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 p-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 p-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 p-globin gene in adult erythroid cells in vivo. The possible basis for the selective transcriptional activation by sodium butyrate in this system is discussed.

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MATERIALS AND METHODS

Animals. White leghorn chickens were treated with 1-acetyl-2-phenylhydrazine to induce anemia and with cytosine arabinoside (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-aminom-butric acid (Sigma) was injected intramuscularly (1M) at a dose of 50 mg/kg every eight hours.

Quantitation and blot hybridization of RNA. Isolation of polyclonal cytoplasmic RNA, whole cell RNA, or poly(A) + RNA; electrophoresis, transfer to nitrocellulose, and hybridization to [32P] labeled, nick-translated embryonic p-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) HincII fragment from β1BH4.5

Nuclear runoff transcription assays. Nuclei were prepared from peripheral reticuloocytes as previously described. Transcription of nuclei was performed with modifications of published procedures. Hybridization to Southern blot 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-cloride, pH 7.5, for five minutes. Hybridization mixtures contained 5 x 10^7 cpm embryonic p-globin gene or adult β asymmetric [32P] labeled cRNA probe, 50% deionized formamide, 2× SSC, 5× Denhardt’s solution, 10% dextran, 100 µg/mL salmon sperm, and 1 µg/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 reticuloocyte RNA or 1 µg of poly(A) + RNA from each treatment sample was hybridized overnight to 10⁵ cpm of labeled cRNA under the conditions described previously by Melton et al. After digestion of RNase at 40 µg/mL and RNase T-1, 2 µg/mL, for 30 minutes at 37°.

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RESULTS

The embryonic \( \beta \)-globin gene is activated at the transcriptional level. As previously described, adult white leghorn chickens were rendered anemic by phenylhydrazine injection, treated with daily IM injections of 5-aza-CR for seven days, and then injected for five more days comitantly with sodium butyrate. When doses of sodium butyrate greater than 500 mg/kg were administered every eight hours, the amount of \( \beta \)-globin RNA was sevenfold higher than in 5-aza-CR–only samples. The coinciding appearance of a 5' DNase I-hypersensitive site was felt to be most consistent with a transcriptional event.21

To test this hypothesis, in vitro runoff transcription assays were performed by using nuclei from five-day-old chick embryos and from phenylhydrazine- and 5-aza-CR–only–, and 5-aza-CR plus sodium butyrate–treated adult animals. In Fig 1A, lanes A contain a 1.2-kb 3' HpaII restriction fragment specific for adult \( \beta \)-globin gene transcripts, and lanes B contain a 0.4-kb 3' PstI restriction fragment specific for \( \beta \)-globin gene transcripts.22 As expected, transcripts from five-day-old nuclei hybridized only to the \( \beta \)-globin gene fragment and not to the adult \( \beta \)-globin gene fragment. Adult \( \beta \)-globin–specific transcripts were detectable in all the treatment groups (lanes A). \( \beta \)-Globin gene–specific transcripts were not detectable in phenylhydrazine-only–treated cells. However, a low level of \( \beta \)-globin gene transcription could be detected in 5-aza-CR–only–treated cells, and a much higher level of transcription was seen in 5-aza-CR plus sodium butyrate–treated cells (lanes B).

The relative amounts of \( \beta \)-globin gene transcription in the five-day–only and the various treatment groups were quantitated by densitometric scanning of the autoradiograms in Fig 1. The amount of \( \beta \)-globin gene–specific transcripts in the 5-aza-CR plus sodium butyrate sample was about sevenfold higher than in the 5-aza-CR–only samples. The amount of specific \( \beta \)-globin transcripts was equal in both samples, which suggests that there was no reciprocal downregulation of transcription of the \( \beta \)-globin gene by sodium butyrate and that the difference in \( \beta \)-globin gene transcription is not due to technical variability between transcription assays.

These data correlate directly with the amount of \( \beta \)-globin mRNA present in each treatment group.23 Therefore transcriptional upregulation is sufficient to account for the increase in steady-state globin mRNA, although our studies do not preclude any posttranscriptional mechanism(s).

