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Murine erythroleukemia cell differentiation: DNase I hypersensitivity and DNA methylation near the globin genes

(erythrocyte differentiation/gene expression/chromatin structure)

MICHAEL SHEFFERY, RICHARD A. RIFKIND, AND PAUL A. MARKS

Laboratory of Developmental Cell Biology, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, New York 10021

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ABSTRACT **The sensitivity to digestion by DNase I of chromatin containing the α- and β_{major}-globin genes and the pattern of DNA methylation near these genes was examined during hexamethylenebisacetamide (HMBA)-mediated erythroid differentiation of murine erythroleukemia cells (MELC). In uninduced and induced cells, the chromatin regions containing the α- and β-$_{major}$-globin genes are more sensitive to digestion by DNase I than is the region containing an immunoglobulin gene (Igα) not expressed during erythroid differentiation. However, at low concentrations of DNase I, a 6- to 10-fold increase in site-specific cleavages was generated in chromatin regions near both the α- and β_{major}-globin genes in cells induced to differentiate by HMBA. The DNase I hypersensitive site near the β_{major}-globin gene maps to a small region near the 5′ terminus of the gene. No detectable change in the pattern of DNA methylation around either the α- or β-globin genes was observed during HMBA-mediated erythroid differentiation. Of the potentially methylated sites assayed and mapped near the β_{major}-globin gene, one site is fully methylated, one is partially methylated, and one is unmethylated both in uninduced and induced cells. Many (but not all) sites assayed near the α-globin genes are unmethylated in both uninduced and induced cells. These results show that specific alterations of chromatin structure occur during MELC differentiation and suggest that these changes may not involve alterations in the pattern of DNA methylation.**

Murine erythroleukemia cells (MELC) are transformed cells that can be induced by several agents to undergo a differentiation process that closely resembles normal murine erythroid differentiation (1). During induced differentiation, there is a ≈10-fold increase in the rate of accumulation of globin mRNAs (2). The molecular mechanisms responsible for this change in MELC gene activity are not known. Work in several laboratories has established that changes in chromatin structure, assayed by the sensitivity of specific genes to digestion by DNase I, are associated with the activation of chromosomal domains (3, 4). More recent work has demonstrated that, within DNase I-sensitive regions, there may be sites that are "hypersensitive" to digestion (5–9). DNase I-hypersensitive sites are usually found immediately proximal to the 5′ end of active (or potentially active) genes (5–9). Although the sensitivity to DNase I digestion of active chromatin domains appears to depend on the binding of high mobility group (HMG) proteins 14 and 17 (10), the factors that allow selective binding of these proteins to chromatin regions are not understood. The biochemical basis for hypersensitivity to DNase I digestion is also unknown.

The pattern of DNA methylation might provide a heritable molecular mechanism to control or distinguish active and inactive DNA sequences (see ref. 11 for a recent review). This possibility is supported by several recent observations that show a relationship between DNA hypomethylation and gene expression (9, 12–17). For example, during chicken erythrocyte development, globin gene expression is accompanied by a decrease in gene methylation and an increase in the sensitivity of the corresponding chromatin to DNase I (9). Other experiments, using 5-azacytidine incorporation into DNA to inhibit methylation (18), have shown that this treatment can heritably activate specific genes, such as the mouse metallothionein I gene (12) and unexpressed endogenous avian viral sequences (13). In the avian system, gene activation after demethylation is accompanied by an increased sensitivity of the corresponding chromatin to digestion by DNase I and the appearance of specific DNase I-hypersensitive sites (13). Not all potential sites within a domain, however, need be unmethylated for gene activation. In the rabbit globin domain, demethylation of a few specific sites correlates with gene activity (14).

In MELC, a small but consistent decrease in overall DNA methylation has been described during induction of erythroid differentiation by several agents, including hexamethylenebisacetamide (HMBA) (19). In addition, chromatin regions containing the globin genes are reported to be relatively sensitive to DNase I both in uninduced and induced MELC (20). The present studies were designed to examine the relationship between globin gene methylation and the sensitivity to DNase I. The results show that whereas both uninduced and induced chromatin containing the α- and β-globin genes are relatively sensitive to digestion by DNase I compared to chromatin containing a gene not expressed during erythroid differentiation, discrete DNase I-hypersensitive sites develop near both the α- and β-globin genes during HMBA-mediated differentiation. This change in chromatin occurs in the absence of detected change in the pattern of DNA methylation in the vicinity of these genes.

