

The mixed lineage leukemia fusion partner AF9 binds specific isoforms of the BCL-6 corepressor

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The mixed lineage leukemia (*MLL*) gene at chromosome band 11q23 is commonly involved in reciprocal translocations that are detected in acute leukemias. Evidence suggests that the resulting *MLL* fusion genes contribute to leukemogenesis. *AF9* is a common *MLL* fusion partner in acute myeloid leukemia. The *AF9* protein functions as a transcriptional activator in artificial reporter gene assays and a structurally related protein in yeast, *ANC1/TFG3*, is a component of the *SWI/SNF* complex. Apart from these observations, little is known about the biologic function of *AF9* in mammals. We have found that a recently described transcriptional repressor, *BCL-6* corepressor (*BCoR*), interacts with the carboxy-terminus of *AF9*. The interaction of *AF9* with *BCoR* has been confirmed by independent *in vitro* and *in vivo* protein-binding studies. The *BCoR* gene is expressed as several alternatively spliced transcripts. *AF9* only binds *BCoR* isoforms that contain a unique 34 aa sequence located in the mid-portion of the protein. In artificial reporter gene assays, a *BCoR* isoform that binds *AF9* efficiently suppresses *AF9* transcriptional activity, while a nonbinding isoform does not. These results indicate that different isoforms of *BCoR* have unique biologic properties and that cell function may be partly determined by the different isoforms that are present within the cell.

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Introduction

Recurrent chromosomal translocations involving specific loci are a hallmark of leukemia. Abundant data suggest that translocated genes actively participate in leukemic transformation and are not merely markers of an unstable genome. Among the more commonly

rearranged sites detected in acute leukemias is chromosome band 11q23. Translocations involving 11q23 are found in 5% of acute myelogenous leukemias and 10% of acute lymphoblastic leukemias (Rowley, 1993; Downing and Look, 1996). The mixed lineage leukemia (*MLL*) gene, also known as human trithorax, has been independently identified and cloned from the 11q23 breakpoint of acute leukemias. *MLL* is a ubiquitously expressed 3969 amino-acid (aa) nuclear protein whose function is incompletely understood. *MLL* shares sequence homology with the *Drosophila* trithorax (*Trx*) gene product in at least two regions, a centrally located zinc-finger domain and a carboxy-[C]-terminal SET domain. In *Drosophila*, *Trx* functions to maintain the expression of homeotic genes involved in body pattern development. Studies in mice have shown that *MLL* has similar functions in regulating *Hox* gene expression and segmental identity in mammals (Djabali *et al.*, 1992; Gu *et al.*, 1992; Tkachuk *et al.*, 1992; Yu *et al.*, 1995).

Analysis of acute leukemias with 11q23 translocations has shown that *MLL* is fused to as many as 30 different chromosomal regions to generate chimeric genes with 'in-frame' coding sequences. At least 15 of the *MLL* fusion partners have been cloned, and fusion gene transcripts and protein products have been consistently detected in patient specimens and leukemia cell lines. Significantly, neither truncated versions of *MLL* nor out-of-frame fusions with translocation partners have been described in leukemias with 11q23 breakpoints. These observations suggest that the chimeric *MLL* proteins, and not simply the amino-[N]-terminus of *MLL*, act as leukemogenic factors (reviewed in Ayton and Cleary, 2001).

Reciprocal chromosome translocations involving 11q23 are remarkable for their heterogeneity despite their association with a specific disease. 11q23 leukemias have a distinct gene expression profile that reflects an origin from hematopoietic progenitor cells and that clearly distinguishes the 11q23 leukemias from either ALL or AML that lack this rearrangement. Furthermore, gene expression profiles of a panel of 11q23 leukemias including five different reciprocal translocations indicate that a unique subset of about 250 genes is consistently expressed or repressed (Armstrong *et al.*, 2002). The relation between the numerous and

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seemingly unrelated *MLL* fusion partners and the development of a biologically distinct type of acute leukemia remains enigmatic.

Is there a common characteristic that defines the *MLL* fusion partners? Many but not all of the known *MLL* translocation partners participate in the regulation of gene expression including CBP and p300. Five other *MLL* partners, namely AF9, ENL, AF4, AF10, and AF17 have transcriptional activity although their physiological functions are not fully understood. Evidence of shared structures or functions becomes more difficult to discern when one considers other known *MLL* fusion partners. Among them is ELL, an RNA polymerase II elongation factor; the septins SEPTIN6, CDCREL1, and MSF; and gephyrin, a glycine receptor-associated neuronal protein (Shilatifard *et al.*, 1996; Ida *et al.*, 1997; Sobulo *et al.*, 1997; Ayton and Cleary, 2001; Kuwada *et al.*, 2001; Ono *et al.*, 2002).

To begin to address the role of chimeric *MLL* proteins in leukemogenesis, we have focused on one of the most commonly encountered fusion partners, AF9. Chimeric *MLL*-AF9 proteins are detected in acute leukemias with t(9;11)(p22;q23) (Joh *et al.*, 1996). Moreover, *MLL*-AF9 knockin mice have been shown to develop leukemia that is phenotypically similar to naturally occurring acute leukemias in humans (Corral *et al.*, 1996). Both AF9 and another *MLL* fusion partner, ENL, share sequence similarity and function as transcriptional activators when targeted to a reporter gene promoter (Rubnitz *et al.*, 1994). The transactivation domain of AF9 is contained within the C-terminus and it is this portion of the protein that is fused to *MLL* in t(9;11)(p22;q23) leukemias. However, it remains to be demonstrated whether AF9 actually contributes to the transcriptional machinery in mammals.

Here we show that the C-terminus of AF9 physically interacts with the mouse BCL-6 corepressor molecule (mBCoR). Human BCoR was originally identified as a protein that interacts with the BCL-6 oncoprotein and augments its activity as a transcriptional repressor (Huynh *et al.*, 2000). Mouse BCoR is expressed as at least four splice variants, the largest of which is predicted to produce a protein of 1759 amino-acids. We find that only two of the four corresponding mBCoR isoforms, each of which contains a 34 amino-acid sequence absent in the other isoforms are capable of binding AF9. Although the different isoforms function in transcriptional repression, they vary in their ability to interact with AF9 and form distinct subnuclear complexes. Thus, AF9 may be recruited to a transcriptional repressor complex by specific isoforms of mBCoR. Alternative splicing of mBCoR may serve as a mechanism to regulate the activity of AF9.

Results

Isolation of mouse BCoR by two-hybrid selection of AF9 interacting proteins

When fused to *MLL*, the carboxy-terminus of the AF9 protein and its homolog, ENL, is capable of experi-

mental transformation of hematopoietic cells (Corral *et al.*, 1996; Slany *et al.*, 1998). In a previous yeast two-hybrid selection for proteins that bind the Polycomb protein MPC3, we identified the C-terminus of AF9 as an MPC3 binding partner (Hemenway *et al.*, 2001). We hypothesized that, in addition to MPC3, other proteins physically interact with the AF9 C-terminus and that these proteins may play a role in leukemogenesis. Therefore, we undertook a yeast two-hybrid selection to identify AF9 C-terminal binding proteins.

A yeast two-hybrid 'bait' comprised of the Gal4 DNA-binding domain fused to the C-terminal 94 aa of mouse AF9 was used to select proteins expressed from a mouse 11-day embryo cDNA library. In all, 18 Ade⁺ His⁺ colonies were selected from 1.0×10^6 transformed yeast cells. Library plasmid DNA was isolated from these colonies. Upon subsequent analysis, three cDNA clones were identified encoding proteins that interacted specifically with AF9 in yeast two-hybrid assays. MPC3 was not isolated indicating that the selection was not saturated. One of the three clones that was recovered, the subject of this report, contained a 1.0 kb cDNA fragment whose sequence was nearly identical to the human BCoR (Accession No. NM017745) (Huynh *et al.*, 2000). We designated the corresponding mouse gene mBCoR. The complete open reading frame and 3' untranslated region (UTR) of mBCoR was isolated from a mouse cDNA library by a combination of conventional PCR and RACE. The open reading frame encodes a 1759 aa protein that, excluding sequence gaps, is 89% identical to the amino-acid sequence of human BCoR (Figure 1).

