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

Wagner V. Yotov¹ and René St-Arnaud²

Genetics Unit, Shriners Hospital, and Departments of Surgery and Human Genetics, McGill University, Montréal, Québec, Canada H3G 1A6

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 α NAC. 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 α NAC prevented C2C12 differentiation and 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 in the regulation of myoblast fusion. Our data demonstrate that differential splicing converts α 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.

[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 fac-

tors 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 activation 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

¹Present address: Centre de Cancérologie Charles Bruneau, Hôpital Ste-Justine, Montréal, Québec, Canada H3T 1C5.

²Corresponding author.

inhibited in myoblasts overexpressing the *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 α NAC cDNA (W.V. Yotov, A. Moreau, F.H. Glorieux, and R. St-Arnaud, in prep.). NAC (nascent polypeptide-associated complex) was recently purified as a heterodimeric complex binding the newly synthesized polypeptide chains as they emerge from the ribosome (Wiedmann et al. 1994). The β -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 α NAC as the transcription factor BTF3b is somewhat puzzling and suggests a putative role for α 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 α NAC in adult tissue. We have detected α 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 α 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 α NAC may function as a transcriptional activator. We have identi-

fied 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 data bases 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 α 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 α 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

NAC expression in adult tissues and muscle

Figure 1, A and B, shows the expression pattern of the α NAC mRNA in various adult tissues. The 900-bp α NAC transcript was detected in all tissues postnatally. Interestingly, a larger-sized transcript (7.0 kb) was identified in skeletal muscle and, at reduced levels, in 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 α NAC protein (W.V. Yotov, A. Moreau, F.H. Glorieux, and R. St-Arnaud, in prep.) recognized the 35 kD α NAC 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 7.0-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 α NAC 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 α NAC; similarly, the last 192 amino acids correspond to the remainder of the α NAC protein sequence, suggesting that the skNAC isoform arose from splicing-in of additional sequence between residues 23 and 24 of the α NAC 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 that is spliced-out in most tissues but inserted in differentiated muscle cells (data not shown).

The protein sequence features an EF-hand motif, a selective and high affinity Co^{2+} -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 αNAC 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 α NAC or skNAC could bind DNA with sequence specificity. Figure 3A shows that two rounds of SAAB with the recombinant α NAC protein were sufficient to detect specific DNA binding, and a third round of amplification further enhanced the binding affinity of α NAC for the selected sites. Extracts from bacteria transformed with the empty expression vector

