

# Structural basis for the heterodimeric interaction between the acute leukaemia-associated transcription factors AML1 and CBF $\beta$

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**Mutations in the genes encoding the interacting proteins AML1 and CBF $\beta$  are the most common genetic abnormalities in acute leukaemia, and congenital mutations in the related AML3 gene are associated with disorders of osteogenesis. Furthermore, the interaction of AML1 with CBF $\beta$  is essential for haematopoiesis. We report the 2.6 Å resolution crystal structure of the complex between the AML1 Runt domain and CBF $\beta$ , which represents a paradigm for the mode of interaction of this highly conserved family of transcription factors. The structure demonstrates that point mutations associated with cleidocranial dysplasia map to the conserved heterodimer interface, suggesting a role for CBF $\beta$  in osteogenesis, and reveals a potential protein interaction platform composed of conserved negatively charged residues on the surface of CBF $\beta$ .**

**Keywords:** chromosomal translocation/cleidocranial dysplasia/core binding factor/mutation/osteogenesis

## Introduction

Leukaemias are characterized by the presence of recurrent chromosomal translocations (Rabbitts, 1994). The genes associated with these chromosomal breakpoints in acute leukaemias frequently encode transcription factors that play pivotal roles in normal development and in leukaemogenesis (Cleary, 1991; Rabbitts, 1991). The core binding factors (CBFs) are representative of this phenomenon. These heterodimeric transcription factors consist of a DNA-binding  $\alpha$ -subunit, and a non-DNA-binding  $\beta$ -subunit (Ogawa *et al.*, 1993b). Three mammalian genes encode the  $\alpha$ -subunit: AML1/CBFA2/PEPBP2 $\alpha$ B (herein called AML1), AML2/CBFA3/PEBP2 $\alpha$ C and AML3/CBFA1/PEBP2 $\alpha$ A/Osf2 (herein called AML3). All  $\alpha$ -subunits share an evolutionarily conserved region of 128 amino acids known as the Runt domain, which mediates both DNA binding and heterodimerization to the  $\beta$ -subunit. The *Drosophila* gene *runt*, which is the founding member of the  $\alpha$ -subunit family, is required for segmentation, sex determination and neurogenesis. Only one gene (CBFB) is known to encode the mammalian  $\beta$ -subunit, CBF $\beta$ , which associates with all three  $\alpha$ -subunits (Ogawa *et al.*, 1993a). However, two

CBFB homologues, *brother* and *big brother*, have been identified in *Drosophila* (Golling *et al.*, 1996).

The AML1 gene encodes a 453 amino acid protein with an N-terminal transcriptional inhibitory domain (residues 1–49), the Runt domain (residues 50–177), and C-terminal transcriptional inhibition (residues 178–290) and activation (residues 291–453) domains (Ito, 1999). It was cloned from one of the most frequently acquired cytogenetic abnormalities in acute myeloid leukaemia (AML), the translocation t(8;21)(q22;q22), and was subsequently shown to be involved in the recurrent chromosomal translocation t(12;21)(p13;q22) associated with childhood acute lymphoblastic leukaemias, and the translocation t(3;21)(q26;q22) associated with therapy-related leukaemias and myelodysplasia (reviewed in Look, 1997). In all of these translocations, the AML1 Runt domain becomes fused with new protein domains encoded by exons from the partner chromosome, thereby retaining the ability to heterodimerize with the CBF $\beta$  protein. Additionally, nonsense, missense and frameshift mutations in the AML1 gene are associated with sporadic AML (Osato *et al.*, 1999), and congenital mutations in AML1 have been described in individuals with the rare autosomal dominant disease, familial platelet disorder (FDP), in which there is a congenital predisposition to the development of AML (Song *et al.*, 1999). Interestingly, these disease-associated mutations in the sporadic and congenital disorders are clustered within the Runt domain of AML1. In man, mutations in AML3 are associated with cleidocranial dysplasia (CCD), an autosomal dominant disorder of skeletal morphogenesis (Lee *et al.*, 1997; Mundlos *et al.*, 1997), and again, the majority of the point mutations associated with this disorder cluster within the Runt domain (Lee *et al.*, 1997; Quack *et al.*, 1999; Zhou *et al.*, 1999). Aml3 is essential for osteoblast differentiation and bone development in the mouse (Komori *et al.*, 1997; Otto *et al.*, 1997). Thus, the conserved Runt domain of this family of transcription factors is a key target for disease-associated mutations in man.

It is significant that the gene encoding CBF $\beta$ , the  $\beta$ -subunit of the core binding factors family, is also involved in chromosomal translocations in AML as a result of inv(16)(p13q22), t(16;16) and del(16)(q22), which fuse the N-terminal 165 amino acids of CBF $\beta$  in-frame with a C-terminal portion of the smooth muscle myosin heavy chain in 15% of AML (Liu *et al.*, 1993). Thus, together, the heterodimeric CBF transcription factor genes AML1 and CBFB are the most frequently mutated genes in human acute leukaemia, accounting for 25% of AML and 20% of paediatric common B-cell acute lymphoblastic leukaemia (Look, 1997).

AML1 binds as a monomer to the core DNA sequence TGT/cGGT, which is present in a number of different viral and cellular promoters and enhancers, as well as

**Table I.** Data collection, structure determination and refinement statistics

Data collection and MIR phasing statistics							
Data set	Resolution (Å)	Observations/ unique reflections	Completeness (last shell) %	$R_{\text{merge}}^a$ (last shell)			
SeMet $\lambda_1$	3.2	78580/60509	88.9 (84.1)	0.047 (0.15)			
SeMet $\lambda_2$	3.2	86114/63424	92.8 (85.4)	0.066 (0.25)			
Native	2.6	259515/71198	99.3 (99.2)	0.084 (0.28)			
Structure refinement statistics							
Resolution (Å)	Protein atoms	Waters	$R_{\text{cryst}}^b$	$R_{\text{free}}^b$ (% data used)	R.m.s.d. from ideality <sup>c</sup>		Dihedrals
25.0–2.6	9551	121	26.46	29.43 (3)	Bonds	Angles	Dihedrals
					0.0072	1.3921	24.76

<sup>a</sup> $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I_i(\text{hkl}) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I_i(\text{hkl})}$ .

<sup>b</sup> $R_{\text{cryst}}$  and  $R_{\text{free}} = \frac{\sum |F_{\text{obs}} - F_{\text{calc}}|}{\sum F_{\text{obs}}}$ ;  $R_{\text{free}}$  calculated with the percentage of the data shown in parentheses.

<sup>c</sup>R.m.s.ds for bond angles and lengths in regard to Engh and Huber parameters.

haematopoietic cell-specific genes (Rodan and Harada, 1997). The Runt domain binds to the major groove of DNA (Thornell *et al.*, 1988), and dimerization with CBF $\beta$  enhances the DNA-binding activity of AML1 without itself contacting DNA (Kamachi *et al.*, 1990). Binding of CBF $\beta$  to the Runt domain protects Cys81 from oxidation by diamide, but does not hinder access of much larger reducing molecules to this site (Akamatsu *et al.*, 1997a). These data suggest that the Runt domain undergoes a conformational change on binding to CBF $\beta$ , which results in enhanced DNA binding and alters the susceptibility of Cys81 to oxidation. However, direct evidence for this has not been obtained.

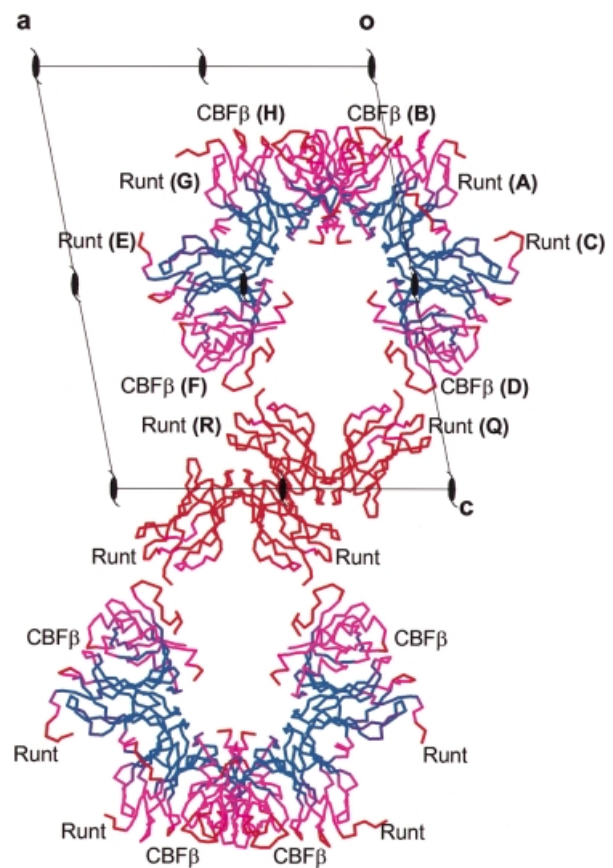
We have determined the structure of the Runt domain of AML1 bound to CBF $\beta$  to understand the mode of interaction between the two subunits, to investigate the mechanism whereby CBF $\beta$  enhances DNA binding by the Runt domain, and to understand the molecular consequences of physiologically relevant mutations. We describe the 2.6 Å resolution crystal structure of the human AML1–CBF $\beta$  complex, providing insights into the significance of human disease mutations associated with acute leukaemia and cleidocranial dysplasia.

