with this buffer containing 0.10 M, 0.25 M, 0.50 M and 3.0 M NaCl. The percentage of protein eluted in each peak is shown in the upper panel.

in the presence of the 0.5 M QAE fraction hybridized efficiently with histone cDNA ($Cr_0t_{1/2} = 4 \times 10^{-1}$).

As discussed above, when G_1 chromatin is reconstituted in the presence of various amounts of added S phase chromosomal proteins, there is a dose-dependent but saturable activation of histone gene transcription. Specifically, transcripts from G_1 chromatin reconstituted in the presence of 1000 μg of S phase total chromosomal protein per mg of G_1 DNA (as chromatin) contain approximately 10 times more histone mRNA sequences than transcripts from the same amount of G_1 chromatin reconstituted in the presence of 100 μg of these proteins. Since the 0.5 M QAE fraction containsl only approximately 8% of the total chromosomal protein, one would anticipate that 100 μg of the 0.5 M QAE fraction should activate histone gene transcription from G_1 chromatin to the same degree as 1000 μg of the total S phase HeLa chromosomal protein. As can also be seen in Fig. 8a, there are no significant differences in the kinetics of hybrid formation with histone cDNA between transcripts from G_1 chromatin reconstituted in the presence of 100 μg

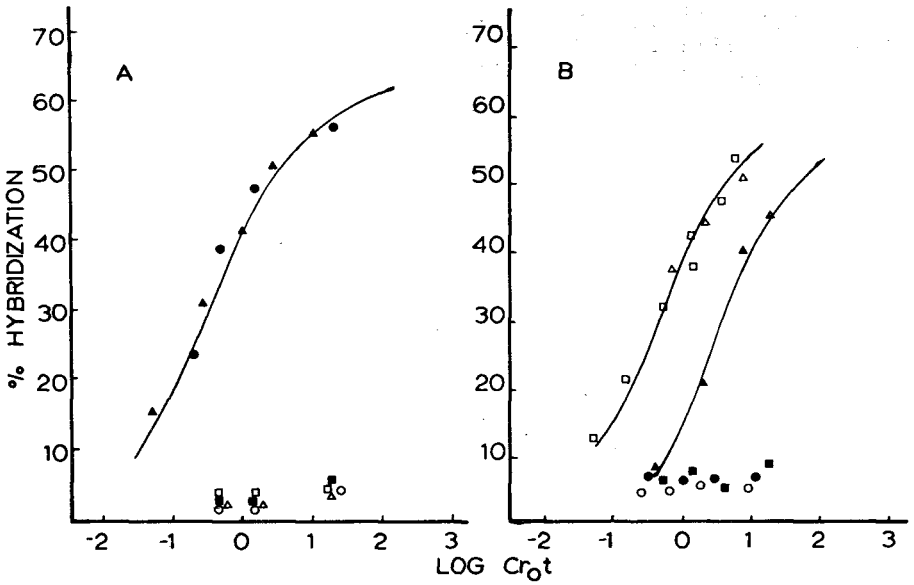


Fig. 8. Hybridization of histone cDNA to *in vitro* transcripts from G_1 HeLa chromatin reconstituted in the presence of S phase HeLa cell chromosomal protein fractions. A) Transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 100 μg of S phase chromosomal proteins eluted from QAE Sephadex A-25 by 5 M urea-10 mM Tris (pH 8.3) containing 0 M (□), 0.1 M (■), 0.25 M (○), 0.5 M (●) and 3.0 M (△) NaCl or in the presence of 1000 μg of phase total chromosomal proteins (▲). B) Transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 10 μg of S phase chromosomal proteins eluted from SP-Sephadex by 5 M urea-0.2 M sodium acetate (pH 5.2) containing 0.1 M (○), 0.2 M (●), and 0.4 M (□) NaCl and by 5 M urea-10mM Tris (pH 8.3)-3 M NaCl (■) or in the presence of 1 μg (△) or 0.1 μg (▲) of the 0.4 M fraction.

of the 0.5 QAE fraction and 1000 μg of the total HeLa chromosomal proteins per mg of G_1 DNA (as chromatin), indicating that *at least* a 10-fold purification of the S phase nonhistone chromosomal protein(s) involved in transcription of histone genes has been achieved.

