Lymphoid Enhancer Factor-1 Links Two Hereditary Leukemia Syndromes through Core-binding Factor α Regulation of ELA2*

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Two hereditary human leukemia syndromes are severe congenital neutropenia (SCN), caused by mutations in the gene ELA2, encoding the protease neutrophil elastase, and familial platelet disorder with acute myelogenous leukemia (AML), caused by mutations in the gene AML1, encoding the transcription factor core-binding factor α (CBFα). In mice, CBFα regulates the expression of ELA2, suggesting a common link for both diseases. However, gene-targeted mouse models have failed to reproduce either human disease, thus prohibiting further in vivo studies in mice. Here we investigate CBFα regulation of the human ELA2 promoter, taking advantage of bone marrow obtained from patients with either illness. In particular, we have identified novel ELA2 promoter substitutions (−199 C to A) within a potential motif for lymphoid enhancer factor-1 (LEF-1), a transcriptional mediator of Wnt/β-catenin signaling, in SCN patients. The LEF-1 motif lies adjacent to a potential CBFα binding site that is in a different position in human compared with mouse ELA2. We find that LEF-1 and CBFα co-activate ELA2 expression. In vitro, the high mobility group domain of LEF-1 interacts with the runt DNA binding and proline-, serine-, threonine-rich activation domains of CBFα. ELA2 transcript levels are up-regulated in bone marrow of an SCN patient with the −199 C to A substitution. Conversely, a mutation of the CBFα activation domain, found in a patient with familial platelet disorder with AML, fails to stimulate the ELA2 promoter in vitro, and bone marrow correspondingly demonstrates reduced ELA2 transcript. Observations in these complementary patients indicate that LEF-1 cooperates with CBFα to activate ELA2 in vivo and also suggest the possibility that up-regulating promoter mutations can contribute to SCN. Two hereditary AML predisposition syndromes may therefore intersect via LEF-1, potentially linking them to more generalized cancer mechanisms.

Two hereditary human bone marrow failure syndromes leading to leukemia are severe congenital neutropenia (SCN) and familial platelet disorder with acute myelogenous leukemia (FDP/AML).

SCN consists of low numbers of neutrophils, the primary phagocytic white blood cell (1, 2). Examination of the bone marrow reveals that maturation of the myeloid lineage fails at the promyelocytic stage, before the formation of terminally differentiated neutrophils (3). Many patients (4–6) with SCN develop myelodysplasia or AML. The most common genetic cause of SCN is heterozygous mutation of ELA2 (7), encoding neutrophil elastase (NE). NE (8) is a serine protease, mainly found in the granules of neutrophils, and ELA2 mutations also cause a related disorder, cyclic neutropenia (9).

In contrast, FDP/AML is an autosomal dominant bleeding disorder composed of low numbers of functionally defective platelets that also frequently evolves to myelodysplasia or AML (10). FPD/AML results from mutations in AML1 (11), on chromosome 21, encoding the transcriptional regulator core-binding factor α (CBFα). Somatic mutations of CBFα (12, 13) and the t(8;21) translocation, producing the AML1/ETO fusion gene, are among the most common abnormalities of AML (14, 15). The translocation t(12;21), generating the TEL/AML1 fusion gene, and AML1 gene amplification (16) appear often (17, 18) in acute lymphoblastic leukemia. Somatic AML1 mutations are also frequent in myelodysplasia (19). CBFα contains a domain homologous to the Drosophila pair rule gene runt (20), directing promoter recognition and heterodimerization with the β subunit of core-binding factor, CBFβ, thus stabilizing DNA binding.

ELA2 appears to be a downstream target of CBFα, suggesting a possible clue for why leukemia is a feature common to both syndromes. In mice, CBFα regulates (21) expression of the murine neutrophil elastase promoter (mELA2) through a binding site adjacent to recognition sites for the factors PU.1, c-Myc, and C/EBP. The latter three cooperatively regulate the mELA2 promoter in vitro (22, 23).

Attempts to model these two leukemia syndromes in mice have failed, however. Knock-in mice targeted with an SCN-causing human ELA2 mutation demonstrate normal hematopoiesis (24). Knock-out mice targeted for heterozygous disruption of AML1 also lack thrombocytopenia, platelet abnormalities, and leukemia (25–27). A possible explanation for the shortcomings of the mouse

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1 The abbreviations used are: SCN, severe congenital neutropenia; AML, acute myelogenous leukemia; FPD, familial platelet disorder; NE, neutrophil elastase; CBFα, core-binding factor α; LEF-1, lymphoid enhancer factor-1; C/EBP, CCAAT/enhancer-binding protein; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; HMG, high mobility group; PST, proline-, serine-, and threonine-rich; CREB, CAMP-response element-binding protein.
models is that there may be differences in transcriptional control of hematopoiesis. In particular, a CBFA binding site does not appear conserved at the corresponding position in the human neutrophil elastase (hELA2) promoter, and there is evidence for the involvement only of PU.1 (28) and C/EBP (29) but not c-Myc (30).

To define the regulation of the hELA2 promoter in vitro toward the goal of investigating a potential transcriptional linkage between these two leukemic syndromes, we here utilize bone marrow from patients with these two diseases. One sample comes from an SCN patient demonstrating a novel ELA2 promoter sequence substitution, whereas the other derives from a FPD/AML patient with an AML1 activation domain mutation. We find that CBFA directly interacts with lymphoid enhancer factor-1 (LEF-1). LEF-1 is an effector of Wnt signaling (31), a pathway entrenched in cancer mechanisms. The cooperative activation of ELA2 by LEF-1 and CBFA suggests that there may be a common link between both syndromes that may, in turn, connect them to more general cancer mechanisms.

