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5′-Flanking Sequences Mediate Butyrate Stimulation of Embryonic Globin Gene Expression in Adult Erythroid Cells

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A stable transfection assay was used to test the mechanism by which embryonic globin gene transcription is stimulated in adult erythroid cells exposed to butyric acid and its analogs. To test the appropriate expression and inducibility of chicken globin genes in murine erythroleukemia (MEL) cells, an adult chicken β-globin gene construct was stably transfected. The chicken β-globin gene was found to be coregulated with the endogenous adult mouse α-globin gene following induction of erythroid differentiation of the transfected MEL cells by incubation with either 2% dimethyl sulfoxide (DMSO) or 1 mM sodium butyrate (NaB). In contrast, a stably transfected embryonic chicken β-type globin gene, ρ, was downregulated during DMSO-induced MEL cell differentiation. However, incubation with NaB, which induces MEL cell differentiation, or α-amino butyrate, which does not induce differentiation of MEL cells, resulted in markedly increased levels of transcription from the stably transfected ρ gene. Analysis of histone modification showed that induction of ρ gene expression was not correlated with increased bulk histone acetylation. A region of 5′-flanking sequence extending from −569 to −725 bp upstream of the ρ gene cap site was found to be required for both downregulation of ρ gene expression during DMSO-induced differentiation and upregulation by treatment with NaB or α-amino butyrate. These data are support for a novel mechanism by which butyrate compounds can alter cellular gene expression through specific DNA sequences. The results reported here are also evidence that 5′-flanking sequences are involved in the suppression of embryonic globin gene expression in terminally differentiated adult erythroid cells.

Stage-specific expression of the individual members of vertebrate β-type globin gene clusters occurs during the normal process of development, so that embryonic genes are switched off and adult genes are switched on. In the adult, the circulating red cell mass is supported by dividing and maturing erythroid progenitor cells, and in a manner which parallels the developmental switch, immature erythroid cells appear to express fetal or embryonic globin genes which are subsequently suppressed as maturation and maximal expression of the adult globin genes occur (38, 39). Thus, terminally differentiating adult erythroid cells may provide a system for understanding the molecular mechanisms that control the developmental switching of globin genes. Furthermore, models that involve reversing the developmental hemoglobin switch in adult animals should be useful for gaining insight into the mechanisms controlling this process in normal development.

We have previously described a model of reversed globin gene switching in adult chickens (20). In the avian β-type globin cluster (5′-ρ-β^H-β-ε-3′) (Fig. 1), the switch-off of the embryonic ρ gene concomitant with the switch-on of the adult β-gene occurs at 5 days of embryonic development (5). In the adult chicken, the embryonic globin genes are nontranscribed (23, 28) and are fully methylated in erythroid cells (34). When adult anemic chickens are treated with 5-azacytidine (5-aza-CR), an S-phase-active agent known to block DNA methylation, the normally silent embryonic ρ gene is expressed. That 5-aza-CR but not other S-phase-active drugs is able to reverse the developmental globin gene switch (6, 20) suggests that DNA methylation is an important regulatory mechanism affecting control of globin gene expression. When adult anemic chickens are treated with 5-aza-CR in combination with either sodium butyrate (NaB) (20) or α-amino butyrate, but not with butyrate compounds alone or in combination with other S-phase-active agents, the amount of ρ gene transcription is severalfold higher than in animals treated with 5-aza-CR alone (6). This synergism suggests a mechanism of action for butyrate and its analogs that is distinct from that of 5-aza-CR or other cytotoxic agents.

There are currently several experimental systems in which upregulation of fetal globin genes in mammals is induced by S-phase-active cytotoxic drugs. Examples include reversal of the hemoglobin switch in baboons treated with 5-aza-CR (16), cytosine arabinoside (40), and hydroxyurea (30) as well as reversal of the hemoglobin switch in humans treated with 5-aza-CR (9, 31, 32), hydroxyurea (47), and cytosine arabinoside (51). The mechanism of action of the S-phase-active agents in these systems is as yet undetermined. Current evidence suggests that the hematopoietic stress and resultant regeneration of the red cell mass induced by these agents may be critical factors. As mentioned, immature adult erythroid progenitor cells are able to express fetal/embryonic globin genes, suggesting that they lack the tightly controlled adult developmental program of globin gene expression (38). In the regeneration of hematopoiesis after suppression caused by 5-aza-CR or other S-phase-active drugs, erythroid progenitors are shunted toward altered erythroid maturation, so that the fetal/embryonic program persists in cells producing large amounts of globin chains (39). Alternatively, it has also been shown that 5-aza-CR may have an additional

