FEBS 27410

FEBS Letters 547 (2003) 15–19

Functional interaction of the subunit 3 of RNA polymerase II (RPB3) with transcription factor-4 (ATF4)

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Received 6 March 2003; revised 26 May 2003; accepted 27 May 2003

First published online 17 June 2003

Edited by Gianni Cesareni

Abstract **RPB3 is a core subunit of RNA polymerase II (pol II) that, together with the RPB11 subunit, forms the heterodimer considered as a functional counterpart of the bacterial α subunit homodimer involved in promoter recognition. We previously employed the yeast two-hybrid system and identified an interaction between RPB3 and the myogenic transcription factor myogenin, demonstrating an involvement of this subunit in muscle differentiation. In this paper we report the interaction between RPB3 and another known transcription factor, ATF4. We found that the intensity of the interaction between RPB3 and ATF4 is similar to the one between RPB3 and myogenin. This interaction involves an RPB3 specific region not homologous to the prokaryotic α subunit. We demonstrated that RBP3 is able to enhance ATF4 transactivation, whereas the region of RPB3 (Sud) that contacts ATF4, when used as a dominant negative, markedly inhibits ATF4 transactivation activity. Interestingly, ATF4 protein level, as reported for its partner RPB3, increases during C2C7 cell line muscle differentiation.**

Key words: RNA polymerase II; RPB3; ATF4; Muscle differentiation

1. Introduction

The eukaryotic heteromeric enzyme RNA polymerase II consists of 12 different subunits [1,2]. Despite the fundamental role of pol II in initiation, elongation, and termination of mRNA transcription, little is known about the specific functions of its individual subunits, associations between subunits, or possible contacts between subunits and holoenzyme components and transcription factors. Indeed, in addition to the role of the two largest subunits, RPB1 and RPB2, which are homologous to β^1 and β subunits of the bacterial enzyme and contain the main catalytic center [3], the contributions of the 10 additional smaller subunits to the enzymatic functions are still not well characterized [4–7].

We have previously cloned two subunits of human pol II core, RPB11 and RPB3, and have shown the involvement of these proteins in doxorubicin (dox)-mediated cellular toxicity and cellular differentiation [8–11]. RPB11 and RPB3 contain two amino acid sequences (α motif) with limited homology to the α subunit of *Escherichia coli* RNA polymerase, and they form a heterodimer considered as the functional counterpart of the bacterial α subunit homodimer involved in promoter recognition [1,12–15]. In *Saccharomyces cerevisiae* these two subunits form with another pol II subunit, RPB2, an intermediate core subassembly analogous to the $\alpha_2\beta$ complex in the assembly of *E. coli* RNA polymerase [16]. Interestingly, mediator complex/pol II interactions are centered on the RPB11/RPB3 heterodimer [17], and both subunits resulted highly expressed in adult human skeletal muscle and heart, suggesting additional role(s) for the heterodimer in these tissues [8,9]. Moreover, multiple alignments of RNA polymerase α homolog subunits from several organisms have revealed two additional conserved regions present only in eukaryotic RNA polymerase and thus considered to be involved in functions specific for higher organisms [18].

Recently we reported the identification of the myogenic factor myogenin as a novel partner for RPB3 [11]. We showed that ectopic expression of RPB3 increased myogenin transactivation activity, whereas an RPB3 dominant negative mutant strongly inhibited myogenin activity and muscle differentiation. Notably, we found that RPB3 expression is regulated during murine C2C7 myoblast cell line differentiation [11].

In a search for additional proteins interacting with RPB3 in muscle cells, we screened a cDNA library derived from human skeletal muscle. Three positive interacting clones were identified to be ATF4, a member of the large ATF/CREB transcription factor family not previously shown to directly interact with pol II. ATF/CREB proteins include CREB (cAMP (cyclic adenosine monophosphate) responsive element binding protein), CREM (CRE modulator), ATF1, ATF2, ATF3 and ATF4 [19,20]. These proteins bind to DNA via their basic region and dimerize via their leucine zipper domain to form a large variety of homo- and/or heterodimers coordinating signals from different cell pathways [19,20]. In particular, ATF4 was proposed to be a positive regulator of transcription [21–23], increasing the expression of genes, such as somatostatin, serotonin, or interleukin-2 [24–26]. We showed that RPB3 directly binds to ATF4 both in vitro and in vivo and enhances ATF4 transactivating activity. Moreover, we found that ATF4 expression as well as RPB3 is modulated during murine C2C7 myoblast differentiation. Thus, RPB3/ATF4 interaction may play an important role in gene regulation by ATF4 and in muscle differentiation.