## Results

### Formation of the Runt domain–CBF $\beta$ complex, crystallization and structure determination

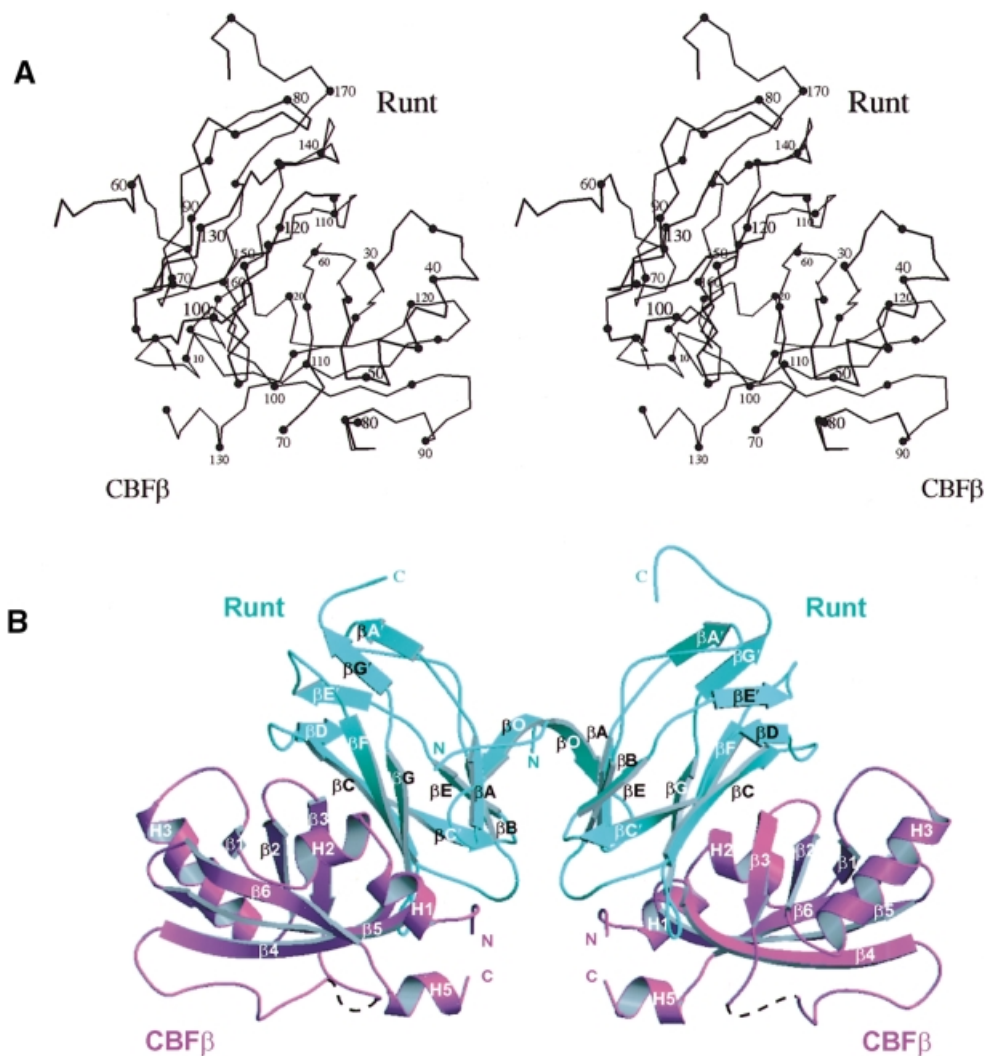
Fragments of the human AML1 and CBF $\beta$  proteins were co-expressed in *Escherichia coli*. The strategy of co-expression was crucial to obtaining a soluble functional heterodimeric complex without a requirement for mutagenesis or refolding. The expressed fragments of human AML1 (residues 50–183, out of 451), corresponding to the Runt domain, and CBF $\beta$  (residues 2–135, out of 182) form a stable complex in solution that is fully active in sequence-specific DNA binding as determined by electrophoretic mobility shift assays (data not shown).

Two crystal forms of the complex were obtained, one with *P*61 symmetry and the second with *P*21 symmetry. The *P*21 symmetry crystals diffracted to higher resolution and were used in the structure determination. The structure was solved by the method of multiple anomalous dispersion (MAD) (Hendrickson *et al.*, 1990), using isomor-



**Fig. 1.** *B*-factor distribution and crystal packing of Runt domain–CBF $\beta$  heterodimers. Six Runt domain subunits and four CBF $\beta$  subunits are packed in alternating layers in the crystal. One layer is composed of two dimers of heterodimers (F+E, G+H; B+A, C+D), and the second comprises a single Runt domain homodimer (Q+R). The subunits are represented as  $C_{\alpha}$  traces, and are coloured according to temperature (*B*) factors. Colours are graded blue ( $\leq 30$  Å<sup>2</sup>) through to red ( $\geq 70$  Å<sup>2</sup>). Runt domain subunits are labelled A, C, E, G, Q and R; CBF $\beta$  subunits are labelled B, D, F and H. The view is down the *b*-axis with *a*, *c* and crystallographic 2<sub>1</sub> axes indicated.

phous crystals produced from seleno-methionine (SeMet)-substituted protein. Crystallographic phases were determined using data sets collected at two wavelengths,



