THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 14, Issue of April 3, pp. 7941–7948, 1998

Printed in U.S.A.

Members of the Meis1 and Pbx Homeodomain Protein Families Cooperatively Bind a cAMP-responsive Sequence (CRS1) from Bovine *CYP17**

(Received for publication, January 13, 1998)

Larry J. Bischof‡§, Norio Kagawa‡, John J. Moskow¶‖, Yoshitaka Takahashi‡, Akihiro Iwamatsu‡‡, Arthur M. Buchberg¶, and Michael R. Waterman‡**

From the ‡Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, ¶Kimmel Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania 19107-5541, and ‡‡Kirin Brewery Co., Ltd., Central Laboratories for Key Technology, 1-13-5 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 221, Japan

The mammalian Pbx homeodomain proteins provide specificity and increased DNA binding affinity to other homeodomain proteins. A cAMP-responsive sequence (CRS1) from bovine *CYP17* has previously been shown to be a binding site for Pbx1. A member of a second mammalian homeodomain family, Meis1, is now also demonstrated to be a CRS1-binding protein upon purification using CRS1 affinity chromatography. CRS1 binding complexes from Y1 adrenal cell nuclear extract contain both Pbx1 and Meis1. This is the first transcriptional regulatory element reported as a binding site for members of the Meis1 homeodomain family. Pbx1 and Meis1 bind cooperatively to CRS1, whereas neither protein can bind this element alone. Mutagenesis of the CRS1 element indicates a binding site for Meis1 adjacent to the Pbx site. All previously identified Pbx binding partners have Pbx interacting motifs that contain a tryptophan residue amino-terminal to the homeodomain that is required for cooperative binding to DNA with Pbx. Members of the Meis1 family contain one tryptophan residue amino-terminal to the homeodomain, but site-directed mutagenesis indicates that this residue is not required for cooperative CRS1 binding with Pbx. Thus, the Pbx-Meis1 interaction is unique among Pbx complexes. Meis1 also cooperatively binds CRS1 with the Pbx homologs extradenticle from *Drosophila melanogaster* and ceh-20 from *Caenorhabditis elegans*, indicating that this interaction is evolutionarily conserved. Thus, *CYP17* CRS1 is a transcriptional regulatory element containing both Pbx and Meis1 binding sites, which permit these two homeodomain proteins to bind and potentially regulate cAMP-dependent transcription through this sequence.

Homeobox genes encode proteins that contain a conserved 60-amino acid DNA binding domain, the homeodomain, and function as transcriptional regulators (1, 2). One specific class of homeobox genes is the PBC class, which includes five related genes (3). The members of this class are the *Caenorhabditis elegans* gene *ceh*-20 (4), the *Drosophila melanogaster* gene *extradenticle* (*exd*)[1] (5, 6), and the mammalian genes *Pbx1*, *Pbx2*, and *Pbx3* (7). The proteins encoded by these genes contain highly conserved atypical 63-amino acid homeodomains belonging to a three amino acid loop extension (TALE) superclass (8), as well as regions of identity amino-terminal to the homeodomain (3). The functional role of the ceh-20 protein is not known. However, in *Drosophila*, genetic studies have demonstrated that *exd* is required for normal development, and based on these studies, *exd* was proposed to function in parallel with genes of the homeotic complex (5, 9). The homeotic complex contains a clustered set of evolutionarily conserved homeobox genes involved in segmental identity in the developing embryo, and these genes are referred to as the *HOM* complex in *Drosophila* or the *Hox* complex in mammals (10, 11). *exd* in collaboration with specific *HOM* genes is required for expression of specific endogenous and transgenic reporter genes in the *Drosophila* embryo (12, 13). A biochemical basis for this activity is that although the protein exd binds DNA weakly by itself and many of the HOM proteins also bind DNA with low affinity and specificity, exd and HOM proteins interact to cooperatively bind DNA (12, 14). In addition, exd cooperatively binds DNA with engrailed, a homeodomain protein that is not encoded by a *HOM* gene (14). Therefore, a proposed role for exd action is that it serves to provide specificity of binding and increased affinity for DNA to other homeodomain proteins (15).

Pbx1 was first identified as a site of t(1;19) chromosomal translocation leading to the production of an E2A-Pbx1 fusion protein in a subset of pre-B cell acute lymphoblastic leukemias (16, 17). Similar to the exd and HOM cooperative DNA binding, *in vitro* studies have confirmed that Pbx proteins can also bind DNA cooperatively with many Hox proteins and mammalian engrailed at artificially selected DNA binding sites (18–21). All proteins binding cooperatively to DNA with Pbx or exd contain a tryptophan residue amino-terminal to the homeodomain that is required for interaction with Pbx, although the distance from the tryptophan to the homeodomain and its sequence context varies between these proteins (22–27). Although multiple Hox proteins and engrailed interact with Pbx to cooperatively bind artificially selected DNA sites *in vitro*, only three transcriptional regulatory elements have been reported to be binding sites for Pbx. The rhombomere 4 regulatory region from the mouse *Hoxb-1* gene contains three sites required for enhancer

* This work was supported by National Institutes of Health Grants GM07347 (to L. J. B.), DK28350 and ES00267–31 (to L. J. B., N. K., Y. T., and M. R. W.), and CA21124 (to J. J. M. and A. M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Tel.: 615-343-1373; Fax: 615-322-4349; E-mail: Bischolj@ctrvax.vanderbilt.edu.

‖ Present address: Duke University Medical Center, Durham, NC 27710.

** Present address: Department of Pharmacology, Kanazawa University School of Medicine, 13-1 Takara-Machi, Kanazawa, Ishikawa 920, Japan.

[1] The abbreviations used are: exd, extradenticle; CRS1, cAMP-responsive sequence 1; EMSA, electrophoretic mobility shift assay; *Mrg*, Meis1-related gene.

activity, two of which are bound by Pbx1 and Hoxb-1 *in vitro* (28). The element TSEII regulates expression of the somatostatin gene and is cooperatively bound by Pbx and the homeodomain protein STF-1 (29). The third regulatory binding site for Pbx1, and the first such site discovered, is a cAMP-responsive sequence (CRS1) from the bovine *CYP17* gene (30, 31). *CYP17*, which encodes 17α-hydroxylase/17,20-lyase cytochrome P-450 required for cortisol and androgen biosynthesis, is expressed in adrenal glands and gonads and is transcriptionally regulated by cAMP (32). Pbx1a and Pbx1b were purified as CRS1-binding proteins and demonstrated by overexpression studies to enhance the cAMP transcriptional response conferred through this element (31, 33).

