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# Myogenic Vector Expression of Insulin-like Growth Factor I Stimulates Muscle Cell Differentiation and Myofiber Hypertrophy in Transgenic Mice*

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**The avian skeletal α-actin gene was used as a template for construction of a myogenic expression vector that was utilized to direct expression of a human IGF-I cDNA in cultured muscle cells and in striated muscle of transgenic mice. The proximal promoter region, together with the first intron and 1.8 kilobases of 3′-noncoding flanking sequence of the avian skeletal α-actin gene directed high level expression of human insulin-like growth factor I (IGF-I) in stably transfected $C_2C_{12}$ myoblasts and transgenic mice. Expression of the actin/IGF-I hybrid gene in $C_2C_{12}$ muscle cells increased levels of myogenic basic helix-loop-helix factor and contractile protein mRNAs and enhanced myotube formation. Expression of the actin/IGF-I hybrid gene in mice elevated IGF-I concentrations in skeletal muscle 47-fold resulting in myofiber hypertrophy. IGF-I concentrations in serum and body weight were not increased by transgene expression, suggesting that the effects of transgene expression were localized. These results indicate that sustained overexpression of IGF-I in skeletal muscle elicits myofiber hypertrophy and provides the basis for manipulation of muscle physiology utilizing skeletal α-actin-based vectors.**

Insulin-like growth factor I (IGF-I),[1] a peptide growth factor that is structurally related to proinsulin (1–4), has a primary role in promoting the differentiation and growth of skeletal muscle. The effects of IGF-I on myogenic cells include stimulation of myoblast replication, myogenic differentiation, and myotube hypertrophy (see Refs. 5 and 6 for review). *In vivo*, up-regulation of IGF-I expression in skeletal muscle is coincident with myotube formation in the developing embryo (7), stretch-induced myofiber hypertrophy (8) and muscle regeneration following injury (9), suggesting that IGF-I serves as an autocrine/paracrine mediator of these processes in skeletal muscle. Increased biosynthesis and extracellular secretion of IGF-I from cultured mammalian myoblasts has been shown to be coincident with myoblast alignment, withdrawal from the cell cycle, and fusion (5, 6). In addition, inclusion of IGF-I in the media of primary cultures of avian myofibers has been shown to elicit larger fiber diameters, a near doubling in myosin content and substantial increases in protein stability and synthesis in comparison to untreated cultures (10)

The effects of IGF-I overexpression have previously been studied in cultured cells and in transgenic mice, but these earlier studies did not address the effects of IGF-I expression on skeletal muscle specifically (11, 12). Mathews *et al.* (11) utilized the metallothionein promoter to drive expression of an hIGF-I cDNA in transgenic mice resulting in IGF-I overexpression in a broad range of visceral internal organs and increased concentrations of IGF-I in serum. These transgenic mice exhibited an increase in body weight and organomegaly, but only a modest improvement in muscle mass. Thus, in order to test the effects of IGF-I overexpression on muscle growth and physiology *in vivo*, we reasoned that it would be necessary to target its overexpression specifically to striated muscle.

The α-skeletal actin gene is a member of the actin multigene family which, in vertebrates, is made up of three distinct classes of actin isoforms termed as "cytoplasmic," "striated," and "smooth muscle" on the basis of their cellular distribution and pattern of expression in adult tissues (13–15). The striated actins, α-cardiac and α-skeletal, are co-expressed in embryonic heart and skeletal muscle, and examination of the switching of actin gene expression studied during myogenesis of birds and small mammals indicated that paired vertebrate sarcomeric cardiac and skeletal α-actin genes are up-regulated sequentially during early muscle development, whereas only skeletal α-actin is maintained at high levels in adult skeletal muscle but reduced in cardiac tissue (13, 15, 16). At adulthood, skeletal α-actin accounts for approximately 8% of the poly(A) RNA in avian skeletal muscle (16) and is expressed in all classes of myofibers (17). To determine how the avian skeletal α-actin gene was regulated during striated muscle differentiation, our laboratory (18) and others (19) identified the rough 5′ regulatory boundary at −202 base pairs (bp) which harbors evolutionarily conserved regulatory elements that accurately initiate skeletal α-actin transcripts and induce transcripts from reporter genes in differentiated skeletal muscle cells and cardiomyocytes. Mouse transgenic studies by Petropulous *et al.* (20) indicated that the conserved sequences in the proximal 200 bp of the promoter region were primarily responsible for the avian skeletal α-actin gene's restricted expression pattern in heart and skeletal muscle. However, these studies also revealed a high degree of ectopic expression suggesting that additional regulatory elements from the skeletal α-promoter are required for strict striated muscle-specific expression. Recent research implicates the contiguous 3′-flanking region of the human skeletal α-actin gene in directing correct temporal and spatial expression of skeletal actin-based transgenes in

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[1] The abbreviations used are: IGF, insulin-like growth factor; hIGF, human IGF; bp, base pair(s); kb, kilobase(s); IGFBP, insulin-like growth factor-binding protein(s).

mice (21), and we have recently observed in transfection assays *in vivo* that potent activating sequences are located immediately upstream of the core promoter in the region spanning −424 to −202 bp upstream of the transcription start site (22).

We describe here the construction of a myogenic expression vector containing regulatory elements from both the 5′- and 3′-flanking regions of the avian skeletal α-actin gene and its application for overexpression of an hIGF-I cDNA in cultured muscle cells and transgenic mice. Our study indicates that IGF-I overexpression in cultured muscle cells caused precocious alignment and fusion of myoblasts into terminally differentiated myotubes and elevated the levels of myogenic basic helix-loop-helix factors, intermediate filament, and contractile protein mRNAs. Transgenic mice carrying a single copy of this hybrid skeletal α-actin/hIGF-I transgene had hIGF-I mRNA levels that were approximately half those of the endogenous murine skeletal α-actin gene on a per allele basis, while conferring tissue-specific expression activity. In transgenic mice we observed that elevated levels of IGF-I in skeletal muscle caused uniform muscle hypertrophy without increases in overall body weight or circulating IGF-I concentration.

