 Human Molecular Genetics, 1999, Vol. 8, No. 12 **2311–2316**

CBFA1 mutation analysis and functional correlation with phenotypic variability in cleidocranial dysplasia

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Received July 19, 1999; Revised and Accepted September 7, 1999

Cleidocranial dysplasia (CCD) is a dominantly inherited skeletal dysplasia caused by mutations in the osteoblast-specific transcription factor *CBFA1*. To correlate *CBFA1* mutations in different functional domains with the CCD clinical spectrum, we studied 26 independent cases of CCD and a total of 16 new mutations were identified in 17 families. The majority of mutations were *de novo* missense mutations that affected conserved residues in the runt domain and completely abolished both DNA binding and transactivation of a reporter gene. These, and mutations which result in premature termination in the runt domain, produced a classic CCD phenotype by abolishing transactivation of the mutant protein with consequent haploinsufficiency. We further identified three putative hypomorphic mutations (R391X, T200A and 90insC) which result in a clinical spectrum including classic and mild CCD, as well as an isolated dental phenotype characterized by delayed eruption of permanent teeth. Functional studies show that two of the three mutations were hypomorphic in nature and two were associated with significant intrafamilial variable expressivity, including isolated dental anomalies without the skeletal features of CCD. Together these data show that variable loss of function due to alterations in the runt and PST domains of *CBFA1* may give rise to clinical variability, including classic CCD, mild CCD and isolated primary dental anomalies.

INTRODUCTION

The core binding factor-α1 gene, *CBFA1*, is one of three mammalian homologs of the *Drosophila runt* gene (1,2). It encodes a transcription factor required for osteoblast cell fate commitment (3,4). CBFA1 contains an N-terminal stretch of consecutive polyglutamine and polyalanine repeats (Q/A domain), a runt domain and a C-terminal proline/serine/threonine-rich (PST) activation domain (Fig. 1) (5–7). The runt domain is a 128 amino acid polypeptide motif originally described in the *Drosophila runt* gene that has the unique ability of independently mediating DNA binding and, in some cases, protein heterodimerization (8). We and others have shown that mutations in *CBFA1* result in cleidocranial dysplasia (CCD) (5,6). CCD is a dominantly inherited skeletal dysplasia with high penetrance and variable expressivity characterized by hypoplastic clavicles, large fontanelles, dental anomalies and delayed skeletal development (9). The phenotype suggests that the primary defect is due to alteration of both intramembranous and endochondral bone formation. Heterozygous disruption of the *Cbfa1* allele in mice produces a CCD phenotype while homozygous mice die at birth and have complete absence of osteoblasts and bone (10,11). These, and studies showing direct activation of osteoblast-specific genes by CBFA1, demonstrate that CBFA1 is so far the only described transcriptional regulator of osteoblast differentiation (10–12).

Based on the biological function of CBFA1, the clinical phenotype of CCD and the co-localization of the respective genetic loci, we and Mundlos *et al*. identified deletion, insertion and missense mutations in *CBFA1* in CCD families (5,6). To further delineate the range of *CBFA1* mutations in CCD and their functional consequences and to better understand the molecular mechanism by which *CBFA1* controls osteoblast differentiation, we have analyzed the *CBFA1* gene in 26 independent cases of CCD and evaluated their effect on CBFA1 binding and transactivation, and correlated these findings with phenotypic expression.

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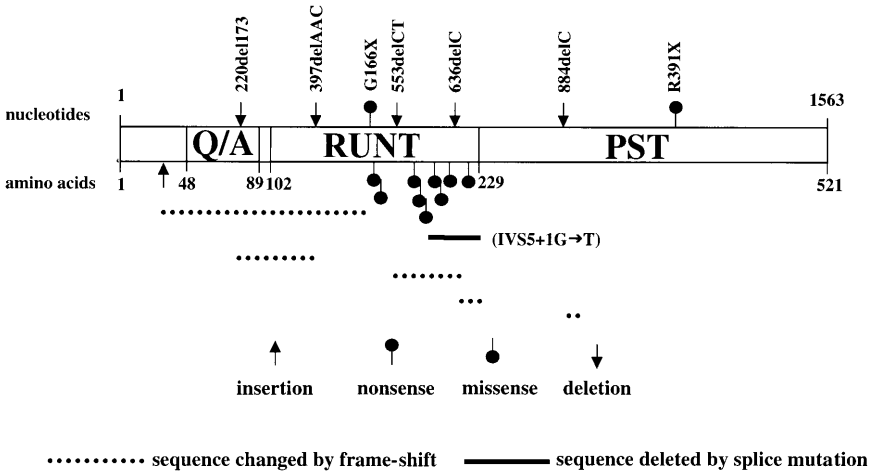

