MOZ-TIF2 Alters Cofactor Recruitment and Histone Modification at the RARβ2 Promoter

DIFFERENTIAL EFFECTS OF MOZ FUSION PROTEINS ON CBP- AND MOZ-DEPENDENT ACTIVATORS

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Hilary M. Collins1, Karin B. Kindle1, Sachiko Matsuda1, Colm Ryan1, Philip J. F. Troke3, Eric Kalkhoven1,2, and David M. Heery1

From the 1School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, United Kingdom and the 2Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands

MOZ-TIF2 and MOZ-CBP are leukemogenic fusion proteins associated with therapy-induced acute myeloid leukemia. These proteins are thought to subvert normal gene expression in differentiating hematopoietic progenitor cells. We have previously shown that MOZ-TIF2 inhibits transcription by CREB-binding protein (CBP)/p300-dependent activators such as nuclear receptors and p53. Here we have shown that MOZ-TIF2 associates with the RARβ2 promoter in vivo, resulting in altered recruitment of CBP/p300, aberrant histone modification, and down-regulation of the RARβ2 gene. In contrast, MOZ-CBP up-regulated transcription mediated by the MOZ/MYST3-dependent activator AML1/RUNX1. Both wild type MOZ and MOZ-TIF2 were found to colocalize with AML1, and MOZ-TIF2 was recruited to an AML1 target promoter. A MOZ-CBP fusion protein showed similar functions to MOZ-TIF2 in that it inhibited retinoic acid receptor-mediated transcription but enhanced AML1 reporter activation. Although it contains almost the entire CBP sequence, MOZ-CBP does not appear to associate with PML bodies. In summary, our results indicate that leukemogenic MOZ fusion proteins have differential effects on the activities of CBP-dependent and MOZ-dependent activators because of their ability to alter cofactor recruitment and chromatin modification at target promoters.

Acute myeloid leukemia (AML) is associated with recurrent, reciprocal chromosomal translocations in hematopoietic progenitor cells. Genes identified at the breakpoints of these translocations include transcriptional regulators such as the DNA binding transcription factor AML1 and/or genes encoding transcriptional cofactors or chromatin modifiers such as MLL, CBP/p300, MOZ, MORF, and TIF2. Oncogenic fusion proteins resulting from these events are likely to cause both global and promoter-specific alterations in chromatin modification, thus perturbing gene expression programs required for myeloid differentiation, leading to leukemia.

Chromosomal translocations that fuse the gene encoding the histone acetyltransferase MOZ (monocytic leukemia zinc finger protein; also known as MYST3 or ZNF220) with genes encoding the nuclear receptor coactivators CBP (CREB-binding protein)/p300 or TIF2 (transcriptional intermediary factor) are associated with M4/M5 subtypes of AML (1–6). Recent studies have confirmed that MOZ-CBP and MOZ-TIF2 fusion proteins have the ability to transform cell lines or recapitulate AML in vivo, and some of the molecular mechanisms involved have been revealed (7–10). Retrovirally expressed MOZ-TIF2 immortalized lineage-deficient murine bone marrow cells in vitro (7, 8) and induced acute myeloid leukemia in mice in bone marrow transplant assays (8). The CBP-binding domain (AD1) of MOZ-TIF2 was found to be required to extend the proliferative potential of hematopoietic progenitors in vitro and induce AML in vivo (7, 8). Previous results from our laboratory have demonstrated that MOZ-TIF2 interacts directly with CBP in vivo and as a consequence inhibits transcription by p53 and nuclear receptors, including the retinoic acid receptors (7). Expression of MOZ-TIF2 in hematopoietic progenitors and various cell lines depletes cellular levels of CBP protein and mislocalizes CBP from PML bodies. Thus, the expression of genes that require CBP/p300 function, such as retinoic acid receptor target genes, are likely to be adversely affected.

MOZ has been shown to acetylate histones in vitro (11, 12) and has been shown to be associated with a multiprotein complex containing AML1 (Runx1/CBFα2/PEBP2β) and CBFB, which are important regulators of hematopoiesis (9). Exogenously expressed MOZ was reported to enhance transcriptional activation by Runx family proteins such as AML1 and Runx2 (9, 13), whereas a fusion protein resembling MOZ-CBP repressed AML1 activity (9). However, it is not known whether AML1 transcriptional activity is adversely affected by MOZ-TIF2 or whether MOZ-TIF2 can associate with AML1.

To further understand the molecular mechanisms of MOZ-TIF2 inhibition of CBP-dependent gene expression, we have examined the effect of MOZ-TIF2 on histone modification and coactivator recruitment at the RARβ2 gene promoter in vivo. We also investigated whether MOZ and MOZ fusion proteins can modulate the transcriptional activity of AML1. Our results indicate that MOZ fusion proteins have differential effects on CBP/p300-dependent or MOZ-dependent activators, which may have important implications for the pathogenesis of these proteins in AML.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Expression vectors (based on pcDNA3.1) for epitope-tagged MOZ, MOZ-TIF2, TIF2, MOZ-N, and MOZ-TIF2ΔAD1 have been described previously (7). FLAG-tagged MOZ G657E and MOZ-TIF2ΔH/PHD (deleting amino acids 63–240) constructs were generated by site-directed mutagenesis using PCR, and the
FLAG-MOZ-CBP expression vector was made by PCR generation of a HindIII site in the MOZ sequence to allow in-frame ligation of fragments released from existing vectors (MOZ-(1–1117) and CBP-(28–2004)). The pcDNA4/TO YFP-HA-MOZ-TIF2 construct was generated by ligation of a Nhel/XbaI fragment into a modified pcDNA4/TO vector (Invitrogen). The following plasmids were obtained as gifts: pT109 (3xAML1-luc), pRep4-RARE-Luc (A. Zelent), pcmv5-AML1b (D. E. Zhang), and pRARβ2-luc (G. Folkers).

