Differentiation-dependent mechanisms of transcriptional regulation of the catalytic subunit of phosphorylase kinase

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The amount of phosphorylase kinase in skeletal muscle is exquisitely sensitive to developmental signals such as differentiation and innervation, and is clearly regulated in such a manner so as to always maintain the γ catalytic subunit under the control of its regulatory α , β and γ subunits. To identify how the transcription of the γ subunit is regulated, we have analysed 3.8 kb of the upstream regulatory region using a luciferase reporter system. A complex sequence of interdependent regulations is evident. The γ catalytic subunit gene contains two inhibitory controls with very dominant features. Also evident are an array of multiple positive regulatory elements, prominent amongst which are four E-boxes, of which two are downstream, one is upstream and one is in the middle of the CAAT-TATA core promoter. Differentiation-dependent positive regulation arises as a consequence of both E-box regulation and the

activation of at least one other regulatory element. The primary mode of transcriptional regulation of the γ catalytic subunit gene appears to occur by the relief of regulation of an otherwise default inhibitory status. It is noteworthy that such a mode of regulation mirrors the regulation of the enzymic activity of many protein kinases, including phosphorylase kinase. With phosphorylase kinase, both its transcriptional regulation as well as the regulation of the protein itself, are primed to maintain the γ catalytic subunit either unexpressed or inactivate respectively, until a positive signal occurs to override an otherwise dominant default inhibitory condition.

Key words: E-boxes, integrated signalling, multi-subunit complexes, MyoD, transcriptional regulation.

INTRODUCTION

Phosphorylase kinase (PhK) is a key regulatory enzyme involved in the glycogenolytic pathway, catalysing the activation, by phosphorylation, of phosphorylase. This hexadecameric enzyme, with subunit composition $(\alpha\beta\gamma\delta)_4$, serves to integrate both hormonal and neuronal signals, leading to the breakdown of intracellular glycogen stores [1,2]. Physiological activation of the holoenzyme occurs as a result of either the phosphorylation of the α and β subunits by the cAMP-dependent protein kinase and/or the binding of Ca^{2+} to the intrinsic calmodulin subunit (i.e., δ). Activation of the enzyme *in vitro* may also occur as a consequence of phosphorylation of the regulatory α and β subunits by the catalytic γ subunit or by the Ca^{2+} -dependent binding of exogenous calmodulin to the α and/or β subunits. These various covalent and allosteric modifications of the α , β and δ regulatory subunits are thought to induce a conformational change in the protein that relieves their inhibition of the γ catalytic subunit. Within the PhK holoenzyme these regulatory mechanisms are tightly integrated as a consequence of subunit-subunit interactions (see [1,2] and references cited therein).

In vitro, in the absence of the regulatory subunits, the γ subunit is constitutively active and displays a level of activity equivalent to, or greater than, the fully activated holoenzyme [3–6]. Were free, and thereby unregulated, γ subunit to occur *in vivo*, uncontrolled glycogenolysis would occur at a very high rate with deleterious consequences for the cell. Several studies indicate, however, that there is a highly efficient co-ordination of the expression of the multiple subunits such that free γ subunit does not occur physiologically. This has been explored in a number of *in vivo* systems, including examination of PhK subunit

and mRNA levels during normal skeletal-muscle development [7], in regenerating muscle following bupivacaine-induced muscle degeneration [8] and during the process of re-innervation of muscle following nerve transection [7,9]. In all circumstances when the total level of PhK increased, there was a concomitant expression of the catalytic γ subunit and its regulatory subunits with the result that holoenzyme formation accompanied subunit expression ensuring control of γ subunit catalytic activity. When PhK activity was markedly reduced by nerve transection, the initial response was a decline in both γ subunit protein and mRNA levels, followed, after a brief delay, by a similar decrease in the levels of α and β subunit proteins. Interestingly, there was an extended delay before the levels of the α and β subunit mRNAs decline, thus maintaining a greater capacity for α and β subunit formation and ensuring that the γ subunit is only present as part of the holoenzyme [7]. The level of PhK holoenzyme, and its constituent subunits, appears to be governed by the transcriptional regulation and expression of the γ subunit.

Transcription of the γ subunit gene has also been shown to be critically influenced by muscle differentiation. In this respect, muscle-specific transcription factors, such as those in the MyoD family, have been shown to play key regulatory roles in dictating the profile of genes expressed during the transition from undifferentiated myoblasts to fully differentiated myotubes [10–15]. Several consensus motifs specific for these factors have been identified in the upstream regulatory region of a number of genes induced during myogenesis.

The present study has probed the mechanisms of regulation of γ subunit gene transcription. To do so, we have furthered our previous characterization of the γ subunit gene [16]; an elaborate array of transcriptional regulatory elements is now evident.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; UTR, untranslated region; ORF, open reading frame; SV40, simian virus 40; MCS, multiple cloning site; DTT, dithiothreitol; PhK, phosphorylase kinase; PKA, cAMP-dependent protein kinase; YY-1, Yin Yang-1.

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Cloning of a 3.8 kb region of the promoter, including 1.3 kb upstream of the transcription start site and 2.4 kb of the upstream intron A, into a luciferase reporter system allowed the identification of both positive and negative *cis*-acting transcriptional controls, including a pattern of four E-box consensus motifs (-CANNTG-), responsive to the MyoD family of transcription factors.

MATERIALS AND METHODS

Cell culture

The C2C12 mouse muscle cell line was acquired from the American Type Culture Collection. The RMo cell line was established as a rat myogenic line capable of differentiating into contracting myotubes in culture (a gift from Dr G. Merrill, Oregon State University, Corvallis, OR, U.S.A.) [17]. Both cell lines were maintained as proliferating myoblasts in a growth media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin (Life Technologies) and 20% fetal-calf serum (FCS). To differentiate the myoblasts to myotubes, cells were grown to $\approx 95\%$ confluency in this growth media, washed with PBS, and then switched to a differentiation media (DMEM supplemented with either 2% FCS or 5% horse serum). Fusion to multinucleated myotubes occurred in 48 h. NIH-3T3 cells were maintained in the DMEM-based growth media with 10% FCS. Following transfection with MyoD expression vectors the cells were switched to differentiation media.

