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Barrier to Autointegration Factor Interacts with the Cone-Rod Homeobox and Represses Its Transactivation Function*

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Crx (cone-rod homeobox) is a homeodomain transcription factor implicated in regulating the expression of photoreceptor and pineal genes. To identify proteins that interact with Crx in the retina, we carried out a yeast two-hybrid screen of a retinal cDNA library. One of the identified clones encodes Baf (barrier to autointegration factor), which was previously shown to have a role in mitosis and retroviral integration. Additional biochemical assays provided supporting evidence for a Baf-Crx interaction. The Baf protein is detectable in all nuclear layers of the mouse retina, including the photoreceptors and the bipolar cells where Crx is expressed. Transient transfection assays with a rhodopsin-luciferase reporter in HEK293 cells demonstrate that overexpression of Baf represses Crx-mediated transactivation, suggesting that Baf acts as a negative regulator of Crx. Consistent with this role for Baf, an E80A mutation of CRX associated with cone-rod dystrophy has a higher than normal transactivation potency but a reduced interaction with Baf. Although our studies did not identify a causative Baf mutation in retinopathies, we suggest that Baf may contribute to the phenotype of a photoreceptor degenerative disease by modifying the activity of Crx. In view of the ubiquitous expression of Baf, we hypothesize that it may play a role in regulating tissue- or cell type-specific gene expression by interacting with homeodomain transcription factors.

Development and maintenance of photoreceptor function in mammalian retina requires the expression of photoreceptor-specific or photoreceptor-enriched genes. Under- or over-expression of these genes, such as the visual pigment rhodopsin (1, 2), can lead to a developmental defect or photoreceptor degeneration. The molecular mechanisms regulating photoreceptor gene expression involve interactions of *cis*-acting elements in the promoter region with *trans*-acting transcription factors (3, 4). Among these factors, Crx (cone-rod homeobox) (5, 6) and Nrl (neural retina leucine zipper) (7) are reported to be essential for photoreceptor development and function (8, 9).

Crx is a member of the Otd/Otx homeodomain protein family expressed predominantly in the rod and cone photoreceptors of the retina and pinealocytes of the pineal gland (5, 6, 10). Crx regulates the expression of several photoreceptor genes (5) as well as pineal genes involved in melatonin synthesis (11) by binding to their promoters. It acts synergistically with Nrl (5), a bZIP transcription factor expressed specifically in rod photoreceptors (12). The Crx protein includes a homeodomain (HD)[1] near its N terminus, followed by a glutamine-rich (Gln) region, a basic region, a WSP (SI*WSP*ASESP) region, and an Otx tail region that all share homology with corresponding regions of Otx1 and Otx2 (5). The Crx HD is of the K_{50} subtype (with lysine at its 50th residue) of the paired-like class (5), and it is responsible for binding to target DNA (5, 13), the nuclear localization of the Crx protein (14), and mediating a physical and functional interaction with Nrl (15). *In vitro* protein-DNA binding assays demonstrated that the Crx HD binds to at least three target sites in the rhodopsin promoter, all with a (C/T)TAATCC consensus sequence, including a high affinity site called BAT-1 and two low affinity sites called Ret-1 and Ret-4 (5). Transient transfection assays in HEK293 cells demonstrated that the C-terminal region of Crx (between amino acids 107 and 284) contains the transactivation domains AD-1 and AD-2, which are important for its ability to activate promoters (13).

Mutant mice that are homozygous for a null allele of *Crx* ($Crx^{-/-}$) fail to develop outer segments of the photoreceptors, which subsequently undergo progressive degeneration (8). The expression levels of many photoreceptor genes are altered in the $Crx^{-/-}$ mouse retina, indicating that these genes are either direct or indirect targets of Crx (8, 16, 17). Consistent with

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[1] The abbreviations used are: HD, homeodomain; Baf, barrier to autointegration factor (different from the BAF proteins in the SWI/SNF-like BAF chromatin remodeling complexes); dbd, DNA-binding domain; CREB, cAMP-response element-binding protein; GST, glutathione *S*-transferase; NTA, nitrilotriacetic acid; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; PIC, preintegration complex; contig, group of overlapping clones; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; BBS1, Bardet-Biedl syndrome-1.

these studies, over a dozen *CRX*[2] mutations are associated with human retinal degenerative diseases, such as autosomal dominant cone-rod dystrophy (CORD2) (18–21), retinitis pigmentosa (20), and Leber congenital amaurosis (20, 22–24). *In vitro* functional analyses of some of these mutations demonstrated reduced binding to and/or *trans*-activation of the rhodopsin promoter (13, 21, 23). These results, combined with the mouse studies, provide strong evidence that Crx is required for both the development and maintenance of photoreceptors by acting as an important regulator of photoreceptor gene expression.

Several studies have identified Crx-interacting proteins, such as Nrl (15), CREB-binding protein/p300 (25), phosducin and phosducin-like proteins (PhLP1 and PhLOP1) (26), the nonhistone high mobility group protein HMGA1 (formerly HMG19Y) (27), and ataxin-7 (28). Among these, Nrl, HMGA1, and CREB-binding protein/p300 enhance, whereas Phd (PhLPs) and ataxin-7 repress, the transactivation activity of Crx. To further enhance our understanding of Crx function, we carried out a protein-protein interaction screen of a bovine retinal cDNA library in yeast with Crx as bait. Here, we report the identification of barrier-to-autointegration factor (Baf) as a Crx-interacting protein and a detailed characterization of the physical and functional interaction of Crx and Baf. Our studies suggest a novel cellular function for Baf that is directly linked to transcriptional regulation of tissue-specific genes in addition to its reported role in chromatin decondensation and nuclear envelope assembly during mitosis (29–31).

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Interaction Assays—The two-hybrid assays in yeast (32) were carried out using the Matchmaker Two-Hybrid System 2 (BD-Biosciences CLONTECH, Palo Alto, CA) with a dual reporter strain, Y190, as described previously (15, 28, 33). The "bait" construct Crx-HD-pAS2 contains the bovine Crx homeodomain and its flanking sequences (amino acid residues 34–107) fused in frame with the Gal4-DNA binding domain (dbd) in the pAS2-1 vector. A bovine retinal cDNA library (34) generated in pACTII (the prey vector with a Gal4 activation domain) was used for screening Crx-HD-interacting clones. The reporter strain Y190 was transformed with the bait vector Crx-HD and tested for a basal expression level of the dual reporter genes *His3* and *lacZ* using 3-amino-1,2,4-triazole (3-AT; a competitive inhibitor of the His3 protein) and a colony lift X-gal filter assay, respectively, as described in the CLONTECH manual. The bait-containing Y190 cells were subsequently transformed with 20 μg of DNA from the retinal cDNA library. Colonies that grew on SD-Trp$^-$, Leu$^-$, His$^-$ medium supplemented with 15 mM 3-AT were considered as "positives," and they were verified using X-gal filter assays. Y190 transformants containing the known interacting protein partners, Snf1 (in pAS1) and Snf4 (in pACTII), were used as controls for a positive interaction (35). Yeast DNA harboring a mixture of the bait and prey plasmids was prepared from each of the clones that tested positive by the dual reporter assay. The prey plasmids in the positive colonies were recovered by electroporation of the yeast DNA into *Escherichia coli* strain DH5α, selection of *E. coli* colonies containing the plasmids, and subsequent amplification and purification of plasmid DNA. False positives were further eliminated by retransforming the prey DNA to the original bait strain and a strain harboring the unrelated bait Snf1. Library clones that were positive for interaction with Crx-HD but not with Snf1 were sequenced and characterized.

To confirm the interaction of Crx and the product of the *Baf* gene identified by the yeast two-hybrid screening, an insert swap between the bait and prey was performed. The *Baf* insert was PCR-amplified and cloned into the pAS2 bait vector at the *Nde*I site (filled in) with the predicted open reading frame fused in-fame with Gal4-dbd. The full-length coding region of bovine *Crx* was cloned in-frame with Gal4 activation domain in pACTII at the *Bam*HI (5′) and *Xho*I (3′) site. The resulting Baf bait and Crx prey constructs were co-transformed into the yeast Y190 for 3-AT and X-gal assays as described above.

Constructs for Recombinant Protein Expression—To express and purify the bovine Baf (bBaf) protein from *E. coli*, a PCR-amplified cDNA corresponding to the open reading frame of *bBaf* was cloned in frame with the His_6 tag of pTrcHisA (Invitrogen) at the *Bam*HI (5′) and *Eco*RI (3′) site. For mammalian expression and *in vitro* transcription/translation, a PCR-amplified cDNA containing the *bBaf* coding region fused in frame with an N-terminal Myc tag was generated and cloned into pcDNA3.1(+) (Invitrogen) at the *Hin*dIII and *Xba*I site (*bBaf*-pcDNA3.1(+)/myc). All of the PCR amplifications were performed using the high fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The frame of each fusion protein was confirmed by sequencing using an ABI Prism DNA sequencing kit and ABI Prism 310 Genetic Analyzer (PerkinElmer Life Sciences). The bacterial expression vector Crx-HD-GST containing the Crx homeodomain fused with the GST tag in pGEX-4T-2 (Amersham Biosciences), the mammalian expression vectors carrying the coding cDNA of the human (*hCRX*) and bovine (*bCrx*) *Crx* and its deletion series, in frame with the Xpress tag in pcDNA3.1/HisC (Invitrogen), and the *hCRX* constructs carrying missense mutations in the HD were described previously (5, 15).

Purification of Bacterially Expressed Recombinant Proteins and Antibody Collection—The GST and Crx-HD-GST proteins were expressed in *E. coli* and purified using glutathione-Sepharose beads as described previously (5). The His_6-tagged Baf (Baf-His) and the His_6 tag alone (His) were expressed in the *E. coli* BL21 strain (Stratagene) and purified using Ni^{2+}-NTA-agarose resin (Qiagen, Valencia, CA) under denaturing conditions with 6 M guanidine-HCl according to the manufacturer's instructions with some modifications. In brief, bacterial cells were lysed in a lysis buffer (0.1 M NaH_2PO_4, 10 mM Tris-Cl, 6 M guanidine HCl, pH 8.0), bound to the Ni^{2+}-NTA resin using the batch method, washed with the lysis buffer supplemented with 5 mM imidazole, and eluted with an imidazole gradient (20–700 mM) in the lysis buffer. The fractions containing either Baf-His or His were pooled after SDS-PAGE analysis of each fraction. The affinity-purified proteins were renatured by dialysis against a storage buffer containing 25 mM Hepes, pH 7.6, 60 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride. The resulting protein preparations were quantified using Bio-Rad Protein Assay Kit II and analyzed by SDS-PAGE and immunoblots with the anti-polyhistidine monoclonal antibody (Sigma-Aldrich) and an anti-Baf antibody (see below). Each protein preparation was also analyzed for possible DNA contamination by UV spectrum measurement (at 260 and 280 nm) and agarose gel electrophoresis (1% with ethidium bromide). P261, a rabbit polyclonal antibody to Crx, was described previously (28). A polyclonal antibody against human BAF was generated in rabbits using purified recombinant human BAF expressed in *E. coli*. This antibody and its control serum (preimmune) were kindly provided by Robert Craigie.