EXPERIMENTAL PROCEDURES

Patients—Bone marrow aspirates from patients and controls, subjected to Institutional Review Board-approved protocols, represent dis- carded portions of samples obtained during care. The 199 C to A ELA2 substitution appears in two SCN cases. One is an 11-year-old girl with SCN presenting at age 4 years following recurrent aphthous stomatitis; other family members were unavai- lable. Mutations in other known and candidate SCN genes, including Gf1, were not detected in either SCN patient. IV/2 pedigree 3 of Michaud et al. (47) is a 19-year-old man with the Y200X AML1 mutation developing M5 AML. Bone marrow was aspirated during first complete remission, when blood cell counts were normal.

Vectors and Site-directed Mutagenesis—Human and mouse ELA2 promoters were PCR-amplified from peripheral blood mononuclear cell DNA and subcloned into the pGL3 firefly luciferase reporter (Promega). Human LEF-1-1HA, dnLEF-1-1HA, and pt-β-catenin expression constructs have been described (32, 33). Human CBFA and CBFB pCNA3 (Invitrogen) expression vectors were gifts of Dr. H. Scott (Walter and Eliza Hall Institute). cDNA encoding human PU.1, cEBP, and c-Myc were reverse transcription (RT)–PCR-amplified from HL-60 cells and sub- cloned into pCS2+ expression vectors (34). LEF-1 cDNA was subcloned into pCS2+ for in vitro transcription/translation. Mutagenesis of oligonucleotide cassette was used to construct ELA2 promoter and LEF-1 and CBFA mutations. LEF-1 and CBFA mutants were subcloned into pCDNA3.1 expression vector. pET-23b expression vector (Novaga- nes) was used to generate His-tagged LEF-1 and CBFA.

Cell Lines and Cultivation—HEK293T, HeLa, NIH-3T3, U-937, KG-1, and HL-60 cells were purchased from ATCC and grown in Dul- becco’s modified Eagle’s medium plus 10% fetal bovine serum (first three) or RPMI plus 12.5% fetal bovine serum (latter three). RT-PCR and Real Time PCR Analysis—RNA was isolated from bone marrow with QiAamp and from cell culture with RNeasy (Qiagen). RT-PCR was performed with TaqMan Gold (ABI) using random priming (or oligo(dT), as indicated) for 25 or 40 cycles, as noted. Real time PCR was performed using the ABI 7900HT system in a 50-μl volume for 40 cycles: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 sec; 60 °C for 1 min with 5′-FAM-5′-BHQ-1 ELA2 probe and VIC glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probe. ELA2 transcript was normalized to GAPDH using the standard curve method (manufactur- er’s protocol). Expression analysis of LEF-TCF factors used specific human primers with oligo(dT)-primed cDNA for 30 PCR cycles to pro- duce products of the indicated sizes. LEF-1, 251 bp; TCF-1, 247 bp; TCF-3, 250 bp; TCF-4, 264 bp.

Transient Expression of Recombinant Proteins and Luciferase Assay—For luciferase assays, 2 × 10⁵ or 4 × 10⁵ cells were seeded per well into 12- or 6-well tissue culture dishes, respectively, cultured overnight, and then transiently transfected using LipofectAMINE Plus (Invitro- gen) or oligo(dT)–primed DNA (unless stated otherwise), respectively, according to the manufacturer’s instructions. Renilla luciferase (pRL- TK; Promega) was co-transfected as an internal control, and the firefly activities were normalized to Renilla activity. In 12-well dish transfections, a total of 1 μg of the tested activators, 50 ng of reporter and 5 ng of pRL-TK were used (unless stated otherwise), and quantities per well were doubled for 6-well plates. Luminesmetry (Berthold Technologies) scored luciferase activity 24 h after transfection using the Dual-Lucif- erase assay (Promega). Transfections were performed in triplicate and repeated three times, and S.E. was reported. Empty vector was added to normalize the total amount of DNA in every transfection, and each transfection was performed in triplicate. 0.1 μg of the 280-bp ELA2 promoter was used as luciferase reporter in all of the assays (unless stated otherwise).

Recombinant Protein Expression—His-tagged LEF-1 and CBFA were produced in Escherichia coli. cDNAs were subcloned into pET-23b (Novagen) and expressed in the pLysS strain with nickel affinity chromato- graphy purification. In vitro transcription/translation was performed using TNT coupled reticulocyte lysate (Promega) with or without [35S]methionine (Amersham Biosciences).

