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ARTICLES

Extracellular Matrix Is Required for Skeletal Muscle Differentiation But Not Myogenin Expression

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Abstract Skeletal muscle cells are a useful model for studying cell differentiation. Muscle cell differentiation is marked by myoblast proliferation followed by progressive fusion to form large multinucleated myotubes that synthesize muscle-specific proteins and contract spontaneously. The molecular analysis of myogenesis has advanced with the identification of several myogenic regulatory factors, including myod1, myd, and myogenin. These factors regulate each other's expression and that of muscle-specific proteins such as the acetylcholine receptor and acetylcholinesterase (AChE). In order to investigate the role of extracellular matrix (ECM) in myogenesis we have cultured myoblasts (C_2C_{12}) in the presence or absence of an exogenous ECM (Matrigel). In addition, we have induced differentiation of myoblasts in the presence or absence of Matrigel and/or chlorate, a specific inhibitor of proteoglycan sulfation. Our results indicated that the formation of fused myotubes and expression of AChE was stimulated by Matrigel. Treatment of myoblasts induced to differentiate with chlorate resulted in an inhibition of cell fusion and AChE activity. Chlorate treatment was also found to inhibit the deposition and assembly of ECM components such fibronectin and laminin. The expression of myogenin mRNA was observed when myoblasts were induced to differentiate, but was unaffected by the presence of Matrigel or by culture of the cells in the presence of chlorate. These results suggest that the expression of myogenin is independent of the presence of ECM, but that the presence of ECM is essential for the formation of myotubes and the expression of later muscle-specific gene products.

Key words: skeletal muscle differentiation, chlorate, myogenin, matrigel, proteoglycans

The fusion of mononucleated myoblast cells to form multinucleated myotubes is a central event in skeletal muscle development. The terminal differentiation of myoblasts begins with their withdrawal from the cell cycle and synthesis of a new set of proteins. The mononucleated cells undergo a series of events including cell fusion and the synthesis and assembly of myofibrillar proteins to form muscle. 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 cells have been identified. Thus, when myogenesis begins, myogenic regulatory genes belonging to the MyoD family [Emerson, 1990; Olson, 1990; Weintraub, 1993] which includes MyoD1 [Davis et al., 1987], myogenin [Wright et al., 1989], Myf5 [Braun et al., 1989], and Mrf4 [Braun et al., 1990] are activated. It has been shown that the activation of myogenin is under the control of fibroblast growth factor (FGF) [Brunetti and Goldfine, 1990].

In addition several lines of evidence demonstrate the importance of extracellular matrix (ECM) molecules as part of the signaling mechanism in myogenesis [Buck and Horwitz, 1987]. It has been shown that an inhibitor of collagen synthesis [Nandan et al., 1990; Saitoh et al., 1992] inhibits expression of myogenin affecting differentiation of cultured myoblasts. The addition of RGDS (arg-gly-asp-ser) peptide or antibodies to integrin receptors to myoblast cultures [Menko and Boettiger, 1987] also inhibits fusion and further differentiation. Thus, interactions of myoblasts with ECM appear to influence the course of myoblast fusion.

Abbreviations used: AChE, acetylcholinesterase; CPC, cetylpyridinium chloride; ECM, extracellular matrix; FGF, fibroblast growth factor; SDS-PAGE, polyacrylamide gel electrophoresis.

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Several ECM components and their receptors have been identified and purified, which has allowed for an examination of ECM function at the molecular level [Adams and Watt, 1993]. Proteoglycans are major components of ECM and evidence suggests that they play important roles in cell proliferation [Fritze et al., 1985], cell matrix adhesion [Saunders and Bernfield, 1988], binding of growth factors [Yayon et al., 1991], and overall ECM architecture [Höök et al., 1984]. One interesting and potentially important property of proteoglycans is their ability to bind other matrix constituents such as laminin, fibronectin, thrombospondin, and collagen [Koda et al., 1985; Saunders and Bernfield, 1988; Sun et al., 1989; Carey and Stahl, 1990]. Many of these interactions are mediated by the highly sulfated glycosaminoglycan chains of the proteoglycans [Kjellén and Lindahl, 1991]. In skeletal muscle the expression of some of these ECM components is regulated during differentiation. It has been shown, using a mouse derived skeletal muscle cell line (C_2C_{12}), that the synthesis of laminin [Olwin and Hall, 1985] and at least two proteoglycans, decorin [Brandan et al., 1991] and glypican [Campos et al., 1993; Brandan et al., 1995], is up-regulated during myogenesis.

While these findings indicate that ECM is vital for myogenesis, the exact mechanisms through which it modulates this process remain to be established. To elucidate the role of ECM in myogenesis we investigated the effect of chlorate, a specific inhibitor of proteoglycan sulfation, on the expression of myogenin, a skeletal muscle specific regulatory protein, and acetylcholinesterase (AChE), a skeletal muscle differentiation marker. We report that chlorate inhibits ECM deposition, resulting in a decrease of myoblast fusion and the time course of appearance of AChE. These effects were abolished by the addition of Matrigel, an exogenous ECM. In contrast to these above results, neither chlorate nor Matrigel had any affect on the expression of myogenin. Our observations suggest that the presence of ECM is essential for myogenesis, but is not required for induction of myogenic regulatory genes in the initial phase of muscle cell differentiation.

METHODS

Cell Culture

The mouse skeletal muscle cell line C_2C_{12} [Yaffe and Saxel, 1977] was cultured as described [Brandan et al., 1991]. Cells were plated at a density of 6,000 per square centimeter in growth medium, which consisted of Dulbecco's modified Eagle's (DME) medium with 1 g/l glucose supplemented with 20% fetal calf serum (Sigma Chemical Co., St. Louis, MO) and 0.5% chick embryo extract (GIBCO-BRL, Gaithersburg, MD). On day 3 after plating, when the cells were about 80% confluent, the cultures were switched to differentiation medium, which consisted of DME with 1 g/l glucose supplemented with 2% horse serum (Sigma Chemical Co., St. Louis, MO). The medium was changed daily, thereafter.

Sodium chlorate (final concentration 30 mM) was added to the cultures at the time of plating (day 0) or on day 3 after plating. Matrigel (Collaborative Research, Bedford, MA) was added to the cultures on day 0 or day 3. The medium was removed and 30 ul/cm^2 of Matrigel (diluted 1:5 in DME-Ham's F12) was added below and over (day 0) or only over the cells (day 3) and allowed to polymerize for 2 hours at 37°C. Fresh growth or differentiation medium was then added to the plates containing the polymerized gel.