Figure 1. Relative positions of *CBFA1* mutations in CCD patients. Nonsense and deletion mutations are indicated above the gene product, insertion and missense mutations below. The portions of the protein product altered by frameshift mutations and splice mutation are also indicated below.

Table 1. *CBFAI* mutations in CCD patients arranged by mutation type

Mutation[a]	Type	Location	Clinical severity[b]
Missense mutations			
R169Q	familial	runt	C
M175R[5]	*de novo*	runt	C
R190Q	*de novo*	runt	C
S191N[5]	*de novo*	runt	C
R193C	*de novo*	runt	C
L199F	*de novo*	runt	C
T200A	familial	runt	D/C
Q209R	*de novo*	runt	C
R225Q	*de novo*	runt	C
Deletion, insertion and splice mutations			
ΔN133 (397delAAC)	*de novo*	runt	C
220del173	*de novo*	Q/A domain (stop in runt)	C
553delCT	familial	runt (stop in runt)	C
636delC	*de novo*	runt (stop in runt)	C
884delC	familial	PST (stop in PST)	C
90insC	familial	N-terminal (stop in runt)	D/M
IVS5+lG→T	familial	in-frame exon skipping in runt	C
Nonsense mutations			
G166X	familial	runt	C
R391X	*de novo*	PST	C

[a]Mutation numbering begins with the ATG codon as number 1 (5).
[b]C, delayed closure of fontanelle, hypoplastic clavicles, with or without dental anomalies; M, delayed fontanelle closure, minimal effects on axial skeleton; D, primary dental anomalies including delayed loss of primary teeth, delayed eruption of permanent teeth and/or supernumerary teeth.

RESULTS

Clinical data

Patients were recruited via genetic consultation services from the USA, Canada and Europe. After informed consent for DNA testing was obtained, 26 sporadic and familial cases were ascertained with ages ranging from 1 to 67 years. Summaries of clinical genetics evaluations were obtained. A classic CCD phenotype was defined as the presence of hypoplastic clavicles and delayed closure of the anterior fontanelle in addition to observation of classic craniofacial features. Due to the age-related penetrance of dental anomalies, this was not fixed as a criterion, but was consistently observed in older patients in this category. Mild CCD was defined as minimal or absent clavicular findings. The clinical description of isolated primary dental anomalies was applied to cases who had delayed loss of primary teeth, delayed eruption of permanent teeth and/or supernumerary teeth without evidence of cranial or axial skeletal anomalies on X-ray (Table 1). Specifically, in the T200A family, individuals had supernumerary teeth, dental misalignment and delayed eruption of permanent teeth. In the 90insC family, affected individuals had small teeth and delayed eruption of permanent teeth.

Mutation analysis and phenotypic correlations

The *CBFA1* gene was screened for mutations in 26 unrelated CCD patient families. A total of 16 mutations were identified in 17 probands. One mutation was found to recur in two unrelated families. Mutation types included missense, deletion/splice/insertion variants resulting in premature termination and nonsense mutations. All *de novo* mutations were confirmed to be absent in parents. Familial mutations were confirmed to segregate in affected individuals. For those mutations (T200A) that did not have a demonstrable effect on DNA binding or transactivation, their presence as polymorphic variants were excluded in 150 control chromosomes (data not shown).