We now report the copurification of a member of another homeodomain family, Meis1, by CRS1 affinity chromatography. The first identified member of the *Meis1* homeobox gene family is also referred to as *Meis1*, and this locus was originally identified as a site of retroviral integration associated with development of myeloid leukemia in BXH-2 mice (34). The other two mammalian members of the *Meis1* family are *Meis1-related gene 1* (*Mrg1*) and *Meis1-related gene 2* (*Mrg2*), and the proteins encoded by these genes contain atypical 63-amino acid homeodomains also belonging to the TALE superclass (35, 36). CRS1 is the first regulatory element identified as a binding site for a member of the Meis1 family. Both Pbx1 and Meis1 are components of CRS1 binding complexes from mouse adrenal cell Y1 nuclear extract. Furthermore, Meis1 is demonstrated to cooperatively bind CRS1 with Pbx1 as well as with the Pbx homologs exd and ceh-20. Mutagenesis of the CRS1 element identifies a Meis1 binding site adjacent to a Pbx binding site. The cooperative DNA binding between Pbx1 and Meis1 does not require a tryptophan residue amino-terminal to the Meis1 homeodomain. This study therefore reveals that members of the Meis1 family are Pbx binding partners on CRS1, of which the interactions with Pbx are distinct from all previously reported binding partners.

EXPERIMENTAL PROCEDURES

Purification of CRS1 Binding Proteins and Peptide Sequencing—Purification of nuclear proteins from Y1 mouse adrenal cells by CRS1 affinity chromatography and peptide sequencing was described previously (31).

Plasmids—Human Pbx1a and Pbx1b cDNAs were subcloned in pBK-RSV (Stratagene) (31). Human Pbx1 cDNAs were used for these studies, but human and mouse Pbx1b proteins are identical (17, 31). The complete coding sequences of Meis1a and Meis1b (34) were subcloned into pBluescript (Stratagene). Extradenticle cDNA in pBluescript (5) was kindly received from Cordelia Rauskolb and Eric Weischaus. Lambdaphage SHLX2 containing ceh-20 insert was kindly received from Lucinda Fulton, and this phage was originally obtained from the *C. elegans* cDNA library produced by Chris Martin (4). The phage was converted to plasmid using recombination between lox sites flanking the insert, and the ceh-20 cDNA was then subcloned into pBK-RSV using a *Sac*I site at the 5′ end and a *Hin*dIII site at the 3′ end.

In Vitro Transcription and Translation—Proteins were produced using the TnT-coupled reticulocyte lysate system (Promega) according to the manufacturer's specifications using either T7 or T3 polymerase. Proteins were produced simultaneously both in the presence and absence of [^{35}S]methionine (NEN Life Science Products). Proteins translated in the presence of [^{35}S]methionine were analyzed following 10% SDS-polyacrylamide gel electrophoresis using a PhosphorImager (Molecular Dynamics).

Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assay (EMSA)—Y1 nuclear extract was prepared essentially as described (37). Wild type CRS1 oligos used for EMSA contained the following sequence from bovine *CYP17* (*CYP17* sequence in uppercase letters, flanking restriction sites in lowercase letters): 5′-cGAGACGTTGATGGACAGTGAGCAAGg-3′ and the complementary oligo 5′-tcgacCTTGCTCACTGTCCATCAACGTCTCgagct-3′. The three mutant CRS1 oligonucleotides comprised the following (mutant bases underlined): mutant A, 5′-GAGACGTTGCGGGACAGTGAGCAAG-3′; mutant B, 5′-GAGACGTTGATGCTCAGTGAGCAAG-3′; and mutant C, 5′-GAGACGTTGATGGAGCGTGAGCAAG-3′. Complementary matching oligos were synthesized, and mutant oligos contained similar flanking sites as wild type oligo. Oligos were end-labeled with T4 polynucleotide kinase (New England Biolabs) using [γ^{32}P]ATP (NEN Life Science Products). For EMSA using reticulocyte lysate, a total of 2 μl of lysate was mixed on ice with 10,000 cpm end-labeled CRS1 in a total volume of 20 μl consisting of 10 mM Hepes (pH 7.9), 0.2 mM EDTA, 0.5 mM dithiothreitol, 4% Ficoll (molecular weight, 400,000), 2% glycerol, 0.2% Nonidet P-40, 5 mM $MgCl_2$, 75 mM KCl, 100 ng of poly(dI-dC) (Amersham Pharmacia Biotech), and 2 μg of tRNA. After mixing, reactions were electrophoresed on a prerun 5% polyacrylamide, 0.5× TBE (1× TBE = 90 mM Tris borate, 2 mM EDTA), 0.5% Ficoll gel at 250 volts (38), and dried gels were analyzed using a PhosphorImager. For EMSA with nuclear extract, 2 μg of nuclear extract was used, with conditions similar to those for lysate reactions except that 1 μg of poly(dI-dC) and 10 μg of tRNA were used. For supershift experiments, antibodies were added just before labeled oligonucleotides.

Site-directed Mutagenesis of Meis1 to Convert Trytophan 213 to Alanine—Mutagenesis of codon 213 from tgg to gcg was accomplished by the QuikChange site-directed mutagenesis protocol (Stratagene). Complimentary oligos used for production of the mutant codon were as follows (mutated bases are underlined): 5′-CTGACCAGCCCTCTGCGAATAGAGACCATG-3′ and 5′-CATGGTCTCTATTCGCAGAGGGCTGGTCAG-3′. Meis1 cDNA was amplified with mutant oligos using native *Pfu* polymerase (Stratagene). An *Apa*I fragment containing the mutation was excised and subcloned back into wild type Meis1a cDNA, from which the wild type *Apa*I fragment had been removed. This eliminated the possibility of errors in other regions of the cDNA or plasmid. The *Apa*I fragment obtained by mutagenesis was completely sequenced to confirm that the only mutation in this fragment was at codon 213.

Production of Pbx and Meis1 Antibodies—The complete coding sequence of human Pbx1b was subcloned into pGEX-4T-2 (Amersham Pharmacia Biotech) for production of glutathione *S*-transferase-Pbx1b fusion protein. Glutathione *S*-transferase-Pbx1b was produced in *Escherichia coli* strain JM109, and expression of the fusion protein was induced by isopropyl β-D-thiogalactoside. Glutathione *S*-transferase-Pbx1b was purified using glutathione-Sepharose chromatography as per the manufacturer's instructions (Amersham Pharmacia Biotech). Approximately 600 μg of glutathione *S*-transferase-Pbx1b was emulsified with complete Freund's adjuvant and used for injection into a rabbit. The rabbit was boosted with the same amount of protein in incomplete Freund's adjuvant. The IgG fraction of the Pbx immunoreactive serum was purified from a protein A-Sepharose CL-4B column (Amersham Pharmacia Biotech).