At least four splice variants of mouse BCoR are expressed

Patterns of BCoR gene expression in the mouse were examined by RT-PCR. Transcripts were detected by this method in nearly all tissues examined with relatively high levels found in liver, lung, and spleen (Figure 2a). These results are in general agreement with those of Huynh *et al.* (2000) for human BCoR.

Initial inspection of the RT-PCR product following agarose gel electrophoresis indicated that several PCR amplicons were present. Agarose gel electrophoresis was repeated overnight at low voltage. Under these conditions, four distinct PCR amplicons were visible (Figure 2b). The individual bands were excised from the gel, cloned, and sequenced. The largest amplicon was identical to the sequence found in the original yeast two-hybrid clone. The other amplicons matched the mouse BCoR sequence but contained in-frame deletions, suggesting that they were amplified from alternatively spliced transcripts. We designated the sequence corresponding to the largest amplicon splice variant a. The human BCoR cDNA sequence that has been characterized (Huynh *et al.*, 2000) corresponded to the third largest amplicon, and we have designated it splice variant c. Splice variant b was assigned to the second largest amplicon. The sequence of the smallest amplicon revealed it to be a combination of splice variants a and c (Figures 1 and 2c).

1 MLSATPLYGNVHSMNSERVRCMCGTSEDRKIPVNDGDASKARLELREETPLSHSVVDTSGAHRIDGLAALSMDRT 75
 76 GLIREGLRVPGNIVYSGLCGLGSEKGREATPSSLSGLGFSSERNPEMQFKPNTPETVEASAVSGKPNPNGFSAIYK 150
 151 TPPGIQKSAVVTAESLSLDRPASDKQSPNLNINGASYLRLPWNPNYMEGATPAIYPPFLDSPNKYSLNMYKALLPQQ 225
 226 SYGLAQLYSPVCTSGERFLYLPPIPHYVNPPISSLASPMRLSTPSASAAIPPLVHCSDKSLPWKMGVNPNGNVPD 300
 301 SHSYPHIQNSKQPRVTSKAVNSGLPGDTALLPSPRPSARVHLPTQPAAEITYSEFHKKHYPRISTSPSVTLTKP 375
 376 YMTANSEFSTSRLSNGKYPKALDGGDCAQSMGPHTRKTTVQDRKDDGGSPPLLEKQTVTKDVTDKPLDLSKVDA 450
 451 DASKGDHMKMMAPTVLVHSRAASGLVLSGSEIPKETLSPPGNGCSIYRSEIISTAPSSWVVPGPSNEENNGKSL 525
 526 SLKNKALDWAIQQORSSSCPRMGGTDAVVTVNSGVSSSSRPASASAPNANANADGKTSTRSSVDTTPSVIQHV 600
 601 GQPSSTPAKHGGSTSSKGAKANPEPSFKASENGLPPTSIFLSPNEAFRSPAIPYPRSYLPYAAPEGIALSPSLSLH 675
 676 GKGPVYPHPVLLPNSGLFPGHLAPKPLPYGLHTRSRPEFVTYQDALGLGMVHPMLVPHTPIEITKEEKPERRSR 750
 751 HERARYEDPTLRSRFSEMLEASSTKLHPEVPTDKNLKPNSSWNQGGKTGVKSDKLVYVDLLREEDTKTDAGAPKA 825
 826 GLVAENVGQDTEATKPSADPVIQQORREFISLREELGRITDFHESFTFKQASSQPVFSLGKDSGAAGTNKENLGVQ 900
 901 VATPFLLETALGSEGPVTFGKTQEDPKPFVCGAPPNMDVTPAYTKEGTDEAESNDGKVLKPKPSKLAKRIANSA 975
 976 GYVGDRLFVCVTTELYADSSQLSREQRALQMEGLQEDSILCLPAAAYCERAMMRFSELEMKEREGSHPATKDSVCK 1050
 1051 FSPADWERLKGNGQEKPKSVTLEEAIADQNDSECEYSTGNKHDLFEAPEDKDLVPEKYFLERPPVSEPPSDQGV 1125
 1126 VDTPHSPTLRLDRKRKLSGSDSTHTETAEEEAEDPLKAKRRRISKDDWPEREMTNSSSNHLEDPHCNELTNLKV 1200
 1201 IELTGLHPKQRHLLHLRERWEQQVSAAESKPGRQSRKEVAQVQPEVTSQGTNITEEKPGRKAEEKGNRGWSE 1275
 1276 ESLKSCDNEQGLPVLSGSPPMKLSSTNASGKKQTQPSCTPASRLPAKQKIKESQKTDVLTCTGEDEDCQAASPL 1350
 1351 QKYTDNIEKPSGKRLCKTKHLLPQESRRSLQITGDYVENTDTKMTVRRFRKRPEPSSDYDLSPPAQEPKPFGR 1425
 1426 LQQLLPATQATQLPRNSNPQETTQSRMPPEARRLIVNKNAGETLLQRAARLGYYEVVLYCLENKVCVNVHRDNA 1500
 1501 GYCALHEACARLNIVRHLLLEYGADVNCSAQDGRPLHDAVENDHLEIVRLLLSYGADPTLATYSGRITIMKMT 1575
 1576 SELMEKFLTDYLNLDLQGRSEDDTSGAWEFYGSVCEPDESVDYDLANPPGPEDPDEEEDTYSDLFEFEFAESSL 1650
 1651 LPCYNIQVSVAGQPRNWLSDVLLKLMSSRIFRSNFPNLEIVTIAEAEFYRQVSTSLLFSCPKDLEAFNPESK 1725
 1726 ELLDLVEFTNELQTLGSSVEWLHPSDTGHENYW• 1759

Figure 1 The deduced amino-acid sequence of mouse BCoR. Amino-acid sequences absent from specific isoforms are underlined. The GenBank accession numbers of alternatively spliced mouse BCoR cDNA sequences are AY161170–AY161173

PCR amplification of human leukocyte cDNA was performed with primers that flank the alternatively spliced sites of mouse BCoR. PCR yielded four distinct amplicons suggesting that the BCoR mRNA is also alternatively spliced in human cells (Figure 2d). Next, RNA isolated from the human T-cell leukemia cell line MOLT-4 was amplified by one-step RT-PCR with primers that flank the 3' alternatively spliced exon. As expected from the mouse BCoR cDNA sequence, the reaction produced two amplicons. The bands were removed from the gel, cloned, and analysed by DNA sequencing. DNA sequence of the smaller PCR product matched the human BCoR sequence precisely. However, the largest amplicon contained a 102 nucleotide sequence absent from the cDNA molecule originally described by Huynh *et al* (2000), but present in the mouse BCoR cDNA. The human cDNA FLJ3804 fis (Accession No. AK095360) also contains this 102 nucleotide sequence and is identical to the largest amplicon. The predicted amino-acid sequence encoded by these 102 nucleotides is 94% identical to the mouse sequence (Figure 2e). We conclude that alternative splicing of BCoR occurs in both mice and humans and that the sequences of the splice variants predict that homologous protein isoforms exist in both species.

In order to begin to determine whether specific splice variants are preferentially expressed in different cell types, neoplastic hematopoietic cells were analysed for the presence of transcripts containing or lacking the 102 nucleotide sequence. A human BCoR specific primer pair that flanks this sequence was used to amplify BCoR either by one-step RT-PCR of total RNA as described in 'Materials and methods'. Employing RT-PCR to test for the presence of specific BCoR transcripts, pro-B cell acute leukemia (RS4;11), myelomonocytic acute leukemia (MV-4-11), T-cell acute leukemia (MOLT-4), and

B-cell lymphoma (Raji) cell lines all express alternatively spliced BCoR mRNAs (Figure 3a).

