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DNA methylation and regulation of the human β-globin-like genes in mouse erythroleukemia cells containing human chromosome 11

(5-azacytidine/hypomethylation/gene regulation)

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ABSTRACT **The human β-globin gene is expressed—but the human fetal (γ) and embryonic (ε) globin genes are not—in an induced mouse erythroleukemia cell line (M11-X) that contains most of human chromosome 11. A 24-hr exposure of M11-X cells to 5-azacytidine before induction causes "global" DNA hypomethylation but selective activation of the human γ-globin genes. Genomic DNA is remethylated 2–3 days after exposure to 5-azacytidine, but sequences near the human and mouse globin genes remain hypomethylated, suggesting that the remethylation process is inhibited in these regions.**

The human β-globin-like genes (5′-ε-^{G}γ-^{A}γ-δ-β-3′) are found on the short arm of human chromosome 11 and are expressed consecutively (5′ → 3′) during gestation (1). The mechanisms responsible for the sequential activation of these genes are unknown, but several studies have related changes in chromatin structure (2, 3) and DNA methylation (4–6) to periods of gene activity.

Several studies (4–6) have noted a correlation between DNA hypomethylation and the active expression of individual globin genes during different developmental periods. Busslinger *et al.* (7) recently demonstrated that *in vitro* methylation of cytosine residues near the 5′ end of a human γ-globin gene prevented expression of this gene in mouse L cells; methylation of coding sequence DNA did not affect expression. Despite this strong association of hypomethylation and human globin gene activity, the overall role of DNA methylation in gene regulation is not yet clear (8, 9).

Previous studies demonstrated that the human β- but not γ-globin genes could be induced in tetraploid mouse erythroleukemia cells (MEL cells) [cells with an "adult" erythroid phenotype (10)] fused to human fibroblasts (11). The human chromosomes are rapidly segregated from such hybrid cells, however, so manipulation of human gene expression is difficult. M11-X cells (12) were developed to circumvent this problem. A human t(11;X) chromosome (13) is stably maintained in these cells (see below); human β-globin mRNA and protein (as well as mouse α- and β-globin) accumulate with induction, but the human γ- and ε-globin genes are not expressed (12). DNA near the γ- and ε-globin genes is heavily methylated, as it is in adult bone marrow cells (5, 6).

We carried out this study to determine whether the human γ- and ε-globin genes could be reactivated in M11-X cells by hypomethylating DNA near these genes. This can be accomplished with 5-azacytidine (azaC), a cytidine analogue that inhibits the enzymatic methylation of newly synthesized DNA (14, 15). azaC causes reactivation of a variety of repressed genes in tissue culture cells (9) and specifically augments fetal hemoglobin ($\alpha_2\gamma_2$) synthesis in patients with β-thalassemia or sickle cell anemia by unknown mechanisms (16–18). In this report, we demonstrate that incubation of M11-X cells with azaC, followed by induction with hexamethylenebisacetamide (HMBA), leads to selective activation of human γ-globin gene expression.

MATERIALS AND METHODS

The M11-X cell line (12) was created by fusing tetraploid (2S) HGPRT$^-$ mouse erythroleukemia cells with human fibroblasts (GM 3552) known to contain the translocation chromosome t(11;X) (13). Hybrid cells containing the t(11;X) chromosome could be grown in hypoxanthine/aminopterin/thymidine medium because of complementation of the HGPRT$^-$ MEL cells by the *HGPRT* gene on the X portion of the human t(11;X) chromosome. The translocation chromosome, therefore, became a stable part of the M11-X cell, and all other human chromosomes were lost after several months of continuous culture [this general strategy was devised by Deisseroth and Hendrick to study the behavior of human α-globin genes in MEL cells (19)]. G-banding and C-banding of M11-X cells (12) generally revealed a tetraploid complement of mouse chromosomes and a single human chromosome, t(11;X). Southern blot analyses of M11-X cell DNA demonstrated the presence of all the human β-globin-like genes and the human insulin and c-Ha-*ras1* genes, also located on the short arm of chromosome 11 (20, 21).

