Myogenic Differentiation Triggered by Antisense Acidic Fibroblast Growth Factor RNA

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Acidic fibroblast growth factor (FGF) and related family members regulate differentiation in organisms as diverse as *Xenopus laevis* and mammals. We utilized a well-characterized model of myogenic development to directly assess the importance of endogenously produced FGF in controlling differentiation. A role for endogenous FGF is suggested by the previous finding that acidic and basic FGF abundance in cultured myocytes decreases during differentiation. In this study we inhibited the endogenous production of FGF in murine Sol 8 myoblasts by using antisense RNA and observed precocious myogenic differentiation. Exogenously supplied acidic FGF rescues this phenotype. Further results suggest that the effect of FGF on myogenic differentiation is mediated in part through inhibition of myogenin expression. These results demonstrate a direct role for endogenously synthesized growth factors in regulating myogenesis and provide support for a general role for related proteins in mammalian development.

Acidic and basic fibroblast growth factors (FGFs) are members of the heparin-binding growth factor family of proteins with mitogenic, angiogenic, mesoderm-inducing, and neuroectoderm-modulating activities (2, 4). Acidic and basic FGFs are found in a broad range of organisms and tissues and are thought to have important (though incompletely defined) roles in embryogenesis and differentiation in organisms as diverse as *Xenopus laevis* and mammals (13, 14, 21). With respect to myogenic differentiation, exogenously administered FGFs suppress differentiation of myoblasts in culture (3, 6, 11, 15, 16, 23). Recently, acidic and basic FGF mRNA and protein were demonstrated to be expressed in cultured skeletal myoblasts (12, 19, 24), and expression was down-regulated during myogenesis in skeletal muscle and in cultured myocytes (1, 12, 19). A cause-and-effect relationship between decreases in either acidic or basic FGF expression and the occurrence of myogenic differentiation has yet to be established. The present study was designed to explore the role of acidic FGF in regulating skeletal myogenesis. A well-characterized model of myogenic development that utilizes a cultured murine myocyte cell line, Sol 8 (18), was examined to determine whether inhibition of acidic FGF synthesis by antisense RNA affects the differentiation program of these cells.

The results of this study demonstrate that the expression of antisense acidic FGF RNA in myoblasts results in a significant reduction in cellular FGF activity and triggers precocious myogenic differentiation. Exogenously supplied acidic FGF rescues this phenotype. Furthermore, the results suggest that the effect of FGF on myogenesis may be mediated in part through the suppression of myogenin expression, as the level of myogenin mRNA was increased in proliferating myoblasts expressing antisense acidic FGF mRNA and normal expression was restored with the addition of exogenous acidic FGF. Taken together, these results demonstrate a direct role for endogenously synthesized FGFs in regulating myogenesis and provide support for a general role for related proteins in mammalian development.

MATERIALS AND METHODS

Expression vectors, transfection, and selection of stable cell lines. An expression vector containing the cytomegalovirus immediate-early promoter and the full-length murine acidic FGF cDNA coding sequence in the antisense orientation (pCMV/ASaFGF) was constructed (Fig. 1). The eukaryotic expression vector used was pBC12/CMV/IL2 (a gift of Bryan Cullen, Duke University) (7), which contains the cytomegalovirus promoter, a human interleukin 2 coding sequence, and rat preproinsulin II genomic sequences (which do not code for a functional peptide but enhance expression of sequences inserted upstream). A *HindIII*-*Bam*HI fragment containing human interleukin 2 sequences was removed, and the vector was blunt-end ligated to produce the control vector pCMV/Ø. The antisense murine acidic FGF expression vector pCMV/ASaFGF was prepared by blunt-end ligation of an *XhoI*-*XbaI* fragment from murine acidic FGF clone A1 (a gift of Gail Martin, University of California, San Francisco) (13) containing the full-length murine acidic FGF coding sequence into the pCMV/Ø vector. The orientation of the insert was confirmed by restriction endonuclease mapping.

