Molecular Biology of the Cell
Vol. 20, 3543–3551, August 1, 2009

Induction of *HoxB* Transcription by Retinoic Acid Requires Actin Polymerization

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Submitted February 9, 2009; Revised May 18, 2009; Accepted May 19, 2009
Monitoring Editor: Richard K. Assoian

We have analyzed the role of actin polymerization in retinoic acid (RA)-induced *HoxB* transcription, which is mediated by the *HoxB* regulator Prep1. RA induction of the *HoxB* genes can be prevented by the inhibition of actin polymerization. Importantly, inhibition of actin polymerization specifically affects the transcription of inducible *Hox* genes, but not that of their transcriptional regulators, the RARs, nor of constitutively expressed, nor of actively transcribed *Hox* genes. RA treatment induces the recruitment to the *HoxB2* gene enhancer of a complex composed of "elongating" RNAPII, Prep1, β-actin, and N-WASP as well as the accessory splicing components p54Nrb and PSF. We show that inhibition of actin polymerization prevents such recruitment. We conclude that inducible *Hox* genes are selectively sensitive to the inhibition of actin polymerization and that actin polymerization is required for the assembly of a transcription complex on the regulatory region of the *Hox* genes.

INTRODUCTION

The presence of β-actin in the nucleus was documented for the first time many years ago (Egly *et al.*, 1984; Scheer *et al.*, 1984), but the connection between β-actin and gene transcription has only recently been demonstrated (Olave *et al.*, 2002; Pederson and Aebi, 2002; Fomproix and Percipalle, 2004; Grummt, 2006; Jockusch *et al.*, 2006; Percipalle and Visa, 2006; Obrdlik *et al.*, 2007). Nuclear β-actin associates with components of the ATP-dependent chromatin-remodeling complexes (Olave *et al.*, 2002; Bettinger *et al.*, 2004), with RNP particles (Percipalle and Visa, 2006), and with the three RNA polymerases in the eukaryotic cell nucleus, both in vitro and in vivo (Hu *et al.*, 2004; Philimonenko *et al.*, 2004; Kukalev *et al.*, 2005; Wu *et al.*, 2006). The above studies have shown that nuclear actin is a component of large protein complexes that include RNA polymerases, transcription elongation factors, and accessory splicing factors that can be recruited to the RNAPII carboxy terminal domain and hence to promoters.

Interestingly, a number of studies have shown the nuclear presence of proteins that stimulate actin polymerization, e.g., N-WASP (neuronal Wiskott-Aldrich Syndrome Protein) and Arp2/3 (Wu *et al.*, 2006; Yoo *et al.*, 2007), raising the possibility that actin polymerization may occur in this cellular compartment. The observation in a recent FRAP analysis that ~20% of the total nuclear actin pool is in the polymeric state supports this idea (McDonald *et al.*, 2006). Importantly transcription is affected by the down-regulation of N-WASP or by inhibiting actin polymerization either through the expression of polymerization-deficient actin mutants or by using high concentrations of specific drugs, such as cytochalasin D (CytD) or latrunculin A (LatA; McDonald *et al.*, 2006; Wu *et al.*, 2006; Yoo *et al.*, 2007; Ye *et al.*, 2008). These observations indicate that actin polymerization is necessary for gene transcription. However, it is not known whether all genes are equally sensitive to inhibition of actin polymerization. Also it is not known whether actin polymerization is required for the recruitment of active transcription complexes to transcription regulatory sites. This article deals with the role of actin in the induction of *Hox* gene transcription by retinoic acid (RA). Cell fate–determining clustered *Hox* genes are expressed colinearly in the same order as their location on the genome, generating anterior-to-posterior identities (Krumlauf, 1994). Several lines of evidence have demonstrated the importance of RA receptors (RARs) for patterning and basal expression of *Hox* genes in the vertebrate neural tube (Marshall *et al.*, 1994, 1996; Gould *et al.*, 1998; Dupe *et al.*, 1999). However, expression of anterior *HoxB* genes also depends on an auto-regulatory circuit involving the HoxB1 protein and the Prep1–Pbx1 complex (Marshall *et al.*, 1996; Maconochie *et al.*, 1997; Dupe *et al.*, 1999; Jacobs *et al.*, 1999; Ryoo *et al.*, 1999; Ferretti *et al.*, 2000, 2005; Huang *et al.*, 2002). *Prep1* and *Pbx1* are essential genes in embryonic development (Selleri *et al.*, 2001; Waskiewicz *et al.*, 2002; Deflorian *et al.*, 2004; Penkov *et al.*, 2005; Ferretti *et al.*, 2006; Di Rosa *et al.*, 2007). Unlike monomeric Prep1 and Pbx1, dimeric Prep1–Pbx1 complexes bind DNA and interact with HoxB1 to activate *HoxB1* and *HoxB2* transcription

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–02–0114) on May 28, 2009.

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(Berthelsen *et al.*, 1998; Ryoo *et al.*, 1999; Ferretti *et al.*, 2000, 2005; Huang *et al.*, 2002).

Colinear transcription can be reproduced in the NT2-D1 teratocarcinoma cell line in which RA induces multilineage differentiation. In these cells, time-course experiments show that the expression of the *HoxB* cluster initiates at the 3′ end with the *HoxB1* gene and time-dependently proceeds toward the 5′ end, transcribing all genes colinearly with their chromosomal location (Simeone *et al.*, 1990).

We have recently shown that Prep1 specifically copurifies and coprecipitates not only with its dimeric partner Pbx1 and with RNAPII, but also with nuclear (but not cytoplasmic) β-actin (Diaz *et al.*, 2007a). As the Prep1 complex is required for transcriptional induction of the *HoxB* genes (Ferretti *et al.*, 2000, 2005; Waskiewicz *et al.*, 2002; Deflorian *et al.*, 2004), the association of Prep1 to β-actin prompted us to analyze the role of actin polymerization in *HoxB* transcriptional induction. Here, we report that three different approaches to block actin polymerization: the use of CytD or LatA inhibitors, down-regulation of the actin polymerization stimulator N-WASP, and a dominant-negative actin mutant—all inhibit the induction of *HoxB* genes by RA. Our studies with CytD demonstrate that actin polymerization is required for the colinear expression of *HoxB* genes at the time of transcription initiation. Importantly, we show that the inhibition of actin polymerization has no effect on genes whose transcription has already started, indicating that induced genes are more sensitive to β-actin polymerization inhibition than constitutively transcribed genes. Although CytD has no effect on the expression of RARs, actin polymerization is required for the RA-induced recruitment of a number of proteins to the regulatory regions of the *HoxB2* gene, such as Prep1, β-actin, the elongating form of the RNAPII phosphorylated in serine 2 of the carboxy-terminal domain (RNAPII-S2p), N-WASP, and the p54/Nrb–PSF complex that was previously shown to interact with N-WASP (Wu *et al.*, 2006). Treatment with CytD totally blocks the recruitment of these proteins to the regulatory region of *HoxB2*, supporting a direct functional role of actin polymerization in the induction of the HoxB cluster.

