

# Ethyl-3,4-dihydroxybenzoate Inhibits Myoblast Differentiation: Evidence for an Essential Role of Collagen

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**Abstract.** To study the role of (pro)collagen synthesis in the differentiation of rat L6 skeletal myoblasts, a specific inhibitor of collagen synthesis, ethyl-3,4-dihydroxybenzoate (DHB), was utilized. It is shown that DHB reversibly inhibits both morphological and biochemical differentiation of myoblasts, if it is added to the culture medium before the cell alignment stage. The inhibition is alleviated partially by ascorbate, which along with  $\alpha$ -ketoglutarate serves as cofactor for the enzyme, prolyl hydroxylase. DHB drastically decreases the secretion of procollagen despite an in-

crease in the levels of the mRNA for pro $\alpha$ 1(I) and pro $\alpha$ 2(I) chains. Probably, the procollagen chains produced in the presence of DHB, being underhydroxylated, are unable to fold into triple helices and are consequently degraded in situ. Along with the inhibition of procollagen synthesis, DHB also decreases markedly the production of a collagen-binding glycoprotein (gp46) present in the ER. The results suggest that procollagen production and/or processing is needed as an early event in the differentiation pathway of myoblasts.

**T**HE terminal differentiation of myoblasts begins with their withdrawal from the cell cycle and synthesis of a new set of proteins. The mononucleated cells then undergo a series of events including cell fusion and synthesis and assembly of myofibrillar proteins to form muscle (for review see Sanwal, 1979; Pearson, 1981; Wakelam, 1985). Controlling the onset and progression of this process is a complex set of interactions between myoblasts and their environment. Some of the regulatory proteins induced within the cell have recently been identified (Wright et al., 1989), but little is known of the environmental clues or signals at the membrane that allow differentiation to proceed.

Several types of evidence point to the importance of extracellular matrix molecules as part of the signaling mechanism in myogenesis. (a) Collagen coating of tissue culture dishes enhances the differentiation of primary avian myoblasts (Hauschka and Konigsberg, 1966; de la Haba et al., 1975). (b) The addition of RGDS (arg-gly-asp-ser) peptide to myoblast cultures (which interferes with binding to several matrix molecules) or an antibody to cell surface matrix receptors (anti-CSAT) inhibits myoblast fusion and further differentiation (Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987; Menko and Boettiger, 1987). (c) The addition of exogenous fibronectin decreases fusion (Podleski et al., 1979). Thus, matrix components supplied externally influence the course of myoblast fusion.

There is also indirect evidence that myoblasts play an active role in shaping their matrix and this may be important for differentiation. Myoblasts do synthesize several different matrix molecules including collagen types I and III, and fibronectin (Chiquet et al., 1981; Mayne and Sanderson,

1985; Nusgens et al., 1986). The form of the fibronectin matrix is altered concomitant with fusion, and integrin receptors are also redistributed (Damsky et al., 1985). Treatment of cultures with transforming growth factor  $\beta$  results in inhibition of fusion which may be related to the action of the growth factor in causing increased deposition of extracellular matrix (Florini et al., 1986; Massague et al., 1986; Olson et al., 1986). Furthermore, analogues of proline that inhibit collagen formation also inhibit fusion (de la Haba and Bricker, 1981). Taken together, these observations point to an active role for the myoblasts in forming the matrix.

Our recent studies of a collagen-binding protein (gp46) have also suggested a requirement for collagen synthesis in myogenesis: mutants with decreased levels of the protein (and abnormal collagen synthesis) lack the ability to fuse (Cates et al., 1984, 1987). The recent discovery of compounds that specifically inhibit collagen hydroxylation (reviewed in Kivirikko et al., 1989) offered a different approach to this question of the need for endogenous collagen. In this report, we have examined the effects of one such inhibitor on myogenesis in culture; the results support the idea of a critical role for the newly made matrix molecules early in myogenesis.

## Materials and Methods

### Cells and Cell Culture

A highly myogenic subclone of the L6 rat skeletal myoblast cell line originally isolated by Yaffe (1968) was cultured in MEM obtained from Gibco Laboratories (Grand Island, NY). The medium was supplemented with

10% horse serum, 50  $\mu\text{g/ml}$  of gentamycin, and 16 mM glucose. Myoblast fusion was quantitated by determining the proportion of nuclei within myotubes as described by Morris and Cole (1972).