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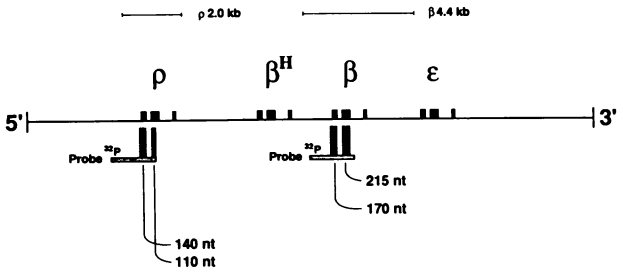

FIG. 1. Schematic illustration of the chicken β-type globin gene cluster. Shown is the 5′ to 3′ orientation of the four β-type globin genes (5′ρ-β^H-β-ε-3′). The bars above the gene map illustrate the size and orientation of ρ- and β-globin constructs used. The bars below represent the templates for the ^{32}P-labeled antisense RNA used in the RNase protection assays, and the vertical hatchmarks show the regions of hybridization. The length of these protected fragments in nucleotides (nt) is shown. The ρ and β probes each generate two protected fragments for correctly initiated and spliced mRNA.

independent effect on fetal globin gene expression in primates, analogous to the effect mediated by demethylation in the avian model (25).

Similarly, butyric acid and butyrate analogs have been shown to be capable of altering the developmental expression pattern of globin genes in a number of mammalian systems. Examples include the stimulation of fetal hemoglobin expression in baboons treated with α-amino butyrate (13) and the delay of the normal fetal-to-adult hemoglobin switch in fetal sheep treated with NaB infusion (43). Butyrates are also active in increasing fetal globin chain production in human erythroid progenitors (41, 42).

In addition to effects on fetal globin genes, NaB has been shown to produce diverse effects on a variety of genes in a number of cultured cell lines (27). The mechanisms by which butyrate exerts its effects are unclear. The major known pharmacologic action of butyrate in culture is to increase histone acetylation by inhibiting histone deacetylase (7). This action results in generalized changes in chromatin structure, and hence, a causative relationship has been suggested (22, 45). However, the specificity of butyrate's action in modulating the expression of only a few, select genes suggests that in some cases the observed generalized chromatin changes may be an epiphenomenon. In the avian model of reversed hemoglobin switching, for example, only the ρ gene is induced by butyrates. The other embryonic globin gene, ε, is not upregulated (6), nor is the chicken β-hatching (β^H) gene (21). Based on this specificity of embryonic ρ-globin gene activation in the anemic adult chicken model, we have proposed that butyrates alter developmental globin gene expression by a mechanism that involves specific regulatory DNA sequences (6).

To evaluate the molecular action of butyrate on embryonic ρ-globin gene expression in terminally differentiating adult erythroid cells, cloned avian embryonic ρ-globin genes were stably transfected into murine erythroleukemia (MEL) cells. MEL cells are arrested at the proerythroblast stage of adult erythroid maturation and can be induced to terminal differentiation by treatment with a variety of agents, including dimethyl sulfoxide (DMSO) (18) and NaB (29, 33). Our results indicate that *cis*-acting DNA sequences upstream of the embryonic ρ gene are a major determinant of ρ gene expression during MEL cell differentiation and that these sequences mediate the stimulatory action of butyrate compounds upon ρ gene transcription. These data are evidence for a new and perhaps general paradigm for the mechanism by which butyric acid and related compounds alter cellular gene expression. In addition, these results are supportive evidence for a strong evolutionary conservation of the DNA sequences involved in the developmental regulation of vertebrate globin gene expression.

MATERIALS AND METHODS

Transfection. Stable MEL cell transfectants were generated by electroporation with a BTX Transfector 100 (Biotechnologies and Experimental Research, Inc., San Diego, Calif.) and a 1.9-mm gap electrode (BTX P/N 470); 1.5×10^7 MEL cells were transfected in a single, ethanol-sterilized, semi-micro cuvette (VWR 58017-847) in 0.5 ml of serum-free RPMI 1640, with cells electroporated at 250 V and a single discharge with a 1-s pulse decay time. Approximately 20 μg of linearized chicken globin gene DNA (described below) was electroporated per sample, and RSV-*neo* was cotransfected at a 1:50 (*neo*/globin gene) molar ratio to convey G418 resistance. Double-stranded sonicated salmon sperm DNA, approximately 150 μg/ml, was added as a carrier, and after each transfection, cells were incubated on ice for 10 min before being transferred to tissue culture flasks.

For all experiments, the stably transfected cells represent the heterogeneous populations generated when 1.5×10^7 cells were transfected and selected in bulk with G418.

