THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 47, Issue of November 24, pp. 37264–37270, 2000

Printed in U.S.A.

Functional Domains of the Cone-Rod Homeobox (CRX) Transcription Factor*

Received for publication, March 31, 2000, and in revised form, September 11, 2000
Published, JBC Papers in Press, September 12, 2000, DOI 10.1074/jbc.M002763200

Kai-Yin Chau‡, Shiming Chen§, Donald J. Zack¶, and Santa Jeremy Ono‡‖‡‡**

*From the ‡Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02114, the §Department of Ophthalmology and Visual Science, Washington University School of Medicine, St. Louis, Missouri 63110, the ¶Departments of Ophthalmology, Molecular Biology and Genetics, and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, and the ‖Division of Rheumatology, Immunology, and Allergy, Department of Medicine, Brigham & Women's Hospital, and the **Committee on Immunology, Harvard Medical School, Boston, Massachusetts 02115*

The paired-like homeodomain transcription factor CRX (cone-rod homeobox) is involved in regulating photoreceptor gene expression and rod outer segment development. Mutations in *CRX* have been associated with several retinal degenerative diseases. These conditions range from Leber congenital amaurosis (a severe cone and rod degeneration of childhood onset) to adult onset cone-rod dystrophy and retinitis pigmentosa (an adult onset condition that primarily affects rods). The goal of this study is to better understand the molecular basis of CRX function and to provide insight into how mutations in CRX cause such a variety of clinical phenotypes. We performed deletion analysis in conjunction with DNA binding and transient transfection-based transactivation studies to identify the functional domains within CRX. DNA binding requires a complete homeodomain. Furthermore, truncated proteins that did not contain an intact homeodomain failed to demonstrate detectable expression in tissue culture upon transfection. Transactivation analysis indicated that both the OTX tail and the WSP domain are important for controlling positive regulatory activity of CRX. Interestingly, the mapped CRX transactivation domains were also critical when coexpressed with NRL. Specifically, the synergy between CRX and NRL was constant regardless of which CRX variant was used.

CRX (cone-rod homeobox), a transcription factor implicated in photoreceptor gene expression, belongs to the *OTD/OTX* homeobox gene family (1–5). Similar to other family members (see Fig. 1), it possesses a paired-like homeodomain followed by a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the *OTD* and *OTX* genes, expression of CRX is restricted to developing and adult retinal photoreceptors and cells within the pineal gland (1–3, 6). *In vitro* studies demonstrate that CRX binds specifically to regulatory elements in the promoters of several photoreceptor-specific genes including rhodopsin; and in transient transfection assays, CRX appears to transactivate the expression of these genes (1, 3). Work is still ongoing to determine unequivocally what genes are regulated by CRX and the mechanism of control (7–11). In the case of the rhodopsin promoter, CRX acts synergistically with the bZIP transcription factor NRL (neural retinal leucine zipper) (3, 12–15).

CRX has been implicated in retinal development *in vivo*. Retrovirus-mediated CRX overexpression appears to increase photoreceptor number, and expression of a putative dominant-negative form of CRX interferes with rod outer segment formation (1). *CRX* knockout mice generated by Cepko *et al.* fail (16) to develop normal photoreceptor outer segments and have reduced visual function. The expression of many photoreceptor-specific genes, including rhodopsin, is reduced. In addition, gene mutations in *CRX* have been associated with several forms of human retinal degeneration. R41W, R41Q, E80A, A158T, V242M, a 1-bp[1] deletion in codon 168 (E168Δ1bp), and 196/7Δ4bp mutations have been identified in patients with autosomal dominant cone-rod dystrophy (2, 4, 17), a generally adult onset disease causing degeneration of both rod and cone photoreceptors. The R41Q mutation has been reported in patients with autosomal dominant retinitis pigmentosa (17), a generally late-teen to adult onset disease that affects primarily rods, but does affect cones in late stages. Additional mutations have been found in patients with Leber congenital amaurosis, a severe childhood degeneration that affects both rods and cones (17–20). In each of these examples, *CRX* mutations were only identified in one allele; the other allele was wild-type in all cases. This was initially surprising in terms of the Leber congenital amaurosis patients since this disease is generally thought to be autosomal recessive. Analysis of the parents, however, demonstrated that the mutations were *de novo* since, although paternity was confirmed, none of the parents carried the *CRX* mutations. Indeed, Silva *et al.* (19) recently reported that a patient heterozygous for the *CRX* null allele is affected with Leber congenital amaurosis, although her father, who has normal vision, is heterozygous for the same mutation. This suggests that haplo-insufficiency of CRX is not sufficient to cause retinal disorders (19). In addition, a family with Leber congenital amaurosis has been identified in which the disease phenotype does appear to be due to a recessive CRX R90W mutation (20).

* This work was supported by National Institutes of Health Grants GM49661 (to S. J. O.) and EY09769 (to D. J. Z.); the Lucille P. Markey Foundation (to S. J. O.); a postdoctoral fellowship from the Fight for Sight Foundation (to K.-Y. C.); the Foundation Fighting Blindness; the Rebecca P. Moon, Charles M. Moon, Jr., and Dr. P. Thomas Manchester Research Fund; the Mrs. Harry Duffey Research Fund; and a career development award from the Research to Prevent Blindness, Inc. (to D. J. Z. and S. C.). 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: Schepens Eye Research Inst., 20 Staniford St., Boston, MA 02114. Tel.: 617-912-2521; Fax: 617-912-0127; E-mail: sjono@vision.eri.harvard.edu.

[1] The abbreviations used are: bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase.

The mutated *CRX* gene associated with retinal diseases may express a functionally impaired CRX. Indeed, point mutations of the homeodomain (*i.e.* R41W and R90W) were demonstrated to directly or indirectly interfere with CRX binding to its regulatory sequence and/or functioning (15, 20). Frameshift mutations of the C-terminal portion of CRX will lead to alteration of the encoded sequence and loss of the OTX tail, which may affect interactions with other factors, *e.g.* NRL, RX, p300/CBP, and phosducin (3, 15, 21–24).

