Butyrate Induces Selective Transcriptional Activation of a Hypomethylated Embryonic Globin Gene in Adult Erythroid Cells

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An animal model of hemoglobin switching has been developed in which anemic adult chickens are treated with 5-azacytidine and sodium butyrate or α-aminobutyric acid, thereby resulting in activation of the embryonic β-globin gene in adult erythroid cells. In vitro nuclear runoff transcription assays using erythroid nuclei from treated birds show that the mechanism of activation of the β-globin gene is transcriptional whereas no transcriptional activation of the embryonic ε-globin gene occurs. The action of 5-azacytidine appears to be as an inhibitor of DNA methylation because other S-phase active cytotoxic drugs, when substituted for 5-azacytidine, do not cause demethylation of the embryonic globin genes, nor do they allow transcriptional activation to occur. Embryonic p-globin gene activation in this model is not due to selection of primitive erythroid cells since a subpopulation of primitive erythroid cells is not evident either morphologically or when cells are probed for embryonic and adult globin RNA by in situ hybridization. These studies show that demethylation by 5-azacytidine is a prerequisite but not sufficient cis-regulatory event for a high level of transcriptional activation of the embryonic β-globin gene in adult erythroid cells in vivo. The possible basis for the selective transcriptional activation by sodium butyrate in this system is discussed.

Materials and Methods

Animals. White leghorn chickens were treated with 1-acetyl-2-phenylhydrazine to induce anemia and with cytokine arabinosylcytidine (Ara-C), 5-azacytidine, and neutral sodium butyrate as previously described. Hydroxyurea (HU) (Sigma Chemical Co, St Louis) was dissolved in sterile normal saline immediately before use and injected intravenously at a dose of 15, 30, 60 or 100 mg/kg/d. 1-α-aminono-butyric acid (Sigma) was injected intramuscularly (IM) at a dose of 50 mg/kg every eight hours.

Quantitation and blot hybridization of RNA. Isolation of polysonal cytoplasmic RNA, whole cell RNA, or poly(A) + RNA; electrophoresis, transfer to nitrocellulose, and hybridization to [32P] labeled, nick-translated embryonic β-globin gene and adult β-specific globin gene probes were as previously described.

A 3'-end embryonic ε-globin gene–specific probe was prepared by subcloning the 1.9-kilobase (kb) HindIII fragment from β1BH4.10

Nuclear runoff transcription assays. Nuclei were prepared from peripheral reticulocytes as previously described. Transcription of nuclei was performed with modifications of published procedures. Hybridization to Southern blot Nytran (Schleicher & Schuell, Inc, Keene, NH) filters containing 2 µg of restriction enzyme–digested globin gene DNA per lane was at 43°C in 50% formamide for three days.

In situ hybridization. In situ hybridization was performed with modifications of published procedures. Smears of whole blood from 5-day-old embryos and treated animals were made on glass slides and fixed for two minutes at room temperature in absolute methanol and rehydrated in 0.1 mol/L glycine and 0.2 mol/L Tris-chloride, pH 7.5, for five minutes. Hybridization mixtures contained 5 × 107 cpm embryonic β-globin gene or adult β asymmetric [35S] labeled cRNA probe, 50% deionized formamide, 2× SSC, 5× Denhardt's solution, 10% dextran, 100 µg/mL salmon sperm, and 1 mg/mL tRNA.

Nuclease protection assay. Single-stranded labeled RNA complimentary to a region extending from the second exon to 900 nucleotides 5' upstream from the cap site of the p-globin gene was generated by the SP-6 polymerase reaction in the presence of [α-32P] guanosine triphosphate. Either 50 µg of total reticulocyte RNA or 1 µg of poly(A) + RNA from each treatment sample was hybridized overnight to 106 cpmp of labeled cRNA under the conditions described previously by Melton et al. After digestion with RNase at 40 µg/mL and RNase T1, 2 µg/mL, for 30 minutes at 37°C,
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RESULTS

The embryonic 

process in an 8-mol/L urea, 6% acrylamide gel that was then dried and autoradiographed with Kodak XAR x-ray film.