Immunoblots with Whole Cell Extracts—Crude protein lysates were prepared by homogenization of frozen tissue samples or cell pellets in a 3-fold volume of a sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 200 mM dithiothreitol, 10% glycerol, and 0.001% bromphenol blue using a Pro250 homogenizer (PRO Scientific Inc., Monroe, CT), followed by immediately boiling for 10 min. Eight μl of each protein sample were resolved by SDS-PAGE (15% gel) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), which was probed with the primary antibody against Baf at a 1:1000 dilution, and the signal was detected by a horseradish peroxidase-conjugated anti-rabbit-IgG secondary antibody at a 1:1000 dilution and the ECL kit (Amersham Biosciences).

Co-immunoprecipitation and Pull-down Assays—Co-immunoprecipitation assays with *in vitro* translated proteins were carried out essentially as described previously (28) with minor modifications. 1% Triton X-100 was included in the wash buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100), and 2–4 μl of a specific antibody were used for co-immunoprecipitation. Antibodies used for co-immunoprecipitation include anti-Crx P261 (28), anti-Myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Chx10 (a gift of Dr. Connie Cepko), and anti-Nrl (12). Co-immunoprecipitated proteins were resolved by SDS-PAGE and quantified using a Storm 860 PhosphorImager system and ImageQuant 5.0 analytic program (Amersham Biosciences). For co-immunoprecipitation assays with tissue extracts, whole cell extracts were prepared by homogenizing tissue samples in a 3-fold volume of a whole cell lysis buffer (50 mM Tris-Cl (pH 7.5), 450 mM NaCl, 1% Triton X-100, and 10% glycerol with a mixture of protease inhibitors (Roche Molecular Biochemicals)). After a brief centrifugation for 5 min at 10,000 $\times$ *g*, 200 μl of the supernatants were incubated with 2 μl of the anti-Crx P261 antibody for 2 h at 4 °C, followed by the addition of 45 μl of 50% Protein A-Sepharose beads and gentle mixing on a rotator at 4 °C overnight. After being washed five times with the wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100), the bound proteins were

[2] Per guidelines of the Human Gene Nomenclature Committee, the names of human genes and proteins are represented by capital letters, whereas only the first letter is capitalized for those from other species.

eluted and analyzed by SDS-PAGE and immunoblots with the anti-Baf antibody.

For pull-down assays, 100 ng of the purified Crx-HD-GST protein was gently mixed with 45 μl of Ni^{2+}-NTA beads coupled with the Baf-His or His protein in 100 μl of a binding buffer (1× PBS, 0.01% Nonidet P-40) at 4 °C overnight. In a reciprocal approach, 50 ng of the purified Baf-His protein in 100 μl of the binding buffer was incubated with glutathione-Sepharose beads coupled with Crx-HD-GST or GST. Proteins bound to the beads were washed five times with the wash buffer, eluted from the beads by boiling in 20 μl of a SDS-PAGE loading buffer, resolved by SDS-PAGE (11–15% gel), and detected by immunoblots with the anti-GST antibody (Sigma) (for assays with the His protein beads) or anti-Baf antibody (for assays with the GST protein beads).

Electrophoretic Mobility Shift Assays (EMSAs)—For EMSAs with Crx-HD peptides, the GST tag was removed from the Crx-HD-GST proteins by digestion with thrombin protease (Amersham Biosciences) at a concentration of 2 units/μg of fusion protein (room temperature for 4 h). EMSAs with recombinant proteins and bovine retinal nuclear extracts were performed as described (5). For supershift EMSAs, the Crx-HD peptides or the bovine retinal nuclear extract were preincubated with increasing amounts (in μl) of the Baf-His protein or the His tag control in the reaction buffer for 10 min on ice prior to the addition of the probes. The reactions were incubated on ice for an additional 30 min and resolved by native PAGE (5% gel).

Cell Culture and Transient Transfection Assays—HEK293 cells were cultured on 35-mm plates, transfected using the calcium phosphate method, and analyzed using dual luciferase assays as described by Chen *et al.* (13). Typically, a total of 3.0 μg of DNA was used for each transfection, including 2 μg of the rhodopsin-luciferase reporter pBR130-luc (36), 1 ng of the *Renilla* luciferase reporter pRL-CMV (Promega, Madison, WI) as an internal control for transfection efficiency, 100 ng of a mammalian vector expressing the transcription activator Crx (b*Crx*-pcDNA3.1/HisC), and/or Nrl (pMT-*NRL*), 50–800 ng of the Baf expression vector (b*Baf*-pcDNA3.1(+)/myc), and various amounts of the carrier DNA (pcDNA3.1/HisC) to keep the amount of total DNA constant. Each sample was done in duplicate, and at least four independent experiments were performed. The significance of the results was calculated using Student's *t* test, and it was assumed that each pair of samples under comparison had equal variances. For analyzing the effect of Baf on transactivation activity of the Gal4 fusion proteins and c-Jun/c-Fos, two different luciferase reporters were used: a Gal4-responsive luciferase construct pFR-luc (Stratagene) for assays with Gal4dbd-Crx-(111–299) or Gal4-VP16 (13) and a collagenase promoter-luciferase construct (36) for assays with c-Jun/c-Fos.

Immunocytochemistry and Confocal Microscopy—HEK293 cells were cultured on poly-D-lysine (100 μg/ml; Sigma)-coated glass coverslips and co-transfected with 1 μg of each of the mammalian cell expression constructs *bCrx*-pcDNA3.1/HisC and *bBaf*-pcDNA3.1(+)/myc. At 24 h after transfection, the cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS, pH 7.4) for 15 min, washed three times with PBS, permeabilized in a blocking buffer (5% fetal calf serum, 0.5% Triton X-100, 0.5% glycine in PBS) for 30 min at room temperature, and stained for Baf and Crx. The double staining was performed by sequentially probing with the following antibodies in PBS buffer with 1% bovine serum albumin: anti-Myc (1:400; Santa Cruz Biotechnology), the monoclonal antibody to the expression tag of bBaf, Alexa 488 goat anti-mouse secondary antibody (1:400; Molecular Probes, Inc., Eugene, OR), anti-Crx antibody P261 (1:200), and Rhodamine Red goat anti-rabbit secondary antibody (1:1000; Molecular Probes). For reciprocal double staining, rabbit anti-Baf (1:1000) and mouse anti-Xpress (1:1000), the expression tag of Crx, were used as the primary antibodies. The coverslips were washed three times with the PBS buffer containing 1% bovine serum albumin, mounted on Superfrost/Plus slides (Fisher) with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, California), and examined using a scanning confocal microscope (Model 410; Carl Zeiss, Thornwood, NY). The digitized images were processed and analyzed using Adobe Photoshop version 6.0.

Preparation of Ocular Tissue Sections and Immunohistochemistry—Eyes collected from adult and postnatal day 0–5 (P0–P5) albino mice BALB/c were fixed in 4% paraformaldehyde/PBS, pH 7.4, overnight and processed for routine paraffin histology. Eyes were embedded for sagittal sectioning and cut on a rotary microtome at 5 μm. Sections were mounted on Superfrost/Plus slides and allowed to dry overnight before staining. For embryonic eyes, the whole embryos of embryonic days 12.5, 14.5, and 18.5 were dissected from sacrificed timely pregnant BALB/c mothers, treated as described above, embedded with the top of the head down in the block, and serially sectioned. Sections containing the eyes were saved for future staining. The pineal gland dissected from the brain of an adult Lewis rat (Charles River Laboratories, Wilmington, MA) was processed in a similar manner. For immunostaining of Baf, sections were deparaffinized in xylene and 100% alcohol, blocked with 3% hydrogen peroxide (H_2O_2) in methanol for 30 min to remove endogenous peroxidase, and rehydrated in 95 and 70% alcohol and distilled water, followed by heat-induced epitope retrieval. Heat-induced epitope retrieval was performed in citrate buffer (pH 6.0) using a Decloaking Chamber (Biocare Medical, Walnut Creek, CA). Heat-induced epitope retrieval-treated slides were rinsed in distilled water and placed in PBS, blocked with 20% normal donkey serum for 30 min, and probed with rabbit anti-Baf (1:1000) or its control serum overnight at 4 °C. The Baf labeling was detected using Vectastain Elite Rabbit IgG ABC kit (Vector Laboratories Inc., Burlingame, CA) and DAB peroxidase substrate (Sigma) according to the manufacturer's instructions. After color development, the slides were washed in PBS, dehydrated in alcohol, cleared in xylene, and coverslipped with a resinous mounting medium (Permount; Fisher). The results were examined by light microscopy (Olympus BH-2) and photographed using a Spot SP100 Cooled Color Digital Camera (Diagnostic Instruments Inc.). Immunostaining of Crx was performed similarly as described above except using the Crx antibody P261 at a 1:800 dilution and hematoxylin (Harris Formula; Surglpath Medical Industries, Inc.) for counterstaining of nuclei of retinal sections (*light blue*). The retinal sections used for Crx staining were made from an eye of the adult C57BL/6 mouse (wild type) or a mutant mouse homozygous for a null allele of *Crx* ($Crx^{-/-}$) at 14 days of age (kindly provided by Drs. Takahisa Furukawa and Connie Cepko).

Bioinformatics Analysis of the Human BAF Gene—Similarity searches were performed by using BLAST software (37) to scan the human genome data base from NCBI (available on the World Wide Web at www.ncbi.nlm.nih.gov) and from Celera Corp. (available on the World Wide Web at www.celera.com). Matching output sequences from the BLAST analyses as well as STS marker sequences were positioned on the genome sequence using the resources provided by the NCBI human genome Web site (www.ncbi.nlm.nih.gov/genome/guide/human/). The software ClustalW (38) (available on the World Wide Web at www2.ebi.ac.uk/clustalw) was used for both sequence alignment and for computing phylogenic relationships among sequences, which were graphically elaborated by the program DRAWTREE (39) (available on the World Wide Web at bioweb.pasteur.fr/seqanal/interfaces/drawtree-simple.html).