To gain further insight into the role of CRX in regulating photoreceptor gene expression and development as well as its involvement in human retinal disease, we have performed a structure and function analysis of the factor. Similar approaches have been used to study other homeobox proteins, with particular attention to the basic and C-terminal conserved regions of homeodomain protein families (25–27). As a first step in this process, we generated and characterized two panels of C-terminal deletions of CRX to examine for 1) their DNA-binding activity with the BAT-1 site on the rhodopsin promoter (3), 2) their expression pattern in tissue culture, and 3) their transactivation of the rhodopsin proximal promoter in isolation of other synergistic factors and in cooperation with NRL. Here, we present the results of this analysis and discuss their implications for understanding the molecular basis of CRX-related retinal dystrophies.

EXPERIMENTAL PROCEDURES

Cell Culture—293 human embryonic kidney cells were maintained as described (3).

Plasmid Constructions—A wild-type bovine CRX expression plasmid was cloned as described previously (3) with an N-terminal fusion of 6-His and an Xpress peptide tag (Invitrogen, Carlsbad, CA). Truncated forms of CRX were amplified by polymerase chain reaction utilizing primers with appropriate added restriction enzyme recognition sites and subcloned into *Bam*HI/*Eco*RI-digested pcDNA3.1/HisC mammalian expression vector (Invitrogen). All deletions are in-frame. Sequences were confirmed by DNA sequencing (Physiology Department, Tufts DNA Sequencing Facility, Boston, MA). Plasmid DNA for transfection was prepared with a QIAGEN plasmid kit.

In Vitro Expression of CRX and Its Truncated Forms—In vitro transcribed and translated proteins were prepared using the T7 RNA polymerase TnT kit (Promega, Madison, WI) and [^{35}S]methionine (1000 Ci/mmol; ICN, Costa Mesa, CA) as described by the manufacturer. Labeled lysates were resolved by SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Blue and destained and then dried and exposed to x-ray film (Fuji Rx) for autoradiography.

Electrophoretic Mobility Shift Assays (EMSAs)—The BAT-1 oligonucleotides used were as follows: 5′-GTGAGGATTAATATGATTAATA-ACGCCCC-3′ and its exact complement (synthesized by Genosys Biotechnologies, Inc., The Woodlands, TX). Double-stranded probe was ^{32}P-radiolabeled with polynucleotide kinase (New England Biolabs Inc., Beverly, MA) and gel-purified. Radiolabeled probe was mixed with *in vitro* translated proteins, and EMSA was performed following a modified protocol as described (3). Four μg of poly(dG-dC) (Amersham Pharmacia Biotech) was used, and the binding reaction was carried out in a total volume of 30 μl.

Western Blot Analysis of CRX and Its Truncated Forms—293 cells were transfected with equal amounts of expression plasmids for CRX and its truncated forms as described above. Cells were harvested and washed with Dulbecco's phosphate-buffered saline (Life Technologies, Inc.), and extracts from both the nuclear and cytoplasmic compartments of the transfected cells were prepared as described (28). Extracts were then resuspended in SDS-PAGE sample buffer. Equal amounts of lysates from each sample were resolved by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech) following the standard protocol. Western blots were blocked overnight at 4 °C in 0.2% I-Block (Western-Light kit, Tropix Inc., Bedford, MA) in phosphate-buffered saline containing 0.05% Tween 20. Anti-Xpress monoclonal antibody (Invitrogen) was used as suggested, followed by the chemiluminescence method of detection (Western-Light kit).

Transient Transfections and Luciferase and Chloramphenicol Acetyltransferase (CAT) Assays—Transfections of 293 cells were performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instruction. Cells were seeded onto 35-mm diameter dishes at 30–50% confluence. The pbRho−130 rhodopsin/reporter (0.3 μg) plasmid (13) was mixed with 0.3 μg of expression plasmid for CRX or its mutants created here, 0.3 μg of either the NRL expression plasmid or an empty vector, 1 μg of pCAT control vector (Promega), and 6 μg of LipofectAMINE and incubated at room temperature for 30 min, and then the mixture was added to 293 cells in 1 ml of Dulbecco's modified Eagle's medium (Life Technologies, Inc.). After 6 h, 1 ml of Dulbecco's modified Eagle's medium with 20% fetal bovine serum (Life Technologies, Inc.) was added. Cells were grown and harvested 40 h post-transfection, washed with Dulbecco's phosphate-buffered saline, lysed in 150 μl of lysis buffer (Promega), and detached using a rubber policeman. The lysis mixture was frozen, thawed, and centrifuged. A 50-μl aliquot was measured for luciferase activity by mixing with 50 μl of luciferin (Promega), and luminescence was measured in a Monolight luminometer (Pharmingen, San Diego, CA) for 20 s. CAT activity was determined using a CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) on 50 μl of cell lysate. Subsequently, luciferase activities were normalized relative to the CAT activity of each sample.

Data Analysis—Values are presented as means ± S.E. To determine statistical significance, Student's *t* test was conducted.

RESULTS

Generation of CRX Deletion Constructs—A panel of C-terminal truncations of bovine CRX was generated to test its functional domains responsible for transactivation. The guiding principles in generating the original set of deletions were the positions of conserved sequences within the OTD/OTX family and the locations of the known naturally occurring *CRX* mutations (1–4, 17, 18, 20, 29, 30). Mutant CRX-(1–285) encodes CRX without the OTX tail (Fig. 1). This construct would allow us to assess the role of the OTX tail, which is believed to encode a transactivation domain of the factor. CRX-(1–107) preserves essentially only the paired-like homeodomain and lacks the C-terminal region of the molecule, including the OTX tail, the WSP domain, and the basic region. This truncated CRX polypeptide would permit a comparison with the previous CRX deletion, probing the contributions of the WSP domain and the basic region of CRX to transactivation. CRX-(1–79) contains only the first two helices of the homeodomain, with the recognition helix (helix 3) completely deleted. CRX-(1–54) has only an N-terminal fragment of the homeodomain. The last two constructs would allow us to probe the functional roles of the CRX homeodomain.

