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Transcriptional Upregulation of γ-Globin by Phenylbutyrate and Analogous Aromatic Fatty Acids

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ABSTRACT. Phenylbutyrate has been shown recently to induce fetal hemoglobin (HbF) production in patients with sickle cell anemia and β thalassemia. We have now examined related aromatic fatty acids in order to define the range of active structures and identify plausible mechanisms of action. Structure–function analysis revealed that for effective stimulation of HbF in erythroid precursors: (1) the ideal length for the aliphatic side chain is four carbons; (2) oxygen or sulfur substitutions in the carboxylic chain are allowed, as evidenced by the equal or increased activity of phenoxypropionate, benzylthioglycolate, and benzyloxyacetate compared with phenylbutyrate; and (3) blocking the carboxylate group by conversion to the amide form greatly reduces potency. Molecular analysis indicated that the prototype agent, phenylbutyrate, increases HbF production through transcriptional activation of the γ-globin gene. The latter contains a butyrate responsive promoter known to up-regulate transcription in the presence of short-chain fatty acids of three to five carbons. To determine whether stimulation of an element in this promoter by phenylbutyrate and its analogues might contribute to their mechanism of action, we used a transient expression system involving K562 erythroleukemia cells transfected with a luciferase reporter gene driven by the minimum γ-globin promoter. Transcriptional activation in this experimental system correlated well with the capacity of an aromatic fatty acid to increase HbF production in erythroid precursors ($r = 0.94$). Our studies identify potent analogues of phenylbutyrate for the treatment of β-chain hemoglobinopathies, and suggest that stimulation of a butyrate responsive promoter may be responsible for their activity. BIOCHEM PHARMACOL 52;8:1227–1233, 1996.

KEY WORDS. erythroid precursor; hemoglobin F; β-hemoglobinopathies; aromatic fatty acids; phenylbutyrate; butyrate responsive promoter

Phenylbutyrate and its metabolite, phenylacetate, enhance HbF† production in erythroid precursor cells *in vitro* [1, 2] and in human subjects [3, 4]. Consequently, phenylbutyrate is currently being developed as an oral drug for the treatment of inherited β-chain hemoglobinopathies [4, 5]. This approach to the treatment of patients suffering from sickle cell disease is feasible because HbF-containing erythrocytes have lower concentrations of HbS. Moreover, HbF directly inhibits polymerization of HbS, accounting for the lower propensity of such cells to undergo sickling [6]. In β thalassemia, the elevated γ-chains of hemoglobin partially compensate for the deficiency in β-chains relative to α-chains [5]. Several pharmacologic agents such as hydroxyurea [7], 5-azacytidine [8], and butyrate [9] have been shown to increase HbF in patients with these diseases. Although hydroxyurea benefits adults with sickle cell anemia, it has not been used in children because of unacceptable myelosuppression [10]. Likewise, the clinical use of 5-azacytidine has been hindered by its toxicity and concerns of potential carcinogenesis [11]. While butyrate is less toxic, its very short plasma half-life in patients (about 6 min) [12] requires continuous intravenous infusion, which limits its use. Oral phenylbutyrate may provide an attractive alternative, considering its longer plasma half-life (1–2 hr) and low toxicity profile [5]. Results of early clinical trials are encouraging, showing activity in patients with sickle cell anemia and β thalassemia at well-tolerated doses [4, 5].

The promise of phenylbutyrate for the treatment of β-chain hemoglobinopathies has prompted our studies of its mechanism of action and the search for more active analogs. Phenylbutyrate and phenylacetate were found to increase the levels of γ-globin mRNA in treated cells [2]. This response may result from transcriptional control, as

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† *Abbreviations:* HbF, fetal hemoglobin; HbS, sickle hemoglobin; BRP, butyrate responsive promoter; and IC_{50}, inhibitory concentration causing 50% reduction in cell proliferation.

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the 5′ end of the $^{A}\gamma$-globin gene contains a shortened segment, −437 to −1, the minimal promoter, known to be activated by butyrate and other short-chain fatty acids [13], and thus termed a BRP.