One day before induction, 2.5 × 10^4 M11-X cells maintained in continuous logarithmic-phase growth were seeded in each milliliter of Dulbecco's improved minimal essential medium (with 10% fetal calf serum) and were grown in 5% CO_2/95% air at 37°C. On day 0, cells were treated with nothing (group 1), 5 mM HMBA (group 2), or 2.5 μM azaC (groups 3 and 4). Cells in groups 3 and 4 were spun out of medium 24 hr later, washed with phosphate-buffered saline, resuspended in medium, and induced with nothing (group 3) or with 5 mM HMBA (group 4). azaC was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute.

RNA spot blots (22) and S1 nuclease analyses (23, 24) were carried out as described. Analyses of DNA methylation patterns also used previously described strategies (5, 16). Details of these analyses are given in the figure legends.

RESULTS

Characteristics of Cellular Induction. Characteristics of cellular growth, viability, and hemoglobin synthesis are shown in Table 1. With induction by 2.5 μM azaC, the number of cells present on day 4 was approximately one-half that of control cells or HMBA-treated cells, suggesting that this dose of azaC causes cellular toxicity or a decrease in the rate of cellular division. In addition, 10%–20% of cells treated with azaC were multinucleated and strikingly larger than

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Abbreviations: HMBA, hexamethylenebisacetamide; azaC, 5-azacytidine.

Table 1. Cellular characteristics of induction

	Group 1 (control)	Group 2 (HMBA)	Group 3 (azaC)	Group 4 (azaC → HMBA)
Cells per ml × 10^{-5}	4.5 ± 2.4	3.3 ± 1.0	2.4 ± 1.2	1.0 ± 0.2
% giant cells	0.3 ± 0.5	0.2 ± 0.5	26 ± 6.0	12 ± 6.1
% viable cells	80 ± 3.0	71 ± 28	84 ± 14	77 ± 20
% benzidine-positive cells	2.0 ± 2.7	84 ± 24	26 ± 16	50 ± 10

Means ± 1 SD are shown for each parameter. Values represent determinations on day 4 from at least three experiments. The giant cells formed in groups 3 and 4 were 20–30 μm in diameter and contained multiple nuclei; only 10%–20% of these cells were benzidine positive on day 4. The parental line of tetraploid MEL cells also formed giant cells with azaC induction. Creusot *et al.* (14) have also demonstrated giant cell formation with diploid Friend erythroleukemia cells incubated in 1 μm azaC; in their study, the giant cells did not give rise to colonies in semisolid medium, implying that they are formed because of disordered cell divisions (14). RNA obtained from a purified population of giant cells induced with azaC and HMBA contained barely detectable amounts of human γ-globin mRNA (data not shown). Cell viability was determined by the ability of cells to exclude trypan blue. Benzidine positivity implies cellular hemoglobinization.

cells from groups 1 or 2 (14) (see Table 1 legend). Cells treated with 2.5 μM azaC and retreated 1 day later with HMBA (group 4) accumulated even more slowly. Despite differences in the rates of cell growth, most cells from all groups were viable (i.e., excluded trypan blue) on day 4. Active division (with cell doubling times of 1–2 days) was noted for all three treated groups between days 3 and 4, but it declined rapidly thereafter.

RNA Accumulation with Induction. The steady-state levels of RNAs from each of the human and mouse β-globin-like genes (as well as the human insulin and c-Ha-*ras1* genes) were assayed by RNA spot blotting and S1 nuclease mapping (Fig. 1; Table 2). Control cells (group 1) harvested on day 4 contained ≈250 copies of mouse β-globin mRNA and 7 copies of human β-globin mRNA; no human ε- or γ-globin mRNA was detected. ras and insulin mRNAs were likewise undetectable (data not shown).

Cells induced with 5 mM HMBA (group 2) accumulated ≈9000 copies of mouse β-globin mRNAs (Fig. 1, mouse β^{maj} probe, lane 2), an amount similar to that previously described for MEL cells (28) and for human globin mRNAs in adult erythroid precursors (≈15,000 copies of human α- and β-globin mRNA per cell) (27). Human β-globin mRNA also accumulated with HMBA induction, but at much lower levels than mouse β-globin mRNA (3%–4%). However, the relative increase in mouse and human β-globin mRNA levels with HMBA induction (30- to 40-fold) was similar. Human insulin, ras, and ε-globin mRNAs were not found in HMBA-induced cells, but a very low level (0.5 copy per cell) of human γ-globin mRNA was detected (Fig. 1, γ probe, lane 2).