For the production of the Meis1 antibody, a peptide corresponding to the first 16 amino acids of this protein was synthesized by MacroMolecular Resources. This peptide was used to generate a rabbit polyclonal antibody by Cocalico Biologicals Inc. This antibody is specific for Meis1a and Meis1b.[2]

RESULTS

A Member of the Meis1 Homeodomain Family Is a CRS1-binding Protein—During previous studies to determine the mechanism by which CRS1 from bovine *CYP17* activates transcription in a cAMP-dependent manner, four CRS1-binding proteins from mouse adrenal Y1 cells were purified by CRS1 DNA affinity chromatography, and peptide sequences were obtained from each (31). The size of one of the purified proteins was approximately 43 kDa, two proteins were in the range of 53 kDa, and one protein was 60 kDa. Based on peptide sequence, the 43-kDa protein and one of the 53-kDa proteins were determined to be the homeodomain proteins Pbx1b and Pbx1a, respectively, which are produced by differential splicing of *Pbx1* mRNA (31). The identity of the 60-kDa protein is being investigated. The sequence for one peptide was obtained from the second 53-kDa protein. This sequence matches an amino acid stretch contained within the Meis1 homeodomain family (Fig. 1*A*). The three mammalian members of the *Meis1* gene family are *Meis1*, *Mrg1*, and *Mrg2* (34–36). Based on the sequence data, this peptide most closely matches amino acids 182–192 of

[2] S. Steelman, unpublished observations.

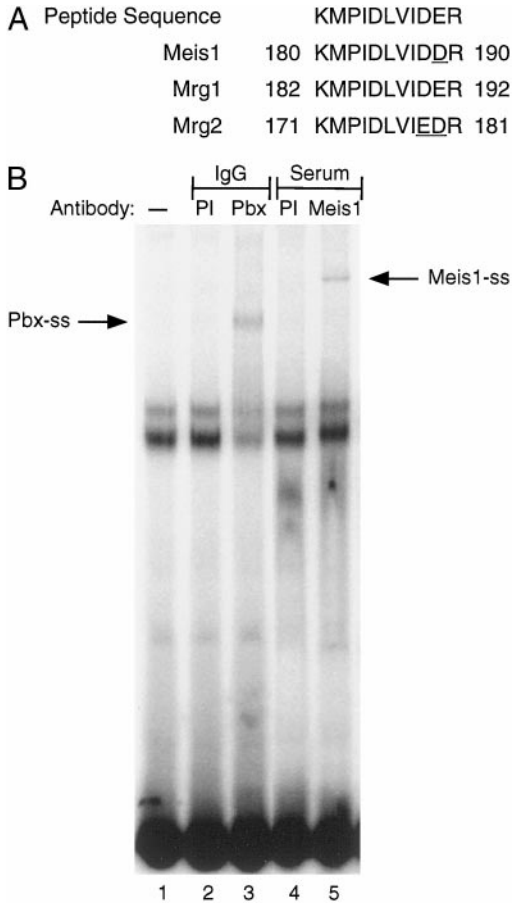

FIG. 1. **A member of the Meis1 homeodomain family binds CRS1.** *A*, shown is the peptide sequence obtained from one of the 53-kDa proteins purified by CRS1 affinity chromatography. Below this sequence is a comparison to matching regions of murine Meis1, Mrg1, and Mrg2. The positions of the first and last amino acids of these regions are indicated. Differences from the peptide sequence are *underlined*. *B*, Pbx and Meis1 antibodies were used to assess the effect of Y1 nuclear extract binding CRS1 by EMSA. The antibodies used in these reactions as indicated above each lane were as follows: no antibody (−), preimmune (*PI*) IgG, anti-Pbx (*Pbx*) IgG, preimmune serum, and anti-Meis1 (*Meis1*) serum. Pbx supershift (*Pbx-ss*) and Meis1 supershift (*Meis1-ss*) are indicated.

Mrg1 (Fig. 1*A*), but the specific isoform that was purified cannot be determined unambiguously from this one peptide.

To further test whether a member of the Meis1 family can bind CRS1, the EMSA was tested with Y1 nuclear extract. An antibody specific for the amino terminus of Meis1 was used to determine whether Meis1 binds CRS1. There are two isoforms of Meis1 produced by the *Meis1* gene due to differential splicing of the mRNA (34). These proteins, referred to as Meis1a and Meis1b, differ in their carboxyl termini, and therefore, the Meis1 antibody will recognize both isoforms. Two CRS1 binding complexes are formed within Y1 nuclear extract as revealed by EMSA (Fig. 1*B, lane 1*) (30). Neither preimmune IgG nor serum affected these CRS1 binding complexes (Fig. 1*B, lane 1 versus lanes 2* and *4*). Anti-Pbx IgG reduced the intensity of the CRS1 complexes and produced a supershift (Fig. 1*B, lane 3*). Anti-Meis1 serum produced a supershift complex although it did not greatly reduce the overall intensity of the two CRS1 complexes (Fig. 1*B, lane 5*). One possible reason for the minimal effect of Meis1 antiserum on CRS1 binding may be the presence of other isoforms of this family, such as Mrg1 and Mrg2, contained within this complex, that would not react with this specific antibody. This experiment confirms that Meis1 is a CRS1-binding protein contained within Y1 nuclear extract.

Homeodomain Proteins Pbx1 and Meis1 Cooperatively Bind CRS1—Because CRS1 binding complexes from Y1 nuclear extract contain both Pbx1 and Meis1, *in vitro* translated Meis1 was used to test whether this protein could bind CRS1 either individually or by interacting with Pbx1. Pbx1 has previously been demonstrated not to bind DNA as a monomer or homodimer to other DNA sequences (18, 19), and likewise, Pbx1 alone does not bind CRS1 *in vitro*.[3] The proteins encoded by the two mRNA splicing variants of *Pbx1*, Pbx1a and Pbx1b, as well as the two proteins encoded by the mRNA splicing variants of *Meis1*, Meis1a and Meis1b, were used to analyze CRS1 binding. These proteins were translated *in vitro* in a reticulocyte lysate and predominately full-length proteins were produced for both isoforms of Pbx1 and Meis1 (Fig. 2*A*). Binding to CRS1 was examined using EMSA (Fig. 2*B*). Neither of the Pbx1 isoforms and neither of the Meis1 isoforms could bind CRS1 alone (Fig. 2*B, lanes 1–5*). However, when either Pbx1 isoform was incubated with either Meis1 isoform, unique complexes formed that bound CRS1 (Fig. 2*B, lanes 6–9*). These complexes migrated corresponding to the size of the Pbx1 and Meis1 isoforms included in each reaction, which indicates that the complexes contained both Pbx1 and Meis1 isoforms. Specifically, Pbx1a and Meis1b, the largest isoforms, produced the slowest migrating CRS1 complex. Likewise, Pbx1b and Meis1a, the smallest isoforms, produced the fastest migrating CRS1 complex. The other two combinations of isoforms produced complexes of intermediate migration. In addition, an isoform of both Pbx1 and Meis1 are required for cooperative binding because neither a mixture of Pbx1 isoforms nor a mixture of Meis1 isoforms could interact to bind CRS1 (Fig. 2*B, lanes 10* and *11*).