MATERIALS AND METHODS

Cell Culture and Treatments

Human NT2-D1 cells were grown in DMEM (Cambrex BioScience, Milan, Italy) and used at 70% confluence. *Trans*-RA was from Sigma-Aldrich (Milan, Italy). Controls were treated with 0.1% DMSO. CytD was from EMD Biosciences (Darmstadt, Germany).

Antibodies

The following antibodies were used: monoclonal anti-β-actin (Sigma-Aldrich); polyclonal anti-total RNAPII, anti-RNAPII-S2p, and anti-Pbx1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Prep-1 polyclonal antibody (Berthelsen *et al.*, 1998) and CH12.2 monoclonal were prepared by standard techniques. Polyclonal anti-N-WASP was published previously (Rohatgi *et al.*, 1999). Polyclonal anti-PSF and monoclonal anti-p54/Nrb antibodies were kind gifts of Drs. A. Krainer (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and J. Patton (Vanderbilt University, Nashville, TN), respectively.

RNA and Protein Extraction, Coimmunoprecipitation, Immunoblotting, and Electrophoretic Mobility Shift Assay

RNA was extracted with Qiagen mini columns and RNeasy mini kit-250 (Qiagen GmbH, Hilden, Germany). Nuclear extracts were prepared as described (Dignam *et al.*, 1983).

Coimmunoprecipitations were performed with protein G-Sepharose (Zymed Laboratories, San Francisco, CA). Nuclear proteins were diluted in 10 mM Tris, pH 8, 0.2% NP-40, and 150 mM NaCl, precleared, incubated with 5 μg antibodies overnight at 4°C, and pulled down with protein G.

Electrophoretic mobility shift assay (EMSA) for Prep1–Pbx1 was carried out with the ^{32}P-labeled O1 oligonucleotide 5′CACCTGAGAGTGACAGAAGGAGGCAGGGAG3′ (Berthelsen *et al.*, 1998).

N-WASP Silencing

hN-WASP-1 (CGGCAAGAAAUGUGUGACUAUGUCU; Invitrogen, Milan, Italy) was transfected in NT2-D1 cells with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. At 24 h cells were passed to new medium with RA (1 μM) or DMSO (control) and grown for 16 h. We also used the SC36006 oligonucleotide from Santa Cruz Biotechnology, which totally abolished N-WASP expression.

PCR and Real-Time PCR

RNA, 1 μg, oligodT, 0.5 μg, and SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen) were used. Primers were as follows: GADPH: ACCACCTGGTGCTCAGTGTA (sense) and ACATCATCCCTGCCTCTACTG (antisense); HoxB1: GCATCTCCAGCTGCCTCCTT (antisense) and CCTTCTTAGAGTACCCACTCTG (sense); and HoxB2: AGTGGAATTCCTTCTCCAGTTCC (antisense) and TCCTCCTTTCGAGCAAACCTTCC (sense).

cDNA, 5 ng, was amplified (in triplicate) with TaqMan PCR Mastermix and TaqMan Gene expression assay (Applied Biosystems, Foster City, CA) and measured in the ABI/Prism 7900 HT Sequence Detector System (Applied Biosystems), using a pre-PCR step of 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. RNA without reverse transcriptase was used as negative control. 18S rRNA and GAPDH were used as standards. Proprietary primers from Applied Biosystems were used.

For real-time PCR of the *HoxB2* expression levels in actin mutant transfections, the reverse-transcribed RNA was amplified in a light cycler (Roche, Indianapolis, IN) using a FastStart DNA mix SYBR Green I kit (Roche). PCR conditions were as follows: for *HoxB2* mRNA: denaturation and DNA polymerase activation step, 95°C for 10 min; second denaturation step, 95°C for 15 s; annealing step, 56°C for 6 s; and extension step, 72°C for 20 s. GAPDH conditions were as follows: first denaturation and DNA polymerase activation step, 95°C for 10 min; second denaturation step, 95°C for 15 s; annealing step, 57°C for 6 s; extension step, 72°C for 20 s. The amount of *HoxB2* mRNA was normalized to GAPDH mRNA. The primers are reported above.

Pyrene Actin Polymerization Assay

Nuclear extracts from NT2-D1 cells were dialyzed against G buffer (5 mM Tris-HCl, pH 7.8, 0.2 mM ATP, 1 mM DTT, 0.1 mM $CaCl_2$, and wt/vol, 0.01% NaN_3) for 4 h. Actin polymerization was measured by the increase in fluorescence of 10% pyrenil-labeled actin, as described (Disanza *et al.*, 2006) after addition of 0.1 M KCl, 1 mM $MgCl_2$, and 0.2 mM EGTA to a solution of Ca-ATP-G-actin (2 μM-10% pyrene) containing 80 μl of extract, and 10 μg of glutathione *S*-transferase (GST) or GST-vascular cell adhesion (VCA) fusion protein. Purified Arp 2/3 complex (10 nM), supplemented with GST or GST-VCA, was used as internal control.

Chromatin Immunoprecipitation

Chromatin from *p*-formaldehyde (1%) cross-linked cells was prepared and sonicated as described (Ferrai *et al.*, 2007). Aliquots were immunoprecipitated overnight with 1 μg of antibody, and DNA was extracted after reversion of cross-linking by heating at 65°C for 5 h. Purified immunoprecipitated DNA was quantitated by QT-PCR in a light cycler (Roche): denaturation and DNA polymerase activation, 95°C for 10 min; denaturation, 95°C, 15 s; annealing, 54°C (HoxB2 enhancer) and 60°C (intergenic region) for 6 s; extension, 72°C for 20 s. The relative enrichment was determined by calculating the ratio of immunoprecipitated DNA to input DNA.

The following primers were used: primers for HoxB2 Enhancer: Fw-TGGCTGTTCGCTCTGCTTTCC; Rev-AGGGCACAAGACTCTGAGCC; primers for the uPA intergenic region: Fw-CAGTAATCTGGCCTTGCCTTTCC; Rev-GAGGAATCGAGAGGCTTGTAAATTC.

