Published online 26 November 2007

Nucleic Acids Research, 2008, Vol. 36, No. 2 ***435–443***
doi:10.1093/nar/gkm1058

Retinoic acid modulates chromatin to potentiate tumor necrosis factor alpha signaling on the *DIF2* promoter

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Received July 25, 2007; Revised October 18, 2007; Accepted November 9, 2007

ABSTRACT

Transcriptional activation by nuclear hormone receptors is well characterized, but their cooperation with other signaling pathways to activate transcription remains poorly understood. Tumor necrosis factor alpha (TNFα) and all-*trans* retinoic acid (RA) induce monocytic differentiation of acute promyelocytic leukemia (APL) cells in a synergistic manner. We used the promoter of *DIF2*, a gene involved in monocytic differentiation, to model the mechanism underlying the cooperative induction of target genes by RA and TNFα. We show a functional RA response element in the *DIF2* promoter, which is constitutively bound by PML/RARα in APL cells. RA stimulates release of corepressors and recruitment of chromatin modifying proteins and additional transcription factors to the promoter, but these changes cause only a modest induction of *DIF2* mRNA. Co-stimulation with RA plus TNFα facilitates binding of NF-κB to the promoter, which is crucial for full induction of transcription. Furthermore, RA plus TNFα greatly enhanced the level of RNA Pol II phosphorylation on the *DIF2* promoter, via synergistic recruitment of TFIIH. We propose that RA mediates remodeling of chromatin to facilitate binding of transcription factors, which cooperate to enhance Pol II phosphorylation, providing a mechanism whereby nuclear receptors interact with other signaling pathways on the level of transcription.

INTRODUCTION

Nuclear receptor mediated transcription is a multi-step process involving an allosteric change in receptor conformation followed by an exchange of corepressors and histone deacetylases (HDACs) for coactivators with histone acetyl transferase (HAT) activity (1). These co-activators are thought to regulate transcription through local histone modifications, as well as further recruitment of complexes with ATP-dependent chromatin remodeling activities. These actions can result in the formation of a mature preinitiation complex and induction of target gene transcription (2). The physiological relevance of tight control of nuclear receptor target genes is underscored by the association of malfunctional mutant receptors with disease, such as the link between APL and the expression of the PML/RARα oncoprotein, which blocks transcription of RA target genes via an increased affinity for corepressors (3,4). Pharmacologic concentrations of RA release corepressors from PML/RARα, induce transcription of RARα target genes and thereby stimulate granulocytic differentiation.

Previously, we and others have found that RA and TNFα can act in synergy to promote differentiation of leukemia cells (5–7) as well as normal monocytes (8). Other signaling pathways including that of cyclic AMP (9) can also act in synergy with RA to achieve differentiation. However, the exact mechanisms through which these biologic effects are achieved remain largely unknown. A likely point of convergence for these pathways is the synergistic activation of common target genes, although few studies have investigated the mechanisms whereby nuclear hormone receptor pathways synergize

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with other signaling pathways to induce immediate early genes.

To study the mechanism of transcriptional synergy between RA and TNFα in leukemia cells, we used the promoter of the *DIF2* gene (also known as *IER3*, *IEX-1* or *PRG1*) as a model. *DIF2* encodes an anti-apoptotic protein that is specifically expressed in monocytes (10,11), and may be directly involved in the differentiation process stimulated by RA in combination with TNFα (5,6). The promoter of this gene contains binding sites for several transcription factors, including an NF-κB responsive element (11,12) and a putative retinoic acid response element (RARE) (13). In the APL cell line NB4, *DIF2* induction by TNFα alone is modest, consistent with the very limited differentiation induced by TNFα in these cells (6).

We now present evidence that PML/RARα can bind to the *DIF2* promoter and, in the presence of RA, induce chromatin changes associated with active transcription. In this setting, co-treatment with TNFα induces substantial NF-κB binding, Pol II phosphorylation and gene induction on this promoter. Importantly, similar results were found in non-APL cells, indicating that this mechanism is not limited to the PML/RARα oncoprotein, but also applies to wild-type RARα.

We propose that the chromatin remodeling effects of liganded RARα can facilitate binding of transcription factors from other signaling pathways and that the cooperative actions of these activators direct phosphorylation of Pol II, thereby stimulating a synergistic activation of transcription.

MATERIALS AND METHODS

Cell culture and reagents

NB4 and U937 cells were cultured in RPMI-1640 supplemented with 10% FBS. Trichostatin A (TSA, Sigma) was used at a final concentration of 200 nM. RA (Sigma) was used at a concentration of 1 μM. TNFα (Peprotech) was used at 10 ng/ml. The IKKβ inhibitor compound A (14) was used at 5 μM.

Northern blotting

Ten microgram of RNA was electrophoresed on a 1% formaldehyde agarose gel and blotted onto Zeta probe (Bio-Rad) transfer membranes. cDNA probes were labeled by random priming (Pharmacia-Amersham). Hybridization and autoradiography was performed as previously described (6). *DIF2* full-length cDNA was a kind gift of Dr G. Schmitz.