Screening of Patients with Photoreceptor Degeneration for Mutations in BAF—Most of the patients were recruited from the Berman-Gund Laboratory for the Study of Retinal Degenerations, where they were diagnosed through eye examinations including funduscopy and electroretinography. Patients with Bardet-Biedl syndrome, Leber congenital amaurosis, and retinitis pigmentosa were included in this study. Inheritance patterns were inferred from family history. Informed consent was obtained from all participants, in accordance with the tenets of the Declaration of Helsinki, before they donated 10–30 ml of blood for this research. DNA was purified from peripheral blood leukocytes by standard procedures. Blood samples were also obtained from control individuals with no visual symptoms of and no known blood relatives with hereditary retinal degeneration.

The human *BAF* coding regions were PCR-amplified from the leukocyte DNA samples from patients and from controls using the following primers: exon 2, primer 4546 (5′-GCCCTAATCTGCCTTTTTTTTGGG-3′) and 4547 (5′-GCACTAGGTACACGCAGCCACCCC-3′); exon 3, 4548 (5′-AGCAGCACGCTCCTTCCTTTTCCC-3′) and 4549 (5′-TGGATGAGGGCTGGGGATTGAGAG-3′), according to previously published methods (40). ^{33}P-Radiolabeled PCR products were scanned for mutations by single-stranded conformation polymorphism analysis in two nondenaturing acrylamide gels, one with and one without 10% glycerol. Samples with an abnormal single-stranded conformation polymorphism analysis pattern were amplified a second time and analyzed by direct sequencing in both sense and antisense directions.

RESULTS

Baf Interacts with Crx in Yeast Two-hybrid Assays—To identify Crx-interacting proteins, the yeast two-hybrid assay was employed using a set of bait constructs that would produce fusion proteins containing wild-type bovine Crx with or without N- and C-terminal deletions (13). Upon transformation to the reporter yeast strain Y190, only the homeodomain (Crx-HD) bait showed no autoactivation of the *His3* and *lacZ* reporter genes (data not shown), suggesting that other regions of Crx,

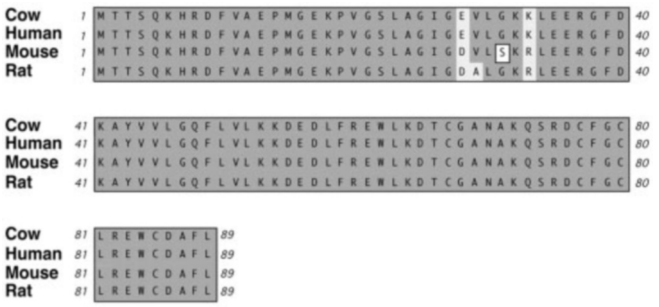

FIG. 1. **Comparison of amino acid sequences of Baf from cows, humans, mice, and rats.** The predicted amino acid sequence from an open reading frame (89 amino acids) of the bovine cDNA clone described here is shown at the *top*. Residues that are identical in all four species are *shaded* in *dark gray*, whereas conservative changes are *shaded* in *light gray*. A nonconservative change is indicated by a *white box*.

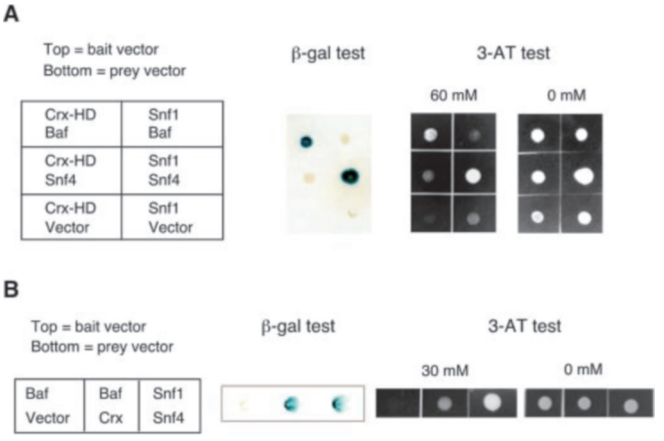

FIG. 2. **Baf interacts with Crx in yeast two-hybrid assays.** *A*, using Crx-HD as bait; *B*, using bovine Baf as bait. The indicated bait and prey vectors were cotransformed into the yeast Y190 cells and analyzed for the reporter activity of *lacZ* and *His3* using β-galactosidase (*β-gal*) and 3-AT tests as described under "Experimental Procedures." A positive interaction is scored by growing on 3-AT medium and producing a *blue color* on an X-gal filter.

particularly the C-terminal region, contain a transactivation domain(s). This is consistent with the results of deletion analysis of Crx in mammalian cells (13, 41).

Crx-HD was subsequently used as bait for screening a bovine retinal cDNA library (34). After screening 1.7×10^6 co-transformed yeast cells, eight clones grew on a His-minus medium with 15 mM 3-AT (a competitive inhibitor of His3) and produced a blue color on X-gal filters. Sequence analysis revealed that one of the dual positive clones encoded phosducin (Phd), a photoreceptor protein already known to interact with Crx (26). Two other clones that were positive in both reporter assays harbored an identical cDNA insert of 0.6 kb with a predicted open reading frame of 89 amino acids (accession number AF529228) plus an additional 28 amino acids derived from the 5′-untranslated region at its N terminus fused in-frame with the Gal4 activation domain. Sequence comparison showed that the open reading frame encoded a protein with a predicted amino acid sequence identical to that of the human barrier-to-autointegration factor BAF (42) (Fig. 1). Baf is highly conserved among different mammalian species. At the amino acid level, bovine Baf shares 100, 97, and 97% homology, respectively, with its orthologs in humans, mice, and rats. At the nucleotide level, bovine *Baf* shares 93% homology with human *BAF* in the coding region (data not shown).

To test the specificity of Baf-Crx-HD interaction, Y190 cells were co-transformed with the bBaf prey plasmid and each of the pAS bait plasmids, Crx-HD, the empty vector, and Snf1 (unrelated bait as a negative control), respectively. Double transformants were analyzed by the dual reporter assays. Fig. 2*A* shows that expression of the *His3* and *lacZ* reporter gene was activated only in yeast cells harboring both the Crx-HD bait and Baf prey or the positive control partners Snf1 and Snf4 (35). The presence of either the Snf1 or empty bait vector with the Baf prey did not yield a positive result under identical conditions, suggesting that Baf does not interact with Gal4-dbd and that the interaction of Baf and Crx-HD is specific.

To further establish the specificity of a Baf-Crx interaction, an insert swap between the bait and prey plasmid was performed; the coding region of *bBaf* was cloned in frame with Gal4-dbd in the pAS2 bait vector, whereas the full coding region of bovine *Crx* (*bCrx*) was cloned in-frame with the Gal4 activation domain in the pACTII prey vector. The resulting bait and prey plasmids were co-transformed into Y190 cells and analyzed using the dual reporter assay. Fig. 2*B* shows that a positive interaction was detected with the Baf bait and Crx prey but not with the empty prey vector, demonstrating that Baf interacts with the full-length Crx in yeast.

Baf Binds Directly to Crx in Vitro—To test whether Baf binds to Crx directly, bacterially expressed His_6-tagged Baf (Baf-His), His_6 alone control (His), GST-tagged Crx-HD (Crx-HD-GST), and GST alone control proteins were purified using Ni^{2+}-NTA and glutathione-Sepharose beads, respectively, and used for pull-down assays. Purified Baf-His shows a doublet on an SDS-PAGE gel with an apparent molecular mass of 10 kDa, which is consistent with the calculated molecular mass for the fusion protein with a His_6 tag (3 kDa for the His tag alone) (Fig. 3*A*, *lane 3 versus lane 2*). It is unclear if the doublet resulted from degradation or contamination of other proteins. Fig. 3*B* shows that the Baf-His protein was pulled down by the Crx-HD-GST beads but not the GST control beads (*lane 2 versus lane 1*). In a reciprocal experiment, the Crx-HD-GST protein was pulled down by the Baf-His beads but not the His control beads (Fig. 3*C*, *lane 2 versus lane 1*). These results suggest that Baf interacts with the Crx homeodomain directly.

To determine whether other regions of Crx could mediate Crx-Baf interaction, co-immunoprecipitation assays with *in vitro* translated proteins were performed using a ^{35}S-labeled-Baf, a polyclonal antibody P261 against Crx (28), and either a full-length Crx or its deletion mutant Crx-(111–299), where the homeodomain and its flanking 38 amino acids at the N terminus are removed (13). Fig. 3*D* shows that Baf was co-immunoprecipitated with the full-length Crx (Crx-(1–299)) by the Crx antibody (*lane 1*), which did not occur in the absence of either the Crx protein (*lane 2*) or the Crx antibody (*lane 3*). This result, consistent with that obtained from the yeast two-hybrid assays, provides further evidence that Baf interacts directly with the full-length Crx. In contrast, Baf was not co-immunoprecipitated with the N-terminal truncation mutant Crx-(111–299), (*lane 4*), suggesting that the homeodomain (plus its N-terminal flanking sequence) is necessary for Crx to bind to Baf.

Baf Binds to Several Homeodomain Transcription Factors, but Not the bZIP Protein Nrl—Since the homeodomain of Crx is highly homologous to that of Otx1 (88%) and Otx2 (86%) (5), it seemed likely that Baf could interact with Otx1 or Otx2. To test this possibility, we performed co-immunoprecipitation assays with *in vitro* translated Otx1 or Otx2 (^{35}S-labeled) and a Myc-tagged Baf using an anti-Myc antibody. As predicted, both Otx1 and Otx2 can be co-immunoprecipitated with Baf (data not shown), suggesting that Baf can interact directly with Otx1 and Otx2.