Helix 3 of the Homeodomain Is Required for DNA-binding Activity—To assess the binding of CRX and its deletion derivatives to its responsive element, we chose the BAT-1 site, which shows the highest affinity for CRX binding among other *cis*-elements on the rhodopsin proximal promoter, *i.e.* Ret-1 and Ret-4 (3). Individual CRX constructs of the panel created were expressed in the presence of radiolabeled methionine using an *in vitro* transcription and translation system, and the different forms of truncated CRX produced were confirmed by SDS-PAGE to be of expected size and at similar levels (Fig. 2*A*). Identical transcription and translation reactions were then carried out with unlabeled methionine, and the resulting products were used for EMSAs with radiolabeled BAT-1 oligomers as probes. The results shown in Fig. 2*B* demonstrate that CRX with a C-terminal truncation removing helix 3 (the recognition helix of the homeodomain), as in CRX-(1–79) (or further deleting almost the entire homeodomain, as in CRX-(1–54)), resulted in a total loss of DNA-binding ability. The presence or absence of other portions of the CRX molecule (*i.e.* the OTX tail, WSP domain, and basic region, as observed with wild-type CRX, CRX-(1–285), and CRX-(1–107)) did not affect the BAT-1 EMSA results, suggesting that the CRX homeodomain acts independently of other parts of the molecule in mediating DNA binding. A complementary EMSA experiment was performed in which equal amounts of radiolabeled wild-type and trun-

