THE ROLE OF DNA METHYLATION IN THE DIFFERENTIATION OF FRIEND ERYTHROLEUKEMIA CELLS

BY

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1979

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5-22-79
date

Chairman of Examining Committee

5/22/79
date

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ABSTRACT

THE ROLE OF DNA METHYLATION IN THE DIFFERENTIATION OF FRIEND ERYTHROLEUKEMIA CELLS

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The role of DNA methylation in eukaryotes is unknown. Several lines of evidence have suggested that DNA methylation may play a role in cellular differentiation. However, there is not direct evidence as to the biological function of eukaryotic DNA methylation. Friend erythroleukemia cells provide a model system for studying differentiation. These cells undergo many of the changes which have been shown to accompany normal erythroid differentiation. Previous findings in our laboratory have shown that the nuclear DNA purified from Friend erythroleukemia cells grown in the presence of L-ethionine, dimethylsulfoxide, butyric acid or hexamethylene bisacetamide is hypomethylated. Thus, a correlation has been established between the methylation of DNA and the expression of differentiated cell function. This study was undertaken to determine the cause of hypomethylation of nuclear DNA in Friend erythroleukemia cells and its relationship to differentiation.

DNA methylation was studied at the chromatin level. It was shown that the restrictions operating in the whole

cell which prevent the methylation of cytosine residues are operating at the level of chromatin. The strongest evidence that these restrictions are due to chromatin proteins, which interfere with DNA methylation, came from comparing the in vitro methylation of chromatin DNA with the methylation of DNA purified from chromatin. It was shown that, irrespective of the differentiated state of the cells from which chromatin is prepared, 0.35 - 0.46 pmoles of methyl are transferred to 1 A_{260nm} unit of chromatin by either chromatin-bound or added DNA methyltransferases during a 30 minute incubation period at 37°C. In contrast, previous results in our laboratory have shown that under the same reaction conditions, 5-7 pmoles of methyl are transferred to 1 A_{260nm} unit of DNA purified from untreated Friend erythroleukemia cells, and 15-25 pmoles of methyl are transferred to 1 A_{260nm} of DNA purified from dimethylsulfoxide-treated Friend erythroleukemic cells.

Chromatin fractionation procedures were used to separate actively-transcribed and non-transcribed DNA. It was shown that, regardless of the differentiated state of the cell from which chromatin was prepared, the sequences of actively- transcribed DNA were poor methyl acceptors in vitro, indicating that these sequences were virtually fully methylated in vivo. In addition, there were low levels of DNA methyltransferase activity associated with this DNA. In contrast, virtually all of the cytosine residues which were not modified in vivo are clustered in the template-inactive DNA. The majority of the DNA methyltransferase activity is also associated with this fraction.

While these findings are not inconsistent with several proposed mechanisms whereby DNA methylation may play a role in gene control during differentiation, a relationship has not been established. Since it has also been shown that chromatin proteins restrict the access of the DNA methyltransferases to sites in the DNA, the possibility arises that chromatin proteins can regulate methylation of nuclear DNA. Thus, a restriction of DNA methylation might be the result of a change in chromatin configuration which is a consequence of, rather than a cause of, cellular differentiation.

ACKNOWLEDGEMENTS

My graduate studies represent the culmination of efforts on the behalf of many persons, to all of whom I am deeply indebted.

I would like to thank my sponsor, Dr. Kurt Hirschhorn, for providing me with the opportunity to study human genetics in his laboratory. Dr. Hirschhorn's expert scientific abilities, kindness, and friendship will always be remembered.

I am indebted to Drs. George Acs and Judith Christman for providing critical insight and invaluable guidance throughout the course of this research project. In addition to their keen scientific insights, their warmth and compassion were a constant source of support for me.

I would also like to thank my friend, Dr. Peter Price, for generously giving of his own time and readily sharing his expertise in the laboratory.

In addition, I must acknowledge Ms. Francine Creusot for making her findings available to me.

Finally, I wish to thank my wife, Debbie, without whose love and encouragement this project could never have been completed.

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LIST OF ABBREVIATIONS

Absorbance	A
Counts per minute	CPM
Deoxyribonuclease	DNase
Ethylenediamine tetraacetic acid	EDTA
Friend erythroleukemia cell	FL cell
Lymphoid Leukosis Virus	LLV
Non-histone chromatin proteins	NHCP
Phenylmethylsulfonylfluoride	PMSF
Reticulocyte standard buffer	RSB
Messenger ribonucleic acid	mRNA
Transfer ribonucleic acid	tRNA
Ribosomal deoxyribonucleic acid	rDNA
Spleen Focus Forming Virus	SFFV

LIST OF DEFINITIONS

Total chromatin	- Chromatin prior to nuclease digestion.
Undigested chromatin	- That fraction of the chromatin remaining undigested after digestion with nuclease.
Chromatin DNA or Endogenous DNA	- DNA with associated chromatin proteins.
Purified DNA	- Chromatin DNA which has been deproteinated, ribonuclease treated, and extracted with isoamyl alcohol:chloroform.

INTRODUCTION

The biochemical and morphological events which are associated with cellular differentiation are numerous. Erythroid differentiation has been well studied, so that a number of these events have been elucidated. Friend erythroleukemia (FL) cells provide a model system for studying erythroid differentiation *in vitro*, since these cells undergo many of the changes known to occur during erythropoiesis within mammalian bone marrow. Cellular differentiation and the regulation of metabolic processes in the cell are dependent upon the production of new macromolecular species. They are also dependent upon the modification of pre-existing cellular proteins and nucleic acids, by the processes of acetylation, phosphorylation and methylation.

Several lines of evidence have indicated that DNA methylation may play a role in cellular differentiation. It has been shown that when Friend erythroleukemia cells are induced to differentiate by growth in the presence of ethionine, dimethylsulfoxide (Me_2SO), hexamethylene bisacetamide (HMBA), or butyric acid, the nuclear DNA is hypomethylated. Based on these findings of a correlation between DNA hypomethylation and the expression of a differentiated function, studies were undertaken to determine the cause of DNA hypomethylation in Friend erythroleukemia cells. In addition, it was of interest to determine whether there is a correlation between the extent to which DNA is methylated and its template activity.

BACKGROUND: CHROMATIN

I. Cellular Chromatin Structure

A. Subunit Structure

DNA in the eukaryotic cell nucleus is present as a nucleoprotein complex called chromatin. Biochemical (Garel and Axel, 1976; Cech et al., 1978) and electron microscopic (Kornberg, 1974; Olins and Olins, 1974; Finch et al., 1977) studies have established that most of the eukaryotic chromatin is organized in a repeating subunit structure. This subunit structure, or nucleosome, contains approximately 160 base pairs of DNA associated with a histone octameric core comprised of two molecules of histones H2A, H2B, H3 and H4. This DNA is folded 6 times in length (Griffith, 1975). Nucleosomes are separated by a linker DNA. The length of internucleosomal DNA varies with cell type (Keichline and Wassarman, 1977; Kornberg, 1977) and the differentiated state of the cell (Weintraub, 1978). The fifth histone (H1) has been localized to the internucleosomal DNA (Simpson and Whitlock, 1976; Noll and Kornberg, 1977).

Nucleosome core structure produced after limit digestion of chromatin with micrococcal nuclease (EC 3.1.4.7), is comprised of 140 base pairs of DNA associated with the histone octamer (Chambon, 1978). Electron micrographic and X-ray crystallographic studies on purified core particle crystals show the particle to be disc-shaped, with a diameter of 110Å and a thickness of 57Å. It is arranged into two symmetrical "layers" along its short axis. The nucleosomal DNA is located

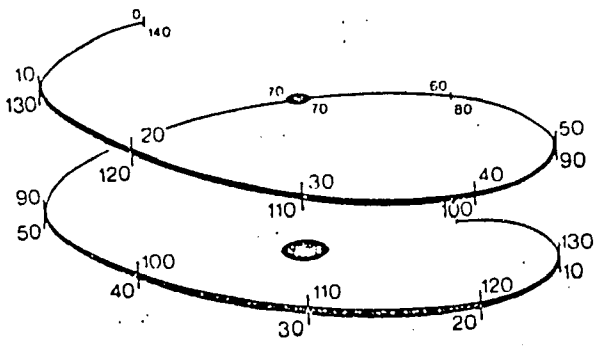
on the outside of the histone core. This DNA is wound in a negative superhelix with a 28Å pitch and 1.75 turns with 80 base pairs per turn (Finch et al., 1977). A diagram of the arrangement of the 140 base pairs of DNA in the nucleosome core is shown in Figure 1.

The model deduced by Finch and co-workers (1977) from X-ray diffraction studies of nucleosome core crystals does not explain how histones are organized within the nucleosome core. Mirzabekov et al., (1978) have used histone cross-linking methods to determine the sequence of histones on nucleosomal single-stranded DNA. Mirzabekov labelled cross-linked histones with I^{125}. To measure the relative lengths of single-stranded DNA fragments which are cross-linked to radiolabeled histones, two-dimensional electrophoresis was performed. Electrophoresis in the first dimension was carried out under DNA denaturing conditions, thereby separating according to the size of the DNA which is cross-linked to histones. DNA is then hydrolyzed, and histones were identified by electrophoretic separation in the second dimension. Mirzabekov has determined the sequence of histones on nucleosomal DNA directly from an autoradiogram of this two-dimensional electrophoresis (Fig. 2A). Mirzabekov's model for the symmetrical arrangement of histones on double-stranded nucleosomal DNA is also presented (Fig. 2B).

B. Higher Ordered Structure of Chromatin

There is evidence (Benyajati and Worcel, 1976) that in Drosophila interphase chromatin, the supercoiled DNA of the nucleosomal filament is still further supercoiled into

FIGURE 1.


0
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110
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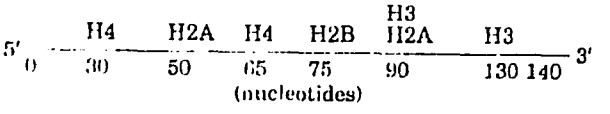
Legend to Figure 1.

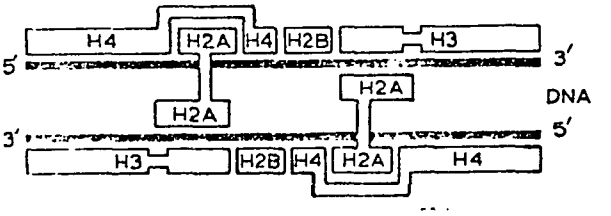
The arrangement of the 140 base pairs of DNA in the nucleosome core was deduced by Finch and coworkers (1977), based on their X-ray crystallographic studies.

FIGURE 2.

2A.



2B.



Legend to Figure 2.

2A. I^{125}-labelled histones were cross-linked to nucleosomal DNA and then electrophoresed in the first dimension which separates according to the length of cross-linked DNA. The DNA was then hydrolyzed and 4 histones were separated by electrophoresis in the second dimension. This sequence of histones along single-stranded DNA was deduced by Mirzabekov et al., (1978) from an autoradiogram of the two dimensional electrophoresis.

2B. Model proposed by Mirzabekov et al., (1978) for the symmetrical arrangement of histones on double-stranded nucleosomal DNA.

chromosome-sized loops. Three such loops are found in a Drosophila interphase chromosome. On the average, a loop is comprised of 85 kilobases of DNA. It is postulated that the conformation of these loops is stabilized by DNA-RNA and DNA-protein crosslinks. It is not known whether internucleosomal histones play a role in maintaining the structure of the loop. Although their structures are somewhat different, DNA loops have been detected in cultured human cells (Cook and Brazell, 1975) and murine cells (Ide et al., 1975).

The architecture of the metaphase chromosome has been described. A structure which is morphologically similar to the metaphase chromosome is obtained after sucrose density gradient centrifugation of metaphase chromosomes depleted of histones by pretreatment with polyanions. This structure is referred to as a scaffold, and is comprised of 30 nonhistone chromatin proteins of molecular weight 50,000 or greater and DNA. Contractile proteins, such as tubulin and actin, have not been found within this group of proteins (Adolph et al., 1977a).

Electron microscopic studies of histone-depleted metaphase chromosomes indicate that the scaffold is associated with a halo of DNA organized into loops, very similar to those observed in interphase chromosomes (Benyajati and Worcel, 1976). Metaphase loops contain between 30 and 90 kilobases of DNA, and are anchored at both ends to the scaffold structure. These loops are further compacted by histones to yield the 300Å fiber of the metaphase chromosome.

It has been possible to isolate the scaffold after

micrococcal nuclease digestion (Adolph et al., 1977b). Taken together, these data were interpreted as indicating that the scaffold is an independent structure which is biochemically distinct from the rest of the chromosome (Paulson and Laemmli, 1977).

II. Actively Transcribed and Non-Transcribed DNA Sequences in Cellular Chromatin.

A. Physical and Biochemial Differences

The repeating subunit model of chromatin structure contains no inherent structural features which could account for differences between actively transcribed and non-transcribed chromatin. Yet, there is much evidence indicating that the structure of actively transcribed chromatin differs from that of silent chromatin. Thermal denaturation and circular dichroism studies suggest that active chromatin is in a more extended configuration (McConaughy and McCarthy, 1972; Polacow et al., 1973; Gottesfeld et al, 1974b) and more susceptible to nuclease degradation (Berkowitz and Doty, 1975; Gottesfeld et al., 1975). DNA in transcriptionally-active chromatin is more easily sheared (Frenster et al., 1963; Simpson and Reeck, 1973; McCarthy et al., 1973). Active chromatin is enriched in nonhistone chromosomal proteins, and somewhat depleted in histone content (Marushige and Bonner, 1971; Berkowitz and Doty, 1975). The sedimentation coefficients of template-active and template-inactive chromatin subunits are different. (Gottesfeld et al., 1975). Transcriptionally-active chromatin has a higher in vitro template activity using E.

coli RNA polymerase than silent chromatin (Simpson, 1974; Gottesfeld et al., 1974; Berkowitz and Doty, 1975). Both actively transcribed and non-transcribed chromatin are associated with nucleosomes. In contradistinction to the nucleosomes containing non-transcribed DNA sequences, the nucleosomes associated with actively transcribed sequences contain nascent RNA (Lacy and Axel, 1975; Gottesfeld et al., 1975).

B. Deoxyribonucleases as Probes of Chromatin Structure

1. DNase I (EC 3.1.4.5)

In a limited digestion of chromatin, DNase I preferentially digests gene sequences within chromatin which are actively transcribed. Sequences coding for globin mRNA but not for ovalbumin mRNA are digested in chick erythyrocytes (Weintraub and Groudine, 1976). In contrast, ovalbumin genes are preferentially digested in hen oviduct nuclei, but not in rat liver (Garel and Axel, 1976). Active gene transcription is probably nct required for preferential DNase I digestion. Increasing evidence suggests that genes which have the potential for transcription during development and cellular differentiation are also sensitive to digestion by DNase I (Mayfield et al., 1978; Miller et al., 1978). These findings indicate that sequences of DNA which are actively transcribed are in a conformation which differs from transcriptionally-inactive DNA. Flint and Weintraub (1977) compared the sensitivity to DNase I of integrated adenovirus genes that are transcribed to the sensitivity of adjacent viral DNA sequences which are not transcribed in transformed hamster cells. The authors

were not only able to show that the transcribed sequences were more sensitive to DNase I, but were able to establish that DNase I cleaves within 2-3 nucleosomes at the 5' end of the transcriptional unit and between 2-15 nucleosomes at the 3' end.