## Collagen Assays

For the determination of collagen production, cells were plated at an initial density of  $10^5$  cells per 60-mm plate. After 48 h of growth the incubation medium was replaced by fresh medium containing DHB. After 3 h of preincubation with DHB, 50  $\mu\text{Ci}$  of L-[2,3- $^3\text{H}$ ]proline (specific activity, 29.1 Ci/mmol) was added and labeling was continued for 13 h. At the end of this period, medium was collected and supplemented with a solution of protease inhibitors (0.5 mM PMSF, 2  $\mu\text{g}$  leupeptin/ml, and 1 mM EGTA). After collecting the medium, cells were washed extensively with cold PBS and lysed by adding 1 ml of hot  $1\times$  Laemmli sample buffer (Laemmli, 1970) directly on the culture plate. Lysed contents were passed three times through a 0.5-in, 27-gauge needle and cleared by centrifuging in a microfuge for 10 min at room temperature. Aliquots of the medium and cell lysates were precipitated with cold TCA (10%, final concentration). BSA was added to the cell lysates to facilitate TCA precipitation of proteins. After incubation for 30 min on ice, the precipitated material was recovered by centrifugation. Precipitates were washed three times with cold 10% TCA and then hydrolyzed in 6 N HCl in evacuated sealed tubes for 18 h at 115°C, dried using a flash evaporator, and dissolved in 0.2 M sodium citrate buffer, pH 2.2. The hydrolyzates were analyzed on an autoprogrammed single-column analyzer (Beckman Laboratories Inc., Palo Alto, CA). Peaks corresponding to proline and hydroxyproline were counted for radioactivity.

To study the synthesis of procollagens, myoblasts were preincubated with 0.2 mM ethyl-3,4-dihydroxybenzoate (DHB)<sup>1</sup> for 3 h, 25  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]proline was then added to the medium and labeling was continued for 6 h. At the end of this period, medium was collected and supplemented with a mixture of protease inhibitors. Procollagens were precipitated by adding ammonium sulfate to 30% of saturation. After overnight precipitation in the cold, precipitates were recovered by centrifugation. These were then washed twice with cold 70% ethanol. Finally, precipitates were vacuum dried and dissolved in 500  $\mu\text{l}$  of  $1\times$  Laemmli sample buffer. Procollagens were separated by SDS-PAGE as described by Laemmli (1970).

To examine incorporation of proteins into extracellular matrix, cells labeled with [ $^3\text{H}$ ]proline were rinsed and extracted with a urea-containing buffer as described (Igotz and Massague, 1986). Samples were run on SDS-PAGE and autoradiographed.

## Immunoprecipitation

Cells were seeded at an initial density of  $10^5$  per 60-mm tissue culture plate. After 48 h of growth, the desired amount of DHB was added directly to the medium. After 48 h cells were labeled with 50  $\mu\text{Ci/ml}$  of [ $^3\text{S}$ ]methionine (1,131 Ci/mmol) in methionine-free medium for 2 h without DHB. At the end of the labeling period cells were washed three times with an excess of ice-cold PBS. Cells were lysed directly on the tissue culture dish by adding 50  $\mu\text{l}$  of twofold concentrated RIPA buffer (20 mM sodium phosphate, 0.3 M NaCl, 0.2% SDS, 2% sodium deoxycholate, and 2% Triton X-100) containing the protease inhibitors. Cells were scraped with a rubber policeman, passed three times through 27-gauge needle and left on ice for 20 min to ensure proper extraction of proteins. Insoluble material was cleared by centrifugation. 200  $\mu\text{l}$  of supernatant was diluted by adding an equal volume of distilled water and supplemented with BSA to 0.5% (final concentration). The mixture was precleared with 25  $\mu\text{l}$  of rabbit antimouse-coated protein A-Sepharose and then incubated with suitably diluted antibody for 90 min on ice. A further aliquot (25  $\mu\text{l}$ ) of coated protein A-Sepharose was added and incubation continued for 30 min. The protein A-Sepharose complexes were recovered by centrifugation, washed three times with cold  $1\times$  RIPA buffer and the bound proteins recovered by heating the complexes with 200  $\mu\text{l}$  of Laemmli sample buffer in boiling water bath for 3 min. Released proteins were electrophoresed on 8.5% SDS-polyacrylamide gels. The gels were stained, destained, treated with EN<sup>3</sup>HANCE (DuPont Canada, Inc., Mississauga, Ontario), dried, and exposed to x-ray film.