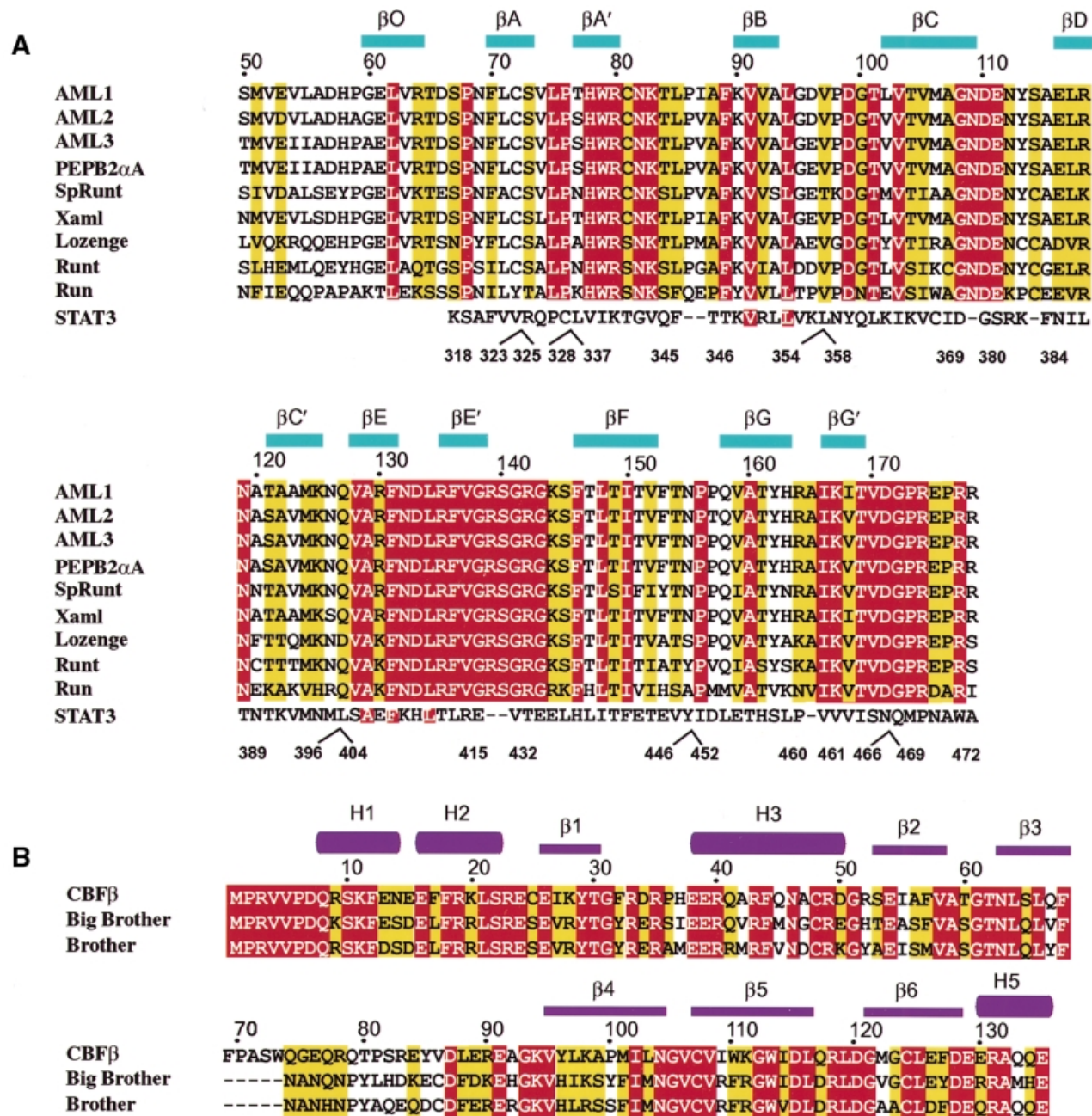
**Fig. 2.** Structure of the Runt domain–CBF $\beta$  heterodimeric complex. **(A)** A stereoscopic diagram of the  $C_{\alpha}$  trace of the AML1 Runt domain, residues 54–178, bound to CBF $\beta$  residues 2–135, prepared with MOLSCRIPT (Kraulis, 1991). The numbering corresponds to the amino acid sequences of human AML1 (Miyoshi *et al.*, 1991) and human CBF $\beta$  (Liu *et al.*, 1993). **(B)** Ribbon diagram of a dimer of Runt domain–CBF $\beta$  heterodimers (two per asymmetric unit). Runt domain, cyan; CBF $\beta$ , magenta. The Runt domain  $\beta$ -strands are labelled  $\beta O$  to  $\beta G$ , consistent with the established immunoglobulin fold nomenclature and with Nagata *et al.* (1999), except for the extensions to strands  $\beta A$  and  $\beta G$ , which have been labelled  $\beta A'$  and  $\beta G'$ , respectively. The  $\beta$ -strands of CBF $\beta$  are labelled  $\beta 1$ – $\beta 6$ , and the helices are numbered H1–5, consistent with previous nomenclature (Goger *et al.*, 1999). Only one hydrogen bond corresponding to a short potential  $3_{10}$ -helix was seen in the region corresponding to helix H4, which was therefore not represented. CBF $\beta$  residues 73–78 are disordered and are shown as a dashed line.

measured at the ESRF, Grenoble, from crystals maintained at 100 K (Table I). Phases were extended to 2.6 Å resolution, and the resulting electron density map was of sufficient quality to build the initial model. The structure has been refined to a free  $R$ -factor of 29.4%, with no residues in disallowed regions of the Ramachandran plot (Table I).

#### **Overall structure of the Runt domain–CBF $\beta$ heterodimeric complex**

The asymmetric unit of the crystal contains six Runt domain subunits and four CBF $\beta$  subunits arranged as two dimers of Runt domain–CBF $\beta$  heterodimers [(Runt domain–CBF $\beta$ ) $_2$ ], and a Runt domain homodimer (Figure 1). The molecules pack in alternate layers of heterodimers and homodimers. The final atomic model is most complete for the heterodimer CD, which includes

residues 54–177 of AML1, and residues 2–135 from the CBF $\beta$  subunit. Residues 52–53 and 178–183 of the Runt domain are disordered, as are residues 75–80 from CBF $\beta$ , and are not included in the model. One-hundred and twenty-one water molecules are also included. The Runt domain homodimer QR (Figure 1) has electron density at the interfaces where CBF $\beta$  binds in the heterodimers, but not sufficient to indicate an ordered CBF $\beta$  subunit. Attempts to refine with a model fitted into this density resulted in a higher free  $R$ -factor. This density may represent partial occupancy by CBF $\beta$  subunits. We have noted the formation of (Runt domain–CBF $\beta$ ) $_2$  dimers mediated by interactions between the Runt domain N-terminal residues in two different crystal forms ( $P21$  and  $P61$  symmetry) and in three independent examples in the asymmetric unit of the  $P21$  crystal form. Gel filtration studies (data not shown) suggest that the formation of

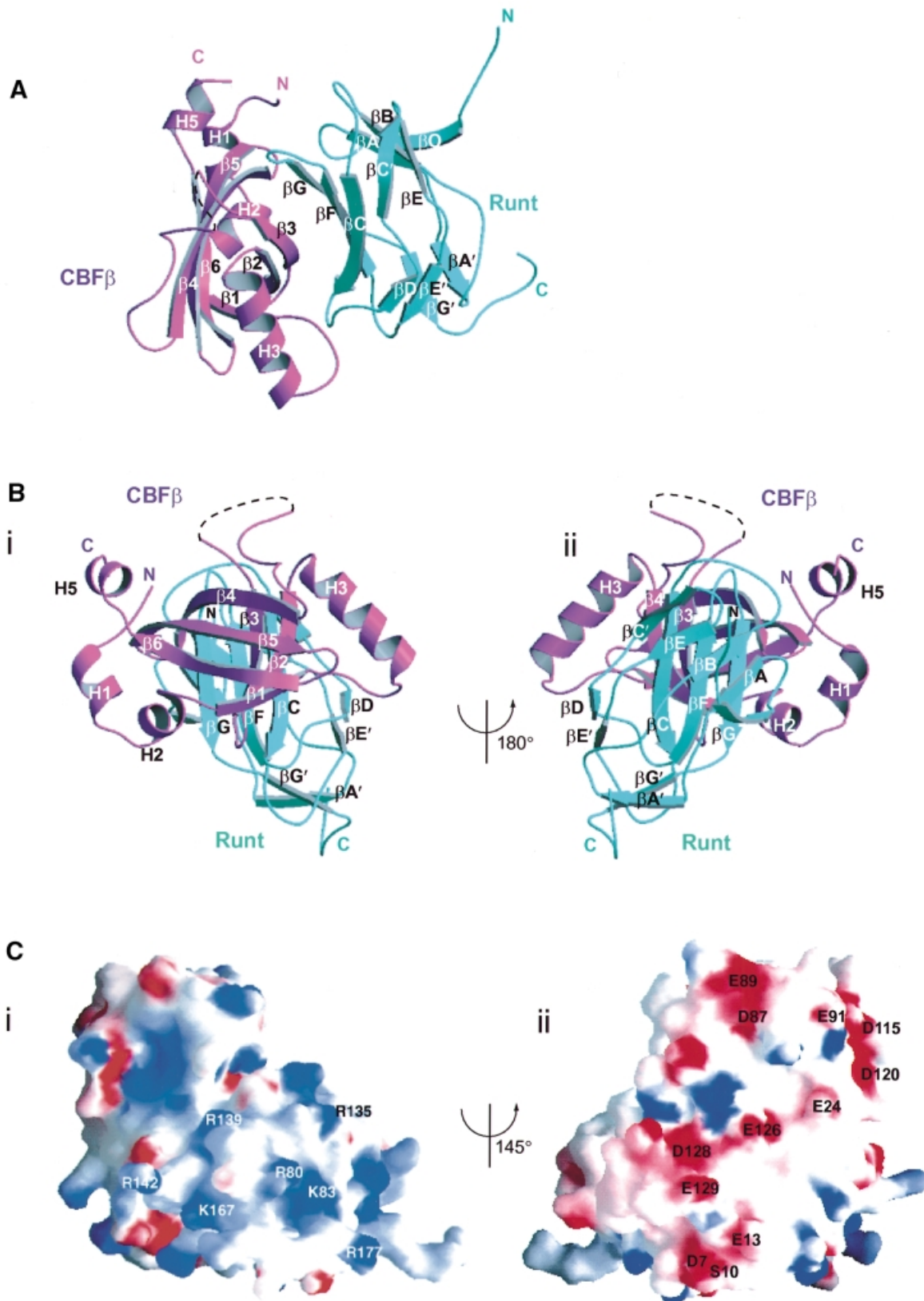


**Fig. 3.** Amino acid sequence alignment of the Runt domain and CBF $\beta$ . (A) The amino acid sequence accession numbers for the Swiss-Prot and DDBJ/EMBL/GenBank databases are given in parentheses. The sequence information is derived from human AML1 (Q01196), residues 50–178; human AML2 (Q13761), residues 54–182; human AML3 (Q08775), residues 102–220; murine PEBP2 $\alpha$ A (Q08775, D14636), residues 50–178; sea-urchin SpRunt-1 (Q26628), residues 57–185; frog Xaml1 (O73725), residues 50–178; fruit-fly Lozenge (Q24183), residues 278–406 and Runt (Q24709), residues 106–234; nematode Run (O01834), residues 10–138. The last line of the alignment shows the structural similarity of the Runt domain to murine STAT3 $\beta$  (P42227), residues 318–472, with manually introduced sequence gaps indicated. Identical residues are highlighted in red, conservatively substituted residues are highlighted in yellow. Sequences were aligned using CLUSTALW (Thompson *et al.*, 1994) and manually adjusted. (B) Sequence alignment of human CBF $\beta$  (Q13951), residues 1–135, and the fruit-fly homologues Brother (Q24039) and Big Brother (Q24040). Residues 70–74 in CBF $\beta$  have no equivalent in *Drosophila*, and are indicated by dashed lines.