Antibodies against Pbx1 and Meis1 were used to further confirm the presence of these proteins in the complex. The formation of the CRS1 binding complex in the presence of Pbx1b and Meis1a was not affected by the addition of preimmune IgG (Fig. 2*C, lane 2*). However, the addition of anti-Pbx IgG reduced formation of the CRS1 binding complex (Fig. 2*C, lane 3*). A faint supershift was seen, and in addition, the anti-Pbx IgG disrupted binding so that much less complex was formed. Because this is a polyclonal antibody, the loss of complex formation could be due to disruption of the Pbx1 and Meis1 interaction, to that of Pbx1 and CRS1, or both. The Meis1 antibody also disrupted formation of the complex formed by Pbx1 and Meis1 and produced a supershift complex. Therefore, the use of these antibodies demonstrates that both Pbx1 and Meis1 are binding CRS1 together in a complex.

Mutations within CRS1 Disrupt Binding of Both Nuclear Proteins as Well as in Vitro Translated Pbx1 and Meis1—Mutant CRS1 binding sites were designed to compare binding requirements by *in vitro* translated Pbx1b and Meis1a to Y1 nuclear proteins. Three CRS1 mutants were designed that each had two base pair changes (Fig. 3). Mutant A contained this mutation within a region of CRS1 (5′-TTGAT-3′) that corresponds to a polymerase chain reaction identified high affinity Pbx binding site (39, 40), and this mutation within CRS1 has been demonstrated to eliminate binding of nuclear proteins to this element (33). A CRS1 mutant 3′ to this Pbx binding site has also previously been demonstrated to show greatly reduced binding in Y1 nuclear extract (33), and based on Pbx and Hox DNA binding models (24, 41, 42), the proposed Meis1 binding site would be adjacent to the Pbx site on the 3′ side. Therefore, CRS1 mutants B and C were designed in the predicted Meis1 binding site. Binding to the wild type and mutant CRS1 oligonucleotides was analyzed by EMSA (Fig. 3). In Y1 nuclear extract, binding to mutant A was undetectable (Fig. 3, *lanes 1*

[3] Y. Takahashi, unpublished observations.

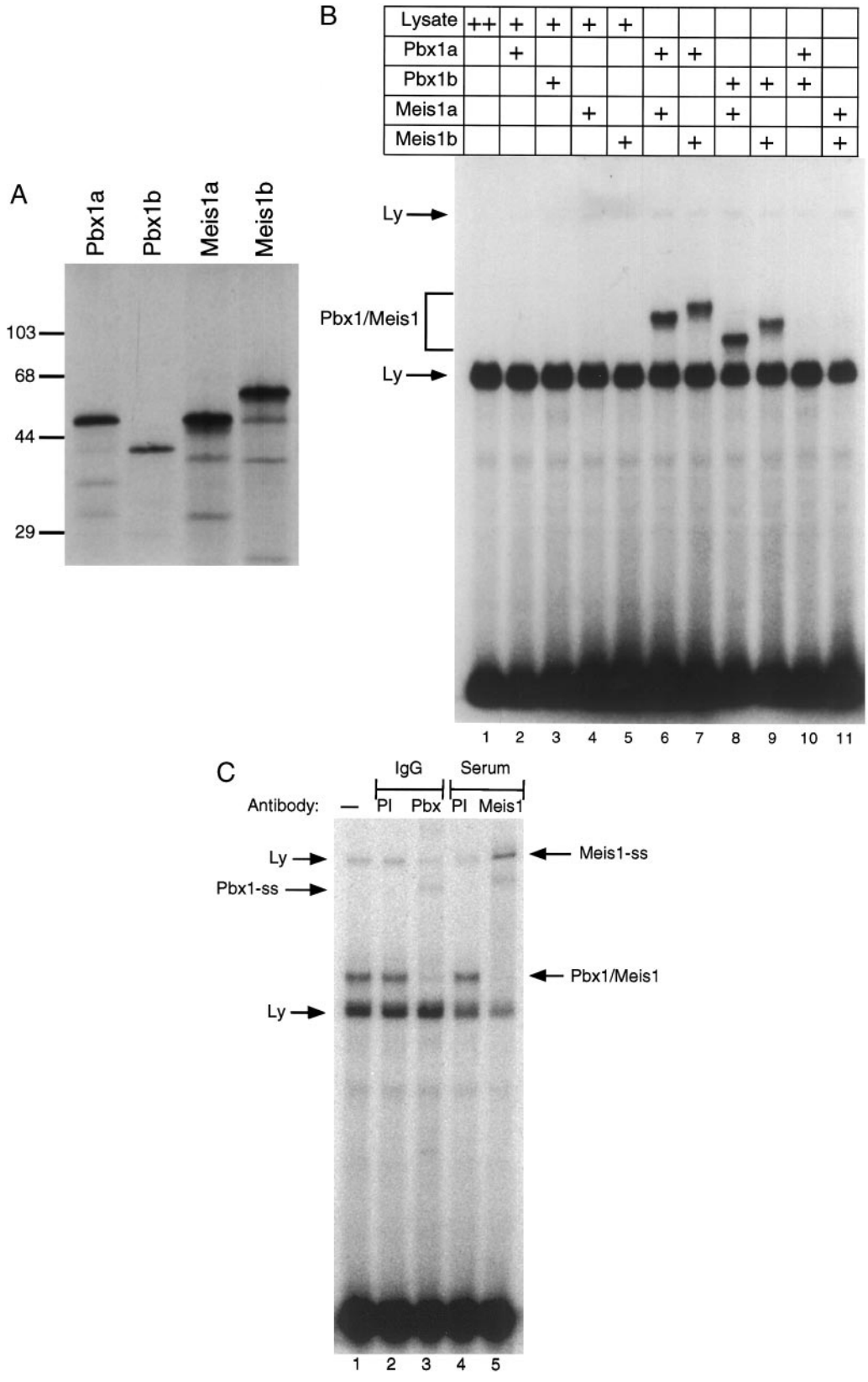

FIG. 2. **Pbx1 and Meis1 cooperatively bind CRS1.** *A*, Pbx1a, Pbx1b, Meis1a, and Meis1b isoforms translated in reticulocyte lysates in the presence of [^{35}S]methionine were separated by 10% SDS-polyacrylamide gel electrophoresis. Sizes of molecular mass markers are indicated (kDa). *B*, Pbx1 and Meis1 isoforms were analyzed for binding to CRS1 by EMSA. Proteins used for analysis are indicated above each lane. A total of 2 μl of lysate was used in each reaction; each + equals 1 μl of lysate. Specific Pbx1 and Meis1 complexes are indicated by *bracket*. Ly are complexes endogenous to the lysate. *C*, the effect of anti-Pbx IgG on Pbx1 and Meis1 binding CRS1 was analyzed by EMSA. All reactions contained 1 μl of Pbx1b and 1 μl of Meis1a lysates and antibodies, indicated above each lane as described in the legend to Fig. 1*B*. Pbx supershift (*Pbx-ss*) and Meis1 supershift (*Meis1-ss*) are indicated. The Meis1 supershift migrates with the upper nonspecific lysate complex.