Extensive digestion of the nucleosome core produces DNA fragments which are integral multiples of 10 nucleotides along the nucleosomal DNA (Sollner-Webb and Felsenfeld, 1977). It has been demonstrated that the enzyme cleaves nucleosomal DNA so that staggered cuts on opposite strands are separated by 8 nucleotides with the 3' hydroxyl group recessed, and by 2 nucleotides so that a 5' phosphate is recessed. It has been suggested that the recognition and cutting sites of the enzyme do not coincide (Sollner-Webb and Felsenfeld, 1977).

2. DNase II (EC 3.1.4.6)

Limited digestion of chromatin by DNase II preferentially releases nucleosomes containing transcriptionally-active sequences, although nucleosomes containing transcriptionally-inactive sequences are also released (Gottesfeld et al., 1974a, 1975). While the nucleosomal core DNA is resistant to DNase II, extensive digestion results in fragments which are integral multiples of 10 nucleotides (Sollner-Webb et al., 1976). It has been shown that DNase II does not cut at the same site as DNase I. When nucleosomal DNA is cleaved by DNase II, the nearest cuts on opposing strands are separated by 4 nucleotides, with the 3' end extending (Sollner-Webb et al., 1978).

3. Micrococcal Nuclease (EC 3.1.4.7)

Nucleosomes can be converted to core subunits by digestion with micrococcal nuclease (Noll, 1974). Under condi-

tions of limited digestion, micrococcal nuclease cleaves internucleosomal DNA. Extensive digestion of nucleosomal core DNA by micrococcal nuclease produces the 10 nucleotide repeat as observed by DNase I and DNase II digestion. Micrococcal nuclease produces staggered cuts on opposite strands of nucleosomal DNA, separated by 2 nucleotides with a 5' phosphate extended (Sollner-Webb et al., 1978).

C. Chromatin Fractionation

Several methods have been developed for at least partially fractionating chromatin into transcriptionally-active and inactive fractions. DNase II digestion of chromatin releases nucleosomes which contain both actively transcribed and non-transcribed DNA sequences. Nucleosomes containing silent genes are precipitated by divalent cations,and are separated by differential centrifugation (Marushige and Bonner, 1971). The Mg^{++}-soluble fraction has been shown to possess many of the characteristics associated with transcriptionally-active-chromatin (Elgin and Weintraub, 1976). The DNA in this fraction is enriched in nonrepetitive DNA sequences coding for cellular RNA (Gottesfeld et al., 1974). The transcriptionally-active chromatin fraction contains a different subset of nonrepetitive genome sequences depending upon the tissue of origin (Gottesfeld et al., 1976). Nascent RNA and RNA polymerase are copurified with this fraction (Bonner et al., 1973; Marushige and Bonner, 1971). The fraction is enriched in nonhistone chromosome proteins and depleted in histone H1 (Marushige and Bonner, 1971; Gottesfeld et al., 1975).

Although DNase I destroys DNA from the template-active fraction, it can be used to fractionate chromatin proteins. As a consequence of the ability of this enzyme to distinguish between template-active and template-inactive DNA, chromatin proteins bound to template-active DNA are released into the soluble fraction following DNase I digestion. These proteins may then be separated from protein bound to template-inactive DNA.

III. Post-Synthetic Modification of Chromatin Components.

A. Histones

Early findings by Stedman and Stedman (1943) led these authors to postulate that tissue-specific histones were responsible for the repression of gene expression. However, several findings have provided strong evidence against assigning histones the function of specific gene repressors. The primary structure of histones has been highly conserved during evolution. The amino acid sequence of histone 4 from calf thymus and pea seedlings differ by only two residues (DeLange et al., 1969). Immunological studies have failed to demonstrate a tissue or organ specificity for histones with one exception. Histone 5 is present only in avian, amphibian and icthyoid cell nuclei (Bustin and Stoller, 1973). The amount of histone in cell nuclei does not appear to vary during differentiation. The quantity of histone present is the same in the nuclei of rapidly proliferating avian erythrocytes and in the non-dividing, differentiated polychromatic avian erythrocyte (Appels et al., 1972).

Although their primary structure has been conserved evolutionarily, histone side chains are extensively modified post-transcriptionally. These modifications of histones include phosphorylation, acetylation, methylation and ADP ribosylation (Elgin and Weintraub, 1975). Acetylation and phosphorylation of histones precedes or occurs concomitantly with the activation of transcription (Stein et al., 1978). These processes have been shown to occur during erythroid cell maturation (Allfrey, 1979; Sung et al., 1977), after partial hepatectomy (Pogo et al., 1968; Lee and Paik, 1972), at selected times during the mammalian cell cycle (Marks et al., 1973), during lymphocyte activation (Allfrey et al., 1966), after hormone administration (DeVilliers et al., 1973; Langan, 1969), and in adeno-virus infected cells (Sarkander et al., 1975). It has been suggested that the regulatory role of histones is related to the extent of their modification (DeLange and Smith, 1971). This is supported by evidence that chemical acetylation of chromatin changes its configuration so that it resembles actively-transcribed chromatin in its sensitivity to nucleases (Sealy and Chalkley, 1978) and solubility in Mg++ (Wallace et al., 1977). It has been suggested that sequential removal of acetate and phosphate groups from histones may provide a mechanism for repression of activated genetic sequences (Stein et al., 1978).

B. Post-Synthetic Modification of Non-Histone Chromatin Proteins (NHCP).

The NHCP content of transcribable DNA is higher than in

template-inactive DNA (Marushige and Bonner, 1971; Berkowitz and Doty, 1975). The results of *in vitro* transcription studies demonstrate that NHCP increase template activity as well as restore histone-inhibited transcription (Spellsberg and Hnilica, 1969); Enea and Allfrey, 1973). NHCP have been implicated in the specific restriction of transcription in chromatin (Paul and Gilmour, 1968; Kostraba and Wang, 1972).

The NHCP undergo extensive post-synthetic modification as a result of methylation (Friedman *et al.*, 1969), acetylation (Pogo *et al.*, 1966), and phosphorylation (Kleinsmith *et al.*, 1966). Whether alterations in NHCP by post-translational modification are a prequisite for gene expression remains to be established (Stein *et al.*, 1978). There is evidence indicating that gene expression is modulated by the phosphorylation of NHCP (Kleinsmith *et al.*, 1966; Gershey and Kleinsmith, 1969; Jungman and Schweppe, 1972). It has been well documented, however, that factors which regulate gene expression, such as hormones, influence the amount and the modification of NHCPs. The appearance of specific chromosomal proteins was observed after the administration of cortisol (Shelton and Allrey, 1970), insulin (Buck and Schauder), glucagon (Enea and Allfrey, 1973), aldosterone (Swaneck *et al.*, 1970), and diethylstilbestrol (Spelsberg et al., 1973). The turnover of NHCPs and their phosphate groups has been measured in Friend erythroleukemia cells (Neumann *et al.*, 1978), in splenic tissue during the early and late phases of erythropoiesis (Spivak 1975), in activated lymphocytes (Johnson *et al.*,

1974), and after partial hepatectomy (Kostraba and Wang, 1970). Immunological studies have shown that neoplasia changes the tissue specificity of the NHCP-DNA complex to a form which is characteristic of malignant growth (Wakabayashi and Hnilica, 1973).

C. Post-Synthetic Modification of Cellular DNA.

While nuclear DNA is modified to a significant extent (0.7-2 moles methyl/100 moles of bases; Vanyushin et al., 1970; Dawid, 1974), the role of DNA methylation in eukaryotes is unknown. The only base which is modified in eukaryotic DNA is cytosine (Lawley et al., 1972). In prokaryotes, methylation at the N-6 position of adenine or the C-5 position of cytosine within a specific base sequence confers protection against cleavage by restriction endonucleases (Meselson et al., 1972). Scarano (1977) and Holliday and Fugh (1975) have postulated that methylated cytosine may play a role in gene control. Several lines of evidence implicate 5-methylcytosine in the control of cellular differentiation: (a) The 5-methylcytosine content of sea urchin DNA rises during development (Adams, 1973), (b) DNA from different tissues of the same animal has a differing content of 5-methylcytosine (Vanyushin et al., 1973), (c) The DNA sequences found to be methylated appear to be highly conserved in vertebrate evolution (Browne and Burdon, 1977; Grippo et al., 1968; Brown et al., 1977), (d) Polyoma-transformed BHK cells contain twice as many 5-methylcytosine residues as do untransformed cells (Nass, 1973; Rubery and Newton, 1973).

The distribution of 5-methylcytosine residues within chromatin have been studied. Anti-nucleoside antibody studies have indicated that the centromeric heterochromatin of human chromosomes 1, 9, 11, 15p and the distal portion of the Y chromosome are enriched in 5-methylcytosine (Lubit et al., 1976). Since centromeric heterochromatin is not transcribed (Comings, 1973), these findings would indicate that template-inactive chromatin DNA is enriched in 5-methylcytosine. However, DNA which is template-active, as determined by its rapid solubilization following DNase I digestion, is enriched two fold in 5-methylcytosine content (Adams et al., 1977). Eukaryotic DNA which is resistant to micrococcal nuclease degradation has been shown to be enriched in 5-methylcytosine (Razin and Cedar, 1977). The distribution of 5-methylcytosine residues in DNA has been analyzed by studies on pyrimidine tracts. It has been shown that the sequences in which 5-methylcytosine are present in the main band DNA and satellite DNA are different. In the main band, over 90% of the 5-methylcytosine residues are contained in the sequence purine-5-methylcytosine-purine, while in satellite DNA, about 50% of the residues are in pyrimidine tracts (Harbers et al., 1975).

Christman and coworkers (1977) have shown a correlation between methylation of nuclear DNA and the expression of a differentiated function. These authors demonstrated that when Friend erythroleukemia cells are grown in the presence of inducers of hemoglobin synthesis, the nuclear DNA purified from induced cells is hypomethylated. This DNA is capable of accepting more methyl per unit weight in vitro than DNA

which has been purified from uninduced Friend erythroleukemia cells.

IV. Friend Erythroleukemia Cells (FL Cells)

The origin and biology of tissue culture lines derived from virus-induced murine leukemia cells has recently been reviewed (Marks and Rifkind, 1978). In 1957, Charlotte Friend isolated a virus which was shown to produce splenomegaly, hepatomegaly, and to induce an erythroleukemia in susceptible strains of mice (Friend, 1957). Transplantable tumors could be elicited in syngeneic mice following subcutaneous implantation of splenic fragments from mice rendered erythroleukemic after innoculation with Friend virus (Friend and Haddad, 1960). These cultured tumor explants are the progenitors of the Friend tissue culture cell lines.

The Friend leukemia virus is comprised of two viruses, a spleen focus-forming virus (SFFV) and lymphoid leukosis virus (LLV). SFFV is defective for replication,with LLV acting as a helper virus. Susceptibility is controlled by two independently segregating genes (Tooze, 1973). The virus is apparently able to transform a committed erythroid precursor stem cell (Rauscher, 1962; Chirigos and March, 1974; Odaka, 1969; Steeves et al., 1969; Tambourin and Wendling, 1971). The transformed stem cell is able to differentiate into proerythroblasts in the absence of erythropoietin (Mirand et al., Sassa et al., 1968). Friend erythroleukemia cells (FLC) resemble bone marrow proerythroblasts (Friend et al., 1966). A low percentage of the population undergo spontaneous differentiation and pro-

duce enough hemoglobin to stain positively with benzidine (Friend et al., 1971). When FLC are grown in the presence of 1-2% Me_2SO, they are induced to terminally differentiate into cells resembling erythrocytes. A number of changes identified with normal erythropoiesis are undergone by FL cells exposed to Me_2SO (Harrison, 1976). Erythrocyte membrane antigens appear (Ibawa et al., 1973), heme synthesis and iron uptake increase (Friend et al., 1974), delta-amino levulinic acid synthetase activity increases (Ebert and Ikawa, 1974), globin mRNA accumulates (Ross et al., 1972; Orkin et al., 1975; Conkie et al., 1974) and hemoglobin synthesis is induced, with 60-95% of the cells becoming benzidine positive after 5-7 days (Friend et al., 1971).

DNA hybridization studies have shown that induction of hemoglobin synthesis in FL cells is not the result of gene amplification (Bishop et al., 1972; Packman et al., 1972; Harrison et al., 1974). However, this finding did not exclude the possibility that the accumulation of globin in differentiating FL cells was due to a more efficient processing of globin mRNA. There is some evidence to suggest that induced globin synthesis is associated with an increased transcription of globin genes. Sherton and Kabat (1976) have demonstrated that there is a progressive decrease in total cellular RNA in differentiating FL cells by 24 hours. Nudel et al., (1977) detect an increase in the accumulation of globin mRNA within 6-24 hours of culture. Marks and Rifkind (1978) have interpreted these findings as indicating that upon induction of

FL cell differentiation, there is a transcriptional activation of globin sequences in these cells. However, the authors have noted that these findings do not exclude the possibility that the accumulation of globin mRNA in differentiating FL cells may be due to a stabilization of existing molecules of globin mRNA.

Studies have indicated that FL cell differentiation is associated with changes in chromatin structure. Chromatin prepared from undifferentiated and differentiating FL cells differ with respect to acridine orange binding and thermal denaturation of DNA (Darzynkiewcz et al., 1976). A 25,000 Dalton protein (Peterson and McConkey, 1976; Keppel et al., 1977) and a 65,000 Dalton protein (Neumann et al., 1978), which are not present in chromatin prepared from untreated FL cells, have been found to be associated with the chromatin prepared from Me_2SO-treated FL cells. It is unknown whether the 25,000 Dalton protein described by Peterson and McConkey and by Keppel and coworkers is the same protein. It has been shown that histone H2A appears to influence the responsiveness of FL cells to inducers (Blankenstein and Levy, 1976). Butyrate, a potent inducer of FL cell differentiation, alters the degree of acetylation of histone H4 while Me_2SO does not (Riggs et al., 1977).

Chromatin prepared from undifferentiated and differentiating FL cells has been fractionated using the DNase II/Mg^{++}-solubility method. It appears that the extent of enrichment of globin sequences within the transcriptionally-active sub-

fraction depends upon the clone being studied. Gottesfeld and Partington (1977) report an increase of globin sequences in the template-active, Mg^{++}-soluble chromatin fraction prepared from differentiating FL cells. Wallace et al., (1977), have shown an enrichment of globin genes in the template-active chromatin fractions prepared from undifferentiated and differentiating FL cells. Lau et al., (1978) were unable to detect major differences in the distribution of globin sequences between the template-active and template-inactive chromatin fractions.

RATIONALE

The biological significance of DNA methylation in eukaryotes is unknown. Several investigators have postulated that DNA methylation may play a role in gene regulation during cellular differentiation. By using Friend erythroleukemia cells as a model system for studying cellular differentiation, a correlation has been established between the hypomethylation of nuclear DNA and the expression of a differentiated function. Studies were undertaken to determine whether sites in the DNA of differentiating FL cells are in some way unique. The results of such studies have shown that the distribution of unmethylated sites between highly-repetitive, middle-repetitive, and unique sequence DNA is the same whether DNA is isolated from undifferentiated or differentiating FL cells. In addition, when the distribution of 5-methylcytosine between satellite and main band DNA were compared in these cells, it was shown that the distribution of sites which are methylated *in vivo* are identical. The distribution of 5-methylcytosine residues in pyrimidine isostichs,isolated from undifferentiated and differentiating FL cells,was also shown to be the same (Christman, 1978).

These findings indicate that the distribution of sites does not change during cellular differentiation. The differences in 5-methylcytosine which are observed during cellular differentiation are probably due to a modulation in the activity of a single DNA methyltransferase,rather than an alteration in the site specificity of DNA methyltransferases.