## MATERIALS AND METHODS

*Construction of α-Skeletal Actin / IGF-I Hybrid Genes*—Organization of the avian skeletal α-actin gene (14, 23) and identification of 5′ regulatory boundaries have been described previously (18, 24). In order to construct hybrid skeletal α-actin/IGF-I genes, the 5′ core promoter, upstream activating sequences (−202 to −424), natural capsite, 5′-UTR (exon 1), first intron, and portions of exon 2 up to the initiation ATG were obtained from 2.3 kb of α-SK cloned into M13mp18 (25). Site-directed mutagenesis was utilized to replace the sequences at the initiation ATG of α-SK733 and the hIGF-I cDNA (26) with an *Nco*I site based on the methodology of Kunkel (27) utilizing single-stranded uridine-substituted M13 DNA from the BW313 (*dut*⁻, *ung*⁻) host upon superinfection with helper phage m13K07. Two oligonucleotides were synthesized such that each contained a central *Nco*I site flanked by 12 nucleotides on each side complementary to the non-coding strand produced by the phagemid. The oligonucleotides were annealed to the uridine-substituted template and extended with the Klenow fragment of DNA polymerase I. Double-stranded plasmids were sealed with T4 DNA ligase, and the synthetic strands carrying the *Nco*I mutations were selected upon transformations of a *dut*⁺ *ung*⁺ *Escherichia coli* host, DH5α. The *Nco*I-containing mutants were then sequenced to verify the mutation and the integrity of adjoining sequences. SK733 IGF-I was created by subcloning the full-length hIGF-I cDNA (26), including the native IGF-I 3′-UTR and poly(A) addition site, into this construction on an *Nco*I/*Hin*dIII fragment adjacent to the skeletal α-actin *Nco*I site. A *Nae*I/*Hin*dIII fragment containing the skeletal α-actin 3′-UTR and contiguous 3′-noncoding region was excised from the avian skeletal α-actin genomic clone and then subcloned into *Eco*RV/*Hin*dIII-digested pBluescript II to form SK 3′. SK733 IGF-I 3′-SK was then created by subcloning the actin promoter and hIGF-I cDNA, minus the 3′-UTR and poly(A) addition site from SK733 IGF-I on a *Bam*HI fragment into the *Bam*HI site of SK 3′.

*Cell Culture*—The murine myoblast line $C_2C_{12}$ (28) was utilized for generation of stable transfectants. Stable transfection of myoblasts was achieved by co-transfection of skeletal α-actin/IGF-I constructs with the drug selectable-vector EMSV-hygromycin. The EMSV-hygromycin vector was created by cloning the hygromycin resistance gene (29) into the EMSV expression plasmid (30). Transfection and selection with hygromycin B (150 μg/ml) were performed as described previously (31). Stable transfectant myoblasts were maintained in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, 25 μg/ml gentamycin sulfate, and 150 μg/ml hygromycin B (Boehringer Mannheim, Mannheim, Germany). Myoblasts were switched to Dulbecco's modified Eagle's medium containing 0.05% (w/v) bovine serum albumin to permit myogenic differentiation and study of the expression activity of the skeletal α-actin/IGF-I hybrid gene.

*Generation of Transgenic Mice*—One-cell mouse embryos resulting from matings of FVB strain mice (Taconic, Germantown, NY) were microinjected with approximately 2 pl of linearized, gel-purified DNA (2 ng/ml) representing the gene constructs of interest as described previously (32). After microinjection, embryos were transferred to pseudopregnant females. Founder mice and subsequent generations were screened for the presence of transgenes by Southern blot analysis of genomic DNA (33). Transgene copy number was determined by comparing the hybridization signal for 10 μg of mouse genomic DNA to that of known quantities of purified DNA constructs diluted in 10 μg of salmon sperm DNA using dot-blot hybridization. Hybridization signal was quantitated using a Betascope model 603 blot analyzer (Betagen, Waltham, MA).

*RNA Isolation and Northern Blot Analysis*—Total RNA was isolated from cells and tissues by selective precipitation from phenol-extracted, guanidine thiocyanate homogenates as described previously (34). Northern blots were prepared by size fractionation of total RNA samples on 1% agarose, 2.2 M formaldehyde gels, and subsequent capillary transfer to GeneScreen nylon membranes (DuPont NEN). After transfer, RNA was cross-linked to the membrane by exposure to approximately 120,000 μJ UV (254 nM), and the membranes were then baked at 80 °C for 2 h. All prehybridizations and hybridizations were performed in 50% (v/v) formamide, 5 × SSPE, 5 × Denhardt's (0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) ficoll), 1% (w/v) SDS, 10% (w/v) dextran sulfate, 200 μg/ml sheared salmon sperm DNA. Northern blots were hybridized with either $^{32}$P-labeled DNA or antisense RNA probes as denoted for specific probes (see figure legends). Hybridizations with DNA probes were carried out for 16 h at 45 °C with $10^6$ cpm/ml probe. Membranes were subsequently washed 2 × 30 min in 2 × SSPE, 1% (w/v) SDS at 68 °C and then 1 × 30 min in 0.5 × SSPE, 0.1% (w/v) SDS at 55 °C. Blots were subsequently exposed to x-ray film (Kodak X-Omat AR) at −70 °C with intensifying screens.

*Immunohistochemistry and IGF-I Radioimmunoassay*—IGF-I concentrations in conditioned media were determined by radioimmunoassay as described previously (35). Immunostaining of $C_2C_{12}$ myoblasts was performed using a 1:200 dilution of rabbit anti-hIGF-I antiserum (35) as the primary antibody and a goat anti-rabbit IgG immunoperoxidase staining kit (Vectastain, Burlingame, CA). IGF-I concentrations in acid extracts of skeletal muscle and acid/ethanol extracts of serum were determined using a radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). Acid extracts of skeletal muscle were prepared essentially as described previously (4). Briefly, frozen hind limb muscle was powdered under liquid $N_2$ and then homogenized 1:5 (w/v) in 1 M acetic acid using a Polytron tissue homogenizer. Homogenates were incubated on ice for 2 h and then centrifuged at 3,000 × *g* for 15 min at 4 °C. The supernatant fraction was removed to a new tube, and the pellet was re-extracted by disbursing into 5 × volume of 1 M acetic acid and incubation on ice for 2 h. The mixture was centrifuged at 3,000 × *g* for 15 min at 4 °C. Both supernatant fractions were then pooled and lyophilized. The lyophilized extract was reconstituted in 0.1 × volume of 50 mM Tris-Cl (pH 7.8) and then clarified by centrifugation prior to radioimmunoassay.

