Weich, Nadine S.

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by

Nadine Weich

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date

Executive Officer

Supervisory Committee

The City University of New York

ABSTRACT

DNA METHYLATION IN DIFFERENTIATING MOUSE ERYTHROLEUKEMIA CELLS

by

Nadine Weich

Adviser: Professor George Acs

Significant amounts of 5-methylcytosine have been shown to occur in all eucaryotic DNAs examined. This minor base is produced by the enzymatic transfer of methyl groups from S-adenosylmethionine to specific cytosine residues in the DNA polymer. The biological function of DNA methylation in eucaryotes is unknown. However, recent studies indicate that it may be involved in the regulation of gene expression.

I have found that mouse erythroleukemia cells grown in the presence of either hypomethylating agents which cause their differentiation (L-ethionine and 5-azacytidine) or known inducing agents which are not general inhibitors of transmethylation reactions (dimethylsulfoxide, butyrate, hexamethylene-bisacetamide or pentamethylene-bisacetamide) synthesize undermethylated DNA. A direct correlation between the extent to which DNA becomes hypo-methylated and the number of cells in the population which

become committed to differentiation is not observed. Nevertheless, the two processes are connected. Hypomethylated DNA is synthesized only in cells chemically induced to differentiate. DNA isolated from a dimethylsulfoxide resistant clone grown in the presence of that agent is not undermethylated, while DNA prepared from these cells after exposure to agents which induce their differentiation is undermethylated.

The relationship between DNA methylation and changes in gene expression during differentiation was further examined by determining whether loss of methyl groups occurs at specific sites within and around globin genes during mouse erythroleukemia cell differentiation. Although it was possible to demonstrate differences in the patterns of methylation of C methylation-sensitive restriction endonuclease (Hpa II and Hha I) sites in the α-like globin gene regions of two separate mouse erythroleukemia cell clones, the methylation of Msp I, Hpa II and Hha I sites located within the globin gene regions was not found to be altered during erythroid differentiation.

Thus, while the results of this study suggest that hypomethylation of DNA is an integral part of the process of cell differentiation, it is clear that the degree of methylation of many sites within a gene remains unchanged even during a period when the expression of that gene is enhanced.

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LIST OF ABBREVIATIONS:

5-azacytidine	5-aza-CR
5-aza-2'-deoxycytidine	5-aza-CdR
base pairs	bp
β-globin^{major}	$_{\beta}^{maj}$
β-globin^{minor}	$_{\beta}^{min}$
benzidine positive	B+
bovine serum albumin	BSA
bromodeoxyuridine	BrdUrd
deoxyribonuclease	DNase
dimethylsulfoxide	DMSO
dithiothreitol	DTT
Eco RI β-globin^{major} gene fragment	βM_2 DNA
Eco RI α-globin gene fragment	α_1 DNA
ethylenediamine tetracetric acid	EDTA
fetal calf serum	FCS
hexamethylene-bisacetamide	HMBA
high pressure liquid chromatography	HPLC
hour	hr
intervening sequence	IVS
kilobases	kb
metallothionein-I	MT-I
methyl	Me
5 methylcytosine	5MeC
minimal Eagle's medium	MEM
mouse erythroleukemia cell	FL cell

pentamethylene-bisacetamide	PMBA
phenylmethanesulfonylflouride	PMSF
picomoles	pmol
ribonuclease	RNase
S-adenosylmethionine	S-AdoMet
sodium dodecyl sulfate	SDS
volume	vol

INTRODUCTION

The minor base 5-methylcytosine (5MeC) has been shown to occur to a significant extent in all eucaryotic DNAs examined. Although this modified base was discovered over 30 years ago (Hotchkiss, 1975), its biological function remains unknown. Early reports of changes in DNA methylation levels during embryonic development (Adams, 1973) and tissue specific variations in 5MeC content (Vanyushin et al., 1970) led to the speculation that methylation of C residues in DNA may play a role in regulating gene activity (Holliday and Pugh, 1975). A correlation between DNA methylation and gene expression was established with the observation that mouse erythroleukemia cells (FL cells) while undergoing chemically induced differentiation synthesize hypomethylated DNA (Christman et al., 1977). Further evidence that a relationship exists has been provided by studies using restriction enzymes to analyze the extent of methylation of specific gene sequences (Mandel and Chembon, 1979; McGhee and Ginder, 1979; Shen and Maniatis, 1980; van der Ploeg and Chambon, 1980; Compere and Palmiter, 1981; Jones et al., 1981; and Weintraub et al., 1981). These experiments showed that specific sites within a gene are undermethylated in the DNA of tissues where it is expressed as compared to the level of methylation in tissues where it is never expressed. Also, agents known to inhibit transmethylation reactions, such as L-ethionine and

5-azacytidine, were found to induce differentiation in cultured cell systems (Christman et al., 1977; Constantinides et al., 1978; and Creusot et al., 1982).

Based on the observations that during in vitro erythroid differentiation of FL cells a stimulation of globin mRNA synthesis is seen as well as a decrease in the level of methylation of total genomic DNA (Christman et al., 1977), a study was undertaken to determine (a) whether the 5MeC content of DNA is affected in cells exposed to inducing agents to which they are insensitive, and (b) whether changes occur in the pattern of methylation of specific base sequences in the α-like globin, β-globin^{major} and β-globin^{minor} genes during differentiation. The findings presented here indicate that the cells synthesize hypomethylated DNA only when exposed to agents which cause their differentiation and that this loss of methyl groups does not occur in experimentally accessible sites located immediately adjacent to the globin genes. However, a difference was found in the methylation pattern of the α-like globin gene regions of two FL cell clones.

BACKGROUND

I. GLOBIN GENE EXPRESSION IN THE MOUSE

A. Mouse Erythroleukemia Cells

1. Origin

In 1957, Friend reported the recovery of a virus from the spleen of a Swiss mouse previously inoculated with filtrate from Ehrlich ascites tumor cells. The virus causes a disease in susceptible mice, which in its early phase (≈ 2 weeks) is characterized by erythroblastosis and enlargement of the spleen and liver. After 2 to 3 months, the spleen, liver, bone marrow, and peripheral blood become infiltrated with immature cells and the animals die.

Tumor formation results at the site of subcutaneous implantation of fragments of spleen or liver from animals in the advanced stages of Friend virus-induced leukemia (Friend and Haddad, 1960). These tumors are serially transplantable in DBA/2J mice, continue to carry virus, and appear to be devoid of mature erythroid elements. Cells released from tumor minceates were used to establish tissue culture lines (Friend *et al.*, 1966). These cell lines grow in suspension and have been maintained in continuous culture for years. The cell cultures appear to be pleomorphic, for when single cells from mass cultures were cloned in semisolid media, the progeny of each clone consisted of both immature cells and differentiating erythroblasts. A low level (≈1%) of FL cells in culture undergo spontaneous stimulation of hemoglobin

production as determined by benzidine staining (Patuleia and Friend, 1967)a finding suggesting the FL cells have the potential for erythroblastic differentiation.

2. Characteristics of Induced Differentiation

The process of normal erythroid cell differentiation begins with an undifferentiated stem cell that gives rise to mature erythrocytes within 7 to 10 days. Little is known about the molecular events occurring when stem cells become committed to erythropoiesis. However, it is during these early phases of differentiation that the potential for expression of the globin genes, as well as other specialized features of erythroid cells are determined. Also, at this time, an expanding compartment of erythroid precursors is formed. Erythropoietin promotes terminal maturation of committed erythroid stem cells and proerythroblasts, thereby increasing the population involved in hemoglobin synthesis (Neinhuis and Benz, 1977). FL cells when exposed to this hormone continue to proliferate. They do not, however, mature beyond the proerythroblast stage.

In 1971, Friend demonstrated that erythroid differentiation of FL cells could be stimulated by the addition of dimethylsulfoxide (DMSO) into the culture medium (Friend, et al., 1971). The cells express a program of differentiation that is similar morphologically and biochemically to that observed in normal, erythropoietin-stimulated erythropoiesis, i.e., 1) The mature cells morphologically resemble

orthochromatophilic normoblasts. They are smaller and have a lower nuclear to cytoplasmic ratio than the cells from cultures which do not have DMSO in the medium. 2) After 5 days growth in 2% DMSO, the frequency of benzidine positive cells is increased to 95% and the amount of hemoglobin synthesized increases up to 40 fold. 3) The cells display a decrease in tumorgenicity and reduction in their proliferating ability (Friend et al., 1971). 4) Membrane-specific antigens that cross-react with antiserum directed against adult DBA/2J mouse erythrocyte membrane-specific antigens appear (Ferusawa et al., 1971).

One of the most studied of the above phenomena is the stimulation of hemoglobin synthesis. Upon exposure of FL cells to DMSO, an increase in iron accumulation and incorporation into heme and hemoglobin is observed. The formation of α- and β-globin chains occurs in a one to one ratio, and the hemoglobin which accumulates is indistinguishable from adult DBA/2J hemoglobin (Boyer et al., 1972). The rise in the rate of hemoglobin synthesis is accompanied by a differential increase in the amounts of α- and β-globin mRNA synthesized during FL cell differentiation. α-globin mRNA is present in excess of β-globin mRNA through the early phase or first 30 hours of induction. However, the α/β-globin m RNA ratio approaches one as differentiation proceeds. (α/β= 1, at 96 hours) (Orkin et al., 1975a)

Variant FL cell clones resistant to induction by DMSO

have been isolated by selection in medium containing the inducer (Ohta et al., 1978). Most FL cells when exposed to DMSO undergo terminal differentiation. Only those cells which are resistant to induction continue to proliferate. Variant clones retain their sensitivity to other chemicals that induce erythroid differentiation in FL cells.

B. The BALB/c Mouse

1. Expression and Characterization of β-Globin and β-Like Globin Genes

During intrauterine development of the mouse, two β-like (Y and Z) embryonic globins are synthesized (Fantoni et al., 1976). They are replaced in the final week of gestation by the adult form of β-globin chains[β-globin^{major} (β^{maj}) and β-globin ^{minor} (β^{min})] (Russell and McFarland, 1974). Recombinant DNA technology has been used to clone and sequence both the β^{maj} and β^{min} genes of the BALB/c mouse (Tilghman et al., 1977; Tiemeier et al., 1978; Konkel et al., 1979). This work has revealed how the structures of the β-globin genes are related to one another.

The β^{maj} gene sequence is contained in a 7 kilobase (kb) Eco RI fragment. It has two intervening sequences (IVS, noncoding regions) that are spliced out of a 15S globin mRNA precursor during formation of the mature 9S transcript (Kinniburgh et al., 1978; Kinniburgh et al., 1979). The shorter IVS1 is 116 base pairs (bp) long and is situated between codons 30 and 31. The longer IVS2 consists of 650 bp

and is positioned between codons 104 and 105. The β^{min} gene lies between two Eco RI sites that are 14 kb apart. It is also interrupted by two intervening sequences and shares segments with extensive homology with the β^{maj} gene (the proteins differ at 9 aa). The β^{min} short IVS1 and long IVS2 are equal in length to and located in the identical positions as the β^{maj} intervening sequences. Only 3 out of the 116 bp in the IVS1s differ in the two genes. The two long noncoding sequences, however, have considerable lack of homology. An interesting and unexpected finding to come out of the research done with these cloned genes is that although only 4 β-like globin proteins (2 embryonic and 2 adult) are synthesized in the BALB/c mouse, at least 6 discrete β-like globin coding regions exist, two of which are silent (Leder et al., 1980).

2. Expression and Characterization of α-Globin and α-like Globin Genes

Three different α-like globins are synthesized in the mouse. One embryonic globin (x) is produced in the nucleated red blood cells of the embryonic yolk sac while 2 adult globins (α_1 and α_2) are made in both the embryonic red blood cells and in nonnucleated erythrocytes (Fanconi et al., 1967). Except for one aa (position 68) which is a serine in α_1 and a threonine in α_2, the adult globins are identical (Popp, 1967). They do not however have many aa residues in common with the x-globin.

Six α-like globin coding regions have been discovered in the BALB/c mouse genome (Leder et al., 1980). The physical arrangement of the active coding regions places the embryonic and adult genes on chromosome 11 in the following order: 5' embryonic sequence -α_1 sequence -α_2 sequence 3' (Leder et al., 1981). Although little has been published about the α_2-globin gene and the embryonic α-like gene, a great deal of information concerning the α_1-globin gene has been obtained. Cleavage of the BALB/c genome with Eco RI resulting in the formation of a 9.7 kb fragment that contains the α_1 gene made it possible to clone this segment of DNA into λgtWES (Leder et al., 1978). Studies of the cloned gene revealed that it is encoded in 3 discontinuous segments of DNA and that the 2 short intervening sequences interrupting the α_1 coding regions are located at positions strikingly similar to those of the β IVS1s and IVS2s. Also, small areas of homology that are most likely to be signals necessary for transcription are preserved among the highly divergent flanking regions of the α_1 and β genes. (Nishioka and Leder, 1979).

Two inactive α-like genes (pseudogenes) have been shown to reside on 2 separate chromosomes 15 and 17 (Leder et al., 1981). These pseudogenes consist of segments of DNA that are extensively homologous to the α coding sequence but contain missense and/or nonsense mutations which render them nonfunctional. One pseudogene (α-ψ_3) located between

2 Eco RI sites that are 4.7 kb apart has an extremely unusual feature. It completely lacks the 2 intervening sequences that interupt all of the globin coding segments investigated (Nishioka et al., 1980). A similar type of α-like gene has been isolated from the genome of CD1 Swiss mice (Vanin et al., 1980). The second inactive α-like region (α-ψ_4) is situated between 2 Eco RI sites that are separated by 2.4 kb and contains mutations analogous to those found in hemoglobins associated with certain human blood disorders.

II. DNA METHYLATION AND GENE EXPRESSION

A. DNA Methylation

DNA methylation occurs by an enzymatic transfer of methyl groups from S-adenosyl methionine (S-AdoMet) to specific bases in the DNA polymer (Srinivasan and Borek, 1964). In procaryotes, the DNA is methylated in both the N-6 position of adenine and the C-5 position of cytosine (Dunn and Smith, 1958). Although the role of the majority of these methylated bases is still undefined, methylation at some sites has been shown to be involved in bacterial restriction-modification (Arber, 1974). Two types of restriction enzymes are known. Type I enzymes are complex multimeric proteins that generally require ATP, S-AdoMet and Mg^{++} as cofactors. They function as both a modification methylase and as a restriction endonuclease. They recognize

specific sequences in the DNA, but cleavage by these endonucleases is random and at a distance from the recognition site. Type II enzyme systems modify and cleave DNA at a specific recognition sequence. Shortly after a new strand of DNA is synthesized in bacteria, it is methylated by a restriction enzyme (Type I or Type II) at positions complementary to sites where the parental strand is methylated. A pattern characteristic of the particular strain is formed by this modification. Foreign DNA which lacks methyl groups in the appropriate positions will be cleaved by Type I enzymes at nonspecific sites located away from the recognition sequence or by Type II nucleases at the unmodified sites. The methylation of a single cytosine in bacteriophage ØX174 appears to serve a function in the phage replication process. The methyl group, which is located near the termination site of DNA replication is a signal to a specific endonuclease which mediates the excision of the one unit length viral genome (Friedman and Razin, 1976).

In eucaryotes, the nuclear DNA is methylated shortly after DNA synthesis. Only the C-5 position of cytosine is methylated (Vanyushin et al., 1970), and this modification occurs most frequently on residues in the sequence CpG (Doskocil and Sorm, 1962). Inhibition of this reaction results in the hypomethylation of the newly replicated DNA. This appears to be the only means by which a loss of methyl groups from DNA can occur, since demethylase enzymes have

never been located in cells. The function of 5MeC in eucaryotes is unknown. However, certain observations indicate that this methylated base may be involved in the regulation of cell differentiation. This theory is based on data from laboratories showing variations in the 5MeC content of DNA during embryo development (Adams, 1973) and expression of the differentiated phenotype (Christman et al., 1977). In addition, differences in the degree of methylation of specific gene sequences have been observed when comparing DNAs from tissues where the genes are expressed with DNA from tissues in which the same genes are never expressed (Mandel and Chambon, 1979; McGhee and Ginder, 1979; Shen and Maniatis, 1980; Compere and Palmiter, 1981; Jones et al., 1981; and Weintraub et al., 1981).

B. Relationship Between DNA Hypomethylation and Gene Expression

1. Effect of Inhibitors of Transmethylation reactions on Cell Differentiation

a. 5-Azacytidine

The antileukemic drug 5-azacytidine (5-aza-CR) has a nitrogen in place of carbon at the fifth position in the pyrimidine ring of cytidine (Von Hoff et al., 1976). Inhibition of protein, RNA and DNA synthesis is observed in cells exposed to high levels of the drug (Cihak, 1974). It also appears to interfere with S-AdoMet dependent transfer of methyl groups of cytosine residues in DNA and RNA polymers. Specific

inhibition of liver 5MeC; tRNA methyltransferase has been demonstrated in mice injected with 5-aza-CR and tRNA isolated from the livers of these animals was deficient in 5MeC (Lu and Randerath, 1979). In Escherichia coli (E. coli) treated with the drug loss of 5MeC;DNA methyltransferase activity occurred, while the DNA (N^6 adenine) methyltransferase activity remained unaffected (Friedman, 1979).

Addition of 5-aza-CR or the more potent 5-aza-2'-deoxycytidine (5-aza-CdR) to the culture medium of C3H/10T½ CL8 mouse embryo cells at concentrations which inhibit DNA methylation brings out the formation of contractile striated muscle cells (Jones and Taylor, 1980). Expression of the differentiated phenotype was eventually seen in 14% of the cell population as long as exposure to the drugs took place during early S phase of the cell cycle (Constantinides et al., 1978). The above findings suggest that both DNA hypomethylation and cellular differentiation may be linked to the incorporation of 5-aza-C into DNA and that 5-aza-C might cause heritable changes in DNA methylation.

Insight into the mechanism by which 5-aza-CR and 5-aza-CdR influence DNA methylation and the induction of cell differentiation was obtained by our laboratory by treating FL cells with these analogs. Only a small portion of the cell population was observed to differentiate after exposure to these agents (10-15%). However, they caused rapid loss of active DNA methyltransferase from these cells >80% in 4 hr., and

therefore the synthesis of severely undermethylated DNA. It was also found that 5-aza-C must be incorporated to mediate the inactivation of the methyltransferase. Removal of the analogs from the culture medium was followed by a gradual restoration of enzyme activity, and most of the affected sites were remethylated as the 5-aza-C residues became diluted out during successive DNA replications and new enzyme was made (Creusot et al., 1982).

b. L-Ethionine

L-Ethionine, an ethyl analog of the amino acid methionine, when activated by ATP to S-adenosyl ethionine inhibits S-AdoMet dependent transmethylation reactions (Moore and Smith, 1969; and Byvoet and Baxter, 1975). Exposure of cells to this compound results in the synthesis of undermethylated tRNA (Moore and Smith, 1969; Wainfan et al., 1975a; and Wainfan et al., 1975b), DNA (Sneider et al., 1975) and histones (Copp, 1981). L-Ethionine also induces FL cells to differentiate (Christman et al., 1977). When the cells are grown in the presence of this agent, an accumulation of globin mRNA precedes an enhancement of hemoglobin production. Both DNA and tRNA synthesized in cells exposed to concentrations of L-ethionine which are sufficient to induce differentiation are hypomethylated, as measured by their ability to accept methyl groups in vitro.

2. Effect of FL Cell Inducing Agents on DNA Methylation

The discovery in our laboratory that an inhibitor of

transmethylation reactions induces differentiation in FL cells prompted the testing of other compounds which are not hypomethylating agents, but are known FL cell inducers for their ability to inhibit in vivo methylation. It was observed that compounds such as DMSO and butyrate inhibit the methylation of DNA and not of tRNA (Christman et al., 1977). The hypomethylation of DNA can be detected early enough in the differentiation process (24 hours after the addition of an inducing agent to the culture medium) to allow its possible involvement in commitment. If DMSO induced differentiation is inhibited by concurrently exposing cells to bromodeoxyuridine (BrdUrd), DNA from these cells accepts the same amount of methyl groups in vitro as DNA from untreated cells, (i.e., it does not become hypomethylated). When up to 40% of the thymidine residues have been substituted by BrdUrd, the 5MeC to cytosine ratio in the BrdUrd substituted DNA equals that of unsubstituted FL cell DNA. Therefore, BrdUrd does not affect the ability of DNA to accept methyl groups in vivo (Christman et al., 1980).

In an attempt to localize the region in the genome where hypomethylation occurs during FL cell differentiation, further studies were carried out. The findings are as follows: 1) The distribution of unmethylated sites in total DNA isolated from untreated and differentiating FL cells appear identical. This was determined by methylating the DNA in vitro with radiolabelled S-AdoMet and then fractionating

the DNA into (a) main band and satellite, (b) highly repetitive, middle repetitive and unique sequences, and (c) pyrimidine tracts. In all cases, DNA from differentiating cells accepted more methyl groups than DNA from untreated cells, however, the distribution of radiolabelled methyl groups in DNA from both populations of cells was the same (Christman, 1979). Limited digestion of chromatin with DNAse II produces a Mg^{++}-soluble fraction which contains bound RNA and is therefore considered an actively transcribed region of the genome (Marushige and Bonner, 1971). DNA isolated from the active fraction of chromatin accepts less methyl groups *in vivo* and *in vitro* than DNA from the Mg^{++}-soluble or inactive fraction. The lower content of CpG doublets in structural genes could account for this observation.

C. Methylation of Specific Base Sequences and Gene Expression

1. Detection of 5MeC in a Specific Gene Sequence

Bird and Southern introduced a very useful technique for determining the extent of methylation of a specific sequence at a precise location in total genomic DNA (Bird and Southern,1978). The method evolved from the findings that (a) approximately 90% of the 5MeC residues in eucaryotic DNA are located in the dinucleotide CpG (Doskocil and Sorm, 1962) and (b) several Type II restriction enzymes contain CpG in their recognition sequence (Roberts, 1978).

The recognition cleavage sequences of some of these enzymes are listed in Table I. Each of these endonucleases is unable to cleave at its recognition site if one of the C residues in that site is methylated. This provides a means by which to distinguish between methylated and unmethylated sequences in specific regions of DNA. After digestion of the genome with the appropriate endonuclease, the resulting fragments are separated according to size by agarose gel electrophoresis, transferred by blotting to a nitrocellulose filter and hybridized with a labelled probe (RNA or cloned DNA) which is complementary to the DNA region being studied (Southern, 1975). Upon exposure of the nitrocellulose filter to x-ray film a specific band pattern is visualized. The extent of methylation of restriction sites in the gene sequence is determined by comparing the genomic band pattern to the one obtained when a cloned fragment of DNA containing the site under investigation in an unmethylated form is digested with the same enzyme.

Using this procedure, Bird and Southern showed that in rDNA of Xenopus laevis most Hpa II and Hha I sites were methylated, however, one particular site was unmodified in about 50% of the rDNA repeats. In contrast, amplified rDNA from this animal contains no detectable 5MeC. This method has since been employed to study the methylation patterns of several gene regions in various animal tissues.

