Modulation of muscle creatine kinase promoter activity by the inducible orphan nuclear receptor TIS1

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TIS1, an inducible orphan nuclear receptor, was originally isolated as a tumour-promoter-inducible gene in mouse 3T3 cells and later shown to be induced by growth factors and other extracellular stimuli. We show here that TIS1 mRNA was expressed in proliferating C2C12 mouse skeletal muscle cells but that the level of TIS1 expression increased during muscle differentiation. Overexpression of TIS1 transactivated muscle creatine kinase (MCK) reporter genes containing as little as 80 bp of the proximal 5' flanking region. In contrast, a promoterless TIS1 construct and a frameshift mutant TIS1 construct were unable to transactivate the MCK reporter gene.

Moreover, the effect exerted by TIS1 appeared to be selective for the MCK promoter. Treatment of C2C12 cells with forskolin, which is known to induce TIS1 expression, also stimulated MCK reporter gene activity. Interestingly, *in vitro* translated TIS1 protein failed to bind to the MCK promoter region, suggesting that the transactivation effect of TIS1 may be mediated without direct interaction of the protein with the MCK promoter DNA. Collectively, these results suggest that changing levels of TIS1 may help to modulate the expression of MCK, and perhaps other muscle-specific genes, in response to physiological changes.

INTRODUCTION

TIS1 (also called nur77 or N10) is a primary response gene that was originally identified by its rapid induction by the tumour promoter phorbol 12-myristate 13-acetate (PMA) in Swiss mouse 3T3 cells [1–3]. Like the other primary response genes, TIS1 can be induced in diverse cell types by a wide range of stimuli, including growth factors, differentiation-inducing factors, membrane depolarization and calcium influx [2,4–6]. For example, the rat homologue of TIS1 was identified as the nerve growth factor-inducible gene NGFIB in PC12 cells [7], while nur77/TIS1 was also isolated as a serum-inducible primary response gene in mouse 3T3 cells during the G0/G1 transition [2]. The protein encoded by TIS1 has a high degree of identity with members of the thyroid/steroid nuclear receptor superfamily [2,3,7]. Members of this superfamily are known to act as ligand-dependent transcription factors capable of regulating the expression of their target genes, and available evidence suggests that TIS1 can indeed act as a potent transcriptional activator, perhaps even in the absence of any ligand [8–10]. Interestingly, several findings suggest that TIS1 might also play important roles during animal growth and development. We have shown, for example, that the expression of TIS1 was regulated in a tissue- and developmental stage-specific manner [11], while the human homologue of TIS1, NAK1, was identified and isolated from a human fetal muscle cDNA library [12]. Moreover, both TIS1 (nur77) and a distinct but highly related gene, RNR1, were shown to be rapidly induced in regenerating liver [13]. In spite of all these findings, however, relatively little is known about the potential downstream targets of the TIS1 protein. Thus far, the only gene implicated to be regulated by TIS1/NGFIB in a physiological manner is the gene coding for steroid 21-hydroxylase in mouse Y1 adrenocortical cells [14]. Whether and how TIS1 functions in other cell and tissue types is largely unknown.

In this regard, our previous finding that a gradual increase in TIS1 mRNA occurs in mouse skeletal muscle tissues during postnatal development [11], together with our recent studies demonstrating that TIS1 can be induced by growth factors and other agents in the established mouse skeletal muscle cell line C2C12 [15,16], hinted at a potentially important role for TIS1 in skeletal muscle cells. In the present paper, using C2C12 cells as a model system, we demonstrate that the TIS1 mRNA is in fact expressed at a detectable level in proliferating myoblasts not subjected to additional stimulation. Furthermore, a 3–5-fold increase in the TIS1 mRNA level was observed during the muscle differentiation process in culture, and the expression of TIS1 could be further enhanced by *in vitro* treatment of differentiated C2C12 cells with agents believed to act as co-transmitters in neuromuscular junctions. In addition, co-transfection studies showed that the TIS1 protein can transactivate a reporter gene (–80MCKCAT) that is regulated by the proximal promoter region of the mouse muscle-specific creatine kinase (MCK) gene (–80 to +7 relative to the transcriptional start site). Treatment with forskolin, which has been shown to induce the expression of TIS1 in C2C12 cells, also resulted in an increase in –80MCKCAT activity, suggesting that activation of the endogenous TIS1 gene may be able to modulate MCK promoter function. Interestingly, activation of the MCK promoter did not seem to require direct binding of the TIS1 protein to the MCK promoter region, suggesting that TIS1 action on MCK may be mediated either by indirect interaction through other cellular proteins or through the secondary activation of other cellular genes. Taken together, the data presented in this paper raise the possibility that TIS1 may serve as an accessory factor in conjunction with other muscle-specific and ubiquitous transcription factors to fully activate the expression of the MCK gene. The highly inducible nature of TIS1 in response to hormonal stimulation and membrane depolarization [2,5] suggests that the

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; NGFIB, nerve growth factor-inducible protein; MCK, muscle creatine kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; CGRP, calcitonin-gene-related peptide.