RNA isolation and Northern blot analysis

Total RNA was isolated from cultured C2C12 myoblasts or myotubes using TRIzol Reagent (Life Technologies) and 5 μ g of total RNA was then fractionated on a 1%-agarose/formaldehyde gel and transferred by capillary electrophoresis to a membrane (Duralon-UV; Stratgene) using the procedures detailed previously [8]. Blots were fixed by UV irradiation and hybridized with a radiolabelled PhK- γ probe that corresponded to the 81 bp coding sequence of the C-terminus of the γ subunit plus 107 bp of the 3'-untranslated region (3'-UTR). An actin probe was also used as a control. Pre-hybridization was carried out at 68 °C for 30 min using QuikHyb (Stratagene) followed by hybridization with the probe at 68 °C for 2 h. Filters were then washed in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) for 15 min, followed by high-stringency washing in $0.2 \times$ SSC with 0.1% SDS at 60 °C for 1 h. Filters were exposed to Kodak X-OMAT film at -70 °C for 3 days.

Construction of luciferase expression plasmids

Regions of the 5'-UTR from the PhK- γ gene were cloned into either a pGL2-Basic vector (Promega), which encodes the luciferase open reading frame (ORF) without a promoter, or into the pGL2-Promoter vector (Promega), which contains the luciferase ORF under the control of the simian-virus-40 (SV40) promoter. Regions of the γ 5'-UTR, including the endogenous PhK- γ promoter, were cloned into the pGL2-Basic reporter construct, while regulatory regions outside the PhK- γ promoter were used in conjunction with the SV40 promoter construct. The potential PhK- γ regulatory elements in addition to the PhK- γ promoter were derived from a 3.8 kb *SacI/SacI* fragment of the γ G6 PhK- γ genomic clone [16]. This fragment spans from -1367 to +2500 (Figure 1, upper panel; constructs designated A1-I1 in Figure 3). It includes a major part of intron A, but does not contain the PhK- γ translation initiation start codon (Figure 1,

upper panel). The CAAT and TATA boxes of the PhK- γ promoter are located at -155 to -151 and -31 to -28 bp respectively. Some constructs were created from a modified 3.8 kb *SacI/SacI* fragment containing an upstream insertion of the multiple cloning site (MCS) of pBluescript KS 11(+). The two parent p-Luc plasmids containing the original PhK- γ 3.8 fragment and the MCS-PhK- γ 3.8 adduct [designated here as p(PhK- γ 3.8)Luc and p(MCS-PhK- γ 3.8)Luc respectively], were both sequenced in both directions across the -1367 to +670 region to confirm their identity and orientation, as well as to extend the partial sequence that has been previously obtained [16].

Restriction-enzyme digestion of the parent p-PhK- γ -Luc plasmids, using one or a combination of *BstEII*, *NcoI*, *BstXI*, *HincII* and *EcoRI*, followed by purification and re-ligation, allowed the generation of the following plasmids: pMCS(-1367/+870)Luc (E1), pMCS(-1367/+440)Luc (F1), p(-1367/+440)Luc (G1), pMCS(-1367/+357)Luc (H1) and p(-1367/+180)Luc (I1) (numbers refer to the PhK- γ gene sequence; MCS denotes the presence of the relevant portion of the pBluescript MCS). Each of these constructs contains the basic minimal PhK- γ -Core promoter region (-163/+24) that includes the transcription initiation site (+1) and the CAAT and TATA boxes. Similar procedures were used to generate the constructs designated in Figure 3, lower panel, as constructs C2-G2 respectively: p(-1367/-470)SV40-Luc, p(+350/+2500)SV40-Luc, p(+870/+2500)SV40-Luc and p(+350/+870)SV40-Luc. Each of these latter plasmids contains the denoted PhK- γ sequences upstream of the SV40 promoter, but does not contain the PhK- γ promoter itself. *KpnI-BstX* digestion of p(MCS-PhK- γ 3.8)Luc and subsequent progressive exonuclease III digestion was used to create the sequence of 5' nested deletion reporter plasmids: p(-1367/+357)Luc, p(-1234/+357)Luc, p(-1098/+357)Luc, p(-980/+357)Luc, p(-955/+357)Luc, p(-744/+357)Luc, p(-484/+357)Luc, p(-355/+357)Luc, p(-248/+357)Luc, p(-116/+357)Luc and p(-59/+357)Luc. Their sequences were confirmed by sequencing and are illustrated schematically in the relevant Figures.

Three E-box sequences, known consensus elements important for muscle-specific transcription, are present in close proximity to the PhK γ subunit promoter, with the canonical -CANNTG-sequences located at -114 to -109 (denoted here as Ep), +273 to +278 (denoted E₁) and +302 to +309 (denoted E₂) respectively. The Ep sequence lies within the minimum PhK- γ -Core promoter between the CAAT and TATA boxes. Using p(PhK- γ 3.8)Luc as the template, a number of fragments were generated by PCR containing appropriate restriction-enzyme digestion sites for cloning into the luciferase reporter constructs. Using site-directed mutagenesis with overlapping PCR primers the canonical E-box sequences of Ep and E₁ were mutated to TTAAGTTCT and AAGACGTAT respectively, denoted herein as Ep* or E₁*. Together these techniques were used to generate the following: p(-159/+23)Luc, p(-159/Ep*/+23)Luc, p(-159/+347)Luc, p(+105/+347-159/+23)Luc, p(-159/+299)Luc, p(-159/E₁*/+347)Luc, p(-159/Ep*/+347)Luc and p(+105/+347-159/Ep*/+2)Luc (and are illustrated schematically on the relevant Figures). In the present paper, for convenience, and to denote function, these reporter plasmids are termed respectively CP-Ep-Luc, CP*-Luc, CP-EpE₁E₂-Luc, E₁E₂-CP-Ep-Luc, CP-EpE₁-Luc, CP-EpE₂-Luc and CP*-E₁E₂-Luc. CP-Ep represents the core promoter containing the single intervening Ep E-box. CP* refers to this same core promoter with the intervening E-box mutated. CP-EpE₁E₂ contains the intact core promoter in addition to two E-boxes located downstream called E₁ and E₂ as in the wild-type PhK- γ gene. The

Upper panel: a schematic drawing of the 3.8 kb *SacI*/*SacI* fragment is shown. The constructs used in the present study were derived from a 3.8 kb *SacI*/*SacI* fragment of the γ G6 PhK- γ genomic clone [16]. This fragment spans from -1367 to +2500 (numbering relative to the transcription start site +1). It includes a major part of intron A, but does not contain the PhK- γ translation-initiation start codon. Lower panel: nucleotide sequence analysis of the Rat PhK- γ promoter region. The sequence was determined in both strand directions by the procedures defined in the Materials and methods section. As denoted, within the promoter are several putative *cis*-acting elements identified by sequence comparison. The TATA and CAAT boxes are also identified in italics.