The monocytic leukemia cell line THP-1 is known to express an *MLL-*AF9** fusion gene (Odero *et al.*, 2000). As the *MLL-*AF9** chimeric protein is a potential target of BCoR, BCoR transcripts in THP-1 cells were compared to other leukemia cell lines. The presence of alternatively spliced transcripts is also apparent in THP-1 cells, although BCoR may be expressed at a lower level in this cell line compared to other acute leukemia cell lines (Figure 3b).

AF9 binds to the mid-portion of the BCoR molecule and only to specific isoforms of mBCoR

The cDNA clone originally isolated by two-hybrid selection encodes aa 921–1272 of mBCoR. The AF9-binding domain was further mapped by testing fragments of mBCoR in a yeast two-hybrid assay. The portion of mBCoR from aa 1127 to 1251 (mBCoR_{1127–1251}) produces a strong two-hybrid signal; however, further C-terminal truncation to aa 1221 abolishes the signal.

The minimal AF9-binding fragment, mBCoR_{1127–1251}, contains a 34 aa sequence that is absent from isoform c as a consequence of alternative splicing. To determine whether this 34 aa sequence is important for AF9 binding, mBCoR isoforms were tested for their ability to bind AF9 in a two-hybrid assay using hybrid molecules that corresponded roughly in size to the original mBCoR clone (aa 921–1272 vs 938–1272). Isoforms a and b display a robust two-hybrid signal, while isoform c fails to interact with AF9 under the assay conditions (Figure 4). These results indicate that the 34 aa sequence absent from isoform c makes an essential contribution to the structure of the AF9-binding interface of mBCoR.

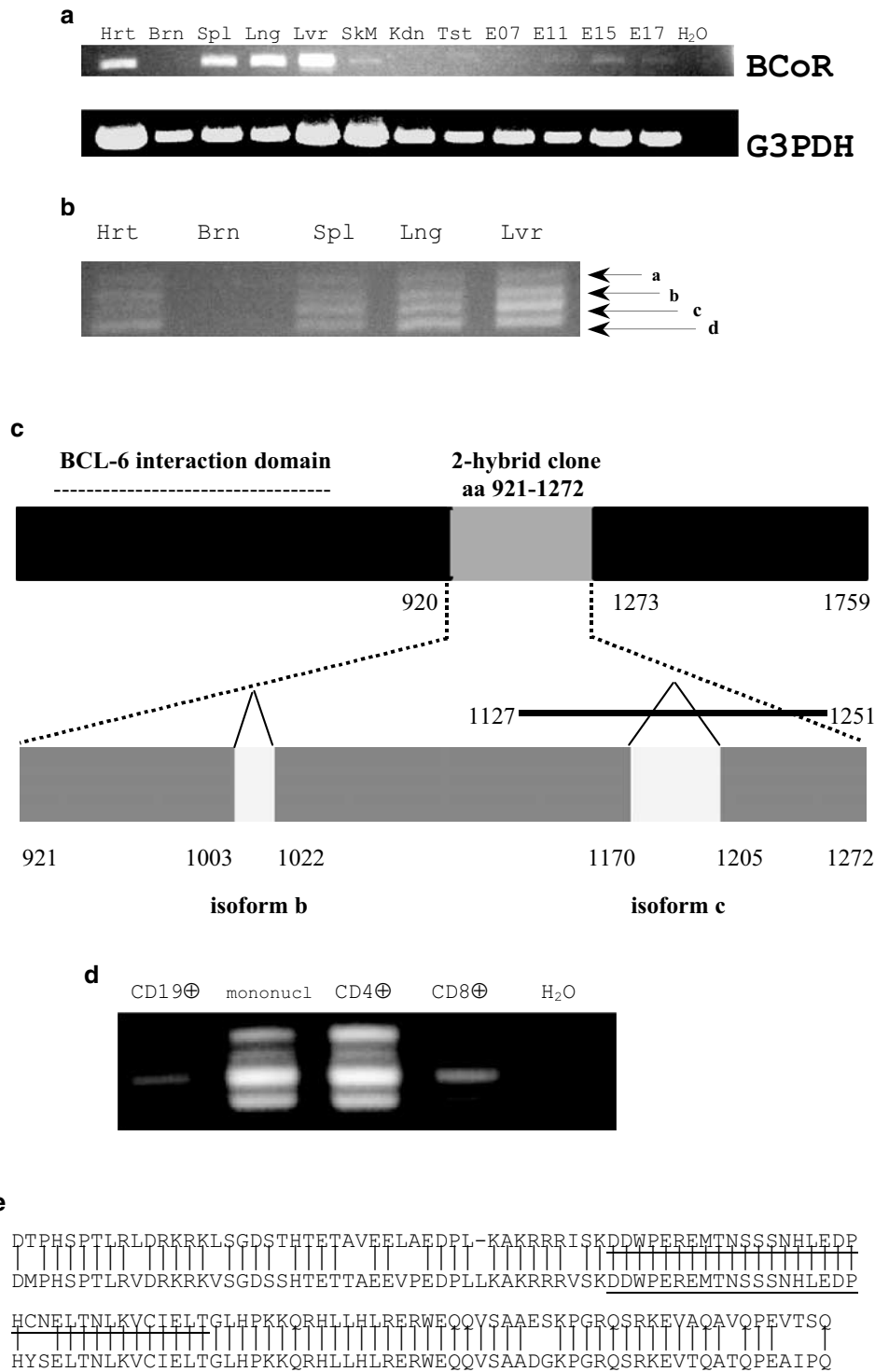


Figure 2 Gene expression and splice variants of BCoR. **(a)** mBCoR was amplified by PCR from cDNA obtained from mouse tissues: Hrt = heart; Brn = brain; Spl = spleen; Lng = lung; Lvr = liver; SkM = skeletal muscle; Kdn = kidney; Tst = testes. The PCR products were then analysed by agarose gel electrophoresis. G3PDH is included as a control. **(b)** Higher resolution separation of the PCR products reveals four distinct bands (a–d) corresponding to alternatively spliced transcripts. **(c)** A diagrammatic representation of the mBCoR isoforms and functional domains. Amino-acids 1127–1251 (solid bar) encompass the minimal AF9-binding domain. **(d)** Human BCoR is also expressed as multiple splice variants. BCoR was amplified from cDNA prepared from human leukocytes and analysed by agarose gel electrophoresis. The PCR primer pair was as in (a) but modified to match the human sequence. **(e)** The deduced mouse (top) and human (bottom) BCoR amino-acid sequence corresponding to residues 1127–1251 of mouse BCoR

Yeast two-hybrid data were independently tested by *in vitro* assays of AF9 binding to mBCoR affinity matrices. Recombinant proteins consisting of GST fused to aa 925–1759 of mBCoR-a and aa 925–1725 of mBCoR-c were isolated from *Escherichia coli* and immobilized on a glutathione matrix. Biotinylated AF9, synthesized by *in vitro* transcription and translation, was applied to the matrix. The matrix was washed extensively and was then analysed for the retention of biotinylated AF9 by SDS-PAGE and Western blot. Figure 5a demonstrates that AF9 is retained by an affinity matrix containing GST-BCoR-a, but is not retained by GST-BCoR-c or by GST alone. The outcome of this experiment is consistent with the results

of the yeast two-hybrid assay, and emphasizes that mBCoR isoforms are distinguishable based on their ability to bind AF9.

mBCoR isoform a associates with AF9 in vivo

Results of yeast two-hybrid assays as well as GST ‘pull-down’ experiments indicate that the -a isoform of mBCoR binds AF9. The distribution pattern of fluorescent protein-tagged mBCoR and AF9 was examined to test whether mBCoR-a associates with AF9 *in vivo*. Employing digital deconvolution fluorescence microscopy, red fluorescent protein-tagged AF9 is found within distinctive speckles within the nuclei of cells. This observation is consistent with others studies of AF9 (Erfruth *et al.*, 2000, submitted; Hemenway *et al.*, 2001). GFP-tagged mBCoR also congregates within small subnuclear structures (Figure 6). Importantly, the distribution of mBCoR-a coincides precisely with AF9. Analysis of multiple cell nuclei revealed similar patterns in which >90% of the mBCoR and AF9 foci match. This indicates that the two proteins are in close proximity or directly contacting one another (Figure 6, left-hand column).