Labeling of the Cultures and Analysis of Proteoglycans

35 mm^2 dishes containing C_2C_{12} cells at different stages of differentiation were radiolabeled by incubation in medium containing 100 uCi [^{35}S]-Na_2SO_4 (carrier free; New England Nuclear, Boston, MA) for 18 h. The cells were lysed with 0.5% Triton X-100 in PBS (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.5) and removed with a cell scraper. Incorporation of [^{35}S]-Na_2SO_4 into macromolecules was measured by cetyl pyridinium chloride (CPC) precipitation [Brandan and Inestrosa, 1987]. Incorporation of radioactive precursors for protein ([^{35}S]-methionine, 6,889 Ci/mmol; New England Nuclear, Boston, MA) and phosphoproteins ([^{32}P]-phosphorous, 9,000 Ci/mmol; New England Nuclear, Boston, MA) was evaluated by incubating differentiated myotubes in medium containing the radioactive precursors for 18 hours in the presence or absence of 30 mM sodium chlorate. The incorporation of radioactive sulfate was nearly linear for the entire incubation period. At the end of the incubation period the medium was removed, the cells were washed twice with cold PBS and proteins were precipitated with 12% cold TCA. The precipitates were washed three times with cold

TCA, resuspended in 1.0 ml of 1.0 N NaOH, and transferred to a scintillation vial which contained 0.25 ml of 4 N HCl. Ten ml of scintillation cocktail was added and the radioactivity was determined in a Beckman LC 100 scintillation counter.

Immunoanalysis

Cells to be immunostained were grown on glass coverslips. The medium was removed and the plates were rinsed with PBS. For staining of ECM proteins the cells were incubated with primary antibodies for 1 h at 4°C before fixation. Anti-laminin (GIBCO-BRL, Gaithersburg, MD), anti-fibronectin (Sigma Chemical Co., St. Louis, MO), anti-chondroitin sulfate (Sigma Chemical Co., St. Louis, MO), and anti-α-tubulin (Sigma Chemical Co., St. Louis, MO) antibodies were used according to the manufacturer's recommendations. After rinsing, the cells were fixed with 3% paraformaldehyde for 30 min at room temperature. The cells were rinsed with Blotto (20 mM Tris-HCl, pH 7,4, 0,1 M NaCl, 5% Carnation nonfat dry milk) and then incubated for 30 minutes at room temperature with affinity purified fluorescein-conjugated secondary antibodies (Sigma Chemical Co., St. Louis, MO) diluted 1:20 in Blotto. After rinsing, the cover slips were mounted on glass slides. Fluorescein was visualized using a Nikon Diaphot inverted microscope equipped for epifluorescence.

Control and chlorate treated cells were washed three times with PBS containing 1.0 M NaCl to remove any bound fibronectin and incubated with DME medium alone for 18 h. The incubation media and material associated with the cells were obtained and aliquots equivalent to 1.25 ug of DNA were electrophoresed on a 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) as described [Carlson and Wight, 1987]. After electrophoresis, proteins were transferred to nitrocellulose, incubated in Blotto (20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 5% nonfat dry milk) at room temperature for 1 h to block nonspecific binding, and incubated with rabbit anti fibronectin antiserum (Sigma Chemical Co., St. Louis, MO), diluted 1/100 in Blotto or monoclonal anti α-tubulin (Sigma Chemical Co., St. Louis, MO), diluted 1/500 in Blotto. The bound antibody was visualized with affinity-purified alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO).

AChE Activity

AChE activity was determined by the method of Ellman, as described previously [Inestrosa et al., 1982; Brandan et al., 1985]. AChE was assayed in the presence of 0.1 mM ethopropazine, a specific inhibitor of butyrylcholinesterases. The activity was expressed as units/ug of DNA.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from cell cultures at the indicated times by guanidium thiocyanate/phenol/chloroform extraction and isopropanol precipitation as described [Chomczynski and Sacchi, 1987] using RNAzol B (Biotecx Laboratories, INC. Houston, TX). RNA samples (10 ug/lane) were electrophoresed through 1.0% agarose/formaldehyde gels, transferred to Nylon membranes (Sigma Chemical Co., St. Louis, MO), and hybridized with a random-primed ^{32}P-labeled cDNA probe (GIBCO-BRL, Gaithersburg, MD) for myogenin. A plasmid, EMSVscribe, containing the human myogenin cDNA (a generous gift of Dr. Eric N. Olson, University of Texas, USA) was used to generate a cDNA probe corresponding to the most highly conserved region between the rat and human sequences. This was obtained by PCR amplification using primers corresponding to bases 719–739 and 31–51 of the human sequence. Hybridization was performed in 1.0 M NaCl, 1% sodium dodecyl sulfate, 10% dextran sulfate, 100 ug/ml denaturated salmon testes DNA at 65°C overnight. Hybridized membranes were washed twice at 65°C in 0.2X SSC, 0.1% sodium dodecyl sulfate for 5 minutes and exposed to Kodak X-ray film.

DNA and Protein Determination

DNA was determined in aliquots of cell extracts as described [Labarca and Paigen, 1980]. Protein was determined as described [Bradford, 1976].

RESULTS

Chlorate Inhibits ECM Deposition in Skeletal Muscle Cells

To study the effect of sodium chlorate on ECM deposition, skeletal muscles cells were induced to differentiate in the presence of the inhibitor. Data presented in Table I indicated that sulfation of cell and matrix associated proteoglycans was inhibited more than 90% com-

TABLE I. Effect of Chlorate on the Incorporation of [^{32}P]-Phosphorous, [^{35}S]-Methionine, and [^{35}S]-Sulfate Into Macromolecules Synthesized by Differentiated Myotubes*

	[^{32}P]-Phosphorous		[^{35}S]-Methionine		[^{35}S]-Sulfate	
	c.p.m./ug DNA	Total	c.p.m./ug DNA	Total	c.p.m./ug DNA	Total
Control	100.0 ± 5.2	100.0 ± 5.2[a]	100.0 ± 6.3	100.0 ± 6.3[c]	100.0 ± 7.6	100.0 ± 7.6[e]
Chlorate	99.7 ± 6.5	81.0 ± 4.8[b]	100.1 ± 5.7	82.1 ± 4.2[d]	7.6 ± 3.4	5.6 ± 2.3[f]
Matrigel + Chlorate	N.D.	N.D.	N.D.	N.D.	3.7 ± 1.9	5.5 ± 2.3[g]

