

RNA Polymerase II subunit 3 is retained in the cytoplasm by its interaction with HCR, the psoriasis vulgaris candidate gene product

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Summary

Here, we show that the subcellular localization of α -like RNA polymerase II core subunit 3 (RPB3) is regulated during muscle differentiation. We have recently demonstrated that the expression of RPB3 is regulated during muscle differentiation and that, inside RNA polymerase II (RNAP II), it is directly involved in contacting regulatory proteins such as the myogenic transcription factor Myogenin and activating transcription factor ATF4. We show for the first time, that RPB3, in addition to its presence and role inside the RNAP II core enzyme, accumulates in the cytoplasm of cycling myogenic cells and migrates to the nucleus upon induction of the differentiation program. Furthermore, using human RPB3

as bait in a yeast two-hybrid system, we have isolated a novel RPB3 cytoplasmic interacting protein, HCR. HCR, previously identified as α -helix coiled-coil rod homologue, is one of the psoriasis vulgaris (PV) candidate genes. In cycling myogenic C2C7 cells, we show that the RPB3 protein directly interacts with HCR within the cytoplasm. Finally, knocking down *HCR* expression by RNA interference, we demonstrate that HCR acts as cytoplasmic docking site for RPB3.

Key words: RPB3, RNAP II, HCR, SBP, Psoriasis vulgaris, Muscle differentiation

Introduction

Regulation of RNA polymerase II (RNAP II) transcription is a highly dynamic process requiring the ordered binding of distinct protein complexes that catalyse initiation, elongation, termination and coupled mRNA processing events (Sims et al., 2004; Woychik and Hampsey, 2002; Gnatt, 2002; Cramer et al., 2000). The RNAP II core enzyme consists of at least 12 different subunits that associate with several mediator proteins and general transcription factors to form the holoenzyme complex (Bushnell et al., 2003; Gnatt et al., 2001; Davis et al., 2002; Myers et al., 2000; Schaller et al., 1999). Several auxiliary factors can control RNAP II activity, conferring specificity for selected loci and determining transcriptional responsiveness within different cell types and diverse developmental and environmental signals (Jeronimo et al., 2004; De Angelis et al., 2003; Howe, 2002; Corbi et al., 2002; Fanciulli et al., 2000; Fanciulli et al., 1996).

We have previously cloned two human RNAP II subunits, RPB11 and RPB3 (Fanciulli et al., 1996; Fanciulli et al., 1998), which contain two α -like-motif amino acid sequences, with limited homology to the α subunit of bacterial RNA polymerase (Woychik et al., 1993; Tan et al., 2000). RPB11 and RPB3 form a heterodimer that is considered to be the functional counterpart of the bacterial α -subunit homodimer

involved in promoter recognition. Significantly, the Rpb3-Rpb11 heterodimer plays a central role in the interaction between RNAP II and the mediator complex, suggesting functional conservation from prokaryotes to eukaryotes (Davis et al., 2002).

We found that RPB11 and RPB3 are expressed at high levels in adult human skeletal muscle and heart (Fanciulli et al., 1996; Fanciulli et al., 1998), and that RPB3 expression is regulated during murine C2C7 myoblast differentiation (Corbi et al., 2002). In addition, the involvement of RPB3 in tissue-specific transcription was demonstrated by its direct contact with transcription factors including ATF4 (a member of the ATF/CREB family) and Myogenin (a member of the MyoD gene family) (De Angelis et al., 2003; Corbi et al., 2002). ATF4 binds to DNA via its basic region and dimerizes via its leucine-zipper domain to form a wide range of homo- and/or heterodimers that coordinate signals from different pathways (Hai and Hartman, 2001). ATF4 expression is modulated during C2C7 myoblast differentiation and is enhanced by RPB3 overexpression. Thus, the RPB3-ATF4 interaction might play an important role in the regulation of gene transcription driven by ATF4, with particular reference to muscle differentiation (De Angelis et al., 2003). RPB3 contacts Myogenin in correspondence of the highly conserved helix-

loop-helix (HLH) domain but does not bind any other myogenic HLH family protein, such as MyoD, Myf5 and MRF4 (Molken and Olson, 1996; Jones, 2004). We showed that ectopic overexpression of RPB3 in C2C7 cells slightly increased Myogenin transactivation activity, whereas a dominant-negative RPB3 mutant that misses the RNAP II signatures but retains Myogenin-binding ability, strongly inhibited Myogenin activity and muscle differentiation. The interaction between RPB3 and Myogenin strongly reinforces the concept of a direct link between tissue-specific transcription and RNAP II (Corbi et al., 2002). These findings are consistent with RPB3's proposed role as the acceptor of activation signals from transcription factors.

Here, we show that the subcellular localization of RPB3 is finely tuned during muscle differentiation. In addition to its presence inside RNAP II as a fundamental component of the core enzyme, RPB3 accumulates within the cytoplasm of cycling myogenic cells and migrates to the nucleus upon induction of the differentiation programme. Using yeast two-hybrid screening, we have isolated the RPB3 cytoplasmic interacting protein previously identified as HCR. The *HCR* gene was identified within the 111 kb telomeric region of the *HLA-C* gene, a crucial region for psoriasis vulgaris (Oka et al., 1999; Suomela et al., 2003). The direct involvement of HCR in psoriasis is currently being discussed (Asumalahti et al., 2000; O'Brien et al., 2001; Asumalahti et al., 2002; Elomaa et al., 2004). Using *HCR*-transgenic mice, a recent report suggested that *HCR* is a potential candidate gene for psoriasis susceptibility (Elomaa et al., 2004). Sugawara et al. (Sugawara et al., 2003) identified a further role for HCR, which they called SBP, in promoting steroidogenesis by direct interaction with steroidogenic acute regulatory protein (StAR). Here, we characterize the RPB3-HCR interaction during muscle differentiation and provide evidence that HCR acts as a RPB3 cytoplasmic docking site.