Cells induced with 2.5 μM azaC (group 3) accumulated ≈1/4th as much human and mouse β-globin mRNA as HMBA-induced cells (Fig. 1, mouse β^{maj} probe, lane 3; β

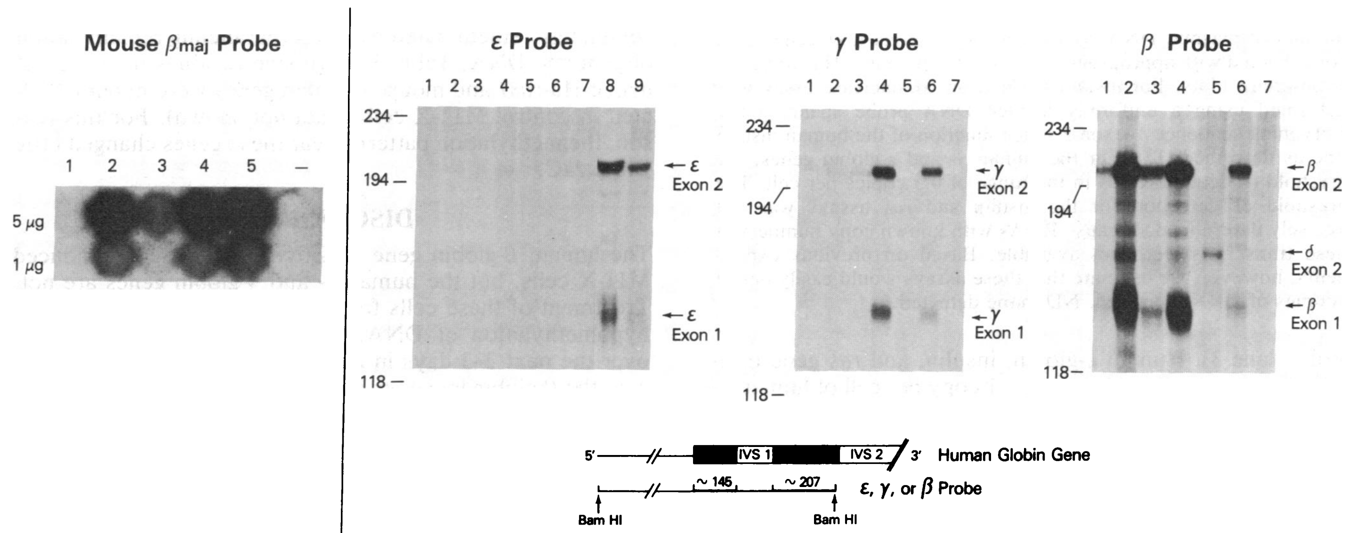

FIG. 1. Globin RNA production in day-4 M11-X cells. The left-most panel represents an RNA spot blot hybridized with a nick-translated probe made from the 1.9-kilobase-pair (kbp) *Eco*RI/*Bam*HI fragment containing the 5′ end of the mouse β^{maj}-globin gene (25). Indicated amounts of nondenatured total cellular RNA were spotted. Lane 1, control cells; lane 2, 5 mM HMBA; lane 3, 2.5 μM azaC; lane 4, 2.5 μM azaC followed 1 day later by HMBA; lane 5, total bone marrow RNA obtained from a patient with sickle cell anemia; lane 6 (−), no RNA. The three right-hand panels represent analyses of steady-state RNA transcribed from the human ε-, γ-, and β-globin genes. The uniformly labeled probes used in these analyses were obtained from M13mp7-globin gene recombinants containing the 5′ end of each of these genes (ε-globin, 0.67 kbp; $^{G}\gamma$-globin, 2.7 kbp; and β-globin, 1.9 kbp). The total length of each probe differs, but the lengths of probe fragments protected from S1 nuclease degradation by correctly initiated and processed mRNA are nearly the same for each gene, as shown in the stick diagram. In each panel, the positions of correctly processed mRNA species are indicated on the right. The sizes of simultaneously run DNA size markers (in nucleotides) are shown at the left. Analysis of day-4 M11-X cell RNAs are shown in the first four lanes of each panel. Identical 20-μg aliquots from the same experiment were used in each analysis. Lane 1, control cells; lane 2, 5 mM HMBA; lane 3, 2.5 μM azaC; lane 4, 2.5 μM azaC followed by 5 mM HMBA. The remainder of the lanes in each panel represent analyses of RNAs obtained from other controls or from Cos cells transfected with expression vectors containing normal human γ-, δ-, or β-globin genes (26). These cells contain correctly initiated and processed RNA transcribed from each of these genes and, therefore, authenticate probe specificity. ε probe: lane 5, 10 μg of human bone marrow RNA from a patient with sickle cell anemia; lane 6, 20 μg of Cos cell RNA containing human β-globin mRNA; lane 7, 20 μg of Cos cell RNA containing human γ-globin mRNA; lane 8, 2.5 μg of RNA obtained from K562 cells; lane 9, 0.25 μg of K562 cell RNA. γ probe: lane 5, 20 μg of Cos cell RNA containing human β-globin mRNA; lane 6, 20 μg of Cos cell RNA containing human γ-globin mRNA; lane 7, RNA from HMBA-induced tetraploid MEL cells. β probe: lane 5, 20 μg of Cos cell RNA containing human δ-globin mRNA [the 167-nucleotide band is caused by cross-hybridization of the human β-globin probe with correctly processed human δ-globin mRNA sequences from exon 2 (24, 26)]; lane 6, 20 μg of Cos cell RNA containing human β-globin mRNA; lane 7, 20 μg of Cos cell RNA containing human γ-globin mRNA.