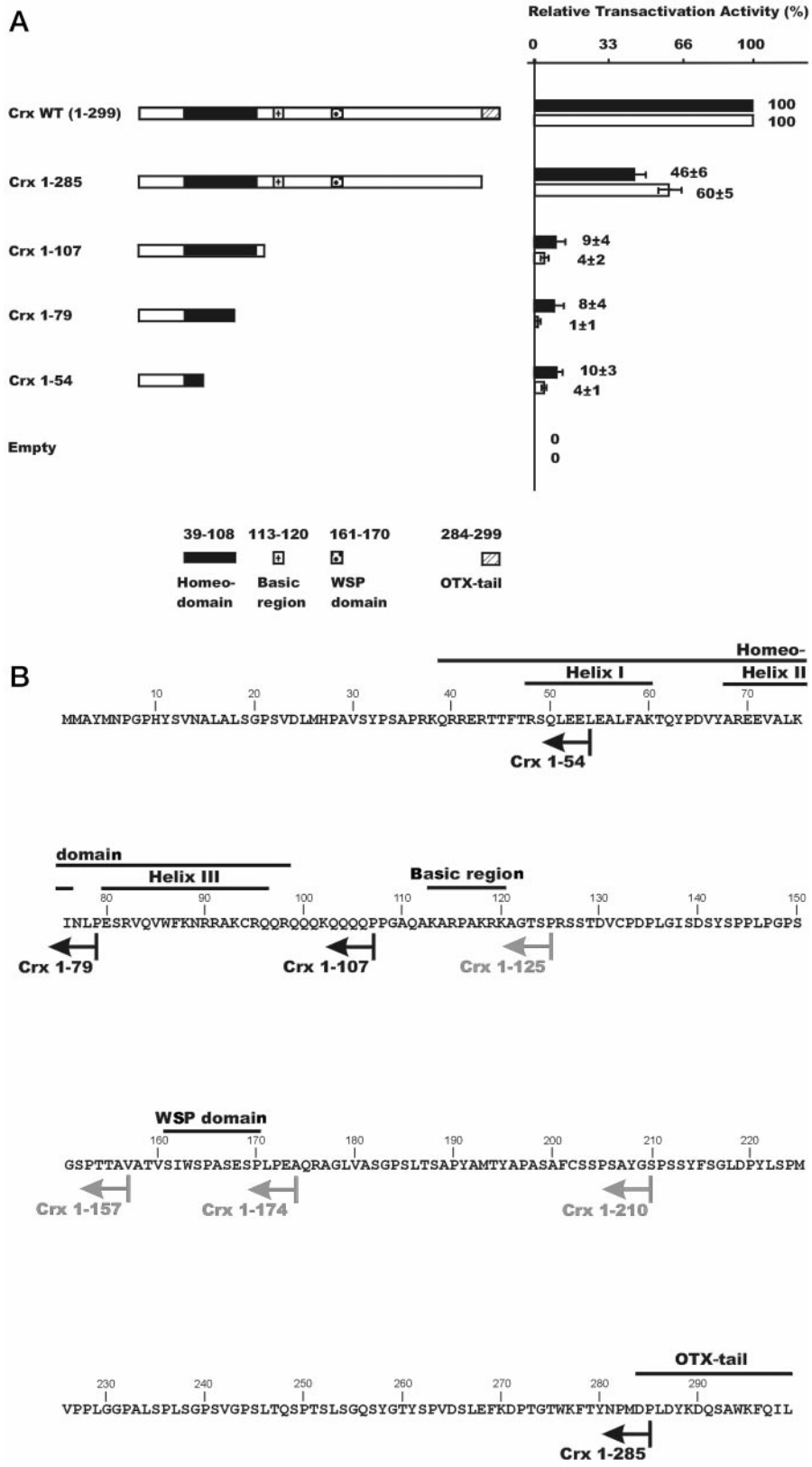

FIG. 1. **Structures of the CRX protein and the C-terminal deletion mutants constructed for this study and their transactivation activities.** *A*, schematic illustrations of expressed wild-type CRX (*Crx WT*) and its mutants are shown in the *left panel*, in which the homeodomain, basic region, WSP domain, and OTX tail are indicated. The *right panel* shows the transactivation activities of wild-type CRX and its truncation mutants in the presence (*white bars*) or absence (*black bars*) of NRL expression. 293 human embryonic kidney cells were cotransfected with a fusion construct consisting of the bovine rhodopsin proximal promoter (−130 to +70 bp) linked to a luciferase reporter cassette (pbRho−130) and with a plasmid expressing wild-type CRX protein (CRX-(1–299)), one of its truncated forms, or an empty expression vector. NRL was expressed heterologously in 293 cells by transfecting the NRL expression construct *versus* an empty vector. A plasmid expressing CAT (pCAT control vector) was cotransfected as well, and CAT activities were determined by CAT enzyme-linked immunosorbent assay. In all transfections, the same amount of plasmid DNA was delivered. Rhodopsin promoter activity was read as luciferase activity after normalization with CAT activity for transfection efficiency. -Fold induction was calculated as the -fold increase in rhodopsin promoter activity upon cotransfecting CRX or its mutant compared with that receiving the empty vector. The relative transactivation activity is expressed as the percentage of the -fold induction given by the wild-type CRX-mediated transactivation of the rhodopsin promoter (set as 100%) compared with that contributed by the empty vector (set as 0%, the base line). The data represent means ± S.E. from six independent experiments. *B*, shown is the amino acid sequence of bovine CRX protein. Truncation boundaries are marked by *horizontal arrows* for each CRX mutant construct. Additional CRX C-terminal truncation mutants (CRX-(1–210), CRX-(1–174), CRX-(1–157), and CRX-(1–125)), described in the legends to Figs. 3 and 4, are shown in *gray*.

cated CRX proteins were incubated with unlabeled BAT-1 probe, and identical results were obtained (data not shown).

In Vivo Expression of CRX Mutants—Wild-type CRX and the C-terminal mutants were transiently expressed in 293 human embryonic kidney cells. Before assessing the various constructs for transactivation activity, their levels of expression were compared. Both the nuclear and cytoplasmic fractions of the cells transfected with each of the constructs were isolated and analyzed by Western blotting using an antibody against the Xpress peptide tag. As shown in Fig. 2*C*, wild-type CRX and the CRX-(1–285) and CRX-(1–107) mutants were expressed at similar levels in the nuclei of the transfected cells. In contrast, no expressed protein was present in transfectants with the CRX-(1–79) and CRX-(1–54) expression vectors. None of the ex-

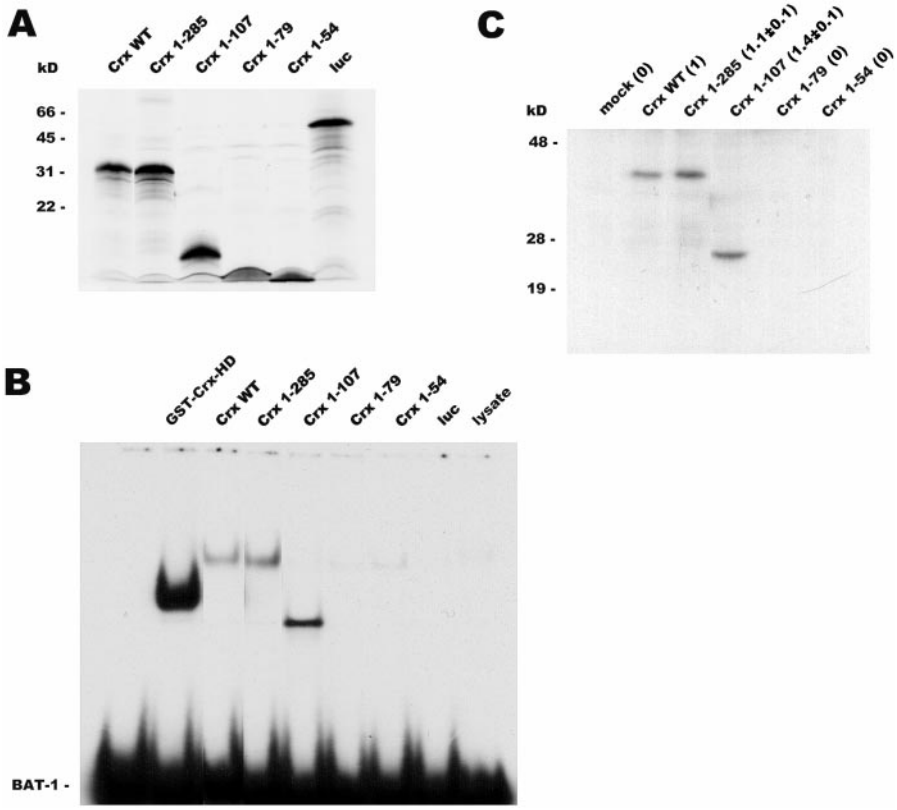

FIG. 2. **Analysis of the DNA-binding activity and *in vivo* expression of CRX and its truncation mutants.** *A*, wild-type CRX (*Crx WT*) and its deletion mutants were produced by *in vitro* transcription and translation in the presence of [^{35}S]methionine, size-fractionated by SDS-PAGE, and autoradiographed. A luciferase control (*luc*) was also expressed. The mobility positions of molecular mass standards are shown. *B*, DNA-binding activity of *in vitro* expressed wild-type CRX and its mutants. Equal amounts of unlabeled CRX and the truncation mutants were incubated with ^{32}P-labeled BAT-1 probe and then subjected to EMSA. Bacterially expressed glutathione *S*-transferase fusion protein containing only the homeodomain region of CRX (*GST-Crx-HD*; CRX-(34–108)) was included as a positive control, whereas expressed luciferase and reticulocyte lysate (*lysate*) served as negative controls. The position of the free probe is indicated. The experiment was performed three times with identical results. The results suggest that the CRX homeodomain is necessary and sufficient for DNA-binding activity. *C*, expression constructs for wild-type CRX and its truncated forms were transiently transfected into 293 cells and then examined by Western blot analysis. Both the nuclear and cytoplasmic (not shown) fractions of the transfected cells were prepared, resolved by SDS-PAGE, transferred to membrane, and then probed with monoclonal antibody to the Xpress leader peptide encoded by the pcDNA3.1/HisC expression vector. Equal amounts of the expression plasmids were transfected. A mock transfection (*mock*) served as a negative control. Molecular mass standards are shown. Expressed proteins are of the expected size. Each lane contained an equal amount of total protein loaded. The results, obtained from two separate transfection experiments and duplicate Western procedures, were quantified by densitometry, and the readings (shown in *parentheses* above the lanes as mean density ± S.E.) are presented relative to that of wild-type CRX, which is set as 1.

pressed CRX forms were retained in the cytoplasm (data not shown). The expression levels of different forms of CRX in the nucleus were quantified by densitometry. From this analysis, no significant variability ($p > 0.6$) in the abundance of the CRX mutant proteins was detected in the nuclei of transfected cells.

