Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene *Crx*

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Background: Terminal differentiation of many cell types is controlled and maintained by tissue- or cell-specific transcription factors. Little is known, however, of the transcriptional networks controlled by such factors and how they regulate differentiation. The paired-type homeobox transcription factor, Crx, has a pivotal role in the terminal differentiation of vertebrate photoreceptors. Mutations in the human *CRX* gene result in either congenital blindness or photoreceptor degeneration and targeted mutation of the mouse *Crx* results in failure of development of the light-detecting outer segment of photoreceptors.

Results: We have characterized the transcriptional network controlled by Crx by microarray analysis of gene expression in developing retinal tissue from *Crx*$^{+/+}$ and *Crx*$^{-/-}$ mice. These data were combined with analyses of gene expression in developing and adult retina, as well as adult brain. The most abundant elements of this network are ten photoreceptor-specific or -enriched genes, including six phototransduction genes. All of the available 5′ regulatory regions of the putative Crx targets contain a novel motif that is composed of a head-to-tail arrangement of two Crx-binding-element-like sequences. Analysis of the 5′ regions of a set of mouse and human genes suggests that this motif is specific to Crx targets.

Conclusions: This study demonstrates that cDNA microarrays can be successfully used to define the transcriptional networks controlled by transcription factors in vertebrate tissue *in vivo*.

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Background

Studies of neural development have highlighted the role of cell- and tissue-specific transcription factors in regulating both cell fate determination events and the later morphological stages of neuronal differentiation [1]. Little is known, however, about the gene expression or transcriptional networks regulated by these factors or those controlling cell fate determination.

In the developing vertebrate retina, several transcription factors have been implicated in the differentiation of specific cell types, including the paired-type family member Chx10 (bipolar neurons; [2]) and the POU-domain transcription factor family member Brn-3b (subtype of ganglion cells [3]). The transcription factor Crx (cone, rod homeobox) has a pivotal role in the morphological differentiation of both rod and cone photoreceptors [4,5]. Mutations in human *Crx* result in either congenital blindness or photoreceptor degeneration [6–9] and targeted mutation of mouse *Crx* results in failure of growth of the light-detecting outer segment of photoreceptors and absence of phototransduction [10]. In addition, *Crx*$^{-/-}$ mice have an altered photoentrainment phenotype [10].

Crx is normally expressed in both cone and rod photoreceptors after the precursors of these cells have exited the cell cycle, but prior to overt morphological differentiation. It is expressed in these two cell types and pinealocytes throughout the life of the organism [4,5]. Crx is a paired-type homeodomain-protein that was identified by a number of independent approaches, including its ability to bind to a photoreceptor-specific promoter fragment from the upstream region of the rhodopsin gene in a yeast one-hybrid assay [5]. Several studies have indicated the presence of functional consensus Crx-binding sites in the upstream regions of photoreceptor genes, including rhodopsin and arrestin [4,5], and several of these genes are expressed at low levels in the neural retina in mice homozygous for mutations in *Crx* [10].

Delineation of the transcriptional networks controlled by transcription factors is a major goal of functional genomics [11,12]. To date, this approach has been used successfully in yeast and in mammalian cell lines *in vitro* [12–17]. As most vertebrates have genomes with approximately twenty times as many genes as yeast, it will be important to determine if the genomics approaches developed for model organisms can be successfully applied to vertebrates such as mice and humans. It will be particularly useful if these approaches can be applied to tissue taken directly from an animal, as cells typically exhibit changes in both gene expression and genome structure upon cultivation *in vitro*.

Rod photoreceptor differentiation is a particularly tractable system in which to carry out such studies in vertebrates. Rod photoreceptors make up over 70% of the cells of the rodent retina [18] and undergo terminal differentiation over a short window of time after birth [19]. This means that retinal tissue samples from early postnatal time points represent a relatively synchronized population of differentiating rod photoreceptors. A considerable number of photoreceptor-specific genes have been cloned in mammals and their upstream regions sequenced and characterized [5,20–22], thus enabling gene identification and promoter sequence analysis. Most importantly for the current study, characterization of the phenotype of the *Crx* mutant mouse indicated that several photoreceptor-specific genes are down-regulated in the absence of Crx function [10]. These studies also demonstrated that photoreceptor degeneration in the *Crx* mutant does not begin until several weeks after birth and identified postnatal day 10 (P10) as a time when *Crx*$^{–/–}$ and *Crx*$^{+/+}$ retinae appear equivalent histologically. This then enabled a study of the transcriptional targets of *Crx* without the confounding effects of photoreceptor degeneration.