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00659-8

2. Materials and methods

2.1. Yeast two-hybrid selection

For two-hybrid screening, the complete open reading frame (ORF) of human RPB3 was cloned into the *Bam*HI restriction site of the vector pGBKT7 (Clontech, Palo Alto, CA, USA) in frame with the *GAL4* binding domain (BD), and used to screen a human skeletal muscle cDNA library (Clontech) as previously described [11]. The recovered library-derived plasmids were analyzed as positive candidates. Liquid Y187 yeast cultures were assayed for β-galactosidase activity to quantify two-hybrid interactions according to the method of Schneider et al. [27].

2.2. Plasmids

cDNA coding for the full-length ATF4, isolated by two-hybrid screening, was cloned into a pCS2-MT myc-tagged mammalian expression vector, and into pGEX4T3 (Amersham) to generate a glutathione-S-transferase (GST)-ATF4 fusion protein. Full-length human RPB3 and constructs carrying different RPB3 deletions, Gal4-Luc reporter expression vector and pM3-VP16 expression vector containing the GAL4 DNA BD fused to the VP16 AD were already described [11]. To investigate the ATF4 domains involved in the RPB3 interaction, four GST fusion proteins were constructed in pGEX4-T1 vector. These four constructs carried different ATF4 deletions: GST 88–351 (from amino acid 88 to amino acid 351), GST 172–351 (from amino acid 172 to amino acid 351), GST 264–351 (from amino acid 264 to amino acid 351), and GST 1–265 (from amino acid 1 to amino acid 265). The reporter containing three binding sites for ATF4 was kindly provided by Dr. Tsonwin Hai (Ohio State University). All constructs produced were sequenced by Sequenase reaction (Amersham) according to the manufacturer's instructions.

2.3. Pull-down analysis

BL21 bacterial strains were transformed with GST fusion protein constructs and the proteins purified on glutathione-Sepharose resin (Pharmacia). For in vitro binding assays, comparable amounts of resin-bound GST fusion proteins were incubated with 500 μg of cell lysates in NETN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1% NP-40) for 1 h at 4°C. The resins were then pelleted and extensively washed in the same buffer. The bound proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the gels were analyzed by Western blot using anti-RPB3 polyclonal antibody [11]. Immunoreactivity was detected by enhanced chemiluminescence (ECL) reaction (Amersham). In vitro transcription and translation were carried out with tetranitroblue tetrazolium (TNT) coupled reticulocyte lysate systems (Promega) and L-[^{35}S]methionine (>1000 Ci/mmol; Amersham) as previously described [11].

2.4. Cell culture and transfections

HeLa human cells and NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). C2C7 mouse myoblasts were grown in DMEM supplemented with 20% fetal bovine serum (GM) until they reached confluence. Differentiation was induced by switching to differentiation medium (DM), consisting of DMEM containing 2% horse serum. Transient transfections were performed using lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. Transfection efficiency ranged between 40 and 60% of cells. The transfection medium was replaced by DMEM supplemented with 10% FCS 18 h after transfection. After incubation for 48 h, luciferase was assayed using reagents from Promega according to the manufacturer's instructions. β-Galactosidase was assayed using a β-galactosidase assay kit (Tropix).

2.5. Co-immunoprecipitations and immunoblotting

Cells were rinsed three times with ice-cold phosphate buffered saline (PBS), harvested, centrifuged at 4°C and cell pellets lysed by incubation at 4°C for 30 min in 300 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 100 mM NaF, 3 mM Na_3VO_4, 10 nM okadaic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin) supplemented with 1%

