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Opposing Early Inhibitory and Late Stimulatory Effects of Insulin-like Growth Factor-I on Myogenin Gene Transcription

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Abstract Insulinlike growth factors (IGFs) stimulate skeletal muscle cell differentiation in association with an increase in the mRNA of myogenin, a member of the MyoD family of skeletal muscle–specific transcription factors that plays an essential role in the differentiation process. However, this is a relatively late effect, requiring treatment periods of >24 h. In contrast, IGFs initially inhibit skeletal muscle cell differentiation, associated with a marked reduction in myogenin mRNA. The mechanisms by which IGF-I initially inhibits and subsequently stimulates myogenin expression are unknown. In the first 24 h, we find that IGF-I inhibits myogenin gene transcription by >80% but has no effect on myogenin mRNA stability. Similarly, in the first 24 h, IGF-I markedly inhibits myogenin promoter activity; the sequence −145 to −9 of the myogenin gene is sufficient to confer this inhibitory effect of IGF-I. In contrast, 48 h of treatment with IGF-I results in an increase in myogenin promoter activity that parallels the increase in myogenin steady-state mRNA. This increase in promoter activity is completely prevented in constructs lacking the sequence −1,565 to −375 of the myogenin gene. These data indicate that the early inhibitory and late stimulatory effects of IGF-I on myogenin expression are mediated at the level of transcription, and that these time-dependent, opposing effects of IGF-I on myogenin transcription are mediated by distinct regions of the myogenin gene. To our knowledge, this is the first demonstration of a gene whose promoter activity is initially inhibited and subsequently stimulated by IGF-I. J. Cell. Biochem. 78:617–626, 2000.

Key words: IGF-I; skeletal muscle cells; myogenic differentiation

Skeletal myoblasts are inherently programmed to leave the cell cycle and begin the differentiation process after withdrawal of exogenous growth factors [Molkentin and Olson, 1996]. Although growth factors, in general, inhibit the differentiation of skeletal muscle cells, IGFs are unique in that they are the only known mitogens that initially inhibit and subsequently stimulate skeletal muscle cell differentiation [Ewton and Florini, 1981; Florini et al., 1991; Rosenthal and Cheng, 1995; Silverman et al., 1995]. IGF stimulation of skeletal muscle cell differentiation is associated with an increase in the steady-state mRNA levels of myogenin [Florini et al., 1991; Rosenthal and Cheng, 1995; Silverman et al., 1995], a member of the MyoD family of skeletal muscle–specific transcription factors that plays an essential role in muscle differentiation [Hasty et al., 1993; Nabeshima et al., 1993]. Studies demonstrating IGF-induced increases in myogenin mRNA, however, involved relatively long treatment periods of >24 h. In contrast, we have

Abbreviations used: IGF, insulin-like growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEF2, myocyte enhancer factor 2.

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recently shown that early (<24 h) IGF treatment of skeletal myoblasts markedly inhibits myogenin mRNA [Rosenthal and Cheng, 1995].

In this study, we have examined the mechanisms by which IGF-I initially inhibits and subsequently stimulates myogenin mRNA. Using a clonally derived skeletal muscle cell line, we find that the early inhibitory and late stimulatory effects of IGF-I on myogenin steady-state mRNA are mediated at the transcriptional level, and that these time-dependent, opposing effects of IGF-I on myogenin transcription are mediated by distinct regions of the myogenin gene.

MATERIALS AND METHODS

Materials

The following materials were purchased: [α-^{32}P]deoxy-CTP (3,000 Ci/mmol) and [α-^{32}P] UTP (6,000 Ci/mmol) from DuPont New England Nuclear (Boston, MA), pGL3 (pLuc) and pRL-CMV plasmids and Dual Luciferase Reporter Assay System from Promega (Madison, WI), tissue culture medium components and fetal bovine serum (FBS) (HyClone) (Logan, UT) from the Cell Culture Facility (University of California, San Francisco) and bovine serum albumin (BSA) and actinomycin D (ActD) from Sigma Chemicals (St. Louis, MO). Des(1-3)IGF-I was a gift from Genentech (South San Francisco, CA). The myogenin promoter and cDNA were provided by E. N. Olson (University of Texas Southwestern Medical Center, Dallas). A plasmid containing a GAPDH cDNA was a gift from G. Chazenbalk (Department of Veterans Administration Medical Center, San Francisco).

Cell Culture

Rat L6E9 skeletal myoblasts were provided by B. Nadal-Ginard [Nadal-Ginard, 1978]. These cells express the biochemical markers and morphologic features of primary skeletal muscle cells and have been used extensively as a model system to study the events that regulate muscle cell proliferation and differentiation [Nadal-Ginard, 1978; Rosenthal and Cheng, 1995; Silverman et al., 1995; Engert et al., 1996; Kaliman et al., 1996, 1998]. These myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/ 1% glutamine and antibiotics supplemented with 20% FBS. For IGF treatment studies, cells were placed in serum-free medium/1% BSA supplemented with vehicle (0.1 M acetic acid) or the IGF peptide.