Co-immunoprecipitation, Immunoblotting, and in Vitro Association Assay—Transiently co-transfected cells were lysed in 400 μl of iced lysis buffer (20 mM Tris-HCl, pH 8.0, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, and 10% glycerol), followed by 5 × 5 sec sonication. Lysates were cleared by centrifugation at 15,000 × g for 30 min at 4 °C, adjusted for protein concentration, and incubated with 2 μg of goat polyclonal anti-CBFA antisera (C-19; Santa Cruz Biotechnology) or rat monoclonal anti-hemagglutinin (anti-βA) antisera (Roche Applied Science) at 4 °C overnight with rotation, followed by the addition of 10 μl of protein G-Sepharose (Sigma) with 1 h of further incubation. Immunoprecipitates were then washed three times with 1 ml of ice-cold lysis buffer. 20 μl of loading buffer were added to the drained beads and then resolved by SDS-PAGE with immunoblot detection using ECL (Amersham Bio- sciences). The concentrations of antibodies for immunoblot were as follows: 3 μg/ml mouse monoclonal antibody to human LEF-1 (REMB1, Exalphi Biologicals); 1,500 C-19; 1,500 mouse monoclonal antibody to GAPDH (6C5; Biosciences); 1,000 anti-HA; 1,000 mouse monoclonal anti-FLAG antiserum (Sigma). Secondary detection employed 1:10,000 peroxidase-conjugated antibody (Jackson ImmunoResearch) re- active to the primary antibody. In vitro association assays used 25 μl of vector-programmed TNT extract. [35S]Labeled CBFA- or LEF-1-HA-con- taining extracts were mixed with unlabeled LEF-1 or CBFA extracts with or without HA or CBFB. Antibodies 5 μg of immunoprecipitated DNA served with 10 μl of iced lysis buffer. 20 μl of protein G beads (Sigma) were added and rotated for 1 h, and immunoprecipitates were subjected to SDS-PAGE with autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)—1 μg (unless stated otherwise) of nucleic affinity-purified, bacterially expressed His-tagged LEF-1 or CBFB was mixed with 1 ng of 35P-5′-radioabeled annealed double-stranded oligonucleotide probe in an equal volume of 2× buffer (40 mM Hepes, pH 7.6, 100 mM KCl, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and 1 mg/ml poly(dI-dC)), incubated for 30 min on ice, and then electrophoresed on a 4% polyacrylamide gel. Competitive oligonucle- otides, at indicated excess, were added 5 min before probe, and antibody of immunoprecipitated DNA served with rotation overnight at 4 °C. 20 μl of protein G beads (Sigma) were added and rotated for 1 h, and immunoprecipitates were subjected to SDS-PAGE with autoradiography.

Antisense Morpholino Oligonucleotide Knockdown—LEF-1, AML1, or scrambled sequence control antisense morpholino oligonucleotides (GeneTools) were transfected to HL-60 or U937 cells according to the manufacturer’s protocol. 17 h later, cells were extracted, and NE activity was assayed (34).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described (35) using U937 cells. 1 μg of mouse monoclonal antibody to human LEF-1 (REMB1) or goat polyclonal antibody to CBFA (N-20; Santa Cruz Biotechnology) were used to precipitate pro- tein-DNA complexes. About 5 μg of immunoprecipitated DNA served as a template in each reaction in a total volume of 50 μl and corresponded to about 1:10,000 to 1:20,000 of the genomic DNA isolated from 1.5 ml of soluble chromatin.

RESULTS

ELA2 Promoter Substitutions in SCN—ELA2 mutations af- fecting the protein sequence cause the majority of SCN cases (7); promoter mutations have not previously been sought. In 4 of 41 SCN patients lacking ELA2 coding abnormalities, we identified two different single nucleotide substitutions in the promoter after sequencing ~300 upstream base pairs. Two patients demonstrate a heterozygous C to A transition 15 bases upstream of the transcription start site in a potential GC-box for the general transcription factor Sp1 (Fig. 1A). Two
other SCN patients possess a heterozygous C to A transversion at position −199, within a potential LEF-1/TCF motif that differs from the consensus only by a G to T transition (Fig. 1A).

Neither variant appears in any of 47 SCN patients with ELA2 coding mutations. Among 260 controls, the −15 C to A substitution does not occur. The −199 C to A substitution is present in just one of these controls. SCN is usually a monogenic disorder; however, there is a suggestion that in some circumstances multiple inherited factors might be contributory (2).

Regardless of their potential contributions to disease, these variants afford an opportunity to investigate ELA2 transcriptional regulation in vivo.

Elevated ELA2 Expression in an SCN Patient with the Potential LEF-1/TCF Binding Site Substitution—To determine whether the −199 C to A substitution affects ELA2 expression, we performed RT-PCR (Fig. 1B) and real time RT-PCR (Fig. 1C) on bone marrow obtained from one of these SCN patients.

The ELA2 transcript shows an over 4-fold increase in the SCN patient compared with two normal controls. These results suggest that the −199 C to A substitution found in the LEF-1/TCF binding site of the ELA2 promoter up-regulates expression.

**Fig. 1. Elements of the ELA2 promoter.** A. Alignment of human and mouse ELA2 promoters, with gaps as needed, and identities underlined. The −15 and −199 C to A substitutions are shown in italic type. Transcription factor sites are highlighted. B. Elevated ELA2 expression, compared with GAPDH, in bone marrow of an SCN patient with the −199 C to A promoter substitution versus control, by RT-PCR, primed randomly. C. Real time RT-PCR results with ELA2 expression normalized to GAPDH, primed randomly or with oligo(dT). D. RT-PCR analysis of LEF-1/TCF expression in human bone marrow. E. RT-PCR analysis of LEF-1 expression in HL-60 and U-937 cells.
encoding LEF-1 and TCF-3, but neither TCF-1 nor TCF-4, in human bone marrow (Fig. 1D) and LEF-1 expression (Fig. 1E) in human HL-60 promyelocytes (39) and U-937 promonocytes (40), both of which (8) express ELA2. (Immunoblot analysis also confirms expression of LEF-1 in HL-60 cells, shown below in Fig. 3A.) We therefore have focused on LEF-1 as probably contributing to the transcriptional effects of the −199 C to A substitution.