In the present study, we prepared a series of aromatic fatty salts and compared their potential to activate the γ-globin promoter and to increase HbF production. Promoter activation was tested using a transient expression assay, involving human erythroleukemia K562 cells transfected with the minimal $^{A}\gamma$-globin promoter linked to a luciferase reporter gene. The effect of drugs on HbF production was determined in normal human erythroid precursors cultured according to published methods [2, 10, 14, 15]. The results identify several active derivatives of phenylbutyrate, and indicate that activation of the γ-globin BRP by this class of aromatic fatty acids may be responsible for an increase in the rate of γ-globin transcription and HbF accumulation in the developing erythroid cells.

MATERIAL AND METHODS

Reagents

Except where specified, all chemicals were obtained from Aldrich (Milwaukee, WI). For addition to cell medium, acid reagents were dissolved in water containing an equivalent of sodium hydroxide. The amide of phenylbutyrate was produced as described previously [16]. Briefly, the sodium salt of phenylbutyric acid (Elan Pharmaceutical Research Corp., Gainesville, GA) was heated with a small excess of thionylchloride followed by the addition of ice-cold concentrated ammonia. The reaction mixture was acidified, extracted with hexane, and the aqueous phase taken to dryness. Phenylbutyramide was extracted from the dried residue and purified by recrystallization from boiling water. Benzyloxyacetic acid was prepared by hydrolysis of benzyloxyacetylchloride (Aldrich) in water containing a slight excess of sodium hydroxide. The reaction solution was extracted with diethyl ether, acidified, and extracted with hexane. The hexane layer was removed, taken to dryness, and the residue dissolved in boiling hexane, which was then filtered and chilled on ice. Benzyloxyacetic acid was collected as a separate phase that was dried under vacuum. Sodium phenylacetate and sodium phenylbutyrate (Elan Pharmaceutical) were of pharmaceutical grade purity; phenylpropionic, phenoxypropionic, and phenylvaleric acids (Aldrich) were specified as minimum 99% pure, 3-phenylbutyric acid (Aldrich) as 98% pure, and benzylthioglycolic acid as 97% pure. Concerning the synthesized compounds, the identity and purity of phenylbutyramide were confirmed by melting point and mass spectrum analysis [16]; the purity of ben-zyloxyacetic acid was estimated by titration (98% pure) and the identity was confirmed by mass spectrometry. These compounds exhibited only the expected molecular ions.

Erythroid Cell Cultures

Erythroid progenitors from the peripheral blood of normal volunteers were grown in two-phase liquid culture protocol [2, 10, 14, 15]. Mononucleated cells, isolated by Ficoll-Hypaque centrifugation, were seeded in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) (both from GIBCO, Grand Island, NY), 1 μg/mL cyclosporin A (Sandoz, Basel, Switzerland), and 10% 5637 bladder-carcinoma cell conditioned medium. Cultures were maintained in a 5% CO_2, 100% humidity atmosphere at 37°. After 7 days in phase I culture, nonadherent cells were collected and cultured in phase II medium containing α-MEM, 30% FBS, 1% deionized bovine serum albumin, 1×10^{-5} M β-mercaptoethanol, 1.5 mM glutamine, 1×10^{-6} M dexamethasone, and 1 U/mL human recombinant erythropoietin (Ortho Pharmaceutical Co., Raritan, NJ). Compounds to be tested were added to the cultures on day 7 of phase II. The cells were harvested on day 13. At this time, 95% of the erythroid cells were judged, morphologically, to be orthochromic normoblasts following staining by May-Grunwald Giemsa.

Resolution and Determination of Hemoglobins

Cells were washed three times in phosphate-buffered saline, and lysed in distilled water. Lysate was pelleted by a 1-min microfuge spin, and the supernatant was collected and stored at 4°. Hemoglobins in the lysate were resolved by cation-exchange HPLC (Maximum 820, Waters Chromatography Division, Millipore Co., Milford, MA), using a 250 × 4.6 mm Syncropak CM300 column (Synchron Inc., Lafayette, IN) and eluting with 30 mM bis-Tris buffer (pH 7.4). The percentage of HbF out of the total Hb was calculated using "Maxima" software. The percentage of HbF in the treated cells was divided by the percentage of HbF in the untreated cells to obtain the fold increase. Hb standards (Isolab Inc., Akron, OH) were used for reference.