E₁E₂-CP-Ep essentially contains the same elements but with the E₁ and E₂ boxes having been translocated upstream of the core PhK- γ promoter. CP-EpE₁, CP-EpE₂ and CP-E₁E₂ are constructs with only two of the three E-boxes intact. For all of these constructs the PCR products to create them were purified by Wizard DNA purification resin (Promega) and the integrity of their construction verified by DNA sequencing.

Transient transfections and reporter assays

C2C12, RMo and 3T3 cells were seeded into 12-well plates at a density of 1×10^4 cells/well. At 24 h later when the cells had reached 70–80 % confluency in preparation for transfection they were washed once with Opti-MEM reduced serum medium (Life Technologies). Transfection of cells was accomplished using LIPOFECTAMINE[™] (Life Technologies). Plasmid DNA and LIPOFECTAMINE[™] were pre-incubated for 30–60 min prior to transfection to allow formation of lipid–DNA complexes. Each well was then transfected by carefully layering the lipid/DNA mixture on to the cells with 2 μ g of luciferase reporter plasmid, 1 μ g of pRSV-*lacZ* plasmid (to measure transfection efficiency) and 5 μ l of LIPOFECTAMINE[™] in a total volume of 200 μ l of Opti-MEM media. At 6 h post-transfection, 200 μ l of DMEM supplemented with 40 % FCS was added to the cells and transfection continued for a further 18 h. At 24 h post-transfection the DNA/lipid mix was aspirated, the cells were washed with DMEM/20 % FCS, and then fresh growth medium was added to each well. Cells were grown for a further 24 h, during which time the myoblasts reached confluency. Upon reaching confluency the cells were either lysed as myoblast cultures or switched to a differentiation medium with the FCS reduced to 2 % and incubated for a further 2–3 days until myotube formation was complete. Cell extracts of either myoblasts, myotubes or 3T3 cells were obtained by washing the cells three times in PBS, scraping them into Microfuge tubes, lysing them by incubation in 100 μ l of lysis buffer (Analytical Luminescence Laboratory, formerly Dynex Technologies, now Thermo Labsystems, Franklin, MA, U.S.A.) and the cell debris then removed by centrifugation at 12000 *g* for 5 min. A 20 μ l portion of the supernatant was added to each well of an opaque 96-well microtitre plate (Microlyte; Dynatech, Chantilly, VA, U.S.A.) and luciferase activity was determined using a luciferase reporter assay kit (Analytical Luminescence Laboratory). Each assay sample was read in a 96-well luminometer (ML3000; Dynatech). To correct for variations in transfection efficiencies within a single experiment, the luciferase activity of an extract was normalized to the β -galactosidase activity. β -Galactosidase activity was measured using a luminescence-based reporter detection kit (Tropix, Bedford, MA, U.S.A., now Applied Biosystems, Foster City, CA, U.S.A.). All transfections were carried out a minimum of four times in triplicate with at least three different preparations of DNA, purified using Qiagen columns (Qiagen, Valencia, CA, U.S.A.). In the set of experiments indicated, PhK- γ Luc reporter constructs were co-transfected into 3T3 cells with pEMSVscribe₂ expression plasmids that encoded one of the four members of the MyoD family of transcription factors. MyoD, myogenin, myf5 and MRF4 were provided by Dr Andrew Lassar (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, U.S.A.), Dr Eric Olson (Department of Molecular Biology, University of Texas, Southwestern Medical Center at Dallas, TX, U.S.A.), Dr Hans-Henning Arnold (Department of Cell and Molecular Biology, Technical University of Braunschweig, Braunschweig, Germany) and Dr Stephen Konieczny (Department of Biological Sciences, Purdue University, West Lafayette, IN, U.S.A.) respectively and their characteristics have

been detailed in references [11,18–20]. In these experiments 1.5 μ g of Luc reporter plasmid and 0.5 μ g of MyoD plasmid were used in the transfection mixture together with 1 μ g of the pRSV-*lacZ* galactosidase plasmid. In a set of test experiments a comparison was made between the effect of transfection in myotubes that were differentiated either before or after transfection. The results obtained were similar with both methods, except that the level of transfection achieved was less with already differentiated cells. All of the experiments reported were undertaken with cells that were transfected prior to inducing differentiation.

Preparation of nuclear extracts

Nuclear extracts of C2C12 myotubes were prepared as described by Dignam et al. [21], as modified by Lee et al. [22]. Briefly, fully differentiated myotubes ($\approx 2 \times 10^6$ cells) were washed in PBS and resuspended in 1 packed-cell vol. of Buffer A [10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM EGTA, and 0.5 mM PMSF, pH 7.8], containing leupeptin (0.5 μ g/ml), pepstatin (1 μ g/ml), aprotinin (1 μ g/ml) and 0.1 mM benzamide added fresh before use. The cells were allowed to swell on ice for 30 min and then passed through a 25-gauge needle attached to a 1 ml syringe. Cells were drawn slowly into the syringe and rapidly ejected in a single stroke. This procedure was repeated five times to achieve 80 % lysis. The homogenate was then centrifuged at 14000 *g* for 20 s. The crude nuclear pellet was then resuspended in two-thirds of the original packed-cell volume in Buffer C [20 mM Hepes, 25 % (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA and 0.5 mM PMSF, pH 7.8] containing protease inhibitors as described for Buffer A and incubated for 30 min at 4 °C on a shaking platform. The nuclear debris was removed by centrifugation at maximum speed for 5 min and the supernatant was dialysed against 100 vol. of Buffer D [20 mM Hepes, 20 % (v/v) glycerol, 0.1 M KCl, 0.5 mM DTT, 0.2 mM EDTA, pH 8, and 0.5 mM PMSF] with protease inhibitors for 2 h. Following determination of the protein concentration, the nuclear extract was divided into portions, snap-frozen in liquid nitrogen and stored at –70 °C.