MPGEATVETVP	ATOEQLPQPC	AETAVLPMSS	ALKVAAGVQP	GTPTPSSSLGP	QQSPIVTAHQ	60
PSPLPSSVSS	TPFEVPFAQP	ITAEATLSPG	TAPPPTPTFLP	HLIIGPISPA	ALALASPMIG	120
LAQKGARSSS	ASLGLSVALAP	HSATQSSVCP	PHPLTSPPSA	AGALGELTA	SIPPLEPKTS	180
TSQVSPQGLT	NLKGATPCFP	DVVRAPBSHL	ENPLASVGP	LMSCPQLTSLN	TSPPKGVVIS	240
SALTQSRSLG	NLKGQVSPPA	RNTAAPSIDL	APSTSLGCHL	SLDKHSSVDS	PIQPPGQSGL	300
AVNSTPTVGH	SGIAASCPSNE	RCVPLVPLFSR	LVAADGSAAP	EDDGSSASV	NELCSPPGSS	360
NVAGTSLSPK	ASLIVAKPFSV	ALQPLVTQVF	ASQKTGLKEI	PVSCIGATHH	ALDNPSAISF	420
APATHVPPPT	SSGLVSSKDP	ASFVTLVLVP	AAHKQFPAPF	ASATLGVVPS	PLPATBGLKN	480
LPISALVNVG	APVSPAQAPG	PTKRDTTLGP	LAPIALRESP	SSQASASLEV	LSEDTVTWKI	540
TGQAPFVVRP	AIAGVAITTS	LRAADSPFVNI	RADSCSSPNT	VSPQLKRSVT	DPAMAPRTAK	600
NTAPSTPSL	VLPASEGCVF	ASSMSPSLP	AVSEVTSALAL	SPETPKSVF	PDPLAEISF	660
SNARKDAVS	HMESSGSSRQ	GHPDASVTAK	GTVVCLADSS	LDTSVSASKG	SALSGASSPL	720
YPLEVSPLEP	AGLAVQCPKG	SLNKLSPSTP	SKSGAPVPT	GAPSPFKGAP	IVPTESSISS	780
KQVPAEILPS	PKPTPEVTAS	RELIAASVPE	VDPIMSDVL	TSPEKTSATA	VPKDTSATLS	840
LQSPVATPSL	SPKAPVAPS	NEATIVTEPI	PLTSLNALAA	ATPCKTLATS	IPKTSVSPSQ	900
KTPKSVSLKG	APAMTSKKAT	EIAASKDVSF	SQPFKEVPLL	QHVPPTSPPK	SPVSDTLGSA	960
LTSPSSKGGP	ATLAETPTVP	KKSPKPAASK	KTPATPSPLG	VTAVPLEIIP	CSSKAKPTAA	1020
PKSSSATPSS	KRAKTPATVK	EIPSKGVAAL	PLEISLPLKE	TKSAPTPGKE	SASSPKRSFK	1080
TAGKPTPTPG	VATVPPEIS	LPKPEPTQNA	PTNESLAASS	QKSPKTSVP	KATTPGGVTA	1140
MPLETPASQ	KAPKTAVPKQ	IPTDEDATVI	LAGSLPSPKK	ASKTAAPKEA	PATPSGVVIA	1200
VSGEISPSPK	KTSKTAAPK	ENSATLPFKRS	PKTAARKPET	ATSSSEGVTA	PSEISSPSPT	1260
PASKGVPVTL	TKGAPNALA	EPASPSKVPK	KTAAPETST	KTPSQKIPKV	AGPKREASPT	1320
PSKKTPKTAV	PKETSAPSEB	VTAVPLEIIP	SPRKAPKTA	PKETPAESPE	GATTAVPQIP	1380
PSPRKGSKKA	GKPTPTTPPS	PEGVTAAPLE	IKISSKKTSS	MASKPETLVT	PSKKLSLGT	1440
APKSTLEGA	TAETLEIPPS	HKKAPKTVDP	KQVLPTPSPK	DAPTTLAESP	SPFKKAPKTA	1500
GPPSERVTV	PPEKPAFTK	AGSTTKVAS	VPAETQEVAV	SRETPVTPTA	VPVKNPNSSH	1560
KKTSKTIELK	EAPATLPPSP	TKSPKIPSSK	KAPRTSAPKE	FPASPSIKVP	TTSLAQTAFF	1620
AAQAPSTTI	PKENLAAPAV	LEVSSKSPAA	PARASASSTP	ATAAPQTAPK	EATTIPSCBK	1680
SLKPTPTIET	TPASHLSEAP	KETSETSVSK	VLMSSSPPKKA	SSSKRASTLT	ATPLPSLKEA	1740
SVLSPATSS	GKDHSHSPVS	DACSTGTTTP	QASEKLSPSK	GPTAFTMLA	APPAPESALAI	1800
TAPIQKSPGA	NNSNASSPKC	PDPSKKDKTK	GLPSAVALAP	QTVPVKEOTS	KAIBETLVLP	1860
AKGSDCLHSP	KGVLVPSQVAT	PLAAFTSDKV	PPFAVSASVA	PKPAPAASLT	LAFPSFVAPLT	1920
RPQPLLESAP	GSVLSPSKAL	PFAEDELPL	PLIIPPEAVSG	GEQFPQILVN	MPAPKPAAGT	1980
APAPSAKQPV	LKNNKSGQTE	SDSDESVPPE	ERODSTOTAT	QOAPLAAAE	IDEEPVSXAK	2040
QSSSEKKAKK	AMSKILGRQV	TGVTVTITRK	SKNLIJEVTK	PDVVKSPASD	TVIVGEGAKI	2100
LDPLQOAOAL	AAEKFKVQGE	AVSNIQENTQ	TPTVOBESSE	EEVDETGVEV	KDILVMSQA	2160
NVSRKAKVRA	LKNNSDNVN	AMIELMT				2187