(Runt domain–CBF $\beta$ )<sub>2</sub> dimers is not a consequence of crystal packing, but reflects homodimerization of the Runt domain–CBF $\beta$  complex in solution.

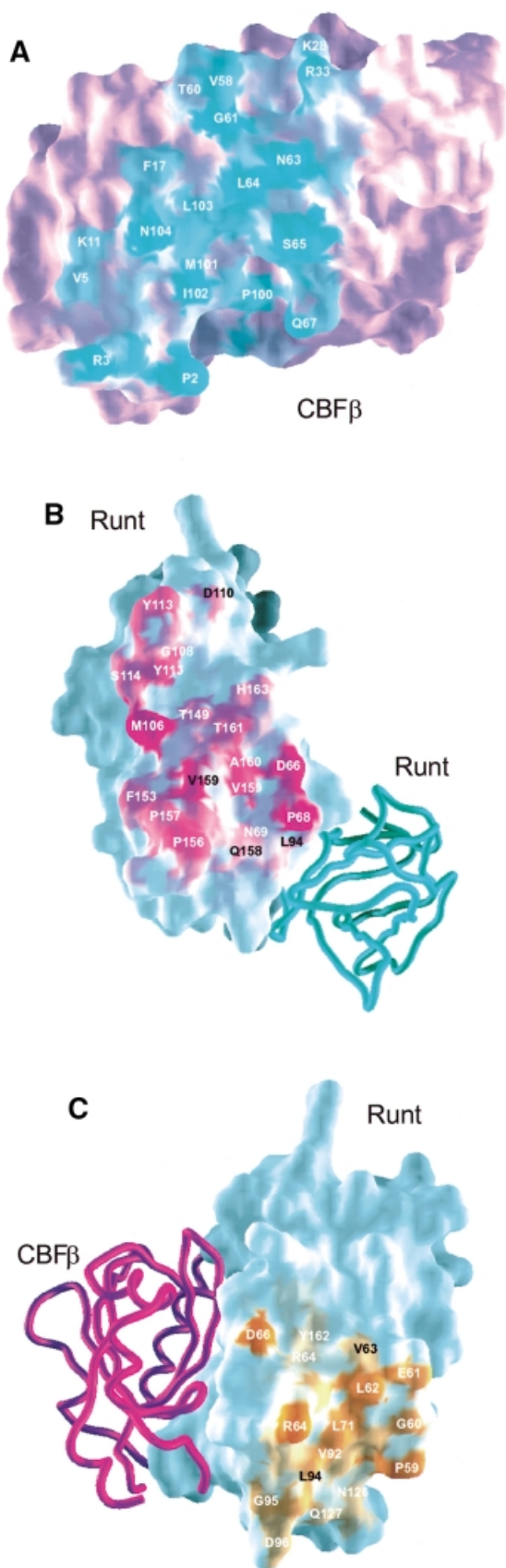
The overall structure of the (Runt domain–CBF $\beta$ )<sub>2</sub> dimer is shown in Figure 2. Although the fold of the individual subunits of the complex is consistent with recent NMR studies (Berardi *et al.*, 1999; Goger *et al.*, 1999; Huang *et al.*, 1999; Nagata *et al.*, 1999), specific

regions of the Runt domain differ in conformation (discussed later). The Runt domain of AML1 forms a 12-stranded (10 antiparallel and two parallel strands)  $\beta$ -barrel that adopts an s-type immunoglobulin (Ig) fold (Bork *et al.*, 1994). CBF $\beta$  is a mixed  $\alpha/\beta$  structure, consisting of a partly open six-stranded  $\beta$ -barrel with  $\alpha$ -helices packed against the top and bottom. Although structural comparisons with the DALI database show that



**Fig. 4.** The mode of interaction between the Runt domain and CBF $\beta$ . (A) Runt domain (cyan) and CBF $\beta$  (magenta) viewed perpendicular to the long axis of CBF $\beta$ . The concave surface of the Runt domain  $\beta$ -sheet, formed from strands  $\beta$ G,  $\beta$ F and  $\beta$ C, packs against the complementary convex strand  $\beta$ 3 of CBF $\beta$ . (B) Two views of the Runt domain–CBF $\beta$  structure (i and ii), related by a 180° rotation about the vertical axis. The long axes of the CBF $\beta$  and Runt domain  $\beta$ -barrels are orthogonal to one another. (C) Electrostatic surface potential of the Runt domain–CBF $\beta$  heterodimer. The two views are related by a 145° rotation about the vertical axis. Positive areas are shaded blue; negative areas are shaded red. This figure was prepared using GRASP (Nicholls *et al.*, 1991). (i) Positive surface. Labelled residues are mutated in cleidocranial dysplasia, familial platelet disorder and sporadic acute myeloid leukaemia. (ii) Negative surface. The evolutionarily conserved, negatively charged residues that are labelled are all located on the surface of CBF $\beta$ .

the  $\beta$ -barrel component of CBF $\beta$  has overall structural similarity to a number of functionally unrelated proteins, it appears that the fold is distinct, as opposed to a possible relationship to the OB fold (Goger *et al.*, 1999). As



classified in the SCOP protein structure database (Murzin *et al.*, 1995), the OB fold consists of a five-stranded  $\beta$ -barrel, with Greek key topology and a shear number of 8 or 10. CBF $\beta$  forms a partly opened six-stranded  $\beta$ -barrel with a unique combination of a meandering up and down topology of the  $\beta$ -strands and a shear number of 10. Consequently, this particular  $\beta$ -barrel structure can be classified as a novel fold from the distinct combination of topology and shear number.

There are extensive heterodimeric contacts at the interface between the Runt domain and CBF $\beta$  subunits, and homodimeric contacts between the N-termini of the Runt domains, but no contacts between the CBF $\beta$  subunits. The temperature factors for the (Runt domain-CBF $\beta$ )<sub>2</sub> structure (Figure 1) suggest that the Runt domain forms a relatively stable core, whereas the CBF $\beta$  subunit is more mobile. Figure 3 shows the secondary structure elements for the conserved Runt domain and CBF $\beta$  determined from our structure, aligned to the protein sequences of various family members.