Quantitative PCR

cDNA was prepared from 5 μg total RNA, using Superscript II reverse transcriptase (Invitrogen). Quantification of *DIF2* gene expression was performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following primers: 5′-CGCTCTGGACCTCAGCAC TT-3′ (forward) and 5′-TGTTTCTTTGTGGTTTTTCG GATT-3′ (reverse) and SYBR® Green based detection. Amplification was performed with 40 cycles of 95°C for 15 s and 60°C for 60 s. GAPDH was used as the endogenous control.

Western blotting

Thirty microgram of nuclear extracts, prepared as previously described (6), were used for western blotting to detect nuclear p65 and Pol II protein levels. Both antibodies were from Santa Cruz biotechnologies.

Chromatin immunoprecipitation (ChIP)

The Upstate biotechnology protocol was used with minor modifications. A total of 2×10^6 cells were treated with RA and/or TNFα or TSA for the indicated time periods. Protein–DNA complexes were immunoprecipitated overnight at 4°C with antibodies against SMRT, AIB1 (Affinity Bioreagents), Pol II phosphoserine-5 (Covance), SNF5 (a kind gift of Dr G. Crabtree), CBP, HDAC1, PML, Pol II, RARα, TBP, TFIIH (p62), p50, p65, PU.1 and SP1 (Santa Cruz), acetyl histone H4, H3 di/trimethyl-lysine 4, dimethyl-arginine 17 (Upstate) and CARM1/PRMT4 antibodies (a gift of Dr S. Richard). Antibody complexes were pulled down for 4 h with 60 μl protein A sepharose slurry (Upstate). To reverse the formaldehyde-induced cross-linking, NaCl was added at a final concentration of 0.2 M and incubated overnight at 65°C. DNA was phenol/chloroform extracted followed by ethanol precipitation using tRNA as a carrier. Oligonucleotides used for amplification of different areas of the *DIF2* gene were as follows: #1a 5′-GGAAATCGAGAC CACCCTGG-3′, #1b 5′-CAGTCTCGTTCGGTCG CCA 3′, #2a 5′-GTGAGGGATCCTGTGGCTAA-3′, #2b 5′-CGGGTCCTTCTAACTCCTCC-3′, #3a 5′-CCT GGGTGACAGTGAAATCC-3′, #3b 5′-TCAGCTCACT GCAACCTCTG-3′, #4a 5′-CCACTCCTTTCCAGCCA TTA-3′, and #4b 5′-GAGGCTTCATGGAAGAGT GG-3′. Primers surrounding the NF-κB site of the IL8 promoter region were as previously described (15). For quantitative PCR, amplification was performed using primers 2a and 2b with 40 cycles of 95°C for 15 s and 60°C for 60 s. Input DNA was used as the endogenous control. Each treatment was performed in duplicate, amplifications were performed in triplicate, and results are represented as means of six measurements ±SEM.

RESULTS AND DISCUSSION

Identification of an RAREs that may mediate enhancement of *DIF2* expression by RA

By northern blotting, we showed an early induction of *DIF2* mRNA production after treatment of PML/RARα positive NB4 cells with either RA or TNFα. Induction by TNFα alone was significantly stronger than with RA alone, but co-treatment with both drugs was required for maximal induction. Quantitative RT-PCR verified that RA alone triggers a weak induction of *DIF2* expression, but strongly potentiates its induction by TNFα (Figure 1A).