Figure 2. Deduced amino acid sequence of the muscle-specific skNAC protein. Shared residues with α NAC 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.

failed to bind the probes (Fig. 3A, lane 1). Sequencing of 33 independent clones bound by the recombinant protein established the following consensus binding site:

Mutagenesis of this consensus sequence revealed that changing the A residues at positions 3 and 5 (in bold characters) inhibited binding (data not shown).

To determine whether skNAC could bind the site identified using α NAC in the SAAB assay, we in vitro-translated the skNAC cDNA and used the programmed lysate to test specific DNA-binding activity in the gel mobility shift assay. Figure 3B shows that the in vitro-translated skNAC protein bound a labeled oligonucleotide containing two copies of the consensus NAC-binding site (lane 2). However, skNAC could not bind to a probe in which the critical A residues of the consensus binding site were mutated to T residues (lane 5). The control unprogrammed reticulocyte lysate did not bind to either of the probes (lanes 3,6). These data indicate that both forms of the α NAC protein specifically bind to the same consensus DNA sequence.

Figure 3. α NAC and skNAC bind DNA with sequence specificity. (A) Gel retardation assay of selected binding sites using SAAB methodology. The arrow points to the specific α -NAC-DNA complexes. The first round yielded weak signal and is not shown here. (Lane 1) Probe from the third round of SAAB was assayed with an extract from bacteria transformed with the empty GST vector pGEX-4T-3 (Pharmacia). (B) Gel retardation assay showing binding of in-vitro translated skNAC to the NAC-binding site. The protein did not bind to the mutated NAC site (see Materials and methods for sequence of the wild-type and mutant probe). The unprogrammed reticulocyte lysate was used as negative control (lanes 3,6).

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 (Fig. 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 (Fig. 3B) were subcloned upstream of the heterologous promoter (Fig. 4A, plasmid 2X mu tk luc). Similarly, skNAC did not stimulate the expression of the reporter

gene from the parental tk luc vector (Fig. 4A). The α NAC protein had no effect in this assay (data not shown), suggesting that despite its capacity to bind the NAC-binding site (Fig. 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 (Fig. 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 (Fig. 4C, luc reporter). When mutations were engineered at the critical A residues of the skNAC-binding sites using site-specific mutagenesis (Fig. 4B), the skNAC-dependent stimulation of the expression from the myoglobin promoter was abolished (Fig. 4C, mut. myoglobin-luc reporter), demonstrating that skNAC activated transcription via binding to its cognate sites in the promoter. The lower molecular weight α NAC isomer did not stimulate the expression from either the wild-type or the mutant myoglobin reporter constructs (Fig. 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 α NAC 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 αNAC 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 αNAC 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.

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

The results presented above, as well as the observation that αNAC can bind the same consensus DNA sequence as skNAC in gel retardation assays (Fig. 3A), suggested that αNAC 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-

Figure 5. Overexpression of α NAC or skNAC in myoblasts perturbs differentiation. (A) Immunoblotting of stable clones of C2C12 myoblasts transfected with the PGK-neo selection vector alone (vector) or in combination with CMV-driven expression vectors for α NAC or skNAC and probed with the anti- α NAC antibody. Note the increased levels of protein in the transfected clones compared with the control, vector-transfected clone. (B–D) Immunostaining for myosin heavy chain using the MF-20 monoclonal antibody. Confluent cultures of control-expressing (B), α NAC (C), or skNAC-expressing (D) clones were stained. Representative clones are shown; identical results were obtained with each of the four clones derived for each vector.

ing amounts of α NAC had no effect on the capacity of skNAC to stimulate transcription from the myoglobin promoter. As observed previously, α NAC did not increase the expression of the reporter gene from the parental luc vector or the myoglobin-luc plasmid (Fig. 6). Levels of expression from each NAC vector were tested by immunoblotting assays (data not shown).