*C_2C_{12} cells were induced to differentiate on day 3 in the presence or absence of chlorate and/or Matrigel. On day 9 the cells were incubated with the radioactive precursors and the incorporation evaluated as explained in Methods. Total incorporation corresponds to the total incorporated precursors per culture. Values for specific and total incorporation for each precursor were: [^{32}P]-phosphorous, 595 and 19,861; [^{35}S]-methionine, 42,558 and 1,420,850; [^{35}S]-sulfate, 11,921 and 397,922 cpm/ug DNA and cpm/plate, respectively. The values in the table are expressed as percents of control values and correspond to the means ± standard deviations of five different plates.
[a,b,c,d]$P < 0.005$ (Student's t test).
[e,f,g]$P < 0.001$ (Student's t test).

TABLE II. Amount of DNA in C_2C_{12} Cells Induced to Differentiate Under Different Conditions*

	Total (ug)	%
Control	33.31 ± 1.88[a]	100.0
Chlorate	26.98 ± 1.46[b]	80.9
Matrigel	43.64 ± 3.33[c]	131.0
Matrigel + Chlorate	35.21 ± 2.28[d]	105.7

*Cells were induced to differentiate under the indicated conditions on day 3. On day 9 total DNA was determined as described in Methods. The amounts indicated correspond to the mean value ± standard deviation of five 100 mm plates.
[a,b]$P < 0.05$ (Student's t test).
[a,c]$P < 0.04$ (Student's t test).
[a,d]$P < 0.1$ (Student's t test).

pared to control cultures. In contrast, incorporation of [^{35}S]-methionine and [^{32}P]-phosphorous was not affected at all. A 20% inhibition on the total incorporation (per culture) of [^{35}S]-methionine and [^{32}P]-phosphorous was observed by chlorate treatment, probably due to an inhibitory effect of chlorate on cell proliferation (see below Table II). Thus, chlorate treatment resulted in a specific inhibition of proteoglycan sulfation, with a small effect on total protein synthesis.

Previous studies have shown that inhibition of proteoglycan synthesis results in an inhibition of ECM deposition in vascular smooth muscle and Schwann cell cultures [Carey et al., 1987; Hamati et al., 1989]. We used indirect immunofluorescence staining with specific antibodies to determine the effect of inhibition of proteoglycan sulfation on ECM protein deposition in skeletal muscle cultures. Control cultures stained with anti-laminin antibodies showed bright and specific fibrillar staining of the ECM over the differentiated myotubes (Fig. 1A). As shown in Figure 1B, chlorate treatment strongly decreased the staining with this antibody. Figure 1C shows staining with anti-fibronectin antibodies. A well defined fibrillar network within the ECM was seen in control cells. Chlorate treatment altered this pattern, producing a less dense fibrillar pattern (Fig. 1D). In Figure 1E the staining pattern obtained with anti-chondroitin sulfate antibodies is shown. Again, a fibrillar network was seen in control cultures, that was essentially absent in chlorate treated cultures (Fig. 1F). In contrast, no effect of chlorate (Fig. 1H) was seen on the staining of α-tubulin compared to untreated cells (Fig. 1G). Next, the amount of fibronectin synthesized by control and chlorate treated cells (day 9 of differentiation) was evaluated by Western blot analysis. As is shown in Figure 2A, chlorate did not significantly affect the synthesis of fibronectin, although a slight increase in the amount of fibronectin in the incubation media of chlorate treated cells compared to control cells was observed; concomitant a small decrease of the cell-associated fibronectin in chlorate treated cells was detected when compared to control cells. Furthermore to evaluate if chlorate had any toxic effect on the synthesis of proteins, the level of α-tubulin was determined in control and treated cells. As is shown in Figure 2B chlorate did not affect the level of expression of this cytoskeletal protein. These results demonstrated that inhibition of proteoglycan sulfation leads to a decreased deposition of both proteoglycans and glycoproteins into ECM, resulting in a less extensive and less organized matrix.