The antisense and control vectors were separately cotransfected with a plasmid containing the *neo* gene into Sol 8 murine myoblasts (18) as follows. Cells maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 20% fetal bovine serum (FBS; Hazleton) were grown to 30% confluence, washed twice with phosphate-buffered saline (PBS), and incubated overnight in Opti-MEM (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) containing 10 µg of Lipofectin reagent (Bethesda Research Laboratories, Inc.) per ml and 2.5 µg of plasmid DNA per ml (10:1 expression vector/*neo* vector ratio). After 16 h, the lipofection solution was removed, and the cells were cultured in selection medium consisting of DMEM, 20% FBS, and 0.8 mg of G418 (Geneticin; Gibco) per ml. G418-resistant cells were selected by this method, and stably transfected cell populations were isolated. In one series of transfections, clonal populations of cells were isolated by harvesting single colonies of cells from the original transfection plate and then expanding these colonies in selection medium. In another series of transfections, colonies of cells were harvested and expanded in pools. These transfected cell populations will hereafter be referred to as

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FIG. 1. Construction and expression of antisense acidic FGF and control expression vectors. (A) Schematic representation of the expression vector pCMV/ASaFGF. The human interleukin 2 coding sequences were removed from pBC12/CMV/IL2, which contains the cytomegalovirus immediate-early promoter (CMV) and rat genomic preproinsulin II sequences. Next, either the vector was blunt-end ligated to produce the control vector pCMV/Ø or the full-length murine acidic FGF coding sequence was inserted in the antisense orientation (pCMV/ASaFGF) by blunt-end ligation. The orientation of the insert was confirmed by restriction endonuclease mapping. UTR, untranslated region. (B) RNase protection analysis of antisense acidic FGF (aFGF) vector-transfected cells. Sol 8 cells were stably transfected with the acidic FGF antisense expression vector, pCMV/ASaFGF, or with the control vector, pCMV/Ø. Total RNA (30 µg) from representative monoclonal populations of pCMV/Ø- and pCMV/ASaFGF-transfected myoblasts cultured in DMEM containing 33% FBS and pCMV/Ø- and pCMV/ASaFGF-transfected myotubes cultured in 5% horse serum was analyzed by RNase protection analysis for expression of the antisense acidic FGF RNA transcript. The radiolabeled sense probe is 220 nt in length, and hybridization to the mRNA protects a 200-nt fragment. (C) Similar to panel B, except that the RNA was from three polyclonal populations of Sol 8 myoblasts transfected with the antisense acidic FGF expression vector pCMV/ASaFGF. Total RNA (20 µg) from each cell line (cultured in DMEM–33% FBS and harvested as confluent myoblasts beginning to form myotubes) was analyzed as for panel B. Parallel samples from two polyclonal control-transfected cell lines contained no detectable antisense transcript. The autoradiogram was digitally scanned, and the image was edited with Photoshop software (Adobe) on a Macintosh Centris 650 computer (Apple) to include lane markers and labels.

monoclonal and polyclonal cell lines, respectively, in reference to their origins and modes of isolation.

In order to maximally suppress differentiation and ensure log-phase growth in both the antisense vector- and control vector-transfected cell lines, transfected populations were routinely maintained in DMEM containing 33% FBS following their initial selection. Subsequent experiments with the transfected cell lines were conducted under these conditions, except for the differentiation time course experiments, which were conducted in DMEM containing 10% FBS (see below).

Extraction of RNA from cells and RNase protection analysis. Six monoclonal and 22 polyclonal pCMV/ASaFGF-transfected cell lines were screened for the presence of the acidic FGF antisense transcript by RNase protection analysis (17). RNA was extracted from cells by the method of Chirgwin et al. (5). A [³²P]UTP-labeled sense acidic FGF transcript (220 nucleotides [nt]) was prepared by T3 RNA polymerase transcription of a *Bam*HI-digested murine acidic FGF plasmid (described above). Hybridization and RNase digestion result in the protection of 200 nt of the probe RNA by the antisense RNA transcript synthesized by pCMV/ASaFGF-transfected cells. Hybridization was carried out in a solution containing 50% formamide, 4 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.7), 40 mM NaCl, and 0.1 mM EDTA at 42°C for 16 h. The hybridization reaction mixture was digested with a mixture of RNases A (10 µg/ml) and T₁ (450 U/ml) for 10 min at 37°C and subjected to denaturing polyacrylamide gel electrophoresis and autoradiography. Two monoclonal and three polyclonal populations expressed abundant antisense transcripts and were characterized in comparison with one monoclonal and two polyclonal cell lines that had been transfected with a control plasmid lacking FGF sequences (pCMVØ).