To understand the factors which regulate DNA methyltransferase activity during cellular differentiation, my aim was to examine FL cell DNA methylation at the chromatin level. The purpose of these experiments was to determine the cause of DNA hypomethylation in the FL cells. In addition, it was of interest to localize the undermethylated sites in chromatin. Chromatin fractionation techniques were used to ascertain whether these sites are clustered in template-active or template-inactive DNA. These experiments were not only undertaken to gain an additional understanding of DNA methylation, but also of the mechanisms which may regulate cellular differentiation.

MATERIALS AND METHODS

I. Tissue Culture Media

Minimal Earle's medium was prepared by reconstituting dehydrated powder (Grand Island Biological Company, F15) with glass-distilled H_2O. Liquid media was then filter sterilized using a Millipore filtering apparatus. Media was distributed into aliquots into sterile 500 ml bottles which were stored at 4°C until used. Media was supplemented with 10% fetal bovine serum, 250 units/ml penicillin and 0.2 mg/ml streptomycin (Grand Island Biological Company).

II. Culture Conditions

Strain 745A, a murine erythroleukemia cell line, provided by Dr. Charlotte Friend, was the only clone used for these experiments. The cell line was maintained in minimal Earle's essential medium(see above) at 37°C in borosilicate glass roller bottles (670 cm^2 growth area, Bellco) which were rotated on a Bellco roller apparatus. Inducing agents were added to cultures which had been seeded at a density of 1 x 10^5 cells/ml. Certified reagent grade Me_2SO (MCB) was added directly to the medium. All other solutions were prepared and filter-sterilized before use. The percentage of hemoglobin-containing cells was determined by the benzidine reaction described by Orkin et al., (1975). All determinations were done in triplicate, examining at least 100 cells.

III. Materials

Unless otherwise indicated, all chemicals were purchased from Fisher Scientific Company. S-adenosyl-L-(methyl ^3H) meth-

ionine purchased from either ICN Corporation or Amersham/ Searle. During the course of these experiments, batches of S-adenosyl-L-[methyl^{3}H]methionine with an increase in specific activity became available for use. The S-adenosyl[methyl^{3}H] methionine used in early experiments had a specific activity of 2.2 Ci/mmole, whereas the S-adenosyl[methyl ^{3}H] used in later experiments had a specific activity between 11 and 16 Ci/mmole. The specific activity of the radiolabel used for each experiment is given in the text.

IV. Isolation of Nuclei

A. Method A

Cells grown to a density of 1 x 10^6/ml were harvested for nuclear isolation. Cell viability, as determined by Trypan Blue exclusion, was always greater than 90%. Cells were collected by centrifugation at 2000 rpm for 10 minutes at 4°C. Cells were washed once with cold saline, gently resuspended in reticulocyte standard buffer (RSB: 10 mM Tris-Cl, pH 7.4; 3 mM $MgCl_2$, 10 mM NaCl) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF) by pipetting up and down several times, and collected by centrifugation at 1800 rpm for 10 minutes at 4°C. This procedure was repeated once. Following this series of washing, cells were lysed by adding 40 ml of lysis buffer (0.5% NP-40 (Fluka) in RSB containing 0.1 mM PMSF) per 1 x 10^9 cells, and then pipetting the cell suspension up and down 10 times. Disrupted cells were kept on ice for 5 minutes, and then again resuspended by pipetting. The presence of intact, non-aggregated nuclei was determined by examining an aliquot of cell lysate under the phase contrast microscope.

B. Method B

Cells were collected by centrifugation at 1000 rpm for 10 minutes in a refrigerated IEC centrifuge at 4°C, and washed once with cold saline. Cells were lysed by the addition of 5.1 ml of lysis buffer (10 mM Tris HCl, pH 7.8, .32M sucrose, 3 mM $MgCl_2$, and 0.3% Triton X-100) per 1 x 10^9 cells, and then resuspended by pipetting. Cell lysates were maintained on ice for 5 minutes, and then aliquots were examined under a phase-contrast microscope for the presence of intact, non-aggregated nuclei.

V. Chromatin Isolation

A. Method A

Chromatin was prepared according to the method of Gottesfeld and Partington (1977). Nuclei, isolated as described above, were recovered by centrifugation at 1800 rpm for 5 minutes in an IEC refrigerated centrifuge at 2°C. The supernatant was gently removed, and the nuclear pellet washed by resuspending in 20 ml of Hewish and Burgoyne's (1973) Buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM Tris-Cl, pH 7.5) adjusted to 0.25 M sucrose (Schwarz Mann Ultra Pure). Nuclei were washed an additional time in Buffer A, resuspended in 0.25 mM EDTA, pH 8.0, and lysed with 10-15 strokes of a loose-fitting ball type Dounce homogenizer. Chromatin preparations had a minimal amount of nuclear contamination as judged by phase-contrast microscopy.

B. Method B

Chromatin was prepared according to the method of Huang and Huang (1969). Nuclei, isolated as described above (Method B), were recovered by centrifugation at 1500 rpm for 5 minutes at 4°C. Nuclei were resuspended in 1.0 ml of buffer (.075 M NaCl, .025 M EDTA, pH 8.0) per 1 x 10^9 cells, and lysed with 50 strokes of a loose-fitting homogenizer. The nuclear lysate was centrifuged at 8000 rpm for 10 minutes at 4°C in a Sorvall RC2 Superspeed centrifuge equipped with an SS-34 rotor. The pellet was resuspended in 5 volumes of .05 M Tris, pH 8.0, and homogenized as described above using 300 strokes. The homogenate was transferred to a glass centrifuge tube, and centrifuged as described. The pellet was resuspended in 5 volumes of .01 M Tris, pH 8.0, homogenized, and then centrifuged at 8000 rpm for 20 minutes. The supernatant was carefully removed and discarded. The gelatinous pellet was resuspended in 4 mM NH_4OH, and then dispersed by slow stirring in 10 volumes of 4mM NH_4OH at 4°C.

VI. Chromatin Fractionation

A. DNase II Digestion/Magnesium Precipitation Method.

Chromatin was prepared as described in Method A and fractionated according to the method of Gottesfeld and Butler (1977). Chromatin in 0.25 mM EDTA, pH 8.0 was adjusted to a concentration of 10 A_{260nm} units/ml. The zwitterionic buffer (2[N-Morpholino]ethane sulfonic acid (MES), was added to chromatin to a final concentration of 10 mM. The pH of the solution was measured using a pH meter (Fisher), and then adjusted to pH 6.6 using dilute HCl. The solution was warmed

to 24°C, and 5 enzyme units of DNase II (Worthington, HDAC) was added for each A_{260nm} unit of chromatin. Solutions of DNase II were prepared by diluting 20,000 units of enzyme in 1.0 ml of glass-distilled H_2O, and stored at -20°C until used. The chromatin solution was incubated with DNase II for 5-20 minutes at 24°C. After incubation, the digestion mixture was adjusted to pH 7.5 using 100 mM Tris, pH 9.0. At this pH, DNase II is inactive (Gottesfeld and Butler, 1977). The digestion mixture was then centrifuged at 6,000 rpm for 5 minutes at 4°C in a Sorvall RC5 centrifuge equipped with an SS-34 rotor. The pellet (P1) contains the undigested chromatin. The supernatant (S1) was adjusted to 2 mM $MgCl_2$, using a stock solution of millipore-filtered 100 mM $MgCl_2$, and then incubated at 0°C for 30 minutes. Following precipitation with Mg^{++}, the S1 fraction was centrifuged at 12,000 rpm for 10 minutes at 4°C in a Sorvall RC5 centrifuge. The supernatant (S2) and pellet (P2) fractions are enriched in template-active and template-inactive DNA, respectively.

B. Fractionation of Chromatin by Column Chromatography on Biogel A5m.

Nucleosomes were isolated from solubilized chromatin by column chromatography on Biogel A5m according to the method of Goodwin et al., (1977). Biogel beads (100-200 mesh) were preswollen in buffer (10 mM Tris, pH 8.0; .7 mM Na EDTA, pH 7.5; 0.5 mM dithiothreitol. and 0.1 mM PMSF). The slurry was degassed before use. A jacketed Pharmacia column (2.4 x 45 cm) was used. The jacket temperature was maintained at 3°C by a

solution of polyethylene glycol which was cooled and pumped using a Buchler refrigerated fraction collector. Undifferentiated FL cell chromatin was digested with DNase II as described. An S1 fraction was prepared, loaded on the column, and eluted with the buffer described above. The elution profile of the column effluant was monitored using an ultraviolet detector equipped with a chart recorder (ISCO UA2 or UA5). The A_{260nm} of the fractions containing nucleosomal and nonnucleosomal material were determined using 1.0 cm quartz cuvettes in a Beckmann spectrophotometer. The DNA methyltransferase activity of each of the fractions was determined as described below.

C. DNase I Method

Nuclei or chromatin were fractionated with DNase I according to the method of Weintraub and Groudine (1976). Chromatin in 0.25 mM EDTA, pH 8.0 was adjusted to 10 mM Tris, pH 7.4, 10 mM NaCl, 4 mM $MgCl_2$. Nuclei were prepared in a similar buffer (RSB consists of 10 mM Tris, pH 7.4; 10 mM NaCl, 3 mM $MgCl_2$). The nuclear suspension and chromatin solution were adjusted to a concentration of 10 A_{260nm} units/ml with RSB, warmed to 37°C, and 17 enzyme units of DNase I (Worthington) were added for each A_{260nm} unit of material in the digestion mixture. Solutions of DNase I were prepared by diluting 28,000 units of enzyme in 1.0 ml of glass-distilled H_2O, and stored at -20°C until used. Nuclei and chromatin were treated with DNase I for 10 minutes at 37°C. The digestion mixture was chilled at 0°C, and then centrifuged at 7000 rpm for 5

minutes at 4°C in a Sorvall RC5 centrifuge with an SS-34 rotor. The supernatant and pellet fractions were assayed immediately in buffer with a sufficient concentration of EDTA to inhibit any contaminating DNase I.

VII. Preparation of Nuclear DNA Methyltransferases.

Nuclei, isolated as described above (Method B), were recovered by centrifugation at 1500 rpm for 3 minutes at 4°C. The supernatant was discarded, and the nuclear pellet was resuspended in lysis buffer by gentle pipetting. The nuclear suspension was centrifuged as described, and the pellet then resuspended in 0.5 ml of buffer (.32 M sucrose, 2 mM $MgCl_2$, 1 mM KPO_4, pH 6.8). Nuclei were then transferred to freezing ampules (NUNC Denmark), and disrupted by 3 repetitions of freezing in a dry ice-acetone mixture and thawing in H_2O at 0°C. Ampules were immersed in dry ice-acetone for 30 seconds, and then placed in an ice bath until the nuclear suspension had thawed. The nuclear lysate was adjusted to 0.35 M NaCl (Fluka Ultra Pure), and then kept on ice for 10 minutes, Using a 1.0 ml plastic pipet, the lysate was resuspended several times, and then transferred to plastic centrifuge tubes (Beckmann Spinco). Salt-extracted nuclear lysates were centrifuged at 47,000 rpm for 4 hours at 4°C using a 75Ti rotor (Beckmann Spinco).

The supernatant was carefully removed and transferred to dialysis tubing which had been rinsed, and then stored in glass-distilled H_2O for 1 hour prior to use. The supernatant was dialyzed against buffer (20 mM Tris-HCl, pH 7.5; 20 mM NaCl, 0.5 mM dithiothreitol) for 4 hours at 4°C. The dialyzed salt-extract was used as a source of DNA methyltransferases.

VIII. Assay Method for DNA Methylation

The assays of DNA methylation were performed according to the method of Roy and Weissbach (1975). Reaction mixtures contained 100 mM Imidazole buffer, 0.5 mM dithiothreitol, 20 mM EDTA adjusted by pH 7.5 with HCl, 10 µCi of S-adenosyl-L-(methyl ^{3}H)methionine (specific activity varied, see above), 0.2-0.4 A_{260nm} units of chromatin or 0.2 A_{260nm} units of DNA, and 25 µg of DNA methyltransferases. The final volume of the reaction mixture was 200 µl. The mixtures were incubated at 37°C for 30 minutes. Proteinase K (Beckmann) was then added to a final concentration of 400 µg/ml, and the mixtures were incubated for an additional 20 minutes at 37°C. The incorporation of methyl groups into DNA was determined by two methods which are described below.

A. Method A

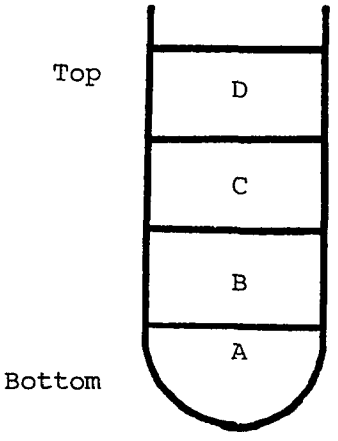
Following incubation with Proteinase K, RNA in the mixture was hydrolyzed in alkali by adding 1.0 ml of 0.5 NaOH, and then incubating at 65°C for 10 minutes. The mixture was then chilled on ice, and an equal volume of cold 20% trichloroacetic acid (TCA) was added. The acid precipitate was collected on Whatman GF/A 2.4 cm glass fiber filters which had been presoaked in 5% TCA, washed twice with 10 ml of cold 5% TCA, and then placed under a heat lamp until completely dry. Dried filters were placed in borosilicate glass scintillation vials to which 10 ml of OCS (Amersham/Searle) scintillation fluor was added. Radioactivity was determined in a Nuclear Chicago liquid scintillation counter. Method A was used for routine assays.

B. Method B

Following treatment with Proteinase K, the mixture was adjusted to 0.6% Sarcosyl (NL Laboratories). The methylated DNA was isolated from protein and RNA by centrifugation on a $CsCl/Cs_2SO_4$ isopycnic density gradient according to the method of Shaw and Mueller (1975). This method is presented below.

1. Preparation of the gradient

The $CsCl/Cs_2SO_4$ density gradient used in these experiments in discontinuous, consisting of four layers A, B, C and D, with densities of 1.82, 1.72, 1.5 and 1.45, respectively. The composition of a gradient is shown below



COMPOSITION OF THE LAYERS

	CsCl Stock Added (µl)	Cs_2SO_4 Stock Added (µl)	H_2O Added (µl)	Total Volume
D	278	—	722	1.0 ml
C	556	—	444	1.0 ml
B	806	—	194	1.0 ml
A	820	110	70	1.0 ml

The stock solution of ultra-pure CsCl (Beckmann) was prepared by thoroughly mixing 49.6 grams of CsCl with 26.6 ml of glass-distilled H_2O. The stock solution was diluted with H_2O to prepare the four layers described above. A saturated solution of Cs_2SO_4 was prepared, millipore-filtered, and then

diluted with glass-distilled H_2O to a final density of 1.84. Densities were determined by measuring the refractive indices of each solution, and then referring to a standard plot of refractive index versus density for known concentrations of CsCl and Cs_2SO_4.

The gradient has a volume of 4.0 ml. To construct the gradient, 1.0 ml of solution A is pipetted into a 5 ml polyallomer tube (Beckmann Spinco). Next, 1.0 ml of solution B was hand-layered onto the first layer. This process was repeated using 1.0 ml of solutions C and D.

Following <u>in vitro</u> methylation and deproteinization, the reaction mixture was adjusted to 0.6% Sarcosyl (NL Laboratories). The mixture was increased to a final volume of 400 µl, and then hand-layered on top of the gradient.