Materials and Methods

Polyclonal-antibody production and purification

Human *HCR* cDNA fused to 6×His tag into the pQE-30 vector was expressed in bacteria and purified according to the manufacturer's protocol (Qiagen). For antiserum production, New Zealand white rabbits were immunized four times with 0.5 mg purified fusion protein at 3 week intervals with Freund's adjuvant (Difco). Antisera were collected 20 days after the last injection (Harlow and Lane, 1988). The specific polyclonal antibody was purified by affinity chromatography using recombinant fusion protein coupled to activated CH Sepharose, according to the manufacturer's instructions (Pharmacia). For the mouse polyclonal antibody, mice were immunized twice at an interval of 1 week with 100 µg purified fusion protein in Freund's adjuvant. The antiserum was collected 10 days after the last injection (Harlow and Lane, 1988). The high degree of homology exhibited by human and murine HCR proteins allowed the production of polyclonal antibodies that specifically recognize both human and murine HCR proteins.

The rabbit polyclonal antibodies against human RPB11 and RPB3 have been described previously (Fanciulli et al., 1996; Corbi et al., 2002).

Plasmids

The pGEX-RPB3 and myc-tagged pCS2-RPB3 constructs have been described previously (Corbi et al., 2002). Full-length human RPB3

and RPB3 deletion mutants were generated by PCR and cloned into the pEGFP-C1 vector (Clontech). Full-length human *HCR* was subcloned into the Flag-tagged mammalian expression vector pCMV-Tag2A (Stratagene). The pSuper-HCR constructs were generated as described in the RNA interference (RNAi) section. GeneLab Service (Enea-Casaccia) sequenced all constructs.

Immunofluorescence

Immunofluorescence was performed to detect RPB3, RPB11 and HCR proteins in GM- and DM-cultured C2C7 cells (see Cell culture and transfections section below). Briefly, cells were fixed in 3:7 methanol-acetone for 20 minutes at -20°C , air dried and then preincubated with 3% bovine serum albumin (BSA) in PBS. To detect endogenous RPB3, RPB11 and HCR expression, cells were incubated for 1 hour at room temperature (RT) with a 1:100 dilution (in PBS containing 2% BSA) of polyclonal antibodies specific for RPB3, HCR and RPB11. Immunoreactivity was visualized using rhodamine-conjugated anti-rabbit immunoglobulin (Ig) secondary antibody (Pierce). To monitor exogenous myc-RPB3 expression during differentiation, cells were analysed by immunofluorescence using the monoclonal antibody anti-myc 9E10. In particular, 12 hours after transfection with pCS2-Myc-RPB3 construct, C2C7 myoblasts were treated with trypsin and half kept in GM by avoiding cell-cell contact and the remaining half cultured to confluence and transferred to DM for an additional 48 hours. Cells were incubated with a 1:3 dilution of 9E10 hybridoma-conditioned medium in PBS containing 3% BSA for 1 hour at RT. Bound antibody was visualized using a fluorescein-conjugated anti-mouse Ig secondary antibody (Pierce).

Colocalization of endogenous RPB3 and HCR was detected using the mouse polyclonal anti HCR antibody together with rabbit polyclonal anti-RPB3 antibody. To detect colocalization, cells transfected with a fusion between enhanced green fluorescent protein and RPB3 (EGFP-RPB3) and Flag-HCR were fixed in 4% paraformaldehyde for 10 minutes at RT, permeabilized with 0.1% Triton X-100 for 5 minutes at RT and incubated for 1 hour with mouse monoclonal anti-Flag antibody (1:200 dilution; Sigma). Bound antibody was visualized using rhodamine-conjugated anti-mouse Ig secondary antibody. EGFP and the EGFP-RPB3 chimera were directly visualized after cell fixation. Nuclei were visualized by staining with Hoechst 33258 ($1\ \mu\text{g}\ \text{ml}^{-1}$) (Sigma).

Subcellular fractionation

C2C7 myoblasts (GM and 2 days DM, at a density of 2×10^6 cells) were washed three times in PBS and allowed to swell for 15 minutes in 400 µl buffer A (20 mM Hepes, 2 mM MgCl_2 , 0.2 mM EDTA, 25 mM NaF, 5 mM β -glycerophosphate, 0.1% Triton X-100, 5% glycerol, supplemented with protease inhibitors). Cells were scraped and centrifuged at 1200 g for 2 minutes and the supernatants were centrifuged at 8000 g for 15 minutes and saved as the cytoplasmic fraction. The pellet obtained after the first centrifugation was lysed in 200 µl buffer B (20 mM Hepes, 2 mM MgCl_2 , 0.2 mM EDTA, 0.4 M NaCl, 25 mM NaF, 5 mM β -glycerophosphate, 0.1% Triton X-100, 5% glycerol, supplemented with protease inhibitors), incubated on ice for 30 minutes and centrifuged for 10 minutes at 15,000 g and the supernatant saved as the nuclear fraction. All procedures were performed at 0°C . Both fractions were reconstituted to an equal volume containing a final concentration of $1 \times$ Laemmli sample buffer and analysed by SDS-PAGE and western blotting.