To detect myogenin mRNA, a ³²P-labeled myogenin antisense transcript (200 nt) was prepared by T3 RNA polymerase transcription of an *Eco*RI-digested subclone of a murine

myogenin plasmid (a gift of Eric Olson, Baylor College of Medicine) (8). Hybridization and RNase digestion result in the protection of a 140-nt probe fragment which migrates electrophoretically as a doublet. To assess α-skeletal actin expression, a ³²P-labeled α-skeletal actin antisense transcript (220 nt) was prepared by T3 RNA polymerase transcription of an *Eco*RI-digested murine α-skeletal actin plasmid (a gift of Margaret Buckingham, Institut Pasteur) (20). Hybridization and RNase digestion result in the protection of a 60-nt probe fragment.

To assess the effect of antisense expression on immunoreactive acidic FGF content, lysates were prepared from confluent pCMV/Ø- and pCMV/ASaFGF-transfected myoblasts, partially purified by heparin affinity chromatography (24), and subjected to Western (immunoblot) analysis using antisera against recombinant human acidic FGF (a gift of Michael Jaye, Rorer Biotechnology). Despite the known immunoreactivity between this antiserum and murine acidic FGF in mouse tissues, immunoreactive murine acidic FGF could not be detected in lysates from 5 × 10⁸ control or antisense acidic FGF-expressing myoblasts (sensitivity of detection, <100 pg). The acidic FGF content of Sol 8 myoblasts may be less than that of other myogenic cell lines such as rat L6 (24) and may account for the effectiveness of the antisense mRNA in influencing differentiation.

The functional consequences of antisense acidic FGF RNA

expression were then assessed. The content of biologically active FGF in cells was measured by the mitogenic response of BALB/c 3T3 fibroblasts (CCL 163; American Type Culture Collection) to heparin affinity-purified extracts (24) prepared from pCMV/Ø- and pCMV/ASaFGF-transfected myoblasts. Subconfluent myoblasts cultured in DMEM containing 33% FBS were chilled on ice, rinsed in ice-cold PBS, and harvested by scraping in a small volume of 2 M NaCl–10 mM Tris HCl (pH 7.4) containing the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (0.1 mM), and phenanthroline (0.1 µg/ml). Harvested cells were diluted with Tris buffer containing these protease inhibitors in order to decrease the NaCl concentration to 0.5 M and were homogenized in a tight-fitting Thomas homogenizer. The nondenaturing, zwitterionic detergent CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate} was added to a 0.1% final concentration, and extracts were incubated briefly on ice. Membrane debris was then pelleted by centrifugation at $12,000 \times g$ for 30 min at 4°C. The total protein concentration was then determined by colorimetric assay (Bio-Rad), and equal amounts of total cell protein (about 750 µg per cell line) were applied to a heparin-agarose affinity column (Bio-Rad) and allowed to bind for 2 h at 4°C. After being washed with 0.5 M NaCl–10 mM Tris HCl–0.1% CHAPS, the columns were eluted with 2 M NaCl–10 mM Tris HCl–0.1% CHAPS. Aliquots of each eluate were then added in quadruplicate to confluent BALB/c 3T3 cells seeded in 96-well plates. The cells were grown in DMEM containing 10% calf serum and then withdrawn from serum stimulation by incubation in medium containing 0.5% calf serum for >24 h prior to the addition of Sol 8 extracts. Following incubation of 3T3 fibroblasts with Sol 8 extracts for 16 h, 1 µCi of [³H]thymidine (410 Ci/mmol; Amersham) was added per well, and the cells were incubated for an additional 2 h. Medium was then removed, and cells were lysed with 0.5 N NaOH. Lysates were collected by suction onto glass microfiber filters and washed extensively. The filters were dried and counted in a liquid scintillation counter, and data were expressed as disintegrations per minute per well.