Analysis of histone acetylation. Nuclei were isolated from reticulocytes of control or treated animals as described. Nuclei were sonicated and differentially extracted with 0.4 N H$_2$SO$_4$ as described by Covault and Chalkley. Acid-precipitated histones were resuspended and subjected to electrophoresis in a 6% polyacrylamide, acid-urea-trition gels that were then stained with Coomassie’s brilliant blue G (Sigma).

DNA sequences

Fig 1. Nuclear runoff transcription assays carried out with nuclei from five-day-old embryos, 5-aza-CR, and 5-aza-CR plus sodium butyrate–treated animals (A) and with nuclei from HU or Ara-C plus sodium butyrate–treated animals (B). Transcripts were hybridized to Southern blot filters containing highly specific restriction fragments from genomic subclones of the β-type globin gene cluster at the bottom of the figure. Lanes A, 1.2-kb adult β-globin–specific DNA; Lanes B, 0.4-kb embryonic ρ-globin–specific DNA.

Demethylation of the p-globin gene is a requirement for transcriptional activation. Since other S-phase active drugs have been shown to increase fetal globin gene expres-
Fig 2. Demethylation of the embryonic p-globin gene in 5-aza-CR-treated animals. Reticulocyte DNA was extracted and digested with HindIII followed by either HpaII or MspI. HpaII will digest the DNA only at C-C-G-G sites in which the dinucleotide CpG is unmethylated, whereas MspI digestion occurs regardless of methylation status at C-C-G-G sites. Digests were subjected to electrophoresis in 1% agarose gels, transferred to nitrocellulose, and hybridized to a specific p-globin gene probe. Lanes A, phenylhydrazine only; lanes B, 5-aza-CR only; lanes C, 5-aza-CR plus sodium butyrate; lanes D, HU only; lanes E, HU plus sodium butyrate; lanes F, Ara-C only; lanes G, Ara-C plus sodium butyrate.

The embryonic p-globin gene is transcriptionally activated in the majority of reticulocytes from induced animals. To rule out the possibility that a subpopulation of reticulocytes that express the embryonic p-globin gene was being induced or selected for, asymmetric [35S] cRNA probes specific for the adult β-globin and embryonic p-globin genes were used to perform in situ hybridizations on blood smears of red cells from five-day-old embryos and reticulocytes from phenylhydrazine-treated controls and 5-aza-CR plus sodium butyrate–treated animals (Fig 4). RBCs from five-day-old embryos hybridized strongly to the p-globin gene probe (Fig 4A). Reticulocytes from phenylhydrazine-treated animals hybridized to the adult β-globin probe (Fig 4C) and not to the p-globin gene probe (Fig 4B). As such, the small number of silver grains in Fig 4B reflect nonspecific background hybridization. All reticulocytes from 5-aza-CR plus sodium butyrate–treated animals hybridized strongly to the adult β-globin gene probe (Fig 4E) and also uniformly but much less intensely to the embryonic p-globin gene probe (Fig 4D). Thus there was no apparent subpopulation of reticulocytes containing only RNA that hybridized to the embryonic p-globin gene probe, nor was a subpopulation of morphologically primitive erythroid cells evident.

Activation by sodium butyrate is selective for the embryonic p-globin gene. The embryonic p- and e-globin genes are coexpressed in primitive red cells so that induction of a primitive erythroid globin expression phenotype should result in the activation of both genes. To test for this possibility, polysomal cytoplasmic RNA was isolated from reticulocytes of 5-aza-CR plus sodium butyrate–treated animals. The resulting RNA samples were analyzed by blot hybridization with highly specific globin probes. The filter was hybridized to a specific p probe, washed free of probe, and hybridized to a specific e probe. As shown in Fig 5, polysomal RNA from five-day-old embryo red cells hybridized to both p and e probes (lanes A). In contrast, p-globin RNA was present in 5-aza-CR and sodium butyrate–treated animals, but no e-globin RNA could be detected (lanes B).