In contrast to isoform a, the -c isoform of mBCoR does not bind AF9 in yeast two-hybrid assays or in a GST affinity matrix. Consistent with these findings, GFP-tagged mBCoR-c does not colocalize with AF9, but illuminates a distinct array of subnuclear speckles in all nuclei examined (Figure 6, right-hand column). Thus, under these experimental conditions, mBCoR isoforms a and -c occupy different subnuclear domains. The distribution of the isoforms appears to be determined by the ability of the protein to bind AF9.

The association of mBCoR-a and AF9 *in vivo* was further analysed by attempting to co-precipitate epitope-tagged versions of the proteins. A mammalian expression vectors was produced that encodes the entire AF9 protein sequence fused to an N-terminal triplicated FLAG epitope. Another vector was employed that encodes the HA epitope fused to the N-terminus of

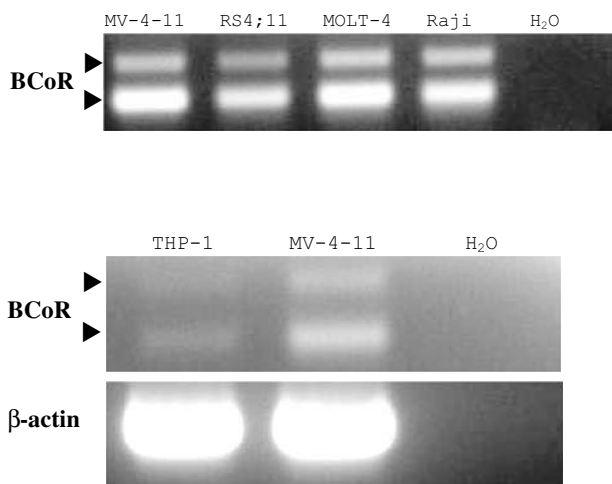


Figure 3 BCoR is alternatively spliced in human leukemia cell lines. RT-PCR was performed with total RNA isolated from leukemia cell lines MV-4-11 (myelomonocytic), RS4;11 (pro-B cell), MOLT-4 (T-cell), Raji (B-cell), and THP-1 (monocytic with t(9;11)(p22;q23)). PCR primers flank the alternatively spliced 3' exon. Ethidium bromide-stained agarose gels reveal two amplicons as predicted from the alternatively spliced mouse sequences. The MOLT-4 RT-PCR amplicons were cloned and sequenced to verify the human BCoR sequence

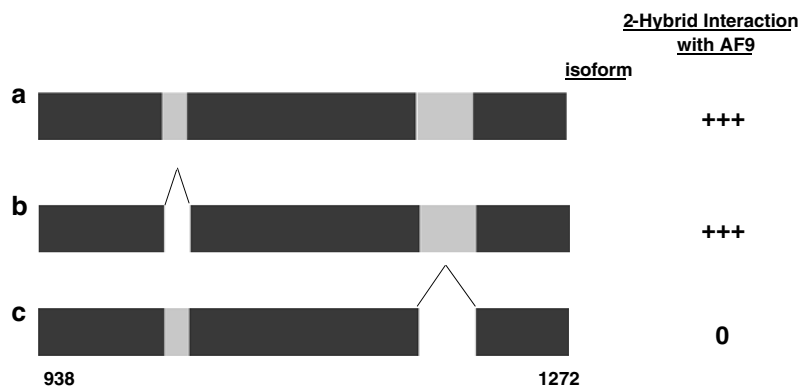


Figure 4 mBCoR isoforms a and b interact with AF9 in yeast two-hybrid assays; isoform c does not. Portions of mBCoR isoforms fused to the Gal4-binding domain were tested for their ability to interact with the C-terminus of AF9 fused to the Gal4 activation domain. Interaction of the two hybrid molecules was measured by growth of yeast strain PJ69-4A on adenine-deficient medium. Growth of the yeast colonies on adenine-deficient medium relative to adenine-containing medium is reported from +++ to 0.

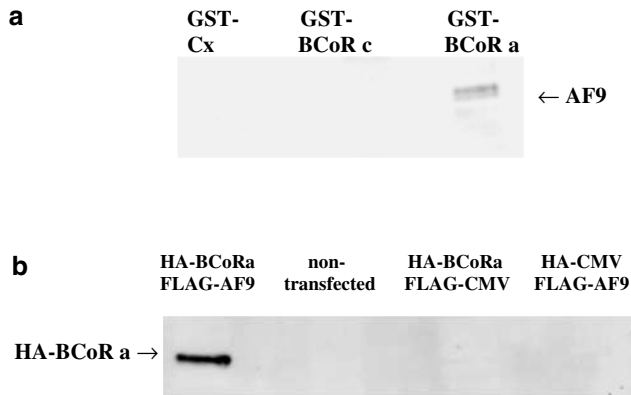


Figure 5 AF9 binds BCoR isoform a *in vitro* and *in vivo*. **(a)** *GST affinity matrix*: Recombinant GST fusion proteins were produced in *E. coli* and immobilized on a glutathione matrix. After extensive washing, *in vitro* transcribed/translated biotinylated AF9 is retained by GST-BCoR isoform a (aa 925–1759) but not by isoform c (aa 925–1725) or GST alone. Following SDS–PAGE, biotinylated AF9 is revealed by colorimetric detection of a streptavidin–alkaline phosphatase conjugate. A second SDS–PAGE was performed in parallel. The gel was stained with Coomassie stain to confirm expression and retention of the recombinant GST proteins. **(b)** *Immunoprecipitation*: NIH 3T3 cells were transfected with plasmids expressing FLAG-tagged AF9, HA-tagged mBCoR isoform a (aa 921–1272), or control plasmid vectors as indicated. Cell lysates were prepared and immune complexes were formed with anti-FLAG antibody M2. Immune complexes were recovered with Protein-A agarose beads and analysed by SDS–PAGE followed by Western blot with anti-HA antibodies. FLAG-AF9 immune complexes contain HA-BCoR isoform a. HA-BCoR-a is not recovered in the absence of FLAG-AF9

amino-acids 921–1272 of mBCoR-a. NIH 3T3 cells were transfected with these plasmid vectors and total cellular protein was isolated. When immune complexes are formed with anti-FLAG antibodies, HA-tagged BCoR-a co-precipitates with FLAG-AF9 (Figure 5b). Collectively, the *in vitro* and *in vivo* data indicate that AF9 and the -a isoform of mBCoR are found together within small structurally stable domains in the nucleus.

BCoR-a and -c cooperate with BCL-6 to repress gene transcription

BCoR was originally identified as a binding partner of the BCL-6 transcriptional repressor. The BCoR molecule that was first described, corresponding to isoform c, cooperates with BCL-6 to repress transcription of a BCL-6-responsive reporter gene. In addition, when expressed alone as a Gal4-BCoR chimera, BCoR represses a Gal4-responsive reporter gene (Huynh *et al.*, 2000). These findings suggest that BCoR interferes with transcription when targeted to a promoter, either through binding a sequence-specific transcriptional repressor (BCL-6) or by artificial fusion to another sequence-specific DNA-binding protein (Gal4).