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TIS1 protein might be important in modulating the expression of muscle-specific genes in response to physiological alterations.

MATERIALS AND METHODS

Cell culture

Both C2C12 and 10T $\frac{1}{2}$ cells were cultured at 37 °C under a humidified atmosphere of 5% CO $_2$ in air. All cultures were routinely maintained on gelatin-coated culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 50 μ g/ml gentamycin. Differentiation of C2C12 cells was induced at approx. 80% confluence by rinsing the cultures twice with Puck's saline G followed by exposure to a differentiation-permissive medium (DMEM containing 5% horse serum).

Immunostaining

Cells grown on 150 mm dishes were washed twice with PBS and fixed by incubation in acetic acid/formaldehyde/ethanol (5:3:70, by vol.) at room temperature for 1 min. The fixed cells were incubated in 1% horse serum in PBS for 1 h to block non-specific binding and then incubated for 30 min with MF20 anti-myosin heavy chain) monoclonal antibody (1:20 dilution). Biotinylated goat anti-mouse IgG (1:200 dilution) was used as the secondary antibody. After each antibody application, the cells were washed with PBS five times for 5 min each. The immunoperoxidase reaction was performed using the Vectastain ABC kit as described by the manufacturer (Vector Lab).

Plasmids

The MCK cDNA plasmid pHO.4 and the reporter plasmids p-3300MCKCAT and p-80MCKCAT were kindly provided by Dr. Jean Buskin and Dr. Stephen Hauschka (University of Washington, Seattle, WA, U.S.A.). The isolation and derivation of these plasmids have been described previously [17]. The TIS1 plasmid pTIS1 was produced by ligating the full-length TIS1 cDNA into the *Eco*RI site of PUC18, and does not contain a functional eukaryotic promoter. The TIS1 expression vector pTIS1EV was constructed by ligating the pTIS1 *Eco*RI restriction fragment comprising the full-length cDNA into the unique *Eco*RI site of pEMSV α 2, kindly provided by Dr. Andrew Lassar (Harvard Medical School, Cambridge, MA, U.S.A.) [18]. The orientation of the TIS1 insert in pEMSV α 2 was determined by restriction enzyme digestion. A TIS1 frame-shift mutation construct (pTIS1 Δ BalI) was made by deleting a *Bal*I fragment from pTIS1EV, thus destroying the correct reading frame of TIS1 beyond the first 38 amino acids. pSV2 β -galactosidase was purchased from Promega. The pCHOB plasmid, originally isolated from Chinese hamster ovary cells and recognizing a constitutively expressed mRNA [19], was used as a control in Northern blot analysis to normalize for variations in RNA loading and transfer.

RNA isolation and Northern blot analysis

Total RNA was isolated using a modified guanidine thiocyanate acid phenol extraction method as described in [20]. Poly(A) RNA was isolated by using the FASTTRACK kit from Invitrogen. RNA was fractionated by electrophoresis on a 1.1% agarose gel with 6% formaldehyde and transferred to a nitrocellulose membrane (Schleicher & Schuell). Radioactive cDNA probes for TIS1, MCK and CHO B were prepared by labelling with [α - ^{32} P]dCTP (NEN) to a specific radioactivity of about 1×10^8 c.p.m./ μ g using the Klenow fragment of *Po*II and random

oligonucleotide primers. RNA membrane blots were pre-hybridized at 42 °C for 2–10 h in a solution of 50% formamide, 5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50 mM sodium phosphate, pH 6.5, 250 μ g/ml denatured salmon sperm DNA, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll and 1% glycine. Membranes were hybridized with ^{32} P-labelled probes at a concentration of 1×10^6 c.p.m./ml at 42 °C for at least 18 h in a solution containing 50% formamide, 5 $\times$ SSC, 20 mM sodium phosphate, pH 6.5, 100 μ g/ml denatured salmon sperm DNA, 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 10% dextran sulphate. Membranes were then washed in 2 $\times$ SSC with 0.2% SDS at 50 °C for 30 min, followed by another 30 min wash with 0.2 $\times$ SSC containing 0.2% SDS at 55 °C, and then exposed to Kodak XAR-5 film with an intensifying screen at -70 °C.