Additional purification of the S phase nonhistone chromosomal protein(s) involved with transcription of histone genes was obtained by chromatography on SP-Sephadex C-25. The proteins eluted from QAE-Sephadex by 0.5 M NaCl were titrated to pH 5.3 with 1 M sodium acetate (pH 4.5), dialyzed against 5 M urea-0.1 M NaCl-0.2 M sodium acetate (pH 5.2), and loaded on a column previously equilibrated with the same buffer. The proteins were eluted with two column volumes each of 5 M urea-0.2 M sodium acetate (pH 5.2) containing 0.2 M and 0.4 M NaCl and then with two column volumes of 5 M urea-3 M NaCl-100 mM Tris (pH 8.3). Total recovery of protein from the column was approximately 50% To assay the ability of the SP fractions to render histone genes available for transcription, 1 mg of DNA as chromatin was dissociated and then reconstituted in the presence of 10 μg of each of the fractions. Only the transcripts from the chromatin reconstituted in the presence of the 0.4 M SP fraction showed a significant level of hybridization with histone cDNA ($Cr_0t_{1/2} = 2 \times 10^{-1}$), (Fig. 8b). The 0.4 M SP fraction contains 10% of the protein loaded on the column. Since as shown in Fig. 8b, 1 μg of the SP fraction is as effective as 10 μg of this same fraction in dictating availability of histone genes for transcription from G_1 chromatin, it is evident that the extent to which histone genes can be rendered transcribable by this fraction is saturable. This result would not be expected if endogenous histone mRNA sequences were present in the SP fraction and were not responsible for the observed hybridization with the histone cDNA.

To ascertain the molecular weight of the component of the 0.4 M SP fraction which affects transcription of histone genes, we chromatographed the fraction on a 1.5 × 27 cm column of Sephadex G-100. The proteins were titrated to pH 8.3 with 1 M Tris and solid NaCl was added to a final concentration of 3 M. The protein were eluted from the column with 5 M urea-3 M NaCl-10 mM Tris (pH 8.3), and fractions were assayed for their ability to render histone genes transcribable. The activity was contained in the fractions with elution constants between 0.16 and 0.27, corresponding to an apparent molecular weight of 40,000-60,000 daltons. The Sephadex G-100 column was calibrated using bovine serum albumin, ovalbumin, and whale skeletal muscle myoglobin in 5 M urea 3 M NaCl 10 mM Tris (pH 8.3).

It is important to determine whether molecules which are involved in the regulation of histone gene transcription from chromatin are protein or nucleic acid in nature. Therefore, the 0.4 M SP fraction was centrifuged in 5 M urea-10 mM Tris (pH 8.3) containing 0.41 mg of CsCl/ml for 80 hours at 37,500 rpm in a Beckman SW 50.1 rotor, conditions which separate greater than 99.5% of the nucleic acids from chromosomal proteins. Each component of the gradient was then assayed for ability to influence availability of histone genes for transcription in G_1 chromatin. We found that the activity resided solely with the material contained in the protein region of the gradient material with a density between 1.25 and 1.28 g/cc (Park *et al.* 1977b). While this result is consistent with the component responsible for transcription of histone mRNA sequences from chromatin being a protein, we cannot dismiss the possibility that small pieces of nucleic acid, covalently or otherwise tightly bound to nonhistone chromosomal proteins, are involved with regulation of histone gene readout.