*Staining of Myofibers for Morphometric Analysis*—The superficial gluteus and gastrocnemius (female only) muscles were dissected immediately after sacrifice and affixed to a 1-cm diameter cork sheet with OCT (Miles) freezing media with the aid of a dissecting microscope for proper orientation. Muscle samples were then frozen by immersion into a container of isopentane cooled in liquid $N_2$ for 10 s. Frozen muscle samples were stored in humidified (*i.e.* one small chip of wet ice) glass scintillation vials at −70 °C until sectioning in the cryostat. Tissues were allowed to warm up to approximately −15 °C in the cryostat prior to sectioning, and sections were taken at 6 μm. Staining for succinate dehydrogenase activity was performed as described previously (36). Following staining for succinate dehydrogenase activity the sections were fixed in formol saline, washed in distilled water, and coverslipped using an aqueous mounting medium. Morphometric analysis of succinate dehydrogenase-stained myofibers was conducted with the aid of a computer-based image analysis system running Optimas image analysis software (Bioscan, Edmonds, WA). Four fields per animal, encompassing at least 290 total myofibers, were analyzed. Myofibers in the superficial gluteus muscle exhibited three distinct staining intensities for succinate dehydrogenase and were thus classified as high, medium, or low for data analysis (37).

## RESULTS

*Skeletal α-Actin 3′-UTR Enhances the Accumulation of IGF-I mRNA through mRNA Stabilization*—In order to examine these parameters, we constructed two IGF-I expression vectors based on the avian skeletal α-actin gene which is normally activated during withdrawal from the cell cycle and myoblast fusion (16). Schematic representations of the two α-actin/hIGF-I hybrid constructions that were utilized for the studies

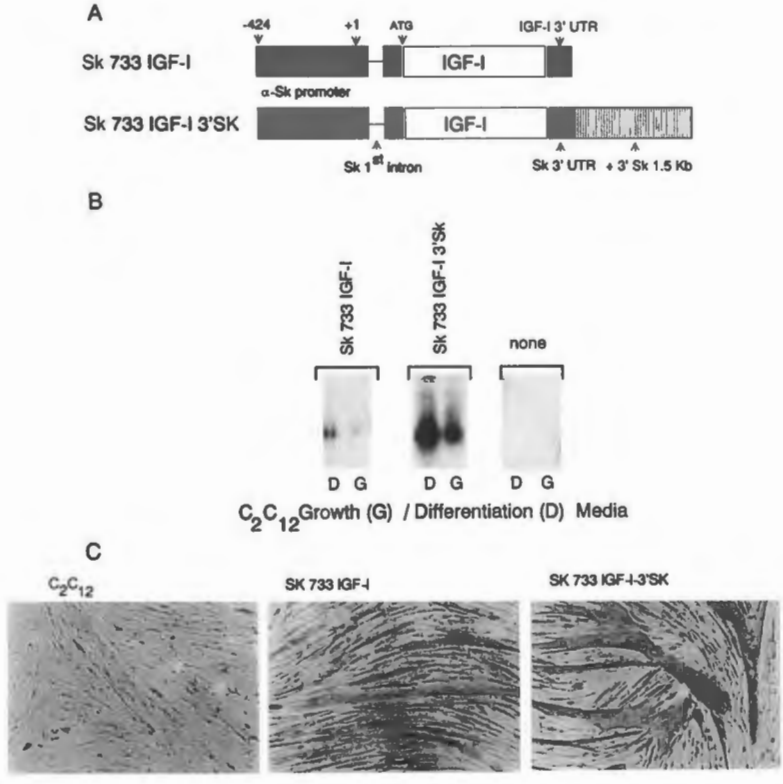


FIG. 1. **The avian skeletal α-actin 3′-UTR and contiguous 3′-noncoding region fostered robust expression of skeletal α-actin/IGF-I hybrid genes in differentiated $C_2C_{12}$ myoblasts.** *A*, schematic diagram of the skeletal α-actin/IGF-I hybrid gene constructions show the avian skeletal α-actin promoter −424 to +1 (*angled lines*), the natural capsite (+1), and the 5′-UTR, (exon 1, *cross-hatch*, 60 bp), first intron (*line*, 123 bp), portions of exon 2 up to the initiation ATG (*cross-hatch*, 15 bp), and human IGF-I cDNA (*open box*, 504 bp). The SK733 IGF-I construct contains the IGF-I 3′-UTR (*filled box*, 240 bp), while the SK733 IGF-I 3′-SK construct contains the skeletal α-actin 3′-UTR (*mini boxes*, 310 bp) and contiguous 1.5 kb of noncoding sequences (*vertical lines*) as shown above, but not drawn to scale. *B*, Northern blots of total RNA samples (10 μg) isolated from $C_2C_{12}$ myoblasts cultured in either growth media (*G*) or differentiation media (*D*) were hybridized with a $^{32}$P-labeled hIGF-I cDNA probe (26). RNA samples isolated from myoblasts cultured in differentiation media were harvested 48 h after switching from growth to differentiation media. *C*, stably transfected $C_2C_{12}$ myoblasts carrying the skeletal α-actin/IGF-I hybrid genes described in *A* were grown to approximately 80% confluence in growth media and then switched to differentiation media and cultured for 4 days. Differentiated cultures were immunostained with a rabbit anti-hIGF-I antiserum (generous gift of Dr. Ray Hintz, Stanford University) and a goat anti-rabbit IgG immunoperoxidase kit (Vectastain, Burlingame, CA) and then photographed at equal magnification. Conditioned media from a population of stable transfected myoblasts were assayed for IGF-I (Table I).