MATERIALS AND METHODS

Cell Culture. MELC strain DS19 was isolated and maintained in α medium (minus nucleosides; Gibco) as described (21). At the initiation of experiments, exponentially growing cells were inoculated at 1×10^5 cells per ml either in the presence or absence of inducer (4 mM HMBA). Cells were subsequently grown for 48 hr. At this time, more than 90% of cells treated with inducer are committed to terminal erythroid differentiation (22).

Isolation and Digestion of Nuclei. After 48 hr of growth in either the presence or absence of inducer, MELC nuclei were

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Abbreviations: MELC, murine erythroleukemia cells; HMBA, hexamethylenebisacetamide; kb, kilobase(s); bp, base pairs.

purified as described (23). Isolated nuclei (9×10^7 nuclei per ml) were digested with various concentrations of DNase I (Worthington) for 10 min at room temperature. Digestion was terminated by addition of EDTA to 50 mM and sodium dodecyl sulfate to 0.2%.

Purification and Restriction of DNA. Nuclear lysates were incubated with Proteinase K (0.1 mg/ml; Boehringer Mannheim) for at least 2 hr, and nucleic acids were purified by successive organic extractions, followed by precipitation with ethanol at −20°C (24). Ethanol precipitates were recovered by centrifugation (60 min at 16,000 × *g* at −15°C in a Sorvall HB-4 rotor), dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 7.4, and incubated for 60 min at 37°C with RNase A (50 μg/ml; Worthington) that previously had been heated to 80°C for 10 min. Samples were reextracted with organic solvents several times (as described above) and reprecipitated with ethanol. Purified DNA preparations were dissolved in 6 mM Tris·HCl/6 mM NaCl/0.1 mM EDTA, pH 7.5. Digestions with restriction endonucleases (Bethesda Research Laboratories or New England BioLabs) were carried out in the buffers recommended by the suppliers. Purified DNA samples obtained from nuclei digested with DNase I were digested with restriction endonucleases, extracted with organic solvents (as described above), precipitated with ethanol, and redissolved in 10 mM Tris·HCl/1 mM EDTA, pH 7.4. DNA concentration was measured by absorbance at 260 nm, and equal amounts of DNA were applied to each gel electrophoresis lane.

Electrophoresis, Transfer of DNA to Nitrocellulose, and Hybridization. DNA samples were subjected to electrophoresis through 0.7% agarose gels (SeaKem) in a Tris acetate/EDTA buffer (25). After electrophoresis, DNA was denatured and transferred to nitrocellulose filters (Schleicher and Schuell, BA 85) in 1.5 M NaCl/0.15 M sodium citrate, pH 7.4, as described (26). DNA adhering to the filters was hybridized against denatured DNA probes radioactively labeled *in vitro* with ^{32}P by nick translation (ref. 27; specific activity, $0.7–2 \times 10^9$ cpm/μg) as described (6). After hybridization, filters were washed (6) and exposed to x-ray film (Kodak, XAR-2) at −80°C for 1–5 days. All procedures using recombinant DNA were performed by National Institutes of Health guidelines.

RESULTS

DNase I Sensitivity of Chromatin Containing the β_{major}- and α-Globin Genes. Previous studies of the globin genes in MELC by liquid hybridization suggested that uninduced globin genes are in an "active" configuration because they are relatively sensitive to DNase I (20). In the present experiments, we first compared the sensitivity to DNase I of chromatin containing the α- and β_{major}- globin genes to that containing the Igα gene.

Nuclei from uninduced MELC were digested with various concentrations of DNase I; the DNA was purified, digested with *Eco*RI, and analyzed by the method of Southern (26) with probes specific for the β_{major}-globin gene (28) or the Igα gene (generous gift of S.-P. Kwan and M. D. Scharff). The β_{major}-globin gene in uninduced MELC was relatively more sensitive to digestion by DNase I than was the Igα gene (Fig. 1 *A* and *B*). No β_{major}-globin gene was detectable after digestion with DNase I (3 μg/ml) for 10 min, whereas the Igα gene was still detected after equivalent digestion. Similar results were obtained for the α-globin genes (data not shown). These results confirm the observations of Miller *et al.* (20) and suggest that some stable alteration in chromatin structure in the globin domains already has occurred in the developmental history of these transformed erythroid precursor cells.

