Differential splicing-in of a proline-rich exon converts αNAC into a muscle-specific transcription factor

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NAC (nascent polypeptide-associated complex) was recently purified as an α/β heterodimeric complex binding the newly synthesized polypeptide chains as they emerge from the ribosome. We have identified, cloned, and characterized a muscle-specific isoform of αNAC. The 7.0-kb mRNA arises from differential splicing-in of a 6.0 kb-exon giving rise to a proline-rich isoform that we termed skNAC. The skNAC protein was specifically expressed in differentiated myotubes but not in myoblasts. We have identified a specific DNA binding site for skNAC and shown that it can activate transcription through that element. The murine myoglobin promoter contains three putative skNAC-binding sites. skNAC was shown to activate transcription from the myoglobin promoter, and site-specific mutation of the skNAC response elements abrogated skNAC-dependent activation. We also examined the role of the NAC isoforms in the myogenic program. Whereas overexpression of αNAC prevented C2C12 differentiation and myotube formation, the overexpression of skNAC in C2C12 myoblasts led to early fusion of the cells into gigantic myosacs, suggesting that skNAC may be involved in normal differentiation along the myogenic lineage and in the regulation of myoblast fusion. Our data demonstrate that differential splicing converts αNAC into a tissue-specific DNA-binding activator and suggest that this regulation may be an important event in the proper control of gene expression during myogenic differentiation.

[Key Words: Transcription; myoblast differentiation; myoglobin expression]

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The differentiation of skeletal myoblasts into functional myotubes has been characterized extensively at the molecular level, which has led to the identification of a transcriptional regulatory cascade implicating the myogenic transcription factors of the basic-helix-loop-helix (bHLH) family, MyoD, myogenin, myf5 and MRF4, as well as the MEF2 [myocyte enhancer factor-2] proteins (for review, see Olson and Klein 1994; Olson et al. 1995). The myogenic bHLH factors interact with ubiquitous HLH proteins, such as E12 and E47, to form functional heterodimers binding the E box consensus sequence [Murre et al. 1989; Lassar et al. 1991]. The MEF2 factors contain the 56 amino acid MADS domain [Shore and Sharrocks 1995] and bind DNA as homodimers or heterodimers between the various MEF2 family members [Yu et al. 1992]. Both the E box site and the MEF2-binding site have been identified, in various combinations, in the promoter region of many muscle-specific genes [Gosset et al. 1989; Weintraub et al. 1991]. Recent work has demonstrated that either MEF2 or myogenic bHLH factors can act as cofactors to potentiate the transcriptional activity of the other (Molkentin et al. 1995). These results have revealed novel mechanisms of gene activation during myogenesis and have shed light on the paradoxical observation that myogenic bHLH proteins can activate transcription of muscle-specific genes lacking E boxes in their promoter region.

In addition to the activation of the skeletal muscle differentiation program initiated by the myogenic transcription factors, terminal differentiation of myoblasts into functional myotubes requires intercellular fusion of myoblasts. This process is initiated by intercellular recognition of myoblasts followed by membrane fusion [Knudsen and Horowitz 1977; Wakelam 1985]. Several adhesion macromolecules have been implicated in myoblast fusion, including neural cell adhesion molecule [NCAM] [Dickson et al. 1990; Knudsen et al. 1990a], N-cadherin [Knudsen et al. 1990b], very late antigen 4 [VLA-4] [Rosen et al. 1992], vascular cell adhesion molecule 1 [VCAM-1] [Rosen et al. 1992], and meltrin-α [Yagami-Hiromasa et al. 1995]. Among others, manipulations that inhibit protein kinase C (Lin et al. 1987) or attachment to extracellular substratum [Daniels and Sandra 1990] affect myoblast fusion. Moreover, fusion is
inhibited in myoblasts overexpressing the \textit{c-myc} proto-oncogene (Crescenzi et al. 1994), a site-specific DNA-binding transcriptional activator (Lüscher and Eisenman 1990). This suggests that fusion involves a signal transduction pathway, possibly mediated through protein kinase C, linking extracellular recognition events to regulated gene expression.

While searching for genes expressed in developing bone, we have recently cloned the murine \textit{aNAC} cDNA (W. V. Yotov, A. Moreau, F. H. Glorieux, and R. St-Arnaud, in prep.). NAC [nascent polypeptide-associated complex], a site-specific DNA-binding transcriptional activator (Liischer and Eisenman 1990), is recently purified as a heterodimeric complex binding the newly synthesized polypeptide chains as they emerge from the ribosome (Wiedmann et al. 1994). The \( \beta \)-NAC subunit has been identified as BTF3b (Wiedmann et al. 1994), a protein involved in regulating transcription in yeast (Hu and Ronne 1994) and in higher eukaryotes (Zheng et al. 1990). The identification of the heterodimerization partner of \( \alpha \)NAC as the transcription factor BTF3b is somewhat puzzling and suggests a putative role for \( \alpha \)NAC in transcriptional control (W. V. Yotov, A. Moreau, F. H. Glorieux, and R. St-Arnaud, in prep.).

In this study we have examined the pattern of expression of \( \alpha \)NAC in adult tissue. We have detected \( \alpha \)NAC mRNA in all tissues examined postnatally. Interestingly, a larger size transcript was identified in skeletal muscle and heart. Cloning of the cDNA corresponding to this 7.0-kb mRNA revealed that it arises from differential splicing-in of a 6.0-kb exon giving rise to a proline-rich isoform of \( \alpha \)NAC that we termed skNAC. The 220-kD skNAC protein was specifically expressed in differentiated myotubes but was not detected in myoblasts.