Isolation of Nuclei and Nuclear Run-On Transcription Assay

Nuclei were isolated as described [17–19]. Briefly, erythroid precursor cells that had been treated with drug for 72 hr were collected, washed with 10 mM Tris (pH 8.4):14 mM NaCl:1.5 mM $MgCl_2$ (RNA buffer) and lysed with 10 mM Tris (pH 8.4):14 mM NaCl:1.5 mM $MgCl_2$:1% Nonidet P-40. The nuclei were pelleted by centrifugation at 1000 *g* for 5 min at 4°, dispersed by passage through a 21-gauge needle, and isolated on a 1 M sucrose cushion in RNA buffer. The rate of gene transcription was determined by standard procedures [14, 15]. Briefly, purified nuclei, 1×10^7 per data point, were incubated at 30° for 15 min in labeling solution as follows: pelleted nuclei were resuspended in 2.0 mM Tris:2.0% glycerol:14 mM KCl:1.4 mM $MgCl_2$:0.1 mM $MnCl_2$:1.4 mM mercaptoethanol:33 mM each of ATP, CTP, and GTP:10 mg/mL phosphocreatine kinase:1 M phosphocreatine:300 mCi [α-^{32}P]UTP (New England Nuclear, Boston, MA; 760 μCi/mol). The reaction was stopped by the addition of 0.8% SDS:0.02 M EDTA:

0.05 M Tris (pH 7.5). The labeled mRNA was extracted and hybridized to excess globin or β-actin probes (2 μg each) immobilized onto nitrocellulose. Responses were determined by densitometry of the resulting autoradiograms. For each treatment, responses were standardized against the actin probe, and the result was compared with that from control cells to obtain the fold increase. The amount of hybridization to a plasmid DNA control was negligible (≤0.005% of γ-globin, untreated cells).

Cell Transfection with the Luciferase-γ Globin Gene Promoter Chimera

The minimal ^{A}γ-globin gene promoter (5′ −380 to +34), described by Lumelsky and Forget [20], was inserted into the upstream *Xho*I cloning site of the promoterless luciferase plasmid vector pXP2 by blunt end ligation as described previously [14]. No other elements were used. We have demonstrated correct ^{A}γ-promoter initiation in this luciferase construct. Plasmid DNA was prepared by cell lysis and cesium chloride centrifugation. The plasmid DNA was introduced into K562 cells by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA). In this procedure, cells were mixed with 25 μg plasmid DNA and 10 μg of sonicated salmon sperm DNA in a volume of 200 μL. The cells were electroporated at 240 V and 960 μF at 0°. Cells from several electroporation procedures were pooled, divided into aliquots, and placed into medium containing drug or into control medium without drug. Incubation continued for 48 hr at 37°. The luciferase assay was performed on lysates containing 0.25 mM ATP and 0.2 mM luciferin, using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA).

RESULTS

Nuclear Run-on Transcription of Globin Genes in Cells Treated with Phenylacetate and Phenylbutyrate

In previous experiments, we have shown that phenylacetate and phenylbutyrate increased γ-globin mRNA content in precursor cells [1, 2]. To determine the level at which control occurred, we examined the rate of nRNA synthesis. Nuclei isolated from treated erythroid precursor cells actively transcribed globin genes. Desitometric analysis of hybridized dot blots indicated that after normalization for actin, 7.5 mM phenylacetate stimulated γ-globin nRNA synthesis 2.2-fold and 1.5 mM phenylbutyrate stimulated a 2.4-fold increase (Fig. 1) while leaving α- and ε-globin nRNA synthesis rates nearly unchanged (data not shown). By contrast, β-globin nRNA declined 38% in response to the phenylacetate treatment and 49% in response to phenylbutyrate. These nuclear run-on experiments demonstrate that the aromatic fatty acids may enhance HbF synthesis by a selective increase in γ-globin gene transcription.