The OTX Tail Is Important for Transactivation Activity—The various CRX constructs were then tested for their ability to transactivate a rhodopsin promoter/luciferase reporter in transiently transfected cells. Photoreceptor-like cells (*e.g.* Y79 and WERI retinoblastoma cells, pineal cells, and primary retinal cultures) commonly express CRX in a predominant and constitutive manner, and mutated forms of CRX did not exhibit dominant-negative effects on endogenously or heterologously expressed wild-type CRX in these cells (data not shown). For this reason, we could not dissect functional domains of CRX in any of these cell systems. 293 human embryonic kidney cells were employed instead, as they have been used extensively for studies of CRX bioactivity (3, 20, 21). CRX (and NRL; see below) is not expressed in these cells, and the activity of the proximal rhodopsin promoter (−130 to +70 bp harboring the BAT-1, NRE, and Ret-4 sites) can be readily transactivated (3–6 fold) (data not shown) by CRX heterologous expression. Under the conditions used, maximal transactivation by wild-type CRX was set at 100% transactivation activity (Fig. 1*A*, *right panel*, *black bars*). The C-terminal mutant CRX-(1–285) (without the OTX tail) demonstrated 46% of the activity of the full-length protein, and the decrease was statistically significant ($p = 10^{-5}$), with it noted above that the two CRX species showed equivalent DNA-binding activity (Fig. 2*B*) and nuclear expression (Fig. 2*C*). Mutant CRX-(1–107), which lacked most of the molecule C-terminal to the homeodomain and again possessed a similar DNA-binding activity and was expressed at a similar level in the nucleus compared with wild-type CRX, demonstrated minimal, if any (9%), transactivation activity ($p = 0.0007$). Therefore, removing the region of CRX between amino acids 108 and 285 significantly depleted its transcription activation level. As expected, the homeodomain deletions that eliminated DNA binding and showed minimal nuclear expression (CRX-(1–79) and CRX-(1–54)) had little, if any, transactivation activity.

Since it is well established that NRL cooperates with CRX in mediating rhodopsin gene activation (3, 15), we extended our CRX transactivation analysis in 293 cells to cells coexpressing NRL. The cooperative transactivation activity with NRL dropped to 60% ($p = 10^{5}$) upon deletion of the OTX tail of CRX (*i.e.* CRX-(1–285)) (Fig. 1*A*, *right panel*, *white bars*). Further deletion of the C-terminal tail of CRX to amino acid 107 caused a 4% residual activity (in conjunction with NRL; $p = 10^{-6}$).

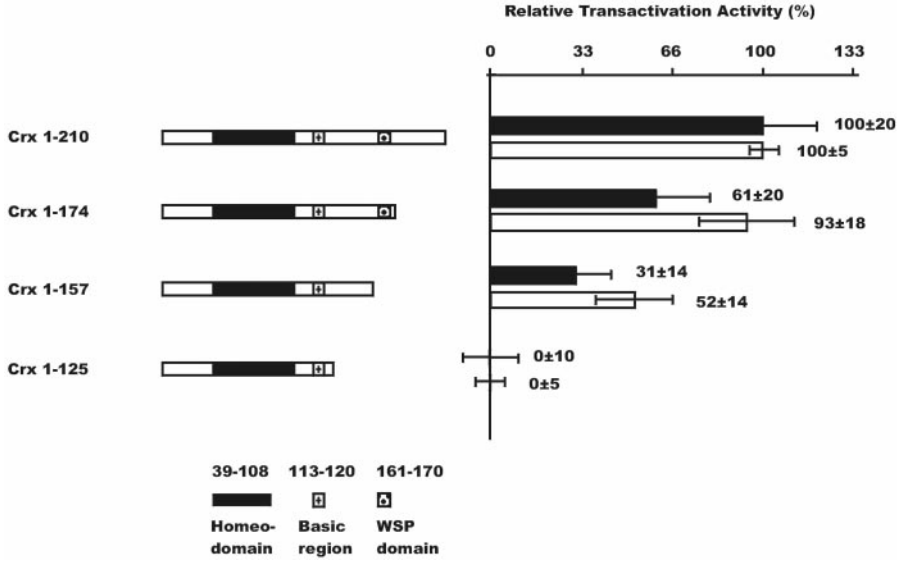

FIG. 3. **Structures of additional CRX C-terminal truncation mutants (CRX-(1–210), CRX-(1–174), CRX-(1–157), and CRX-(1–125)) and their transactivation activities.** *Left panel*, schematic illustration; *right panel*, comparison of the transcription activation levels of the four additional C-terminal deletion mutants for the rhodopsin proximal promoter. Transactivation activities were first adjusted for transfection efficiencies and then normalized to the longest (100%) and shortest (0%) constructs, CRX-(1–210) and CRX-(1–125), respectively. *Black bars* represent activities contributed by CRX and its mutants alone, whereas *white bars* show the synergistic transactivation activities of CRX and mutants in the presence of NRL expression. The results (mean ± S.E.) are a summary of six independent experiments.

The finding that CRX-(1–285) had substantial decreased transactivation activity for the proximal rhodopsin promoter compared with wild-type CRX, yet bound with a similar affinity to the *cis*-element and was expressed at a similar level, suggested that the OTX tail is involved in transactivation. Indeed, the OTX tail appears to account for one-half of the transactivation activity of CRX with respect to the rhodopsin promoter. The difference in activity between mutants CRX-(1–285) and CRX-(1–107) might indicate the presence of other transactivation domain(s) between amino acids 285 and 107.