Gradients were centrifuged at 30,000 rpm for 15-18 hours at 26°C using either an SW 50.1 or SW 56 rotor (Beckmann Spinco). A model L3-50 or L5-75 ultracentrifuge (Beckmann Spinco) was used for gradient centrifugation.

Following centrifugation, gradients were fractionated from the top using a pump (Auto Densi-Flow II C, Searle) which was attached to a timer so as to deliver 0.5 ml fractions. An equal volume (0.5 ml) of glass-distilled H_2O was added to each fraction, which was then vortexed intermittently. The $A_{260:280}$ of each fraction was measured using 1.0 ml quartz cuvettes in a Zeiss spectrophotometer. The DNA, which sediments to a position in the middle of the gradient (fractions 5-7), is detected as an increase in absorbance at 260_{nm}. Fractions were preci-

pitated with 1.0 ml of cold 20% TCA. The acid precipitate was collected on Whatman GC/A 2.4 cm glass fiber filters which had been pre-soaked in 5% TCA, washed twice with 10 ml of cold 5% TCA, and then placed under a heat lamp until completely dry. Dried filters were placed in borosilicate glass scintillation vials to which 10 ml of OCS (Amersham/Searle) scintillation fluor was added. Radioactivity was determined in a Nuclear Chicago liquid scintillation counter.

IX. Isolation and Purification of FL Cell DNA

A. Purification of DNA from Ethionine-Treated Cells for Use as Substrate for Chromatin-Associated DNA Methyltransferases

FL cells were suspended at a density of 5×10^4 cells/ml in minimal essential media adjusted to 10% fetal bovine serum, 4 mM L-ethionine (Sigma Chemical Co.) and maintained at 37°C in roller bottles for 120 hours. Cell cultures grew to a density of 1.86×10^6 cells/ml. Greater than 95% of the cells were viable as determined by the trypan blue exclusion method, and 23% of the cell population were benzidine positive. Nuclei were isolated from these cells according to the procedure described above (Method A). The nuclear pellet was suspended in 5.0 ml of RSB containing 0.1 mM PMSF, and stored at -20°C for 17 days. Frozen nuclei were warmed to 23°C, and then adjusted to a final concentration of 10 mM Tris-Cl, pH 7.4; 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4; and 100 mM NaCl. A solution of 20% sodium dodecyl sulfate (SDS) was added to adjust the nuclear suspension to a final concentra-

tion of 0.5% SDS. When SDS is added, most of the nuclei are lysed. The suspension is vortexed intermittently for 5 minutes to ensure complete nuclear lysis and to solubilize the nuclear lysate. Proteinase K was then added to a final concentration of 0.1 mg/ml to the nuclear lysate, which was transferred to a 37°C water bath and incubated for 3 hours.

To extract the digested protein, an equal volume of Tris buffer-saturated phenol-cresol-chloroform was added to the nuclear lysate. The lysate-phenol mixture was vortexed intermittently for 3 minutes. The aqueous and phenol phases were then separated by centrifugation at 10,000 rpm for 10 minutes at 25°C using a Sorvall RC2 superspeed centrifuge equipped with an Hb-4 rotor. The aqueous phase was carefully separated from the phenol phase, and then transferred to a clean centrifuge tube to which an equal volume of the phenol-cresol-chloroform solution was added for re-extraction. This process was repeated until the interphase between the aqueous phase and phenol phase was free of protein. The aqueous layer was then combined with 2 volumes of cold 95% ethanol. Nucleic acids were recovered from the aqueous phase by spooling on a glass rod. The spooled material was dried in vacuo and then redissolved in .01 x SSC (1.5 mM sodium chloride, .15 mM sodium citrate, pH 6.8). The solution was then incubated with ribonucleases for 30 minutes at 37°C. (Final concentrations: 0.1 mg/ml pancreatic; 20 units/ml T1 in 1 x SSC, boiled for 10 minutes. Enzymes purchased from Sigma Chemical Company). Following incubation with ribonucleases, the DNA solution was

incubated with 0.1 mg/ml pronase (Calbiochem Corporation) for 30 minutes at 37°C.

After treatment with ribonuclease and pronase, the solution was re-extracted with chloroform:isoamyl alcohol (24:1) as described above. Extractions were repeated until there was no visible protein precipitate at the aqueous:chloroform interphase. The aqueous phase was carefully removed, and combined with 2 volumes of cold 95% ethanol to precipitate DNA in the solution. The precipitated DNA was spooled on a glass rod, dried in vacuo and redissolved in 0.01 x SSC (1.5 mM sodium citrate, pH 6.8) at a concentration of 9.55 A_{260nm} units/ml.

B. Isolation and Purification of DNA from Chromatin Fractions Prepared Using the DNase II Digestion/ Magnesium Precipitation Chromatin Fractionation Procedure.

Chromatin was prepared and fractionated as described above. DNA was isolated from chromatin according to the method of Gottesfeld and Partington (1977). Each fraction was adjusted to a final concentration of 150 mM NaCl, 100 mM EDTA, 50 mM Tris-Cl (pH 7.5), 0.5% SDS, and then incubated with Proteinase K (Beckmann) at a concentration of 100 µg/ml for 3 hours at 37°C. Following treatment with Proteinase K, solutions were transferred to a glass round-bottom flask to which an equal volume of chloroform:isoamyl alcohol (24:1) was added. Flasks were then attached to a mechanical shaker (New Brunswick Scientific), and shaken for 30 minutes at 24°C. Following

extraction, the solution was transferred to glass centrifuge tubes. The aqueous and chloroform phases were then separated by centrifugation at 10,000 rpm for 10 minutes at 25°C using a Sorvall RC5 centrifuge equipped with an SS-34 rotor. The chloroform phase was re-extracted with buffer. The aqueous phases were combined with 2 volumes of cold 100% ethanol to precipitate DNA in the solution. The precipitates were collected by centrifugation at 10,000 rpm for 45 minutes at 4°C in a Sorvall RC5 centrifuge equipped with an SS-34 rotor. The supernatant was carefully removed and discarded. The precipitates were dried in vacuo, and dissolved in buffer containing 150 mM NaCl, 100 mM EDTA, 50 mM Tris-Cl (pH 7.5). Ribonucleases were added, and the solution was incubated for 1 hour. (Final concentration: 1.0 mg/ml pancreatic; 20 units/ml T1 in 1 x SSC, boiled 10 minutes. Enzymes were purchased from Sigma Chemical Company).

Following ribonuclease treatment, the solution was incubated for 2 hours at 37°C with SDS and Proteinase K at a final concentration of 0.5% and 40 µg/ml respectively, and then extracted with chloroform:isoamyl alcohol (24:1) as described. The aqueous layers were combined and precipitated with 2 volumes of 100% ethanol. The precipitates were collected by centrifugation. The supernatants were discarded, the precipitates were dried in vacuo, and dissolved in .1 x SSC (15 mM sodium chloride, 1.5 mM sodium citrate, pH 6.8).

RESULTS:

I. Methylation of Chromatin.

A. Self-Methylation: Endogenous DNA is Methylated by Chromatin-Associated DNA Methyltransferases In Vitro.

DNA methyltransferase is tightly bound to chromatin and can be found in association with the DNA (Roy and Weissbach, 1975). It has been shown in a variety of systems that the enzyme is released from DNA using a salt concentration high enough to dissociate non-histone proteins (Roy and Weissbach, 1975; Turnbull and Adams, 1976; Simon et al., 1978). Friend erythroleukemia cell DNA methyltransferase prepared in this manner is capable of methylating purified FL cell DNA in vitro (Christman et al., 1978). DNA purified from FL cells induced to differentiate following growth in dimethylsulfoxide or ethionine accepts more methyl groups per A_{260nm} units in vitro from an FL cell DNA methyltransferase than the DNA purified from untreated cells, presumably because it is incompletely methylated in vivo.

Although there are a prodigious number of changes accompanying cellular differentiation which could affect DNA methylation, I concentrated on those mechanisms which might result in a failure of induced cell DNA to be methylated in vivo: (1) Alteration in DNA methyltransferase activity. This could result from either a lowered level of DNA methyltransferase or a change in the site specificity of the enzyme. (2) Reduced levels of S-adenosylmethionine. As a consequence of either impaired synthesis or acceleration of breakdown,

less methyl donor is available for methyl transfer reactions. (3) Accumulations of a specific inhibitor. (4) A change in the amount or distribution of chromatin associated DNA methyltransferases. (5) Variation in chromatin conformation such that sites which were available to DNA methyltransferase in the undifferentiated cell chromatin are rendered inaccessible to DNA methyltransferase in the differentiating cell chromatin.

Experiments in progress in our laboratory made possibilities 1-3 appear unlikely since (1) it could be shown that there was little detectable difference in the level and specificity of DNA methyltransferases extracted from whole nuclei. (2) The levels of S-adenosylmethionine in differentiating cells were sufficient for normal methylation of tRNA and histones. (3) Mixing experiments with nuclear extracts did not reveal any inhibitory activity. However, DNA methylation at the chromatin level had not been investigated. Consequently, it was of interest to determine (1) whether DNA methyltransferase activity could be demonstrated in a preparation of isolated chromatin and whether this enzyme would be capable of methylating endogenous DNA <u>in vitro</u> and (2) if a difference in the enzyme activity could be detected between chromatin isolated from undifferentiated and differentiated cells.

Chromatin isolated by either of two methods((1) Gottesfeld and Partington, 1977; (2) Huang and Huang, 1969) was incubated with buffer and tritium-labeled S-adenosylmethionine as described in Methods. The reaction was terminated by incuba-

ting the reaction mixture with Proteinase K, RNA was hydrolyzed with alkali to nucleotides, and the incorporation of radioactive methyl into DNA was measured by precipitating the DNA with trichloracetic acid (TCA) and counting it. This procedure is referred to as Method A. Data are presented in Table 1.

Although significant incorporation of radiolabeled methyl groups into TCA precipitable material was observed utilizing Method A, the following experimental procedure was followed to exclude the possibility that incorporation was due to contamination by incompletely digested labeled protein. After in vitro methylation, chromatin was treated with Proteinase K and detergent, and then subjected to $CsCl/Cs_2SO_4$ isopycnic density gradient centrifugation. This technique separates DNA, RNA and protein on the basis of differences in their densities. Gradients were fractionated, and the optical densities of each fraction determined at 260 and 280 nm. The DNA, which sediments to a position in the middle of the gradient, is detected by an increase in absorbance at 260 nm. Methylation is measured as incorporation of radiolabeled methyl into these TCA precipitable materials. This method is referred to as Method B. Fig. 3 shows a schematic representation of Method B and data obtained using Method B for examining chromatin methylation from undifferentiated FL cells are presented in Fig. 4. It is clear that by using this method, I have removed some non-DNA material which is methylated in vitro. However, the results obtained

TABLE 1.

METHYLATION OF CHROMATIN DNA

BY ENDOGENOUS DNA METHYLTRANSFERASE

METHOD A

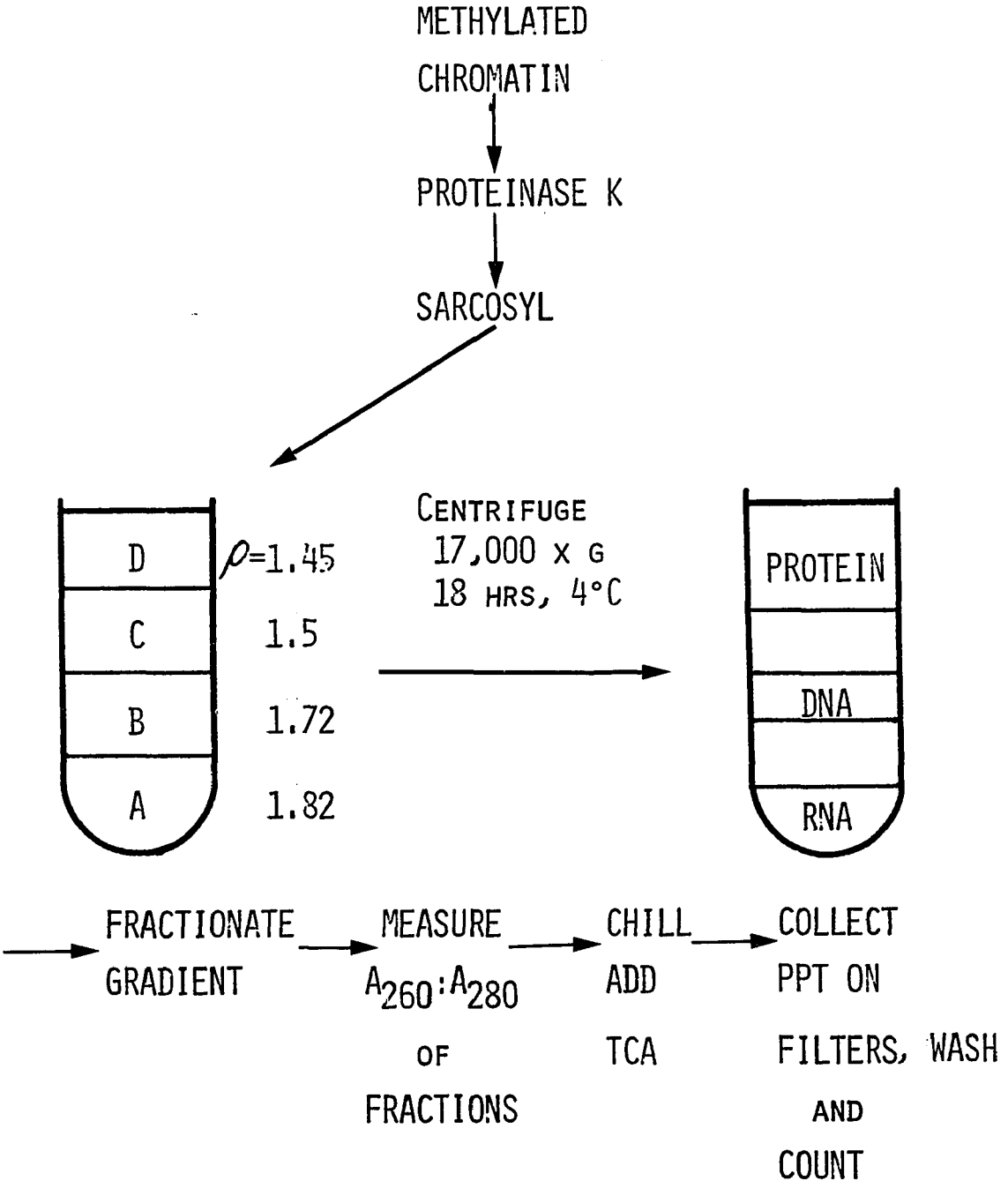
Treatment	CPM/A_{260nm} (Input)
Untreated	2103
Me_2SO (240mM)	2259

Legend to Table 1.

Chromatin was prepared according to Huang and Huang (1968). Chromatin (1.0-1.5 A_{260nm} units) was incubated with buffer and S-adenosyl-L-[methyl-^{3}H] methionine (2.2 Ci/mmole) and incorporation of [^{3}H]-methyl determined as described in Methods and the text for chromatin DNA methylation, Method A. Data are an average of results obtained from three different preparations of chromatin prepared from untreated and dimethyl-sulfoxide-treated FL cells.

FIGURE 3.