β-Catenin-independent Enhancer Function of the Potential LEF-1 Element—We addressed whether the potential LEF-1 binding site contributes to in vitro ELA2 promoter activity by constructing derivative ELA2 promoters, containing either 560 or 260 bp of the upstream regulatory region, driving a luciferase reporter gene (Fig. 2A). We assayed activity in transiently transfected HEK293T kidney cells, commonly used to investigate Wnt/β-catenin signaling, with or without co-transfection of a LEF-1 expression vector. As shown in Fig. 2B, LEF-1 stimulates the wild type ELA2 promoter (NEP260WT). In contrast, reporters containing a deletion (NEP260ΔLEF-1) or substitutions (NEP260ΔLEM-1BM) within the LEF-1 binding site only weakly react to LEF-1, confirming that this sequence functions as a LEF-1-responsive element in vitro. (Assays in HeLa and NIH-3T3 cells produced similar results (not shown).) TCF-3 also activated the expression of ELA2 (data not shown).

Wnt signaling can proceed through noncanonical β-catenin-independent pathways (41), and LEF-1 can activate transcrip-
tion independently of β-catenin (42, 43). To determine whether β-catenin influences ELA2 transactivation, we tested the effects of pt-β-catenin, a stabilized form in which four amino-terminal serine and threonine residues are substituted with alanines (44). Co-expression of pt-β-catenin and LEF-1 did not significantly increase reporter activity (Fig. 2B). A dominant negative form of LEF-1 (dnLEF-1) lacking the β-catenin binding domain stimulated the ELA2 promoter as efficiency as the full-length pt-β-catenin (data not shown), indicating that LEF-1 activates the ELA2 promoter largely independently of β-catenin.

Elevated ELA2 Expression with the –199 C to A Substitution in HEK293T Cells—To determine whether the effect of the –199 C to A ELA2 substitution can be reproduced in vitro, we prepared reporters containing this substitution fused with either 560 bp (NEP560C>A) or 260 bp (NEP260C>A) of the upstream regulatory region (Fig. 2A). This substitution confers increased transcription upon both lengths of sequence in HEK293T cells, whereas there is no significant difference in the basal level (Fig. 2C). The effect is not as strong as what is apparent in the bone marrow of the SCN patient with the corresponding sequence change. There are at least two reasons for this difference. First, the reporter may not include all upstream elements required for co-activation of the more proximal promoter region present in the reporters. Second, HEK293T cells, derived from kidney, are unlikely to express an appropriate repertoire of co-activating transcription factors. Nevertheless, we can conclude that the elevated ELA2 transcription in bone marrow from an individual with this substitution probably results from this single base substitution, as opposed to the possibility of an undetected, additional DNA sequence change. There are at least two reasons for this possibility. First, the reporter may not include all upstream elements required for co-activation of the more proximal promoter region present in the reporters. Second, HEK293T cells, derived from kidney, are unlikely to express an appropriate repertoire of co-activating transcription factors.

LEF-1 Binding in Electrophoretic Mobility Shift Assays—To corroborate the function of the LEF-1 binding site in the ELA2 promoter, we carried out EMSAs employing recombinant LEF-1 (Fig. 2D). The probes consisted of 32P-5′-labeled double-stranded oligonucleotides spanning the region from –210 to –176 of the ELA2 promoter, corresponding to either the wild type sequence or the –199 C to A substitution. Fig. 2D shows that LEF-1 forms a retarded complex with the wild type sequence (compare lane 1 with lane 4). Anti-LEF-1 antibody (lane 3), but not control IgG (lane 2), supershifts the retarded complex. LEF-1 therefore binds specifically to the sequence found in the ELA2 promoter. LEF-1 also binds specifically to a probe containing the –199 C to A substitution (lane 10). When the cold –199 C to A substituted probe is used as a competitor to 32P-labeled wild type probe (compare lane 6 with lane 5), it out-competes the cold wild type probe (compare lane 7 with lane 6), suggesting that the effect of the sequence change is to increase the strength of LEF-1 binding. Similarly, the cold –199 C to A substituted probe also out-competes wild type probe in the assay employing labeled probe containing the –199 C to A substitution (compare lane 9 with lane 8). These results are consistent with both the in vivo and in vitro determinations of increased ELA2 expression arising from the –199 C to A substitution. To confirm that the –199 C to A substitution confers a significant change in LEF-1 binding affinity, we performed a quantitative “titration-EMSA” experiment with limiting concentrations of identical quantities of radiolabeled wild type or –199 C to A probes (6,000 cpm of each 32P-5′-end-labeled probe). As shown in Fig. 2E, in the presence of equal amounts of LEF-1, the –199 C to A probe forms more DNA-protein complex compared with the wild type.

Antisense LEF-1 Reduction of ELA2 Expression in Myeloid Cells—To determine whether LEF-1 activates ELA2 in myeloid cells in vitro, we used antisense morpholino oligonucleotides (45) to deplete endogenous LEF-1 protein in HL-60 and U-937 cells, which also express ELA2. Immunoblot (Fig. 3A) reveals that LEF-1 antisense (LEF-1MO) depresses LEF-1 protein quantities while leaving levels of GAPDH protein, serving as an internal control, unchanged. The LEF-1MO antisense oligonucleotides decreased ELA2 expression as judged by RT-PCR in both cell lines (Fig. 3B) and reduced concentration-dependent NE enzymatic activity in HL-60 cell extracts (Fig. 3C). These results confirm that LEF-1 functions as a transcriptional activator of the ELA2 promoter.