reported herein are shown in Fig. 1*A*. These IGF-I expression vectors were evaluated for developmental expression, IGF-I biosynthesis, and their influence on myogenesis in stably transfected myogenic $C_2C_{12}$ cells. Examination of IGF-I mRNA content was evaluated by hybridizing Northern blots with a $^{32}$P-labeled human IGF-I cDNA probe to total RNA isolated from myoblasts that were switched from growth to differentiation media as displayed in Fig. 1*B*. An approximate 1 to 2 orders of magnitude increase in IGF-I mRNA accumulation was detected in fused $C_2C_{12}$ myotubes containing SK733 IGF-I 3′-SK in comparison to the expression of SK733 IGF-I. The avian skeletal α-actin promoter displayed developmentally restricted expression with the IGF-I 3′-UTR, but replacement with the skeletal α-actin 3′-UTR and contiguous 3′-noncoding sequences led to dramatically increased expression of IGF-I mRNA upon myogenic differentiation.

The steady state levels of different mRNAs reflect the balance between the rate of synthesis of new mRNA and the rate of mRNA degradation. We asked if one of the regulatory roles of the skeletal α-actin 3′-UTR is to impart mRNA stability. A transcription blocker, actinomycin D (8 μg/ml), was added to differentiated myogenic cultures in order to measure relative mRNA stabilities by monitoring total mRNA content after inhibiting RNA synthesis. Timed samples were removed for Northern blot analysis as shown in Fig. 2. Transcripts containing the natural IGF-I 3′-UTR were found to turn over rapidly (half-life of less than 1 h) in contrast to skeletal α-actin transcripts which had greater stability with a half-life estimated to be more than 6–8 h under conditions with actinomycin D, a highly toxic drug (see Schwartz (38)). These data indicate that the avian skeletal α-actin 3′-UTR conferred increased mRNA stability to a heterologous RNA species.

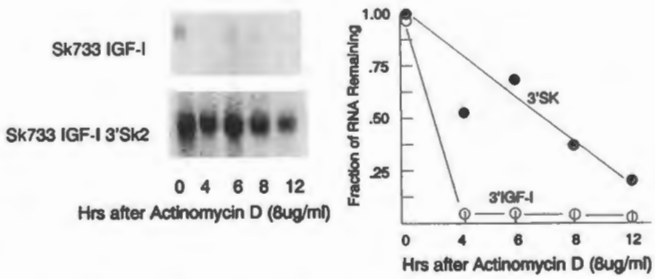


FIG. 2. **The avian skeletal α-actin 3′-UTR increased the half-life of hIGF-I mRNA in $C_2C_{12}$ myoblasts transfected with skeletal α-actin/IGF-I hybrid genes.** Pooled populations of $C_2C_{12}$ myoblasts stably transfected with the actin/IGF-I constructs described in Fig. 1 were grown to approximately 80% confluence and then switched to differentiation media. After 48 h, actinomycin D (8 μg/ml) was added followed by isolation of total RNA at 0, 4, 6, 8, and 12 h later. Northern blots of total RNA samples (10 μg) were hybridized with a $^{32}$P-labeled hIGF-I cDNA probe (26). Hybridization signal was quantified by densitometry and expressed relative to the hIGF-I mRNA levels at the time of actinomycin D addition for each construct to depict rates of hIGF-I mRNA turnover.

*Expression of IGF-I in Stably Transfected Myoblasts Causes*

TABLE I
*IGF-I concentrations in media conditioned by stable transfectant muscle cells*

| Construction | Days in serum-free media | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| | *ng/ml IGF-I* | | | |
| EMSV hygromycin | 0.6 | 0.4 | 0.6 | 0.4 |
| SK733 IGF-I | 0.9 | 1.0 | 1.3 | 4.4 |
| SK733 IGF-I 3′-SK | 22.2 | 30.1 | 59.4 | 79.4 |

*Precocious Myoblast Fusion and Enhances Myogenic Gene Expression*—Previous studies indicate that the progression of myoblasts through the terminal differentiation pathway might be directed by the autocrine/paracrine action of IGFs (39, 40). We observed in confluent cultures that myoblasts containing SK733 IGF-I and SK733 IGF-I 3′-SK exhibited a greater degree of fusion after switching to differentiation media as compared to control myoblasts (Fig. 1*C*). Immunoperoxidase staining of myogenic cultures revealed increased production of immunologically reactive IGF-I in myotubes of stably transfected myoblasts, but not in control $C_2C_{12}$ myoblasts stably transfected with an EMSV-hygromycin resistance gene (Fig. 1*C*). The most intense staining resided with the expression of the SK733 IGF-I 3′-SK construct. These data provide qualitative evidence that increased IGF-I expression coincided with enhanced myotube formation. After determining that SK733 IGF-I and SK733 IGF-I 3′-SK were effective in driving IGF-I expression in muscle cells, we quantified the biosynthesis of this factor by radioimmunoassay of conditioned tissue culture media (Table I). Levels of IGF-I in control cultures were no greater than 0.6 ng/ml. In comparison, cultures stably transfected with the vector SK733 IGF-I 3′-SK had accumulated levels of IGF-I in culture media that were at least 150 times greater than control myoblasts after 4 days in culture. Cultures stably transfected with SK733 IGF-I produced IGF-I at approximately 5% the level of cultures containing the SK733 IGF-I 3′-SK vector. These results suggest that substitution of the skeletal α-actin 3′-UTR and contiguous noncoding region for the native IGF-I 3′-UTR significantly enhanced IGF-I expression.

Under serum-free culture conditions, we wanted to determine how IGF-I influenced myogenic gene activity. Northern blots of total RNA isolated from myoblasts cultured in growth media with IGF-I demonstrated enhanced expression of the myogenic basic helix-loop-helix factors such as MyoD and myogenin in general agreement with Florini and co-workers (40). As shown in Fig. 3, we observed a correspondence between elevated IGF-I expression and increased expression of myogenic specified gene products, such as the intermediate filament protein desmin, and skeletal α-actin.