In addition, in vitro nuclear runoff transcription assays were performed in an effort to detect specific e-globin gene transcripts in nuclei from five-day-old embryos and 5-aza-CR plus sodium butyrate–treated animals. In Fig 6, lanes A
Probes

5'  p  3'

Fig 4. In situ hybridization analysis of red cells from five-day-old embryos (A) and reticulocytes from phenylhydrazine (B and C) and 5-aza-CR plus sodium butyrate (D and E)-treated animals. Five-day embryo cells were hybridized with the strand-specific p-globin gene probe described in Methods. phenylhydrazine, and reticulocytes with the same specific p-globin gene probe (B) or an adult β-globin gene probe (C), and 5-aza-CR plus sodium butyrate-treated reticulocytes were hybridized with a specific p-globin gene probe (D) or an adult β-globin gene probe (E).

contain the 1.2-kb specific adult β-globin fragment and lanes B the 0.4-kb specific p-globin fragment as described earlier. Lanes C contain a 0.6-kb HpaII restriction fragment from the second intron of the ε gene that reflects a true level of specific ε-globin gene transcription. The other fragments in lanes C most likely reflect cross-hybridization to p-globin transcripts since these fragments share a high degree of sequence homology. As expected, transcripts from five-day-old primitive red cell nuclei hybridized to both the p- and ε-specific gene fragments in a ratio of about 3:1, but not to the specific adult β-globin gene fragment. P-Globin gene transcripts were detectable in reticulocyte nuclei from 5-aza-CR plus sodium butyrate-treated animals at levels previously described (lanes B). However, no specific ε-globin gene transcripts were detected (lanes C). Thus, transcription of the ε gene is either absent or at least tenfold less than p transcription.

Bulk acetylation of reticulocyte histones is not affected by butyrate treatment. Sodium butyrate is a known inhibitor of histone deacetylase, and its action in this system could be related to altering histone acetylation in the chromatin of erythroid cells. To test for the type of bulk histone acetylation effect observed in butyrate treated cultured cells, histones were extracted from reticulocyte nuclei of control and butyrate-treated animals and analyzed by electrophoresis in acid-urea-triton gels. As shown in Fig 7 there was no detectable change in bulk acetylation of reticulocyte histones from treated animals.

DISCUSSION

Our earlier studies with this model system suggested that at least two separate steps are involved in the high-level induction of the normally silent embryonic p-globin gene in erythroid red cells of anemic chickens treated with 5-aza-CR

Probes

5'  p  3'

Fig 5. Northern blot hybridization analysis of p- and ε-globin gene RNA. A sample of 5 μg of glyoxylated cytoplasmic polysomal mRNA from five-day-old embryo cells was loaded in lane A and 50 μg of mRNA from 5-aza-CR plus sodium butyrate–treated adult reticulocytes in lane B of a 1% agarose gel. After capillary transfer to a nitrocellulose membrane the blot was hybridized to the p-globin gene probe illustrated at the bottom left of the figure, washed, and rehybridized to the ε-globin gene probe shown at the bottom right.

DNA sequences

Lane A: β 3' 1.2 kb
Lane B: p 3' 0.4 kb
Lane C: εIVS 0.6 kb

Fig 6. Nuclear runoff transcription assays carried out with nuclei from five-day-old embryos and 5-aza-CR plus sodium butyrate treated animals as indicated above the respective autoradiograms. Transcripts were hybridized to filters containing the following DNAs: lanes A, 1.2-kb adult β-globin–specific DNA; lanes B, 0.4-kb embryonic p-globin–specific DNA; lanes C, 0.6-kb embryonic ε-globin–specific DNA.
and sodium butyrate. Although 5-aza-CR treatment causes demethylation in the majority of C-C-G-G and G-C-C-G sites within and surrounding the \( \beta \)-globin gene, treatment with butyrate is required to produce a high level of \( \beta \)-globin gene expression.