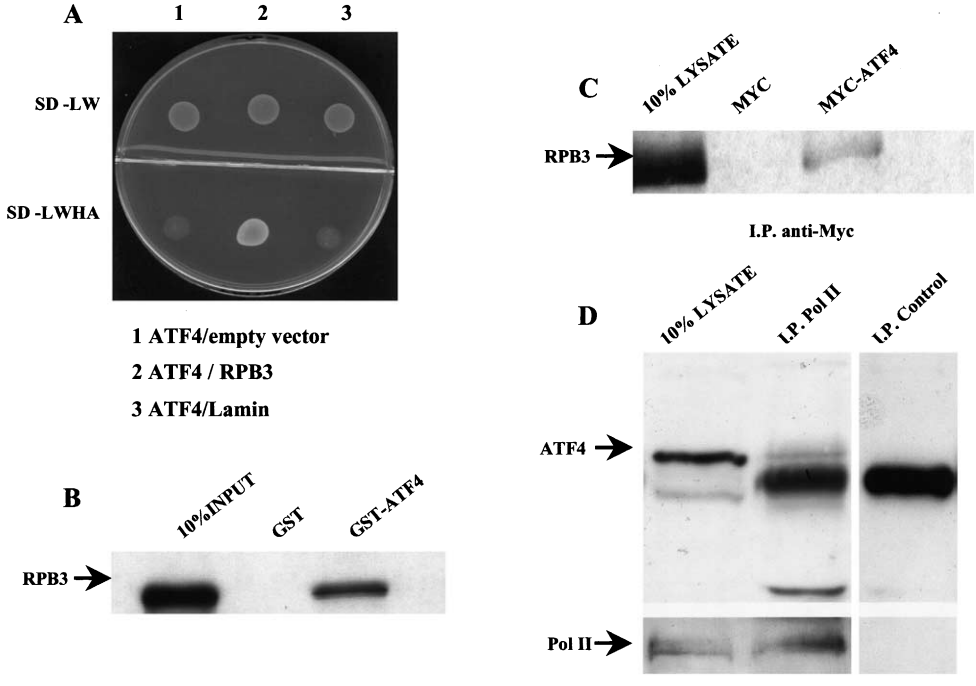

Fig. 1. RPB3 interacts with ATF4. A: AH109 yeast cells were cotransformed with the indicated constructs and plated onto SD media lacking leucine and tryptophan (−LW) to verify the expression of both bait (W^+) and prey (L^+) plasmids; or onto media lacking leucine, tryptophan, histidine and adenine (LWHA) for examining the interaction between bait and prey proteins. B: Labeled RPB3 was subjected to GST pull-down analysis using GST or GST-ATF4 beads. C: Whole cell extracts of HeLa cells transfected with myc-ATF4 or empty myc-tag expression vectors were immunoprecipitated with anti-myc monoclonal antibody and analyzed by Western blot using anti-RPB3 polyclonal antibody. D: Whole NIH 3T3 cell extracts were immunoprecipitated with anti-pol II polyclonal antibody or with an irrelevant antibody as control, and analyzed by Western blot using anti-ATF4 polyclonal antibody.

NP-40. Supernatants were cleared by centrifugation, pre-cleared using 20 μl protein A/protein G beads (Santa Cruz, CA, USA), and immunoprecipitated by standard procedures using anti-RNA pol II (C-21, Santa Cruz, CA, USA) polyclonal antibody or anti-myc (9e10, Invitrogen) monoclonal antibody [11]. Western blots were prepared by standard procedures and polyclonal antibodies directed against RPB3 [11] and ATF4 (Santa Cruz, CA, USA) were used to detect this protein by chemiluminescence reaction (Amersham). C2C7 mouse myoblasts were rinsed three times with ice-cold PBS, harvested, centrifuged at 4°C and cell pellets lysed by incubation at 4°C for 1 h in lysis buffer. Supernatants were cleared by centrifugation, normalized for total protein content, subjected to SDS–PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Blots were incubated with polyclonal antibodies specific for RPB3 and ATF4, and a monoclonal antibody specific for α-tubulin (Sigma).

3. Results and discussion

3.1. RPB3 binds ATF4

Using the yeast two-hybrid system, a cDNA expression library derived from human skeletal muscle was screened using RPB3 as bait. Of an estimated 2.5×10^6 transformants screened, 30 clones were able to proliferate on media lacking histidine and adenine and stained positive for β-galactosidase. These clones were isolated and further characterized. As expected, several independent clones contained the RPB3 partner, thus confirming the specificity of the performed screening. Three independent positive clones encoded for the whole ORF of the transcription factor ATF4. The remaining clones encoded for other transcriptional factors such as the basic helix-loop-helix myogenic transcription factor myogenin [11] or were unknown. The specificity of RPB3/ATF4 interaction was confirmed in a two-hybrid assay cotransforming ATF4 with either RPB3, or with pGBKT7 empty vector or lamin control vector (pLAM). As shown in Fig. 1A, RPB3 interacted with ATF4 whereas lamin and pGBKT7 did not show any significant interaction, indicating that the observed binding of RPB3 with ATF4 was specific.