The WSP Domain and N-terminal Flanking Region Are Responsible for the Residual Transactivation Activity—To identify additional transactivation domains responsible for residual transactivation activity between amino acids 108 and 285, additional deletion constructs (CRX-(1–210), CRX-(1–174), CRX-(1–157), and CRX-(1–125)) were generated (Figs. 1*B* and 3). We (4, 30) and others (2, 17, 29) suspected that the WSP domain of CRX may be responsible for rhodopsin transactivation. The new series of constructs created would allow us to determine whether this is in fact the case. All four new constructs demonstrated efficient expression *in vitro* (coupled transcription and translation system) (Fig. 4*A*). In EMSAs with the BAT-1 site, the four deletions showed very similar DNA-binding activities (Fig. 4*B*). This verifies our above observation that any C-terminal portion of CRX is independent of the homeodomain in binding its *cis*-element. Expression of the four deletion mutants in 293 cells demonstrated that their levels were comparable and not significantly different ($p = 0.2–0.4$) (Fig. 4*C*). We could therefore directly compare their transcription activation potentials.

With or without NRL (Fig. 3), a decrease in transactivation potential from CRX-(1–210) to CRX-(1–174) was observed (to 61 and 93%, respectively), but was not significant ($p = 0.2$ and 0.7). Further C-terminal deletions that removed the WSP domain in construct CRX-(1–157) caused a substantial and statistically significant decrease in activity (down to 31 and 52% (with NRL) as compared with CRX-(1–210)). Additional loss of transactivation activity was seen in construct CRX-(1–125) (eliminating the WSP domain plus a region of 32 N-terminal amino acids), and the overall difference in the level of transcription activation between CRX-(1–125) and CRX-(1–210) was significant ($p < 0.01$) with or without an NRL background. Therefore, it appears that the WSP domain and its N-terminal flanking region (amino acids 126–174) harbor the residual transactivation domain. Importantly, the mapped activation domains were identical in the absence or presence of NRL expression.

DISCUSSION

In view of the critical role that CRX plays in photoreceptor gene expression and development, as well as its involvement in human blindness, we felt that a detailed analysis of its structure and function would be worthwhile both to provide further insight into its mechanism of action and also to facilitate future molecular approaches aimed at the therapy of CRX-related and perhaps other retinal dystrophies. As a first step in this process, we generated two panels of C-terminal deletions of CRX with the aim of identifying the domains required for sequence-specific DNA binding, expression, and transcription activation. It is worth mentioning that in the OTD/OTX homeobox gene family consisting of *OTD* and *OTX* genes and their homologs (31–33), no functional dissection of any of the factors has been carried out. Therefore, our work represents the first domain mapping of a member of the OTD/OTX homeobox protein family.

DNA Binding of CRX Requires a Complete Homeodomain—Wild-type CRX as well as eight different truncated forms were all expressed *in vitro* and resulted in stable proteins. Testing of these *in vitro* synthesized proteins by EMSA, using double-stranded oligonucleotides spanning the BAT-1 site of the bovine rhodopsin proximal promoter as probes, revealed that helix 3 (recognition helix) of the homeodomain is required for sequence-specific DNA binding. In fact, it has been demonstrated that the R90W mutation, which falls in this recognition helix of the homeodomain in CRX, disrupts its DNA-binding activity and results in diminished function (20). CRX with an N-terminal deletion leaving only helix 3 of the homeodomain lost its ability to bind to the BAT-1 site (data not shown). This suggests that helices 1 and 2 of the homeodomain contribute to CRX binding to DNA as well and that mutations of this region (*e.g.* R41W and R41Q) would hinder CRX from binding DNA correctly. This is supported by a recent report showing that the R41W and R90W mutations exhibit reduced DNA binding, transcriptional synergy, and interaction with NRL (15). Taken together, it appears that an intact homeodomain is required for CRX to bind to its target DNA elements for proper functioning. As discussed below, an incomplete homeodomain also gives rise to failure in expressing stable CRX. Within the sensitivity of the assay employed, other domains within CRX did not seem to

