We have previously identified a muscle-specific enhancer within the first intron of the human β enolase gene. Present in this enhancer is an A/T-rich box that binds MEF-2 protein(s) and a G-rich box (AGTGGGG-...). Present in this enhancer are an A/T-rich box that functions as a repressor of a Kruppel-like zin finger protein, termed β enolase repressor factor 1, that binds in a sequence-specific manner to the G-rich box and functions as a repressor of the β enolase gene transcription in transient transfection assays. Using fusion polypeptides of β enolase repressor factor 1 and the yeast GAL4 DNA-binding domain, we have identified an amino-terminal region responsible for the transcriptional repression activity, whereas a carboxyl-terminal region was shown to contain a potential transcriptional activation domain. The expression of this protein decreases in developing skeletal muscles, correlating with lack of binding activity in nuclear extract from adult skeletal tissue, in which novel binding activities have been detected. These results suggest that in addition to the identified factor, which functionally acts as a negative regulator and is enriched in embryonic muscle, the G-rich box binds other factors, presumably exerting a positive control on transcription. The interplay between factors that repress or activate transcription may constitute a developmentally regulated mechanism that modulates β enolase gene expression in skeletal muscle.

The muscle-specific or β isoform of the glycolytic enzyme enolase (EC 4.2.1.11) is encoded by a member of the enolase gene family, the expression of which is regulated in a tissue-specific and development-specific manner (1–4). The gene is primarily expressed in the cardiac and skeletal muscles, where, during embryonic development, the β isoform progressively replaces the nearly ubiquitous α isoform (5). Indeed, the message for the β enolase is first detectable in the cardiac tube and in the myotome (11); expression remains extremely low in skeletal primary fibers, whereas a striking increase occurs in the second generation of fibers at the fetal stage of development (6, 11), and a further increase is observed after birth. In the adult, β enolase is expressed in both cardiac and skeletal muscles, with higher levels of expression detected in fast-twitch fibers than in slow-twitch fibers (11). In vitro studies on myogenic cell lines and primary myoblasts have shown that the level of β enolase expression increases with terminal differentiation but, at variance with the majority of the muscle-specific genes, expression already occurs in proliferating myoblasts (6–8). Interestingly, β enolase, like desmin (9), belongs to a relatively small group of muscle-specific genes expressed in proliferating myoblasts, as well as in differentiated myotubes, and it has been suggested that it might be a marker of adult satellite cells in humans (8, 10).

From these data, it can be presumed that regulation of the β enolase gene expression may take place at multiple levels and involve complex molecular mechanisms, making the gene a suitable model to investigate various aspects of muscle-gene transcriptional control.

In the last few years, a great number of transcription factors and DNA regulatory elements have been identified as contributors to the activation of the muscle differentiation program. In skeletal muscle, the MyoD family of basic helix-loop-helix proteins (MyoD, myogenin, Myf-5, and MRF4), the ectopic expression of which can activate skeletal muscle gene expression in a wide range of non-muscle cell types, plays a pivotal role during development (12). The MEF-2 family of MADS-box transcription factors, which bind an A/T-rich element found in the promoters and enhancer of the majority of skeletal and cardiac muscle genes, is involved both in a direct and indirect mechanism of transcriptional activation (13, 14).

Recently, the scenario has become more complex, because several reports have outlined the importance of ubiquitously expressed factors in association with tissue-restricted factors to maintain tissue-specific expression. Functional cooperation between elements that bind ubiquitous factors and tissue-restricted factors has been demonstrated for the regulatory regions of both cardiac and skeletal muscle genes, and in almost all cases, these sequences are located in relatively close proximity (less than 60 bp), suggesting that protein-protein interactions might be involved in the cooperation (reviewed in Ref. 15). Furthermore, it has recently been reported that an apparently ubiquitous binding activity consists itself of a complex...
BERF-1 is a negative regulator of β enolase gene

1 The abbreviations used are: BEE-1, β enolase element 1; BERF-1, β-enolase repressor factor 1; dpc, day(s) postcoitum; kb, kilobase; bp, base pair; GAPD, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase gene; PCR, polymerase chain reaction; TK, thymidine kinase; ORF, open reading frame.

The protein with several scripts and proteins expression in myogenic cells and tissues, followed by plaque purification. The pBluescript phagemid was excised from infection assays, indicating that MEF-2 and BEE-1 binding factors are each necessary for tissue-specific expression of the β enolase gene in skeletal muscle cells. Furthermore, sequences homologous to the BEE-1 site in association to a MEF-2 binding site are present in the transcriptional regulatory regions of several skeletal and cardiac muscle-specific genes (15), suggesting the existence of a widespread pathway of muscle-gene transcriptional control.

This article reports the isolation and characterization of a protein that binds in a sequence-specific manner to the BEE-1 element. The deduced amino acid sequence revealed the presence of four C2H2 zinc finger domains, which identify the protein as belonging to the family of transcription factors resembling the Kruppel segmentation gene product of Drosophila (17), and high similarity with a human zinc finger protein, which has been shown to bind the promoter of the gene for the enolase repressor factor 1 (BERF-1) and the expression of enolase gene in skeletal muscle cells. Furthermore, sequences homologous to the BEE-1 site in association to a MEF-2 binding site are present in the transcriptional regulatory regions of several skeletal and cardiac muscle-specific genes (15), suggesting the existence of a widespread pathway of muscle-gene transcriptional control.

