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Activation of Histone Gene Transcription in Quiescent WI-38 Cells or Mouse Liver by a Nonhistone Chromosomal Protein Fraction from HeLa S_3 Cells†

W. Park, R. Jansing, J. Stein, and G. Stein*

ABSTRACT: Using *Escherichia coli* RNA polymerase, histone genes can be transcribed in vitro from chromatin of S phase HeLa S_3 cells and WI-38 human diploid fibroblasts, but not from chromatin of G_1 phase cells. Histone gene transcription is assayed by hybridization of a ^3H-labeled single-stranded DNA complementary to histone mRNA. Using the technique of chromatin reconstitution, we have recently presented evidence which suggests that this cell-cycle difference in histone gene transcription from HeLa and WI-38 cell chromatin can be accounted for by a component or components of the S phase nonhistone chromosomal proteins which has the ability to render histone genes available for transcription. In the present study we examine the ability of chromosomal proteins from S phase HeLa cells to activate histone gene transcription in chromatin from contact-inhibited WI-38 human diploid fibroblasts or from mouse liver. These results suggest that, when chromatin from either contact-inhibited WI-38 cells or mouse liver is dissociated and then reconstituted in the presence of chromosomal proteins from S phase HeLa cells, the histone genes are rendered transcribable. When the chromosomal proteins from S phase HeLa cells are fractionated with QAE-Sephadex, only one fraction which represents approximately 10% of the total chromosomal protein is found effective in activating histone gene transcription from chromatin of G_1 phase HeLa cells, contact-inhibited WI-38 fibroblasts, or mouse liver. Reconstitution of mouse liver chromatin in the presence of S phase HeLa cell chromosomal proteins does not bring about transcription of mouse globin genes, suggesting that the activation of histone genes does not effect a general activation of gene readout.

In HeLa S_3 cells the synthesis of histones is restricted to the S phase of the cell cycle (Spalding et al., 1966; Borun et al., 1967; Gallwitz and Breindl, 1972; Jacobs-Lorena et al., 1972), and cell-free translation (Borun et al., 1967; Gallwitz and Breindl, 1972; Jacobs-Lorena et al., 1972; Pederson and Robbins, 1970; Borun et al., 1975) as well as nucleic acid hybridization data (Stein et al., 1975a) have indicated that histone mRNA is associated with the polysomes only at this time. When chromatin isolated from S phase cells is transcribed in vitro, histone sequences are efficiently transcribed. In contrast, histone sequences are not available for transcription in G_1 chromatin, even though the total amount of RNA transcribed is similar (Stein et al., 1975b). Using the technique of chromatin reconstitution, we have recently presented evidence suggesting that it is the nonhistone chromosomal protein portion of the chromatin that is responsible for this difference in in vitro transcription of the histone genes and that this difference can be accounted for by a component or components of the S phase nonhistone chromosomal proteins having the ability to activate histone gene transcription from G_1 chromatin (Stein et al., 1975c; Park et al., 1976).

Since the expression of histone genes is an integral part of cell proliferation, examining the regulation of histone gene transcription may provide an avenue for understanding the control of the proliferative process. To this end, we have examined the regulation of histone gene transcription not only in continuously dividing HeLa S_3 cells, but also in contact-inhibited WI-38 human diploid fibroblasts which have been stimulated to proliferate (Jansing et al., 1977). When nondividing WI-38 fibroblasts are stimulated to divide, histone

† From the Department of Biochemistry and Molecular Biology (W. P., R. J., and G. S.) and the Department of Immunology and Medical Microbiology (J. S.), University of Florida, Gainesville, Florida 32610. *Received April 12, 1977.* These studies were supported by National Institutes of Health Grant GM20535 and National Science Foundation Grant BMS7518583.

mRNAs become associated with polysomes during the activation of DNA synthesis, and the ability of chromatin from these cells to serve as a template for the in vitro transcription of histone mRNA sequences parallels the onset of DNA replication. Furthermore, recent evidence from our laboratory suggests that the mode of histone gene regulation in nondividing WI-38 fibroblasts that have been stimulated to proliferate is analogous to that observed in continuously dividing HeLa S_3 cells (Jansing et al., 1977). Results from chromatin reconstitution experiments suggest that it is the nonhistone chromosomal protein portion of the WI-38 chromatin which is responsible for the regulation of histone gene transcription in vitro. Such a role for the nonhistone chromosomal proteins in the regulation of histone gene transcription is consistent with the findings of other investigators that the nonhistone chromosomal 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 steroid hormone-dependent transcription of ovalbumin genes (Tsai et al., 1976a,b).

While the nonhistone chromosomal proteins have been shown to play a major role in the regulation of histone gene transcription in both continuously dividing HeLa S_3 cells and contact-inhibited WI-38 cells, it is not known whether the mechanism by which histone gene transcription is regulated is the same in other tissues and species. It is of particular interest to determine whether a highly transformed, continuously dividing cell, such as HeLa, contains all of the components necessary to activate transcription of histone genes from other cells which have greater degrees of growth control. To examine these questions, chromatins from both contact-inhibited WI-38 human diploid fibroblasts and mouse liver have been dissociated and then reconstituted in the presence of added chromosomal protein from S phase HeLa cells. In vitro transcripts from these reconstituted chromatins were assayed for histone mRNA sequences by hybridization with histone complementary DNA.

Materials and Methods

Cell Culture. Exponentially growing HeLa S_3 cells were maintained in suspension culture in Joklik-modified Eagle's MEM[1] supplemented with 7% calf serum. Cells were synchronized as described previously (Stein and Borun, 1972). S phase cells were obtained by synchronization with two cycles of 2 mM thymidine block. Three hours after release from the second thymidine block cells were collected; 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 absence of S phase cells.

Human diploid WI-38 fibroblasts were grown in monolayer culture in Eagle's basal medium (BME) containing 10% fetal calf serum. The cells were cultivated in roller bottles at 37 °C in an atmosphere of 5% CO_2. Seven days after plating the cells were confluent and were stimulated to proliferate by replacing the exhausted growth medium with fresh BME containing 20% fetal calf serum. It has been shown previously that under these conditions approximately 60% of the contact-inhibited cells are stimulated to replicate DNA (Rhode and Ellem, 1968; Rovera and Baserga, 1971). The cells used in this study ranged from passage 28 to 30.