To further examine the possibility that nucleic acids associated with chromosomal proteins are responsible for determining availability of histone genes for transcription, the activity of the 0.4 M NaCl fraction from the SP-Sephadex columns was assayed for sensitivity to micrococcal nuclease. Under the conditions we employed for nuclease digestion, the enzyme effectively degrades RNA and both single and double-stranded DNA. Since the enzyme activity is Ca^{2+}-dependent, it is readily inactivated by EGTA. 3.4. μg of the SP fraction was incubated for 30 min at 37°C with 0.06 μg of micrococcal nuclease (in a parallel reaction containing the SP fraction, 3 μg of ^{3}H-labeled λ DNA were rendered 99% TCA-soluble). After the incubation EGTA was added to a final concentration of 5 mM and 0.3 μg of the nuclease-treated fraction was then reconstituted with 1 mg of G_1 DNA as chromatin in the presence of 2 mM EGTA. As shown in Fig. 9a, there is no significant difference in the kinetics of hybridization with histone cDNA of transcripts from G_1 chromatin reconstituted in the presence of 0.3 μg of nuclease-treated 0.4 M SP fraction and transcripts from G_1 chromatin reconstituted in the presence of 0.3 μg of untreated 0.4 M SP fraction. To examine the possibility that increased histone gene trancription G_1 chromatin was due to the action of EGTA or of the nuclease itself, G_1 chromatin was dissociated and then reconstituted in the presence of EGTA or EGTA-inactivated micrococcal nuclease. Transcripts from these reconstituted preparations do not hybridize to a significant extent with histone cDNA (Fig. 9a). These results suggest that the component of the 0.4 M SP fraction which has the ability to render histone genes transcribable is not a nucleic acid. However, we cannot eliminate the possibility that the 0.4 M SP fraction contains a small amount of nucleic acid which is complexed with protein or is in a configuration such that it is not susceptible to digestion by micrococcal nuclease.

To approach directly the question of whether the component of the SP-Sephadex fraction which renders histone genes transcribable in G_1 chromatin is a protein, we examined the sensitivity of the 0.4 M SP fraction to chymotrypsin (Fig. 9b). 3.4 μg of the fraction were incubated for 60 min at 22°C with 30 μg of chymotrypsin covalently bound to agarose beads (Miles Laboratories) in 2 M urea-0.1 M Tris (pH 8.3). conditions which result in the digestion of at least 50 μg of chromosomal proteins. After incubation the immobilized enzyme was removed by filtration and phenyl-