Yeast two-hybrid selection

For two-hybrid screening, the complete open reading frame (ORF) of human *RPB3* was cloned into the pGBKT7 vector (Clontech) in frame with the GAL4-binding domain (BD). Yeast strain AH109, bearing the synthetic upstream activating sequences (UASg) UASg-*His3*,

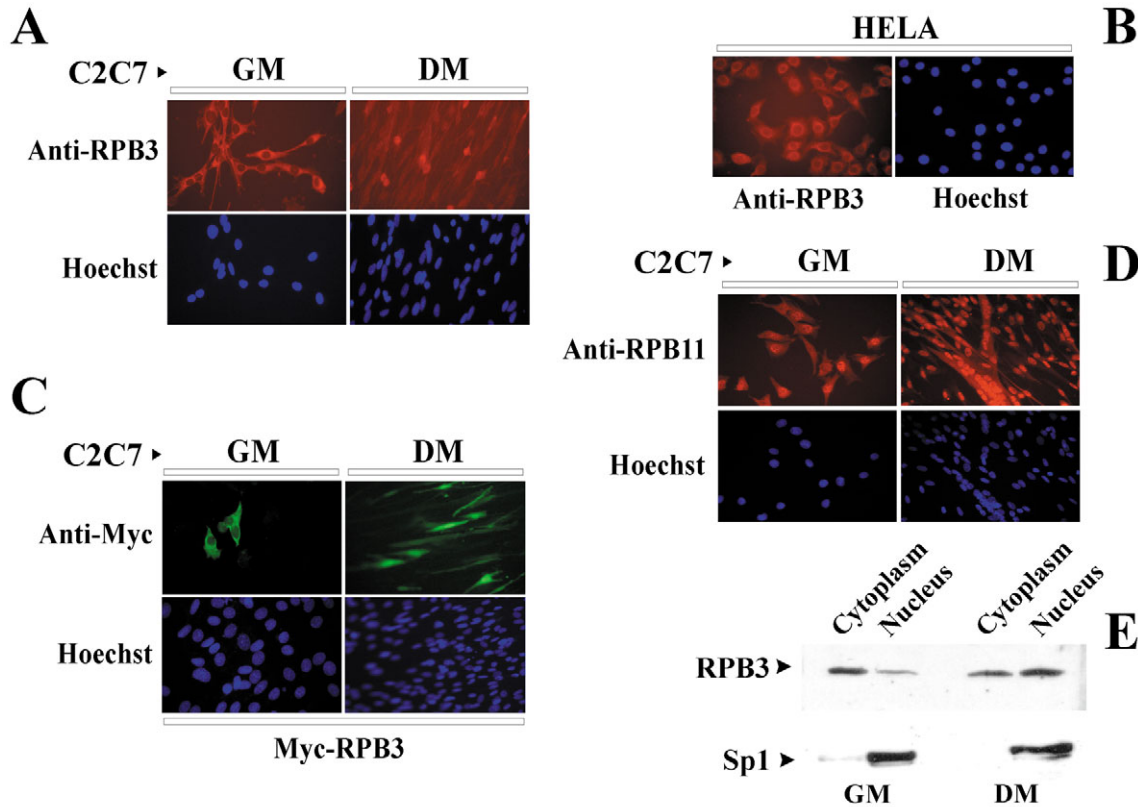


Fig. 1. RPB3 subcellular localization. (A) Endogenous RPB3 in cycling (GM) and differentiated (DM) myogenic C2C7 cells. (B) Endogenous RPB3 in HeLa cells. (C) Transfected Myc-RPB3 in cycling (GM) and differentiated (DM) myogenic C2C7 cells. (D) Endogenous RPB11 in cycling (GM) and differentiated (DM) myogenic C2C7 cells. Indirect immunofluorescence (A-D) was performed with the indicated antibodies. Hoechst-stained nuclei relative to the same fields are shown. (E) Western-blot analysis of subcellular cytoplasmic and nuclear fractions derived from GM and DM myogenic C2C7 cells. The blot was incubated with anti-RPB3 antibody to determine nuclear/cytoplasmic localization. To verify fraction quality, the same extracts were blotted and incubated with anti-Sp1 antibody.

UASg-*ADE2* and UASg-*LacZ* as reporter genes, was co-transformed with the bait pGBKT7-RPB3 and an adult human skeletal-muscle cDNA library (Clontech) fused to the GAL4 activation domain (AD) in the vector pGAD10. Transformation was performed using the lithium-acetate method (Gietz et al., 1992). Cells were plated directly on minimal synthetic defined (SD) medium [2% glucose, 0.67% Bacto yeast nitrogen base (Difco)], supplemented with the required bases and amino acids except tryptophan (Trp), leucine (Leu), adenine (Ade) and histidine (His). Plates were incubated for 7 days at 30°C, at which time Ade⁺His⁺ transformants were isolated. The Ade⁺His⁺ colonies were replica plated on SD -Leu -Trp -His -Ade medium and LacZ⁺ clones identified by a filter-lifting assay for β -galactosidase activity (Gietz et al., 1992). Plasmid DNA was prepared from candidate clones and electroporated into *Escherichia coli* XL1-BLUE competent cells (Stratagene). The recovered library-derived plasmids were then analysed as positive candidates. Liquid cultures of Y187 yeast strain were assayed for β -galactosidase activity to quantify two-hybrid interactions (Schneider et al., 1996).