5-Aza-CR has variable effects on tumor progression and antigen expression and has been shown to selectively induce certain genes.\(^22,24\) Several other repressed genes have been shown to require 5-aza-CR as a primary stimulus in combination with another agent as a secondary stimulus for gene activation.\(^27\) Many of these effects of 5-aza-CR are felt to be due to demethylation of DNA. 5-Aza-CR has successfully been administered to baboons and to human patients with sickle cell anemia and thalassemia and resulted in a rapid and prolonged increase in HbF production.\(^24\) However, similar “reversed hemoglobin switches” have been shown to be induced by HU and Ara-C, other S-phase–specific drugs that do not significantly inhibit methylation.\(^69\) It remains unclear, therefore, whether induction of fetal hemoglobin is caused by a loss of DNA methylation at specific loci or whether 5-aza-CR acts either as a cytotoxic agent that allows repopulation by cells that express embryonic genes or by some as yet undefined reprogramming mechanism.\(^70\) In regard to the latter possibility, we have recently presented evidence that both 5-aza-CR and Ara-C can allow rapid transcriptional activation of suppressed HLA class I genes by interfering with RNA synthesis.\(^13\)

To clarify further the mechanism by which 5-aza-CR facilitates \( \beta \)-globin gene transcription in the avian model, either HU or Ara-C was substituted for 5-aza-CR in the induction treatments. No demethylation of the \( \beta \)-globin gene loci could be detected in DNA from animals treated separately with each drug. Embryonic \( \beta \)-globin RNA could not be detected at any dose of HU alone or in combination with butyrate, and transcription assays did not detect any \( \beta \)-globin gene transcripts in nuclei from Ara-C– or HU-treated animals. In situ hybridization of five-day-old embryonic RBCs and reticulocytes from phenylhydrazine-treated and 5-aza-CR plus butyrate–treated animals verified that there was no distinct subpopulation of cells expressing the \( \beta \)-globin gene. On the basis of these results it is unlikely that the mechanism by which 5-aza-CR facilitates \( \beta \)-globin gene expression is some nonspecific cytotoxicity or reprogramming related to its S-phase action. In addition, when 5-aza-CR treatment was withdrawn during the five-day administration of sodium butyrate to allow time for nearly complete turnover of peripheral reticulocytes, the level of \( \beta \)-globin gene transcription was unchanged when compared with simultaneous 5-aza-CR plus sodium butyrate treatment.\(^7\) Therefore, the continued presence of 5-aza-CR is not required, further supporting the hypothesis that DNA hypomethylation, a heritable \( \textit{cis} \)-acting effect of 5-aza-CR, is a necessary prerequisite for activating embryonic \( \beta \)-globin gene transcription in adult erythroid cells. Thus the data presented here establish a direct link between the loss of site-specific methylation and transcriptional activation of a developmentally silent embryonic gene in the normal chromosomal milieu of an adult erythroid cell in vivo.

The embryonic \( \epsilon \)-globin gene is transcribed at a level of at least 25% that of the \( \beta \)-globin gene in 5-day-old embryo cells\(^22\) in accord with the known relative amounts of globin protein in these cells.\(^9\) The \( \epsilon \) gene is demethylated after 5-aza-CR treatment and resides in DNaseI-sensitive chromatin in red cells of untreated adult animals.\(^29\) Thus, while by virtue of all currently known measures of chromatin structure the potential for \( \epsilon \)-globin gene transcription exists, we were unable to detect any \( \epsilon \)-globin gene steady-state mRNA or nuclear runoff transcripts in cells from treated gene animals. One possible explanation for this discrepancy is that, in contrast to the situation in embryonic erythroid cells where the adult \( \beta \)-globin gene is silent, the transcriptional activity of the \( \beta \)-globin gene in adult erythroid cells may selectively inhibit transcription of the downstream \( \epsilon \)-globin gene by some type of transcriptional interference.\(^30\) However, it is also possible that the differential activation of the \( \beta \)-globin gene is a consequence of some level of specificity of the regulatory signal induced by sodium butyrate treatment.