Since the homeodomain of Otd/Otx family belongs to the K_{50} subtype of the paired-like class, we decided to evaluate whether Baf could interact with Chx10, a Q_{50} subtype of the paired-like

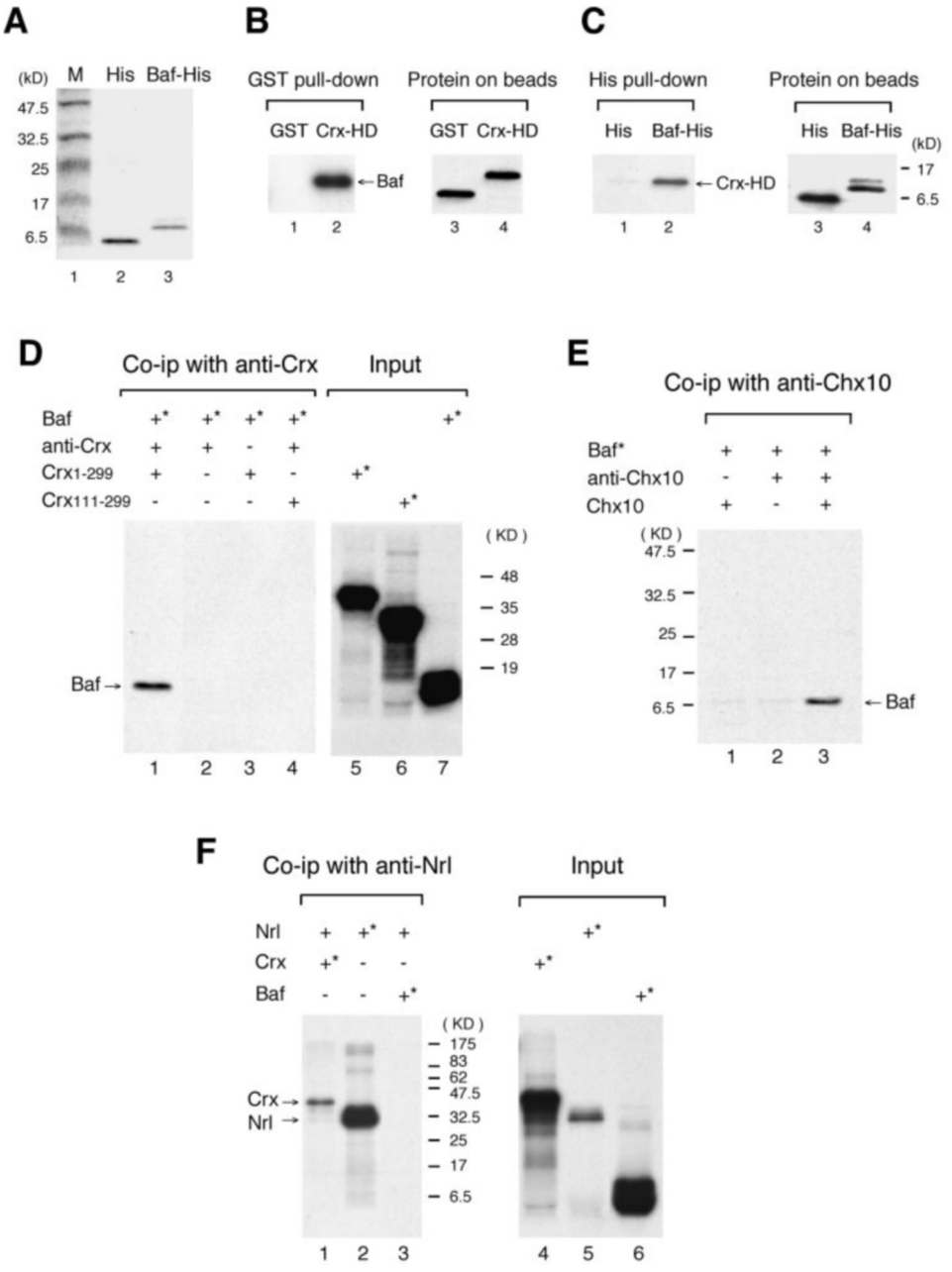

FIG. 3. **Baf directly interacts with Crx *in vitro* via the homeodomain.** *A*, SDS-PAGE (12% gel) demonstrating the purification of bacterially expressed bovine Baf-His and His proteins. A proximal 100 ng of the indicated protein and a prestained protein marker M (New England Biolabs) were loaded on the gel and stained with Coomassie Blue. The apparent molecular mass of each marker band in kilodaltons is shown on the *left*. *B*, GST pull-down assays. 50 ng of the purified Baf-His protein was incubated with glutathione-Sepharose beads coupled with either the GST (*lane 1*) or Crx-HD-GST (*lane 2*) protein. After stringent washes, bound proteins were eluted from the beads, resolved by SDS-PAGE (12% gel), and immunoblotted with an anti-Baf antibody. The presence of GST and Crx-HD-GST protein on the beads was confirmed by SDS-PAGE and immunoblots with an anti-GST antibody (Sigma) (*lanes 3* and *4*). *C*, His pull-down assays. 100 ng of the Crx-HD-GST protein was incubated with Ni^{2+}-NTA beads (Qiagen) coupled with either the His (*lane 1*) or Baf-His (*lane 2*) protein. Proteins bound to the beads were treated as described for *B* and analyzed with the anti-GST antibody. The presence of the purified His and Baf-His protein on the beads was confirmed by SDS-PAGE and immunoblots with an anti-polyhistidine antibody (Sigma) (*lanes 3* and *4*). *D*, co-immunoprecipitation (*co-ip*) assays using *in vitro* translated Baf and Crx proteins and a Crx antibody. A Myc-tagged bovine Baf, the full-length bovine Crx (Crx-(1–299)), and its N-terminal deletion (Crx-(111–299)) were generated using a TnT T7 Quick Coupled Transcription/Translation kit (Promega) in the presence (marked by an *asterisk*) and absence of [^{35}S]methionine. The indicated translation products were used for co-immunoprecipitation by the anti-Crx antibody P261 (*lanes 1–4*). *Lanes 5–7*, the input controls without co-immunoprecipitation (one-twentieth of the volume used for co-immunoprecipitation). *E*, co-immunoprecipitation of Baf with Chx10. Co-ip was performed using *in vitro* translated Baf (^{35}S-labeled; *asterisk*), Chx10, and a Chx10 antibody. *Lanes 1* and *2*, are negative controls without the addition of the Chx10 antibody or protein, respectively. *F*, Baf does not co-immunoprecipitate with Nrl. The indicated ^{35}S-labeled (*asterisk*) and unlabeled *in vitro* translated proteins were analyzed by co-immunoprecipitation assays with the anti-Nrl antibody (*lanes 1–3*). *Lanes 4–6*, the input controls (one-twentieth of the volume used for co-immunoprecipitation).

homeodomain protein expressed in the retina (43). Fig. 3*E* shows that Baf was co-immunoprecipitated with Chx10 by an antiserum to Chx10, suggesting that Baf also interacts directly with Chx10. In addition, *in vitro* co-immunoprecipitation assays also detected an interaction of Baf with Pax-6, an S_{50} subtype of the paired-like homeodomain (44) (data not shown). Thus, Baf interacts with all three subtypes of paired-like homeodomain proteins.

To determine the specificity of Baf-homeodomain interactions, we searched for evidence that Baf might bind to the Maf

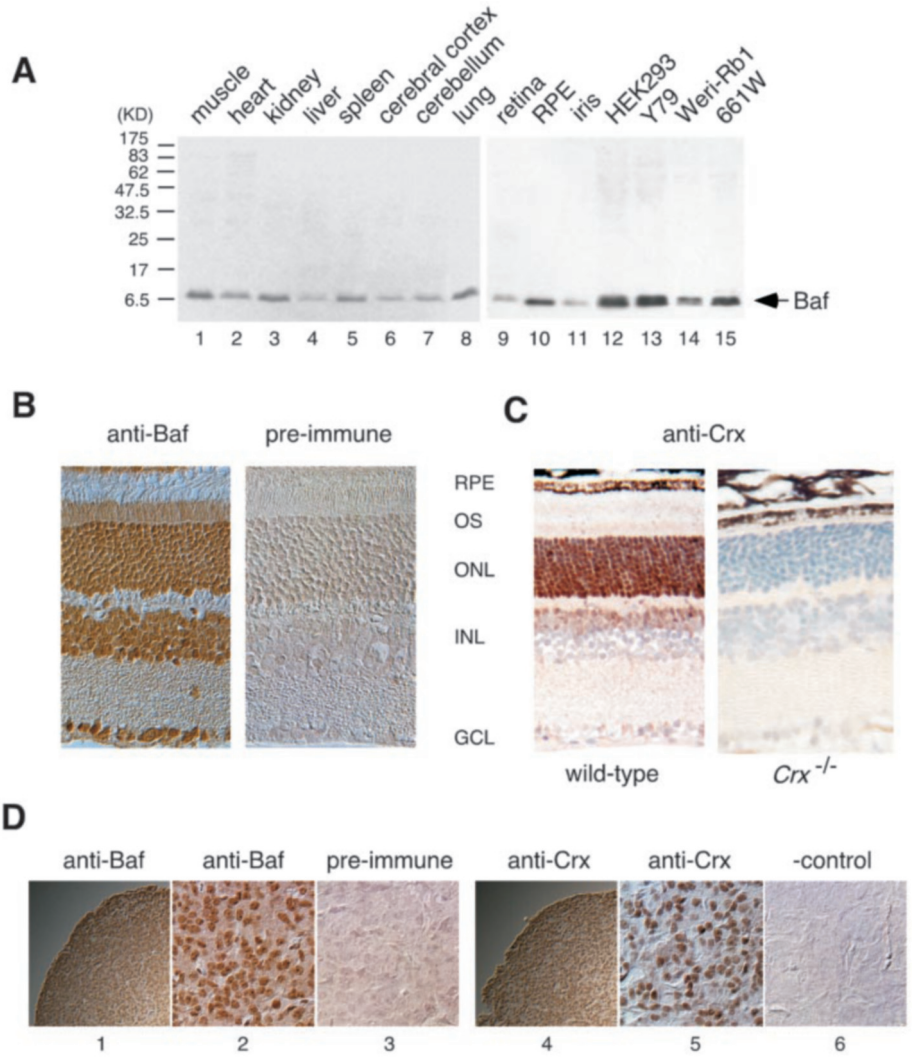

FIG. 4. **Baf is ubiquitously expressed, including neurons in the retina and pinealocytes of the pineal gland that express Crx.** *A*, immunoblot analysis. 8 μl of whole cell extracts were separated by SDS-PAGE (15% gel), transferred to polyvinylidene difluoride membranes (Bio-Rad), and probed with a rabbit polyclonal antibody against Baf at a 1:1000 dilution. *Lanes 1–8*, extracts made from the indicated tissues of a rat (Brown Norway; Charles River Laboratories). *Lanes 9–11*, extracts made from the indicated tissues dissected from a bovine eye; *RPE*, retina pigment epithelium. *Lanes 12–15*, extracts made from cultured cell lines: HEK293 (human embryonic kidney; ATCC), Y79 (human retinoblastoma; ATCC), Weri-Rb-1 (human retinoblastoma; ATCC), and 661w (SV40 T-antigen-transformed 661 mouse photoreceptor cell line) (71). *B*, immunostaining of Baf on retinal sections from adult mice (BALB/c) using the anti-Baf or its control (preimmune) serum. The cell layer labels are as follows. *RPE*, retinal pigment epithelium; *OS*, outer segments; *ONL*, outer nuclear layer; *INL*, inner nuclear layer; *GCL*, ganglion cell layer. *C*, immunostaining of Crx (*brown color*) on retinal sections from a wild-type (C57BL/6) and a homozygous Crx knock-out mouse ($Crx^{-/-}$) using anti-Crx antibody P261. The sections were counterstained with hematoxylin to show nuclei (*light blue*). *D*, immunostaining of Baf (D1, D2) and Crx (D4, D5), respectively, on pineal gland sections from an adult Lewis rat. D3 received the control serum for Baf (preimmune), whereas D6 did not receive any primary antibody. D2, D3, D5, and D6 represent a higher magnification (×400) of D1 and D4 (×100).

family neural retina leucine zipper protein Nrl (7), which is specifically expressed in the rod photoreceptor cells (12). Nrl contains a basic motif-leucine zipper domain that is thought to mediate its binding to DNA (45) and interaction with Crx (15). As shown in Fig. 3*F*, an anti-Nrl antibody failed to bring down Baf with Nrl in a co-immunoprecipitation assay (*lane 3*), although it co-immunoprecipitated Crx with Nrl in a control experiment under similar conditions (*lane 1*). Furthermore, no interaction between Baf and Nrl was detected using a yeast two-hybrid assay (data not shown).