Table 2. Various RNAs (copies per cell) in M11-X cells harvested 4 days after induction

Gene	Control	HMBA	azaC	azaC → HMBA
Mouse β-globin	250	9000	2750	10,500
Human β-globin	7	285	40	210
Human γ-globin	ND	0.5	1.0	21
Human ε-globin	ND	ND	ND	0.4
c-Ha-*ras1*	ND	ND	ND	ND
Human insulin	ND	ND	ND	ND

Values shown were obtained by performing densitometric tracings on the bands and spots shown in Fig. 1. The RNA spot blot of Fig. 1 was washed using nonstringent conditions (42°C, 50% formamide), allowing complete cross-hybridization of the human and mouse β-globin sequences. Since human β-globin mRNA comprises only a very small part (<5%) of the total β-globin mRNA in induced M11-X cells, the intensity of the hybridization spots from M11-X cell RNA predominantly represents hybridization with mouse β-globin mRNA. Concentrations of human globin mRNAs were calculated by comparing the density of bands from known amounts of M11-X cell RNA to known amounts of control RNAs under conditions of probe excess. The standard for β- and γ-globin mRNA levels was total bone marrow RNA obtained from a patient with sickle cell anemia; this bone marrow sample contained >80% erythroid cells. The α/β globin biosynthetic ratio of these bone marrow cells was 1.0, and the γ/β biosynthetic ratio was 0.02. We assumed, therefore, that this patient's erythroid bone marrow cells contained ≈15,000 copies of α- and β-globin mRNA (27) and ≈300 copies of γ-globin mRNA. K562 cell RNA was used as a control for human ε-globin mRNA copy number; we assumed that K562 cells contain ≈1000 copies of mature human ε-globin mRNA (E. Benz, personal communication). c-Ha-*ras1* assay was performed using RNA spot blotting with the nick-translated rat c-Ha-*ras1* gene as the probe. Sensitivity of this assay was estimated by using known amounts of plasmid DNA containing the rat c-Ha-*ras1* gene. This probe reacted with appropriate species of the human c-Ha-*ras1* gene on Southern blots. For insulin mRNA, an S1 nuclease assay was performed using a uniformly labeled DNA probe spanning the intervening sequence 1–exon 2 splice junction of the human insulin gene as described (12). For the human γ- and ε-globin genes, the threshold of detection was in the range of 0.5 copies per cell. The threshold of detection for the insulin and *ras* assays was not precisely determined, because RNAs with known copy numbers of these transcripts were not available. Based on previous experiments, however, we estimate that these assays would easily detect 5 copies of mRNA per cell. ND, none detected.

probe, lane 3). Human ε-globin, insulin, and *ras* gene transcripts did not accumulate, but ≈1 copy per cell of human γ-globin mRNA was detected.