We wished to test whether other isoforms of mBCoR differ in their ability to repress transcription. Reporter gene assays were performed in NIH 3T3 cells using different combinations of mBCoR isoforms and BCL-6

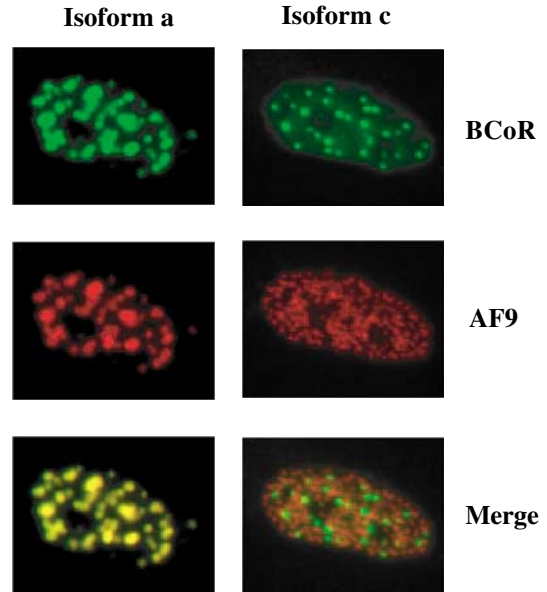


Figure 6 mBCoR-a colocalizes with AF9, while mBCoR-c does not. GFP was fused to the N-terminus of mBCoR isoforms. Red fluorescent protein was fused to the N-terminus of AF9. NIH 3T3 cells were transfected with expression vectors encoding these proteins and were then observed using digital deconvolution fluorescence microscopy. The merged images are pseudocolored yellow to depict colocalization of green and red fluorescence. The cell nucleus is included in each image; no fluorescence was observed in the cytoplasm

in the presence of a luciferase reporter plasmid containing BCL-6-binding sites. In the absence of exogenously expressed BCL-6, mBCoR-a and -c, respectively, suppress luciferase activity to 74 and 72% of baseline. Expressed alone, BCL-6 reduces luciferase activity to 60% of baseline. In contrast, coexpression of BCL-6 and mBCoR-a suppresses luciferase activity to 32% of baseline, while BCL-6 and mBCoR-c coexpression suppresses activity to 21% (Figure 7a). Thus, a synergistic repression of the reporter gene is observed when either isoform of BCoR is expressed with BCL-6. Based on these results, we conclude that both isoforms of BCoR function as BCL-6 corepressor molecules.

mBCoR isoform a but not isoform c represses transactivation by AF9

The BCL-6-binding site has been mapped to the N-terminal half of BCoR (Huynh *et al.*, 2000). This region of the molecule is not affected by alternative splicing, thus it is not unexpected that isoforms -a and -c may each functionally cooperate with BCL-6. However, our experiments also reveal unique protein interactions and suggest that mBCoR-a and -c might differ in their ability to modulate the transcriptional activity of AF9. Consistent with experiments employing the AF9 homolog ENL (Slany *et al.*, 1998), we found that a Gal4 fusion protein containing the C-terminal 94 aa of AF9 activates luciferase expression from a minimal promoter containing Gal4-binding sites. In NIH 3T3 cells containing the

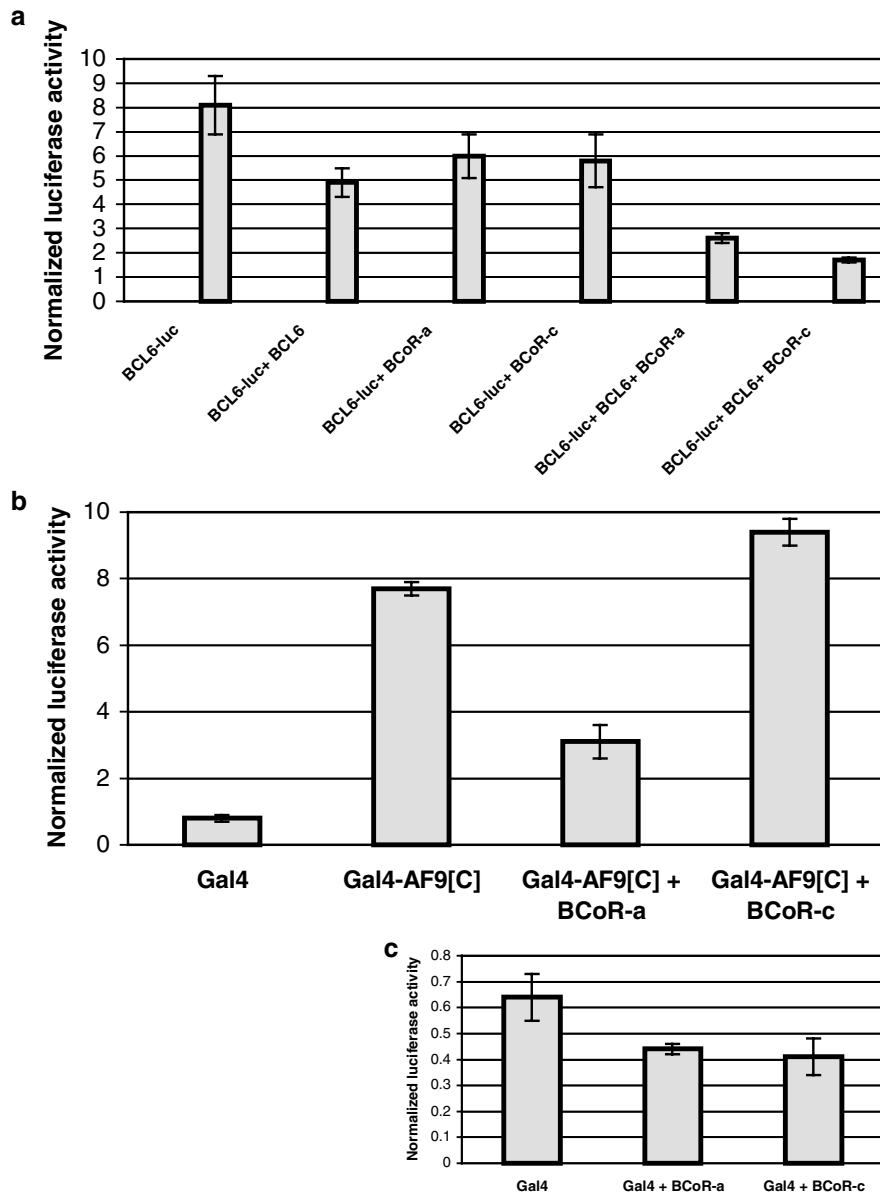
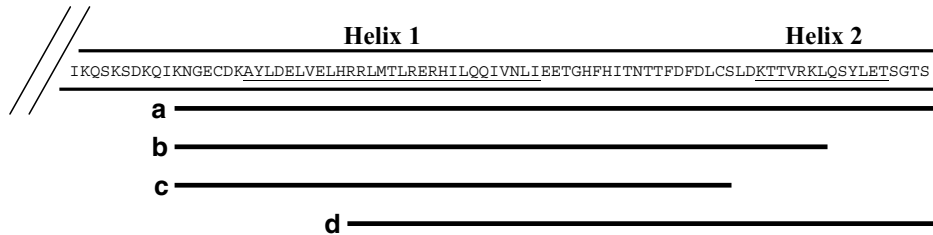


Figure 7 (a) mBCoR-a and -c cooperate with BCL-6 as transcriptional repressors. NIH 3T3 cells were transiently transfected with 200 ng of BCL-6-responsive luciferase reporter (pBCL6rep-luc), 200 ng of a BCL-6 expression plasmid (pcDNA3.BCL6), 200 ng of a BCoR expression plasmid (pcDNA3.BCoR-a or pcDNA3.BCoR-c), and 200 ng of a *Renilla* luciferase expression plasmid. pcDNA3 was substituted as appropriate to achieve a quantity of 800 ng of DNA for all transfections. Normalized luciferase activity is the ratio of firefly luciferase activity to the constitutively expressed *Renilla* luciferase activity. Data are presented as the value derived from four independently transfected populations of cells. (b) mBCoR-a represses the transcriptional activity of AF9, but mBCoR-c does not. NIH 3T3 cells were transiently transfected with 400 ng of Gal4 responsive luciferase reporter (pGal4act-luc), 400 ng of a Gal4-binding domain expression plasmid or the same plasmid expressing Gal4 fused to the C-terminus of AF9 (pBind or pBindAF9 (This plasmid also encodes *Renilla* luciferase for data normalization.)), and 400 ng of a BCoR expression plasmid (pcDNA3.BCoR-a or pcDNA3.BCoR-c). pcDNA3 was substituted as appropriate to achieve a quantity of 1.2 μ g of DNA for all transfections. Luciferase activity was normalized as described in (a) above. Data are presented as the mean value derived from four independently transfected populations of cells