RESULTS

Actin-depolymerizing Drugs Block HoxB Induction

We analyzed the role of actin polymerization in RA-induced transcription of the *HoxB* gene cluster in NT2-D1 cells. We measured the levels of various *HoxB* mRNAs by real-time PCR at different times after RA (1 μM) addition and explored the effect of the actin-capping agent CytD (100 nM). Table 1 shows that, in the absence of CytD, *HoxB1*, and *HoxB2* mRNAs were already induced 16 h after RA addition, whereas *HoxB3* and *HoxB6* were induced at 48 and 72 h, respectively (as expected) (Simeone *et al.*, 1990). CytD was added to the cells 16 or 24 h before mRNA level determination (i.e., at t = 0 for the 16-h measurement, at t = 24 h after RA for the 48-h measurement, and at t = 48 h after RA for the 72-h measurement; see Table 1). A scheme of the experiment is presented in Supplemental Figure S1. When added

Table 1. CytD inhibits RA-induced Hox gene expression in NT2–1 cells

Genes	Control 0 h	RA 16 h	RA 16 h + CytD	RA 48 h	RA 48 h + CytD	RA 72 h	RA 72 h + CytD
HoxB1	0	1.0 (1.22–0.82)	0.177 (0.21–0.15)	1.0 (1.07–0.94)	0.996 (1.02–0.97)	1.0 (1.22–0.821)	1.07 (1.25–0.925)
HoxB2	0	1.0 (1.052–0.95)	0.05 (0.061–0.041)	1.0 (1.12–0.89)	1.102 (1.12–1.08)	1.0 (0.876–1,14)	1.154 (1.095–1.22)
HoxB3	0	0	0	1.0 (1.19–0.84)	0.5 (0.62–0.40)	1.0 (1.2–0-83)	0.8 (0.93–0.69)
HoxB6	0	0	0	0	0	1.0 (1.13–0.89)	0.377 (0.172–0.83)

The data are the average of triplicate quantitative PCR experiment performed at least twice. Time in hours (h) refers to the exposure to 1 μM RA. When present, CytD (100 nM) was added before the measurement, i.e., at t = 0 for the 16-h, at t = 24 for the 48-h, and at t = 48 for the 72-h measurement. The level of mRNA expression after induction (in the absence of CytD) is set equal to 1.0; the values in parentheses represent the confidence interval at 95%.

with RA at t = 0, CytD completely inhibited *HoxB1* and *HoxB2* induction, as measured at 16 h (Table 1; raw data obtained by real-time PCR are shown in Supplemental Figure S2). Transcription of *HoxB3* and *HoxB6*, which were induced at 48 or 72 h, respectively, was inhibited significantly when CytD was added 24 or 48 h after RA, respectively (Table 1). However, when CytD was administered after transcription of *HoxB1*, *HoxB2*, or *HoxB3* had already started, i.e., at 24 or 48 h after RA, respectively, no transcriptional inhibition was observed (Table 1). These results suggest that, at the concentration used (100 nM), CytD inhibits the initiation of *HoxB* transcription, but that it does not affect transcription that has already initiated. Consequently, genes may be differentially sensitive to CytD, depending on their expression state.

We also measured the CytD sensitivity of other basally expressed or inducible genes after RA treatment (Table 2). The expression of three RA-inducible genes, *HoxA1, HoxA2,* and *Meis1 was* not affected by CytD, confirming that not all inducible genes are necessarily inhibited by 100 nM CytD. *Eps8*, which is constitutively expressed independently of RA, likewise, was not affected by CytD (Table 2).

We used another inhibitor of actin polymerization, LatA, and measured HoxB3 mRNA levels 48 h after RA addition to confirm our data. Indeed, LatA inhibited HoxB3 transcription by 75% (data obtained by Q-PCR, Supplemental Table S1). For reasons not fully understood, the effect of LatA on HoxB1 and HoxB2 early expression was not as strong as CytD. Similarly to CytD, LatA had no effect on the transcription of *HoxB1* and *HoxB2* after their expression had been induced, but inhibited well HoxB3 and HoxB6 expression. We also compared the effects of 100 nM CytD to that of two F-actin–stabilizing drugs, jasplakinolide and phalloidin, on the RA-induction of *HoxB1* and *HoxB2* by performing RT-PCR at 24 h (drugs added together with RA). At the concentrations used, these two drugs had a slight stimulatory effect on *HoxB1* and *HoxB2* transcription (Supplemental Figure S3). Overall, our data support the interpretation that the CytD-dependent inhibition of transcription is due to a block in actin polymerization.

The inhibition of actin polymerization can certainly also affect the properties of cells, for example, neuronal differentiation after RA addition. However, the effect of CytD on *HoxB* expression seems to be specific and we believe is unlikely to be secondary to changes in cell morphology or cell division. In fact, both CytD or LatA do not drastically change the cell shape (even at the low concentrations used) and have no effect on NT2-D1 cell division (data not shown).

N-WASP Down-Regulation Inhibits the Expression of the HoxB Cluster

We next tested the effect of RA on actin polymerization mediated by the ARP2/3 complex. N-WASP is an effector protein for actin polymerization that is also present in the nucleus (Wu *et al.,* 2006). The WCA domain of N-WASP can promote actin nucleation and its subsequent polymerization by simultaneously associating with the ARP2/3 complex and monomeric actin present in the nuclear extracts. The formation of this tripartite unit allows the thermodynamic barrier of actin nucleation to be overcome (Pantaloni *et al.,* 2000, 2001). We reasoned that the addition of RA may facilitate the formation of the actin–ARP2/3 complex, thus accelerating the polymerization of actin in the presence of exogenous WCA. We thus incubated purified C-terminal WCA domain of N-WASP with pyrenil-labeled actin and

Table 2. CytD does not inhibit transcription of inducible *HoxA* and *Meis1* genes or of constitutively expressed Eps8 gene in NT2-D1 cells

Gene	Untr.	Untr. CytD at t = 0	RA 16 h	RA 16 h CytD at t = 0	RA 48 h	RA 48 h CytD t = 24 h	RA 72 h	RA 72 h CytD t = 48 h
HoxA1	0	0	1.0 (0.94–1.06)	1.07 (1.03–1.11)	1.0 (1.13–0.88)	0.946 (1.16–0.77)	ND	ND
HoxA2	0	0	0	0			ND	ND
Meis1	0.1 (0.114–0.1)	0.15 (0.12–0.20)	0	0	1.0 (1.25–0.8)	0.90 (0.98–0.82)	ND	ND
Eps8[a]	1.33 (1.6–1.1)	ND	ND	ND	1.0 (1.22–0.82)	0.885 (1.02–0.77)		
Eps8[a]	0.762 (0.65–0.9)	ND	ND	ND			1.0 (0.79–1.27)	0.92 (0.79–1.06)

The data are the average of triplicate quantitative PCR experiment performed at least twice. Time in hours (h) refers to the exposure to 1 μM RA. When present, CytD (100 nM) was added before the measurement, i.e., at t = 0 for the 16-h, at t = 24 for the 48-h, and at t = 48 for the 72-h measurement. The level of mRNA expression after induction (in the absence of CytD) is always set equal to 1.0; the values in parentheses represent the confidence interval at 95%. Untr., untranslated.