A CBFA Element in the ELA2 Promoter Upstream From That of the Mouse—The locations of motifs for the transcription factors PU.1, C/EBP, and c-Myb, all demonstrated to regulate mouse ELA2 expression, are nearly identical in both the murine and human ELA2 promoters; however, a documented recognition site for CBFα (5′-GGCCACA-3′) at –66 to –72 of the mELA2 promoter, which differs from the consensus (5′-PuAC-CPuCA-3′) by an A to G transition at the second position, is disrupted (21) at the corresponding position in the human ELA2 promoter (Fig. 1A). Note that a potential CBFα binding site at positions –185 to –192 (5′-AATCGCA-3′) better matches the consensus sequence, differing only by a C to T transition at position 3, and sits immediately downstream of the LEF-1 element. It is possible that the human promoter may therefore also be CBFα-responsive but that the binding site could be in a different place relative to that of the mouse.

We tested this potential alternative site. In transient transfection assays in both HEK293T (Fig. 4A) and HeLa (data not shown) cells, a 260-bp fragment of the ELA2 promoter (NEP260WT) responds to co-expression of CBFα, along with its partner, CBFβ, but mutation (NEP260AML1BM) of the proposed site abolishes the effect. Since the proposed CBFα motif differs from the consensus sequence by a C to A transition at the third position, we tested whether improving its match with the consensus would have an effect. We mutated the T to C to form a consensus site (AML1BC) and found that it did not further improve upon the ability of CBFα to activate the promoter (Fig. 4A). Next, we performed EMSA using recombinant CBFα protein and probes spanning the human ELA2 promoter from –210 to –176, corresponding to either the wild type sequence or the substituted sequence (AML1BM) found to be inactive in the above reporter assays. CBFα binds wild type probe to form a single retarded complex (Fig. 4B, lane 1). Excess amounts of unlabeled wild type probe, but not the substituted probe, inhibit complex formation (compare lanes 2 and 3). The complex supershifts upon the addition of CBFα antibody but not control IgG (compare lanes 6 and 5), confirming specific binding of CBFα to the proposed sequence. Experiments with a mutant probe mutated to match the consensus sequence by a C to T transition at the third position appeared to demonstrate a comparable level of affinity to CBFα (data not shown). Finally, we mutated a runt domain cysteine required for DNA binding activity (46) to serine at conserved position 72 (AML1bcC72S). The C72S substitution only weakly activates the ELA2 promoter, either alone or upon co-transfection with CBFβ (Fig. 4C). Thus, CBFα activates ELA2 through the upstream element situated next to the LEF-1-responsive site.

Reduced ELA2 Expression in Vito and in Vivo from an Activation Domain Mutation in AML1 Causing FPD/AML—Heterozygous AML1 deletions and point mutations within the runt domain abrogating DNA binding, nuclear translocation, or heterodimerization cause FPD/AML (11, 47). We previously identified an FPD/AML family (47) resulting from a novel nonsense substitution (Y260X) in the CBFA transcription domain. Y260X does not activate ELA2 either alone or with
co-expression of CBFβ upon transient transfection in HEK293T cells (Fig. 4C). To determine whether this mutation functions in a dominant negative manner, we coexposed Y260X with a wild type expression vector and found that Y260X repressed the activation of the wild type CBFβ. If CBFβ contributes to physiologic regulation of ELA2, as predicted from in vitro studies, then bone marrow from FPD/AML individuals should show decreased levels of ELA2 protein after transfecting CBFβ morpholino oligonucleotide, compared with GAPDH control, as judged by RT-PCR for 30 cycles, with or without reverse transcriptase (RT) as control. F, time-dependent decrease in NE activity after transfection of HL-60 cells with CBFβ morpholino oligonucleotide, assayed in cell extracts; a representative result is shown.

**Antisense CBFα Reduction of ELA2 Expression in Myeloid Cells**—We verified that CBFβ transactivates ELA2 in HL-60 cells by depleting endogenous CBFβ protein (Fig. 3D) with an antisense morpholino oligonucleotide (AML1MO) directed against AML1. As predicted, this resulted in reduced levels of ELA2 transcript (Fig. 3E), confirmed by corresponding time-dependent decreases in NE enzymatic activity (Fig. 3F).

**ELA2 and CBFα Immunoprecipitation from ELA2 Promoter Chromatin**—To test whether LEF-1 and CBFα bind to their recognition sites in the ELA2 promoter in vivo, we performed ChIP. We cross-linked DNA-bound protein in vivo in human U937 promyelocytes, which express LEF-1, CBFα, and NE. LEF-1 and CBFα antibodies were used to immunoprecipitate protein-associated DNA from sheared chromatin, in parallel with mouse and goat IgG as negative control sera. PCR of a specific ELA2 promoter (−35 to −250) fragment confirmed the in vivo binding of LEF-1 and CBFα to the ELA2 promoter (Fig. 4F).

**Synergistic Activation of ELA2 by LEF-1 and CBFα**—Well separated LEF-1 and CBFα motifs are present in the human TCRα enhancer, where these two factors act synergistically via an indirect interaction (48, 49). In the ELA2 promoter, the LEF-1 and CBFα elements flank one another (Fig. 1A), raising the possibility of a direct interaction. As a first step addressing this possibility, we determined whether these two factors act synergistically on the ELA2 promoter. We thus tested the effect of co-transfected LEF-1 and CBFα expression vectors upon wild type or mutant ELA2 reporters in transiently transfected HEK293T cells. LEF-1 and CBFα independently activate the wild type ELA2 promoter (NEP260WT) by about 10-fold apiece (Fig. 5A); together, however, there is an approximately 30-fold stimulation. The addition of CBFβ further potentiates activation. A confirming result is that the use of reporters bearing mutations in either the LEF-1 or the CBFα element (NEP260LEF-1BM or NEP260AML1BM, respectively) eliminates synergy.