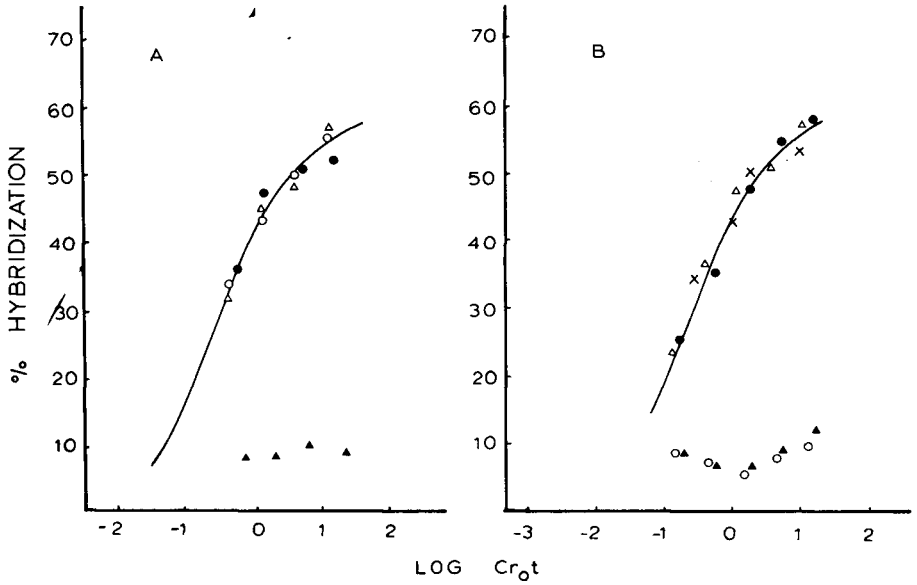


Fig. 9. A) Hybridization of histone cDNA to *in vitro* transcripts from HeLa cell G_1 chromatin reconstituted in the presence of micrococcal nuclease-treated S phase HeLa cell nonhistone chromosomal proteins. Transcripts from 1 mg of G_1 DNA as chromatin reconstituted with 0.3 μg of nuclease-treated (○) or untreated (●) 0.4 M SP Sephadex fraction. G_1 chromatin was also reconstituted in the presence of EGTA and EGTA-inactivated micrococcal nuclease in the absence of additional chromosomal protein (▲) or with 0.3 μg of the 0.4 M SP Sephadex fraction (△). B) Kinetics of annealing of histone cDNA to *in vitro* transcripts from HeLa cell G_1 chromatin reconstituted in the presence of chymotrypsin-treated S phase HeLa cell nonhistone chromosomal proteins. Transcripts from 1 mg of G_1 DNA as chromatin reconstituted with: 0.3 μg of chymotrypsin-treated (○), or untreated (×) 0.4 M SP Sephadex fraction; PMSF-containing buffer which had been incubated with immobilized chymotrypsin either in the absence of additional chromosomal protein (▲) or in the presence of 0.3 μg of untreated 0.4 M SP Sephadex fraction (△); or 0.3 μg of the SP Sephadex nonhistone chromosomal protein fraction that had been incubated with PMSF-inactivated chymotrypsin (●).

methylsulfonyl fluoride (PMSF) was added to the 0.4 M SP fraction to a final concentration of 1 mM to inactivate any remaining enzyme. PMSF was not added during reconstitution and transcription. RNA transcripts from G_1 chromatin reconstituted in the presence of 0.3 μg of untreated 0.4 M SP fraction hybridized with ^3H histone cDNA with a $Cr_0t_{1/2}$ of 3×10^{-1}, while transcripts from G_1 chromatin reconstituted with the same amount of chymotrypsin-treated fraction did not hybridize with histone cDNA to a significant level. The ability of the 0.4 M SP fraction to influence transcription of histone genes in G_1 chromatin was not affected when G_1 chromatin was reconstituted in the presence of 0.4 M SP fraction which had been incubated with PMSF-inactivated chymotrypsin. Treatment of the fraction with buffer which had been incubated in the presence of immobilized chymotrypsin also did not reduce the effectiveness of the fraction in rendering histone sequences transcribable. Reconstitution of G_1 chromatin in the presence of buffer which had been incubated with immobilized chymotrypsin and then treated with PMSF did not stimulate transcription of histone mRNA sequences. Taken together these results suggest that the component of the 0.4 M SP fraction which renders histone genes transcribable from G_1 chromatin is a protein.

Activation of histone gene transcription in chromatin from human diploid cells or mouse liver by a nonhistone chromosomal protein fraction from hela S_3 cells

It is not known whether the mechanism by histone gene transcription is regulated is the same in