Baf and Crx Are Co-localized in the Nucleus and Can Be Co-immunoprecipitated from a Retinal Extract—To gain insights into a possible relevance of a Baf-Crx interaction *in vivo*, the expression pattern of the Baf protein was examined using immunoblots of whole cell extracts probed with a polyclonal antibody against the human BAF. Fig. 4*A* shows that the Baf antibody recognized a doublet with an apparent molecular mass of ~7 kDa in protein extracts made from all tissues (*lanes 1–11*) and cell lines (including those with retinal and nonretinal origins; *lanes 12–15*) examined, including retina, retinal pigment epithelium, and iris of the eye. The size of the detected protein is consistent with the predicted molecular weight of Baf and with the reported molecular weight of purified human BAF (42). Although the doublet can be observed in all of the *lanes*, it is more apparent with cultured cell lines that contain a mixture of cells in various stages of the cell cycle. It is unclear whether this doublet results from a post-translational modification of Baf or protein degradation. The ubiquitous expression of Baf suggests that it may be essential for cellular function in both dividing and nondividing (terminally differentiated) cells. To examine Baf expression in the retina, immunohistochemical studies were performed using the Baf antibody and retinal sections from adult and embryonic mouse eyes. As shown in Fig. 4*B*, the anti-Baf antiserum stained all of the nuclear layers of the adult retina. A control serum (preimmune) did not produce any signal under the same conditions, suggesting that the staining by the anti-Baf antiserum is specific. In contrast, the anti-Crx antibody P261 stained intensely the outer nuclear layer (*ONL*) of the wild-type mouse retina, where the photoreceptor nuclei are localized, and also stained lightly the outer part of the inner nuclear layer (*INL*), where bipolar cells reside (Fig. 4*C*). For a negative control, P261 did not stain the retina of a mutant mouse ($Crx^{-/-}$) that is homozygous for a null allele of *Crx* (8), suggesting that the staining observed for the wild-type retina by P261 is specific to Crx. Furthermore, double labeling with P261 and a Chx10 antibody confirmed that those inner nuclear layer cells positive for Crx expression are actually bipolar cells (data not shown). These results demonstrated that Crx and Baf are co-expressed in the nuclei of photoreceptor cells as well as in bipolar cells of the retina. The Baf immunoreactivity was also found in the nuclei of the developing mouse retina at all of the developmental stages tested (as early as embryonic day 12.5; data not shown), suggesting that Baf may play an important role in the development and main-

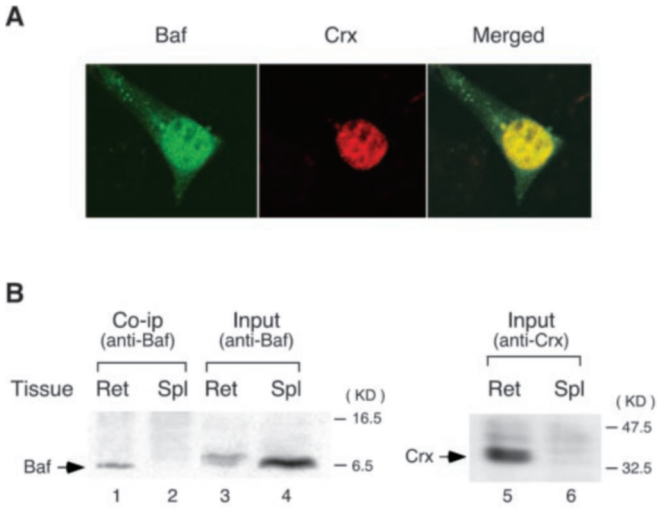

FIG. 5. **Baf and Crx are co-localized in the nucleus of co-transfected 293 cells and can be co-immunoprecipitated from a bovine retinal extract.** *A*, immunostaining of Crx (*red*) and Baf (*green*) in HEK293 cells co-transfected with their expression vectors using antibodies to Xpress (the expression tag of Crx) and Baf. *B*, co-immunoprecipitation (*Co-ip*) of Baf with Crx from a retinal extract. 200 μl of whole cell extracts made from bovine retina (*Ret*) and spleen (*Spl*) were incubated with the anti-Crx antibody P261, precipitated by Protein A beads, and immunoblotted with the anti-Baf antibody (*lanes 1* and *2*). *Lanes 3–6*, input controls (without immunoprecipitation) loaded with 10 μl of the indicated extracts and immunoblotted with either anti-Baf (*lanes 3* and *4*) or anti-Crx P261 antibody (*lanes 5* and *6*).

tenance of the retina by acting in the nucleus. In addition, to examine whether Baf is co-expressed with Crx by pinealocytes in the pineal gland, we performed immunostaining of Baf and Crx using paraffin sections from a rat pineal gland. As shown in Fig. 4*D*, both the anti-Baf antiserum and anti-Crx antibody stained pinealocytes intensely. The staining patterns are very similar and appear to be nuclear, suggesting that Baf may also interact with Crx in the pineal gland and play a role in pineal function.

Since both anti-Baf and anti-Crx antibodies were generated in rabbits, double staining of Crx and Baf in the retinal or pineal sections is technically difficult to perform. To confirm that Crx and Baf are co-localized in the nucleus of cells that express both proteins, HEK293 cells were co-transfected with constructs overexpressing an Xpress-tagged Crx and a Myc-tagged Baf. The subcellular localization of the two recombinant proteins in co-transfected cells was examined using immunocytochemistry with double labeling and confocal microscopy. Two pairs of rabbit/mouse antibodies to the recombinant Crx and Baf were used for double labeling, either rabbit anti-Crx/mouse anti-Myc (Baf) or rabbit anti-Baf/mouse anti-Xpress (Crx). Double staining with both pairs of antibodies yielded very similar results. As shown in Fig. 5*A*, Crx (*red*) and Baf (*green*) are co-localized in the nucleus of the co-transfected cells, although a small amount of Baf is also seen in the cytoplasm.

To further examine Crx-Baf interaction, co-immunoprecipitation assays were performed using the Crx antibody P261 and protein extracts made from the bovine retina and other tissues. The presence of Baf in the immunoprecipitated protein complex was revealed by immunoblots with the anti-Baf antibody. Fig. 5*B* shows that the Crx antibody co-immunoprecipitated Baf from the retinal extract (*lane 1*) but not from the spleen extract (*lane 2*) that does not contain Crx (demonstrated by the immunoblot with anti-Crx, *lane 6*), despite a higher amount of Baf detected in the spleen than in the retinal extract (*lane 4 versus lane 3*). These results, combined with the co-localization of both proteins in the nucleus of co-transfected cells and retinal photoreceptor cells, suggest that Baf and Crx interact *in vivo*.

Baf Represses the Transactivation Activity of Crx—To address the physiological relevance of a Baf-Crx interaction, we examined whether overexpression of Baf has any effect on the transactivation activity of Crx using transient transfection assays. It has been established that Crx is able to *trans*-activate the expression of a rhodopsin promoter-luciferase reporter (pBR130-luc), either on its own or in combination with Nrl, in transiently transfected HEK293 cells (5). We then included a mammalian vector expressing the Baf, in various ratios to the vector(s) expressing Crx, Nrl, and Crx plus Nrl, respectively, in the transactivation assays. The resulting activities of transactivation were compared with those obtained from samples receiving the empty vector (100%). Consistent with the previous reports (5), the rhodopsin reporter alone had little if any activity in HEK293 cells (data not shown). The promoter activity was increased by 3–6-fold when co-transfected with either Crx or Nrl alone and by 60–80-fold with both together. In contrast, co-transfection of the Baf expression vector alone with the reporter did not produce any detectable effect on rhodopsin promoter activity (data not shown), suggesting that Baf, on its own, does not regulate rhodopsin promoter activity. However, when both the Crx and Baf expression vectors were included in the co-transfection assays, Baf significantly decreased the Crx-dependent transactivation activity in a concentration-dependent manner, ranging from 34% ($p = 0.00025$) at a *Baf/Crx* ratio of 0.5:1 to 60% ($p = 5.4 \times 10^{-7}$) at a ratio of 4:1 (Fig. 6*A*). In contrast, Baf did not significantly ($p > 0.05$) repress the Nrl-dependent activity under similar conditions, even at the higher *Baf/Nrl* ratio tested (4:1) (Fig. 6*A*). To examine whether the repression effect of Baf is dependent on the Crx homeodomain, we used a fusion protein construct, Gal4dbd-Crx-(111–299), lacking the homeodomain in transfection assays. This construct can transactivate a Gal4-responsive promoter-luciferase reporter in 293 cells (13). The addition of increasing amounts of the Baf expression vector with this Gal4dbd-Crx fusion construct did not repress Crx-mediated transactivation (data not shown), suggesting that the homeodomain is needed for Baf to repress Crx. Furthermore, Baf failed to show any effect on transactivation activity of Gal4-VP16 for the Gal4-responsive promoter-luciferase reporter or on the activity of c-Jun and c-Fos for a collagenase promoter-luciferase reporter (data not shown). These results suggest that Baf is not a general repressor of transcription and that its repression effect on Crx requires an interaction with the homeodomain. Consistent with this, Baf also decreased the synergistic activity of Crx plus Nrl in a concentration-dependent manner, ranging from 26% ($p = 0.025$) at a Baf/activator ratio of 1:1 to 64% ($p = 0.00028$) at a ratio of 4:1 (Fig. 6*A*).

