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The interaction of nuclear proteins with essential promoter element of the chicken cardiac myosin light chain 2 gene is involved in muscle-specific transcription

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A quantitative microinjection procedure has been developed to demonstrate muscle-specific transcription of the myosin light chain 2-A (MLC2-A) promoter in differentiated chicken primary breast muscle cells. Nuclear protein binds to the distal region of the required promoter sequence but not to a mutated version of this sequence. The functional significance of this specific DNA-protein interaction for the promoter activity is demonstrated by 'in vivo' competition of microinjected MLC-CAT reporter construct together with excess of synthetic oligonucleotides encompassing the protein binding sites.

Muscle-specific gene expression; Nuclear protein; Binding competition

1. INTRODUCTION

The differentiation of muscle cells is an excellent model system for studying tissue-specific gene regulation since the morphological transition from myoblastic precursor cells to differentiated, multinucleated myotubes is accompanied by the accumulation of newly synthesized contractile proteins and their corresponding mRNAs [1,2]. The vast increase of muscle-specific mRNAs is primarily controlled at the level of transcription [3]. Heterokaryon experiments have elegantly demonstrated the involvement of diffusable, trans-activating factors in the regulation of muscle gene expression [4]. Understanding the mechanisms which determine the cell-specific activation of muscle gene promoters requires therefore the elucidation of interactions of cellular factors with gene sequences governing cell type-specific expression. Binding properties of trans-acting factors have been determined in numerous instances (review [5]). Only a few studies, however, have demonstrated the functional relevance of these interactions in reconstituted in vitro transcription assays [6]. We here demonstrate by nuclear microinjection that a fragment of the chicken cardiac myosin light chain 2-A promoter extending from nucleotides −135 to +25 is sufficient to confer myotube-specific expression. We furthermore show that the distal sequence of this fragment specifically binds to nuclear factors. The functional significance of this factor-DNA interaction is illustrated by in vivo competition with protein-binding oligonucleotides which leads to the effective inhibition of the MLC2-A promoter activity in the expressing cells.

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2. EXPERIMENTAL

Hybrid genes containing promoter fragments of the chicken MLC2-A gene were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene as described [7].

Primary cells for transfection or microinjection experiments were isolated from 12-day embryonic chicken pectoralis muscle [8] and cultured on collagen-coated cover slips. For each nuclear injection experiment 50 nuclei of primary myoblasts (Mb) or myotubes (Mt) were microinjected with equal volumes

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(approx. 10 fl) of CAT expressing reporter plasmid (1 mg/ml) [9,10] plus competing oligonucleotides. CAT activity was determined as in [10,11]. For gel mobility-shift assays, radiolabeled MLC2-A promoter fragment (−135/+25) or the synthetic oligonucleotide DPE was incubated with various concentrations of nuclear extracts prepared from 12-day embryonic chicken hearts according to [12]. Poly dI/dC (80 μg/ml) and salmon sperm DNA (6 μg/ml) were included as unspecific carrier. The assay was performed as described [13].

3. RESULTS

We have previously shown that the chicken MLC2-A gene promoter linked to the bacterial CAT reporter gene is transcriptionally active when transfected into chicken primary breast and cardiac muscle cells but inactive in primary skin fibroblasts and several myogenic murine cell lines [7]. Although this earlier observation established the fact that the MLC2-A promoter exhibits muscle specificity, it did not formally distinguish whether transcription was activated in myoblasts or in fused myotubes. In primary muscle cultures differentiation proceeds spontaneously during the culturing period, hence both types of myogenic cells are always present. We therefore devised a transient expression assay in which MLC2-CAT chimeras were directly microinjected into nuclei of either unfused, mononucleated myoblasts or into

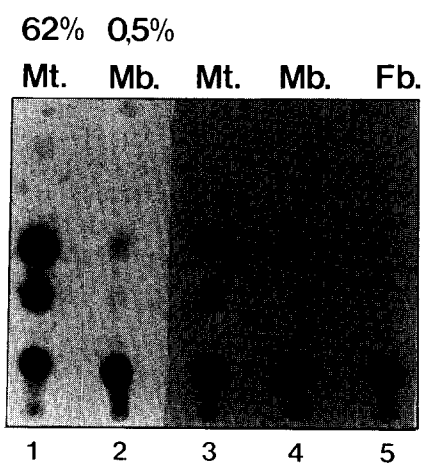

Fig.1. Myotube-specific expression of the chicken MLC2-A promoter fragment of plasmid LC-pUC-CAT (−135/+25) [7] linked to the CAT reporter gene. The percentage of chloramphenicol conversion is indicated. Two independent injection experiments are shown. Mb, myoblasts; Mt, myotubes; Fb, fibroblasts.

