Activation of a chicken embryonic globin gene in adult erythroid cells by 5-azacytidine and sodium butyrate

(gene activation/DNA methylation/globin gene switching/chromatin structure)

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ABSTRACT Adult White Leghorn chickens were rendered anemic by injection with 1-acetyl-2-phenylhydrazine and then treated with parenteral 5-azacytidine, and levels of embryonic globin RNA in circulating reticulocytes were measured. A very small but detectable amount of correctly initiated embryonic ρ-type globin RNA was detected in reticulocytes from birds treated with 5-azacytidine, while none was detected in reticulocytes from those receiving only phenylhydrazine or phenylhydrazine plus 1-β-D-arabinofuranosylcytosine (cytotoxic pyrimidine analog). An attempt to increase embryonic globin RNA induction by treatment with parenteral sodium butyrate after 7 days of 5-azacytidine administration resulted in a 5- to 10-fold increase in the level of embryonic globin RNA. However, sodium butyrate did not induce embryonic gene expression when given alone or after treatment with cytotoxic arabinonucleoside. Sodium butyrate treatment also caused a DNase I-hypersensitive site to be exposed at the 5′ end of the ρ-globin gene only after 5-azacytidine induced demethylation of several CpG sites in and around the gene. The implications of this model of gene activation in vivo are discussed in the context of multistep gene regulation.

The chicken β-globin gene cluster offers a useful model system to study developmental gene regulation, and in particular those features of DNA and nucleoprotein structure that differ in active versus inactive genes, since essentially pure populations of stage-specific erythroid cells can be readily isolated. The embryonic ρ and ε globins are active until the fifth day of embryogenesis, but no embryonic globin is detectable in adult red cells (1). Conversely, the adult β-globin gene is inactive until the sixth day of development. The embryonic genes are highly methylated in adult erythroid cells (2,3) and do not have DNase I-hypersensitive sites at their 5′-proximal ends as do active adult globin genes (4).

A number of lines of evidence suggest that DNA methylation plays an important role in higher eukaryotic gene expression (5,6). An inverse correlation has been demonstrated between methylation and expression of globin and other eukaryotic genes (refs. 5–10; refs. 5 and 6 are reviews), and several studies in which cloned eukaryotic genes were methylated in vitro have demonstrated a direct inhibitory effect on transcription (11–14). In addition, a variety of studies in cell culture systems have shown that 5-azacytidine, a cytotoxic pyrimidine analog that inhibits DNA methylation, can induce cellular differentiation (15) and either directly activate (16) or allow activation of specific genes (17). More recently it has been demonstrated that 5-azacytidine causes selectively increased expression concomitant with specific demethylation of the fetal γ-globin genes in anemic baboons (18) as well as human patients with β-thalassemia (19) or sickle cell anemia (20). In these latter studies there remains some question as to whether increased γ-globin gene expression is due to gene demethylation, cell selection, or both, since 5-azacytidine is known to have a variety of toxic effects on cells, and since γ-globin expression can be stimulated in adults by several types of bone marrow stress.

As part of ongoing studies aimed at elucidating mechanisms that regulate globin gene expression, we have attempted to study the effects of demethylation of embryonic globin genes in anemic adult chickens. We report here that while 5-azacytidine treatment causes nearly complete demethylation of the ρ embryonic globin gene in adult erythroid cells, only a minimal amount of ρ-globin RNA is detectable, and the surrounding chromatin structure, as assayed by DNase I digestion, does not differ from untreated controls. On the other hand, pharmacologic doses of sodium butyrate greatly increase the amount of embryonic ρ-globin RNA in adult reticulocytes and result in the exposure of a 5′ DNase I-hypersensitive site in some of the reticulocyte nuclei, but only when the gene has first become demethylated by 5-azacytidine treatment.

MATERIALS AND METHODS

Treatment of Animals and Blood Collection. Adult White Leghorn hens (Yoder, Kalona, IA) were rendered anemic by five daily intramuscular injections with 1-acetyl-2-phenylhydrazine (Sigma) at 20 mg/kg or by phlebotomy of 10 ml of whole blood per day to achieve a hematocrit of 18–22% (normal 35–40%). Reticulocyte counts were maintained at 75–90% (normal less than 5%) as determined by staining red cells with new methylene blue. 5-Azacytidine and 1-β-D-arabinofuranosylcytosine (cytotoxic arabinonucleoside; Sigma) were dissolved in distilled water immediately before use and injected at 3 mg/kg per day. Neutral sodium butyrate, made by titrating 2 M butyric acid with sodium hydroxide to pH 7.4, was injected intramuscularly in equally divided doses of 650 mg/kg every 8 hr for 5 days. At least three birds were included in each experimental or control treatment, and birds were crossed over to different treatment regimens. Unless otherwise noted, peripheral reticulocytes for biochemical analyses were collected by venipuncture and diluted into cold sterile phosphate-buffered saline containing 5 mM sodium butyrate and 5 mM EDTA (21).