[a] Eps8 was measured in two experiments, with different times of induction and CytD addition.

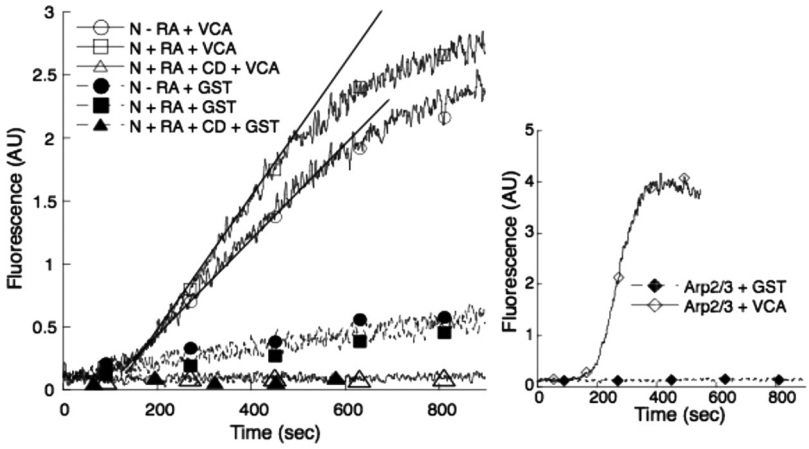

Figure 1. RA increases the polymerization rate in NT2-D1 nuclear extracts, in vitro. Nuclear extracts from NT2-D1 cells were prepared as described in *Materials and Methods*. Left, equal amounts of extracts were mixed with GST or GST-VCA (10 μg), as indicated, and subjected to the pyrene actin polymerization assay. The lines represent the initial rate of polymerization. Right, purified Arp2/3 complex (10 nM) was used as an internal control for the reaction. N, nuclear extract; RA, retinoic acid; CD, cytochalasin D.

nuclear extract of cells treated or not with RA. Remarkably, the rate of N-WASP–dependent actin polymerization was increased in nuclear extracts of RA-treated cells in a CytD-dependent manner (Figure 1).

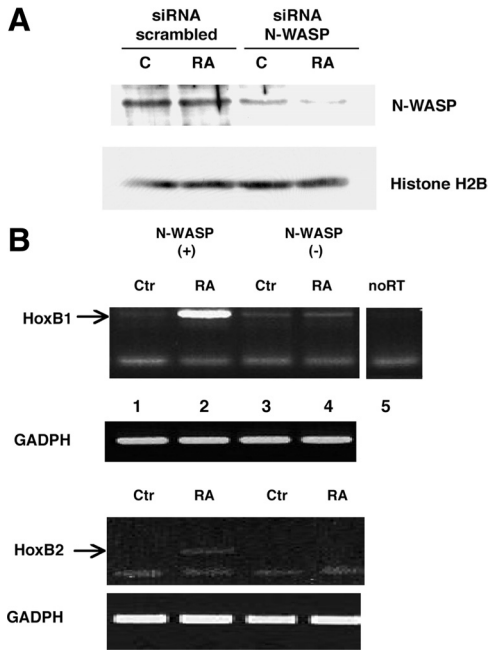

Figure 2. Down-regulation of N-WASP prevents *HoxB1* and *HoxB2* induction by RA. (A) Immunoblotting analysis to assess the down-regulation of N-WASP in NT2-D1 cells. Cells were transfected with an h-N-WASP–interfering oligonucleotide (siRNA) or with a scrambled oligonucleotide (see *Materials and Methods*). After 24 h, 1 μM RA was added, and 16 h thereafter proteins were isolated and immunoblotted with anti N-WASP and Histone 2b antibodies. (B) N-WASP down-regulation prevents *HoxB1* induction. RNA isolated in parallel from the same experiment of A, was subjected to semiquantitative RT-PCR with primers specific for the *HoxB1* and *GADPH* mRNA (see *Materials and Methods*). Lane 1, control, N-WASP$^+$ cells; lane 2, RA-treated N-WASP$^+$ cells (16 h) in which *HoxB1* is induced; lane 3, control N-WASP$^-$ cells; lane 4, RA-treated N-WASP$^-$ cells; lane 5, control transcription of the sample used in lane 2 performed in the absence of reverse transcriptase. At the bottom, the same RNA samples used for lanes 1–4 were used to measure *HoxB2* (with primers specific for HoxB2) and *GAPDH* RNA (see *Materials and Methods*). This experiment was carried out in duplicate with identical results.

We then decided to down-regulate N-WASP and to study the RA-induction of *HoxB1* and *HoxB2*. Transfection of an h-N-WASP RNAi oligonucleotide into NT2-D1 cells strongly reduced the level of N-WASP in total cell extracts (Figure 2A). The expression level of an internal control, histone H2B, was unaffected. We then compared by RT-PCR the induced level of *HoxB1* and *HoxB2* mRNAs at high (i.e., normal) and low levels of N-WASP. Figure 2B shows an experiment in which, when N-WASP is down-regulated, the induction of *HoxB1* and *HoxB2* mRNAs by RA (at 16 h) is abolished, whereas transcription of the constitutive GAPDH gene is not affected. These results confirm that actin polymerization is required at least for the transcription of the 3′ *HoxB* genes. They further provide a functional connection between RA-induced *HoxB* transcription and N-WASP. The different levels of *HoxB1* versus *HoxB2* mRNA in this experiment are expected, because at the time of measurement (24 h) *HoxB1* is already fully induced, whereas *HoxB2* transcription is at an earlier phase of induction (Simeone *et al.*, 1990).

We obtained identical results when we used N-WASP–specific siRNA oligonucleotides (SC36006), which totally abolished N-WASP expression (not shown).