Nuclear Run-on Transcription Assays

At 60–70% confluence, cells maintained in 20% serum-supplemented medium were either harvested or placed in serum-free medium with 1% BSA in the absence or presence of des(1-3)IGF-I for 12 h. Nuclei were then prepared from 10^8 cells by washing in phosphate-buffered saline followed by resuspension in lysis buffer [Groudine et al., 1981] containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40, and incubation on ice for 5 min. Nuclear pellets were obtained by centrifugation at 800*g* at 4° C for 5 min. Nuclei were resuspended in storage buffer (40% glycerol, 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA) and used immediately or stored at −80°C. Five micrograms of the plasmid containing the coding sequence of either myogenin or GAPDH were linearized, purified, and denatured in 0.1 M NaOH, and applied to Nitrocellulose membranes using a slot blot minifold (Schleicher & Schuell) (Keene, NH). Each slot was washed with 0.5 ml 6x SSPE (20x SSPE is 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA-Na_2, pH 7.4). DNA was fixed onto the membranes with a UV Stratalinker (Stratagene, La Jolla, CA) and by baking the membranes under vacuum at 80°C for 2 h.

Transcription was initiated by suspending 5×10^7 nuclei in a 200-μl reaction mixture containing 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM dithiothreitol, 50 U/ml RNase inhibitor, 0.5 mM each GTP, CTP, and adenosine triphosphate, and 200 μCi of [α-^{32}P] UTP and incubating at 26°C for 30 min. Total RNA products were then purified using DNase I, proteinase K in the presence of sodium dodecyl sulfate (SDS), salt precipitation, and subsequent phenol-chloroform extraction [Groudine et al., 1981]. Equal numbers of cpm from each sample were reconstituted into a final volume of 1.5 ml and hybridized to slot-blotted cDNAs for 36 h at 65°C. After hybridization, membranes were washed sequentially in 1x SSPE/0.1% SDS at 65°C for 90 min, 0.1x SSPE/0.1% SDS at 65°C for 20 min, 2x SSPE with 1.25 μg/ml RNase A at 37°C for 15 min, and 2x SSPE at room temperature for 10 min. Membranes were then exposed to a Phospho-

rImager screen (Molecular Dynamics) (Sunnyvale, CA) for quantitation of run-on activity.

RNA Isolation and Northern Blotting

At the indicated times, cells were harvested and total RNA was isolated by extraction in Trizol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's recommendations. RNA was quantitated by spectrophotometric determination at 260 nm, and 25 μg of RNA per sample were denatured in formaldehyde, subjected to electrophoresis in 1% agarose gels, transferred to nylon membranes (Amersham, Piscataway, NJ), and fixed by UV cross-linking. Myogenin cDNA was labeled using random primers to 10^9 cpm/μg. Nylon membranes were prehybridized and hybridized in Express Hyb solution (Clontech) (Palo Alto, CA) according to the manufacturer's recommendations, washed as previously described [Sambrook et al., 1989], and exposed to a PhosphorImager screen for quantitation followed by autoradiography using Biomax MS film (Kodak, Rochester, NY).

mRNA Stability Studies

L6E9 cells were placed in serum-free medium/1% BSA for 12 h, then switched to fresh serum-free medium/1% BSA with des(1-3)IGF-I (100 ng/ml) or vehicle. Four hours later (time 0) ActD was added to a final concentration of 1 μg/ml. At the times indicated after ActD treatment, cells were harvested for Northern analysis and quantitation by PhosphorImaging, as described above. After quantitation of myogenin mRNA, membranes were stripped by boiling twice in distilled water for 2 min, and reprobed for detection of 18S ribosomal RNA. Values of myogenin mRNA were then adjusted for sample loading by dividing them by the corresponding values of 18S ribosomal RNA in each lane then plotted as percentages.

Analysis of Promoter Activity

Promoter activity of deletional constructs of the 5′-flanking region of the myogenin gene was studied by transient transfection assays. Myogenin-luciferase reporter genes were generated by subcloning restriction fragments or polymerase chain reaction–derived DNA fragments into the promoterless pGL3 basic plasmid (p0Luc) just upstream of the firefly luciferase gene. For each recombinant, the orientation and nucleotide sequence were validated before use.

Transfections and luciferase assays were performed using Qiagen's (Chatsworth, CA) Superfect or Effectene transfection reagents and Promega's Dual Luciferase Assay kit, respectively, according to the manufacturers' recommendations. Briefly, L6E9 cells were seeded in 24-well plates for 24 h, then washed and placed in 400 μl DMEM/20% FBS containing 1 μg of myogenin-luciferase plasmid and 20 ng of pRL-CMV plasmid that were incubated with the Qiagen transfection reagent for 15 min at room temperature. After recovery from transfection, cells were washed and placed in serum-free medium with or without the IGF-I peptide. Fourteen or 48 h after IGF-I treatment, cells were lysed and luciferase activity was measured using an automated Monolight 1500-B Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). To control for transfection efficiency, cells were cotransfected with a Renilla Luciferase expression vector (pRL-CMV, Promega) in which the cDNA encoding for Renilla Luciferase is inserted downstream of a CMV promoter. Firefly Luciferase and Renilla Luciferase are distinct enzymes with dissimilar substrate requirements. Accordingly, the activity of each enzyme can be measured independently using the Dual Luciferase Assay system (Promega). In each experiment, values of firefly Luciferase activity, which reflect myogenin promoter activity, were adjusted for efficiency of transfection by dividing them by the corresponding values of Renilla Luciferase activity in each sample. In addition, in cells treated for 48 h, the results were adjusted for cell number because cell number increased during the 48-h IGF treatment studies but not during the 14-h studies.