Gal4-responsive luciferase reporter gene, Gal4-AF9 stimulates luciferase activity 9.6-fold (Figure 7b). We reasoned that mBCoR-a, by binding the C-terminus of AF9, would suppress luciferase activity. As expected, cotransfection of an mBCoR-a expression vector suppresses AF9-stimulated luciferase activity to 40% of the baseline value. In contrast, cotransfection of an

mBCoR-c expression vector does not suppress luciferase activity mediated by AF9 transactivation (Figure 7b). Expression of either mBCoR-a or -c suppresses basal luciferase activity modestly; however, no significant difference in suppression of basal reporter gene activity by mBCoR-a is observed when compared to mBCoR-c (Figure 7c). Importantly, significant and specific

**Two-hybrid interactions:**

- a = mBCoR-a₉₂₁₋₁₂₇₂, MPc3₂₀₂₋₃₆₂, mFMR2₇₂₈₋₈₁₄: all positive
 b = mFMR2₇₂₈₋₈₁₄: positive; mBCoR-a₉₂₁₋₁₂₇₂, MPc3₂₀₂₋₃₆₂: negative
 c = all negative
 d = all negative

Figure 8 Protein interactions with the C-terminus of AF9. The amino-acid sequence of the AF9 C-terminus is depicted and the predicted α helices are underlined. Portions of the AF9 C-terminus corresponding to the segments labeled a, b, c, and d were expressed as chimeric proteins in a yeast two-hybrid assay. These fragments of the AF9 protein were tested for their ability to interact with mouse BCoR-a, Pc3, and FMR2 (subscripts indicate the amino-acid positions included in the respective chimeric two-hybrid proteins). mBCoR-a, MPc3, and mFMR2 all interact with the AF9 C-terminus that encompasses both α helices. mFMR2 is unique in its ability to bind a portion of AF9 that is partially truncated at the second helix

differences in repression of the transactivation domain of AF9 are observed for isoforms a and -c under these assay conditions. These differences correlate with the ability of the isoforms to bind AF9.

FMR2 and BCoR-a bind distinct regions of the AF9 C-terminus

In addition to mBCoR-a, the initial yeast two-hybrid selection (described above) identified FMR2 as an AF9 interacting protein (Erfruth *et al.*, 2000, submitted). Mutations in *FMR2* are responsible for some cases of the Fragile X mental retardation syndrome. Furthermore, FMR2 is a homolog of two other MLL fusion partners, AF4 and AF5 (Gecz *et al.*, 1996; Gu *et al.*, 1996). When expressed in a yeast two-hybrid assay, amino-acids 728–814 of mouse FMR2 interact with the C-terminus of AF9. We wished to test whether mBCoR-a and FMR2 can simultaneously bind AF9 or whether binding is mutually exclusive. FMR2_{728–814} and mBCoR_{921–1272} were expressed as yeast two-hybrid proteins in strain PJ69-4A. Under ordinary assay conditions, these proteins do not interact. However, when full-length AF9 is simultaneously expressed from the yeast expression vector pBEVY, FMR2_{728–814} and mBCoR_{921–1272} generate a strong positive ‘three-hybrid’ signal as measured by adenine prototrophy (Table 1). We interpret these results to indicate that FMR2 and mBCoR do not directly associate. Rather, through simultaneous binding to AF9, FMR2_{728–814} and mBCoR_{921–1272} form a multiprotein complex that can be detected in a yeast three-hybrid assay. Thus, binding of mBCoR-a and FMR2 to AF9 is not a mutually exclusive event, but can occur simultaneously under these experimental conditions.

We previously reported that the Polycomb protein MPc3 binds the C-terminus of AF9 (Hemenway *et al.*, 2001). Using the approach described above, we tested

Table 1 FMR2, AF9, and mBCoR-a form a stable ternary complex in a yeast ‘three-hybrid’ assay

<i>Gal4BD</i>	<i>AF9</i>	<i>Gal4AD</i>	<i>Ade⁺</i>
FMR2 _{728–814}	(–)	mBCoRa _{921–1272}	No
FMR2 _{728–814}	(+)	mBCoRa _{921–1272}	Yes
Empty	(+)	mBCoRa _{921–1272}	No
FMR2 _{728–814}	(+)	Empty	No
mBCoRa _{921–1272}	(–)	FMR2 _{728–814}	No
mBCoRa _{921–1272}	(+)	FMR2 _{728–814}	Yes
Empty	(+)	FMR2 _{728–814}	No
mBCoRa _{921–1272}	(+)	Empty	No
mBCoRa _{921–1272}	(+)	MPc3 _{202–362}	No
MPc3 _{202–362}	(+)	mBCoRa _{921–1272}	No

Portions of the FMR2, mBCoR-a, and MPc3 molecules were expressed as chimeras fused to the Gal4 DNA-binding domain or to the transactivation domain in yeast strain PJ69-4A. Full-length AF9 was expressed from a yeast expression vector. Transformed yeast cells were tested for adenine prototrophy on synthetic medium lacking adenine (indicated as Ade⁺). In this yeast strain, adenine prototrophy indicates activation of the reporter gene and a stable interaction between the two chimeric molecules. Note that coexpression of AF9 is required for the chimeric molecules to interact.

whether mBCoR-a and MPc3 simultaneously bind AF9. Amino-acids 202–362 of MPc3 were expressed with mBCoR_{921–1272} as yeast two-hybrid proteins in the presence or absence of exogenously expressed AF9. In contrast to FMR2, MPc3 does not appear to form a stable complex with AF9 and mBCoR-a (Table 1).

Mapping studies of the C-terminus of AF9 reveal slightly different binding requirements of mBCoR-a, FMR2, and MPc3. The C-terminus of AF9 is predicted to contain two α helices. Based on results of experiments performed with the AF9 homolog ENL, both α helices are required for neoplastic transformation (Slany *et al.*, 1998). Portions of the AF9 C-terminus were tested in yeast two-hybrid assays for mBCoR-a, FMR2, and MPc3 binding (Figure 8). Both mBCoR-a and MPc3 require the entire AF9 C-terminus containing both α

helices for binding. While FMR2 requires the N-proximal α helix (helix 1) for binding, partial truncation of the second helix does not abolish its ability to bind AF9. Although none of the three AF9-binding proteins tolerate complete deletions of the α helices, FMR2 is able to bind AF9 despite deletion of a portion of helix 2. The ability of mBCoR-a and FMR2 to occupy simultaneously sites within the AF9 C-terminus is consistent with the differing binding requirements of mBCoR-a and FMR2.

Discussion

Among the numerous MLL fusion partners, *AF9* is one of the most commonly rearranged genes encountered in patients with acute leukemia. The t(9;11)(p22;q23) translocation that results in the fusion of *MLL* to *AF9* is particularly common in secondary leukemias resulting from prior exposure to epipodophyllotoxins (Pui *et al.*, 1989; Smith *et al.*, 1994). The N- and C-termini of AF9 are highly conserved in another MLL fusion partner, ENL, and in a budding yeast protein, ANC1/TFG3 (Welch and Drubin, 1994; Cairns *et al.*, 1996). With regard to leukemogenesis, it is the C-terminus of AF9 and ENL that are of significance. Analysis of the breakpoint region of t(9;11)(p22;q23) leukemias indicates that the C-terminal 91 amino-acids of AF9 render the MLL–AF9 chimera leukemogenic (Nakamura *et al.*, 1993). Similarly, in an experimental model of MLL–ENL leukemic transformation, it has been demonstrated that the C-terminal 84 amino-acids of ENL are sufficient for immortalization of primary hematopoietic cells. The C-termini of AF9 and ENL are predicted to form two α helices and deletion of either of the helices from ENL abolishes the ability of MLL–ENL chimeras to immortalize hematopoietic cells (Slany *et al.*, 1998).