Isolation of Nuclei and Chromatin. Nuclei and chromatin were isolated from WI-38 fibroblasts and HeLa S_3 cells at 4 °C as described previously (Stein and Farber, 1972). Cells were harvested, washed three times with 80 volumes of spinner salts (Grand Island Biological Co.), and lysed with 80 volumes of 80 mM NaCl–20 mM EDTA–1% Triton X-100 (pH 7.2). Nuclei were pelleted by centrifugation at 1000*g* for 3 min and washed two times in 0.15 M NaCl–10 mM Tris (pH 8.0) and collected by centrifugation at 1000*g* for 3 min. Nuclei isolated in this manner are free of cytoplasmic contamination when observed by phase contrast microscopy. Nuclei were lysed in 60 volumes of double-distilled water by gentle homogenization. The chromatin was allowed to swell in an ice bath for 20 min and was then pelleted by centrifugation at 12 000*g* for 20 min.

To obtain mouse liver nuclei and chromatin, 10 adult animals were sacrificed and a total of 12.5 g of liver was removed and minced into 20 volumes of 2.2 M sucrose–4 mM $MgCl_2$. The minced tissue was then homogenized ten strokes with a motor driven Potter-Elvehjem Teflon homogenizer and filtered through one layer of Miracloth. The homogenate was centrifuged for 1 h at 113 000g_{max} (25 000 rpm) in a Beckman SW-27 rotor at 4 °C. The pellet was washed twice in 0.15 M NaCl–10 mM Tris (pH 8.0) followed each time by centrifugation at 1000*g* for 3 min. Nuclei were lysed in 60 volumes of double-distilled water by gentle homogenization. The chromatin was allowed to swell in an ice bath for 20 min and was then pelleted by centrifugation at 12 000*g* for 20 min.

Preparation of Total Chromosomal Protein. Total chromosomal proteins were prepared by dissociating chromatin in 3 M NaCl–5 M urea–10 mM Tris (pH 8.3) at 4 °C at a DNA concentration of 0.5 mg/mL. To facilitate dissociation of chromatin, solid NaCl and urea were added to the chromatin pellet. Nucleic acid was removed by centrifugation of the dissociated chromatin at 150 000*g* for 36 h.

Fractionation of S Phase HeLa Chromosomal Protein. Chromosomal proteins, which have been dissociated from chromatin by high concentrations of salt and urea and from which nucleic acid has been removed by ultracentrifugation, are dialyzed against four changes of 10 volumes of 5 M urea–10 mM Tris (pH 8.0) at 4 °C. The proteins are loaded on a column of QAE-Sephadex A-25 (which has been equilibrated with this same buffer) at a flow rate of 15–30 mL cm^{-2} h^{-1}, using approximately 1 g of Sephadex per 10 mg of protein. The column is then eluted with 2.5 column volumes each of 5 M urea–10 mM Tris (pH 8.3) containing 0, 0.1, 0.25, 0.5, or 3 M NaCl at the same flow rate (Figure 1). The total recovery of protein is 50–85%.

Reconstitution of Chromatin. Chromatin at a DNA concentration of 0.25 mg/mL was reconstituted by a slight adaptation of the gradient dialysis procedure of Bekhor et al. (1969). The chromatin which had been dissociated in 3 M NaCl–5 M urea–10 mM Tris (pH 8.3) at 4 °C was dialyzed for 4 h each against 20 volumes of 5 M urea–10 mM Tris (pH 8.3) containing successively 1.5, 1.0, 0.8, 0.6, 0.4, 0.3, 0.2, and 0.1 M NaCl. It was then dialyzed for two changes against 5 M urea–10 mM Tris (pH 8.3) without NaCl. The chromatin was recovered by centrifugation at 30 000*g* for 60 min. The gelatinous chromatin pellet was then resuspended in water, allowed to swell in ice for 30 min, and spun at 30 000*g* for 30 min. The reconstituted chromatin recovered contained approximately 75% of the input DNA and had the protein-to-DNA ratio of native chromatin (1.8–2.0) over the range of protein-to-DNA inputs used in these studies. Evidence for the

[1] Abbreviations used: MEM, minimum Eagle's medium; BME, Eagle's basal medium; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

fidelity of chromatin reconstitution has been reported (Bekhor et al., 1969; Paul and More, 1972; Stein et al., 1975d). Transcription of globin (Paul et al., 1973; Barrett et al., 1974), ovalbumin (Tsai et al., 1976a), and histone (Stein et al., 1975c) genes as well as total poly(A)$^+$ mRNA sequences from native and reconstituted chromatin preparations have been shown to be indistinguishable.

Transcription of Chromatin. Chromatin was transcribed using fraction V *E. coli* RNA polymerase prepared according to the method of Berg et al. (1971). Transcription was carried out for 60 min at 37 °C in a Dounce homogenizer fitted with a wide clearance pestle, and the reaction mixture was periodically homogenized to maintain chromatin solubility. The incubation mixture in a final volume of 3.5 mL contained: 40 mM Tris (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 20 μM EDTA, 0.008% β-mercaptoethanol, 0.4 mM each of ATP, CTP, UTP, and GTP, 150 μg/mL of DNA as chromatin, and 200 U of RNA polymerase. 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 2 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 10 mM sodium acetate–0.1 M NaCl–1 mM EDTA (pH 5.4). RNA was precipitated with 2 volumes of ethanol. For hybridization analysis, the RNA was resuspended in 25 mM Hepes–0.5 M NaCl–1 mM EDTA (pH 7.0).