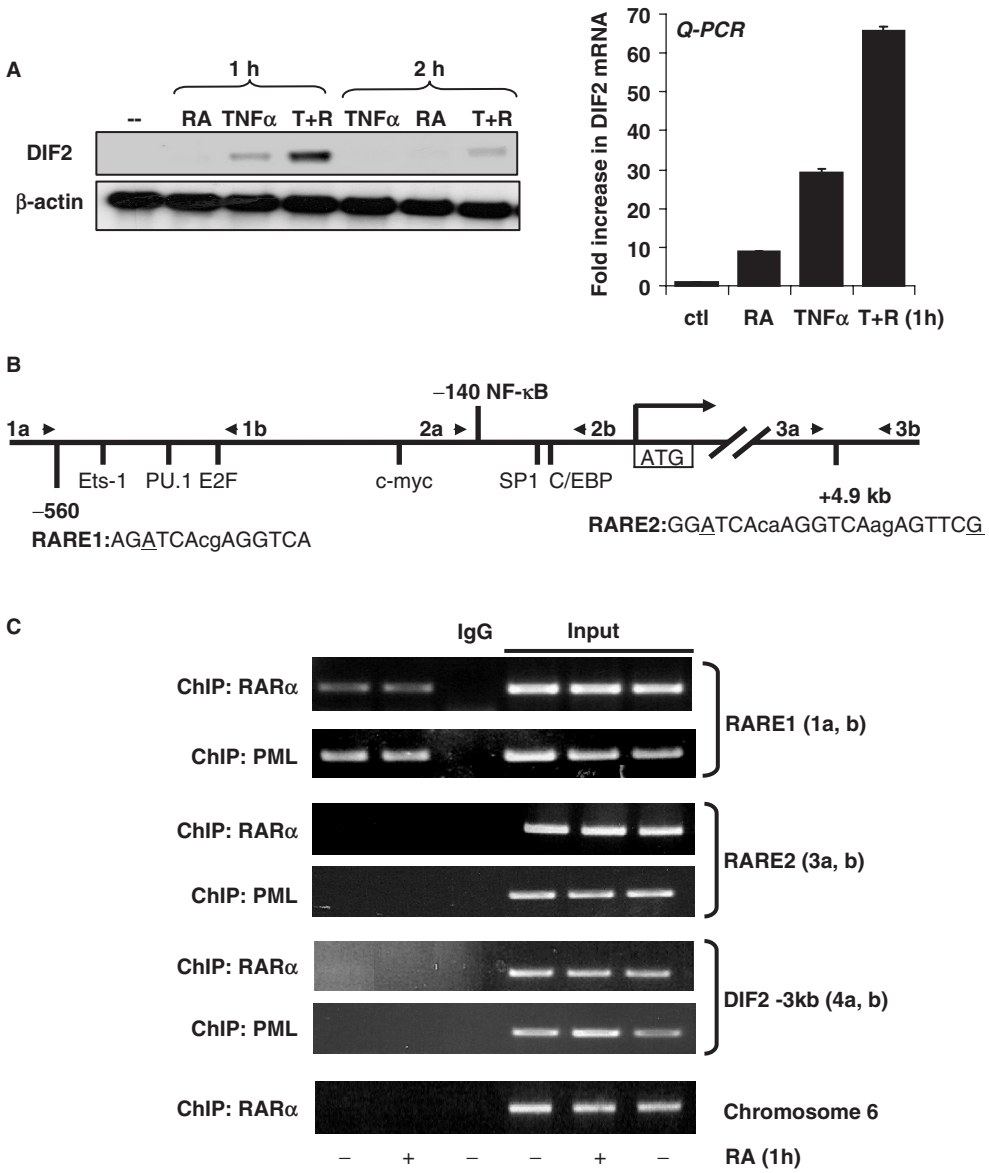

Figure 1. TNFα and RA cooperate to activate *DIF2* transcription. (**A**) Northern blot showing *DIF2* mRNA levels in response to RA and TNFα. T + R designates co-treatment with TNFα and RA. The graph to the right shows relative induction of *DIF2* as determined by Q-PCR of NB4 cells treated for 1 h. (**B**) Overview of the *DIF2* promoter region showing putative transcription factor binding sites. Arrows represent position of primers employed in ChIP experiments. Underlined bases indicate deviations from a perfect, consensus RARE sequence (**C**) ChIP assays showing association of PML/RARα with RARE1, but not RARE2, in the *DIF2* promoter. Immunoprecipitation with mouse or rabbit IgG was used as a control for antibody specificity. Primers encompassing a region 3 kb upstream of the *DIF2* start site and chromosome six primers corresponding to a region nonadjacent to the *DIF2* gene were used to show specificity of the amplification.

To identify putative RAREs in the *DIF2* gene that may mediate the effects of RA, we screened 5 kb upstream and downstream of the transcriptional start site for direct repeats (DR) of the half-site (A/G)G(G/T)TCA, using the net-based programs CONSITE and TRANSFAC® TFBlast. The maximal deviation from the consensus sequence was restricted to one position. Upstream of the *DIF2* start site, we identified a putative DR2 RARE at position −560 (named RARE1, Figure 1B) having the sequence AGATCAcgAGGTCA. Two overlapping DR2 elements were also found downstream of the start site, at position +4912 (named RARE2). Of note, a putative NF-κB site was found close to RARE1, at position −140, while no NF-κB site was found in the vicinity of RARE2. By ChIP analysis using antibodies selective for RARα or PML, we showed that PML/RARα associates with RARE1 *in vivo*. The receptor was found to be constitutively bound to this site and the presence of ligand did not alter the association (Figure 1C). EMSA experiments also showed PML/RARα binding to this sequence *in vitro* (data not shown). In contrast, no association of PML/RARα with RARE2 was detected, in the absence or presence of RA (Figure 1C). Using primers specific to an area 3 kb upstream of the *DIF2* start site and primers to a region

of chromosome six nonadjacent to the *DIF2* gene, we confirmed specificity of the PML/RARα association with RARE1 (Figure 1C).