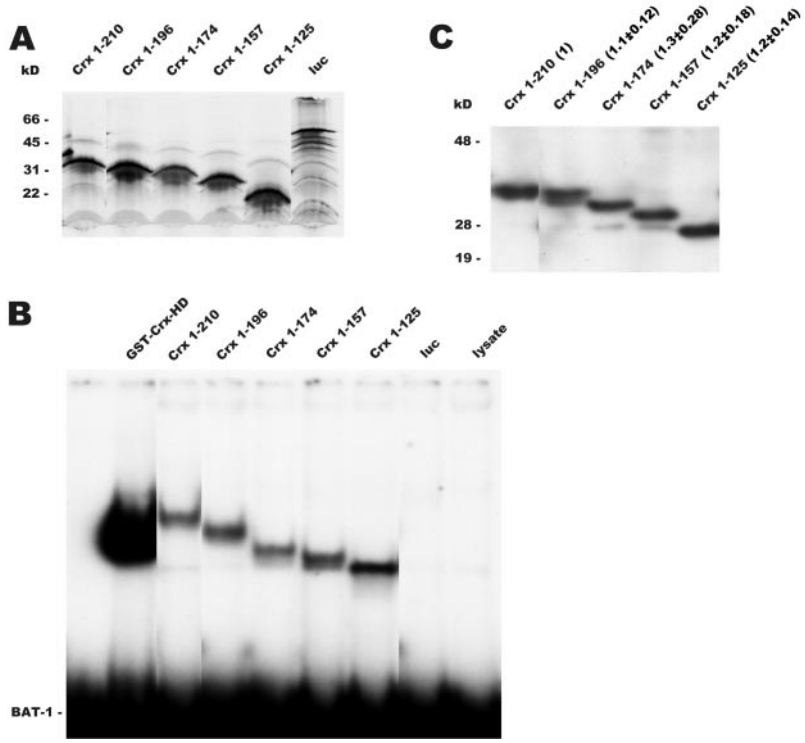

FIG. 4. **Characterization of the four additional CRX C-terminal truncation mutants: CRX-(1–210), CRX-(1–174), CRX-(1–157), and CRX-(1–125).** *A*, the additional deletion mutants were synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine, size-fractionated by SDS-PAGE, and autoradiographed. A luciferase control (*luc*) was also expressed. The mobility positions of molecular mass standards are shown. *B*, shown is the DNA-binding activity of the *in vitro* expressed additional CRX mutants as measured by EMSA, performed as described in the legend to Fig. 2*B*. The position of the free probe is indicated. The experiment was performed three times with identical results. *C*, shown is the *in vivo* expression of the additional CRX deletion mutants, performed as described in the legend to Fig. 2*C*. Results, obtained from two separate transfection experiments and duplicate Western procedures, were quantified by densitometry, and the readings (shown in *parentheses* as mean density ± S.E.) are presented relative to that of CRX-(1–210), which is set as 1.

modulate (positively or negatively) the affinity of CRX for the BAT-1 site.

Truncated Proteins That Do Not Contain an Intact Homeodomain Fail to Demonstrate Detectable Expression—In contrast to the *in vitro* expression results, when the panel of truncated forms of CRX was expressed by transiently transfecting the corresponding expression plasmid in 293 cells, only those constructs encoding an intact homeodomain resulted in detectable nuclear expression. In the subset of constructs (CRX-(1–79) and CRX-(1–54)) not producing stable protein in the nuclei, no protein was detected in the cytoplasmic fraction, suggesting that this phenomenon was not due to failure of nuclear translocation of the expressed protein due to removal of a nuclear targeting sequence. Instead, it is likely affecting the mRNA stability and/or translation that requires an intact homeodomain. This statement is supported by the fact that an N-terminal deletion of CRX lacking helices 1 and 2 of the homeodomain also inhibited CRX expression (data not shown). However, stable protein expression may require sequences within the paired-like homeodomain for nuclear translocation. This is reminiscent of the case with the beta cell homeodomain PDX-1, where the nuclear localization signal resides in helix 3 of its homeodomain (34). Upon inspection of the CRX paired-like homeodomain, no sequence appears similar to this or any other known nuclear localization signal. Thus, CRX may harbor a novel nuclear localization signal. Ongoing mutagenesis of the CRX homeodomain will hopefully help identify any nuclear localization signal within the paired homeodomain.

The OTX Tail, WSP Domain, and Its N-terminal Flanking Region Are Important for Significant Positive Regulatory Activity—The series of C-terminal truncations suggest that CRX does not contain a single well defined region that is both necessary and sufficient for transactivation activity. Rather, more than one region may be important, although whether these separate sequences yield one three-dimensional structure through protein folding remains to be determined. Our data support the previously asserted hypothesis, based on sequence conservation, that the OTX tail plays a major role in CRX-mediated transactivation (2, 4, 17, 29, 30). In view of this, mutations in *CRX* that resulted in loss of the OTX tail (*e.g.* E168Δ1bp and 196/7Δ4bp) when expressed would be expected to affect photoreceptor-specific gene expression. Our data also demonstrated that deletion of the WSP domain and its N-terminal flanking region did have a dramatic effect on transcription activity as well, and this was responsible for any residual transactivation activity.

Very recently, Mitton *et al.* (15) determined that the interaction between CRX and NRL requires the homeodomain of CRX with an extended C-terminal region including the glutamine-rich and basic regions. Interestingly, this basic region did not influence DNA-binding activity in our hands (as assessed in EMSAs), and we therefore believe that the region acts as a traditional activating surface. Since our C-terminal deletion left minimal transactivation activity in construct CRX-(1–125), in which the basic region was preserved, we could not address the functional difference that may occur regarding this basic region.

The reporter construct used in this study spanned the −130 to +70 bp region of the rhodopsin promoter that contained the BAT-1, NRE, and Ret-4 sites, but was devoid of the Ret-1 site. We specifically mutated the BAT-1 site of this reporter and observed that CRX totally lost its ability to transactivate the rhodopsin promoter (data not shown). This suggests that although CRX was demonstrated previously to bind to BAT-1 and Ret-4 sites *in vitro*, the BAT-1 site is chiefly responsible for CRX transactivation *in vivo*.

Our transfection experiments were performed in 293 cells to measure CRX-mediated transactivation of the rhodopsin promoter, both alone and in combination with NRL. 293 cells were used since other photoreceptor cell systems exhibit saturating levels of CRX expression, obviating such experiments. Since the 293 cell system has been used extensively to probe CRX activity, we elected to use this system in these experiments. Our mapping of CRX activation domains in this study validates the usefulness of this approach.

The experiments with NRL were important since NRL acts synergistically with CRX, as would occur in a *bona fide* photoreceptor cell. We have observed a 3–7-fold synergy between

CRX and NRL (consistent with the original report of Zack and co-workers (3)), underscoring the importance of both factors in rhodopsin gene expression. We show here that this synergy remains constant irrespective of the CRX variant used. Since NRL does not interact with the activation domains mapped in this study (15), it is significant that NRL can convey its synergistic effect on whatever part of the CRX activation domain is left intact. We also note that the current data are significant with respect to our recent demonstration that high mobility group I (HMG-I) proteins participate in CRX-dependent transcription in photoreceptor cells (35).

Even though our results are consistent with the data of others, it should be stressed that our experiments (as well those of others) using cell lines are likely to miss important phenotypic changes, such as those that may occur upon CRX mutation during early photoreceptor development. The full assessment of the biological consequences of CRX mutation will best be accomplished by adding back mutated *CRX* transgenes to the background of CRX-deficient mice.

Acknowledgments—We thank the members of our laboratories for stimulating discussions and support during this line of investigation. We are grateful to Professor Colyn Crane-Robinson and Dr. Christian Parry for insight into the structural basis of CRX/DNA interaction.