**Antibodies**—For Western blotting and immunofluorescence, the following primary antibodies were used: aML1 N20, anti-CBP A22 (Santa Cruz), anti-αH3K9, anti-αH3K14 (Upstate), anti-α-tubulin, anti-β-actin, anti-FLAG (Sigma), anti-GFP (Abcam), and anti-HA (Roche Applied Science). Secondary antibodies were used as anti-rabbit IgG peroxidase, anti-mouse IgG peroxidase, anti-goat IgG peroxidase (Santa Cruz), anti-goat IgG Alexa Fluor 594, anti-rabbit IgG Alexa Fluor 594, and anti-mouse IgG Alexa Fluor 488 (Invitrogen). The primary antibodies used in ChIP assays were anti-RARα, anti-RARβ, anti-RARγ, anti-p300 N15, anti-MOZ N19 (Santa Cruz), anti-HA (Roche Applied Science), anti-GFP (Abcam), anti-CBP CT, anti-AML1 ab-1 (Calbiochem), anti-diMeH3K17, anti-diMeH4R3, anti-acH3K9, anti-acH3K14, and anti-acH4K8 (Upstate).

**Cell Culture and Transient Transfections**—U2OS and COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 μM glutamine at 5% CO2. For AML1 reporter assays, U2OS cells were seeded 6 h prior to transfection in 12-well plates, and the cells were transiently transfected with 100 ng of β-galactosidase reporter pCH110, 500 ng of the AML1-responsive reporter pT109-luc, 100 ng of AML1, and the indicated amounts of MOZ expression plasmids and variants thereof wherever applicable, along with empty vector to standardize the quantity of DNA in each well. For nuclear receptor (NR) transient transfections, COS-1 or U2OS cells were seeded 6 h prior to transfection in 12-well plates, and the cells were transiently transfected with 100 ng of β-galactosidase reporter pCH110, 500 ng of luciferase reporter (RARα-luc, RARβ2-luc, or pGal4Δ-1E1bΔ-luc), expression vectors (100 ng unless otherwise stated), and empty vector to standardize the quantity of DNA in each well. In the case of p(Gal4)3Δ1E1bΔ-luc, fresh medium containing 10−7 M all-trans-retinoic acid (ATRA) or vehicle was added 24 h post-transfection and the cells incubated for an additional 24 h before harvesting. Transfections were carried out using the CalPhos mammalian transfection kit (Clontech), and cells were harvested 48 h post-transfection and assayed as follows. Cells were resuspended in 100 μl of lysis buffer (Dual Light system kit; Applied Biosystems); 5 μl of extract were assayed (in duplicate) for luciferase activity and the results normalized using β-galactosidase activity.

**Immunofluorescence**—Cells were plated onto coverslips in 6-well plates at 1–3 × 105 cells/well and transfected 24 h later using TransFast (Promega) according to the manufacturer’s protocol. After 48 h the cells were fixed in 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Cells were washed, and after blocking in 3% bovine serum albumin/phosphate-buffered saline, incubated with primary antibodies (1:50 dilution for AML1b, 1:200 for FLAG, and 1:10 for CBP). After 1 h of incubation, the cells were washed and incubated with the secondary antibody (1:500 dilution). DNA was stained using 0.5 μg/ml of Hoechst 33258. The samples were viewed using a confocal microscope (LSM510 Meta; Zeiss).

**Generation of a Stable Cell Line for Inducible Expression of MOZ-TIF2**—U2OS TReX cells (which contain the regulatory plasmid pcDNA6/TR) were transfected with 2.5 μg of pcDNA4/TO YFP-HA-MOZ-TIF2 using TransFast. After 48 h, cells were washed with phosphate-buffered saline and cultured in Dulbecco’s modified Eagle’s complete medium that contained antibiotics (400 μg/ml of Zeocin™ and 200 μg/ml of Hygromycin) in order to select cells that contain both the regulatory plasmid (pcDNA6/TR) and the inducible plasmid (pcDNA4/TO YFP-HA-MOZ-TIF2). The cells were maintained with antibiotics at all times. Once cells were selected, YFP-HA-MOZ-TIF2 was induced by incubation with 2 μg/ml of doxycycline for up to 5 days. Because of the short half-life of doxycycline (48 h), the appropriate amount of doxycycline was added every 2 days.

