Effect of Histones and Nonhistone Chromosomal Proteins on the Transcription of Histone Genes from HeLa S_3 Cell DNA†

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ABSTRACT: To elucidate the manner in which histones and nonhistone chromosomal proteins interact to render histone genes transcribable in HeLa S_3 cells, we have examined transcription of histone mRNA sequences from DNA, as well as from several DNA–chromosomal protein complexes. Histone mRNA sequences were assayed by hybridization to a ^{3}H-labeled single-stranded DNA complementary to histone mRNAs. Our results indicate that DNA is an effective template for transcription of histone mRNA sequences and that histones by themselves inhibit transcription from DNA, including transcription of histone genes, in a dose-dependent, nonspecific manner. When complexed with DNA alone, nonhistone chromosomal proteins do not affect the transcription of histone mRNA sequences. However, when associated with DNA in the presence of histones, nonhistone chromosomal proteins are capable of selectively rendering histone genes transcribable. These results suggest a possible role for nonhistone chromosomal proteins in mediating the interactions of histones with DNA to render histone genes transcribable.

Most models of eukaryotic gene regulation are predicated on the complete complement of genetic information being present in all diploid cells of an organism, as well as on the cell and tissue specific transcription of limited components of the information encoded in the DNA. In eukaryotic cells the genome contains two principal classes of chromosomal proteins: histones and nonhistone chromosomal proteins, both being purported to play important roles in dictating structural and transcriptional properties (Stein et al., 1974b; Hnilica, 1972; Elgin and Weintraub, 1975; Stein and Kleinsmith, 1975). For some time it has been known that histones are repressors of DNA-dependent RNA synthesis (Huang et al., 1962; Allfrey et al., 1963) and that these basic chromosomal proteins are involved in "packaging" of the genome (Olins and Olins, 1974; Rill and Van Holde, 1973; Kornberg, 1974; Weintraub and Van Lente, 1974). Several lines of evidence point to the nonhistone chromosomal proteins as a class of macromolecules containing components that may render defined genetic sequences available for transcription (Stein et al., 1974b; Hnilica, 1972; Elgin and Weintraub, 1975; Stein and Kleinsmith, 1975; Paul and Gilmour, 1968; Paul et al., 1973; Teng et al., 1971; Kostraba and Wang, 1973; Spelsberg and Hnilica, 1970; Kleinsmith et al., 1970; Stein and Farber, 1972; Stein et al., 1972; Gilmour and Paul, 1970). Recently these nonhistone chromosomal proteins have been shown to be responsible for the tissue specific transcription of globin genes (Paul et al., 1973; Barrett et al., 1974; Chiu et al., 1975) and the cell cycle stage specific transcription of histone genes (J. Stein et al., 1975; Stein et al., 1975c). In the present study, transcription of histone genes from DNA, as well as from several DNA–chromosomal protein complexes was examined. The objective of these studies was to better understand the manner in which histones and nonhistone chromosomal proteins interact to render a specific set of genes transcribable.

Materials and Methods

Cell Culture and Cell Synchronization. Exponentially growing (log phase) HeLa S_3 cells were maintained in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 3.5% each of calf and fetal calf serum. Cells were synchronized as previously described (Stein and Borun, 1972). S-phase cells were obtained by synchronization with two cycles of 2 mM thymidine block. Three hours after the release from the second thymidine block, cells were harvested; at this time 98% of the cells were in S phase as determined by autoradiographic assessment of [^{3}H]thymidine labeled nuclei. G_1 cells were obtained 3 h after selective detachment of mitotic cells from semiconfluent monolayers. Ninety-seven percent of the cells were in the G_1 phase of the cell cycle; incorporation of [^{3}H]thymidine into nuclei could not be detected autoradiographically, reflecting the complete absence of S-phase cells.

Isolation of Chromatin and DNA. Nuclei were obtained by washing cells in 80 volumes of Earle's balanced salt solution and lysing the cells in 80 volumes of 80 mM NaCl–20 mM EDTA–1% Triton X-100 (pH 7.2). The nuclei were washed three times with the lysing medium and then twice with 0.15 M NaCl–0.01 M Tris (pH 8.0). Nuclei isolated in this manner are largely free of cytoplasmic material when examined by phase-contrast and electron microscopy. Lysis of nuclei was achieved by suspending the nuclear pellet in triple glass-distilled water. The chromatin was allowed to swell at 4 °C for 30 min, pelleted at 20 000g for 15 min, resuspended in distilled water, and again pelleted at 20 000g. DNA was prepared by the method of Marmur (1961), treated with pancreatic ribonuclease A (50 μg/ml for 30 min at 37 °C) and Pronase (50 μg/ml for 2 h at 37 °C), and then extracted twice with phenol before use.