EXPERIMENTAL PROCEDURES

Southwestern (DNA-protein) Screening of the Skeletal Muscle cDNA Expression Library—A λ Zap II expression cDNA library was prepared with mRNA from limbs of 12-day mouse embryos using a cDNA synthesis kit (Stratagene). The amplified expression library was screened by the method of Vinson et al. (19) using as a probe double-stranded BEE-1 oligonucleotides (sense, 5′-AGCTGTTCTGAGGGGAAGGGGG-3′; antisense, 5′-AGCTGTTCTGAGGGGAAGGGGG-3′) that had been end-labeled and ligated into con-tamers. One positive clone out of 2.0 × 10^9 plaques survived three rounds of plaque purification. The pBluescript phagemid was excised from the λ Zap expression vector by helper phage coinfection (R408 strain) according to the instructions of the manufacturer (Stratagene). The isolation of additional cDNAs was carried out by standard methods using the cDNA identified by Southwestern screening as a probe. DNA Sequencing and Expression of the Cloned cDNAs by in Vitro Transcription and Translation—The nucleotide sequence of the cDNA clones were determined on both the sense and antisense strands by the dideoxynucleotide chain-termination method using modified T7 DNA polymerase (Sequenase; United States Biochemicals). In vitro transcription of the isolated clones was carried out with 2 μg of linearized pBluescript plasmid using an mRNA capping kit (Stratagene). In vitro translation was performed with a commercially available rabbit reticulocyte lysate system according to the instructions of the manufacturer (Promega), and when needed, 35S-methionine was added to the translation mixture.

RNA isolation and Northern blot analysis—Total RNA was extracted from cultured cells and from limbs and hearts of adult mice or embryos isolated at 12, 14, and 16 days postcoitum (dpc) by the guani-dine isothiocyanate method (20). Mouse multiple tissue Northern blot was prepared by CLONTECH. Fifteen micrograms of total RNA were fractionated by electrophoresis on denaturing agarose gel, transferred to nylon membranes, and hybridized as described previously (6). A 2.3-kb (kilobase) BamHI fragment containing the most extreme coding region was isolated from the A22 cDNA and used as a probe. As a control of the amount of RNA loaded per lane and to check differentiation of myogenic cells in cultures, filters were rehybridized with a chicken β actin cDNA (6) and/or with a human glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA (ATCC 7501).

Generation of Polyclonal Antibodies and Immunoblot Analysis—DNA fragments encoding different portions of the ZF22 protein were subcloned into the bacterial expression vector pGEX-2T (Pharmacia). A 344-bp BstXI-AacI fragment and a 884-bp ClaI-BamHI fragment were isolated from the A22 cDNA, whereas a 2-kb EcoRV-BamHI fragment was excised from the A21 cDNA encoding the truncated ZF21 polypeptide. The three glutathione S-transferase-ZF22 fusion proteins, which consisted of the amino-terminal region from amino acid 26 to amino acid 111, the zinc finger region from amino acid 76 to amino acid 319 and the carboxyl-terminal region from amino acid 430 to amino acid 740, respectively, were overexpressed in Escherichia coli and affinity purified by binding to glutathione-linked Sepharose beads (21). Rabbit anti-ZF22 polyclonal antiserum were raised against the purified fusion proteins, and antibodies were immunopurified on columns containing the respective glutathione S-transferase-fusion protein used as immunogen according to established procedures (22). For immunoblot analysis, nuclear proteins (5 μg), prepared as described in the following section, or total cell lysates (30 μg) obtained by extraction in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxy-collate, 0.1% SDS, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, were resolved by electrophoresis on an SDS-7% polyacrylamide gel and electrod blotted to a nitrocellulose membrane (Hybond-C, Amersham Corp.). The membrane was incubated with affinity purified anti-ZF22 antibody (5 μg/ml) and with a secondary antibody coupled to horseradish peroxidase (Pel-Freez) (1:5000). The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL kit, Amersham Corp.).

Preparation of Nuclear Extracts and EMSSAs—Nuclear extracts were prepared from cultured cells and embryonic muscle tissue (limbs isolated from 12-dpc mouse embryos), as described previously (15). Preparation of nuclear extracts from skeletal muscle tissue isolated from adult mice was according to Zahradadka et al. (23). The following double-stranded oligonucleotides were used as probes and competitors in electrophoretic mobility shift assays (EMSSAs): BEE-1w (5′-AGCTGTTCTGAGGGGAGGGGGCTGCGCCTGC-3′), wild-type consensus (15); BEE-1m (5′-AGCTTCTTCTAGTGGGACTCTAGGCTGCGCCTGC-3′), mutated consensus (15); Sp1 site (5′-ATTGATCGGGGCCGCGGGCAATG-3′), canonical Sp1 binding site (24); Eco-Box (5′-TTTACCCAGACATGTGGCTGCC-3′), left Eco-Box from muscle creatine kinase enhancer (25).