We next compared the effects of DNase I digestion on nuclei prepared from uninduced and induced MELC. Fig. 2*C* shows

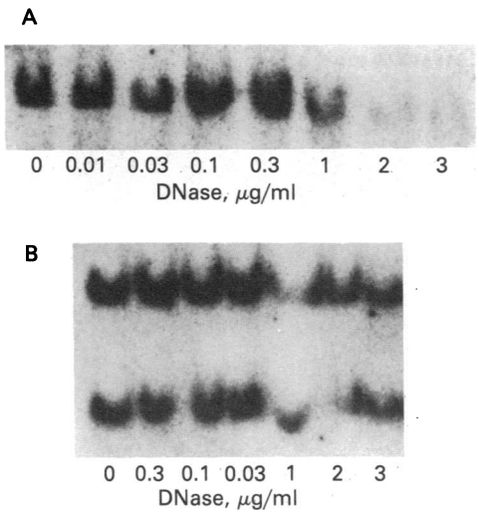

FIG. 1. Relative sensitivity of chromatin containing the β_{major}-globin gene and the Igα gene to digestion by DNase I in uninduced MELC. Nuclei from uninduced MELC were purified and digested with the concentrations of DNase I indicated below each lane. Purified DNA was digested by *Eco*RI and analyzed for the presence of the β_{major}-globin gene (*A*) or the Igα gene (*B*). *Eco*RI digestion produces two Igα gene fragments of ≈2.5 and ≈1.8 kb.

an abbreviated restriction endonuclease map of the β_{major}-globin gene (29) and the location of the cloned probe specific for β_{major}-globin gene that was used in these experiments (28). The β_{major}-globin gene in MELC is contained in *Xba* I and *Eco*RI fragments of approximately 9 and 7 kilobases (kb) respectively. The cloned sequence used as a probe specific for the β_{major}-globin gene is located 3′ (rightward) of the gene sequence. The 3′ end of the probe is defined by the *Xba* I site located 2 kb from the β_{major}-globin gene cap site, and it extends ≈400 base pairs (bp) toward the 5′ end (leftward in Fig. 2*C*). Nuclei were digested at several concentrations of DNase I. Purified DNA from these nuclear digests in turn was digested by *Xba* I and analyzed with the probe specific for β_{major}-globin gene. During induced differentiation, a small chromatin region near the β_{major}-globin gene became particularly sensitive to digestion by DNase I (Fig. 2*A*). The cut at this DNase I "hypersensitive site" generated a somewhat diffuse subfragment of 2–2.2 kb. The same filter, of which the autoradiograph is shown in Fig. 2*A*, was washed (30) and rehybridized with the Igα-specific probe. Chromatin around the Igα gene remained relatively resistant to digestion by DNase I in both uninduced and induced cells, and no Igα-specific subfragments were generated in HMBA-induced cell nuclei (data not shown).

Because the cloned probe used to identifiy the β_{major}-globin gene is bounded at its 3′ end by the *Xba* I site (28), we used the size of the DNase I-generated fragment (Fig. 2*A*) to determine the location of the inducible β_{major}-globin gene DNase I-hypersensitive site (5). The DNase I-hypersensitive site mapped to a small region (≈200 bp) beginning 50 bp 5′ of the β_{major}-globin gene cap site (Fig. 2*C*). This map position was confirmed by digesting DNA (purified from a completely separate DNase I digestion series) with *Eco*RI and analyzing with the β_{major}-globin gene-specific probe. In this case, we found the DNase I cleavage near the 5′ end of the β_{major}-globin gene, producing an *Eco*RI subfragment of ≈6 kb in the DNA of chromatin from induced MELC (Fig. 2*B*).

We also examined the sensitivity to DNase I of chromatin containing the α-globin genes. DNA purified from DNase I-digested nuclei was digested with *Sac* I, blotted by the method