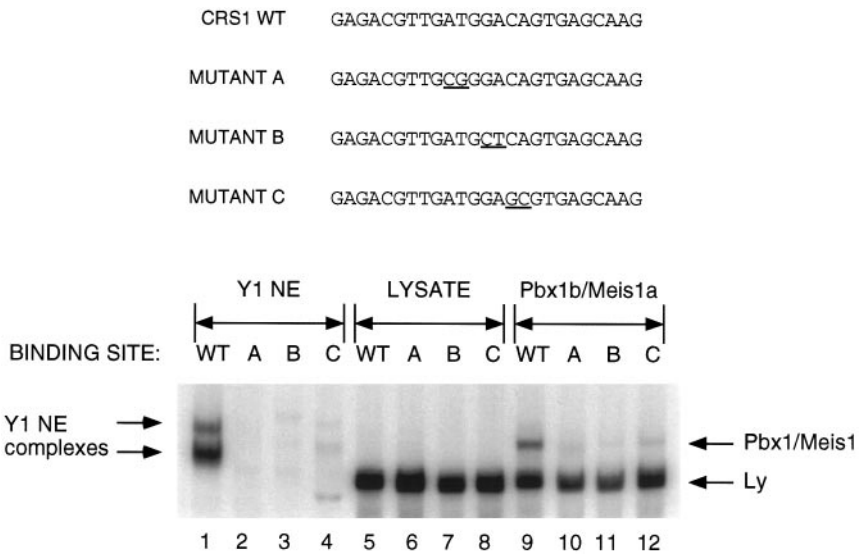

FIG. 3. **Mutagenesis within CRS1 disrupts both Pbx1 and Meis1 binding, as well as binding of endogenous nuclear proteins.** CRS1 wild type (*WT*) is shown at top aligned with three mutant CRS1 sites with mutations underlined. For EMSA experiments, 2 μg of Y1 nuclear extract was used in *lanes 1–4*, 2 μl of control reticulocyte lysate in *lanes 5–8*, and 1 μl each of Pbx1b and Meis1a in *lanes 9–12*. The CRS1 binding site used in each reaction is indicated above the lanes. *Arrows* on the *left* indicate CRS1 binding complexes in Y1 nuclear extract (*NE*). *Ly arrow* on the *right* indicates nonspecific complexes formed from lysate; *Pbx1/Meis1 arrow* on the *right* indicates specific complexes formed from translated proteins.

and *2*). Binding to mutants B and C was greatly decreased compared with wild type but detectable with prolonged exposure of reactions (Fig. 3, *lanes 3* and *4*). Nonspecific binding by lysate was not affected by the mutant oligonucleotides (Fig. 3, *lanes 5–8*). Also, binding of either Pbx1b or Meis1a individually to the wild type or mutant CRS1 sites was not detectable (Fig. 2*A* and data not shown). *In vitro*-translated Pbx1b and Meis1a incubated together exhibited greatly decreased but detectable binding to all the CRS1 mutants compared with the wild type (Fig. 3, *lanes 9–12*). This experiment indicates that bases within CRS1 that decrease binding by Pbx and Meis1 also decrease binding of endogenous Y1 nuclear proteins. This evidence further corroborates Meis1 proteins as CRS1 binding partners of Pbx1 within Y1 cells. In addition, it identifies a putative Meis1 binding site within CRS1, because the mutants B and C are outside of the Pbx binding site.

The Tryptophan Residue Amino-terminal to the Meis1 Homeodomain Is Not Required for Meis1 to Cooperatively Bind CRS1 with Pbx1—All previously characterized binding partners of Pbx have a tryptophan residue amino-terminal to the homeodomain that is required for cooperative DNA binding with Pbx. There are three classes of tryptophan-containing motifs found in these interacting proteins. Most of the homeotic genes, as well as STF-1, contain a hexapeptide motif (consensus, IYPWMK) (3) that includes a tryptophan required for Pbx cooperative DNA binding (22, 43). The homeotic proteins Hoxd-9 and Hoxa-10 do not contain hexapeptides, but each has the sequence ANW amino-terminal to the homeodomain, in which the tryptophan is required for cooperative binding with Pbx to DNA (25). Finally, both *Drosophila* and mouse homologs of engrailed have a conserved motif amino-terminal to the homeodomain referred to as EH2, and within this motif is the sequence WPAW. Mutation of the first tryptophan to lysine greatly reduces cooperative DNA binding with Pbx, whereas mutation of the second separately to lysine abolishes this interaction (26). There is one tryptophan amino-terminal to the homeodomain that is conserved among members of the Meis1 family, but it cannot be categorized into any of the three previously described Pbx interacting motifs. To determine whether this tryptophan was required for Pbx1 and Meis1 cooperative binding to CRS1, tryptophan 213 of Meis1a, contained within the amino acid sequence, PSWNR was mutated to alanine. A similar tryptophan to alanine mutation in the hexapeptide of Hoxa-5 abrogates cooperative DNA binding with Pbx (22). Both wild type Meis1a and Meis1a(W213A) were translated and found to be produced in approximately equal amounts, although Meis1a(W213A) migrated slightly slower on SDS-polyacrylamide gel electrophoresis than did the wild type protein (Fig. 4*A*). Because no other mutations exist in Meis1a(W213A), the W213A mutation must slightly alter the electrophoretic mobility of this protein. EMSA was used to test whether Meis1a(W213A) could cooperatively bind CRS1 with Pbx1 (Fig. 4*B*). Like Meis1a, Meis1a(W213A) did not bind CRS1 alone (Fig. 4*B*, *lanes 3* and *4*). The mutant protein retained the ability to cooperatively bind CRS1 with Pbx1b to a degree similar to wild type Meis1a (Fig. 4*B*, *lanes 5* and *6*). Therefore, this single tryptophan amino-terminal to the Meis1 homeodomain is not required for cooperative binding with Pbx1 to CRS1. This indicates that Meis1 contains a Pbx interacting motif distinct from other known Pbx binding partners.

Pbx Homologs Extradenticle and ceh-20 Interact with Meis1 to Cooperatively Bind CRS1—To determine whether the interaction of Pbx and Meis1 families extends beyond mammalian isoforms, the Pbx homologs from *Drosophila* and *C. elegans* were tested for cooperative CRS1 binding with Meis1. An amino acid sequence alignment of Pbx1, exd, and ceh-20 showing the identity between these proteins is presented (Fig. 5*A*). These proteins are highly conserved in the homeodomain and in a large region amino to the homeodomain but diverge in the extreme amino and carboxyl termini. Exd and ceh-20 were translated into full-length proteins (Fig. 5*B*). Cooperative binding to CRS1 by Meis1a and both Pbx homologs was tested using EMSA. Similar to Pbx1, neither exd nor ceh-20 bound CRS1 alone (Fig. 5*C, lanes 1* and *2*). However, in the presence of Meis1a, both exd and ceh-20 could cooperatively bind CRS1 in a manner analogous to Pbx1 (Fig. 5*C, lanes 3–5*). Therefore, similar to the evolutionary conservation of the Pbx and Hox interactions in mammals and exd and HOM interactions in *Drosophila*, the cooperative binding mediated by members of the Pbx and Meis1 families may also be conserved from insects to mammals. A Meis1 homolog named homothorax was recently identified from *Drosophila* and was demonstrated to interact with exd *in vitro*, which supports these results (44). Also, ceh-25 has been identified as a Meis1 homolog from the *C. elegans* cosmid *ceh-T28F12* (35, 45). This homeodomain protein from *C. elegans* is therefore predicted to be an endogenous binding partner of ceh-20.