Preparation of DNA–Chromosomal Protein Complexes. Chromatin was dissociated in 3 M NaCl–5 M urea–0.01 M Tris (pH 8.3), and the DNA was pelleted at 150 000g for 36 h. Proteins were fractionated into histone and nonhistone chromosomal protein fractions by the QAE-Sephadex method of Gilmour and Paul (1970). DNA–chromosomal protein

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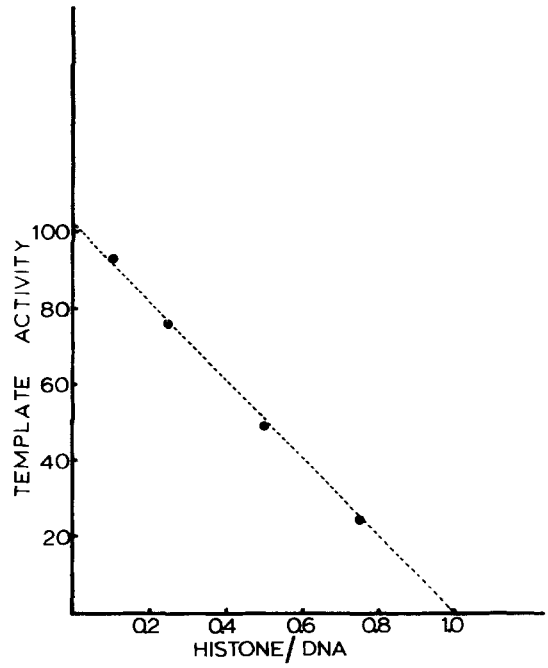

FIGURE 1: Template activity of histone–DNA complexes. Histone–DNA complexes were reconstituted as described under Materials and Methods with histone/DNA ratios of 0.75, 0.50, 0.25, and 0.10. Transcription of these reconstituted nucleohistone complexes was carried out under conditions where the template was rate limiting, using Fraction V *E. coli* RNA polymerase (Berg et al., 1971) in the presence of 0.1 μCi of [^{14}C]ATP. Template activity refers to the relative availability of DNA as template for RNA synthesis in histone–DNA complexes compared with DNA (100%).

complexes were reconstituted by the gradient dialysis procedure of Bekhor et al. (1969). The details of these methods (Stein and Farber, 1972; Stein et al., 1975c) and evidence for fidelity of chromatin reconstitution (Bekhor et al., 1969; Paul and More, 1972; Chiu et al., 1975; Stein et al., 1975a,c) have been reported.

RNA Transcription and Nucleic Acid Hybridization. RNA was transcribed from DNA, chromatin, and various chromosomal protein–DNA complexes using Fraction V *Escherichia coli* polymerase prepared according to the method of Berg et al. (1971). Transcription was carried out for 70 min at 37 °C in a Dounce homogenizer fitted with a wide-clearance pestle, and the reaction mixture was periodically homogenized to maintain solubility. The incubation mixture in a final volume of 3.5 ml contained: 0.04 M Tris (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02 mM EDTA, 0.008% β-mercaptoethanol, 0.4 mM each of ATP, CTP, UTP, and GTP, 150 μg/ml of DNA, and 200 units of RNA polymerase.[1] RNA was extracted as follows. The reaction was brought to a concentration of 1% sodium dodecyl sulfate–0.1 M NaCl–10 mM sodium acetate–1 mM EDTA (pH 5.4) and incubated at 37 °C for 15 min. Following two extractions with equal volumes of phenol and chloroform–isoamyl alcohol (24:1, v/v) and two extractions with chloroform–isoamyl alcohol, nucleic acids were precipitated with 3 volumes of ethanol. The pellet was resuspended in 10 mM Tris–0.1 M NaCl–5 mM $MgCl_2$ (pH 7.4) containing 40 μg/ml of DNase I and incubated at 37 °C for 60 min. Following one extraction with phenol–chloroform–isoamyl alcohol and two with chloroform–isoamyl alcohol, the aqueous phase containing the RNA transcripts was chromatographed on Sephadex G-50 fine and eluted with 0.1 M NaCl–10 mM sodium acetate–1 mM EDTA (pH 5.4). RNA was precipitated with 2 volumes of ethanol and resuspended in 25 mM Hepes–0.5 M NaCl–1 mM EDTA, pH 7.0.