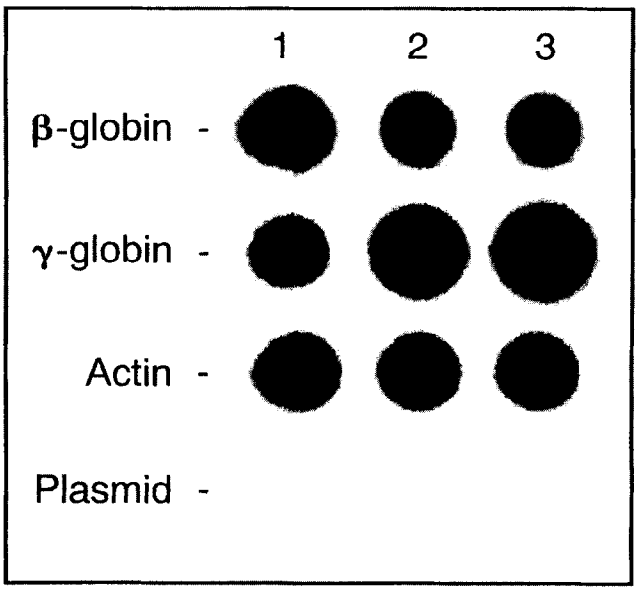

FIG. 1. Nuclear run-on transcription of globin genes from cells treated with phenylacetate or phenylbutyrate. Nuclear run-on assay was performed on treated erythroid precursors as described in Materials and Methods. Nuclear globin gene transcripts were labeled with [^{32}P]uridine triphosphate. Lane 1: control; lane 2: 7.5 mM phenylacetate; and lane 3: 1.5 mM 4-phenylbutyrate.

Stimulation of HbF Synthesis by Phenylbutyrate and Its Analogs

Confirming previous reports [1,2] phenylacetate and phenylbutyrate stimulated HbF synthesis. The fold increase induced by phenylbutyrate varied from donor to donor depending on the individual responsiveness and the amount of HbF being produced before stimulation. Stimulation by 4.0 mM phenylbutyrate of cells from four different donors, used successively, resulted in fold increases of 6.0, 14.4, 9.1, and 29.7, while HbF in untreated cells ranged from 1.1 to 2.4% of total. To demonstrate that the comparative responses to different analogs were not dependent on donor individualities, fold increases produced by the analogs were normalized against the phenylbutyrate response of the cells from the same donor. In this series of experiments, phenylbutyrate was about fifteen times more active than phenylacetate (Fig. 2). Also much more active were phenoxypropionate, benzyloxyacetate, and benzylthioglycolate, all of which (like phenylbutyrate) have a three atom spacer between the aromatic phenyl group and the carboxyl group. At 4 mM, phenylbutyrate and phenoxypropionate were about equally active, indicating that oxygen substitution at the fourth position of the carboxylate side-chain has little effect. On the other hand, substitution of the carbon at the third position with oxygen or sulfur, such as occurs in benzyloxyacetate or benzylthioglycolate, increased activity about 2-fold. Phenylpropionate at 4 mM was about a third as active as 4-phenylbutyrate at 4 mM, demonstrating that a three-carbon carboxylate chain is not as stimulatory. The 3-phenylbutyrate, in which a methyl group occupies the