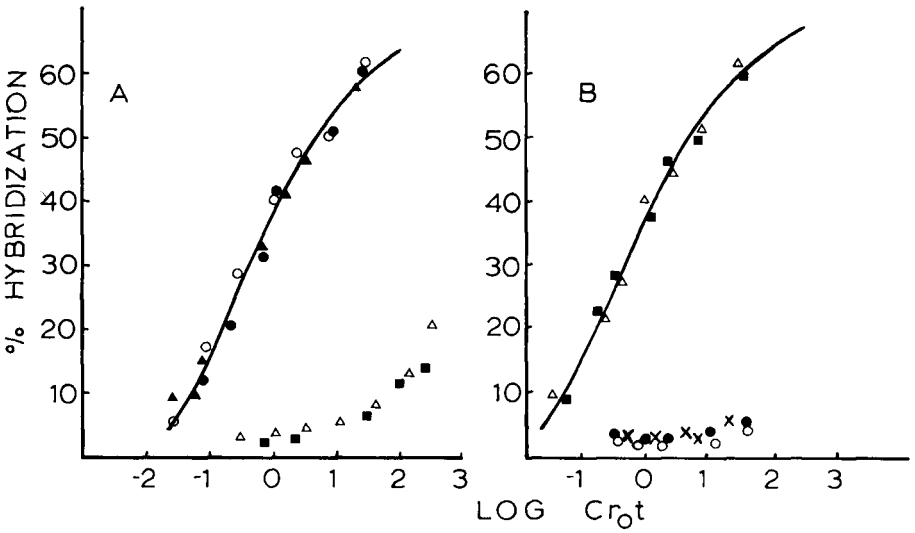


Fig. 10. A) Effect of S phase HeLa cell chromosomal proteins on transcription of histone mRNA sequences from chromatin of WI-38 human diploid fibroblasts. ^{3}H-histone cDNA was annealed to transcripts from 1 mg of DNA as chromatin from contact-inhibited WI-38 cells reconstituted with no additional chromosomal proteins (△), 100 μg of the 0.5 M QAE-Sephadex fraction of S HeLa chromosomal proteins (○) or 1000 μg of total chromosomal proteins from S phase WI-38 cells (●). ^{3}H-cDNA was also annealed to transcripts from native chromatin of contact-inhibited WI-38 cells (■) or S phase WI-38 cells (▲). B) Effect of S phase HeLa cell chromosomal proteins on transcription of histone mRNA sequences from mouse liver chromatin. ^{3}H-cDNA was annealed to transcripts from 1 mg of DNA as chromatin from adult mouse liver (○) and from 1 mg of adult mouse liver chromatin dissociated and reconstituted in the presence of no additional protein (●), 100 μg of S phase HeLa cell total chromosomal proteins (△), 100 μg of the S phase HeLa cell 0.5 M QAE-Sephadex fraction (■), or 100 μg of the S phase HeLa cell 0.25 QAE-Sephadex fraction (×).

different tissues and species. It is of particular interest to determine whether a transformed, continuously dividing cell such as HeLa contains components which can render histone genes transcribable from other cells which have greater degrees of growth control. To examine these questions, chromatin preparations from both contact-inhibited WI-38 human diploid fibroblast and adult mouse liver (both non-proliferating) were dissociated and then reconstituted in the presence of added chromosomal proteins from S phase HeLa cells; the reconstituted chromatin were then transcribed *in vitro* and the transcripts assayed for histone mRNA sequences by hybridization with histone cDNA. These studies show that S. phase HeLa cell nonhistone chromosomal proteins can render histone genes of chromatin from contact-inhibited WI-38 human diploid fibroblasts (Fig. 10a) or from nondividing mouse liver (Fig. 10b) available for transcription (Park *et al.* 1977a). Specifically, these studies show that when the S phase HeLa chromosomal proteins are fractionated on QAE-Sephadex in the presence of 5 M urea, only the fraction eluted by 0.5 M NaCl can activate histone gene transcription from chromatin of G_1 HeLa celss, contact-inhibited WI-38 fibroblasts or mouse liver-indicating that activation of histone genes in heterologous chromatins is not elicited by S phase nonhistone chromosomal proteins in general. Several lines of evidence also suggest that activation of histone gene transcription in mouse liver chromatin by S phase HeLa nonhistone chromosomal proteins is not a random phenomenon. Addition of the HeLa proteins to mouse liver chromatin does not significantly modify chromatin template activity. More specifically, the HeLa chromosomal proteins do not render mouse globin sequences transcribable (assayed by hybridization of chromatin transcripts with mouse globin ^{3}H cDNA).

It is well established that histone proteins are

similar in different mammalian species and in different cell types of the same species. Our data would seem to suggest that the mechanism by which the transcription of histone genes in chromatin is regulated by the nonhistone chromosomal proteins in HeLa cells. WI-38 cells and mouse liver may be the same or similar. This can be accounted for by postulating that the DNA sequences with which certain nonhistone chromosomal interact, perhaps regulatory sequences, are conserved between mouse and humans. Alternatively, the DNA sequences involved with activation of histone gene transcription may differ between mouse and humans, but both types of sequences may be recognized by the HeLa nonhistone chromosomal proteins. However, our results do illustrate that a transformed, continuously dividing cervical carcinoma cell such as HeLa contains components necessary to make the histone genes of contact-inhibited tissue culture cells or nondiving cells from an intact organism available for transcription from chromatin by *E. coli* RNA polymerase.

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