The importance of the C-terminus of AF9 and ENL in 11q23 leukemias is supported by experimental evidence. However, the function of these proteins in normal and diseased cells is not yet clear. Targeted disruption of the *AF9* and *ENL* loci in mice has recently been accomplished and the lethal phenotype of nullizygous embryos (*ENL*) or newborn pups (*AF9*) indicates that each gene plays an essential role in embryonic development (Collins *et al.*, 2002; Doty *et al.*, 2002). Artificial reporter gene assays indicate that the C-termini of the proteins promote transcription but, apart from these assays, there is little direct evidence that AF9 and ENL are *bona fide* transcription factors (Rubnitz *et al.*, 1994; this report). In contrast, the *Saccharomyces cerevisiae* protein ANC1/TFG3 is a component of TFIID and TFIIF complexes and is thus intimately linked to the transcription of genes by RNA polymerase II (Henry *et al.*, 1992, 1994). ANC1 has also been isolated from the yeast SWI/SNF complex suggesting that it also plays a role in transcriptional activation through chromatin remodeling (Cairns *et al.*, 1996). Moreover, there is evidence that MLL as well as MLL–ENL can be found in a multiprotein complex that includes GADD34 and a component of the human SWI/

SNF complex hSNF5 (Adler *et al.*, 1999). Based on these findings, there is accumulating support for the proposal that AF9 and ENL, like yeast ANC1, promote transcription as elements of a SWI/SNF chromatin remodeling complex, but it must be emphasized that the evidence is indirect and far from conclusive.

More recently, a Polycomb group (PcG) protein, Pc3, has been found to associate with the C-termini of both AF9 and ENL (García-Cuellar *et al.*, 2001; Hemenway *et al.*, 2001). PcG proteins assemble as large multiprotein complexes that silence gene expression and antagonize the function of Trithorax proteins (reviewed in Mahmoudi and Verrijzer, 2001). PcG complexes are heterogeneous and can be distinguished based on biochemical activity. An EED-containing PcG complex interacts with histone deacetylase and methylates histone H3 at lysine 27, while the PRC1 complex blocks SWI/SNF-mediated chromatin remodeling (Shao *et al.*, 1999; van der Vlag and Otte, 1999; Cao *et al.*, 2002). Despite differing mechanisms, the activities of the PcG complexes are consistent with their role in gene silencing.

This report demonstrates that the C-terminus of AF9 interacts with another repressor of gene transcription, BCoR. BCoR associates with HDACs, and like Pc3 it interacts with other proteins through multiple independent protein interaction domains (Huynh *et al.*, 2000). In addition, only some isoforms of BCoR bind AF9. This situation is reminiscent of Pc3 and its homologs. Pc3 is unique in its ability to bind AF9 when compared to its Pc homolog, M33 (Hemenway *et al.*, 2001). A potential role for both BCoR and Pc3 is to repress the transcriptional activity of AF9. The finding of multiple isoforms of BCoR as well as homologs of Pc3 introduces the possibility of tightly controlled AF9-mediated transcription based on differential splicing of AF9 and preferential expression of individual Pc homologs. This proposal is speculative, however, particularly in the absence of an established function for AF9.

As BCoR binds the C-terminus of AF9, it seems likely that BCoR will also bind chimeric MLL–AF9 proteins. Slany (García-Cuellar *et al.*, 2001) has previously commented on the implications of recruiting a transcriptional repressor to a site that is normally occupied by wild-type MLL. DNA-binding AT-hook motifs are located at the N-terminus of MLL and are preserved in chimeric MLL proteins. It is postulated that MLL–AF9 (and other MLL chimeras) bind the same DNA regulatory regions that are controlled by wild-type MLL. If this is the case, MLL–AF9 could position BCoR-a (or Pc3) at a site that is normally maintained in a transcriptionally active state by wild-type MLL. MLL is clearly important in the regulation of hematopoiesis as indicated by the severe hematologic abnormalities in *MLL* null mice (Hess *et al.*, 1997). As transcriptional repressors, BCoR or Pc3 bound to MLL–AF9 might interfere with the expression of genes required for normal hematopoiesis. This model is limited by a lack of data demonstrating that BCoR and/or Pc3 actually bind chimeric MLL–AF9 protein and that *BCoR* splice variants a and b and/or *Pc3* are expressed in primary leukemias with t(9;11)(p22;q23) rearrangements.

Thus, it is equally plausible that neither BCoR-a, BCoR-b, nor Pc3 are found in t(9;11) acute leukemias and that leukemic transformation is due, in part, to tethering the AF9 transactivation domain to MLL under conditions in which the transcriptional activity of the chimeric protein is unchecked by specific repressors. In either of these circumstances, the stage may be set for dysregulated gene expression and subsequent leukemic transformation. Additional complexity is introduced by the finding that other proteins may bind the C-terminus of AF9. FMR2, a family member of the MLL fusion partners AF4, AF5, and LAF4 binds to AF9 through a domain that is highly conserved among the four family members. Ultimately, the activity of AF9 and MLL-AF9 may depend on the relative proportions of AF9-binding isoforms of BCoR, Pc3, and FMR2/AF4/AF5/LAF4 present in the cell. Testing of these models awaits development of antisera for the reliable detection of MLL-AF9 and BCoR proteins as well as a source of primary leukemia cells with t(9;11)(p22;q23).

Materials and methods

Yeast two-hybrid selection and assays

Yeast cells were grown and transformed with plasmid DNA using standard conditions and protocols. A yeast two-hybrid selection was performed with cDNA encoding aa 475–569 of mouse AF9 as 'bait' (Hemenway *et al.*, 2001). The cDNA was cloned in the yeast expression vector pGBT9. A mouse 11-day embryo cDNA library cloned in vector pGAD10 (Clontech) was selected in yeast strain PJ69-4A. Following incubation on synthetic medium lacking adenine, leucine, and tryptophan, Ade⁺ colonies were isolated and tested for histidine prototrophy on synthetic medium containing 1 mM 3-aminotriazole and lacking histidine, leucine, and tryptophan. Library DNA was isolated from Ade⁺ His⁺ colonies and the phenotype was confirmed by transforming yeast cells with the bait and the selected library 'prey' plasmid DNA. The specificity of the two-hybrid interaction was tested using a lamin bait vector.

Protein interaction domain mapping experiments were performed by testing yeast strain PJ69-4A for adenine and histidine prototrophy. Two-hybrid expression plasmids were produced by cloning restriction endonuclease fragments or PCR amplicons into either pGBT9 (bait) or pGAD424 (prey). Yeast three hybrid experiments were conducted as outlined above but with the addition of the yeast expression vector pBEVY-U with, or without, the complete AF9 open reading frame. Selective media were as described above but also lacked uracil.

Mouse BCoR cDNA cloning

The 3' end of mouse BCoR cDNA was amplified by RACE from an adaptor-ligated mouse 11-day embryo cDNA pool (Clontech). An adaptor-specific primer from the commercial kit was used with the BCoR-specific reverse primer: 5'-GCCATTGCCGACCAGAATGACAGTGAGAGA-3' to produce a 2.4 kb PCR amplicon. A cDNA molecule encoding the entire mouse BCoR open reading frame (5.5 kb) was obtained by PCR employing a forward primer derived from the human BCoR 5' UTR sequence: 5'-GCTTGGGGGAAAACCTTCAAAGAGCCGGATCGCAGG-3' and a reverse primer identical to the mouse sequence: 5'-GGGTGGGCGGGGTAGGTGGCTACCAG-3'. *Pfu* Turbo

polymerase (Stratagene) was used for the PCR amplification and the nucleotide sequences of the PCR amplicons were verified by automated sequence analysis.