CK assay. pCMV/Ø- and pCMV/ASaFGF-transfected myoblasts grown in DMEM containing 33% FBS were harvested with trypsin and counted with either a Coulter Counter (Coulter Electronics, Hialeah, Fla.) or a hemacytometer. Cells were then seeded at low density (10^4 cells per cm²) in DMEM containing 10% FBS in 24-well culture plates, and the cells from three to four wells were harvested daily over a 4- to 5-day period starting 24 h after seeding. The medium was replaced every other day. Groups of wells were washed twice with PBS and harvested by scraping and homogenizing in a solution containing 25 mM Tris HCl (pH 7.4), 1 mM dithiothreitol, and 0.1% Nonidet P-40. Samples were stored at –70°C until assayed; neither the presence of nonionic detergent (Nonidet P-40) nor frozen storage affected creatine kinase (CK) activity in cell extracts or purified preparations of CK (Boehringer). Total CK activity was measured through the generation of NADPH in a combined enzymatic reaction dependent upon CK by using a spectrophotometric assay as previously described (9). The reaction rate of each sample was calculated by measuring the time-dependent change in the A_{340} of the reaction mixture and normalized to the amount of total cell protein (22) in each sample.

RESULTS

Antisense acidic FGF expression accelerates myoblast differentiation. Sol 8 myoblasts transfected with an expression vector containing a murine acidic FGF minigene in the anti-

sense orientation (Fig. 1A) expressed the acidic FGF antisense RNA transcript as determined by RNase protection assay (Fig. 1B and C). The transcript was present in both antisense vector-transfected myoblasts and antisense vector-transfected myotubes but was not present in control myoblasts or myotubes. The phenotype of cells expressing antisense acidic FGF was characterized by the ability of these cells to express differentiated functions under conditions of mitogen exposure that support continued growth and prevent or delay differentiation in control cells. No significant differences between the proliferative rate of pCMV/Ø-transfected cells and that of pCMV/ASaFGF-transfected cells were observed during log-phase growth in DMEM containing 33% FBS (data not shown). pCMV/ASaFGF-transfected cells did form occasional foci of multinucleated, fusiform cells resembling myotubes, but the majority of the cells maintained a normal morphology in high concentrations of FBS. All of the cells continued to proliferate after being plated in DMEM–10% FBS, although some of the antisense cell lines proliferated more slowly than when plated in DMEM–33% FBS and were observed to form large, multinucleated myotubes more readily and at lower cell densities than control cells (data not shown).

Antisense nucleic acids interfere with the function of mRNA target sequences by a variety of possible mechanisms. These include targeting the mRNA for premature degradation, interfering with nuclear processing and transport to the cytoplasm, and inhibition of translation. Multiple attempts to quantify the levels of acidic FGF mRNA in transfected Sol 8 cells by RNase protection analysis were inconclusive because of the very low abundance of this transcript in these cells. Efforts to quantify the gene product by immunoblotting were similarly unsuccessful (see Materials and Methods). Therefore, in order to demonstrate the functional consequences of antisense expression in terms of FGF activity, extracts of antisense and control cell lines were partially purified by heparin affinity chromatography and analyzed for heparin-binding growth factor activity as measured by their mitogenic stimulation of BALB/c 3T3 fibroblasts. This assay is commonly used to measure the content of biologically active FGF in cells and tissue. The heparin-binding growth factor activity of these cells eluting from heparin-agarose at 2 M NaCl consists predominantly (and perhaps exclusively) of acidic and basic FGFs. The amounts of biologically active FGF in four antisense acidic FGF RNA-expressing cell lines were decreased compared with those in control transfected cells and averaged half of those contained in two control cell lines (Fig. 2). The results of these experiments show that expression of antisense acidic FGF mRNA is associated with a significant decrease in total cellular FGF activity, thus demonstrating the ability of an antisense RNA strategy to inhibit FGF production.

As a marker of differentiation, the time courses of induction of muscle CK enzymatic activity in antisense and control cells growing in the presence of 10% FBS were measured. Two monoclonal cell lines expressing antisense acidic FGF were compared with one monoclonal control cell line (Fig. 3A), and three polyclonal lines expressing antisense acidic FGF were compared with two polyclonal control cell lines (Fig. 3B). In both cases, the cell lines expressing the antisense transcript displayed a more rapid accumulation of CK activity over time than did control cells. Similar results were obtained when the differentiation medium was DMEM–20% FBS (data not shown). The time course of differentiation of the pCMV/ASaFGF-transfected cells in DMEM–10% FBS was comparable to that of either pCMV/Ø-transfected cells or non-transfected Sol 8 cells cultured in differentiation medium (DMEM–5% horse serum; data not shown). The maximum