Preparation and Properties of Histone cDNA. To detect the presence of histone-specific RNA sequences, a single-stranded DNA complementary to histone mRNAs was synthesized using RNA-dependent DNA polymerase as described previously (Thrall et al., 1974). RNA (7–11S) was isolated from polysomes of S phase HeLa S_3 cells and poly(A)-containing RNAs were removed by oligo(dT)-cellulose chromatography. The remaining 7–11S RNAs directed only the synthesis of all five classes of histones in a cell-free protein synthesizing system from wheat germ, although histone H1 is synthesized in lower amounts than the other histones (Thrall et al., 1974). This mRNA preparation has also been shown to lack internal methylated ribonucleotides, indicating the absence of ribosomal RNA (Stein et al., 1977). Poly(A) (an average of 35 AMP residues) was added to the 3′-OH termini of the histone mRNAs with an ATP-polynucleotidylexotransferase isolated from maize seedlings. The isolation and properties of this enzyme have been reported (Mans and Huff, 1975). The polyadenylated RNAs were then transcribed with RNA-dependent DNA polymerase from avian myeloblastosis virus using dT_{10} as a primer. The mean sedimentation coefficient of the cDNA in alkaline sucrose was 6.1 S which corresponds to an average size of approximately 400 nucleotides (McEwen, 1967; Studier, 1965). When the cDNA is annealed to the histone mRNA preparation isolated from the polysomes of S phase HeLa cells at 52 °C in the presence of 50% formamide, the reaction proceeds with a $Cr_0t_{1/2}$ of 1.7×10^{-2} to a maximum of 63% hybridization as estimated by a double-reciprocal plot (Birnstiel et al., 1972). Since the molar concentration of the nucleotide sequences in solution determines the rate of hybridization, comparison of the $Cr_0t_{1/2}$ of the histone mRNA to that of a kinetic standard such as globin mRNA (complexity of 1200 bases (Labrie, 1969) and $Cr_0t_{1/2}$ in similar conditions of 3.8×10^{-3} (Young et al., 1974)) yields a calculated sequence complexity of approximately 5400 which is two times greater than expected for the total complexity of the five histone messages. This is, however, within the range of variation found for the rate of RNA–DNA hybridization (Birnstiel et al., 1973; Straus and Bonner, 1972; Bishop, 1969, 1972). When cDNA and histone mRNA are hybridized in the absence of formamide at 75 °C, the $Cr_0t_{1/2}$ is 5×10^{-3}. From the $Cr_0t_{1/2}$ of the globin mRNA in these conditions ($Cr_0t_{1/2} = 2.0 \times 10^{-3}$) (Barrett et al., 1974; Gulati et al., 1974) the sequence complexity of the histone mRNA can be estimated to be 3000 bases. When the probe is annealed with *E. coli* RNA in either of the above conditions, no significant level of hybrid formation is detected. The low level of S_1 nuclease-resistant, Cl_3CCOOH-precipitable radioactivity may be accounted for by a limited amount (3%) of [^{3}H]cDNA which is not digested by S_1 nuclease in the incubation conditions used. When the hybrids are formed at 52 °C in the presence of 50% formamide, thermal denaturation curves of the histone mRNA–cDNA hybrids exhibit a single transition with a T_m of 67 °C in 50% formamide–0.5 M NaCl–25 mM Hepes (pH 7.0)–1 mM EDTA. This T_m value is consistent with the recorded base composition of histone mRNA of 54% GC (Adesnik and Darnell, 1972).

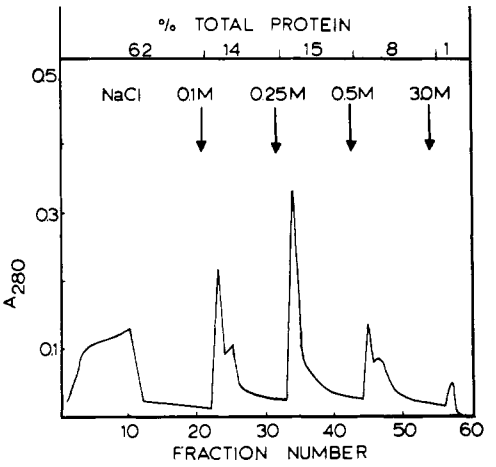

FIGURE 1: Elution profile of S phase HeLa chromosomal proteins from QAE-Sephadex. Proteins were loaded in 5 M urea–10 mM Tris (pH 8.3) and were eluted with this buffer containing 0.10, 0.25, 0.50, and 3.0 M NaCl. The percentage of protein eluted in each peak is shown in the upper panel.

Additional evidence for specificity of the histone cDNA is its ability to form hybrids with total polysomal RNA isolated from intact S phase HeLa cells ($Cr_0t_{1/2} = 1.8$) and its lack of hybrid formation with G_1 polysomal RNA (Stein et al., 1975a). From these findings it is reasonable to conclude that ribosomal (5S, 18S, and 28S) and transfer RNA complementary sequences are not present in the histone cDNA. Furthermore, the polysomal RNA isolated from HeLa cells in which histone and DNA synthesis have been blocked by cytosine arabinoside does not form hybrids with the histone cDNA (Stein et al., 1975c). These results are consistent with data from several laboratories (Thrall et al., 1974; Breindl and Gallwitz, 1973; Butler and Mueller, 1973; Gallwitz and Mueller, 1969) which indicate that histone mRNA is not present on the polyribosomes of HeLa cells treated with inhibitors of DNA synthesis and additionally rule out the possibility that the cDNA contains detectable amounts of sequences complementary to other S phase specific, nonpoly-

adenylated RNAs which have been reported to be insensitive to cytosine arabinoside (Borun et al., 1975). We cannot exclude the possibility that our cDNA preparation contains material other than histone sequences, particularly if the RNA from which this material was reverse transcribed does not translate in a cell free system. If such material is present, however, it has all of the properties discussed above that one would expect for histone sequences and, in addition, its transcription from chromatin is apparently regulated by the nonhistone chromosomal proteins in the same fashion as the histone sequences.

Hybridization Analyses. Histone [^{3}H]cDNA and in vitro transcripts 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 (25 000 dpm/ng), and various amounts of RNA. Reactions were carried out in conditions of RNA excess. *E. coli* RNA was included in the reaction mixtures when necessary so that the final amount of RNA was 3.5 μg. 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 nuclease digestion was determined by trichloroacetic acid precipitation.

Hybridization analyses to detect mouse globin mRNA were performed with ^{3}H-labeled globin cDNA at 65 °C in the presence of 0.45 M NaCl as previously described (Ross, 1976). Control experiments to ensure that there were no inhibitors of hybridization in the chromatin transcript were performed as follows: A known quantity of authentic mouse globin mRNA was mixed with chromatin transcript RNA, and the mixture was incubated with ^{3}H-labeled globin cDNA. The presence of chromatin transcript RNA did not interfere with hybridization of the mouse globin mRNA.

Isolation of Polysomal RNA from Mouse L929 Cells. Mouse L929 cells were grown in monolayer culture in 32-oz prescription bottles in Eagles' medium containing 10% calf serum. Four days after cells were subcultured, nonconfluent cultures were harvested with a rubber policeman, washed 3 times with Earles' balanced salt solution, and pelleted by centrifugation at 1000*g* for 3 min. The pellet was drained well and resuspended in 10 volumes of 10 mM KCl–10 mM Tris–1.5 mM $MgCl_2$ (pH 7.4). The cell suspension was transferred to a Dounce homogenizer and after 20 min the cells were lysed by homogenization with a tight fitting pestle. The homogenate was centrifuged at 15 000*g* for 15 min to pellet nuclei and mitochondria. The supernatant was transferred to 8-mL polycarbonate tubes and centrifuged at 100 000*g* for 90 min. All glassware and solutions were treated with diethyl pyrocarbonate and then autoclaved.