A Nuclear-Targeted Dominant Negative Actin Mutant Blocks HoxB Induction by RA

To further demonstrate a direct role of actin polymerization in gene regulation, we used two actin mutant constructs that contain a nuclear localization signal (NLS) that allows the nuclear localization of the mutant actin forms (Chuang *et al.*, 2006; Dundr *et al.*, 2007). Of these, mRFP-NLS-G13R is deficient in actin polymerization and blocks F-actin polymerization in a dominant-negative manner (Chuang *et al.*, 2006), whereas mRFP-NLS-S14C favors nuclear actin polymerization (Chuang *et al.*, 2006). We transfected these constructs into NT2-D1 cells and confirmed by immunofluorescence the nuclear localization of both RFP-actin mutants (not shown). We measured *HoxB2* mRNA levels by real-time PCR after treating cells with RA for 24 h. Figure 3 shows that the G13R mutant decreased the level of RA-induced *HoxB2* expression in a dose-dependent manner. Conversely, the S14C mutant, which favors F-actin polymerization, had a slight enhancing effect on *HoxB2* expression. This result implicates actin polymerization in the process of transcriptional activation of the HoxB genes. Importantly the use of the nuclear targeted dominant-negative construct that blocks F-actin polymerization excludes the possibility that transcriptional inhibition of the HoxB genes could simply be

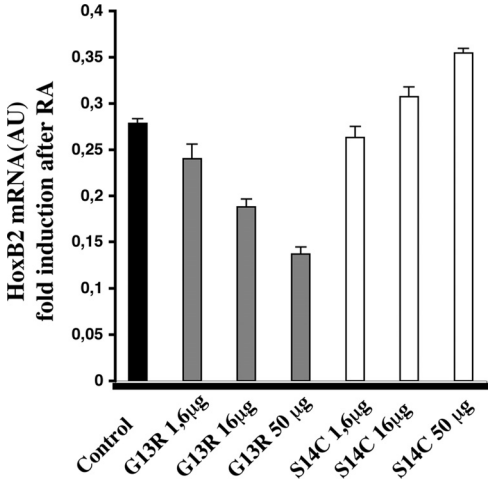

Figure 3. Effect of a dominant-negative actin mutant on the RA-induced *HoxB2* transcription. NT2-D1 cells were transfected with 1.6, 16, or 50 μg of DNA encoding either of two variants of actin containing a nuclear localization signal, the G13R mutant that is polymerization-defective (dominant-negative) and the S14C mutant, which is facilitated in polymerization. Cells were treated with RA for 24 h, after which RNA was extracted and HoxB2 mRNA measured by quantitative PCR. The data are the average of at least two independent experiments performed in triplicate. Error bars, SD.

a secondary effect resulting from an alteration in cytoplasmic actin polymer levels.

Overall, our data show, using three distinct and independent approaches, that a block of actin polymerization inhibits the RA-dependent transcription of *HoxB* genes.

The Role of RA Receptors and Prep1 in Actin-dependent HoxB Induction by RA

Because RARs are important in *HoxB* expression in the neural region (Marshall *et al.*, 1994, 1996; Gould *et al.*, 1998), we tested whether treatment with CytD affected the expression of any of these receptors. As shown in Table 3, CytD did not affect the expression levels of any of the RAR and retinoid X receptor (RXR) transcription factors, either in the absence or in the presence of RA. Therefore, the effect of CytD on *HoxB* transcription cannot be due to interference with the expression of the RARs. Our results here also confirm that not all inducible genes are sensitive to CytD (see RARα, Table 3).

Expression of the 3′ *HoxB* genes requires Prep1 both in cell culture (including NT2-D1 cells) and in vivo (Ferretti *et al.*, 2000, 2005; Deflorian *et al.*, 2004; Diaz *et al.*, 2007b). We

Table 3. Lack of effect of 100 nM CytD on the expression of RARs in RA-treated NT2-D1 cells

Gene	Control	16 h, RA	16 h, CytD	16 h, RA + CytD
RARα	1.0	5.5	0.855	5.01
RARβ	Absent	Absent	Absent	Absent
RARγ	1.0	1.1	1.35	1.15
RXRα	1.0	1.09	1.35	1.15
RXRβ	1.0	1.1	1.37	1.42
RXRγ	Absent	Absent	Absent	Absent

Cells were treated (or not) with 10 μM RA and/or 100 nM CytD at t = 0, and the level of mRNA was assessed after 16 h.

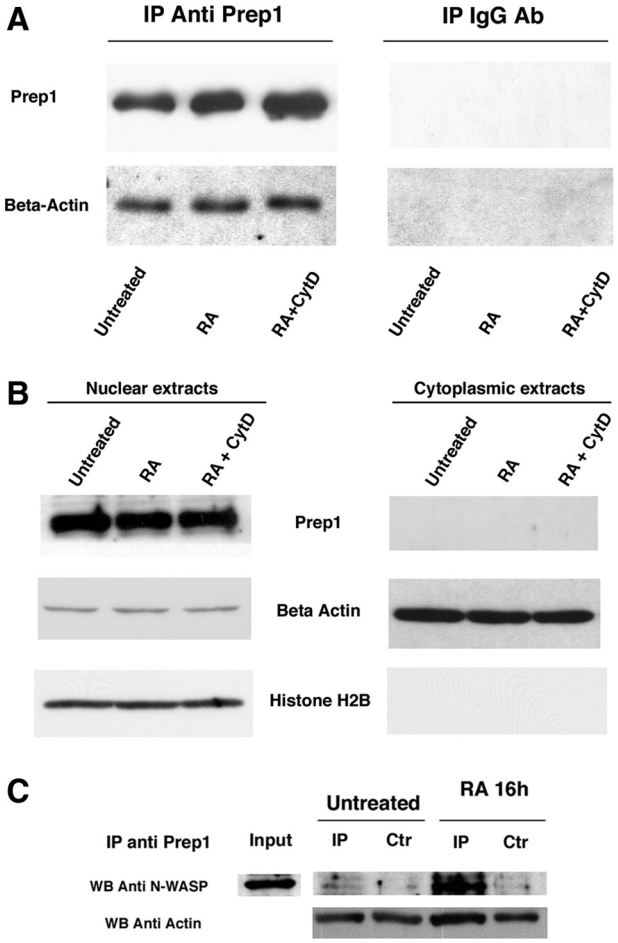

Figure 4. Prep1 interaction with actin and N-WASP. (A) Nuclear extracts of untreated, 1 μM RA- and RA + CytD-(100 nM) treated NT2-D1 cells were immunoprecipitated with an anti-Prep1 antibody and blotted against a β-actin antibody. Right panel, the results with an irrelevant antibody. (B) RA and CytD do not modify the nuclear localization of Prep1. Nuclear or cytoplasmic extracts of NT2-D1 cells treated as indicated were immunoblotted with the indicated antibodies. An anti-histone and an anti-β-actin 2B antibody was used to assess equal loading of extracts. (C) RA-dependent interaction of Prep1 with N-WASP. Nuclear extracts from untreated or RA-treated (24 h) cells were immunoprecipitated with anti-Prep1 (IP) or control (Ctr) antibodies and blotted with N-WASP and actin antibodies.