Because proline-rich domains have been identified as transcriptional activation domains, we have examined the possibility that the skNAC isoform of \( \alpha \)NAC may function as a transcriptional activator. We have identified the specific DNA-binding site of skNAC and have shown that skNAC can activate the expression of a reporter gene driven by a heterologous promoter containing two copies of the skNAC-binding site. A search of the DNA databases has revealed that the murine myoglobin promoter contains three sites similar to the skNAC-binding site. skNAC was shown to activate transcription from the myoglobin promoter, and site-specific mutation of the skNAC response elements abrogated skNAC-dependent activation. Gain-of-function mutations were engineered in C2C12 myoblasts: whereas overexpression of the short form of the protein \( \alpha \)NAC prevented myotube fusion, the overexpression of skNAC in C2C12 myoblasts led to early fusion of the cells into gigantic myosacs, suggesting that skNAC may be involved in normal differentiation along the myogenic lineage and may be implicated in the signal transduction cascade regulating myoblast fusion. Our data demonstrate that differential splicing converts \( \alpha \)NAC into a tissue-specific DNA-binding activator and suggest that this regulation may be an important event in the proper control of gene expression during myogenic differentiation.

Results

\textit{NAC expression in adult tissues and muscle}

Figure 1. A and B, shows the expression pattern of the \( \alpha \)NAC mRNA in various adult tissues. The 900-bp \( \alpha \)NAC transcript was detected in all tissues postnatally. Interestingly, a larger-sized transcript (7.0 kb) was identified in skeletal muscle and heart (Fig. 1B).

We used the immunoblot assay to determine whether the long NAC transcript coded for a larger NAC protein isoform in skeletal muscle cells. As shown in Figure 1C,
the anti-NAC polyclonal antibody raised against the recombinant aNAC protein (W.V. Yotov, A. Moreau, F.H. Glorieux, and R. St-Arnaud, in prep.) recognized the 35-kD aNAC protein in nuclear extracts from osteoblastic MC3T3-E1 cells, P19 embryonal carcinoma cells, and C2C12 myoblasts and myotubes. However, the antibody also recognized an abundant protein of 220 kD specifically expressed in differentiated C2C12 myotubes (Fig. 1C, lane 4). The size of the protein recognized by the antibody in skeletal myotubes corresponded to the size of the in vitro translation product from the large 70-kb NAC cDNA [data not shown]. We named the 220-kD isoform skNAC to denote its expression in skeletal myotubes. Expression of the skNAC protein was also detected by immunohistochemistry in sections from the tongue [data not shown].

Cloning of the skNAC cDNA

We probed a mouse skeletal muscle cDNA library with an aNAC probe to clone the 7.0-kb skNAC cDNA. Figure 2 shows the deduced amino acid sequence derived from the full-length skNAC cDNA. The first 23 residues are identical to the amino-terminal sequence of aNAC; similarly, the last 192 amino acids correspond to the remainder of the aNAC protein sequence, suggesting that the skNAC isoform arose from splicing-in of additional sequence between residues 23 and 24 of the aNAC protein. This was confirmed by the cloning and sequencing of genomic NAC clones. The additional 1972 residues in skNAC are encoded by a large 5916-bp third exon (Fig. 2).

The protein sequence features an EF-hand motif, a selective and high-affinity Co²⁺-binding motif named after the E- and F-helices of parvalbumin (for review, see Heizmann and Hunziker 1991). A putative nuclear targeting sequence is also present [Fig. 2]. These structural motifs are shared with the aNAC isoform (W.V. Yotov, A. Moreau, F.H. Glorieux, and R. St-Arnaud, in prep.). No additional structural motifs could be identified in the deduced skNAC protein sequence, apart from a relatively large proportion [18%] of proline residues in the sequence encoded by the spliced-in third exon (Fig. 2).

Because proline-rich domains have been characterized as transcriptional activation domains (Mermod et al. 1989), we examined the possibility that skNAC might function as a site-specific activator in skeletal myotubes.

skNAC binds DNA with sequence specificity

We used the selection and amplification of binding sites (SAAB) technique [Blackwell and Weintraub 1990] to determine whether aNAC or skNAC could bind DNA with sequence specificity. Figure 3A shows that two rounds of SAAB with the recombinant aNAC protein were sufficient to detect specific DNA binding, and a third round of amplification further enhanced the binding affinity of aNAC for the selected sites. Extracts from bacteria transformed with the empty expression vector

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**Figure 2.** Deduced amino acid sequence of the muscle-specific skNAC protein. Shared residues with aNAC are underlined, putative nuclear localization signal [residues 2043–2050] and calcium-binding EF hand [residues 2101–2113] are doubly underlined. Proline residues in the spliced-in exon 3 are in boldface type.