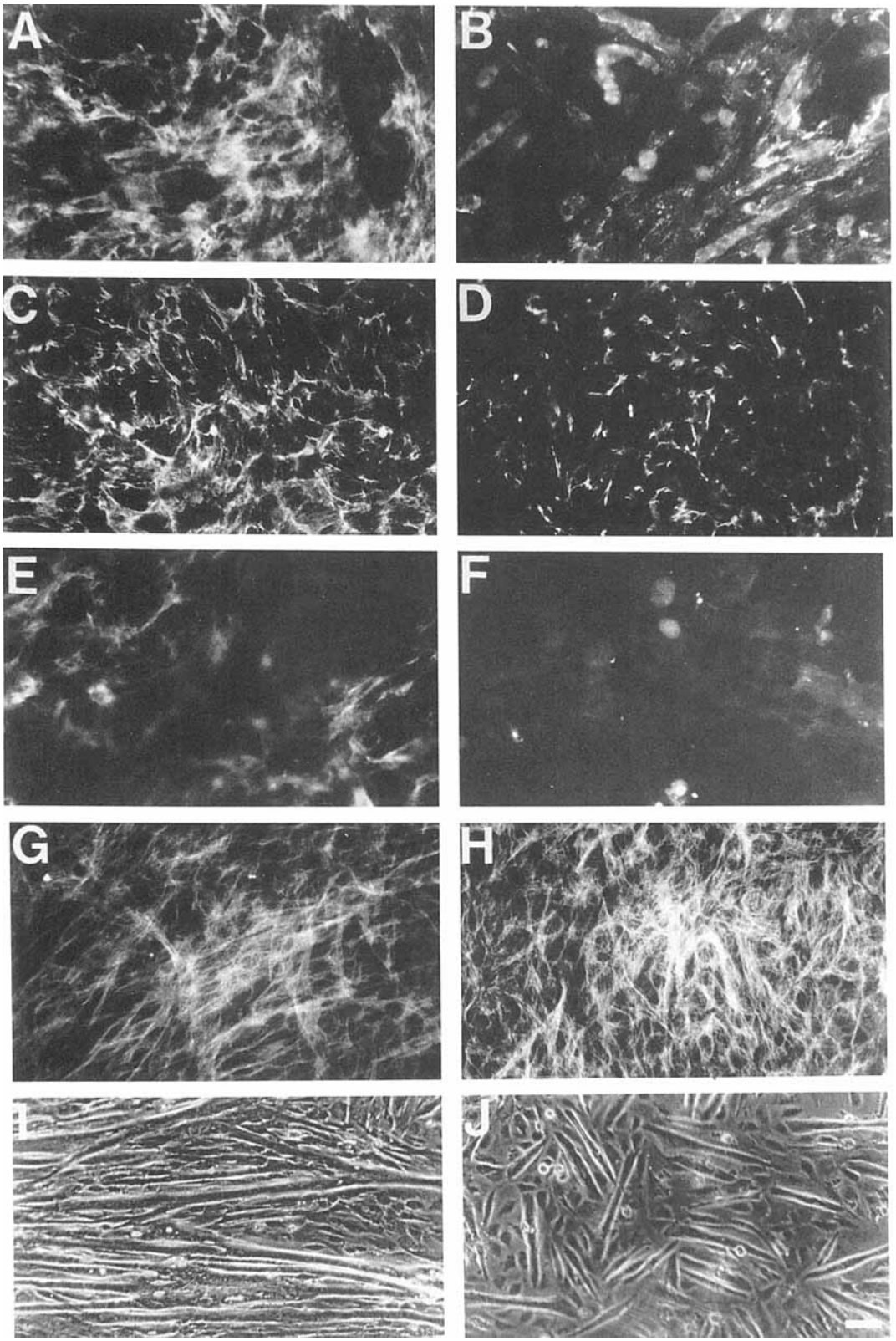

Fig. 1. Effect of chlorate on accumulation of ECM proteins. C_2C_{12} cells were induced to differentiate on day 3 of culture without (**A, C, E, G, and I**) or with (**B, D, F, H, and J**) 30 mM chlorate. After 6 days (day 9 of culture) the cells were processed for indirect immunofluorescent staining with anti-laminin antibodies (A and B), anti-fibronectin antibodies (C and D), anti-chondroitin sulfate antibodies (E and F), anti-α-tubulin antibodies (G and H), or phase contrast (I and J). Fields were photographed and printed under identical conditions. For panels the scale bar corresponds to 25 μm except for panels E–F, which correspond 12.5 μm.

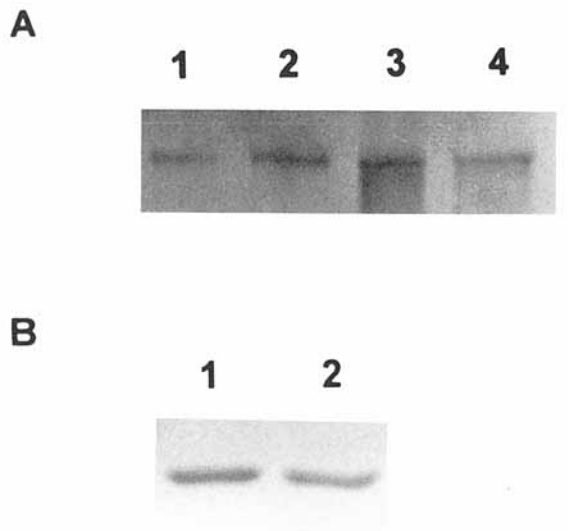

Fig. 2. Effect of chlorate on the synthesis of fibronectin and α-tubulin. C_2C_{12} cells were induced to differentiate in the presence or absence of 30 mM chlorate. On day 9 the cells were washed with three times with PBS containing 1.0 M NaCl and incubated in DME in the absence of serum for 18 hours. The medium was collected and the material associated with the cells removed with the aid of a cell scraper. Aliquots equivalent in DNA content were separated by SDS-PAGE as explained in Methods to determine fibronectin (**A**) and α-tubulin (**B**). A: *Lane 1,* incubation media from control cells; *lane 2,* incubation media from chlorate treated cells; *lane 3,* cell associated material from control cells; *lane 4,* cell associated material from chlorate treated cells. B: *Lane 1,* cell associated material from control cells; *lane 2,* cell associated material from chlorate treated cells. The molecular weights determined for fibronectin and α-tubulin were 220 and 57 KdA respectively.

Effects of Chlorate on Skeletal Muscle Cell Fusion

An important aspect of skeletal muscle cell differentiation is the transformation of myoblasts to elongated, multinucleated myotubes. To examine the effect of chlorate on the ability of the cultured skeletal muscle cells to carry out this morphological transformation we counted the number of nuclei inside recognizable myotubes. As shown in Figure 3, in day 9 differentiated control cultures (bar A), nearly all the nuclei were present within myotubes. As expected, few multinucleated cells were observed when the cells were maintained in the growth medium without inducing differentiation (Fig. 3, bar F). Chlorate treatment resulted in a significant inhibition of myoblast fusion, as indicated by a striking decrease in the percentage of nuclei present in myotubes. This effect was time dependent. Maximal inhibition was observed when chlorate was added on day 3 of culture, i.e., at the beginning of the induction of differentiation (Fig. 3, bar B). Addition of chlorate on successive days thereafter resulted in a progressively smaller degree of inhibition (Fig. 3, bars C–E). The effect of chlorate on cell proliferation was evaluated by determining the amount of DNA at day 9 of cell culture. Table II indicates that chlorate affected cell proliferation by 20% compared to untreated cells. As shown in Fig. 1J, chlorate treatment decreased the number and the length of fused myotubes compared to control cultures (Fig. 1I).