RESULTS AND DISCUSSION

Early IGF-I Treatment Inhibits Myogenin Gene Transcription

To determine whether the early inhibitory effects of IGF-I on steady-state myogenin mRNA in skeletal myoblasts [Rosenthal and Cheng, 1995] are mediated at the level of gene transcription and/or mRNA stability, we carried out nuclear run-on and mRNA half-life studies. Des(1-3)IGF-I, a naturally occurring IGF-I analog that has markedly reduced affin-

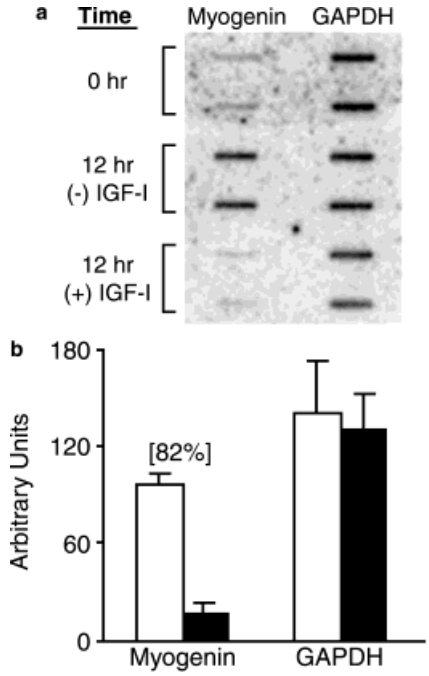

Fig. 1. **a:** Nuclear run-on assays for myogenin and GAPDH transcription in the absence and presence of des(1-3)IGF-I for 12 h. Slot-blot hybridization (in duplicates) was performed using labeled total RNA synthesized by nuclei from L6E9 myoblasts in 20% fetal bovine serum–supplemented medium (time 0), and nuclei from cells incubated for 12 h in serum-free medium/1% bovine serum albumin in the absence or presence of des(1-3)IGF-I (100 ng/ml). A representative of four independent experiments is shown. **b:** A quantitative representation of the rate of myogenin and GAPDH gene transcription in the absence (open bars) or presence (closed bars) of des(1-3)IGF-I (100 ng/ml) for 12 h (mean ± SD of four measurements). The percent decrease in myogenin transcription in IGF-treated versus control cells is indicated.

ity for IGF binding proteins [Bagley et al., 1989] but has an affinity for the IGF-I receptor comparable to that of native IGF-I [Ballard et al., 1987], was used to minimize any potential effects of IGF binding proteins [Silverman et al., 1995]. In nuclear run-on assays, myogenin transcription was virtually nondetectable in proliferating myoblasts maintained in 20% FBS-supplemented medium (time 0), as expected [Brunetti and Goldfine, 1990; Edmondson et al., 1992] (Fig. 1a). After 12 h in serum-free medium in the absence of IGF peptide, myogenin transcription was induced (Fig. 1a). In contrast, the induction of myogenin gene transcription was inhibited by >80% after 12 h in serum-free medium in the presence of des(1-3)IGF-I (100 ng/ml) (Fig. 1a,b). Transcription of the GAPDH gene, used as a control, was not affected by serum withdrawal or by IGF treatment (Fig. 1a,b).

To determine whether treatment with the IGF-I analog affects myogenin mRNA stability, myogenin mRNA half-life studies were carried out using ActD, an inhibitor of mRNA synthesis. Because previous studies of mRNA stability have used ActD at doses ranging from 2 to 10 μg/ml [Levine et al., 1986; Saitoh et al., 1990; Yoshizumi et al., 1993; Lofquist et al., 1995], we initially carried out dose–response studies to determine a dose of ActD that completely inhibits myogenin transcription. Because the increase in steady-state myogenin mRNA in serum-free medium [Florini et al., 1991; Rosenthal and Cheng, 1995; Silverman et al., 1995] is a consequence of increased transcription (Fig. 1a), we used Northern analysis to evaluate the ability of ActD to inhibit myogenin gene expression in serum-free medium. As seen in Figure 2, myogenin mRNA was minimally detectable in myoblasts maintained in 20%FBS-supplemented medium, and was induced after 12 h in serum-free medium, as expected. Myogenin gene expression was completely inhibited in the presence of ActD (1–10 μg/ml) (Fig. 2). The ActD vehicle (dimethylsulfoxide) had no effect on myogenin gene expression. Subsequent myogenin mRNA stability studies were carried out with 1 μg/ml ActD.

To assess the effects of IGF-I on myogenin mRNA stability, myoblasts were initially placed in serum-free medium for 12 h to allow for adequate induction of myogenin mRNA.