RA recruits co-activators and chromatin modifying complexes to the *DIF2* promoter region and induces changes associated with active transcription

Having established that *DIF2* is cooperatively induced by RA and TNFα within 1 h, we deemed it to be a useful model system to investigate the mechanism whereby the two pathways integrate on a transcriptional level. Since PML/RARα was found constitutively bound to RARE1 in the *DIF2* promoter, and RA induced some transcription, we first investigated the ability of RA to recruit different co-stimulatory molecules to this promoter region. RA stimulation has been demonstrated to recruit co-activators and chromatin modifying activity to RAR target genes within 2 h (16–18), and in agreement with this, we found that the corepressor SMRT dissociated from the *DIF2* promoter and was replaced by co-activators CBP, TRAP220 (MED1) and ACTR (AIB1, p/CIP, SRC3) within 1 h of RA treatment (Figure 2A). These changes further verified the presence of a functional RARE in the promoter. We did not find the histone methyltransferase CARM1 to be recruited to the promoter, nor was its target, arginine 17 of histone 3, methylated upon RA stimulation (data not shown). This is consistent with a previous report suggesting that CARM1 or ACTR are recruited to the oestrogen receptor in mutually exclusive complexes (19).

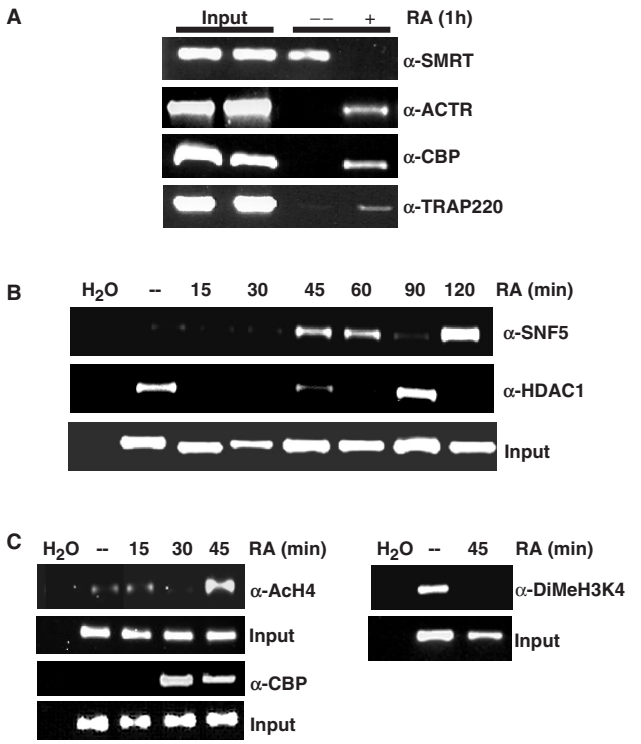

Figure 2. RA treatment leads to cofactor exchange on the *DIF2* promoter. (**A**) Chromatin was isolated from NB4 cells treated with RA as indicated, and amplifications of ChIP product using primers 1a,b show that RA stimulation leads to recruitment of ACTR, TRAP220 and CBP to the *DIF2* upstream region while causing a release of SMRT. (**B**) ChIP analysis demonstrating inverse temporal cycling of SNF5 and HDAC1 in response to RA. (**C**) ChIP analysis of specific histone modifications showing an increase in H4 acetylation and a concurrent decrease in H3K4 methylation after 45 min of RA treatment.

A previous study using *in vitro* reconstituted chromatin found an ordered recruitment of protein complexes to the RARα prior to transcriptional activation (20). It was found that components of the SWI/SNF complex could also augment RAR-driven transcription when added to the reaction subsequent to complexes having acetyltransferase activity. Therefore, we looked at SWI/SNF association with the *DIF2* promoter in response to RA, using antibodies toward the SNF5 catalytic subunit of the complex. The chromatin remodeler was seen complexed with the *DIF2* promoter within 45 min of RA treatment and its binding was inversely correlated with the repressor HDAC1 (Figure 2B). Interestingly, SNF5 cycled on and off the *DIF2* promoter in a manner similar to that previously reported for the BRG1 component of the same complex on the oestrogen-responsive pS2 promoter (19). These data indicate that activation of PML/RARα results in cycling of corepressors and activator proteins, as has been observed with wild-type nuclear receptors (18,21). Of note, even though the *DIF2* promoter displayed many aspects of a transcriptionally active gene upon RA treatment, only a modest increase in mRNA was observed in the absence of TNFα (Figure 1A), indicating that chromatin remodeling in response to RA is not enough to fully activate transcription of this gene.