Legend to Table I.

Restriction enzyme cleavage sites are from Roberts, 1978. The effects of methylation are from Mann and Smith, 1977; Bird and Southern, 1978; Sneider, 1980.

Table 1. Restriction Endonucleases Sensitive to 5 Methyl Cytosine

Endonuclease	Recognition Sequence Cleaved	Not Cleaved
Hha I	GCGC	GMeCGC
Hpa II	CCGG, MeCCGG	CMeCGG
Msp I	CCGG, CMeCGG	MeCCGG

2. Tissue-Specific Methylation Patterns of Active Genes

a. Globin Genes

One of the early reports on the DNA methylation patterns of globin genes demonstrated that certain Hpa II sites in the area of the chicken β-globin gene were unmethylated in erythrocyte DNA, but were only partially methylated in tissues where the gene is not expressed (McGhee and Ginder, 1979). When the extent of methylation at chicken β-globin Hha I sites was investigated, a lower level of methylation in tissues where the gene is active was seen (Mandel and Chambon, 1979). Similar results were obtained in an extensive study of the human γ-δ-β-globin locus (Van der Ploeg and Flavell, 1980). Some important observations were made during this research and are as follows:

(i) The extent of methylation in tissues where the genes are not active ranges from almost complete modification of the β-globin region to essentially none at all.

(ii) In the DNA of tissues expressing one or a combination of the genes, the area including and immediately surrounding the genes is methylated to a lesser extent than neighboring DNA regions.

These findings as well as the analogous ones obtained in a study of the rabbit β-globin region (Shen and Maniatis, 1980) suggest that if methylation plays a role in globin gene expression the sites where methyl group loss occurs will be very few and extremely specific.

An examination of the methylation pattern of the adult chicken α-globin locus revealed an undermethylation of a subdomain of this region in tissues synthesizing hemoglobin. The subdomain includes the coding sequences for the genes, the spacer DNA between the genes and the flanking sequence located to the 3' side of the genes. An association of the hypomethylated subdomain with the endogenous RNA polymerase II was demonstrated (Weintraub et al., 1981).

b. Non globin Genes

The methylation patterns in the regions of several genes other than globin genes were investigated. The following observations have been reported:

(i) The genes coding for ovalbumin, ovotransferrin (conalbumin) and ovomucoid appeared to be less methylated in the oviduct of laying hen than in tissues where these proteins are not synthesized (Mandel and Chambon, 1979).

(ii) Three Hpa II sites located in chicken δ-crystallin genes from embryonic lens were found to be undermethylated when compared to the same sites in tissues not expressing the genes (Jones et al., 1981).

(iii) Treatment of a mouse cell line resistant to cadmium induction of metallothionein-I with the hypomethylating agent 5-azacytidine resulted in the loss of methyl groups from all the Hpa II sites within the metallothionein-I gene in a population of cells selected for their inducibility (Compere and Palmiter, 1981).

c. Viral Genes

Restriction enzyme analysis of methylation levels of specific sequences has been applied to viral DNAs. The results of the research were quite similar to those seen when eucaryotic genes were studied. The findings are as follows:

(i) An inverse correlation appears to exist between the levels of methylation of specific integrated adenovirus type 12 DNA sequences and the extent of their expression in transformed cells (Sutter and Doerfler, 1980).

(ii) Herpes saimiri DNA sequence present in cell lines which do not produce detectable virus are extensively methylated. This same viral DNA when present in virus-producing lines is completely unmethylated (Desrosiers et al., 1979).

(iii) Proviral DNA of sarcoma virus is methylated in a nonpermissive cell line, but not in permissive cells (Guntaka, et al., 1980)

(iv) An inactive ev-1 endogenous retrovirus locus in chicken cells becomes both hypomethylated and transcriptionally active after exposure of the cells to 5-azacytidine (Groudine et al., 1981).

It should be noted, that at certain sites located within or around a gene, the degree of methylation cannot be correlated with the expression of the gene (Mandel and Chambon, 1979; van der Ploeg and Flavell, 1980).

3. In Vitro Methylation of Specific DNA Sequences and Gene Expression.

In order to study the effect of methylation on the biological activity of a gene, cloned thymidine kinase genes that were methylated in vitro with Hpa II methylase were introduced into thymidine kinase deficient cells. The efficiency with which methylated DNA transformed cells was found to be much lower than that of unmethylated DNA, although both DNAs had the same frequency of incorporation (Wigler et al., 1981). It was also observed that the in vitro modification of vector DNA was not well maintained by the cells. Only 10% of the clones contained methylated Hpa II sequences in the acquired gene (Pollack et al., 1980).

MATERIALS AND METHODS

I. Mouse Cells

A. FL Cell Culture

FL cells of strain 745A and its clones 5-86 (Scher and Friend, 1978), DS-19 and DR-10 (Ohta et al., 1978) were kindly provided by Dr. C. Friend, Dr. W. Scher, Dr. M.Terada and Dr. P. Marks. The cell lines were maintained in minimal Eagle's medium (MEM) (Grand Island Biological Co.) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories), 250 units/ml penicillin (Pfizer) and 0.2mg/ml streptomycin (Lilly). Inducing and hypomethylating agents were added to cultures that were inoculated with 0.5-1 x 10^5 cells/ml. Certified reagent grade DMSO (Sigma Chemical Co.) and butyric acid (Fisher) were added, unsterilized, to the medium in the concentrations given in the text. The other compounds were dissolved in the medium at concentrations 10 times that of the final ones indicated, filter-sterilized (0.2μ Millipore filter) and then diluted. The cultures were grown at 37°C in an atmosphere of 5% CO_2 plus 95% air. DR-10 cells were routinely passed through medium containing 240mM DMSO. DS-19 cells which are extremely sensitive to induction by DMSO were grown in the presence of 240mM DMSO for 4 days prior to harvesting the cells and isolating the DNA. Treated 745A cells in which the methylation of specific gene sequences was investigated were grown in the presence of either 4mM DL-ethionine for 4 days or 10μM 5-aza-CR (Sigma

Chemical Co.) for 20 hours (hr). Those cultures allowed to recover from 5-aza-CR treatment were separated from the compound by centrifugation (200 x g, 3min.), washed twice with sterile phosphate buffered saline (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 1mM $CaCl_2$, 1mM $MgCl_2$) (PBS), and resuspended in MEM 10% FCS at 4 x 10^5cells/ml for 48 additional hr. To determine the percentage of the cell populations containing hemoglobin, 100µl of 0.4% benzidine base and 2% hydrogen peroxide in 12% acetic acid was added to 200µl of the cell culture. The cells staining blue were counted (Orkin et al., 1975).

B. Preparation of Cellular DNA

FL cell nuclei were isolated by disruption of the cells with a nonionic detergent (nonidet P-40, Schlesinger Chemical Corp.) and centrifugation at 1000 x g for 10 minutes. The DNA was prepared from these nuclei using a procedure described by Jeffreys and Flavell,(1977). The nuclei were resuspended in 20 volumes (vol) of cold 150mM NaCl, 100mM ethylenediamine tetracetic acid (EDTA) (pH 8.0). The suspension was brought to a final concentration of 1% with sodium dodecyl sulfa e (SDS)and sodium perchlorate was added to a final concentration of 1M. The nuclei were then gently agitated with a glass rod for 30 minutes at room temperature. The lysate was extracted two times with an equal vol of chloroform-isoamylalcohol (24:1). The DNA was precipitated with 0.1 vol of 2M sodium acetate (pH5.6) and 1 vol of

isopropanol, spooled onto a glass rod, washed with 70% ethanol and dissolved into 0.1 x 50mM Tris HCl (pH 7.5), 100mM NaCl, 5mM EDTA, (TNE). The DNA solution was incubated with 20µg/ml of pancreatic ribonuclease (RNase) (Type II-A, Sigma Chemical Co.) for 1 hr at room temperature and then with proteinase K (100µg/ml in 1 x TNE, 1% SDS) (Merck) for 2 hr at 37°C. The solution was extracted with an equal vol of a phenol-cresol-8 hydroxyquinoline (100:14:0.1) mixture until a clear interphase was obtained. The DNA was precipitated with 0.1 vol of 2M sodium acetate (pH 5.6) and 0.54 vol of cold isopropanol, spooled, and redissolved in a buffer containing: 0.5M Tris HCl (pH 7.5), 0.1mM EDTA.

Adult DBA/2J mouse livers and kidneys were washed in cold saline (0.9% NaCl) and then cut into large pieces with a scissors. The tissues were homogenized with 0.33M sucrose, 4mM $CaCl_2$ (1g tissue/3ml) using a motor driven Teflon-glass Potter-Elvehjem homogenizer. The nuclei were collected by centrifugation at 600 x g for 10 minutes at 4°C. They were resuspended in the following solution: 150mM NaCl, 100mM EDTA (pH 8.0), 1% SDS, so that the final vol equaled 20mls. 100µg/ml of proteinase K was added to the nuclear suspension and it was incubated for 2 hr at 37°C. The proteinase K treatment was repeated a second time. Sodium perchlorate crystals were added to a final concentration of 1M, and the preparations were extracted with an equal vol of chloroform-isoamylalcohol. The DNA was precipitated with

0.1 vol of 2M sodium acetate (pH 5.6) and 1 vol of isopropanol, spooled, washed with 70% ethanol and dissolved in 0.1 x TNE. The rest of the DNA isolation procedure is identical to that described for FL cell DNA.

C. Isolation of DNA Methyltransferase and In Vitro Methylation of DNA

Cells were washed with PBS and then suspended in the following buffer: 10mM Tris HCl (pH 7.8), 320mM sucrose, 3mM $MgCl_2$, 0.3% Triton X-100 (Sigma Chemical Co.). Lysis was achieved by gentle pippetting of the cells. Nuclei were pelleted, washed two more times with the same buffer, and quick-frozen in a solution consisting of 0.32M sucrose, 1mM $MgCl_2$, 1mM potassium phosphate (pH 6.8). The procedures of Roy and Weissbach,(1975), were used to isolate the DNA methyltransferase and assay its activity. After thawing and adding NaCl to a final concentration of 0.3M, the nuclei were left on ice for 15 minutes. Further dissociation of the enzyme from chromatin is accomplished by homogenization in a ground glass dounce homogenizer. The suspension was centrifuged at 220,000 x g for 4 hr and the supernatant was then dialyzed for 4 hr with one buffer change, against 2 liters of the following buffer: 20mM Tris HCl (pH 7.4), 20mM NaCl, 0.5mM dithiothreitol (DTT) (Sigma Chemical C0.). Phenylmethanesulfonlyfluoride (PMSF) (Sigma Chemical Co.) is added to the enzyme solution which is stable for approximately two weeks at 4°C.

The DNA methyltransferase assay mix containing 0.1M Imidazole HCl (pH 7.5), 20mM EDTA, 0.5mM DTT, 5µg DNA, 10µCi (^{3}H) S-AdoMet (3-10Ci/mmole) and varying amounts of crude enzyme solution in a total vol of 0.2ml is incubated for 30 minutes at 37°C. Proteinase K (80µg) is then added to the reaction mix which is incubated for 20 more minutes at 37°C. Deproteinization is achieved by treatment with 0.5M NaOH for 10 minutes at 60°C. DNA is then precipitated with perchlorate, washed onto glass fiber filters (Whatman GFA, 2.4cm), and counted in a liquid scintillation counter.

D. Base Analysis of FL Cell DNA by High Pressure Liquid Chromatography (HPLC)

FL cell DNA purified from 2 x 10^8 cells was methylated in vitro. The addition of sarkosyl to 0.6% terminated the reaction after 1 hr. It was then treated with 20µg/ml of RNase at 30°C for 30 minutes. The DNA was reisolated from the incubation mixture by banding in a CsCl/$CsSO_4$ (The Harshaw Co.) gradient at 88,500 x g for 18 hr. (Shaw et al., 1975). The DNA was hydrolyzed in 250µl of 70% perchloric acid by heating at 100°C for 1 hr. The acid was neutralized with 5N KOH and $KClO_4$ was removed by centrifugation. A sample containing approximately 4300 cpm was diluted to 100µl with distilled H_2O and loaded into a 0.4 x 25cm Partisil SCX column (Whatman Co.) which had been preequilibrated with 0.02M NH_4PO_4 (pH2.3). Isocratic elution at 500 psi and ambient temperature (23°C) with a flow rate of 1.25ml/min gave the following elution times: thymine, 6.5 min; guanine, 11.2 min;

cytosine, 14.5 min; adenine, 19 min; and 5-methylcytosine, 23 min. Fractions of column effluent containing 1ml were collected and assayed for radiolabel in a liquid scintillation counter using a water-accepting fluor (Aquafluor, National Diagnostics). Approximately 4170 cpm coeluted with marker 5MeC and no other peak of radiolabelled material was observed. An ISCO Model UA-5 detector (Instrumentation Specialities Co.) with a low-volume flow cell was used to monitor column effluent at 245nm.

II. Cloned Globin Genes

The α_1-globin and β-globin^{major} genes from the BALB/c mouse genome have been cloned into the arms of λgtWES· B (Tilghman et al., 1977; and Leder et al., 1978). The genes which are carried on two different Eco RI fragments (α_1-9.7kb and β^{maj}-7kb) have been sequenced in the areas including and immediately surrounding the coding regions (Konkel et al., 1979; and Nishioka et al., 1979) and have been generously provided by Dr. P. Leder.

A. Transfection with Recombinant Phage DNA

Bacteriophage λgtWES·$\lambda\beta$ is propagated in E. coli strain LE392. Transformation of LE392 with phage DNAs was carried out using a procedure described by Tiemeier (Tiemeier et al., 1977). Sterile techniques and solutions were used throughout the transfection procedure and except where indicated, all steps were done at 4°C. An overnight culture of LE392 was diluted 1/100 in fresh L broth (1% tryptone, 0.5% yeast extract, (DIFCO), 0.1% D-

glucose (Merck) plus 50µg/ml thymidine (Sigma Chemical Co.) and grown to a density of 2 x 10^8 cells/ml (A_{590}=0.6) at 37°C with vigorous shaking. The cells were collected by centrifugation at 4000 x g for 5 minutes and washed with an equal vol of the following buffer: 25mM Tris HCl (pH7.5), 10mM NaCl. They were then recollected by centrifugation at 4000 x g for 3 minutes, resuspended in a buffer containing 0.5 vol of 25mM Tris HCl (pH7.5), 10mM NaCl, 50mM $CaCl_2$ and incubated on ice for 20 minutes. After centrifugation at 2000 x g for 10 minutes and resuspension in 0.2 vol of the above $CaCl_2$ solution, the 'competent' cells were immediately transfected. 0.4ml of the cells were added to 0.2 ml of the following buffer: 25mM Tris HCl (pH7.5), 10mM NaCl, 50mM $CaCl_2$, which contained 60-80ng of the recombinant DNAs. The suspensions were incubated for 60 minutes on ice and then for 60 seconds at 30°C. One drop of a freshly grown LE392 culture (5 hrs) was plated with the transfected cells. The plates (L broth plus 2% agar (Difco)) were incubated overnight at 38°C. Under these condition the transfection efficiency of intact DNA is 500 to 2000 plaques/ng DNA.

B. Preparation of Phage Stocks

Freshly grown LE392 cells (5ml) were collected by centrifugation at 2000 x g for 10 minutes and resuspended in 10mM $MgSO_4$. A single plaque was transferred from a plate as an agar plug with a sterile pasteur pippette to a 15ml sterile glass screw capped tube containing 50µl of the above

cell suspension and the tube was placed at 30°C for 5 min. L broth (2.5ml) brought to a final concentration of 2mM with $MgSO_4$ (sterile) was added to the tube which was incubated with vigorous shaking at 38°C for 12hrs. Three drops of chloroform were then added and the cultures were shaken for an additional 5 minutes at 38°C. Debris was spun out of the cell lysate at 5000 x g for 5 minutes. Sterile $MgSO_4$ was added to a final concentration of 10mM and the recombinant phage solutions were stored at 4°C for a maximum of 1 week.

A freshly grown culture of LE392 was resuspended in 10mM $MgSO_4$, and 20ml of the suspension was inoculated with 4 x 10^8 phage particles for 15 minutes at 30°C before diluting the cells into one liter of L broth containing 2mM $MgSO_4$. The bacteria and phage were grown for 12 hr at 38°C with vigorous shaking. Phage lysis was enhanced by the addition of 20 ml of chloroform and NaCl to a concentration of 0.6M and 10 more minutes of shaking at 38°C. Debris was pelleted during a 10 minute centrifugation at 4000 x g in 150ml glass bottles (Corex, Du Pont Instruments) and discarded. Polyethylene glycol 6000 (Baker) was dissolved into the supernatant which was brought to a final concentration of 12mM $MgSO_4$ and was then left to sit at 4°C for 90 minutes. The resulting precipitate was collected by centrifugation and resuspended in a buffer made up of 50mM Tris HCl (pH7.5), 10mM $MgSO_4$. The phage suspension was extracted with an equal vol of chloroform to remove the polyethylene glycol and then

0.75μg of CsCl was added per ml. During centrifugation for 18 hr at 178,000 x g the phage band in the CsCl gradients that are formed. Approximately 1 to 5 x 10^{11} phage particles make up this band which served as the stock solution of λgtWES· plus inserted gene. The solution is stable for at least 6 months at 4°C.

C. Phage DNA Isolation

The phage suspension which has been removed from the CsCl gradients into a syringe was dialyzed against 1 liter of buffer consisting of 20mM Tris HCl (pH7.5), 100mM NaCl, 10mM $MgSO_4$, for 2 hr. The buffer was changed at that time and dialysis continued for 2 more hr. Deoxyribonuclease (DNase) I (10μg/ml, Worthington DPFF Electrophoretically pure) was added and the mixture was incubated for 30 min at 37°C. The enzyme reaction was stopped with the addition of 30mM EDTA. Pronase (1mg/ml, Calbiochemicals) was added to the phage suspension which was then dialyzed against 1 liter of a buffer containing 20mM Tris HCl (pH7.5), 500mM NaCl, 2.5mM EDTA for 90 min. The DNA was extracted 2 times with an equal volume of a phenol-cresol-chloroform mixture, precipitated with 2 vol of cold ethanol, and suspended in the following buffer: 20mM Tris HCL (pH7.5), 0.1mM EDTA.

D. Restriction and Electrophoresis of FL Cell, Cloned Globin and Phage DNAs

Restriction endonuclease cleavage of phage DNA and cloned gene fragments was performed with 0.5 to 1μg of DNA

and 0.5 to 10 units of enzyme (New England Biolabs) in 10µl for 1 hr at 37°C. The enzymes and conditions used were as follows: Bam HI-6mM Tris HCl (pH7.9), 6mM $MgCl_2$, 150mM NaCl, Eco RI-100mM Tris HCl (pH7.2), 5mM $MgCl_2$, 50mM NaCl, 2mM β-mercaptoethanol, Hha I-6mM Tris HCl (pH7.4), 6mM $MgCl_2$, 50mM NaCl, 6mM β-mercaptoethanol, Hind III-20mM Tris HCl (pH7.5), 7mM $MgCl_2$, 60mM NaCl, Hpa II-10 Tris HCl (pH7.4), 7mM $MgCl_2$, 1mM DTT, Msp I-10mM Tris HCl (pH7.4), 10mM $MgCl_2$, 6mM KCl, 1mM DTT, Sac I-6mM Tris HCl (pH7.4), 6mM $MgCl_2$, 6mM β-mercaptoethanol, and Taq I-6mM Tris HCl (pH7.4), 6mM $MgCl_2$, 6mM NaCl, 6mM β-mercaptoethanol, 100µg/ml gelatin (incubated at 60°C). Electrophoresis of DNA was in 1% agarose (Seakem ME, Marine Colloids) horizontal slab gels at 50mAmps for 4 hr with buffer E (40mM Tris Acetate (pH7.9), 5mM sodium acetate, 1mM EDTA). The gel was then stained with ethidium bromide (Biorad) and the DNA was visualized under a UV lamp.

FL cell DNA was first digested with Eco RI and then with either Msp I, Hpa II, or Hha I under the conditions described above. A typical reaction mixture contained 100µg of DNA and 400 to 1000 units of enzyme in a volume of 1 ml. Hha I digestions were performed in a 2 ml vol. The enzyme was allocated such that half of the final amount was added at the beginning of the 4 hr incubation and half was added 2 hr after the reaction had begun. To monitor the extent of the digestion, a sample (2.5µg of DNA) of the mixture was removed after each addition of enzyme and pBR322 DNA (0.5µg) was

added (pBR322:FL cell DNA=1:5). Cleavage of the pBR322 DNA was analyzed by agarose gel electrophoresis. Once the digestion was determined to be complete, the sample was extracted with phenol-cresol-chloroform and brought to 0.3M with sodium acetate (pH5.6). The DNA was precipitated with 2 vol of cold ethanol. The DNA which was to be digested with two enzymes, was precipitated two times after the first digestion. The first precipitation was with sodium acetate and 2 vol of ethanol. Following this the DNA was suspended in distilled H_2O, brought to a final concentration of 2M with ammonium acetate and then reprecipitated with 3 vol of cold ethanol. All FL cell DNAs were suspended in buffer E.

E. Preparation of Globin Gene Hybridization Probes

λgtWES·βM_2 DNA was digested with Eco RI and λgtWES·α_1 DNA was digested with Eco RI and then Hind III. The DNA was then extracted with phenol-cresol-chloroform, precipitated with sodium acetate and ethanol and dissolved in buffer E. The digested DNA (maximum of 200 μg DNA/gel) were electrophoresed in 0.7% low gelling temperature agarose gels (Marine Colloids) with buffer E for 18 hr at 15mAmps. The gel was stained with ethidium bromide (1μg/ml) and the band consisting of the cloned gene was cut out. The band was heated at 69°C until the gel was melted, cooled to 37°C, diluted to a final concentration of 0.3% agarose with a buffer containing 50mM Tris HCl (pH8.0), 0.5mM EDTA and extracted with a 0.75 vol of freshly distilled phenol (37°C)

saturated with the above Tris-EDTA buffer. The DNA was precipitated by bringing the solution to a final concentration of 0.3M with sodium acetate (pH5.6) and then adding two vol of cold ethanol. It was dissolved into 100µl of distilled H_2O and reextracted with phenol. The DNA was precipitated again with sodium acetate and ethanol, dissolved into distilled H_2O, and reprecipitated with 2M ammonium acetate and 3 vol of ethanol. The 7.0kb Eco RI β^{maj} DNA fragment (100µg) was digested with Taq I and 100µg of the 9.7kb Eco RI α_1 DNA fragment was digested with Sac I. The digests were brought to 0.4% with sarkosyl, and electrophoresed on low gelling temperature agarose gels. The appropriate DNA fragments were isolated as described above. Using this procedure, 60 to 70% of the cloned DNA fragment was recovered.