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**Figure 3.** Putative DNA-binding site for skNAC. (A) The sequence of a skNAC–DNA complex is shown. The shaded areas are the consensus DNA sequence. The underlined A residues are the regulatory sites. The shaded boxes indicate putative calcium-binding EF hands. (B) A sequence-specific transcription factor

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**Table 1.** Myosite-specific transcription factor

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**Table 2.** skNAC and skNAC isoforms

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**Table 3.** skNAC and skNAC isoforms

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**Table 4.** skNAC and skNAC isoforms

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**Table 5.** skNAC and skNAC isoforms

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**Figure 4.** skNAC binding to DNA. (A) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (B) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (C) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (D) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (E) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (F) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (G) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (H) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (I) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (J) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (K) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (L) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (M) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (N) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (O) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (P) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (Q) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (R) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (S) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (T) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (U) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (V) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (W) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (X) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (Y) DNA mobility shift analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6). (Z) Competition analysis shows that skNAC specifically binds to the consensus target sequence (lane 1). Other DNA targets are also shown (lanes 2–6).
A natural target promoter of skNAC

We next addressed whether the NAC proteins could directly trans-activate via their cognate DNA-binding site. Two copies of the consensus binding site described above were subcloned upstream of the minimal thymidine kinase promoter driving the luciferase reporter gene to generate the plasmid 2X tk luc. Cotransfection of this reporter construct with a skNAC expression vector resulted in very strong stimulation of the expression of the reporter gene [Figure 4A]. The empty vector had virtually no effect in this assay, demonstrating the specificity of the skNAC-dependent activation of transcription [Figure 4A]. The stimulation of the expression of the reporter gene was entirely dependent on the presence of the cognate skNAC-binding sites, as no increase in expression was measured using a reporter construct in which two mutant skNAC-binding sites that fail to bind skNAC [Figure 3B] were subcloned upstream of the heterologous promoter [Figure 4A, plasmid 2X mu tk luc]. Similarly, skNAC did not stimulate the expression of the reporter gene from the parental tk luc vector [Figure 4A]. The αNAC protein had no effect in this assay [data not shown], suggesting that despite its capacity to bind the NAC-binding site [Figure 3A], it does not act as a transcriptional activator. This observation was confirmed by the data presented below.

A search for natural promoters that could contain the skNAC-binding site revealed that the proximal promoter of the murine myoglobin gene contains three such putative sites. Figure 4B lists the sequence of the promoter from residues -280 to +38. The three putative skNAC-binding sites are highlighted: a first site corresponding to positions -142 to -135 on the noncoding strand; a second site at positions -130 to -121; and a third site covering positions -120 to -110, both on the coding strand. Alignment of these putative sites with the skNAC consensus binding site is shown above the sequence [Figure 4B]. We subcloned the first 900 bp of the myoglobin promoter upstream of the luciferase reporter gene [plasmid myoglobin-luc] and assayed whether it would respond to skNAC. Figure 4C shows that coexpression of skNAC resulted in transcriptional activation of the myoglobin promoter leading to a reproducible 12-fold induction of the expression of the reporter gene. This induction was specific as the empty vector plasmid had no effect. Moreover, the skNAC expression vector did not affect the basal expression level of the promoterless luciferase control plasmid [Figure 4C, luc reporter]. When mutations were engineered at the critical A residues of the skNAC-binding sites using site-specific mutagenesis [Figure 4B], the skNAC-dependent stimulation of the expression from the myoglobin promoter was abolished [Figure 4C, mut. myoglobin-luc reporter], demonstrating that skNAC activated transcription via binding to its cognate sites in the promoter. The lower molecular weight αNAC isomer did not stimulate the expression from either the wild-type or the mutant myoglobin reporter constructs [Figure 4C], confirming that it does not exhibit intrinsic transcriptional activating function. Immunoblotting assays confirmed that equivalent levels of expression were achieved from each NAC vector in all assays [data not shown].

Overexpression of skNAC in myoblasts perturbs differentiation

To address the putative role of skNAC in myogenesis, we overexpressed the protein in various cell lines. The phenotype of C3H10T1/2 embryonic fibroblasts was unaffected by skNAC overexpression [data not shown], suggesting that skNAC cannot activate the program for skeletal muscle differentiation like the myogenic bHLH factors. However, overexpression of NAC isoforms in skeletal myoblasts affected their differentiation pattern. Figure 5A shows representative examples of expression levels for αNAC and skNAC detected by immunoblotting in stable clones transfected with the respective expression vector. Four independent clones derived from each vector were analyzed; four clones transfected with the selection plasmid alone served as controls.
Figure 4. skNAC is a sequence-specific transcriptional activator. (A) Transient transfection assays of cytomegalovirus (CMV)-driven skNAC with reporter constructs in which wild-type (2X tk luc) or mutated (2X mu tk luc) NAC-binding sites have been subcloned upstream of the parental tk luc reporter vector. (B) Sequence of the proximal murine myoglobin promoter. The three NAC-binding sites are shaded and aligned with the NAC consensus binding site above; mutated residues are in boldface type below the sequence. Also indicated are the transcription start site (bent arrow), the TATAA box (bold), and sequences homologous to previously identified control elements from the human gene (CCAC box and A/T element; Bassel-Duby et al. 1992). (C) Transient transfection assays of CMV-driven aNAC and skNAC with reporter constructs in which the wild-type (myoglobin-luc) or mutated (mut. myoglobin-luc) proximal myoglobin promoter have been subcloned upstream of the luciferase (luc) reporter gene. (Vector) Empty CMV expression plasmid. The expression levels detected in cells transfected with the tk luc (A) or luc (C) reporters alone were arbitrarily ascribed a value of 1. Results are expressed as mean fold induction ±S.E.M. of four independent transfections.