Baf Does Not Abolish the DNA Binding Activity of Crx—Since overexpression of Baf did not show any effect on either the basal activity of the rhodopsin promoter or the transactivating activity of Nrl and Gal4-VP16, the mechanism by which Baf represses Crx is likely to be specific to Crx. One such mechanism could be to block the ability of Crx to bind to its targets, especially considering that Baf interacts with the Crx homeodomain. We decided to test this possibility using EMSAs with recombinant Crx and Baf proteins. Due to technical difficulties in the expression and purification of a full-length recombinant Crx from *E. coli*, we carried out EMSAs using the purified Crx-HD peptides and DNA probes carrying three Crx target sites (BAT-1, Ret-1, and Ret-4) of the rhodopsin promoter, in the absence and presence of a His_6-tagged recombi-

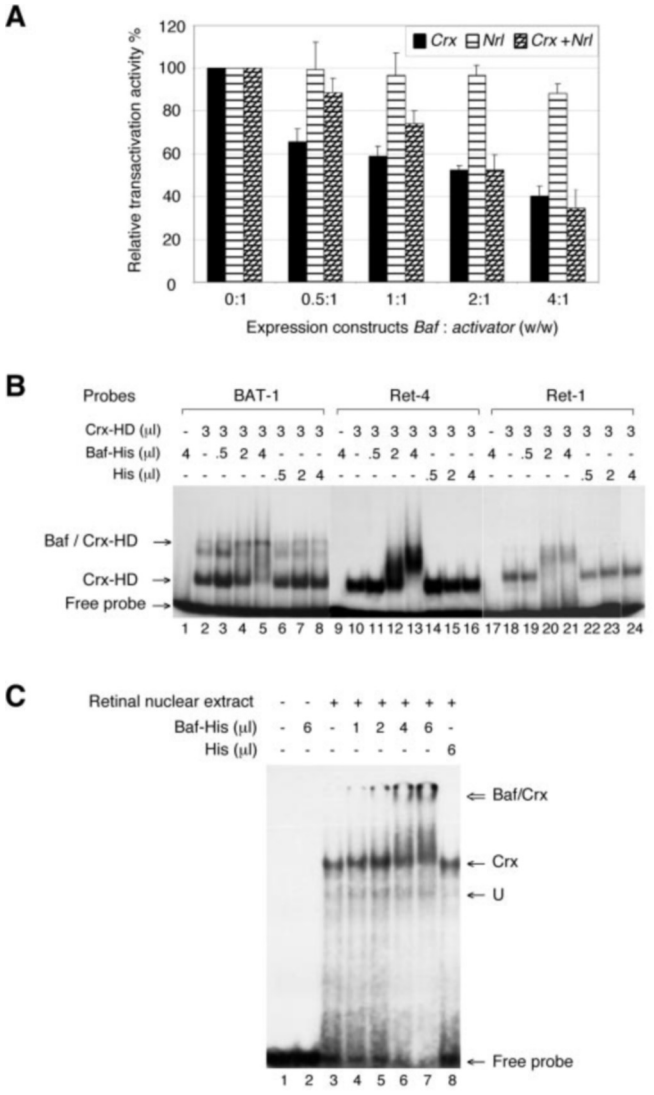

FIG. 6. **Baf binds to Crx-DNA complexes and represses Crx-mediated transactivation of the rhodopsin promoter in HEK293 cells.** *A*, Baf represses the ability of Crx, but not Nrl, to transactivate a rhodopsin-luciferase reporter. HEK293 cells were co-transfected with a total of 3 μg of DNA, including 2 μg of pBR130-luc containing the bovine rhodopsin promoter (−130 to +70 bp) fused to a luciferase reporter (36); an internal control plasmid pRL-CMV (Promega) for transfection efficiency; 100 ng of expression vector carrying the coding region of the transcription activator Crx (*bCrx*-pcDNA3.1/HisC, *solid black*), Nrl (pMT-*hNRL*, *horizontal lines*), or Crx plus Nrl (*hatched pattern*); and the indicated amount (presented as a ratio relative to the total amount of the activator DNA with 0:1 for the samples without Baf) of the Baf expression vector *bBaf*-pcDNA3.1(+)/myc. Transactivation activity (-fold activation) for each transfection was calculated by comparing the relative luciferase activity (after normalized with the internal control) with that obtained from the pBR130-luc reporter alone (0-fold). The data are presented as mean values of percentage of transactivating activity relative to the values obtained from those samples without the *Baf* vector (100%). The *error bars* represent the S.E. from four independent experiments ($n = 4$). *B*, Baf binds to Crx-HD-DNA complexes. EMSA reactions were carried out using a Crx-HD peptide and three ^{32}P-labeled oligomer probes harboring Crx target sites from the bovine rhodopsin promoter, BAT-1 (*lanes 1–8*), Ret-4 (*lanes 9–16*), and Ret-1 (*lanes 17–24*) (5). The indicated amount (μl) of the Baf-His (*lanes 3–5*, *11–13*, and *19–21*) or the His control (*lanes 6–8*, *14–16*, and *22–24*) protein was preincubated with the Crx-HD peptide in the reaction buffer for 10 min on ice before mixing with a 1 nM concentration of the indicated probes. The reactions were then incubated on ice for an additional 30 min and resolved by native PAGE (5% gel). The concentration of each purified protein was about 40 ng/μl for Baf-His and His alone and 10 ng/μl for Crx-HD. *C*, Baf binds to a native Crx-DNA complex. EMSAs were carried out using 5.0 μg of a bovine retinal nuclear extract and 0.5 nM ^{32}P-labeled Ret-4 oligomer as the probe. The presence of protein-DNA complexes is indicated by the *arrows*, marked *Crx* and *U* for the binding activity of Crx and a ubiquitously expressed protein, respectively, as demonstrated previously (5). The indicated amount (in μl) of the Baf-His (*lanes 4–7*) or the His control (*lane 8*) protein was preincubated with the retinal nuclear extract in the reaction buffer for 10 min on ice prior to the addition of the probe. A supershift containing the Baf-Crx-DNA complex observed with *lanes 4–7* is marked by a *double arrow*. *Lane 1* did not receive any protein, whereas *lanes 2* and *3* received only the Baf-His protein or the retinal nuclear extract, respectively.

nant Baf protein (Baf-His) (Fig. 6*B*). In the absence of Baf, Crx-HD produced a band shift(s) with each probe, as reported previously (13) (*lanes 2*, *10*, and *18*). However, preincubation of Crx-HD with increasing amounts of recombinant Baf resulted in supershifts of Crx-HD/DNA bands (*lanes 3–5 versus lane 2*; *lanes 11–13 versus lane 10*; *lanes 19–21 versus lane 18*). The supershifts did not occur with the His control under the same conditions (*lanes 6–8*, *14–16*, and *22–24*). In addition, the Baf protein alone, even at the highest concentration tested, did not produce a visible shift with each of the three probes (*lanes 1*, *9*, and *17*). To test whether this phenomenon could occur with a native Crx in the retina, supershift EMSAs were carried out using a bovine retinal extract and the Ret-4 probe in the presence of increasing amounts of the recombinant Baf. Fig. 6*C* shows that, in the absence of the recombinant Baf (*lane 3*), the retinal nuclear extract produced two shifted bands with the Ret-4 probe as demonstrated previously (5). The major band (labeled *Crx*) represents the binding activity of Crx, whereas the minor band (labeled *U*) represents the binding activity of a ubiquitously expressed protein. Preincubation of the retinal nuclear extract with the increasing amounts of the recombinant Baf (Baf-His) supershifted the Crx band to a higher position(s) (labeled *Baf/Crx*) without affecting the U band in a concentration-dependent manner (*lanes 4–7 versus lane 3*). This supershift was not observed with the His-negative control (*lane 8*). Together, these results suggest that interacting with Baf does not abolish the ability of Crx (or Crx-HD) to bind to its DNA targets *in vitro*.

E80A, a Homeodomain Mutation of Crx Associated with Autosomal Dominant Cone-Rod Dystrophy, Interferes with Both the Physical and Functional Interaction of Crx and Baf—Since Baf interacts with the homeodomain of Crx, it is possible that mutations in the Crx homeodomain could have some effect on Crx-Baf interaction. To test this possibility, we carried out *in vitro* protein-protein interaction assays using recombinant Baf and five mutant forms of human CRX carrying homeodomain mutations identified from photoreceptor degenerative diseases. Among the five CRX mutations, four (R41Q, R41W, E80A, and R90W) are likely to be pathogenic, since they co-segregate with either autosomal dominant cone-rod dystrophy (CORD2) or Leber congenital amaurosis, whereas one (A56T) is of uncertain pathogenicity because it has not been shown to co-segregate with a disease (18, 21, 23). Previous biochemical analysis of these mutants has demonstrated that A56T does not cause any detectable defects in CRX function and therefore is likely to be a non-disease-causing variant (13). In contrast, the four mutations co-segregating with disease cause abnormalities in the DNA binding and/or transactivation activity of CRX (13). Interestingly, the E80A mutation leads to a "hyperactive" CRX with a transactivation activity 2-fold greater than wild-type CRX (13). To test whether these mutant forms of CRX could interact with Baf in a similar manner as wild-type CRX, *in vitro* co-immunoprecipitation assays were carried out using a Myc-tagged Baf, anti-Myc antibody, and ^{35}S-labeled CRX proteins carrying each of the five homeodomain mutations. Fig. 7*A* demonstrates that the E80A mutant had a lower binding affinity to Baf than the wild-type CRX and the non-disease-causing