The pelleted polyribosomes were resuspended in 1% sodium dodecyl sulfate–0.1 M NaCl–10 mM sodium acetate–1 mM EDTA (pH 5.4). Polysomal RNA was extracted twice with 1 volume of purified phenol plus 1 volume of chloroform–isoamyl alcohol (24:1, v/v), followed by two extractions with 1 volume of chloroform–isoamyl alcohol. The RNA was precipitated with 2 volumes of ethanol, centrifuged at 3000*g* for 15 min, and resuspended in 25 mM Hepes–0.5 M NaCl–1 mM EDTA (pH 7.0) for hybridization analysis.

Determination of Melting Temperature of Histone cDNA–RNA Hybrids. In order to determine the melting temperature of the hybrids formed, histone cDNA and transcripts from the various chromatin preparations were hybridized at 52 °C to a Cr_0t at which 80% of maximal hybridization was observed with a concentration of transcripts such that this required approximately 10 h. The samples were then heated for 10 min at the indicated temperature and quick cooled in an ice–water slurry. The amount of hybrids remaining was assayed with S_1 nuclease.

Results

Activation of G_1 Phase HeLa Chromatin by an S Phase HeLa Chromosomal Protein Fraction. In order to assay the availablity of histone genes for transcription, chromatin can be transcribed in a cell-free system by *E. coli* RNA polymerase, the transcripts isolated, and the presence of histone mRNA sequences assayed by hybridization to histone cDNA. We have previously shown that transcripts from chromatin isolated from S phase cells hybridize with histone cDNA with a $Cr_0t_{1/2}$ of 2.0×10^{-1} compared with a $Cr_0t_{1/2}$ of 1.7×10^{-2} for the histone mRNA–cDNA hybridization (Stein et al., 1975b,c). That the histone mRNA sequences detected represent new synthesis rather than endogenous histone sequences isolated along with the chromatin is shown by the lack of significant hybrid formation when RNA polymerase is omitted from the reaction mixture and RNA is isolated either with or without the addition of *E. coli* RNA equal to the amount of RNA that is normally transcribed (Stein et al., 1975c; Park et al., 1976). When chromatin isolated from G_1 cells is transcribed, however, even though the total amount of RNA transcribed is similar to that from S phase chromatin, there is no significant hybrid formation between transcripts from G_1 chromatin and histone cDNA, even at a Cr_0t of 100 (Stein et al., 1975b,c; Park et al., 1976).

Utilizing the technique of chromatin reconstitution, we have presented evidence which suggests that it is the nonhistone chromosomal protein portion of chromatin which is responsible for the difference in in vitro transcription of histone sequences from G_1 and S phase chromatin (Stein et al., 1975c). In further studies we have shown that this difference can apparently be accounted for by a component(s) of the S phase nonhistone chromosomal proteins which has the capacity, when reconstituted in the presence of G_1 chromatin, to make the histone genes available for transcription in a dose-dependent fashion (Park et al., 1976). These studies have also shown that removal of contaminating endogenous nucleic acid by buoyant density centrifugation in cesium chloride in the presence of 5 M urea (Park et al., 1976) does not diminish the ability of the S phase nonhistone chromosomal proteins to make histone genes available for transcription from G_1 chromatin. Reconstitution of G_1 chromatin in the presence of additional G_1 chromosomal proteins does not render the histone genes transcribable suggesting that the S phase nonhistone chromosomal proteins have specificity.

In order to determine which component of the S phase HeLa chromosomal proteins is responsible for the dose-dependent activation of histone gene transcription from G_1 chromatin, the S phase chromosomal proteins have been fractionated in the presence of 5 M urea on QAE-Sephadex as shown in Figure 1. Chromosomal proteins from which nucleic acids have 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 2 column volumes each of 5 M urea–10 mM Tris (pH 8.3) containing 0, 0.1, 0.25, 0.5, and 3.0 M NaCl. As shown in Figure 2, the histones and approximately 10% of the nonhistone chromosomal proteins are not bound and are eluted in the void volume, whereas a

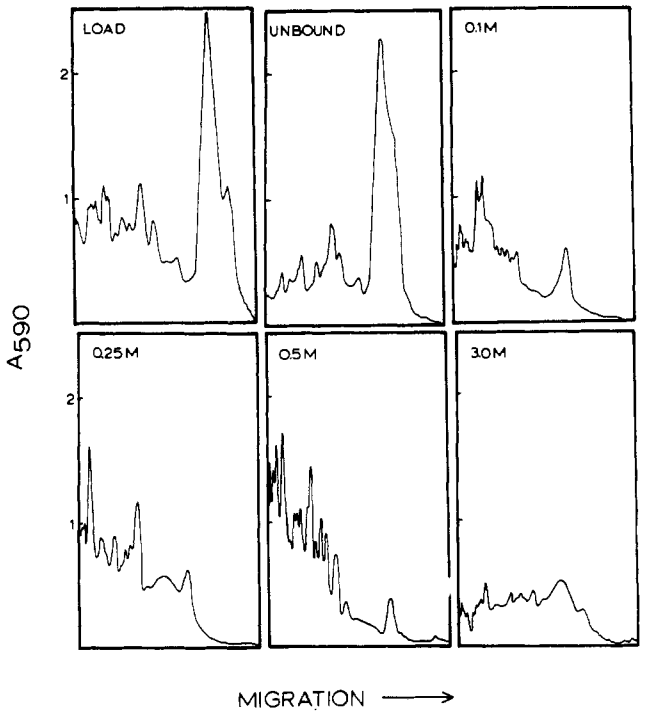

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoretic profile of S phase chromosomal proteins fractionated with QAE-Sephadex. The proteins were heated for 30 min at 60 °C in 1% sodium dodecyl sulfate-1% β-mercaptoethanol-0.01 M $NaPO_4$ (pH 7.0) and were dialyzed against 0.1% sodium dodecyl sulfate-0.1% β-mercaptoethanol-0.01 M $NaPO_4$ (pH 7.0). Aliquots (20-50 μg) were electrophoresed for 7 h at 8 mA/gel on 0.6 × 7.5 cm, 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Bhorjee and Pederson (1973). The gels were fixed overnight in 12% trichloroacetic acid-40% ethanol-7% acetic acid, stained for 5 h at 37 °C in 0.25% Coomassie brilliant blue in 40% ethanol-7% acetic acid, and diffusion destained in 10% ethanol-7% acetic acid. The gels were then scanned at 600 nm in a Beckman Acta 2 spectrophotometer equipped with a linear transport. Details of the procedure have been reported by Krause et al. (1975).