These results show that α NAC does not inhibit the *trans*-activating activity of skNAC *in vivo*. The mechanism by which α NAC affects myotube differentiation remains to be elucidated.

Discussion

We have identified and cloned a differentially spliced isoform of α NAC specifically expressed in differentiated

Table 1. Differentiation of α NAC- and skNAC-expressing C2C12 clones

Clones	Growth medium ^a		Differentiation medium ^b	
	myotubes	myosacs ^c	myotubes	myosacs ^c
neo	2.3 $\pm$ 0.5	0	31.1 $\pm$ 2.8	0
α NAC	0.9 $\pm$ 0.3	0	2.5 $\pm$ 0.6	0
skNAC	0	6.7 $\pm$ 0.3	0	4.0 $\pm$ 0.4

Results are expressed as mean $\pm$ 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).

^a12% fetal bovine serum.

^b2% horse serum.

^cArbitrarily 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 α NAC 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

Figure 6. α NAC does not influence the skNAC transcriptional activating function. Transient transfection assays are shown, in which the CMV-driven skNAC expression vector was cotransfected with increasing quantities of CMV-driven α NAC. The myoglobin-luc plasmid was used as reporter; the empty parental luc reporter served as control. The expression level detected in cells transfected with the luc reporter alone was arbitrarily ascribed a value of 1. Results are expressed as mean fold induction $\pm$ S.E.M. of three independent transfections.

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. α NAC 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 α NAC 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 α NAC 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 1972 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 α NAC recognized the same DNA sequence in gel mobility shift assays (Fig. 3). However, all attempts to demonstrate that α NAC could directly *trans*-activate through its cognate response element have been unsuccessful (Fig. 4C; data not shown). We do not yet know whether α NAC 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 α NAC does not competitively inhibit the transcriptional activating function of skNAC *in vivo* (Fig. 6) suggests that the ability of α NAC 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 trans-

fection 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 α NAC 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 (Bassel-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 (Bassel-Duby et al. 1994). Interestingly, MNF is also a proline-rich transcriptional activator (Bassel-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 α NAC in skeletal myoblasts generated the opposite phenotype by inhibiting fusion. Although we detected low levels of α NAC protein in myoblasts and fused myotubes using immunoblotting assays (Fig. 1C), we estimate the ratio of skNAC to α NAC mRNA transcripts at 20:1 based on the relative proportion of cDNA clones for α NAC that we detected in the skeletal muscle library during the cloning of skNAC (not shown). Thus, overexpression of α NAC 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 α NAC 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 α NAC.

As mentioned previously, BTF3b has been identified as the heterodimerization partner of α NAC (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 α NAC 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 con-

tribution 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 α NAC cDNA using conventional protocols.

Immunoblotting

The murine α NAC cDNA was subcloned into the pGEX-4T-3 vector (Pharmacia Canada, Baie d'Urfé, Québec, Canada), and the glutathione *S*-transferase (GST)- α NAC fusion protein was purified according to the manufacturer's instructions. Polyclonal antibodies were raised by immunization of rabbits with GST- α NAC. 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; Candelieri et al. 1996).

Cloning of skNAC

The PCR-labeled full-length α NAC 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'-TCGTGGCCCT-3') were used to generate the double-stranded labeled probes used in the SAAB procedure (Blackwell and Weintraub 1990). One microgram of GST- α NAC 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 α NAC-binding site (underlined), 5'-CTAGCCGACACACAGGAGGG-CCACGACACACACG-3', into the expression vector tk luc (Glass et al. 1988). An oligonucleotide in which the α NAC binding sites were mutated, 5'-CTAGCCGACTCTCAGGAGGGC-

CACGACTCTCACG-3' (mutated residues in boldface type), was used to generate the related vector 2X μ 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 μ l of the LipofectAMINE reagent (GIBCO BRL, Gaithersburg, MD) and a total of 5 μ g of DNA according to the instructions of the manufacturer; cells were harvested 24 hr post-transfection. All assays included a fixed amount (0.1 μ g) 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 α NAC 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

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

W V Yotov and R St-Arnaud

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