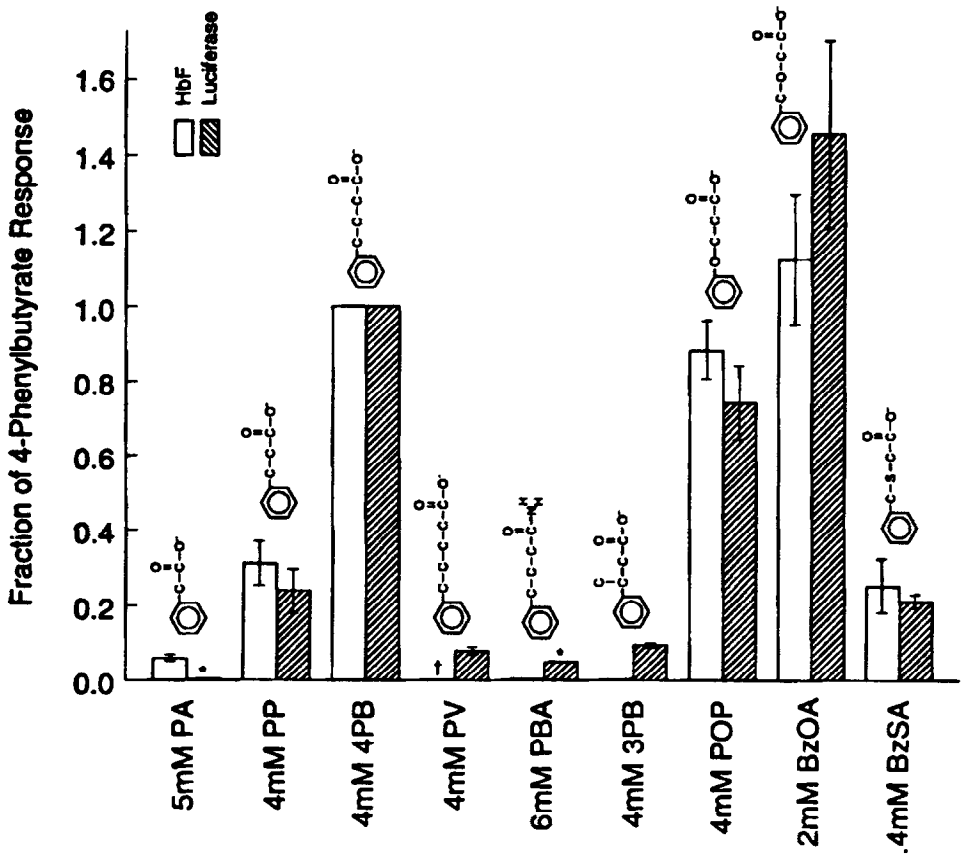

FIG. 2. Comparison of HbF production in erythroid precursors with luciferase activity induced by stimulation of the γ-globin promoter. Data from three independent experiments (except where noted * or †) were normalized against the 4-phenylbutyrate response in each experiment (hence no standard deviations for 4-phenylbutyrate). Abbreviations: PA, phenylacetate; PP, phenylpropionate; POP, phenoxypropionate; PB, phenylbutyrate; BzOA, ben-zyloxyacetate; BzSA, benzylthioglycolate; PV, phenylvalerate; and PBA, phenylbutyramide. Key: (*) one determination only; and (†) cytostatic in erythroid precursors.

third carbon of the propionate side chain, was still less active. 4-Phenylbutyramide was inactive even at 6 mM, illustrating that a free carboxyl group is essential for activity. The latter observation is supported by studies comparing the cytostatic activity of butyrate and butyramide that found a 25-fold attenuation of activity in the amide form [21]. However, we might expect that this disparity would not be observed in cells that can rapidly hydrolyze amide groups. Phenylvalerate, with a five-carbon carboxylate side chain, did not produce HbF at 1 mM (data not shown) and was cytostatic above 1 mM. It appears, therefore, that an aliphatic chain length of four carbons (or in the case of oxygen or sulfur substitutions, three carbons plus the substituted atom) may be optimal (Fig. 2). At 7.5 mM, phenylacetate produced 2 to 3-fold increases (donor variation) in erythroid precursor HbF (data not shown) which is consistent with the 2.2-fold increase in γ-globin nRNA found in the nuclear run-on experiments, and at 1.5 mM phenylbutyrate produced 2 to 5-fold increases in HbF which is not inconsistent with a 2.4-fold increase in γ-globin nRNA synthesis.

Activation of the γ-Globin Gene Promoter

We next compared the effects of the aromatic fatty acids and of aliphatic butyrate on the BRP. K562 cells were transfected with a plasmid-based expression vector in which the γ-globin minimal promotor containing a butyrate response region was ligated to the luciferase reporter gene [13, 20] as described in Materials and Methods. The capacity to activate transcription through stimulation of the promoter was measured in terms of increased synthesis of luciferase. Transfection efficiency varied from experiment to experiment. Fold increases stimulated by 4 mM 4-phenylbutyrate in successive experiments were 22.5, 103, 94.6, and 54. In the experiment in which phenylbutyrate produced a 22.5-fold increase, butyrate at 2.5 mM produced a 37-fold increase. Stimulation by the analogues of phenylbutyrate was normalized against the phenylbutyrate response in each experiment (Fig. 2). Phenylacetate produced little activation at 5 mM. This result is in agreement with previous work showing that phenylacetate at 5 mM is about one-thirtieth as active as 2.5 mM butyrate [13]. Phenylbutyrate and its structural analogues phenoxypropionate, benzyloxyacetate, and benzylthioglycolate were strong stimulators of the BRP. In potency, 4 mM phenylbutyrate and 2 mM benzyloxyacetate were about equivalent to 2.5 mM butyrate (data not shown). Phenylpropionate was less than a fourth as active as 4-phenylbutyrate on a molar basis, and its analogue 3-phenylbutyrate was still less active. Phenylvalerate was not toxic to the K562 cells; however, it demonstrated only