Transient transfection and chloramphenicol acetyltransferase (CAT) assay

Transfection of plasmid DNA was carried out by conventional calcium phosphate precipitation [21]. Briefly, plasmid DNA dissolved in 248 mM CaCl $_2$ was added slowly to an equal volume of 2 $\times$ HBS (280 mM NaCl, 50 mM Hepes, 1.5 mM Na $_2$ HPO $_4$, 10 mM dextrose, pH 7.05) and precipitates were allowed to form for 30 min at room temperature. Calcium phosphate/DNA precipitates were then added to proliferating cells cultured in growth medium (DMEM+10% FBS). After 16–24 h, the cultures were osmotically shocked by incubation in 15% glycerol in 1 $\times$ HBS for 4 min and then fed with either growth medium (DMEM+10% FBS) or differentiation-promoting medium (DMEM+5% horse serum). At different times after transfection, the cells were harvested and assayed for β -galactosidase activity to normalize for transfection efficiency among different samples. Cell extracts containing the same amount of β -galactosidase activity were assayed for CAT activity [22], which was monitored by TLC. The CAT activity was quantified by excising ^{14} C-labelled spots from TLC plates and counting the radioactivity in scintillation fluid. The transfection assays were performed on multiple sets of cultures over a period of several months, with at least two different DNA preparations for each plasmid to minimize the possibility of artifacts arising from peculiarities in either the cell cultures or the DNA preparations.

In vitro translation

Full-length TIS1 cDNA cloned into the *Eco*RI site of the pGEM4 vector (Promega) was used to express the TIS1 protein *in vitro* by coupled transcription and translation with the SP6 TnT reticulocyte lysate system (Promega), according to the manufacturer's instructions.

Electrophoretic mobility shift assay

The -80MCKCAT plasmid was digested with *Hind*III and *Bst*EII to produce a fragment encompassing the -80 to +7 region of the MCK promoter. The DNA fragment was filled in with the Klenow fragment of polymerase I in the presence of [α - ^{32} P]dCTP and other unlabelled dNTPs. A radiolabelled DNA fragment containing an authentic TIS1/NGFIB binding site was generated in a similar manner from a reporter plasmid consisting of the TIS1 binding site placed upstream of a simian virus 40 (SV40) promoter. The radiolabelled MCK promoter fragment was isolated by NuSieve agarose gel electrophoresis on to DEAE paper as described [23]. The specific radioactivity of the labelled probe was (2–8) $\times 10^7$ c.p.m./ μ g. Aliquots of the transcription/

translation mixture with or without added TIS1 plasmid (5 μ l) were preincubated at room temperature for 15 min with 10 μ l of binding buffer [7.5mM Tris/HCl, pH 7.5, 75 mM NaCl, 1.5 mM dithiothreitol, 7.5 mM $MgCl_2$, 15% glycerol and 0.5 μ g of poly(dI·dC)]. The samples were incubated for an additional 20 min at room temperature after the addition of ^{32}P -labelled DNA fragments (15000–25000 c.p.m.) and analysed by electrophoresis on non-denaturing 5% polyacrylamide gels in $0.5 \times TBE$ (45 mM Tris/45 mM boric acid/1 mM EDTA). The gels were dried and exposed to XAR-5 film at $-70^\circ C$ with an intensifying screen.

RESULTS

TIS1 mRNA is expressed in proliferating skeletal muscle cells

TIS1 was initially identified as a PMA-inducible gene in mouse 3T3 fibroblasts, and was subsequently shown to be rapidly and transiently induced by a variety of growth factors and cAMP mobilizing agents in several cell systems [2,24]. Previous studies showed that the level of TIS1 mRNA in unstimulated cells was below the limit of detection when total RNA was analysed. To determine if the TIS1 gene was nevertheless expressed at a low basal level in exponentially growing myoblasts, proliferating C2C12 cells cultured in DMEM + 10% FBS were harvested at 70–80% confluence and the poly(A)-containing RNA was isolated and examined by slot-blot analysis. As shown in Figure 1, an appreciable level of TIS1 mRNA was clearly present in proliferating myoblasts when as little as 0.7 μ g of RNA was loaded. The intensity of the TIS1 hybridization signal was comparable with that detected with 25-fold less poly(A) RNA from cells that had been stimulated with forskolin, as determined by densitometry of the autoradiograph. Accurate loading of the RNA was confirmed by re-analysis of the same blot for the constitutively expressed CHOB mRNA, which showed the same hybridization intensity when equal amounts of RNA from stimulated and unstimulated cells were loaded. Based on this estimate, the TIS1 mRNA in proliferating unstimulated myoblasts was expressed at about 4% of the level detected after 1 h of forskolin stimulation.