## Isolation and Analysis of RNA

Total RNA was prepared by using a modification of the procedure of Chirgwin et al. (1979). Various concentrations of DHB were added to the culture

medium 48 h after plating in 100-mm plates. Either 24 (day 3) or 72 h (day 5) after DHB treatment, the cells were scraped into guanidine thiocyanate buffer (5 M guanidine thiocyanate, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 10% freshly prepared 2-mercaptoethanol), and homogenized by passing the extract through an 18-gauge needle. DNA and denatured protein were separated by centrifugation through a cesium chloride cushion (5.7 M CsCl and 25 mM sodium acetate, pH 7.5) in a rotor (TLA 100.2; Beckman Instruments, Inc.) at 100,000 rpm for 4 h. The RNA pellets were resuspended in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1% *N*-lauroylsarcosine and extracted successively with phenol/chloroform and chloroform, and the RNA was precipitated by ethanol. Northern blot analysis was done to show the specificity of the  $\alpha 1\text{R1}$  and  $\alpha 2\text{R2}$  probes. Total RNA was denatured with deionized glyoxal and size separated through a 0.9% agarose gel buffered with 10 mM phosphate (Maniatis et al., 1982). After electrophoresis, the RNA was transferred to BioTrans nylon membranes (Thomas, 1980). For quantitation, various concentrations of each RNA sample were slot blotted onto BioTrans nylon membranes using a Minifold II (Schleicher & Schuell, Inc., Keene, NH). The filters from both the Northern transfer and the slot blots were prehybridized at 42°C for 3 h and then hybridized at 42°C for 24 h in a solution containing 50% formamide,  $5\times$  sodium saline citrate (SSC) ( $1\times$  SSC = 150 mM NaCl and 15 mM sodium citrate),  $5\times$  Denhardt's solution (Denhardt, 1966), and denatured salmon sperm DNA. The hybridization solution also contained 0.1% SDS and denatured  $^{32}\text{P}$ -labeled probes. Blots were washed twice in  $2\times$  SSC containing 0.1% SDS at room temperature for 5 min each. The blots probed with the collagen probes were then washed twice in  $0.1\times$  SSC containing 0.1% SDS at 60°C for 10 min each. The myosin light chain 2 probed blots were washed twice in  $0.1\times$  SSC and 0.1% SDS at 42°C for 20 min each. Filters were exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY) with intensifying screens at  $-70^\circ\text{C}$  for various lengths of time. Various exposures were scanned by laser densitometry to quantitate the levels of RNA.

## DNA Probes

Rat collagen pro- $\alpha 1$ (I) ( $\alpha 1\text{R1}$ ) and pro- $\alpha 2$ (I) ( $\alpha 2\text{R2}$ ) cDNA clones were obtained from Dr. David Rowe, University of Connecticut Medical Center, Farmington, CT (Genovese et al., 1984). The plasmid  $\alpha 1\text{R1}$  consists of a 1.3-kb insert in the Pst I/Bam HI site of pUC18 encoding amino acids 400-846 in the triple helical domain of the pro- $\alpha 1$ (I) chain. The  $\alpha 2\text{R2}$  plasmid contains a 900-bp insert in the Pst I site of pUC18 encoding the COOH-terminal extension peptide of pro- $\alpha 2$ (I). A cDNA clone for rat myosin light chain 2 (pMLC2-18) was obtained from Dr. B. Nadal-Ginard, Harvard University, Boston, MA (Garfinkel et al., 1982). The plasmid pMLC2-18 contains a 500-bp insert in the Pst I site of pBR322 which codes for 102 amino acids of the COOH terminus.

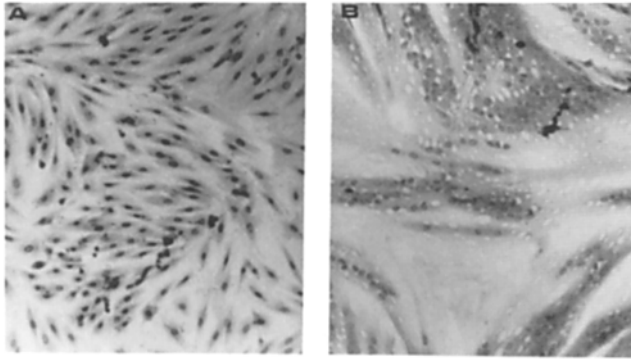
## Preparation of DNA Probes

Recombinant plasmids were propagated in *Escherichia coli* HB101. Plasmid DNA was extracted using alkaline lysis (Maniatis et al., 1982) followed by equilibrium centrifugation in cesium chloride and ethidium bromide. The plasmids were digested with the appropriate restriction endonucleases. Each preparation was subsequently size fractionated on 0.8% agarose gels. Fragments were isolated by electrophoretic transfer on to NA-45 membranes (Schleicher & Schuell, Keene, NH). DNA probes were generated using a random-primed labeling kit supplied by Promega Biotec (Madison, WI) (Feinberg and Vogelstein, 1983).