Preparation of Hybridization Probes and Restriction Mapping. A 3′-end-specific ρ-globin gene probe was prepared by subcloning the 1.1-kilobase (kb) Ave I fragment from the plasmid p2H2 (22). The adult β-globin 1.4-kb Msp I fragment was derived from pCAβG1 (23). Nick-translations of hybridization probes to specific activities of 2–4 × 106 cpm/μg were carried out as described (7). The 5′ nucleotide protection assay probe was prepared by isolating and 5′-end-labeling the 0.7-kb HindII fragment derived from pβ2H2 with T4 polynucleotide kinase (P-L Biochemicals) and [γ-32P]ATP (Amersham). Restriction mapping with Hpa II.

Abbreviations: kb, kilobase(s); bp, base pair(s).
**RESULTS**

5-Azacytidine Causes Extensive Demethylation of Embryonic Globin Genes in Adult Red Cells. Adult White Leghorn chickens were rendered anemic by phenylhydrazine injection and then treated with 10⁹ reticulocytes either by cell lysis followed by digestion of polyoma cytoplasmic RNA as described (23) or by whole cell disruption in 6 M guanidinium isothiocyanate (Eastman Kodak) followed by extraction with neutral phenol (Mallinkrodt) at 65°C and then precipitation with ethanol. For filter hybridization studies, 20 μg of each RNA was electrophoresed and transferred to nitrocellulose as described by Thomas (24). Filters were hybridized to nick-translated p-globin or adult β-globin gene probes in 50% (vol/vol) formamide/0.6 M NaCl/0.06 M sodium citrate/10% dextran sulfate/100 μg of salmon sperm DNA per ml/50 mM sodium phosphate, pH 6.8, at 46°C for 18–24 hr. Quantitation of the various bands was carried out by scanning the resulting autoradiograms on a Beckman DU-8 absorbance scanner.

Analysis of RNA by S1 nuclease protection assay was performed by a minor variation of published methods (25, 26). Approximately 10⁹ dpm of end-labeled 0.7-kb HindII p globin gene probe was hybridized separately to 25 μg of each RNA sample in 50% (vol/vol) formamide buffer (23) for 4 hr at 37°C. Each sample was then incubated with 600 units of S1 nuclease (Bethesda Research Laboratories) for 30 min at 37°C. The samples were electrophoresed in 6% polyacrylamide/8 M urea gels, which were dried and exposed to Kodak XAR-5 x-ray film at −80°C for 12–96 hr in the presence of Lightning Plus intensifying screens (Dupont).

DNase I Titration of Nuclei. DNase I titration experiments were carried out by minor modifications of the procedure described by McGhee et al. (21). Approximately 3 A₂₆₀ units of nuclei were incubated at 37°C for 30 min with pancreatic DNase I (Worthington) at 0.1–2.0 mg/ml and the reactions were terminated by addition of EDTA to 0.1 M, proteinase K (Boehringer Mannheim) to 0.1 mg/ml, and 0.6 M Na₂SO₄ (Sigma) to 0.2%, followed by phenol extraction and ethanol precipitation of DNA. DNA samples were redissolved in distilled water and 20 μg of each DNA was digested for 4 hr at 37°C with 100 units of restriction endonuclease HindIII (Bethesda Research Laboratories or Amersham) followed by electrophoresis in a 1.0% agarose gel in 10 mM sodium phosphate pH 7.0 buffer. The DNA was transferred to nitrocellulose filters (Schleicher & Schuell) and hybridized to specific nick-translated probes as described (7).

**Fig. 1.** Demethylation of the embryonic p-globin gene in 5-azacytidine-treated chickens. Reticulocyte DNA was extracted and digested with EcoRI followed by either HpaII or MspI, or with HindIII followed by HhaI, as indicated. Digests were electrophoresed in 1.2% agarose gels and transferred to nitrocellulose (7). The filters were hybridized to an embryonic p-globin gene probe, pJRho 3', lane A, butyrate only; lane B, cytosine arabinonucleoside and butyrate; lane C, 5-azacytidine only; lane D, 5-azacytidine and sodium butyrate.