F. Nick Translation

DNase I (Sigma Chemical Co., DNEP, Electrophoretically pure) was dissolved into 10mM HCl (1mg/ml). The solution was divided into 50µl aliquots and frozen at -20°C. On the day of the assay, one aliquot was thawed into 450µl of a buffer containing 10mM Tris HCl (pH7.5), 5mM $MgCl_2$, 1mg/ml bovine serum albumin (BSA), and incubated at 4°C for 2 hr. This solution was diluted again immediately before it was added to the reaction mixture, with the Tris-$MgCl_2$-BSA buffer so that the final concentration of enzyme was 133ng/ml.

The following procedure is based on one described by

Rigby et al., (1977). When nick translating the 3.8kb Taq I βM_2 DNA fragment, either 50pmol of α^{32}P-dCTP (Amersham 2000-3000 Ci/mmole) dissolved in 50% ethanol was vacuum dried and redissolved into the reaction mixture or 50 pmol of α^{32}P-dCTP (New England Nuclear, 850Ci/mmole) dissolved in an aqueous solution was added to the reaction mixture. When nick translating the 3.0kb Sac I α_1 DNA fragment, either 50 pmol of α^{32}P-dCTP and α^{32}P-TTP (Amersham, 2000-3000Ci/mmole) dissolved in 50% ethanol were vacuum dried and redissolved into the reaction mixture or α^{32}P-dCTP and α^{32}P-TTP (New England Nuclear, 850Ci/mmole) dissolved in aqueous solutions were added to the reaction mixture. The reaction mixture which was assembled on ice and had a vol of 25µl after all the enzymes were added, consisted of: 50mM Tris HCl (pH7.5), 5mM $MgCl_2$, 25µM dATP, 25µM dGTP, 3µM dCTP, 3µM TTP (25µM when the DNA was the 3.8kb Taq I βM_2 DNA fragment) and 250ng DNA. DNase I (2µl of 133ng/ml solution) was added to the reaction mixture which was then incubated at 37°C for 30 seconds. The tube was put back into ice and 3 units of E. coli DNA Polymerase I (Boehringer Mannheim, grade 1) were added to the reaction before it was incubated at 14°C for 4 hr. The nick translation was stopped by the addition of 0.1% SDS, 12.5mM EDTA. Unincorporated deoxyribonucleotides were separated from the radiolabelled DNA by Sephadex-G50 chromatography (Pharmacia) (column, 16cm x 1cm) in a buffer containing 10mM Tris HCl (pH7.5), 10mM NaCl, 0.1mM EDTA, and the excluded

fractions were pooled. A specific activity of 10^8 cpm/μg 3.8kb Taq I βM_2 DNA and 2 x 10^8 cpm/μg 3.0kb Sac I λ_1 DNA were generally obtained.

The radiolabelled probe was denatured by adding NaOH to a final concentration of 0.3M, and it was neutralized by the addition of HCl. The pH of the solution was checked on indicator paper(Fisher) before using in an hybridization reaction.

G. DNA Transfer and Hybridization

The procedure described by Wahl et al., (1979) was used. Restriction endonuclease digested DNA was electrophoresed in agarose horizontal slab gels (10cm x 15cm x 0.9cm) in buffer E for 18 hr at 15mAmps. The gel was placed in a pan at room temperature with 250ml of 0.25M HCl for 15 minutes. The liquid was decanted and the acid wash was repeated. The gel was briefly washed with distilled H_2O to remove any residual acid, and then was washed with two 250ml portions of a solution containing 0.5M NaOH, 1M NaCl for 15 minutes each. The gel was neutralized in 250ml of the following buffer: 0.5M Tris HCl (pH7.4), 3M NaCl, for 30 minutes. The liquid was decanted and the procedure was repeated.

Transfer of the DNA from agarose gels to nitrocellulose (Schleicher and Schuell, 0.45μ ;was performed at room temperature for 20 hr in the following manner: two sheets of Whatman 3MM paper (15cm x 15cm), saturated with a solution containing 3M NaCl and 0.3M sodium citrate was

placed on top of a sheet of plastic wrap; the gel was placed on top of the saturated paper; the plastic wrap was placed around the edges of the gel to prevent contact between the dry paper to be placed above the gel and the wet paper beneath the gel; the nitrocellulose (10cm x 13cm) was placed on top of the gel and all air bubles between the two were removed; and then two sheets of dry Whatman 3MM (15cm x 15cm), a 3 inch layer of paper towels and a light weight were place on top of the nitrocellulose.

The nitrocellulose was briefly washed with 200ml of a solution containing 0.3M NaCl and 0.03M sodium citrate, air dried and then baked in a vacuum oven at 80°C for 4 hr. It was placed into a plastic pouch (Kopak/Scotchpak, 6½" x 8") which was heat sealable with 10ml of the following pre-hybridization solution: 50% formamide (Fluka), 750mM NaCl, 75mM sodium citrate, 5 times Denhardt's reagent (Denhardt's reagent contains 0.02% wt/vol each of BSA, polyvinyl pyrrolidone (Sigma Chemical Co.), and Ficoll (M_r 400,000, Pharmacia)),50mM sodium phosphate (pH6.5), 1% glycine and 500µg of sonicated, denatured salmon sperm DNA (Sigma Chemical Co.) per ml. The pouch was incubated at 42°C for 8 hr and then the liquid was removed from the pouch.

The following hybridization mixture (10ml) was prepared: 50% formamide, 750mM NaCl, 75mM sodium citrate, Denhardt's reagent, 20mM sodium phosphate (pH6.5), 100µg of sonicated, denatured salmon sperm DNA per ml and 10% sodium

dextran sulfate 500 (Pharmacia). The solution was vortexed and 9ml were added to the pouch. The remaining ml was heated to 65°C for 5 minutes to facilitate mixing with the probe, and 5 x 10^6 cpm of radiolabelled probe was added to the heated hybridization solution. The material was vortexed and added to the pouch which was then sealed near the nitrocellulose without trapping air bubbles. The pouch was incubated at 42°C for 12-14 hr.

The nitrocellulose was washed with two 250ml portions of a solution containing 300mM NaCl, 30mM sodium citrate, 0.1% SDS for 15 minutes each at room temperature. It was next washed with two 250ml portions of the following solution: 15mM NaCl, 1.5mM sodium citrate, 0.1% SDS, at 50°C for a total of 60 minutes, followed by 2 washes in the same solution at room temperature for 5 minutes each. The nitrocellulose was air dried and exposed to x-ray film (Kodak XR-5) at -70°C using Du Pont Lightning Plus intensifying screens for 24-96 hr.

The chemicals used in this study, if not already specified, were from Fisher or Sigma Chemical Co.

RESULTS

I. In Vitro Methylation of DNA from FL Cells

A. Strain 745A and Clone 5-86

DNA methylation occurs a short time after replication (Burdon and Adams, 1969). Thus, DNA prepared from logarithmically growing cells is not completely methylated *in vivo* and can accept methyl groups from methyltransferase *in vitro*. (Table 2) Ten to twelve pmoles (pmol) of methyl groups were transferred to 100 µg of FL cell DNA during a 30 minute incubation, using (^{3}H) S-AdoMet and a 0.3M NaCl extract as a source of enzyme. However, the DNA isolated from cells grown in the presence of L-ethionine, butyrate, DMSO, hexamethylene-bisacetamide (HMBA) or pentamethylene-bisacetamide (PMBA), compounds which induce a considerable number of the cells to synthesize hemoglobin (measured by the percentage of cells staining positive with benzidine (B+)), accepts 3 to 5 times as many methyl groups (30-50 pmol). The data extends the results of previous studies done using only strain 745A which showed that the cells treated with chemicals causing their differentiation synthesize DNA which is a better substrate for *in vitro* methylation than DNA from untreated cells (Christman *et al.*, 1977). It should be noted that a direct correlation does not exist between the level of FL cell DNA hypomethylation and FL cell differentiation.

Legend to Table 2.

DNA methyltransferase assays were as described in Materials and Methods. Each value represents the average incorporation for three to give assays on at least three separate DNA preparations from cells grown in the presence of the indicated inducing agent for 3-4 days. At most, the variation from these values was ± 5 pmol. Analysis of acid hydrolysates of the DNA reisolated after in vitro methylation (see Materials and Methods) by high pressure liquid chromatography demonstrated that all recovered radiolabel from (^{3}H) Me-SAdoMet was present as 5MeC.

ND, not determined.

B+, benzidine positive cells in cultures exposed to the inducing agents for 5 days.

Table 2. In Vitro Methylation of Mouse Erythroleukemia Cell DNA by Mouse Erythroleukemia Cell DNA Methyltransferase.

Treatment of cells before DNA isolation	DNA Source: Strain 745 A — pmol of CH_3/100 µg of DNA	Strain 745 A — % B+	Clone 5-86 — pmol of CH_3/100 µg of DNA	Clone 5-86 — % B+
None	10.8	1-2	12.3	1
DMSO (240 mM)	41	78	40.6	80
L-Ethionine (4 mM)	54.6	40	45	35
Butyrate (2 mM)	36.2	82	ND	ND
HMBA (4 mM)	30.7	95	33.5	93
PMBA (4 mM)	31.8	90	ND	ND

B. Clone DR-10

Results obtained with DNA from a DMSO-resistant clone, DR-10, are significantly different from those obtained with strain 745A and clone 5-86 (Table 3) After 4 days growth in medium containing L-ethionine, HMBA, and PMBA, up to 80% of the DR-10 cells accumulate hemoglobin, and DNA isolated from these cells accepts 30-40 pmol of methyl groups per 100 µg of DNA, the same range observed with strain 745A. However, when the cells are grown in the presence of DMSO for 4 days, hemoglobin is synthesized by only 1 to 2% of the cell population. Even though the cells take up DMSO (Ohta et al., 1978), the ability of DNA to act as a methyl acceptor in vitro is the same as seen in untreated cells.

If DR-10 cells are grown in the absence of DMSO for an extended period of time, they become sensitive to the agent. After 4 days growth in the presence of DMSO, fifty-five percent of the cells in the culture stain positive with benzidine, and hypomethylation of the DNA is observed once more (34 pmol of methyl groups/100 µg of DNA). These results indicate as did previous ones with inhibitors of differentiation (Christman et al., 1980), that only when an agent induces differentiation in a cell will the DNA synthesized in that cell become hypomethylated.

Legend to Table 3.

All details as in Table 2. Stocks maintained in medium containing 240mM DMSO were grown in the absence of DMSO for 1-2 weeks before beginning the experiment. DNA from stock cultures accepted 10.5 pmol of methyl groups per 100µg of DNA. Methyltransferase used in these experiments was isolated from FL cells of strain 745A, however, similar results were obtained with enzyme from DR-10 cells.

Table 3. In Vitro Methylation of DNA from DR-10 Cells

Treatment of cells before DNA isolation	pmol of CH_3/100 µg of DNA	% B+
Cells from cultures maintained in DMSO		
None	11.5	1-2
DMSO (240 mM)	12.8	1-2
L-Ethionine (6 mM)	35.5	80
HMBA (4 mM)	41.4	55
PMBA (4 mM)	44	60
Cells from cultures maintained 6 mo without DMSO		
None	10.8	1
DMSO (240 mM)	34.5	55

II. Methylation of the α- and β-Globin Gene Regions of Mouse Cells.

Since it was established that during FL cell differentiation a change in methylation levels of DNA occurred, an attempt was made to localize the region in the genome where the loss of methyl groups occurred. Classical fractionation techniques were employed to separate the DNA from both un-induced and induced cells into (a) main band and satellite; (b) highly repetitive, middle repetitive, and unique sequences; and (c) pyrimidine tracts (Christman, 1979). The amount of 5MeC in each of these fractions was measured by HPLC and it was found to be the same whether the DNA was isolated from differentiated or undifferentiated cells. When the DNA was methylated *in vitro* and then fractionated, a higher level of methyl acceptance was observed in all the fractions of DNA from treated cells, but the distribution of radiolabelled methyl groups in the various DNA fractions was identical in both cell populations (Christman, 1979; and Christman *et al.*, 1980). It was apparent that the methods employed were not sensitive enough to locate specific areas in the genome where undermethylation occurs during FL cell differentiation. Restriction endonucleases which are inhibited by methylation of cytosine in their recognition sequences seemed more likely to provide a sensitive enough tool for investigating the loss of specific 5MeC residues from FL cell DNA. As described below, some of these enzymes were used to determine

the patterns of methylation in and around genes whose expression is augmented during FL cell differentiation (globin genes), to see if these regions become hypomethylated as the cells mature along the erythroid pathway.

A. Localization of Hpa II (Msp I) and Hha I Restriction Endonuclease Sequences in Two Cloned Mouse Globin Genes

1. Restriction Endonuclease Digestion and Mapping of the β-Globin^{major} Gene

A 7kb Eco RI*DNA fragment containing the mouse β-globin^{major} (β^{maj}) gene has been inserted into λgtWES 'arm' to form the recombinant phage λgtWES.βM_2 (Tilghman et al., 1977) (Figure 1). Digestion of this phage with Eco RI and electrophoresis in a 1.0% agarose gel results in the separation of three fragments corresponding to the two λgtWES 'arms' (21kb and 14kb) and the 7kb β^{maj} gene fragment (βM_2 DNA) (lane a). βM_2 DNA can be isolated from low gelling temperature gels (lane b), as described in Materials and Methods. One Hpa II (Msp I) site (lane c) and one Hha I site (lane f) are located near the center of the gene fragment. Cleavage by either one of these enzymes results in two pieces of DNA of approximately 3.5kb, indicating that the two recognition sites lie close together. Taq I digestion of βM_2 DNA produces 4 pieces with the following sizes: 3.8kb, 1.8kb, 1.2kb and 0.2kb (lane d). Double digests with Taq I and Hpa II (Msp I) (lane e) or Taq I and Hha I (lane g) reveal

*The recognition sequences of the restriction endonucleases used in the following studies are listed in Tables 1 and 4.

Legend to Table 4.

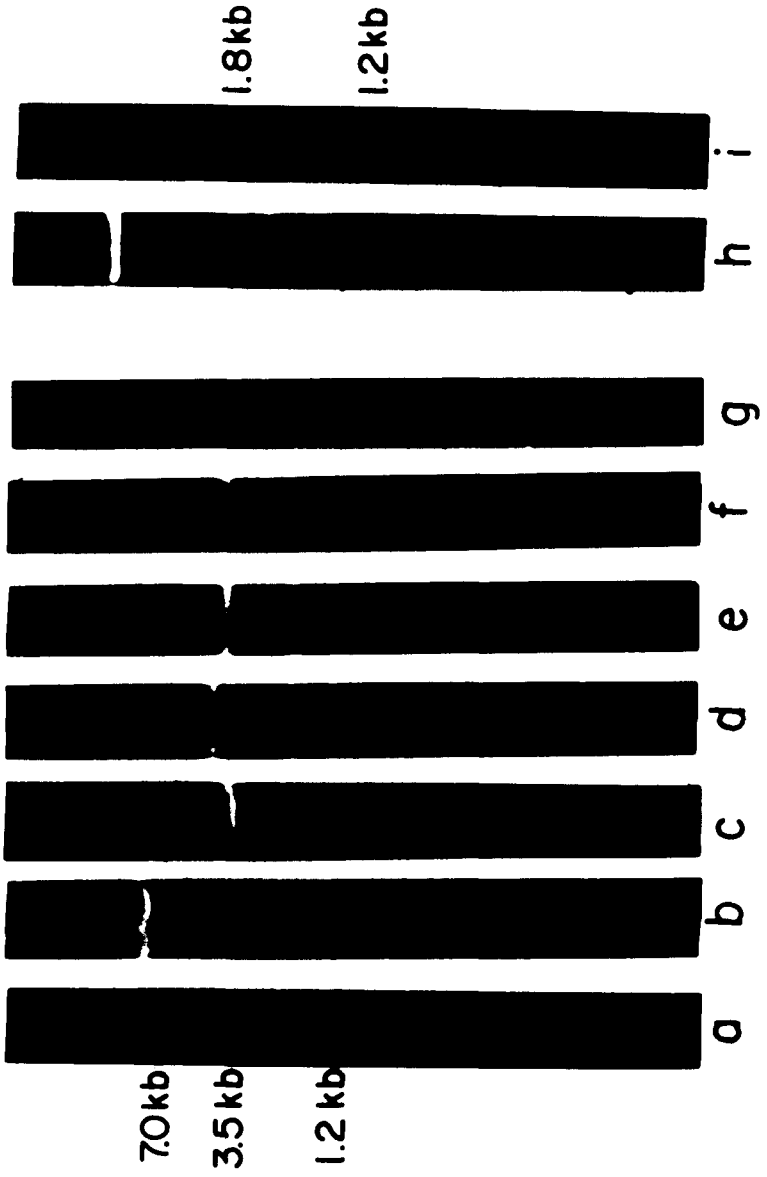
Restriction enzyme recognition sites are from the following sources: Bam HI-Wilson and Young, 1975 and Roberts et al., 1977; Eco RI-Hedgpeth et al., 1972, Dugaiczyk et al., 1974 and Greene et al., 1974; Sac I-Roberts, 1978; Taq I- Sato et al., 1977.

Table 4. Restriction Endonucleases and Their Recognition Sequences

Endonuclease	Recognition Sequence
Bam HI	G↓GATCC
Eco RI	G↓AATTC
Sac I	GAGCT↓C
Taq I	T↓CGA

Figure 1. Restriction Endonuclease Digestion of λgtWES·βM_2 DNA and βM_2 DNA.

DNA was cleaved and electrophoresed on a 1% agarose slab gel as described in Materials and Methods. The DNA is visualized by staining with ethidium bromide. (Lane a) Eco RI digest of gtWES·βM_2 DNA; (lane b) purified βM_2 DNA. (Lane c) Msp I (the residual 7kb band is due to incomplete digestion); (lane d) Taq I; (lane e) Taq I and Msp I; (lane f) Hha I; (lane g) Taq I and Hha I; (lane h) Bam HI; and (lane i) Taq I and Bam HI.


1.8kb
1.2kb
i
h
g
f
e
d
c
b
a
7.0kb
3.5kb
1.2kb

that the Hpa II and Hha I sites are located within the largest Taq I fragment (3.8kb) approximately 600bp in from one end. Digestion with Bam HI shows a single Bam HI site situated 2.2kb in from one end of βM_2 DNA. Since restriction by both Bam HI and Taq I leads to the disappearance of the band corresponding to a 3.8kb Taq I piece of DNA (lane h) and to the creation of 2 fragments corresponding to DNA pieces of 1.6kb and 1.2kb (lane i), the Bam HI site is within the 3.8kb Taq I fragment.

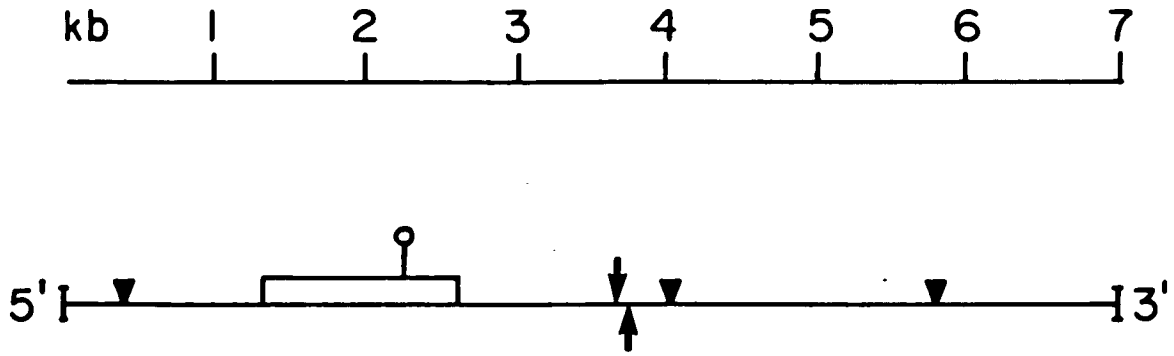
By combining the information obtained from the restriction endonuclease digestions of βM_2 DNA and the published sequence of the β^{maj} gene (Konkel et al., 1978; and Konkel et al., 1979), a restriction map of the 7kb Eco RI fragment could be constructed (Figure 2). The coding sequence of the β-globin^{major} gene begins approximately 1.2kb in from the 5' end of the βM_2 DNA and lies between the 2 Taq I sites which are separated by 3.8kb. The Bam HI site is located 1kb to the 3' side of the start of the structural gene sequence. Therefore, both the Hpa II (Msp I) and Hha I sites are approximately 1kb downstream from the 3' end of the coding segments.

2. Restriction Endonuclease Digestion and Mapping of the Mouse α_1-Globin Gene

The α_1-globin gene is located on a 9.7kb Eco RI fragment which has been cloned within λgtWES·α_1 (Figure 3).

Figure 2. Restriction Endonuclease Map of the Eco RI Fragment Containing the β-Globin^{major} Gene.

The positions of cleavage sites were determined by both restriction endonuclease digestion of βM_2 DNA (see Figure 1) and sequencing of the β-globin^{major} gene (Konkel et al., 1978; Konkel et al., 1979).



I Eco RI

Bam HI

Msp I (Hpa II)

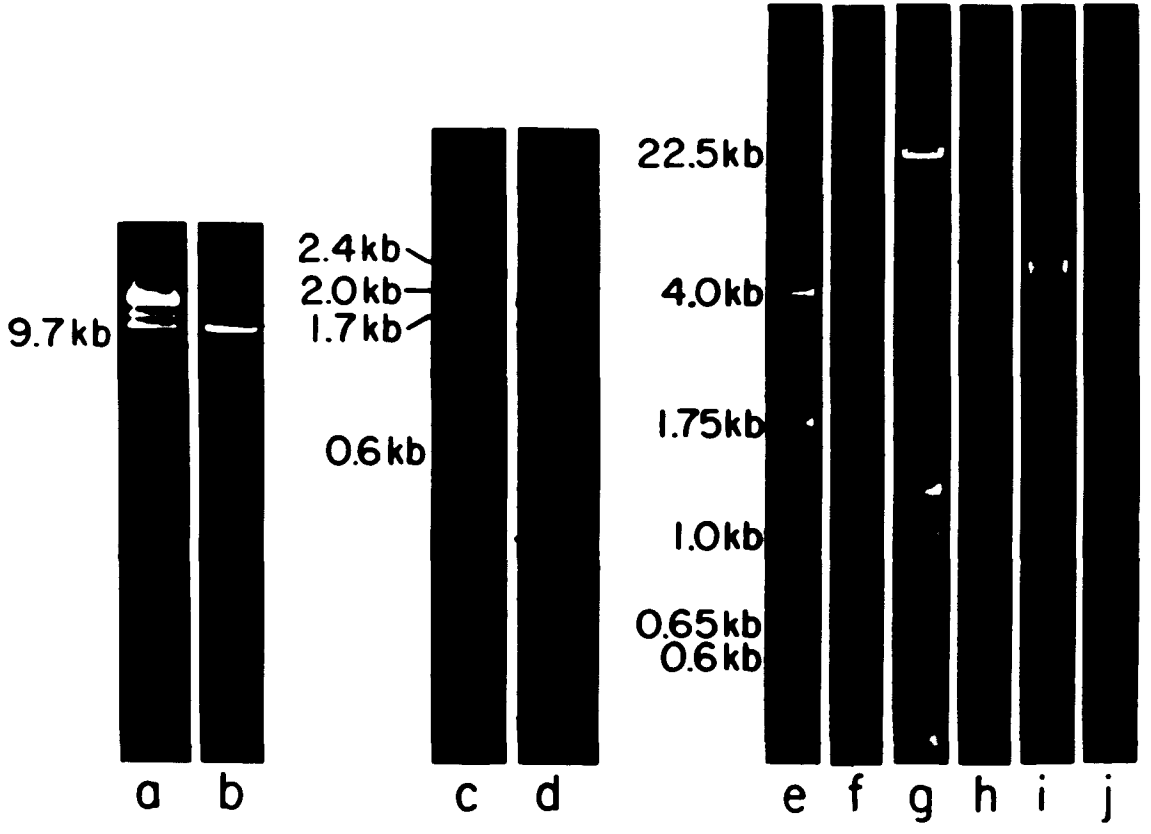
Hha I

Taq I

Coding sequence

Figure 3. Restriction Endonuclease Digestion of λgtWES·αβ$_1$ DNA and αβ$_1$ DNA.