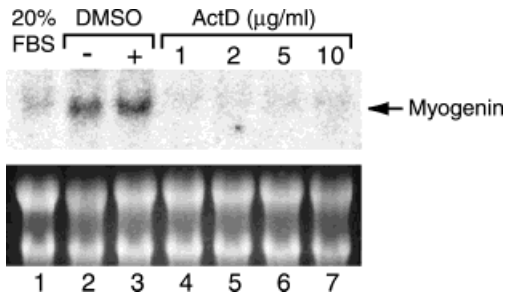

Fig. 2. Effect of actinomycin D (ActD) on myogenin mRNA: dose–response. Myogenin mRNA was examined by Northern blots in L6E9 myoblasts in 20% fetal bovine serum (FBS)-supplemented medium (**lane 1**) and after 12 h in serum-free medium/1% bovine serum albumin in the absence (**lane 2**) or presence of increasing doses of ActD (**lanes 4–7**) or vehicle (**lane 3**). The arrow indicates myogenin mRNA. Ethidium bromide staining of the gel is shown below. A representative of three independent experiments is shown. DMSO, dimethylsulfoxide.

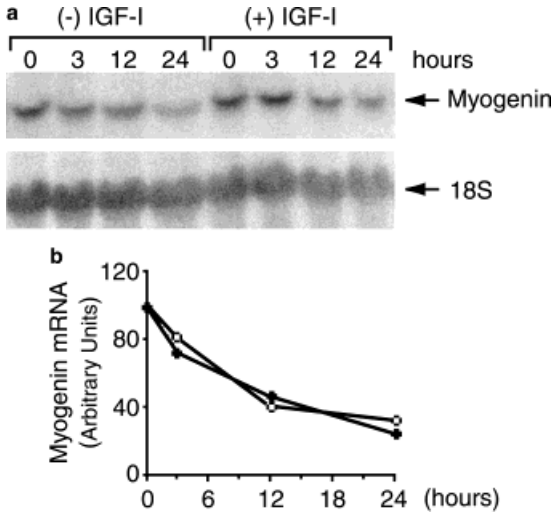

Fig. 3. Effect of des(1-3)IGF-I on myogenin mRNA stability. L6E9 cells were placed in serum-free medium/1% bovine serum albumin for 12 h, then treated with vehicle [open circles, (**b**)] or des(1-3)IGF-I (100 ng/ml) [closed circles, (**b**)]. Four h later (time 0), cells were either harvested or treated with actinomycin D (ActD) (1 μg/ml) for up to 24 h to inhibit myogenin mRNA synthesis. At time 0 and at 3, 12, and 24 h after addition of ActD, total RNA was harvested for Northern blot analysis (**a**) and quantitation of myogenin mRNA (**b**) as described in Materials and Methods. A representative of two independent experiments is shown.

Cells were then treated with vehicle or the IGF-I analog (100 ng/ml). Four hours later (time 0), cells were either harvested or treated with ActD for up to 24 h to inhibit myogenin mRNA synthesis. The IGF-I analog was added 4 h before ActD treatment to allow for possible IGF-associated transcriptional events that might be required for potential IGF regulation of myogenin mRNA stability. At time 0, and at 3, 12, and 24 h after addition of ActD, myogenin mRNA levels were determined by Northern blotting and quantitated by PhosphorImager analysis as described in Materials and Methods. In control cells, myogenin mRNA half-life was approximately 12 h. As seen in Figure 3, myogenin mRNA half-life was not affected by the IGF-I analog because the rate of mRNA decay was identical in the absence or presence of IGF-I. Previous studies of myogenin mRNA stability in mouse and rat skeletal muscle cells demonstrated half-lives that differed from each other and from the results in the present study [Saitoh et al., 1990; Rotwein et al., 1995]. In a previous study in L6E9 cells, myogenin mRNA half-life was found to be 3–4 h [Saitoh et al., 1990]. The difference in myogenin mRNA half-life in L6E9 cells between the earlier and present studies may reflect differences in experimental design. In the studies by Saitoh et al., ActD was added after 48 h in differentiation medium supplemented with 5% horse serum, whereas in the current study, ActD was added after 16 h in serum-free medium.

In addition to IGF-I, basic (b) and acidic fibroblast growth factor (FGF) and transforming growth factor (TGF)-β, known inhibitors of skeletal myoblast differentiation, also inhibit the gene expression of myogenin [Brunetti and Goldfine, 1990; Heino and Massagué, 1990; Brennan et al., 1991; Fox et al., 1994]. Like IGF-I, bFGF inhibits transcription of the myogenin gene [Brunetti and Goldfine, 1990]. In addition, bFGF may inhibit the muscle differentiation process by phosphorylating a conserved site in the DNA-binding domain of myogenin and inhibiting its binding to DNA [Li et al., 1992]. In the presence of TGF-β, constitutively expressed myogenin translocated to the nucleus and bound DNA but was unable to activate the muscle differentiation process [Brennan et al., 1991; Martin et al., 1992]. Whether IGF-I modifies myogenin binding to DNA or its ability to activate the myogenic program is not yet known. Even if IGF-I is able to posttranslationally modify myogenin or modify its transcriptional activity, such effects are unlikely to play a role in IGF-I-induced inhibition of muscle differentiation, because myogenin mRNA remains undetectable during the period in which IGF-I inhibits the muscle differentiation process [Rosenthal and Cheng, 1995].