**Evidence of Ternary DNA Complex Formation with LEF-1 and CBFα**—To further determine how LEF-1 and CBFα cooperate, we investigated whether they form an interacting complex with DNA. As before, EMSA experiments employed the DNA probe spanning from −210 to −176 and containing the adjacent LEF-1 and CBFα motifs. As shown in Fig. 5B, LEF-1 and CBFα each bind the probe independently and in combination (lanes 1–3, respectively). There is reduced formation of the apparent LEF-1-CBFα-DNA complex in the presence of excess competitive probes containing either the unlabeled wild type sequence (WT, lane 4) or disrupted LEF-1 (LEF-1B mAML1B WT, lane 5) or CBFα (LEF-1B WT/AML1B M, lane 6) binding...
sites but not with both sites mutated (LEF-1B M/AML1B M, lane 7). We conclude that LEF-1 and CBFα can each bind their recognition sites in the presence of the other factor, although the data are insufficient to demonstrate simultaneous binding.

Direct Interaction between LEF-1 and CBFα—Both the synergistic activation and the formation of a ternary DNA complex support the possibility of a physical interaction between LEF-1 and CBFα. We employed co-immunoprecipitation experiments to directly test this possibility. We transiently co-transfected HEK293T cells, which do not express CBFα/H9251 (50), with vectors expressing HA epitope-tagged LEF-1 (LEF-1-HA) and CBFα alone or together with CBFα/H9252. After 24 h, we immunoprecipitated transfected cell lysates with rat anti-HA antibody and immunoblotted the precipitates with goat anti-CBFα antibody (or, vice versa). LEF-1 indeed co-immunoprecipitates (Fig. 5C) with CBFα and (with reversal of antibodies). Notably, the interaction between LEF-1 and CBFα is stronger in the presence of CBFβ, but LEF-1 does not form a complex with CBFβ alone, indicating that CBFβ stabilizes the interaction between LEF-1 and CBFα.

We sought confirmation by determining whether the endogenously expressed proteins interact. We prepared nuclear extracts from nontransfected HL-60 cells, human KG-1 myeloblasts (51), or normal human bone marrow. For co-immunoprecipitation experiments, we employed mouse anti-LEF-1 antibody and mouse IgG as a control, followed by immunoblotting with goat anti-CBFα antibody. As shown in Fig. 5D, the endogenous proteins form an immunoprecipitable complex and verify interaction between LEF-1 and CBFα.

Finally, to determine whether LEF-1 and CBFα directly interact or if other factors mediate the relationship, we performed association assays using 35S-labeled in vitro translated proteins. We mixed radiolabeled in vitro synthesized CBFα with unlabeled in vitro synthesized LEF-1 and then immunoprecipitated with mouse anti-LEF-1 antibody and mouse IgG as a control, followed by immunoblotting with goat anti-CBFα antibody. As shown in Fig. 5E, labeled CBFα immunoprecipitates LEF-1 (and vice versa). No immunoprecipitate forms with control mouse and goat IgG. We conclude that LEF-1 and CBFα directly interact.

Interaction of LEF-1 with CBFα through the HMG Domain—LEF-1 contains two regions responsible for transcriptional enhancer activity. A context-dependent activation domain stimulates transcription in the presence of other enhancer-binding proteins (52), and a high mobility group (HMG) domain induces a DNA bend (48). To map the region of LEF-1 responsible for
interaction with CBFα, we generated a series of deletion mutants of LEF-1 tagged with a carboxyl-terminal HA epitope (Fig. 6A) and transiently co-transfected these constructs in HEK293T cells together with a CBFα expression vector containing a carboxyl-terminal FLAG tag. We immunoprecipitated cell lysates with mouse anti-FLAG antibody and immunoblotted the products with rat anti-HA antibody. Immunoblotting of cell lysates with anti-HA reveals that expression of all truncated proteins occurs at comparable levels (not shown). All but three of the LEF-1 derivatives interact with CBFα: deletion of the HMG DNA binding domain (ΔHMG), deletion of everything but the context-dependent activation domain (CAD), and deletion of everything but part of the HMG domain (Δ1–350). An intact HMG domain is therefore required for CBFα interaction.

Interaction of CBFα with LEF-1 through the Runt or Proline-, Serine-, and Threonine-rich (PST) Domains—To define the complementary region of CBFα interacting with LEF-1, we created deletions of CBFα fused to a carboxyl-terminal FLAG tag (Fig. 6B), co-transfected them into HEK293T cells with HA-tagged LEF-1, immunoprecipitated cell lysates with anti-HA, and immunoblotted with anti-FLAG. Immunoblotting of cell lysates with anti-FLAG reveals that expression of all truncated proteins occurs at comparable levels (not shown). Surprisingly, only the smallest construct, runt-(136–177), containing just part of the carboxyl-terminal portion of the runt DNA-binding domain, fails to interact with LEF-1. Deletion of either the entire runt domain or the PST activation domain preserves association with LEF-1, indicating a requirement for either, but not both, of these domains.