Cell cultures. MEL cells (ATCC CCL 745) before and after transfection were cultured in RPMI 1640 medium containing 10% fetal calf serum and supplemented with glutamine, penicillin, and streptomycin. Gentricin (G418; GIBCO) was added to the medium as the selection agent after transfection. Cells were induced to differentiate in either 2% DMSO, 1 mM neutral NaB (made by titrating butyric acid to pH 7.4 with sodium hydroxide), or 50 mM L-α-amino-*n*-butyric acid (Sigma, St. Louis, Mo.) for 5 days.

Globin gene constructs. For the embryonic β-type globin gene ρ, a 2-kb *Stu*I restriction fragment was obtained from a ρ 4.6-kb *Hin*dIII fragment (20). The fragment contains DNA sequences extending 725 bp upstream of the cap site, the entire coding sequence, and approximately 200 bp of DNA extending 3′ from the polyadenylation site. This fragment was subcloned into the *Sma*I site of pUC19, forming the pUC19-based ρ 2.0-kb plasmid. Approximately 536 bp of the 5′ region of the 2-kb *Stu*I fragment were deleted by digestion of the ρ 2.0-kb plasmid with *Eco*RI and *Bsp*MII to form the truncated ρ 1.4-kb plasmid. The intermediate-length constructs ρ 1.8 kb and ρ 1.6 kb were generated by polymerase chain reaction amplification with appropriate 5′ and 3′ primers and with the ρ 4.6-kb *Hin*dIII fragment as the template. These were cloned into the plasmid pGEM-2 (Promega, Madison, Wis.). All plasmid constructs were linearized with *Eco*RI prior to transfection.

For the adult β-globin gene, a 4.4-kb *Eco*RI-*Bam*HI fragment that includes the 3′ erythroid-specific enhancer (12, 36) was subcloned into pGEM-2 and linearized with *Bam*HI prior to transfection.

RNase protection assay. To obtain RNA, MEL cells were lysed in Nonidet P-40 (NP-40) buffer (0.5% NP-40, 140 mM NaCl, 10 mM Tris hydrochloride [pH 8.4], 1.5 mM magnesium chloride), nuclei were removed by centrifugation at 10,000 × *g*, and cytoplasmic RNA was extracted with neutral phenol and then with chloroform and then ethanol precipitated in sequence as described previously (11). Single-stranded, labeled cRNA was generated by using SP6

RNA polymerase and [α-^{32}P]GTP (35) in the presence of the following templates. For the chicken ρ gene, a 1.9-kb *Sac*I fragment extending from the second exon to 900 nucleotides upstream from the cap site was subcloned into the plasmid SP65 (35); for the chicken adult β gene, a 2.1-kb *Hin*dIII-*Eco*RI 5′ fragment of the gene was subcloned into pGEM-1 (Promega); for the *neo* gene, a 360-bp *Pvu*II fragment was subcloned into pGEM-1; and for the mouse α-globin gene, a 3′ portion of the α-globin gene subcloned into the plasmid SP65 was kindly provided by Peter Curtin.

From 10 to 75 μg of MEL cell cytoplasmic RNA was hybridized overnight to 10^6 cpm of labeled cRNA under conditions described previously (6). After digestion with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) for 30 min at 37°C, protection products were deproteinized and subjected to electrophoresis in an 8 M urea–6% acrylamide gel, which was then dried and autoradiographed.

Nuclear run-on transcription assay. Nuclei were isolated for transcription reactions by a modification of procedures previously described (11). ρ 2.0-kb-transfected MEL cells were treated for 5 days with 2% DMSO, 1 mM NaB, or 50 mM α-amino butyrate, and approximately 10^8 cells were harvested in each treatment group.

Transcription reactions were as described before (11), with approximately 3×10^7 nuclei per reaction. Reactions were run at 26°C for 10 min and terminated by treatment with 0.4 μg of RNase-free DNase I (Bethesda Research Labs, Gaithersburg, Md.) per μl for 5 min at 26°C, followed by lysis and deproteinization in 1 × SET buffer (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 10 mM Tris-HCl [pH 7.5]) with 0.1 μg of proteinase K per μl at 37°C for 30 min. Transcripts were extracted with neutral phenol-chloroform and precipitated with sodium acetate-ethanol, and the air-dried pellets were resuspended in 100 μl of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and separated from remaining unincorporated radioactivity on a gravity-feed Sephadex column (Nick columns; Pharmacia LKB, Piscataway, N.J.).