In our patient cohort, we identified seven missense mutations [R169Q, R190Q, R193C, L199F, T200A, Q209R and R225Q (Fig. 1 and Table 1)] and one single amino acid deletion (ΔN133). All the mutations occur in the highly conserved runt domain, and the R190Q and R193C mutations affect an ATP binding consensus motif (8). In all cases, a stable polypeptide is predicted and all associated phenotypes except for the familial T200A mutation were consistent with classic CCD. Interestingly, the T200A mutation occurs in a Mennonite family with classic CCD in two of four children,

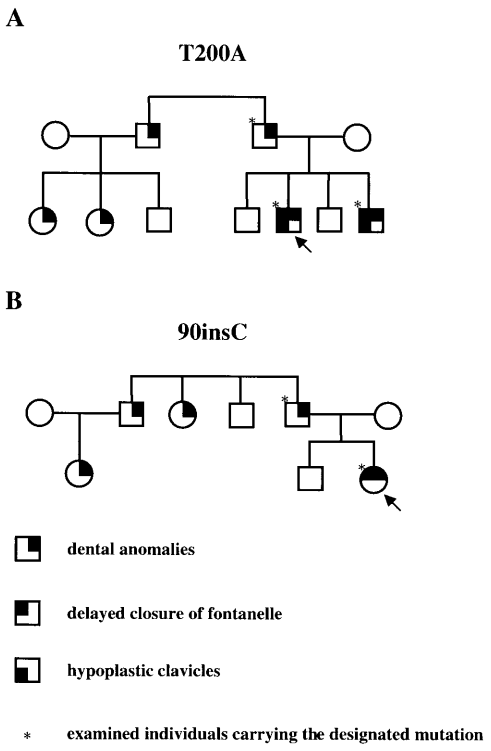

Figure 2. Abbreviated pedigrees of the CCD families carrying the T200A (**A**) and 90insC (**B**) mutations with the phenotypic variability indicated.

while the father, who also harbors the mutation, only has dental anomalies, including delayed eruption of permanent teeth, misalignment and multiple dentures (Fig. 2A). He does not have evidence of CCD on skeletal radiographs. Even though the mutation affects a highly conserved amino acid immediately adjacent to a described L199F mutation, we tested and did not find this mutation in 100 unrelated control chromosomes and 50 Mennonite control chromosomes (data not shown).

We identified one splicing mutation in a family with CCD. The mutation IVS5+1G→T causes in-frame skipping of exon 5 and was confirmed by RT–PCR on RNA from patient lymphoblastoid cells (data not shown). The mutation segregates with all affected individuals with classic CCD in this family. Skipping of exon 5 would be expected to eliminate one-third of the runt domain and would likely abolish DNA binding of the mutant protein (Fig. 1). Four additional deletion mutations (553delCT, 636delC, 884delC and 220 del173) were found associated with classic CCD and each results in premature termination either in or immediately after the runt domain. Interestingly, we have identified an insertion mutation (90insC) early in the *CBFA1* transcript between two potential translation start positions (1 and 39) which is associated with mild CCD in the proband and only dental anomalies in the father who also carries the mutation (Fig. 2B). While utilization of the upstream Met is preferred by Kozak consensus and would predict premature termination in the runt domain, utilization of the downstream Met would result in an intact polypeptide missing an N-terminal sequence which has been shown in other studies to exhibit only a partial decrease in transactivation (13).

We have identified one familial, nonsense mutation (G166X) that segregates with the CCD phenotype and causes premature termination in the runt domain. As in the deletion/frameshift mutations, it would be expected to completely abolish DNA binding. In addition, we identified a *de novo* nonsense mutation outside the runt domain (R391X) that is associated with classic CCD. If this message was stable, this mutation would be expected to delete approximately half of the PST activation domain. In this cohort, mutations were not identifiable in nine probands. Eight of these were *de novo* cases while the last derived from a small family with an affected daughter and mother.

These data show that a majority of *CBFA1* mutations in patients with classic CCD affect the runt domain. The majority of these mutations were missense mutations that are expected to abolish DNA binding or deletion/nonsense mutations that cause premature termination in the runt domain. The mutations that can be anticipated to result in haploinsufficiency were invariably associated with classic CCD. However, exceptions, including 90insC and T200A, are associated with mild CCD, isolated dental anomalies and significant intrafamilial variability, raising the question of whether hypomorphic/neomorphic effects and genetic modifiers alter the clinical expressivity of these mutations.