Early Inhibitory Effect of IGF-I on Myogenin Transcription Is Mediated by Sequence(s) Contained from −145 to −9 of the Myogenin Gene

To identify a potential region in the myogenin gene that confers the early inhibitory effect of IGF-I on myogenin transcription, we have examined a 1.6-kb upstream region (−1,565 to +18) of the myogenin gene. This region has been previously shown to mediate the inhibitory effect of serum on myogenin transcription [Edmondson et al., 1992]. In promoter-reporter transfection assays, we found that treatment with des(1-3)IGF-I (100 ng/ml) for 14 h markedly inhibited the activity of the myogenin promoter in a construct that contained the entire 1.6-kb region (p-1565) (Fig. 4). To further de-

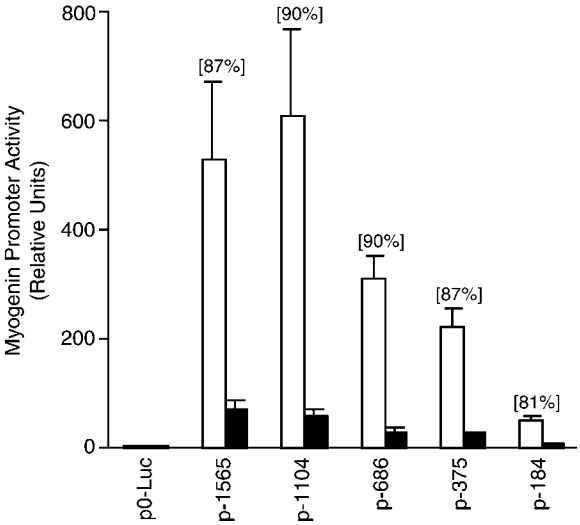

Fig. 4. Early inhibitory effect of des(1-3)IGF-I on myogenin promoter activity in skeletal myoblasts. L6E9 cells were transiently transfected with a promoterless vector (p0Luc) or with a reporter plasmid construct containing serial 5′ deletional DNA segments of the myogenin promoter. The 3′ end of all segments corresponds to position +18 of the myogenin gene. After recovery from transfection, cells were placed in serum-free medium/1% bovine serum albumin in the absence (open bars) or presence (closed bars) of des(1-3)IGF-I (100 ng/ml) for 14 h. Cells were subsequently harvested and luciferase assays were carried out. Transfections were performed in quadruplicate and results are expressed as mean (±SD) × 10^{-4}. Numbers in brackets above the bars indicate the percent decrease in myogenin promoter activity in IGF-treated versus control cells. A representative of four independent experiments is shown.

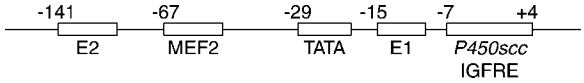

Fig. 5. A schematic representation of the sequence −184 to +18 of the myogenin promoter showing the essential elements that are required for myogenin expression [Edmondson et al., 1992] and the region with homology to the *P450scc* IGF-response element (IGFRE) [Urban et al., 1994].

fine the cis-elements that confer the inhibitory effect of IGF-I on the activity of the myogenin promoter, we studied a series of 5′ deletional constructs of the 1.6-kb region in reporter transfection assays. As seen in Figure 4, the IGF-I analog inhibits the activity of the myogenin promoter by 80–90% for all of the constructs tested. This suggests that an IGF response element (or elements) is contained in the region from −184 to +18, which is common to all of the constructs. Of note, the decrease in activity of the p-184 construct in serum-free conditions is consistent with previous findings of Edmondson et al. These authors have also shown that this construct contains elements that are essential for myogenin promoter activity [Edmondson et al., 1992] (Fig. 5). These elements include a binding site for MEF2 at −67 to −59, and two E boxes at −141 to −136 and −15 to −10, consensus sequences that bind basic helix–loop–helix transcription factors dimerized with E2A gene products [Murre et al., 1989]. Loss of the distal E box decreased myogenin promoter activity by half (compared to the intact construct −184 to +18), and mutation of the MEF2 site or deletion of the proximal E box resulted in a significant decrease in promoter activity in differentiated myotubes [Edmondson et al., 1992]. Similar results were found after deletions of the corresponding MEF2 site and proximal E box in the chick myogenin promoter [Malik et al., 1995]. Accordingly, we ceased 5′ deletions at −145, because further 5′ deletions would significantly decrease the basal activity of the myogenin promoter, rendering it difficult to evaluate an inhibitory effect of IGF-I. Sequence analysis of the region −145 to +18 revealed the presence of a GC-rich 11-bp region (−7 to +4) in which 10 bases are identical to a recently characterized IGF-I response element in the porcine *P450* side-chain cleavage (*P450scc*) gene, which mediates a stimulatory effect of IGF-I on the expression of this gene [Urban et al., 1994] (Fig. 5). However, a 3′ deletional construct of the myogenin gene (p-145/-9), which lacks this GC-rich region, conferred an inhibitory effect of IGF-I on myogenin promoter activity comparable to that seen with the p-145/+18 construct (Fig. 6). These studies suggest that an inhibitory IGF response element (or elements) is contained in the region −145 to −9 of the myogenin gene.