Exposure to differentiation-permissive conditions increases the level of TIS1 expression

If TIS1 is involved in cell proliferation, its level of expression might be expected to decrease as the cells withdraw from the cell

Figure 1 Expression of TIS1 in proliferating C2C12 cells

Poly(A)-containing RNA was isolated from actively growing C2C12 cells that either received no additional treatment (unstimulated) or were stimulated by treatment with 20 μ M forskolin (FK) for 1 h. The abundance of TIS1 mRNA was determined by slot-blot hybridization to ^{32}P -labelled TIS1 cDNA. The indicated amounts of RNA from stimulated or unstimulated cells were loaded. The slot blot was stripped following autoradiography and rehybridized to a CHOB cDNA probe corresponding to a constitutively expressed mRNA first identified in Chinese hamster ovary cells [19] and used here as a standard for RNA loading.

Figure 2 The level of TIS1 mRNA increases during differentiation of C2C12 cells

C2C12 cells were either maintained under proliferation conditions or induced to differentiate by exposure to differentiation-permissive medium for 4 days, as described in the Materials and methods section. Poly(A)-containing RNA was isolated from cells cultured under both conditions and analysed (A). The abundance of TIS1 and MCK mRNA was determined by Northern hybridization with either ^{32}P -labelled TIS1 full-length cDNA or MCK cDNA. A sample of 15 μ g of poly(A) RNA was loaded in each lane. The same blots were also hybridized with pCHOB cDNA. Parallel dishes were immunostained with anti-(myosin heavy chain) antibody to assess the degree of muscle differentiation (B). P, proliferation conditions; D, differentiation conditions.

cycle and become differentiated. To test this theory, C2C12 myoblasts were grown to 80% confluence and induced to become terminally differentiated by exposing them to differentiation-promoting medium (DMEM + 5% horse serum) for 4 days. The level of TIS1 mRNA was examined and compared with that in proliferating cultures. The same blot was reprobed with MCK cDNA to assess the degree of muscle differentiation. Sister dishes were immunostained with an anti-(myosin heavy chain) monoclonal antibody (MF20). As expected, MCK was expressed at very high levels under the differentiating culture conditions, but was almost completely undetectable under the proliferating culture conditions (Figure 2A). Morphological and immunocytochemical staining likewise revealed a substantial degree of differentiation under the differentiation-permissive conditions and little or no differentiation under the proliferating culture conditions (Figure 2B). Contrary to our expectations, however, the level of TIS1 mRNA was increased 3–5-fold in the differentiated culture as compared with the proliferating culture (Figure 2A). Interestingly, the intensity of the CHOB signal appeared to be lower under differentiation conditions, even though ethidium bromide staining of the RNA indicated that roughly the same amount of RNA was loaded in each lane. Since we did not detect a decrease in the CHOB hybridization signal when total RNA from differentiated cultures was analysed (results not shown), the discrepancy is most probably due to the fact that many muscle-specific poly(A)-containing RNAs become abundantly expressed following differentiation, which would in effect decrease the relative proportion of CHOB even if CHOB expression did not change on a per cell basis. If the TIS1 mRNA level was normalized

Figure 3 TIS1 expression is stimulated by ATP and CGRP

C2C12 cell cultures were allowed to differentiate for 4 days (for CGRP experiments) or 6 days (for ATP experiments) by incubation in the differentiation-permissive medium consisting of DMEM and 5% horse serum. The differentiated muscle cell cultures were then stimulated by the addition of 0.1 μ M CGRP, 0.1 μ M PMA or 0.1 mM ATP in the presence of 10 μ g/ml cycloheximide as indicated. Control lanes (-) represent samples treated with 10 μ g/ml cycloheximide alone. Total RNA (10 μ g) was analysed by Northern hybridization to a ^{32} P-labelled TIS1 cDNA probe. Ethidium bromide staining confirmed equivalent amounts of RNA loading and transfer for each sample (not shown).

to the CHOB signal, the increase in expression under differentiation conditions would be even more pronounced.

TIS1 expression in differentiated C2C12 cells is further increased by treatment with ATP or calcitonin-gene-related peptide (CGRP)

The apparently increased expression of TIS1 during muscle differentiation suggests that it might play some functional role in differentiated muscle fibres. Since TIS1 is known to be induced rapidly by a variety of stimuli, one might infer that TIS1 could be involved in mediating transcriptional responses to external signals in skeletal muscle tissues. Since skeletal muscle properties and functions are known to be regulated by nerve activities, we examined whether the level of TIS1 expression in differentiated muscle cultures might be induced by agents that are believed to act as co-transmitters at the neuromuscular synapses [25–27]. C2C12 cells were placed under differentiation conditions for 4–6 days, and then challenged with either 0.1 mM ATP or 0.1 μ M CGRP in the presence of 10 μ g/ml cycloheximide. Parallel cultures were stimulated with 100 nM PMA as positive controls. RNA was harvested after 3 h of stimulation and subjected to Northern blot analysis. We found that both ATP and CGRP stimulated TIS1 expression in C2C12 cells to a substantial level, comparable with that seen with PMA stimulation (Figure 3). TIS1 expression in skeletal muscle tissues *in vivo* might likewise be elevated in response to release of co-transmitters at the nerve terminal, and the increased expression might contribute to the physiological responses of muscle fibres to nerve stimulation.