## Results

### Effect of DHB on Myoblast Fusion

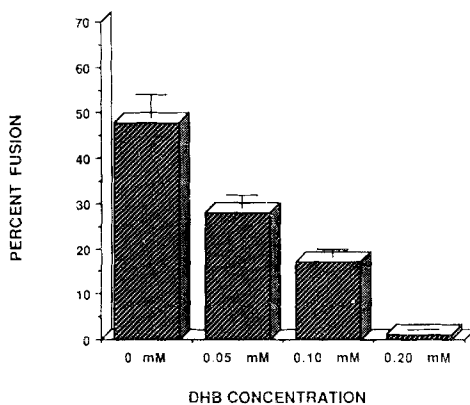
Addition of DHB to the medium after 48 h of growth inhibits myoblast fusion. The cells continue to grow until a monolayer is formed and do not show noticeable abnormalities (Fig. 1) aside from the lack of fusion. The dose dependence of fusion on DHB concentrations is shown in Fig. 2. About 40% inhibition of myotube formation was obtained with a concentration of DHB as low as 0.05 mM. Complete inhibition of fusion occurred in the presence of 0.2 mM. It should be mentioned that DHB did not have any significant effect on the rate of growth of myoblasts. Thus, when cells were plated



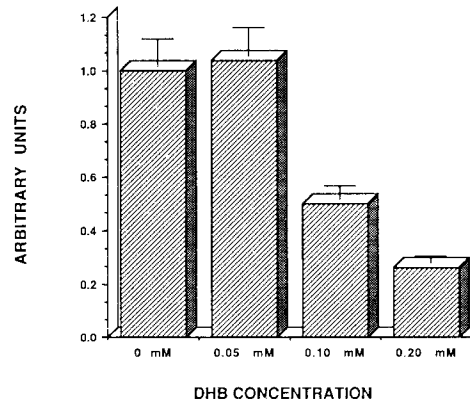
**Figure 1.** Effect of DHB on L<sub>6</sub> myoblast morphology. After 48 h of growth, myoblast cultures were placed in fresh medium with or without DHB. After a further 3 d in culture the cells were fixed, stained, and photographed. *A*, with 0.2 mM DHB; and *B*, without DHB. The apparent vacuoles in the myotubes are an artifact of the staining process.

at a density of  $10^5$  per 60-mm plate with and without the inhibitor (0.2 mM), their number increased to  $1.6 \times 10^6$  in control plates after 4 d and to  $1.4 \times 10^6$  in DHB-containing cultures. The effect of DHB was reversible. Under our culture conditions, L<sub>6</sub> myoblasts show >90% fusion 6 d after plating. When 0.2 mM DHB was included in the medium, fusion did not occur by day 7, but when DHB was withdrawn on day 6 by changing the medium, a burst of fusion resulted 24 h later.

The effect of DHB was dependent on the stage of differentiation of myoblasts. In horse serum, myoblasts rapidly divide and begin to align themselves after 72 h of growth. If DHB is added to the cultures after the alignment stage, even at a concentration of 0.4 mM fusion occurs normally. This suggests that only early events in myogenesis are influenced by DHB. The effect of DHB on myoblast fusion can be partially reversed by ascorbate. Thus, when 1 mM ascorbate was added in the medium along with 0.1 mM DHB after 48 h of growth, and the amount of fusion scored on day 5, the inhibition was ~70% in the presence of DHB alone and ~40% in the presence of both DHB and ascorbate (data not shown).



**Figure 2.** Effect of DHB on myoblast fusion. After 48 h of growth, culture medium was changed to medium containing the appropriate concentration of DHB. After a further 3 d of growth triplicate cultures were fixed and stained, and fusion was quantitated. Bar represents standard error.