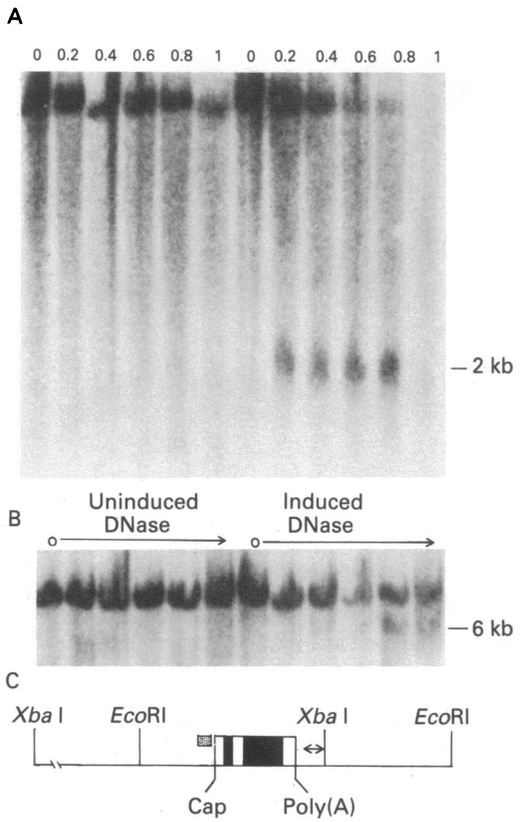

FIG. 2. Hypersensitivity to DNase I of a chromatin region near the 5′ end of the β_{major}-globin gene in induced MELC. (*A*) Nuclei were prepared from uninduced (*Left*) or induced (*Right*) MELC and digested with the concentration of DNase I in μg/ml indicated above each lane. DNA was purified, digested with *Xba* I and analyzed by the method of Southern (26) with a β_{major}-globin gene-specific probe (see *C*). A DNase I-generated subfragment of 2 kb is indicated. (*B*) DNA, purified from another DNase I digestion series, was digested with *Eco*RI and analyzed with the β_{major}-globin gene probe. Increasing concentrations (from zero) of DNase I are indicated by the arrows above the lanes. A DNase I-generated subfragment of ≈6 kb, produced from induced cell nuclei, is indicated. (*C*) A simplified restriction map around the β_{major}-globin gene showing the location of adjacent *Eco*RI and *Xba* I sites. The 5′ and 3′ ends of the map (based on the direction of transcription) are to the left and right, respectively. The size and location of the β_{major}-globin gene-specific probe sequence (28) is indicated by the double-headed arrow extending from the 3′ *Xba* I site. The probe is located in the 3′ flanking sequence of the β_{major}-globin gene and does not cross-react with the β_{minor}-globin gene. The β_{major}-globin gene is represented by the open (exon) and solid (intron) boxes. The cap and poly(A) addition sites are indicated. ▨, The DNase I-hypersensitive region, mapped from data presented in *A* and *C*.

of Southern (26), and hybridized with an α-globin gene cDNA cloned probe (31). DNase I-generated subfragments of 4 and 1.2 kb were present (Fig. 3) in DNA from induced MELC. We did not map the DNase-hypersensitive sites near the α-globin genes. Whereas the 4-kb subfragment must be generated from one of the large *Sac* I fragments, the source of the 1.2-kb subfragment is ambiguous. If, in fact, the 1.2-kb subfragment is generated from the 3.2-kb *Sac* I fragment containing the α_1-globin gene (32), then this DNase I-hypersensitive site is located just 5′ proximal to the α_1-globin gene.

Small amounts of globin gene-related subfragments also were produced by DNase I digestion of uninduced cell nuclei. The intensity of radioautograph bands corresponding to the DNase I-generated subfragments from induced MELC, as determined by densitometry, was 6–10 times that of the faint subfragment bands generated from nuclei prepared from uninduced cells.

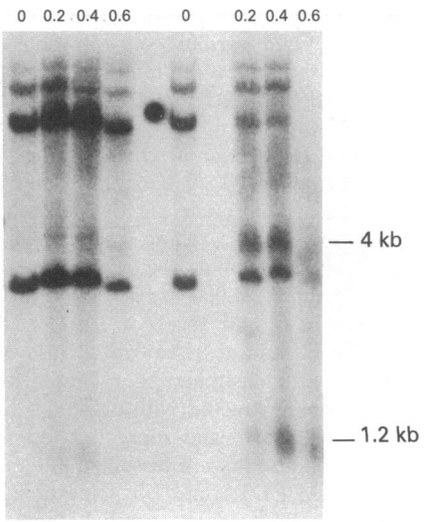

FIG. 3. Hypersensitivity to DNase I of chromatin regions near the α-globin genes. Nuclei from uninduced (*Left*) and induced (*Right*) MELC were digested with the concentrations of DNase I in μg/ml indicated above each lane. Purified DNA was digested with *Sac* I, prepared as in Fig. 2, and analyzed with an α-globin gene-specific probe. Subfragments of ≈1.2 kb and ≈4 kb, generated by DNase I digestion, are indicated.