a weak stimulation of luciferase synthesis. Likewise, phenylbutyramide was very weak compared with phenylbutyrate. These results agree with those of the stimulation of HbF in erythroid precursors; blocking the carboxylate group of phenylbutyrate by conversion to the amide greatly reduced activation of the γ-globin promoter, and the ideal length for the carboxylate side chain on the aromatic ring is four carbons, with oxygen or sulfur substitutions allowed. Figure 2 shows the capacity of the agents to stimulate HbF production compared with their γ-globin promoter activation, demonstrating a direct correlation ($r = 0.94$) between these activities in the two experimental systems. In general, the K562 cells transfected with the γ-globin minimal promoter-luciferase chimera were four times more responsive to fatty acid stimulation than were the erythroid precursors. We attribute this difference to a number of factors including difference in cell type, the possible loss of a transcriptional inhibitor or, conversely, RNA destabilizing sequences, and differences in the cellular stabilities of the two translation products.

DISCUSSION

In this paper, we demonstrate a strong correlation between the capacity of aromatic fatty acids to activate the BRP of the γ-globin gene and their capacity to stimulate HbF synthesis in erythroid precursors ($r = 0.94$). We also show that structural considerations are central to the potency of these lipids. These observations may have clinical significance in the treatment of β thalassemias and sickle cell anemia.

In erythroid precursor cells, HbF synthesis was stimulated most effectively by aromatic fatty acids having a free carboxyl group on a carboxylate side-chain of about four carbons in length. For example, synthesis was strongly induced by the aromatic fatty acid 4-phenylbutyrate, which bears a four-carbon carboxylate side chain, and by analogues of phenylbutyrate in which the β or γ carbon has been replaced with oxygen, e.g. phenoxypropionate and benzyloxyacetate. By contrast, those with two- or three-carbon chains, phenylacetate, phenylpropionate, and 3-phenylbutyrate, were less active, and phenylvalerate, with a five-carbon carboxylate side chain, was inactive at non-toxic concentrations. Phenylbutyramide, in which the carboxylate function is blocked, was inactive. Considering the possibility that the capacity to induce fetal hemoglobin is related to cytostatic activity, we point out that both phenylvalerate and phenylbutyramide are cytostatic in tumor cell lines, with phenylvalerate being about as active as phenylbutyrate (IC_{50} values of 1.1 ± 0.1 and 0.9 ± 0.1 mM, respectively, in melanoma 1011 cells) and phenylbutyramide being about half as active [16]. However, both were much less potent than phenylbutyrate in stimulating HbF synthesis and activating the γ-globin promoter, suggesting that the capacity to induce hemoglobin is not dependent on non-specific, cytostatic activity. In addition, the HbF synthesis did not result from developmental arrest but was observed in cells uniformly in the orthochromic normoblast stage of development.