Isolation of histone mRNA's from polyribosomes of S phase HeLa S_3 cells as well as synthesis of the complementary [^{3}H]DNA ([^{3}H]cDNA) using [^{3}H]dCTP and [^{3}H]dGTP were carried out as previously described (Thrall et al., 1974; J. Stein, et al., 1975; Stein et al., 1975c). Poly(A) was added to the 3′-OH termini of the histone mRNA's with an ATP-polynucleotidylexotransferase isolated from maize seedlings (Mans and Huff, 1975), and the polyadenylated mRNA's were then transcribed with RNA-dependent DNA polymerase from avian myloblastosis virus using dT_{10} as a primer. Characteristics of the histone cDNA probe have been reported (Thrall et al., 1974; J. Stein et al., 1975; Stein et al., 1975c). It should be noted that RNA complementary to histone mRNA's has been prepared by McCarthy and co-workers using Qβ replicase (Obinata et al., 1975). [^{3}H]cDNA 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, 25 mM Hepes (pH 7.0), 1 mM EDTA, 0.04 ng of cDNA, and RNA as indicated. The reaction mixtures were assayed for hybrid formation using fraction IV, single-strand specific S_1 nuclease isolated from *Aspergillus oryzae* (Vogt, 1973). Each sample was incubated for 20 min in 2.0 ml of 30 mM sodium acetate, 0.3 M NaCl, 1 mM $ZnSO_4$, 5% glycerol (pH 4.6), containing S_1 nuclease at a concentration sufficient to degrade at least 95% of the single-stranded nucleic acids present. The amount of radioactive DNA resistant to digestion was determined by trichloroacetic acid precipitation.

Results

Histones and Transcription of Histone Messenger RNA Sequences. Direct evidence that histones are biological repressors of DNA-dependent RNA synthesis was initially provided by studies carried out in Bonner's (Huang and Bonner, 1962) and in Allfrey's (Allfrey et al., 1963) laboratories. Huang and Bonner (1962) demonstrated that addition of histones to DNA brings about an inhibition of transcription, while Allfrey et al. (1963) showed that extraction of histones from nuclei results in a marked increase in RNA synthesis. The data in Figure 1 clearly indicate that when histone–DNA complexes are reconstituted with an increasing histone to DNA ratio, a decreasing template activity for RNA synthesis is observed. An inverse linear relationship between template activity and the ratio of histones to DNA is apparent. Consistent with the findings of Huang and Bonner, total repression of transcription is evident at a histone to DNA ratio of 1—the histone to DNA ratio of the isolated genome (chromatin). These results provide little information concerning the interaction of histones with defined genetic sequences; furthermore, they do not necessarily reflect the effects of histones on the transcription of specific genes.

The influence of histones on the transcription of specific genes from DNA was examined by assessing the presence of histone messenger RNA sequences in RNA transcripts from the same histone–DNA complexes examined in Figure 1. RNA was transcribed from these histone–DNA complexes using fraction V *E. coli* RNA polymerase (Berg et al., 1971), and the ability of these RNA transcripts to form hybrids with a ^{3}H-labeled single-stranded DNA complementary to histone messenger RNA sequences was assayed under conditions of

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; NHCP, nonhistone chromosomal proteins.

TABLE I: Template Activities of DNA, Chromatin, and DNA-Chromosomal Protein Complexes.[a]

Sample	μg of DNA	cpm of [^{14}C]ATP/ μg of DNA	% DNA Template Transcribable
DNA	40	1240.0	100.0
DNA	40	1190.0	100.0
DNA	20	1230.0	100.0
DNA	20	1299.0	100.0
Native chromatin	40	83.5	6.7
Native chromatin	40	86.6	7.0
Reconstituted chromatin	40	85.4	6.9
Reconstituted chromatin	40	89.3	7.2
DNA-NHCP[b]	40	1172.0	95.0
DNA-NHCP[b]	40	1248.0	101.0
DNA-histone	40	1.4	0.11
DNA-histone	40	2.0	0.16