FIG. 2. Biologically active FGF in extracts of transfected myoblasts. Four different cell lines (one monoclonal and three polyclonal) expressing the antisense acidic FGF transcript and two cell lines transfected with the control expression vector were cultured at subconfluent density in medium supplemented with 33% FBS. Cells were harvested, and lysates were partially purified by heparin affinity chromatography. Aliquots of the fraction eluted with 2 M NaCl were added in quadruplicate to 96-well plates containing quiescent BALB/c 3T3 fibroblasts and incubated for 16 h prior to the addition of [³H]thymidine. After being labeled for 2 h, the cells were lysed with NaOH and filtered through glass microfiber filters, and the level of incorporation of [³H]thymidine into DNA was determined by scintillation counting. The activity in each of the four antisense construct-expressing cell lines was less than that in each of the two control-transfected cell lines. The bars represent the pooled means $\pm$ standard deviations for all wells for the antisense cell lines ($n = 4$, four wells each) and the control cell lines ($n = 2$, four wells each).

CK activities measured differed according to the clonal origins of the cell lines. Monoclonal cell lines displayed nearly twice the maximum CK specific activity of polyclonal cell lines. In both series of experiments, the induction of CK activity was accompanied by morphologic changes characteristic of differentiation (i.e., fusion to form multinucleated myotubes). In addition, the induction of CK activity in this system correlated well with an independent marker of biochemical differentiation, the expression of acetylcholine receptor as measured by the binding of radiolabeled α -bungarotoxin (unpublished observation).

Antisense acidic FGF mRNA stimulates the expression of myogenin in myoblasts. The ability of the pCMV/ASaFGF-transfected myoblasts to express differentiation-specific gene products such as muscle CK in mitogen-rich media suggested that these cells had been released from control mechanisms that normally prevent such expression under growth conditions. Previous studies demonstrate that FGFs negatively regulate the expression of the myogenic determination gene MyoD1 (25) and suppress activation of the muscle gene program even in the presence of constitutive expression of myogenic determination genes (26). If endogenously produced acidic FGF is normally responsible (either directly or indirectly) for suppressing myogenin transcription in proliferating Sol 8 myoblasts, then inhibition of acidic FGF expression should be permissive for myogenin expression despite maintenance in mitogen-rich media. No myogenin mRNA was detectable in control-transfected myoblasts grown in DMEM containing 33% FBS, while antisense acidic FGF vector-transfected myoblasts expressed high levels of myogenin mRNA (Fig. 4). Antisense vector- and control-transfected cell lines accumulate myogenin mRNA to similar extents (comparable to the amounts accumulated by nontransfected Sol 8 cells) when induced to differentiate in mitogen-poor media (DMEM containing 5% horse serum). These data demonstrate that inhibition of acidic FGF expression leads to induction of myogenic determination gene expression even in the presence of serum factors normally capable of suppressing such tran-

FIG. 3. Differentiation of transfected myoblasts cultured in 10% FBS. (A) Two monoclonal cell lines expressing the antisense acidic FGF construct (pCMV/ASaFGF) were compared with a monoclonal cell line transfected with the control vector (pCMV/ $\emptyset$). Cells were seeded on day 0 at low density (10^4 cells per cm^2) and cultured in DMEM containing 10% FBS for up to 4 days. Cells from triplicate wells were harvested daily, and levels of CK activity were determined and normalized to total cell protein levels. CK activities are expressed as the means $\pm$ standard deviations of the reaction rates, in nanomoles of NADPH produced per minute per microgram of total cell protein. (B) Three polyclonal cell lines expressing the antisense acidic FGF construct (pCMV/ASaFGF) were compared with two polyclonal cell lines transfected with the control vector (pCMV/ $\emptyset$). Cells were seeded and cultured as for panel A for up to 5 days. Cells from quadruplicate wells were harvested daily, and levels of CK activity were determined and normalized to total cell protein levels. CK activities are expressed as the means $\pm$ standard deviations of the reaction rates, in nanomoles of NADPH produced per minute per microgram of total cell protein.