There are several possible explanations for the action of sodium butyrate in this model system that are based on its known biochemical actions.\(^31\) Sodium butyrate is a potent inhibitor of histone deacetylase,\(^22,23\) and its action could be related to altering histone acetylation in the chromatin around the \( \beta \)-type globin cluster. However, we have demon-
strated directly that there is no change in bulk acetylation of histones derived from reticulocytes of sodium butyrate-treated animals. This does not preclude some postsynthetic alteration in nonhistone chromosomal proteins that is induced by butyrate or minor changes in histone acetylation in the immediate vicinity of the $p$-globin gene. On the other hand, even relatively localized histone or high-mobility group protein modifications would be expected to affect both the $p$-gene and $e$-globin gene loci, which reside within 15 kb of contiguous DNA sequence. Moreover, detailed studies of this overall nuclease accessibility of the $p$- and $e$-globin genes in adult red cell nuclei have demonstrated no clear differences between the chromatin configuration of the two genes. Recent results from cell fusion experiments have shown that globin genes are not irreversibly repressed in differentiated cells and that trans-acting regulatory factors in erythroid cells are capable of activating globin genes in a stage-specific and tissue-specific manner. The differential activation of the $p$-globin gene in the avian system could result from such a sequence-specific trans-acting factor or factors induced or modified by butyrate or one of its metabolites.

The relationship between the activation of the embryonic $p$-globin gene in this adult animal model and the augmentation of fetal globin expression in primates, including humans, deserves further consideration. It would appear that 5-aza-CR acts somewhat differently in the two systems in that in primates 5-aza-CR alone causes substantial elevations in fetal $\gamma$-globin expression and this effect can be mimicked by other S-phase-active drugs. While such data do not preclude hypomethylation as a contributing factor, they do make it unlikely that the sole effect rests there and suggest either another role for S-phase-active drugs or that not all S-phase-active agents act via the same mechanism. In the avian model on the other hand, the effect of 5-aza-CR alone is minimal, and most importantly, the potentiating effect appears to depend on the loss of methylation of the $p$-globin gene template since neither HU nor Ara-C allows $p$-globin gene activation. We take this as strong evidence that methylation does serve as a negative regulating factor in normal erythroid tissues in vivo. It is possible that the activation mechanism of HU or Ara-C for $\gamma$-globin genes in primate adult erythroid cells overrides the negative effect of methylation, or alternatively, it is possible that critical sites in the $\gamma$-globin gene–regulatory regions are already demethylated in adult erythroid cells.

In contrast to the effects of 5-aza-CR, the effects of sodium butyrate and its metabolite alpha-amino butyric acid are more clearly analogous between the avian and primate systems. Recent evidence regarding the delayed fetal-to-adult globin gene switch in fetuses of diabetic mothers suggests that this effect could be due to high serum levels of alpha-amino butyric acid, a known metabolite of butyrate. In addition, a direct effect of alpha-amino butyric acid on fetal hemoglobin has been demonstrated in human neonatal erythroid progenitors. In this regard it is interesting to note that alpha-amino butyric acid is capable of stimulating $p$-globin gene transcription in our model at about a tenfold lower concentration than is sodium butyrate (see Fig 3). The avian model system may prove particularly useful in attempts to define this globin gene switching effect at a molecular level and could thus facilitate attempts to safely modulate fetal globin expression in certain hemoglobinopathies as suggested previously.

In summary, we have established that demethylation of embryonic globin genes in adult erythroid cells is a prerequisite, but not sufficient stimulatory event for the transcriptional activation of these developmentally regulated and tissue-specific genes. The basis for the preferential activation of the $p$-globin gene remains to be explained but it appears to be a consequence of a selective effect of sodium butyrate or its metabolites on the transcription potential of the embryonic $p$-globin gene.

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