[a] DNA and DNA-chromosomal protein complexes were transcribed in the in vitro RNA synthesizing system described under Materials and Methods under conditions where the template was rate limiting. Transcription was carried out utilizing *E. coli* RNA polymerase (Berg et al., 1971) in the presence of 0.1 μCi of [^{14}C]ATP. Details of the template assay have been reported (Stein and Farber, 1972; Stein et al., 1975). "Percent DNA Template Transcribable" refers to the relative availability of DNA as template for RNA synthesis in chromatin and in chromosomal protein-DNA complexes compared with DNA. All chromatin components were from log-phase HeLa cells. Although template activity for DNA has been arbitrarily assigned a value of 100% it should be noted that, in vitro, as well as in vivo, not all DNA sequences are transcribable. Chromatin was reconstituted by combining DNA, NHCP and histone in 3 M NaCl-5 M urea-0.01 M Tris (pH 8.3)(1:1.5:1.5) followed by gradient dialysis as described under Materials and Methods. DNA-chromosomal protein complexes were similarly reconstituted by combining DNA with nonhistone chromosomal proteins (1:1.5, w/w) or with histones (1:1.5, w/w) followed by gradient dialysis. Recovery of DNA as chromatin and as DNA-chromosomal protein complexes was between 80–85%. Protein:DNA ratios of the preparations were as follows: native chromatin 1.94, reconstituted chromatin 1.92, DNA-nonhistone chromosomal protein complexes 0.89, and DNA-histone complexes 1.10. [b] Nonhistone chromosomal protein.

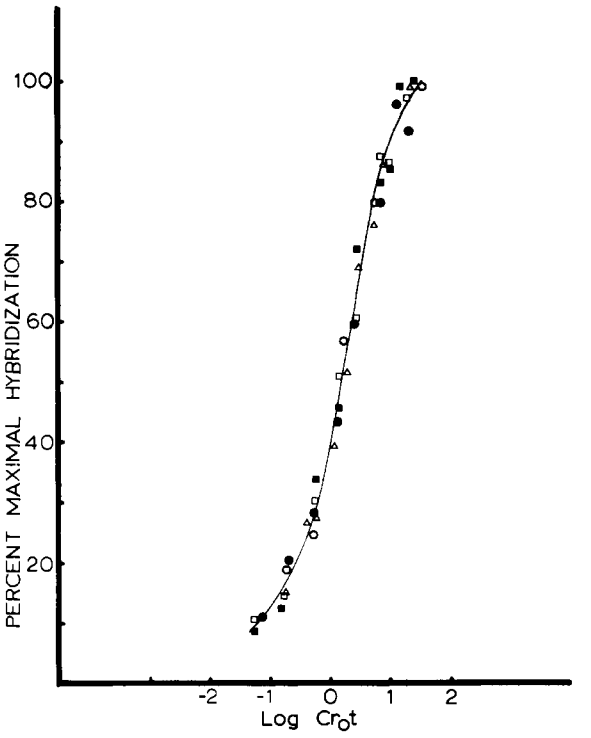

FIGURE 2: Kinetics of hybridization of RNA transcripts from DNA and histone-DNA complexes. Histone-DNA complexes were reconstituted as described under Materials and Methods with histone/DNA ratios of 0.75 (●), 0.50 (○), 0.25 (■), and 0.10 (□). Transcripts (0.8–1.0 μg) from these histone-DNA complexes and from DNA (△) were hybridized to 0.04 ng of histone [^{3}H]cDNA (27 000 dpm/ng) in the presence of 3.5 μg of *E. coli* RNA. Cr_0t = mol of ribonucleotides s l.$^{-1}$.

RNA excess. The properties of histone cDNA and hybridization conditions have been reported (Thrall et al., 1974; J. Stein et al., 1975; Stein et al., 1975c). Similar kinetics were observed for each of the hybridization reactions between histone cDNA and RNA transcripts from the histone-DNA complexes reconstituted at histone/DNA ratios of 0.1, 0.25, 0.5, and 0.75 (Figure 2). The $Cr_0t_{1/2}$ of the hybridization reactions was 1.8—the same as that observed for the hybridization reaction between histone cDNA and transcripts from DNA (Figure 2). Although the availability of template for RNA transcription varies in DNA and in the histone-DNA complexes, the relative amounts of histone messenger RNA sequences in the RNA transcripts are constant, suggesting that in these reconstituted chromatin complexes histones are not preferentially associated with histone genes. Since histone-DNA complexes reconstituted at a histone/DNA ratio of 1 were ineffective as templates for in vitro RNA synthesis, hybridization with histone cDNA could not be examined. When the kinetics of the histone cDNA-histone mRNA hybridization reaction ($Cr_0t_{1/2} = 1.7 \times 10^{-2}$) are compared with those of the hybridization reactions of histone cDNA with RNA transcripts from DNA ($Cr_0t_{1/2} = 1.8$) and from the four histone-DNA complexes ($Cr_0t_{1/2} = 1.8$), it is evident that histone messenger RNA sequences comprise approximately 1% of the transcript from DNA, as well as from histone-DNA complexes. The presence of 1% histone mRNA sequences in transcripts from DNA is consistent with approximately 8% of the transcripts from S-phase chromatin being histone mRNA sequences, since, as indicated in Table I, the template activity of chromatin is 7% that of DNA.