Functional consequences of *CBFA1* mutations

While mutations which abolish DNA binding would be expected to produce the classic CCD phenotype by haploinsufficiency, we hypothesized that hypomorphic mutations may give rise to significant clinical variability. To address this question, we compared the functional consequences of several mutations associated with classic and milder CCD-like phenotypes on DNA binding and transactivation. All four missense mutations described in this study and the single amino acid deletion mutation ΔN133 affect highly conserved residues within the runt domain and would be expected to impair DNA binding. To test this hypothesis, we generated mutant runt domains by site-directed oligonucleotide mutagenesis and tested their *in vitro* binding to the target OSE2 element derived from the osteocalcin promoter (14). DNA binding determined by electrophoretic mobility shift assay (EMSA) of wild-type and mutant (ΔN133, R190Q, L199F, T200A and R225Q) runt domains showed that, except for T200A, all mutant runt domains abolished binding to the consensus CBFA1-binding sequence. In contrast, the T200A mutant runt domain exhibited binding to OSE2, albeit with slightly altered mobility (Fig. 3). This mutation was associated with significant intrafamilial variability and primary dental anomalies in one family.

To further evaluate the effect of various *CBFA1* mutations on transcriptional activation of the target osteocalcin promoter p6OSE2luc by CBFA1, we generated *CBFA1* full-length expression constructs containing several CCD mutations for co-transfection studies (14). These constructs included two previously described (M175R and S191N) mutations which abolished DNA binding and two currently described missense mutations (L199F and T200A) (5). In addition, we generated the R391X premature termination mutation which was associated with classic CCD. Transactivation studies showed that the

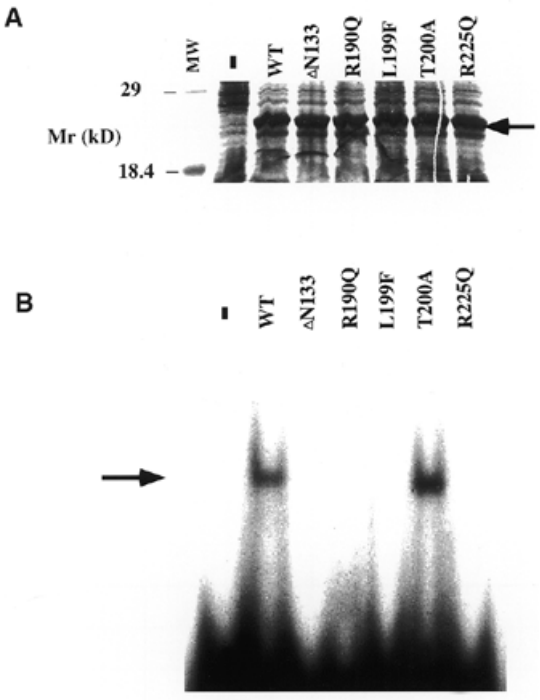

Figure 3. DNA binding by wild-type and mutant runt polypeptide domains of CBFA1. (**A**) SDS–PAGE of bacterial extracts. The induced recombinant runt polypeptide is indicated by an arrow. (**B**) EMSA of missense and single amino acid deletion mutant runt domains. The specific DNA–probe complex is indicated by the arrow. All mutations except T200A abolish DNA binding in EMSA.