The root mean square deviations (r.m.s.ds) of the C $\alpha$  backbone traces between different Runt domain subunits in the asymmetric unit range between 0.15 and 0.35 Å, and for the four CBF $\beta$  subunits, the values range between 0.16 and 0.36 Å. The overall dimensions of a single Runt domain-CBF $\beta$  heterodimer are  $\sim 41 \times 50 \times 27$  Å. The Runt domain and CBF $\beta$  interact along a large continuous curved interface (Figure 4A), and are oriented such that the long axes of the two  $\beta$ -barrel domains are orthogonal to one another (Figure 4B). When viewed from the perspective of Figure 4B, with the C-terminus of the Runt domain oriented downward, it is evident that CBF $\beta$  makes contact only with the upper part of the Runt  $\beta$ -barrel. CBF $\beta$  makes no direct contact with Runt domain loops  $\beta$ A'-B,  $\beta$ E'-F or the C-terminus. Residues within these loops have been shown biochemically to be essential for DNA binding (Kagoshima *et al.*, 1996; Osato *et al.*, 1999). The  $\alpha$ -helices H5, H1 and H2 of CBF $\beta$  lie on the right lateral aspect of the upper half of the Runt  $\beta$ -barrel, and helix H3 lies on the upper left lateral aspect (Figure 4Bii). Our data differ from the proposed mode of interaction between the Runt domain and CBF $\beta$ , based on chemical shift analysis (Nagata *et al.*, 1999). It was suggested that CBF $\beta$  is oriented in the heterodimer with helices H1, H2 and H5 up, and helix H3 down, placing CBF $\beta$  residues Gln74, Gln79 and Arg83 on the same face of the heterodimer as the proposed DNA-binding surface of the Runt domain (loops  $\beta$ A'-B and  $\beta$ E'-F and the C-terminus). In fact, the crystal structure demonstrates that the CBF $\beta$  is rotated by 90° relative to the previous proposal (Figure 4B), so that the evolutionarily non-conserved CBF $\beta$  loop  $\beta$ 3- $\beta$ 4 (residues

**Fig. 5.** Interaction surfaces within the (Runt domain-CBF $\beta$ )<sub>2</sub> complex. (A) Heterodimerization surface of CBF $\beta$ . Solvent-accessible surface is shown in purple; residues buried in the Runt domain surface are shown in cyan. All interface residues apart from Q67 and P100 are conserved (see also Figure 3). (B) Heterodimerization surface of the Runt domain. Solvent-accessible surface is shown in cyan; residues buried in the interface with CBF $\beta$  are shown in magenta. The Runt domain homodimer binding partner is shown in worm representation (cyan). (C) Homodimerization surface of the Runt domain. Solvent-accessible surface is shown in cyan; buried residues are shown in orange. The related heterodimeric CBF $\beta$  subunit is shown in worm form (magenta).

68–93) makes no contribution either to the heterodimer interface or to the DNA binding surface of the molecule.

The conformation of the C-terminus of the Runt domain (residues 169–177), which is essential for DNA binding, is clearly defined in the crystal structure. The C-terminus forms a loop that extends towards the N-terminus of the Runt domain, passing below strand  $\beta A'$  (Figure 4B). All the loops on the lower face of the Runt domain  $\beta$ -barrel, as orientated in Figure 4B ( $\beta C$ –D,  $\beta E'$ –F, the C-terminus and  $\beta A'$ –B), are linked to one another and are well buttressed on one side as a result of the interaction of loop  $\beta C$ –D with CBF $\beta$ . Runt domain loop  $\beta C$ –D, which has not been implicated in DNA binding, makes a number of contacts with CBF $\beta$  through the side chain of Tyr113. Finally, we find that the three Cys residues in CBF $\beta$  (Cys25, Cys107 and Cys124) are not related to the heterodimer interface as proposed (Huang *et al.*, 1999), suggesting that these residues are not directly related to the modulation of oxidation state-dependent behaviour of AML1.

### **Electrostatic surface potential of the Runt domain–CBF $\beta$ heterodimer**

There are two contrasting surfaces on the heterodimer in terms of the electrostatic surface potential (Figure 4Ci and ii). The strongly positive surface corresponds to the position of loops  $\beta A'$ –B,  $\beta E'$ –F and the C-terminus of the Runt domain. This supports the biochemical and human mutation data, which directly implicate these regions of the Runt domain in DNA binding (Lenny *et al.*, 1995; Kagoshima *et al.*, 1996; Osato *et al.*, 1999). In contrast, rotation by 145° from this region reveals a strikingly negative surface (Figure 4Cii), corresponding to the upper outer surface of CBF $\beta$ . Five areas on the surface of CBF $\beta$  make up this charged surface: the end of strand  $\beta 6$  and the residues prior to helix H5; residues in loop  $\beta 3$ – $\beta 4$ ; the  $\beta 5$ – $\beta 6$  loop; strand  $\beta 1$ ; and helix H1. The majority of these residues are conserved in evolution (Figure 3B), suggesting a conserved biological function.

### **The Runt domain–CBF $\beta$ interface**

The Runt domain and CBF $\beta$  subunits interact over a large continuous curved interface (Figure 4A), such that a total of 1900 Å<sup>2</sup> in solvent-accessible surface area is buried [assuming default radii of GRASP and a 1.4 Å solvent probe (Nicholls *et al.*, 1991)]. The interaction surface of the Runt domain is concave, and packs against a complementary convex surface on CBF $\beta$ . The curved heterodimerization surface of the Runt domain shown in Figure 4A involves loops  $\beta F$ –G,  $\beta O$ –A and  $\beta B$ –C at the top of the  $\beta$ -barrel; strands  $\beta C$ ,  $\beta F$  and  $\beta G$ ; and loop  $\beta C$ –D at the bottom of the  $\beta$ -barrel. The regions of CBF $\beta$  involved in heterodimerization are the N-terminal loop and helix H1; strand  $\beta 1$  and loop  $\beta 1$ –H3; strands  $\beta 2$ ,  $\beta 3$  and the connecting loop  $\beta 2$ – $\beta 3$ , which together form the central convex interaction surface; strand  $\beta 4$  and the proximal part of loop  $\beta 4$ – $\beta 5$ .

Figure 5A shows the residues on the surface of CBF $\beta$  that are buried in the Runt domain at the interface. Comparing interface residues between human CBF $\beta$  and the *Drosophila* homologues, 13 residues are identical and six involve similar substitutions (Figure 3B). Only two residues have non-conservative substitutions between *Drosophila* and human. Mutagenesis studies demonstrated

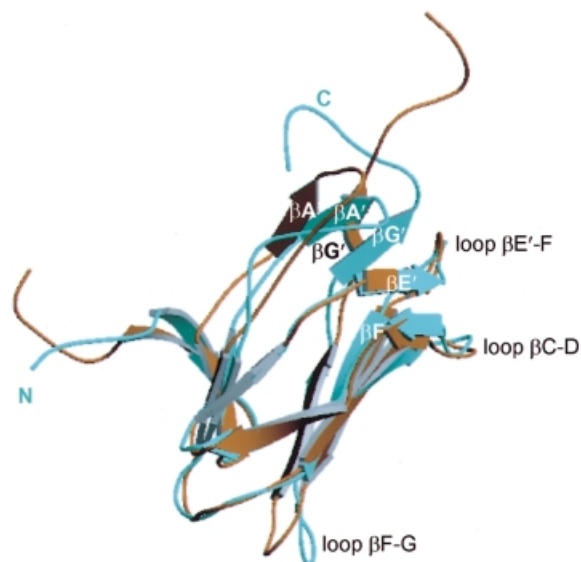
a requirement for the N-terminal 5–6 residues of CBF $\beta$  for heterodimerization to the Runt domain (Golling *et al.*, 1996; Kagoshima *et al.*, 1996). We find that the Runt domain–CBF $\beta$  interaction involves the N-terminus of CBF $\beta$  (residues 2–5). The Runt domain loop  $\beta O$ –A, loop  $\beta B$ –C and strand  $\beta G$  are all involved in contacts with the N-terminus of CBF $\beta$ , with four potential hydrogen bonding interactions mediated by CBF $\beta$  Arg3. Significant chemical shifts at Gln74, Gln79 and Arg83 were noted on binding of a Runt domain–DNA complex to CBF $\beta$  (Goger *et al.*, 1999), but the crystal structure demonstrates that this is not a result of burying these residues at the heterodimer interface, and that they do not come to lie proximal to the putative DNA-binding loops  $\beta E'$ –F or  $\beta A'$ –B of the Runt domain (see below).

Two-thirds of the residues on the surface of the Runt domain that are buried in the CBF $\beta$  subunit (Figure 5B) are either conserved or conservatively substituted between members of the  $\alpha$ -subunit family (Figure 3A). Structurally, the conserved *cis*-Pro156 induces a kink in Runt domain loop  $\beta F$ –G, which makes important contacts with residues in CBF $\beta$ . The Runt domain strand  $\beta G$  (residues 159–161) pairs with CBF $\beta$  strand  $\beta 4$  (residues 102–104) to form a short antiparallel  $\beta$ -sheet extension. The  $\beta$ -sheet extension between the two subunits is stabilized by a cluster of conserved hydrophobic residues (Runt domain Val159; CBF $\beta$  Met101, Ile102 and Leu103). At the opposite end of the heterodimer interface, Runt domain Ser114 and Tyr113 provide a large surface area of interaction with CBF $\beta$ . These residues lie in a solvent-accessible polar environment, consistent with previous spectroscopic analysis (Crute *et al.*, 1996). CBF $\beta$  residues Glu111 and Asp110 also contribute to this polar environment. Runt domain Met106 makes a significant contribution to the buried surface area in the central part of the heterodimer interface. The functional importance of this residue to the interaction is supported by biochemical studies, which demonstrate that a M106V mutation abolishes the interaction between AML1 and CBF $\beta$  *in vitro* (Akamatsu *et al.*, 1997b). There are a total of 42 contacts between the two subunits, 10 of which represent potential hydrogen-bonding interactions. Bridging contacts are also mediated indirectly via water molecules near the interface. We have identified eight water molecules buried at the heterodimer interface, the role of which is presumably to optimize the complementarity of the interaction interfaces.