Effects of Chlorate on AChE Expression

The results presented above demonstrated that inhibition of proteoglycan sulfation affected the fusion and morphology of skeletal muscle cells. To determine whether chlorate treatment had any effect on other aspects of the phenotypic differentiation of the skeletal muscle cells we evaluated the appearance of a specific skeletal muscle marker, AChE. As shown in Figure 4A, the expression of AChE was strongly inhibited by the presence of chlorate during differentiation. As was observed for the effect of chlorate on myoblast fusion, the extent of inhibi-

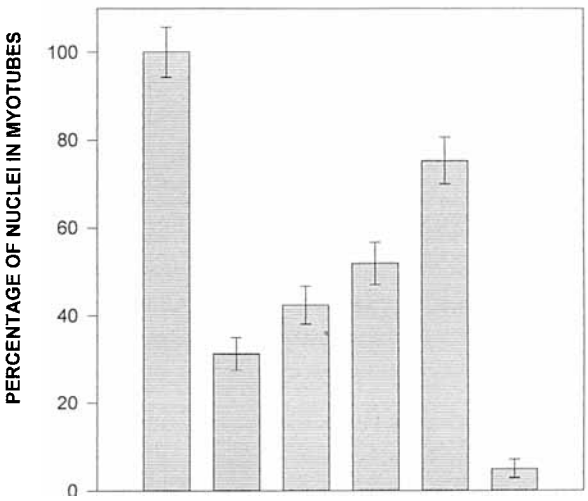

Fig. 3. Effect of chlorate on the fusion of skeletal muscle cells induced to differentiate. C_2C_{12} cells were induced to differentiate on day 3. Thirty mM sodium chlorate was added to the cultures on day 3 (**B**), 4 (**C**), 5 (**D**) or 6 (**E**), or not added (**A**). The number of nuclei inside myotubes was determined on day 9. The bar labeled **F** corresponds to undifferentiated muscle cells. Cells were fixed in 3% paraformaldehyde and stained with Giemsa. Nuclei were counted in 10 random fields. The values correspond to the means ± standard deviations of five plates.

tion was time dependent. When chlorate was added to the cultures on day 3, i.e., when differentiation was initiated, an 80% inhibition of AChE activity was seen on day 11, however when chlorate was added at later times (day 9) only a 20% inhibition was observed. The strong inhibitory effect of chlorate on AChE activity cannot be explained by the effect observed on cell proliferation (Table II).

We also measured the time course of appearance of AChE activity in the absence or presence of chlorate (Fig. 4B). In the control cultures detectable AChE activity was observed on day 7, and maximal AChE activity was observed on days 9–11. In chlorate treated cultures the time course of appearance of AChE activity was similar, but reached a level that was only 30% of the activity in control cultures. Additional control experiments demonstrated that chlorate (up to 50 mM) had no detectable direct effect on AChE activity (data not shown). Because the AChE activity expressed by these cells correspond to several different molecular forms present in different cellular compartments [Inestrosa et al., 1982], we examined the effect of chlorate on AChE activity present in conditioned medium, detergent extracts, and detergent insoluble fractions of cells induced to differentiate in the presence or absence of chlorate. The same percentage of inhibition was found in all the fractions tested (data not shown).

Exogenous ECM Prevents the Inhibitory Effect of Chlorate on Skeletal Muscle Differentiation

As described above, one of the effects of inhibition of proteoglycan sulfation by chlorate is an inhibition of ECM deposition by skeletal muscle cells. If the lack of this ECM is responsible for the effects of chlorate on skeletal muscle cell morphology, fusion, and expression of AChE, it should be possible to prevent these effects by providing the cells with exogenous ECM. For these experiments we used Matrigel, a basement membrane-like ECM obtained from the mouse EHS sarcoma. To examine the effects of exogenous ECM on skeletal muscle cell differentiation, the cells were induced to differentiate in the presence or absence of Matrigel. The cells grown with Matrigel fused more extensively and more rapidly than the control cells. The cultures grown with Matrigel plus chlorate were indistinguishable from those grown with Matrigel alone (data not shown). As is shown in Table II, Matrigel had an effect on proliferation (30%) but this effect was not observed in cells grown in Matrigel and chlorate, probably due to the inhibitory effect on proliferation of the latter.

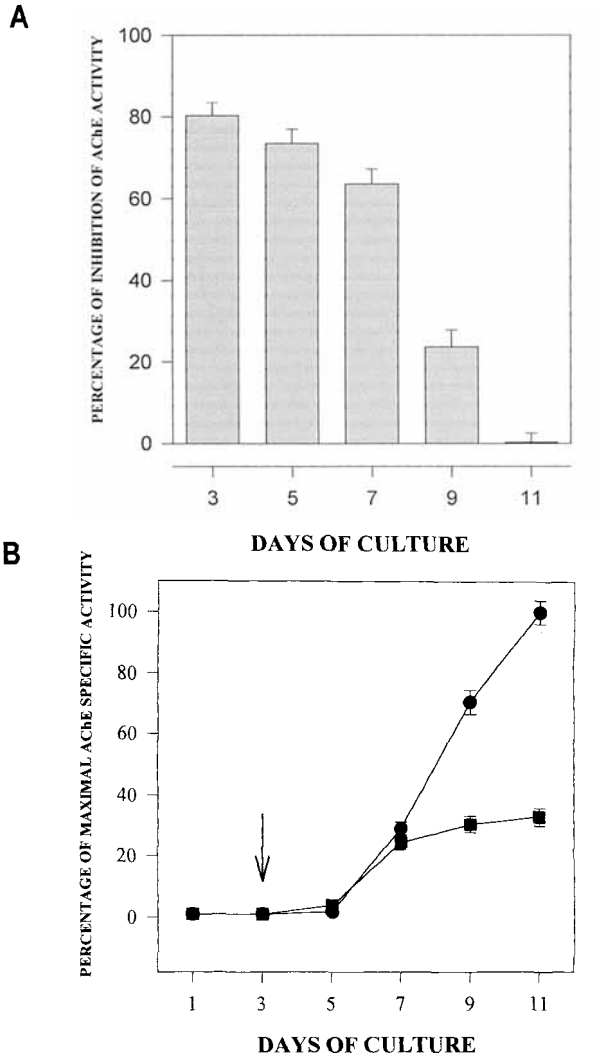

Fig. 4. Effect of chlorate on the expression of AChE. **Panel A:** Plates containing myoblasts were induced to differentiate on day 3. On days 3, 5, 7, 9, and 11, 30 mM chlorate was added to the cultures and total cell associated AChE activity determined on day 11 of culture. For the value corresponding to day 11, chlorate was added just before processing the samples. The values are expressed as percentages of inhibition of AChE activity compared to day 11. **Panel B:** Cells were incubated beginning on day 3 in the absence (*circles*) or presence (*squares*) of 30 mM chlorate. On the indicated days total cell associated AChE activity was determined. The values are expressed as percentages of the maximal control values. Each value corresponds to the mean ± standard deviation of five plates. The maximal control value corresponds to 1.41 ΔOD_{430nm}/ug of DNA. The *arrow* indicates when differentiation was triggered.