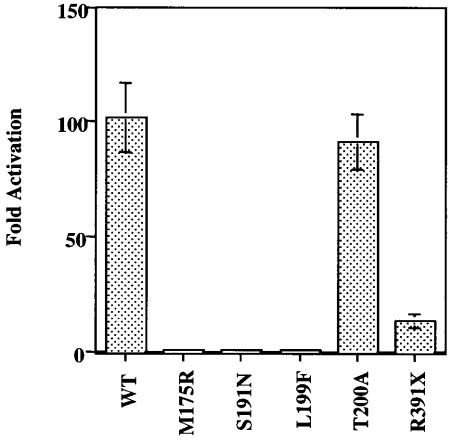

Figure 4. Transactivation ability of wild-type and mutant CBFA1. COS7 cells were transfected with p6OSE2luc as a reporter plasmid, full-length wild-type or mutant *CBFA1* [cloned in expression vector pcDNA3.1(–)] as effector plasmid and pSV2βgal as an internal control of transfection efficiency. Data are presented as fold activation relative to the activity obtained with the pcDNA3.1(–) empty vector plasmid. Bars represent the average ratios of luciferase to β-galactosidase activity. The standard deviations obtained from three independent transfections of one representative experiment are represented by error bars.

missense mutations which abolished DNA binding on EMSA exhibited negligible transactivation of the luciferase reporter. In contrast, the T200A mutation, which had detectable DNA binding *in vitro*, also exhibited normal transactivation compared with wild-type CBFA1. Interestingly, the R391X mutation exhibited detectable transactivation, ~20% of that of wild-type CBFA1, but correlated with a classic CCD phenotype (Fig. 4). In these studies, the majority of missense mutations in the runt domain abolished binding to the target DNA sequence and consequent transactivation. The putative hypomorphic mutations (R391X and T200A) were associated with classic CCD and a milder CCD phenotype, respectively, with significant clinical variability, including isolated dental anomalies.

DISCUSSION

Mutations in murine and human *CBFA1* suggest that it fulfills a unique function during osteoblast differentiation (4–6,10–12). The phenotypes of *Cbfa1*$^{+/-}$ mice, *Ccd* radiation-induced mutant mice characterized by heterozygous deletion of the *Cbfa1* locus and CCD patients with microdeletion of 6p21 together support the conclusion that the CCD phenotype arises from a mechanism of haploinsufficiency (6,11,15–17). Loss of function of one allele results in delayed intramembranous and endochondral ossification since both these pathways have in common osteoblast cell fate commitment and differentiation as a final step in matrix ossification. It is therefore not unexpected that the most affected skeletal components include the skull and the clavicles, as the skull is the primary site of intramembranous ossification while the clavicle is a unique bone which begins to form at two sites of intramembranous ossification and then completes ossification via a cartilagenous anlagen, i.e. endochondral process (18,19). However, the specific mechanism by which haploinsufficiency is generated by subtle mutations and the clinical consequences of hypomorphic mutations have yet to be completely delineated.

Our current studies show that human *CBFA1* mutations primarily cluster within the runt domain. They either directly substitute conserved amino acid residues or cause frameshift and premature termination within this domain. The majority of mutations are associated with complete abolition of DNA binding to target promoter sequences and consequent transactivation *in vitro*. The pathogenic nature of the mutations described are supported by: (i) *de novo* occurrence in the patient; (ii) segregation with the clinical phenotype in familial cases; (iii) absence in unrelated, control chromosomes; and (iv) alteration of DNA binding and transactivation. In general, those mutations that result in complete loss of function of the mutant protein are associated with classic CCD with delayed closure of the fontanelles, hypoplastic clavicles and dental abnormalities (the latter depending on age of ascertainment).

However, in two families with mild CCD and intrafamilial clinical variability, putative hypomorphic (and/or neomorphic) mutations have been identified. The T200A mutation is associated with classic CCD and also with isolated, primary dental anomalies in the same family. While the dental anomalies in the CCD phenotype, including delayed loss of primary teeth, delayed eruption of permanent teeth and/or supernumerary teeth, contribute the majority of morbidity in CCD patients, they are the phenotypic features exhibiting highest age-related penetrance in our cohort. *In vitro* studies of this mutation show the presence of DNA binding and normal transactivation. However, the effect of this mutation *in vivo* is unknown. It may

indeed be hypomorphic in nature, with the implication that the *in vitro* binding and transfection studies are not of sufficient sensitivity to demonstrate this effect. Alternatively, the mutation may be neomorphic in nature and might affect an alternative function of the runt domain. For example, the runt domain has been shown to participate in heterodimerization, best exemplified by the interaction between a mammalian homolog Cbfa2 and its partner Cbfβ (8). There is genetic evidence that alteration of function of either Cbfa2 or Cbfβ results in a similar phenotype (20–23), directly supporting their *in vivo* interaction in regulating specific hematopoietic pathways. CBFA1 does not heterodimerize with CBFβ in this fashion (13). However, it is possible that the T200A mutation may instead alter a hypothesized CBFA1 interaction with an as yet unidentified partner, thereby producing a CCD-like phenotype.