Conditions are the same as described in Figure 1. (Lane a) Eco RI digest of λgtWES·αβ$_1$ DNA; (lane b) purified αβ$_1$ DNA. All of the following digests are of αβ$_1$ DNA. (Lane c) Hha I; (lane d) Hha I and Sac I; (lane e) Msp I; (lane f) Sac I; (lane g) Molecular weight markers - Hind III cI857 DNA fragments: 22kb, 9.4kb, 4.7kb, 2.3kb, and 1.9kb. Hae III ΦX 174 DNA fragments: 1.35kb, 1.07kb, 0.87kb, and 0.6kb; (lane h) Msp I and Sac I; (lane i) Bam HI; and (lane j) Bam HI and Msp I.

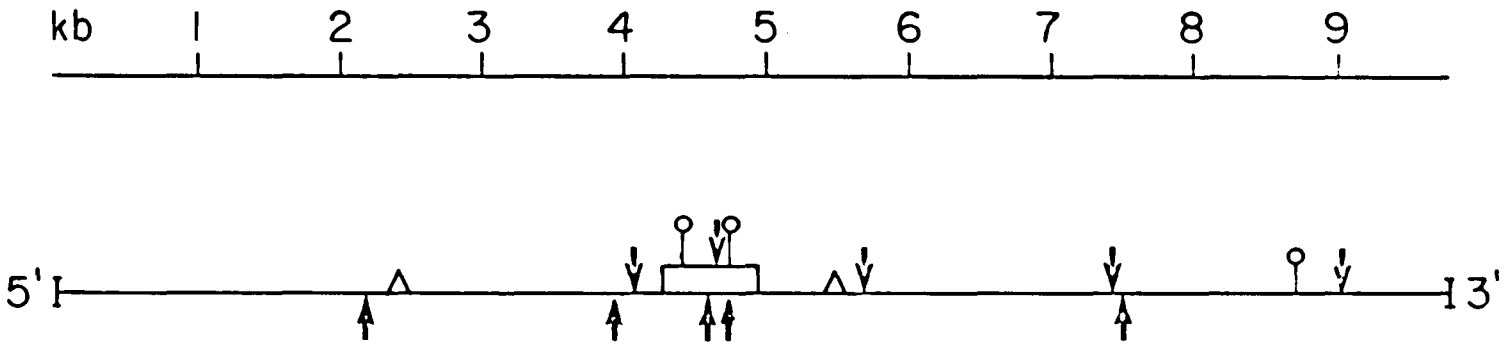

9.7kb
2.4kb
2.0kb
1.7kb
0.6kb
22.5kb
4.0kb
1.75kb
1.0kb
0.65kb
0.6kb
a
b
c
d
e
f
g
h
i
j

Eco RI digestion of the phage releases the inserted gene fragment (α_1 DNA) (lane a, bottom band) from the λgtWES 'arms' (top bands). When the isolated 9.7kb piece (lane b) is treated with Hpa II (Msp I) (lane c) six bands are seen on the agarose gel indicating that the enzyme cleaves at 5 sites. However, double digestion with Hpa II (Msp I) and Bam HI reveals that there are actually 6 Hpa II sites. The 1.75kb Hpa II band contains 2 DNA fragments of similar size, since Bam HI cleavage of the DNA in this band yields 3 pieces: 1.75kb, 1.45kb and 0.3kb. Digestion of α_1 DNA with Sac I creates 6 fragments of the following sizes: 3.0kb, 2.4kb, 2.0kb, 1.3kb, 0.7kb and 0.4kb (lane f). Two of the Hpa II sites are found to be situated with the 3.0kb Sac I fragment since treatment of the Sac I digest with Hpa II (lane h) results in the formation of 3 smaller fragments, 1.7kb, 0.7kb and 0.6kb, from the 3.0kb DNA fragment. Digestion with Hha I plus Sac I (lane d) produces 4 fragments from the 3.0kb Sac I fragment, (0.65kb, 0.63kb, 0.6kb and 0.12kb). Thus, 3 of the 7 Hha I sites (lane c) in the Eco RI fragment lie between 2 Sac I sites that are separated by 3.0kb.

Figure 4 presents a restriction endonuclease map of the 9.7kb Eco RI fragment containing the α_1-globin gene. Only those sites that are relevant to this study have been presented. The coding sequence of the gene begins approximately 2.2kb downstream from the 5' end of the Eco RI

Figure 4. Restriction Endonuclease Map of the Eco RI Fragment Containing the α_1-Globin Gene.

The positions of cleavage sites were determined by both restriction endonuclease digestion of α_1 DNA (see Figure 2) and sequencing of the α_1-globin gene (Nishioka and Leder, 1979).



I Eco RI

Δ Sac I

Msp I

Hha I

Bam HI

Coding sequence

fragment and lies between the 2 Sac I sites that are 3.0kb apart. Sequencing of the gene (Nishicka and Leder, 1979) has revealed 3 Hha I sites located within and immediately surrounding the structural sequence. One site is 335bp upstream from the 5' end of the coding segments and the other two sites are downstream (320bp and 430bp). One Hpa II site was detected 210bp 5' to the gene, while a second Hpa II site was discovered 360bp within the gene sequence.

A combination of single and double digests of α_1 DNA allowed the positioning of the other Hha I and Hpa II sites seen on the map. The Hpa II sites are 0.3kb, 2kb and 3.75kb downstream from the 3' end of the structural gene and the Hha I sites are 2kb to the 3' side and 2kb to the 5' side of the coding segments.

B. Detection of 5MeC in the Globin Gene Regions of Differentiating FL Cells

1. Restriction Endonuclease Digestion of FL Cell DNA

High molecular weight FL cell DNA was prepared by making use of a procedure by Jeffreys and Flavell, (1977) which allowed for the purification of a large quantity of DNA containing a minimum amount of breaks. Lysis of nuclei was achieved by gentle agitation in the presence of SDS and sodium perchlorate. Nuclear proteins were eliminated from the preparation by proteinase K treatment and extraction with a phenol-cresol-chloroform mixture, and the DNA was isolated by spooling upon alcohol precipitation. Electrophoresis

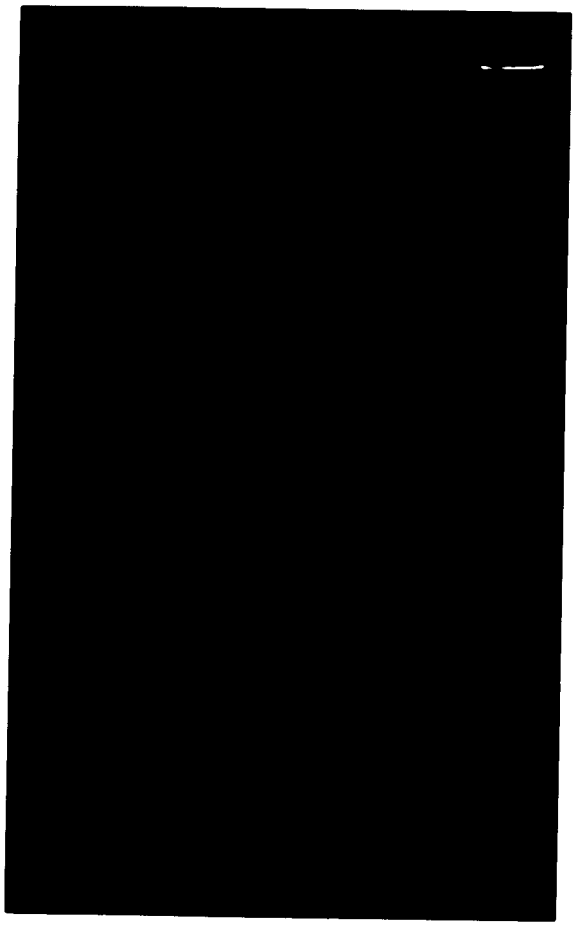
revealed that no small fragments were contained in this preparation.

FL cell DNA was digested with C methylation-sensitive restriction enzymes (Table 1), so that the levels of modification at specific sequences could be determined. Defined regions were investigated by first cutting the DNA with Eco RI, an enzyme whose cleavage is not affected by C methylation and with a C methylation-sensitive endonuclease. To insure complete cleavage of the genomic DNA, up to a 10 fold excess of enzyme was used and the extent of digestion was monitored with internal controls of pBR322 DNA. In lane c of Figure 5, an example of incompletely digested 745A DNA is presented. The lack of total digestion is indicated by the presence of a band of pBR322 DNA in the position corresponding to the supercoiled (uncut) form (middle band). One Eco RI site exists in this plasmid, so that complete cleavage results in the appearance of a single band on a gel (lane b). No restriction endonuclease reaction was taken as complete unless the pBR322 DNA was fully cut.

Figure 6 presents a typical analysis of the extent of FL cell DNA digestion by the C methylation-sensitive enzymes. Fully cut pBR322 DNA is visualized in lanes b (Msp I), c (Hpa II) and d (Hha I). Digestion of the DNA from FL cells with Msp I (lanes e and h), Hpa II (lanes f and i) and Hha I (lanes g and j) is considered complete, since the pBR322 DNA in these same reactions is totally cut.

Figure 5. Eco RI Digestion of FL Cell DNA and pBR322 DNA.

Conditions are the same as described in Figure 4. Lanes a and b contain 0.5μg of pBR322 DNA. Lanes c-f contain Eco RI digests of 0.5μg pBR322 DNA and 2.5μg of cellular DNA. (Lane a) undigested DNA; (lane b) Eco RI digest; (lane c) DNA from untreated 745A cells; (lane d) DNA from ethionine treated 745A cells; (lane e) DNA from untreated DS-19 cells; and (lane f) DNA from DMSO treated DS-19 cells.



a b c d e f

Figure 6. Msp I, Hpa II and Hha I Digestion of FL Cell DNA and pBR322 DNA.

DNA was cleaved and electrophoresed on a 1% agarose slab gel as described in Materials and Methods. The DNA is visualized by staining with ethidium bromide. Lanes a-d contain 0.5µg of pBR322 DNA. Lanes e-g contain 2.5µg of DNA from untreated 745A cells. Lanes h-j contain 0.5µg of pBR322 DNA plus 2.5µg of DNA from untreated DS-19 cells. (Lane a) undigested DNA; (lanes b, e and h) Msp I digests; (lanes c, f and i) Hpa II digests; and (lanes d, g and j) Hha I digests.

a b c d e f g h i j

Cleavage of the FL cell DNA with Hpa II and Hha I is inhibited to a much greater degree than is cleavage with Msp I which would indicate extensive methylation of the CMeCGG and GMeCGC sequences. The lack of digestion of these sequences does not appear to be due to problems with the digestions, since when Eco RI is used FL cell DNAs are observed to be extensively cut (Figure 5).

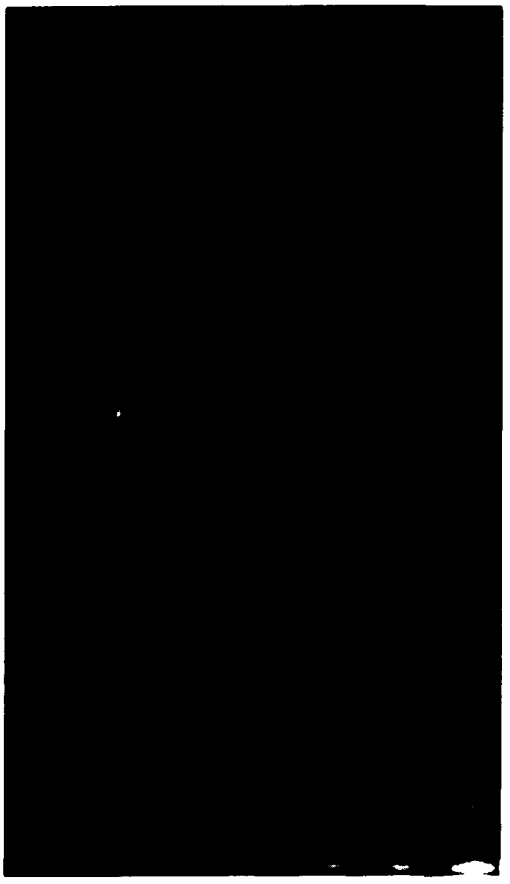
Double digestions of FL cell DNAs with Eco RI and the C methylation-sensitive enzymes produce patterns which reflect the extent of methylation of the recognition sequences in the genome. Since most Hpa II and Hha I sites are methylated, digestion of DNAs from the 745A cells (Figure 7, lanes c and d) and DS-19 cells (lanes e and f) with both Eco RI and Hha I did not change the overall profiles observed in samples cleaved with only Eco RI. Total cleavage of the pBR322 DNA with Hha I (lane b) was seen when the plasmid was included as an internal standard in the second of these cellular digestions, indicating that all unmethylated GCGC sequences had been cut.

2. Blot Hybridization of FL Cell DNA with Cloned Mouse Globin DNA

As described previously in the introduction, the extent of methylation of a specific sequence in FL cell DNA can be examined by cleavage of the DNA with a C methylation-sensitive enzyme followed by hybridization of the digested DNA with a radiolabelled probe which recognizes the regions of interest. Using a modification of the Southern procedure

Figure 7. Eco RI-Hha I Digestion of FL Cell DNA and pBR322 DNA.

Cellular DNA was previously digested with Eco RI. Conditions for Eco RI and second restriction endonuclease digestions are as described in Materials and Methods. Agarose gel electrophoresis is as described in Figure 5. Lanes a and b contain 0.5µg of pBR322 DNA. Lanes c-f contain Hha I digests of 0.5µg of pBR322 DNA and 2.5µg of cellular DNA. (Lane a) undigested DNA; (lane b) Hha I digest; (lane c) DNA from untreated 745A cells; (lane d) DNA from ethionine treated 745A cells; (lane e) DNA from untreated DS-19 cells; and (lane f) DNA from DMSO treated DS-19 cells.



a b c d e f

(Wahl et al., 1979), digested DNA was transferred to nitrocellulose filters and hybridized with λgtWES·βM_2 DNA or λgtWES·α_1 DNA ^{32}P-labelled by nick-translation. The filters were then exposed to x-ray film so that the bands corresponding to genome fragments which are complementary to βM_2 DNA and α_1 DNA could be seen. The autoradiogram shown in figure 8 was obtained when λgtWES·βM_2 DNA was used as the hybridization probe. All three FL cell DNA samples (DNA from untreated 745A cells; DNA from ethionine treated 745A cells and DNA from DMSO treated DS-19 cells.) were digested with Eco RI and Hha I. In each lane corresponding to digested FL cell DNA, one faint band is seen within a high level of background markings. The band represents hybridization of radiolabelled λgtWES·βM_2 DNA with a 7kb Eco RI genome fragment which contains the β^{maj} gene. Since the Hha I site located on the fragment was not cut, this site must be methylated in the cellular DNAs.

The experiments also revealed the presence of a repeated sequence in βM_2 DNA. The high level of background grains which appeared only in the lanes where the DNA had been, indicated the existence of a sequence in βM_2 DNA which hybridizes with sequences repeated in other regions of the genome. This postulate was confirmed by Edgell and his co-workers (Haigwood et al., 1981) who located one repeated sequence approximately 2kb 3' to the β^{maj} gene.

Repeated sequences are also found in α_1 DNA. The

Figure 8. Hybridization of FL Cell DNA with λgtWES·βM_2 DNA.

15μg of FL cell DNA digested with Eco RI and Hha I was electrophoresed on a 1% agarose gel, blotted into nitrocellulose and hybridized with ^{32}P-labelled λgtWES·βM_2 DNA as described in Materials and Methods. (Lane a) DNA from untreated 745A cells; (lane b) DNA from ethionine treated 745A cells; and (lane c) DNA from DMSO treated DS-19 cells.

a b c

autoradiogram in Figure 9 presents the results in duplicate of a hybridization of ^{32}P-labelled λgtWES·α_1 DNA with Eco RI Msp I double digested DNA from untreated 745A cells. Even though hybridization of λgtWES·α_1 DNA with α_1 DNA which has been digested with Eco RI plus Msp I results in a clear banding pattern (lane a), the background level in the two lanes (b and c) of FL cell DNA is so high that the bands produced by the blotting procedure are barely visible. This implies that more than one repeated sequence exists in the 9.7kb Eco RI cloned fragment containing the α_1-globin gene. It was obvious from these last two findings (Figure 8 and 9) that fragments of DNA made up of unique sequences specific for the mouse globin genes were needed as probes for studying methylation of the globin gene regions of FL cells.

3. Isolation and Characterization of Specific Globin Gene Probes

a. 3.8kb Taq I βM_2 DNA Fragment

The 3.8kb Taq I fragment containing the β-globin^{major} gene, one Msp I site and one Hha I site was isolated to use as a probe with the expectation that it would not contain a repeated sequence. In Figure 10, lane c, it can be seen that the 3.8kb fragment was isolated. The recovered DNA was analyzed by Msp I digestion (lane d) and Hha I digestion (lane e). In this same figure βM_2 DNA (lane a) and a $^{\beta}M_2$ DNA Taq I digest (lane b) are visualized.

Hybridization of the ^{32}P-labelled 3.8kb Taq I DNA

Figure 9. α_1 DNA and FL Cell DNA Hybridized with λgtWES·α_1 DNA.

Conditions are the same as described in Figure 8, except DNA was hybridized with λgtWES·α_1 DNA. (Lane a) Msp I digest of α_1 DNA; and (lane b and c) Eco RI-Msp I digest of DNA from untreated 745A cells.

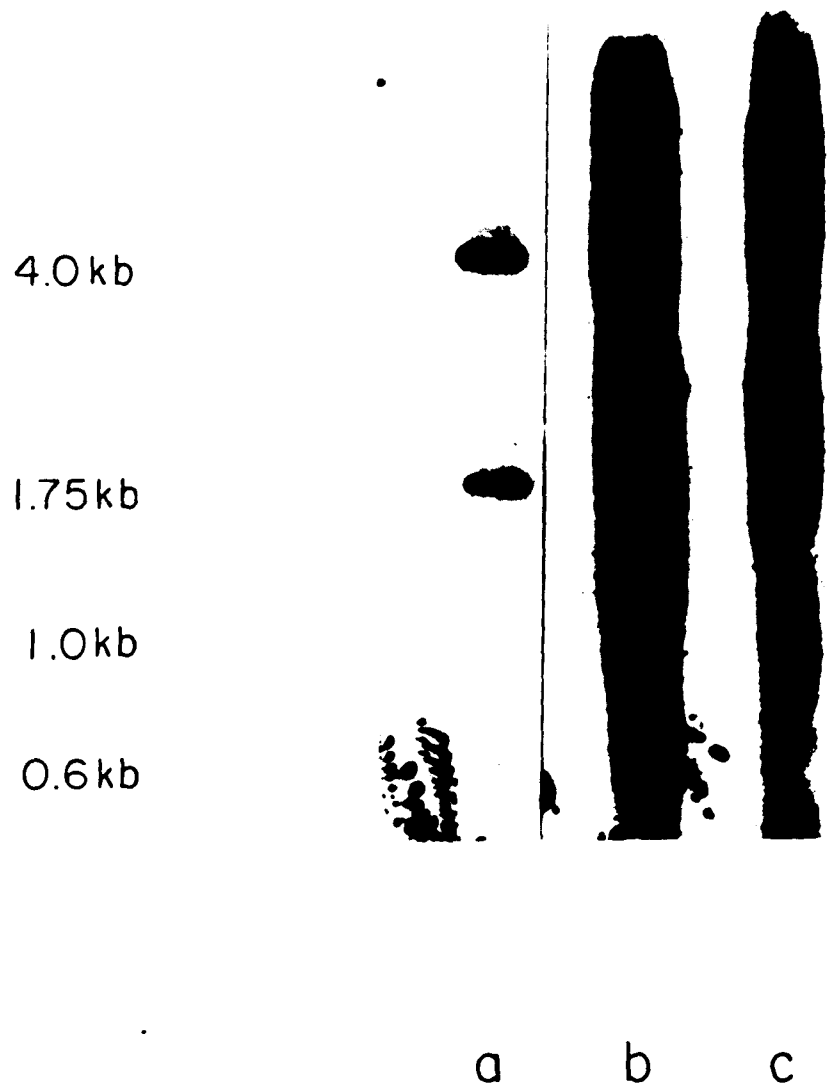
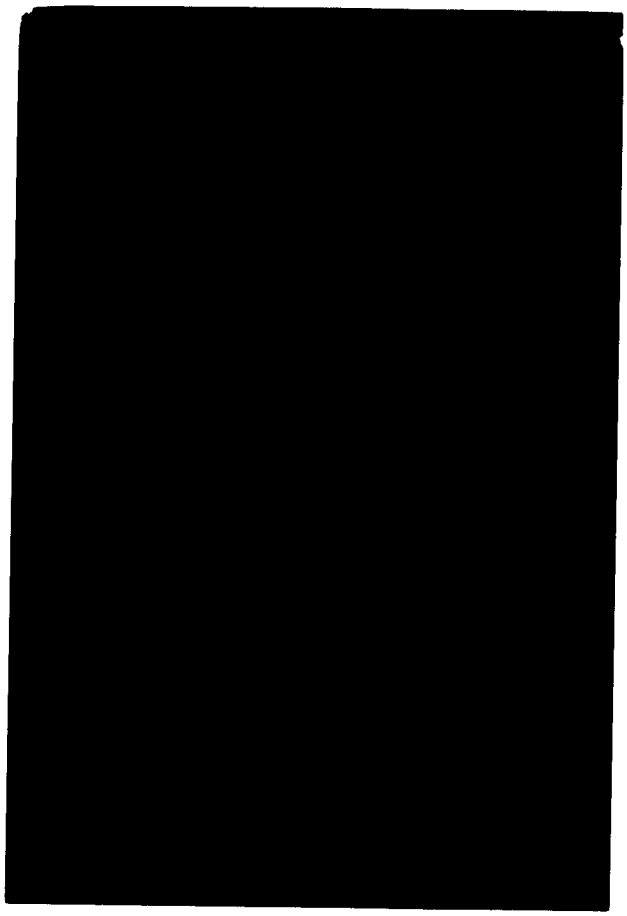

4.0kb
1.75kb
1.0kb
0.6kb
a
b
c

Figure 10. Restriction Endonuclease Digestion and Agarose Gel Electrophoresis of the 3.8kb Taq I βM_2 DNA Fragment.