FIG. 4. Expression of myogenin and α -skeletal (α -sk.) actin in transfected myoblasts cultured in mitogen-rich media. Sol 8 cells were stably transfected with the acidic FGF antisense expression vector, pCMV/ASaFGF, or with the control vector, pCMV/ $\emptyset$. Total RNA (10 μ g) from clones of pCMV/ $\emptyset$ - and pCMV/ASaFGF-transfected myoblasts cultured in DMEM containing 33% FBS was analyzed by RNase protection analysis for myogenin and α -skeletal actin mRNA expression. The radiolabeled myogenin probe is 200 nt in length, and hybridization to the mRNA protects a 140-nt doublet. The 32 P-labeled α -skeletal actin probe is 220 nt in length, and the mRNA protects a series of fragments of ≤ 60 nt.

scriptional activation. As further evidence for progression along the differentiation pathway, α -skeletal actin mRNA abundance is greater in pCMV/ASaFGF-transfected myoblasts than in pCMV/ $\emptyset$ -transfected myoblasts (Fig. 4).

Exogenously administered acidic FGF rescues the phenotype of antisense transcript-expressing myocytes. If the antisense phenotype (i.e., fusion to form myotubes and induction of CK activity in mitogen-rich media) is a consequence of, rather than merely associated with, the inhibition of acidic FGF production, then supplementation of the antisense transcript-expressing cells with exogenously supplied acidic FGF should reverse this phenotype. Recombinant acidic FGF (30 ng/ml [~ 1.7 nM; a gift of Michael Jaye, Rorer Biotechnology]) plus heparin (5 U/ml; Upjohn) added to a monoclonal antisense cell line suppressed the induction of CK activity in media containing 10% FBS (Fig. 5). Similar results were obtained with a second monoclonal antisense cell line (data not shown). The monoclonal control cell line expressed only low levels of CK activity during the course of the experiment; this phenomenon was not influenced by supplementation with acidic FGF.

To further test the ability of exogenous acidic FGF to correct the precocious differentiation phenotype of the cells expressing the antisense transcript, the levels of myogenin and α -skeletal actin expression in pCMV/ASaFGF- and pCMV/ $\emptyset$ -transfected cells incubated in the presence of 30 ng of acidic FGF per ml plus heparin were measured. Exposure of the cells to acidic

FIG. 5. Differentiation of transfected myoblasts cultured in 10% FBS and rescue of the antisense phenotype by exogenous acidic FGF. Antisense (pCMV/ASaFGF) and control (pCMV/ $\emptyset$) cells were seeded at low density (10^4 cells per cm^2) in DMEM containing 10% FBS and incubated for 1 to 4 days in the absence ($-a$ FGF) or presence ($+a$ FGF) of 30 ng of recombinant human acidic FGF per ml and 5 U of heparin per ml. Media and additives were replaced daily. Cells from triplicate wells were harvested daily, and levels of CK activity were determined and normalized to total cell protein levels. CK activities are expressed as the means $\pm$ standard deviations of the reaction rates, in nanomoles of NADPH produced per minute per microgram of total cell protein.

FGF for 6 h reversed the transcriptional activation of myogenin and α -skeletal actin in monoclonal pCMV/ASaFGF-transfected cells to levels comparable to those of monoclonal pCMV/ $\emptyset$ -transfected cells (Fig. 6).

DISCUSSION

Previous studies document coordinate decreases in acidic FGF mRNA abundance and acidic FGF protein abundance during myogenic differentiation in cultured myogenic cells, including Sol 8 cells (1, 12, 19), but stop short of demonstrating that changes in endogenous acidic FGF production can influence myogenic differentiation. In the present study, myoblasts expressing antisense acidic FGF mRNA and maintained in mitogen-rich media (containing 33% FBS) contained less FGF (measured as total heparin-binding mitogenic activity) than control-transfected cells. Under these culture conditions, the antisense transfectants have already entered the differentiation pathway as determined by the transcriptional activation of myogenin and α -skeletal actin expression, while control transfectants have not. In addition, maintenance of transfected myoblasts in 10% FBS inhibited differentiation of control-transfected cells but was permissive for biochemical differentiation of cells expressing antisense acidic FGF mRNA, as evidenced by the progressive accumulation of CK activity and the formation of myotubes in these cells. The addition of acidic FGF to the culture medium rescues the differentiated phenotype of antisense transcript-expressing cells, providing further support for the theory that inhibition of endogenous FGF production causes precocious differentiation of these cells.