EMSSAs were performed by incubating end-labeled probes (0.1 ng, about 40,000 cpm) with nuclear extracts (4–8 μg) or in vitro-translated proteins (3 μl) as described previously (15). When the Eco-Box was used as a probe, incubation was carried out in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 15% glycerol as described by Ferrari et al. (26). DNA-protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel in 25 mM TBE (25 mM Tris, pH 8.3, 20 mM boric acid, 0.5 mM EDTA) and visualized by autoradiography. For antibody interference, EMSSAs were performed under the conditions described above except for the addition to the reaction mixture of 1 μg of antibodies. Anti-Sp1 and anti-Sp3 antibodies (Santa Cruz Biotechnology) were kindly provided by Dr. H. Schoeler (European Molecular Biology Laboratory, Heidelberg, Germany), and anti-MNF serum (27) was a generous gift of Dr. R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX).

Nuclear extract from cells transfected with constructs expressing various GAL-ZF22 fusion proteins were prepared according to Hoppe-
Results

Cloning of BEE-1-binding Proteins: Isolation of cDNAs Encoding the Zinc Finger Polypeptides ZF21 and ZF22—The BEE-1 binding site is a G-rich element required to support muscle-specific expression of the β enolase gene together with an adjacent MEF-2 site. For isolation of BEE-1 binding factors, an embryonic muscle cDNA expression library was screened by the Southwestern method using a concatenated BEE-1 probe. One single clone (A21) that displayed sequence-specific binding to the BEE-1 probe was isolated (Fig. 1A). The nucleotide sequence of the cDNA insert revealed the presence of an open reading frame (ORF) of 960 bp, potentially encoding a polypeptide of 320 amino acid residues with a calculated molecular mass of 35 kDa (Fig. 1B, ZF21). Additional clones were isolated by screening the cDNA library with 5′ and 3′ fragments of the cDNA originally identified by Southwestern screening. One cDNA (A22) was sequenced and appeared to be identical to the one encoding ZF21 except for the presence of a longer 5′-untranslated region and a one-nucleotide deletion. This deletion would result in a frameshift (Fig. 1A) and the creation of an ORF of 2382 bp encoding a 794-amino acid polypeptide with a predicted molecular mass of 89 kDa (Fig. 1B, ZF22). The same deletion was found in six independent clones, suggesting that the originally isolated cDNA encoding ZF21 might be representative of a rare message or be the result of a reverse-transcriptase error that occurred during the preparation of the library.

A search in the GenBank data base revealed a high similarity (79% at the nucleotide level and about 89% at the amino acid level) with a human cDNA encoding a CACCC-box-binding protein called hTβ, previously identified as one of the factors binding to the proximal promoter of the gene for the Vβ8.1 chain of the T-cell receptor (18). The cDNA encoding hTβ contains an ORF of 1362 nucleotides, predicting a protein of 49 kDa, and the sequence showed several transitions and two insertions that would shift the frame and create several stop codons not present in the cDNA encoding ZF22. Recently, while this manuscript was in preparation, the isolation of the rat homologue of ZF22 was reported, and the factor, termed ZBP-89, was shown to bind the human gastrin promoter (37); independently, other authors reported the isolation of a mouse cDNA encoding an amino-terminal shorter form of ZF22, named BFCCOL1, which binds the proximal promoters of the two mouse type I collagen genes (38). In this last report, the isolation of a partial cDNA for human hTβ was also described, the sequence of which shows a continuous ORF and suggests that the major form of the factor in human cells might be a polypeptide with a predicted molecular mass of 89 kDa, as has been found for mouse and rat.

The amino acid sequence is extremely well conserved among mouse, rat, and human proteins (the mouse and rat sequences are more than 99% similar, and the amino-terminal 400 amino acids of htβ share about 95% similarity with the other sequences) and displays several distinctive features (Fig. 1B). These include the presence of four C2H2 Kruppel-like zinc finger motifs, an amino-terminal acidic domain, and two basic domains (both containing a nuclear localization signal (39), located upstream and downstream of the zinc finger cluster.)

Cell Culture, Transfection, and CAT Assays—C2C12 myogenic cells (33) were cultured as proliferating myoblasts in a growth factor-rich medium at 37°C in 5% CO2 and 95% air. Briefly, 10^5 cells in a 50-mm-diameter culture dish. Cells were harvested 48 h later and subjected to β-galactosidase and CAT assays as described previously (15). All transfections were performed on multiple sets of cultures with at least two different DNA preparations for each plasmid.

Results

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The amino acid sequences of ZF22, BFCOL1, and ZBP-89 also show the presence of a PEST domain (NSSDVPETQSE) that has been identified in a number of eukaryotic proteins characterized by a short half-life (40) (Fig. 1B). Alignment of the nucleotide sequences encoding ZF22, ZBP-89, htβ, and BFCOL1 showed that the latter is shorter at the 5'-end, where three in-frame AUG codons are present in close proximity. According to a recent report (41) no one of these putative ORFs is indicated on the right. C, comparison among the ZF22, ZF21, BFCOL1, ZBP-89, and htβ amino-terminal amino acid sequences. Dashed lines indicate amino acid identity. The putative initiator methionine of BFCOL1 (38) is indicated by a vertical arrow.