Conditions are the same as described in Figure 1. Lanes a and b contain βM_2 DNA. Lanes c, d and e contain the 3.8kb Taq I βM_2 DNA fragment. (Lane a) undigested DNA; (lane b) Taq I digest; (lane c) undigested DNA; (lane d) Msp I digest (Residual band at 3.8kb is due to an incomplete digestion.); and (lane e) Hha I digest. (Fluresence seen above the 3.8kb band in lanes c, d and e is an artefact caused by the DNA isolation procedure.)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a b c d e

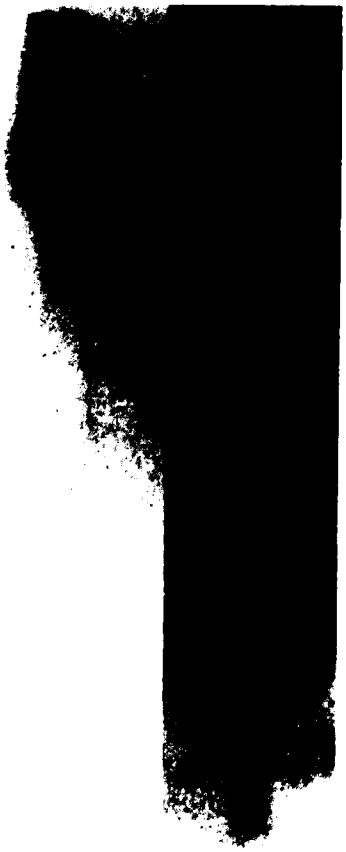
with Eco RI digested DNA from FL cells produces a low level of background grains on an autoradiogram, signifying the absence of any repeated sequences. Also, two different sized genome fragments that are comprised of sequences which are complementary to the probe are seen on the film (Figure 11, lane b). The 7kb band represents the fragment containing the β^{maj} gene and the 14kb band represents the fragment containing the β^{min} gene.

b. 3.0kb Sac I α_1 DNA Fragment

Sac I digestion of α_1 DNA produces six visible bands on an agarose gel, the largest of which is 3.0kb in size and contains the coding segments of the α_1 gene (Figure 12a, lane a and see Figure 4). The fragment was isolated for use as a probe since it is devoid of reiterated sequences (Leder, A., Personal communication) (Figure 12a, lane b). When the 3.0kb fragment is hybridized with Eco RI digested DNA from either untreated 745A cells (Figure 11, lane c) or DMSO treated DS-19 cells (lane d), five bands are seen on an autoradiogram. Thus the 3.0kb Sac I fragment anneals as expected with the α_1-globin gene (9.7kb Eco RI fragment) and the α_2-globin gene (11kb Eco RI fragment), but also has sufficient homology with three other α-like sequences to anneal with the Eco RI fragments on which they are located (6.5kb, 4.3kb and 2.4kb). These three α-like sequences most likely are pseudogenes. The data shows that ^{32}P-labelled, intact α_1 DNA recognizes all of the fragments produced by Msp I digestion of α_1 DNA

Figure 11. Hybridization of the 3.8kb Taq I βM_2 DNA Fragment and the 3.0kb Sac I α_1 DNA Fragment with Eco RI Digested FL Cell DNA.

Conditions are the same as described in Figure 8, except FL cell DNA was digested only with Eco RI and was hybridized with either the 3.8kb Taq I βM_2 DNA fragment (lanes a and b) or with the 3.0kb Sac I α_1 DNA fragment (lanes c and d). (Lane a) 50 pg of βM_2 DNA; (lanes b and c) DNA from untreated 745A cells; and (lane d) DNA from DMSO treated DS-19 cells.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a b c d

(Figure 9, lane a). The 3.0kb Sac I α_1 DNA probe anneals with the 4.0kb, 1.0kb and 0.6kb pieces, but not with the 1.75kb and 0.65kb pieces (Figure 12b). In addition, it hybridizes most efficiently with the 9.7kb α_1 DNA fragment. When the cloned 9.7kb fragment is digested with a 10 fold excess of Msp I to a point where no undigested α_1 DNA can be visualized by ethidium bromide staining, a strong band appears on an autoradiogram which corresponds to the intact fragment. This band is more intense than the one representing the 4.0kb Msp I piece of DNA, a fragment which has 1.6kb in common with the probe. The other two pieces of Msp I digested α_1 DNA which are recognized by the α-globin probe are barely visible. This may be due in part (especially in the case of the 0.6kb fragment) to the fact that smaller pieces of DNA transfer less efficiently onto nitrocellulose.

C. Methylation of Hpa II (Msp I) and Hha I Sites Surrounding the Globin Genes of FL Cells and FL cell Differentiation

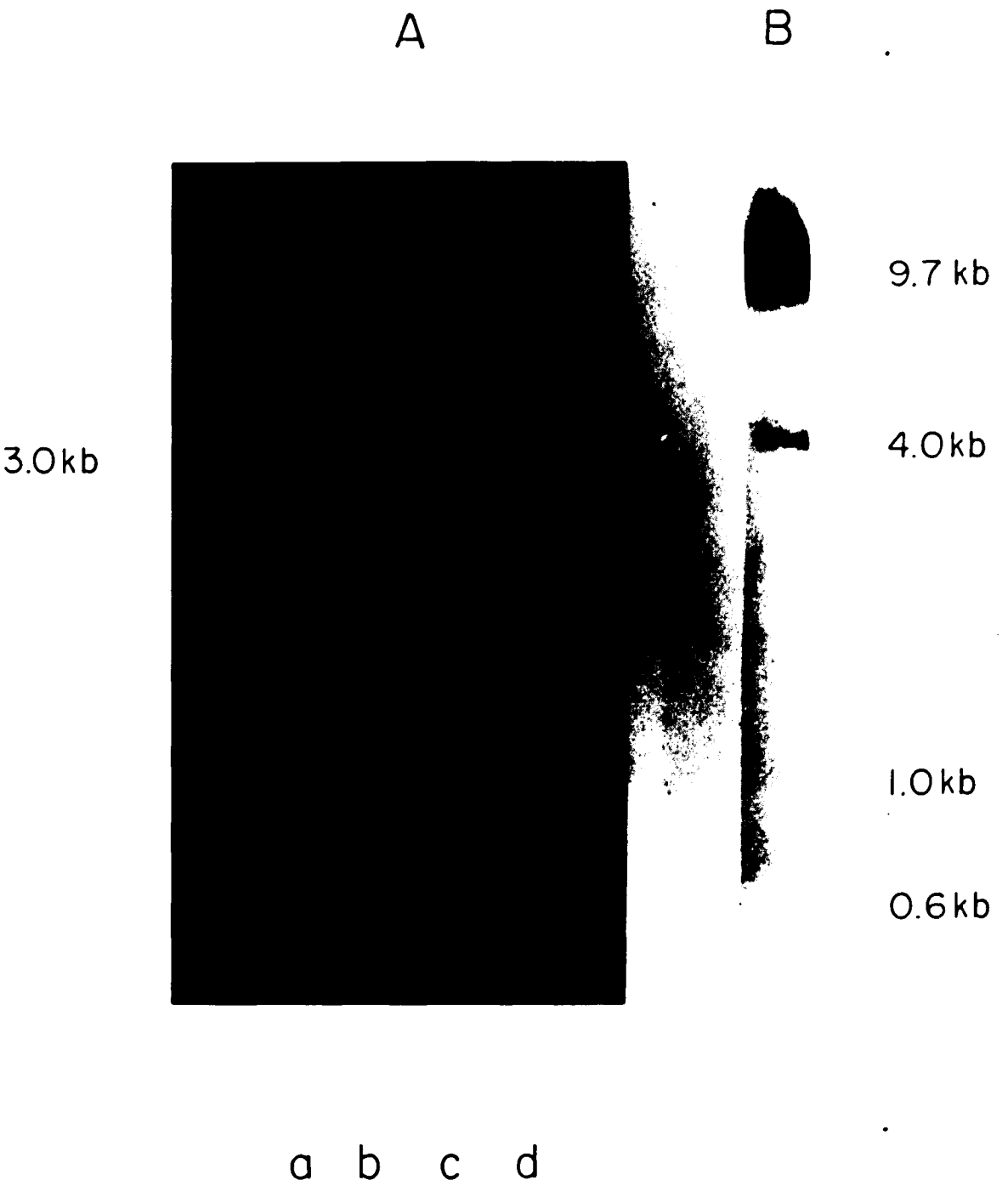
1. DMSO Induced Differentiation of DS-19 Cells

a. Methylation of the β-Globin Gene Regions

One Hpa II site is located between the two Eco RI sequences that surround the mouse β-globin^{major} gene (see Figure 2). When this CCGG sequence is totally unmethylated and mouse DNA is cut with Eco RI and either Msp I or Hpa II, a DNA fragment 3.5kb in size results. The presence of a methyl group on the outer or inner C residue inhibits cleavage

Figure 12. Agarose Gel Electrophoresis of the 3.0kb Sac I α_1 DNA Fragment and Hybridization with α_1 DNA.

(a) Conditions are the same as described in Figure 1. (Lane a) Sac I digest of α_1 DNA (Bands larger than 3.0kb are due to incomplete digestion.); (lane b) undigested 3.0kb Sac I α_1 DNA fragment (Band larger than 3.0kb is most likely to be a dimer.); (lane c) Molecular weight markers-Hae III ΦX174 DNA fragments, see Figure 3; and (lane d) Molecular weight markers-Hind III c1857 DNA fragments, see Figure 3. (b) Conditions are the same as described in Figure 8 except, α_1 DNA was digested with Msp I and hybridized with the 3.0kb Sac I α_1 DNA fragment.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a b c d

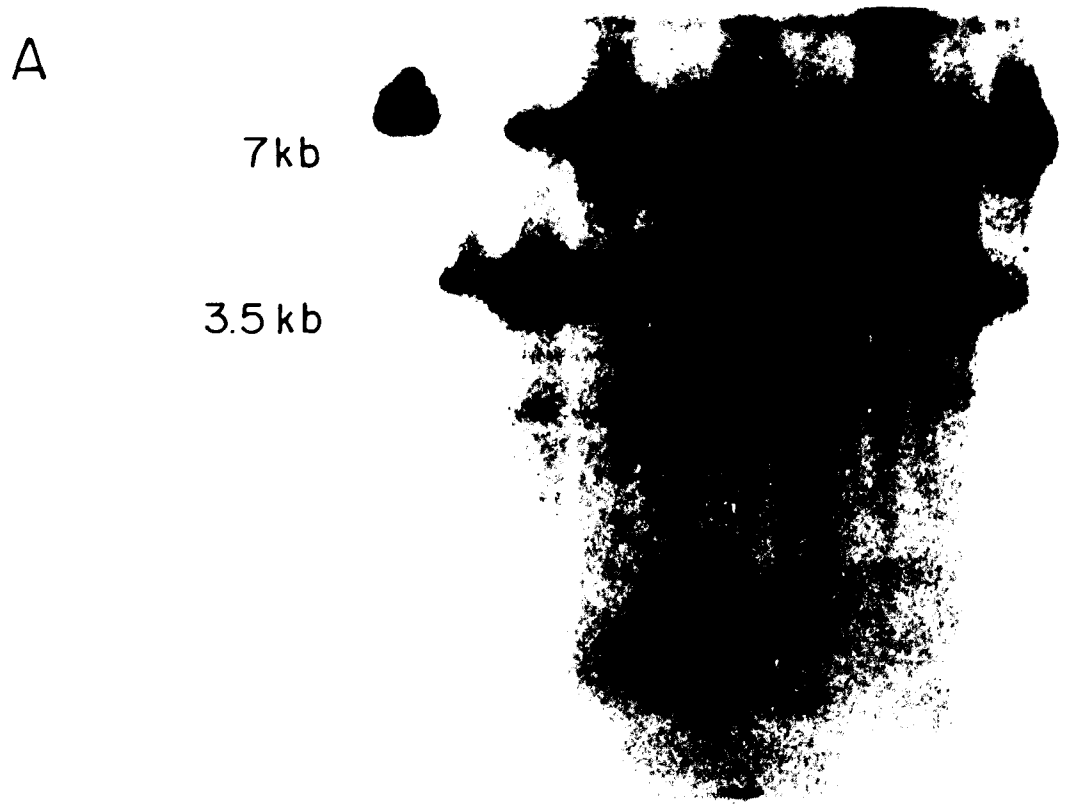
of this site by Msp I or Hpa II, respectively, leaving the 7kb Eco RI fragment containing the sequence intact. Two bands are detected on an autoradiogram when DNA from both untreated and DMSO treated DS-19 cells is digested with Eco RI and Msp I (Figure 13a, lanes g and i). The bottom band corresponds to the 3.5kb DNA fragment that is produced by cleavage of the site located 3' to the β^{maj} gene. (Figure 13b) The top band corresponds either to the Eco RI β^{maj} gene fragment which is uncut due to methylation of the Msp I sequence (MeCCGG) or to a 7kb fragment that contains the β-globin^{minor} gene. Eco RI and Hpa II double digestion reveals that the Hpa II site positioned near the β^{maj} gene is fully methylated in the two DS-19 DNAs (lanes h and j). A 7.6kb fragment which most likely contains β^{min} coding sequences is also visualized in both DS-19 DNA digests. Since, the β^{min} gene is found on a 14kb Eco RI fragment, the band pattern indicates that at least one Hpa II site surrounding the gene is cleaved due to the lack of methylation of the CCGG sequence. If the 7kb Eco RI-Msp I fragment does contain the β^{min} gene, than one Hpa II site on the 14kb piece of DNA is never cut and always methylated. Single Msp I digests of DNA from untreated (Figure 14, lane g) and DMSO treated (lane i) DS-19 cells produce a 3.8kb fragment. An intense band is seen on an autoradiogram when this fragment is bybridized with the β-globin^{major} probe. Since digestion of the FL cell DNA with Msp I alone results in the formation of a DNA fragment containing the gene that is slightly larger than

the fragment produced when the genome is cut with Eco RI plus Msp I, an Msp I sequence must lie just to the 5' side of the Eco RI site that is located 5' to the β^{min} gene. A weak band corresponding to a 7kb Msp I fragment is detected in the Msp I digested DS-19 DNAs. Taking into consideration the fact that the probe also recognizes a 7kb piece of DNA in the Eco RI-Msp I double digests of these DNAs, it is probable that both bands represent the same Msp I fragment on which the β^{min} gene is located. All of the Hpa II sites surrounding the β^{maj} gene from DMSO induced (Figure 14, lane j) and uninduced (lane h) DS-19 cells are fully methylated. It can be seen from the double digests that one Hpa II site located in the vicinity of the β^{min} gene is unmethylated in both DS-19 cell populations. All of the other sites, however, that are positioned near the coding sequences and can be detected by the 3.8kb Taq I βM_2 DNA probe are methylated. Therefore, these sites cannot be cleaved by the enzyme and the Hpa II fragments containing the genes are large. Since such pieces of DNA do not transfer onto nitrocellulose very well, they are barely detectable.

The restriction endonuclease Hha I cleaves the sequence GCGC only when the internal C is unmethylated. One Hha I sequence is found near the center of the 7kb Eco RI DNA fragment on which the β^{maj} gene resides. Therefore, by digesting the DNAs from DMSO treated (Figure 15, lane e) and untreated (lane d) DS-19 cells with both Eco RI and Hha I,

Figure 13. Methylation of CCGG Sequences Located Between the Eco RI Sites Surrounding the β-Globin Genes.

(a) Conditions are the same as described in Figure 8 except, FL cell DNA was digested with Eco RI and Msp I (lanes c, e, g and i) or Eco RI and Hpa II (lanes d, f, h and j) and was hybridized with the 3.8kb Taq I βM_2 DNA fragment. (Lane a) undigested βM_2 DNA; (lane b) Msp I digested βM_2 DNA; (lanes c and d) DNA from untreated 745A cells; (lanes e and f) DNA from ethionine treated 745A cells; (lanes g and h) DNA from untreated DS-19 cells; and (lanes i and j) DNA from DMSO treated DS-19 cells. (b) Map of Hpa II (Msp I) site located near the β^{maj} gene. The Hpa II site is indicated by an arrow pointing downwards; and Eco RI is indicated by lines. The line below the map represents the fragment detected by the nick-translated probe.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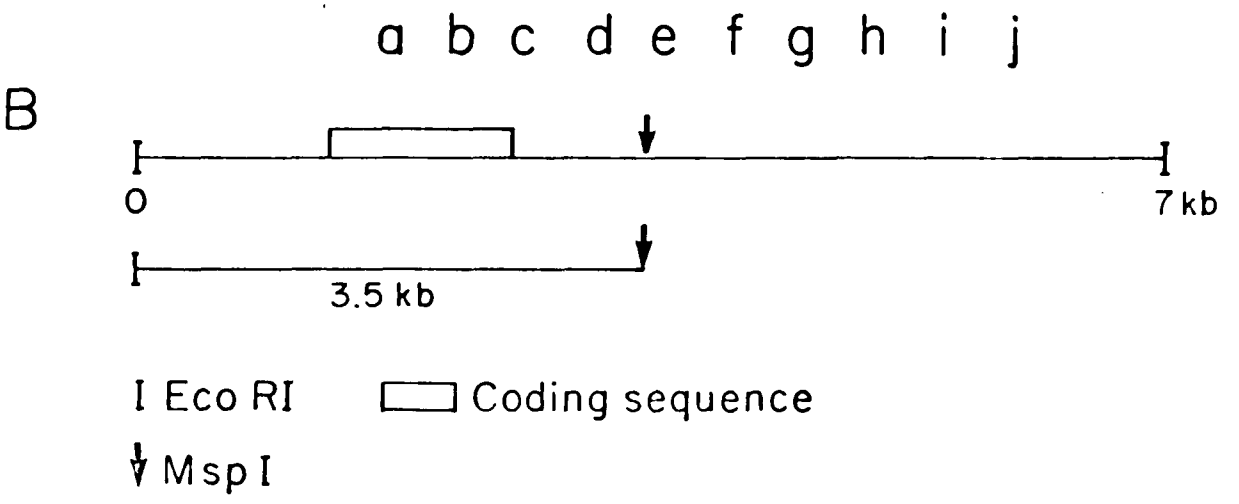
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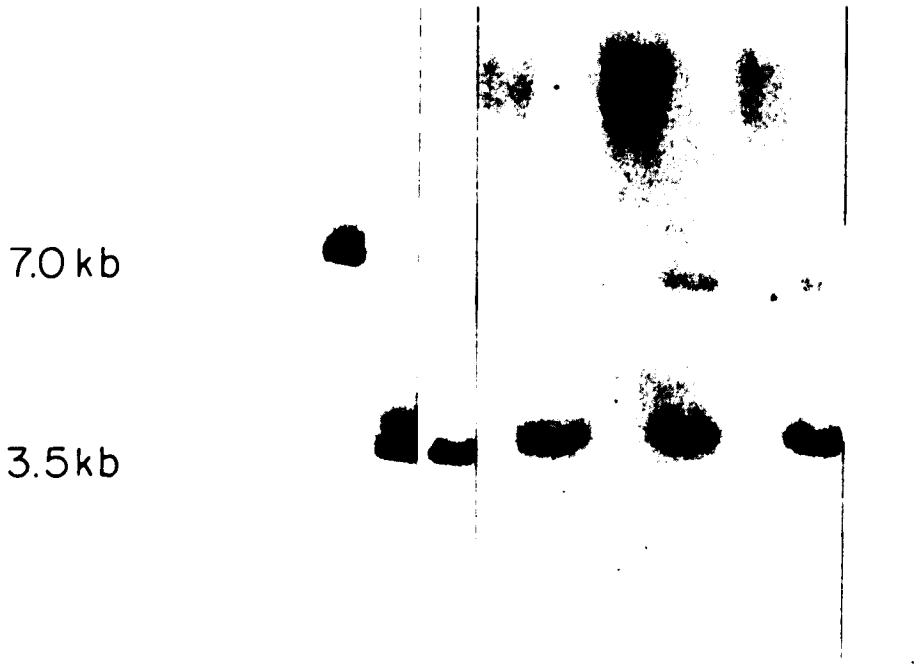
0
7 kb
3.5 kb
I Eco RI
Coding sequence
Msp I

Figure 14. Methylation of CCGG Sequences Surrounding the β-Globin Genes.

15μg of FL cell DNA digested with either Msp I (lanes c, e, g and i) or Hpa II (lanes d, f, h and i) was electrophoresed on a 0.7% agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labelled 3.8kb Taq I βM_2 DNA fragment as described in Materials and Methods. Lanes a and b contain 50pg of βM_2 DNA. (Lane a) undigested DNA; (lane b) Msp I digest; (lanes c and d) DNA from untreated 745A cells; (lanes e and f) DNA from ethionine treated 745A cells; (lanes g and h) DNA from untreated DS-19 cells; and (lanes i and j) DNA from DMSO treated DS-19 cells.


7.0kb
3.5kb
a b c d e f g h i j

the extent of methylation at this site when the β^{maj} gene either is or is not being expressed could be determined. However, whether the cells have been induced to differentiate or not, the sequence was apparently completely modified, since a band corresponding to the uncut 7kb Eco RI fragment is visible on an autoradiogram in both DS-19 DNA lanes. Also detected in these double digests was an 8kb piece of DNA which is represented by the top band in lanes d and e of Figure 15. As the probe, recognizes both the β^{maj} and β^{min} genes, it is likely that this 8kb DNA sequence includes the coding segments of the β-globin ^{minor} gene. No change in the pattern of methylation of the Hha I sites located within the 14kb Eco RI β^{min} DNA fragment was found after DMSO induced differentiation. Digestion of DNA from the two DS-19 cell populations with Hha I alone gives results that are similar to those of the double digests (Figure 16, lanes e and f). The extent of methylation of the Hha I sites surrounding the β^{maj} and β^{min} genes is identical in DMSO induced and uninduced cells. Most of these sites appear to be fully modified, as the markings seen on the film corresponds to large genome fragments.

b. Methylation of the α-like Globin Gene Regions

Ten α-like globin genes, four of which have been cloned and sequenced (Nishioka et al., 1979; Nishioka et al., 1980; and Leder et al., 1980), were discovered in the BALB/c mouse genome (Leder et al., 1981). As shown above, the

Figure 15. Methylation of GCGC Sequences Located Between the Eco RI Sites Surrounding the β-Globin Genes.