Pull-down assay

In-vitro transcription and translation assays were performed using L-[³⁵S]-methionine (>1000 Ci mmol⁻¹; Amersham) and the TNT (T7 quick coupled transcription/translation)-coupled reticulocyte lysate system (Promega), as directed by the manufacturer. *E. coli* BL21 (Stratagene) were transformed with glutathione-S-transferase (GST) fusion protein constructs and the proteins purified on glutathione-Sepharose resin (Pharmacia). For protein-protein interaction assays,

comparable amounts of resin-bound GST fusion proteins were incubated with in-vitro-translated proteins in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) for 1 hour at 4°C. Resins were pelleted and extensively washed in the same buffer, bound proteins were separated by SDS-PAGE, the gel fixed, incubated in EnLightening solution (DuPont), dried and exposed for fluorography.

Cell culture and transfections

NIH 3T3 mouse fibroblasts and human HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum. C2C7 mouse myoblasts were grown in DMEM supplemented with 20% foetal bovine serum (GM) up to confluence. Differentiation was induced by switching to a differentiation medium (DM) consisting of DMEM containing 2% horse serum. Transient transfections were performed using Lipofectamine reagent (Gibco) according to the manufacturer's instructions. Total amounts of transfected DNA were equalized using empty vector DNA.

Immunoblotting

C2C7 at different times of differentiation were rinsed twice with PBS and lysed for 10 minutes in ice-cold lysis buffer [50 mM Tris.HCl, pH 8.0, 250 mM NaCl, 5 mM MgCl₂, 5 mM ATP, 5 mM EDTA, 5 mM β -glycerophosphate, 0.1 mM NaVO₄, 1 mM dithiothreitol, 50 mM NaF, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg ml⁻¹ leupeptin, 10 mg ml⁻¹ aprotinin]. After high-speed

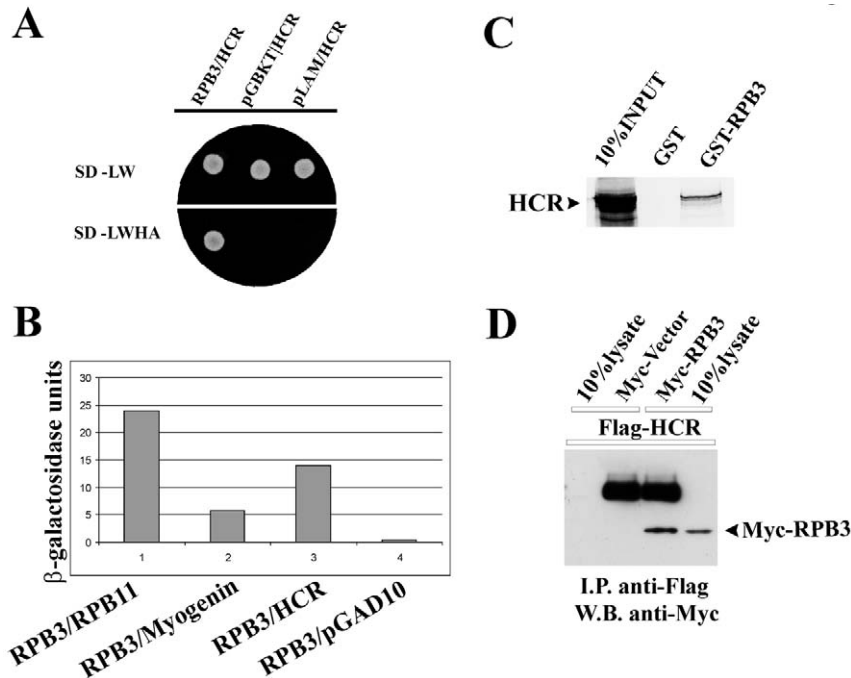


Fig. 2. RPB3 interacts with HCR. (A) AH109 yeast cells were co-transformed with the indicated constructs and plated onto SD medium lacking leucine and tryptophan (–LW) to verify the expression of both bait (W⁺) and prey (L⁺) plasmids, or onto medium lacking leucine, tryptophan, histidine and adenine (–LWHA) to examine the interaction between bait and prey proteins. (B) Y187 yeast cells were co-transformed with the indicated constructs and assayed for β -galactosidase activity. (C) In-vitro translated and S³⁵-labelled HCR was subjected to GST pull-down analysis using GST or GST-RPB3 beads. (D) Whole-cell extracts of NIH 3T3 cells transfected with both Flag-HCR and Myc-RPB3, or with Flag-HCR and empty Myc-tag vectors (Myc-Vector) were immunoprecipitated with anti-Flag monoclonal antibody and analysed by western blot using an anti-Myc monoclonal antibody.

centrifugation, the lysates (normalized for total protein content) were subjected to SDS-PAGE and transferred to nylon membranes. Blots were incubated with the following antibodies: polyclonal rabbit antibody specific for HCR (1:1000 dilution); polyclonal rabbit antibody specific for RPB3 (1:1000 dilution); polyclonal antibody specific for RPB11 (1:1000 dilution); monoclonal antibody specific for Myogenin clone IF5D (1:20 dilution) (Corbi et al., 2002); monoclonal antibody, clone MF20, specific for myosin heavy chain (1:5) (Corbi et al., 2002); monoclonal antibody specific for α -tubulin (1:500 dilution) (Sigma). After incubation with horseradish-peroxidase-conjugated secondary antibodies (Bio-Rad), blots were developed using the enhanced chemiluminescence (ECL) detection method (Pierce).