To further address this issue, we investigated the functionality of the co-activators recruited by RA. We found a functional HAT complex to be recruited by RA as evidenced by a marked increase in levels of histone acetylation surrounding RARE1 (Figure 2C, left panel). Histone acetylation was observed shortly after recruitment of the histone acetyltransferase CBP and concurrently with SNF5 recruitment to the *DIF2* promoter (Figure 2B and C), consistent with a previous report showing that SWI/SNF can augment RARα induction of transcription on chromatin templates when added subsequent to histone acetylation (20). Studies in yeast have also indicated that recruitment of the SWI/SNF complex may require histone acetylation (22). Of note, amplification with primers 3a, b and 4a, b detected no increase in histone acetylation around RARE2 or further upstream of the *DIF2* start site (data not shown). We further assessed changes in histone lysine methylation after 45 min of RA treatment, when acetylation was seen to be increased. In contrast to reports showing an increase in H3 lysine 4 methylation upon RA stimulation (23), we observed a decrease in dimethylated H3 lysine 4 without an increase in trimethylated H3 lysine 4 (Figure 2C, right panel, and data not shown). This is similar to what was seen in a *xenopus* oocyte system with the thyroid receptor (24). A recent report also demonstrated that recruitment of histone demethylases and a transient decrease in histone methylation is required for ligand-dependent induction of transcription by nuclear receptors (25).

The above data demonstrate that RA activation of *DIF2* in NB4 cells is associated with the induction of many

hallmarks of a fully transcriptionally active promoter and are consistent with previous reports of nuclear receptor function on their target promoters.

To determine if histone acetylation is sufficient for SNF5 recruitment and synergy with TNFα, we asked whether HDAC inhibition by TSA could recapitulate the effects of RA. TSA stimulation increased both histone 4 acetylation and SNF5 recruitment, as expected (Figure 3A). However, northern blotting showed that TSA did not significantly increase *DIF2* mRNA levels when combined with either RA or TNFα, indicating that an increase in total acetylation on this promoter, such as is seen as a result of either RA or TSA action, cannot alone mediate synergy with TNFα (Figure 3B). Other HDAC inhibitors were also tested, with similar results (data not shown).

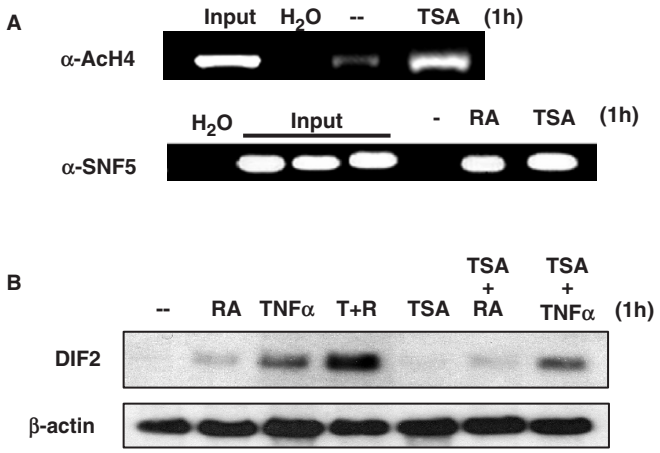

Figure 3. The HDAC inhibitor TSA does not enhance *DIF2* expression induced by TNFα or RA. (**A**) Treatment of NB4 cells with 200 nM TSA results in increased histone acetylation and SNF5 recruitment that is comparable to that induced by RA. (**B**) Northern blot indicating no enhancement of *DIF2* mRNA levels when cells were treated with TSA plus TNFα or RA.

RA facilitates recruitment of NF-κB by TNFα without altering nuclear translocation of p65/RelA

Induction of transcription by TNFα is mediated by the transcription factor NF-κB (26). In unstimulated cells, the NF-κB complex is sequestered in the cytoplasm by IκB proteins. Upon TNFα stimulation, IκB is rapidly phosphorylated by IκB kinase beta (IKKβ) and subsequently degraded, thereby allowing release and activation of the NF-κB complex. Activated NF-κB translocates into the nucleus where it binds DNA and activates transcription (27). TNFα-induced transcription is thus dependent on IKKβ and can be inhibited by a selective inhibitor, such as compound A (14). To test if the effect of RA on *DIF2* transcription is also dependent on NF-κB/IKKβ, we assessed induction of *DIF2* expression by RA and/or TNFα in the absence or presence of compound A. As expected, the inhibitor completely blocked induction of *DIF2* by TNFα. In contrast, it had no effect on RA-induced expression, but reduced the superinduced levels seen with RA plus TNFα to that seen with RA alone (Figure 4A). Thus, RA action on the *DIF2* promoter appears to be independent of NF-κB, while augmenting the NF-κB-dependent activity of TNFα. Interestingly, a recent paper presented a detailed study of the *HAS2* (hyaluronan synthase 2) promoter, which like the *DIF2* promoter contains binding sites for both RARα and NF-κB, as well as SP1 (28). Treatment with either RA or