SCHEMATIC REPRESENTATION OF METHOD B
FOR MEASURING CHROMATIN DNA METHYLATION
BY ENDOGENOUS DNA METHYLTRANSFERASES

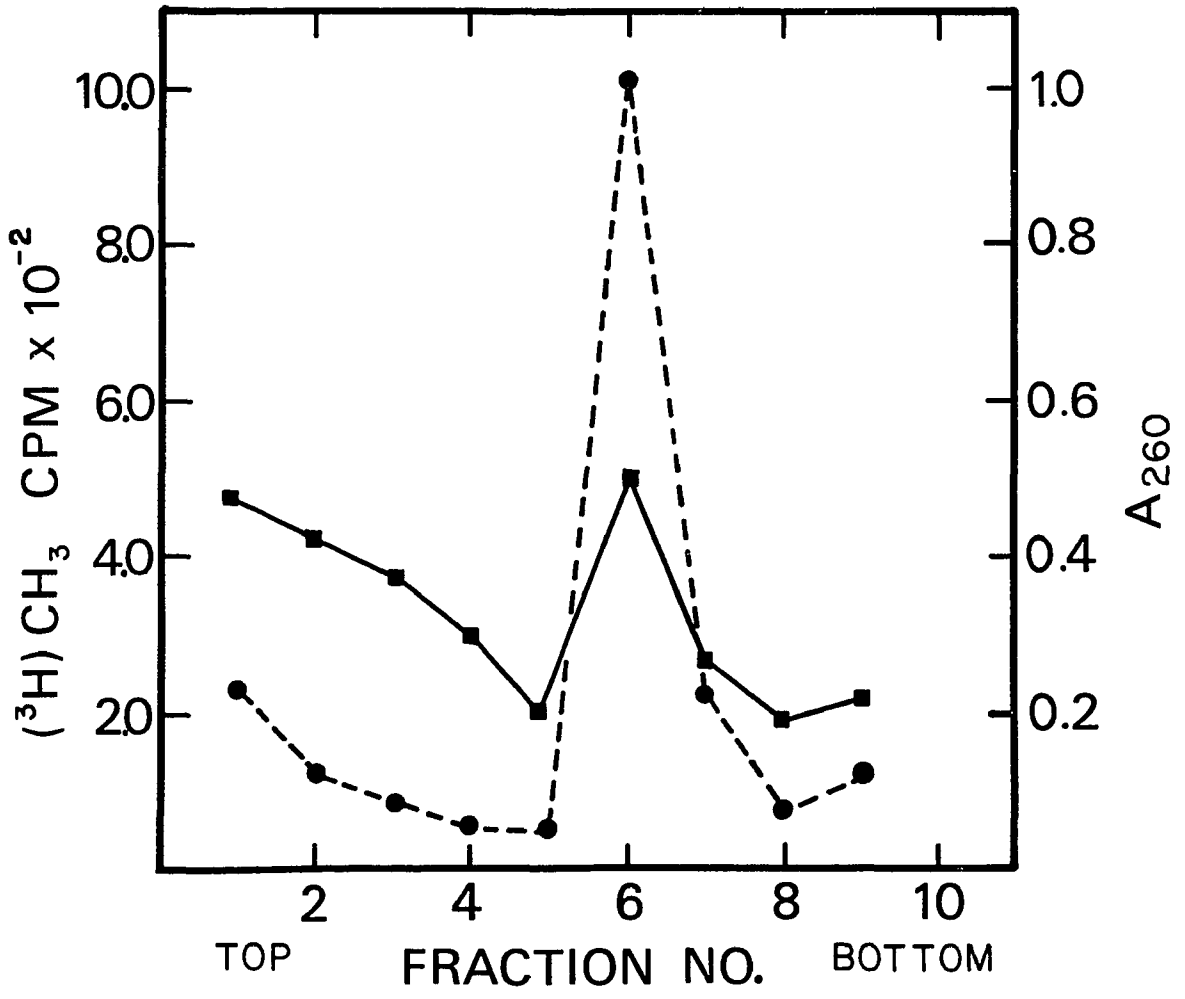


Legend to Figure 3.

Chromatin was prepared from undifferentiated FL cells according to the method of Huang and Huang (1968). Chromatin was methylated as described in Methods. Approximately 1.0 A_{260} unit of methylated chromatin was incubated with Proteinase K (400ug/ml) for 30', adjusted to 0.6% Sarcosyl and then centrifuged as described in the figure. Gradients were fractionated from the top into 0.5 ml fractions using an Amersham DensiFlow pump. An equal volume of glass-distilled water was added to each fraction, which was then vortexed and the A_{260} : A_{280} measured. Fractions were then precipitated with 1.0 ml of cold 20% TCA. Precipitates were collected on glass-fiber filters (Whatman) which were then washed with cold 5% TCA and dried under a heat lamp. Dried filters were transferred to glass scintillation vials, non-aqueous scintillation fluor was added (10ml) to each of the vials, and radioactivity determined in an ISOCAP 500 scintillation counter (Nuclear Chicago).

FIGURE 4.

METHYLATION OF CHROMATIN DNA
BY ENDOGENOUS DNA METHYLTRANSFERASES
METHOD B



Legend to Figure 4.

Data were obtained as described in Fig. 3 and Legend to Fig. 3. The incorporation of ^{3}H-methyl into TCA precipitable material (————) and the A_{260} (--------) are shown for each fraction. Radioactivity determinations are as described in the Legend to Fig. 1. Optical density measurements were made with 1 cm light path quartz cuvettes using a Beckmann spectrophotometer.

Although the data presented in Fig. 4 were obtained by using chromatin from undifferentiated FL cells, similar results were obtained when chromatin from differentiating cells were used (data not shown).

from the two procedures (Table 2) are comparable. With both methods, the incorporation of radiolabelled methyl/A_{260} is slightly lower for the chromatin prepared from differentiating FL cells than from undifferentiated cells. The data in Table 2 represent the results of a single experiment. Other preparations show virtually the same degree of incorporation using Method A. As an example, compare the chromatin methylation (Column 3) in Table 4.

To ensure that the acid precipitable material in fractions 5-7 (Fig. 4) was DNA, the fractions were pooled and dialyzed against .1xSSC (15mM sodium chloride, 1.5mM sodium citrate) overnight. Aliquots were then assayed for sensitivity to DNase, RNase and Proteinase K. The data presented in Table 3 show that the material in these fractions is sensitive to DNase, but not susceptible to digestion by either ribonuclease or Proteinase K.

These findings indicate that the $CsCl/Cs_2SO_4$ density gradient method shown in Fig. 3 is an effective means of separating DNA from contaminating protein and RNA. By using this procedure, it was shown that the amount of methylation in the two chromatin preparations is low and approximately equal, so that the hypomethylation of differentiated cell DNA is not detectable in intact chromatin.

The data presented in Table 2 show that 1.1-1.2 pmole of methyl are transferred to 1 A_{260nm} unit of endogenous chromatin DNA by bound enzyme *in vitro*. This degree of methylation is considerably less than the *in vitro* methyla-

TABLE 2.

A COMPARISON OF THE METHODS USED TO MEASURE METHYLATION OF CHROMATIN DNA BY ENDOGENOUS DNA METHYLTRANSFERASES

Cell Treatment Prior to Chromatin Isolation	Method A	Method B		
	CPM/A_{260nm} Input DNA	Fract. 5-7	CPM/A_{260}	pMoles/A_{260nm} units of DNA recovered
None	2210		1414	1.2
2% (v/v) Me_2SO (96 hrs)	1738		1239	1.1

Legend to Table 2.

Chromatin was prepared according to Huang and Huang (1968). Chromatin (1.0-1.5 A_{260nm} units) was incubated with buffer and S-adenosyl-L-[methyl-^{3}H] methionine (2.2 Ci/mmole) and incorporation of [^{3}H]-methyl determined as described in Methods and the text for chromatin DNA methylation, Methods A and B.

TABLE 3.

IDENTIFICATION OF METHYLATED MATERIAL IN GRADIENT FRACTIONS 5-7 AS DNA.

Acid Precipitable CPM (Fractions 5-7)	Total CPM
No Treatment[a]	2500
+ DNase[b] (30',37°C)	30
+ RNase[c] (30',37°C)	2410
+ Proteinase K[d] (90',37°C)	2480

Legend to Table 3.

[a] The incorporation of radiolabeled methyl into the pooled, dialyzed material was measured as described in the text (Method A).

[b] Stock solutions of DNase I (Sigma) were prepared (14,000 enzyme units/500 ul glass-distilled H_2O, 2839 units/mg) and aliquots digested at a concentration of 5µg enzyme/A_{260} of material as described in the table.

[c] RNase solution was prepared at a 100 fold concentration (10mg/ml pancreatic; 2000 units T_1 in 1XSSC, boiled 10'), diluted, and added to aliquots which were digested as described in the table.

[c] Proteinase K was added to a final concentration of 0.1 mg/ml.

tion of purified DNA from either undifferentiated or differentiated FL cells by added enzyme (Christman et al.; Table 6, this text). Therefore, it was of interest to determine (1) whether the activity of chromatin-bound DNA methyltransferases are substantially lower than the activity of extracted DNA methyltransferases and (2) whether sites which are capable of being methylated by FL cell DNA methyltransferases are masked by chromatin proteins.

B. Methylation of Exogenous DNA by Chromatin

To determine the activity of chromatin-associated enzyme from Me_2SO-tereated and untreated cells, it was necessary to demonstrate that bound enzyme could methylate added or exogenous FL cell DNA. For this purpose, I prepared chromatin according to the method of Gottesfeld and Partington (1977), and incubated it with S-adenosyl-L-[methyl-^{3}H] methionine and DNA isolated from FL cells grown in L-ethionine. It is not possible to physically separate chromatin DNA from added DNA by the assay methods described above. Consequently, to measure the methylation of added DNA by chromatin-associated DNA methyltransferases, the following experiments were performed. The degree to which chromatin DNA is methylated in vitro by bound enzymes was determined by incubating chromatin with buffer and S-adenosyl-L-[methyl-^{3}H] methionine in the absence of added DNA, and then measuring the incorporation of radiolabeled methyl groups into chromatin DNA. To determine the degree to which both chromatin DNA and added DNA are methylated by bound enzymes, the incorporation of radiolabeled

TABLE 4.

METHYLATION OF ADDED FL CELL DNA BY CHROMATIN ASSOCIATED DNA METHYLTRANSFERASES

Chromatin Source	[^{3}H]Methyl Transferred		
	Chromatin + DNA	Chromatin	Δ (CPM/A_{260} Added DNA)
745A Untreated	6506	2361	4145
2% (v/v) ME_2SO (96 hours)	3033	1716	1317

Legend to Table 4.

Chromatin was prepared according to the method of Gottesfeld and Partington (1977). Reaction mixtures containing 0.2 A_{260nm} units of chromatin,or chromatin and 0.2 A_{260nm} units of DNA purified from ethionine-treated FL cells, were incubated under standard conditions as described in Methods. The incorporation of [^{3}H]-methyl groups into DNA was determined as described in Methods and the text for chromatin DNA methylation, Method A.

methyl groups into these DNAs was also determined. If the value obtained for the incorporation of radiolabeled methyl groups into chromatin DNA is subtracted from these data, then the difference is a measure of the incorporation of radiolabeled methyl into the added DNA.

These results are presented in Table 4, and indicate that the chromatin-associated FL cell DNA methyltransferases can methylate exogenous DNA. In addition, these findings suggest that the chromatin-associated enzyme of undifferentiated cells is more active on exogenous DNA than the chromatin-associated enzyme from differentiated cells. However, when I extracted DNA methyltransferase from the chromatin of undifferentiated and differentiated cells, and assayed each preparation on purified DNA, I found that the enzyme activities were approximately equal (Table 5). The finding that chromatin preparations with the same amount of bound enzyme differ in their ability to methylate added FL cell DNA may indicate that differences in the conformation of chromatin from differentiated and undifferentiated cells affect the interaction of enzyme with added DNA.

C. Chromatin DNA Methylation by an Exogenous DNA Methyltransferase.

The findings which I presented in Sections A and B show that preparations of chromatin isolated from undifferentiated and differentiated FL cells have similar amounts of chromatin bound DNA methyltransferase activity. The results also indicate that undifferentiated and differentiated cell chromatin contain a limited and equivalent number of unmethylated

TABLE 5.

ACTIVITY OF DNA METHYLTRANSFERASES PREPARED FROM CHROMATIN OF UNTREATED AND TREATED FL CELLS.[a]

Chromatin Source	[^{3}H]Methyl Transferred CPM/10^9 Cells
745A Untreated	316,860
2% (v/v) Me_2SO	355,240

Legend to Table 5.

[a]DNA methyltransferase was extracted from chromatin which was isolated from untreated and dimethylsulfoxide-treated FL cells as described in Methods. Values extrapolated from range of linear response to added enzyme protein in standard assay. The background incorporation of methyl groups with enzyme alone was subtracted from the data presented in the table.

cytosine residues which are accessible to bound DNA methyltransferase *in vitro*. To ascertain whether chromatin proteins or chromatin configuration also limit the accessibility of these sites to added enzyme, the ability of chromatin DNAs from undifferentiated and differentiated FL cells to be methylated *in vitro* by an added DNA methyltransferase was tested under two conditions. Intact chromatin, or DNA purified from the same preparation of chromatin, was incubated with a partially purified DNA methyltransferase. The data in Table 6 indicate that additional sites in the DNA of intact chromatin are methylated when DNA methyltransferase is added. However, by comparing the differences in the degree to which purified DNA and chromatin DNA were methylated, it can be seen that a large number of sites in chromatin DNA remain inaccessible, even to the added enzyme.

The findings presented in Sections A and B provided strong evidence that chromatin proteins can affect the degree to which chromatin DNA is methylated. The number of sites within the chromatin DNA of undifferentiated and differentiating FL cells which are accessible to the DNA methyltransferases is small but equal. By adding enzyme, some additional sites are methylated, but chromatin DNA is still not efficiently methylated. Hypomethylation of chromatin DNA was detected only after deproteinization. Almost three times as many unmethylated sites can be detected in purified DNA from differentiating FL cells as compared with undifferentiated cell DNA. This indicates that sites which were inaccessible to the chromatin-associated or added DNA methyltransferase in intact chromatin are methylated in the purified DNA by added enzyme.

TABLE 6.

IN VITRO METHYLATION OF FL CELL CHROMATIN DNA

BY ADDED DNA METHYLTRANSFERASES

Additions to Methylation Reactions[a]	Cell Treatment Prior to Chromatin Isolation			
	NONE		ME_2SO (96 Hours)	
	CPM/A_{260}[b]	pmoles methyl transferred/A_{260}	CPM/A_{260}[b]	pmoles methyl transferred/A_{260}
Chromatin	3,700	.46	2,720	.34
Chromatin and DNA Methyltransferase	14,890	1.86	13,010	1.62
DNA and DNA Methyltransferase	59,925	7.4	173,780	21.7

Legend to Table 6.

[a]Three reaction mixtures were prepared. All three mixtures contained 10 µCi S-adenosyl-L-(methyl-^{3}H) methionine (16 Ci/mmole) and incubation buffer (described in Methods). In addition, the first reaction mixture (line 1) contained 0.4 A_{260nm} units of chromatin; the second reaction mixture (line 2) contained 0.4 A_{260nm} units of chromatin and 25 µg of DNA methyltransferase isolated from untreated FL cells; the third reaction mixture (line 3) contained 0.2 A_{260nm} units of DNA from ethionine-treated cells and 25 µg of DNA methyltransferase isolated from untreated FL cells. The final volume of a reaction mixture was 200 µl. Conditions of incubation are described in Methods.

[b]Assay Method B was used to determine the incorporation of radiolabeled methyl groups into DNA. Data provided are for DNA recovered by CsCl density gradient centrifugation.