previously showed that β-actin was specifically copurified with Prep1 from the nucleus (but not from the cytoplasm) of NIH-3T3 cells (Diaz *et al.*, 2007a). We immunoprecipitated nuclear extracts from control, RA- (1 μM), or RA + CytD– (100 nM) treated cells with anti-Prep1 antibodies, and the cells were immunoblotted with actin antibodies. Figure 4A confirms this interaction between Prep1 and β-actin and demonstrates that it is constitutive and unaffected by CytD. The interaction of Prep1 and actin is most likely indirect because purified recombinant Prep1 and polymerized actin did not cosediment after high-speed ultracentrifugation (90,000 $\times$ *g*; not shown).

Previous experiments showed that the use of drugs that inhibit actin polymerization control the nuclear translocation of the MAL transcription factor (Vartiainen *et al.*, 2007). To test whether CytD affects Prep1 nucleocytoplasmic distribution, we analyzed nuclear and cytoplasmic extracts from control, RA-, or RA + CytD-treated cells by immuno-

blotting. Our data show that these treatments do not affect Prep1 levels or its nuclear localization (Figure 4B). It is therefore unlikely that inhibition of actin polymerization acts via the sequestration of Prep1.

Finally, because both Prep1 (Diaz *et al.*, 2007b) and N-WASP (see above) are required for HoxB induction in NT2-D1 cells, we tested for the presence of a Prep1–N-WASP interaction in NT2-D1 nuclear extracts by immunoprecipitating with anti-Prep1 and blotting with anti-N-WASP antibodies. N-WASP was immunoprecipitated by anti-Prep1 antibodies only in extracts of RA-treated cells (Figure 4C). Overall, these results suggest that RA induces actin polymerization by recruiting N-WASP into a complex that may already contain actin and Prep1.

In the nucleus, N-WASP is associated with p54Nrb–PSF (Wu *et al.*, 2006), a protein complex that interacts with both RNAPII and the splicing machinery (Shav-Tal and Zipori, 2002). We therefore tested the association of Prep1 with p54Nrb–PSF. An anti-Prep1 antibody pulled down both p54Nrb and PSF from nuclear extracts of untreated NT2-D1 cells (Figure 5A), in addition to the known Prep1 interactor Pbx1 (not shown). Prep1 was, likewise, immunoprecipitated by PSF antibodies (not shown). We also confirmed (not shown) that PSF antibodies pulled down actin and N-WASP, as previously shown (Wu *et al.*, 2006).

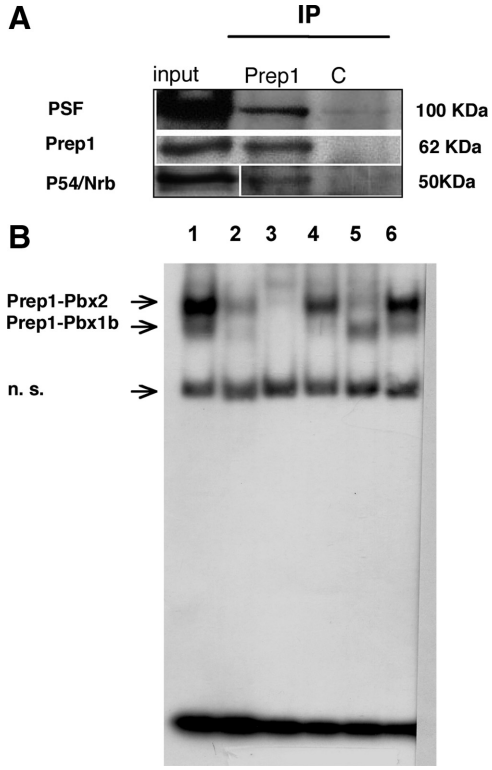

Figure 5. DNA-binding Prep1 is associated with p54Nrb and PSF. (A) Nuclear extracts of untreated NT2-D1 cells were immunoprecipitated with a monoclonal anti-Prep1 or a control (C) antibody and blotted using polyclonal PSF, Prep1, or p54Nrb antibodies. (B) P54Nrb is present on the DNA-binding Prep1–Pbx complexes. EMSA analysis of HeLa nuclear extracts with the specific Prep1–Pbx-binding oligonucleotide (see *Materials and Methods*). n.s., nonspecific band. The nuclear extract from HeLa cells (lanes 1 and 6) forms two specifically retarded bands. Prep1 antibodies prevent the formation of both bands (lane 2). The lower band is inhibited by the anti-Pbx1 antibodies (lane 4), whereas the upper band is inhibited by the anti-Pbx2 (lane 5) antibodies. Therefore the two bands are identified as Prep1–Pbx1b (lower band) and Prep1–Pbx2 (upper band) as previously reported (Berthelsen *et al.*, 1998). Formation of both bands is inhibited by anti-p54Nrb antiserum (lane 3).

In preliminary experiments in HeLa cells (in which Prep1 and p54Nrb and PSF are rather abundant), we found that the Prep1–Pbx complex is bound to the p54Nrb component of the p54Nrb–PSF complex (not shown). We also showed that p54Nrb, at least, is part of the Prep1–Pbx DNA-binding complex. We previously showed that, in nuclear extracts of HeLa cells, Prep1 is bound to either of the two isoforms of Pbx, Pbx1b, and Pbx2, forming DNA-binding complexes (Berthelsen *et al.*, 1998). We performed EMSA to test whether p54Nrb is associated with these DNA-binding complexes. As shown in Figure 5B the two bands that formed with the specific labeled oligonucleotide (lane 1) correspond to Prep1–Pbx1a and Prep1–Pbx2 complexes, as expected. Indeed, anti-Prep1 antibodies inhibited the formation of both complexes (lane 2); anti-Pbx1 antibodies inhibited formation of only the lower complex (lane 4), whereas anti-Pbx2 inhibited only the upper complex (lane 5). Importantly, anti-p54Nrb antibodies inhibited the formation of both complexes (lane 3), whereas preimmune IgGs had no effect (lane 6). We conclude that the DNA-binding Prep1–Pbx complex is constitutively associated with p54Nrb.