*Actin/IGF-I Transgene Expression in Mice Results in a Localized Increase in IGF-I and Myofiber Hypertrophy*—A line of mice harboring a single copy of the skeletal α-actin/IGF-I transgene (SK733 IGF-I 3′-SK), which was shown to be strongly expressed in differentiated cultures of myogenic $C_2C_{12}$ cells (see Figs. 1 and 3), was utilized for study of the effects of overexpression of IGF-I in skeletal muscle. The level and tissue specificity of transgene expression in this line of mice was similar to that of the endogenous skeletal α-actin gene being restricted to striated muscle and more abundant in skeletal muscle than in heart (Figs. 4 and 5). An abundant 1.1-kb hIGF-I mRNA was detected on Northern blots of total RNA from skeletal muscle of transgenic mice that was not present in control mice (Fig. 5*A*). Expression of the transgene in this single copy line of mice was approximately 50% that of the endogenous skeletal α-actin gene in hind limb muscle of adult mice (Fig. 5*C*). High level transgene expression in the present study resulted in concentrations of IGF-I in acid extracts of

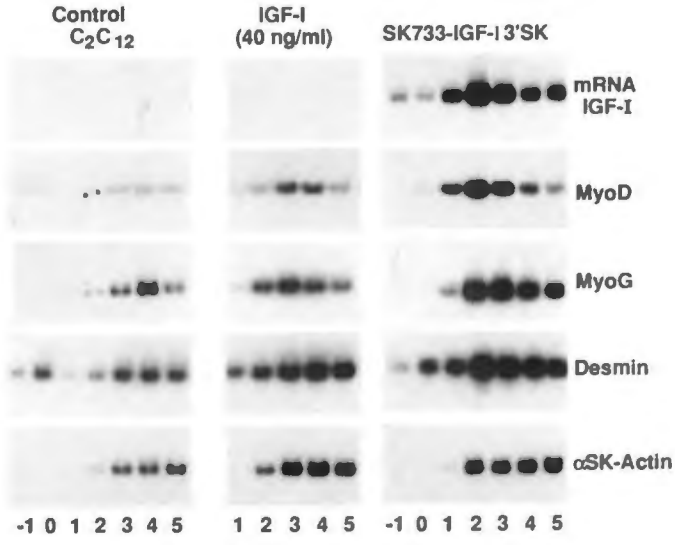


FIG. 3. **Overexpression of IGF-I in muscle cell-stimulated myogenic gene activity.** Control $C_2C_{12}$ myoblasts and myoblasts stably transfected with the actin/IGF-I hybrid gene SK733-IGF-I 3′-SK were grown to approximately 80% confluence and then switched to differentiation media (day 0). Replicate plates of control $C_2C_{12}$ myoblasts were switched to differentiation media (day 0) with and without 40 ng/ml hIGF-I. Total RNA was isolated at −1, 0, 1, 2, 3, and 4 days after switching to differentiation media. Northern blots of total RNA samples (10 mg) were probed for human IGF-I (26), murine MyoD (51), murine myogenin (MyoG, 52), murine desmin (53), and murine skeletal α-actin (54) mRNAs using $^{32}$P-labeled cDNA probes. *DMEM*, Dulbecco's modified Eagle's medium; *BSA*, bovine serum albumin.

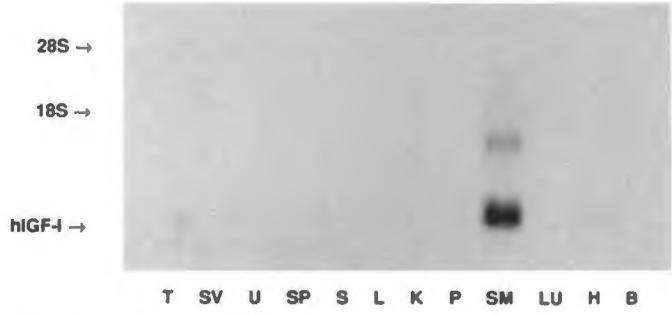


FIG. 4. **The SK733 IGF-I 3′-SK hybrid gene is expressed specifically in striated muscle of transgenic mice.** A Northern blot of total RNA (20 μg) isolated from individual tissue pools of 2 female and 2 male (except as noted below) SK733 IGF-I 3′-SK transgenic mice was hybridized to a $^{32}$P-labeled DNA probe derived from the 3′-UTR of the avian skeletal α-actin gene (16). Abbreviations for individual tissues are: *T*, testis ($n$ = 2 male mice); *S*, seminal vesicles ($n$ = 2 male mice); *U*, uterus ($n$ = 2 female mice); *SP*, spleen; *S*, stomach; *L*, liver; *K*, kidney; *P*, pancreas; *SM*, skeletal muscle; *LU*, lung; *H*, heart; and *B*, brain. Hybridization and washing conditions are described under "Materials and Methods."

skeletal muscle from transgenic mice being at least 47-fold greater than in wild type mice (Table II). Interestingly, IGF-I concentrations in serum of transgenic mice were not elevated relative to age-matched wild type mice (Table II) leading us to conclude that the hIGF-I resulting from transgene expression did not enter the circulation in appreciable quantities. This conclusion is further supported by the lack of an effect of transgene expression on body weight (Table II) or endogenous IGF-I expression in liver (Fig. 5*A*).