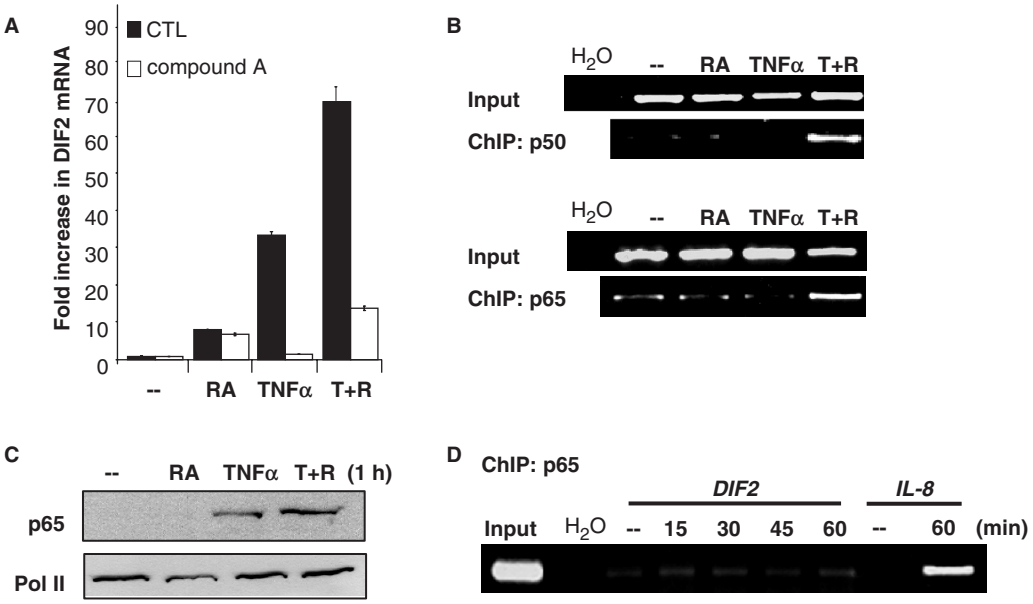

Figure 4. Full activation of *DIF2* transcription by RA and TNFα is NF-κB dependent. (**A**) Q-PCR analysis of *DIF2* expression in NB4 cells treated with RA and/or TNFα in the absence or presence of an IKKβ inhibitor (compound A). (**B**) ChIP analysis showing that RA facilitates TNFα-induced binding of NF-κB subunits p50 and p65 (primers 2a, b). (**C**) Western blot analysis of nuclear extracts showing that nuclear translocation of p65 is unaffected by RA. Equal loading was demonstrated by Pol II levels. (**D**) ChIP analysis of TNFα-induced p65 binding to the *DIF2* promoter (primers 2a, b) and the *IL8* promoter.

TNFα caused recruitment of co-activators to this promoter. In addition, both drugs increased *HAS2* mRNA in an NF-κB-dependent manner, but co-treatment did not provide any additive effect (28). This may suggest that the NF-κB independent activity of RA on the *DIF2* promoter is crucial for its ability to cooperate with TNFα to induce transcription.

We therefore investigated whether RA can stimulate binding of NF-κB transcription factors to the *DIF2* promoter. We found that neither RA nor TNFα alone could stimulate a detectable increase in binding of the NF-κB subunits p50 and p65 to the NF-κB site found at –140 (Figure 1B). However, RA plus TNFα caused a large increase in binding of both p50 and p65 to this site (Figure 4B), while no increased binding to another NF-κB site located at –3 kb was observed (data not shown). This increase could not be explained by enhanced nuclear shuttling of NF-κB, as levels of p65 in the nucleus were found to be equal in cells treated with TNFα alone versus RA plus TNFα (Figure 4C). The lack of an observable increase in NF-κB promoter binding induced by TNFα alone was surprising, as TNFα stimulation of NB4 cells does result in an accumulation of p65 in the nucleus, and also a significant increase in *DIF2* mRNA. This failure to fully activate NF-κB binding is promoter dependent, since TNFα alone stimulates p65 binding to the IL-8 promoter in NB4 cells (Figure 4D).

RA stimulates binding of unrelated transcription factors to the *DIF2* promoter

As shown in Figure 1B, the *DIF2* promoter region has several putative transcription factor binding sites. Since we found that RA could stimulate TNFα-induced binding of NF-κB, we hypothesized that RA-induced histone modifications and SNF5 recruitment result in a change of chromatin conformation that facilitates transcription factor binding in general. Indeed, RA activation of the *DIF2* promoter resulted in increased binding of both PU.1 and SP1 within 1 h of treatment (Figure 5A). These results indicate that RA stimulation facilitates transcription factor binding over a range encompassing the RARE and further downstream, proximal to the transcription start site. Mechanistically, these data support the idea that integration of TNFα and RA signals lead to binding of transcriptions factors including PML/RARα, PU.1, SP1 and NF-κB that may interact to form a fully mature enhancesome, which is apparently NF-κB dependent. This is similar to another NF-κB target, *BFL1*, where c-Rel, c-Jun, C/EBP beta and HMG-IC cooperate in an NF-κB-dependent manner to activate transcription (29).