Electrophoretic-mobility-shift assays

The CP-Ep sequence (–159/+23) was amplified by PCR, purified by agarose-gel electrophoresis and then digested with *Dde*I (–125) and *Ava*II (–67) to give a 58 bp oligonucleotide probe corresponding to the E-box at position –114/–109. A second probe containing the E-box pair located at position +270 (E₁) and +299 (E₂) was also generated by PCR to yield a 101-bp product (+247/+347), which was then digested to an 81 bp probe using *Dde*I (+266) and the engineered *Bgl*II site (+347). Both of these digests generated overhangs that were end-labelled with [³²P]deoxynucleotides (dGTP and dCTP) using the Klenow fragment of *Escherichia coli* DNA polymerase I. Each probe (2 $\times 10^5$ c.p.m.) was incubated with 10 μ g of nuclear extract in a binding reaction mixture containing 10 mM Hepes, pH 7.8, 40 mM NaCl, 1 mM EDTA, 4 % (v/v) glycerol with 10 mM DTT and poly(dI-dC) (1 mg/ml) to a final volume of 20 μ l. The reaction mixtures were incubated at room temperature for 20 min, followed by electrophoretic separation on a 5 % (w/v)-polyacrylamide gel run in 0.25 $\times$ TBE (1 $\times$ TBE is 45 mM Tris/borate/1 mM EDTA) at 220 V for 2 h and analysed by autoradiography. These two probes are designated Pr-Ep and Pr-E₁E₂ respectively.

RESULTS

Cultured muscle cells, such as the C2C12 and RMo cell lines used in the present study, have the capacity to go through the complex program of differentiation from myocytes to myotubes. Upon appropriate change of medium, myocytes within a 3-day period acquire the characteristic myotube morphology. PhK activity, subunit proteins and subunit transcripts are virtually undetectable in myocytes, but, coincident with differentiation, both subunit transcript and subunit protein accumulate. By 3 days after the change in medium the subunit proteins reach a stable level that is about 3–5% of that of adult skeletal muscle [23]. Increases beyond that level, as occur in normal developing skeletal muscle, requires neural input, as occur with innervation during normal muscle development [7]. As illustrated for C2C12 cells, induced differentiation from myoblasts to myotubes under the conditions used in the present study occurs with a prompt induction of the PhK γ mRNA (Figure 2).

As the first step in investigating the potential control elements that might exist in the γ subunit gene, we extended the sequence analysis of the γ subunit gene to encompass the region from

–1387 to +670 (Figure 1, lower panel). On the basis of sequence homologies, several putative regulatory elements were identified within this region. A total of four E-box transcriptional elements are evident in the γ subunit gene, located at positions +303 to +309, +273 to +278, –114 to –109 and –966 to –954. Each of these has high homology (85–92%) with E-boxes established as regulatory elements in the genes of other muscle developmentally regulated proteins [10–12,14–16,24,25]. The first two of these E-boxes in PhK- γ are located within intron A, which is internal to the transcription start site. The third of these is within the primary PhK- γ promoter sequence between the CAAT and TATA boxes that are located at –144 to –140 and –32 to –26 bp respectively. Multiple other *cis*-regulatory sites that are apparent from the PhK- γ sequence (Figure 1, lower panel), include an MEF-2 site at –213 and three GATA-4 sites at –1027, –895 and –551, which in other systems have been shown to be important for skeletal- and cardiac- muscle development [26,27].

Analysis of regulatory elements of the γ subunit gene: identification of a major negative regulatory element downstream of the PhK- γ promoter

To test for potential PhK- γ gene regulatory elements, a set of plasmid constructs were created containing the luciferase ORF, the PhK- γ or SV40 promoters and segments of the 3.8 kb *SacI/SacI* fragment of the γ G6 PhK- γ genomic clone [16] (see the Materials and methods section for details and plasmid nomenclature). These were tested by transient transfection into C2C12 and RMo cells that were grown as myoblasts or induced to differentiate into myotubes. Luciferase activities were measured 48 and 96 h later and were normalized to β -galactosidase activity. Activities of each of the deletion constructs are shown as fold induction over basal activity obtained with the promoter-less construct. A construct containing a minimal intact PhK- γ core promoter sequence (CP-Ep-Luc), designated B1 in the schematic diagram in Figure 3, upper panel, was capable of inducing a 10-fold increase in luciferase activity in myoblasts and a 20-fold increase in myotubes, above that obtained with promoter-less construct pGL2-Basic (A1) (Figure 3, upper panel). CP-Ep-Luc contains 187 bp of the PhK- γ that includes the transcription initiation site, the TATA and CAAT boxes, and one E-box (Ep) intervening between these two promoter elements. Luciferase expression, however, was ablated down to the level of the promoter-less pGL2-Basic construct with two parent plasmids, p(PhK- γ 3.8)Luc (C1) and p(MCS- PhK- γ 3.8)Luc (D1), each of which contains the entire 3.8 kb region *SacI/SacI* fragment of γ G6 PhK- γ genomic clone including this minimum PhK- γ promoter sequence (Figure 3, upper panel). This ablation strongly suggested the presence of a negative element(s) within a 3.8 kb sequence of PhK- γ , outside of the core γ -promoter. Serial deletion of the downstream region located the negative regulatory element to between +357 to +440 (Figure 3, upper panel), as especially evidenced by the difference in luciferase expression between the p(PhK- γ –1367/+440)Luc (G1) and the p(PhK- γ –1367/+357)Luc (H1) vectors. It is noteworthy that the expression of luciferase with both p(–1367/+180)Luc (I1) and CP-Ep-Luc (B1) was differentiation-dependent, with the expression in myotubes being 2.5–3-fold higher than in myoblasts. Results similar to those shown in Figure 3 for C2C12 cells were also observed with RMo cells (results not shown).