All control clones behaved like the parental C2C12 cells: Upon reaching confluency, they fused to form elongated myotubes when placed in low-serum differentiation medium (Table 1); fusion into myosin heavy chain-positive myotubes could also be detected at high serum concentrations in confluent cultures [Fig. 5B, Table 1]. On the contrary, all clones overexpressing the aNAC isoform, which is normally expressed at very low levels in myoblasts and myotubes (Figs. 1B and 5A), were inhibited in their differentiation program and did not fuse at confluency [Fig. 5C], even when placed in differentiation medium [Table 1]. The clones overexpressing skNAC exhibited an opposite phenotype with the myoblasts from confluent cultures [and sometimes even before reaching confluency] differentiating into gigantic, myosin heavy chain-positive, multinucleated myosacs, even in the presence of high concentrations of serum in the culture medium [Fig. 5D]. All four clones overexpressing skNAC exhibited the same phenotype [Table 1; data not shown]. Thin myotubes were not detected in the cultures of skNAC-expressing clones (Table 1). The overall number of myosacs did not change significantly when the cultures were fed with the differentiation medium [Table 1]. These data suggest that the differential splicing of the NAC gene in skeletal myotubes may represent a key regulatory event and that skNAC could be implicated in the regulation of the myogenic differentiation program.

aNAC is not a dominant-negative inhibitor of the skNAC trans-activating function

The results presented above, as well as the observation that aNAC can bind the same consensus DNA sequence as skNAC in gel retardation assays (Fig. 3A), suggested that aNAC could act as a dominant-negative inhibitor of the skNAC trans-activating function by competitively blocking the NAC-binding site. We tested this possibility using transient transfection assays with the myoglobin-luc reporter plasmid. As shown in Figure 6, increas-
Table 1. Differentiation of αNAC- and skNAC-expressing C2C12 clones

| Clones       | Growth medium* | Differentiation medium* |
|--------------|----------------|-------------------------|
|              | myotubes       | myosacs                 | myotubes | myosacs |
| neo          | 2.3 ± 0.5      | 31.1 ± 2.8              | 0        | 0       |
| αNAC         | 0.9 ± 0.5      | 2.5 ± 0.6               | 0        | 0       |
| skNAC        | 0              | 6.7 ± 0.3               | 0        | 4.0 ± 0.4 |

Results are expressed as mean ± S.E.M. of number of MF-20-positive cells in 4 microscope fields from four independent clones of each type, in duplicate [32 fields counted for each type of clone].
*12% fetal bovine serum.
*2% horse serum.
*Arbitrarily defined as structures with a diameter more than fourfold the diameter of myotubes.

skeletal myotubes. This protein isoform, termed skNAC, arises from splicing-in of a large proline-rich exon that confers transcriptional activating function to the protein. Although both αNAC and skNAC were shown to specifically bind DNA, only the muscle-specific skNAC isoform displayed transcriptional activating capacity. The murine myoglobin promoter was identified as a natural promoter responsive to skNAC. Interestingly, gain-of-function experiments in which skNAC...
was overexpressed in skeletal myoblasts perturbed the differentiation sequence of the cells and led to early fusion of myoblasts into gigantic multinucleated structures called myosacs. These results suggest a critical role for skNAC in the regulation of the myogenic program.

Alternative splicing is a prevalent mechanism utilized to generate specific protein isoforms in muscle tissue. This has been observed for sarcomeric proteins (for review, see Nadal-Ginard et al. 1991), plasma membrane enzymes (Brandt and Vanaman 1994), and transcriptional regulatory factors (Martin et al. 1994). The case at hand is somewhat unusual in light of the large size of the alternately spliced exon and the apparent dramatic change of function conferred to the protein by the additional sequence. αNAC has been associated previously with translational control and binding to the ribosome (Wiedmann et al. 1994; Powers and Walter 1996). However, the dimerization partner of αNAC is the transcription factor BTF3b (Zheng et al. 1990; Hu and Ronne 1994; Wiedmann et al. 1994), raising concern about the translational regulatory function of the NAC heterodimer or suggesting a putative involvement of αNAC in transcriptional control. Recent results from our laboratory support such a role (W.V. Yotov, A. Moreau, F.H. Glorieux, and R. St-Arnaud, in prep.).

The large proline-rich domain tagged onto the skNAC protein prompted us to examine the possibility that it could function as a transcriptional activator. Proline-rich activation domains have been identified in several transcription factors including CTF/NF-1 (Mermod et al. 1989) and AP-2 (Williams and Tjian 1991). The relative proportions of proline residues within the 100 amino acid activation domain of CTF/NF-1 and the 67 amino acid activating region of AP-2 are 25% and 22%, respectively (Mermod et al. 1989; Williams and Tjian 1991). The spliced-in exon 3 of skNAC shows an overall proportion of 18% of proline residues over a very large domain of 192 amino acids; subregions of the protein show proline concentrations as high as 28% over stretches of 90 amino acids (Fig. 2). Thus, the sequence and structure of the proline-rich domain of skNAC was entirely consistent with a transcriptional activating function.