(a) Conditions are the same as described in Figure 14 except, the DNAs are digested with Eco RI and Hha I. (Lane a) 50pg of undigested βM_2 DNA; (lane b) DNA from untreated 745A cells; (lane c) DNA from ethionine treated cells; (lane d) DNA from untreated DS-19 cells; (e) DNA from DMSO treated DS-19 cells. (b) Map of Hha I site located near the β^{maj} gene. The Hha I site is indicated by an arrow pointing upwards; and Eco RI sites are indicated by lines. The line below the map represents the fragment detected by the nick-translated probe.

A

B

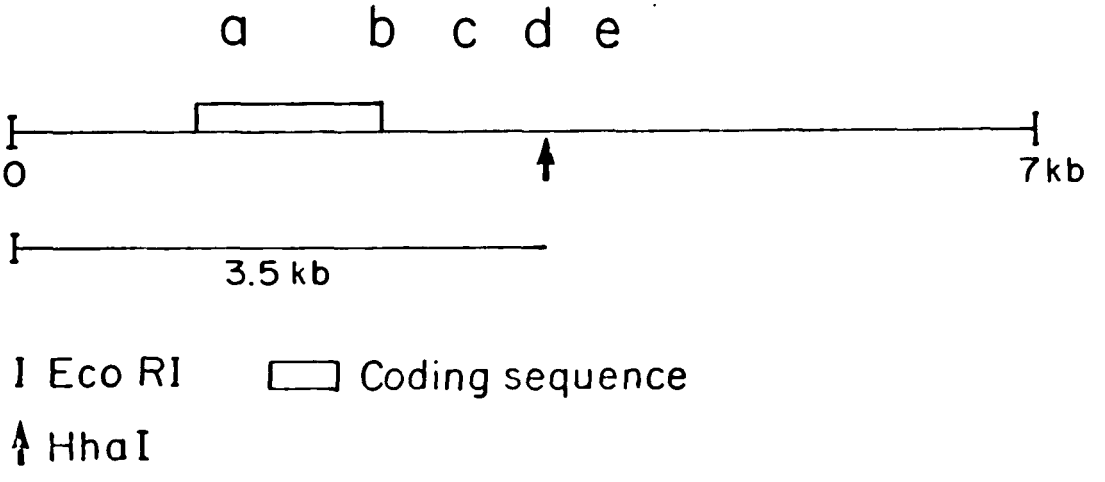


Figure 16. Methylation of GCGC Sequences Surrounding the β-Globin Genes.

Conditions are the same as described in Figure 14 except, the DNAs are digested with Hha I. (Lane a) 50pg of undigested βM_2 DNA; (lane b) DNA from untreated 745A cells; (lane c) DNA from ethionine treated 745A cells; (lane d) DNA from untreated DS-19 cells; and (lane e) DNA from DMSO treated DS-19 cells.

a b c d e

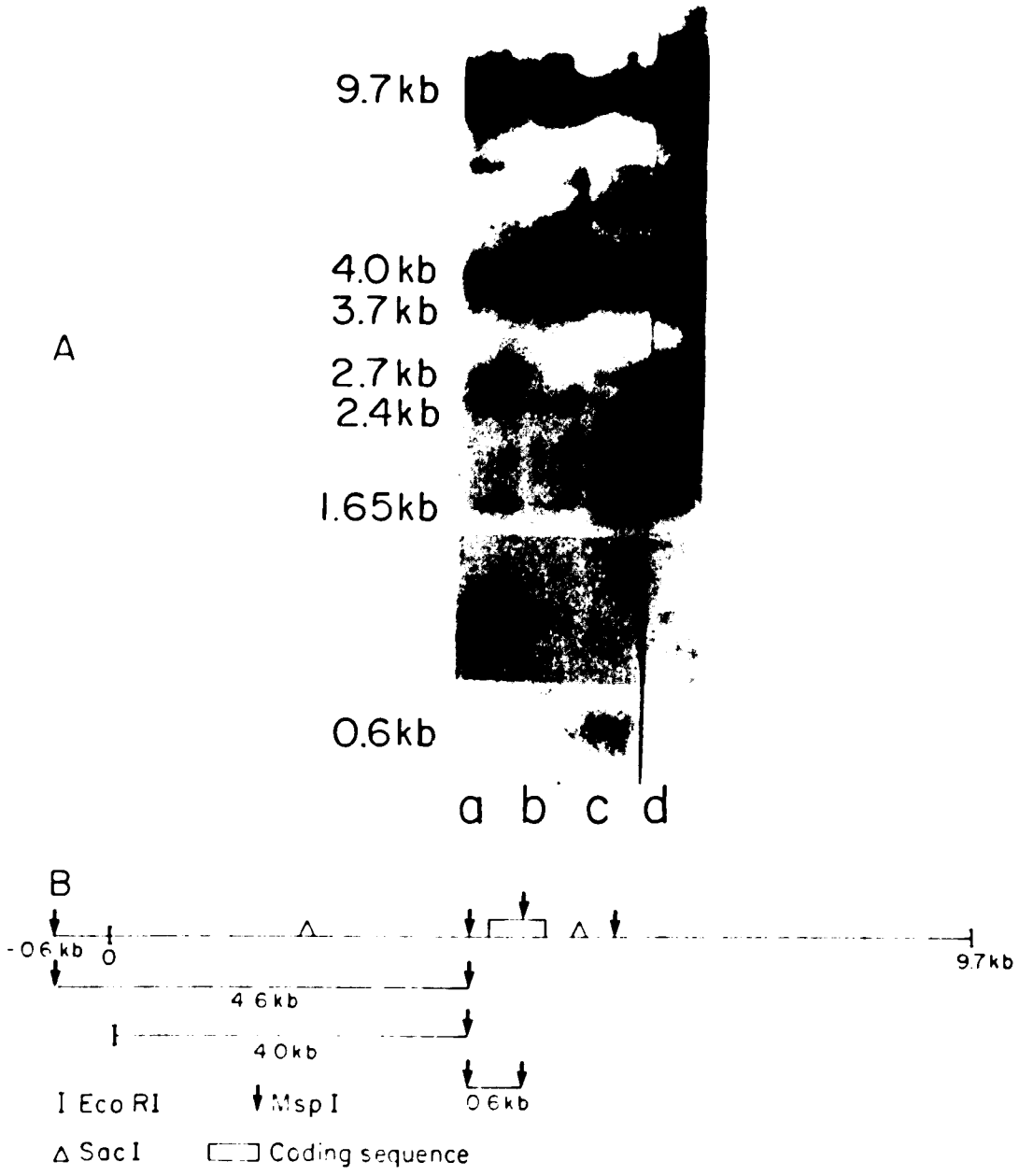
probe I used hybridizes with five α-like sequences. Thus, bands visualized with this probe could arise from any of these genes and might even consist of similarly sized fragments from more than one α-like sequence.

Changes in the sequence of the α-like genes, which often result in the loss of recognition sites, occur among animal strains (Vanin et al., 1980), and fragments which are expected to be produced in one strain by the cleavage of sites that were located in a second strain are not always formed. Therefore, the restriction map of the BALB/c mouse α_1-globin gene (Figure 4) identifies fragments that might be formed during cleavage of FL cell DNA. Differences in sequence have also been observed within an α-like globin gene family from one strain (Nishioka et al., 1980).

The 3.0kb Sac I α_1 DNA probe gives identical banding patterns on an autoradiogram when it is hybridized with Eco RI-Msp I digested DNA from either DMSO induced or uninduced DS-19 cells (Figure 17, lanes a and c). The same result is obtained when the DNAs are digested with Msp I alone (Figure 18a, lanes a and c). Therefore, no change in the pattern of methylation of the Msp I sites in the α-like regions is detected during DMSO induced differentiation. The top band in lanes a and c of Figure 17 corresponds to a 9.7kb DNA fragment which is most likely to be undigested Eco RI fragments containing the α_1-globin gene. All of the Eco RI-Msp I FL cell DNA digests were carried out

Figure 17. Methylation of CCGG Sequences Located Between the Eco RI Sites Surrounding the α-Like Globin Genes in DS-19 Cells.

(a) Conditions are the same as described in Figure 13 except, the DNA was hybridized with the 3.0kb Sac I α_1 DNA fragment. (Lane a) Eco RI-Msp I digested DNA from untreated DS-19 cells; (lane b) Eco RI-Hpa II digested DNA from untreated DS-19 cells; (lane c) Eco RI-Msp I digested DNA from DMSO treated DS-19 cells; (lane d) Eco RI-Hpa II digested DNA from DMSO treated DS-19 cells. (b) Map of Hpa II (Msp I) sites located near the α_1 gene. The Hpa II sites are indicated by an arrow pointing downwards; Sac I sites are indicated by triangles; and the Eco RI sites are indicated by lines. The lines below the map represent the fragments detected by the nick-translated probe.


A
9.7kb
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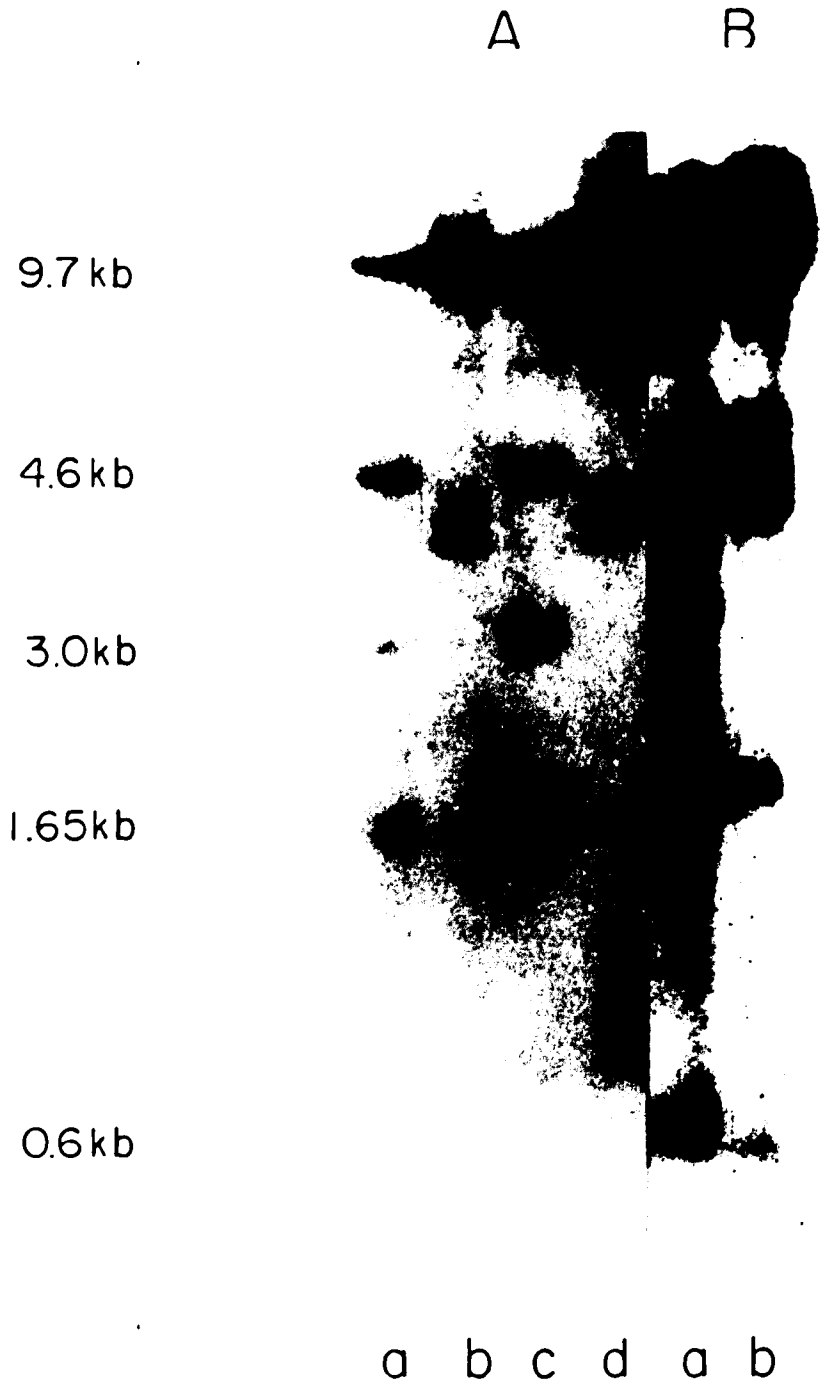
3.7kb
2.7kb
2.4kb
1.65kb
0.6kb
a b c d
B
-0.6kb
0
9.7kb
4.6kb
4.0kb
0.6kb
I Eco RI
Msp I
Δ Sac I
Coding sequence

Figure 18. Methylation of CCGG Sequences Surrounding the α-Like Globin Genes in DS-19 Cells.

Conditions are the same as described in Figure 14 except, the DNAs were electrophoresed on a 1% agrose gel and hybridized with ^{32}P-labelled 3.0kb Sac I α_1 DNA fragment. (a) FL cell DNA fragments smaller than 1kb were not transferred onto nitrocellulose. (Lane a) Msp I digested DNA from untreated DS-19 cells; (lane b) Hpa II digested DNA from untreated DS-19 cells; (lane c) Msp I digested DNA from DMSO treated DS-19 cells. (b) FL cell DNA fragments of all sizes were transferred. (Lane a) Msp I digested DNA from DMSO treated DS-19 cells; and (lane b) Hpa II digested DNA from DMSO treated DS-19 cells.


A
B
9.7kb
4.6kb
3.0kb
1.65kb
0.6kb
a b c d a b

with a 10 fold excess of the methylation-sensitive enzyme, and completeness of the reaction was monitored with pBR322 DNA as an internal standard. It appears that even when a tremendous excess of enzyme is used, a tiny amount of the 9.7kb Eco RI fragment remains undigested by Msp I. As the probe hybridized efficiently with intact α_1 DNA, an extremely strong band whose intense radiographic image does not correlate to the amount of nuclear DNA involved in its formation is produced on film. The vast majority of the genomic DNA, however, has been completely cut, as determined by the digestion profiles observed on an agarose gel of both nuclear DNA and the internal standard. Single digestion with Msp I creates a 9.7-9.8kb piece of DNA which anneals very efficiently with the α-globin probe suggesting that the entire 3.0kb Sac I sequence is contained within the Msp I fragment (Figure 18a, lanes a and c). It could be comprised in whole or part, however, of another α-like sequence located between two Msp I sites, 9.7-9.8kb apart, that is complementary to the probe. An intense band representing a 4.0kb Eco RI-Msp I fragment, most likely to be derived from cleavage of the Eco RI site and Msp I site located 5' to the α_1-globin coding sequence is seen in Figure 17, lanes a and c. The band corresponding to a 2.7kb piece of DNA also appears to originate from the cleavage of an Eco RI and an Msp I site located in one of the other α-like sequences detected by the probe, since in single Msp I digests of DS-19 DNAs both the 4·0kb and the 2.7kb fragments are replaced by 4.6kb and 3.0kb fragments, respectively (Figure 18a, lanes

a and b). The increase in size of the 4.0kb fragment indicates that 600bp 5' to the Eco RI site upstream from the α_1 gene sequence an Msp I site is positioned. The 1.65kb, 0.68kb and 0.6kb fragments produced during the digestion with Eco RI and Msp I presumably are bordered by Msp I sequences which in the intact genome lie between Eco RI sites that surround the α-like genes, since the same size fragments are created when the DS-19 DNAs are digested with Msp I alone (Figure 18a, lane a). Considering the fact that restriction mapping of the BALB/c α_1-globin gene revealed the presence of two Msp I sites that are separated by 0.6kb (Figure 4), there is an excellent possibility that the genomic 0.6kb fragment originates from the FL cell α_1 gene. Eco RI digestion of DS-19 DNA results in the formation of a 2.4kb fragment (Figure 11, lane d) which apparently corresponds to the 2.4kb inactive α-like sequence in the BALB/c mouse. Although the presence of an Msp I site could be expected in this piece of DNA (One has been located within the BALB/c mouse α_1-globin gene, Figure 4.),the fragment is not cleaved by this enzyme. This could be due to either methylation of a Msp I site located on the FL cell 2.4kb fragment or the lack of any CCGG sequence within this piece of DNA. In the BALB/c mouse, inactive α-like genes have been found to contain many differences in sequence when compared to the α_1-globin gene. Some of the changes in the pseudogenes were seen to result in the loss of Msp I (Hpa II) sites.

Whether the DNA was isolated from DMSO induced or uninduced DS-19 cells, the patterns of methylation of the α-like globin gene regions were the same. This result was seen when the DNAs were digested with either Eco RI plus Hpa II (Figure 17a, lanes b and d) or Hpa II alone (Figure 18a, lanes b and c). Since the CCGG sequence when present in the mouse genome is methylated at the internal C residue to a much greater extent than at the external C residue (Singer et al., 1979), it was of no surprise that the band patterns observed on autoradiograms reflected a higher level of methylation of Hpa II sites than of the Msp I sites in DS-19 DNA. The top bands produced by digestions of the DS-19 DNAs with Hpa II followed by hybridization with the α-globin probe indicate the existence of two unmethylated Hpa II sites 9.7-9.8kb apart surrounding an α-like sequence, which could be the α_1-globin gene or one of the other four α-like sequences detected in the FL cell genome. The presence of 9.7kb fragments in Eco RI-Hpa II digests are probably the result of methylation of Hpa II sequences within the piece of DNA, although the contribution of small amounts of incompletely digested fragments cannot be ruled out. Methylation of CCGG sequences in the α-like regions of DNA that has been digested with Eco RI inhibits the cleavage by Hpa II so that no 2.7kb fragment appears and the amounts of 1.65kb and 0.6kb fragments are less than those seen in Msp I digestions. In single digestions of DS-19 DNA, methylation of Hpa II sites blocks

the formation of both the 4.6kb and 3.0kb fragments which were found during Msp I digestions, and again reduced amounts of 1.65kb and 0.6kb pieces of DNA are produced as compared to the amounts formed in Msp I digestions. The fact that the 4.6kb fragment is not formed in single digests indicates that the CCGG sequence located 600bp upstream from the Eco RI site 5' to the α_1-globin gene is likely to be extensively methylated at the internal C residue. These single and double digests suggests that the Hpa II (Msp I) site located 4.0kb downstream from the Eco RI site mentioned above (see Figure 17b) is unmethylated to some degree at both of the C residues, since a 4.0kb fragment is found in both double digests (Eco RI-Msp I and Eco RI-Hpa II). However, the very strong Eco RI-Hpa II band might be representing two totally different 4.0kb pieces of DNA, since a 4.0kb fragment which cannot be part of the α_1-globin gene is visualized in the single Hpa II digests. A fragment appearing in the Hpa II digested DNA which is 3.8kb in size, most likely contains an Eco RI site, since double digestion with Eco RI plus Hpa II produces a 3.7kb fragment instead. Cleavage of both a Hpa II site and an Eco RI site also seems necessary for the formation of the 6.2kb fragment found only in double digests. If no CCGG sequences exist in the 2.4kb fragment produced by Eco RI digestion of the DS-19 DNA, than cutting the same DNA with Hpa II following the Eco RI treatment should leave the 2.4kb piece of DNA intact, and infact it does.

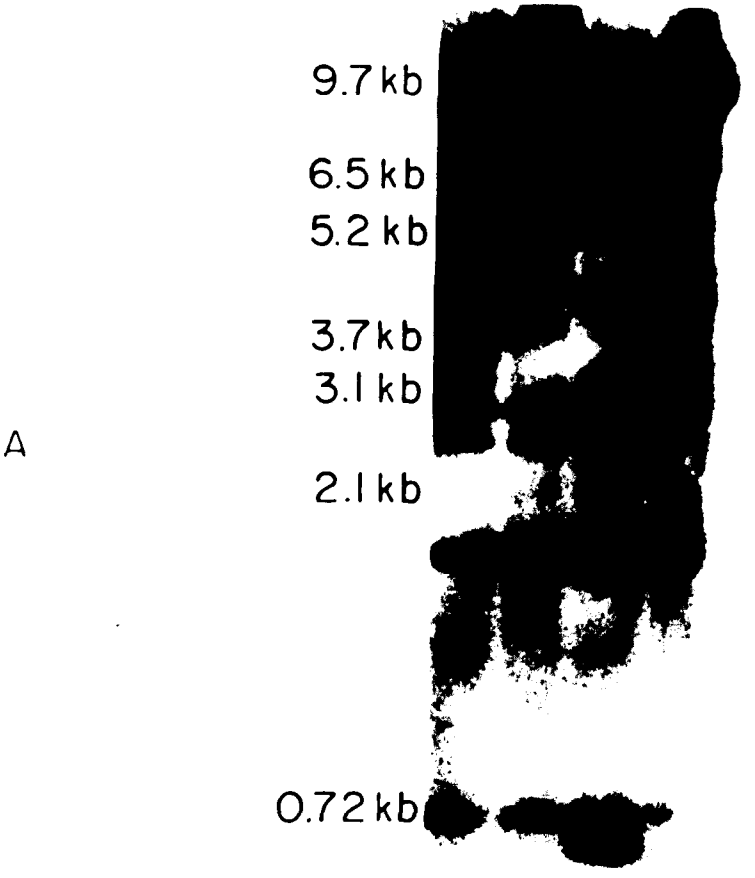
A similar situation was observed when genomic DNA was digested with Eco RI plus Hha I, i.e., no change was found in the pattern of methylation during differentiation (Figure 19, lanes c and d). Since no enzyme is known which will cut the Hha I sequence when it is methylated (GMeCGC), the DS-19 DNA digestion profile of the α-like globin regions obtained with Eco RI plus Hha I could not be compared to the profile obtained when all the sites within these defined regions are cleaved. However, comparisons could be made with Eco RI-Hha I fragments obtained from digestion of the cloned α_1-globin gene and Eco RI single digests of genomic DNA. Both 6.5kb and 2.4kb fragments are found in Eco RI digests and are not cleaved any further by Hha I, indicating the presence of possible Hha I sites within these fragments which are methylated. It is probable that the 1.7kb piece of DNA appearing on film is formed when two Hha I sites positioned 5' to the FL cell α_1-globin coding sequence (Figure 19b) are unmethylated and therefore cut by the enzyme. Cleavage of unmethylated Hha I sites located 0.32kb 5' and 0.4kb 3' to the start of the α_1-globin gene would produce a 0.72kb fragment and one is found in the DS-19 Eco RI Hha I digests.

2. Induction of 745A Cells by Hypomethylating Agents

Treatment of FL cells with either L-ethionine or 5-azacytidine results in a significant undermethylation of the DNA synthesized. Thus, it was of interest to see if these known hypomethylating agents, which have been shown

Figure 19. Methylation of GCGC Sequences Located Between the Eco RI Sites Surrounding the α-Like Globin Genes.