Morphometric analysis was performed on cross-sections of the superficial gluteus muscle stained for the mitochondrial enzyme succinate dehydrogenase (Table III, Fig. 6). Myofibers were grouped based on their succinate dehydrogenase staining

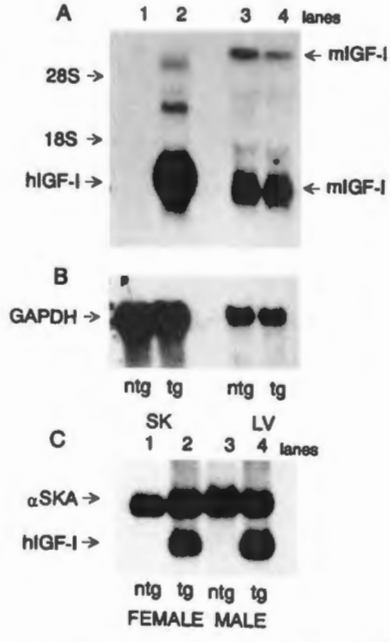


FIG. 5. **Expression of the single-copy SK733 IGF-I 3′-SK hybrid gene in transgenic mice is approximately one-half of the level of the endogenous murine skeletal α-actin gene.** *A*, total RNA (20 μg) from pooled hind limb muscle and liver of transgenic (*tg*) and nontransgenic littermate control (*ntg*) mice was hybridized to a $^{32}$P-labeled hIGF-I cDNA probe (26). *A* and *B* show blots of RNA from ntg (*lane 1*) and tg (*lane 2*) skeletal muscle and from ntg (*lane 3*) and tg (*lane 4*) liver tissues. The hybridization signal in liver depicts expression of the endogenous mIGF-I gene. *B*, the blot depicted in *A* was stripped and subsequently hybridized with a $^{32}$P-labeled murine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe to demonstrate approximately equal RNA loading. *C*, total RNA pooled ($n$ = 2) from skeletal muscle of age-matched control mice (*ntg*) and adult transgenic (*tg*) from female (*lanes 1* and *2*) and male (*lanes 3* and *4*) mice was hybridized simultaneously to murine skeletal α-actin (54) and hIGF-I (26) cDNA probes labeled to approximately the same specific activity by polymerase chain reaction. Analysis of the hybridization signal by direct counting on a Betascope (Betagen, Waltham, MA) indicated that expression of the SK733IGF-I 3′-SK hybrid gene in skeletal muscle was approximately 50% that of the endogenous murine skeletal α-actin gene on a per allele basis. Hybridization and washing conditions are described under "Materials and Methods."

TABLE II

*Body weight and IGF-I concentrations in serum and skeletal muscle of SK733 IGF-I 3′-SK and age-matched control mice*

Data presented are mean ± S.D. for adult male transgenic and age-matched control mice.

| | $n$ | Control | Transgenic |
|---|---|---|---|
| Body weight (g) | 4 | 31.2 (.35) | 32.1 (2.5) |
| Serum IGF-I (ng/ml) | 4 | 475 (47.7) | 453 (40.3) |
| Skeletal muscle IGF-I (ng/g) | 4 | 5.68 (5.26)[a] | 269 (57.5) |

[a] Means are significantly different ($p < 1.01$) by Student's $t$-test.

TABLE III

*Morphometric analysis of succinate dehydrogenase-stained myofibers in the superficial gluteus muscle of SK733 IGF-I 3′-SK and littermate control mice*

Data presented are mean ± S.D. for two male SK733 IGF-I 3′-SK and two male littermate control mice and mean for one female SK733 IGF-I 3′-SK and one female littermate control.

| | Succinate dehydrogenase staining intensity | | |
|---|---|---|---|
| | High | Intermediate | Low |
| Myofiber area (μm²)[a] | | | |
| Female | | | |
| Control | 674 | 1256 | 1975 |
| SK733 IGF-I 3′-SK | 1447 | 2435 | 3518 |
| Male | | | |
| Control | 945 (226) | 1595 (272) | 3899 (559) |
| SK733 IGF-I 3′-SK | 1279 (2.12) | 2203 (44.5) | 4568 (291) |
| Percent fiber number[b] | | | |
| Female | | | |
| Control | 10.9 | 17.3 | 71.7 |
| SK733 IGF-I 3′-SK | 31.4 | 16.4 | 52.1 |
| Male | | | |
| Control | 16.4 (6.1) | 17.8 (3.4) | 65.8 (2.7) |
| SK733 IGF-I 3′-SK | 24.6 (2.0) | 17.0 (4.6) | 58.4 (6.6) |
| Percent cross-sectional area[c] | | | |
| Female | | | |
| Control | 4.3 | 12.7 | 83.0 |
| SK733 IGF-I 3′-SK | 16.9 | 14.8 | 68.2 |
| Male | | | |
| Control | 5.1 (1.6) | 9.4 (1.7) | 85.6 (.07) |
| SK733 IGF-I 3′-SK | 9.4 (.78) | 11.2 (3.2) | 79.5 (4.0) |

[a] Myofiber area is given in μm² as determined using Optimas image analysis software (Bioscan, Edmonds, WA).

[b] Percent fiber number represents the percentage of all fibers measured that were classified as either high, intermediate, or low with regard to succinate dehydrogenase staining intensity.

[c] Percent cross-sectional area represents the sum of myofiber cross-sectional areas for myofibers classified as either high, intermediate, or low with regard to succinate dehydrogenase staining intensity expressed as a percentage of the sum of all myofiber cross-sectional areas measured.

intensity as either high, medium, or low intensity as has been described previously (37). Results indicate that overexpression of IGF-I in skeletal muscle resulted in hypertrophy of myofibers regardless of succinate dehydrogenase staining intensity. This was evidenced by increases in the cross-sectional area of myofibers that ranged from 115% for high intensity succinate dehydrogenase fibers in a female transgenic to 17% for low intensity succinate dehydrogenase fibers in male transgenic mice as compared to wild type littermates. In addition to an increased cross-sectional area of myofibers, morphometric analysis revealed a trend toward a higher proportion of high intensity succinate dehydrogenase myofibers (*e.g.* 31.4% *versus* 10.9% in female mice) and a lower proportion of low intensity succinate dehydrogenase myofibers (*e.g.* 52.1% *versus* 71.7% in female mice) in transgenic *versus* littermate wild type mice. A previous study (37) has established that high intensity succinate dehydrogenase staining of myofibers correlates with fibers classified as either 2A or 2X based on myosin ATPase staining, whereas medium intensity succinate dehydrogenase staining correlates with type 1 fibers and low intensity succinate dehydrogenase staining corresponds to type 2B fibers. These data indicate that IGF-I overexpression induced a shift toward more oxidative fiber types (likely types 2A and 2X), in addition to increasing the relative cross-sectional area of all classes of myofibers.