We first attempted to determine whether skNAC could bind DNA with sequence specificity. To our surprise, both skNAC and αNAC recognized the same DNA sequence in gel mobility shift assays (Fig. 3). However, all attempts to demonstrate that αNAC could directly trans-activate through its cognate response element have been unsuccessful (data not shown). We do not yet know whether αNAC actually binds DNA in vivo in mammalian cells or whether this function is restricted to in vitro assays using recombinant protein produced in bacteria. The observation that αNAC does not competitively inhibit the transcriptional activating function of skNAC in vivo (Fig. 6) suggests that the ability of αNAC to bind to the consensus NAC-binding site may be masked in vivo.

The transcriptional activating function of skNAC, however, was readily measurable using transient transfection assays. Reporter constructs linking either chimeric or natural promoter elements were shown to respond to skNAC, and this transcriptional response was strictly dependent on the capacity of skNAC to bind its cognate response element, as mutations that inhibited binding of the protein to DNA concomitantly inhibited transcriptional activation. Further experiments will be required to analyze the structure–function relationships of the skNAC protein to delineate the precise boundaries of the activation domain as well as to locate the DNA-binding domain. The observation that αNAC has inherent DNA-binding activity in vitro reveals that this function resides in the common structural domains of the two isoforms, that is, the amino-terminal 23 residues or the last 192 amino acids of the proteins.

We have identified the myoglobin promoter as a natural promoter responding to skNAC. It is interesting to note that myoglobin expression remains low during embryonic development, whereas it is markedly up-regulated in heart and skeletal muscle after birth (Weller et al. 1986; Parsons et al. 1993). This postnatal increase in expression correlates with the onset of the expression of skNAC (not shown). Previous studies that addressed the control of the expression of the human myoglobin gene have identified two main sequence elements involved in the developmental and tissue-specific expression of the myoglobin gene: an A/T-rich sequence located at positions –160 to –169, and the CCAC box centered around position –216 (Bassell-Duby et al. 1992). The A/T element was shown to bind two nuclear factors, an unidentified protein of 35 kD, and members of the MEF-2 gene family (Grayson et al. 1995), whereas the CCAC box binds the myocyte nuclear factor (MNF), a winged-helix transcription factor showing multiple isoforms of 65, 68, and 90 kD (Bassell-Duby et al. 1994). Interestingly, MNF is also a proline-rich transcriptional activator (Bassell-Duby et al. 1994). Although skNAC is distinct from any of these previously identified regulators of myoglobin expression, it is worth pointing out that the murine myoglobin promoter does not exhibit a complete sequence similarity with the human gene at the A/T and CCAC elements (Fig. 4B). However, the skNAC-binding sites that we identified are conserved in the human myoglobin promoter (Grayson et al. 1995). Moreover, the human myoglobin promoter fragment that was used in transgenic mice to recapitulate the developmental expression pattern of myoglobin encompassed the three skNAC-binding sites (Parsons et al. 1993). Thus, the previous data concerning the regulation of myoglobin gene expression do not contradict the results presented here and skNAC may well represent another factor involved in the proper developmental and tissue-restricted expression of myoglobin.

In addition to the putative role of skNAC in the control of myoglobin transcription, the gain-of-function experiments presented here also support a role for skNAC in the regulation of the myogenic differentiation program. Although the skNAC activator did not behave like the myogenic bHLH factors in inducing myogenesis [not shown], overexpression of the protein in skeletal myo-
blasts dramatically perturbed their differentiation sequence, leading to early fusion into large myosacs. Overexpression of meltrin-α, a transmembrane protein implicated in myoblast fusion, has also been shown to induce myosac formation (Yagami-Hiromasa et al. 1995). Similarly, inhibition of protein kinase C, as well as inhibition of substrate attachment, leads to an identical phenotype (Lin et al. 1987; Daniels and Sandra 1990). Thus, it is conceivable that interactions of myoblasts with the extracellular matrix via transmembrane receptors trigger a protein kinase C-dependent signaling cascade leading to myotube fusion and that skNAC may be implicated in this transduction pathway.

Overexpression of αNAC in skeletal myoblasts generated the opposite phenotype by inhibiting fusion. Although we detected low levels of αNAC protein in myoblasts and fused myotubes using immunoblotting assays (Fig. 1C), we estimate the ratio of skNAC to αNAC mRNA transcripts at 20:1 based on the relative proportion of cDNA clones for αNAC that we detected in the skeletal muscle library during the cloning of skNAC [not shown]. Thus, overexpression of αNAC may perturb the regulation of myoblast fusion normally controlled by skNAC. The results presented in Figure 6 show that this does not occur via a dominant-negative effect on the skNAC trans-activating function. It is possible that αNAC inhibits myoblast fusion through its hypothesized role in translational control and interactions between ribosomes and the endoplasmic reticulum membrane [Wiedmann et al. 1994; Powers and Walter 1996]. Further studies will be required to delineate the relative importance of the proposed transcriptional [W.V. Yotov, A. Moreau, F.H. Glorieux, and R. St-Arnaud, in prep.] and translational [Wiedmann et al. 1994; Powers and Walter 1996] regulatory activities of αNAC.

As mentioned previously, BTF3b has been identified as the heterodimerization partner of αNAC (Wiedmann et al. 1994). Initially identified as an alternatively spliced form of the general transcription factor BTF3 purified from HeLa cells (Zheng et al. 1990), the function of BTF3b has been studied primarily in yeasts, where it was shown to act as a negative regulator of RNA polymerase II-dependent gene transcription [Parthun et al. 1992; Hu and Ronne 1994]. We tested the effect of BTF3b on skNAC-mediated transcriptional activation. Increasing amounts of BTF3b had no effect on the transcriptional activating function of skNAC tested using two different promoters [myoglobin-luc and 2X tk luc] in two different cell lines [data not shown]. These results suggest that the function of BTF3b in mammalian cells may differ from its function identified in yeast. Alternatively, skNAC may not interact with BTF3b, a parameter that remains to be tested.