Co-immunoprecipitations

Transfected NIH 3T3 cells were lysed in a buffer containing 50 mM Tris.HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 5 mM EDTA, 0.2% NP-40, 1 mM PMSF, 10 mg ml⁻¹ leupeptin, 10 mg ml⁻¹ aprotinin. The supernatants were precleared with 20 μ l protein-A/protein-G bound to agarose beads (Santa Cruz Biotechnology) and immunoprecipitated using the M2 monoclonal anti-Flag antibody (Sigma). Western blots were prepared by standard procedures and incubated with monoclonal antibody against EGFP (Clontech; 1:1000 dilution) used to detect EGFP chimera proteins by chemiluminescence (Pierce).

RNAi

To obtain RNAi of *HCR*, a specific targeting sequence was cloned into the pSuper vector system, which directs synthesis of small interfering RNAs (siRNAs) (Brummelkamp et al., 2002). The selected 19 nucleotide specific *HCR* target sequence was: 5'-GACCTCGCT-GCAGCAGAAG-3'. This sequence comprises nucleotides 260-279 (from the ATG start codon) of the human *HCR* cDNA (accession number AB029331) and is completely conserved between human and mouse (accession number BC031416). In siRNA experiments, EGFP-RPB3 and pSuper constructs (empty or with the *HCR* RNAi) were transfected at a 1:15 ratio, respectively. 60 hours after transfections, cells were processed for either western blot or immunofluorescence analysis.

Results

RPB3 subcellular localization

Using indirect immunofluorescence, we have investigated the subcellular localization of RPB3 in several cycling cell lines and have detected in all of them an unexpected cytoplasmic localization. In Fig. 1A,B, we show the subcellular localization of RPB3 in murine C2C7 myogenic cells and in human HeLa cells. In cycling C2C7 cells cultured in GM, the RPB3 cytoplasmic staining was particularly intense and slightly polarized. Notably, this signal moves to the nucleus in association with differentiation (induced by DM). As shown in Fig. 1C, we confirmed this RPB3 localization by overexpressing in both cycling and differentiated C2C7 cells Myc-RPB3, which can be visualized using an anti-myc monoclonal antibody. On the basis of these results, we extended the subcellular localization analysis in C2C7 cells to the other α -like subunit, RPB11, which forms a heterodimer with RPB3. By indirect immunofluorescence using polyclonal anti-RPB11 antibody, RPB11 in both cycling and differentiated C2C7 cell shows an invariant nuclear localization (Fig. 1D). We also investigated the subcellular localization of RPB3 by western-blot analysis of cytoplasmic and nuclear fractions from GM- and DM-cultured C2C7 cells. Blots were incubated with anti-RPB3 antibody and then with anti-Sp1 antibody in order to verify cellular fractionation (Fig. 1E). In cycling C2C7 cells, RPB3 exhibited an intense signal in the cytoplasmic fraction and a faint signal in the nuclear fraction. Upon differentiation, RPB3 signal intensity was diminished in the cytoplasm and strongly augmented in the nuclear fraction.

RPB3 interacts with HCR

In order to identify candidate proteins that interact with RPB3 in the cytoplasmic compartment, yeast two-hybrid experiments were performed, using RPB3 as bait to screen a cDNA library prepared from human skeletal muscle. Of an estimated