DISCUSSION

CRS1 was the first transcriptional regulatory element identified as a Pbx1 binding site (31). It has now been established that members of the Meis1 homeodomain family also bind

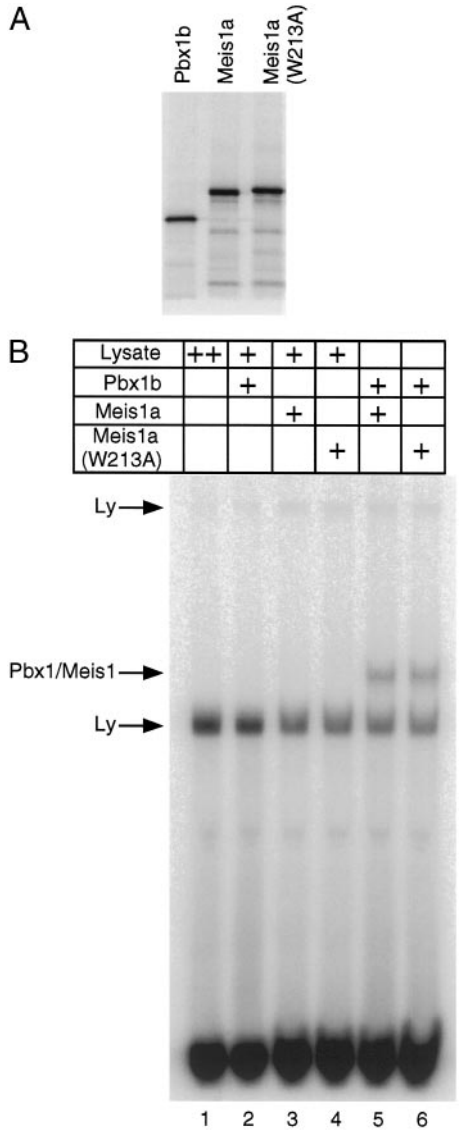

FIG. 4. **Tryptophan 213 of Meis1 is not required for cooperative binding to CRS1 with Pbx1.** Tryptophan 213 of Meis1a, which is amino-terminal to the homeodomain, was mutated to alanine. *A*, proteins translated in the presence of [^{35}S]methionine were separated by SDS-polyacrylamide gel electrophoresis. Meis1a(W213A) was translated to a level similar to that of as Meis1a, but it had a slightly altered electrophoretic mobility. *B*, the ability of Meis1a(W213A) to cooperatively bind CRS1 with Pbx1b was analyzed by EMSA. Each reaction contained a total of 2 μl of lysate; each + equals 1 μl of lysate. Meis1a(213A) cooperatively bound CRS1 with Pbx1b to the same degree as wild type Meis1a. *Ly* indicates binding to CRS1 by lysate alone.

CRS1, the first regulatory element identified as a binding site for this family. Although neither Pbx1 nor Meis1 can bind CRS1 individually, they are demonstrated to bind this element cooperatively. Pbx1 and a member of the Meis1 family were copurified, indicating that they may interact within Y1 cells as they do *in vitro*. In addition, by EMSA both Pbx1 and Meis1 are demonstrated to be components of the CRS1 binding complex in Y1 nuclear extract. Contained within CRS1 is the sequence 5′-TTGATGGACAG-3′. The 5′ half of this site, 5′-TTGAT-3′, was initially defined as a Pbx binding site using polymerase chain reaction-based site selection (39, 40). Mutagenesis within CRS1, both within the Pbx site and at bases within 5′-GGACAG-3′, decreases both binding of *in vitro*-translated Pbx1 and Meis1 and binding by proteins in Y1 nuclear extract. This further supports Meis1 as a Pbx1 binding partner on CRS1 within nuclear extract and also indicates a putative Meis1 binding site adjacent to the Pbx site. This arrangement would agree with a model for Pbx-Hox DNA binding in which the Hox site is 3′ and immediately adjacent to the Pbx site (24, 41, 42). Recent published work (46) also supports this analysis. In studies of the interaction between Pbx and Meis1 on a polymerase chain reaction-generated DNA sequence, a site selection assay was utilized to identify a Meis1 binding site as 5′-TGACAG-3′ (46). Also recently, Meis1 was demonstrated to interact with and stabilize DNA binding by Abdominal-B-like Hox proteins in which a Meis1 DNA binding site was also identified as 5′-TGACAG-3′ (47). Therefore, these studies support the mutagenesis data shown here and indicate the 5′-GGACAG-3′ within CRS1 as the Meis1 binding site.

CRS1 positively regulates transcription of reporter genes in response to cAMP and is believed to regulate expression of bovine *CYP17* (30). Therefore, Pbx and Meis1 are two homeodomain protein families that may function in the cAMP response through CRS1. The basis for this cAMP response is not yet well understood. Pbx1 enhances the cAMP activated transcriptional response mediated by protein kinase A on CRS1 regulating a reporter gene (31, 33). There are several possible mechanisms by which protein kinase A could regulate the activity of CRS1. First, the levels of one or more of the CRS1-binding proteins could be regulated, such as at the level of gene expression or translation. Alternatively, Pbx1 or Meis1 could be posttranslationally modified by phosphorylation, which could affect DNA binding, dimerization ability, or transactivation function. For example, protein kinase A phosphorylation of the homeodomain protein thyroid transcription factor 1 is involved in the expression of the surfactant protein B gene promoter by this homeodomain protein (48). An additional possibility is that protein kinase A modifies a non-DNA-bound protein that may interact with the Pbx-Meis1 complex. Finally, concerning CRS1 activity, there may be other components of the CRS1 binding complex, such as the 60-kDa protein that was copurified (31). Preliminary data indicate that this protein may be the homeodomain protein, Pknox1, which is related to Meis1 (49).[4] The identification of this protein, as well as how protein kinase A may regulate transcription through this element, is being investigated.

The peptide sequence of the Meis1 family member that was purified from mouse adrenal Y1 cells did not allow unambiguous determination of which isoform was purified. Meis1 was demonstrated to be a component of the CRS1 binding complex in Y1 nuclear extract, but other isoforms may also be present. All members of the Meis1 family are predicted to cooperatively bind CRS1 with Pbx1 due to their overall conserved identity (35, 36), but functionally, there may be a specific isoform of the Meis1 family that is required to interact with Pbx1 to mediate the cAMP response. Regarding expression of the specific isoforms, Meis1 expression appears to be ubiquitous, whereas Mrg1 and Mrg2 have more restricted expression but are still found in multiple tissues (35, 36). In addition to Meis1 serving as a DNA binding partner for Pbx, a recent report indicates that in *Drosophila*, a Meis1 homolog, homothorax, is required for nuclear transport of the Pbx homolog exd from the cytoplasm (44). Therefore, Meis1 proteins may have two distinct roles in interacting with Pbx proteins: nuclear translocation and cooperative participation in transcriptional regulation.