In the other family, the 90insC mutation was found associated with mild CCD in the proband and isolated dental anomalies in the father. By history, the report of additional individuals affected with primary dental anomalies in these two families suggests that somatic mosaicism is not the basis for the findings in the probands' fathers (Fig. 2). While utilization of the preferred translational start site based on Kozak consensus would predict early premature termination of the mutant peptide and haploinsufficiency, the mild clinical phenotype and variable expressivity raise the possibility of utilization of the downstream methionine. This would result in a polypeptide missing 38 N-terminal residues, but with intact Q/A, runt and PST domains. Transactivation studies with a similar peptide in another study exhibited residual, but decreased, transactivation of a reporter gene consistent with the presence of an activation domain in this region of the protein (13). At least *in vitro*, we have observed a translation product which corresponds to this secondary start site (13). Whether this might occur *in vivo* to produce a hypomorphic effect on skeletogenesis and odontogenesis is unclear and untestable without clinical material.

In nine patients with classic CCD, we did not find subtle mutations or deletions of the coding region. However, *CBFA1* is a large gene estimated to be >120 kb with large intronic regions (7). Deletions of intronic regions and/or regulatory sequences may account for some of these patients' mutations. Based on linkage studies published to date, there is no evidence of locus heterogeneity in classic CCD (15,16,24,25).

In summary, we have extended the range of *CBFA1* mutations observed in cases of CCD and demonstrated their functional consequences with *in vitro* assays of DNA binding and transactivation. Whereas most mutations affect the runt domain and result in haploinsufficiency, yielding classic CCD, we provide evidence that mutations outside this domain may result in hypomorphic states with significant clinical variability. Interestingly, primary dental anomalies observed in two families with significant clinical variability are associated with mutations that in one case exhibits no observable effects *in vitro*, but which may be either hypomorphic or neomorphic in action *in vivo*. An alternative function regulating epithelial–mesenchymal interactions in odontogenesis that differs from the initiative role in osteoblastogenesis has been proposed for CBFA1 (26). This difference may underlie the high penetrance of the tooth phenotype in the spectrum of *CBFA1* mutations. Moreover, it suggests the potential for other isolated tooth anomalies as correlations for alteration of CBFA1 function.

MATERIALS AND METHODS

Mutation analysis

Genomic DNA from patients was PCR amplified using intron- and exon-specific primers as described (5). PCR products were directly sequenced by dye primer chemistry on an ABI 377 automated sequencer. Putative mutations were confirmed by cloning and sequencing of each allele. Segregation in familial cases was confirmed by allele-specific oligonucleotide (ASO) hybridization using wild-type and mutant oligonucleotides as probes for PCR-amplified exons (27). Similarly, the absence of familial mutations in control chromosomes was confirmed by ASO hybridization.

Mutagenesis and DNA binding analysis

The wild-type runt domain (amino acids 102–229) was PCR amplified with primers HR1 (5′-GCGGCCGCCCCAC-GACAACCGCA-3′) and HSEQ1 (5′-CGGGGACGTCATCT-GGCTC-3′). Except for R225Q, all mutations were introduced into the runt domains of *CBFA1* by means of two-step PCR amplification (28) and confirmed by DNA sequencing. The mutant primers used for the first step PCR were HR1 with MUT3 and HSEQ1 with MUT5, and the second step PCR amplification was performed with HR1 and HSEQ1. For the respective mutations, the sequences of the primers were as follows (nucleotide mutations are underlined and positions of deletion are indicated by asterisks):