Identification of ZF22 as the Major BEE-1-binding Protein in C2C12 Nuclear Extracts—To confirm the predicted ORF of both A21 and A22 cDNAs and to check the ability of the encoded polypeptides to bind the BEE-1 element, the pBluescript plasmids carrying the two cDNAs were in vitro transcribed and translated. The major product of the ZF22-encoding plasmid was a polypeptide migrating with an apparent molecular mass of about 110–120 kDa, whereas two major polypeptides of about 110–120 kDa, whereas two faster migrating DNA-protein complexes were resolved with ZF21 (Fig. 2A, lane 5). This result was consistent with the heterogeneity of the translation products. The specificity of the binding was confirmed by addition of a molar excess of the oligonucleotide containing either a wild-type BEE-1 consensus or a mutated consensus site (Fig. 2A, lanes 3 and 6 and lanes 4 and 7, respectively). These results indicated that both ZF22 and ZF21 contain a DNA-binding domain, presumably the four zinc fingers, and suggested that ZF22 corresponds to the predominant BEE-1-binding protein detected in nuclear extracts by EMSA.

To confirm that the ZF22 zinc finger protein represents a component of the endogenous BEE-1 binding activity in skeletal muscle cells, antibodies were raised against three different glutathione S-transferase-ZF22 fusion proteins and subsequently examined for their ability to interfere with the BEE-1 binding activity in C2C12 nuclear extracts. All three antisera, directed against the amino-terminal region of the protein, the zinc finger cluster, and the carboxyl-terminal region, respectively, when added to the myotubes nuclear extract, diminished the major BEE-1 binding activity and gave rise to a supershifted complex (Fig. 2B, lanes 2, 4, and 6); preimmune sera had no detectable effect (Fig. 2B, lanes 3, 5, and 7). Because the core sequence of the BEE-1 element, GGGAGG, has been shown to be an Sp1-like site (24), we decided to test whether Sp1 and Sp3 proteins bind to the β enolase G-rich box using...
specific Sp1 and Sp3 antibodies in EMSA experiments followed by separation of the DNA-protein complexes on a more discriminating polyacrylamide gel (compare Fig. 2B and 2C). Fig. 2C shows that three closely migrating complexes and a fourth, faster mobility complex (a) were resolved. The most abundant complex was due to binding of ZF22 as confirmed by the addition of specific antibodies and the consequent appearance of a supershifted complex at the top of the gel (Fig. 2C, lane 3). Similarly, the fastest migrating complex (a), the intensity of which varied among different experiments, was supershifted in the presence of anti-ZF22 antibodies (Fig. 2C, lanes 3, 6, 8, and 9), indicating that a related protein or a breakdown product of the zinc finger factor might be part of the complex. The other two minor complexes, one slightly more retarded than the BEE-1/ZF22 complex, the other migrating relatively faster, were not reduced in the presence of the anti-ZF22-antiserum. Addition of specific anti-Sp3 antibody in the binding reaction resulted in reduction of the most retarded complex (Fig. 2C, lane 4), whereas addition of Sp3 antibody resulted in the absence of the faster migrating complex (lane 5). Preincubation with both Sp1 and Sp3 antibodies resulted in the absence of both complexes (lane 7). In agreement with data reported for Sp1 and Sp1-like consensus site identified in other genes (43, 44), the faster migrating complex is due to Sp3 binding, whereas the most retarded complex is indeed composed of two very closely retarded bands due to specific interaction with Sp1 and Sp3. The concurrent addition of the three antisera, anti-Sp1, anti-Sp3, and anti-ZF22, resulted in the change of mobility of all the DNA-protein complexes (Fig. 2C, lane 9). When a canonical Sp1 consensus site was used as a probe, no DNA binding activity due to ZF22 was detected, as assessed by inclusion of anti-ZF22 antibodies in the binding reaction (data not shown). Taken together, these results confirmed that ZF22 is the major component of the endogenous BEE-1 binding activity in C2C12 myotubes.