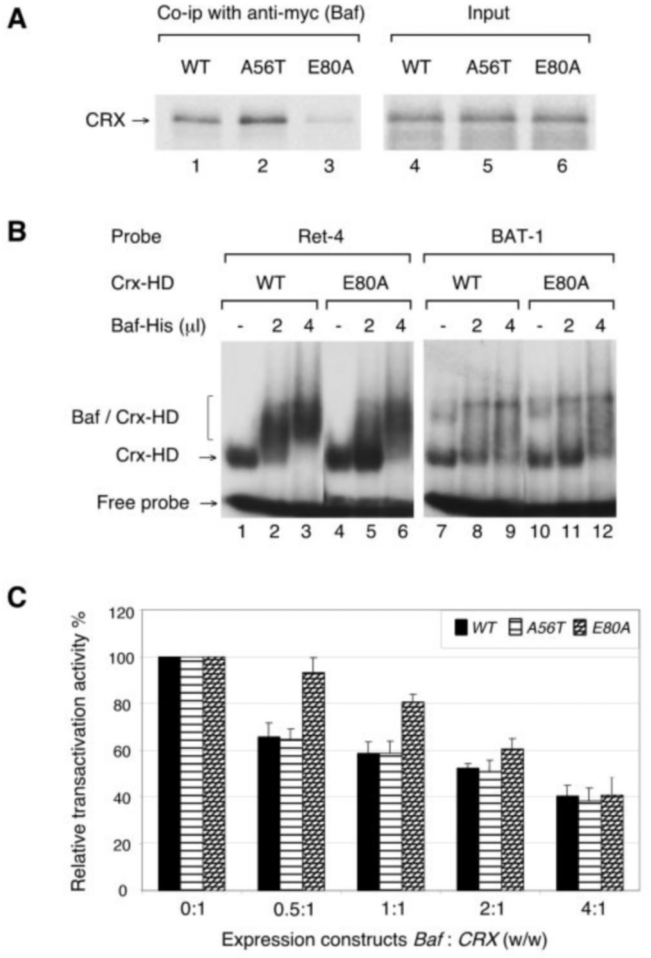

FIG. 7. **E80A, a homeodomain mutation of CRX, reduces the strength of CRX-Baf interaction.** *A*, E80A interferes with the physical interaction of CRX and Baf: co-immunoprecipitation assays. *In vitro* translated, ^{35}S-labeled human CRX (wild type (*WT*); *lane 1*) or its mutants E80A (*lane 3*) and A56T (*lane 2*) were incubated with a Myc-tagged Baf and immunoprecipitated using an anti-Myc antibody (Santa Cruz Biotechnology, Inc.). *Lanes 4–6* are input controls (one-twentieth volume used for co-immunoprecipitation) for *in vitro* translated CRX proteins. *B*, E80A interferes with the physical interaction of Crx and Baf: supershift EMSAs. Equal amounts of the wild-type and mutant (E80A) Crx-HD peptide were incubated with the Ret-4 and BAT-1 probe in the presence of the indicated amount of the Baf-His protein. *C*, E80A interferes with the functional interaction of CRX and Baf: co-transfection assays. The potency of Baf to repress the wild-type and two mutant CRXs (E80A and A56T) was measured by transient co-transfection assays in HEK293 cells. 100 ng of the *hCRX* expression vector and an increasing amount of the *Baf* expression vector (presented as a ratio of *Baf*/*CRX*) were included in each co-transfection assay with the rhodopsin promoter-luciferase construct pBR130-luc as the reporter. The results were analyzed as described in the legend to Fig. 6*A* and presented as percentage of transactivation activity relative to those of samples without the *Baf* vector (*Baf*/*CRX* = 0:1, 100%). The *error bars* represent S.E. from four independent experiments ($n = 4$).

variant A56T (*lane 3 versus lanes 1* and *2*). Further analysis of three independent experiments using PhosphorImager assays showed that the E80A mutation reduces the ability of CRX to bind to Baf by 50% (data not shown). None of the other three CRX mutants (R41Q, R41W, and R90W) had an affinity for Baf that was significantly different from wild-type CRX (data not shown). To confirm these results, supershift EMSAs with two different probes were carried out using recombinant Baf and purified Crx-HD wild-type and E80A peptides. As shown in Fig. 7*B*, the minimal amount of the Baf protein required for supershifting Crx-HD is higher for the E80A mutant than that for the wild-type Crx (*lanes 4–6 versus lanes 1–3* and *lanes 10–12 versus lanes 7–9*), supporting a reduction in the affinity of Crx for Baf by the E80A mutation. To see whether this reduction in the physical interaction of Crx and Baf caused by the E80A mutation would affect the functional interaction, we also tested whether the E80A mutation has any effect on the sensitivity of CRX to Baf-mediated repression using transient transfection assays in HEK293 cells. Consistent with a previous study (13), in the absence of a recombinant Baf, E80A acted as a "hyperactive" mutant, showing a 100% increase in transactivation activity compared with the wild-type CRX (data not shown). A56T, in contrast, showed the same activity as the wild-type CRX (data not shown). An increasing amount of the Baf expression vector was then added to these co-transfection assays. The transactivation activities obtained from these experiments, presented as percentages of those obtained in the absence of Baf (100%), are shown in Fig. 7*C*. The results indicated that the E80A mutant is less sensitive to Baf-mediated repression than the wild-type CRX and the A56T mutant. The sensitivity difference was significant when the *Baf*/*CRX* DNA ratio was less than 2:1 ($p = 0.0071$ at the ratio of 0.5:1, and 0.004 at the ratio of 1:1, respectively, for wild type *versus* E80A). As an example, at a ratio of 0.5:1, Baf did not significantly repress the *trans*-activating activity of E80A ($p = 0.30$; comparing the samples with Baf *versus* without Baf), although it did significantly repress both the wild-type CRX and A56T by about 34% under the same conditions. These results, combined with the physical interaction data, suggest that the E80A mutation interferes with both the physical and functional interaction of Crx and Baf.

The Human BAF Gene Tentatively Maps to the Chromosomal Region Containing the Bardet-Biedl Syndrome-1 (BBS1) Locus—The finding that Baf forms a physical and functional interaction with Crx and that a Crx mutation reduces this interaction raised the possibility that mutations of Baf itself might cause a disease of the retina. We therefore determined the genomic structure and chromosomal location of the human *BAF* gene. A BLAST search of the human genome databases from the NCBI and Celera Corp. revealed the presence of six human sequences that were similar to the sequence of the bovine cDNA fragment obtained from the yeast two-hybrid experiments (Fig. 8*B*). Of these six sequences, two were possibly ancient pseudogenes with truncated reading frames and a low level of homology with the bovine probe (sequences B4 and B5 in Fig. 8*B*), and three carried longer, intronless reading frames producing higher homology scores with the bovine open reading frame and were considered more recent pseudogenes (B1, B2, and B6). One sequence (B3) corresponded to a putative three-exon gene encoding a predicted protein that is 100% identical to bovine *Baf* (Fig. 8*A*); we interpreted this to be the human *BAF* homolog. The chromosomal structure of the human *BAF* gene was determined by comparison between its mRNA (GenBank™ accession number NM_003860) and a clone containing a human DNA region from chromosome 11q13 (GenBank™ accession number AP001191). In particular, the human *BAF* locus appeared to lie within chromosome 11q13, in a region containing the gene responsible for BBS1 (OMIM: 209901) (46, 47). However, the precise location of sequence AP001191 differs among the different human genome databases. Besides 11q13 (Celera; available on the World Wide Web at www.celera.com), some assemblies of the human genome show, for example, that this sequence may be found in contigs that are thought to be derived from chromosome 2q (Celera; Ensembl; available on the World Wide Web at www.ensembl.org) or 14q (NCBI; available on the World Wide Web at www.ncbi.nlm.nih.gov).

BBS1 is a multisystem, autosomal recessive disorder that

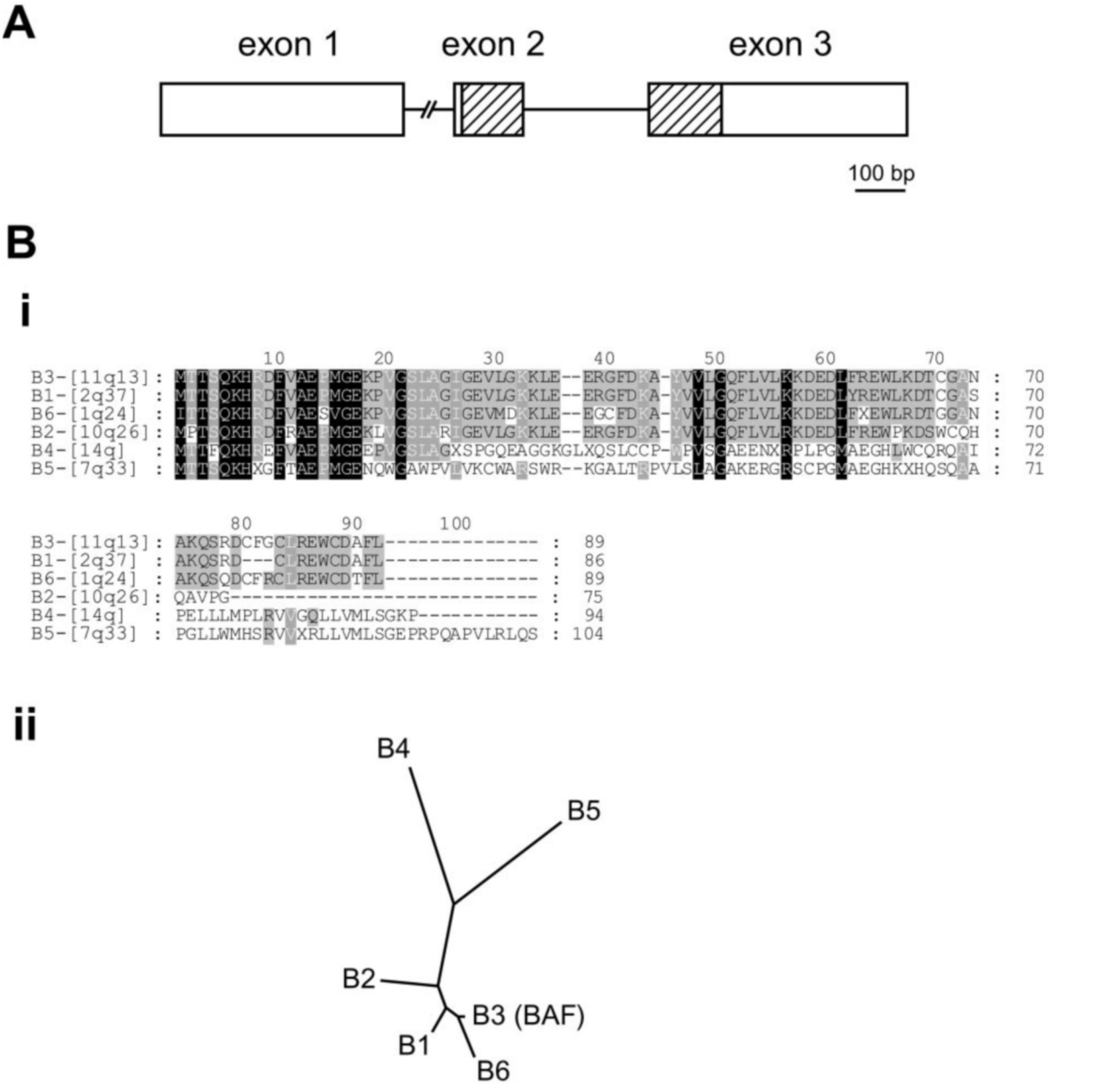

FIG. 8. **Analysis of the human *BAF* gene(s).** *A*, chromosomal structure of the human *BAF* gene. The coding region is *hatched*. *B*, human paralogues of the bovine Baf protein. *B* (*i*), The actual human BAF, encoded by the gene depicted in *A*, corresponds to sequence B3 and is 100% identical to the predicted bovine protein. All other sequences are from intronless open reading frames, possibly belonging to retropseudogenes. The chromosomal location of such sequences is indicated in *brackets*. Stop codons are indicated by *X*. *B* (*ii*), phylogenetic tree of the sequences depicted in *B* (*i*) (see "Experimental Procedures").

includes retinitis pigmentosa as one of its manifestations. Because of the possible location of *BAF* within 11q13, we considered *BAF* a candidate for BBS1. However, an analysis of leukocyte DNA from 43 unrelated patients with Bardet-Biedl syndrome did not reveal any sequence variations in the coding exons or flanking intron sequences. Since ~40% of families with Bardet-Biedl syndrome show linkage to the BBS1 region (46, 48, 49), it is highly likely that we had cases caused by BBS1 in our set of 43 unrelated patients from families in which linkage analysis had not been performed. Thus, this negative result strongly suggests that *BAF* is not the BBS1 gene. Because of the ambiguity in the chromosomal localization of *BAF* and the possibility that *BAF* mutations might cause some other form of retinal degeneration whose locus has not yet been mapped to the *BAF* locus, we extended our mutation survey and screened 51 unrelated patients with Leber congenital amaurosis and 37 with autosomal recessive retinitis pigmentosa. Again, no sequence variations were found.