Late Stimulatory Effect of IGF-I on Myogenin Expression Is Mediated by Sequences Contained from −1,565 to −375 of the Myogenin Gene

To determine whether the late stimulatory effect of IGF-I on myogenin mRNA is associated with increased activity of the myogenin promoter, we carried out similar reporter transfection studies in which the cells were treated with IGF-I for 48 h. We found that des(1-3)IGF-I (100 ng/ml) caused a onefold increase in myogenin promoter activity in the construct p-1565 compared to untreated cells

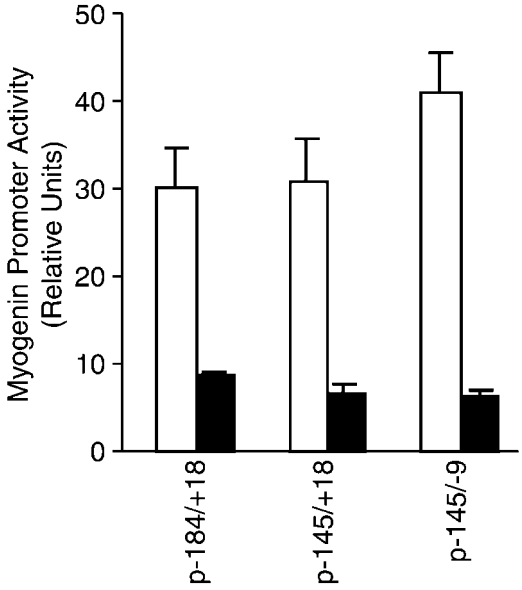

Fig. 6. Early inhibitory effect of des(1-3)IGF-I (100 ng/ml) on myogenin promoter activity: comparison with a 3′ deletional construct lacking the region of homology with the putative IGF-response element in the *P450scc* gene [Urban et al., 1994]. Transient transfections and luciferase assays were carried out as described for Figure 4. Transfections were performed in quadruplicate, and results are expressed as mean (±SD) $\times 10^{-4}$. A representative of three independent experiments is shown.

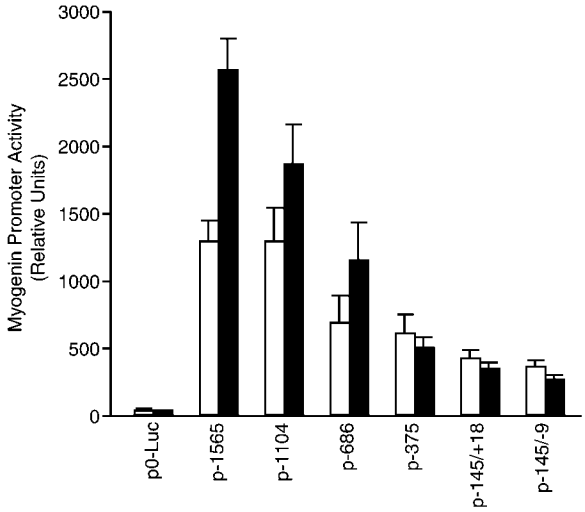

Fig. 7. Late stimulatory effect of des(1-3)IGF-I on myogenin promoter activity in skeletal myoblasts. L6E9 cells were transiently transfected with reporter plasmids described for Figures 4 and 6, and placed in serum-free medium/1% bovine serum albumin in the absence (open bars) or presence (closed bars) of des(1-3)IGF-I (100 ng/ml) for 48 h. Cells were subsequently harvested, and luciferase assays were carried out. Transfections were performed in quadruplicate and results are expressed as mean (±SD) $\times 10^{-4}$. A representative of three independent experiments is shown.

(Fig. 7). This increase in promoter activity occurs in parallel with an approximately onefold increase in steady-state myogenin mRNA in these cells [Rosenthal and Cheng, 1995], making it unlikely that changes in mRNA stability play a role in mediating the late stimulatory effect of IGF-I on myogenin gene expression. These results are consistent with a recent report in which 48-h exposure to IGF-I also caused a onefold increase in myogenin promoter activity in differentiated L6E9 skeletal muscle cells [Musarò and Rosenthal, 1999].

To determine whether the same region of the myogenin gene that mediates the early inhibitory effect of IGF-I also mediates the late stimulatory effect, we carried out reporter transfection studies using the same deletional constructs used in Figures 4 and 6. We found that IGF-I treatment for 48 h caused an increase in myogenin promoter activity in the constructs p-1104 and p-686 that was consistently less than the increase seen in the p-1565 construct (Fig. 7). However, IGF-I for 48 h had no effect on promoter activity in the constructs lacking sequences upstream of −375 (Fig. 7). This suggests the presence of a stimulatory IGF response element or elements within the sequence −1,565 to −375, and demonstrates that the early inhibitory and late stimulatory effects of IGF-I on myogenin transcription are mediated by different regions of the myogenin gene.

Despite the large number of genes that are regulated by IGFs, IGF-response elements have been identified in only a small number of genes [Alemany et al., 1990; Kamikubo et al., 1990; Rich et al., 1993; Wolfe et al., 1993; Urban et al., 1994]. In the chicken δ1 crystallin [Alemany et al., 1990], the rat thyroglobulin [Kamikubo et al., 1990], rat elastin [Rich et al., 1993, Wolfe et al., 1993], and the porcine *P450scc* [Urban et al., 1994] genes, IGF response elements have been characterized to varying degrees and are thought to mediate a stimulatory effect of IGF-I on gene transcription. Comparative analysis of these stimulatory IGF-response elements with the sequence −1,565 to −375 of the myogenin gene revealed three regions with varying degrees of homology to the response elements in the elastin and thyroglobulin genes. These regions represent potential targets for IGF action that may me-

diate IGF-I-induced stimulation of myogenin promoter activity.