**Chromatin Immunoprecipitation Assay**—ChIP assays were performed essentially as described (14). In brief, U2OS-TReX cells (referred to as U2OST) or U2OST with stably integrated MOZ-TIF2 DNA (referred to as U2OST-MT2) were grown in phenol-red free Dulbecco’s modified Eagle’s medium supplemented with 5% charcoal-dextran-stripped fetal bovine serum for 72 h. For ChIP studies of the RARβ2 promoter, cells were treated overnight with ATRA (unless otherwise stated) at a final concentration of 10−7 M. The cells were fixed with 1% formaldehyde for 10 min at room temperature, washed three times with ice-cold phosphate-buffered saline, resuspended in 1.5 ml of collection buffer (100 mM Tris-HCl, pH 9.4, 100 mM dithiothreitol), and then placed on ice for 15 min and incubated for a further 15 min at 30 °C. The cells were then collected and washed with 1.5 ml of buffer 1 (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5) and then with buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5). The cells were resuspended in 350 μl of ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 0.5%), supplemented with EDTA-free protease inhibitor mixture (Roche Applied Science), and sonicated twice for 15 s on and 30 s off at power setting 12 μ (yielding fragments of 200–1000 bp). DNA content was determined by measuring the OD 260/280, and 25 μg of DNA was used for each immunoprecipitate. Immunoprecipitation was performed with 10 μg of a specific antibody overnight at 4 °C. The beads were washed twice for 10 min with TSE 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl) and then with buffer III (0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The immunoprecipitate was eluted by incubation in 100 μl of elution buffer (1% SDS, 0.1 mM NaHCO3) for 30 min. The supernatants were combined and the cross-link reversed by incubating at 65 °C overnight. DNA was purified through QIAquick PCR purification kit eluting into 50 μl of H2O. 1 μl of DNA was used in a 25-μl PCR reaction. Primers were designed to amplify the region —383 to —31, which contains a COUP-TF binding site, the canonical RARE, and the TATA box, to detect recruitment of factors at the endogenous RARβ2 promoter. For ChIP studies of the AML1 reporter, U2OST-MT2 cells were transfected with pT109-luc and an AML1 expression vector prior to induction of MOZ-TIF2 with doxycycline. ChIP assays were performed as outlined above, and primers were designed to amplify a 249-bp fragment containing the three AML1 binding sites in pT109-luc. PCR reactions were replicated several times and samples analyzed after 25–35 cycles to optimize the signal/noise ratio.

**RESULTS**

**MOZ-TIF2 Alters Cofactor Recruitment at the RARβ2 Promoter**—We have previously demonstrated that MOZ-TIF2 inhibits the transcriptional activity of CBP-dependent activators such as RARs, peroxisome proliferator-activated receptors, and p53 in a variety of cell lines, including human embryonic kidney 293, U2OS, SaOS2, and U937 cells (7). This inhibition of transcription factor activity is dependent on the interaction of the MOZ-TIF2 AD1 domain with CBP. To further investigate the molecular mechanisms underlying MOZ-TIF2 action, we...
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FIGURE 1. MOZ-TIF2 disrupts cofactor recruitment at the RARβ2 promoter. A, the RARβ2 promoter is represented schematically to show the primers (indicated by arrows), the position of the canonical RARE (DR5, −131 to −115), and the transcription start site. Cells were grown in the presence (+) or absence (−) of all-trans retinoic acid (ATRA), and transcription factor/coactivator recruitment at the RARβ2 promoter was studied under these conditions. ChIP experiments were performed using U2OST cells. B, transient transfection experiments using a RARβ2 promoter luciferase reporter, pRARβ2-luc. The effect of MOZ-TIF2 on transcription from the RARβ2 promoter is shown. 10⁶ U2OS cells were co-transfected with 500 ng of pRARβ2-luc and 100 ng of pCH110. The amount of transfected DNA (ng) of reporter is indicated. Data are represented as -fold induction luciferase reporter activity over the control values. Representative experiments are shown; the data are presented as averages, and S.E. of the mean from triplicate samples are shown. C, epifluorescence microscope images and Western blot analysis of YFP-HA-MOZ-TIF2 in the U2OST-MT2 cell line following induction with doxycycline for 5 days (upper panel) or 12 h (lower panel). D, cofactor recruitment at the RARβ2 promoter following induction of YFP-HA-MOZ-TIF2. ChIP experiments were performed using U2OST-MT2 cells. Upper panel, recruitment of CBP to the RARβ2 promoter. U2OST-MT2 cells were grown in the absence (−) or presence (+) of doxycycline for 12 h to induce YFP-HA-MOZ-TIF2, followed by treatment with ATRA as indicated. Lower panel, recruitment of CBP and p300 to the RARβ2 promoter following 5 days of doxycycline induction. E, recruitment of YFP-HA-MOZ-TIF2 to the RARβ2 promoter following 5 days of doxycycline induction. F and G, Western blot analysis of CBP in whole cell extracts from U2OST-MT2 cells expressing MOZ-TIF2 with and without ATRA. Levels of β-tubulin and α-tubulin are shown as protein loading controls.

generated a stable U2OS cell line enabling the inducible expression of YFP-HA-MOZ-TIF2 in vivo. We have previously confirmed that YFP-HA-MOZ-TIF2 behaves in a similar fashion to MOZ-TIF2 in terms of subcellular localization, protein interactions, and transcriptional inhibition (7). Stable hygromycin/zeocin-resistant cells that displayed inducible expression of YFP-HA-MOZ-TIF2 upon treatment with 2 μg/ml of doxycycline for 2–5 days were selected for further experiments (see Fig. 1C).