RA and TNF synergistically recruit TFIIH and stimulate phosphorylation of RNA polymerase II

We next investigated how the coordinated recruitment of different transcription factors, in association with co-activators and chromatin-modifying enzymes, can promote transcriptional synergy. A likely candidate would be enhanced recruitment of basal transcription factors such as TBP or RNA Polymerase II (Pol II). ChIP analysis showed a low level of TBP bound to the *DIF2* promoter in untreated NB4 cells. RA and TNFα both increased this binding, but a synergistic recruitment of TBP by the two drugs together was only observed after 120 min of treatment, well after a significant enhancement of transcription is seen (Figure 5B). Pol II was undetectable at the *DIF2* promoter in untreated cells, but was recruited by either RA or TNFα, within 30 min (Figure 5B). RA plus TNFα induced a slight increase in Pol II recruitment, however, similar to TBP, a synergistic increase was not detected until 120 min post-treatment. Thus, enhanced recruitment of Pol II may partially explain how RA and TNFα cooperate to stimulate *DIF2* transcription, and synergistic recruitment of both TBP and Pol II after 120 min may further explain how transcription is maintained for a longer time in the presence of both drugs (Figure 1A and data not shown). Of note, the recruitment of TBP and Pol II seen in response to RA or TNFα was cyclical and showed similar kinetics to SNF5 recruitment. These data are consistent with previous reports that found cyclical recruitment of co-activators and the polymerase complex in response to oestrogen, corresponding with progressive rounds of transcription (19). PML/RARα itself was not found to cycle, but was constitutively bound to the promoter, independently of ligand, at all time points tested (Figure 1C and data not shown). It is thus not clear what is the role of the receptor itself in the cycling process. We postulate that at the end of each round of transcription, represented in our data by the 90 min-time-point, there is an exchange of ligand bound for unliganded receptor, a release of co-activators and transcriptional machinery (Figure 5B), and re-recruitment of corepressors, including HDAC1 (Figure 2B). We base this model on previous studies, which have shown that proteasomal degradation and 'recycling' of nuclear receptors as well as transcriptional cofactors are required for transcriptional activation and probably occur at each round of transcription (30–32).

Phosphorylation of the C-terminal repeat (CTR) of Pol II plays a crucial role in transcriptional elongation and it has been reported that a mature enhancesome (33) or NF-κB binding (15) can stimulate Pol II phosphorylation. Therefore, we investigated the status of Pol II phosphorylation at the *DIF2* promoter, postulating that this may be a target of synergistic activation. Despite the fact that both drugs increased the total level of Pol II at the promoter, we could detect no increase in serine 5-phosphorylated Pol II in NB4 cells treated for 1 h with either RA or TNFα alone. In contrast, the combination of TNFα and RA caused a significant increase, indicating a potential synergistic action of the two drugs at this level (Figure 5C). Concurrently, with this increase in phosphorylation, we observed a recruitment of the p62 subunit of the Pol II kinase TFIIH (Figure 5C), which represents a mechanism whereby Pol II phosphorylation and, in turn, a heightened rate of elongation may occur in response to the combination of TNFα and RA. Taken together, our data suggest that although the combination of RA and TNFα may increase the recruitment of Pol II to the *DIF2* promoter, the main cooperation exerted by these drugs occur at the level of TFIIH recruitment and Pol II