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least 50–70% of the reticulocytes the C-C-G-G and G-C-G-C sites immediately surrounding the p-globin gene are completely demethylated. Similar methylation site mapping studies using a chicken ovalbumin probe (kindly provided by S. McKnight) also showed some demethylation of that gene in the red cells from 5-azacytidine-treated birds, but much less than around the p-globin gene. In addition, demethylation around at least one site in the 5' region of the embryonic e-globin gene was observed after 5-azacytidine treatment (data not shown).

Embryonic RNA Induction Is Greatly Enhanced by Sodium Butyrate Treatment. To examine the effect of demethylation upon expression of the embryonic globin genes, approximately 5 × 10⁹ reticulocytes were collected from animals treated with the various drug regimens, and RNA was isolated either by cytoplasmic polysome extraction or by whole cell extraction in guanidinium thiocyanate. The resulting RNA samples were analyzed by 5' S1 nuclease mapping and blot hybridization. For S1 nuclease mapping, a 5' end-labeled probe derived from a 0.7-kb HindII fragment extending from the first exon of the p-globin gene to about 600 nucleotides upstream from the cap site was hybridized to RNA. As illustrated in Fig. 2, lane F, the reticulocyte RNA from the 5-azacytidine-treated bird contains a very small (relative to the 5-day embryo red cell RNA control, lane B) but measurable amount of embryonic p-globin RNA, while phenylhydrazine only (lane A) and cytosine arabinonucleoside-treated controls (lane G) have none detectable. While this result suggests that 5-azacytidine can activate an embryonic globin gene in adult red cells, the change in p-globin RNA production is clearly not proportional to the percentage of cells in which complete demethylation is measured around the p-globin gene. Thus, it appears that even in differentiated normal erythroid cells, factors other than DNA methylation must be involved in determining the full activation of a particular globin gene.

While 5-azacytidine did not cause a large amount of expression of the p-globin gene in these experiments, a large body of data suggests that DNA methylation may inhibit transcription (27) and that loss of methylation, at least in the 5' regions of a gene, may be necessary for full activation (5, 6). Because of this, we sought to extend this animal model by attempting to further induce the embryonic globin genes. We chose to use sodium butyrate, which is a naturally occurring fatty acid with a wide variety of demonstrated pharmacologic effects in cultured cells (28). Among these effects is its ability to induce erythroid differentiation and globin gene
expression in Friend murine erythroleukemia cells (29), avian erythroblastosis virus-transformed chicken erythroid cells (30), and some clones of the human K562 cell line (31).

Interestingly, when high doses of sodium butyrate were administered concomitantly with 5-azacytidine, a marked increase in β-globin RNA was detected. Lane E of Fig. 2 shows the increase in embryonic β-globin RNA from the same bird as in lane F after butyrate treatment. This experiment also demonstrates that the induced β-globin RNA apparently has the same 5' map coordinates as bona fide β-globin mRNA from 5-day embryonic red cells (lane B). It is not yet certain whether the 110-nucleotide protected fragment seen in both control and experimental samples represents incomplete S1 nuclease digestion or true heterogeneity of transcriptional initiation as has been described in the chicken β-globin gene in vivo (32). S1 nuclease mapping studies done with a 3' end β-globin probe yielded the same results as the 5' probe (data not shown). It can also be seen that treatment with the same sodium butyrate dose in the absence of 5-azacytidine (lane D) results in no detectable embryonic globin RNA in this assay.

Another method used to analyze the induction of embryonic globin RNA was blot hybridization analysis. For this assay RNA was denatured and electrophoresed in a 1% agarose gel and transferred to nitrocellulose filters, which were then hybridized to a probe containing the second intron and 3' exon of the β-globin gene (22). The results depicted in Fig. 3 again show a striking increase in β-globin RNA in red cells from birds receiving 5-azacytidine plus sodium butyrate compared to 5-azacytidine alone, while butyrate alone resulted in no detectable β-globin RNA. To control for the relative amount of mRNA each sample as well as for any selective loss of RNA, each filter was washed free of probe and hybridized to an adult β-globin gene probe. Fig. 3B demonstrates that there is about the same amount of adult β-globin RNA in samples from the butyrate-treated, 5-azacytidine-treated, or 5-azacytidine-plus-butyrate-treated birds.

The relative amounts of globin RNA resulting from each treatment were further quantitated by densitometric scanning of the autoradiograms in Fig. 3. As shown in Fig. 4A the amount of β-globin RNA in the 5-azacytidine plus butyrate sample is about 7-fold higher than in the 5-azacytidine only sample. However, Fig. 4B illustrates that the probe pβL.4 detects the same amount of adult β-globin RNA in all samples. This suggests that there is no reciprocal down-regulation of the β-globin gene by butyrate and that the differences in β-globin RNA are not due to selective loss of mRNA in the butyrate only or 5-azacytidine only samples.