The structure of the heterodimer interface is consistent with the *in vitro* Runt domain mutants M106V, G108R and N109D, which show loss of heterodimerization, but preservation of DNA-binding activity (Akamatsu *et al.*, 1997b). Mutations in the Runt domain residues 66–69 located on the  $\beta O$ –A loop also abolish heterodimerization (Lenny *et al.*, 1995). Recent *in vivo* experiments in *Drosophila* demonstrated that the G108R Runt protein mutant was dysfunctional in several *in vivo* assays, showing that the interaction of Runt with the *Drosophila* CBF $\beta$  homologues is essential *in vivo* for the function of the transcription complex (Li and Gergen, 1999).

### **Homodimer interface of the Runt domain**

The crystal structure identifies two homodimeric interactions between the Runt domains, one interface between



**Fig. 6.** The Runt domain complexed to CBF $\beta$  has a novel conformation. Least squares superposition of the  $C_{\alpha}$  trace of the Runt domain crystal structure onto the most closely related NMR conformer [PDB code 1cmo, number 38, (Nagata *et al.*, 1999)]. The analysis was performed for all the NMR conformers, and similar results were obtained. The NMR structure is shown in brown and the crystal structure is shown in cyan. The crystal and NMR structures differ in the relative positions of the  $\beta A'-\beta G'$  sheet and the  $\beta F-G$  loop. The C-terminus is well defined in the crystal structure.

the Runt domains in the dimer of heterodimers, and a smaller interface involving a subset of these interactions within the homodimer QR (890  $\text{\AA}^2$  solvent-accessible surface area buried as opposed to 1300  $\text{\AA}^2$ ). The residues buried at the interface are shown in Figure 5C. The N-terminal residues (59–66) make the most prominent contribution to this interface, which is stabilized further by contacts between strands  $\beta A$  and  $\beta B$ , and loops  $\beta B-C$  and  $\beta C'-E$ . Runt domain Asp66 contributes to both the heterodimer and homodimer interfaces. The homodimeric interaction in the dimer of heterodimers is mediated by a short edge to edge anti-parallel  $\beta$ -sheet formed from the pairing of residues 60–62 at the N-terminal end of the Runt domain, and is stabilized by a hydrophobic cluster involving Pro59, Leu62, Val63, Leu71, Val92 and Leu94 from each subunit. The hydrophobic core of the interface shows strong sequence conservation (Figure 3A).

#### **Comparison with NMR data shows conformational differences in the Runt domain**

Interaction with CBF $\beta$  is essential for the *in vivo* function of AML1 (Wang *et al.*, 1996). *In vitro*, CBF $\beta$  decreases the dissociation constant ( $K_d$ ) of the isolated Runt domain for its cognate DNA-binding site 5- to 10-fold (Kagoshima *et al.*, 1996). The molecular basis for this enhanced DNA-binding affinity of the Runt domain in the presence of CBF $\beta$  is not yet established. The availability of structures of the Runt domain–CBF $\beta$  binary complex (this work), together with the co-ordinates for the NMR structures of the Runt domain (performed in the presence of DNA) (Nagata *et al.*, 1999) and the bundle of NMR structures of the uncomplexed CBF $\beta$  subunit (Goger *et al.*, 1999; Huang *et al.*, 1999), allows us to address this issue.

Although the experimental restraints for the NMR structures are not available, examination of the bundle of NMR structures gives some idea of the flexibility of the structure and the accuracy of the model. We found no significant differences in the crystal structure of CBF $\beta$  with respect to the available NMR co-ordinates (Goger *et al.*, 1999; Huang *et al.*, 1999) (PDB codes 2jhb and 1cl3), apart from the disorder in the flexible non-conserved loop  $\beta 3-\beta 4$  (residues 75–80) in the heterodimer. The  $C_{\alpha}$  traces for the structures of the CBF $\beta$ - and DNA-bound forms of the Runt domain superimpose well, except in the region of the putative DNA-binding loops, where the structures differ significantly (Figure 6). In the Runt domain–CBF $\beta$  complex, the C-terminal residues of the Runt domain are ordered, defining the conformation of this essential DNA-binding region. The parallel  $\beta$ -sheet,  $\beta A'-\beta G'$ , is shifted significantly towards the  $\beta E'-F$  loop, and is stabilized in this position by the potential main-chain hydrogen bonding interactions between strand  $\beta G'$  (Ile168) and loop  $\beta E'-F$  (Arg139 and Gly141). Cys81 forms potential main- and side-chain hydrogen bonding interactions linking loop  $\beta A'-B$  (Cys81) both to strand  $\beta G'$  (Thr169) and to strand  $\beta E'$  (Val137). Relative to the DNA-bound Runt domain conformation, Cys81 shows the largest change in relative position of any  $C_{\alpha}$  (7.2  $\text{\AA}$ ). This change in chemical environment may alter the susceptibility of Cys81 to oxidation (Akamatsu *et al.*, 1997a).

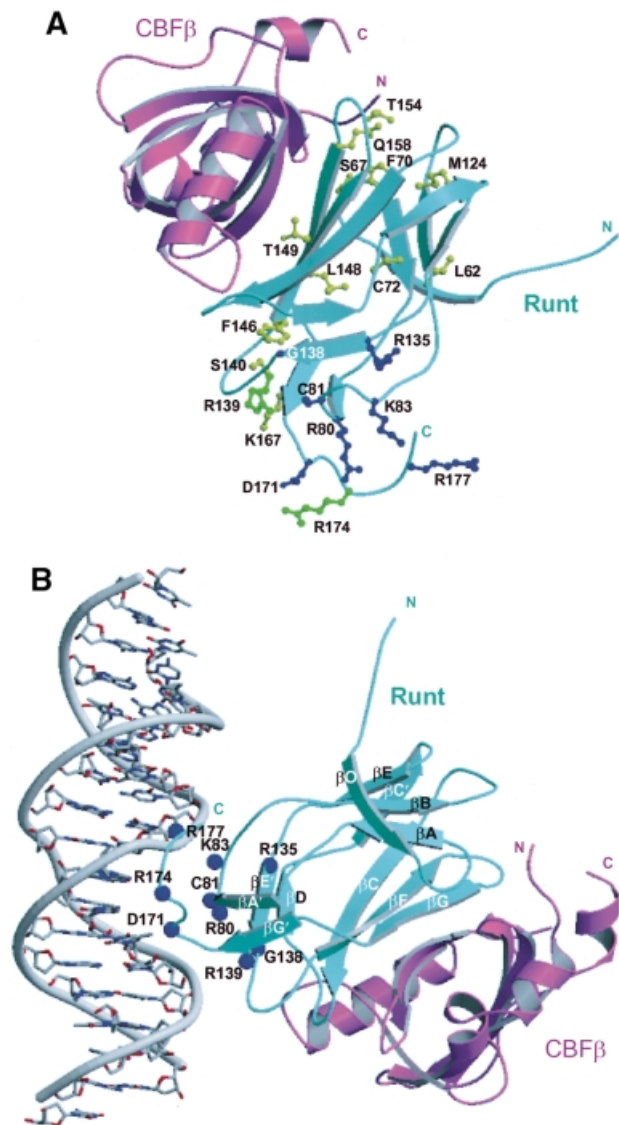
To accommodate the movement of the  $\beta A'-\beta G'$  sheet, there is a displacement in the main-chain conformation of residues 85–89 (Pro86 is shifted by 5.9  $\text{\AA}$ ), and an upward kink at Ala165 (shifted by 6.5  $\text{\AA}$ ), relative to the NMR structure. The kink at Ala165 is stabilized by main-chain potential hydrogen bonding with Tyr162. Tyr162 projects into the hydrophobic core of the molecule, but the residues immediately adjacent (Thr161 and His163) are buried in the CBF $\beta$  subunit at the heterodimer interface, suggesting a mechanism for induced conformational change transmitted from the heterodimer interface by stabilization of the orientation of the Tyr162 side chain. There is also a change in the conformation of the Runt domain  $\beta F-G$  loop (residues 153–159), which is twisted through a 90° angle in the crystal structure, resulting in displacement of Pro156 by 6.15  $\text{\AA}$  relative to its position in the NMR structures. This region of the Runt domain forms extensive contacts with CBF $\beta$  (Figures 4A and 5B).