Hybridization to electroblotted DNA probes on Nytran was carried out at 65°C for 48 h in 3× saline sodium citrate (SSC)–10× Denhardt's solution–0.4% SDS, 40 μg of yeast tRNA per ml–0.5 μg of salmon sperm DNA per ml–0.5 μg of blank vector (pUC19) DNA per ml (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; 1× Denhardt's solution contains 0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll). Filters were washed twice for 10 min at 65°C in 2 × SSC–0.1% SDS, then washed twice in 0.1× SSC–0.1% SDS, and autoradiographed.

The DNA probes used for run-on transcription hybridization were as follows: a 1.1-kb *Ava*I fragment from the 3′ end of the ρ coding sequence, the 3′ end of the mouse α-globin coding sequence, and an α-actin cDNA probe from plasmid pHM2-A1 (24). These DNA fragments were separated on a 1.5% agarose gel and electroblotted to Nytran in 1× TAE, and the Nytran filters were UV-crosslinked in a Stratalinker (Stratagene La Jolla, Calif.).

Analysis of histone acetylation. MEL cell nuclei were isolated by cellular lysis in NP-40 buffer and were washed, sonicated, and differentially extracted with 0.4 N H_2SO_4 as described by Covault et al. (14). Acid-precipitated histones were resuspended and subjected to electrophoresis in a 12.5% polyacrylamide–acid–urea–Triton gel (14) that was then stained with Coomassie's brilliant blue G (Sigma).

RESULTS

Coregulation of the avian β-globin gene and the endogenous mouse α-globin gene in MEL cells. While studies of endogenous ρ-globin gene expression in anemic animals have provided a physiological model for demonstrating the potency and level of action of butyrate compounds on globin gene expression (6, 20), it has been technically difficult to define the precise mechanism(s) of butyrate action in erythroid cells. For this reason, a cell culture assay system was developed. The MEL cell line was chosen because it is the only well-established, phenotypically stable, and continuously cultured erythroid cell line with an adult hemoglobin program and because it has been widely used to study *cis*-acting regulatory sequences of mammalian globin genes (10, 15, 33, 49). Previous evidence showing a high degree of conservation between avian and mammalian tissue-specific regulatory elements, such as the GATA binding site (17, 37, 50), led us to speculate that vertebrate globin gene developmental control sequences might also be conserved. In line with this speculation, MEL cells were stably co-transfected with either cloned adult β-globin gene or embryonic ρ-globin gene constructs along with the drug selection plasmid RSV-*neo*. Bulk populations selected for resistance to G418 were then tested for the expression of the transfected chicken globin genes, and expressing populations were treated with either 1 mM NaB, 50 mM α-amino butyrate, or 2% DMSO. Since the transfected chicken globin genes are cloned and not methylated, 5-aza-CR treatment, which is necessary for embryonic globin gene induction in the chicken model (6, 20), had no effect in the cell culture system (data not shown) and was omitted.

To test the expression and inducibility of the adult chicken β-globin gene in MEL cells, the β 4.4-kb clone, which contains the β-globin gene plus flanking sequences that include the 3′ erythroid-specific enhancer (12, 36), was stably transfected into MEL cells, and populations of these cells were treated with DMSO, NaB, and α-amino butyrate. Figure 2 shows the results of RNase protection assays with RNA obtained from these treated cell populations. In these assays, the MEL cell RNA was probed simultaneously with the chicken β-globin gene cRNA probe and the *neo* gene cRNA probe. The *neo* gene serves as a constitutive control. In these experiments, quantitation of *neo* gene expression correlated directly with the quantitation of the RNA obtained by ethidium bromide staining of cytoplasmic RNA from uninduced and from DMSO-, NaB-, and α-amino butyrate-treated MEL cells (data not shown). As shown in Fig. 2, the adult chicken β-globin gene is expressed at a low level in uninduced MEL cells, and expression increases approximately 8- to 10-fold upon induction with 2% DMSO. Expression of the β-globin gene increases twofold to threefold with NaB, and there is no change with treatment with α-amino butyrate. The lower panel confirms that the MEL cells were induced toward erythroid differentiation by DMSO and NaB treatment, as judged by the marked increase in endogenous mouse α-globin gene transcripts. These data are consistent with the increased number of benzidine-positive cells in these samples (data not shown) and with previous reports showing that α-globin gene expression is a reliable indicator of MEL cell differentiation (19, 29, 33, 48). As shown here and as reported previously, α-amino butyrate treatment does not induce MEL cell differentiation (29). Thus, the transfected chicken adult β-globin gene is regulated in parallel with the endogenous