A second set of expression vectors, in which luciferase expression was under control of the SV40 promoter and the PhK γ promoter was absent, confirmed the location of the prominent negative regulator downstream of the PhK- γ transcription start

Figure 2 Expression of PhK γ -subunit in C2C12 myoblasts and myotubes

C2C12 cells, grown as undifferentiated myoblasts, or differentiated myotubes, were analysed for the presence of PhK- γ -catalytic-subunit mRNA and an actin mRNA probe using the procedures described in the Materials and methods section. Top right and top left panels: myoblasts (left) or myotubes 48 h following induction (right). Lower panels: PhK- γ mRNA, actin mRNA or total RNA from either myoblasts (MB) or myotubes (MT) following indicated period of induction.

Figure 3 Identification of a major negative determinant in the PhK- γ promoter

The 3.8 kb *SacI*/*SacI* fragment of the 5'-UTR of the PhK- γ gene (as detailed in Figure 1), and regions of it generated by restriction digests or by PCR, were cloned into either the pGL2-Basic or the pGL2-SV-40 luciferase reporter plasmids with transcription under the control of PhK- γ and SV40 promoters respectively. Luciferase expression was determined in either proliferating C2C12 myoblasts or differentiated myotubes (MT) and normalized to β -galactosidase expression. Details are presented in the Materials and methods section. Results are means $\pm$ S.D. relative to the basal level of activity observed with the promoter-less construct. Each set of results are typical for at least six independent experiments. The presence of the upstream MCS was tested with some of the vectors and shown to make no difference in the level of expression. Upper panel: the induction of luciferase activity with the PhK- γ promoter-luciferase constructs. Left-hand side, schematic organization of the constructs; right-hand side, luciferase activity. Lower panel: the induction of luciferase activity with SV40 promoter-luciferase constructs. Left-hand side, schematic organization of the constructs; right-hand side, luciferase activity.

Figure 4 Functional activity of the E-boxes within the PhK- γ promoter in muscle-cell cultures

Upper panel: a series of constructs created with the Ep (bp -114 to -109), E1 (bp +273 to +278) and E2 (bp +303 to +309) E-boxes arranged or selectively mutated and constructed into the luciferase reporter plasmid pGL2-Basic. Details are presented in the Materials and methods section. Lower panel: luciferase expression in either proliferating C2C12 myoblasts or differentiated myotubes (MT), normalized to β -galactosidase expression. The results are typical of those from six independent experiments.

site (Figure 3, lower panel). The core SV40 promoter (pSV40-Luc) (B2) alone efficiently supported luciferase activity. This expression was not effected by the presence of the +870/+2500 bp downstream region of the PhK- γ promoter, but extending the vector to +370 with the plasmid p(+350/

+2500)SV40-Luc (D2) markedly diminished expression. Expression was equivalently depressed with just the presence of the short +350 to +870 bp sequence. The upstream region of the PhK- γ promoter, in the plasmid p(-1367/-470)SV40-Luc (F2), did not effect SV40-driven luciferase expression. The p(-1367/

Figure 5 Functional activity of the E-boxes within the PhK- γ promoter in 3T3 fibroblasts co-transfected MyoD (upper panel) and other proteins of the MyoD family (lower panel)

Upper panel: luciferase activity with the E-box constructs depicted in Figure 4 transiently transfected in the non-myogenic cell line NIH-3T3, either with or without co-transfection with a MyoD expression plasmid. Details are provided in the Materials and methods section. The results are representative of those from six independent experiments. Lower panel: luciferase expression following transient transfection into 3T3 fibroblasts of combinations of expression vectors encoding each of the four members of the MyoD family of transcription factors (MyoD, myogenin, myf5 and MRF4) co-transfected with the E-box-luciferase constructs. The results are typical of those from five independent experiments. Abbreviation: RLU, relative luciferase units of activity.

+357)SV40-Luc (G2) construct, which contains both the SV40 and PhK- γ promoters, but in which the negative upstream 350–470 region is absent, gave the same level of expression as the SV40 promoter alone. The combination of these data of Figure 3, upper and lower panels, show that a negative regulatory

element resides in the PhK- γ gene between bp +350 and +440. A highly likely candidate for this is the established negative regulator Yin Yang-1 (YY-1) [28], a binding site for which is evident from the sequence analysis at position +361 to +379 (Figure 1, upper panel).

Figure 6 Binding of C2C12 myotube nuclear proteins to the PhK- γ promoter E-boxes

The two probes Pr-Ep (bp -127 to -67) and Pr-E1-E2 (bp +266 to +347), corresponding to the respective E-boxes, were synthesized by PCR and radiolabelled as described in the Materials and methods section. Each radiolabelled oligonucleotide was incubated with 10 μ g of myotube nuclear extract from either C2C12 (lanes 2 and 5) or RMO cells (lanes 3 and 6). Lanes 1 and 4, controls; lanes 1, 2 and 3, Pr-Ep; lanes 4, 5 and 6, Pr-E1-E2. The location of the free and bound probe are indicated.

Analysis of regulatory elements of the γ subunit gene: exploration of the regulatory role of the E-boxes proximal to the promoter site

E-box consensus binding motifs are present in the genes of many muscle proteins and are the site of binding of the helix-loop-helix myogenic transcription factors of the MyoD family [11,12]. Other members of this family include myogenin [29], myf5 [30] and MRF4 [20]. Acting in concert or individually, these factors have been implicated in the regulation of expression of an array of muscle-specific genes, including muscle creatine kinase [11], myosin light chain [31], α 7-integrin gene [32] and the acetylcholine receptor α and δ subunits [24]. The regulatory potential of the three E-boxes closely proximal to the PhK- γ promoter at positions -114 to -109 (denoted here as Ep), +273 to +278 (denoted E₁) and +303 to +309 (denoted as E₂) was tested by the construction of pLuc plasmids containing varying complements of these sites. The nomenclature and structure of these plasmids are defined in the Materials and methods section and their composition illustrated schematically in Figure 4, upper panel. The studies with these plasmids demonstrated both an E-box-enhanced and an E-box differentiation-dependent expression of the reporter (Figure 4, lower panel). Luciferase expression with the CP*-Luc construct, which contained the core PhK- γ promoter elements, but with the intervening Ep-box eliminated by mutation, was no greater than with the promoter-less pGL2-Basic plasmid (Figure 4, lower panel). Alone, therefore, in the absence of an E-box, the core elements of the PhK- γ promoter do not support transcriptional expression in muscle cells. When Ep is present within this core (construct CP-Ep), however, there is a markedly enhanced transcription. This enhancement is also differentiation-dependent with an 8- and 20-fold increase observed in myoblasts and myotubes respectively (Figure 4, lower panel; CP-Ep comparison of hatched and black bars). When tested in myoblasts the presence of the additional downstream E₁- and E₂-boxes, either alone or in combination, produced no