## DISCUSSION

*Targeting IGF-I Expression to Striated Muscle*—In most vertebrates, skeletal α-actin is the predominant striated actin isoform expressed in adult skeletal muscle, whereas it is expressed at a much lower level in cardiac muscle in which the α-cardiac isoform predominates (5). Results from previous studies utilizing transient transfection assays have suggested that the regulatory elements responsible for cell type restricted and developmental expression reside within the proximal 202 bp of the avian skeletal α-actin gene promoter (18, 19), and, in general, these observations were corroborated in earlier transgenic mouse studies (20, 41, 42). However, Petropoulos *et al.* (20) also reported that transgenes driven by either the avian skeletal α-actin proximal promoter (−197 to +27 bp) or more extensive 5′-flanking sequence (−2200 to +27 bp) exhibited ectopic expression in several transgenic lines in addition to increased expression in adult heart relative to skeletal muscle. We observed in the present study that inclusion of the skeletal α-actin 3′-UTR, along with approximately 1.5 kb of contiguous

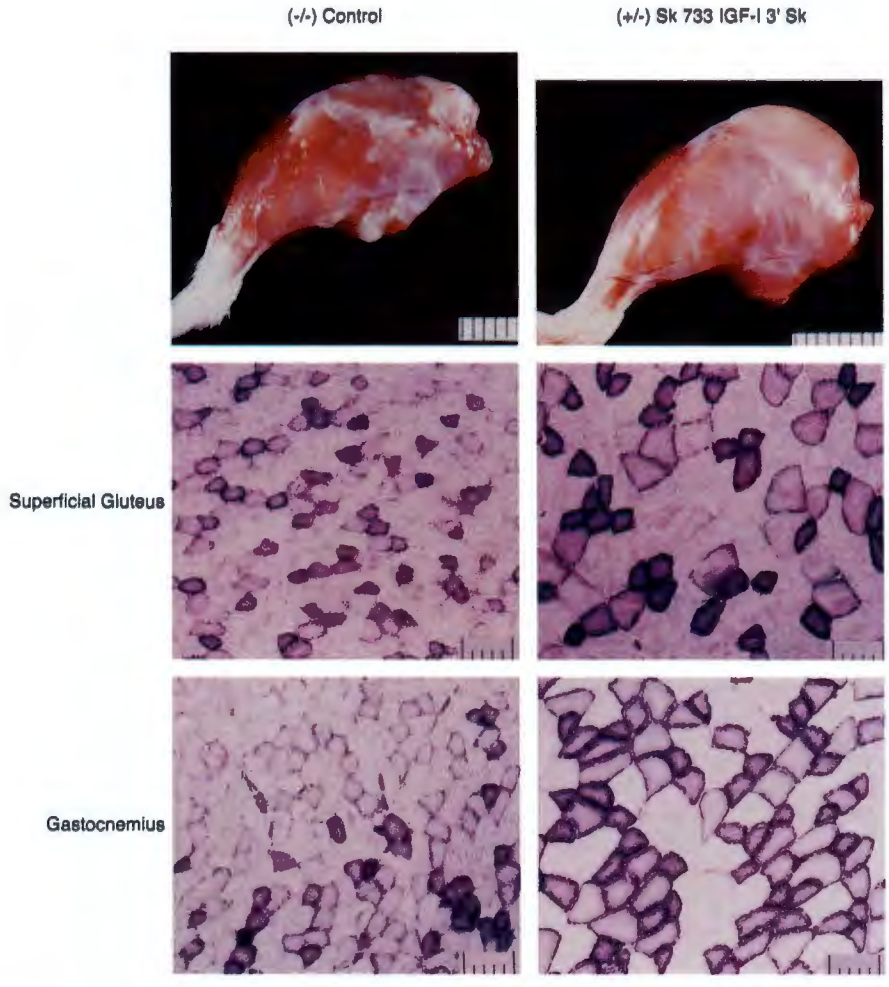


FIG. 6. **Expression of the SK733 IGF-I 3′-SK hybrid gene in transgenic mice-induced muscle hypertrophy.** Hind limbs from adult (8 months of age) littermate transgenic and control mice (female) were skinned and photographed with a ruler marker in millimeters at equal magnification prior to dissection. Cross-sections were taken from the approximate midpoint of the superficial gluteus and gastrocnemius muscles, stained for succinate dehydrogenase (36), and then photographed at the same magnification (see *ruler* in photographs). Data for morphometric analysis of fibers in the superficial gluteus muscle are presented in Table III.

3′-flanking sequence in a chimeric actin/IGF-I transgene dramatically increased its expression in cultured myotubes and resulted in expression being restricted to striated muscle in transgenic mice. Moreover, this transgene's level of expression in skeletal muscle was similar to that of the endogenous murine skeletal α-actin gene. Together, these data suggest that the elements required to mimic the level and tissue specificity of expression for the endogenous murine skeletal α-actin gene are contained within the SK733 IGF-I 3′-SK construct.