Expression of TIS1 transactivates a reporter gene directed by the MCK proximal promoter

To determine if increased TIS1 expression might have functional significance in differentiated muscle, we co-transfected into C2C12 cells a TIS1 expression vector (pTIS1EV) and a CAT reporter gene directed by a 3.3 kb 5' fragment of the mouse MCK gene (–3300MCKCAT), and measured reporter gene activity after allowing the cells to differentiate. Previous studies have shown that the MCK gene is transcriptionally activated during muscle differentiation and that the regulatory region resides within the 3.3 kb region used in our reporter gene [28]. As shown in Figure 4(A) (gel 1), overexpression of TIS1 caused an average 7-fold increase in CAT reporter gene activity (based on three independent experiments with duplicate samples). To determine

the minimal region necessary for the action of TIS1, the ability of TIS1 to transactivate the CAT reporter gene containing the –80 to +7 fragment of the MCK gene (–80MCKCAT) was examined. As shown in Figure 4(A) (gel 2), deletion of the –3300 to –80 region did not abolish the transactivation by TIS1 (an average 4-fold increase from three independent experiments).

Since muscle-specific regulatory factors such as myoD and myogenin have been shown to interact with and activate the MCK gene though enhancer elements located in the –1256 to –1020 region [29–34], the result shown in Figure 4(A) suggests that muscle-specific factors might not be required for TIS1-induced transactivation from the proximal promoter region. To address this question further, the ability of TIS1 to transactivate –80MCKCAT was determined in mouse embryonic fibroblast 10T $\frac{1}{2}$ cells and in C2C12 cells under proliferating conditions. The results in Figure 4(B) show that TIS1 still retained its ability to transactivate –80MCKCAT in both systems (averages of 9- and 7-fold enhancement respectively, each based on three independent experiments). These data confirmed that TIS1 could transactivate the –80MCK proximal promoter region in the absence of muscle-specific regulatory factors. As expected, overexpression of TIS1 alone was insufficient to activate –3300MCKCAT in non-myogenic cells or in C2C12 cells under non-differentiating conditions (results not shown). Apparently, the presence of the muscle-specific enhancer region was able to suppress the ability of TIS1 to transactivate MCK. It is important to note, however, that this suppressive effect was readily reversed in the differentiated cultures (Figure 4A), presumably due to the presence of functional myogenic regulatory factors. Taken together, these results suggest that TIS1 might play a supportive or modulatory role in the expression of MCK in differentiated skeletal muscle cells.

Transactivation of MCKCAT requires expression of the complete coding region of TIS1, and the effect exerted by TIS1 is specific for the MCK promoter

To confirm that the transactivation effect exerted by TIS1 is mediated by the TIS1 protein, we introduced a frameshift mutation into TIS1 by deletion of a *Bal*I fragment, which effectively destroyed the open reading frame of the cDNA beyond the first 38 amino acids; we then tested the ability of this mutant construct to transactivate –80MCKCAT. Figure 4(C) shows that, in contrast to the wild-type TIS1, the mutated TIS1 (pTIS1 Δ *Bal*I), which should no longer produce functional TIS1 protein, failed to transactivate –80MCKCAT in C2C12 cells. To further demonstrate that production of the TIS1 protein was necessary for the transactivation effect, we co-transfected MCKCAT with a TIS1 plasmid (pTIS1) lacking any eukaryotic promoter sequence and examined the level of CAT expression. As shown in Figure 4(C) (gel 1), MCKCAT activity was not altered in response to the promoterless TIS1 construct. Transactivation of MCK is therefore likely to be mediated through the TIS1-encoded protein, and is not simply due to the presence of TIS1 DNA or RNA sequences. Similarly, deletion of the MCK promoter region abolished both basal and TIS1-stimulated CAT activity (basic-CAT in Table 1), indicating that the transactivating effect of TIS1 was dependent on the MCK promoter region and was not mediated through some cryptic promoter in the plasmid. Moreover, ligating the MCK promoter region to a different CAT reporter vector conferred TIS1 regulation to the new vector, suggesting that TIS1 was not acting on a cryptic enhancer site in the MCKCAT plasmid sequence (results not shown). Finally, the transactivation effect of TIS1 appeared to be