Requirement for Interaction between LEF-1 and CBFα for the Cooperative Activation of the ELA2 Promoter—To establish whether the cooperative activation of LEF-1 and CBFα is mediated through their interaction, we separated their two binding sites by insertion of an extra 10 base pairs. EMSA shows that each of the factors still binds its recognition site (data not shown). Reporter assays demonstrated that although LEF-1 and CBFα alone can still activate a promoter containing the 10-base pair insertion (NEP260LEF1–10bp-AML), they no longer do so cooperatively (Fig. 5A). Presumably, the 10-base pair separation, about one helical turn, is a large enough distance to abolish the direct interaction required for cooperative activation but is too short to allow for an interaction requiring DNA bending. Next, we tested whether the domains required
for the interaction between LEF-1 and CBFα, as defined above, are sufficient for cooperative transactivation. As shown in Fig. 7A, neither the HMG domain of LEF-1 nor the runt or PST domain of CBFα alone activates the promoter (NEP260WT). The HMG domain of LEF-1 or the PST domain of CBFα is not sufficient for cooperating with CBFα or LEF-1. Surprisingly, the runt domain of CBFα showed moderate cooperative activation with LEF-1, indicating that the DNA binding of the runt domain may increase the DNA bending mediated by LEF-1.

Synergistic Activation of ELA2 by PU.1, c-Myb, and C/EBPα—Independently of LEF-1 and CBFα—PU.1, C/EBPα, and c-Myb synergistically activate transcription of the mELA2 promoter (22). PU.1 and C/EBPα cooperates with CBFα in the activation of the promoter driving the macrophage colony-stimulating factor receptor gene (53). We therefore addressed whether PU.1, C/EBPα, and c-Myb similarly contribute to regulation of the human ELA2 promoter and whether those factors cooperate with LEF-1 and CBFα. As before, the assay consisted of transient co-transfection of HEK293T cells with expression vectors testing activation of a ELA2 promoter fragment (NEP260WT). Fig. 7 indicates representative results at nonsaturating conditions. PU.1, C/EBPα, and c-Myb activate ELA2 synergistically among themselves but only additively with LEF-1 and CBFα. LEF-1 or CBFα alone, additively and together, showed synergistic activation in the presence of PU.1, C/EBPα, and c-Myb. Thus, PU.1, C/EBPα, and c-Myb contribute to the regulation of human ELA2 separately from LEF-1 and CBFα.

DISCUSSION

In vivo observations in two complementary patients, one with SCN and an ELA2 promoter substitution and another with FPD/AML caused by an AML1 mutation, along with corroborating in vitro studies, indicate that these two leukemia syndromes act through an intersecting circuit. The −199 C to A substitution in the ELA2 promoter improves LEF-1 binding and leads to increased expression of NE. CBFα synergistically co-activates expression of ELA2 through an adjacent binding site via a direct interaction between its runt or PST domains and the HMG domain of LEF-1. Thus, NE is a transcriptional target of CBFα, and involvement of LEF-1 unexpectedly connects the pathway to Wnt signaling.

Possibility of ELA2 Promoter Mutations as a Cause of SCN—The identification of the −199 C to A ELA2 substitution in a potential LEF/TCF-responsive element in SCN patients, otherwise lacking NE protein sequence mutations, led us to investigate the physiologic contributions of this sequence to gene regulation. There are presently insufficient genetic epidemiologic data to substantiate this sequence change as causative of SCN. However, its absence among SCN patients with ELA2 mutations altering the polypeptide sequence and its greatly increased frequency in individuals with SCN indeed warrant further study. An additional two SCN patients demonstrating a different promoter variant, −15 C to A at a potential Sp1 motif, also suggest that regulatory mutations may cause SCN.

The mechanism of SCN remains unknown. Not all mutant
forms of NE demonstrate perturbed enzymatic activity (34). If confirmed through more extensive genetic association studies, then the identification of promoter mutations would indicate that up-regulated expression of NE contributes to the etiology of this disorder. The up-regulation of \textit{ELA2} in the SCN patient reported here is consistent with data indicating that mutant forms of the enzyme found in SCN may be excessively misrouted to membrane-bound compartments (54), leading to elevated proteolytic activity in certain subcellular locations. Moreover, it is consistent with the finding that inactivating mutations of the transcriptional repressor Gfi1 also cause SCN through the overexpression of \textit{ELA2} (55). We have proposed that SCN and cyclic neutropenia might result from a defective feedback circuit. In this scenario, NE, as a product of terminally differentiated myeloid cells, inhibits further differentiation and proliferation of myeloid precursors (Fig. 8). Support for this hypothesis comes from the observation that a cellular fraction enriched for membrane compartments containing NE inhibits myeloid colony formation \textit{in vitro}, but NE inhibitors abolish its effects (56).

Independently of potential pathogenic contributions of promoter variants, the discovery of the −199 C to A substitution within a LEF-1 binding site affords a unique opportunity to determine factors physiologically regulating \textit{ELA2} expression in \textit{humans in vivo}.

\textbf{Synergistic Interaction between LEF-1 and CBFα—}Here we demonstrate that LEF-1 and CBFα cooperate to activate \textit{ELA2} through adjacent binding sites; both elements function as enhancers in HEK293T cells. Each element independently binds their respective factor in EMSA and ChIP assays, and both factors together form a ternary complex with DNA. Sequence variation within the LEF-1 motif causes up-regulation of \textit{ELA2} in human bone marrow and in HEK293T cells. Antisense “knockdown” of either factor in HL-60 and/or U-937 cells reduces \textit{ELA2} expression. Human bone marrow containing mutant \textit{AML1} demonstrates reduced \textit{ELA2} transcript. The two factors physically associate through the HMG domain of LEF-1 and the runt or PST domains of CBFα. Transactivation of \textit{ELA2} by these two factors occurs independently of regulation of this gene by PU.1, c-Myb, and C/EBP. Recently, a remarkably similar interaction between the HMG domain of LEF-1 and the runt DNA-binding domain of the highly related family member CBFα has also been found to occur on adjacent elements in the osteocalcin gene promoter (57).