Our results support a role for the skNAC isoform of αNAC in the control of gene transcription in skeletal myotubes as well as in the regulation of the myogenic differentiation program. Experiments aimed at creating targeted mutations in the muscle-specific form of NAC through homologous recombination in embryonic stem cells are in progress and should help elucidate the contribution of skNAC to the normal process of myogenesis in vivo.

Materials and methods

Northern blot hybridization

The commercially available mouse multiple tissue Northern blot (Clontech Laboratories, Palo Alto, CA) was probed with the full-length, PCR-labeled αNAC cDNA using conventional protocols.

Immunoblotting

The murine αNAC cDNA was subcloned into the pGEX-4T-3 vector [Pharmacia Canada, Baie d’Urfé, Québec, Canada], and the glutathione S-transferase (GST)–αNAC fusion protein was purified according to the manufacturer’s instructions. Polyclonal antibodies were raised by immunization of rabbits with GST–αNAC. The polyclonal antisera [1:1000 dilution] were used to probe immunoblots of nuclear extracts from various cell lines using standard protocols [Sambrook et al. 1989]. Nuclear extracts from C2C12 myoblasts and myotubes were a generous gift of Dr. M. Nemer [Institut de Recherches Cliniques, Montréal, Canada], nuclear extracts from osteoblastic MC3T3-E1 cells and P19 embryonal carcinoma cells have been described previously [St-Arnaud and Moir 1993; Candeliere et al. 1996].

Cloning of skNAC

The PCR-labeled full-length αNAC cDNA was used to probe a commercially available mouse skeletal muscle cDNA library [Clontech Laboratories, Palo Alto, CA]. The 5′ ends of partial skNAC cDNA clones obtained in this fashion were used as probes to rescreen the library until a full-length clone was identified. The 7.0-kb full-length skNAC cDNA clone was sequenced entirely on both strands using the Sequenase 2.0 kit as directed by the manufacturer [Amersham Life Sciences Canada, Oakville, Ontario].

SAAB

Fully degenerated 10-mer oligonucleotides flanked by specific primers [5′-CTTGATTGCC-3′ and 5′-TGTTGGCCCT-3′] were used to generate the double-stranded labeled probes used in the SAAB procedure [Blackwell and Weintraub 1990]. One microgram of GST–αNAC fusion protein was incubated with the labeled probes for 30 min at room temperature and separated on a 6% nondenaturing gel as described elsewhere [St-Arnaud and Moir 1993]. The retarded complexes were cut directly from the dried gel, and the DNA was reamplified and used in subsequent rounds of gel retardation.

Gel mobility shift assay

The skNAC cDNA was in vitro transcribed and translated using the TNT-coupled reticulocyte lysate system following the instructions of the manufacturer [Promega Corp., Madison, WI]. Gel mobility shift assays were performed as described previously [St-Arnaud et al. 1995] using a Klenow-labeled oligonucleotide corresponding to the skNAC-binding site.

Transient transfection assays

The 2X tk luc expression vector was constructed by inserting an oligonucleotide encoding two copies of the consensus αNAC-binding site [underlined], 5′-TCAGCCGACACACAGGAGGGG-CACACGCACAGG-3′, into the expression vector tk luc [Glass et al. 1988]. An oligonucleotide in which the αNAC-binding sites were mutated, 5′-CTAGCCGACTCTCAGGAGGGG-CACACGCACAGG-3′, was used to generate the double-stranded labeled probes used in the SAAB procedure [Blackwell and Weintraub 1990]. One microgram of GST–αNAC fusion protein was incubated with the labeled probes for 30 min at room temperature and separated on a 6% nondenaturing gel as described elsewhere [St-Arnaud and Moir 1993]. The retarded complexes were cut directly from the dried gel, and the DNA was reamplified and used in subsequent rounds of gel retardation.

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CACGACTCTCACG-3' (mutated residues in boldface type), was used to generate the related vector 2X mu tk luc. For experiments using the myoglobin promoter, the proximal region was cloned from a murine genomic library (Stratagene Corp., LaJolla, CA) using PCR with primers corresponding to residues −902 to −887 and +20 to +38 of the published sequence [Blanchetot et al. 1986]. The amplified fragment was cloned into the pGL2basic vector (Promega Corp., Madison, WI) to yield the plasmid myoglobin-luc. To generate the mutant plasmid mut myoglobin-luc, point mutations (see Fig. 4B) were introduced by inverse PCR [Ochman et al. 1988]. The mutations were confirmed by the sequencing of both strands. All NAC expression plasmids were generated by cloning the corresponding full-length cDNAs into the pCI vector (Promega Corp., Madison, WI). Plasmid vectors were constructed using standard molecular biology procedures, and full details and sequences are available on request. Embryonic fibroblasts of the C3H10T1/2 line (Taylor and Jones 1979) were transfected using 5 μl of the LipofectAMINE reagent (GIBCO BRL, Gaithersburg, MD) and a total of 5 μg of DNA according to the instructions of the manufacturer. Cells were harvested 24 hr post-transfection. All assays included a fixed amount (0.1 μg) of plasmid pSV6tkCAT [Courey and Tjian 1988] to monitor for fluctuations in transfection efficiency. CAT and luciferase activities were assayed as described previously [St-Arnaud and Moir 1993].