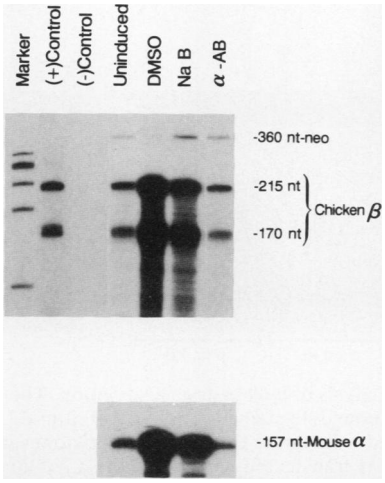

FIG. 2. RNase protection assay of RNA from MEL cells stably transfected with the adult chicken β-globin gene (β 4.4 kb; Fig. 1). The upper part of the figure shows the autoradiogram obtained from hybridization of cytoplasmic RNA (35 μg) from MEL cells, treated as indicated, to the ^{32}P-labeled β-globin cRNA probe, as depicted in Fig. 1, and the ^{32}P-labeled *neo* cRNA probe. The 360-nt protected fragment corresponds to the *neo* gene transcript and serves as an internal standard for mRNA integrity and quantity. The positive control corresponds to protection of 6-day chicken embryo reticulocyte whole-cell RNA (2 ng) and demonstrates the 215-nt and 170-nt protected fragments corresponding to correctly initiated and processed β-globin mRNA. The negative control is a protection assay containing tRNA only. The lower part of the figure shows the autoradiogram obtained from hybridization of aliquots of the same MEL cell RNA (10 μg) to the mouse α-globin cRNA probe.

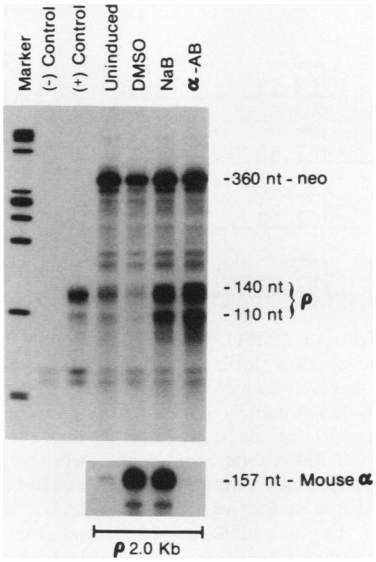

FIG. 3. RNase protection assay of RNA from MEL cells stably transfected with the embryonic chicken β-type globin gene ρ (ρ 2.0 kb; Fig. 1). The upper part of the figure shows the autoradiogram obtained from hybridization of cytoplasmic RNA (40 μg) from MEL cells, treated as indicated, to the ^{32}P-labeled ρ-globin cRNA probe, as indicated in Fig. 1, and the ^{32}P-labeled *neo* cRNA probe. The 360-nt protected fragment corresponds to the *neo* gene transcript and serves as an internal standard for mRNA integrity and quantity. The positive control corresponds to protection of 5-day chicken embryo reticulocyte whole-cell RNA (2 ng) and demonstrates the 140-nt and 110-nt protected fragments corresponding to correctly initiated and processed ρ-globin mRNA. The negative control is a protection assay containing tRNA only and shows a lower-molecular-weight doublet that is present in all hybridization assays containing the ρ probe. The lower part of the figure shows the autoradiogram obtained from hybridization of aliquots of the same MEL cell RNA (10 μg) with the mouse α-globin cRNA probe.

mouse adult α-globin gene in uninduced as well as in DMSO-, NaB-, and α-amino butyrate-treated MEL cells.

5'-Flanking sequences determine modulation of embryonic ρ gene expression in MEL cells. In order to test the effects of adult erythroid differentiation and butyrate exposure on embryonic ρ gene expression, a plasmid containing the ρ gene and 725 bp of 5'-flanking DNA (ρ 2.0 kb) was stably cotransfected along with RSV-*neo* into MEL cells. Populations of MEL cells expressing the ρ-globin gene were then exposed to either DMSO, NaB, or α-amino butyrate. Figure 3 shows the results of RNase protection assays with RNA obtained from these variously treated cell populations. In these assays, MEL cell RNA was probed simultaneously with the ρ gene cRNA probe and the *neo* gene cRNA probe, which again served as a constitutive control. The observed protected fragments that migrate between the ρ 140-nucleotide (nt) fragment and the 360-nt *neo* fragment most likely represent incompletely protected *neo* cRNA, since these bands are never present in assays in which the same MEL cell RNA is probed with the ρ probe alone (data not shown). Again, as in Fig. 2, the endogenous mouse α-globin mRNA control serves as an indicator of terminal erythroid differentiation. As can be seen, treatment with NaB or α-amino butyrate stimulates ρ-globin gene expression. In contrast, DMSO treatment suppresses the level of ρ gene expression. Erythroid differentiation is observed after incubation with either DMSO or NaB, as expected (29), but there is no evidence of erythroid differentiation after exposure to α-amino butyrate, as determined by the lack of any increase in expression of the endogenous mouse α-globin gene.