II. Fractionation of FL Cell Chromatin DNA:

A. The Methylation of Chromatin DNA is Correlated With Its Template Activity.

I have presented evidence that chromatin DNA in both undifferentiated and differentiating FL cells contains unmethylated cytosine residues which are not accessible to either endogenous or added DNA methyltransferases. Furthermore, I demonstrated that once chromatin proteins are removed, these sites can be methylated, and reconfirmed the observation that DNA derived from differentiating FL cells contains more unmethylated sites than DNA from undifferentiated FL cells. It was of interest to determine whether the hypomethylation of differentiating FL cell DNA is the outcome of a series of chance events,in which cytosine residues become randomly blocked by chromatin proteins and are not modified, or whether a correlation exists between the degree to which DNA is methylated and its template activity.

The enzyme DNase II (EC 3.1.4.6) cleaves high molecular weight chromatin into fragments without destroying actively transcribed DNA sequences. Sequences of chromatin DNA which are undergoing transcription can be distinguished from those which are quiescent on the basis of physical and chemical differences (Gottesfeld et al., 1976; Wallace et al., 1977). It has been shown that as a consequence of these differences,

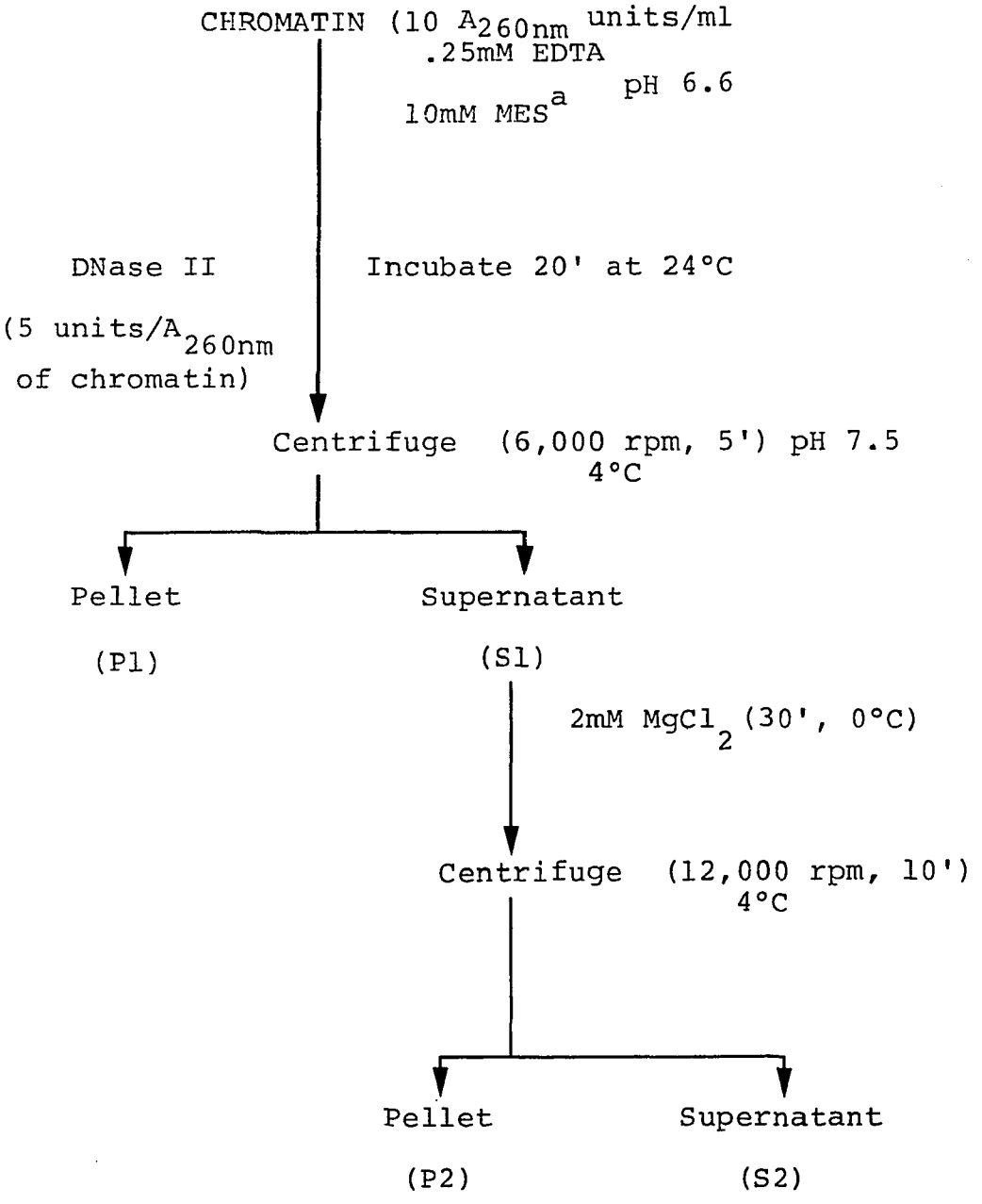
actively-transcribed sequences are soluble in 2mM Mg^{++} while non-transcribed sequences are insoluble (Gottesfeld, 1977). By taking advantage of this difference in solubility, chromatin subfractions may be prepared which are enriched in actively-transcribed or non-transcribed sequences. This protocol is presented schematically in Figure 5.

Chromatin was prepared from undifferentiated and differentiating FL cells and fractionated according to this protocol. The DNA from each fraction was purified as described by Gottesfeld and Partington (1977), and assayed for the ability to accept methyl groups in vitro. If failure to modify cytosine residues during differentiation is a random process, then the hypomethylated sites should be uniformly distributed between template-active and inactive chromatin. If hypomethylation is non-random,and associated with template activity, one might expect an enrichment of the hypomethylated sites in one of the chromatin subfractions.

Data on the methylation of FL cell DNA prepared from Mg^{++}-soluble (S2) and Mg^{++}-insoluble (P2) chromatin subfractions and total chromatin(P1) are presented in Table 7. These data show that the DNA of the Mg^{++}-soluble, template-active fraction (S2) is a relatively poor methyl acceptor in vitro. Moreover, it appears that with the exception of the Mg^{++}-soluble fraction prepared from ethionine-treated cells, the DNA from each of the Mg^{++}-soluble fractions is methylated to approximately the same extent. Ethionine is not only an inducer of cellular differentiation in Friend

FIGURE 5.

FRACTIONATION OF DNase II SOLUBILIZED CHROMATIN



Legend to Figure 5.

Chromatin was prepared according to the method of Gottesfeld and Partington (1977), and fractionated as described by Gottesfeld and Butler (1977).

The only modification of the fractionation procedure was that FL cell chromatin was digested in 10 mM MES (2[N-Morpholino]ethane sulfonic acid), pH 6.6.

TABLE 7.

IN VITRO METHYLATION OF DNA ISOLATED FROM CHROMATIN FRACTIONS

Treatment of Cells Prior to Chromatin Isolation	Chromatin fraction[a] cpm/A_{260nm}[b] Mg^{++}-sol. (S2)	Mg^{++}-insol. (P2)	Total Chromatin
none	12,963	64,310	59,930
DMSO (240mM)	14,788	157,570	167,578
HMBA (4mM)	18,920	157,740	169,185
L-ethionine (4mM)	90,189	310,137	312,230

Legend to Table 7.

[a] Chromatin was prepared according to the method of Gottesfeld and Butler (1977), and digested in the presence of 5 units of DNase II/A_{260nm} units of chromatin at 25°C for 15 minutes. Material which remained in the supernatant (S1) after centrifugation at 10,000 x g for 10 minutes was brought to 2 mM with $MgCl_2$, and recentrifuged at 27,000 x g for 20 minutes to yield Mg^{++}-soluble (S2) and Mg^{++}-insoluble (P2) fractions. DNA was isolated from total chromatin, S2 and P2 as described in Methods.

[b] Assay Method A was used to determine the incorporation of radiolabeled methyl groups into DNA. Results are given as CPM/A_{260nm} input DNA.

erythroleukemia cells (Christman et al., 1977), but in the form S-adenosylethionine is a general inhibitor of methyltransferases, causing an undermethylation of DNA, tRNA, and histones. From these data, it seems probable that unless the DNA methyltransferase is inhibited, such as when cells are grown in ethionine, virtually all of the sites within template-active chromatin DNA are modified in vivo. The data in Table 7 also shown that the degree of undermethylation of total chromatin DNA is reflected in the DNA purified from the Mg^{++}-insoluble (P2) fraction. These results indicate that cytosine residues in chromatin DNA which are unmodified in vivo are concentrated in the Mg^{++}-insoluble, template-inactive (P2) chromatin fraction.

B. DNA Methyltransferase Activity is Associated with Template-Inactive DNA.

In the previous sections, I presented evidence that regardless of the differentiated state of the cells, the DNA of the template-active chromatin fraction is almost fully methylated in vivo, and that the unmethylated sites in chromatin DNA are clustered in the template-inactive chromatin fraction. This fractionation, based on DNase II digestion, allowed me to determine whether the FL cell DNA methyltransferase is also preferentially associated with template-active or template-inactive chromatin DNA.

Chromatin was isolated from both untreated and Me_2SO-treated cells, and each of the preparations were fractionated as described. The DNA methyltransferases bound to total

chromatin, and to the S1, P1, S2, and P2 chromatin fractions were assayed for the ability to methylate endogenous and added FL cell DNA. The data obtained on the methylation of endogenous DNA and added DNA are shown in the 3rd and 4th columns, respectively, of Tables 8 and 9. If the enzymatic activity associated with the total chromatin (both Tables, line 1) is compared with the activity of each of the chromatin fractions (both Tables, lines 2-5), it can be seen that (1) after digestion, at least 70% of the DNA methyltransferase activity associated with chromatin is recovered in the Mg^{++}-insoluble, template-inactive (P2) chromatin fraction. (2) The specific activity of the enzyme associated with the Mg^{++}-insoluble, template-inactive fraction (P2) is higher than that of the other chromatin fractions. (3) The transcriptionally-active chromatin fraction (S2) has the lowest level of DNA methyltransferase activity. (4) The same distribution of chromatin-associated enzyme is found in differentiating FL cells (Table 9).

It can be seen from the data in Table 10 that after DNase II digestion, the recovered enzymatic activity was sometimes greater than 100% of the enzymatic activity in the total chromatin. It cannot be ruled out that nuclease digestion may loosen chromatin configuration, thereby allowing for a more efficient methylation of exogenous substrate. However, it is also possible that these differences represent error introduced by the extrapolation required to calculate total activity from DNA methyltransferase activity in the range of linear response to added chromatin. In

TABLE 8.

DNA METHYLTRANSFERASE ACTIVITY OF CHROMATIN FRACTIONS PREPARED BY DNASE II DIGESTION OF CHROMATIN FROM UNTREATED CELLS

Fraction[a]	[^{3}H] Methyl Transferred[b] Chromatin + DNA	Chromatin (Endogenous DNA)	Δ (CPM/Added DNA)	CPM per A_{260nm} of Input DNA
Chromatin	16,712,450	7,260,100	9,452,350	22,150
P1	2,239,200	1,291,680	947,250	16,283
S1	13,316,800	6,353,360	6,963,440	21,256
S2	342,125	148,750	193,375	4,094
P2	11,715,750	4,407,750	7,308,000	27,262

Legend to Table 8.

[a]Chromatin fractions were prepared as shown in Fig. 5.

[b]Activity was determined in incubation mixtures containing 0.3-0.6 A_{260nm} units of chromatin isolated from untreated FL cells, or 0.3-0.6 A_{260nm} units of chromatin and 0.2 A_{260nm} units of DNA which was purified from cells grown in the presence of L-ethionine. All incubation mixtures contained 10 µCi of S-adenosyl-L-(methyl-^{3}H)methionine (16 Ci/mmole) and incubation buffer (described in Methods). The total volume of the incubation mixture was 200 µl. Reactions were incubated as described in Methods. Radiolabel incorporated into DNA was determined by using assay Method A.

Note that P1 and S1 = 94% of input A_{260nm} and that P2 + S2 = 96% of S1 from which they were prepared.

TABLE 9.

DNA METHYLTRANSFERASE ACTIVITY OF CHROMATIN FRACTIONS PREPARED BY DNASE II DIGESTION OF CELL CHROMATIN FROM DIMETHYLSULFOXIDE-TREATED CELLS

Fraction[a]	[^{3}H] Methyl Transferred[b]			
	Chromatin + DNA	Chromatin (Endogenous DNA)	Δ (CPM/Added DNA)	CPM per A_{260nm} of Input DNA
Chromatin	6,508,152	4,419,576	2,088,576	12,790
P1	1,662,500	978,000	684,500	17,408
S1	6,719,931	2,781,077	3,938,854	18,973
S2	240,033	189,448	50,585	3,801
P2	7,227,000	3,047,550	4,179,450	22,932

Legend to Table 9.

[a]Chromatin fractions were prepared as shown in Fig. 5.

[b]Activity was determined in incubation mixtures containing 0.3-0.6 A_{260nm} units of chromatin isolated from Me_2SO-treated FL cells, or 0.3-0.6 A_{260nm} units of chromatin and 0.2 A_{260nm} units of DNA which was purified from cells grown in the presence of 4 mM L-ethionine. All incubation mixtures contained 10 µCi of S-adenosyl-L-(methyl-^3H)methionine (16 Ci/mmole) and incubation buffer (described in Methods). The total volume of the incubation mixture was 200 µl. Reactions were incubated as described in Methods. Radiolabel incorporated into DNA was determined by using assay Method A.

Note that P1 + S1 = 90% of input A_{260nm} and P2 + S2 = 96% of S1 from which they were prepared.

addition, the necessity of using particulate enzymes such as DNA methyltransferase in the chromatin-bound form made accurate kinetic studies difficult.

Under conditions of limited digestion, DNase II preferentially solubilizes actively-transcribed chromatin DNA. Therefore, in the first minutes of digestion, almost all of the chromatin is Mg^{++}-soluble (Gottesfeld et al., 1975). With increased digestion time, inactive chromatin is also released, and the proportion of enzyme solubilized chromatin which is Mg^{++}-soluble decreases. If FL cell DNA methyltransferase is bound to template-inactive DNA, one would predict that as the extent of chromatin digestion by DNase II increases, increasing amounts of DNA from template-inactive chromatin, along with bound enzyme, should be released into the S1 fraction until the specific activity of this fraction approaches that of the P2. One would also predict that the small amount of enzyme associated with the template-active DNA would be released within the first minute or two of digestion, and that the proportion of solubilized enzyme activity remaining soluble after Mg^{++}-precipitation should decrease with time.

The data presented in Table 10 indicate that (1) at zero time, with 14% of chromatin solubilized, only 2% of the activity is released into S1. (2) As a consequence of increasing the time of digestion, both the total and specific activities of the S1 increase substantially, and the proportion of this activity which is Mg^{++}-soluble decreases. (3)

TABLE 10.

A. TIME COURSE FOR SOLUBILIZATION OF DNA METHYLTRANSFERASE BY DNase II.

Time of Digestion (minutes)	% A_{260nm} Released [a]	% Activity Released
0	14	2.2
1	28	23
5	45	31

B. DISTRIBUTION OF DNA METHYLTRANSFERASE.

Time of Digestion	Mg^{++} Soluble CPM/10^9 Cells	%Sl	Mg^{++}-Insoluble CPM/10^9 cells	%Sl
0	33,668	54	30,896	46
1	27,713	21	102,538	79
5	57,478	11	462,909	89

Legend to Table 10.