In the TCRα enhancer, LEF-1 and CBFα also cooperatively activate transcription (48, 49), but they do so through an indirect association. LEF-1 induces a helical bend that allows CBFα to contact the cAMP-response element-binding protein, CREB, for which there is an element in the TCRα promoter, via an intermediate interaction with the ETS-1 transcription factor. Another ETS family member, MEF, also interacts directly with CBFα (58). \textit{ELA2} does not contain an apparent CREB-responsive element. We do find a role for another ETS transcription factor family member, PU.1, but the addition of CBFα in a test of its ability to co-activate \textit{ELA2} in the presence of c-Myb and C/EBP yielded no effect. In distinction to the TCRα enhancer, the ETS family factor is transcriptionally uncoupled from CBFα and does not appear to be required for association of LEF-1 and CBFα.

At least two additional transcription factors interact with CBFα. In the context of the macrophage colony-stimulating factor promoter, CBFα directly associates with C/EBP (59). As is the case with PU.1, evidence of synergistic cooperation between CBFα and C/EBP in the \textit{ELA2} promoter is absent. A co-activator, ALY, lacking intrinsic transactivation and DNA binding activities of its own, also couples LEF-1 and CBFα, as
demonstrated in the TCRα enhancer (60). We tested for a potential contribution by AYL but found that it failed to further stimulate transcription of ELA2 by LEF-1 or CBFα (data not shown).

The LEF-1 and CBFα binding sites sit adjacent in the ELA2 promoter but remain separated from the cluster of the proximally situated PU.1, c-Myb and C/EBP elements. This geometry might explain why the latter three factors do not appear to synergize with LEF-1 and CBFα on the ELA2 promoter, even when they do so for other genes.

These observations support the finding that the HMG domain of LEF-1 and the runt or PSt domains of CBFα forge an association between these factors during their activation of ELA2. DNA binding alone fails to account for the range of activities attributable to HMG domain-containing factors. The HMG box additionally mediates protein-protein interactions with a wide variety of proteins, including the recombination activation gene protein RAG1, the p53 tumor suppressor protein, and an assortment of general and tissue-specific transcription factors (61). Recent experiments using phage display find that the HMG box promiscuously binds many apparently unrelated proteins through the recognition of short peptide sequences (62). Conversely, the interactions of CBFα with other proteins, specifically ETS family members and C/EBP, occur through the runt domain, which, in addition to its DNA binding function, also governs protein-protein interactions, specifically heterodimerization with CBFβ (63), MEF (58), and other uncharacterized factors (64).

LEF-1 activation of ELA2 takes place largely in the absence of a requirement for contributions from β-catenin, and the β-catenin binding domain of LEF-1 is not required for interaction with CBFα. The interaction of β-catenin with LEF-1 appears to accentuate DNA binding (32). The association of CBFα with LEF-1 could conceivably at least partially replace the function of β-catenin in this regard. The supporting data for this suggestion is our observation that the runt domain of CBFα moderately cooperates with LEF-1, indicating that the DNA binding of the Runt domain may increase the DNA binding mediated by LEF-1. If this were the case, then the bent DNA might spontaneously facilitate access of other transcription factors such as CBFβ.

Relevance to Leukemia—The determination that LEF-1 is expressed in myeloid lineages and participates in transcriptional control of their differentiation extends the role of this factor to broader aspects of hematopoiesis. Perturbed Wnt signaling is a feature of colorectal and other malignancies as evidenced by frequent mutation of the gene CTNNB1, encoding β-catenin as well as additional components of the pathway, including the tumor suppressor gene APC and the Wnt inhibitor Axin (65). Defective Wnt/β-catenin signaling appears in acute lymphoblastic leukemia (38, 66), but chromosomal translocation and somatic mutation involving CBFα are common to both acute lymphoblastic leukemia and AML. The finding of an interaction here between CBFα and LEF-1, capable of acting as a terminal mediator of Wnt/β-catenin signaling, suggests that common factors may contribute to both forms of leukemia.

As a transcription factor, CBFα regulates the expression of many genes (14, 30) in addition to ELA2. Presumably, its leukemogenic potential arises because of the widespread developmental changes induced by its aberrant expression. However, ELA2 stands out; hereditary mutations in ELA2 and AML1 represent the only human leukemia syndromes in which there is neither a generalized risk of cancer, a DNA repair deficiency, nor pervasive developmental abnormalities (67). How might the transcriptional connection between these two syndromes make sense with regard to the origins of leukemia?

Loss of CBFα activity in FPD/AML, as shown here, leads to lower levels of ELA2 expression. NE, whose concentration is a function of the mass of mature neutrophils, might provide negative feedback for further myeloid proliferation and differentiation (Fig. 8). A low inhibitory signal, arising from a reduced quantity of NE, could inappropriately lead to potentiation of proliferative signals that might otherwise be held in check as well as create cellular environments in the bone marrow selecting for mutations causing constitutive signaling in growth pathways.

An intriguingly supportive observation is that just two potential mutations involving AML1, both involving chromosome 21 aneuploidy, are known in SCN-induced AML. Instead of inactivating AML1, one case demonstrated trisomy 21 in the leukemic clone, whereas the other revealed pentasomy 21, resulting in a remarkable 5-fold amplification of an apparently normal copy of the AML1 gene (68).

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