HMBA induction of cells treated with azaC (group 4) led to mouse and human β-globin mRNA accumulation similar to that observed with HMBA induction alone (Fig. 1, mouse β^{maj} probe, lane 2 vs. 4; β probe, lane 2 vs. 4). A significant quantity (21 copies per cell) of human γ-globin mRNA was detected in cells treated with both inducers (γ probe, lane 4), compared with 0.5 copies in cells induced only with HMBA. A very small amount (0.4 copies per cell) of human ε-globin mRNA was detected in group 4 cells, but transcripts from the human insulin and *ras* genes were not detected.

"Global" Hypomethylation of DNA with azaC. The genomic frequency of 5-methylcytosine (29) changed very little in cells treated with HMBA or with 0.5–1.25 μM azaC (data not shown). However, cells treated with 2.5 μM azaC demonstrated a 35%–40% decrease in the total number of 5-methylcytosine residues within 24 hr of treatment; this level of hypomethylation persisted for an additional day, but it was approaching the baseline value by day 3 (Table 3).

Cytosine residues found in *Hpa* II sites just 5′ to the human ε-, $^{G}\gamma$-, and $^{A}\gamma$-globin genes were nearly 100% methylated in untreated M11-X cells (Fig. 2 *A* and *B*); these sites are similarly modified in adult human erythroid cells (5, 6). The site located just 3′ to the human β-globin gene was 50% modified, as it is normally in adult bone marrow DNA (5). Two days after azaC treatment, there was a marked decrease in the frequency of 5-methylcytosine residues at all these sites and at sites 5′ to the human δ- and mouse β^{maj}-globin genes; no change was noted in DNA samples obtained from control cells or from HMBA-induced cells (Fig. 2). Although most of the genomic DNA was remethylated by days 3–5, hypomethylation near the globin genes persisted 4 days after azaC induction. The extent of hypomethylation on day 4 was equivalent in cells treated with azaC alone or with azaC and HMBA (data not shown).

Table 3. Genomic 5-methylcytosine frequency of M11-X cells incubated for 24 hr with azaC

Day	% 5-methylcytosine	% control
0	3.02 ± 0.13	100
1	1.89 ± 0.32	62.6
2	1.93 ± 0.16	63.9
3	2.66 ± 0.48	88.1
5	2.81 ± 0.16	93.0

Cells were incubated with 2.5 μM azaC from day 0–1, washed, and then resuspended in medium. [6-^{3}H]Uridine was added to the cells 24 hr before harvest for 5-methylcytosine determinations (29). Analyses were done in triplicate as described (29). Numbers represent the percent mean ± 1 SD. % 5-methylcytosine = (5-methylcytosine)/(5-methylcytosine + cytosine) × 100.

DNA near the human insulin gene was nearly completely methylated in control M11-X cells, as it is in normal human bone marrow cells (Fig. 3). The number of methylcytosine residues found in *Hpa* II sites near this gene had decreased 2 days after treatment with azaC, but these sequences were completely remethylated by day 4 (paralleling remethylation of genomic DNA; Table 3). Cytosine residues near the human c-Ha-*ras1* and mouse α-globin genes were hypomethylated in control M11-X cells (data not shown). For this reason, the methylation patterns near these genes changed little with azaC.

DISCUSSION

The human β-globin gene is actively expressed in induced M11-X cells, but the human ε- and γ-globin genes are not. Treatment of these cells for 1 day with azaC causes global hypomethylation of DNA, but remethylation takes place over the next 2–3 days in culture. However, remethylation near the β-globin loci does not occur. azaC treatment is associated with accumulation of human γ- but not ε-globin mRNA, despite a similar degree of hypomethylation near these two genes. γ-globin gene activation may well be mediated at the level of RNA transcription, because human γ-globin mRNA appears to be stable in both MEL cells (32, 33) and adult human erythroid cells (1). An *in vitro* nuclear transcription assay would be required to confirm this hypothesis.

The relationship between DNA methylation and human globin gene expression is further characterized by these studies. azaC causes DNA hypomethylation in M11-X cells, but this treatment alone led to minimally increased levels of human γ-globin mRNA. Maximal γ-globin mRNA accumulation was observed in hypomethylated cells induced with HMBA, an agent known to cause DNase I hypersensitive sites to appear near the 5′ ends of the mouse globin genes of MEL cells after induction (34, 35). These findings support the previously stated hypothesis that hypomethylation is necessary, but not sufficient, for active γ-globin gene expression (5).