Previous work demonstrated the ability of exogenous FGF to suppress the differentiation of myogenic cells in the absence of serum factors required to support FGF-stimulated mitogenesis, establishing distinct pathways for FGF in myogenic differentiation and proliferation (6). The present study demonstrates the converse, i.e., that inhibition of endogenous FGF activity can induce myogenic differentiation in the presence of

FIG. 6. Reversal of myogenin and α -skeletal actin expression in transfected myoblasts cultured in mitogen-rich media supplemented with acidic FGF. pCMV/ $\emptyset$ - and pCMV/ASaFGF-transfected myoblasts were cultured in DMEM containing 33% FBS at low density. Cells were harvested prior to (t_0) or 6 h following the addition of 30 ng of recombinant human acidic FGF per ml plus 5 U of heparin per ml (+aFGF) or heparin alone ($-aFGF$). Total RNA (10 μ g) from each sample was analyzed by RNase protection analysis for myogenin and α -skeletal (α -sk) actin mRNA expression as described in the legend to Fig. 4.

serum factors that normally support cell proliferation. This observation demonstrates a direct role for endogenous FGF in the transcriptional control of myogenic differentiation.

Transcriptional regulatory factors are normally present in very small amounts, and their activity is carefully regulated in a wide variety of cell systems. Acidic FGF may behave in a similar manner, as evidenced by our inability to detect this protein in Sol 8 cells by a standard immunoblotting procedure. Although the precise mechanism of antisense mRNA inhibition of intracellular FGF activity was not revealed by the present study, several possibilities are suggested by these and previously published data. The most straightforward mechanism would invoke a direct inhibition of the production of acidic FGF protein through interference with mRNA processing and transport or by arrested translation. This mechanism seems plausible, since acidic FGF mRNA and protein are normally present in low abundance in myogenic cells. An alternative and perhaps complementary mechanism involves the disruption of a positive autocrine feedback loop, whereby acidic FGF protein positively regulates the steady-state abundance of acidic FGF mRNA. A feedback loop in Sol 8 cells involving exogenously supplied FGF has been demonstrated by our laboratory (10). The theory that endogenously produced FGF can participate in a feedback loop that has been demonstrated to operate with exogenously supplied FGF is further supported by the ability of exogenously supplied FGF to correct the phenotype associated with antisense mRNA inhibition of endogenously produced FGF.

Sol 8 cells express basic FGF as well as acidic FGF, and it

has been shown that both acidic and basic FGFs can participate in the positive feedback loop operating to increase the mRNA abundance of the genes for both of these proteins in Sol 8 cells (10). Such cross talk at the protein level suggests another potential mechanism to account for the antisense phenotype. A decrease in the synthesis of acidic FGF might interfere with an autocrine feedback loop for basic FGF, resulting in lowered production of this growth factor as well. Finally, there is enough homology between the primary DNA sequences of these two proteins that it is conceivable that the antisense acidic FGF mRNA might directly interfere with the activity of basic FGF mRNA, thus leading to another potential mechanism that could account for these observations. Each of these potential mechanisms is compatible with the finding of diminished total FGF mitogenic activity in the cell lines expressing antisense acidic FGF mRNA.

The observation of activation of myogenin transcription in the cells expressing the antisense mRNA suggests a potential mechanism through which FGF might exert regulatory influence over the myogenic differentiation program. Myogenin and the other members of the basic helix-loop-helix family of myogenic determination genes are known to activate the program of muscle-specific gene expression when expressed constitutively in nonmuscle cells. The inhibition of endogenous FGF activity by the antisense mRNA provokes myogenic differentiation and is associated with transcriptional activation of myogenin; reconstitution with exogenous acidic FGF restores the undifferentiated state and reverses myogenin transcriptional activation. Endogenous FGF may thereby regulate myogenic differentiation by acting as a negative regulator of myogenin gene expression.

The broad phylogenetic and ontogenetic distribution of members of the FGF family, their recognized participation in diverse developmental pathways (13, 14, 21), and the demonstration in this study that members of this family can regulate a complex process (myogenesis) suggest a central role for these proteins in coordinating vertebrate development. Further studies are required to define the roles of other members of this polypeptide growth factor family in regulating complex developmental processes such as myogenesis.

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