To demonstrate the direct interaction between RPB3 and ATF4 we performed binding studies between recombinant RPB3 and ATF4 proteins in vitro. For this purpose, the ATF4 cDNA insert was cloned in frame into bacterial expression GST plasmid to obtain GST-ATF4 fusion protein. Then, GST-ATF4 was tested for the ability to bind in vitro translated ^{35}S-labeled RPB3. Data shown in Fig. 1B confirmed a specific, direct physical interaction between ATF4 and RPB3.

To provide evidence that RPB3 also associates with ATF4 in vivo, ATF4 cDNA insert was cloned into myc-tagged mammalian expression vector and transfected into HeLa cells. As shown in Fig. 1C, negative control vector Myc-tag showed no binding to RPB3, whereas immunoprecipitation of tagged ATF4, followed by Western blot analysis of the precipitates for the presence of RPB3, indicated a clear interaction between these two proteins. Next, to evaluate whether ATF4 could be recruited on pol II through its association with RPB3, we performed a co-immunoprecipitation from NIH 3T3 cell lysates with a polyclonal antibody against the largest subunit of pol II (RPB1), clearly demonstrating the presence of ATF4 in pol II complex (Fig. 1D).

The strength of ATF4/RPB3 interaction was further investigated by yeast LacZ assay comparing its intensity with the strong structural interaction between RPB11 and RPB3 heterodimeric α-like subunits and with the weaker binding of RPB3 to another transcription factor, myogenin [11]. As expected, the intensity of the interaction was lower than that of the two pol II α heterodimeric subunits, but it was comparable to RPB3/myogenin interaction (Fig. 2A). Similar results were obtained when HeLa cells were transfected with myc-RPB3 and cell lysates were incubated with GST-RPB11, GST-myogenin, GST-ATF4 or GST beads (Fig. 2B). Notably, RPB3 strongly bound to RPB11, but both interactions with the transcription factors resulted much weaker, supporting in such way the hypothesis of a possible role for these interactions in transcriptional regulation.

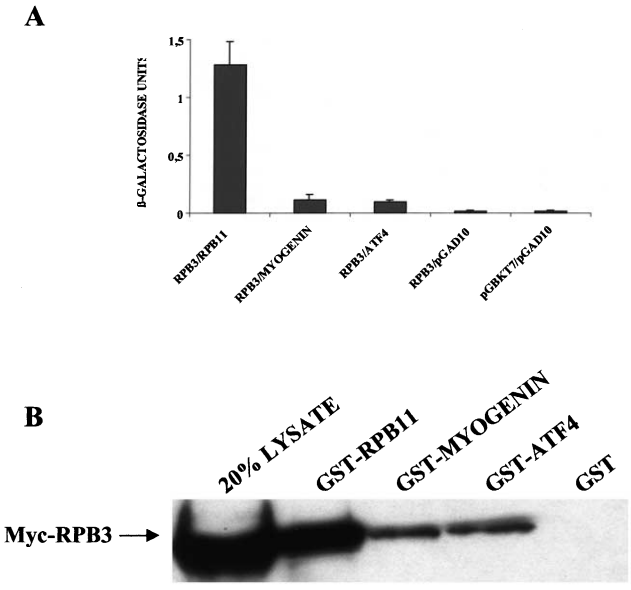

Fig. 2. Interaction analysis of RPB3 with ATF4. A: Y187 yeast cells were cotransformed with the indicated constructs and assayed for β-galactosidase activity. B: Whole cell extracts of HeLa cells transfected with myc-RPB3 were incubated in NETN buffer in the presence of GST, GST-RPB11, GST-myogenin and GST-ATF4 beads as indicated. Recovered interacting proteins were then blotted and incubated with anti-myc monoclonal antibody.