Figure 4 Expression of TIS1 positively regulates CAT reporter gene activity directed through 5'-proximal promoter regions of the MCK gene in C2C12 and 10T_{1/2} cells

(A) Plasmid DNAs were co-transfected into proliferating C2C12 cells cultured in DMEM + 10% FBS by calcium phosphate precipitation and briefly exposed to 15% glycerol as described in the Materials and methods section. Cells were then switched to differentiation-permissive medium (DMEM + 5% horse serum). Cultures were harvested 4 days later, and the level of CAT activity was determined. Representative autoradiographs from the CAT assays are shown. Gel 1: the —3300MCKCAT reporter plasmid (2 μ g) was co-transfected with 8 μ g of either pTIS1EV or the expression vector pEMSV without insert as a control. Gel 2: the —80MCKCAT reporter plasmid (5 μ g) was co-transfected with either pTIS1EV or pEMSV as indicated. The fold induction by TIS1 varied among experiments, with ranges of 3–15-fold for —3300MCKCAT and 2–8-fold for —80MCKCAT. The average induction from three independent experiments, each with duplicate samples, was 7- and 4-fold for —3300MCKCAT and —80MCKCAT respectively. (B) —80MCKCAT was co-transfected with either pTIS1EV or pEMSV by calcium phosphate precipitation into either proliferating C2C12 cells or 10T_{1/2} cells cultured in growth medium. Cells were maintained in growth medium after glycerol shock and harvested for CAT activity assay 2 days after transfection. A representative autoradiogram from the CAT assay is shown. The average induction was 9- and 7-fold for 10T_{1/2} and C2C12 cells respectively (three independent experiments). The amounts of plasmids used were as in (A). (C) The reporter plasmid —80MCKCAT (5 μ g) was co-transfected with either the pTIS1 construct (8 μ g), which contains the complete TIS1 cDNA but does not possess a functional eukaryotic promoter (gel 1), or the pTIS1 Δ BaI construct (8 μ g), in which a frameshift mutation was introduced after the first 38 amino acids (gel 2). Experimental conditions were the same as described in (B). Identical results were observed from two independent experiments. (D) C2C12 cells transfected with —80MCKCAT were stimulated with forskolin (20 μ M) 24 h after glycerol shock (+). Control cultures were left untreated (—). After 20 h, cells were harvested for CAT assay by TLC as described in the Materials and methods section. The amounts of extracts used in all the CAT assays were normalized according to the β -galactosidase activity expressed from a co-transfected pSV2- β -gal plasmid.

Table 1 The modulatory effect of TIS1 is selective for the MCK promoter

Each reporter plasmid was transfected into C2C12 cells with 5 μ g of pTIS1EV or the control pEMSV expression plasmid without insert. CMV, cytomegalovirus; in basic-CAT, the MCK promoter region has been deleted (see the text). Data are presented as the ratio of CAT activity in the presence and absence of the TIS1 plasmid; results are means $\pm$ S.E.M.

Promoter-reporter construct	CAT activity ratio (+ TIS1/— TIS1)
SV40-CAT	1.03 $\pm$ 0.20
CMV-CAT	1.05 $\pm$ 0.10
Basic-CAT	1.10 $\pm$ 0.10

selective for the MCK promoter, since expression of TIS1 had little or no effect on CAT reporter gene activity driven by either an SV40 promoter or a cytomegalovirus promoter (Table 1).

MCKCAT activity is enhanced by agents that stimulate endogenous TIS1 expression

In order to investigate whether induction of endogenous TIS1 might also be able to enhance the expression of —80MCKCAT, C2C12 cells transiently transfected with the —80MCKCAT plasmid were treated with forskolin (20 μ M) 24 h after glycerol shock. The level of CAT activity was assayed 20 h after forskolin addition. Addition of forskolin substantially enhanced CAT reporter gene activity driven by the —80 proximal promoter region of the MCK gene by an average of 3-fold (Figure 4D). Since forskolin treatment of C2C12 cells had previously been

shown to induce a high level of TIS1 expression [15,16], the current finding is consistent with the interpretation that stimulation of endogenous TIS1 expression can transactivate MCKCAT. Since the —80MCK promoter did not contain any consensus cAMP responsive elements, activation of —80MCKCAT and TIS1 expression did not appear to be caused by the coincidental presence of common regulatory elements. However, other mechanisms through which forskolin might have exerted its effect on the MCK promoter have not been completely ruled out.