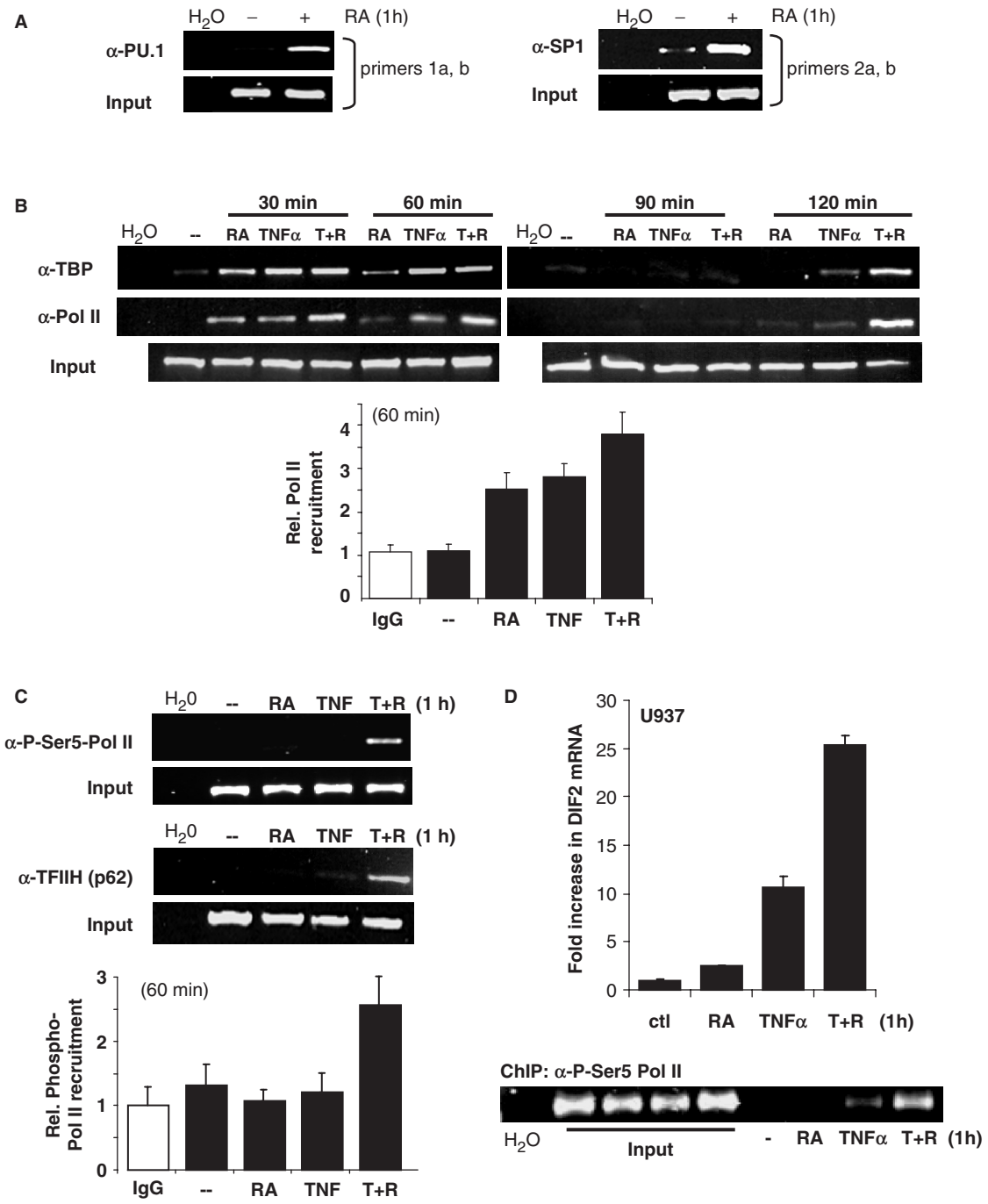

Figure 5. RA stimulation facilitates transcription factor binding and synergistically enhance Pol II phosphorylation in combination with TNFα in both APL and non-APL cells. (**A**) ChIP showing that RA stimulation of NB4 cells increases PU.1 and SP1 binding to the *DIF2* upstream region within 1 h of RA treatment. (**B**) ChIP showing regulation of Pol II and TBP recruitment and cycling of these factors on and off the promoter. NB4 cells were treated as indicated. Bottom panel: Results at 60 min were analyzed by Q-PCR, as indicated in the Materials and Methods section. (**C**) A synergistic enhancement of Pol II phosphorylation and recruitment of the TFIIH component p62 by RA and TNFα at 1 h post treatment was shown in NB4 cells by ChIP. Bottom panel: Q-PCR analysis of phospho-Pol II binding. (**D**) Top panel: Q-PCR showing *DIF2* mRNA induction by RA and TNFα in U937 PML/RARα negative, monoblastic cells. Bottom panel: ChIP analysis of phosphorylated Pol II at the *DIF2* promoter in U937 cells showing increased levels in response to TNFα and RA co-treatment.

phosphorylation. We postulate that this phosphorylation is stimulated via the recruitment of several transcription factors by RA, and dependent on the recruitment of NFκB, which occurs only in the presence of both RA and TNFα.

Finally, we asked the important question whether enhanced transcription of *DIF2* and the synergistic Pol II phosphorylation observed at the *DIF2* promoter in NB4 cells is dependent on the presence of the APL-specific PML/RARα oncoprotein, and thus limited in generality.

Notably, we found a similar induction of *DIF2* mRNA by RA plus TNFα in the PML/RARα negative monoblastic cell line U937, with a concurrent increase in Pol II phosphorylation (Figure 5D). These data suggest a general model for the integration of TNFα and RA signaling, whereby the two pathways contribute to a mature transcriptional platform (enhancesome) that facilitates efficient Pol II phosphorylation. This requirement of two simultaneous signals for transcriptional activation may provide an additional means of spatial and temporal gene regulation and may represent a novel mechanism whereby various signaling pathways can integrate to induce transcription.

ACKNOWLEDGEMENTS

Funding for this work was provided by the Leukemia Lymphoma Society of Canada (to W.H.M.), the Canadian Institute of Health Research (MOP-12863 to W.H.M.), the National Institutes of Health (AI35098 and CA73756 to A.S.B.). M.W. was supported by a predoctoral scholarship from the National Cancer Institute of Canada. W.H.M. and A.S.B. are investigators of the Samuel Waxman Cancer Research Foundation. Funding to pay the Open Access publication charges for this article was provided by the Canadian Institute of Health Research.

Conflict of interest statement. None declared.

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