3.2. Domains of ATF4 and RPB3 involved in the interaction

To define the region(s) of RPB3 interacting with ATF4 we took advantage of RPB3 mutants that were previously described [11]. Using these mutants in a pull-down analysis with ^{35}S-labeled ATF4, we found that two regions of RPB3, Sud (amino acids 74–138) and Ovest (amino acids 139–197) (Fig. 3B), were able to contact ATF4 (Fig. 3A). Notably, Sud contains a Cysteine-rich region that resembles a potential zinc-finger motif [28] and it is necessary for the binding of RPB3 to myogenin [11], whereas the Ovest region contains a potential EF-hand calcium BD [9,29]. These regions are conserved only in eukaryotic pol II and are not present in the corresponding subunits of pol I and pol III, thus they can be considered to be involved in function(s) specific for pol II [1]. Furthermore, it has been demonstrated in yeast RPB3 that these regions are components of a single determinant specifically required for activator-dependent transcription, but not for activator-independent basal transcription [18]. Then, in order to define which domain of ATF4 is required for the interaction with RPB3, three progressive amino-terminal truncated ATF4 derivatives, 88–351, 172–351, and 264–351 (Fig. 3C) were fused to GST and incubated with in vitro translated ^{35}S-labeled RPB3. All three constructs

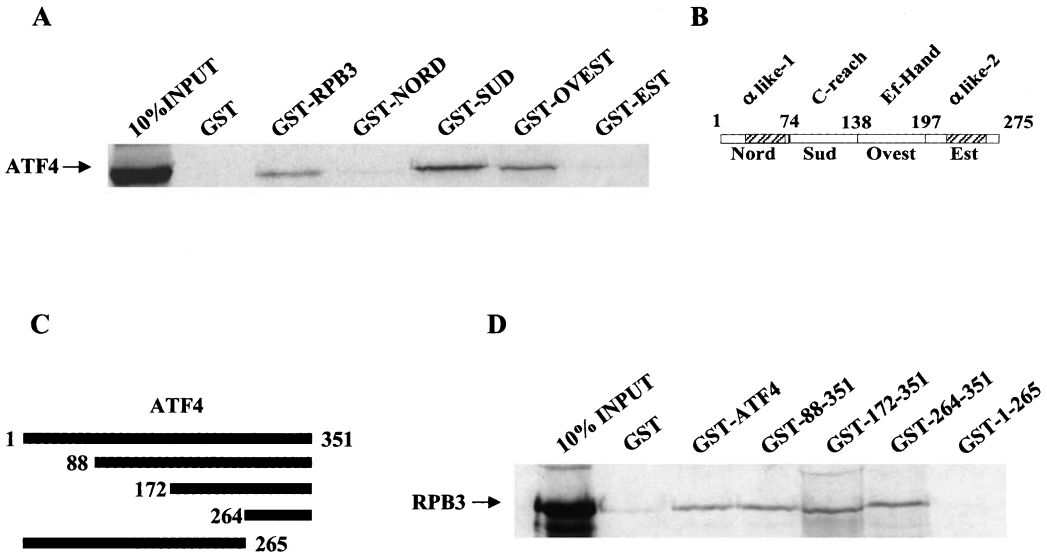

Fig. 3. Domain analysis of RPB3 and ATF4. A: ^{35}S-labeled ATF4 was utilized in pull-down analysis with several deletions of RPB3 fused to GST. B: Schematic representation of the RPB3 deletions employed in the experiments reported in A. C: Schematic representation of ATF4 showing the nested deletion constructs. The horizontal bars represent the amino acid sequence in each construct. D: ^{35}S-labeled RPB3 was utilized in pull-down analysis with the deletions of ATF4 fused to GST depicted in C.

retained RPB3 binding capacity (Fig. 3D). In contrast, a construct lacking the last 86 amino acids (Fig. 3C) was unable to interact with RPB3 (Fig. 3D), indicating that this region is required in ATF4/RPB3 interaction. Interestingly, the highly conserved basic leucine zipper domain (bzip) responsible for transcription activation [21] is present in the carboxy-terminus of ATF4.