Initially, chromatin immunoprecipitation assays were optimized in U2OST cells to assess the effects of ATRA on recruitment of retinoic acid receptors and CBP/p300 to the RARβ2 promoter. As shown in Fig. 1A, RARα and RARβ were found to be associated with the RARβ2 promoter, both in the presence and absence of ligand. This is consistent with a recent report that, in P19 cells, RARα was bound to the RARβ2 promoter in the absence of cognate ligand, leading to corepressor recruitment and gene repression (15). Interestingly, RARγ was also detected on the RARβ2 promoter but only after treatment of the cells with ATRA, and Western blot analysis revealed that this isoform is expressed at very low levels in U2OST cells (data not shown). Similarly, by reducing the number of PCR cycles, we observed that ATRA enhanced recruitment of RARβ protein to the RARβ2 promoter (data not shown), although our data clearly indicate that RARs are constitutively associated with the proximal promoter of the RARβ2 gene. We also examined the effect of ATRA treatment on recruitment of histone acetyltransferases. As shown in Fig. 1A, ATRA treatment strongly induced the association of CBP and p300 proteins with the RARβ2 promoter, consistent with similar analyses in P19 cells (15).

To test the effect of MOZ-TIF2 on transcription from the RARβ2 promoter, we first performed reporter assays on extracts from U2OS cells co-transfected with MOZ-TIF2 and a RARβ2-luciferase reporter. As shown in Fig. 1B, reporter activation was inhibited by MOZ-TIF2 in a dose-dependent manner, indicating that the RARβ2 promoter is
down-regulated by MOZ-TIF2, consistent with our previous studies (7).

The induction of YFP-HA-MOZ-TIF2 expression in doxycycline-treated U2OST-MT2 cells was confirmed by RT-PCR, using primers specific for the fusion junction (data not shown). Consistent with this, YFP-positive cells were detectable 12 h post-doxycycline treatment, although prolonged exposure of up to 5 days was required to detect YFP in >60% of cells. The induced cells displayed YFP proteins in the nucleus in a mesh-like distribution typical for MOZ-TIF2 (Fig. 1C). Western blots confirmed the detection of the recombinant fusion protein at the expected molecular weight (Fig. 1C).

Next, we used ChIP assays to assess whether promoter occupancy in the presence and absence of ATRA was similar in U2OST and U2OST-MT2 cells cultured in the absence of doxycycline, i.e. under conditions where MOZ-TIF2 was not expressed. As expected, RARα and RARβ were constitutively bound to the RARB2 promoter (data not shown). In the absence of doxycycline (and therefore MOZ-TIF2), CBP was not detected on the RARB2 promoter but was recruited in a ligand-dependent manner, (compare 0, 2, and 24 h ATRA treatment; Fig. 1D, upper right panel). However, in cells expressing MOZ-TIF2 (treated with doxycycline for 12 h), CBP was already found to be present on the promoter even in the absence of ATRA, albeit at lower levels. Paradoxically, treatment of these cells with ATRA led to a decrease in CBP levels detected at the RARB2 promoter (Fig. 1D, upper right panel), which is consistent with our previous observations (7). Similar results were obtained for both CBP and p300 where U2OST-MT2 cells were treated with doxycycline for 5 days to obtain maximal MOZ-TIF2 expression (Fig. 1D, lower panel). Thus, we conclude that MOZ-TIF2 expression can perturb the strict ligand-dependent recruitment of transcriptional coactivators to the RARB2 promoter.

To determine whether the MOZ-TIF2 protein itself can associate with the RARB2 promoter in the U2OST-MT2 cells, ChIP assays were performed using an antibody recognizing the N terminus of MOZ. As shown in Fig. 1E, MOZ proteins were detected at the RARB2 promoter in U2OST-MT2 cells treated with doxycycline. Although the antibody used will recognize both wild type and MOZ fusion proteins, the enhanced signal obtained in extracts from cells treated with doxycycline strongly suggests that the MOZ-TIF2 fusion protein is bound to the amplified region. To distinguish between endogenous MOZ proteins and recombinant YFP-HA-MOZ-TIF2, we also performed ChIP assays using antibodies that recognize the YFP and HA tags that confirmed YFP-HA-MOZ-TIF2 associates with the RARB2 promoter region (Fig. 1E). However, in cells treated with ATRA the association of YFP-HA-MOZ-TIF2 with the RARB2 promoter was reduced. Thus, ATRA treatment appears to result in lower levels of promoter-bound MOZ-TIF2 and associated CBP and p300 proteins, and our results indicate a correlation between MOZ-TIF2 and CBP/p300 occupancy on the RARB2 promoter. In a previous study, we demonstrated that CBP levels are depleted in hematopoietic progenitor cells and human embryonic kidney 293 cells expressing MOZ-TIF2 (7). Western blots confirmed that cellular CBP levels were reduced in U2OST-MT2 cells treated with doxycycline (Fig. 1F), and longer exposure revealed that CBP was further reduced in the presence of ATRA (Fig. 1G). In contrast, MOZ-TIF2 expression did not alter the ligand-dependent association of RARs with the RARB2 promoter (data not shown), consistent with its inability to interact directly with nuclear receptors (7).

MOZ-TIF2 Alters Histone Modification at the RARB2 Promoter—Wild type MOZ protein has previously been shown to acetylate histones H3 and H4 in vitro (11, 12). Induced expression of YFP-HA-MOZ-TIF2 in U2OST-MT2 cells increased bulk acetylation of H3, as detected using antibodies specific for acetylation of H3K9 and H3K14 (Fig. 2A). ChIP assays were performed in the U2OST-MT2 cells to assess the effects of MOZ-TIF2 on histone modification at the RARB2 promoter. In the absence of MOZ-TIF2, ATRA was found to induce acetylation of histones H3K9, H3K14, and H4K8 (Fig. 2B, which may be due in part to recruitment of CBP/p300. In addition, arginine methylation of H3R17 and H4R3 was increased after ATRA treatment, and it has been demonstrated in studies of other promoters that these modifications are mediated by the arginine methyltransferases CARM1 and PRMT1, respectively.