(a) Conditions are the same as described in Figure 17 except, the DNA was digested with Eco RI and Hha I. (Lane a) DNA from untreated 745A cells; (lane b) DNA from ethionine treated 745A cells; (lane c) DNA from untreated DS-19 cells; and (lane d) DNA from DMSO treated DS-19 cells. (b) Map of Hha I sites located near the α_1-globin gene. The Hha I sites are indicated by an arrow pointing upwards; the Sac I sites are indicated by triangles; and the Eco RI sites are indicated by lines. The lines below the map represent the fragments detected by the nick-translated probe.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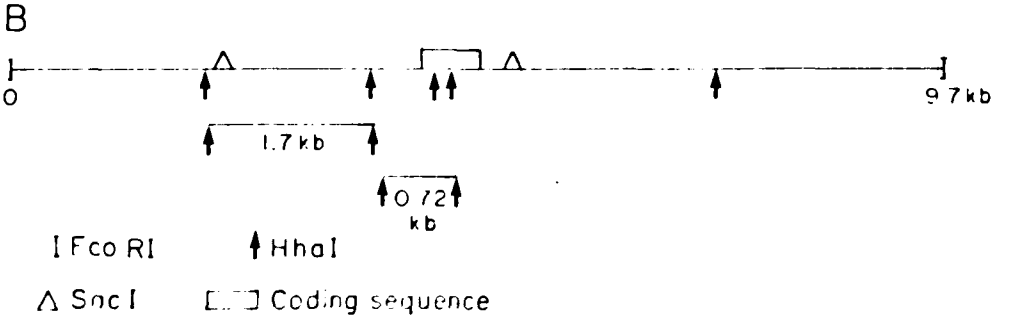

B
0
9.7kb
1.7kb
0.72 kb
EcoRI
HhaI
SncI
Coding sequence

to enhance FL cell globin gene expression, affect the 5MeC content of these genes. Patterns of methylation of CCGG and GCGC sequences located near the α- and β-globin gene regions of 745A cells that were grown in the presence of ethionine or 5-aza-CR were determined.

a. L-Ethionine and 745A Cell Differentiation

i. Methylation Patterns of the β-Globin Gene Regions in Differentiating 745A Cells and DS-19 Cells

Levels of methylation of Hpa II, Msp I and Hha I sites in the β^{maj} and β^{min} gene regions do not change during ethionine induced differentiation of FL cells. When DNA isolated from untreated or ethionine treated 745A cells is digested with Eco RI plus Msp I (Figure 13, lanes c and e) or Eco RI plus Hpa II (lanes d and f), the band patterns observed on film after hybridization with the β-globin probe are identical to those seen when the DS-19 DNAs were cleaved with the same enzymes and hybridized with the same probe. The Hpa II site located 3' to the β^{maj} coding sequence is fully methylated in both 745A cell populations, and the 14kb Eco RI fragment containing the β^{min} gene appears to have at least one Hpa II site that is never methylated and one site that is completely methylated. Single digestions of DNAs from uninduced and induced 745A cells produce the same sized fragments as those found in single digestions of DS-19 cells (Figure 14, lanes c and e). Therefore, the conclusions

drawn concerning the levels of methylation of CCGG sequences surrounding the β-globin genes are the same for both 745A cells and DS-19 cells. Once again these conclusions are that the Hpa II sites in the vicinity of the β^{maj} gene are fully methylated and all but one of the detected Hpa II sites positioned near the β^{min} gene are modified. Cleavage with Eco RI plus Hha I (Figure 17, lanes b and c) or Hha I alone (Figure 16, lanes b and c) reveals that all the sites around the β^{maj} gene are modified, and that changes in methylation levels of Hha I sites in the area of the β^{min} gene do not occur during differentiation of 745A cells or the cloning of DS-19 cells from strain 745A.

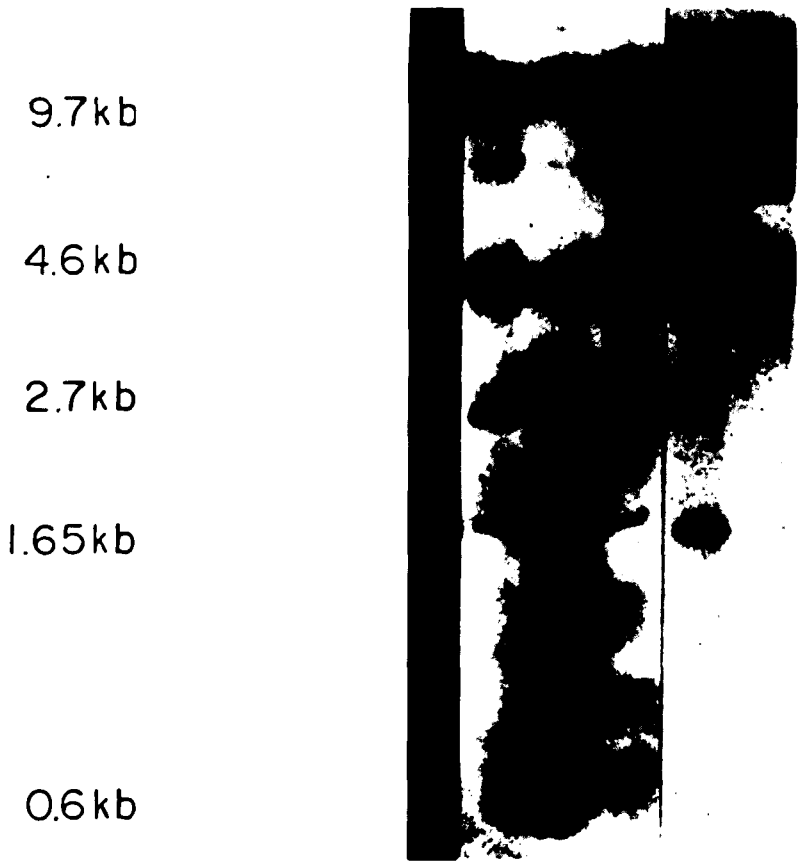
ii. Methylation Patterns of the α-Like Globin Gene Regions in Differentiating 745A Cells and DS-19 Cells

Ethionine induced differentiation does not affect the pattern of methylation of the α-like globin gene regions in 745A cells (Figure 19, lanes a and b, and Figure 20). However, in contrast to the ε-globin genes, the restriction endonuclease digestion fragments of the α-like gene sequences in 745A cells are not identical to the ones seen in DS-19 cells. It appears from these investigations that the pattern of methylation of the α-like globin gene regions is different in these two FL cell genomes.

Digestion of 745A DNA with Eco RI-Msp I (Figure 20,

Figure 20. Methylation of CCGG Sequences Surrounding the α-Like Globin Genes in 745A Cells.

Conditions are the same as described in Figure 19. Lane a, b and e contain DNA from untreated 745A cells. Lanes c, d and f contain DNA from ethionine treated 745A cells. (Lanes a and c) Eco RI-Msp I digest; (lanes b and d) Eco RI-Hpa II digest; (lane e) Msp I digest; and (lane f) Hpa II digest.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a b c d e f

lanes a and c) or Msp I alone (lane e) creates the same fragments detected in the digests of DS-19 DNA. During Eco RI-Hpa II (lanes b and d) and Hpa II (lane f) cleavage, not all of the fragments are formed to the same extent in both cell line DNAs. This was determined by comparing the bands seen consistently on autoradiograms representing hybridizations of digested DNA. A band corresponding to a 3.8kb Hpa II fragment in a single digest and a 3.7kb Eco RI-Hpa II fragment in a double digest are barely seen on films of 745A DNA, but are quite intense on films of DS-19 DNA (Figure 17, lanes b and d; Figure 18a, lanes b and d; and Figure 18b, lane b). This suggests that the Hpa II site which is cut during the formation of this fragment is modified only in the 745A genome.

Several differences exist between the Eco RI-Hha I digestion patterns of 745A DNA (Figure 19, lanes a and b) and DS-19 DNA (lanes c and d). Much more of the 5.2kb fragment was produced from 745A DNA than from DS-19 DNA, while fragments which are 2.1kb and 3.1kb were formed to a much greater extent from DS-19 DNA than from 745A DNA. This suggests that a GCGC sequence located within the 5.2kb fragment might be cut to form the smaller fragments and that if this is so, the sequence is largely methylated and uncut in 745A DNA, and extensively unmethylated and cut in DS-19 DNA. A 6.5kb piece of DNA (which is likely to be an Eco RI fragment, see section II, C, 1, b) is produced only in DS-19 DNA digests. Therefore,

any Hha I sites that may be positioned within this fragment are methylated to some extent in DS-19 DNA and are never all methylated in the same piece of DNA in 745A cells. The presence of a much larger amount of the 3.7kb fragment in 745A DNA digests indicates either a lower level of modification of the Hha I sites bordering or a higher level of modification of the Hha I sites within the 3.7kb DNA sequence when it is located in the 745A genome compared to when it is part of the DS-19 genome. The possibility exists, although it is unlikely to account for all the dissimilarities observed, that the differences seen between DNA digestion patterns of the α-like globin gene regions in the two cell lines are due to sequence changes with resulting loss of restriction endonuclease sites.

b. Treatment with 5-Azacytidine

i. Effect on the Methylation Patterns of the β-Globin Gene Regions

745A cells were grown in the presence of 1.0μm 5-aza-CR for 20 hr. The drug was removed from the medium and the cells were allowed to grow for 48 hr more. The DNA isolated from 5-aza-CR cells was then digested with Msp I, Hpa II, or Hha I and hybridized with the 3.8kb Taq I βM_2 DNA fragment, to see if any changes in methylation patterns had occurred in the β-globin gene regions. The DNA synthesized in cells exposed to 5-aza-CR for 20 hr is severely undermethylated due to inactivation of the DNA methyltransferase

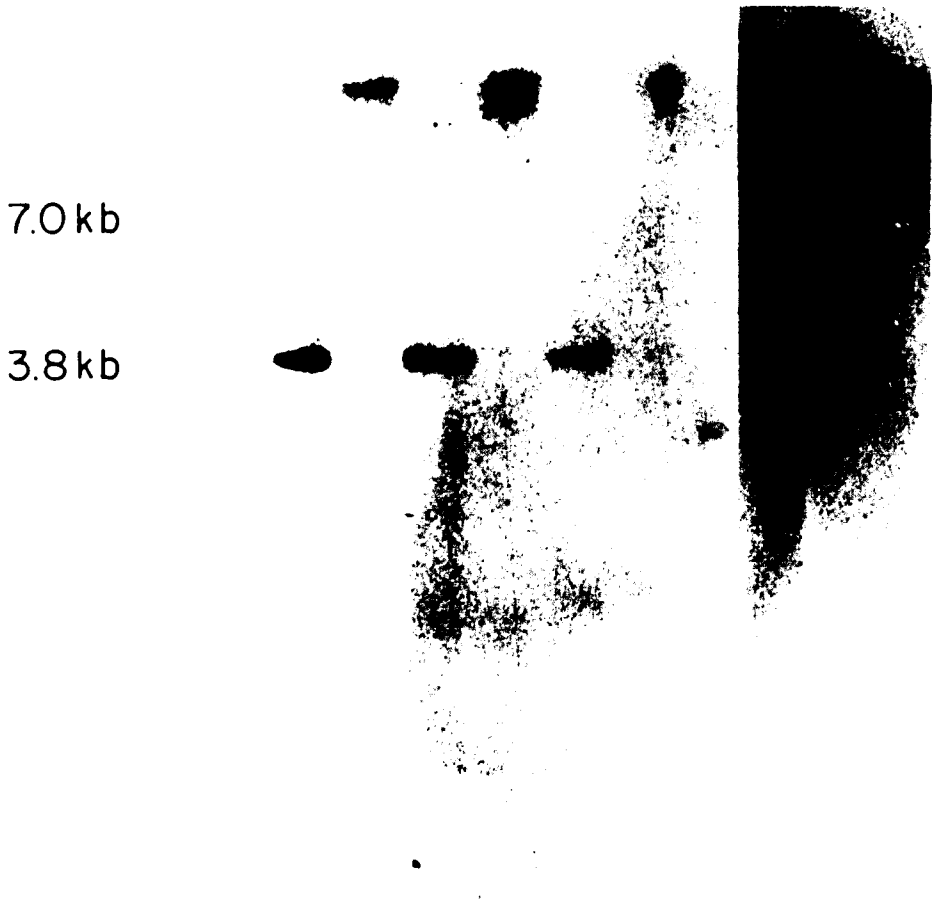
by incorporated 5-aza-CR residues (Creusot et al., 1982). The patterns of methylation of CCGG (Figure 21, lanes a and b) and GCGC (lane g) sequences located in the β-globin gene regions of this DNA are identical to the patterns in untreated 745A DNA (Figure 14, lanes c and d; and Figure 16, lane b). Therefore, a symmetrical loss of methyl groups at the investigated sites cannot be detected. During the 48 hr recovery period from 5-aza-CR exposure, a small number of sites becomes completely unmethylated in the progeny of treated cells. However, no heritable changes are found in the methylation of Msp I, Hpa II or Hha I sequences surrounding the α-globin genes either 24 hr (Figure 21 lanes c, d, and h) or 48 hr (lanes e, f and i) after the cells have been washed free of the drug.

ii. Heritable Changes in the Methylation Pattern of the α-Like Globin Gene Regions

Inhibition of methylation of newly synthesized DNA due to the incorporation of 5-aza-CR, results in the production of hemi-methylated DNA. It has been proposed that DNA modification patterns can be preserved during semi-conservative replication by the methylation of daughter strands at C residues which are opposite to 5MeC residues already existing in the parental strands (Holliday and Pugh, 1975). Therefore, completely unmethylated sites in DNA due to cell exposure to 5-aza-CR, are more likely to be detected after at least one round of replication has occurred in which the

Figure 21. Methylation of CCGG and GCGC Sequences Surrounding the β-Globin Genes in 745A Cells Treated with 5-Azacytidine.

Conditions are the same as described in Figure 13 except, the FL cell DNA was digested with Msp I (lanes a, c and e), Hpa II (lanes b, d and f) or Hha I (lanes g, h and i). Lanes a, b and g contain DNA isolated from cells after a 20 hr exposure to 1.0μM 5-aza-CR. Lanes c, d and h contain DNA isolated from cells 24 hr after 5-aza-CR had been washed out of the medium. Lanes e, f and i contain DNA isolated from cells 48 hr after 5-aza-CR had been washed out of the medium.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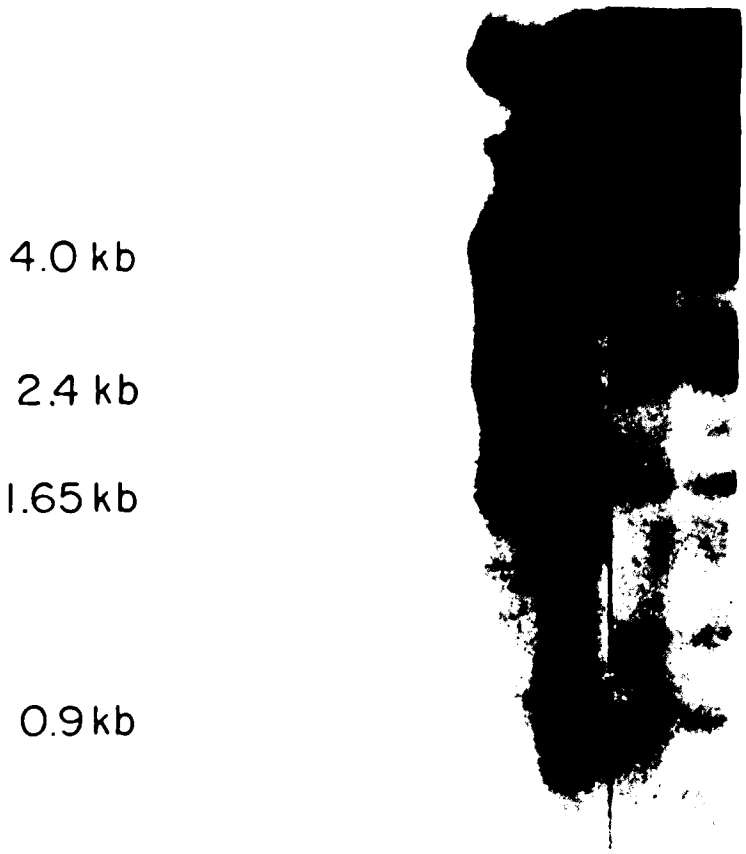
the hemi-methylated DNA synthesized during treatment serves as the template for progeny strands. After cells have been exposed to 5-aza-CR for 20 hr, the rate of DNA synthesis is less than 15% of that in untreated cells. 24 hr after the drug has been removed from the FL cell culture medium, however, the rate of DNA synthesis is approximately 40% that of cells not exposed to the drug. Therefore, methylation patterns were looked at 24 and 48 hr after treatment of the cells. One new fragment which is 0.9kb in size and contains an α-like globin sequence can be detected in Eco RI-Hha I digests of DNA isolated 24 hr after the cells are removed from 5-aza-CR. (Figure 22, lane c). This band is still present at 48 hr eventhough most of the hypomethylated sites produced during the treatment have been remethylated (Creusot et al., 1982). No change in the double digestion patterns (Eco RI-Msp I and Eco RI-Hpa II) of CCGG sequences is seen in the α-like globin gene regions (Figure 22, lanes a and b; untreated 745A DNA - Figure 20, lanes a and b). These results suggest that in one area of the FL cell genome containing both α-like globin and Hha I sequences the pattern of methylation is affected by exposure of the cells to 5-aza-CR.

Figure 22. Methylation of CCGG and GCGC Sequences Surrounding the α-like Globin Genes in 745A Cells Treated with 5-Azacytidine.

Conditions are the same as described in Figure 17 except, the FL cell DNAs in lanes c and d were digested with Eco RI plus Hha I. Lanes a, b and d contain DNA isolated from cells 48 hr after 5-aza-CR had been washed out of the medium. Lane c contains DNA isolated from cells 24 hr after 5-aza-CR had been washed out of the medium. (Lane a) Eco RI-Hpa II digest; (lane b) Eco RI-Msp I digest; and (lanes c and d) Eco RI-Hha I digests.



a b c d

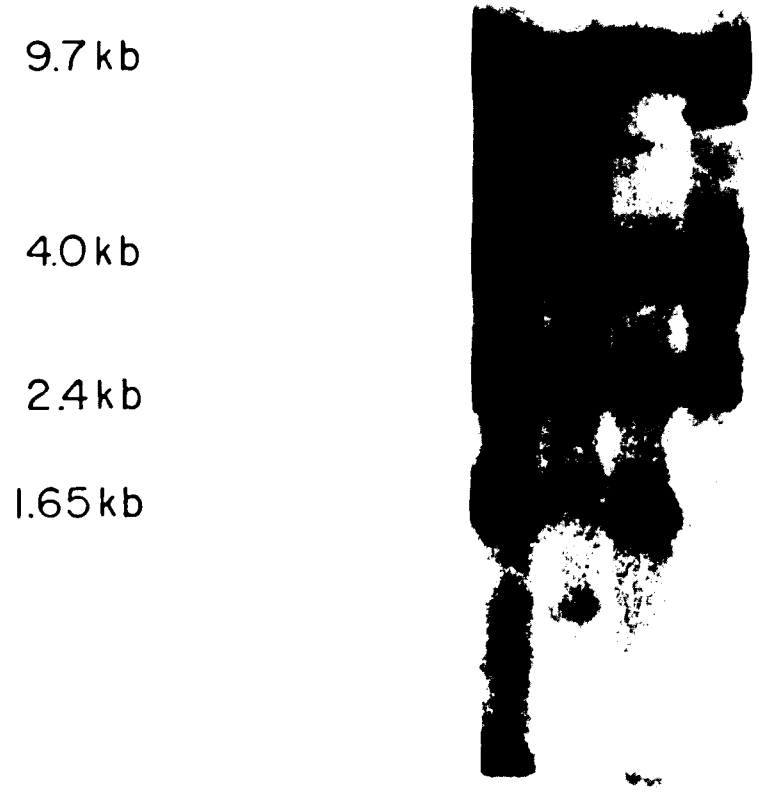
D. Variations in Methylation of CCGG Sequences in the α-Like Globin Gene Regions of FL Cells and Non-expressing Mouse Cells

Low levels of methylation of specific sites in the DNA have been correlated with gene activity. Since I found no changes in DNA methylation of globin genes during chemical induction, it is possible either that they occurred in sites not detected by the restriction enzymes and probes employed or that they took place at an early stage. It was therefore of interest to determine if tissues which do not make globin were more methylated in the α-like gene regions than FL cells. Liver and kidney DNAs were prepared from DBA/2J mice, the strain from which FL cells are derived.

Eco RI-Msp I digests of kidney and liver DNAs produce the same fragments (Figure 23, lanes a and c) formed by the enzymes from FL cell DNA. New fragments (5.8kb and 1.25kb) seen in kidney digests (lane a), most likely result from some methylation of the external C residue of a few CCGG sequences. Double digestions of kidney and liver DNAs with Eco RI plus Hpa II (lanes b and d) suggest that the internal C residues at sites flanking the 1.65kb and 0.6kb fragments are methylated to a greater extent than the ones present in FL cells. One Hpa II site involved in the formation of the 2.7kb fragment is cut to a larger degree in liver DNA than in FL cell DNA, implying that the site in liver has a lower level of modification. The Eco RI-Hpa II

Figure 23. Methylation of CCGG Sequences Located Between the Eco RI Sites Surrounding the α-Like Globin Genes in DBA/2J Mouse Kidney and Liver Cells.

Conditions are the same as described in Figure 18. (Lane a) Eco RI-Msp I digested DNA from kidney cells; (lane b) Eco RI-Hpa II digested DNA from kidney cells; (lane c) Eco RI-Msp I digested DNA from liver cells; and (lane d) Eco RI-Hpa II digested DNA from liver cells.


9.7kb
4.0kb
2.4kb
1.65kb
a b c d

3.7kb fragment found in digests of DS-19 DNAs is also produced from kidney and liver DNAs. Thus, of all the cells investigated kidney has the highest level of methylation of CCGG sequences in the α-like globin gene regions. In contrast, liver has Hpa II sites located in the α-like globin gene regions which are methylated to a greater extent (the sites flanking the 1.65kb and 0.6kb fragments) and to a lesser extent (the site bordering the 2.7kb fragment) than the ones present in FL cells.

DISCUSSION

The results of the in vitro methylation studies presented here support a correlation between FL cell DNA hypomethylation and FL cell differentiation. That is they demonstrate that when FL cells are chemically induced to differentiate, the DNAs isolated from the cells are hypomethylated, and that DNA from a clone which is resistant to induction by a specific agent is normally methylated when the cells are grown in the presence of the agent, but undermethylated when the cells are exposed to compounds which cause their differentiation. Based on these studies, I intended to show whether or not hypomethylation occurred within the α- and β-globin genes during FL cell differentiation. Using C methylation-sensitive restriction endonucleases to look at the patterns of methylation of specific sequences within the gene regions, I could not detect any hypomethylation in the DNA isolated from chemically induced FL cells. This failure could be due to any one of the following reasons:

A. Technical

1. The masking of changes in methylation due to incomplete digestion of DNA.
2. The changes are occurring at sites other than those recognized by the C methylation-sensitive restriction endonucleases.