[a]Chromatin was prepared and fractionated as described in Fig. 5. The length of the digestion period varied, as indicated in the table. Following digestion, the pH of the reaction mixture was raised to 7.5 using 100 mM Tris, pH 9.0. The mixture was then centrifuged at 6000 rpm for 5 minutes at 4°C. The A_{260nm} of the supernatant was measured, and the value obtained was divided by the A_{260nm} units of total chromatin to determine the percent A_{260nm} released.

The distribution of DNA methyltransferase between the Mg^{++}-soluble and Mg^{++}-insoluble fractions was determined by measuring the activity of the enzyme associated with each of these fractions. The activity was measured by determining the incorporation of radiolabeled methyl groups into endogenous DNA, and then subtracting the value from the one obtained for the incorporation of radiolabeled methyl groups into chromatin DNA and added DNA. The difference, which was a measure of the activity of chromatin-associated enzyme on added DNA, is the activities which are shown in the table.

Reaction mixtures contained 10 µCi of S-adenosyl-L-(methyl-^{3}H)methionine (16 Ci/mmole), incubation buffer (described in Methods), and 0.3 A_{260nm} units of solubilized chromatin or 0.3 A_{260nm} units of chromatin and 0.2 A_{260nm} units of DNA purified from ethionine-treated cells. The final volume of the reaction mixture was 200 µl. Conditions of incubation are described in Methods. Assay Method A was used to measure the incorporation of radiolabeled methyl groups into DNA. Data are from a representative experiment.

After 5 minutes digestion, almost 90% of the enzymatic activity in S1 can be recovered in the P2 fraction. Thus, as predicted, the amount of activity in the template-active (Mg^{++}-soluble) fraction is rapidly released and remains constant.

The data presented in Tables 8 and 9 indicate that the Mg^{++}-soluble or template-active regions of the chromatin have little associated DNA methyltransferases. The validity of this interpretation, however, relies on the assumption that the DNA methyltransferases remain bound to chromatin during digestion and fractionation. An artifactual concentration of the enzyme in Mg^{++}-insoluble chromatin fraction could occur if the DNA methyltransferase does not remain bound to chromatin DNA following fractionation, but is insoluble in Mg^{++} and co-precipitates with the transcriptionally-inactive DNA. To rule out this possibility, I performed the following experiments. A preparation of partially purified FL cell DNA methyltransferase was adjusted to 2mM $MgCl_2$, (a concentration in which actively-transcribed DNA is soluble and non-transcribed DNA insoluble), kept on ice for 30 minutes, and then centrifuged at 12,000 rpm for 10 minutes. Under the same conditions used for chromatin, 90% of the DNA methyltransferase activity remained in the supernatant, indicating that the DNA methyltransferase is soluble in the presence of Mg^{++}, and providing evidence that enrichment of the enzyme is the Mg^{++}-insoluble chromatin fraction is not a simple artifact.

I also determined the distribution of DNA methyltransferase in solubilized chromatin which had not been exposed to Mg^{++}, fractionating it by column chromatography on Biogel A5m (Biorad). Biogel A5m is composed of agarose beads, and excludes particles with a molecular weight exceeding 5 million. The DNA methyltransferase activity of nucleosomal material is presented in Table 11. Approximately 80% of the activity and 85% of the A_{260nm} was recovered from the column. All of the recovered activity co-chromatographed with nucleosomal material. No activity was found with soluble proteins, indicating that even in the absence of Mg^{++} almost all of the solubilized DNA methyltransferase is bound to nucleosomes. I cannot rule out the possibility that as a consequence of DNase II digestion, the FL cell DNA methyltransferase is released from internucleosomal DNA and rebinds to nucleosomes. However, this data, combined with that in Table 10, are a strong indication that the FL cell DNA methyltransferase is associated with nucleosomes in inactive-chromatin.

Additional indirect evidence demonstrated that the FL cell DNA methyltransferase is bound to non-transcribed DNA. This evidence was obtained by utilizing a different fractionation procedure which did not rely on Mg^{++}-precipitation i.e., digestion of template-active chromatin DNA with DNase I (EC 3.1.4.5).

Either intact nuclei or chromatin prepared as described above were digested with DNase I. Intact cell nuclei were used since original experiments describing the specificity

TABLE 11.

FRACTIONATION OF S1 ON BIOGEL A5m

	Total Activity[b] (%)	Total A_{260nm} (%)
S1 (Unfractionated)[a]	5,840,000 (100%)	568 (100%)
Column Fractions[c]		
Nucleosomes (Fractions 10-30)	4,656,680 (79%)	347.9 (61%)
Non-Nucleosomal (Fractions 35-50)	N.D.*	142 (25%)
Recovery	(79%)	(86%)

N.D.* = not detected.

Legend to Table 11.

[a]S1 was prepared as described in Figure 5.

[b]The activity was measured by determining the incorporation of radiolabeled methyl groups into endogenous DNA, and then subtracting this value from the one obtained for the incorporation of radiolabeled methyl groups into chromatin DNA and added DNA. The difference was a measure of the activity of chromatin-associated enzyme on added DNA. The activity, which was obtained for the amount of chromatin in the reaction mixture, was then extrapolated to obtain total activity for each fraction.

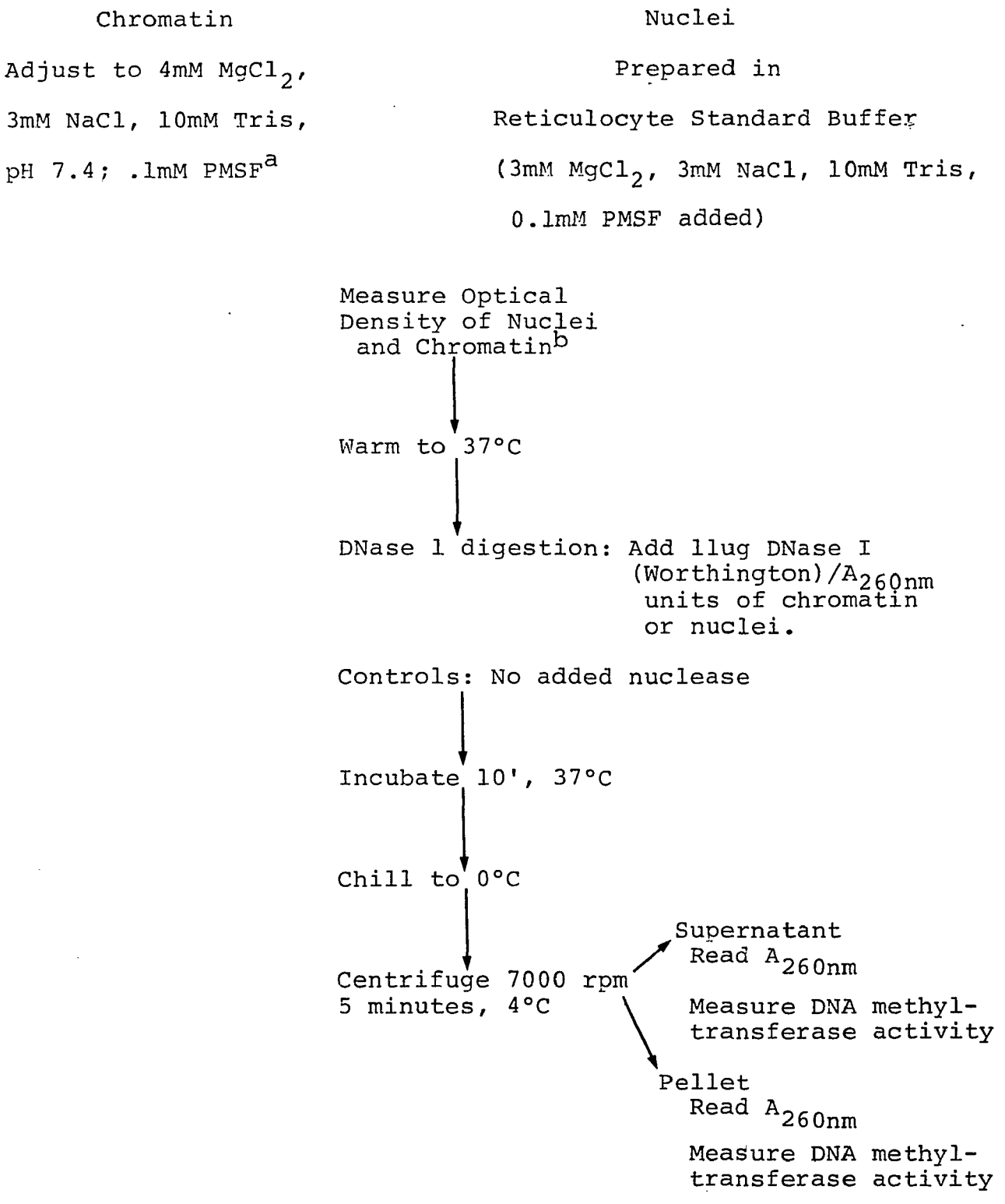
Reaction mixtures contained 10 μCi of S-adenosyl-L-(methyl-^{3}H)methionine (16 Ci/mmole), incubation buffer (described in Methods), and 0.3 A_{260nm} units of chromatin or 0.3 A_{260nm} units of chromatin and 0.2 A_{260nm} units of DNA purified from ethionine-treated cells. The final volume of the reaction mixture was 200 μl. Conditions of incubation are described in Methods. Assay Method A was used to measure the incorporation of radiolabeled methyl groups into DNA. Data are from a representative experiment.

of DNase I were performed in this way (Weintraub and Groudine, 1976). Both methods are shown schematically in Figure 6. Chromatin or nuclei incubated in the absence of added nuclease were used as controls. After incubation, the reaction was terminated by chilling the mixture to 0°C. The mixture was then centrifuged at 7000rpm for 5 minutes at 4°C. Aliquots of the solubilized and insoluble chromatin were immediately assayed for in vitro DNA methyltransferase activity as described above. The incubation buffer contained a sufficient concentration of EDTA to inhibit contaminating DNase I. Data on the extent of chromatin digestion by DNase I, as well as on the incorporation of radiolabeled methyl groups into the DNA of each fraction, are presented in Table 12. These findings indicate the following: (1) Using the method described in Figure 6, FL cell nuclei and chromatin were digested to the same extent by DNase I. (2) The activity of the soluble fraction, which contains the solubilized actively-transcribed chromatin DNA (Weintraub and Groudine, 1976), was between 0.4-0.8% that of the insoluble fraction. (3) The specific activity of the insoluble fraction, which contains the non-transcribed chromatin DNA, was between 130-250 fold greater than that of the soluble fraction.

These data provide additional support that the FL cell DNA methyltransferase is bound to transcriptionally-inactive DNA.

FIGURE 6.

FRACTIONATION OF FL CELL CHROMATIN BY DNASE I.



Legend to Figure 6.

Chromatin was isolated as described by Gottesfeld and Partington (1977). Nuclei were isolated according to the method of Hewish and Burgoyne (1973).

[a] PMSF = Phenylmethylsulfonylfluoride, a protease inhibitor

[b] Aliquots of nuclei or chromatin were adjusted to 0.1N NaOH prior to optical density determinations.

TABLE 12.

DNase I DIGESTION OF FL CELL CHROMATIN

Preparation[a]	A_{260nm}(%)	DNA Methyltransferase Specific Activity[c] (cpm/A_{280})	DNA Methyltransferase CPM/10^9 cells	DNA Methyltransferase % (Total)
Nuclei				
Solubilized	22	206	5,462	(.38)
Insoluble[b]	78	20,322	1,416,000	(99)
Chromatin				
Solubilized	18	416	9,882	(.74)
Insoluble[b]	82	19,052	1,314,625	(99)

Legend to Table 12.

[a] Chromatin was isolated as described by Gottesfeld and Partington (1977). Nuclei were isolated as described in Methods.

[b] Insoluble chromatin is defined as that material which sediments at 7000 rpm after chromatin is digested with DNase I. The complete description of the method used to digest nuclei and chromatin with DNase I is presented in Fig. 6.

[c] Specific activity is defined as the incorporation of radiolabelled methyl groups into DNA/A_{280} input chromatin during a 30' incubation period. Incorporation was determined using assay Method A.

DISCUSSION:

Two models have currently been proposed to explain the control of development. In one model, proposed by Caplan and Ordahl (1978), all genes are active or transcriptionally-accessible during the early stages of development. The differentiated phenotype of a cell results from a progressive and irreversible repression of selected genes.

The model assigns genes to either of two categories, depending upon whether their products are necessary for cell survival and consequently common to all cells (housekeeping genes), or whether they are phenotype specific (luxury genes). Four categories of phenotype specific genes were defined. A) Genes which are irreversibily repressed coinciding with the cessation of their activity. (B) Genes which are irreversibly restricted at some time after the termination of their activity. (C) Genes in which transcription is restricted, but never irreversibly repressed. (D) Genes which are never repressed and whose expression determines phenotype.

The experimental evidence provided by Caplan and Ordahl to support the irreversible gene repression model comes from developmental studies which have shown that as an organism develops and cellular differentiation occurs, individual cells lose the capability of being cloned so as to give rise to a complete organism. As an example, it is well known that at the two cell stage in animal development,

each cell is capable of giving rise to a complete organism. The next cleavage, which produces the four-cell stage, does not restrict developmental potential. After the second or third cleavage, many of the cells are no longer capable of producing normal animals, indicating that restrictions have been imposed on the developmental potential of these cells.

The experimental findings of Gurdon and co-workers (1974) have also been used to support the irreversible gene repression model. Gurdon transplanted cell nuclei into an enucleated fertilized egg. With nuclei from early embryos, many of the recipient eggs develop into normal organisms. With nuclei from more differentiated donor cells, the probability of success of these nuclear transplantation experiments decreased considerably. Caplan and Ordahl interpreted these findings as indicating that during the course of cellular differentiation, selected gene functions are repressed so that the nuclei transplanted from the more differentiated cells could no longer support normal development.

The second model proposes that the entire genome is not transcriptionally active during the early stages of development. Gene control is coordinate, with all genes having the potential for activation or repression as their products are required for cellular growth and differentiation.

Neither of the models, nor the experiments described above, provide a mechanism for gene regulation during differentiation and development. However, several models have been proposed which account for how genes might be controlled

during cellular differentiation.

Scarano (Grippo et al., 1968; Scarano et al. 1977) has proposed a 'synchron model' to explain gene control during differentiation. According to the model, a fraction of the total nuclear genes in higher organisms are organized into ontogenic informational units called synchrons. A synchron is composed of clusters in a hierarchical order. One of the clusters, which contains a specific structural gene called the development programming gene (DPG), is referred to as a control cluster since all other clusters depend upon the product of the development programming gene. The product of the DPG is a DNA-modifying enzyme, or a molecule which binds to a DNA-modifying enzyme and regulates its specificity. This enzyme is controlled allosterically, and consequently, could be activated or inhibited by highly specific allosteric effectors, or hormones and proteins. The DNA modifying enzyme, in turn, regulates the transcriptional activity of its dependent clusters in the following manner. The dependent clusters, which may be located on one or many chromosomes, contain a prepromotor, promotor and several structural genes. The structural genes of a synchron cannot be transcribed unless the prepromotor has a specific conformation that allows RNA polymerase to bind to the promotor. The DNA modifying enzymes effect these necessary changes in prepromotors. The dependent cluster contains one structural gene, whose product regulates the control cluster in a feedback loop by specifically repressing the DPG gene.