RA Recruits Prep1, RNAPII, the Actin-Polymerization Machinery, and the p54Nrb–PSF Complex onto the Enhancer of the HoxB2 Gene

To confirm the above results we tested whether RA was able to recruit to the regulatory regions of the *HoxB* genes a complex containing Prep1, actin, and associated components like RNAPII, p54Nrb, PSF, and N-WASP. We immunoprecipitated sonicated, cross-linked chromatin from untreated, RA-, and RA + CytD-treated NT2-D1 cells with antibodies against Prep1, β-actin, N-WASP, p54Nrb, PSF, or RNAPII-S2p and tested by quantitative PCR whether the *HoxB2* enhancer was enriched in the immunoprecipitated material. Hyperphosphorylation of the C-terminal domain of RNAPII is required for its active engagement in transcription. In particular, the initiation form of RNAPII is phosphorylated at serine 5 (RNAPII-S5p), whereas the elongating form of the enzyme is phosphorylated at serine 2 (RNAPII-S2p). Because RNAPII-S5p has recently been shown to be often associated with genes that are on the point of being, but are not yet, transcribed (Stock *et al.*, 2007; Core and Lis, 2008; Ferrai, unpublished data), we used antibodies that recognize RNAPII-S2p as a mark of active transcription. As shown in Figure 6, in the absence of RA, when *HoxB2* is not transcribed, none of the antibodies immunoprecipitated the *HoxB2* enhancer. However, a strong enrichment of the *HoxB2* enhancer was detected after the immunoprecipitation with all the antibodies (Prep1, β-actin, N-WASP, p54Nrb PSF, and RNAPII-S2p) in cells treated with RA for 24 h, a condition in which *HoxB2* is transcribed. No enrichment was seen using unrelated control antibodies and the specificity of the immunoprecipitation was further confirmed by the absence of signal in experiments performed using an intergenic region (of the *uPA* gene; Figure 6). Importantly, cotreatment of RA-induced cells with 100 nM CytD completely prevented the immunoprecipitation of the *HoxB2* enhancer sequences by all antibodies, including RNAPII-S2p.

This experiment demonstrates that the "active" RNAPII, the specific transcription factor Prep1, the actin polymerization machinery (or, at least, actin and N-WASP) and the two N-WASP– and Prep1-associated factors p54 and PSF are recruited by RA to the enhancer of the *HoxB2* gene. Impor-

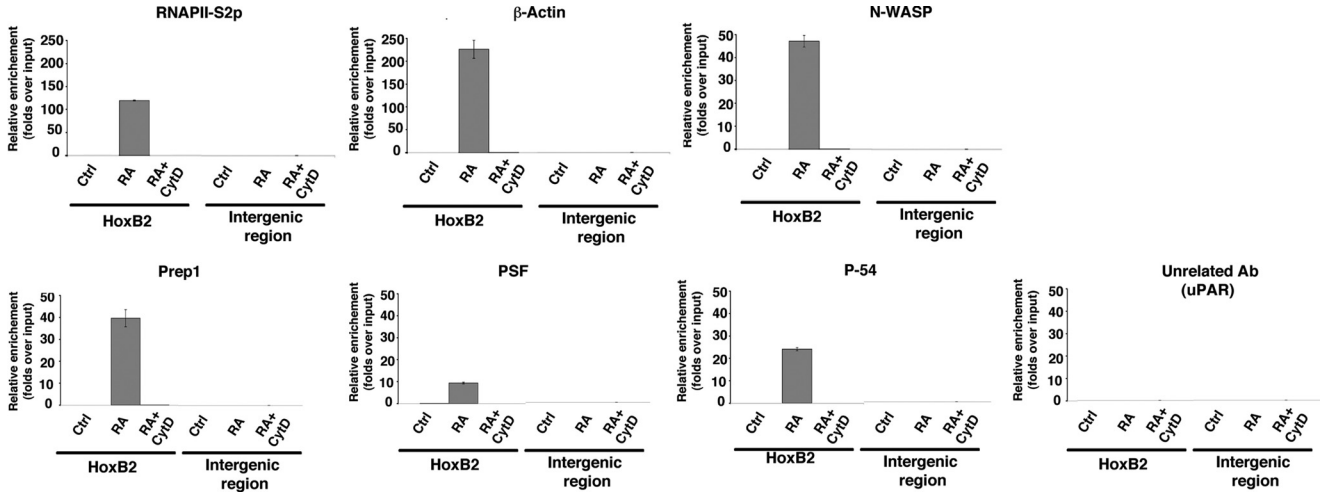

Figure 6. RA treatment induces the recruitment of Prep1, actin, N-WASP, p54Nrb, PSF, and the elongating form of RNAPII on the enhancer of the *HoxB2* gene, but this recruitment is prevented by CytD. Chromatin immunoprecipitation analysis of NT2-D1 cells (untreated, 24-h RA-treated and 24-h RA + CytD-treated). Cross-linked chromatin was immunoprecipitated with the indicated antibodies and the precipitated DNA amplified by quantitative PCR with oligonucleotides specifically identifying the *HoxB2* enhancer or the *uPA* intergenic region (see *Materials and Methods*). The antibodies used are indicated on top. Antibodies to RNPII-S2p recognize the elongating form of the RNAPII enzyme. Results and error bars shown are the average and SD of quantitative PCRs performed in triplicate from three independent chromatin immunoprecipitation experiments. Values are reported as fold enrichment relative to input DNA.

tantly, this recruitment is completely abrogated by CytD. This result not only functionally implicates actin polymerization in the regulation of expression of the *HoxB* genes, but in particular identifies the mechanism by which actin polymerization affects *HoxB* induction. Therefore, we conclude that actin polymerization is functionally implicated in the transcriptional regulation of *HoxB* genes, showing for the first time that actin polymerization is required to recruit the transcription complex onto the regulatory region of *HoxB2*.

DISCUSSION

The role of actin and its participation in transcription by all three forms of RNAP is well documented in vivo and in vitro (Visa, 2005; Percipalle and Visa, 2006). However, despite the importance of nuclear actin, the exact role of actin polymerization and its impact in transcription is still unclear. In this article, we have shown that the RA induction of the *HoxB* genes is inhibited by rather low doses of CytD and LatA, by the down-regulation of N-WASP, and by the use of a dominant-negative actin mutant. On the other hand, F-actin stabilizers jasplakinolide and phalloidin as well as a dominant-positive actin mutant slightly enhance *HoxB* transcription. Overall our data demonstrate that the induction of the *HoxB* genes requires actin polymerization.