greater level of expression than with Ep alone (hatched bars). In contrast, the presence of these E-boxes when tested in myotubes produced an increase in transcription above that obtained with Ep alone (plasmids CP-EpE₁-Luc, CP-EpE₂-Luc, and CP-EpE₁E₂-Luc; Figure 4, lower panel, black bars). Increased transcription also occurred when E₁ and E₂ were located upstream of the core PhK- γ promoter (plasmid E₁E₂-CP-Ep-Luc), rather than downstream as in the native gene, but not to quite the same extent. Since the E₁- and E₂-containing constructs only caused an increase in transcription in myotubes and not in myoblasts, above that seen with Ep alone, these data suggest that their physiologically most important role in transcription is following differentiation. The E₁-E₂ pair could compensate for Ep in both myoblasts and myotubes, however, when Ep was eliminated by mutation (plasmid CP*-E₁E₂-Luc). When all three E-boxes were present, an overall 40-fold increase in expression was observed, with a 5-fold differentiation difference seen between myoblasts and myotubes. Results (not shown) similar to those shown in Figure 4 for C2C12 cells were also observed with RMO cells.

In further evaluation of the regulation of PhK- γ gene transcription by the MyoD family of transcription factors, luciferase expression was also examined in 3T3 fibroblasts. 3T3 fibroblasts do not express any of the MyoD family of proteins. The various constructs were tested in 3T3 cells either alone or co-transfected with the MyoD (Figure 5, upper panel). In the absence of MyoD, the core elements of the PhK- γ promoter enhanced transcription in 3T3 cells above that observed with the promoter-less pGL2-Basic. This level of expression was independent of the presence of a functional E-box (Figure 5, upper panel; comparison of plasmids CP-Ep-Luc and CP*-Luc with pGL2-Basic, in the absence of MyoD). A closely similar level of MyoD-independent expression was observed with all the E-box constructs containing the PhK- γ promoter (hatched bars). These data provide a confirmation that the core elements we have defined as the 'PhK- γ promoter', can serve in this capacity. In the absence of co-expressed myoD, the presence of any combination of E-boxes did not increase expression above that observed with CP*, which contains only the PhK- γ promoter (Figure 5, upper panel, hatched bars). In the presence of MyoD, however, the expression of luciferase in 3T3 fibroblasts was markedly enhanced when E-boxes were present (Figure 5, upper panel, black bars) and closely followed the pattern of E-box-dependent-regulation observed with myotubes (Figure 4). MyoD-dependent expression occurred with the presence of just Ep, or when the absence of Ep was compensated for by the presence of a downstream E₁-E₂ pair. The Ep-dependent expression was enhanced by the simultaneous presence of either E₁ or E₂, with maximal expression occurring when all three E-boxes were present. These data confirm that all of this set of designated E-boxes are sites for muscle-specific transcriptional regulation of the γ subunit by the MyoD.

The 3T3 cell studies also allowed an examination of the potential regulation of PhK- γ by the other members of the MyoD family of transcription factors, namely myogenin, MRF4 and myf5. These were tested either alone or in combination with MyoD with the CP-Ep-Luc, CP-EpE₁E₂-Luc, CP-EpE₁-Luc, and CP-EpE₂-Luc constructs. Each of these myogenic factors presented a somewhat different profile of responses (Figure 5, lower panel). Myogenin stimulated transcription with constructs containing Ep plus E₁, E₂ or both, but not significantly when Ep alone was present. Myogenin alone was not as efficacious as MyoD, and, in combination with MyoD, produced no additional effects. All of myogenin's actions could be accounted for by an interaction restricted to just the downstream E₁-E₂ sites. Myogenin was likely not as effective overall as MyoD, because

Figure 7 For legend see opposite page

maximum transcriptional activity requires binding to the Ep site. MRF4 appeared to be somewhat inhibitory. When present alone luciferase expression was dampened below that in the absence of any added myogenic factors, and it diminished the effects of MyoD when added in combination. Myf5 produced a combination of effects. When present alone it was ineffective. In combination with myoD it produced no effect with the minimal CP-Ep construct, but it markedly enhanced transcription above that of MyoD when either the E_1 or E_2 site was also present. The combination of MyoD and myf5 gave an impressive 160-fold induction of luciferase expression above that of the pGL2-Basic construct. The elevated levels of transcription suggest that optimal transcription activation may occur by a combination of these two factors acting in concert, with MyoD occupying the Ep site and myf5 the E_1 or and/or E_2 sites.

Factors present in myotubes bind to the E-boxes proximal to the PhK- γ core promoter

Confirmation of the presence of protein factors in myotube cells that bind to the Ep and E_1E_2 sites of the PhK- γ gene was obtained by electrophoretic-mobility-shift assays. To undertake this, probes corresponding to the γ gene sequences -125 to -67 and $+266$ to $+347$, and designated Pr-Ep and Pr- E_1E_2 , were created as described in the Materials and methods section. Incubation with nuclear extracts from C2C12 myotubes caused the mobility of these probes to be restricted. Such mobility shifts were demonstrated for C2C12 and RMo myotubes with both P-Ep and P- E_1E_2 (Figure 6). (RMo myotubes were not tested with the P- E_1E_2 construct.) Most likely these represent complexes with one or more of the MyoD family of proteins.