Figure 4 shows the results of nuclear run-on transcription assays which demonstrate that the higher levels of ρ gene mRNA seen after NaB and α-amino butyrate treatment are due to increased transcription rates. For these experiments, ρ 2.0-kb stably transfected MEL cells were treated with NaB or α-amino butyrate for 5 days, and nuclei were isolated. Gene-specific transcripts were quantitated for the ρ gene and the endogenous mouse α-globin and α-actin genes. The mouse α-globin gene again indicates erythroid differentia-

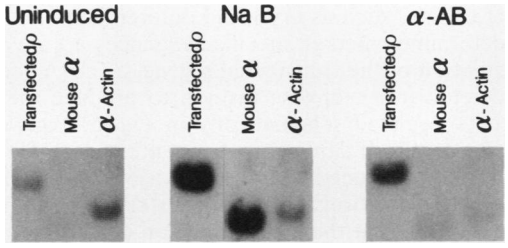

FIG. 4. Nuclear run-on transcription assays performed with nuclei from uninduced MEL cells and from MEL cells treated with NaB or α-amino butyrate (α-AB). This figure shows the autoradiograms obtained from the hybridization of ^{32}P-labeled nuclear run-on transcripts from MEL cells subjected to the indicated treatments to filter-bound DNA probes for the chicken α gene and the mouse α-globin and α-actin genes.

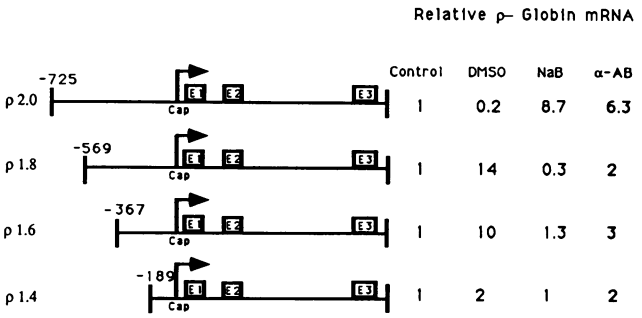

FIG. 5. Effects of DMSO, NaB, and α-amino butyrate (α-AB) treatment on levels of ρ-globin gene expression in bulk populations of MEL cells stably transfected with the ρ-globin gene constructs indicated. For densitometric quantitation, appropriately exposed autoradiograms with signals in the linear range were scanned with a Hoefer model GS 300 scanner, and peaks were integrated by using the Hoefer GS 360 Data System program. For each of the MEL cell transfectants, the value for the uninduced cells was normalized to 1. The values for the integrated signals derived from the autoradiograms from treated cells were reported relative to the value obtained for the corresponding uninduced cell population. Autoradiograms from multiple populations were scanned, and the results were statistically analyzed by Student's *t* test.

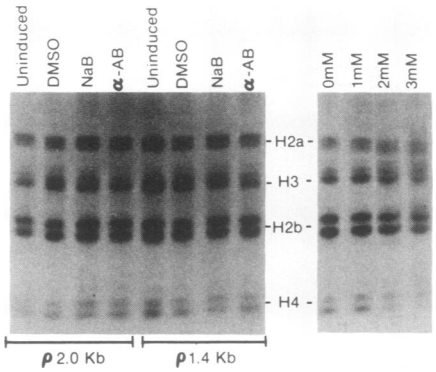

FIG. 6. Analysis of bulk histone acetylation. The left-hand panel shows the Coomassie-stained acid–urea–Triton–12.5% polyacrylamide gel used to separate the major core histones indicated. MEL cell populations transfected with either the ρ 2.0-kb or the ρ 1.4-kb construct were treated as indicated (α-AB, α-amino butyrate). The lower band of each major histone species contains unacetylated histones, and the minor bands seen migrating above the respective major bands represent acetylated variants. The right-hand panel of the figure shows a control gel containing Coomassie-stained histones isolated from MEL cells treated with the concentration of NaB indicated above each lane. This gel illustrates the histone hyperacetylation in these MEL cells at higher concentrations of NaB.

tion, and the α-actin gene serves as a constitutive control. In the uninduced cells, baseline transcription rates are documented for the three genes. In the NaB-treated cells, the rate of ρ gene transcription is elevated compared with the α-actin gene, and the rate of mouse α-globin transcription is also increased, as predicted (1). Lastly, for the α-amino butyrate-treated cells, the rate of ρ gene transcription is increased; but, in this case, the mouse α-globin gene transcription rate remains at the low, uninduced level. Thus, the changes observed in cytoplasmic mRNA levels for these genes are paralleled by changes in transcription rates.