Previously identified partners of Pbx contain a tryptophan residue amino-terminal to the homeodomain that is required for cooperative DNA binding with Pbx. There are three groups of tryptophan motifs that function as Pbx interacting domains. First, the majority of the homeotic proteins interacting with

[4] N. Kagawa, unpublished observations.

A

```
Pbx1b  LQQIMTITDQSLDEAQ.ARKHALNCHRMKPALFNVLCEIKEKTVLSIRGAQEEEPTDPQLMRLDNMLLAEGVAGPEKGGGSAAAAAAAAAS
  exd  -----S-SE-------.----T-----------S--------------NT-----P-----------I------------G----S-----
ceh-20 -DAVLK-NE-T--DNDS-K-QE-Q--P-RQ---D----T------TV-NQVD-T-E-----------V-------D---............

Pbx1b  GGA.....GSDNSVEHSDYRAKLSQIRQIYHTELEKYEQACNEFTTHVMNLLREQSRTRPISPKEIERMVSIIHRKFSSIQMQLKQST
  exd  Q-GSLSID-A--AI---------A-------Q-----------------------------T--------Q---K-------------
ceh-20 ...SLGSDA-GG..DQA---Q--H---VL-NE--R---E------Q--RS--KD--QV---AH-------Y--Q---NG--V------

Pbx1b  CEAVMILRSRFLDARRKRRNFNKQATEILNEYFYSHLSNPYPSEEAKEELAKKCGITVSQVSNWFGNKRIRYKKNIGKFQEEANIYAAK
  exd  ----------------------S---S------------------------R-------------------------A-----L----
ceh-20 ----------------------S-----V------G-------------D--RQ-N-------------------MAKA----SM----
```

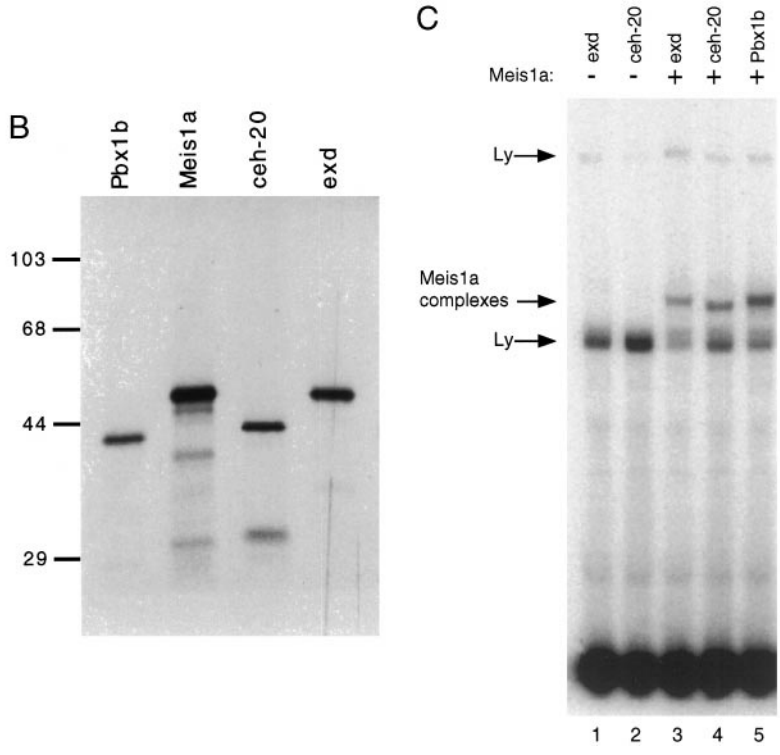

FIG. 5. **Pbx homologs exd and ceh-20 interact with Meis1a to bind CRS1.** *A*, amino acid alignment of the conserved regions of Pbx1b (amino acids 47–308), exd (amino acids 47–313), and ceh-20 (amino acids 13–263) is shown. The full lengths of Pbx1b, exd, and ceh-20 are 347, 376, and 338 amino acids, respectively. The amino and carboxyl termini of these proteins are not conserved. − indicates an amino acid identical to Pbx1b, whereas . is used as a space to optimally align proteins. The homeodomains are indicated in *boldface*. The overall identity among all three proteins in the region shown in the alignment is 68%. Exd and Pbx1b are more similar to each other than to ceh-20; the identity between these two proteins within the alignment is 90%. The overall identity of all three homeodomains is 86%. *B*, exd and ceh-20, translated in the presence of [^{35}S]methionine, were separated by SDS-polyacrylamide gel electrophoresis. Molecular mass markers are indicated to the *left* (kDa). *C*, exd and ceh-20 binding to CRS1 with Meis1 was analyzed by EMSA. Proteins in each reaction are listed above lane. Absence or presence of Meis1a in each reaction is demonstrated by − or +, respectively. All reactions contained a total of 2 μl of lysate. *Ly* indicates binding to CRS1 by lysate alone.

Pbx contain a hexapeptide motif amino-terminal to the homeodomain in which a conserved tryptophan is required for cooperative DNA binding with Pbx (20, 21, 27, 43). Second, Hoxa-10 and Hoxb-9 require the tryptophan within an ANW sequence just amino-terminal to the homeodomain for cooperative DNA binding (24, 25). Third, engrailed interacts with Pbx partially through an EH2 motif that contains two tryptophans, one of which contributes to cooperative DNA binding and the other of which is essential for it (26). Therefore, all previously described partner proteins of Pbx require a tryptophan residue amino-terminal to the homeodomain for interaction with Pbx, although the context of the tryptophan varies. Meis1 represents an unique interaction with Pbx in that the only tryptophan amino-terminal to the homeodomain can be mutated to alanine and the protein can still cooperatively bind CRS1 with Pbx1. Members of the Meis1 family are also distinct from other Pbx binding partners in that they contain atypical 63-amino acid homeodomains rather than the usual 60-amino acid homeodomain contained in other Pbx binding partners. The significance of these three extra amino acids is not clear. The domains within Meis1 that are required for interaction with Pbx and whether the regions of Pbx that interact with Meis1 are the same as those interacting with these other homeodomain proteins await further experimentation.

Pbx binding cooperatively to DNA with both Hox and engrailed are evolutionarily conserved interactions because the Pbx homolog exd also binds cooperatively to DNA with both HOM and engrailed proteins. Likewise, the interaction between Pbx and Meis1 appears to be conserved because Meis1 can interact with the Pbx homologs exd from *Drosophila* and ceh-20 from *C. elegans*. Meis1 homologs have recently been identified from *Drosophila* as homothorax (44), and also from *C. elegans* as ceh-25 (35, 45). Therefore, this newly described interaction raises the likelihood that the Pbx family will be

involved in regulating the expression of an ever-increasing number of genes in multiple species in cooperation with members of the Meis1 family.