The DS19 erythroleukemia cell line consists of a population of erythropoietic precursors, uniformly inducible with a variety of chemical inducers (1, 33). It is unlikely, therefore, that the low level of hypersensitivity to DNase I found in uninduced cells is due to a unique subpopulation of inducible cells. Virtually all MELC strains, including DS19, displayed a low level of spontaneous differentiation (<1%). This may, in part, account for the detection of a low level of globin gene DNase I-hypersensitive sites among uninduced MELC.

Methylation Patterns Around the β_{major}- and α-Globin Genes During HMBA-Mediated Erythroid Differentiation. When total genomic MELC DNA was digested with the restriction endonuclease *Msp* I, subjected to agarose gel electrophoresis, blotted onto nitrocellulose paper (26), and hybridized with the radioactively labeled probe specific for the β_{major}-globin gene, a 3.8-kb fragment was visualized by autoradiography (Fig. 4*A*, lane 3). Sequential digestion of genomic DNA by *Msp* I and *Xba* I generated a 3.5-kb fragment (Fig. 4*A*, lane 4). *Msp* I/*Eco*RI and *Eco*RI/*Xba* I double digests generated fragments of 3.5 and 3.2 kb, respectively (Fig. 4*A*, lanes 5 and 6). These results were used to map one *Msp* I site 0.3 kb to the right of the 3′ distal *Xba* I site; the other *Msp* I site mapped ≈0.3 kb to the left of that *Eco*RI site 5′ to the β_{major}-globin gene (see Fig. 4*B*). Thus, the 9-kb *Xba* I fragment contains only the lefthand *Msp* I site, whereas the 7-kb *Eco*RI fragment has only the right-hand *Msp* I site. The methylation of each of these sites was assayed by monitoring the stability of the 9-kb *Xba* I fragment and the 7-kb *Eco*RI fragment to digestion by the methyl-sensitive isoschizomer of *Msp* I, *Hpa* II (34). The 7-kb *Eco*RI fragment was stable to *Hpa*II digestion in both uninduced and induced cells (compare lanes 8 and 10 in Fig. 4*A*). Thus, the *Msp* I site 3′ to the β_{major}-globin gene is fully methylated in uninduced MELC and remains fully methylated during induced erythroid differentiation.

Methylation at the leftward *Msp* I site was assayed by examining the stability of the 9-kb *Xba* I fragment to *Hpa* II digestion. This site is partially unmethylated in MELC because there was some slight digestion of the 9-kb *Xba* I fragment (Fig. 4*A*, lanes 7 and 9). However, no difference was detected between