The Zinc Finger Factor Expression Is Down-regulated During Myogenesis.—To investigate both the pattern of expression of the ZF22 mRNA in various tissues and the relationship between its expression and that of the β enolase, a Northern blot analysis of RNAs from different sources was performed. Three major transcripts corresponding to 3.4, 4.1, and 7.6 kb were observed in all of the tissues and cell cultures analyzed (Figs. 3 and 4), but other, less abundant messages larger than 7.6 were also detected, as previously reported for hβ and ZBP-89 (18, 37). These multiple bands may be explained by the existence of additional mouse cDNAs containing 3′-untranslated sequences of different length as result of the utilization of different polyadenylation sites (data not shown). The amount of ZF22 transcripts increased from day 12 to day 14 of mouse embryonic limb development, followed by a further decrease in day 14 to day 16 (Fig. 3A, lanes 1–3) and by a further decrease in limb skeletal muscle of newborn and adult mice (Fig. 3A, lanes 4 and 5); similarly, a lower level of expression was observed in adult cardiac muscle tissue when compared with earlier stages (Fig. 3A, lanes 6–8). RNA was also isolated from primary cultures of embryonic (11 dpc) and fetal (16 dpc) myotubes (6, 34). Fig. 3B shows that ZF22 transcripts were present in a relatively large amount in embryonic myotubes, whereas they were barely detectable in fetal myotubes. ZF22 expression both in vitro (during muscle development) and in myogenic primary cultures inversely correlates with β enolase expression (6). Analysis of mRNA from adult tissues showed that the ZF22 message was ubiquitously expressed; however, the relative amount varied greatly among tissues, i.e. the message was more abundant in brain and liver than in skeletal muscle and heart (Fig. 3C). Northern blot analysis of RNA extracted from C2C12 myogenic cells at various times during differentiation induced by withdrawal of growth factors (Fig. 4A) revealed that following differentiation of myoblasts to multinucleated myotubes, the level of ZF22 transcripts slightly decreased (compare lanes 2–4 with lane 1); however, the degree of such down-regulation was much less significant than in vivo during muscle histogenesis (Fig. 3, A and B). Total protein lysates were prepared from C2C12 cells maintained for the same lengths of time in differentiation medium and analyzed by Western blot (Fig. 4B). All three anti-ZF22 antibodies recognized two closely migrating polypeptides, with apparent molecular masses of about 110 and 120 kDa, clearly resolved when electrophoresis was carried out on longer gels (see Fig. 6). Both polypeptides appeared to be slightly more abundant in myoblasts than in

\[ \text{Fig. 2. EMSA analysis of in vitro-translated ZF22/ZF21 proteins and endogenous nuclear proteins. A, a labeled synthetic oligonucleotide corresponding to the \( \beta \) enolase BEE-1 element was incubated with nuclear extract (5 \( \mu \)g) from C2C12 myotubes (lane 1), with in vitro-translated ZF22 (lanes 2–4), or with in vitro-translated ZF21 (lanes 5–7). For competition in the EMSA, a 30-fold molar excess of unlabeled oligonucleotide containing the wild-type or the mutated consensus site (BEE-1w and BEE-1m, respectively) was added to the binding reaction mixture. Specific major ZF22 and ZF21 complexes and the position of the free probe (BEE-1) are indicated. B, effect of ZF22 antibodies on the endogenous binding activity. The BEE-1 probe was incubated with nuclear proteins (4 \( \mu \)g) from C2C12 myotubes in the absence (lane 1) or in the presence of the three anti-ZF22 sera, raised against the amino-terminal region (\( \alpha\)-NH\(_2\), lane 2), the zinc finger region (\( \alpha\)-ZF, lane 4) and the carboxyl-terminal portion (\( \alpha\)-COOH, lane 6) of the protein, or the corresponding preimmune serum (pre, lanes 3, 5, and 7, respectively). The ZF22-specific complexes and the position of the free probe (BEE-1) are indicated. C, effect of Sp1 and Sp3 antibodies on binding activity. EMSA was performed with C2C12 myotubes nuclear extract (6 \( \mu \)g) and a labeled BEE-1 oligonucleotide. The presence of specific antibodies in the binding reaction is indicated above each lane. The anti-ZF22 serum used is the one against the amino-terminal region of the protein. Specific complexes for Sp1, Sp3, ZF22, and ZF22-related proteins (a) are indicated on the left.} \]
myotubes. The specificity of the antibodies was confirmed by use of preimmune sera as a negative control; furthermore, the apparent molecular weight of the two endogenous polypeptides was consistent with the size of the ZF22 protein obtained by in vitro transcription-translation. The two proteins may be the products of alternatively spliced messages or result from post-translational modifications of a single product. Immunofluorescence analysis (not shown) confirmed that ZF22 protein is ubiquitously expressed in the mesoderm of mouse embryos but is no longer detectable in mesoderm of mouse fetuses.

Taken together, these results indicate that the expression of ZF22 is down-regulated during myogenesis in vivo and in vitro but less tightly regulated in C2C12 myogenic cells in culture.

The DNA Binding Activity of the Zinc Finger Factor Is Diminished in Adult Skeletal Muscle Tissue Where Novel BEE-1

**Fig. 3. Northern blot analysis.** A, expression of ZF22 in developing mouse skeletal and cardiac muscles. Total RNA (15 μg) from limbs of 12- and 14-day embryos (lanes 1 and 2), 16-day fetuses (lane 3), and newborn (NB) and adult (MA) mice (lanes 4 and 5, respectively) and from hearts of 12-day embryos (lane 6), 17-day fetuses (lane 7), and adult mice (HA, lane 8) was electrophoresed, transferred to a nylon membrane, and hybridized with a 32P-labeled ZF22 cDNA fragment as described under “Experimental Procedures.” Sizes are indicated in kb. The same filter was washed and rehybridized with a GAPD cDNA to assess the presence of comparable amounts of loaded RNA and with a chicken β-actin cDNA (2) to monitor the pattern of expression of the muscle-specific form (α-actin) and the ubiquitous form (β-actin) (not shown). B, expression of ZF22 in differentiated cultures of embryonic and fetal myoblasts. Myoblasts were isolated from limbs of 11-day embryos and 16-days fetuses, and RNA was extracted after myotube formation on the 5th day of culture (EMt and FMt, respectively). The filter was first hybridized with the chicken β-actin cDNA, stripped, and then reprobed with a ZF22 cDNA fragment. C, expression of ZF22 in adult tissues. Poly(A)+ mRNA (2 μg) was from mouse heart, brain, spleen, lung, liver, skeletal (Sk) muscle, kidney, and testis. The filter was probed with a ZF22 cDNA fragment and then with the GAPD cDNA.