In addition to inhibiting myogenin, IGF-I is also known to inhibit the expression of growth hormone [Yamashita and Melmed, 1987] and the thyroid hormone receptor [Pellizas et al., 1998]; however, an IGF response element that mediates an inhibitory effect on the transcription of the latter genes has not been identified. To our knowledge, our studies with the −145 to −9 region of the myogenin gene represent the first demonstration of a DNA region that confers an inhibitory effect of IGF-I on the transcription of a target gene. This broad region of DNA sequence contains multiple elements that mediate several types of activity; the requirement of the E boxes and MEF2 site (Fig. 5) for myogenin expression in differentiating myotubes [Edmondson et al., 1992; Malik et al., 1995] suggests potential targets for the IGF-I inhibitory effect on myogenin transcription. Studies examining the interactions of nuclear proteins with both the −1,565 to −375 and −145 to −9 regions of the myogenin gene should further elucidate the mechanisms by which IGF-I regulates myogenin transcription.

The initial inhibition and subsequent stimulation of myogenin expression by IGF-I may reflect a divergence in IGF-I-receptor signaling pathways. In a recent study by Coolican et al. [1997], the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI3K) pathways have been implicated in mediating IGF-induced proliferation and differentiation, respectively, in skeletal muscle cells. These authors have shown that IGF-I stimulates myogenin expression through the PI3K signaling pathway. However, a preliminary study from our laboratory indicates that a pathway other than the PI3K or MAPK pathways mediates the inhibitory effect of IGF-I on myogenin mRNA (unpublished data).

In addition to IGF-I's biphasic effect on myogenin expression, we have previously shown that IGF-I also has a time-dependent, biphasic effect on the phosphorylation of the cell cycle regulatory retinoblastoma (Rb) protein [Rosenthal and Cheng, 1995]. Rb is a ubiquitous nuclear factor, the activity of which is regulated by phosphorylation [Buchkovich et al., 1989; Chen et al., 1989]. The phosphorylated form of Rb is thought to inhibit muscle differentiation, whereas the un- (or hypo-) phosphorylated form promotes the terminally differentiated state of myocytes [Gu et al., 1993; Schneider et al., 1994]. Concurrent with the early inhibitory effect of IGF-I on myogenin expression, we have found that Rb is predominantly phosphorylated, potentiating inhibition of muscle differentiation [Rosenthal and Cheng, 1995]. Subsequently, when IGF-I switches to stimulating myogenin expression, Rb becomes dephosphorylated, promoting the differentiated phenotype [Rosenthal and Cheng, 1995]. The signals that determine whether the muscle differentiation response to IGF-I is inhibitory or stimulatory may thus be linked to signals that regulate not only the expression of myogenin, but also the phosphorylation state of Rb.

In summary, our results demonstrate that the early inhibitory and late stimulatory effects of IGF-I on myogenin mRNA in L6E9 skeletal myoblasts are mediated at the level of transcription, and are conferred by different regions of the myogenin gene, with the proximal region (−145 to −9) mediating the inhibitory effect and an upstream region within −1,565 to −375 mediating the stimulatory effect of IGF-I. These time-dependent, opposing effects of IGF-I on the myogenin promoter, together with the previously described biphasic effects of IGF-I on Rb phosphorylation [Rosenthal and Cheng, 1995], suggest a mechanism by which IGF-I initially inhibits and subsequently stimulates the differentiation of L6E9 skeletal muscle cells. To our knowledge, this is the first demonstration of a target gene whose promoter activity is initially inhibited and subsequently stimulated by IGF-I.

REFERENCES

Alemany J, Borras T, de Pablo F. 1990. Transcriptional stimulation of the delta 1-crystallin gene by insulin-like growth factor I and insulin requires DNA cis elements in chicken. Proc Natl Acad Sci USA 87:3353–3357.

Bagley CJ, May BL, Szabo L, McNamara PJ, Ross M, Francis GL, Ballard FJ, Wallace JC. 1989. A key functional role for the insulin-like growth factor 1 N-terminal pentapeptide. Biochem J 259:665–671.

Ballard FJ, Francis GL, Ross M, Bagley CJ, May B, Wallace JC. 1987. Natural and synthetic forms of insulin-like growth factor-1 (IGF-1) and the potent derivative, destripeptide IGF-1: biological activities and receptor binding. Biochem Biophys Res Commun 149:398–404.

Brennan TJ, Edmondson DG, Li L, Olson EN. 1991. Transforming growth factor beta represses the actions of myogenin through a mechanism independent of DNA binding. Proc Natl Acad Sci USA 88:3822–3826.

Brunetti A, Goldfine ID. 1990. Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. J Biol Chem 265:5960–5963.

Buchkovich K, Duffy LA, Harlow E. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58:1097–1105.

Chen PL, Scully P, Shew JY, Wang JY, Lee WH. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58:1193–1198.

Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. 1997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272:6653–6662.

Edmondson DG, Cheng TC, Cserjesi P, Chakraborty T, Olson EN. 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol Cell Biol 12:3665–3677.

Engert JC, Berglund EB, Rosenthal N. 1996. Proliferation precedes differentiation in IGF-I-stimulated myogenesis. J Cell Biol 135:431–440.

Florini JR, Ewton DZ, Roof SL. 1991. Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression. Mol Endocrinol 5:718–724.