Analysis of regulatory elements of the γ subunit gene: deletion analysis of the upstream γ promoter region

To examine the potential existence of further γ gene-regulatory elements, a restriction-enzyme digest and subsequent progressive exonuclease III digestion was used to create the sequence of 5' upstream deletion reporter plasmids: p($-1367/+357$)Luc, p($-1234/+357$)Luc, p($-1098/+357$)Luc, p($-980/+357$)Luc, p($-955/+357$)Luc, p($-744/+357$)Luc, p($-484/+357$)Luc, p($-355/+357$)Luc, p($-248/+357$)Luc, p($-116/+357$)Luc and p($-59/+357$)Luc (illustrated schematically in Figure 7, top panel). Each of these constructs terminate at $+357$ before the major YY-1 negative element located in the intact gene at bp $+361$ to $+379$, which was shown by the data of Figure 3 to block any transcription by the PhK- γ gene promoter. Each of these, excepting p($-116/+357$)Luc and p($-59/+357$)Luc, have the entire γ core promoter. p($-116/+357$)Luc contains the Ep and TATA elements and the transcription initiation site, but lacks the CAAT box. p($-59/+357$)Luc contains only the TATA box and transcription initiation site. The identity of all of these constructs was confirmed by sequencing.

Analysis of this sequence of deletion reporter plasmids with either C2C12 or RMo myoblasts and myotubes cells revealed a complex array of regulation that was closely analogous in both

cell types (Figure 7, middle and bottom panels). The construct p($-59/+357$)Luc, containing only the TATA box, did not support transcription. Addition of sequences up to -484 revealed a strong induction of transcription that was particularly differentiation-dependent. Given that p($-116/+357$)Luc, even in the absence of the CAAT box, also supported modest, but differentiation-dependent, transcription, it is likely that the Ep E-box (bp -114 to -109) is providing the differentiation specificity, with general transcription enhanced by one or more positive elements between bp -115 and -484 . Possible candidates for this include the MEF (bp -215) and GATA-4 (bp -551) sites, which have been shown in other systems to be important for skeletal- and cardiac-muscle development [26,27]. Addition of sequences further upstream of bp -484 led to a potent down-regulation of the transcriptional activity localized to the region spanning -744 to -983 . This region does not contain any known negative regulatory motifs. Addition of a further upstream sequence served to restore positive induction of the reporter gene, counteracting the negative influence of the -744 to -983 region, but not significantly restoring the differentiation signal strongly evident with the shorter p($-484/+357$)Luc, p($-355/+357$)Luc, p($-248/+357$)Luc constructs. Possibly this is a consequence of the E-box at -966 to -954 , which may serve to compete with factors from the E-boxes proximal to the core promoter region and thereby quench the myogenic induction of the γ gene.

DISCUSSION

PhK is a protein the complexity of regulation of which matches its complexity of structure, and it is the site of integration of multiple signal-transduction pathways [1,2]. While currently its only established function is the regulation of glycogenolysis, the discovery of other physiologically important roles appears likely [1]. It is now evident that the basis for regulation of the catalytic activity of broad categories of the protein kinase family of enzymes is that of suppression of the inherent endogenous activity of the catalytic domain. The isolated catalytic subunit or catalytic domain of these protein kinases exhibit a maximum catalytic competence that is kept dormant in the intact protein by the interaction of the pseudosubstrate domain of regulatory regions and subunits. The pseudosubstrate domain mirrors the protein substrate by binding into the catalytic cleft. Activation in response to signal-transduction factors arises by the relief of inhibition via the dissociation of the pseudosubstrate domain and the consequential availability of the catalytic domain to then productively bind the protein substrate. This mechanism was first elucidated for the cAMP-dependent protein kinase (PKA) [33] and subsequently shown to be the mechanism of regulation of many protein kinases [34,35]. This mechanism is abundantly evident in PhK, where the catalytic activity of the Phk γ subunit is markedly reduced by the binding of the α , β and δ subunits when present as the $(\alpha\beta\gamma\delta)_4$ holoenzyme, or in the partial subcomplexes $\alpha\gamma\delta$ and $\gamma\delta$. Physiological activation arises by either the PKA-dependent phosphorylation of the α and β subunits, by the binding of Ca^{2+} to the δ subunit, or by the binding of Ca^{2+} plus exogenous calmodulin to the α and β subunits. Activation might also arise by the autophosphorylation

Figure 7 Deletion analysis of the upstream region of the PhK- γ promoter

Top panel: schematic depiction of a series of 5'-end deletion mutants of the PhK- γ promoter generated as described in the Materials and methods section. The identity of potential *cis*-acting elements (for definitions, see below) is based upon sequence comparisons (Figure 1, lower panel). Luciferase activity was determined following transient transfection of the constructs into C2C12 (middle panel), and Rmo (bottom panel) myoblasts (MB) and myotubes (MT). The luciferase activity (mean $\pm$ S.D., $n = 6$ at least), normalized to β -galactosidase, of each construct is expressed relative to the basal activity seen with the promoter-less pGL-2 construct. Abbreviations: GATA-4, a zinc-finger transcription factor; MEF-2, myocyte enhancer factor 2; NF- κ B, nuclear factor κ B; Nkx2.5, a cardiac homeobox transcription factor; Sp1, stimulating protein-1; STAT1, signal transducer of transcription 1.

of the α and β subunits. Given that, if free Phk γ subunit were to occur *in vivo*, uncontrolled glycogenolysis would occur at a prohibitively high rate, it is of crucial physiological importance to stop this occurring. Several sets of studies indicate that there is a very close co-ordination of expression of the multiple subunits, such that the γ subunit is not expressed in the absence of its regulatory subunits. During normal skeletal-muscle development [7], muscle degeneration and subsequent regeneration following bupivacaine-induced degeneration [8], and the re-innervation of muscle following nerve transection [9] the fully composed holoenzyme is altered dramatically in amount, but the free γ subunit is never produced. It is apparent that the transcription and translation of the α , β and δ subunits is dictated by that of the γ subunit.

The data in this report shows that a complex set of *cis*-acting and *trans*-acting transcriptional controls dictate the transcription of the γ subunit. Two principal negative sites of regulation are evident in the Phk γ gene located within bp regions -744 to -983 (Figure 7) and +350 and +440 (Figure. 3). The negative regulator YY-1 at bp +361 to +379 is a highly likely candidate for the latter. This site is a strongly dominant inhibitor of Phk γ transcription and is of sufficient impact to block the transcriptional expression that might otherwise arise from activation of any of a range of positive *cis*-acting transcriptional elements (Figure 3). Only when the YY-1 site was eliminated could the existence of the various positive sites be identified with luciferase expression (Figures 3, 4 and 7). Others studies have reported a 60% suppression of transcription in 3T3 and C2C12 myoblasts by co-expression with YY-1 cDNA, providing evidence for its involvement in the negative regulation of the COX Vb gene [36]. A number of different negative enhancer mechanisms have been reported [37,38], including competition for binding to the regulatory sequences, repressor-mediated inhibition of the function of activators without inhibiting its binding ability (quenching), and the direct repression by the negative regulatory factor. The presence of such a strongly negative site fits well within the concept that the existence of free γ subunit cannot be physiologically tolerated. Thus the concept that protein kinase activity only arises after the negative influence of the regulatory subunits has been relieved appears to be directly mirrored in the transcriptional regulation of the Phk catalytic subunit where its transcription only occurs after the effects of a strong negative regulator is eliminated. This type of dominant-negative transcriptional control might become evident in the range of protein kinases where free catalytic subunit cannot be tolerated physiologically. Autoinhibitory control of protein kinase activity might thus have an equivalent counterpart in the regulation of the genes from which the active catalytic entities are derived.

A very important positive control of the Phk γ subunit transcription is dictated by its set of E-boxes. E-box elements are bound by heterodimers of the ubiquitously expressed E proteins and members of the MyoD family that are exclusively expressed in muscle cells. Both of these families of proteins have a helix-loop-helix construction. E-box consensus binding motifs are present in the genes of many muscle proteins, including creatine kinase, myosin light chain, $\alpha 7$ integrin gene and the α and δ subunits of the acetylcholine receptor [11,24,31,32]. The three E-boxes surrounding the transcription-initiation site in the Phk γ subunit gene show a high degree of consensus (85–92%) with E-box sequences in other muscle-specific genes, and appear to provide a strongly positive differentiation-dependent regulated expression. When present in combination, Ep, E₁ and E₂ promoted a 40-fold increase in luciferase expression in C2C12 myotubes, with a substantial induction above that observed in myoblasts (Figure 4). This level of differentiation-dependent

expression required some combination of at least two of the three E-boxes, with Ep appearing to play a pivotal role, but some redundancy between the sites is also evident. A similar pattern of E-box-dependent expression of the Phk- γ gene-luciferase constructs was also evident in transfected 3T3 fibroblast, when a source of myogenic factors was also present (Figure 5). As with the expression in myotubes, maximum transcription in 3T3 cells required some combination of at least two of the three E-boxes, producing a 120-fold induction of luciferase expression. Multiple E-boxes working in co-ordination appears to be a common feature in E-box-regulated muscle genes [39]. The fourth Phk- γ E-box located upstream of the core promoter (bp -966 to -954) also has a strong consensus with the sequence in other muscle genes, but its role still needs to be elaborated.

The differentiation-dependent expression evident with the Phk- γ constructs transfected into C2C12 myotubes is almost certainly due to the differentiation-dependent expression of the myogenic family of transcription factors. These factors have a complex pattern of expression with the transition of muscle cells from myocytes to myotubes, with MyoD and Myf5 evident early and MRF4 and myogenin later, in the progression of differentiation. The process of myogenesis can be divided into the following stages: firstly, commitment of the mesodermal precursor cells to the skeletal muscle lineage, followed by withdrawal of myoblasts from the cell cycle and, ultimately, the transcriptional activation of dozens of muscle-specific genes. The myogenic basic helix-loop-helix factors, namely MyoD, myogenin, Myf5 and MRF4, act either individually or in concert at various points in the myogenic lineage in determining the myoblast phenotype and to control terminal differentiation [40]. It appears likely that there is not a single muscle regulatory factor acting to induce transcription, but a core regulatory network composed of various members of the MyoD family. Myf5 and MyoD, for example appear to act jointly as determination genes, and Myf5 and MyoD double knock-outs fail to produce or sustain a significant population of myoblasts [41]. Myogenin, in contrast, appears to function more as a differentiation gene, and myogenin-null mice are highly, though not entirely, deficient in muscle differentiation [41]. A complex pattern of myogenic-factor-dependent expression was evident with the Phk- γ constructs from the *trans*-activation assays with vectors expressing MyoD, myogenin, myf5 and MRF4 transiently in the non-myogenic 3T3 fibroblasts (Figure 5, lower panel). In 3T3 cells, only MyoD and myogenin, when expressed alone, were able to *trans*-activate the E-box constructs, with MyoD being the more potent. Co-operation between two or more E-box sites was evidenced by enhanced transcription resulting from coexpression of both MyoD and myogenin [42]. In contrast, neither myf5 nor MRF4 was capable of inducing transcription when expressed individually, but maximal reporter activity was achieved when both myf5 and MyoD were co-expressed. Interestingly, these are the first transcription factors to be induced during muscle differentiation [41].

In addition to the presence of key negative transcriptional regulators and the notably positive E-box sites, an array of other transcriptional controls are evident in the Phk γ subunit gene (Figure 7). Full detailing of these sites and the possible role in Phk γ subunit expression is still required. Of possibly special significance are the MEF-2 site (bp -215–207) and the heart progenitor cell-specific transcription-factor GATA-4 site (bp -563 to -555). These have been shown to be important for skeletal- and cardiac-muscle development [26,27]. The MEF-2 motif has been shown to regulate the cardiac troponin C gene [43] as well as the myogenin gene itself [44]. In the Phk γ subunit they appear to be providing yet a further differentiation-dependent signal for expression (Figure 7).

The overall set of data provided here suggests strongly that the transcriptional regulation of the Phk γ subunit exhibits considerable complexity. This degree of complexity mirrors the complexity of the PhK protein [1] and therefore is not surprising. Already we know of an elaborate set of covalent and allosteric controls that modulate PhK catalytic activity. The presence of a combination of negative and positive regulators within the γ promoter is likely to be of absolute necessity to restrict the expression of constitutively active kinase catalytic subunit in the absence of expression of the regulatory subunits. In addition to understanding in full the mechanism of regulation of the γ subunit gene, it will also be necessary to elucidate how its regulation is then coupled with that of the unique α and β subunits to ensure tight coupling of holoenzyme formation.

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