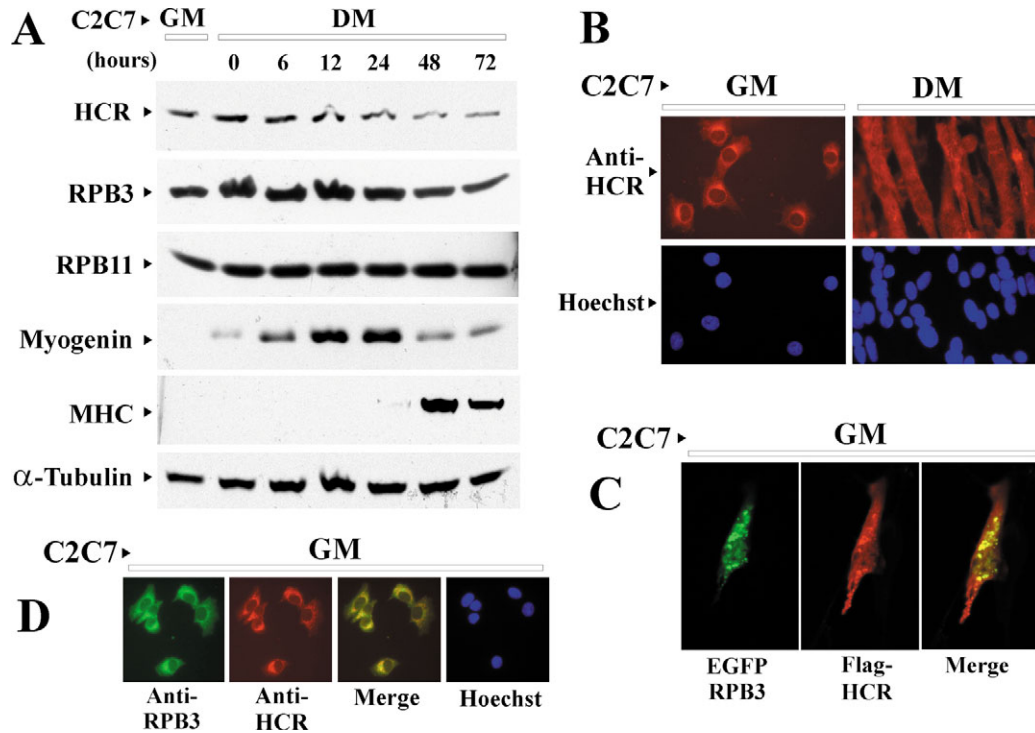


Fig. 3. HCR expression and localization in C2C7 cells. (A) Total lysates from C2C7 myogenic cells incubated in differentiation medium (DM) for the indicated times or maintained in growth medium (GM) were analysed by western blot using the indicated antibodies. α -Tubulin was used to normalize the amount of protein extracts loaded in each lane. (B, top) Indirect immunofluorescence of HCR performed using purified anti-HCR rabbit polyclonal antibody in cycling (GM) and differentiated (DM) myogenic C2C7 cells. (B, bottom) Hoechst-stained nuclei of the same fields as in B, top. (C) Colocalization of overexpressed EGFP-RPB3 and Flag-HCR proteins. (left) Fluorescence of EGFP-RPB3 fusion protein; (middle) indirect immunofluorescence using anti-Flag monoclonal antibody; (right) merge of EGFP-RPB3 and Flag-HCR fluorescence signals. (D) Colocalization of endogenous RPB3 and HCR proteins. (Green) Indirect immunofluorescence using rabbit polyclonal antibody against RPB3; (red) indirect immunofluorescence using mouse polyclonal antibody against HCR; (yellow) merge of RPB3 and HCR indirect immunofluorescence signals; (blue) Hoechst-stained nuclei relative to the same field.

2.5×10^6 transformants, 30 clones proliferated on media lacking histidine and adenine, and stained positive for β -galactosidase. These clones were isolated for further characterization. Several independent clones contained RPB11, previously described to be RPB3 partner (Fanciulli et al., 1998). Two independent positive clones encoded the entire ORF of the basic-HLH myogenic transcription factor Myogenin (Corbi et al., 2002). One clone encoded the entire ORF of the known gene product named HCR or SBP by different research groups (Asumalahti et al., 2000; Sugawara et al., 2003). The specificity of the RPB3-HCR interaction was confirmed in a two-hybrid assay, co-transforming *HCR* with either *RPB3* or empty vector (pGBKT7) and lamin control vector (pLAM). As shown in Fig. 2A, RPB3 interacted with HCR, whereas lamin and pGBKT7 did not. Using the yeast LacZ assay, the strength of the RPB3-HCR interaction was evaluated and compared with interactions between heterodimeric RPB3 and RPB11, and between RPB3 and the transcription factor Myogenin. As shown in Fig. 2B, the intensity of the RPB3-HCR interaction was lower than that of RPB3 and RPB11 but higher than that of RPB3 and Myogenin.

To confirm the RPB3-HCR interaction in a cell-free system, we expressed RPB3 coding sequences in *E. coli* as a GST fusion protein (GST-RPB3) and tested its ability to bind in vitro translated ^{35}S -labelled HCR. Data shown in Fig. 2C confirm a

specific direct physical interaction between RPB3 and HCR. To provide evidence that RPB3 also associates with HCR in mammalian cells, co-immunoprecipitation experiments were performed. Expression vectors for either myc-tagged RPB3 (Myc-RPB3) or myc-tag empty vector (Myc-Vector) were co-transfected together with Flag-tagged HCR (Flag-HCR) into NIH 3T3 mouse fibroblasts. As shown in Fig. 2D, immunoprecipitation using anti-Flag monoclonal antibody followed by western blot analysis of the precipitants with anti-Myc monoclonal antibody clearly demonstrate interaction between Myc-RPB3 and Flag-HCR.

RPB3 and HCR colocalize in the cytoplasm

In order to study the protein production pattern of HCR and its subcellular localization during the onset of C2C7-cell differentiation, a rabbit polyclonal antibody against HCR was produced. Purified anti-HCR antibody was used in western blots to analyse HCR expression during C2C7 differentiation. Fig. 3A illustrates that HCR levels slowly decrease during myogenic differentiation. RPB3, as previously shown (Corbi et al., 2002), was constantly present but its level slightly increased in association with the onset of differentiation. RPB11, the second α -like subunit, was constantly expressed through all differentiation stages. The C2C7 myogenic differentiation