Transactivation of the MCK promoter does not require direct TIS1 binding to the promoter region

Since the —80MCK promoter fragment does not contain a consensus TIS1/NGFIB binding site, we were interested to see whether TIS1 might be able to transactivate MCK by binding to an alternative DNA sequence. Electrophoretic mobility shift assays were performed by incubating *in vitro* translated TIS1 protein with either the —80 to +7 MCK promoter fragment or a DNA fragment containing an authentic TIS1 binding site. Under the experimental conditions used in the assay, we detected no specific binding of TIS1 to the MCK promoter fragment (Figure 5). In contrast, specific protein–DNA complexes were readily observed when the DNA fragment containing the authentic TIS1 binding site was incubated with reticulocyte lysate programmed with the TIS1 plasmid. Thus, under conditions where binding to an authentic TIS1 binding site was observed, the TIS1 protein was unable to bind to the MCK promoter with sufficient affinity to be detected. Taken together, our results suggest that TIS1 might be able to transactivate the MCK

Figure 5 Lack of TIS1 binding to the MCK promoter region

Samples of 5 μ l of TnT reticulocyte lysate transcription/translation mixtures programmed with TIS1 plasmid (+) or without any plasmid (—) were used in electrophoretic mobility shift DNA binding assays with ^{32} P-labelled DNA fragments corresponding either to the -80 to $+7$ MCK promoter region (A) or to a DNA fragment containing an authentic TIS1 binding site (B). The gel was exposed to XAR-5 film for 16 h with an intensifying screen. Longer exposure of the gels (48 h) also failed to reveal any sign of specific binding to the MCK probe (not shown).

promoter without binding directly to DNA sequences located in this region of the gene.

DISCUSSION

TIS1 is a member of the mitogen- and growth-factor-inducible primary response gene family, and encodes a nuclear-receptor-like transcription factor [2,3,8,12]. Like other primary response genes, TIS1 is rapidly and transiently induced in response to a variety of stimuli in a number of biological systems [4,7,24]. Induction of TIS1 expression has been shown to occur through transcriptional activation [5,24]. In addition, TIS1 mRNA has been detected previously in the skeletal muscle tissues of newborn mice, and the expression level increases during postnatal development [11]. Here we present evidence that a substantial level of TIS1 mRNA is present in actively growing mouse C2C12 myoblasts (Figure 1). By using a homogeneous cell line, we have documented the expression of TIS1 in skeletal muscle cells and eliminated the possibility of connective tissue or fibroblast contamination that could complicate analysis of gene expression at the tissue level. In agreement with the postnatal increase in TIS1 mRNA in skeletal muscle tissues, the message level of TIS1 increased when proliferating myoblast cultures were switched to differentiation-permissive conditions (Figure 2).

Increased expression of other primary response genes (e.g. *c-fos*) has been reported during the differentiation of other cell types (reviewed in [35]). Since many of these primary response genes were originally identified by their inducibility in response to growth-stimulatory signals, their increased expression during terminal differentiation may seem to be paradoxical at first glance. This apparent enigma can, however, be readily resolved when one takes into consideration that products of primary response genes may function differently in diverse physiological contexts as a result of different induction kinetics, quantitative differences in the level of expression, different post-translational modifications [4] or heteromolecular complex formation [36–38]. In addition, since multiple primary response genes are often coordinately induced by a stimulus, the precise functional outcome is likely to depend on the exact pattern of gene expression induced by each stimulus. Taking all these factors into consideration, it is conceivable that the TIS1 protein in differentiated

muscle cells could function quite differently than during exponential cell growth. In agreement with this idea, our observation that treatment with agents purported to function as co-transmitters at motor end plates led to a robust induction of TIS1 expression in the differentiated C2C12 muscle cells (Figure 3) suggests that TIS1 expression in differentiated muscle tissue may mediate physiological responses to neurohormonal stimulation rather than growth factor action.

While the biochemical characteristics of the TIS1 protein may implicate it as a likely intermediary factor mediating the transcriptional responses of cells to external stimuli, the precise biological functions of TIS1 remain largely unknown. Studies have shown that the TIS1 homologue NGFIB is capable of transactivating reporter genes through an NGFIB regulatory element, and that a nur77–GAL4 chimera protein containing the GAL4 DNA binding domain can transactivate genes fused to GAL4 binding sites. Results from these studies suggest that the nur77/NGFIB/TIS1 protein can serve as a potent transcriptional activator in the absence of an exogenously added ligand [8–10]. NGFIB/TIS1 has been implicated in the transcriptional activation of steroid 21-hydroxylase in adrenocortical cells in response to adrenocorticotrophic hormone stimulation [14]. Although NGFIB-regulatory-element-like sequences are found in the regulatory regions of many cellular genes [9], the 21-hydroxylase gene was the only gene where a functional response to TIS1 had been demonstrated.