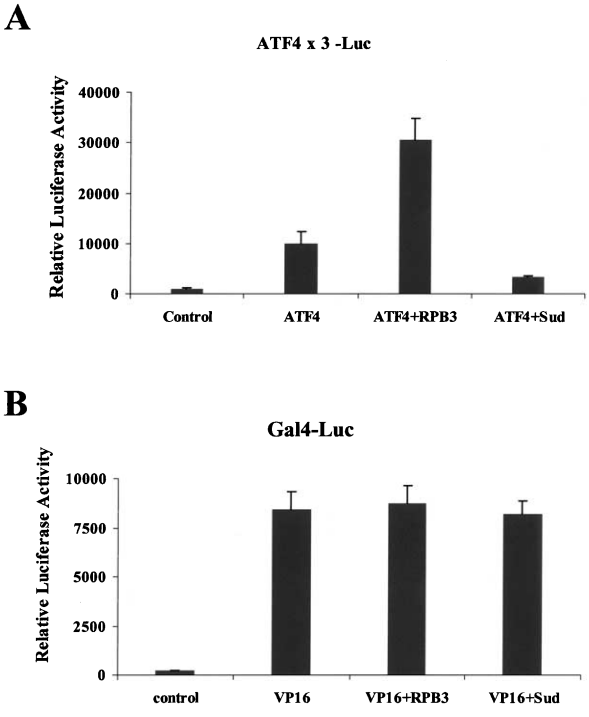

Fig. 4. RPB3 is involved in ATF4-mediated transcription. HeLa cells were transiently transfected with 0.2 μg of the 3xATF4-Luc reporter (A), Gal4-Luc reporter (B), and 1 μg of the indicated expression vectors. Data are presented as the means ± S.D. from three independent experiments performed in duplicate.

3.3. RPB3 enhances ATF4-mediated transactivation

The result that RPB3 contacts the bzip region of ATF4 led us to hypothesize that the interaction between RPB3 and ATF4 can be directly involved in transactivating activity of ATF4. To test this hypothesis, ATF4 and RPB3 mammalian expression plasmids were cotransfected with a luciferase reporter driven by tandem ATF4 sites into HeLa cells. As shown in Fig. 4A, overexpression of ATF4 activated the reporter more than 10-fold whereas cotransfection of RPB3 resulted in a three-fold enhancement of ATF4 activation; no effects were observed when RPB3 was coexpressed with another activator like VP16 together with a Gal4 luciferase reporter (Fig. 4B). Consistent with these results, cotransfection experiments performed using a dominant negative deletion mutant of RPB3, Sud [7], demonstrated that expression of Sud markedly inhibited reporter activation (Fig. 4A), thus confirming an involvement of RPB3 in ATF4-mediated transactivation.

3.4. ATF4 expression is modulated during murine C2C7 myoblast differentiation

We have previously shown that RPB3 is highly expressed in human skeletal muscle [9], and that RPB3/myogenin interaction is important for muscle conversion of murine C3H10T1/2 cells [11]. These observations prompted us to analyze ATF4 protein level during the onset of differentiation of murine C2C7 myoblasts. Fig. 5 shows that ATF4 had the same expression pattern as RPB3 [11]. Indeed, ATF4 was detectable at all stages of differentiation, but with significant different level of expression: the level was low in cycling C2C7 cells, but increased about three-fold in high density orientated cells (concomitantly with the beginning of differentiation program). These data further suggest that the interaction ATF4/RPB3 could play a crucial role in muscle differentiation.

In summary, the data presented in this paper describe a novel interaction between a pol II subunit, RPB3 and the transcription factor ATF4, supporting in such way the hypothesis that RPB3, in addition to the well-described struc-

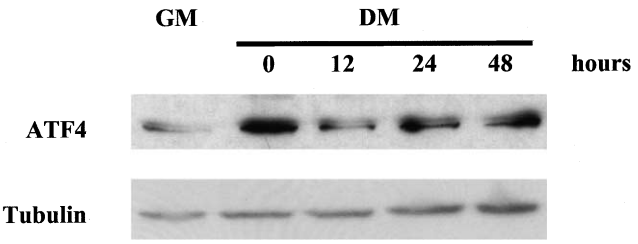

Fig. 5. Expression of ATF4 is modulated during muscle differentiation. Total lysates from C2C7 cells incubated in DM for the indicated times, or maintained in GM, were blotted and incubated with the indicated antibodies.

tural function, is also involved in the regulation of transcription. Indeed, RPB3 is able to directly bind transcription factors and the region involved in these interactions is conserved only in pol II and is not present in the corresponding subunits of pol I and pol III, thus suggesting that it could exert function(s) specific for pol II.

Acknowledgements: We are grateful to Dr. T. Hai for sharing many critical reagents. We thank Miss Annalisa Onori for her precious technical assistance. This work was supported by Ministero della Sanità, Telethon project A160, and MURST 60%. Drs. N.C. and M.D.P. are the recipients of F.I.R.C. fellowships.

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