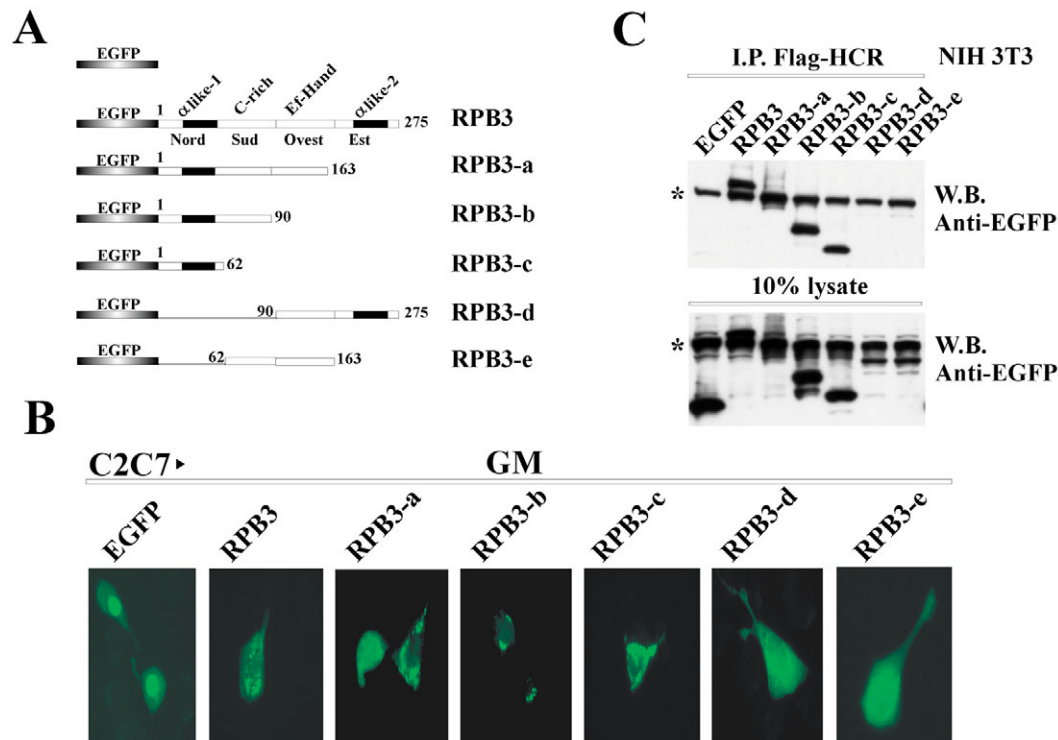


Fig. 4. The α -like-1 RPB3 domain is involved in both cytoplasmic localization and HCR contact. (A) EGFP-RPB3 full-length and relative nested deletion constructs. (B) Subcellular localization of EGFP-RPB3 and indicated deletion construct, overexpressed in cycling (GM) myogenic C2C7 cells. (C) Whole-cell extracts of NIH 3T3 mouse fibroblasts transfected with both Flag-HCR and EGFP-RPB3 deletion constructs were immunoprecipitated with anti-Flag monoclonal antibody and analysed by western blot using anti-EGFP monoclonal antibody. The asterisks indicate a heavy-chain Ig band (top) and a non-specific band (bottom); both partially cover the signal corresponding to the RPB3-a deletion mutant (lane 3).

programme was followed by analysing the expression of Myogenin and the myosin heavy chain (MHC) as myogenic markers. We investigated the subcellular localization of HCR in cycling and differentiated C2C7 cells by indirect immunofluorescence. As shown in Fig. 3B, HCR immunostaining exhibited an intense cytoplasmic signal that was perinuclear and slightly polarized in cycling cells but more diffuse in differentiated myotubes.

To characterize the RPB3-HCR interaction better as a function of subcellular localization in C2C7 cycling cells, we overexpressed Flag-HCR and EGFP-RPB3. As shown in Fig. 3C, the EGFP-RPB3 fluorescence was principally confined to large, discrete spots in the cytoplasm. Flag-HCR indirect immunostaining showed the presence of discrete dots arranged throughout the cytoplasm. Merge analysis of EGFP-RPB3 and Flag-HCR signals clearly shows extensive colocalization of the two proteins. We confirmed this subcellular colocalization with endogenous RPB3 and HCR, using specific mouse anti-HCR antibody. As shown in Fig. 3D, both endogenous proteins showed a similar pattern of expression, confirmed by the merge analysis. These results demonstrate that HCR localizes with RPB3 within the cytoplasm of cycling C2C7 myogenic cells and that the HCR expression pattern is compatible with its presumptive role as a RPB3-cytoplasmic docking site.

RPB3 α -like-1 domain is involved in both cytoplasmic localization and HCR contact

We determined the RPB3 region responsible for cytoplasmic compartmentalization. To this end, as shown in Fig. 4A, we constructed a series of RPB3 deletion mutants fused to the EGFP protein and the resulting constructs were overexpressed in cycling C2C7 cells. Fig. 4B shows that EGFP-RPB3 deletion

mutants (RPB3-d and RPB3-e) missing the region (62 amino acids long) that contains the α -like-1 portion did not retain cytoplasmic localization.

Next, we examined the possibility that the RPB3 α -like-1 region was responsible for both cytoplasmic localization and HCR interaction. For this purpose, we overexpressed Flag-HCR with the EGFP-RPB3 mutant constructs in mouse NIH 3T3 fibroblasts. Whole-cell extracts were immunoprecipitated with anti-Flag monoclonal antibody and analysed by western blot using anti-EGFP monoclonal antibody (Fig. 4C). Flag-HCR coimmunoprecipitated only with EGFP-RPB3 containing the α -like-1 region. These results demonstrate that the α -like-1 region of RPB3 is responsible for both cytoplasmic localization and HCR interaction, supporting the role of HCR as a RPB3-cytoplasmic docking site.

HCR RNAi affects the subcellular localization of EGFP-RPB3

To investigate the functional role of RPB3-HCR protein interactions, we silenced endogenous HCR expression and then checked the localization of EGFP-RPB3 in cycling C2C7 cells. The specific HCR RNAi vector pSuper-HCR was transiently transfected into cycling C2C7 cells at increasing concentrations. Whole cellular lysates were then analysed by western blot using purified anti-HCR antibody. Fig. 5A shows a consistent decrease in HCR signals only upon pSuper-HCR treatment. In Fig. 5B, cycling myogenic C2C7 cells were transiently transfected with empty pSuper vector or with pSuper-HCR interference and with EGFP-RPB3 constructs. EGFP-RPB3 transiently transfected with pSuper vector exhibits the usual, preferentially cytoplasmic, fluorescent pattern (Fig. 5B, top). When EGFP-RPB3 is co-expressed with pSuper-HCR, the fluorescence signal becomes clearly nuclear