**Induction of the β-Globin Gene by Sodium Butyrate Is Associated with an Active Chromatin Configuration.** One feature that appears to accompany and possibly precede transcriptional activation of many eukaryotic genes is the acquisition of nucleosome hypersensitive sites in surrounding chromatin (4, 33). To help define the level at which 5-azacytidine and sodium butyrate act to induce the β-globin gene, we performed DNase I titrations on isolated reticulocyte nuclei by the method of McGhee et al. (21). A map of the 4.6-kb HindIII fragment containing the β-globin gene and demonstrating the positions of the two DNase I-hypersensitive sites demonstrated by Stalder et al. (4) is shown in Fig. 5. The autoradiogram derived from a 5-azacytidine-treated bird shows only the 4.6-kb HindIII parent fragment. The autoradiogram of the butyrate only sample demonstrates a hypersensitive site lying approximately 2 kb upstream from the 5' end of the β-globin gene, as manifested by a 4.0-kb subband. This site, which is not always detected in our experiments, does not correlate with β-globin gene expression but rather

**Fig. 2.** (Lower) S1 nuclease protection map of β-globin RNA. E1 and E2 are exons. Reticulocyte polysomal or cytoplasmic RNA was extracted and 25 μg of each RNA sample was hybridized to the 5'-end labeled HindII fragment of the embryonic β-globin gene as illustrated, followed by S1 nuclease digestion and electrophoresis on a 6% polyacrylamide/8 M urea gel. In other experiments total reticulocyte RNA was isolated, with the same results (data not shown). The samples shown on the resulting autoradiogram (Upper) were derived from reticulocytes of birds receiving the following treatments: lane A, phenylhydrazine only; lane B, 5-day embryonic red cell control; lane C, 12-day embryonic red cell control; lane D, butyrate only; lane E, 5-azacytidine and butyrate; lane F, same bird as in E treated with 5-azacytidine only; lane G, cystosine arabinoside and butyrate. Lanes M are molecular size markers. bp, Base pairs.

**Fig. 3.** Blot hybridization analysis of RNA. In each lane, 20 μg of total reticulocyte RNA from birds receiving the treatment indicated was electrophoresed in a 1.4% agarose gel, transferred to a nitrocellulose membrane, and hybridized to the β-globin gene probe pβL.4 as shown (A). Aza, 5-azacytidine; nt, nucleotides. In the maps, rectangles represent exons.
with adult $\beta$-globin gene expression (4). In contrast, the 5-aza-
cytidine plus butyrate sample demonstrates a faint but
reproducible 2.2-kb subband generated by cleavage at the 5'
hypersensitive site that is located within 200 bp upstream
from the $\rho$-globin transcription initiation site and is charac-
teristically present in active $\rho$-globin genes in 5-day embry-
onic red cells (4).

Since the hypersensitive site appears to be present in only
a minority of the $\rho$-globin reticulocyte chromatin it is not
possible to be sure that the cells that produce $\rho$-globin RNA
are the same cells in which the hypersensitivity site exists.
However, the appearance of the hypersensitivity site does
 correspond to the higher level of $\rho$-globin RNA and is detect-
ed only after both demethylation and sodium butyrate treat-
ment. Moreover, a strong association between DNase I hy-
persensitivity and transcription of the chicken globin genes
has been established by others (4, 21, 34, 35).

**DISCUSSION**

A number of functional studies have shown that 5-aza-
cytidine is capable of inducing gene expression and DNA de-
methylation (15–20). Our experiments demonstrate that 5-
aza-cytidine treatment of anemic chickens causes demethyl-
ation in the majority of C-C-G-G and G-C-G-C sites in the $p$
-globin gene in adult erythroid cells. However, it does not
result in a high level of $\rho$-globin gene transcription or in the
appearance of the 5' DNase I-hypersensitive site that is pres-
ent in the active gene in 5-day embryonic erythroid cells.
Thus, demethylation is not sufficient to cause a high degree
of $\rho$ gene activation even in the fully differentiated red cell,
where presumably many of the factors necessary for effi-
cient globin gene expression are present. This implies that
methylation alone does not account for the inactive state of
the $\rho$-globin gene in adult red cells. However, since our re-
striction enzyme assay does not measure all CpG sites
around the $\rho$-globin gene, it is still possible that the methyl-
ation changes induced by 5-aza-cytidine do not correspond to
the normal state of gene methylation in embryonic red cells.
Since sodium butyrate exerts its activating effect only after the
$\rho$-globin gene has been demethylated in response to 5-
aza-cytidine, demethylation does appear to be a necessary
prerequisite for activation.