DISCUSSION

Baf Interacts with Crx and Four Other Paired-like Homeodomain Transcription Factors—Using a yeast two-hybrid screen, we identified Baf as protein that possibly interacts with Crx. Further evidence supporting a direct interaction of Baf and Crx was provided by pull-down assays, *in vitro* co-immunoprecipitation, and supershift electrophoretic mobility shift assays. We also showed that recombinant Crx and Baf are co-localized in the nucleus of HEK293 cells after co-transfection. Both Crx and Baf proteins are present in the nuclei of the photoreceptors and the bipolar cells of the mature mouse retina as well as in the pinealocytes of the pineal gland, and the two proteins can be co-immunoprecipitated from a bovine retinal extract using a

Crx antibody. These results suggest that Baf and Crx interact *in vivo*. Baf was originally identified as a host cellular factor that plays a role in preventing retroviral DNA from autointegration (integrating into the virus' own genome) and in the formation of preintegration complexes (PICs) during retroviral infection (42, 50, 51). Baf is evolutionarily conserved from *Caenorhabditis elegans* to mammals (52) (Fig. 1), implicating Baf as an important protein for cellular function. Baf is reported to interact directly with the LEM domain[3]-containing proteins, including the nuclear lamin-binding proteins LAP2 (53) and emerin (29, 54). Our study demonstrates for the first time that Baf is also able to bind directly to a paired-like homeodomain transcription factor. Consistent with this, four other paired-like homeodomain proteins (Chx10, Pax-6, Otx1, and Otx2) also have the ability to interact with Baf *in vitro*. NMR and x-ray crystallography studies demonstrate that both Baf and the paired-like homeodomain independently form a higher order α-helical structure important for binding to DNA and for dimerization (52, 55–57). This helical structure may provide a structural base for Baf interaction with a homeodomain. As an example, based on the crystal structure of a complex of a paired-like homeodomain and DNA, it was predicted that residue Glu^{42}, corresponding to Glu^{80} of the Crx protein, is an essential residue for protein-protein interaction (57). Our results support this prediction, since the E80A mutation affects the ability of Crx-HD to bind to Baf but does not affect its binding to DNA. It would be interesting to test whether other classes of the HD and non-HD transcription factors with helical structures, such as helix-loop-helix proteins, also interact with Baf.

Baf is believed to serve as a bridging molecule that binds to DNA randomly *in vitro* (42, 58) and that co-localizes with chromosomal DNA during interphase and mitosis (53). However, our EMSA studies with a recombinant Baf did not detect a Baf-DNA complex with oligomers harboring Crx binding sites. This could be due to our use of EMSA conditions favoring Crx binding specifically to its targets, including the use of a low concentration of probes and a relatively low amount of recombinant Baf. Alternatively, the presence of a His tag at the N terminus of recombinant Baf might reduce its ability to bind DNA. Nevertheless, the interaction of Crx-HD and Baf *in vitro* appears to occur in the absence or presence of double-stranded DNA, since a positive interaction was detected by both pull-down assays (without DNA, as confirmed by UV spectrum and agarose gel analysis of the protein preparations) and *in vitro* co-immunoprecipitation assays in the presence of double-stranded DNA templates. Further experiments are needed to determine whether the interaction of Baf and Crx actually occurs *in vivo* and also to determine the precise location of Crx-Baf complexes in the photoreceptor cells.

Baf Might Regulate Gene Expression in Photoreceptors and/or Pinealocytes—The previously reported interaction of Baf with nuclear lamin-associated proteins raises the possibility that Baf might be indirectly involved in transcriptional regulation, since nuclear lamins have a role in localizing transcription factors or other proteins to the nuclear rim and in repressing transcription (59–61). Our results showing a physical and functional interaction of Baf with Crx suggest that Baf may indeed be involved in transcriptional regulation, at least in the retina and possibly in the pineal gland. Baf also physically interacts with four other paired-like homeodomain transcription factors *in vitro*. Among the four, Otx2 is expressed in several types of neurons in the retina, including photoreceptor cells (62), and it can bind to and *trans*-activate the promoter of the photoreceptor gene *IRBP in vitro* (63, 64). In contrast, Chx10 is expressed in the bipolar cells of the retina and is involved in the proliferation of the retinal progenitor cells and in the determination of bipolar cell fate (43). Chx10 is a repressor of transcription and has been postulated to prevent presumptive bipolar cells from adapting a photoreceptor cell fate during development by repressing Crx-dependent activity.[4] However, since the homeodomain proteins can interact with multiple functional domains, the physiological relevance of Baf interaction with these homeodomain proteins remains to be established.

Increasing evidence suggests that a homeostasis of transcription factor network consisting of activators and repressors is important for controlling expression of tissue- or cell type-specific genes. As for the expression of photoreceptor genes, a high level of Crx expression may be required for overcoming the repressive effect of Baf, Chx10, or other repressors. This requirement may explain why photoreceptor genes are not expressed by bipolar cells although Crx is present at a low level in these neurons (Fig. 4*C*). Our studies also suggest that Baf may participate in transcriptional regulation via its interaction with cell type-specific proteins, although Baf itself is ubiquitously expressed. Future studies with conditional knockouts or overexpression of Baf in specific tissues or cell types are required to decipher the role of Baf *in vivo*.

The mechanism by which Baf represses Crx transactivation activity requires further study. Interaction of Baf with Crx appears not to abolish the binding of Crx to its target DNA based on our EMSA studies. However, the possibility cannot be ruled out that *in vivo*, Baf may act as a general repressor to block transcription by nonspecifically binding to chromosomal DNA or by modifying chromatin structure. Another possibility is that Baf localizes Crx to a specific cellular compartment that prevents Crx from accessing its DNA targets. A recombinant Baf demonstrates both nuclear and cytoplasmic localization in transiently transfected HEK293 cells, whereas Crx localizes primarily to the nucleus regardless of the presence or absence of the Baf expression vector. This would argue that Baf has no effect on the nuclear localization of Crx. However, Crx appears to co-localize with Baf inside the nucleus during interphase, as determined by immunocytochemistry and confocal microscopy. Considering that Baf is associated with nuclear laminae, it is possible that Baf could repress Crx by localizing the Crx protein to a specific nuclear compartment. Alternatively, Baf could lead to a change(s) in the conformation or stability of the Crx protein, or it could antagonize a co-activator or potentiate a repressor of Crx. It is obvious that our understanding of the effect of Baf on Crx activity is limited by the use of transiently transfected HEK293 cells. It would be interesting to determine whether the same effect could be seen in a photoreceptor cell line. Unfortunately, a well characterized and stable photoreceptor cell line is currently unavailable, and only a limited transfection efficiency can be achieved with existing retinoblastoma cell lines or primary retinal cell cultures.

Is BAF Linked to Retinal Diseases?—Loss of Baf in *C. elegans* by using the RNA interference (RNAi) technique leads to an embryonic lethal phenotype due to abnormal segregation of chromosomes during mitosis (58). More recently, it has been found that mutations of emerin and lamin A/C, the two components of nuclear matrix complexes that Baf is associated with, cause muscle and heart diseases such as Emery-Dreifuss muscular dystrophy and lipodystrophy (65). Furthermore, a disease-causing mutant of emerin poorly binds to BAF *in vitro*

[3] The LEM domain is a protein domain with ~40 amino acids that defines a family of nuclear membrane proteins (70), including LAP2 (lamin-associated polypeptide) (53), emerin (54), and MAN1 (70).

[4] R. Bremner, S. Chen, and D. J. Zack, unpublished data.

and fails to assemble into the nuclear envelope *in vivo* (29), suggesting that Baf is required for nuclear envelope reassembly during mitosis and may contribute to the phenotype of tissue-specific diseases caused by mutations in emerin or lamin A/C. Our finding that the interaction of Baf and Crx is affected by a CRX mutant associated with a photoreceptor dystrophy raises the possibility that Baf may also contribute to the phenotype of CRX-associated retinal diseases. In addition to CRX, two other retinal homeodomain transcription factors that interacted with Baf in our study are also linked to human retinal diseases with great heterogeneity, including CHX10 that is associated with microphthalmia (66) and PAX-6 associated with aniridia (67–69). Thus, BAF may be a candidate for a genetic modifier that contributes to these diseases as well. It would be interesting to test whether mutations in either Baf or these Baf interaction partners could affect their physical interaction.

The evidence that Baf interacts with several proteins involved in tissue-specific diseases allows us to hypothesize that *BAF* itself could be a candidate gene that, when mutated, might cause a degeneration or maldevelopment of the retina. As the first step to address this speculation, we considered the fact that the human *BAF* gene may lie in a chromosomal interval (11q13) containing the gene responsible for BBS1, an autosomal recessive disease with multisystem abnormalities, including photoreceptor degeneration (46–48). Although we did not identify BAF mutations in our cohort of patients with BBS or retinopathies, this does not constitute evidence that no *BAF* mutations are associated directly with any retinal disease. Additional mutation screens in the coding exons or the regulatory region of *BAF* in larger populations of patients are needed to address this issue.

Interestingly, we have discovered at least five paraloguous *BAF* sequences from the human genome using the homology searches with the bovine *Baf* cDNA as a query. These sequences appear to be retropseudogenes (*i.e.* pseudogenes that arose by retrotransposition events), since they are devoid of introns. They are scattered through the whole genome. Three of these, B1, B2, and B6, are flanked by poly(A) stretches in their 3′-ends, presumably residua of the original polyadenylated tails from retrotranscribed mRNAs. Their coding sequences exhibit a fairly high degree of homology with the original *BAF* transcript (Fig. 8*B*, *i*). In contrast, sequences B4 and B5 show limited homology with the *BAF* cDNA, and this homology is restricted to the 5′-end of the gene (Fig. 8*B*, *i*). B4 and B5 probably represent more distantly related sequences likely to have originated in the distant past and/or to be the result of a partial integration of retrotranscribed mRNA (Fig. 8*B*, *ii*). A relationship between these pseudogenes and the involvement of BAF in retroviral processes remains a speculative yet intriguing hypothesis.

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