Fox JC, Hsu AY, Swain JL. 1994. Myogenic differentiation triggered by antisense acidic fibroblast growth factor RNA. Mol Cell Biol 14:4244–4250.

Groudine M, Peretz M, Weintraub H. 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. Mol Cell Biol 1:281–288.

Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V, Nadal-Ginard B. 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell 72:309–324.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, Klein WH. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364:501–506.

Heino J, Massagué J. 1990. Cell adhesion to collagen and decreased myogenic gene expression implicated in the control of myogenesis by transforming growth factor beta. J Biol Chem 265:10181–10184.

Kaliman P, Vinals F, Testar X, Palacin M, Zorzano A. 1996. Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. J Biol Chem 271:19146–19151.

Kaliman P, Canicio J, Shepherd PR, Beeton CA, Testar X, Palacin M, Zorzano A. 1998. Insulin-like growth factors require phosphatidylinositol 3-kinase to signal myogenesis: dominant negative p85 expression blocks differentiation of L6E9 muscle cells. Mol Endocrinol 12:66–77.

Kamikubo K, Chai KJ, Nayfeh SN, Chae CB. 1990. Similar nuclear factors mediate stimulation of rat thyroglobulin gene transcription by thyrotropin and insulin-like growth factor-I. Mol Endocrinol 4:2021–2029.

Levine RA, McCormack JE, Buckler A, Sonenshein GE. 1986. Transcriptional and posttranscriptional control of c-myc gene expression in WEHI 231 cells. Mol Cell Biol 6:4112–4116.

Li L, Zhou J, James G, Heller-Harrison R, Czech MP, Olson EN. 1992. FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell 71:1181–1194.

Lofquist AK, Mondal K, Morris JS, Haskill JS. 1995. Transcription-independent turnover of I kappa B alpha during monocyte adherence: implications for a translational component regulating I kappa B alpha/MAD-3 mRNA levels. Mol Cell Biol 15:1737–1746.

Malik S, Huang CF, Schmidt J. 1995. The role of the CANNTG promoter element (E box) and the myocyte-enhancer-binding-factor-2 (MEF-2) site in the transcriptional regulation of the chick myogenin gene. Eur J Biochem 230:88–96.

Martin JF, Li L, Olson EN. 1992. Repression of myogenin function by TGF-beta 1 is targeted at the basic helix-loop-helix motif and is independent of E2A products. J Biol Chem 267:10956–10960.

Molkentin JD, Olson EN. 1996. Defining the regulatory networks for muscle development. Curr Opin Gen Dev 6:445–453.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544.

Musarò A, Rosenthal N. 1999. Maturation of the myogenic program is induced by postmitotic expression of insulin-like growth factor I. Mol Cell Biol 19:3115–3124.

Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S, Nonaka I, Nabeshima Y. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364:532–535.

Nadal-Ginard B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15:855–864.

Pellizas CG, Coleoni AH, Costamagna ME, Di Fulvio M, Masini-Repiso AM. 1998. Insulin-like growth factor I reduces thyroid hormone receptors in the rat liver. Evidence for a feed-back loop regulating the peripheral thyroid hormone action. J Endocrinol 158:87–95.

Rich CB, Goud HD, Bashir M, Rosenbloom J, Foster JA. 1993. Developmental regulation of aortic elastin gene expression involves disruption of an IGF-I sensitive repressor complex. Biochem Biophys Res Commun 196:1316–1322.

Rosenthal SM, Cheng ZQ. 1995. Opposing early and late effects of insulin-like growth factor I on differentiation and the cell cycle regulatory retinoblastoma protein in skeletal myoblasts. Proc Natl Acad Sci USA 92:10307–10311.

Rotwein P, James PL, Kou K. 1995. Rapid activation of insulin-like growth factor binding protein-5 gene transcription during myoblast differentiation. Mol Endocrinol 9:913–923.

Saitoh O, Olson EN, Periasamy M. 1990. Muscle-specific RNA processing continues in the absence of myogenin expression. J Biol Chem 265:19381–19384.

Sambrook T, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Schneider JW, Gu W, Zhu L, Mahdavi V, Nadal-Ginard B. 1994. Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. Science 264:1467–1471.

Silverman LA, Cheng ZQ, Hsiao D, Rosenthal SM. 1995. Skeletal muscle cell-derived insulin-like growth factor (IGF) binding proteins inhibit IGF-I-induced myogenesis in rat L6E9 cells. Endocrinology 136:720–726.

Urban RJ, Shupnik MA, Bodenburg YH. 1994. Insulin-like growth factor-I increases expression of the porcine P-450 cholesterol side chain cleavage gene through a GC-rich domain. J Biol Chem 269:25761–25769.

Wolfe BL, Rich CB, Goud HD, Terpstra AJ, Bashir M, Rosenbloom J, Sonenshein, GE, Foster JA. 1993. Insulin-like growth factor-I regulates transcription of the elastin gene. J Biol Chem 268:12418–12426.

Yamashita S, Melmed S. 1987. Insulinlike growth factor I regulation of growth hormone gene transcription in primary rat pituitary cells. J Clin Invest 79:449–452.

Yoshizumi M, Perrella MA, Burnett JC Jr, Lee ME. 1993. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circulation Res 73:205–209.