MUT5(ΔN133), 5′-GGCGCTGC*AAGACCCTGCCCGTGGCCTTCAAGGT-3′;
MUT3(ΔN133), 5′-ACCTTGAAGGCCACGGGCAGGGTCTT*GCAGAGCC-3′;
MUT5(R190Q), 5′-GATTTGTGGGCCAGAGTGGACGAGGCAAGAGTT-3′;
MUT3(R190Q), 5′-AACTCTTGCCTCGTCCACTCTGGCCCACAAATC-3′;
MUT5(L199F), 5′-GAGTTTCACCTTCACCATAACCGTCTTCACAAAT-3′;
MUT3(L199F), 5′-ATTTGTGAAGACGGTTATGGTGAAGGTGAAACTC-3′;
MUT5(T200A), 5′-GGCAAGAGTTTCACCTTGGCCATAACCGTCTTCACA-3′;
MUT3(T200A), 5′-TGTGAAGACGGTTATGGCCAAGGTGAAACTCTTGCC-3′.

To generate the R225Q mutation PCR was performed using primers HR1 and MUT3(R225Q) (5′-AGTCATCAAGCTTCTG-TCTGTGCCTTCTGGGTTCCTGAGGTCCA-3′). Wild-type and mutant runt coding sequences were cloned in-frame into the pTrcHisA vector (Invitrogen, Carlsbad, CA) and the recombinant proteins were isolated from bacteria and enriched on a Ni–NTA–agarose resin (Qiagen, Valencia, CA). EMSAs were carried out as described previously with 10 fmol of a ^{32}P-end-labeled double-stranded OSE2 oligonucleotide with equivalent amounts of the wild-type and mutant runt polypeptides in a buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Nonidet P-40, 50 mg of bovine serum albumin (BSA) in the presence of 1 μg of poly(dI·dC) (29).

cDNA constructs and transient transfection experiment

To reconstitute full-length mutant *CBFA1* cDNA constructs, a 390 bp *Nco*I–*Hin*dIII fragment containing wild-type runt domain sequence was replaced by sequences corresponding to mutant runt domains M175R, S191N, L199F and T200A without changing any other nucleotide sequences. A 720 bp *Kpn*I–*Xho*I fragment corresponding to the R391X nonsense mutation was generated by PCR using primers MUT5(R391X) (5′-GGAGAGGTACCA-GATGGGACTG-3′) and MUT3(R391X) (5′-AAAGGT-

GGCTCGAGAGTGCATTCATGGGTTG-3′). This replaced a 1283 bp *Kpn*I–*Xho*I fragment in the wild-type full-length *CBFA1* cDNA construct to generate the full-length R391X cDNA. The full-length wild-type and mutant *CBFA1* cDNAs were subsequently inserted into expression vector plasmid pcDNA3.1(–) (Invitrogen). All PCR products were verified by DNA sequencing.

Transient transfection experiments in COS7 cells were performed using lipofectamine-plus according to the manufacturer's recommendations (Gibco BRL, Gaithersburg, MD). Aliquots of 750 ng of expression plasmid containing either wild-type or mutagenized *CBFA1* were co-transfected with 750 ng of a reporter plasmid p6OSE2luc (14). The pSV2βgal plasmid was also co-transfected as an internal control for transfection efficiency. Luciferase and β-galactosidase activities were assayed 24 h after transfection. Luciferase assays were measured with a luminometer using D-luciferin as substrate in luciferase reaction buffer (100 mM potassium phosphate, pH 7.8, 5 mM ATP, 15 mM $MgSO_4$, 1 mM dithiothreitol). β-Galactosidase activities were measured with a chemiluminescent assay kit (Tropix, Bedford, MA). Transfections were performed in triplicate and at three doses to ensure a linear dose–response curve.

ACKNOWLEDGEMENTS

We are grateful to Rossi Dawn for editorial assistance. This work was supported, in part, by National Institutes of Health grants AR44738 (B.L.) and AR 45548 (G.K.), the March of Dimes Birth Defects Foundation (B.L. and G.K.), the Arthritis Foundation (B.L.) and Baylor College of Medicine Child Health Research Center and Mental Retardation Research Center (B.L.). G.Z. is the recipient of a post-doctoral fellowship from the Arthritis Foundation.

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