The human ε-globin gene is not expressed in azaC-treated M11-X cells, even though hypomethylation near the ε- and γ-globin genes appears to be equivalent (Fig. 2). This observation might suggest that the pattern of cytosine methylation

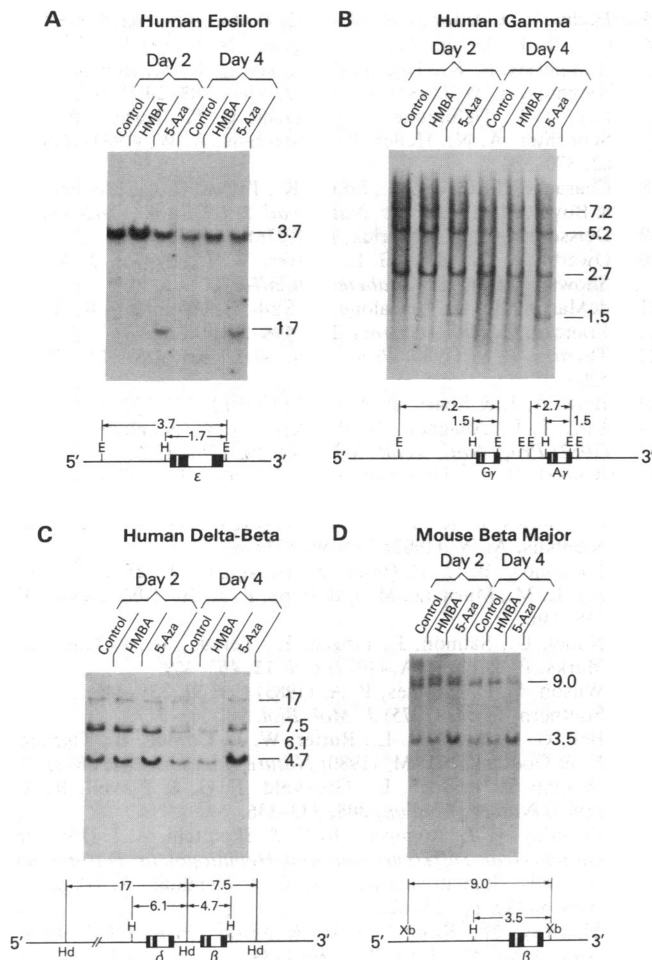

FIG. 2. Southern blot analyses (30) of DNA samples obtained from day-2 and day-4 M11-X cells. Twenty micrograms of total cellular DNA was first digested to completion with a methylation-insensitive enzyme (E, *Eco*RI; Hd, *Hin*dIII; P, *Pvu* II; Xb, *Xba* I) and then with *Hpa* II (H), an enzyme that cuts its recognition sequence C-C-G-G only when the internal C or both cytosine residues are unmethylated. Completeness of digestion was assured for both enzymes, and then the samples were analyzed by Southern blotting as described (5, 16, 30). The restriction enzymes (Bethesda Research Laboratories and New England Biolabs) and the sizes of fragments generated by these enzymes (noted in kilobases) are indicated. Fragments used as nick-translated probes were as follows: (*A*) 0.67-kb *Bam*HI fragment containing the 5' end of the human ε-globin gene; (*B*) 0.9-kb *Bam*HI/*Eco*RI $^{G}\gamma$ intervening sequence II fragment; (*C*) 4.4-kb *Pst* I fragment containing the human β-globin gene; (*D*) 1.9-kb *Eco*RI/*Bam*HI fragment containing the 5' end of mouse β^{maj}-globin gene. Hybridizations and washes were done using stringent conditions, which permitted each probe to be specific for its own sequences. Appropriate mouse and human DNA samples were used to authenticate the origin of all bands in each experiment. The 5.2-kb band in the human γ-globin panel (*B*) is caused by cross-hybridization of the $^{G}\gamma$ probe with the 5.2-kb *Eco*RI fragment containing the human β-globin gene. This fragment contains no *Hpa* II sites. The generation of smaller fragments with azaC treatment indicates hypomethylation of DNA at the indicated *Hpa* II sites.

cis to these genes does not directly influence their expression. Alternatively, hypomethylation near the 5' ends of the γ-globin genes may indeed be necessary for expression; the ε-globin gene may remain "off" because its promoter lies "buried" in chromatin (36), or because M11-X cells lack an embryonic *trans*-acting factor required for active ε-globin gene expression. Similar explanations could account for the lack of expression of the insulin and c-Ha-*ras1*, genes. In any case, DNA methylation does not appear to be the primary