**Fig. 4. Expression of ZF22 in mouse C2C12 myogenic cells.** A, Northern blot analysis of RNA extracted from C2C12 cells grown in differentiation medium for 0 (lane 1), 2 (lane 2) 3 (lane 3), or 4 (lane 4) days. About 15 μg of total RNA were loaded in each lane; for comparison, the same amount of RNA isolated from skeletal muscles (MA) and hearts (HA) of adult mice was electrophoresed on the same gel (lanes 5 and 6, respectively). The same filter was first hybridized with a ZF22 cDNA fragment and then with the chicken β-actin cDNA and the GAPD cDNA. B, Western blot analysis of ZF22-related proteins in C2C12 myoblasts and myotubes. Total protein lysates (30 μg) from C2C12 cells in growth medium (lane 1) or differentiation medium for 2 (lane 2), 3 (lane 3), or 4 (lane 4) days were resolved on an SDS-polyacrylamide gel (7% acrylamide) and transferred to nitrocellulose, and the filter was incubated with affinity-purified ZF22 antibodies against the amino-terminal region of the protein. Molecular size markers are indicated on the left.

**Binding Activities Are Detected**—The discrepancy observed between ZF22 expression in muscle tissues and C2C12 myogenic cells prompted us to investigate the presence of BEE-1 binding activity in skeletal muscle tissues. Nuclear extracts were prepared from embryonic muscle, using as a source limbs of 12-day mouse embryos, and from skeletal muscle of adult mice; both extracts were used in EMSAs (Fig. 5). Strikingly, although the BEE-1-ZF22 complex was easily detected in nuclear extract from embryonic muscle (Fig. 5A, MA, lane 2), it was barely detectable in nuclear extract prepared from adult muscle (Fig. 5A, MA, lane 3). The DNA-ZF22 complex obtained with nuclear extract from embryonic muscle was not distinguishable from the one obtained with C2C12 nuclear extract (Fig. 5A, B, MA, lane 3). The newly detected complexes are specific because they were drastically reduced by addition of an excess of unlabeled probe containing a wild-type BEE-1 consensus site and were not affected by a molar excess of a mutated consensus site (Fig. 5B, lanes 2 and 3). Furthermore, the adult muscle-specific complexes were unaffected by addition of all three anti-ZF22 sera and antibody against MNP (data not shown), a factor reported to bind a CAATC-box within the muscle-specific enhancer of the myoglobin gene (27), indicating that the proteins in the complexes are not related to both factors. An independent assessment of the quality of the extracts is provided in Fig. 6, C and D, which show no significant difference in Sp1 and E-box site binding proteins between nuclear extracts of embryonic and adult muscle; the binding activity detected with the left E-box from the enhancer of the muscle creatine kinase gene was slightly higher in adult muscle than in embryonic tissue (Fig. 6D, lanes...
factor on β enolase gene expression, both ZF22 and ZF21 proteins were overexpressed in transfection assays with CAT reporter plasmids either carrying the β enolase promoter and the entire first intron (nucleotides −172 to +706) with the muscle-specific enhancer in its wild-type location or bearing the minimal enhancer sequence (nucleotides +532 to +611) upstream of the promoter (Fig. 7A, pB10-CAT and pB3–5 PCR1, respectively). In both cases, expression of ZF22 and ZF21 resulted in a consistent and comparable reduction of the CAT activity relative to the activity detected in cells transfected with the parental, insertless expression vector (Fig. 7B). The results shown in Fig. 7 were obtained in transiently transfected C2C12 myotubes, but a similar degree of repression was observed in CH310T1/2 and CV1 cells (data not shown). To further investigate the mechanisms involved in the observed transcriptional repression, different concentrations of the ZF22 expression plasmid were cotransfected with reporter constructs in which CAT gene expression is driven by the β enolase promoter with multiple copies of a wild-type or a mutated ZF22 binding site (Fig. 8A, pB3-BEE-1w4X and pB3-BEE-1m4X) and similar constructs carrying the promiscuous SV40 enhancer downstream of the CAT transcription unit (Fig. 8A, pB35SV-BEE-1w4X and pB35SV-BEE-1m4X). Fig. 8B shows that a dose-dependent transcriptional repression was observed in all cases; ZF22 was able to repress transcription from the reporter plasmids carrying four mutated consensus sites but was quantitatively less repressive on these plasmids than on the reporter plasmids containing four wild-type binding sites. When lower amounts of the ZF22 expression plasmid were transfected (Fig. 8B, 0.5 μg) repression was greater when the reporter plasmid contained the wild-type binding sites, and this difference was more significant when constructs containing the strong SV40 enhancer were used as reporters (Fig. 8B, compare a, b, c, and d). Reporter constructs carrying heterologous promoters, (TK promoter or SV40 early promoter) behaved similarly, suggesting that the observed activity does not depend upon the promoter used (data not shown), although all are TATA-box containing promoters. The results of these transfection experiments indicated that both ZF22 and ZF21 exert a transcriptional repres-