It is unlikely that the mechanism by which 5-aza-cytidine
treatment facilitates $\rho$-globin gene expression in these stud-
ies is by nonspecific cytotoxicity, since another cytotoxic
cytidine analog, cytosine arabinonucleoside, has no such ef-
fect. However, because the exact mechanism(s) of action of
cytosine arabinonucleoside differs from that of 5-aza-cytidine
apart from the effect on DNA methylation, some unique toxic or
selective effect of 5-aza-cytidine cannot be completely ruled out.
We have found that $\rho$-globin gene demethylation and $\rho$
globin RNA in reticulocytes persist at the same level for up to 10
days after cessation of 5-aza-cytidine when sodium butyrate
treatment is continued. Therefore the constant presence of 5-
aza-cytidine is not necessary for continued $\rho$-globin gene
activity.

Of equal or perhaps greater interest than the relationship
between DNA methylation and $\rho$-globin gene expression
described here is the finding that sodium butyrate, which has
been shown to induce increased expression of a variety of
genes in cultured cells (see ref. 28 for review), causes a
marked increase in specific gene expression in animals in
vivo. The precise mechanism by which sodium butyrate in-
creases $\rho$-globin gene expression in these studies remains
unknown. Measurement of an increase in steady-state RNA
levels does not prove that the effect is at the transcriptional
level, but the coinciding appearance of the 5' DNase I-hyper-
sensitive site supports a transcriptional rather than post-
transcriptional event. On the other hand, treatment with bu-
tyrate alone does not confer the active chromatin configu-
ration that induce increased levels of $\rho$-globin RNA.

Given the known activity of sodium butyrate as an inhibi-
tor of histone deacetylase (28, 36) and the frequently sug-
gested association between histone acetylation and tran-
scription (37–39), it is tempting to speculate that increased
histone acetylation plays some role in the activation event. We have analyzed histones from reticulocytes of butyrate-treated birds by electrophoresis in acid/urea/Triton gels (40) and found no gross changes in steady-state histone acetylation (data not shown). However, if only rapid histone acetylation (40) were involved in the initial activation event, one would not expect to see gross changes in acetylation. Once a given gene was activated by some chromatin structural change caused by rapid histone acetylation, it could remain active without any detectable change in bulk histone acetylation. In this regard we have recently detected a strong association between rapid histone acetylation and chicken erythrocyte nucleosomes that are enriched for active globin genes (unpublished observation).

Sodium butyrate has also been shown to affect other modifications of nuclear proteins, including high mobility group (HMG) protein phosphorylation and acetylation (41), and one of these factors could be responsible for its effect in these studies. It is, of course, possible that butyrate induces increased \( \beta \)-globin gene expression in some indirect way or in a manner unrelated to its known effects in cultured cells. For example, it could result in the accumulation of a trans-acting transcriptional enhancing factor analogous to the one encoded by the adenoviral Ela gene (42, 43).

The relationships between DNA methylation, DNase I sensitivity, and RNA production observed in the embryonic \( \beta \)-globin gene in these studies point out the complexity of the interactions between these measurable features of higher eukaryotic gene activity. Neither demethylation nor butyrate treatment alone is capable of inducing DNase I hypersensitivity or high levels of \( \beta \)-globin RNA, so that it appears that demethylation (or at least prior 5-azacytidine treatment) is a necessary prerequisite for embryonic \( \beta \)-globin gene transcription to occur in adult red cells. It has previously been shown by others that in avian erythroblastosis virus-transformed chicken erythroid cell lines (34), and in Roux sarcoma virus-transformed fibroblasts (35) demethylation may not be necessary for globin gene transcription or for development of an active chromatin configuration. This apparent difference from our results may be due to some as yet incompletely defined factor(s) in transformed cells in culture that can somehow replace or override the regulatory mechanisms found in erythroid cells in the bone marrow.

In summary, the induction of the normally silent embryonic \( \beta \)-globin gene in red cells of anemic adult chickens treated with 5-azacytidine and sodium butyrate appears to involve at least two separate steps. Although the mechanism(s) by which sodium butyrate causes increased \( \beta \)-globin gene expression remain to be determined, this experimental model should help improve our understanding of the features of DNA and chromatin that are involved in differential globin gene regulation in erythroid cells in vivo and provide insight into the treatment of certain human hemoglobinopathies.

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