Figure 5 shows the effect of the exogenous ECM on the expression of AChE. In cultures

grown with Matrigel, AChE activity was expressed earlier than in cultures induced to differentiate on plastic. However, the maximal activity achieved was similar (Fig. 5). In cells treated with chlorate in the presence of Matrigel, the expression of AChE activity was similar to cultures treated with Matrigel alone. As expected, chlorate produced a strong inhibition of sulfation by skeletal muscle cells cultured in the presence of Matrigel (Table I). These results indicated that exogenous ECM accelerated the appearance of myotubes and the expression of a skeletal muscle specific marker. In addition, the exogenous ECM blocked the inhibitory effect of chlorate treatment of the ability of the cells to differentiate. The most likely explanation for these findings is that ECM contact is required for skeletal muscle differentiation, and that the inhibitory effect of chlorate derives from its effect on ECM deposition.

Expression of Myogenin Is Independent of the Presence or Absence of the ECM

It is well established that skeletal muscle differentiation, including expression of late skeletal muscle specific markers such as AChE, is dependent of the expression of early myogenic regulators such as myogenin. Myogenin is a DNA binding transcriptional regulatory protein. Myogenin expression induced very soon after differentiation is triggered by removal of growth factors. To determine whether the expression of myogenin was dependent on the presence of ECM, we compared the levels of myogenin mRNA in cells grown in the absence or presence of chlorate. As shown in Fig. 6A, cells triggered to differentiate in the presence or absence of chlorate exhibited the same level of myogenin mRNA, as determined by Northern blot analysis. This lack of effect of chlorate on myogenin mRNA expression was not a result of a delayed effect of chlorate treatment. The same result was obtained if the cells were incubated in the presence of chlorate beginning on day 0 and triggered to differentiate on day 3 (Fig. 6B), or if the cells are maintained in chlorate for 7 days after triggering differentiation (data not shown). Moreover, the effect of chlorate on sulfation was rapid. As shown in Table III, 50% of maximal inhibition of sulfation was obtained after 4 hours of chlorate treatment in myoblasts triggered to differentiate. The same extent of inhibition was seen in cultures of undifferentiated myoblasts (data not shown).

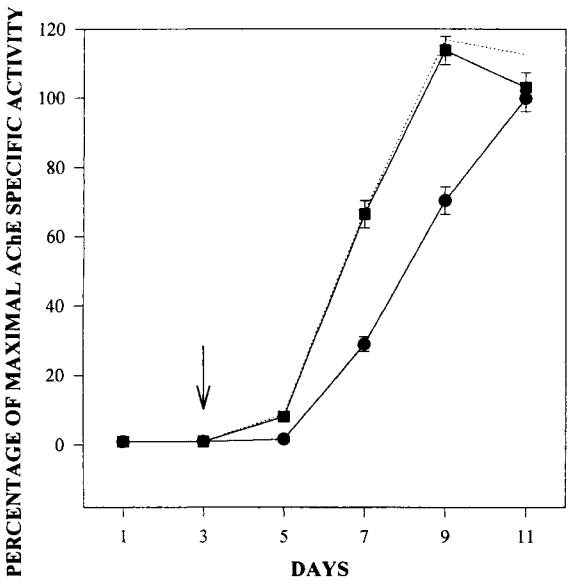

Fig. 5. Effect of exogenous ECM on the expression of AChE. C_2C_{12} cells were induced to differentiate on day 3 (*arrow*) and cultured in differentiation medium in the absence (*circles*) or presence (*squares*) of Matrigel, or with Matrigel and 30 mM chlorate (*dashed line*). At the indicated times cell associated AChE was determined. Each point corresponds to the mean ± standard deviation of five plates. The values are expressed as percentage of maximal control value determined on day 11 and it corresponds to 1.48 ΔOD_{430nm}/ug of DNA.

To determine whether chlorate treatment inhibited the deposition of ECM in the skeletal muscle cultures under conditions used to measure myogenin mRNA induction, the cultures were examined by indirect immunofluorescence with anti-fibronectin antibodies. As shown in Figure 7, in the presence of chlorate (panels B and C), deposition of fibronectin into the ECM was poor compared to control plates (Fig. 7A).

We also measured the levels of myogenin mRNA in cells induced to differentiate in the presence of exogenous ECM. As shown in Figure 8A, the cells expressed the same level of myogenin mRNA independent of the presence or absence of exogenous ECM. The same result was found even when the cells were exposed to the exogenous ECM beginning on day 0 and were induced to differentiate on day 3 (Fig. 8B). Note that myogenin mRNA was only seen when differentiation was triggered by removal of growth factors. Myogenin mRNA was not induced by the presence of the exogenous matrix. These results indicated that the expression of myogenin was independent of the presence or absence of ECM.