The model proposes that there are intermediate and terminal synchrons. An intermediate synchron, which contains genes which are transcribed during the intermediate stages of cellular differentiation, include one or more DPG genes among its structural genes. The terminal synchron, which contains no DPG gene, is comprised of genes which are only transcribed during the terminal stages of differentiation.

Scarano found that more sites were available for methylation in the DNA of nuclei isolated from sea urchin blastulas than in the DNA of nuclei isolated from gastrulas. He also demonstrated the presence of 'minor thymine' (below) in sea urchin embryo DNA. These data provided the experimental basis for his theory that DNA modifying enzymes control gene activity during development.

Scarano concluded from his findings not only that DNA methylation might play a role in sea urchin development, but that the enzymes responsible for methylating DNA may regulate sea urchin development. Scarano also postulated that 'minor thymine' in sea urchin embryo DNA arose by a base transition, in which cytosine is methylated by a DNA-5-methylcytosine methyltransferase and then deaminated by DNA-5-methylcytosine deaminase. Scarano proposed that these DNA modification enzymes could regulate gene activity during development by affecting base transitions in specific regulatory genes. This would result in an alteration in the base sequence of these regions, causing either the activation or repression

of the structural genes they control.

There are two major criticisms of Scarano's synchron model. Since the model implicates DNA modification enzymes as playing a major role in gene regulation during cellular differentiation, Scarano assumes that the 'minor thymine' in DNA is the result of a base transition in which cytosine is enzymatically methylated and then deaminated. Scarano demonstrated the existence of an enzyme in donkey spleen which deaminates 5-methylcytosine at the polymer level. However, Borek (1975) has indicated that there is no evidence that this enzyme is generally present in mammalian tissues as would be expected if the enzyme had an important regulatory function. Moreover, Borek has suggested that the thymine in DNA could result from a salvage pathway by which thymine from the turnover of transfer RNA is made available for incorporation into DNA. Alternatively, chemical or random deamination of 5-methylcytosine could occur during the preparation of DNA.

A second criticism of the synchron model is that if DNA methyltransferases regulate sea urchin embryo development by controlling the rate or extent of DNA methylation, one might expect to observe differences in the 5-methylcytosine content of sea urchin embryo DNA during development. Pollock et al., (1978) using a high pressure liquid chromatographic procedure to measure the 5-methylcytosine content of sea urchin embryo DNA, reported no difference in the extent to which the DNAs of sea urchin blastula or gastrula are methylated.

Holliday and Pugh (1975) have also proposed that the regulation of gene activity during cellular differentiation is modulated by DNA-modifying enzymes. In their model, it is assumed that the modification enzymes control genetic activity by two different mechanisms. (A) DNA-modifying enzymes can effect a change in the nucleotide sequence of DNA as a result of base transitions. If such changes occurred in a regulatory sequence, genes could either be activated or repressed. This aspect of the model is quite similar to the synchron model proposed by Scarano and is therefore open to the same criticisms discussed above. (B) Gene transcription could be controlled directly by DNA methylation without changing base sequence. For this to occur, the authors have postulated that two different DNA methyltransferases are required. These enzymes have been designated E1 and E2. The enzyme E1 methylates one strand of a palindromic sequence. When this DNA is replicated, two different duplexes are produced. Cell division will produce two daughter cells, one of which contains hemimethylated DNA. This DNA could serve as a substrate for E2. The other daughter cell receives unmethylated DNA which could be a substrate for E1. If this daughter cell is missing E1, then as each of these daughter cells replicate, two cell populations will become established. Cells descended from the daughter cell which received the hemimethylated DNA will be modified, while cells descended from the daughter cell which received unmethylated DNA will be unmodified.

The enzymes E1 and E2 are referred to as the 'switch' and 'clock' enzymes, respectively. According to the model, these methylating enzymes could sequentially methylate palindromic sequences which are adjacent to a starter sequence. If the methylation of these sequences were correlated with cell division, this process could be used as a program for such processes as development and even aging. Holliday and Pugh suggest that these modification mechanisms are reversible, and have postulated the existence of a demethylating enzyme. However, a number of investigators have failed to show the existence of such a enzyme.

The recent findings of Bird (Bird and Southern, 1978; Bird, 1978) support at least one aspect of the model proposed by Holliday and Pugh. Bird used two classes of restriction enzymes to investigate the frequency and distribution of 5-methylcytosine in Xenopus rDNA. Both classes of enzyme recognize the sequence CpG in DNA. However, one of the enzymes is unable to cleave the palindromic sequence $\overset{5'CpG}{GpC}$. if either one or both of the strands are methylated. The other enzyme can cleave this palindromic sequence whether the strands are methylated or not.

Bird compared restriction fragments prepared from erythrocyte rDNA, in which 13% of the cytosine residues are methylated, with amplified rDNA which contains no 5-methylcytosine. He showed that nearly all of the CpGs detected in erythrocyte rDNA were methylated. By hybridizing unmethylated and methylated rDNA restriction fragments, Bird showed

that there are no half-methylated sequences in either the DNA-DNA hybrids or in native Xenopus rDNA. Bird observed that one site, located within the nucleotide sequence of erythrocyte rDNA which codes for 28S ribosomal RNA, is 30-60 times less modified than the majority of the other sites in erythrocyte rDNA. He concluded from these findings that Xenopus cells contain a DNA methyltransferase which rapidly completes the methylation of half-methylated DNA and 'reluctantly' methylates unmethylated DNA. Bird also demonstrated that in cultured Xenopus cells, new methyl groups are only added to the progeny strand. These findings indicate that the methylation pattern in the parental strand serves as a template for progeny strands, and that this pattern is copied in a semi-conservative manner.

Although Bird has demonstrated that DNA methylation in Xenopus rDNA follows the pattern expected for a DNA methyltransferase with properties similar to the E2 enzyme described by Holliday and Pugh, there is no direct evidence for the existence of this enzyme. DNA methyltransferase purified from rat liver (Drahovsky and Morris, 1971), HeLa cell nuclei (Roy and Weissbach, 1975) and Krebs II ascites cells (Turnbull and Adams, 1976) are able to methylate both single-stranded and double-stranded DNA in vitro. Moreover, it is unknown whether these enzymes methylate only one or both of the strands in the palindromic sequence $^{5'CpG}_{GpC}$.

Despite the lack of experimental evidence to support both Scarano's synchron model and the Holliday and Pugh model,

there is some evidence for a correlation between DNA methylation and cellular differentiation in organisms or cells other than FL cells. Vanyushin et al., (1973) have shown that DNA isolated from a variety of tissues from the same animal contain different amounts of 5-methylcytosine. Berdishev et al., (1967) have reported differences in the 5-methylcytosine content of DNA among individuals of varying ages. Rubery and Newton (1973) have found differences in the 5-methylcytosine content of DNA from normal and transformed cells.

My own studies were designed to determine the cause of DNA hypomethylation and its relationship to differentiation.

In addition, I was interested in determining whether there was any relationship between the template activity of DNA and the extent to which it is methylated. I provided evidence that in the chromatin-bound form, DNA methyltransferases from undifferentiated cells were more capable of methylating added FL cell DNA than the DNA methyltransferases of differentiating cell chromatin. The chromatin bound FL cell DNA methyltransferases of undifferentiated cells seemed to be more active on exogenous DNA than the chromatin-associated enzyme from differentiating cells. However, when I extracted enzyme from the chromatin of undifferentiated and differentiating cells, and assayed each preparation on purified FL cell DNA, I found that the enzymatic activities were approximately equal.

Darzynkiewicz et al., (1976) have presented evidence that changes in chromatin conformation occur during FL cell differentiation. I have shown that chromatin preparations containing the same amount of bound DNA methyltransferases differ in their ability to methylate added FL cell DNA. I interpret this finding as indicating that the differences in FL cell chromatin affect the interaction of enzyme with DNA.

These findings indicate that the restrictions operating in the whole cell which prevent the methylation of cytosine residues in DNA are operating at the level of the chromatin. The strongest evidence that these restrictions are due to chromatin proteins, which interfere with DNA methylation, comes from comparing the in vitro methylation of chromatin DNA with the methylation of DNA purified from chromatin. Regardless of the differentiated state of the cell from which the chromatin was prepared, less than 1.5 pmoles of methyl are transferred to 1 A_{260nm} unit of chromatin by chromatin-bound enzyme during a 30 minute incubation period at 37°C. If I added approximately 25 micrograms of a partially purified DNA methyltransferase to this reaction mixture, and incubated the mixture under the same conditions, I observed that 1.1-1.2 pmoles of methyl are transferred to 1 A_{260nm} unit of chromatin. Christman and co-workers (1977) showed that DNA purified from undifferentiated FL cells accepts 5-7 pmole methyl/A_{260nm} units of DNA from a partially purified enzyme, as compared with the DNA from Me_2SO-treated cells which accepts 15-25 pmole/A_{260nm} units of DNA under

identical conditions.

Despite the finding that the DNA purified from differentiating FL cells is hypomethylated, I found that chromatin isolated from undifferentiated and differentiating FL cells have an equivalent amount of bound DNA methyltransferases. In addition, the number of sites within each of these chromatins which are accessible to their respective enzymes was small but equivalent. These findings indicate that chromatin proteins may interfere with the methylation of chromatin DNA by bound enzyme. I was also able to show that there are a limited number of sites in undifferentiated and differentiating FL cell chromatin which are accessible to added DNA methyltransferases.

By using procedures which separate actively transcribed and non-transcribed chromatin DNA, I showed that virtually all of the unmethylated sites in chromatin are clustered in the template-inactive chromatin fraction. I demonstrated that the majority of DNA methyltransferase activity is also associated with this fraction. The enzyme was shown to be soluble in 2mM Mg^{++}, indicating that its association with template-inactive DNA was not simply due to being insoluble in Mg^{++}. Lastly, I showed that the DNA methyltransferase is bound to template-inactive nucleosomes.

My findings have shown that, irrespective of the differentiated state of the cell, sequences of actively-transcribed DNA are virtually fully methylated <u>in vivo</u>, as judged by their inability to serve as methyl acceptor <u>in</u>

vitro. The one exception to this finding is the transcriptionally-active DNA prepared from ethionine-treated FL cells. This DNA is hypomethylated, relative to the transcriptionally-active DNAs prepared from Me_2SO-treated, HMBA-treated, and untreated FL cells. Since there is no evidence that the template-active DNA prepared from ethionine-treated cells has a reduced template activity, these findings indicate that methylation of DNA does not appear to be absolutely necessary for transcriptional activity in FL cells.

I have also shown that template-inactive FL cell DNA is hypomethylated, relative to template-active DNA. Electron micrographic studies (Comings, 1973) as well as thermal denaturation and circular dichroism studies (McConaughy and McCarthy, 1972; Gottesfeld et al., 1974b; Polacow and Simpson, 1973) have shown that template-active chromatin is in a more open configuration than template-inactive chromatin. My findings suggest that as a consequence of being in an open configuration, sequences of template-active DNA are more accessible to the DNA methyltransferases and as a result are virtually fully methylated. Template-inactive chromatin is in a more condensed configuration which may serve to restrict the accessibility of the sites to DNA methyltransferase in this DNA.

While most of the unmethylated cytosine residues in chromatin DNA were shown to be localized to the fraction containing template-inactive DNA, it remains to be shown whether these unmethylated sites are contained in the se-

quences of repressed structural genes or regulatory genes. If the unmodified cytosine residues are clustered in repressed structural genes, it would be tempting to speculate that DNA methylation plays a direct role in the activation of genes which code for structural proteins. According to this model, methylated sequences are turned on and unmethylated sequences are turned off. However, if regulatory genes are hypomethylated, a mechanism may be proposed in which hypomethylation occurs only in those regulatory genes which are associated with transcribed structural genes. The undermethylated regulatory sequences may then serve as a binding site for both DNA methyltransferase as well as RNA polymerase.

Adams et al., (1977) have also investigated the methylation of chromatin DNA. Adams prepared micrococcal nuclease digests of nuclei isolated from Chinese hamster ovary cells, and showed that 80% of the DNA methyltransferase activity is released into the soluble fraction during the initial stages of nuclease treatment. Since micrococcal nuclease cleaves internucleosomal DNA (Garel and Axel, 1976; Weintraub and Groudine, 1976), this finding would appear to contradict my results that the DNA methyltransferase is bound to nucleosomes. However, I found that even with limited micrococcal nuclease digestion, nucleosomes are released into the soluble fraction (data not presented). This finding was confirmed by Ms. Francine Creusot in our laboratory. She showed that the FL cell DNA methyltransferase is bound to nucleosomes which sediment more rapidly than the nucleosome

core particle containing 140 base pairs of DNA. These nucleosomes are released from FL cell nuclei with only one minute of micrococcal nuclease digestion. These findings raise the possibility that the FL cell DNA methyltransferase is bound to linker DNA which is adjacent to the nucleosome core.

Other investigators have reported that under conditions of limited digestion in a buffer of low ionic strength, micrococcal nuclease preferentially digests template-active DNA. Thus, Ms. Creusot's finding would appear to contradict my results, which showed that the DNA methyltransferase is bound to template-inactive DNA. One possible explanation for these results would be that DNA methyltransferase is also bound to newly synthesized DNA. Regions of chromatin in which DNA is undergoing replication are presumably in an extended or open configuration to permit replication enzymes to gain access to this DNA. Thus, these sites would be more accessible to nuclease. As a result of micrococcal nuclease digestion, newly synthesized DNA with associated enzymes would be released into the soluble fraction.

Does this hypothesis explain my findings that a majority of the unmethylated cytosine residues, as well as most of the DNA methyltransferase activity, are located in the template-inactive fraction? It has been shown that methylation lags behind synthesis (Adams, 1974), so that it is possible that the DNA methyltransferase binds to newly synthesized DNA, but does not methylate it immediately. If

newly synthesized DNA is not immediately transcribed, it would not be associated with RNA transcripts. As a result, this DNA would become localized to the template-inactive chromatin fraction after DNase II digestion and Mg^{++}-fractionation. One way to determine whether newly synthesized DNA becomes localized to the template-inactive chromatin fraction would be to pulse label cells with radiolabelled DNA precursors, prepare and fractionate chromatin, and show whether the radiolabel is in the DNA of the template-inactive (P2) fraction.

While my findings are not inconsistent with several proposed mechanisms whereby DNA methylation may play a role in gene control during differentiation, it should be recalled that such a relationship has never been established. Since I have also shown that chromatin proteins restrict the access of the DNA methyltransferase to sites in the DNA, the possibility arises that chromatin proteins can regulate methylation of nuclear DNA. Thus a restriction of DNA methylation might be the result of a change in chromatin configuration which is a consequence, rather than a cause of, cellular differentiation.

CONCLUSIONS:

The relationship between DNA hypomethylation and Friend erythroleukemia cell differentiation was investigated. It has been shown that the restrictions operating at the level of the whole cell to prevent the methylation of DNA are present in preparations of isolated chromatin. It could be demonstrated that the enzymatic activity of the DNA methyltransferases bound to chromatin of undifferentiated and differentiating Friend erythroleukemia cells were equal and that the restrictions described above were due to chromatin proteins which restrict the access of DNA methyltransferases to sites in the DNA.

Chromatin fractionation procedures, which enable template-active DNA to be separated from template-inactive DNA, were used to determine whether these hypomethylated sites were clustered in actively-transcribed or non-transcribed genes. It was shown that, irrespective of the differentiated state of the cell, template-active DNA was virtually fully methylated *in vivo* and that the unmethylated sites were localized to the template-inactive DNA. Lastly, the DNA methyltransferase was localized to template-inactive nucleosomal DNA.

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