Gene expression

Tissue-specific expression of BCoR was analysed by PCR amplification of a mouse multiple tissue first strand cDNA panel (Clontech) with the mouse BCoR-specific forward primer: 5'-CTAGCCGGGCAGCCCGCTGCAGGAG-3' and reverse primer: 5'-GTGGGCGGTGCCCTCCAAACATGGA-3'. The reaction yielded four distinct amplicons. The DNA bands were excised from agarose gels and cloned for sequence analysis.

Expression of human BCoR was determined by PCR amplification of a human leukocyte first strand cDNA panel (Clontech) with the human BCoR-specific forward primer: 5'-CTCAGCGGGTTACGTGGGTGACCGA-3' and reverse primer: 5'-CATCTGCTGCCGACACCTGCTGCTC-3'. PCR parameters were as follows: 94°C × 30 s; 30 cycles of 94°C × 5 s, 66°C × 2 min; 66°C × 10 min.

Total RNA for one step RT-PCR was extracted from human leukemia cell lines with the SV Total RNA Isolation System (Promega). Total RNA (100 ng) was reverse transcribed, then PCR amplified with Superscript Platinum RT-PCR mix (Invitrogen) in a single reaction. The human BCoR-specific forward primer was: 5'-GTGGACAGGAAACGCAAAGT-3' and the reverse primer was: 5'-GCTGCTCCCATCGTTCTCTA-3'. RT-PCR parameters were as follows: 48°C × 30 min; 94°C × 3 min; 32 cycles of 94°C × 15 s, 56°C × 30 s, 72 × 1 min; 72°C × 10 min.

GST protein-binding assay

GST-BCoR chimeric proteins were produced by cloning cDNA fragments encoding aa 925 to the C-terminus of BCoR into pGEX-5X-1 (Amersham-Pharmacia). Recombinant protein was isolated from *E. coli* strain BL21 following induction with 0.1 mM IPTG for 4 h at 30°C. Biotinylated AF9 protein was synthesized by coupled *in vitro* transcription/translation employing T7 polymerase and rabbit reticulocyte lysates according to the manufacturer's protocol (TNT Quick/Promega). A *Hind*III restriction fragment of human AF9 cDNA was cloned into pcDNA3 (Invitrogen) and was used as a template for the reaction. This fragment encodes the C-terminal 368 aa of AF9.

Binding assays were performed by mixing 100 μ l bacterial lysate with 50 μ l GSH-agarose (Amersham-Pharmacia) at 4°C for 1 h. The affinity matrix was washed five times with cold binding buffer (PBS pH = 7.4, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.5% NP-40, 1 mg/ml BSA). After washing, the matrix was resuspended in 200 μ l binding buffer. A measure of 10 μ l of the biotinylated protein mixture was added and the matrix was incubated at 4°C for an additional 1 h followed by six washes with cold binding buffer. Protein loading buffer (300 μ l) was added to the matrix, and the mixture was boiled for 5 min. A measure of 20 μ l of the supernatant was separated by SDS-12% polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. After probing the membrane with alkaline phosphatase-streptavidin conjugate, protein was detected by a colorimetric reaction with Western Blue substrate (Promega).

Protein immunoprecipitation

The cDNA encoding amino acids 921–1272 of mBCoR-a was removed from the original yeast two-hybrid isolate and cloned

in-frame into the pHA-CMV expression vector (Clontech). A FLAG epitope-tagged AF9 expression vector was produced as described (Hemenway *et al.*, 2001). 1.0×10^5 NIH-3T3 cells were grown overnight in 100 mm plates containing DMEM supplemented with L-glutamine, 1% nonessential amino-acids, 1% sodium pyruvate, 10% calf serum, and antibiotics. Cells were transfected with 1.5 μ g of each plasmid DNA (3.0 μ g total) using Lipofectamine reagent (Invitrogen). After 48 h, the cells were washed in PBS, collected from plates in 400 μ l of cold PBS plus protease inhibitor cocktail (Sigma) and lysed by sonication. Immune complexes were formed by adding 8 μ g of anti-FLAG M2 monoclonal antibody to the lysates followed by incubation with gentle agitation at 4°C. After 1 h, 50 μ l of protein-A sepharose (Amersham-Pharmacia) was added and incubated for 3 h. The slurry was washed twice with cold TBS pH = 7.5, 1 mM EDTA, 0.1% NP40 and then twice with cold TBS plus 0.1% NP40. After washing, 50 μ l of protein loading buffer was added and the sample was boiled. A measure of 20 μ l of the sample was separated by SDS-10% PAGE. The separated proteins were transferred to a nitrocellulose membrane. The membrane was then probed with anti-HA 12CA5 monoclonal antibody conjugated to horseradish peroxidase (Boehringer Mannheim) and visualized by ECL (Amersham-Pharmacia).

Fluorescence microscopy

The complete open reading frames of mBCoR-a and -c were cloned in-frame into the enhanced GFP expression vector pEGFP-C3 (Clontech). A red fluorescent protein-tagged AF9 expression vector was produced as described (Hemenway *et al.*, 2001). 4.0×10^4 NIH-3T3 cells were seeded in chamber slides containing DMEM supplemented with L-glutamine, 1% nonessential aminoacids, 1% sodium pyruvate, 10% calf serum, and antibiotics. Cells were grown overnight and then transfected with 1.0 μ g of each expression vector (2.0 μ g total DNA) using Lipofectamine 2000 reagent (Invitrogen). After 48 h, the cells were washed in PBS, fixed with 3% paraformaldehyde and observed by fluorescence microscopy. Digital deconvolution images were obtained with a Leica DM R \times A2 microscope equipped with a Cooke CCD Sencisam and Slidebook software (Intelligent Imaging Innovations).

Luciferase assays

Luciferase reporter plasmids were constructed as follows: (i) *pGal4act-luc* – a DNA fragment containing a $5 \times$ Gal4 DNA-

binding sequence adjacent to the adenovirus major late promoter was removed from the *KpnI/BgIII* sites of pG5-luc and cloned into the same sites of pGL3-enhancer (Promega). (ii) *pBCL6rep-luc* – the oligonucleotide 5'-TTGGTACCTTTTCCTAGAATGCACTGACATTTTCCTAGAATGCACTGACATTTTCCTAGAATGCTAGCC-3' and its complementary sequence were annealed to produce a double-strand DNA fragment containing BCL-6-binding sites. The DNA was digested with *KpnI* and *NheI* and cloned into these sites of the pGL3-control vector (Promega).

For the mammalian expression plasmids, (i) pcDNA3.BCoR-a and (ii) pcDNA3.BCoR-c, the complete mBCoR open reading frame was amplified by PCR using forward and reverse primers containing appropriate restriction endonuclease sites for cloning. cDNA was then cloned into pcDNA3 (Invitrogen). Alternatively spliced mBCoR cDNAs cloned in pST-Blue (Novagen) served as the template for the original PCR reactions. For: (iii) pcDNA3.BCL6, the complete BCL-6 open reading frame was amplified by PCR from mouse skeletal muscle cDNA and cloned into pGEM-T-EZ (Promega) and then into pcDNA3 (Invitrogen). For: (iv) pBind-AF9, a yeast two-hybrid clone encoding aa 475–569 of mouse AF9 in vector pGAD10 was cut with *BgIII* to yield a fragment containing the AF9 cDNA. The *BgIII* fragment was cloned in-frame into the *BamHI* site of pBIND (Promega). All cDNA molecules amplified by PCR were verified by sequence analysis.

NIH 3T3 cells were grown in medium consisting of DMEM supplemented with L-glutamine, 1% nonessential amino-acids, 1% sodium pyruvate, 10% calf serum, and antibiotics. Cells were transfected in 24-well plates with Lipofectamine 2000 reagent (Invitrogen). The amount of DNA used for transfection is specified in the figure legends. At 24 h after transfection, cells were washed in PBS. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega). Firefly (experimental) luciferase activity was normalized according to the corresponding *Renilla* (control) activity.

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