To summarize, these three chemical agents exhibit overlapping but distinct activities on ρ-globin gene transcription in this assay system. Both DMSO and NaB act to induce erythroid differentiation, but NaB treatment concomitantly increases embryonic ρ-globin gene expression, while DMSO-induced differentiation suppresses it. Furthermore, α-amino butyrate, which differs from butyric acid by only a single amino group, increases ρ gene expression, but unlike NaB, α-amino butyrate does not induce erythroid differentiation. These results suggest that the ability of butyrate compounds to upregulate ρ gene expression represents a specific transcriptional effect which is separable from more global or indirect effects, such as erythroid differentiation.

To determine whether specific sequences are involved in the regulation of the transfected ρ gene, 5′ DNA sequences were deleted in a stepwise fashion to produce the ρ gene constructs depicted schematically in Fig. 5. Figure 5 also shows the results of densitometric quantitation of the level of induced and uninduced ρ gene expression in independently transfected populations of MEL cells resulting from transfection with each of the ρ gene deletion constructs. Autoradiograms derived from RNase protection assays, as illustrated in Fig. 3, from multiple independent MEL cell populations were used for these determinations.

The top line of the figure reproduces graphically the data from Fig. 3. When multiple, independent MEL cell populations transfected with the ρ 2.0-kb construct were analyzed, it was found that each of the values for the treatments (DMSO, NaB, and α-amino butyrate) showed significant changes from values for the uninduced cells. With DMSO treatment, levels of ρ-globin mRNA are suppressed fivefold from baseline to 0.2 ± 0.1 relative to the control ($P < 0.001$), and with NaB and α-amino butyrate levels are significantly increased (NaB: 8.7 ± 2.1, $P < 0.01$; α-amino butyrate: 6.3 ± 2.6, $P < 0.05$). For the 5′-truncated constructs ρ 1.4 kb, ρ 1.6 kb, and ρ 1.8 kb, the inducibility of ρ gene expression upon exposure to NaB or α-amino butyrate is lost, as is the suppression of ρ gene expression upon exposure to DMSO. Thus, the 156 bp of DNA located from −569 to −725 bp 5′ from the cap site appear to mediate both of these effects.

It is noteworthy that the ρ 1.8-kb and ρ 1.6-kb constructs, which lack the 156-bp 5′ element, are not only not downregulated but actually stimulated by DMSO-induced differentiation. While the basis for this effect is still under study, it is of interest that both the ρ 1.8-kb and ρ 1.6-kb constructs contain an additional GATA binding domain located approximately −200 bp 5′ from the cap site, which may account for induction when unopposed by the 5′-distal 156-bp element.

Lack of change in histone acetylation during butyrate induction of ρ gene expression. Previously, we have shown that there are no detectable changes in bulk acetylation of reticulocyte histones from chickens treated with 5-aza-CR and NaB, but these studies were limited by the fact that the majority of circulating reticulocytes are nontranscribing and metabolically inactive (3, 6). Figure 6 shows the bulk histone acetylation patterns for ρ 2.0-kb-transfected MEL cells and ρ 1.4-kb-transfected MEL cells in the uninduced state and after treatment with DMSO, NaB, or α-amino butyrate. As is best illustrated by the H4 bands, there is detectable histone acetylation even in uninduced MEL cells; however, there are no detectable changes in the level of histone acetylation at the concentrations of NaB and α-amino butyrate that result in ρ gene induction (Fig. 5) compared with the uninduced control. Furthermore, there are no changes in histone acetylation in response to DMSO treatment, which is in agreement with previous reports (7). The right-hand panel

of the figure illustrates that at concentrations of NaB greater than 2 mM, bulk histone acetylation is increased over control levels in the MEL cells used in these studies. This is best illustrated by the nearly complete loss of the unacetylated histone H4 band, so that all H4 histones are acetylated in the MEL cells treated with 3 mM NaB, a result that is in agreement with previous reports (7, 46).

DISCUSSION

It has been shown previously in an avian model that butyric acid can stimulate increased transcription of the embryonic ρ-globin gene in adult erythroid cells (6, 20). In addition, butyrate compounds have been shown to induce persistent high-level expression of fetal globin genes in cultured human erythroid progenitors (41) and ovine fetuses (43) and to stimulate fetal globin gene expression in anemic adult baboons (13). In this report, we present evidence that the effect of butyrate compounds on embryonic avian ρ-globin gene transcription appears to be mediated by 5'-flanking sequences located upstream from the basal globin gene promoter and is not accompanied by alterations in bulk histone acetylation. The data presented here also support the view that 5'-flanking sequences mediate the suppression of the ρ gene in MEL cells induced to terminal differentiation with DMSO.