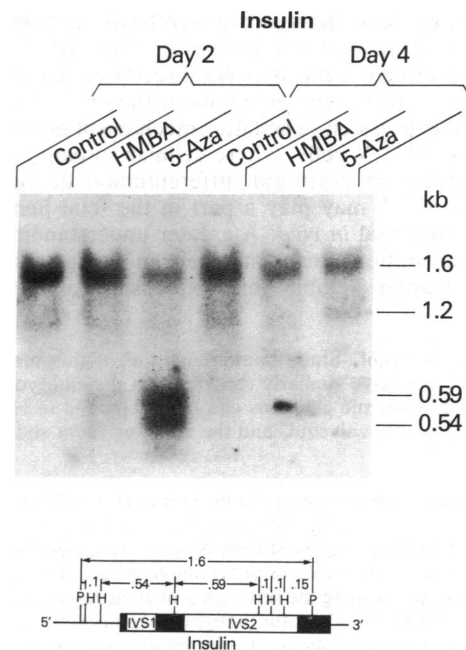

FIG. 3. Analysis of methylation near the human insulin gene of M11-X cells. See Fig. 2 legend for details. Probe: 1.6-kilobase-pair (kbp) *Pvu* II fragment containing the 5' end of the human insulin gene (31). IVS, intervening sequence.

reason why the ε-globin, insulin, and *ras* genes are not expressed in M11-X cells.

It is not clear why DNA at the human β-globin gene locus of M11-X cells remains hypomethylated on day 4, when the majority of genomic DNA is remethylated. DNA initially becomes hypomethylated in M11-X cells because DNA methyltransferase molecules are bound irreversibly by azaC residues inserted into the newly synthesized DNA (14, 15). When azaC is removed from the medium, methyltransferase activity presumably recovers as new enzyme is synthesized and DNA is remethylated (14, 15). During induction, however, changes in chromatin conformation may occur near the β-globin-like genes, or nuclear proteins may associate with this region. These changes may inhibit the facilitated transfer of methyltransferase molecules along newly synthesized DNA strands (37), thereby preventing remethylation in these regions.

In our study, the output of the fibroblast-derived human β-globin gene of M11-X cells was much less than that of the endogenous mouse β-globin genes. Diploid MEL cells containing integrated copies of cloned human β-globin genes also produce less human than mouse β-globin mRNA (32, 38). However, some studies of MEL cells containing intact human chromosomes have noted approximately equivalent outputs of human and mouse globin mRNAs, although outputs may vary from cell line to cell line (19, 33). Despite these quantitative differences, qualitative aspects of globin gene regulation in M11-X cells closely resemble those of normal adult erythroid cells and imply the usefulness of this model for studying aspects of human globin gene switching.

azaC is capable of increasing γ-globin mRNA synthesis in baboons (39) and in the bone marrow cells of sickle cell anemia or β-thalassemia patients treated with this drug (16–18). The reason for increased fetal hemoglobin ($\alpha_2\gamma_2$) synthesis in these patients is not yet clear. This drug is a cytotoxic agent and may act in part by recruiting a subpopulation of early erythroid progenitor cells whose progeny make increased amounts of fetal hemoglobin (40). This hypothesis is supported by recent observations that other cytotoxic agents [e.g., hydroxyurea (41) or cytarabine A (42)] are also capable

of augmenting fetal hemoglobin synthesis in anemic primates; these drugs are not known to affect DNA methylation. Alternatively, azaC may act directly on late erythroid cells to cause DNA hypomethylation, thereby activating γ-globin gene expression. The latter mechanism may be more important in M11-X cells, where cellular subpopulations at different stages of erythroid differentiation do not exist. Both mechanisms may play a part in the fetal hemoglobin activation observed *in vivo*. A greater understanding of the relative contribution of each may help guide further attempts to modify human γ-globin gene expression for therapeutic benefit.

Note Added in Proof. Since communication of this manuscript, Ginder *et al.* (43) have similarly reported that the embryonic β-globin-like gene of anemic chickens can be reactivated *in vivo* by sequential treatments with azaC and the inducing agent sodium butyrate.

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