**Stable clones**

Murine skeletal myoblasts of the C2C12 line were transfected with the selection plasmid PGKneo (Rudnicki et al. 1992) alone or in combination with the αNAC or skNAC expression vectors using the LipofectAMINE reagent (GIBCO BRL, Gaithersburg, MD) according to the instructions of the manufacturer. Individual stable clones were established following selection with G418. Clones were grown in Dulbecco’s Modified Eagle Medium supplemented with 12% fetal bovine serum. For immunostaining with the MF-20 monoclonal antibody directed against myosin heavy chain, confluent cultures were fixed in 90% methanol/phosphate buffered saline (PBS) at −20°C, washed with PBS, and blocked with 5% skim milk powder in PBS for 30 min. Binding of primary antibody (1:10 in blocking solution) was for 1 hr with gentle rocking at room temperature, following washes in PBS, alkaline phosphatase-conjugated secondary antibody (Promega Corp., Madison, WI) was incubated with the cells for 1 hr under the same conditions. Colorimetric detection was according to the instructions of the manufacturer.

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**Note**

The accession number for the sequence reported in this paper is U48364.

**References**

Bassel-Duby, R., M.D. Hernandez, M.A. Gonzalez, J.K. Krueger, and R.S. Williams. 1992. A 40-kilodalton protein binds specifically to an upstream sequence element essential for muscle-specific transcription of the human myoglobin promoter. Mol. Cell. Biol. 12: 5024–5032.

Bassel-Duby, R., M.D. Hernandez, Q. Yang, J.M. Rochelle, M.F. Seldin, and R.S. Williams. 1994. Myocyte nuclear factor, a novel winged-helix transcription factor under both developmental and neural regulation in striated myocytes. Mol. Cell. Biol. 14: 4596-4605.

Blackwell, T.K. and H. Weintraub. 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250: 1104–1110.

Blanchetot, A., M. Price, and A.J. Jeffrey. 1986. The mouse myoglobin gene. Eur. J. Biochem. 159: 469–474.

Brandt, P. and T.C. Vanaman. 1994. Splicing of the muscle-specific plasma membrane Ca²⁺-ATPase isoform PMCA1c is associated with cell fusion in C2 myocytes. J. Neurochem. 62: 799–802.

Candeliere, G.A., P.W. Jurutka, M.R. Haussler, and R. St-Arnaud. 1996. A composite element binding the vitamin D receptor, retinoid X receptor α, and a member of the CTF/NF-1 family of transcription factors mediates the vitamin D responsiveness of the c-fos promoter. Mol. Cell. Biol. 16: 584–592.

Courey, A.J. and R. Tjian. 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel guanine-rich activation motif. Cell 55: 887–898.

Crescenzi, M., D.H. Crouch, and F. Tató. 1994. Transformation by myc prevents fusion but not biochemical differentiation of C2C12 myoblasts: Mechanisms of phenotypic correction in mixed culture with normal cells. J. Cell Biol. 125: 1137–1145.

Daniels, K.J. and A. Sandra. 1990. Cytoskeletal organization and synthesis in substrate-independent and -dependent myogenesis in chick embryos. Anat. Rec. 227: 254–263.

Dickson, G., D. Peck, S.E. Moore, C.H. Barton, and F.S. Walsh. 1990. Enhanced myogenesis in NCAM-transfected myoblasts. Nature 344: 348–351.

Glass, C.K., J.M. Holloway, O.V. Devary, and M.G. Rosenfeld. 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thryroid hormone and estrogen response elements. Cell 54: 313–323.

Gosslett, L.A., D.J. Kelvin, E.A. Sternberg, and E.N. Olson. 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. 9: 5022–5033.

Grayson, J., R.S. Williams, Y.-T. Yu, and R. Bassel-Duby. 1995. Synergistic interactions between heterologous upstream activation elements and specific TATA sequences in a muscle-specific promoter. Mol. Cell. Biol. 15: 1870–1878.

Heizmann, C.W. and W. Hunziker. 1991. Intracellular calcium-binding proteins: More sites than insights. Trends Biochem. Sci. 16: 98–103.

Hu, G.-Z. and H. Ronne. 1994. Yeast BTF3 protein is encoded by duplicated genes and inhibits the expression of some genes in vivo. Nucleic Acids Res. 22: 2740–2743.
Knudsen, K.A. and A.F. Horowitz. 1977. Tandem events in myoblast fusion. Dev. Biol. 58: 328–338.

Knudsen, K.A., S.A. McElwee, and L. Myers. 1990a. A role for the neural cell adhesion molecule, N-CAM, in myoblast interaction during myogenesis. Dev. Biol. 138: 159–168.

Knudsen, K.A., L. Myers, and S.A. McElwee. 1990b. A role for the Ca^{2+}-dependent adhesion molecule, N-cadherin, in myoblast interaction during myogenesis. Exp. Cell Res. 188: 175–184.