FIGURE 2. MOZ-TIF2 alters histone modification. A, Western blot analysis of H3 histone acetylation with the indicated antibodies in whole cell extracts from U2OST-MT2 cells grown in the presence (+) or absence (−) of doxycycline. Levels of β-actin are shown as protein loading controls. B, ChIP experiments were performed on U2OST-MT2 cells grown in the presence (+) or absence (−) of ATRA. Histone modification at the RARB2 promoter was studied in the presence or absence of MOZ-TIF2 (+ or − doxycycline). The effect of the histone deacetylase inhibitor trichostatin A (500 nm for 6 h) on the level of H3K9 acetylation is also shown (lower panel). C, ligand-independent activation of RARE-luc. Reporter activation is represented as fold induction over control value (reporters in the absence of NRs, coactivators, or ligands), and the average of three independent experiments with S.E. is shown.
FIGURE 3. Enhancement of AML1 activity by MOZ and MOZ-TIF2. A, schematic representation of MOZ and MOZ-TIF2 proteins used in conjunction with Western blot analysis showing expressed MOZ proteins. B and C, transient transfection experiments using an AML1-responsive luciferase reporter, pT109-luc. The effect of the indicated MOZ constructs on AML1-mediated reporter activation is shown. 10^6 U2OS cells were co-transfected with 500 ng of pT109-luc, 100 ng of pCH110, and 100 ng of pcmv5-AML1b. The amount of
In cells expressing MOZ-TIF2, we detected high levels of acetylated H3K9, H3K14 at the RARβ2 promoter in the absence of ATRA (Fig. 2B), which may be due to MOZ histone acetyltransferase activity and/or the ligand-independent recruitment of CBP and p300 proteins by MOZ-TIF2. This altered chromatin modification appears to be specific, as in contrast, we did not observe an increase in acetylation of H4K8 in the absence of ligand. In addition, cells expressing MOZ-TIF2 displayed increased dimethylation of H3R17 and H4R3 compared with control both in the absence or presence of ATRA. This may be due to recruitment of CARM1 and PRMT1 proteins to the TIF2 AD2 domain that is present in the fusion protein. However, ChIP assays performed with antibodies against CARM1 were not successful (data not shown); therefore, it remains to be determined whether MOZ-TIF2 recruits CARM1 or PRMT1 to the RARβ2 promoter.

Although MOZ-TIF2 induced ligand-independent acetylation of H3K9 or H3K14, treatment of cells expressing MOZ-TIF2 with ATRA did not lead to further increases in acetylation of H3K9 or H3K14. Indeed, under the conditions used, the levels of acetylation achieved in cells expressing MOZ-TIF2 were equivalent to those obtained by treatment of U2OST-MT2 cells with the histone deacetylase inhibitor trichostatin A (Fig. 2B, lower panel). However, H4K8 acetylation, which was not enhanced by MOZ-TIF2, remained ATRA dependent (Fig. 2B). These results indicate that MOZ-TIF2 perturbs specific histone modifications at the RARβ2 promoter such as H3K9, H3K14, H3R17, and H4R3, whereas others are unaffected. As previous studies have detected no differences in substrate preferences of MOZ and MOZ fusion proteins (9), we suggest that the effects on chromatin modification at the RARβ2 promoter are due to a combination of MOZ-TIF2 acetyltransferase activity and aberrant cofactor recruitment. Although this leads to a weak increase in the activation of a DR5 reporter in the absence of ligand (Fig. 2C and Ref. 7), it does not appear to sustain a full transcriptional response to ligand.

MOZ Stimulates AML1 Transcriptional Activity—A previous report demonstrated that MOZ stimulates AML1-mediated activation of the myeloperoxidase promoter in P19 cells, suggesting that MOZ is a coactivator of AML1 (9). Consistent with this, MOZ was shown to interact with AML1 in vitro, via both N- and C-terminal sequences. To assess the effects of MOZ on AML1 transcriptional activity, U2OS cells were co-transfected with the pT109-luc reporter plasmid (containing three synthetic AML1 response elements) in addition to expression vectors for AML1, MOZ, or derivatives thereof (shown schematically in Fig. 3A). The expression of full-length recombinant MOZ proteins used in these assays was confirmed by Western blot (Fig. 3A). As shown in Fig. 3B, the reporter activity was enhanced 7-fold by AML1. However, this activity was further stimulated to 15-fold above basal activity by co-transfection with the full-length MOZ expression vector, confirming that MOZ enhances AML1 activity (Fig. 3B). To test whether the coactivator function of MOZ was dependent on the acetyltransferase activity of the MYST domain, AML1 was co-transfected with MOZ G657E, as this glycin residue is at the active site of the MYST domain and is required for histone acetyltransferase activity (16, 17). Unexpectedly, MOZ G657E enhanced AML1-mediated reporter activation to similar levels as wild type MOZ, suggesting that the MOZ acetyltransferase activity is not essential to stimulate AML1-mediated transcription. Moreover, a deletion mutant (MOZ-N), which contains the H1/H5, PHD, and MYST domains, did not enhance AML1 activation of the reporter, suggesting that C-terminal sequences are required for MOZ coactivator function. As it has been reported that the C terminus of MOZ can interact with AML1 (9), our results indicate that the N terminus of MOZ, including the MYST domain, is not sufficient to stimulate AML1-mediated transcription in these assays. Co-expression of AML1 with FLAG-MOZ or the derivatives described above revealed that whereas wild type MOZ and MOZ G657E colocalized with AML1, MOZ-N displayed only weak or partial colocalization (Fig. 3F). The levels of AML1 detected in cell-free extracts by Western blotting did not vary significantly due to co-expression with MOZ (Fig. 3B).