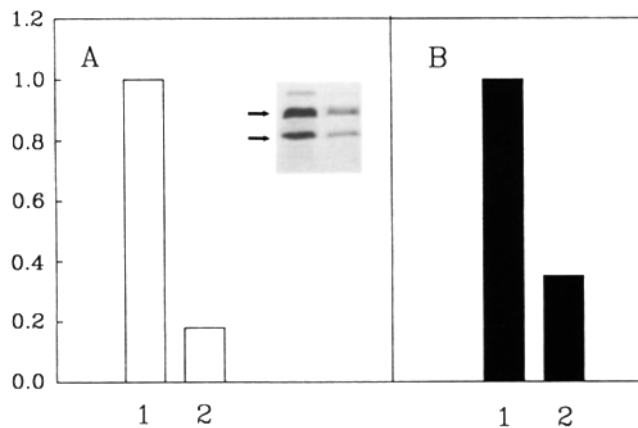
**Figure 3.** Effect of DHB on myosin light chain 2 mRNA levels. L<sub>6</sub> cells were grown for 48 h before the incubation medium was replaced by medium containing DHB. Cells were harvested 72 h after the addition of DHB and total RNA isolated. After blotting and hybridizing with the pMLC2-18 probe, levels were quantitated by laser densitometry and normalized to untreated controls.

### Effect of DHB on Muscle-specific Gene Expression

It is known that some inhibitors of myoblast differentiation affect both morphological as well as biochemical differentiation, while others selectively inhibit fusion, but allow induction of genes coding for muscle-specific proteins (Pearson, 1980; Fiszman et al., 1980). It was thus of interest to determine if DHB inhibits biochemical differentiation along with fusion. Using myosin light chain 2 as a marker of biochemical differentiation, we observed a decrease in the formation of the mRNA for MLC 2 (Fig. 3) in the presence of DHB. This inhibition required higher levels of DHB than inhibition of fusion; however, 0.05 mM DHB effectively decreased fusion but did not affect MLC mRNA (compare Figs. 2 and 3). Thus DHB did show some selectivity.

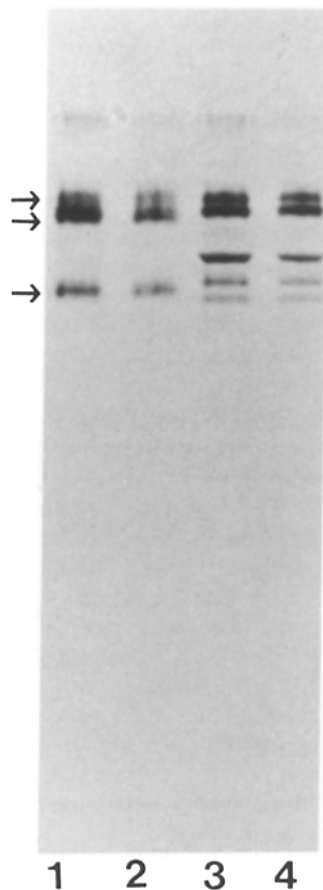
### Inhibition of Synthesis of Collagenous Proteins by DHB

It has been shown by Sasaki et al. (1987) that DHB inhibits prolyl hydroxylase, an enzyme that converts proline into hydroxyproline, and thus interferes with the synthesis of procollagens. In myoblasts, treatment with DHB drastically reduced the secretion of [<sup>3</sup>H]proline-labeled collagenous proteins into the medium (Fig. 4). This decrease was also reflected in a decrease in labeled proteins found in the urea-extractable extracellular matrix fraction (results not shown). Most of the collagenous proteins found in the growth medium were procollagen type I and procollagen type III, as judged by the migration of the bands in SDS-PAGE under reducing conditions and the susceptibility of the bands to collagenase (not shown). To ascertain further that only the procollagen types of proteins were being inhibited by DHB, we determined the levels of [<sup>3</sup>H]hydroxyproline in TCA-precipitable proteins in the medium. As shown in Fig. 4, levels of [<sup>3</sup>H]hydroxyproline were reduced by over 60% in the media of cells treated with 0.2 mM DHB. In contrast, general protein synthesis was not much affected by DHB. Thus, when cells were labeled with [<sup>3</sup>H]tryptophan or [<sup>35</sup>S]-methionine (in methionine-free medium) there was a decrease of only 10–14% in radioactivity incorporated in the