NaB induces terminal differentiation of MEL cells but, in contrast to DMSO, causes increased expression of stably transfected ρ gene templates. In addition, α-amino butyrate, which is at least as effective as NaB in inducing ρ gene transcription in adult bone marrow in vivo (6), is also capable of inducing increased ρ gene expression in transfected MEL cells. The stimulatory effect of α-amino butyrate on ρ gene expression in MEL cells is also mediated by the same 5'-flanking DNA region but, strikingly, does not involve any detectable changes in the state of differentiation of the treated cell populations. Thus, our data are consistent with a model in which embryonic globin gene expression is suppressed during terminal erythroid differentiation via some sequence-specific mechanism while butyrates stimulate embryonic globin gene expression directly and can also override the suppression associated with terminal differentiation.

The fact that butyric acid and α-amino butyrate have the same sequence-specific effect on embryonic ρ-globin gene expression but differ in broader cellular effects suggests that a common metabolite might be the mediator of their shared effect. From our results and the work of Perrine and coworkers (41–43), it is tempting to speculate that such an hypothesized butyrate metabolite might either mimic a circulating physiologic modulator of embryonic/fetal globin gene transcription or alter such a factor.

It remains to be determined whether NaB stimulation of primate and ovine fetal globin gene expression is also mediated by specific *cis*-acting sequences. It has been shown recently that butyrate can upregulate transfected human fetal γ-globin gene promoters and endogenous embryonic mouse β-type globin genes in the GM979 murine erythroleukemia cell line (52), but this cell line is known to express embryonic globin gene transcripts even without butyrate stimulation (4, 26). Perhaps of more relevance, we have found that in the MEL cells used in these experiments, the endogenous murine ε^y gene, which is not expressed in uninduced MEL cells, can be induced by treatment of these cells with 5-aza-CR plus NaB, just as the endogenous ρ gene is transcriptionally activated in avian erythroid cells (20a). In addition, the apparent strong evolutionary conservation of control of avian adult β-globin and embryonic ρ-globin genes in murine erythroid cells demonstrated in the present studies suggests that developmental regulatory sequences, and possibly their cognate binding factors, may indeed be common to birds and mammals. In this context, it is of interest that a *cis*-acting sequence in the distal 5'-flanking region of the human embryonic ε-globin gene has been shown to exert a negative effect on ε gene expression in erythroid and nonerythroid cells (8) and in adult erythroid cells of transgenic mice (44). Thus, it is possible that the 5'-flanking sequences involved in ρ-globin gene downregulation during MEL cell differentiation have counterparts in the human embryonic and/or fetal globin genes.

The studies presented here have major implications regarding the pleiotropic phenotypic effects that NaB has been shown to exert on gene expression in a number of cultured cell lines (27). In all studies reported in which histone analysis has been performed, butyrate-induced changes in the expression level of either transfected or endogenous genes in cultured cells have been found to be accompanied by gross changes in bulk histone acetylation (22, 45). Because of this correlation, a cause-effect relationship has been suggested, although direct causation has been difficult to establish. In contrast, the concentrations of NaB and α-amino butyrate used in the present studies were sufficient to result in marked stimulation of ρ-globin gene expression without causing gross changes in bulk histone acetylation. The method used here does not exclude the possibility of very transient changes in the rapidly acetylated histone pool (3) or another subset of histones. However, taken together with the sequence-specific nature of the stimulatory effect of butyrate and its gene-specific activity in the avian model (6), our data are more consistent with a mode of action that does not involve histone acetylation. To our knowledge, there has been no previously reported direct demonstration of a butyrate-induced effect on tissue-specific cellular gene expression that is mediated by specific DNA sequences, and it is possible that such a mechanism may account for many of the other reported gene-specific effects of butyrate. In this regard, it is of interest that butyrate stimulation of a heterologous reporter gene driven by the human immunodeficiency virus long terminal repeat appears to be mediated by sequences in the basal promoter region of the long terminal repeat (2). In the case of the embryonic ρ gene, the *cis*-acting sequences involved in butyrate stimulation in MEL cells are located upstream of the basal promoter and may be involved in developmental regulation of the gene.

Whether butyrate affects developmental globin gene expression via posttranslational modification of a sequence-specific nuclear factor(s) or by regulating the synthesis of such a factor(s) remains to be determined. The assay system described here should allow these possibilities to be tested directly and should facilitate elucidation of the mechanisms of embryonic globin gene suppression in differentiated adult erythroid cells.

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