Inclusion of the skeletal α-actin 3′-UTR and contiguous 3′-flanking sequence in a chimeric actin/IGF-I construct dramatically increased steady state mRNA and protein levels in cultured myotubes, and our data suggest that a primary mechanism whereby the skeletal α-actin 3′-UTR increased expression of the actin/IGF-I hybrid gene *in vitro* was through increasing mRNA half-life. This was not surprising since we recently demonstrated the selective turnover of the cytoplasmic β-actin *versus* stabilization of striated α-actin mRNAs in primary myogenic cultures, and sequence differences between these evolutionarily conserved actin mRNA isoforms in their 3′-UTRs have been implicated in determining the intrinsic half-life and cellular content of the respective mRNAs (43). However, the degree to which the skeletal α-actin 3′-UTR influences the level of transgene expression *in vivo* is less clear. Brennan and Hardeman (21) reported that substituting a CAT reporter with 3′ SV40 sequences for the human skeletal α-actin coding and 3′ contiguous flanking sequences decreased expression by less that 50%. In addition to putative effects of the 3′-UTR on mRNA half-life, recent research suggests that the 3′-flanking regions of the human striated actin genes contain elements that further restrict expression of transgenes driven by either the human skeletal or cardiac α-actin promoter to striated muscle as well as influencing their developmental pattern of expression (21, 44). We have also observed that the aberrant expression of transgenes driven by the avian skeletal α-actin proximal promoter (*i.e.* −202 to −11 bp) can be eliminated by substituting the skeletal α-actin 3′-UTR and approximately 1.5 kb of contiguous noncoding sequence for the SV40-derived 3′ sequences (data not shown). Thus, in addition to the 5′ promoter and upstream region, the 3′-noncoding regions of the striated actin genes contain regulatory regions necessary for appropriate developmental and tissue-restricted expression of skeletal α-actin-based transgenes, and we conclude that avian skeletal α-actin-based expression vectors comprised of the skeletal α-actin proximal promoter, 3′-UTR and contiguous 3′-flanking sequence can be utilized to target high level expression of heterologous transgenes specifically to striated muscle.

*Overexpression of IGF-I Enhances Muscle Growth*—Numerous studies *in vitro* have established that IGF-I elicits pleiotropic effects on myogenic cells including stimulation of myoblast replication and myogenic differentiation (see Refs. 5 and 6 for review). We observed that myoblasts transfected with SK733 IGF-I 3′-SK expressed higher levels of muscle-specific mRNAs than did myoblasts treated with 40 ng/ml IGF-I even though the concentration of IGF-I in media conditioned by these cells for 48 h was lower. This observation suggests that the IGF-I derived via a sustained release autocrine/paracrine mechanism was more effective as a myogenic stimulus than exogenous IGF-I administered as a bolus. One possible mechanism underlying this observation is that a myoblast-derived insulin-like

growth factor-binding protein(s) (IGFBP) might potentiate the effects of autocrine/paracrine-derived IGF-I. In this regard, we did observe that expression of IGFBP-5, an IGFBP that is specifically induced upon differentiation of $C_2C_{12}$ myoblasts (45), was coordinately up-regulated with overexpression of IGF-I *in vitro* (data not shown). Although beyond the scope of this report, these observations suggest that the model of overexpression of IGF-I in myoblasts may provide novel insights into the potential role of IGFBPs in regulating myoblast proliferation and differentiation.

Recently, genetic truncations of the single murine IGF-I gene (46, 47) and of the type I IGF receptor (48) have provided direct evidence *in vivo* for the ascribed functions of IGF-I in skeletal muscle development. Powell-Braxton *et al.* (46) reported that IGF-I mutant mice showed severe muscular dystrophy and highly reduced myofibrillar organization in both heart and skeletal muscle. They observed that the majority of IGF-I mutant mice die at birth due to respiratory failure likely caused by incomplete maturation of the diaphragm and intercostal muscles. Liu *et al.* (48) reported that mice lacking a functional allele for the type I IGF receptor exhibited generalized cell hypoplasia and specifically had a reduced number of myonuclei and myofibers. In addition to evidence from gene knockout experiments of the role of IGF-I in the development of skeletal muscle, localized up-regulation of IGF-I expression has been implicated as a mediator of stretch-induced myofiber hypertrophy and muscle regeneration (8, 9). Together, these observations lend support to a model in which IGF-I is a central trophic growth factor required for the proliferation of myoblasts, the progression of myogenic differentiation, and subsequent growth and hypertrophy of myofibers. In addition, they suggest that, depending on the developmental timing, overexpression of IGF-I in skeletal muscle *in vivo* could potentially elicit effects at multiple stages of skeletal muscle development. Since expression of the skeletal $\alpha$-actin gene is normally not observed until relatively late in muscle development (*i.e.* after myoblast fusion (15, 16)), overexpression of IGF-I *in vivo* via a skeletal $\alpha$-actin-derived vector is not likely to elicit dramatic effects on myoblast proliferation or differentiation. We observed in the present study that transgenic overexpression of IGF-I elicited pronounced hypertrophy of all classes of myofibers. In addition, results also indicated that overexpression of IGF-I induced a shift in myofiber type toward more oxidative fiber types. This latter observation is consistent with findings from a previous study which indicated that growth hormone treatment of hypophysectomized rats, presumably acting through increased expression of IGF-I, elicited an increase in the relative proportion of type I myofibers (49).

At present, it is not clear what mechanism(s) underlies the observed myofiber hypertrophy in SK733 IGF-I 3′-SK transgenic mice, but based on results from previous studies, it is plausible that overexpression of IGF-I induced hypertrophy through either one or a combination of several mechanisms. First, IGF-I is known to elicit numerous effects on the metabolism of skeletal muscle that are anabolic in nature such as stimulation of amino acid and glucose uptake and enhancement of net myofibrillar protein accretion via combined effects on protein synthesis and degradation (see Refs. 2 and 3 for review). Thus, it could be that the myofiber hypertrophy observed in the present study was primarily due to the cumulative anabolic effects of IGF-I. Another possibility is that overexpression of IGF-I stimulated processes that are normally involved in muscle regeneration and stretch-induced hypertrophy. Previous research has established that expression of IGF-I is increased locally in regenerating muscle (9) and in muscle undergoing stretch-induced hypertrophy (8), and it is hypothesized that this increase in IGF-I drives the hypertrophic response. However, the manifestation of IGF-I's actions on regeneration and hypertrophy of skeletal muscle may also require the action of other regulatory factors. For example, Bischoff and colleagues (50) have observed in studies of single muscle fibers with attached satellite cells *in vitro* that satellite cell proliferation is refractory to an IGF-I challenge unless the cells are first exposed to an as yet unidentified factor present in crushed muscle extract. This suggests exercise or stimuli that naturally result in myofiber hypertrophy may enhance the phenotype observed with overexpression of IGF-I in striated muscle. Currently, studies are ongoing to address these hypotheses.

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