![Fig. 5. Analysis of BEE-1 binding activities in embryonic and adult skeletal muscle. A, EMSA was performed with nuclear extracts (4 μg) from C2C12 myotubes (Mt), embryonic muscle (ME), and adult skeletal muscle (MA) using a labeled oligonucleotide containing the BEE-1 element as probe. B, competition in the EMSA demonstrates the specificity of the binding. A 100-fold molar excess of unlabeled oligonucleotide containing the wild-type or the mutated consensus site (BEE-1w and BEE-1m, respectively) was added to the binding reaction mixture containing nuclear extract (8 μg) from adult skeletal muscle (lanes 2 and 3). Specific major and minor adult muscle-specific complexes (large and small arrowheads, respectively) and the position of the free probe (BEE-1) are indicated. A nonspecific complex probably due to an uneven ions front is indicated by an asterisk.](Image 58x510 to 298x729)

![Fig. 6. Western blot analysis of nuclear extracts with antibody against ZF22. Nuclear proteins (5 μg) from human fibroblasts (PAF, lane 1), from mouse C2C12 myoblasts and myotubes (Mb and Mt; lanes 2 and 3, respectively), embryonic muscle (ME, lane 4), and adult skeletal muscle (MA, lane 5) were resolved on an SDS-polyacrylamide gel (7% acrylamide) and transferred to nitrocellulose, and the filter was incubated with affinity-purified ZF22 antibodies against the amino-terminal region of the protein. Molecular size markers are indicated on the left.](Image 385x532 to 486x729)
C2C12 myotubes cotransfected with 10 m pCDNAI/ZF22 and pCDNAI/ZF21, respectively.

Respond to the expression vector pCDNAI and recombinant constructs

Deletions in the full-length GAL4-ZF22 or fusing different por-
to investigate the presence of other functional domains, a pre-
shown).

Obtained by transfection in C2C12 myogenic cells, but compa-
results were shown). CAT activities, corrected for differences in transfection
represents S.D.

The factor clearly repressed transcription more efficiently when it was targeted to the promoter, although the protein may be able to repress transcription by a mechanism that does not require DNA binding, such as interaction with a component of the basic transcriptional machinery.

ZF22 Contains a Transferable Repression Domain and a Positive Regulatory Domain—Because the BEE-1 element was identified as a positive regulatory element controlling transcription activity on basal as well as activated transcription and suggested that the activity may reside in the amino-terminal half of the protein. The factor clearly repressed transcription more efficiently when it was targeted to the promoter, although the protein may be able to repress transcription by a mechanism that does not require DNA binding, such as interaction with a component of the basic transcriptional machinery.

ZF22 Is a Negative Regulator of β Enolase Gene

Effect of overexpression of ZF22 on basal and activated transcription. A, schematic representation of the reporter plasmids used in transient transfection assays. Each reporter contained four copies of a wild-type or mutated ZF22 binding site (BEEna4X and BEEna4X, respectively). B, CAT assays of C2C12 myotubes cotransfected with 10 μg of the indicated reporter plasmid and 5 μg of the insertless effector plasmid (a) or plasmid expressing the long or the short form of the zinc finger factor (b and c, respectively). CAT activities, corrected for differences in transfection efficiencies, are compared with the activity observed with the insertless effector plasmid, which was arbitrarily set at 100%. The data are averages of at least three independent experiments, and the error bars represent S.D.

To define the boundaries of a putative repression domain and to investigate the presence of other functional domains, a preliminary dissection of ZF22 was performed, either introducing deletions in the full-length GAL4-ZF22 or fusing different por-
tions of the protein to the GAL4-binding domain (Fig. 9B). The resulting chimeric proteins were tested for their abilities to repress or activate the GAL4-dependent reporter gene in C2C12 cells. As a control, the ability of each GAL4-fusion polypeptide to translocate into the nucleus and to display GAL4 DNA binding activity was evaluated by EMSA with nuclear extracts of transfected C2C12 myotubes (data not shown). Deletion of amino acids 665–794 did not affect the intrinsic repression activity (Fig. 9B, compare constructs a, b, and c, and d). A further deletion up to amino acid 185 resulted in a 5-fold decrease of the CAT activity (construct e), whereas additional deletions from amino acid 139 to amino acid 184 and from amino acid 75 to amino acid 138 resulted in a CAT activity twice as much as the activity obtained overexpressing the GAL4 DNA-binding domain alone (Fig. 9B, compare constructs f and g with construct a). This weak activation was consistent with a slightly higher stability of the polypeptides encoded by the constructs spanning the amino-terminal region up to amino acid 138 and might be the result of a longer half-life of the expressed fusion proteins. These results indicate the presence of a strong repression domain between amino acids 138 and 184, as confirmed by the activity of a fusion protein containing only this domain (Fig. 9, construct k). Expression of fusion polypeptides spanning the carboxyl-terminal region of the pro-
protein resulted in a 6–34-fold activation of transcription, suggesting the presence of a putative transcriptional activation domain (Fig. 9B, constructs h, i, and j), as previously reported for BFCOL1 (38).