Demethylation of the \( \beta \)-globin gene is a requirement for transcriptional activation. Since other S-phase active drugs have been shown to increase fetal globin gene expression in adult primates,49 alternative S-phase active drugs, HU and Ara-C were substituted for 5-aza-CR in the treatment protocol. A range of HU doses consisting of 15, 30, 60, and 100 mg/kg/d was used to avoid missing any stimulatory effect that might be negated by marrow suppressive effects of the HU. To test for effects on DNA methylation within and around the \( \beta \)-globin gene, DNA samples from reticulocytes of either control animals or treated animals were digested with the methyl-sensitive restriction enzyme HpaII or its methyl-insensitive isoschizomer MspI and analyzed by Southern blot hybridization. As shown in Fig 2, lanes B and C containing the HpaII-digested DNA, treatment with 5-aza-CR resulted in significant demethylation around the embryonic \( \beta \)-globin gene, but neither HU nor Ara-C affected \( \beta \)-globin gene methylation. Correctly initiated \( \beta \) mRNA was not evident by the RNase protection assay, as shown in Fig 3, for any dose of HU. Nuclear runoff transcription assays did not yield any detectable \( \beta \)-globin transcripts in nuclei from HU– or Aza-C–treated cells (Fig 1B, lanes B). Thus, demethylation changes within and surrounding the \( \beta \)-globin gene induced by 5-aza-CR appear to be a necessary prerequi-
Fig 2. Demethylation of the embryonic $\rho$-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 $\rho$-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.

site for transcriptional stimulation of this embryonic globin gene by sodium butyrate.

The embryonic $\rho$-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 $\rho$-globin gene was being induced or selected for, asymmetric [3H]cRNA probes specific for the adult $\beta$-globin and embryonic $\rho$-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 $\rho$-globin gene probe (Fig 4A). Reticulocytes from phenylhydrazine-treated animals hybridized to the adult $\beta$-globin probe (Fig 4C) and not to the $\rho$-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 $\beta$-globin gene probe (Fig 4E) and also uniformly but much less intensely to the embryonic $\rho$-globin gene probe (Fig 4D). Thus there was no apparent subpopulation of reticulocytes containing only RNA that hybridized to the embryonic $\rho$-globin gene probe, nor was a subpopulation of morphologically primitive erythroid cells evident.

Activation by sodium butyrate is selective for the embryonic $\rho$-globin gene. The embryonic $\rho$- and $\epsilon$-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 $\rho$ probe, washed free of probe, and hybridized to a specific $\epsilon$ probe. As shown in Fig 5, polysomal RNA from five-day-old embryo red cells hybridized to both $\rho$ and $\epsilon$ probes (lanes A). In contrast, $\rho$-globin RNA was present in 5-aza-CR and sodium butyrate–treated animals, but no $\epsilon$-globin RNA could be detected (lanes B).

In addition, in vitro nuclear runoff transcription assays were performed in an effort to detect specific $\epsilon$-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 $5'$ $p$ $3'$ $e$ $3'$

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 $\beta$-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 $\beta$-globin gene probe (E).

Fig 5. Northern blot hybridization analysis of $p$- and $e$-globin gene RNA. A sample of 5 $\mu$g of glyoxylated cytoplasmic polysomal mRNA from five-day-old embryo cells was loaded in lane A and 50 $\mu$g 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 $e$-globin gene probe shown at the bottom right.

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.
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.\textsuperscript{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.\textsuperscript{14} 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 erythrocytes. 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\textsuperscript{22} in accord with the known relative amounts of globin protein in these cells. The \(\epsilon\) gene is demethylated after 5-aza-CR treatment and resides in DNase-I-sensitive chromatin in red cells of untreated adult animals.\textsuperscript{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.\textsuperscript{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.\textsuperscript{31} Sodium butyrate is a potent inhibitor of histone deacetylase,\textsuperscript{23,24} 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 posttranslational alteration in nonhistone chromosomal proteins that is induced by butyrate or minor changes in histone acetylation in the immediate vicinity of the \( \rho \)-globin gene. On the other hand, even relatively localized histone or high-mobility group protein modifications would be expected to affect both the \( \rho \) gene and \( \epsilon \)-globin gene loci, which reside within 15 kb of contiguous DNA sequence. Moreover, detailed studies of this overall nuclease accessibility of the \( \rho \) and \( \epsilon \)-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 \( \gamma \)-actin regulatory factors in erythroid cells are capable of activating globin genes in a stage-specific and tissue-specific manner. The differential activation of the \( \rho \)-globin gene in the avian system could result from such a sequence-specific \( \gamma \)-actin factor or factors induced or modified by butyrate or one of its metabolites.

The relationship between the activation of the embryonic \( \rho \)-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 \( \gamma \)-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 \( \gamma \)-phase-active drugs or that not all \( \gamma \)-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 \( \rho \)-globin gene template since neither HU nor Ara-C allows \( \rho \)-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 \( \rho \)-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 \( \rho \)-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 \( \rho \)-globin gene.

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