Lassar, A.B., R.L. Davis, W.E. Wright, T. Kadesch, C. Murre, A. Vovonova, D. Baltimore, and H. Weintraub. 1991. Functional activity of myogenic bHLH proteins requires heterooligomerization with E12/E47-like proteins in vivo. Cell 66: 305–315.

Lin, Z.X., J.R. Eshelman, S. Forry-Schaudies, S. Duran, J.L. Lessard, and H. Holzer. 1987. Sequential disassembly of myofibrils induced by myristate acid in cultured myotubes. J. Cell Biol. 105: 1365–1376.

Lüscher, B. and R.N. Eisenman. 1990. New light on myc and myb. Part I. Myc. Genes & Dev. 4: 2025–2035.

Martin, J.F., J.M. Miano, C.M. Hustad, N.G. Copeland, N.A. Jenkins, and E.N. Olson. 1994. A Mef2 gene that generates a muscle-specific isoform via alternative mRNA splicing. Mol. Cell. Biol. 14: 1647–1656.

Mermod, N., E.A. O’Neill, T.J. Kelly, and R. Tjian. 1989. The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. Cell 58: 741–753.

Molkentin, J.D., B.L. Black, J.F. Martin, and E.N. Olson. 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell 83: 1125–1136.

Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, J.N. Yan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka, A.B. Lassar, H. Weintraub, and D. Baltimore. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58: 537–544.

Nadal-Ginard, B., C.W.J. Smith, J.G. Patton, and R.E. Breitbart. 1991. Alternative splicing is an efficient mechanism for the generation of protein diversity: Contractile protein genes as a model system. Adv. Enzyme Regul. 31: 261–286.

Ochman, H., A.S. Gerber, and D.L. Hartl. 1988. Genetic applications of an inverse polymerase chain reaction. Genetics 120: 621–623.

Olson, E.N. and W.H. Klein. 1994. bHLH factors in muscle development: Dead lines and commitments, what to leave in and what to leave out. Genes & Dev. 8: 1–8.

Olson, E.N., M. Perry, and R.A. Schulz. 1995. Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. Dev. Biol. 172: 2–14.

Parsons, W.J., J.A. Richardson, K.H. Graves, R.S. Williams, and R.W. Moreadith. 1993. Gradients of transgene expression directed by the human myoglobin promoter in the developing mouse heart. Proc. Natl. Acad. Sci. 90: 1726–1730.

Parthun, M.R., D.A. Mangus, and J.A. Jaehning. 1992. The EGD1 product, a yeast homolog of human BTF3, may be involved in GAL4 DNA binding. Mol. Cell. Biol. 12: 5683–5689.

Powers, T. and P. Walter. 1996. The nascent polypeptide-associate complex modulates interactions between the signal recognition particle and the ribosome. Curr. Biol. 6: 331–338.

Rosen, G.D., J.R. Sanes, R. LaChance, J.M. Cunningham, J. Roman, and D.C. Dean. 1992. Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. Cell 69: 1107–1119.

Rudnicki, M.A., T. Braun, S. Hinuma, and R. Jaenisch. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71: 383–390.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shore, P. and D. Sharrocks. 1995. The MADS-box family of transcription factors. Eur. J. Biochem. 229: 1–13.

St-Arnaud, R. and J.M. Moir. 1993. Wnt-1 inducing factor-1: A novel G/C box-binding transcription factor regulating the expression of Wnt-1 during neuroectodermal differentiation. Mol. Cell. Biol. 13: 1590–1598.

St-Arnaud, R., J. Prud’homme, C. Leung-Hagestein, and S. Dedhar. 1995. Constitutive expression of calreticulin in osteoblasts inhibits mineralization. J. Cell Biol. 131: 1351–1359.

Taylor, S.M. and P.A. Jones. 1979. Multiple new phenotypes induced in 10T 1/2 and 3T3 cells treated with 5-azacytidine. Cell 17: 771–779.

Wakelam, M.I.O. 1985. The fusion of myoblasts. Biochem. J. 228: 1–12.

Weintraub, H., V.J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S.J. Tapscott. 1991. Muscle-specific transcriptional activation by MyoD. Genes & Dev. 5: 1377–1386.

Weller, P.A., M. Price, H. isenberg, Y.H. Edwards, and A.J. Jefreys. 1986. Myoglobin expression: Early induction and subsequent modulation of myoglobin and myoglobin mRNA during myogenesis. Mol. Cell. Biol. 6: 4539–4547.

Wiedmann, B., H. Sakai, T.A. Davis, and M. Wiedmann. 1994. A protein complex required for signal-sequence-specific sorting and translocation. Nature 370: 434–440.

Williams, T. and R. Tjian. 1991. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes & Dev. 5: 670–682.

Yagami-Hiromasa, T., T. Sato, T. Kurisaki, K. Kamiyo, Y.-I. Nabeshima, and A. Fujisawa-Sehara. 1995. A metalloprotease-disintegrin participating in myoblast fusion. Nature 377: 652–656.

Yu, Y.-T., R.E. Breitbart, L.B. Smoot, Y. Lee, V. Mahdavi, and B. Yoldas. 1986. Myoglobin expression: Early induction and subsequent modulation of myoglobin and myoglobin mRNA during myogenesis. Mol. Cell. Biol. 6: 4539–4547.

Zheng, X.M., D. Black, P. Chambon, and J.M. Egly. 1990. Sequencing and expression of complementary DNA for the general transcription factor BTF3. Nature 344: 556–559.
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