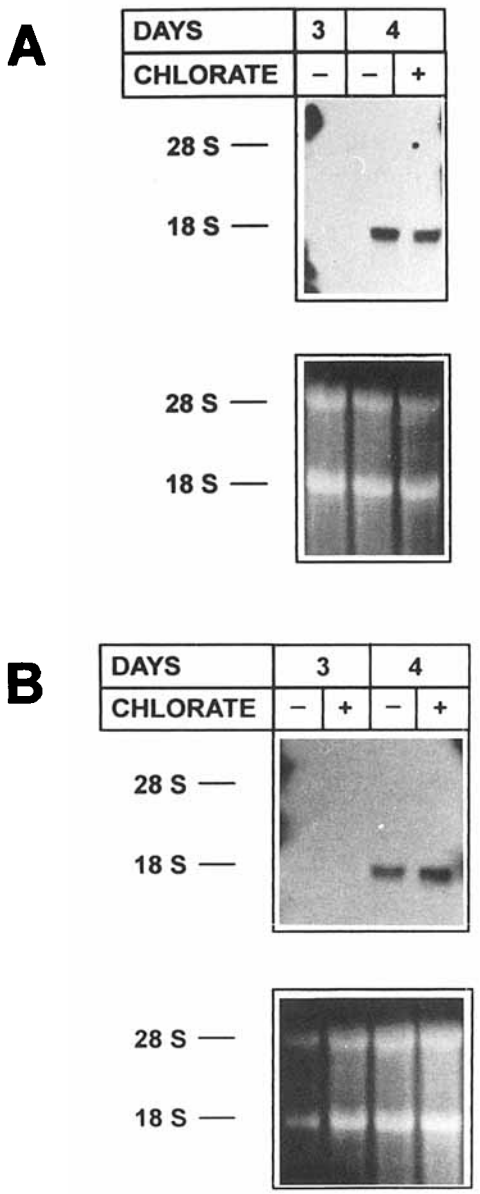

Fig. 6. Chlorate does not affect the expression of myogenin induced during differentiation. **Panel A:** C_2C_{12} cells were induced to differentiate on day 3 in the absence or presence of 30 mM chlorate. **Panel B:** C_2C_{12} cells were grown beginning on day 0 in the absence or presence of chlorate; on day 3 the cells were induced to differentiate. Total RNA was isolated on the indicated days of culture and Northern blot analysis was carried out as described in Methods. The *top half* of each panel shows the myogenin Northern blot autoradiogram; the *bottom half* shows the ethidium bromide stained gel. Positions of 28 and 18s rRNA bands are indicated.

DISCUSSION

In this paper we provide experimental evidence which demonstrates that the ECM is required in order to achieve a successful terminal differentiation of skeletal muscle cells. However, ECM is not required for the activation of the expression of the myogenic regulatory gene, myogenin. In the studies reported here we used sodium chlorate, a specific inhibitor of proteoglycan sulfation, to examine the effects of altered proteoglycan synthesis on the differentiation of skeletal muscle cells. Biochemical analysis showed that sodium chlorate inhibited sulfate incorporation by 90%. Our studies indicated that inhibition of proteoglycan sulfation had profound effects on some aspects of the differentiation of skeletal muscle cells. We observed inhibition of myoblast fusion and AChE activity. These effects appeared to be due to decreased deposition of ECM proteins that occurred in chlorate treated cultures. C_2C_{12} cells grown in the presence of exogenous ECM (Matrigel) in chlorate containing medium exhibited normal fusion and expressed normal levels of AChE. We have found the same results using a Matrigel pre-washed with high salt to remove growth factors associated to it [unpublished results].

TABLE III. Time Course of the Effect of Chlorate on Myoblasts Induced to Differentiate*

Time (hours)	[^{35}S]-Sulfate incorporated (% of control)
0	100.0
4	48.8
8	39.1
12	24.6
24	19.9

*Myoblasts were induced to differentiate on day 3 and immediately incubated with 30 mM chlorate in the presence of [^{35}S]-sulfate. At the indicated times incorporation of radioactivity into cell associated proteoglycans was determined as described in Methods. The values correspond to the average of two plates measured in triplicate.

Several observations suggest that the effects we observed on cell differentiation are not attributable to toxicity of chlorate. First, phosphorylation of proteins was unaltered by the treatment, suggesting that the level of intracellular ATP was probably unaffected by the inhibitor. Also, the incorporation of methionine into newly synthesized proteins was unaffected. Moreover, chlorate had a smaller effect on AChE activity when added to the cells on subsequent days, i.e., during differentiation. In contrast and no effect of chlorate treatment on the level of α-tubulin were observed. Finally, the inhibitory effect of chlorate on AChE activity was totally reverted by the addition of exogenous matrix. The same

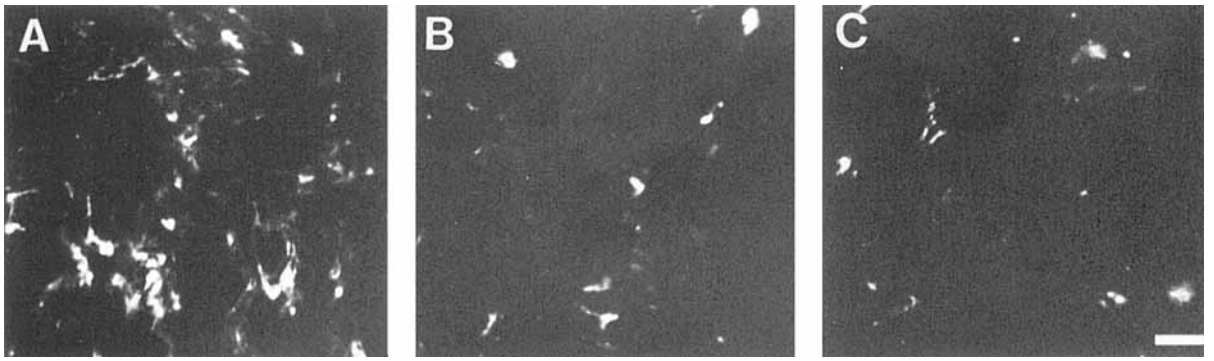

Fig. 7. Chlorate affects ECM accumulation in myoblasts. C_2C_{12} cells were grown in the absence (**A**) or presence of 30 mM chlorate beginning on day 3 (**B**) or day 0 (**C**). Cells were induced to differentiate on day 3 of culture and after 24 hours the cells were processed for indirect immunofluorescent staining with anti-fibronectin antibodies. Fields were photographed and printed under identical conditions. Scale bar, 25 μm.

inhibitory effect of chlorate on sulfation was observed in cells incubated with chlorate in the presence or absence of Matrigel, suggesting that the intracellular level of the inhibitor was the same in both experimental conditions. The effect of chlorate on sulfation is a very rapid process. Fifty percent of maximal inhibition was observed after 4 h of the treatment. This rapid effect is consistent with the known action of chlorate i.e., inhibition of ATP-sulfurylase, a key reaction in the synthesis of PAPS [Burnell and Roy, 1978; Baeuerle and Huttner, 1986; Humphries and Silbert, 1988] which has a high rate of turnover [Urrea et al., 1992].

Our results indicated that an adequate supply of sulfated proteoglycans is necessary for deposition of skeletal muscle cell ECM. Extracellular matrix assembly is a highly cooperative process [Yurchenco et al., 1986] and proteoglycans are essential probably because of their ability to form complexes with other ECM molecules [Hardingham and Fosang, 1992]. Heparan sulfate proteoglycans bind, via their sulfated glycosaminoglycans chains, to ECM proteins such as fibronectin, laminin, collagen, and thrombospondin [Koda et al., 1985; Saunders and Bernfield, 1988; Sun et al., 1989; Carey and Stahl, 1990]. Because of their multiple side chains, proteoglycans serve as multivalent cross-linkers in the ECM [Hamati et al., 1989]. One can expect that inhibition of sulfation of the glycosaminoglycans chains would have a strong effect on the deposition and organization of the ECM, as we have shown in these studies.