**DISCUSSION**

The aim of this work was the identification of factors binding to the G-rich element (AGTTGAGGGGGCTGG, termed BEE-1) that are required, together with an adjacent MEF-2 site, to regulate tissue-specific and differentiation-induced expression of the β enolase gene in skeletal muscle cells. One clone was isolated by screening of a phage expression library and used to isolate additional clones that resulted to encode a Kruppel-like zinc finger factor homologous to a human DNA-binding protein reported to bind a CACCC sequence (18). Northern blot analyses showed that the protein is ubiquitously expressed, but interestingly, expression decreases in limbs and hearts of mouse embryos during development; this down-regulation temporally correlates with the appearance of the second-nerve branch in the mouse (45), and up-regulation is observed in the heart of mouse embryos. In contrast, the factor has been described as a positive regulator of histone H4 expression. Recently, it has been proposed that H4TF1 (47, 48), which binds the histone H4 CTCCC-box, might be related to BERF-1. This hypothesis cannot be proven since neither antibodies nor cDNAs encoding H4TF1 are available; however, the factor has been described as a positive regulator of histone H4 expression. Recently, it has been proposed that H4TF1 might bind a sequence within the distal enhancer of the human vimentin gene that acts as a positive regulatory element (49).

In transfection assays using BERF-1 expression vectors and CAT reporter constructs containing the BEE-1 element, we observed a dose-dependent repression of the reporter gene ac-
activity, but at the same time BERF-1 was able, though at a lower extent, to inhibit promoters lacking its binding site or bearing multiple copies of a mutated consensus site. One possible explanation for these observations is that BERF-1 is a bona fide repressor factor capable of inhibiting basal as well as activated transcription, according to the repression mechanisms proposed for the eukaryotic gene transcriptional regulation (50, 51). Alternatively, the overexpression of BERF-1 results in “squelching” (52), as has been suggested for BFCOL1 (38). Several lines of evidence indicate that our data are consistent with a repression mechanism rather than with a phenomenon of squelching: (i) transcriptional activation was never observed under any experimental conditions we used, regardless the reporter, the cell type, and the concentration of the transfected BERF-1 expression vector; (ii) the amount of the expression plasmid required for repression is within the range that is shown to factor a transferable repression domain (54). The BERF-1 repression domain does not contain alanine-, glutamine-, or proline-rich sequences, which are considered typical features of repression motifs present in suppressor factors like Kruppel and WT1 (55, 56). Recently, the repertoire of the primary amino acid sequences within such domains has expanded as more transcription repressors are characterized (reviewed in Ref. 54). Several reports have indicated high charge as a common features among repression motifs (57–59), and the BERF-1 repression region, which contains a highly basic domain, may fall into this category. The identification of a putative activation domain in the serine-rich carboxyl-terminal region of BERF-1, a feature shared with BFCOL1 (38), leaves open the possibility that the factor or variant forms may be have as activator depending upon the promoter or the cell context.

The BEE-1 element was originally identified as a positive cis-regulatory element that functionally cooperates with the neighboring MEF-2 binding site in conferring muscle-specific transcription to the β enolase gene. Although none of the substitution mutations in the BEE-1 element that we tested inhibited the binding of the repressor factor that we describe in this report and simultaneously result in an increase in the reporter activity in transfected C2C12 myotubes, we propose that at early stage of differentiation activity of the β enolase enhancer is repressed by the abundant presence of BERF-1, which precludes a potential activator from binding to the BEE-1 site or to an overlapping site. During muscle differentiation, the ratio of positive to negative regulatory binding activities changes in favor of the activator due to the developmental down-regulation of BERF-1 and/or by the increasing of different binding activities, as we observed in EMSA experiments with nuclear extracts from adult muscle. The proposed model implies that BERF-1 exerts its activity through competition for binding site occupancy, which is considered a passive transcriptional repression mechanism, but on the other hand, the factor possesses an intrinsic repressing activity that inhibits transcription initiation directly, and this is a mode of action of the active transcriptional repressors (50). However, as more studies contribute to unravelling repression mechanisms, this classification has become loose; for example, it has been recently reported that the human Cut homeodomain protein represses gene expression by both mechanisms: active repression and competition for binding site occupancy (60).

A candidate for the putative positive regulator competing for binding to the BEE-1 site might be Sp1, as has been proposed for the gastrin gERE element, which has been shown to bind both Sp1 and the rat homologue of BERF-1 (37). Although this possibility cannot be entirely excluded, our data indicate that different factors are probably involved. In nuclear extract from adult muscle where BERF-1 binding activity was dramatically reduced, we did not observe a consequent increasing of Sp1 binding activity but rather the appearance of novel DNA-protein complexes. These results are consistent with preliminary Southwestern analyses that indicate the presence of at least one muscle-enriched polypeptide that binds in a sequence-specific manner to the BEE-1 element. Further studies are in progress to identify the positive regulator(s) acting on the BEE-1 element and clarify the molecular mechanisms controlling the developmentally regulated expression of the β enolase gene in skeletal muscle.

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