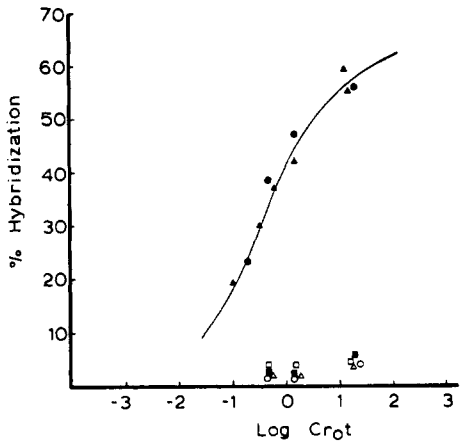

FIGURE 3: Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 HeLa chromatin reconstituted in the presence of S phase HeLa chromosomal protein fractions. ^3H-labeled cDNA was annealed at 52 °C, as described in Materials and Methods, to transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 100 μg of S phase HeLa chromosomal proteins eluted from QAE-Sephadex by 5 M urea-10 mM Tris (pH 8.3) containing 0 (□), 0.10 (■), 0.25 (○), 0.50 (●), and 3.0 M (△) NaCl. ^3H-labeled cDNA was also hybridized to transcripts from the same amount of G_1 HeLa chromatin reconstituted in the presence of 1000 μg of total S phase HeLa chromosomal proteins (▲) (80-100 μg of RNA transcripts was recovered per mg of DNA as chromatin). Cr_0t = moles of ribonucleotides × seconds/liter.

complex but electrophoretically distinct class of nonhistone chromosomal proteins is eluted by each salt concentration. The total recovery of protein from the column is 50-85%. In order to determine the ability of each of the QAE fractions to render histone genes available for transcription, 3 mg of chromatin from G_1 phase cells (containing approximately 1 mg of DNA) was dissociated with 3 M NaCl-5 M urea-10 M Tris (pH 8.3) and was reconstituted in the presence of 100 μg of each of the QAE fractions. The reconstituted chromatin was then transcribed in vitro with *E. coli* RNA polymerase, and the isolated transcripts were assayed for histone mRNA sequences by hybridization to histone cDNA. As shown in Figure 3, 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 from QAE-Sephadex did not show significant hybrid formation with histone cDNA even at a Cr_0t of 30—the same results observed with transcripts from native G_1 chromatin. In contrast, even though the total amount of RNA transcribed was similar, transcripts from G_1 chromatin reconstituted in the presence of the 0.5 M fraction hybridized efficiently with histone complementary DNA ($Cr_0t_{1/2} = 4 \times 10^{-1}$).

We have shown previously that, when G_1 chromatin is reconstituted in the presence of various amounts of S phase chromosomal proteins, there is a dose-dependent activation of histone gene transcription (Park et al., 1976). Specifically, transcripts from G_1 chromatin reconstituted in the presence of 1000 μg of S phase chromosomal protein per mg of G_1 chromatin DNA contain approximately ten 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 NaCl fraction contains only approximately 10% of the protein eluted from the QAE-Sephadex column, one would anticipate that 100 μg of the 0.5 M NaCl fraction should activate histone gene transcription from G_1 chromatin to the same degree as 1000 μg of the total S phase HeLa chromosomal proteins. As can also be seen in Figure 3, there are no significant differences in the kinetics of hybrid formation with histone cDNA of transcripts from G_1 phase chromatin reconstituted in the presence of 100 μg of the 0.5 M NaCl fraction and of transcripts from G_1 chromatin reconstituted with 1000 μg of the total HeLa chromosomal proteins per mg of G_1 chromatin DNA.

Activation of Histone Gene Transcription in Contact-Inhibited WI-38 Cells By an S Phase HeLa Chromosomal Protein Fraction. When WI-38 fibroblasts grow to confluency in monolayer culture, less than 1% of the cells in the confluent monolayer continue to synthesize DNA. When the exhausted growth medium is replaced with fresh medium containing 20% fetal calf serum, an activation of DNA synthesis is evident at 10 h and reaches a maximum at 12 h following stimulation (Jansing et al., 1977; Rhode and Ellen, 1968; Rovera and Baserga, 1971). Using hybridization to a complementary DNA probe for the detection of histone mRNA sequences, we find that histone mRNA becomes associated with polysomes during the activation of DNA synthesis and that the ability of chromatin from WI-38 cells to serve as a template for in vitro transcription of histone mRNA sequences also parallels the onset of DNA replication (Jansing et al., 1977). As shown in Figure 4, transcripts from chromatin of confluent WI-38 cells hybridize only to a limited extent with histone cDNA ($Cr_0t_{1/2} = 180$)—such hybridization most likely being accounted for by the few cells which escape contact inhibition and continue DNA as well as histone synthesis (Jansing et al., 1977). In contrast, the kinetics of hybridization between histone cDNA and RNA transcripts from S phase (12 h) chromatin reveal a 500-fold activation of histone mRNA sequence transcription

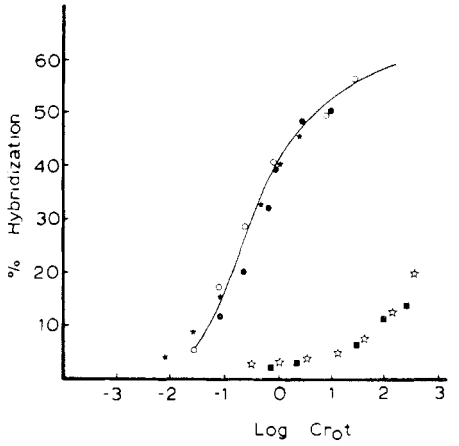

FIGURE 4: Kinetics of annealing of histone cDNA to in vitro transcripts from chromatin of contact-inhibited WI-38 fibroblasts reconstituted in the presence of chromosomal proteins from S phase HeLa cells or from WI-38 cells which have been stimulated to proliferate. ^{3}H-labeled cDNA was annealed at 52 °C, as described in Materials and Methods, to transcripts from 1 mg of DNA as chromatin from contact-inhibited WI-38 fibroblasts reconstituted with no additional chromosomal proteins (☆), 100 μg of the 0.5 M QAE fraction of S phase HeLa chromosomal proteins (○), or 1000 μg of total chromosomal proteins from S phase WI-38 cells (●). ^{3}H-labeled cDNA was also annealed to transcripts from native chromatin of contact-inhibited WI-38 fibroblasts (■) or from WI-38 fibroblasts which had been stimulated to proliferate (★) (80–100 μg of RNA transcripts was recovered per mg of DNA as chromatin); Cr_0t = moles of ribonucleotides × seconds/liter.