MOZ-TIF2 Stimulates AML1 Transcriptional Activity—To test the effect of MOZ-TIF2 on AML1-mediated transcription, reporter assays were performed on extracts from U2OS cells expressing MOZ-TIF2 (and deletion derivatives). As shown in Fig. 3C, AML1 reporter activation was strongly enhanced by MOZ-TIF2 (between 40- and 45-fold above basal levels). As the fusion protein contains the region corresponding to MOZ-N including the MYST domain, we hypothesized that the increased activity may be due to recruitment of CBP/p300 acetyltransferases via the AD1 domain. Consistent with this, a MOZ-TIF2 mutant lacking the AD1 domain, and thus incapable of recruiting CBP/p300, was unable to enhance AML1-mediated reporter activation to the same level (Fig. 3C). In addition, a deletion derivative lacking the histone-like H1/5 domain and part of the PHD domain (MOZ-TIF2ΔH/PHD) also failed to enhance AML1-mediated reporter activation. Expression of the full-length proteins was confirmed by Western blot (Fig. 3A).

To test whether MOZ-TIF2 could associate with an AML1-1-responsive promoter, ChIP assays were performed to detect occupancy of AML1, MOZ-TIF2, and CBP on the pT109-luc reporter. U2OST-MT2 cells were transfected with pT109-luc and AML1-1, and cells were grown in the absence or presence of doxycycline (for 24 h) to induce YFP-HA-MOZ-TIF2. AML1 was found to be associated with a 249-bp fragment of pT109-luc containing AML1 binding sites (Fig. 3D). However, recombinant MOZ-TIF2 was detected at this promoter only under inducing conditions, indicating that MOZ-TIF2 can be recruited to an AML1 target promoter. CBP was also detected at this promoter (albeit weakly) both in the presence and absence of doxycycline. Similar results were detected in ChIP assays using the endogenous protein kinase C promoter (data not shown), which is regulated by AML1 (18). Thus, the enhancement of AML1-mediated transcription of pT109-luc by MOZ-TIF2 appears to involve the recruitment of MOZ-TIF2 to the target promoter. This does not appear to perturb the association of AML1 or CBP proteins with pT109-luc (Fig. 3D). Immunofluorescence experiments revealed that like wild type MOZ, MOZ-TIF2 colocalized with AML1 (Fig. 3F). In contrast, MOZ-TIF2ΔAD1 and MOZ-TIF2ΔH/PHD showed only partial or no colocalization with AML1, respectively. Indeed, MOZ-TIF2ΔH/PHD localized to discrete nuclear foci rather than the typical mesh-like pattern seen for MOZ-TIF2 (Fig. 3F). Thus, both MOZ and MOZ-TIF2 proteins enhance the transcriptional activity of AML1 in a manner that appears to be independent of the MYST domain but requires colocalization with AML1 proteins in vivo. In addition, the ability to enhance AML1 activity requires the H1/5 and PHD transduced DNA (ng) of MOZ constructs is indicated. Data are represented as -fold induction luciferase reporter activity over the control values. Representative experiments are shown; the data are presented as averages, and S.E. of the means from triplicate samples are shown. Western blots indicating the levels of AML1 protein and loading control (a-tubulin) are shown. D, the AML1-responsive promoter in the pT109-luc reporter is represented schematically with the AML1 binding sites and the primers used indicated. ChIP experiments were performed using U2OST-MT2 cells. Cells were transfected with pT109-luc and AML1 and were grown in the absence (-) or presence (+) of doxycycline to induce YFP-HA-MOZ-TIF2. Immunofluorescence experiments revealed that like wild type MOZ, MOZ-TIF2 colocalized with AML1 (Fig. 3F). In contrast, MOZ-TIF2ΔAD1 and MOZ-TIF2ΔH/PHD showed only partial or no colocalization with AML1, respectively. Indeed, MOZ-TIF2ΔH/PHD localized to discrete nuclear foci rather than the typical mesh-like pattern seen for MOZ-TIF2 (Fig. 3F). Thus, both MOZ and MOZ-TIF2 proteins enhance the transcriptional activity of AML1 in a manner that appears to be independent of the MYST domain but requires colocalization with AML1 proteins in vivo. In addition, the ability to enhance AML1 activity requires the H1/5 and PHD
Differential Effects of MOZ-TIF2 on Transcription