B. Conceptual

1. Methylation patterns allowing expression of

the genes already exist in uninduced FL cells.

2. Hypomethylation occurs due to the loss of methyl groups on one strand of DNA during FL cell differentiation.

It is unlikely that I did not detect changes in methylation of the globin genes during a period when their expression was enhanced due to the first technical reason proposed, incomplete digestion of FL cell DNA by restriction endonucleases. The results discussed below indicate that the digestions were extensive and representative of the degree to which each site under analysis is methylated. A procedure was employed to monitor the extent of FL cell DNA digestions which has been used by many other investigators (Mandel and Chambon, 1979; McGhee and Ginder, 1979; Shen and Maniatis, 1980; van der Ploeg and Flavell, 1980; and Jones et al., 1981). Completion of digestion was determined by adding an internal standard to an aliquot of the incubation mixture and then verifying by gel electrophoresis that the standard DNA is totally cleaved. Hybridizations of the restricted DNAs with the α-globin probe, however, revealed that DNA digests in which no undigested DNA could be detected on an agarose gel after ethidium bromide staining contained uncut sites. Therefore, the visualization of required cleavage products signifies most but not all of the recognition sites in DNA are being cleaved by the restriction endonucleases, and thus, the criterion by which

other laboratories judged their digestions to be complete is invalid. All possible measures were then taken to avoid the problem of incomplete digestion. Extra enzyme was added to reactions after they had been determined to be complete by the visualization of totally cleaved internal standards. The amount of endonuclease used per digestion was even increased to an excess of 10 fold. (Both Maniatis and Flavell stressed that the use of a large quantity of restriction endonuclease insures against partial-digestion of DNA; Shen and Maniatis, 1980; and van der Ploeg and Flavell, 1980.) Despite these additional efforts, uncut sites in digested DNAs were seen.

Nevertheless, the fact that I did not detect changes in methylation during FL cell differentiation does not appear to be due to the masking of these changes by incomplete digestions. When several FL cell DNA Msp I digests which were monitored to be incomplete (as determined by the visualization of partially digested internal standards) were hybridized with the α-globin probe, several different banding patterns were formed on film. However, when Msp I FL cell DNA digests which were monitored to be "complete" (as determined by the visualization of totally cleaved internal standards) were hybridized with the α-globin probe, only one banding pattern was produced on autoradiograms (Figure 18a, lane c and Figure 18b, lane a). This same

pattern was formed every time the FL cell DNAs were "completely" digested with Msp I. An analogous situation was observed for Hpa II digests of 745A and DS-19 DNAs and for Hha I digests of 745A and DS-19 DNAs. Thus, the digestions appear to reflect the extent of methylation of the sites under investigation.

The second technical reason changes may not have been seen in methylation of the FL cell globin genes during in vitro differentiation, may be due to the fact that a limited number of sites in the gene regions are experimentally accessible using the restriction enzymes available today. When DNA isolated from FL cells induced to differentiate is methylated in vitro, the enzyme transfers methyl groups to most methylatable sites in the DNA, and therefore, with high specific activity S-AdoMet a small amount of hypomethylation at any type of site can be detected in the DNA. Utilization of C methylation-sensitive endonucleases which recognize only CCGG and GCGC sequences, however, restricts the number of sites analyzed to a small proportion of the total methylatable sites in FL cell DNA. In addition, during these investigations of the globin genes, only those Hpa II (Msp I) and Hha I sites located in the vicinity of the sequences complementary to the probes employed in this study could be examined. Therefore, it is quite possible that changes are occurring in the pattern of globin gene methylation during FL cell differentiation which could not be detected.

The first conceptual reason proposed for why methylation patterns appear identical in the globin gene regions of induced and uninduced FL cells might be that the patterns allowing expression of the genes already exist in uninduced FL cells. Support for this possibility was provided in this study by comparing the results obtained during investigations of the levels of methylation of CCGG sequences in the α-like globin gene regions in adult mouse kidney and uninduced FL cells. Both C residues in this sequence were found to be modified to a greater extent in kidney, a tissue where the genes are never active, than in uninduced FL cells which express the globin genes when grown in an appropriate environment. This finding is similar to the one obtained by Shen and Maniatis (1980), who observed more extensive modification of the Hpa II sites surrounding the α-like globin genes in rabbit kidney than in erythroid tissues. It suggests that any loss of methyl groups from the α-like globin gene regions which may affect activity is most likely to be occurring early in the erythroid differentiation process and that some of the hypomethylation detected by in vitro methylation of DNA from induced FL cells probably represents changes in the methylation patterns of other genes whose expression is augmented during FL cell differentiation.

The last explanation which again is conceptual and would account for my findings is that the loss of a methyl group on only one DNA strand in the globin gene regions may

be all that is needed for gene activity. If this is the case, then significant changes in FL cell DNA methylation would not be revealed by restriction endonuclease methods, since both fully- and hemi-methylated sites are resistant to cleavage by the C methylation-sensitive enzymes.

Evidence for the involvement of hemi-methylation in gene expression was obtained in studies where the relationship between DNA methylation and the inducibility of the mouse metallothionein-I (MT-I) gene in lymphoid cells was investigated (Compere and Palmiter, 1981). It was shown that the loss of methyl groups from only one strand of DNA appeared to allow the expression of the gene, since an hour treatment of the cells with the hypomethylating agent 5-aza-CR, followed by an 8 hour recovery period in the presence of cadmium was sufficient to trigger synthesis of MT-I mRNA. During this 9 hour period, less than a full cycle of DNA replication could occur and therefore, methyl groups could be lost from only one DNA strand (i.e., the newly synthesized strand). When the incorporation of 5-aza-CR into DNA was inhibited, the gene was not inducible by cadmium. This implies that the incorporation of 5-aza-C into DNA can result in the loss of one methyl group from either fully-methylated or hemi-methylated sites, and that some of these undermethylated sites are likely to be functioning in the regulation of MT-I gene activity. Since most

methylated sites in DNA contain methyl groups on both DNA strands (Bird, 1978), it is probable that mostly hemi-methylated sites were created in the treated mouse lymphoid cells.

If chemically induced FL cells also comprise a system in which unsymmetrically methylated sites in DNA are linked to gene expression, then the FL cell DNA hypomethylation which occurs during differentiation should result from the production, at least in part, of hemi-methylated sites. Since this undermethylation is detected by *in vitro* assays in which DNAs from treated cells are methylated with radio-labelled S-AdoMet using DNA methyltransferase, the enzyme would have to be able to both recognize and transfer methyl groups to hemi-methylated sites.

It is postulated (Holliday and Pugh, 1975; and Riggs, 1975), that DNA methylation occurs by two enzyme activities. The first being a "*de novo*" methyltransferase activity which initiates methylation at unmethylated sites and the second, a "maintenance" activity which completes hemi-methylated sites. Bird (Bird, 1978) while studying the distribution of methylation in the ribosomal genes of *Xenopus laevis* obtained some of the first data supporting the actual existence of a "maintenance" enzyme activity. The FL cell methyltransferase preparation used in *in vitro* assays appears reluctant to "initiate" methylation at unmethylated sites, although it is able to achieve a low level of methylation on completely

unmethylated DNAs, cloned $^{\beta}M_2$ DNA and pBR322 DNA (Weich, N. and Schoenbrun, B. Unpublished results). Therefore, it seems probable that many of the sites in treated FL cell DNAs accepting methyl groups from the enzyme during in vitro assays are hemi-methylated, and that some of these sites, although not detectable by C^{1} methylation-sensitive restriction endonucleases, might be located within the FL cell globin gene regions.

Not only do my results confirm that a correlation exists between FL cell DNA hypomethylation and FL cell differentiation, but they also show that new, unmethylated sites can be created without triggering differentiation. An example of this is the heritable change in methylation that was detected in the α-like globin gene regions after treatment with 5-aza-CR. It is unlikely to be involved in globin gene expression, since a change in methylation of a specific site in the DNA of 15% of the cell population (approximately 15% of the cell population synthesizes hemoglobin) would result in the formation of a band on film with much less intensity than the one that was seen.

The possibility that variations in DNA methylation of specific sites might occur in FL cells without being related to differentiation is further supported by the data obtained when comparing methylation of the α-like globin gene regions in DNA from uninduced 745A cells to that in DNA from uninduced DS-19 cells. Two Hpa II sites flanking a 3.8 kb segment of

FL cell DNA were seen to be methylated in 745A cells and not in DS-19 cells. This finding may reflect the overall extent of methylation of Hpa II sequences, since the internal C residue in CCGG sequences was discovered to be modified to a greater degree in 745A DNA than in DS-19 DNA (Christman, J.K. Manuscript in preparation). In contrast, some Hha I sites located in the vicinity of the α-like genes are more methylated in DS-19 DNA than in 745A DNA, (i.e., the sites present within the 6.5 kb Eco RI fragment).

Since DS-19 cells were cloned from strain 745A, one would expect the two cell lines to have the same pattern of DNA methylation. Therefore, it is interesting to speculate on how the variations in methylation of 745A and DS-19 DNA could have arisen. The model below takes into consideration the proposed mode of inheritance of methylation patterns. During reproduction of the parental pattern by methyltransferase on newly synthesized progeny DNA strands

$$\left(\begin{array}{l} 5'\text{-MeCG-}3' \\ 3'\text{-\ \ CG-}5' \end{array} \longrightarrow \begin{array}{l} 5'\text{-MeCG-}3' \\ 3'\text{-GMeC-}5' \end{array}\right)$$

it is possible that occassional, random loss and/or gain of methylation occurs. Thus, a cell population which is constantly dividing, as is the situation with cells in culture, would eventually contain many subpopulations, each containing its own variation of the basic methylation pattern. This then suggests that (1) cultured cells have variable methylation patterns; and (2) when cell line DS-19 was cloned from one 745A cell, only one methylation pattern out of the many likely to be present in the

strain was selected.

The data discussed above indicate that although undermethylation may be an integral part of the process of gene expression, it is unlikely to be the only factor necessary for the activation of transcription. Investigations of the α-like globin genes in mouse liver supported this conclusion by demonstrating that genes which are no longer active retain a low level of methylation within and around their coding sequences. The mouse liver, a tissue which in the fetus is the center of erythropoiesis, no longer contains cells that synthesize globin polypeptides in the adult. However, it appears that the pattern of DNA methylation in the adult liver reflects the fact that globin genes were once expressed in the tissue, since a lower level of methylation is found in the α-like globin gene regions in liver than in a tissue which never expresses the genes (kidney). This situation is analogous to what is observed in the rabbit, where the fetal liver is a primary erythropoietic site, and several Hpa II sequences in the vicinity of the β-like globin genes are found to have a lower level of modification in the adult liver than in tissues which never express the genes (Shen and Maniatis, 1980).

Evidence that changes in chromatin conformation may be important in the regulation of gene activity was provided by investigations concerning DNase I-hypersensitive sites.

These specific points in the chromosome which are usually located to the 5' side of active genes and are preferentially cut by DNase I were observed in the globin gene regions of FL cells only after chemical induction (Rifkind, R., Personal communication). This suggests that certain changes in chromatin conformation which appear to be linked to gene expression cannot occur until the differentiation process is resumed in these cells. Thus, a factor other than methylation seems to be involved in in vitro FL cell differentiation.

Despite the fact that a direct correlation has not been observed between the level of chemically induced FL cell differentiation and the level of FL cell DNA hypomethylation, the studies presented here have established that a definite relationship exists between the two processes. When the mechanism by which methylation functions in differentiation is determined, an understanding of this complicated relationship should be provided.

CONCLUSION

The following conclusions were drawn from the results obtained in this study:

1. The DNAs in two FL cell clones (745A and 5-86) become hypomethylated when the cells are chemically induced to differentiate.
2. Hypomethylated DNA is found only in FL cells exposed to inducing agents which actually cause their differentiation.
3. A loss of methyl groups from Msp I, Hpa II and Hha I sites surrounding the α- and β-globin genes does not occur during *in vitro* differentiation of FL cells.
4. Treatment of FL cells with 5-aza-CR creates new unmethylated sites in the α-like globin gene regions which do not apparently trigger hemoglobin synthesis.
5. Variations exist in the methylation patterns of α-like globin gene regions of two FL cell clones (745A and DS-19).
6. Of all the cells investigated (FL cells, mouse kidney and mouse liver) kidney has the highest level of methylation of CCGG sequences in the α-like globin gene regions. Liver has some Hpa II sites which are methylated to a greater extent and some Hpa II sites which are methylated to a lesser extent than those present in FL cells.

In summary, although changes in methylation of the globin genes are not detected during in vitro differentiation, my results demonstrate that a link between the hypomethylation of FL cell DNA and FL cell differentiation exists.

REFERENCES

Adams, R.P.L., Nature New Biol. 244: 27-29, 1973

Arber, W., Prog. Nucl. Acid Res. and Mol. Biol., 14: 1-37, 1958

Bird, A.P., J. Mol. Biol., 118: 49-60, 1978

Bird, A.P. and Southern, E.M., J. Mol. Biol., 118: 27-47, 1978

Boyer, S.H., Wuu, K.D., Noyes, A.N., Young, R., Scher, W., Friend, C., Preisler, H.D. and Bank, A., Blood, 40: 823-835, 1972

Burdon, R.H., and Adams, R.P.L., Biochim. Biophys. Acta., 174: 322-329, 1969

Byvoet, P. and Baxter, C.S., in Chromosomal Proteins and Their Role in Regulation of Gene Expression, Stein, G.S. and Kleinsmith, L.J., eds., pp. 127-151, Academic Press, New York, 1975

Christman, J.K., Price, P., Pedrinan, L. and Acs, G., Eur. J. Biochem., 8: 53-61, 1977

Christman, J.K. in Transmethylation, Usdin, E., Borchardt, R.T. and Creveling, C.R., eds., pp. 493-502, Elsevier/ North Holland, Inc., New York, 1979

Christman, J.K., Weich, N., Schoenbrun, B., Schneiderman, N. and Acs, G., J. Cell Biol.,86: 366-370, 1980

Cihak, A., Oncology, 30: 405-422, 1974

Compere, S.J. and Palmiter, R.D., Cell, 25:233-240, 1981

Constantinides, P.R., Taylor, S.M. and Jones, P.A., Develop. Biol., 66: 57-71, 1978

Copp, R.P., in Studies of Nuclear Protein Methyltransferase Activity in Friend Erythroleukemia Cells, Ph.D. thesis, New York University, New York, 1981

Creusot, F., Acs, G. and Christman, J.K., J. Biol. Chem., 1982, in press

Desrosiers, R.C., Mulder, C. and Fleckenstein, B., Proc. Natl. Acad. Sci. USA, 76: 3839-3843, 1979

Doskocil, J. and Sorm, F., Biochim. Biophys. Acta, 55: 953-959, 1962

Dugaiczyk, A., Hedgpeth, J., Boyer, H.W. and Goodman, H.M., Biochemistry, 13: 503-512, 1974

Dunn, D.B. and Smith, J.D., Biochem. J., 68: 637-646, 1958

Fantoni, A., Bank, A. and Marks, P.A., Science, 157: 1327-1329, 1967

Ferusawa, M., Ikawa, Y. and Sugano, H., GANN, Proc. Jpn. Acad., 47:220-223, 1971

Friedman, J. and Razin, A.,Nucl. Acids Res., 3: 2665-2675, 1976

Friedman, S., Biochem. Biophys. Res. Commun., 89: 1328-1333, 1979

Friend, C., J. Exp. Med., 105: 307-319, 1957

Friend, C. and Haddad, J.R., J. Natl. Cancer Inst., 25: 1279-1289, 1960

Friend, C., Patuleia, M.C. and deHarven, E., Natl. Cancer Inst., Monogr., 22:505-522, 1966

Friend, C., Scher, W., Holland, J.G. and Sato, T., Proc. Natl. Acad. Sci., USA, 68: 378-382, 1971

Greene, P.J., Betlach, M.C., Goodman, H.M. and Boyer, H.W., Methods Mol. Biol., 7: 87-111, 1974

Groudine, M., Eisenman, R. and Weintraub, H., Nature, 292:311-317, 1981

Guntaka, R.V., Rao, P.Y., Mitsialis, S.A. and Katz, R., J. of Virol., 34: 569-572, 1980

Haigwood, N.L., Jahn, C.L., Hutchinson III, C.A. and Edgell, M.H., Nucl. Acids Res., 9: 1133-1150, 1981

Hedgpeth, J., Goodman, H.M. and Boyer, H.W., Proc. Natl. Acad. Sci., USA, 74: 542-546, 1977

Holliday, R. and Pugh, J.E., Science, 187: 226-232, 1975

Hotchkiss,A., J. Biol. Chem., 175: 315-332, 1948

Jeffreys, A.J. and Flavell, R.A., Cell, 12: 429-439, 1977

Jones, P.A. and Taylor, S.M., Cell, 20: 85-93, 1980

Jones, R.E., DeFeo, D. and Piatigorsky, J., J. Biol. Chem., 265: 8172-8176, 1981

Kinniburgh, A.J., Mertz, J.E. and Ross, J., Cell, 14: 681-693, 1978

Kinniburgh, A.J. and Ross, J., Cell, 17: 915-921, 1979

Konkel, D.A., Tilghman, S.M. and Leder, P., Cell, 15: 1125-1132, 1978

Konkel, D.A., Maizer Jr., J.V. and Leder, P., Cell, 18: 865-873, 1979

Leder, A., Miller, H.I., Hamer, D.H., Seidman, J.G., Norman, B., Sullivan, M. and Leder, P., Proc. Natl. Acad. Sci., USA, 75: 6187-6191, 1978

Leder, A., Swan, D., Ruddle, F., D'Eustachio, P. and Leder, P., Nature, 293, 196-200, 1981

Leder, P., Hansen, N., Konkel, D., Leder, A., Nishioka, Y. and Talkington, C., Science, 209: 1336-1342, 1980

Lu, L.W. and Randerath, K., Cancer Res., 39: 940-948, 1979

Mandel, J.L. and Chambon, P., Nucl. Acids Res., 7: 2081-2103, 1979

Mann, M.B. and Smith, H.O., Nucl. Acids Res., 4: 4211-4221, 1977

Marmur, J., J. Mol. Biol., 3: 208-218, 1961

Marushige, K. and Bonner, J., Proc. Natl. Acad. Sci., USA, 68: 2941-2944, 1971

McGhee, J.D. and Ginder, G.P., Nature, 280: 419-420, 1979

Moore, B. and Smith, R., Canad. J. Biochem., 47: 561-565, 1969

Neinhuis, A.W. and Benz, E.J., New Eng. J. Med., 297: 1318-1328, 1977

Nishioka, Y. and Leder, P., Cell, 18: 875-886, 1979

Nishioka, Y., Leder, A. and Leder, P., Proc. Natl. Acad. Sci., USA, 77: 2806-2809, 1980

Ohta, T., Tanaka, M., Terada, M., Miller, O., Banks, A., Marks, P. and Rifkind, R., Proc. Natl. Acad. Sci., USA, 73: 1232-1236, 1978

Orkin, S.H., Swan, D. and Leder, P., J. Biol. Chem., 250: 8753-8760, 1975a

Orkin, S.H., Harosi, I. and Leder, P., Proc. Natl. Acad. Sci. USA, 72: 98-102, 1975b

Patuleia, M.C. and Friend, C., Cancer Res., 27: 726-730, 1967

Pollack, Y., Stein, R., Razin, A. and Cedar, H., Proc. Natl. Acad. Sci., USA, 77: 6463-6467, 1980

Popp, R.A., J. Mol. Biol. 27: 9-16, 1967

Rigby, P.W.S., Dieckman, M., Rhodes, C. and Berg, P., J. Mol. Biol., 113: 237-251, 1977

Riggs, A.D., Cytogenet. Cell Genet., 14: 9-25, 1975

Roberts, R.J., Wilson, G.A. and Young, F.E., J. Mol. Biol., 97: 123-126, 1975

Roberts, R.J., Gene, 4: 183-193, 1978

Roy, P.H. and Weissbach, A., Nucl. Acids Res., 2: 1669-1684, 1975

Russell, E.S. and McFarland, E.L., Ann. N.Y. Acad. Sci., 241: 25-38, 1974

Sato, S., Hutchison, C.A. and Harris, J.I., Proc. Natl. Acad. Sci., USA, 74: 542-546, 1977

Scher, W. and Friend, C., Cancer Res., 38: 841-849, 1978

Shaw, J.L., Blanco, J. and Mueller, J.C., Anal. Biochem., 65: 125-131, 1975

Shen, C.J. and Maniatis, T., Proc. Natl. Acad. Sci., USA, 77: 6634-6638, 1980

Singer, J., Roberts-Ems, J. and Riggs, A.D., Science, 203: 1019-1020, 1979

Sneider, T.W., Teague, N.N. and Rogachevsky, L.M., Nucl. Acids Res., 2: 1685-1700, 1975

Sneider, T.W., Nucl. Acids Res., 8: 3829-3840, 1980

Southern, E.M., J. Mol. Biol., 98: 503-517, 1975

Srinivasan, P.R. and Borek, E., Science, 145: 548-553, 1964

Sutter, D. and Doerfler, W., Proc. Natl. Acad. Sci., USA, 77: 253-256, 1980

Tiemeier, D.C., Tilghman, S.M. and Leder, P., Gene, 2: 173-191, 1977

Tiemeier, D.C., Tilghman, S.M., Polsky, F.I., Seidman, J.G., Leder, A., Edgell, M.H. and Leder, P., Cell, 14: 237-245, 1978

Tilghman, S.M., Tiemeier, D.C., Polsky, F.I., Edgell, M.H., Seidman, J.G., Leder, A., Enquist, L.W., Norman, B. and Leder, P., Proc. Natl. Acad. Sci., USA, 74: 4406-4410, 1977

van der Ploeg, L.H.T. and Flavell, R.A., Cell, 19: 947-958, 1980

Vanin, E.F., Goldberg, G., Tucker, P. and Smithies, O., Nature, 286: 222-226, 1980

Vanyushin, B.F., Tkacheva, S.G. and Belozersky, A.N., Nature, 225: 948-949, 1970

von Hoff, D.D., Slavik, M. and Muggia, F.M., Ann. Internal Med., 85: 237-245, 1976

Waalwijk, C. and Flavell, R.A., Nucl. Acids Res., 5: 4631-4639, 1978

Wahl, G.M., Stern, M. and Stark, G.R., Proc. Natl. Acad. Sci., USA, 76: 3683-3687, 1979

Wainfan, E. and Maschio,F., Ann. N.Y. Acad. Sci., 255: 567-575, 1975a

Wainfan, E., Moller, M., Maschio, F. and Balıs, M.E., Cancer Res., 35: 2830-2855, 1975b

Weintraub, H., Larsen, A. and Groudine, M., Cell, 24: 333-344, 1981

Wigler, M., Levy, D. and Perucho, M., Cell, 24: 33-40, 1981

Wilson, G.A. and Young, F.E., J. Mol. Biol., 97: 123-126, 1975.