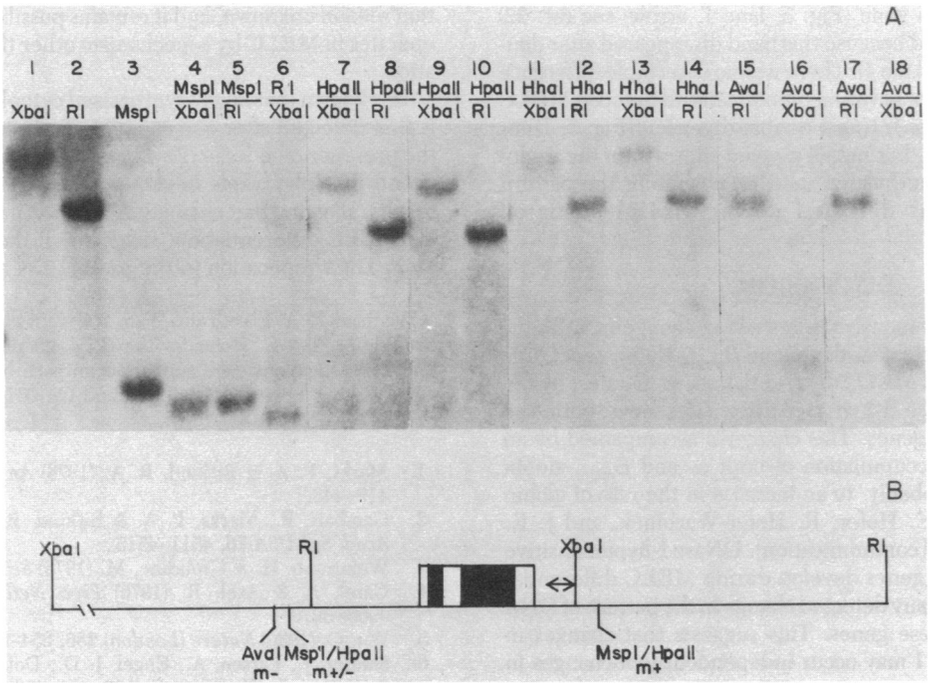

FIG. 4. Pattern of DNA methylation near the β_{major}-globin gene. Total genomic DNA was purified from uninduced MELC or MELC induced by incubation with 4 mM HMBA for 48 hr. (*A*) Samples of DNA were incubated with the restriction enzymes indicated above each lane. Each lane shows the autoradiogram resulting when DNA from uninduced (lanes 1–8, 11, 12, 15, and 16) or induced (lanes 9, 10, 13, 14, 17, and 18) MELC was digested, blotted onto nitrocellulose (26), and hybridized with the radioactively labeled β_{major}-globin gene-specific probe (Fig. 4*B*, double-headed arrow). Lanes 1–6, 7–10, 11–14, and 15–18 are from four separate autoradiograms, and exact comparison of fragment mobilities between these sets of lanes is not possible. (*B*) A simplifed restriction map around the β_{major}-globin gene (as in Fig. 2*C*) showing the *Msp* I (and *Hpa* II) and *Ava* I sites mapped from the data in Fig. 4*A*. m+, Fully methylated site; m+/− partially methylated site; m−, unmethylated site; RI, *Eco*RI.

the patterns produced by digestion of DNA from uninduced or induced cells (compare lanes 7 and 9 in Fig. 4*A*). The *Eco*RI and *Xba* I fragments generated from both uninduced and induced cells were also stable to digestion by two other methyl-sensitive restriction endonucleases, *Hha* I (15) (compare lanes 11 and 12 to lanes 13 and 14 in Fig. 4*A*) and *Sma* I (data not shown). However, although the 7-kb *Eco*RI fragment from uninduced and induced MELC is stable to digestion by the methyl-sensitive restriction endonuclease *Ava* I (ref. 15; compare lanes 15 and 17 in Fig. 4*A*), the 9-kb *Xba* I fragment was cleaved by this enzyme to 3.7 kb in both uninduced and induced MELC (compare lanes 16 and 18 in Fig. 4*A*). Because the probe sequence is bounded on the right by the *Xba* I site (28), the unmethylated *Ava* I site can be placed 3.7 kb to the left of this site, close to the partially methylated *Msp* I described above (Fig. 4*B*). Taken together, these results define the pattern of DNA methylation near the β_{major}-globin gene in MELC (Fig. 4*B*) and show that, for the sites assayed, there is no detectable change in methylation during induced erythroid differentiation.

The methylation pattern around the α-globin genes during induced differentiation was also examined. The probe used to detect α-globin gene fragments was a radioactively labeled α-globin cDNA clone (31). With an experimental design analogous to that described for the β_{major}-globin gene locus, the stability of α-globin gene-specific *Sac* I digestion fragments (Fig. 5, lanes 1 and 5) to digestion by *Hpa* II, *Msp* I or *Hha* I was examined in double digests of uninduced and induced MELC DNA. *Sac* I/*Msp* I double digests (lanes 3 and 7) showed the limit digest pattern expected in the fully unmethylated state. Comparison of the fragments in lanes 1, 2, and 3 shows that several, but not all, *Msp* I sites near the α-globin genes are unmethylated in uninduced cells because all *Sac* I-digestion fragments were also digested by *Hpa* II, but the *Msp* I limit digestion pattern (lane 3) was not produced. When *Sac* I/*Hpa* II double digests of DNA prepared from uninduced and induced cells are compared (Fig. 5, lanes 2 and 6) no change in the methylation pattern is detected. The *Hha* I sites around the 3.2-kb *Sac* I fragment con-