REFERENCES

1. Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997) *Cell* **91,** 531–541
2. Freund, C. L., Gregory-Evans, C. Y., Furukawa, T., Papaioannou, M., Looser, J., Ploder, L., Bellingham, J., Ng, D., Herbrick, J. A., Duncan, A., Scherer, S. W., Tsui, L. C., Loutradis-Anagnostou, A., Jacobson, S. G., Cepko, C. L., Bhattacharya, S. S., and McInnes, R. R. (1997) *Cell* **91,** 543–553
3. Chen, S., Wang, Q.-L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997) *Neuron* **19,** 1017–1030
4. Swain, P. K., Chen, S., Wang, Q.-L., Affatigato, L. M., Coats, C. L., Brady, K. D., Fishman, G. A., Jacobson, S. G., Swaroop, A., Stone, E., Sieving, P. A., and Zack, D. J. (1997) *Neuron* **19,** 1329–1336
5. Sakamoto, K., Oishi, K., Okada, T., Onuma, Y., Yokoyama, K., Sugimoto, K., and Ishida, N. (1999) *Neurosci. Lett.* **261,** 101–104
6. Li, X., Chen, S., Wang, Q.-L., Zack, D. J., Snyder, S. H., and Borjigin, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1876–1881
7. Boatright, J. H., Borst, D. E., Peoples, J. W., Bruno, J., Edwards, C. L., Si, J.-S., and Nickerson, J. M. (1997) *Mol. Vis.* **3,** 15
8. Fong, S.-L., and Fong, W.-B. (1999) *Curr. Eye Res.* **18,** 283–291
9. Bobola, N., Briata, P., Ilengo, C., Rosatto, N., Craft, C., Corte, G., and Ravazzolo, R. (1999) *Mech. Dev.* **82,** 165–169
10. Fei, Y., Matragoon, S., Smith, S. B., Overbeek, P. A., Chen, S., Zack, D. J., and Liou, G. L. (1999) *J. Biochem.* (*Tokyo*) **125,** 1189–1199
11. Livesey, F. J., Furukawa, T., Steffen, M. A., Church, G. M., and Cepko, C. L. (2000) *Curr. Biol.* **10,** 301–310
12. Swaroop, A., Xu, J. Z., Pawar, H., Jackson, A., Skolnick, C., and Agarwal, N. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 266–270
13. Kumar, R., Chen, S., Scheurer, D., Wang, Q.-L., Duh, E., Sung, C. H., Rehemtulla, A., Swaroop, A., Adler, R., and Zack, D. J. (1996) *J. Biol. Chem.* **271,** 29612–29618
14. Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 191–195
15. Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000) *J. Biol. Chem.* **275,** 29794–29799
16. Furukawa, T., Morrow, E. M., Li, T., Davis, F. C., and Cepko, C. L. (1999) *Nat. Genet.* **23,** 466–470
17. Sohocki, M. M., Sullivan, L. S., Mintz-Hittner, H. A., Birch, D., Heckenlively, J. R., Freund, C. L., McInnes, R. R., and Daiger, S. P. (1998) *Am. J. Hum. Genet.* **63,** 1307–1315
18. Freund, C. L., Wang, Q.-L., Chen, S., Muskat, B. L., Wiles, C. D., Sheffield, V. C., Jacobson, S. G., McInnes, R. R., Zack, D. J., and Stone, E. M. (1998) *Nat. Genet.* **18,** 311–312
19. Silva, E., Yang, J.-M., Li, Y., Dharmaraj, S., Sundin, O. H., and Maumenee, I. H. (2000) *Invest. Ophthalmol. Visual Sci.* **41,** 2076–2079
20. Swaroop, A., Wang, Q.-L., Wu, W., Cook, J., Coats, C., Xu, S., Chen, S., Zack, D., and Sieving, P. (1999) *Hum. Mol. Genet.* **8,** 299–305
21. Kimurn, A., Singh, D., Wawrousek, E. F., Kikuchi, M., Nakamura, M., and Shinohara, T. (2000) *J. Biol. Chem.* **275,** 1152–1160
22. Yanagi, Y., Masuhiro, Y., Mori, M., Yanagisawa, J., and Kato, S. (2000) *Biochem. Cell Biol.* **269,** 410–414
23. Zhu, X., and Craft, C. M. (2000) *Mol. Cell. Biol.* **20,** 5216–5226
24. Zhu, X., and Craft, C. M. (2000) *Biochem. Cell Biol.* **270,** 504–509
25. Tanaka, M., and Herr, W. (1990) *Cell* **60,** 375–386
26. Tang, J. K., Singh, S., and Saunder, G. F. (1998) *J. Biol. Chem.* **273,** 7210–7221
27. Singh, S., Tang, H. K., Lee, J.-Y., and Saunders, G. F. (1998) *J. Biol. Chem.* **273,** 21531–21541
28. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
29. Morrow, E. M., Furukawa, T., and Cepko, C. L. (1998) *Trends Cell Biol.* **8,** 353–358
30. Jacobson, S. G., Cideciyan, A. V., Huang, Y., Hanna, D. B., Freund, C. L., Affatigato, L. M., Carr, R. E., Zack, D. J., Stone, E. M., and McInnes, R. R. (1998) *Invest. Ophthalmol. Visual Sci.* **39,** 2417–2426
31. Finkelstein, R., and Boncinelli, E. (1994) *Trends Genet.* **10,** 310–315
32. Simeone, A. (1998) *EMBO J.* **17,** 6790–6798
33. Hirth, F., and Reichert, H. (1999) *Bioessays* **21,** 677–684
34. Hessabi, B., Ziegler, P., Schmidt, I., Hessabi, C., and Walther, R. (1999) *Eur. J. Biochem.* **263,** 170–177
35. Chau, K.-Y., Munshi, N., Keane-Myers, A., Cheung-Chau, K.-W., Tai, A. K.-F., Manfioletti, G., Dorey, C. K., Thanos, D., Zack, D. J., and Ono, S. J. (2000) *J. Neurosci.* **20,** 7317–7324