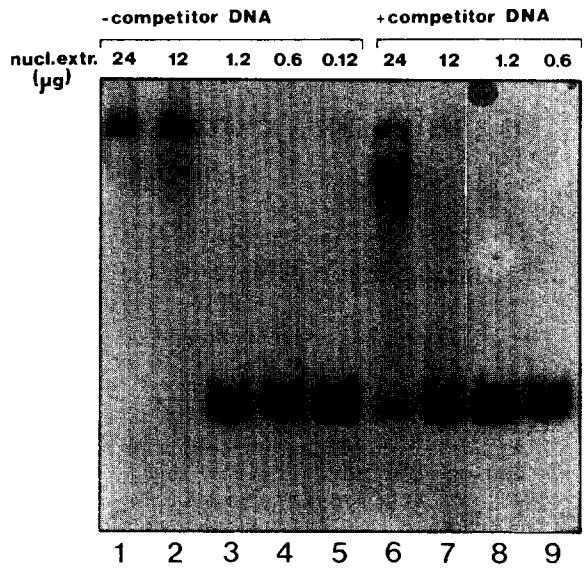

Fig.2. Gel mobility-shift assays with the MLC2-A promoter fragment (−135/+25). Radiolabelled DNA was incubated with the indicated amounts of protein in nuclear extracts (lanes 1–5). A 20-fold molar excess of the same unlabelled fragment was used as specific competitor DNA (lanes 6–9).

nuclei of fully differentiated, multinucleated myotubes. By this approach, the promoter activity could be determined through the CAT activity in groups of cells, which had been selected for microinjection according to their morphology. As illustrated in fig.1 for two independent experiments, transcriptional activity of the 160 bp promoter fragment (−135/+25) of the MLC2-A gene was approx. 100-fold higher in differentiated myotubes than in mononucleated myoblasts. This result indicated that differentiation of muscle cells was required for maximal promoter activity. A slightly higher activity was observed in myoblasts than in fibroblasts which is most probably due to the fact that some of the injected muscle precursor cells had already been postmitotic, although they had not yet been fused to multinucleated fibers.

To elucidate the interaction of the MLC2-A promoter region with nuclear proteins, gel mobility-shift assays were performed [13]. The 160 bp 5′ upstream DNA fragment of the MLC2-A gene (−135/+25) formed a slowly migrating complex when incubated with increasing concentrations of nuclear extracts from chicken embryonic heart muscle (fig.2, lanes 1–5). This complex formation

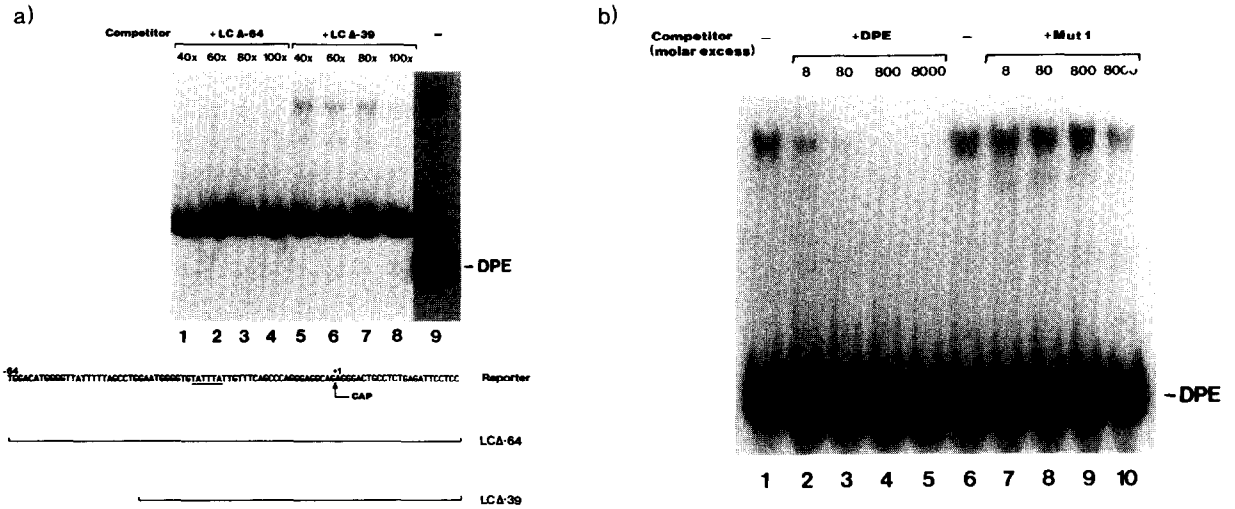

Fig.3. Demonstration of protein binding to the distal promoter element (DPE). (a) The reporter fragment from LCΔ-64 or DPE oligonucleotides were used as radiolabelled probes (lanes 1–8 and 9, respectively). The competing fragments were added at the indicated molar excess over probe. (b) Competition of protein binding to DPE (lanes 1,6) by the addition of an 8–8000-fold molar excess of unlabelled DPE (lanes 2–5) and Mut1 oligonucleotides (lanes 7–10). Both competing oligonucleotides were double stranded. The sequences of DPE and Mut1 are shown in fig.4.