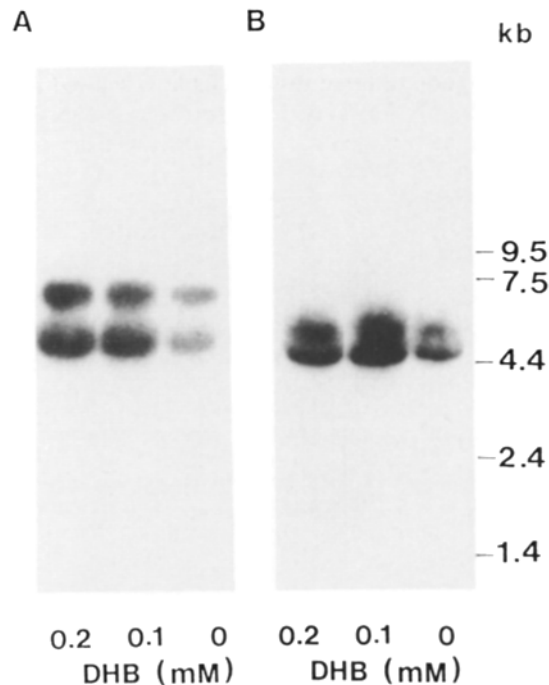


**Figure 4.** Procollagen production in the presence of DHB. *A* shows quantitation of [ $^3\text{H}$ ]proline incorporation into procollagen. An autoradiogram of the separated chains is shown on the right of the panel: upper arrow indicates the procollagen  $\alpha 1(\text{I})$  chain (which also contains small amounts of the procollagen  $\alpha 1(\text{III})$  chain), lower arrow identifies the procollagen  $\alpha 2(\text{I})$  chain. The relative levels of the procollagen chains were quantitated by laser densitometry (both chains combined) and are shown as a bar graph (bar 1) untreated; (bar 2) with 0.2 mM DHB. *B* represents the results of hydroxyproline analysis of secreted procollagen. Bars represent the relative amounts of hydroxyproline per unit cellular protein in the absence (bar 1) or presence (bar 2) of 0.2 mM DHB.

presence of 0.2 mM DHB. Furthermore, when TCA-precipitable proteins from the medium or labeled cells (in the presence or absence of 0.2 mM DHB) were electrophoresed on



**Figure 5.** Effect of ascorbic acid addition on secreted procollagen. After 48 h of growth, cultures were treated with DHB (0.1 mM), ascorbic acid (1 mM), or both together. After 24 h of treatment, the cells were labeled for 6 h with [ $^3\text{H}$ ]proline, and secreted procollagen isolated. Lane 1, no addition; lane 2, DHB; lane 3, ascorbic acid; and lane 4, DHB and ascorbic acid. The top arrow indicates procollagen  $\alpha 1(\text{III})$ , the middle arrow procollagen  $\alpha 1(\text{I})$ , and the lower arrow procollagen  $\alpha 2(\text{I})$ .



**Figure 6.** Northern blot analysis of pro- $\alpha 1(\text{I})$  and pro- $\alpha 2(\text{I})$  mRNA in  $\text{L}_6$  myoblasts treated with DHB. 20  $\mu\text{g}$  of total RNA per lane was run on a 0.9% agarose gel after denaturation with glyoxal. After transfer to nylon membranes, the blots were probed with either  $\alpha 1\text{R1}$  or  $\alpha 2\text{R2}$  probes (*A* and *B*, respectively).

SDS-polyacrylamide gels and autoradiographed, no difference in the distribution of radioactivity was discernible in major protein bands (results not shown). It would thus appear that DHB is relatively specific for the inhibition of procollagen I and III, the only two procollagens known to be present in the myoblasts (Nusgens et al., 1986). As with fusion, ascorbate was able to antagonize the inhibitory effect of DHB on the production of the procollagens. Thus, densitometric scans of the autoradiograms in Fig. 5 show that the level of each procollagen band decreases to  $\sim 40\%$  of the control level in the presence of 0.1 mM DHB, but only to  $\sim 70\%$  in the simultaneous presence of ascorbate.  $\text{L}_6$  myoblasts are not able to process procollagen I in the absence of ascorbate (Fig. 5), but processing is not obligatory for the differentiation of myoblasts. However, both production and processing of collagen are restored when ascorbate is added together with DHB in the medium.