Acknowledgments—We appreciate the gifts of lambda phage SHLX2 containing ceh-20 cDNA from Lucinda Fulton at Washington University, exd cDNA from Cordelia Rauskolb and Eric Weischaus at Princeton University, and Meis1 antibody from Ira O. Daar at NCI-Frederick Cancer Research and Development Center, National Institutes of Health (Frederick, MD).

REFERENCES

1. Scott, M. P., Tamkun, J. W., and Hartzell, G. W., III (1989) *Biochim. Biophys. Acta* **989,** 25–48
2. Gehring, W. J. (1992) *Trends Biochem. Sci.* **17,** 277–280
3. Burglin, T. R. (1994) in *Guidebook to the Homeobox Genes* (Duboule, D., ed) pp. 25–71, Oxford University Press, Oxford
4. Waterston, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shownkeen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berks, M., Du, Z., Thomas, K., Thierry-Mieg, J., and Sulston, J. (1992) *Nat. Genet.* **1,** 114–123
5. Rauskolb, C., Peifer, M., and Wieschaus, E. (1993) *Cell* **74,** 1101–1112
6. Flegel, W. A., Singson, A. W., Margolis, J. S., Bang, A. G., Posakony, J. W., and Murre, C. (1993) *Mech. Dev.* **41,** 155–161
7. Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M. L. (1991) *Mol. Cell. Biol.* **11,** 6149–6157
8. Bertolino, E., Reimund, B., Wildt-Perinic, D., and Clerc, R. G. (1995) *J. Biol. Chem.* **270,** 31178–31188
9. Peifer, M., and Wieschaus, E. (1990) *Genes Dev.* **4,** 1209–1223
10. Akam, M. (1989) *Cell* **57,** 347–349
11. Krumlauf, R. (1994) *Cell* **78,** 191–201
12. Chan, S. K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. S. (1994) *Cell* **78,** 603–615
13. Rauskolb, C., and Wieschaus, E. (1994) *EMBO J.* **13,** 3561–3569
14. van Dijk, M. A., and Murre, C. (1994) *Cell* **78,** 617–624
15. Mann, R. S., and Chan, S. K. (1996) *Trends Genet.* **12,** 258–262
16. Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S. D., and Cleary, M. L. (1990) *Cell* **60,** 535–545
17. Kamps, M. P., Murre, C., Sun, X. H., and Baltimore, D. (1990) *Cell* **60,** 547–555
18. van Dijk, M. A., Peltenburg, L. T., and Murre, C. (1995) *Mech. Dev.* **52,** 99–108
19. Lu, Q., Knoepfler, P. S., Scheele, J., Wright, D. D., and Kamps, M. P. (1995) *Mol. Cell. Biol.* **15,** 3786–3795
20. Phelan, M. L., Rambaldi, I., and Featherstone, M. S. (1995) *Mol. Cell. Biol.* **15,** 3989–3997
21. Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C., and Cleary, M. L. (1995) *Genes Dev.* **9,** 663–674
22. Knoepfler, P. S., and Kamps, M. P. (1995) *Mol. Cell. Biol.* **15,** 5811–5819
23. Shen, W. F., Chang, C. P., Rozenfeld, S., Sauvageau, G., Humphries, R. K., Lu, M., Lawrence, H. J., Cleary, M. L., and Largman, C. (1996) *Nucleic Acids Res.* **24,** 898–906
24. Chang, C. P., Brocchieri, L., Shen, W. F., Largman, C., and Cleary, M. L. (1996) *Mol. Cell. Biol.* **16,** 1734–1745
25. Shen, W.-F., Rozenfeld, S., Lawrence, H. J., and Largman, C. (1997) *J. Biol. Chem.* **272,** 8198–8206
26. Peltenburg, L. T. C., and Murre, C. (1996) *EMBO J.* **15,** 3385–3393
27. Johnson, F. B., Parker, E., and Krasnow, M. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 739–743
28. Popperl, H., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S., and Krumlauf, R. (1995) *Cell* **81,** 1031–1042
29. Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. (1995) *Mol. Cell. Biol.* **15,** 7091–7097
30. Lund, J., Ahlgren, R., Wu, D. H., Kagimoto, M., Simpson, E. R., and Waterman, M. R. (1990) *J. Biol. Chem.* **265,** 3304–3312
31. Kagawa, N., Ogo, A., Takahashi, Y., Iwamatsu, A., and Waterman, M. R. (1994) *J. Biol. Chem.* **269,** 18716–18719
32. John, M. E., John, M. C., Boggaram, V., Simpson, E. R., and Waterman, M. R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4715–4719
33. Ogo, A., Waterman, M. R., and Kagawa, N. (1997) *Arch. Biochem. Biophys.* **338,** 193–200
34. Moskow, J. J., Bullrich, F., Huebner, K., Daar, I. O., and Buchberg, A. M. (1995) *Mol. Cell. Biol.* **15,** 5434–5443
35. Steelman, S., Moskow, J. J., Muzynski, K., North, C., Druck, T., Montgomery, J. C., Huebner, K., Daar, I. O., and Buchberg, A. M. (1997) *Genome Res.* **7,** 142–156
36. Nakamura, T., Jenkins, N. A., and Copeland, N. G. (1996) *Oncogene* **13,** 2235–2242
37. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
38. Kagawa, N., and Waterman, M. R. (1990) *J. Biol. Chem.* **265,** 11299–11305
39. Lu, Q., Wright, D. D., and Kamps, M. P. (1994) *Mol. Cell. Biol.* **14,** 3938–3948
40. van Dijk, M. A., Voorhoeve, P. M., and Murre, C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6061–6065
41. Lu, Q., and Kamps, M. P. (1996) *Mol. Cell. Biol.* **16,** 1632–1640
42. Chan, S. K., and Mann, R. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 5223–5228
43. Neuteboom, S. T., Peltenburg, L. T., van Dijk, M. A., and Murre, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9166–9170
44. Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M., and Mann, R. S. (1997) *Cell* **91,** 171–183
45. Burglin, T. R. (1997) *Nucleic Acids Res.* **25,** 4173–4180
46. Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G., and Cleary, M. L. (1997) *Mol. Cell. Biol.* **17,** 5679–5687
47. Shen, W. F., Montgomery, J. C., Rozenfeld, S., Moskow, J. J., Lawrence, H. J., Buchberg, A. M., and Largman, C. (1997) *Mol. Cell. Biol.* **17,** 6448–6458
48. Yan, C., and Whitsett, J. A. (1997) *J. Biol. Chem.* **272,** 17327–17332
49. Chen, H., Rossier, C., Nakamura, Y., Lynn, A., Chakravarti, A., and Antonarakis, S. E. (1997) *Genomics* **41,** 193–200