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 WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain). Peculiar to the WSP domain is a basic region, a WSP domain, and an OTX tail (generally believed to be the transactivation domain).
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 BamHI/XhoI-digested pCDA3.1HisC 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 T7 kit (Promega, Madison, WI) and [35S]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 (EMSA)**—The BAT-1 oligonucleotide was used as a probe. 5'-GTTAGAATTATGATTAATA-ACGCCCC-3' and its exact complement (synthesized by Genosys Biotechnologies, Inc., The Woodlands, TX). Double-stranded probe was 32P-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(dI-dC) (Amershams 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 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 with 1 µl of lysozyme buffer (Promega), and extracted using a rubber policeman. The lysate 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. 2A). 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. 2B 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 homeodomain (i.e., WSP domain, 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-
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 transfectected with each of the constructs were isolated and analyzed by Western blotting using an antibody against the Xpress peptide tag. As shown in Fig. 2C, 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-
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. 1A, 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. 2B) and nuclear expression (Fig. 2C). 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. 1A, 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; $p = 10^{-6}$).
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 transactivating 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. 1B 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. 4A). In EMSAs with the BAT-1 site, the four deletions showed very similar DNA-binding activities (Fig. 4B). 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. 4C). 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 transactivation 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 R890W 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
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 (31), 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.

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