#### Levels of Procollagen I mRNA in DHB-treated Cells

The decrease in the levels of collagenous proteins in the presence of DHB raised the question of whether this decrease could be due to a decrease in the levels of procollagen mRNAs. Probes for the  $\alpha 1(\text{I})$  and  $\alpha 2(\text{I})$  procollagen mRNAs were used to examine this question. Northern blots of total RNA hybridized with these probes revealed that each probe labeled bands of the expected size and did not cross-hybridize under the conditions used (Fig. 6, Genovese et al., 1984). When steady-state levels of  $\alpha 1(\text{I})$  and  $\alpha 2(\text{I})$  procollagen mRNAs in myoblasts were examined in the presence and absence of various concentrations of DHB, surprisingly, marked elevation in the levels of both procollagen mRNAs

occurred in the presence of 0.1 and 0.2 mM DHB (Fig. 6). Levels of  $\beta$ -actin mRNA did not change significantly (not shown). Quantitation of these results (Table I) showed that mRNAs for both procollagen type I chains were elevated to a similar degree, hence, the ratio of the two chains does not change.

### Inhibition of Biosynthesis of a Collagen-binding Protein by DHB

We had earlier (Cates et al., 1984, 1987) described the characteristics of gp46, a collagen-binding protein from myoblasts. Decreased gp46 levels in some Con A-resistant myoblasts leads to the absence of differentiation in such cells (Cates et al., 1984). Gp46 is localized in the ER (Nandan et al., 1988), and appears to be involved in the assembly of collagen I chains. Similar proteins have been described by others (Kurkinen et al., 1984; Saga et al., 1987). In view of the effect of DHB on collagen mRNA synthesis described above, it was of interest to examine whether biosynthesis of the collagen-binding protein was also affected by DHB. Using an immunoprecipitation procedure, we found that the synthesis of gp46 was inhibited by DHB in a dose-dependent manner (Fig. 7). The inhibition appears to be specific because as described earlier general protein synthesis is not affected by DHB. Inhibition of gp46 by DHB also occurred in a differentiation-defective mutant (Cates et al., 1984) that initially had only 30% of the level of gp46 compared to the wild-type cells (Fig. 7).

### Discussion

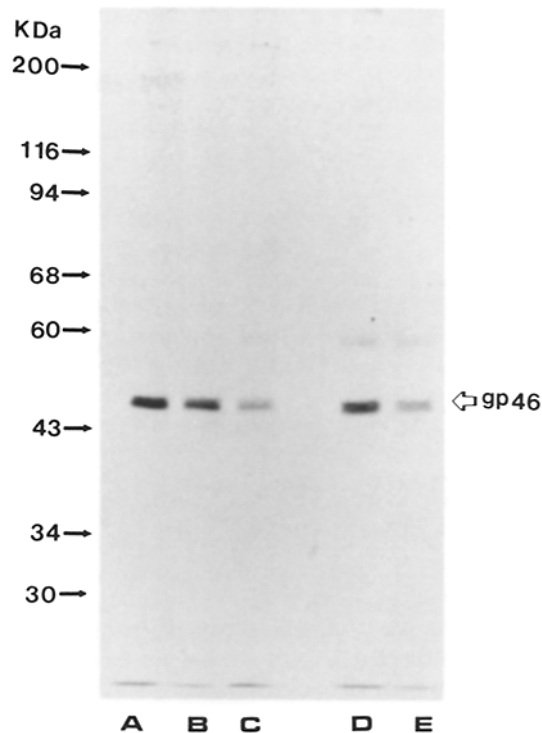
A major enzyme involved in the synthesis of procollagen is prolyl hydroxylase that converts certain proline residues to hydroxyproline during the posttranslational modification of procollagens in the ER (Kivirikko et al., 1989). This modification is essential for the subsequent folding and secretion of collagen. Recently, a class of compounds has been described which are analogues of both ascorbate and  $\alpha$ -ketoglutarate, and serve as effective competitive inhibitors of prolyl hydroxylase (Majamaa et al., 1986). One such inhibitor is the ethyl ester of 3,4-dihydroxybenzoate which not only inhibits the enzyme *in vitro*, but because of its hydrophobic nature can penetrate cells in culture and inhibit drastically the synthesis of collagen in normal and keloid human skin fibroblasts (Sasaki et al., 1987) without affecting their viability, proliferative capacity, or plating efficiency. Fur-

**Table I. Effect of DHB Treatment on mRNA Levels of Procollagen I**

| Concentration of DHB | mRNA species    |                 |
|----------------------|-----------------|-----------------|
| mM                   | $\alpha 1(I)^*$ | $\alpha 2(I)^*$ |
| 0                    | 1.0 (1.0)†      | 1.0 (1.0)†      |
| 0.1                  | 2.2 (2.0)       | 2.0 (2.2)       |
| 0.2                  | 2.4 (3.3)       | 2.1 (3.6)       |

\* The mRNA values are given as the fold increase after treatment of L6 myoblasts for 24 h with the indicated concentrations of DHB. The values were obtained by calculating the average peak area/ $\mu$ g of total RNA, measured by densitometric scans of slot blot data, and normalizing the data to control values.