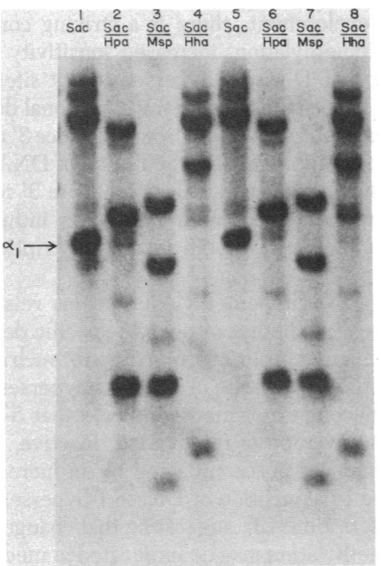

FIG. 5. Pattern of DNA methylation near α-globin genes. Total genomic DNAs from uninduced (lanes 1–4) or induced (lanes 5–8) MELC were digested to completion with the restriction endonuclease(s) indicated over each lane. Digests were sized by electrophoresis in agarose gels, blotted onto nitrocellulose, and hybridized with a radioactive cloned α-globin cDNA sequence. The α_1-globin gene, contained in a 3.2-kb *Sac* I restriction fragment (32), is indicated by the arrow adjacent to lane 1.

taining the α_1-globin gene (Fig. 5, lane 1, arrow; see ref. 32) are also unmethylated because this band disappeared after double digestion (Fig. 5, lane 4). There was no detectable difference in methylation pattern between uninduced and induced MELC as determined by *Sac* I/*Hha* I double digestion (Fig. 5, lanes 4 and 8). Thus, some, but not all assayed sites within the α-globin domain are unmethylated, and no change in the pattern of methylation was detected during HMBA-mediated differentiation.

DISCUSSION

The present study demonstrates a striking change in DNase I sensitivity of chromatin near the α- and β_{major}-globin genes during HMBA-mediated MELC differentiation, as manifest by the appearance of DNase I-hypersensitive sites near both the β_{major}- and α-globin genes. This change is accompanied by an increase in rate of accumulation of both α- and β_{major}-globin mRNAs (2), due, probably, to an increase in the rate of globin gene transcription (E. Hofer, R. Hofer-Warbinek, and J. E. Darnell, Jr., personal communication). DNase I-hypersensitive sites near the globin genes develop during MELC differentiation in the absence of any detected change in the pattern of DNA methylation near these genes. This suggests that changes in sensitivity to DNase I may occur independently of changes in DNA methylation pattern.

The observed DNA methylation patterns near the β_{major}- and α-globin genes are clearly compatible with gene expression during HMBA-mediated differentiation. However, the present studies do not preclude a change in the pattern of DNA methylation near the β_{major}- and α-globin genes during induction because we have examined only those sites accessible to the restriction enzymes used. Discrete rather than widespread changes in DNA methylation may be associated with changes in gene activity as suggested by Shen and Maniatis (14), who have shown that globin gene activation in rabbits is associated with demethylation of a small number of specific sites. These observations and our present studies can be contrasted with the results of a study of chicken erythrocyte development (9). During chicken development, there is a striking correlation between DNA demethylation, increased sensitivity to DNase I, the appearance of DNase I-hypersensitivity sites, and gene transcription. In the chicken, the transcriptional domain of the α-globin genes, which extends for some distance 3′ of the coding sequence (9), also approximates the region of DNA hypomethylation. In MELC, however, at least one site 3′ to the β_{major}-globin gene, remains fully methylated after induction and is located within the β_{major}-globin gene transcriptional domain (28).

In general, DNA hypomethylation seems related to gene activity (9, 12–17). Whether or not site-specific demethylation leads to other changes in chromatin structure, such as sensitivity to DNase I or the appearance of DNase I-hypersensitive sites, is unknown. Recent experiments indicate that 5-azacytidine-mediated demethylation of methylated, inactive, endogenous avian viral sequences is accompanied by an increase in sensitivity to DNase I, acquisition of DNase I-hypersensitive sites, and gene transcription (13), suggesting that changes in the pattern of DNA methylation may be implicated in mediating structural and functional changes in chromatin. The present evidence suggests that development of DNase I-hypersensitive sites near the α- and β_{major}-globin genes in MELC may not always require changes in the pattern of DNA methylation. Although 5-azacytidine is a weak inducer of MELC differentiation (10–15% benzidine-positive colonies 5 days after an 18-hr pulse of the drug; unpublished results; ref. 35), the actual target sequences demethylated during 5-azacytidine-mediated induction remain unknown, and it remains possible that 5-azacytidine operates in MELC by a mechanism other than DNA demethylation.

It is known that newly synthesized cytoplasmic globin mRNA is first detected after one complete S phase of the cell cycle in the presence of inducer (2). This suggests that cell cycle-related events may play a role in globin gene expression. Our present results, showing that changes in chromatin structure occur during MELC differentiation, suggest that these changes may require DNA replication in the presence of an inducing agent.

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