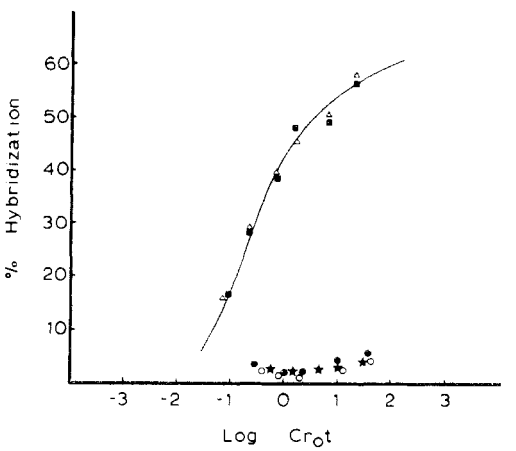

FIGURE 5: Kinetics of annealing of histone cDNA to in vitro transcripts from mouse liver chromatin, or mouse liver chromatin reconstituted in the presence of S phase HeLa chromosomal proteins. ^{3}H-labeled cDNA was annealed at 52 °C as described in Materials and Methods to transcripts from native mouse liver chromatin (○), or transcripts from 1 mg of mouse liver DNA as chromatin reconstituted in the presence of no additional protein (●), 1000 μg of total S phase HeLa chromosomal proteins (△), 100 μg of the 0.5 M NaCl QAE fraction of the S phase HeLa chromosomal proteins (■), or 100 μg of the 0.25 M NaCl QAE fraction of the S phase HeLa chromosomal proteins (★) (80–100 μg of RNA transcripts was recovered per mg of DNA as chromatin); Cr_0t = moles of ribonucleotides × seconds/liter.

($Cr_0t_{1/2} = 4.0 \times 10^{-1}$). This degree of activation is comparable to the increase in DNA synthesis in quiescent cells which have been stimulated to proliferate.

As is shown in Figure 4, when chromatin from contact-inhibited WI-38 cells is dissociated with 3 M NaCl–5 M urea–10 mM Tris (pH 8.3) and is then reconstituted in the absence of additional chromosomal proteins, the same low level of hybrid formation between transcripts of this chromatin and histone cDNA was observed as is seen with transcripts of native, contact-inhibited WI-38 chromatin. In contrast, transcripts from chromatin of contact-inhibited WI-38 cells reconstituted in the presence of 100 μg of the 0.5 M QAE fraction of S phase HeLa chromosomal proteins (per mg of WI-38 DNA as chromatin) hybridize with histone cDNA with a $Cr_0t_{1/2}$ of 4.0×10^{-1}. This degree of histone gene transcription is comparable to that observed when the same amount of contact-inhibited WI-38 chromatin is reconstituted in the presence of 1000 μg of S phase WI-38 chromosomal proteins and that seen from native chromatin of WI-38 cells which have been stimulated to proliferate.

Activation of Histone Gene Transcription from Mouse Liver Chromatin by Chromosomal Proteins from S Phase HeLa S_3 Cells. As shown in Figure 5, when chromatin is isolated from mouse liver and is transcribed in vitro, the transcripts do not hybridize significantly with HeLa histone cDNA even at a Cr_0t of 30. When this chromatin is dissociated with high salt and urea and is then reconstituted in the absence of additional chromosomal protein, again no significant hybrid formation between transcripts and HeLa histone cDNA is observed. In contrast, when mouse liver chromatin is reconstituted in the presence of 1000 μg of total chromosomal proteins from S phase HeLa cells per mg of mouse liver chromatin DNA, the amount of mouse histone mRNA sequences transcribed is increased over 1000-fold ($Cr_0t_{1/2} = 4.0 \times 10^{-1}$).

To rule out the possibility that endogenous HeLa RNA associated with the S phase chromosomal protein at least in part accounts for the formation of hybrids with HeLa histone cDNA, mouse liver chromatin which had been reconstituted in the presence of 1000 μg of HeLa chromosomal protein was placed in the transcription mixture without the addition of RNA polymerase, and the endogenous RNA was extracted by the same procedure used for the isolation of in vitro transcripts. When this endogenous RNA is annealed with HeLa histone cDNA, there is no significant hybrid formation (Figure 5). That hybridization of histone cDNA to transcripts from mouse liver chromatin reconstituted in the presence of S phase HeLa chromosomal proteins is not due to endogenous HeLa RNA sequences is also suggested by the fact that the S phase HeLa chromatin from which these proteins are isolated contains only very low levels of endogenous histone sequences. Although the amount of endogenous histone sequences varies, we generally find less than 10% hybrid formation at a Cr_0t of 100 between histone cDNA and endogenous RNA from S phase chromatin isolated either with or without the addition of carrier RNA. Additionally, it should be pointed out that the HeLa S phase chromosomal proteins contain only very small amounts of nucleic acids (0.2% or less) as estimated by the ratio of absorbance at 280 and 260 nm and by pulse and long-term labeling with [^{3}H]thymidine. We have previously shown that removal of such endogenous nucleic acids by buoyant density centrifugation in cesium chloride in the presence of 5 M urea does not diminish the ability of these HeLa proteins to make the histone genes of G_1 phase HeLa cells available for transcription (Park et al., 1976).