† The values in parentheses represent an independent experiment.



**Figure 7.** Immunoprecipitation with anti-gp46 antibody. Extracts from L<sub>6</sub> myoblasts and the myoblast variant (C8) were immunoprecipitated with monoclonal anti-gp46. Lane A, untreated L<sub>6</sub>; lane B, L<sub>6</sub> + 0.1 mM DHB; lane C, L<sub>6</sub> + 0.3 mM DHB; lane D, untreated C8; and lane E, C8 + 0.3 mM DHB.

thermore, DHB has very little effect on the synthesis of non-collagenous proteins in fibroblasts. These properties made DHB an ideal inhibitor with which to probe the role of collagen in myoblast differentiation.

Our results reveal DHB to be a potent but reversible inhibitor of both morphological and biochemical differentiation. That this inhibition was a result of the drug's effect on collagen synthesis is supported by several lines of evidence. At levels that inhibited differentiation completely, cell growth was only slightly affected and total protein synthesis decreased only by  $\sim$ 10%. Moreover, myoblasts did not fuse even after a much longer time in culture in the presence of the drug. Furthermore, the inhibition could be partially reversed by treatment with ascorbate. (A more complete reversal might require both  $\alpha$ -ketoglutarate and ascorbate, since DHB is competitive with both.<sup>2</sup>) Hence, the inhibition of differentiation points to a role for collagen rather than a nonspecific effect.

The timing of the effect of DHB on fusion suggests that an early event in the differentiation pathway is affected. It is clear that fusion per se is not affected since addition of the drug after the alignment phase has no effect. New collagen may be needed for cell-cell recognition, adhesion, or some other initial step. This finding correlates well with the pattern of collagen synthesis in L6 myoblasts, which peaks just as cells align and drops after fusion (Nusgens et al., 1986). It is also of interest that inhibition of fusion by antibody to

2. We have found that  $\alpha$ -ketoglutarate is toxic at doses that might reverse DHB effects.

CSAT is similarly only effective at an early stage (Menko and Boettiger, 1987). Taken together, these results suggest that the status of the extracellular matrix is tested before any commitment to differentiate is made. This would also account for the inhibition of biochemical differentiation by drugs acting on the extracellular matrix. It should be noted, however, that the selective effect of DHB on fusion at low concentrations of the drug may indicate more than one site of action.

The nature of the requirement for collagen is not clear. It cannot be substituted for by adding exogenous collagen or procollagen, however, nor by plating on collagen-coated dishes (our unpublished observations). It may be that a particular arrangement or location of collagen fibers, which can only be formed using newly synthesized procollagen, is required.

Although DHB drastically decreased secretion of procollagen, levels of its mRNA actually increased. This increase may be reflected in the increased synthesis of procollagen chains which are unable to fold into triple helix and are consequently degraded without leaving the cell (Berg, 1986). In any event, the finding points to a feedback mechanism for the control of procollagen mRNA such that secreted (pro) collagen decreases its own mRNA level. This type of mechanism has been postulated previously (Rowe and Schwartz, 1983; Muller et al., 1986), but was not evident in DHB-treated fibroblasts (Sasaki et al., 1987).

The reduction of gp46 (the collagen-binding protein in the ER (Cates et al., 1984, 1987), which is probably involved in collagen assembly) after drug treatment, is interesting because it suggests secondary effects of alterations in collagen production. Levels of the variety of proteins that are specifically required for procollagen synthesis and secretion may also be regulated by feedback mechanisms. Further experiments will be necessary to determine at what level the regulation of gp46 occurs as well as the generality of the phenomenon.

The extracellular matrix is an extremely important part of the cell's environment and is well known to influence differentiation and development in many cell types (Hay, 1981). Collagen is a basic part of the matrix and our results support earlier work suggesting its importance to myogenic differentiation. In addition, a critical role for the myoblasts in modifying or creating the matrix is indicated by the requirement for the actual synthesis of procollagen. Further work will be necessary to define its role.

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