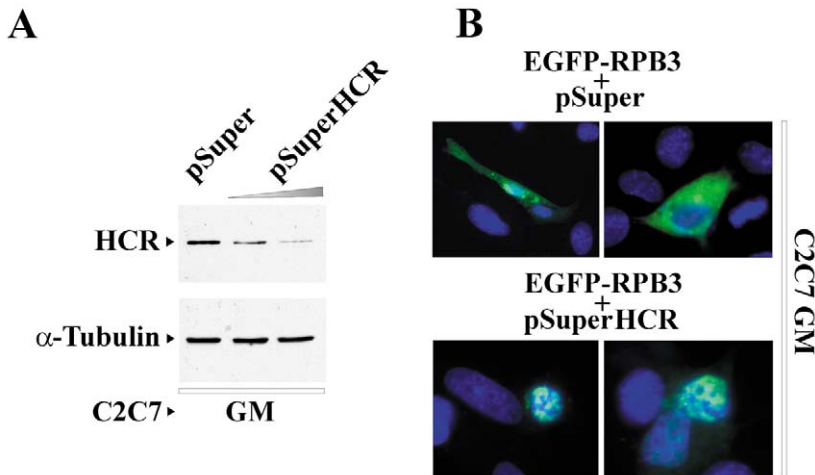


Fig. 5. *HCR* RNAi affects EGFP-RBP3 subcellular localization. (A) Western blot analysis of cycling (GM) myogenic C2C7 cells transfected with pSuper RNAi empty vector and with increasing DNA amounts of pSuper-HCR interference vector. (B) Fluorescence of EGFP-RBP3 fusion protein co-expressed in cycling (GM) myogenic C2C7 cells, either with empty pSuper vector (top) or with pSuper-HCR interference vector (bottom). All nuclei are Hoechst stained.

(Fig. 5B, bottom). This result confirms a role for HCR as a RBP3 cytoplasmic docking site.

Discussion

In addition to its essential structural function in RNAP II, RBP3 displays an active role in tissue-specific transcription. We have previously demonstrated that RBP3 directly contacts the myogenic transcription factor Myogenin and the activating transcription factor ATF4. Thus, RBP3 can be added to the growing list of RNAP II subunits that are involved in specific transcriptional regulation (Lin et al., 1997; Dorjsuren et al., 1998; Petermann et al., 1998; Miyao et al., 1998, Schlegel et al., 2000).

In this paper, we show for the first time that RBP3 exhibits a particular subcellular localization that is regulated during muscle differentiation. In addition to its presence in the RNAP II core enzyme, RBP3 accumulates as an unassembled subunit within the cytoplasm of cycling myogenic C2C7 cells, as well as in the cytoplasm of several other cell lines including HeLa cells (Fig. 1B), NIH-3T3, H293 and COS cells (data not shown). In yeast, the RNAP II subunit 4 (RBP4) was also detected in the cytoplasmic compartment (Kimura et al., 2001; Farago et al., 2003). In C2C7 cells, RBP3 exhibits a massive migration to the nucleus upon induction of myogenic differentiation. Remarkably, we show that the other α -like subunit, RBP11, which forms a heterodimer with RBP3 inside RNAP II, exhibits an invariant nuclear localization in both cycling and differentiated C2C7 cells, and in all other cell lines analysed (data not shown). Several hypotheses could explain such RBP3 compartmentalization. For example, RBP3 is the least abundant subunit in yeast and, together with RBP2 and RBP11, constitutes the core of RNAP II (Kimura et al., 2001), suggesting that the amount of RBP3 could be crucial in defining the amount of RNAP II to be assembled. In mammalian cells, RBP3 cytoplasmic compartmentalization might represent a way to limit transcription levels and its release in the nucleus might contribute to the determination of the proper amount of RNAP II to assemble in response to precise stimuli (e.g. muscle differentiation). Alternatively, RBP3 retained in the cytoplasm by appropriate interactions might undergo specific post-translation modifications that can

allow the assembly of appropriate forms of RNAP II in response to particular stimuli, such as myogenic differentiation.

With the intent to characterize hypothetical RBP3 cytoplasmic interactions in muscle cells, we identified a novel RBP3 partner, HCR. The human *HCR* gene maps to chromosome 6p21, in a region that is crucial for psoriasis vulgaris, located 111 kb towards the telomere of the *HLA-C* gene (Suomela et al., 2003). HCR is expressed differently in lesional psoriatic skin compared with normal skin (Asumalahti et al., 2002). HCR has also been identified as protein partner for StAR and hence has been named SBP (Sugawara et al., 2003). HCR/SBP enhances the ability of StAR to promote steroid-hormone synthesis. Interestingly, we found that HCR contacts RBP3 in correspondence to α -like-1 domain, a domain that is also responsible for cytoplasmic retention of RBP3. In this context, we observed a massive EGFP-RBP3 nuclear migration in cycling C2C7 cells as a direct consequence of HCR knockdown by RNAi. These findings demonstrate that HCR works as a RBP3 cytoplasmic docking site. In double-staining experiments, HCR only partially colocalized with RBP3, suggesting that HCR might be available for additional protein interactions (e.g. with StAR). So far, our attempts to isolate further HCR partners by yeast two hybrid screening have been unsuccessful for the trivial reason that HCR can activate transcription by itself (data not shown). Indeed, the identification of additional proteins that interact with HCR would help to elucidate its biological role(s). It is possible that HCR has a role in the compartmentalization of different proteins that follow a partially overlapping fate, such as specific post-translation modifications and/or shuttling between cellular compartments. Although the role of the RBP3-HCR interaction requires further study, this interaction represents a novel tool to study the control of RNAP II assembly and novel additional levels of transcription regulation.

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