When 1 mg of mouse liver DNA as chromatin is dissociated with high salt and urea, reconstituted in the presence of 1000 μg of total S phase HeLa chromosomal proteins or 100 μg of the 0.5 M QAE fraction of these proteins, and then transcribed in vitro with *E. coli* RNA polymerase, 80–100 μg of RNA can be recovered as purified transcripts. When these mouse liver RNAs are annealed to histone cDNA, as shown in Figure 5, they both hybridize with a $Cr_0t_{1/2}$ of approximately 4×10^{-1}. In contrast, as is also shown in Figure 5, when 1 mg of mouse liver DNA as chromatin is reconstituted in the presence of 100 μg of the 0.25 M NaCl fraction of the HeLa S phase chromosomal protein, even though approximately the same amount

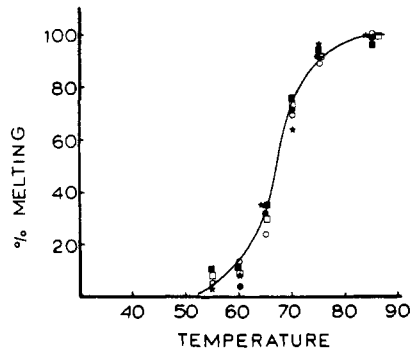

FIGURE 6: Melting profile of hybrids formed between histone cDNA and histone mRNA (□), between histone cDNA and transcripts from 1 mg of DNA as chromatin from mouse liver chromatin reconstituted in the presence of 1000 μg total S phase chromosomal proteins (○), or 100 μg of the 0.5 M NaCl QAE-Sephadex fraction of S phase HeLa chromosomal proteins (●), between histone cDNA and transcripts from 1 mg of DNA as chromatin from contact-inhibited WI-38 fibroblasts reconstituted in the presence of 100 μg of the 0.5 M NaCl fraction of S phase HeLa chromosomal proteins (★), and between histone cDNA and polysomal RNA from mouse L-929 cells (■). The $Cr_0t_{1/2}$ of the hybridization reaction between histone cDNA and mouse L-929 cell polysomal RNA under these conditions is 7.4, which is consistent with this RNA containing approximately 0.23% histone sequences. Melting temperatures were determined as described in Materials and Methods.

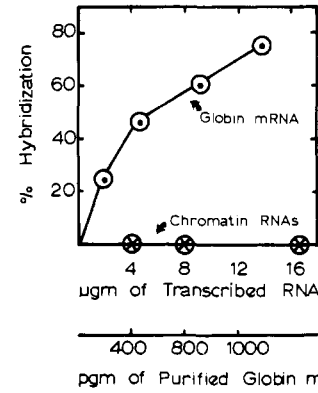

FIGURE 7: Kinetics of annealing of globin cDNA to in vitro transcripts from native adult mouse liver chromatin (X) and from mouse liver chromatin dissociated and reconstituted in the presence of the 0.5 M NaCl QAE-Sephadex fraction from S phase HeLa S_3 cells (135 μg of protein added/mg of DNA as chromatin) (○). Control experiments to ensure that there were no inhibitors of hybridization in the chromatin transcripts were performed as follows: A known quantity of authentic mouse globin mRNA was mixed with chromatin transcript RNA, and the mixture was incubated with [^{3}H]globin cDNA (⊙). The presence of chromatin transcript RNA did not interfere with hybridization of the mouse globin mRNA.

of RNA is transcribed, the transcripts do not show significant hybrid formation with histone complementary DNA.

In Figure 6, the melting profile of the hybrids formed between the HeLa histone cDNA and transcripts from mouse chromatin reconstituted in the presence of either 1000 μg total S phase chromosomal proteins or 100 μg of the 0.5 M QAE fraction of the S phase chromosomal proteins (per mg of mouse liver chromatin DNA) is shown. Within our limits of detection, there are no reproducible differences in the melting profiles of the hybrids formed between HeLa histone cDNA and HeLa histone mRNA, mouse L929 cell polysomal RNA, and transcripts from mouse liver or WI-38 fibroblast chromatin reconstituted in the presence of HeLa chromosomal proteins. It should be noted that small T_m differences would not be detected by this procedure.

Effect of Chromosomal Proteins from S Phase HeLa Cells on Transcription of Mouse Globin Sequences. Fetal liver is an erythropoietic organ which contains globin mRNA sequences, and Gilmour and Paul (1973) have found that chromatin from fetal mouse liver will serve as a template for the in vitro transcription of globin mRNA sequences by *E. coli* polymerase. In contrast, as has been shown previously in birds (Axel et al., 1973) and is shown in Figure 7 for mouse, globin mRNA sequences are not transcribed in vitro by *E. coli* RNA polymerase from chromatin of adult liver, which is not an erythropoietic tissue.

In order to rule out the possibility that chromosomal proteins from S phase HeLa cells make the histone genes of mouse liver chromatin available for transcription nonspecifically, we have examined the effect of HeLa chromosomal protein on the transcription of globin sequences from mouse chromatin. As discussed above, when 1 mg of DNA as chromatin from adult mouse liver is dissociated and then reconstituted in the presence of 1000 μg of S phase total HeLa chromosomal proteins or 100 μg of the 0.5 M fraction of the HeLa chromosomal proteins, the amount of histone mRNA sequences detected in the transcripts is increased over 1000-fold from that found in transcripts from native mouse liver. Significant differences in the overall template activities of these reconstituted chromatin preparations are not observed. As shown in Figure 7, these same transcripts from mouse liver chromatin reconstituted with the 0.5 M NaCl fraction of S phase HeLa chromosomal proteins show no significant hybrid formation with mouse globin complementary DNA. If a mixture of globin mRNA and transcripts from mouse liver chromatin is hybridized with globin cDNA, the globin mRNA anneals with the globin cDNA as expected. Globin mRNA sequences would have been detected in the chromatin transcripts had they been present in as little as 1 part per 10^5.

Discussion

We have previously presented results which suggest that it is the nonhistone chromosomal protein portion of chromatin which is responsible for the difference in in vitro transcription of histone genes from G_1 and S phase HeLa S_3 cell chromatin (Stein et al., 1975c), and that this difference can be accounted for by a component(s) of the S phase nonhistone chromosomal protein which has the ability to render histone genes from G_1 HeLa chromatin available for transcription (Park et al., 1976). The present study suggests that the S phase HeLa chromosomal proteins can also make histone genes from chromatin of contact-inhibited WI-38 human diploid fibroblasts or from nondividing mouse liver chromatin available for transcription. In addition, 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 phase HeLa cells, contact-inhibited WI-38 fibroblasts or mouse liver. Several lines of evidence suggest that activation of histone gene transcription in mouse liver chromatin by S phase HeLa cell nonhistone chromosomal proteins is not a random phenomenon. Addition of the HeLa cell proteins to mouse liver chromatin does not significantly modify chromatin template activity. More specifically, the HeLa chromosomal proteins do not render globin sequences transcribable.