In this study we have identified a second potential target gene for TIS1. We demonstrated by co-transfection studies that MCK reporter genes can be transactivated by expression of TIS1 (Figure 4A). Our results show that the 5' flanking region between positions -80 and $+7$ of the MCK gene was sufficient to mediate the transactivation effect of TIS1 in both proliferating myoblasts and differentiated myocytes (Figure 4B). The effect was dose-dependent with the expression vector being used in the range 0.5–8.0 μ g per transfection (W.-L. Yang and R. W. Lim, unpublished work). Transactivation of the MCK reporter gene was abolished by a frameshift mutation in TIS1 that disrupted its coding sequence (Figure 4C), and could be mimicked by stimulating cells with agents known to stimulate TIS1 expression (Figure 4D). Similar to what has been observed in other systems, no addition of exogenous ligand was required for the transactivation effect.

That the effect of TIS1 could be mediated through the proximal promoter region of MCK was not anticipated. Studies have shown that the MCK gene contains two muscle-specific regulatory regions, one located between positions -1256 and -1020 5' of the transcription start site and another located in the first intron [17,30]. Whereas the upstream regulatory elements have been found to mediate the cell-type- and differentiation-specific expression of creatine kinase, the MCK promoter appeared to be functional in both myoblasts and myotubes, as well as in non-myogenic cell lines, when coupled to appropriate heterologous enhancer elements [30]. Nevertheless, other evidence suggests that the MCK promoter region might not be completely non-specific in function. First, the MCK enhancer exhibits a 3–5-fold preference for the MCK promoter over heterologous promoters such as the SV40 promoter [30]. This promoter–enhancer synergism suggests that the MCK promoter might interact with certain cellular factors necessary to optimize the promoter–enhancer interaction. This notion is supported by the second observation that the MCK -80 promoter region confers a weak but noticeable differentiation-specific activation of a CAT reporter gene in MM14 myocytes [17]. Whether the 3–5-fold increase in TIS1 expression under differentiation conditions (Figure 2A) could account for the MCK promoter–enhancer

synergism and differentiation-specific activation of the proximal promoter region [17,30] remains to be proven.

How does TIS1 transactivate the MCK promoter? We do not yet have a complete answer to this question. The proximal promoter region of MCK does not contain a consensus TIS1 (NGFIB) binding site, and our results indicate that the TIS1 protein does not bind to the DNA sequence in this region with high affinity (Figure 5). The transactivation effect of TIS1 on the MCK promoter thus appears to be mediated without direct DNA binding, although it remains possible that TIS1 might bind indirectly to the promoter region *in vivo* by interacting with other cellular proteins. Similar examples of gene activation without direct DNA binding have been reported for other transcription factors, including other nuclear receptors [39–41]. In addition, members of the nuclear receptor superfamily have been shown to interact with proteins of the transcription initiation complex, including the general transcription factor TFIIB (reviewed in [42]). That TIS1 may stabilize the formation of the initiation complex without high-affinity binding to DNA is an unorthodox but intriguing possibility. Alternatively, TIS1 could regulate the expression of other transcription factors which might then act on the MCK promoter. Recent evidence has also indicated that TIS1 could dimerize with the retinoid X receptor [43], which has been shown to modulate muscle differentiation partly through regulating the expression of muscle-specific regulatory genes [44,45]. Overexpression of TIS1 might thus affect MCK promoter activity indirectly by affecting retinoid X receptor function.

What functional significance might the TIS1–MCK interaction entail? Since little or no effect on the SV40 or cytomegalovirus promoters was seen (Table 1), the transactivation appeared to be selective for MCK and not a generalized effect on gene transcription. The extent of reporter gene induction by TIS1 varied among experiments from 2- to 15-fold. This magnitude of stimulation is less than what is achieved by muscle-specific factors through the MCK upstream muscle-specific enhancer, implying that the increase in TIS1 during muscle differentiation is unlikely to be a determining factor in muscle-specific MCK activation. Instead, the result is consistent with the idea that TIS1 might function in conjunction with the muscle-specific regulatory factors to provide full activation of the MCK gene in differentiated muscle tissues. In addition, since TIS1 expression in differentiated muscle cells can be induced by hormones and putative neural co-transmitters, and has been shown to be responsive to membrane depolarization in other systems [6], its ability to transactivate MCK might reflect a plausible mechanism by which skeletal muscles modulate the expression of MCK, and perhaps other muscle-specific genes, in response to alterations in the extracellular environment, allowing the muscle tissues to adapt to physiological changes.

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