was specifically prevented when unlabelled 160 bp DNA fragment was added in excess as competitor (fig.2, lanes 6–9). A more detailed localization of the protein-binding site was obtained from a gel retardation assay using the 89 bp promoter fragment of the smaller, yet transcriptionally active deletion, LCΔ-64 [7]. As illustrated in fig.3a, protein binding was completely inhibited by a 40–100-fold excess of unlabelled reporter fragment (−64/+25). In contrast, the shorter 5′ deletion fragment (−39/+25), derived from the transcriptionally inactive deletion LCΔ-39 [7] failed to compete for complex formation, even at 80-fold molar excess. From this result it was inferred that the 5′ distal segment of the promoter contains an essential protein-binding site. Indeed, a synthetic oligonucleotide of this distal promoter element (DPE) formed a specific protein complex when incubated with nuclear extracts (fig.3a, lane 9). Footprinting analysis of this DNA region with DNaseI and ExoIII reveals protection of protein-binding sites from nucleotides −61 to −57 and from −54 to −42 (in preparation). The specificity of the protein binding was demonstrated when unlabelled DPE at concentrations between 8- and 8000-fold molar excess over the labelled probe were shown to inhibit effectively the complex formation (fig.3b). In contrast, a mutated oligonucleotide version (Mut1), which contains a sequence that has been altered at 5 nucleotide positions did not compete, suggesting that the protein-binding capacity is sequence specific and involves some or all of the 5 bases that had been mutated (fig.3b).

The attempt to assess the functional importance of the DPE forming a protein complex by in vivo competition experiments was unsuccessful in our hands with DNA transfection techniques. Similar observations have been reported by others for differentiated C2C12 muscle cells [14]. We therefore decided to inject directly the reporter construct LCΔ-64 together with synthetic double-stranded DPE oligonucleotide as competitor into nuclei of myotubes. As illustrated in fig.4, microinjected indicator construct LCΔ-64 alone was highly active (lane 1). Coinjection of increasing concentrations of competing DPE oligonucleotide, however, led to a gradual and significant reduction of the MLC2-A promoter activity (lanes 2–5). When the mutated oligonucleotide Mut1 was coinjected at similar molar concentrations, control levels of CAT reporter gene expression were obtained. These results indicated that the distal promoter element (DPE) which is able to bind to nuclear fac-

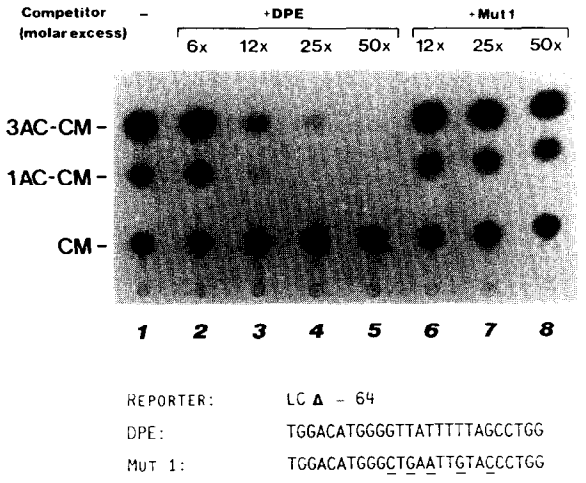

Fig.4. In vivo competition of the transcription of microinjected reporter plasmid LCΔ-64 (sequence shown in fig.3a). Double-stranded DPE (lanes 2–5), and ds-Mut1 (lanes 6–8) were coinjected at the indicated concentrations. The altered bases in Mut1 are underlined.

tors is also functionally required for the transcription of the MLC2-A promoter.

4. DISCUSSION

The aim of this investigation was to demonstrate in vivo the critical interaction of a cis-acting promoter element (DPE) of the muscle-specific myosin light chain 2 gene with positively trans-acting nuclear factor(s). To this end, we have examined the binding properties of an essential 5′ upstream promoter segment, and more importantly, established a procedure by which its functional involvement for transcriptional activation could be demonstrated. The combination of the quantitative transient CAT expression assay and microinjection of reporter DNA plasmids together with competing oligonucleotides into nuclei of differentiated muscle cells yielded quantitative and reproducible results. From these in vivo competition studies it can be concluded that a sequence located in the DPE of the MLC promoter not only binds nuclear components of the muscle cell in a rate-limiting and concentration-dependent fashion but also that this interaction is an essential requirement for the transcriptional activity in living cells. Most significantly, the in vivo competition of this transcriptional activity could not be achieved with a mutated oligonucleotide that also did not bind to nuclear proteins in vitro. These results unambiguously demonstrate the requirement of sequence-specific trans-acting factor(s) for the transcription in an in vivo situation. The method of microinjecting synthetic oligonucleotides in single cell nuclei to compete for limiting protein factors involved in transcriptional activation should allow the determination of various cis- and trans-acting components, even in mixed cell populations of various differentiated cell types.

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