Results from a number of laboratories indicate that histone proteins are similar in different mammalian species and in different cell types of the same species (Delange et al., 1969; Delange and Smith, 1975; Elgin and Weintraub, 1975). Our data would seem to suggest that the mechanism by which the transcription of histone genes is regulated by the nonhistone chromosomal proteins in HeLa cells, WI-38 cells, and in mouse

liver may be the same or similar. This can be accounted for by postulating that the DNA sequences with which certain nonhistone chromosomal proteins interact, perhaps regulatory sequences, are conserved between mouse and human. Alternatively, the DNA sequences involved with activation of histone gene transcription may differ between mouse and human, but both types of sequences may be recognized by the HeLa nonhistone chromosomal proteins. However, our results do illustrate that a highly transformed, continuously dividing cervical carcinoma cell, such as HeLa, contains all of the components necessary to make the histone genes of contact-inhibited tissue culture cells or nondividing cells from an intact organism available for transcription in vitro by *E. coli* RNA polymerase.

It should be noted, however, that even the QAE fraction of the S phase nonhistone chromosomal proteins is a very heterogeneous class of proteins. It is not known which component(s) is responsible for the activation of histone gene transcription or by what mechanism such "activation" occurs. These studies were performed in vitro using *E. coli* RNA polymerase. Although a large amount of evidence has been obtained which indicates that in vitro transcription of chromatin and chromatin reconstitution can be used as tools to examine the regulation of specific genes (Stein et al., 1975c, Park et al., 1976; Paul et al., 1973; Barrett et al., 1974; Chiu et al., 1975; Tsai et al., 1976a,b), it cannot be ruled out that regulatory molecules have been lost or inactivated in the course of isolation, fractionation, and reconstitution of chromatin, or that in vitro transcription is different than that seen in vivo. One might anticipate that there may be other, perhaps more subtle, factors regulating histone gene transcription that interact only with the homologous RNA polymerase or that act in the intact cell. Also caution must be exercised in assuming that a similar mode of histone gene control is operative in all biological situations. For example, during oogenesis and early stages of development, histone mRNA has been shown to be more stable, and it is thought that under such conditions posttranscriptional control may be operative (Farquhar and McCarthy, 1973; Schoultchi and Gross, 1973).

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Modification of Ribonucleic Acid by Vitamin B_6. 1. Specific Interaction of Pyridoxal 5′-Phosphate with Transfer Ribonucleic Acid†

Levy Kopelovich* and Gloria Wolfe

ABSTRACT: Whole tRNA preparation obtained from a human cell line (HT-29) of colon carcinoma and purified specific *Escherichia coli* tRNA were reacted with pyridoxal 5′-phosphate, reduced by sodium borohydride and digested with RNase A and snake venom phosphodiesterase. Two-dimensional chromatography of the pyridoxal 5′-phosphate treated tRNA digest showed that pyridoxal 5′-phosphate binds specifically to GMP, presumably in the form of a Schiff base with the exocyclic amino group of the purine. The reaction of pyridoxal 5′-phosphate with whole tRNA was competitively inhibited by *N*-acetoxy-2-acetylaminofluorene. This suggests that binding occurred primarily to the G_{20} base residue at the unpaired region of the dihydrouridine loop (Fujimura et al., 1972). The modification of tRNA by pyridoxal 5′-phosphate resulted in the inhibition, to varying extent (10–80%), of amino acid acceptance in the aminoacyl-tRNA synthetase reaction. Defects in codon recognition by pyridoxal 5′-phosphate modified amino acid acylated tRNAs in the presence of the corresponding guanine-containing polynucleotide triplets were observed by the ribosomal binding assay.

The extensive modification of tRNA is probably concerned primarily with its function in translation (Miller, 1970; Chambers, 1971; Cramer, 1971; Quigley et al., 1975; Kim, 1976; Sussman and Kim, 1976). It has also been suggested that tRNA may present a critical target causal of cancer development (Farber, 1968; Miller, 1970; Weinstein and Grunberger, 1971, 1974). Although knowledge of the primary and secondary structures of tRNA and its role in protein synthesis is rather extensive (Chambers, 1971; Cramer, 1971; Quigley et al., 1975; Kim, 1976), interaction of higher orders of the tRNA structure in respect of specific biological processes remains to be elucidated. Chemical modifications, those by physiologic compounds in particular, could provide insight into the structural and functional requirements of tRNA.

This report concerns the interaction of vitamin B_6, in the form of pyridoxal 5′-phosphate,[1] with tRNA, a nucleic acid which presents a secondary structure of distinctive open loops and base paired regions. It shows that PLP binds specifically to guanine at position 20 of the dihydrouridine loop and that ligation may involve a conformational change of the tRNA molecule, resulting in the inhibition of amino acid acceptance and ribosomal binding. The results suggest that translation may be modulated, in part, by interaction of PLP with tRNA. A preliminary report of some of these findings has appeared elsewhere (Kopelovich et al., 1976a,b).

Materials and Methods

Isolation of Whole tRNA from a Human Cell Line of Colon Carcinoma. Whole tRNA was obtained from a human cell line of colon carcinoma (HT-29) propagated in this laboratory and purified specific *E. coli* tRNA from Boehringer. Whole tRNA was extracted from cells essentially as described by Fink et al. (1970). Cells were homogenized in 0.01 M Tris-HCl, pH 7.0, 0.2% bentonite, 0.3 M sucrose, and water saturated phenol containing 0.1% 8-hydroxyquinoline. Following centrifugation, 13 000*g* for 25 min at 4 °C, the aqueous layer was reextracted three times with water-saturated phenol containing 0.1% sodium dodecyl sulfate and 0.1% 8-hydroxyquinoline. This was followed by precipitation with 2 volumes of 95% ethanol and 0.1 volume of 2 M sodium acetate, pH 5.0, at −20 °C, solubilization in 1 M NaCl, and an additional precipitation with 95% ethanol at −20 °C. To remove polysaccharides and other low molecular weight contaminants, the tRNA was further purified by DEAE-cellulose chromatography. A DEAE-cellulose column (1.0 × 10 cm) was equilibrated with 0.1 M Tris-HCl (pH 7.2)–0.01 M $MgCl_2$–0.2 M NaCl. A sample of tRNA (about 10 mg) was applied to the column and the column washed three times with the equilibrating buffer. The tRNA was then eluted with 0.1 M Tris-HCl (pH 7.2)–0.8 M NaCl–0.001 M $MgCl_2$ and recovered by ethanol precipitation at −20 °C. The tRNA was resuspended in distilled water containing 0.01 M $MgCl_2$. Stripping of the isolated tRNA (deaminoacylation) was carried out under mild conditions in

† From the Department of Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021. *Received November 22, 1976.* This work was supported by NCI Grants CA-14991 and CA-08748.

[1] Abbreviations used: *N*-acetoxy-AAF, *N*-acetoxy-2-acetylaminofluorene; PLP, pyridoxal 5′-phosphate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet.