Identification of an N-terminal Transactivation Domain of Runx1 That Separates Molecular Function from Global Differentiation Function*

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RUNX1, or AML1, is a transcription factor that is the most frequent target for chromosomal gene translocations in acute leukemias. RUNX1 is essential for definitive hematopoiesis in embryos and profoundly influences adult steady-state hematopoiesis both positively and negatively. To investigate this wide range of normal activities and the pathological role of RUNX1, it is important to define the functions of different domains of the protein. RUNX1, RUNX2, and RUNX3 are highly conserved in their DNA binding runt homology domain and contain divergent sequences of unknown function N-terminal to this domain. Here we analyzed the role of the N-terminal sequence and the α-helix of the runt homology domain of Runx1 in DNA binding, transactivation, and megakaryocyteopoiesis. Both the N terminus and the α-helix were found to reduce DNA binding of Runx1 and be essential for transactivation of the granulocyte-macrophage colony-stimulating factor and IκB promoters by Runx1. The N terminus of Runx1, including the α-helix, was also required for transactivation of a Gal4 reporter when expressed as fusion proteins with a Gal4 DNA binding domain, and the N terminus alone was capable of stimulating transcription when fused to the Gal4 DNA binding domain. The N terminus and the α-helix, however, were not required for megakaryocyte development from embryonic stem cells differentiated in vitro. Thus, our findings define a second transactivation domain of Runx1 that is differentially required for activation of transcription of some Runx1-dependent promoters and megakaryocyteopoiesis.

The RUNX family of transcription factors has three mammalian members that all have important roles in development. RUNX1, also called acute myeloid leukemia 1 (AML1), operates as a key regulator of definitive hematopoiesis in embryos and normal hematopoiesis in adults and is the most common target of chromosomal gene translocations in acute human leukemias (1–3). RUNX2 is essential for skeletal development. Heterozygosity for loss of RUNX2 function gives rise to the skeletal disease cleidocranial dysplasia, and Runx2−/− mice show complete block of bone formation (4). RUNX3 is important for survival and development of certain dorsal root ganglion nerve cells, for establishment of mature CD8+ cytotoxic T lymphocytes, and for antiproliferation and apoptosis of gastric epithelium. RUNX3 is a tumor suppressor; its inactivation is associated with human gastric cancer and with various other human cancers. RUNX3 is also a key component of transforming growth factor-β signaling in dendritic cells, and Runx3−/− mice show spontaneous lung inflammation and inflammatory bowel disease (5–11).

The role of RUNX1 in the hematopoietic system appears to be complex as it depends on both ontogenic stage and differentiation status of the cell. During embryonic development of the hematopoietic system in the mouse, Runx1 is essential for the formation of all definitive hematopoietic cell types (e.g. definitive erythroid cells, granulocytes, macrophages, and lymphoid cells) both in vivo and in embryonic stem (ES) cells differentiated in vitro (1, 3, 12–16). In contrast, deletion of Runx1 from adult hematopoietic stem cells reveals that it is not essential for maintenance of adult hematopoietic stem cells, although several hematopoietic abnormalities do occur (3, 12, 13). For example, such mice reveal pronounced defects in T and B cell development and inefficient platelet formation due to a block in megakaryocyte development (12, 13). Moreover Runx1-deficient mice can also develop a mild myeloproliferative disorder characterized by increased neutrophil counts in peripheral blood and extramedullary hematopoiesis (12). The latter observation might also explain why Runx1 deficiency has been shown to predispose mice to malignant transformation (17). Thus, to understand these pleiotropic and sometimes apparently conflicting normal functions of Runx1 and its role in different malignant diseases, it is important to clarify the roles of different domains of the Runx1 protein.

The RUNX proteins bind to a common DNA motif, TGPy-GGTPy (where Py is pyrimidine), and heterodimerize with a common cofactor, CBFβ (also denoted PEBP2β), which does not itself make contact with DNA directly but instead increases the DNA binding of the RUNX proteins (8, 18). Both the DNA binding and the heterodimerization with CBFβ are mediated by a domain known as the runt homology domain (RD), which is highly conserved both among the three RUNX proteins and across species (18, 19). Elucidation of the structure of the iso-

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lated RD and of RD complexed with DNA and/or CBFβ has shown that most of the RD adopts the fold of an immunoglobulin-like β-barrel similar to that of the DNA binding domains of NFAT (nuclear factor of activated T cells), STAT (signal transducers and activators of transcription), p53, and NF-κB transcription factors. This immunoglobulin (Ig) fold is formed by 12 β-sheets, and in the RD it is preceded (in the N-terminal direction) by an α-helix (residues Ser-50 to Asp-57) that packs against the Ig domain through hydrophobic interactions (20–23). The free Ig fold is in a conformation in which the affinity for DNA is not optimal, and CBFβ binding triggers a switch in the Ig domain structure that stabilizes it in a conformation for sustained DNA binding (8, 21). However, the role of the N-terminal α-helix in the RD is not clear from the structure.

In addition to the RD, RUNX proteins also possess other subdomains, including a large transactivation domain in the C-terminal part that is required for the transcriptional activity of these proteins, and an inhibitory domain at the C-terminal end of the transactivation domain that can down-regulate the transcriptional activity. RUNX proteins also contain a nuclear localization signal at the C-terminal end of the RD and a nuclear matrix-targeting sequence that is required for nuclear transactivation by RUNX1; it is involved in CD4 repression during development of T cells (24–27). In addition, a conserved VWRPY motif at the very end of the C terminus of RUNX proteins mediates the interaction with the corepressor Groucho/TLE (28, 29).

The short RUNX1 sequence N-terminal to the DNA binding RD is a negative element for the DNA binding of the protein (25, 30, 31). The RUNX genes encode isoforms with different N termini due to alternative promoter usage. RUNX isoforms transcribed from the proximal promoters have a slightly shorter N terminus than isoforms transcribed from the distal promoters, and the most N-terminal amino acids differ between the isoforms. Differential expression and some distinct functions have been found for these two forms of RUNX proteins (2, 27, 32–34). In acute myeloid leukemias, approximately one-tenth of all point mutations of RUNX1 have been found around the α-helix of the RD or even more N-terminally (35–37). Molecular characterization and functional analyses of this domain is therefore essential for understanding both the normal and pathological function of Runx1 proteins.

Here we show that the N terminus of Runx1 and the α-helix in the RD reduce DNA binding of Runx1 and are essential for transactivation of the GM-CSF and lδ promoters by Runx1. These domains were also found to be required for transactivation of a Gal4 reporter when expressed as fusion proteins with a Gal4 DNA binding domain but were not essential for in vitro megakaryocytopenosis from mouse embryonic stem cells. Our findings show that the N-terminal sequences constitute a second transactivation domain of Runx1 that is not required for megakaryocyte development.

EXPERIMENTAL PROCEDURES

Cell Lines and Transient Transfections—The human malignant cell lines Jurkat, a T cell line; K562, an early erythroleukemia line; and DG75, an Epstein-Barr virus-negative Burkitt lymphoma, were cultured as described previously (38, 39). Transient transfections were performed as described previously (39) using 4 μg of reporter plasmid, 2 μg of hCMV-β-galactosidase plasmid (reference plasmid for normalization), and 5 μg of each expression plasmid indicated. Where necessary, the corresponding empty expression vector was added to a total of 10 μg of expression plasmids. Approximately 1 × 10⁷ cells were electroporated followed by incubation in 10 ml of medium. The cells were harvested after 20 h of incubation.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously using complementary oligonucleotides with an optimal RUNX1 binding site (5′-GTCGGTTTGGCGTTTGGCGGGA-3′) as DNA probe (40).

Expression and Reporter Plasmids—The expression plasmid for full-length mouse Runx1, pBJ9AML1b; the mouse Runx2 expression plasmid pBJ9PEBP2αA1; and the expression plasmid for a constitutively active deletion derivative of the A subunit of murine calcineurin (CN), ΔCN, have been described previously (41, 42). The Runx1 derivatives with progressive N-terminal deletions, pBJ9Runx1ΔN24 (amino acids 25–451), pBJ9Runx1ΔN50 (amino acids 51–451), pBJ9Runx1ΔN57 (amino acids 58–451), pBJ9Runx1ΔN72 (amino acids 73–451), and pBJ9Runx1ΔN92 (amino acids 93–451), and a corresponding full-length pBJ9Runx1 were generated by PCR with Ffu-Turbo from the full-length cDNA by replacing the deleted sequences with an Ndel site. The amplified products were subcloned as Ndel-Xba1 segments into a modified pBJ9 expression vector, pBJ9-Ndel. Where indicated, the pBJ9-Ndel had a copy of a FLAG sequence upstream of the Ndel site (42).

For studies of retroviral transduction of ES cells, the Runx1 wild type and the N-terminal deletion mutants were subcloned as XhoI-EcoRI fragments from their pBJ9-based plasmid into the MSCV-pac vector (43). For transactivation studies with proteins fused to the Gal4 DNA binding domain (amino acids 1–147), Gal4-Runx1 wild type and the N-terminal deletions Gal4Runx1ΔN24, Gal4Runx1ΔN50, Gal4Runx1ΔN57, Gal4Runx1ΔN72, and Gal4Runx1ΔN92 were generated by the PCR-based overlap extension technique, and the amplified DNA segments were cloned between the Ndel site and BamHI sites of the pB9-Ndel vector. The Gal4Runx1ΔN24, Gal4Runx1ΔN50, Gal4Runx1ΔN57, Gal4Runx1ΔN72, and Gal4Runx1ΔN92 plasmids expressing parts of the N terminus of Runx1 as chimeric proteins with the DNA binding domain of Gal4 were also constructed by PCR amplification followed by cloning between the Ndel and BamHI sites of the pB9-Ndel vector.

Expression plasmids for the ΔN176 and ΔN183 N-terminal deletion derivatives of Runx2 were generated by PCR with Ffu-Turbo from the full-length cDNA by using the upstream primers 5′-CGCGTCTGAGATGTTGGAGATCATCGGG-3′ and 5′-CCGCTCGAGATGCCGGCAGAATGTGCCC-3′, respectively, and the common downstream primer 5′-GAAGATCTTCATATGGCCGCAGACAGACAC-3′. The amplified products were cloned as XhoI-BglII segments into the Ndel sites of the pB9-Ndel vector.

Retroviral transduction of ES cells was performed essentially as described previously using oligonucleotides with an optimal Runx1 binding site (5′-GTCGGTTTGGCGTTTGGCGGGA-3′) as DNA probe (40).
GGGAGACTCGTGCGC-3’ and the downstream primer 5’-GAAGATCTTTAGTAGGAGGTACACGCT-3’ followed by cloning as an XhoI-BglII segment into the pB9 vector.

The pET21a+His-based plasmid for *Escherichia coli* expression of ΔRunx1 (amino acids 1–185 of Runx1) has been described previously (42). Truncated genes encoding the N-terminal deletion derivatives ΔN24 (amino acids 25–185) and ΔN57 (amino acids 58–185) were constructed by PCR using *Pfu* polymerase (Stratagene) by using the upstream primers 5’-ACATGCACTGATGTGGGAGGTACTAGC-3’ and 5’-ACATGCATGGTGGAGATCATGAC-3’, respectively. ΔN24 and ΔN57 were cloned between the Spel and BamHI sites of pET21a+His, creating an N-terminal His tag.

The GM-CSF luciferase reporter plasmid containing a 716-bp segment of the enhancer and a 0.6-kb segment of the promoter of the human GM-CSF gene in a derivative of pGL2-basic has been described previously (44). The Iod1 luciferase reporter plasmid containing the Iod1 promoter (nucleotides −351 to +79) in a derivative of the pGL2-basic reporter plasmid has also been described previously (41, 45). The Gal4 luciferase reporter was pGL2-basic (Promega) with the GAL4 upstream activating sequence upstream of the TATA box of the alkaline phosphatase promoter.

Production and Purification of Recombinant Wild-type and Mutant Runx1 Proteins—Wild-type and mutant Runx1 proteins were expressed in *E. coli* strain BL21(DE3)pLysS (Stratagene) according to the manufacturer’s instructions. After harvesting, cells were lysed by freeze-thawing, then sonicated, and centrifuged. Supernatants were mixed with nickel-nitriolactacid acid-agarose (Qiagen) at 4 °C for 1 h, and the nickel-nitriolactacid acid-agarose was washed according to the manufacturer’s instructions. The proteins were eluted by increasing the imidazole concentration to 250 mM. Purified Runx1 proteins were dialyzed against 25 mM Tris-HCl, pH 7.5, containing 5% glycerol, 10 mM NaCl, and 0.1 mM EDTA, at 4 °C. The protein concentrations were determined with the BCA protein assay kit (Pierce), and protein aliquots were stored at −80 °C.

Production of Retrovirus, Retroviral Transduction of ES Cells, and *In Vitro* Hematopoietic Differentiation Assays—The cells of the packaging cell line EcoPack-293 (BD Biosciences) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum and transfected with MSCV vector, MSCV-Runx1 wild type, or deletion mutants by using the BD CalPhos™ mammalian transfection kit (BD Biosciences). Twenty-four hours posttransfection, the medium of the packaging cells was replaced with DMEM-ES medium (DMEM supplemented with 15% ES cell-qualified fetal calf serum (Invitrogen catalog number 10439-024), 4.5 × 10⁻⁴ M monothioglycerate (Sigma), and 1% conditioned medium from a cell line expressing cDNA for the murine leukemia inhibitory factor). Viral supernatants were collected after 24 h, filtered through a 0.45-μm filter, and used to infect Runx1−/− murine ES cells (kindly provided by Dr. Nancy Specch). These were cultured in DMEM-ES medium in gelatinized T25 flasks without feeders. Retroviral transduction of ES cells was performed as described previously (46, 47).

*In vitro* hematopoietic differentiation assays were performed essentially as described previously (46). Cells were stained with May-Grünwald Giemsa staining kit (VWR catalog numbers 52567-500 and 52566-500) according to the manufacturer’s instructions. Total RNA from ~2 × 10⁵ cells was prepared using TRIzol reagent (Invitrogen) and used for reverse transcription according to the manufacturer’s instructions. For PCRs, 8 µl of the resulting cDNA was used as template and amplified in a final volume of 25 µl using Taq DNA polymerase (New England Biolabs). The pairs of specific oligonucleotide primers used for amplification of GPIbβ and GPIIIα were 5’-AGGAGCAGGACCGCGCATCTCA-3’ and 5’-AGGGCTTCTGGGAGGAAGGGC-3’ and 5’-CTGCCGGAAGAAGCTGTCACTG-3’ and 5’-CATCTCCCTTGTAGCGGAC-3’, respectively (48).

**RESULTS**

The N Terminal and the α-Helix of the RD Reduce DNA Binding of Runx1—The Runx1 protein sequence N-terminal to the DNA binding RD has been shown to be a negative element for the DNA binding of the protein (25, 30, 31). The RD contains one α-helix formed by the first eight amino acids of this domain (residues Ser-50 to Asp-57), and previous work has shown that this α-helix is not part of the DNA-binding Ig fold β-barrel structure (21, 23). To determine whether this α-helix also plays a role in the DNA binding of Runx1, EMSAs were performed using a probe containing an optimal Runx1 binding site (40) and purified heterologously expressed Runx1 proteins. Because full-length Runx1 is highly protease-sensitive and unstable (40, 49), mouse Runx1 with C-terminal truncation after the RD (amino acids 1–185), denoted ΔRunx1, was expressed in *E. coli* and subsequently purified. N-terminal deletion mutant derivatives of ΔRunx1, denoted ΔN24 (amino acids 25–185) and ΔN57 (amino acids 58–185) in which the first 24 and 57 amino acids of the N terminus were deleted, respectively, were also produced and purified. ΔRunx1 with a full-length N terminus bound as expected to the EMSA probe, and a successively higher proportion of the DNA migrated in the ΔRunx1-DNA complex when the amount of ΔRunx1 protein was increased from 0.2 to 100 ng (Fig. 1A, lanes 2–6). Deletion of only 24 amino acids of the N-terminal sequence was sufficient to drastically increase the DNA binding of the protein. The increased binding of the ΔN24 protein to the probe made it necessary to reduce the amount of the protein relative to that of ΔRunx1. This N-terminal deletion mutant exhibited an ~50-fold enhanced ability to bind DNA compared with ΔRunx1 (compare the stronger band with 0.04 ng of ΔN24 in lane 7 and that with 1 ng of ΔRunx1 in lane 3). Interestingly the ΔN57 deletion that also deleted the α-helix of the RD in addition to all sequences N-terminal to this domain enhanced the ability to bind the DNA probe by at least 50-fold (compare the much stronger band with 0.04 ng of ΔN57 in lane 12 and that with 1 ng of ΔRunx1 in lane 3). This result clearly shows that the α-helix created by the first eight amino acids of the RD is not required for DNA binding of Runx1.

Because CBFβ both stabilizes the DNA binding structure of the RD and is required by Runx1 to transactivate genes, we investigated the role of the N terminus in the DNA binding in the presence of CBFβ also. Addition of CBFβ led to formation of a CBFβ-ΔRunx1-DNA triple complex in EMSA for ΔRunx1.
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(Fig. 1B, lanes 3 and 4) and both N-terminal deletion mutants, ΔN24 (lanes 6 and 7) and ΔN57 (Fig. 1B, lanes 9 and 10). Thus, N-terminal deletions including the α-helix in the RD did not affect the CBFβ binding. However, CBFβ increased the DNA binding of ΔRunx1 drastically, whereas the DNA binding of the N-terminal deletion mutants ΔN24 and ΔN57 was not much improved (note that 25-fold more ΔRunx1 than the N-terminal deletion mutants was used). These results suggested that the positive effect of CBFβ on the DNA binding of ΔRunx1 was to a large extent through counteraction of the inhibitory effect of the N-terminal sequences. The N-terminal 24 amino acids are clearly needed for inhibition of DNA binding. However, this analysis could not determine whether a second sequence within the 57 amino acids is also needed for the inhibition of the DNA binding. To analyze the effect of the α-helix in the RD on DNA binding in the presence of an intact N terminus, two ΔRunx1 mutants with point mutations in the α-helix were generated, and the proteins were produced in E. coli. The ΔRunx1-EV/2P mutant with two amino acids replaced with the α-helix-breaking amino acid proline was designed to destroy the α-helix, whereas four amino acids were replaced with the α-helix-stabilizing amino acid glutamine in the ΔRunx1-EVLD/4Q mutant. The ΔRunx1-EV/2P mutant exhibited an ~20-fold increase in DNA binding compared with ΔRunx1 (Fig. 1B, cf. lanes 2 and 14), whereas the ΔRunx1-EVLD/4Q mutant designed to stabilize the α-helix showed a slight reduction in DNA binding (Fig. 1B, cf. lanes 2 and 11). Both mutant Runx1 proteins bound normally to CBFβ because their complexes were supershifted with CBFβ (Fig. 1B, lanes 12 and 13 and lanes 15 and 16). Furthermore CBFβ efficiently enhanced the DNA binding of the four-glutamine mutant, suggesting that the enhancement of Runx1 DNA binding by CBFβ was to a large extent through counteraction of the negative effects of both the most N-terminal sequence of Runx1 and the α-helix in the RD. In summary, the results show that the α-helix within the RD is not required for DNA or CBFβ binding of Runx1 and suggest that the α-helix instead can act as a separate negative element for the DNA binding of Runx1.

The N-terminal Sequences Are Required for Transactivation by Runx1—To investigate whether the N-terminus also has a positive or negative role in transcriptional activation by Runx1, eukaryotic expression plasmids for a series of N-terminal deletion mutants of Runx1 were constructed (Fig. 2A). The promoter/enhancer of the GM-CSF gene is transactivated by Runx1, and the Ca2+/calmodulin-dependent phosphatase calcineurin has been shown to synergize efficiently with Runx1 at this promoter (42). Thus, a reporter plasmid with the GM-CSF promoter/enhancer was used to analyze the effects of N-terminal deletions of Runx1 on the transactivation in the presence and absence of co-expressed constitutively active catalytic subunit of calcineurin (ΔCN). The K562 erythroleukemia cell line was used because it contains very low levels of endogenous RUNX proteins (50, 51). As reported previously (42), the activity of the GM-CSF promoter/enhancer reporter did not increase when co-transfected with the constitutively active calcineurin expression vector ΔCN alone (Fig. 2B). Co-transfection with wild-type Runx1 alone slightly increased the activity of the GM-CSF reporter, and as reported previously (42), a very efficient synergistic stimulation was obtained when the GM-CSF promoter/enhancer reporter was co-transfected with expression plasmids for both wild-type Runx1 and ΔCN (Fig. 2B). Surprisingly despite a 50-fold increase in DNA binding and retained CBFβ interaction, the N-terminal deletions of Runx1, ΔN50 and ΔN57, reduced the activation of the reporter (Fig. 2B). Moreover the synergistic activation in conjunction with ΔCN was not increased with any N-terminal deletion of Runx1. Instead the synergistic activation decreased successively with increasing N-terminal deletions of Runx1 (ΔN24, ΔN50, and ΔN57), and the transactivation was almost completely abolished when 72 or 92 N-terminal amino acids were deleted (Fig. 2B). The effect of the N-terminal sequences on transactivation by Runx1 was also analyzed in other cells, the T cell-derived Jurkat cell line. This cell line has a higher level of endogenous RUNX proteins (50, 51). As reported previously (42), the activity of the GM-CSF promoter/enhancer reporter did not increase when co-transfected with the constitutively active calcineurin expression vector ΔCN alone (Fig. 2B). Co-transfection with wild-type Runx1 alone slightly increased the activity of the GM-CSF reporter, and as reported previously (42), a very efficient synergistic stimulation was obtained when the GM-CSF promoter/enhancer reporter was co-transfected with expression plasmids for both wild-type Runx1 and ΔCN (Fig. 2B). Surprisingly despite a 50-fold increase in DNA binding and retained CBFβ interaction, the N-terminal deletions of Runx1, ΔN50 and ΔN57, reduced the activation of the reporter (Fig. 2B). Moreover the synergistic activation in conjunction with ΔCN was not increased with any N-terminal deletion of Runx1. Instead the synergistic activation decreased successively with increasing N-terminal deletions of Runx1 (ΔN24, ΔN50, and ΔN57), and the transactivation was almost completely abolished when 72 or 92 N-terminal amino acids were deleted (Fig. 2B). The effect of the N-terminal sequences on transactivation by Runx1 was also analyzed in other cells, the T cell-derived Jurkat cell line. This cell line has a higher level of endogenous RUNX proteins, leading to a low level of transactivation by transfected Runx1 alone and also lower but still robust synergistic activation with ΔCN (42). Only transactivation by wild-type Runx1 and the smallest N-terminal deletion, ΔN24, was substantially over background without co-expression of ΔCN (Fig. 2C). Corresponding to the results in K562 cells, the synergistic transactivation of the GM-CSF promoter/enhancer
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together with ΔCN decreased gradually with the successively larger N-terminal deletions in Jurkat cells also (Fig. 2C). To determine whether or not the requirement for the N-terminal sequences in transactivation by Runx1 is specific to the GM-CSF promoter/enhancer, a reporter for another promoter, the immunoglobulin germ line promoter Ix1, which regulates class switching to IgA, was also used. This promoter is activated by Runx1, and it is very efficiently activated by synergy between Runx1 and Smad proteins (45, 52). As expected, transfection of full-length Runx1 alone activated the Ix1 promoter by about 12-fold, and co-transfection with Smad3 expression vector synergistically activated transcription of the promoter ~150-fold (Fig. 2D). In contrast, the successively larger N-terminal deletions resulted in a gradual reduction in Runx1 transactivation of the Ix1 promoter both without co-transfection of Smad3 and in synergy with co-transfected Smad3 (Fig. 2D). The activation of transcription was completely abolished when 72 or more N-terminal amino acids of Runx1 were deleted (Fig. 2D). These results suggest that the dependence of transactivation by Runx1 on N-terminal sequences is neither promoter- nor activator-specific because these sequences were found to be essential for activation of both the GM-CSF promoter/enhancer and the Ix1 promoter and in synergy with different activating proteins.

The reduced transcriptional activation by N-terminally deleted Runx1 was not caused by reduced expression or stability of the proteins because the corresponding effects of the deletions were obtained when N-terminally FLAG-tagged derivatives of Runx1 wild type and the N-terminal deletion mutants were compared, and equal amounts of these N-terminally FLAG-tagged wild type and N-terminal deletion mutants were detected in Western blot (data not shown). Furthermore the negative effects of N-terminal deletions of Runx1 were also obtained for N-terminal Gal4 fusion derivatives (see below).

To investigate the effect of the α-helix in the RD on the transactivation by Runx1 in the presence of an intact N terminus, Runx1 mutants with point mutations or deletion in the α-helix were generated (Fig. 3A), and their transactivation of the GM-CSF promoter/enhancer with and without co-transfection of ΔCN was analyzed in Jurkat and K562 cells. The EV/LD/4Q mutant of Runx1 (Fig. 3A), which showed reduced DNA binding ability (Fig. 1B), also failed to transactivate the reporter in Jurkat cells (Fig. 3B). Two mutants, ED/2Q and VL/2Q, that each contained two of these glutamine mutations (Fig. 3A) retained less than half of the wild-type synergy with ΔCN, and the transactivation in the absence of ΔCN was down to background (Fig. 3B). However, both the α-helix-breaking EV/2P mutant, which showed strongly enhanced DNA binding ability (Fig. 1B), and a Δ52–57 mutant with the α-helix deleted also displayed loss of some of the transactivation ability (Fig. 3B). The analysis of these five mutants of the α-helix was also done in K562 cells, and very similar results were obtained (Fig. 3C).

The promoter either with (filled bars) or without (open bars) co-transfection with expression plasmid for Smad3 in K562 cells. Cells were harvested 20 h after transfection. Bars represent the average luciferase activity expressed from the reporter plasmid in three independent transfections ± S.E. using β-galactosidase expression from an hCMV-β-galactosidase plasmid for normalization.
Thus, alteration of the α-helix either by disruption, stabilization, or deletion led to reduction of transactivation by Runx1. Collectively the results show that although the α-helix is a negative element for DNA binding as seen in EMSA, it is required for the transcriptional activity of Runx1.

RUNX2 and RUNX3, the other two RUNX family members, also have sequences N-terminal to the RD and the α-helix sequence within the RD. Although the sequences N-terminal to the RD differ in both content and length, the α-helix sequence within the RD is highly conserved between RUNX1, RUNX2, and RUNX3. Thus, the effects of N-terminal deletions on activation of the GM-CSF reporter in K562 cells were analyzed for Runx2 and Runx3 also. We have reported previously that Runx2 can synergize with constitutively active calcineurin at the GM-CSF promoter/enhancer, although the synergistic activation was weaker than for Runx1 (42). Analysis showed that human RUNX3 can also synergize with CN in activation of the GM-CSF reporter, and the level of the synergistic activation was intermediate between the level with Runx1 and the level with Runx2 (cf. Fig. 4 and Fig. 2B). For analysis of the possible role of the N-terminal regions of Runx2 and RUNX3 in the transactivation, deletion mutants were generated. The N176 mutant of Runx2 retains the α-helix of the RD, whereas the N183 mutant and the N61 mutant of RUNX3 correspond to the N57 mutant of Runx1 by starting directly after this conserved α-helix sequence. Cells were harvested 20 h after transfection. The bars represent the average luciferase activity expressed from the reporter plasmid in three independent transfections ± S.E. using β-galactosidase expression from an hCMV-β-galactosidase plasmid for normalization.

FIGURE 3. Effects of mutations in the α-helix of the RD on transactivation by Runx1. A, schematic representation of wt Runx1, point mutants of the α-helix in the RD, and the α-helix deletion mutant Runx1-Δ52–57. B and C, Jurkat cells (B) and K562 cells (C) were transfected with wild-type Runx1, the indicated mutant of the α-helix in the RD, or pBJ9 vector control either with (filled bars) or without (open bars) co-transfection with expression plasmid for constitutively active calcineurin (ΔCN). The ΔN176 mutant protein of Runx2 has the α-helix in the RD intact, whereas the ΔN183 mutant and the ΔN61 mutant of RUNX3 correspond to the ΔN57 mutant of Runx1 by starting directly after this conserved α-helix sequence. Cells were harvested 20 h after transfection. The bars represent the average luciferase activity expressed from the reporter plasmid in three independent transfections ± S.E. using β-galactosidase expression from an hCMV-β-galactosidase plasmid for normalization.

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RUNX2 and RUNX3, the other two RUNX family members, also have sequences N-terminal to the RD and the α-helix sequence within the RD. Although the sequences N-terminal to the RD differ in both content and length, the α-helix sequence within the RD is highly conserved between RUNX1, RUNX2, and RUNX3. Thus, the effects of N-terminal deletions on activation of the GM-CSF reporter in K562 cells were analyzed for Runx2 and Runx3 also. We have reported previously that Runx2 can synergize with constitutively active calcineurin at the GM-CSF promoter/enhancer, although the synergistic activation was weaker than for Runx1 (42). Analysis showed that human RUNX3 can also synergize with ΔCN in activation of the GM-CSF reporter, and the level of the synergistic activation was intermediate between the level with Runx1 and the level with Runx2 (cf. Fig. 4 and Fig. 2B). For analysis of the possible role of the N-terminal regions of Runx2 and RUNX3 in the transactivation, deletion mutants were generated. The ΔN176 mutant of Runx2 retains the α-helix of the RD, whereas the ΔN183 mutant and the ΔN61 mutant of RUNX3 start directly after this conserved α-helix sequence. Both transfection of Runx2 and RUNX3 wild type could activate the GM-CSF promoter/enhancer to some extent also in the absence of ΔCN, whereas no transactivation significantly over background was obtained for any of the deletions in the absence of ΔCN (Fig. 4). For Runx2ΔN183, no activation over background
was seen, whereas the corresponding mutant of RUNX3 (RUNX3ΔN61) gave a synergistic activation that was only slightly over the background (Fig. 4). The N-terminal mutant of Runx2 that retained the α-helix (Runx2ΔN176) showed partial loss of the synergistic activation with ΔCN (Fig. 4). Thus, the N-terminal sequences including the α-helix within the RD are required for full transcriptional activity not only for Runx1 but also for Runx2 and RUNX3. The requirement of the N-terminal sequences for transactivation, including the α-helix within the RD, appears to be a conserved characteristic of the RUNX family of proteins.

The N Terminus of Runx1 Can Transactivate Gene Expression in the Gal4 System—The results presented indicate that the N-terminal sequences of Runx1 constitute an autonomous transactivation domain. However, an alternative possibility would be that the transcriptional effect of the N-terminal sequences comes from a positive effect on the DNA binding of the RD that would occur only in the in vivo transcription assay but not in vitro in EMSA. Thus, to determine whether the N-terminal sequences of Runx1 were also important for transcription based on a heterologous DNA binding domain, constructs expressing chimeric proteins containing the DNA binding domain of Gal4 (amino acids 1–147) fused to the N terminus of Runx1 wild type or the various N-terminal deletion mutants were generated. The expression plasmids for Gal4 DNA binding domain–Runx1 fusions were used to co-transfect K562 cells and the B cell line DG75 together with a Gal4 luciferase reporter. The Gal4-Runx1 wild-type fusion protein was capable of stimulating transcription of the Gal4 reporter by 5-fold compared with the Gal4 DNA binding domain alone in K562 cells (Fig. 5A). The transactivation became more and more reduced for fusion proteins of Gal4-Runx1 with successively larger N-terminal deletions and was only completely abolished when 92 amino acids of the N terminus were deleted. Similar results were also obtained using the DG75 cell line. Although the Gal4-Runx1 wild-type fusion protein stimulated transcription of the Gal4 reporter to a lower level in DG75 cells than in K562 cells, transactivation became more and more reduced for fusion proteins of Gal4-Runx1 with successively larger N-terminal deletions in this cell line also (Fig. 5B). These data further support the notion that the N terminus of Runx1 is a bona fide transactivation domain. To find out whether the N terminus of Runx1 is an independent transactivation domain that can function in the absence of the C-terminal transactivation domain and in the absence of a complete DNA binding RD, constructs expressing chimeric proteins containing the Gal4

**FIGURE 5.** The N terminus of Runx1 is essential for activation of transcription from a Gal4 reporter when fused to the Gal4 DNA binding domain. A and B, transfection of K562 cells (A) or DG75 cells (B) with expression vector for Gal4 DNA binding domain (Gal4DBD) alone, Gal4-Runx1 wild type, Gal4 DNA binding domain fusions of the indicated N-terminal deletion mutants, or empty expression vector together with Gal4 luciferase reporter plasmid. C and D, transfection of K562 cells (C) or DG75 cells (D) with expression vector for Gal4 DNA binding domain alone, Gal4 DNA binding domain fusions with the indicated segments from the N terminus of Runx1, or empty expression vector together with the Gal4 luciferase reporter plasmid. Cells were harvested 20 h after transfection. Bars represent the average luciferase activity expressed from the reporter plasmid in three independent transfections ± S.E. using β-galactosidase expression from an HCMV-β-galactosidase plasmid for normalization.
DNA binding domain fused to the isolated N-terminal segments of various lengths from Runx1 were generated and analyzed in co-transfections with the Gal4 luciferase reporter. In both the K562 and DG75 cell lines, Gal4 DNA binding domain-isolated N terminus fusion proteins transactivated the Gal4 reporter more than the Gal4 DNA binding domain alone (Fig. 5, C and D), although the level of transactivation was lower than for the chimeric constructs that also included the C-terminal transactivation domain. Collectively the results that N-terminal deletions led to reduced transactivation by Gal4-Runx1 fusion proteins and that Gal4 fusion proteins containing only N-terminal sequences of Runx1 could activate transcription confirm the presence of a bona fide autonomous transactivation domain at the N terminus of Runx1.

Analysis of N-terminal Deletion Mutants in Runx1-dependent Megakaryocyte Development from Embryonic Stem Cells Differentiated in Vitro—Runx1 is indispensable for development of definitive hematopoietic lineages during embryonic development in vivo and during in vitro differentiation of ES cells (1, 53). Moreover an inducible targeting approach in adult hematopoiesis has revealed the particular importance of Runx1 in the differentiation and maturation of the megakaryocyte/platelet lineage (3, 13). To investigate whether the N terminus contributes to the function of Runx1 during the development of definitive hematopoietic lineages, the Runx1 mutants with progressive N-terminal deletions were introduced into Runx1−/− mouse ES cells by retroviral transduction. Embryoid bodies (EBs) were generated from ES cells transduced with control MSCV retrovirus vector or with retrovirus expressing wild-type (wt) Runx1 cDNA or the various deletion mutants. The EB cells were subsequently cultured with interleukin-3, thrombopoietin, and Steel factor as these growth factors promote megakaryocyte development from hematopoietic progenitor cells. Megakaryocyte development was assessed after 8 days by May-Grünwald Giemsa staining and by expression of the megakaryocyte-specific genes GPIbβ and GPIIIα (48). As expected, no megakaryocytopenesis was observed in the EB cells generated from Runx1−/− ES cells transduced with control vector (Fig. 6, A and G), whereas megakaryocytopenesis occurred in EB cells generated from ES cells transduced with wt Runx1 cDNA (Fig. 6, B and G). Runx1−/− ES cells transduced with Runx1 N-terminal deletion mutant retrovirus MSCV-ΔN50 or MSCV-ΔN57 could also generate megakaryocytes upon in vitro differentiation at least as efficiently as ES cells transduced with wt cDNA (Fig. 6, B, C, D, and G). In contrast, no megakaryocyte development could be observed upon in vitro differentiation of Runx1−/− ES cells transduced with either of the other two N-terminal deletion mutants, ΔN72 or ΔN92 (Fig. 6, E, F, and G).

**DISCUSSION**

The only previously known transactivation domain of RUNX proteins is located C-terminal to the RD and has been shown to be required for the transcriptional activity of these proteins (25, 30, 54). This study shows that the N terminus of Runx1, including the α-helix within the RD, in addition to acting as a negative element for DNA binding, functions as a second distinct transactivation domain that is required for activation of transcrip-
tion of at least some promoters by Runx1. Despite this, however, the N terminus including the α-helix is not required for megakaryocyte development from ES cells differentiated in vitro. This separation of the global function of Runx1 in cell differentiation from specific molecular characteristics, such as DNA binding and regulation of specific genes, might partly explain the wide range of regulatory functions in normal hemato-
poiesis and the pathological role of both translocations and point mutations of the Runx1 gene in different leukemias.

The N terminus was required for transactivation of the GM-
CSF and IκB promoters by Runx1, whereas in vitro hematopoietic differentiation from Runx1+/− mouse ES cells showed that the N-terminal deletion mutants ΔN50 and ΔN57 were capable of rescuing megakaryocyte development. This rescue was not abolished until 72 amino acids at the N terminus were deleted, i.e. a deletion that included important DNA binding sequences in the Ig fold of the RD. This rescue by the N-terminal deletion mutants ΔN50 and ΔN57 shows that this protein region is not required for the relatively complex process that leads to formation of megakaryocytes, but it may have lineage-specific functions and/or functions at later stages of differentiation (e.g. expression of certain lineage-specific genes). Most point mutations in Runx1 associated with malignant development are in the RD and generate a transcriptionally non-functional protein (37, 55). Some mutations in Runx1, associated with myelodysplastic syndrome/acute myeloid leukemia, have also been demonstrated to be unable to rescue megakaryocyte development from Runx1-deficient ES cells (56). However, our results suggest that N-terminal mutations of Runx1 in leukemias (35–37) could generate functional proteins based on their ability to pro-
mote hematopoietic differentiation. Such mutated Runx1 proteins would lead to the generation of large numbers of hematopoietic cells containing Runx1 protein unable to regulate the expression of a number of genes normally regulated by Runx1. A relevant example from a pathological point of view would be the tumor suppressor gene p14ARF, which is important for activation of p53 function. Runx1 binds to the p14ARF promoter and transactivates p14ARF (57), and generation of numerous cells putatively unable to transactivate this gene or genes with similar function would increase susceptibility to leukemic transformation.

Our findings are in line with the existence of isoforms of RUNX1 with different N termini and differences in their prop-
erties. The RUNX genes have the potential to encode such iso-
forms due to alternative promoter usage (2). RUNX isoforms transcribed from the proximal promoters have a slightly shorter N terminus than isoforms transcribed from the distal promoters, and the most N-terminal amino acids differ between the isoforms. Runx1 from the distal promoter (distal Runx1) has been found to be expressed mainly in T lineage cells and hematopoietic stem cells and at lower levels in B cell develop-
ment, whereas proximal Runx1 has been found to predomi-
nate in some myeloid cells (32). This indicates that distal Runx1, which was used in this study, and proximal Runx1 may possess unique properties and have distinct roles in hemato-
poiesis. The proximal Runx1 isoform, but not the distal isoform, has been found to delay mitotic arrest of a myeloid cell line under differentiating conditions (32). This suggests that the N terminus may have important roles in differential regulation of RUNX protein functions and raises the possibility that the N-terminal transcriptional activation domain is responsible for modulating isoform-specific functions of RUNX proteins. Notably megakaryocytopenesis was still found to be intact when not only the isoform-specific part of the N terminus but also its common part and even the conserved α-helix in the RD were deleted.

The in vitro DNA binding analysis showed that partial, or complete, deletion of the N-terminal region increased DNA binding by ~50-fold but did not affect the ability of CBFβ to participate in the complex with Runx1 and DNA. Although the α-helix between amino acids 50 and 57 is part of the RD, it was not required for DNA binding of Runx1. Instead it inhibited the DNA binding because both deletion and disruption of the α-helix increased the DNA binding, whereas stabilization of the α-helix slightly reduced it. How can the α-helix, which is not part of the DNA binding Ig fold of the RD (21, 23), inhibit the DNA binding? The basis of a likely explanation may lie in the finding that the free Ig domain structure is in a conformation in which the affinity for DNA is suboptimal and that CBFβ binding triggers a structural switch that stabilizes the Ig domain in a conformation for sustained DNA binding (8, 21). This ability of the Ig domain to switch between a DNA binding and a subop-
timal structure would make it potentially sensitive to a sur-
rounding protein structure that can shift the Ig domain between the two alternative structures. The possibility that the α-helix in the RD may be capable of this shift is supported by its mobility. Crystallography has shown that the α-helix can make contact with the Ig fold at either of two positions representing a 3.9-Å displacement; in a third crystal structure, the α-helix was so flexible that its position could not be traced (21). Thus, the contacts of the α-helix with the Ig domain might favor the structure that is suboptimal for DNA binding, or perhaps the contacts just increase the rate of switching between the alternative structures so the off rate would increase for the Runx1 complex with DNA.

The N-terminal deletion mutants ΔN24 and ΔN57 of Runx1, with better in vitro DNA binding, functional CBFβ binding, and an intact C-terminal transactivation domain, nevertheless exhibited reduced transactivation ability. This suggests 1) that the DNA binding of the wild type, which is relatively low in vitro, is improved in vivo and 2) that an intact C-terminal transactivation domain is not sufficient for full transactivation ability of Runx1. CBFβ binding, which is needed for Runx1 function in vivo (1), improved the DNA binding of the wild type in vitro (Fig. 1B). Perhaps this CBFβ effect is sufficient in vivo, or it may function in synergy with some protein interaction at the N terminus of Runx1 that might block the negative effect of these sequences on the DNA binding of the Ig domain structure in the RD.

The N terminus of Runx1 and the α-helix in the RD were both found to be important for transactivation of the GM-CSF and IκB promoters by Runx1. A deletion not including the α-helix reduced the transactivation by Runx1, and the α-helix was also important because the transactivation by Runx1 was reduced by point mutations and a small deletion confined to the α-helix. The importance of the N terminus of Runx1 and the
Transactivation Domain in the N Terminus of Runx1

α-helix in the RD for transactivation of both the GM-CSF and Il1α promoters could be the result of a positive interaction with a transcription factor neighbor common to both promoters or the result of a hypothetical positive influence of these sequences on the DNA binding of Runx1 in vivo. However, the N terminus including the α-helix in the RD of Runx1 was also found to be required for transactivation of a Gal4 reporter when Runx1 was expressed as a fusion protein with a Gal4 DNA binding domain, and the isolated N terminus of Runx1 was also capable of stimulating transcription when fused to the Gal4 DNA binding domain. These results confirm the presence of a bona fide autonomous transactivation domain in the N terminus of Runx1.

What protein interaction(s) of the N terminus and the α-helix within the RD might be important for transactivation by Runx1? One candidate could be the p300 co-activator that has been shown to bind to the C-terminal transactivation domain of RUNX1 (58). Acetylation of two lysines in the N terminus of RUNX1, Lys-24 and Lys-43, by p300 has been shown recently to augment the DNA binding activity of RUNX1, and disruption of these two lysines was found to severely impair the DNA binding and reduce the transcriptional activity and transforming potential (59). This ability of p300 to recognize substrates at the N terminus implies that p300 also has some ability to recognize N-terminal sequences. However, we have shown that the N-terminal transactivation domain can also function with a heterologous DNA binding domain (Fig. 5), arguing that much, or all, of the effect of the co-activator is independent of the DNA binding of the RD. Perhaps p300, in addition to the reported C-terminal interaction and improvement of DNA binding, also has an effect on RUNX1 function that is independent of DNA binding through N-terminal interaction. At present, however, we cannot exclude the possibility that the transactivation function of the N-terminal sequences of RUNX1 involves (an)other co-activator(s). The size of the N-terminal transactivation domain and the gradual reduction of the transactivation when deleting or point mutating part of it (Figs. 2, 3, and 5) would indicate that more than one co-activator may interact with this domain.

Our results support the notion that the presence of an N-terminal second transactivation domain is not unique to Runx1. The N terminus and the α-helix in the RD are also essential for transactivation by the other two RUNX family members, Runx2 and RUNX3 (Fig. 4). The three RUNX proteins have C-terminal transactivation domains that have some similarity in sequence and interactions with some common cofactors and also a partial overlap in their biological functions (8, 54, 60). However, the lengths and sequences of the N-terminal transactivation domains are quite different among the RUNX proteins. These differences may be reflected in differences between the properties of the N-terminal transactivation domains that might contribute to the distinct roles that the RUNX proteins have despite the fact that the proteins heterodimerize with a common CBFβ and bind the same consensus DNA sequence.

There are also a number of different C-terminal splice forms among which some isoforms lack part, or all, of the C-terminal transactivation domain, and some isoforms also lack much of the RD. These isoforms may be expressed in the same tissues that also express full-length RUNX1, but they have different functions (27, 32, 34, 61, 62). Runx1 is able to repress CD4 in CD4/CD8 double positive thymocytes, and interestingly the splice forms containing only a short N terminus and part of the RD (32), and even the isolated N terminus, can instead act in a way opposite to wild-type Runx1 by enhancing survival and/or proliferation (27). This suggests that the N terminus of RUNX1 can have significant biological function in the absence of DNA binding and supports the idea that this part of RUNX1 can make important protein interaction(s).

In summary, we have identified the N terminus, including the α-helix in the RD, of Runx1 to be a negative regulator of DNA binding and also an important second transactivation domain that can modulate the function of Runx1 activity. This differential use of the N terminus might be an evolutionary strategy to ensure specific functions of the protein at different development stages.

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