Consistent with previous evidence that histones are nonspecific repressors of DNA-dependent RNA synthesis, the present results suggest that histones repress the transcription of specific genetic sequences—those which code for histones—in a random, nonselective manner.

Nonhistone Chromosomal Proteins and Transcription of Histone Messenger RNA Sequences. We have previously reported that the transcription of histone genes from chromatin is restricted to the S phase of the cell cycle (Stein et al., 1975b,c) and that nonhistone chromosomal proteins are responsible for the transient expression of histone genes at this time (Stein et al., 1975c). The question which then arises is whether nonhistone chromosomal proteins render histone genes transcribable by interacting with DNA alone or by mediating the manner in which histones are associated with DNA. The data in Table I show that the template activity of log-phase nonhistone chromosomal protein-DNA complexes is identical to that of DNA. Furthermore, when RNA transcripts from log-phase nonhistone chromosomal protein-DNA complexes are hybridized to histone cDNA, the $Cr_0t_{1/2}$ of the reaction is identical to that of the reaction between histone cDNA and transcripts from DNA (1.8) (Figure 3). These results indicate that nonhistone chromosomal proteins by themselves do not influence (a) the availability of DNA as template for RNA

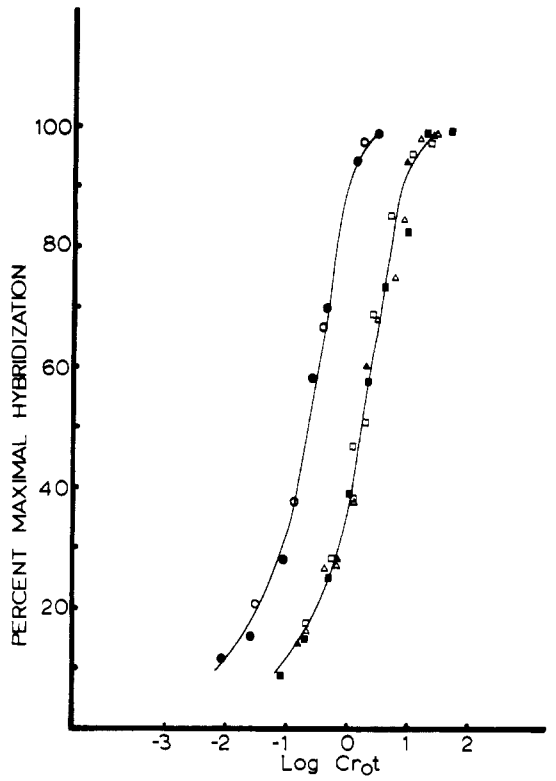

FIGURE 3: Kinetics of hybridization of histone [^{3}H]cDNA with RNA transcripts from native chromatin of log-phase cells (●); chromatin reconstituted with DNA, histone, and nonhistone chromosomal proteins from log-phase cells (○), DNA (△), DNA–log phase nonhistone chromosomal protein complexes (▲), DNA–S phase nonhistone chromosomal protein complexes (■), and DNA–G_1 nonhistone chromosomal protein complexes (□). Transcripts (0.13 μg of chromatin transcripts and 1.0 μg of transcripts from DNA and DNA–nonhistone chromosomal protein complexes) were hybridized with 0.04 ng [^{3}H]cDNA in the presence of 3.5 μg of *E. coli* RNA. Cr_0t = mol of ribonucleotides s l.$^{-1}$.