Importantly because CytD and LatA inhibit *HoxB* induction only when added *before* the start of transcription (Table 1), actin polymerization appears to be required for the initiation of transcription. Moreover, other RA-inducible genes, such as *HoxA1, -A2,* and *Meis1* or the constitutively expressed gene *Eps8* (Tables 1 and 2), are not affected by CytD and LatA. Likewise, although the down-regulation of N-WASP prevents *HoxB1* and *HoxB2* induction, it has no effect on the levels of expression of the housekeeping gene GAPDH (Figure 1). Even the RA-inducible RARα gene was not sensitive to CytD (Table 3). Thus, it appears that specific genes are differentially sensitive to the inhibition of actin polymerization.

Previous data have shown that CytD affects general transcription (McDonald *et al.,* 2006). However, these data were obtained at a concentrations (1 μM) of CytD 10-fold higher than those used in the present article, in which we nevertheless achieved a strong, but incomplete inhibition of actin polymerization (McDonald *et al.,* 2006). The intriguing evidence that different subsets of genes could be differentially sensitive to the inhibition of actin polymerization is new. On the basis of the present and of the literature data, we conclude that actin polymerization is generally required for transcription; however, this requirement may vary from gene to gene. Our data give no indication that different forms of polymeric actin (possibly differentially sensitive to CytD) are involved, although this cannot be excluded.

The possibility that CytD or N-WASP down-regulation might affect *HoxB* transcription by regulating the subcellular localization of Prep1 was considered because CytD inhibits the nuclear translocation of MAL (Vartiainen *et al.,* 2007). However, Prep1 is present in the nucleus of NT2-D1 cells both before and after RA treatment, and its localization is unaffected by CytD (Figure 4B). Furthermore, because a dominant-negative actin mutant with a NLS also inhibits *HoxB* induction, the inhibitory effect of CytD is likely due to its inhibitory effect on nuclear actin polymerization.

We also report that RA treatment slightly affects actin polymerization in nuclear extracts. The conditions we used favor the formation of a tripartite complex between monomeric actin, the WCA domain of N-WASP and Arp2/3, leading to a slight but detectable enhancement of actin polymerization (Figure 1). Although the molecular mechanism through which RA controls this process is unclear, it is conceivable that the assembly of sequence-specific transcription factors and of an actin-polymerization–competent complex is coordinated on at least some RA-dependent promoters, providing spatially confined actin dynamics that aid transcription initiation.

We also found that Prep1 (which is normally present in a DNA-binding complex with Pbx1) is associated with p54Nrb and PSF. These two accessory splicing factors also bind RNAPII and other transcription factors (Shav-Tal and Zipori, 2002) as well as N-WASP (Wu *et al.,* 2006), whose down-regulation prevents *HoxB* induction by RA (Figure 2).

Although in a different cell model (HeLa cells), we show that Prep1 and Pbx1 bind p54Nrb and that the resulting complex still binds DNA (Figure 5B), which indicates that these proteins can be recruited to the same DNA target sequence. Indeed these two proteins are recruited in NT2-D1 cells after induction with RA, together with actin, Prep1, N-WASP, and RNAPII onto the enhancer of the *HoxB2* gene. Importantly, none of these proteins is associated with DNA in the absence of RA induction or when the RA induction of gene expression is prevented by CytD treatment (Figure 6). These data demonstrate that the recruitment of these proteins in vivo to the enhancer of a *HoxB* gene requires both actin polymerization and RA.

RA therefore induces the recruitment of both sequence-specific DNA-binding elements (Prep1 at the very least, but more likely the Prep1–Pbx1 dimer or the Prep1–Pbx1-HoxB1 trimer; Ferretti *et al.*, 2000, 2005), actin, and its polymerizing enzyme(s) N-WASP and RNAPII.

In the chromatin immunoprecipitation experiment of Figure 6, the presence of the active form of RNAPII-S2p on the *HoxB2* enhancer demonstrates that the RA-recruited proteins are actively involved in transcription. These data are therefore in complete agreement with the inhibition of *HoxB* transcription by all conditions that block actin polymerization. The same complex is also likely present on the enhancer of *HoxB1* (because Prep1 also regulates this gene; Ferretti *et al.*, 2000, 2005). The nature of the β-actin–Prep1 interaction is still unknown. Although the two proteins can easily be coimmunoprecipitated (Figure 4A), the interaction is most likely indirect. The protein connecting Prep1 to actin is probably N-WASP, a member of the WASP family of proteins that regulates actin filaments in the cytoplasm and whose nuclear localization can be regulated by its activation state and by phosphorylation (Wu *et al.*, 2004). In the nucleus of 293T cells, N-WASP is associated with the p54Nrb–PSF complex (Wu *et al.*, 2006). Because the p54Nrb–PSF complex binds both N-WASP (Wu *et al.*, 2006) and Prep1–Pbx1 (Figure 5A), it might also be the link between the actin polymerization machinery and Prep1–Pbx1. Preliminary experiments from our laboratory have demonstrated that p54Nrb binds the Pbx1 moiety of the Prep1–Pbx1 complex (Palazzolo and Blasi, unpublished data).

The interaction of actin with Prep1 and its presence on the enhancer of *HoxB2* is functionally relevant because *HoxB2* expression in vivo during embryogenesis depends on the activity of the Prep1–Pbx1 complex (Waskiewicz *et al.*, 2002; Deflorian *et al.*, 2004; Di Rosa *et al.*, 2007). Here we show that actin polymerization is involved in the induction of the *HoxB* genes by regulating the recruitment of the large transcriptional complex on the regulatory sequence. Our results not only reinforce previous data showing that actin plays a key role in transcription, but highlight the participation of actin polymerization in orchestrating transcription and in fine tuning the transcriptome, acting at different levels in different subclasses of genes.

ACKNOWLEDGMENTS

The authors dedicate this article to the memory of Ludovico Faini. We thank Ana Pombo and Tom Misteli for critically reading the manuscript. Flavia Troglio and Maud Hertzog helped with the N-WASP RNAi design and the in vitro actin polymerization. We thank Laura Tizzoni from the IFOM PCR facility for QT-PCR. This work was supported by Italian Association for Cancer Research (AIRC) and Telethon Onlus to F.B. and from AIRC and Ministero della Sanità to M.P.C. V.M.D. was a recipient of an EU Marie Curie fellowship. The authors are indebted to Dr. A. Krainer and J. Patton for their generous gifts of cDNA and antibodies directed against p54 and PSF and to Andrew Belmont (University of Illinois, Urbana, IL) for providing the mRFP-NLS-G13R and mRFP-NLS-S14C actin mutants.

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