These comparisons demonstrate ordering of the C-terminus of the Runt domain in the presence of CBF $\beta$ , and reveal conformational flexibility within the Runt domain that can alter the relative orientation of a specific DNA-binding subdomain. This suggests that CBF $\beta$  may enhance DNA binding by the AML1 Runt domain through the stabilization of this conformation.

#### **Discussion**

The structure demonstrates that the interaction between the AML1 Runt domain and CBF $\beta$  is mediated by highly conserved residues from each subunit, and therefore provides a structural paradigm for heterodimerization by other Runt domain family members. In addition, the molecular consequences of several point mutations associated with human disease can be explained from the structure of the complex. Furthermore, interaction of the





**Fig. 7.** Location of human disease mutations on the AML1–CBF $\beta$  structure. (A) A ribbon diagram of the Runt domain–CBF $\beta$  heterodimer in which residues mutated in cleidocranial dysplasia (CCD) are shown in yellow, familial platelet disorder (FPD) and acute myeloid leukaemia (AML) are shown in blue. Mutations common to CCD and FPD/AML are shown in green. The ribbon representation of the Runt domain is in cyan, and that of CBF $\beta$  is in magenta. (The AML-associated biallelic point mutations G138D, R135G, D171G are a personal communication from P.Fenaux.) (B) Model orienting the Runt domain–CBF $\beta$  heterodimer with respect to B-form DNA. The model was generated by least squares superposition of the co-ordinates of the STAT3 $\beta$ –DNA complex (PDB code, 1bg1) (Becker *et al.*, 1998) onto the AML1 Runt domain. The amino acid residues mutated in human disease mutations are indicated as blue spheres. The Runt domain is shown in cyan, and CBF $\beta$  in magenta. The model is consistent with: the electrostatic surface potential of the heterodimer (Figure 4C); *in vitro* mutagenesis data (Lenny *et al.*, 1995; Kagoshima *et al.*, 1996; Osato *et al.*, 1999); chemical footprinting analysis (Thornell *et al.*, 1988; Melnikova *et al.*, 1993); NOE data from NMR studies (Nagata *et al.*, 1999); and the observations that CBF $\beta$  does not contact DNA directly, or extend the Runt domain footprint on DNA (Kamachi *et al.*, 1990).

Runt domain and CBF $\beta$  appears to have conformational consequences for a region of the Runt domain directly implicated in DNA binding.

### Mutations in cleidocranial dysplasia map to heterodimer interface

Congenital point mutations within the Runt domain of AML1 and AML3 have been attributed to the pathogenesis of familial platelet disorder, which progresses to acute myeloid leukaemia (AML1) (Song *et al.*, 1999), and to a disorder of osteogenesis, cleidocranial dysplasia (AML3) (Lee *et al.*, 1997; Mundlos *et al.*, 1997). Somatic mutations in AML1 have also been demonstrated in acute myeloid leukaemia (Osato *et al.*, 1999).

The majority of point mutations associated with AML involve residues directly implicated in DNA binding. In contrast, recently described missense mutations associated with skeletal dysgenesis map to the heterodimer interface (Quack *et al.*, 1999; Zhou *et al.*, 1999) (Figure 7A). Three mutations affect residues identified in our structure as buried at the heterodimer interface: T149A, Q158R and S67R (Figure 7A), which map to the Runt domain strands  $\beta$ F, strand  $\beta$ G and loop  $\beta$ O–A, respectively. The T149A mutation is associated with variable expressivity in a single family, ranging from classical CCD, to dental anomalies alone, while the Q158R and S67R mutations are associated with classical CCD (Zhou *et al.*, 1999). The T149A mutation would be expected to disrupt a potential hydrogen bond with CBF $\beta$  Asn63. Q158 interacts with CBF $\beta$ , but also stabilizes the conformation of Runt domain loops  $\beta$ O–A and  $\beta$ F–G. The Q158L mutation *in vitro* results in impairment of DNA binding and loss of heterodimerization (Lenny *et al.*, 1995). These mutations demonstrate that the integrity of Runt domain loops  $\beta$ F–G and  $\beta$ O–A is crucial both for interaction with CBF $\beta$  and for DNA binding. This may be due to indirect stabilization of the DNA-binding surface, or to an effect on the overall stability of the Runt domain. The Runt domain M124R mutation associated with CCD (Lee *et al.*, 1997) disrupts DNA binding, but maps to a region away from the potential DNA-binding surface. This mutation introduces a bulky, charged residue into the hydrophobic core of the Runt domain, and would be expected to destabilize the structure.

Our structure defines the conformation of the Runt domain C-terminus, which is essential for nuclear localization and DNA binding (Kagoshima *et al.*, 1996; Osato *et al.*, 1999; Quack *et al.*, 1999). Mapping mutations associated with FPD, AML and CCD onto the structure clearly delineates the potential DNA-binding surface (Figure 7A). One of the mutations described in FPD, R139Q, which is also associated with CCD, has indeed been shown to confer loss of DNA-binding function *in vitro* (Zhou *et al.*, 1999). Arg174 was the residue most frequently affected by mutation in the AML3 Runt domain in a recent CCD study (Quack *et al.*, 1999), which also demonstrated that the mutation R174Q abolishes DNA binding *in vitro*, and that R174Q and R174W disrupt the function of the nuclear localization signal, preventing AML3 accumulation in the nucleus.

### Insight into the DNA-binding function of the AML1–CBF $\beta$ heterodimer

The structural similarity of the AML1 Runt domain to transcription factors p53, NF- $\kappa$ B p52, NF- $\kappa$ B p65, NFAT1, the T-domain and the STAT proteins has been shown (Berardi *et al.*, 1999; Nagata *et al.*, 1999). The co-

ordinates from our crystal structure were used to search the protein database (Dali server), which demonstrated that the Runt domain is most closely related structurally to the transcription factor STAT3 $\beta$ . Structural alignment of the Runt domain with STAT3 $\beta$  gives an r.m.s.d. of 2.1 Å over 82 residues (see Figure 3A). The structural similarity is prominent even within the loop regions of the Runt domain, most strikingly within the C-terminus. The smaller size of the Runt domain DNA-binding module is related to the shorter length of the putative DNA-binding loops. In contrast to STAT3 $\beta$ , which binds DNA as a dimer, the Runt domain appears to bind as a monomer (Ogawa *et al.*, 1993a).

We have generated a model for major groove DNA binding by the Runt domain–CBF $\beta$  heterodimer, by superposition of the STAT3 $\beta$ –DNA co-crystal complex (Becker *et al.*, 1998) onto the AML1 Runt domain (Figure 7B). The orientation of the Runt domain–CBF $\beta$  complex with respect to the DNA is consistent with the location of human disease mutations (Figure 7A) and other data (see Figure 7 legend). Three loops from the Runt domain  $\beta$ -barrel are predicted to make close contacts with the DNA. The  $\beta$ A'–B loop and the C-terminus are predicted to participate in core sequence recognition in the major groove. The conformation of the C-terminus, which interacts with loop  $\beta$ A'–B through the small parallel  $\beta$ A'– $\beta$ G' sheet, is a conserved structural feature between the related Ig fold transcription factors. Our model predicts that the  $\beta$ E'–F loop would make DNA backbone and minor groove contacts. The analogous segment in STAT3 $\beta$  is involved in DNA backbone interactions (Becker *et al.*, 1998).

Berardi *et al.* noted nuclear Overhauser effects (NOEs) to DNA from Runt domain loop  $\beta$ F–G in their NMR studies (Berardi *et al.*, 1999). However, in the crystal structure of the heterodimeric complex, residues on the surface of Runt domain loop  $\beta$ F–G (*cis*-Pro156, Pro157, Gln158) are buried within the CBF $\beta$  subunit (Figure 5B). Our model is compatible with existing footprinting data that demonstrate that the AML1–CBF $\beta$  complex protects ~10 bp of DNA (Thornell *et al.*, 1988). We therefore propose that the core binding factor heterodimer binds DNA through Runt domain loops  $\beta$ E'–F and  $\beta$ A'–B, and the C-terminus.