synthesis or (b) the transcription of histone mRNA sequences. Whatever the interaction between nonhistone chromosomal proteins and DNA, when nonhistone chromosomal proteins as a total class are complexed alone with DNA, there is no indication of an "activator" or "repressor" function, at least when transcription is carried out using *E. coli* polymerase. However, when log-phase nonhistone chromosomal proteins are reconstituted with DNA in the presence of histones, the transcriptional properties of the complex are identical to those of native log-phase chromatin—both template activity (Table I) and transcription of histone mRNA sequences (Figure 3). In comparison with transcription observed from log-phase nonhistone chromosomal protein–DNA complexes, template activity of the reconstituted chromatin preparation is reduced to 7% and the relative representation of histone mRNA sequences is significantly enhanced (from 1 to 8%). To further establish that nonhistone chromosomal proteins by themselves have no effect on transcription of histone genes from DNA, we examined transcripts from G_1 nonhistone chromosomal protein–DNA complexes for the presence of histone mRNA sequences (Figure 3). The $Cr_0t_{1/2}$ of the hybridization reactions between histone cDNA and RNA transcripts from these two DNA–nonhistone chromosomal protein complexes is indistinguishable from that of the hybridization reaction between histone cDNA and transcripts from DNA. Histone mRNA sequences were not detected in our nonhistone chromosomal protein fractions. These findings demonstrate that cell cycle stage specific nonhistone chromosomal proteins, when complexed with DNA alone, do not influence the transcription of histone genes. Since chromatin reconstituted with DNA, histones and S-phase nonhistone chromosomal proteins transcribes histone mRNA sequences while, in contrast, chromatin reconstituted with DNA, histones and G_1 nonhistone chromosomal proteins does not (Park et al., 1976; Stein et al., 1975b,c), it is reasonable to conclude that the presence of both histones and nonhistone chromosomal proteins is essential for the specificity of transcription and particularly for that of histone gene transcription.

Discussion

The present results indicate that DNA is an effective template for the transcription of histone mRNA sequences and that histones by themselves inhibit histone gene transcription from DNA in a dose dependent, nonspecific manner. When complexed with DNA alone, nonhistone chromosomal proteins do not affect the transcription of histone mRNA sequences. However, when associated with DNA in the presence of histones these nonhistone chromosomal proteins are capable of selectively rendering histone genes transcribable. We have previously reported that 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_1 cells is not (Stein et al., 1975c). Hence, it appears that the cell cycle stage specific transcription of histone genes depends upon the source of nonhistone chromosomal proteins. Other studies suggest that histone gene transcription during the S phase is "activated" by a component of the S phase nonhistone chromosomal proteins rather than being "repressed" during the G_1 phase of the cell cycle by a component of the G_1 nonhistone chromosomal proteins (Park et al., 1976). Taken 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 histone from DNA may be brought about by competition of nonhistone chromosomal proteins for 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 may be responsible for cell cycle stage specific variations in the binding of histones to DNA in chromatin are consistent with such reasoning (Stein et al., 1974a,b). 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 the 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 pre-existing chromosomal protein that is enzymatically modified at the onset of S phase to alter its structural and functional properties. Within this context it should be noted that the extent of S-phase nonhistone chromosomal protein phosphorylation has recently been directly shown to influence the transcription of histone genes (Kleinsmith et al., 1976). Such a mechanism would be compatible with the proposal of Gilmour and Paul (1970) that interactions of nonhistone chromosomal proteins with DNA

bring about the transcription of specific genes.

Although evidence has been presented for fidelity of chromatin reconstitution (Bekhor et al., 1969; Chiu et al., 1975; Paul and More, 1972; Stein et al., 1975a,c) there is no absolute proof that reconstituted chromatin or DNA–protein complexes are completely native. Consequently the possibility cannot be excluded that specific repressors of histone gene transcription might be present among the nonhistone chromosomal proteins but that the activity is lost during preparation, dissociation, or reconstitution of chromatin. Caution must be exercised in interpretation of the present results, since transcription was carried out utilizing *E. coli* RNA polymerase. It is reasonable to anticipate that transcription with homologous enzymes and "factors" that render specificity may reveal an additional, more refined level at which transcriptional control is mediated. Recently isolated, genome-associated proteins that appear to influence transcription by homologous polymerases may constitute such macromolecules (Kostraba et al., 1975).

An important question remaining to be answered is which component or components of the complex and heterogeneous nonhistone chromosomal proteins associated with the genome during the S phase of the cell cycle are the macromolecules that render histone genes transcribable. Resolution of this problem is central to understanding the precise mechanism by which regulation of histone genes is mediated. Equally important is the question of whether the mechanism by which the transient expression of histone genes is regulated during the cell cycle is applicable in general to the regulation of gene expression at the transcriptional level.

Acknowledgments

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