Nuclear run-on assays revealed that phenylbutyrate upregulated γ-globin synthesis in erythroid precursors at the transcriptional level while down-regulating β-globin synthesis. The ^{A}γ-globin gene has a transcriptional control region containing a BRP. Previously, Safaya *et al.* [13] using a transient expression system, demonstrated that among the straight chain aliphatic fatty acids, butyrate was the most efficient stimulator of this γ-globin BRP at lower concentrations (2.5 mM), although valerate, a five-carbon fatty acid, gave the greater amplification at higher concentrations of 10 mM. Their data indicate that aliphatic fatty acid chain lengths of four to five carbons are ideal for stimulating the butyrate-like response, whereas three-carbon propionate was weaker. Recently, this order of potency (four > five > three carbons) was observed during induction of γ-globin synthesis in cultured human erythroblasts [22]. Using the assay for transcription-stimulating activity, we demonstrated that the γ-globin promoter was clearly activated by phenylbutyrate and its analogues, and, as we observed in the erythroid precursors, activity varied with structure. As an indication of the correlation between transcriptional activation and erythroid cell response, the most potent activator of the BRP tested, benzyloxyacetate, was also the most potent stimulator of HbF, while the weak stimulators of HbF synthesis, phenylvalerate and phenylbutyramide, were weak effectors of the BRP. Our results demonstrate that the four-carbon length carboxylate side chain (or three carbons with oxygen or sulfur substitution) was optimal in the BRP assay. We note that the bulky phenyl group of phenylbutyrate does not seriously attenuate the BRP response; this is important since the phenyl group imparts a much longer serum half-life in patients, 1–2 h $T_{1/2}$ in the case of phenylbutyrate [5] compared with a 6-min $T_{1/2}$ for butyrate [12], while reducing objectionable odor and taste. It is also interesting that substitutions in the phenylbutyrate side chain with oxygen or sulfur did not block activity. In fact, increased activity was noted in the case of benzyloxyacetate and benzylthioglycolate. These results are consistent with studies that demonstrated that O and S substituted analogues of aliphatic butyrate are good activators of BRPs from HIV and the glucocerebrosidase gene [23].

We do not know the mechanism by which butyrate, phenylbutyrate, and analogs activate the γ-globin gene. The responsive promoter we have studied may not be the one actually driving HbF synthesis, and the stimulation of this γ-globin promoter and HbF synthesis could be distal and unrelated events. It is even possible that this promoter is a general, β-globin family promoter, as some have speculated [22], rather than one specific for γ-globin. However, the exquisite sensitivity of both the promoter and the HbF synthesis in precursor cells to the carboxylic acid side-chain length suggests a common mechanism. In addition, phenylbutyrate decreased β-globin synthesis rather than increasing it. Moreover, the ^{A}γ minimal promoter activated in these experiments contains both the distal and proximal CCAAT boxes and, between them, a segment with a nine

out of twelve base identity with the HIV butyrate response element [24]. In this same region of the minimal promoter, mutations can occur that result in permanently active HbF synthesis in human adults [see review in Ref. 25].

Promoters responding to butyrate are numerous [13, 23, 24, 26, 27], and the butyrate response might be considered by some to be non-specific, but it is not. There are butyrate-insensitive genes that have closely related butyrate-responsive counterparts; examples are: cytotoxic cell proteinase 2 (insensitive) and cytotoxic cell proteinase 1 (sensitive) [26], and placental alkaline phosphatase (insensitive) and placental-like alkaline phosphatase (sensitive) [27]. Promoters for these genes transfer their relative responsiveness to chimeric constructs that were previously unresponsive to butyrate. The large stimulation by butyrate and its analogs (up to 400-fold) of the γ-globin minimal promoter used in our experiments argues against a simple non-specific response by this gene [13]. Further work has indicated that a chimeric gene using bases −52 to +18 of the minimum promoter, and therefore lacking the region controlling the adult expression of HbF and within it the putative butyrate responsive element of the $^{A}\gamma$-globin promoter, is 34 times less responsive to butyrate.[1] This result, together with the results presented here and previously [13], tends to confirm that there is a butyrate-specific responsive region upstream from base −52.

The discovery that phenylbutyrate and its analogues activate a BRP has a significance beyond the induction of HbF. These aromatic fatty acids are also potent cytostatic and differentiating agents with activity against various solid and hematopoietic cancer cells [1, 16, 28–30]. It is possible that activation of a BRP contributes to the anticancer activity of these and other fatty acids. Studies with short-chain fatty acids have demonstrated that the order of their efficacy against human colon carcinoma cells [31] is similar to their capacity to activate the minimal promoter of the γ-globin gene [13]. Currently neither a ligand–receptor system nor binding protein for activating a butyrate response element has been identified. Consequently, the information provided by these experiments concerning the shape and size of efficacious fatty acids may be useful for identifying a butyrate response element activation mechanism, and simultaneously lead to the development of more potent agents for treating β-hemoglobinopathies and, perhaps, some cancers as well. These studies have identified at least two additional stimulators of the γ-globin promoter and HbF synthesis with the therapeutic potential, phenoxypropionate and benzyloxyacetate, which, because of their acceptable odor and taste, deserve further development.

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