Our data also indicated that ECM is not required for activating the myogenic regulatory gene myogenin in the initial phase of muscle cell differentiation. It has been shown that the expression of myogenin is under the control of FGF [Lathrop et al., 1985; Spizz et al., 1986]. Thus, FGF treatment of myoblast inhibits myogenin gene transcription [Brunetti and Goldfine, 1990]. Our results contrast with results obtained using two different inhibitors of collagen synthesis, which were reported to prevent myogenesis in mouse skeletal muscle cells; under these conditions myogenin was not expressed [Saitoh et al., 1992] and with the observation that inhibition of expression of desmin blocks the myogenic differentiation program, inhibiting myoblast fusion. In this case, the normal pattern of expression of MyoD and/or myogenin is also blocked [Li et al., 1994]. Our results clearly indicate that the expression of myogenin does not require the presence of the ECM, thus, when cells were induced to differentiate in the presence of exogenous ECM or chlorate we found that the level and time of expression of this myogenic regulatory product was unaffected. In contrast the expression of AChE and myoblast fusion was affected. When cells induced to differentiate in the presence of Matrigel, we observed that AChE activity was expressed earlier than in control cells, whereas in cells induced to differentiate in presence of chlorate the AChE activity and myoblast cell fusion was inhibited. Therefore the expression of myogenin do not require the presence of an organized ECM. Furthermore the expression of myogenin can be completely abolished by FGF even in the presence of Matrigel [unpublished observation]. These results suggest that an organized ECM is required for late events of myogenesis. The above observations are consistent with the

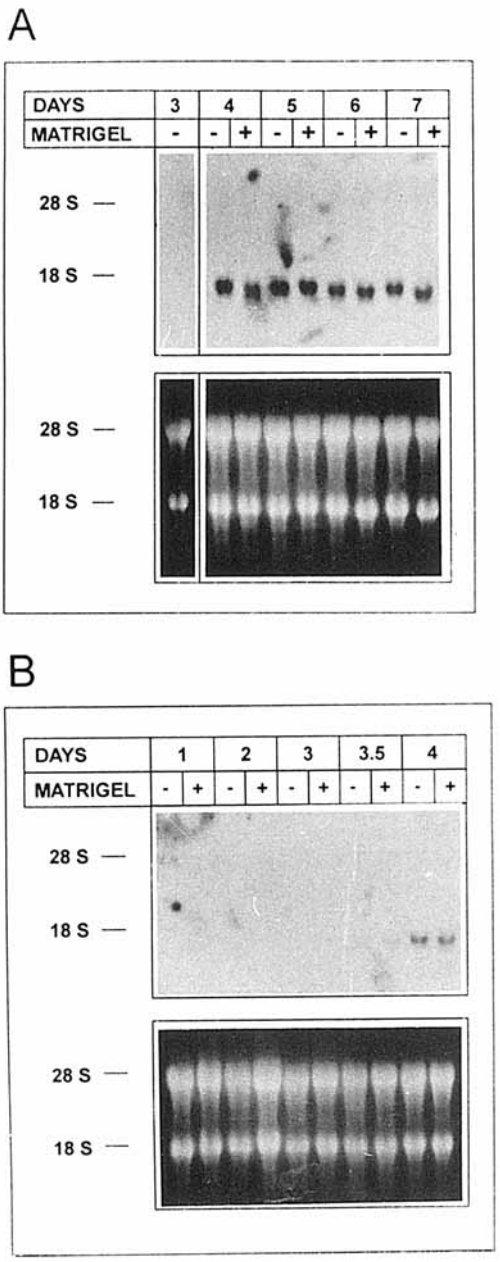

Fig. 8. Exogenous ECM does not affect the expression of myogenin induced during differentiation. **Panel A:** C_2C_{12} cells were induced to differentiate on day 3 in the absence or presence of Matrigel, as indicated. **Panel B:** C_2C_{12} cells were grown beginning on day 0 in the absence or presence of Matrigel; on day 3 the cells were induced to differentiate. Total RNA was isolated 24 hours after induction of differentiation and Northern blot analysis was carried out as described in Methods.

idea that in vivo the expression of FGF receptor decrease during embryonic development whereas an active synthesis of ECM is occurring [Olwin and Hauschka, 1990; Moore et al., 1991].

Exogenously added Matrigel was able to block the chlorate inhibition of differentiation, as evaluated by cell fusion and expression of AChE. Matrigel is produced by a mouse sarcoma. The C_2C_{12} cells used in this study are derived from mouse leg muscle satellite cells. An interesting question, is whether an ECM derived from other sources would be sufficient to block the effects produced by chlorate. Several cell associated receptors for ECM molecules have been described [Buck and Horwitz, 1987; Bernfield et al., 1992; Lin and Bissell, 1993], among them integrins and cell surface proteoglycans [Adams and Watt, 1993]. Because we observed that in the presence of Matrigel and chlorate the fusion and expression of AChE was not affected, it is less likely that cell surface proteoglycans are participating directly as receptors for ECM, since under these experimental conditions the proteoglycans are severely undersulfated and probably will not interact with the ECM constituents [Brauer et al., 1990]. Which of the ligands present in the ECM are sufficient to establish normal skeletal muscle cell fusion and expression of specific markers? We do not know, but an attractive candidate is laminin, which is very abundant in Matrigel [Kleinman et al., 1982; Terranova et al., 1986]. Laminin expression is also regulated during skeletal muscle differentiation [Olwin and Hall, 1985]. Furthermore, it has been shown that the laminin receptor integrin $\alpha 7\beta 1$, is developmentally regulated in skeletal muscle [Collo et al., 1993]. Another attractive question is how these extracellular signals are transmitted across the cell membrane via the transmembrane receptors which recognize ECM molecules. The changes in these receptors, triggered by ligand binding, may cause a rearrangement of the cytoskeletal network and stimulate intracellular signal transduction cascades leading to changes in gene expression and, therefore, the state of the cell differentiation [Adams and Watt, 1993; Clark and Brugge, 1995].

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