### The role of the CBF $\beta$ interaction

The interaction of CBF $\beta$  with the Runt domain enhances its DNA-binding affinity (Kagoshima *et al.*, 1996). We observe significant differences in the conformation of loop  $\beta$ A'–B and the C-terminus of the Runt domain in our structure compared with the NMR data (Nagata *et al.*, 1999). The AML1–CBF $\beta$  heterodimer adopts the same conformation in multiple copies within the asymmetric unit of the crystal, demonstrating that the structure is not an artifact of specific crystal contacts. The shift of 7.2 Å in the position of Cys81 offers a structural explanation for biochemical data that demonstrate protection from oxidation for this residue following binding of the Runt domain to CBF $\beta$  (Akamatsu *et al.*, 1997a). This residue is predicted to contact the DNA backbone (consistent with the C81D mutation that abolishes DNA binding (Akamatsu *et al.*, 1997a), analogous to the role of conserved Cys residues in other Ig fold transcription

factors (Ghosh *et al.*, 1995). This indicates that the conformational changes observed in the heterodimer are biologically relevant.

Conformational differences at the DNA-binding surface are consistent with reported changes in the NMR spectra of the Runt domain upon binding CBF $\beta$  (Berardi *et al.*, 1999). STAT3 $\beta$  superimposes well onto the essential DNA-binding C-terminus of AML1, and NMR studies have demonstrated NOEs between Arg80 and a cytidine residue in the major groove of the DNA, directly implicating Runt domain loop  $\beta$ A'–B in DNA recognition (Nagata *et al.*, 1999). The analogous segment in five other transcription factors binds to DNA in the major groove. Therefore, constraints on the flexibility of the  $\beta$ A'–B loop and the C-terminus would be expected to have an important influence on DNA-binding affinity. Our data therefore support the hypothesis that one role of CBF $\beta$  is to stabilize the relative orientation and conformation of a modular DNA-binding subdomain consisting of loop  $\beta$ A'–B and the C-terminus, in the context of the ternary complex with DNA.

The solvent-exposed surface of CBF $\beta$  in the heterodimer may have additional functions in transcription. CBF $\beta$  may act as a platform to recruit other proteins to modulate the transcriptional activity of AML1 *in vivo*. Our structural data reveal a negatively charged surface composed of conserved residues that may provide a surface for such protein interactions (Figure 4C). The significance of the Runt domain homodimerization in our structure is at present unclear, but may indicate a potential binding site for other proteins *in vivo*. Physical interaction has been demonstrated, for example, between the AML1 Runt domain and the transcription factors C/EBP $\alpha$  and PU.1 (Petrovick *et al.*, 1998). Thus, the structure gives insight into the surfaces of the AML1–CBF $\beta$  heterodimer, which may recruit other tissue-specific factors to stimulate lineage-restricted transcription.

## Materials and methods

### Protein expression and purification

The DNA sequence encoding human AML1, residues 50–183, was amplified from plasmid pET/RM7 (gift of Dr F. Calabi) by the polymerase chain reaction (PCR). The PCR fragment was cloned into the mini-pRSET vector (gift from O. Perisic), adding 16 residues at the N-terminus (MRGSHHHHHGLVPRG). The DNA sequence encoding human CBF $\beta$ , residues 1–135, was amplified by PCR from cDNA synthesized from RNA purified from HEL cells. The fragment was cloned downstream of a Shine–Dalgarno sequence in pBluescript, and subsequently subcloned into the mini-pRSET vector downstream of the AML1 coding sequence. The pmini- $\alpha/\beta$  expression plasmid was transfected into *E. coli* C41 (DE3) cells (Miroux and Walker, 1996). Transformed cells were grown at 37°C in 2 $\times$  TY medium containing ampicillin at 100  $\mu$ g/ml to an OD<sub>600</sub> of 0.6 and induced for 4 h with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. The protein complex was purified on a combination of Ni<sup>2+</sup>-NTA affinity resin (Qiagen), Resource S (Pharmacia) and HiPrep Sephacryl S100 (Pharmacia) columns, with no subsequent cleavage of the His-tag from the AML1 fragment. The protein was concentrated to 12 mg/ml in a solution containing 20 mM HEPES pH 6.6, 300 mM NaCl, 10 mM dithiothreitol (DTT) and 1 mM EDTA, and stored in liquid nitrogen.

### Preparation of SeMet-substituted AML1–CBF $\beta$

SeMet-substituted protein was expressed from the pmini- $\alpha/\beta$  plasmid in the *E. coli* strain 834 (DE3). Log phase cells (OD<sub>600</sub> = 0.3) precultured in 2 $\times$  TY were diluted 1:100 into 2 $\times$  M9 minimal medium supplemented with 0.4% glucose, 19 amino acids at 40  $\mu$ g/ml, seleno-L-methionine

(Sigma) at 40 µg/ml, and vitamins at 1 µg/ml (Ramakrishnan and Biou, 1997). Bacteria grew at 37°C and were induced after 8 h (OD<sub>600</sub> = 0.6). Induction was at 25°C overnight (OD<sub>600</sub> = 1.1). The method of protein purification was identical to that of the unsubstituted protein. SeMet substitution was assayed by MALDI mass spectrophotometry, and was consistent with complete substitution of methionine residues with SeMet, but loss of the N-terminal methionine of CBFβ.

### Crystallization

Crystals of the complex were grown as hanging drops against a reservoir containing 100 mM imidazole, 6% PEG 8000, 5% sucrose and 5 mM DTT at 21°C, using 1 µl of 12 mg/ml protein solution and 1 µl of crystallization solution. Macroseeding was used to obtain diffraction quality crystals, which grew to their final size over 2 weeks. Crystals were harvested by transfer to a cryobuffer containing 30% sucrose, 12% PEG 8000, 5% MPD, 200 mM NaCl, 100 mM imidazole pH 6.5 for 30 s, and cryocooled in liquid nitrogen. Crystals were prepared from SeMet-substituted protein under similar conditions, except that 5% MPD was incorporated as an additive in the initial drops. Crystals belonging to space group *P*61 were also obtained using a crystallization solution with 400 mM NaCl, 50 mM HEPES pH 7.5, 5 mM DTT, 5% PEG 8000. These were cryocooled after transferring to a buffer containing 30% glycerol, 100 mM HEPES pH 7.5, 200 mM NaCl, 5% PEG 8000.

### Data collection and structure determination

Crystals used for native protein data collection were typically 400 × 200 × 40 µm or larger, had *P*21 symmetry, with unit cell dimensions of *a* = 103.2 Å, *b* = 79.4 Å, *c* = 130.1 Å, β = 101.6°, a solvent content of 60%, and diffracted to 2.6 Å. SeMet-substituted crystals had unit cell dimensions *a* = 104.0 Å, *b* = 79.3 Å, *c* = 130.9 Å, β = 101.5°, were somewhat smaller and diffracted to only 2.8 Å resolution. Diffraction data for the native crystal were collected at ESRF beamline ID14-3 (Grenoble) using a MAR CCD detector and the data for the SeMet-substituted crystal were collected at ID14-4 using an ADSC CCD detector, using crystals maintained at 100 K (Table I). Native data were processed using MOSFLM (Leslie, 1992). Each data set was collected as 180° oscillations. For the SeMet-substituted crystal, data sets were collected at two wavelengths, λ<sub>1</sub> = 0.9793 Å and λ<sub>2</sub> = 0.9795 Å, corresponding to the peak and inflection points in the fluorescence spectrum of a SeMet-substituted crystal. Data sets for the SeMet-substituted crystal were integrated with HKL2000 and scaled with SCALEPACK (Otwinowski and Minor, 1997). The native data were scaled and merged with SCALA (CCP4, 1994). Final merging of the SeMet data was carried out with SOLVE (Terwilliger and Berendzen, 1999). The *R*-factor for dispersive differences between data collected at λ<sub>1</sub> and data collected at λ<sub>2</sub> was 0.046. The *R*-factor for anomalous differences was 0.077 for λ<sub>1</sub> and 0.066 for λ<sub>2</sub>. SOLVE (Terwilliger and Berendzen, 1999) was used to locate 21 Se sites. The SOLVE *Z*-scores for the Patterson correlation, cross-validation Fourier, native Fourier and mean figure of merit for the 21-site solution were 7.4, 52, 21 and 9.4, respectively. The overall *Z*-score was 66. The mean figure of merit was 0.47. SeMet data to 3.5 Å resolution were used to provide an initial estimate for the phases of the native data set. Density modification was carried out to improve the phases and extend them to 2.6 Å resolution (Brünger *et al.*, 1998). A model was built into the 2.6 Å resolution electron density maps using the program O (Jones *et al.*, 1991), and refined using CNS (Brünger *et al.*, 1998). The average *B*-factor for all atoms is 55.9 Å<sup>2</sup>. The structure shows good stereochemistry with no residues in disallowed regions of the Ramachandran plot.

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