FIGURE 4. Differential effects of MOZ-CBP on transcription by Gal4-RARα and AML1. A, schematic representation of MOZ, CBP, and MOZ-CBP proteins with confirmation of MOZ-CBP expression using Western blot analysis. B, transient transfection experiments using a Gal4-RARα-responsive luciferase reporter, p(Gal4)5-E1bΔ-luc. 10^6 COS-1 cells were co-transfected with 500 ng of p(Gal4)5-E1bΔ-luc, 100 ng of pCH110, 100 ng of pSGS-TIF2, and 100 ng of Gal4-RARα. The amount of transfected MOZ-CBP DNA (ng) is indicated. Cognate ligand (shaded bars) or an equal volume of vehicle (black bars) was added. Data are represented as fold-induction luciferase reporter activity over the control values. Data from three independent experiments and S.E. of the mean are shown. C, transient transfection experiments using an AML1-responsive luciferase reporter, pT109-luc. 10^6 U2OS cells were co-transfected with 500 ng of pT109-luc, 100 ng of pCH110, and 100 ng of pcmv5-AML1b. The amount of transfected MOZ-CBP DNA (ng) is indicated. Data are represented as fold-induction luciferase reporter activity over the control values. Representative experiments are shown; the data are presented as averages, and S.E. of the mean from triplicate samples are shown. D, subcellular localization of MOZ-CBP. Co-staining of FLAG-tagged MOZ-CBP with endogenous CBP. Scale bar, 5 μm.
domain and the C terminus of MOZ or the CBP binding domain in MOZ-TIF2.

**MOZ-CBP Stimulates AML1 Transcriptional Activity**—The MOZ-CBP protein (shown schematically in Fig. 4A) results from t(8;16)(p11; p13), which has a clinical phenotype that is indistinguishable from the MOZ-TIF2 translocation, inv(8)(p11;q13). To establish whether MOZ-CBP affects the transcriptional activity of retinoic acid receptors, we carried out reporter assays using the GAL4-RAR

CBP affects the transcriptional activity of retinoic acid receptors, we focused on the molecular mechanisms underlying this are poorly understood, and the misregulated genes are unknown. Our previous studies have shown that normal differentiation of myeloid progenitors resulting in AML (7–10).

**DISCUSSION**

There is now substantive evidence that MOZ fusion proteins block normal differentiation of myeloid progenitors resulting in AML (7–10). Although this is likely to result from aberrant gene expression, the molecular mechanisms underlying this are poorly understood, and the misregulated genes are unknown. Our previous studies have shown that MOZ-TIF2 inhibits the transcriptional activity of CBP-dependent activators such as NRs and p53 (7). To further investigate the effects of MOZ-TIF2 on gene transcription, we focused on the RARβ2 gene, which is up-regulated by retinoic acid receptors in response to the cognate ligand ATRA.

Using a stable inducible cell line to express MOZ-TIF2, we have demonstrated that MOZ-TIF2 can associate with the RARβ2 promoter and an AML1-responsive promoter (Figs. 1E and 3D); however, the mechanism of association is unknown. A previous study has reported that a C2HC motif (in the MYST domain) functions as a nucleosome binding motif (19). We note that the N terminus of MOZ contains several domains that potentially facilitate MOZ binding to chromatin, including a sequence with weak homology to histone H1/H5 (the H1/5 domain) and a PHD domain. Further studies will be necessary to investigate the ability of MOZ to interact with DNA/chromatin.

The association of MOZ-TIF2 with the RARβ2 promoter alters the ligand-dependent recruitment of coactivators and also the ATRA dependence of histone modifications (Figs. 1D and 2B). The increase in histone acetylation at H3K9 and H3K14 may be consistent with the aberrant recruitment of CBP and p300 to the promoter in the absence of ligand, as these proteins associate with MOZ-TIF2 in vivo via the AD1 domain (7). However, a recent report has indicated that a histone acetyltransferase complex containing MOZ and ING5 showed specificity for H3K14 acetylation (20); therefore, the MOZ MYST domain may also be responsible for the observed modifications. In addition, the increased arginine methylation observed in our experiments may be catalyzed by CARMA1 and PRMT1 proteins associated with the AD2 domain of MOZ-TIF2, although this remains to be established.

Although MOZ-TIF2 appears to enhance specific histone acetylation and methylation events at the RARβ2 promoter (Fig. 2B), the overall effect of MOZ-TIF2 is to repress RARβ2 transcription (Fig. 1B). This somewhat paradoxical observation probably indicates that aberrant histone modifications are detrimental to ligand-dependent gene transcription. Several studies have shown that ligand-induced recruitment of coactivators to NR target gene promoters is temporally and combinatorially ordered (14, 21, 22), thus allowing sequential chromatin modifications and remodeling events at the promoter (reviewed in Ref. 23).

Therefore, the order and timing of chromatin modifications is crucial to sequential recruitment of coactivator complexes and chromatin remodeling. In the absence of ligand, corepressor complexes can associate with some retinoid and thyroid receptor target promoters, leading to repression of transcription and hypoacetylation of histones. Ligand binding not only displaces the corepressors such as NCoR and SMRT but may also trigger degradation of corepressor complex components via mechanisms involving ubiquitylation and the proteasome (reviewed in Ref. 23). This leads us to propose a model of MOZ-TIF2 action at the RARβ2 promoter, summarized in Fig. 5. MOZ-TIF2 associates with DNA or chromatin elements of the RARβ2 promoter, resulting in recruitment of CBP, p300, CARMA1, and PRMT1 in the absence of ligand. This induces specific acetylation and methylation of histones that alone may be insufficient to lead to sequential recruitment of other coactivators such as TRAP220 and the mediator complex. Thus chromatin modification induced by MOZ-TIF2 and associated factors cannot give the full transcriptional response induced by ligand. In the presence of ATRA, corepressors on the promoter may be degraded by a mechanism involving the proteasome or other proteases, which concomitantly leads to removal of MOZ-TIF2 and CBP from the promoter or their degradation (Fig. 1, D and E). Consistent with this, we have demonstrated that MOZ-TIF2 association with CBP leads to a reduction in CBP protein and that CBP levels are reduced further in the presence of ATRA (Fig. 1, F and G).

Evidence for a role of MOZ as a coactivator is supported by its requirement for Hox gene expression in zebrafish (24). MOZ has been identified with CBP/p300 in a multicomponent complex that co-immunoprecipitates with AML1 (9). The related protein MORF was also found to associate with CBP in a complex containing peroxisome proliferator-activated receptor α (25). MOZ has also been reported to be a coactivator for AML1 (13), which in combination with its heterodimer partner CBFB constitutes the core binding factor complex that regulates hematopoietic development (26, 27). Our results are consistent with this and further show that the coactivator function of MOZ is dependent on C-terminal sequences that are absent in the MOZ-TIF2 and MOZ-CBP fusion proteins (Fig. 3B, compare MOZ and MOZ-N). Moreover, mutation of the acetyl-CoA binding site G657E, which leads to a complete loss of histone acetyltransferase activity in vitro (8), did not abrogate the coactivator potential of MOZ in the reporter assays (Fig. 3B). This is of interest in light of the finding that MOZ-TIF2 containing the G657E mutation retained the ability to induce AML in mice, albeit with a slightly delayed response (8). Thus, the acetyltransferase activity of the MYST domain may not be essential for the ability of MOZ to enhance AML1 activity or for the leukemogenicity of MOZ-TIF2.

Immunofluorescence studies showed that the G657E mutation did not perturb the colocalization of MOZ and AML1 (Fig. 3E). In contrast, however, MOZ-N-(1–1135) did not colocalize with AML1 (Fig. 3E). Previous work by Kitabayashi et al. (9) suggests that MOZ contains two domains that mediate interaction with AML1, the strongest of which was within the C-terminal sequence 1517–2004. Thus, the C terminus...
of MOZ appears to be required for the association of wild type MOZ with AML1.

Surprisingly, reporter assays showed that MOZ-TIF2 increased the activity of AML1/H11011 7-fold, compared with 2.5-fold for wild type MOZ. Given that MOZ-N was unable to enhance AML1 activity, we suggest that the TIF2 sequence or associated proteins may be necessary for the observed enhanced reporter activity. Consistent with this, deletion of the CBP binding domain in MOZ-TIF2 (MOZ-TIF2/H9004 AD1) resulted in much lower reporter activation, although higher than AML1 alone (Fig. 3C). In agreement with this observation, MOZ-TIF2 showed considerable colocalization with AML1 in the nucleus in contrast to MOZ-TIF2/H9004 AD1. A mutant version of MOZ-TIF2 in which the H1/5 and PHD domains were deleted did not enhance the reporter activity above the level of AML1 alone. Interestingly, the subcellular localization of this mutant was distinct from the mesh-like pattern of MOZ-TIF2, as it appeared concentrated in nuclear foci (Fig. 3F). This provides further support for the hypothesis that these domains play a role in the association of MOZ proteins with chromatin.

MOZ-TIF2 and MOZ-CBP proteins are associated with AML of identical phenotypes. This has raised the possibility that the MOZ-TIF2 mimics the molecular action of MOZ-CBP by recruiting CBP to the AD1 domain. However, the strong stimulation of AML1 reporter activity by MOZ-TIF2 is in contrast to the reported inhibitory effect of MOZ-CBP on this activator (9). To establish whether MOZ fusion proteins have differential effects on AML1 transcription and on the activity of NRs, we generated a MOZ-CBP expression vector (Fig. 4A). This adheres to the most common breakpoints reported for the t(8;16) translocation, i.e. fusing MOZ amino acids 1–1117 with amino acids 29–2442 of the human CBP. Reporter assays using the GAL-RARα construct showed that MOZ-CBP inhibits ATRA-dependent reporter activation to a level similar to that observed with MOZ-TIF2 (Fig. 4B). In addition, like MOZ-TIF2, MOZ-CBP strongly stimulated AML1 reporter activation (Fig. 4C). Thus in our hands, MOZ fusion proteins behave similarly with regard to their inhibition of CBP-dependent activators, while they enhanced the activity of the MOZ-dependent activator AML1. We note that the construct used by Kitabayashi et al. (9) was based on a different breakpoint than the one used here. In that study, the construct included an extended N-terminal sequence MOZ-(1–1518) incorporating part of the acidic domain of MOZ reported to function as a “repression domain.” This is a likely explanation for the apparent distinct effects on AML1 reporter activation in the two studies, although promoter context-dependent differences cannot be excluded.

MOZ-TIF2 shows a nuclear localization that is distinct from that of MOZ (7). Moreover, MOZ-TIF2 interacts with CBP, resulting in its mislocalization from PML bodies and depletion of CBP levels through an undefined proteolytic mechanism. Our results suggest that MOZ-
CBP also displays an aberrant subcellular localization as, despite the presence of almost the entire CBP sequence in the fusion protein (29–2442), it does not appear to accumulate in PML bodies (Fig. 4D). This observation reveals a further similarity between the MOZ fusion proteins that may be consistent with the similarity of AML phenotypes.

In this study we have demonstrated that MOZ fusion proteins have differential effects on the transcriptional activities of CBP-dependent retinoic acid receptors and the MOZ-dependent activator AML1. These proteins are likely to alter global histone modification, which can correlate with cancer progression, as in prostate cancer (28), and may also cause aberrant acetylation of non-histone proteins. Future studies will focus on identifying gene targets for MOZ fusion proteins.

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