We have used retinal cDNA microarrays in combination with *Crx* mutant tissue to delineate the transcriptional consequences of lack of *Crx* function and to compare gene expression between the prenatal and adult retina, as well as between the adult retina and adult brain. These comparisons allowed an identification of those genes that fulfill the criteria to be Crx targets.

Results

Comparing gene expression between *Crx* wild-type and mutant mice using cDNA microarrays

In order to identify components of the transcriptional network controlled by *Crx*, we compared gene expression in *Crx* mutant animals with that of wild-type littermates, using a microarray of 960 mouse adult retina cDNA library clones (Figure 1). To probe the arrays, cDNA was synthesized and amplified from postnatal day 10 neural retina from *Crx*$^{+/+}$ and *Crx*$^{–/–}$ animals and labeled by Klenow-mediated incorporation of Cy3–dCTP or Cy5–dCTP respectively (Figure 1). Gene expression was analyzed at this timepoint as it precedes morphogenesis of the outer segment (i.e., overt morphological differentiation) and coincides with the initial point of high expression of known rod photoreceptor genes, including rhodopsin, recoverin and arrestin [23–25].

To test the reproducibility of the cDNA amplification process used, cDNA was prepared from total brain RNA by two independent reverse transcription/SMART amplifications. These two pools of cDNA were then Cy3- and Cy5-labeled and compared to one another and to themselves by hybridizing to the 960-element array (Figure 2). Analysis of the gene expression data from those hybridizations indicated that cDNA pools prepared from two independent reverse transcriptions and amplifications were remarkably similar (correlation between the Cy3 and Cy5 intensities for all spots, r, = 0.97; see also Figure 2 for co-efficient of determination, R^2). Comparison of each cDNA sample to itself by array hybridization gave an indication of the degree of variation introduced by the labeling and hybridization procedures. We found that there was almost no variation introduced (correlation coefficients between the Cy3 and Cy5 intensities for all spots, for each sample, were $r = 0.98$ and 0.99, respectively).

Data from two independent *Crx* wild-type versus mutant hybridizations were analyzed to identify array elements ('spots') whose gene expression ratio between the two conditions differed from the normal distribution, as judged empirically (Figure 2). Spots were chosen for further analysis if their expression ratios differed from the normal distribution in both hybridizations. This analysis identified 38 spots whose ratios differed between the two conditions, with all but one of these being genes that were expressed at a lower level in the mutant retina.
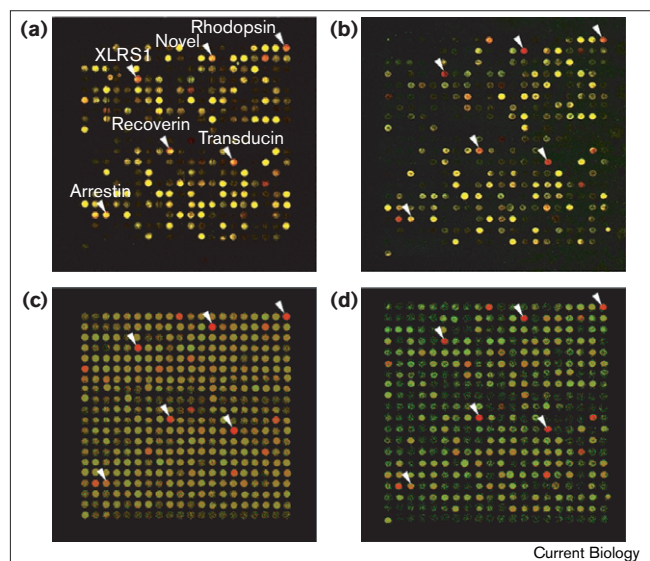
Sequence identity of differentially expressed genes

Sequencing the 38 spots that were identified as differentially expressed on at least two arrays revealed that these represent 16 different genes (Table 1). Of these 16 genes, only one, the α-1H T-type calcium channel subunit [26,27], is expressed at a higher level in the *Crx*$^{–/–}$ retina (see below). Of the 15 genes expressed at a higher level in the wild-type retina, five encode components of the phototransduction pathway: rhodopsin, rod α-transducin, recoverin, rod cGMP phosphodiesterase γ-subunit and arrestin. The sixth encodes a structural protein of the photoreceptor outer segment, peripherin. All of these genes are either photoreceptor-specific or highly enriched in photoreceptors in their expression [28,29].

Of the other three known genes identified as expressed at a higher level in the wild-type retina, two are photoreceptor-specific genes identified by positional cloning as genes underlying retinal degeneration in humans, the X-linked retinoschisis-1 (*XLRS-1*) [30] and retinitis pigmentosa-1 genes [31,32]. The final known gene encodes the aryl hydrocarbon receptor interacting protein (AIP), a widely expressed immunophilin-related protein involved in regulating signaling by the the aryl hydrocarbon receptor (AhR), a protein of the bHLH–PAS family (basic helix-loop-helix–Period-Arnt-Singleminded [33,34]). The other six genes identified as expressed at a significantly lower level in *Crx*$^{–/–}$ neural retina are all transcripts with matches to the expressed sequence tag (EST) database and thus represent transcripts of unknown function.

Sequence analysis of members of the group of transcripts identified as significantly different on only one hybridization
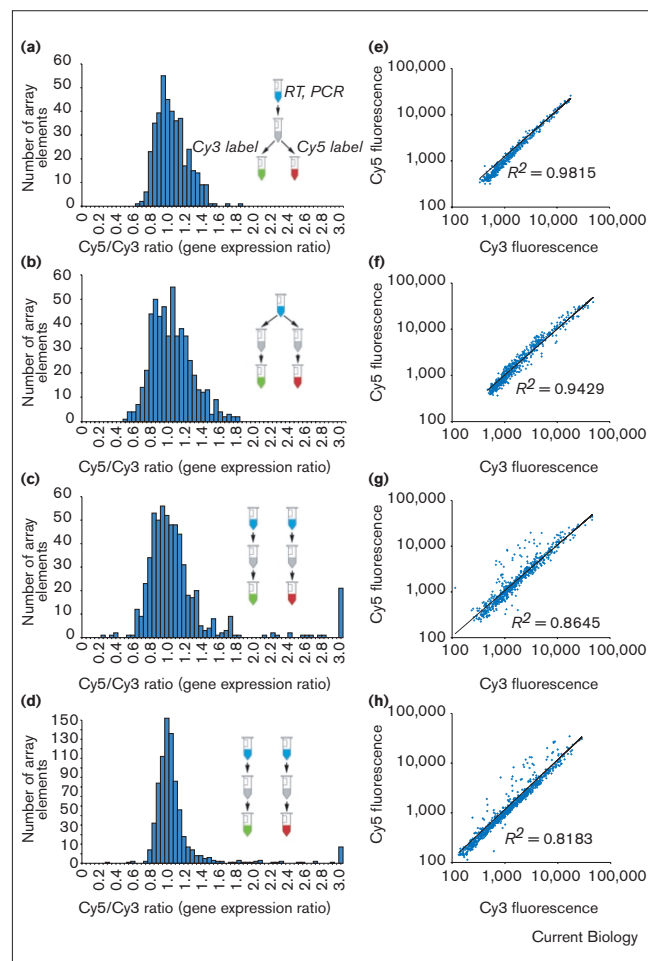
Figure 1

Identification of genes differentially expressed between *Crx⁺/⁺* and *Crx⁻/⁻* neural retina. cDNA microarray hybridizations comparing gene expression between *Crx⁺/⁺* and *Crx⁻/⁻* P10 neural retina. All panels show the same 384-spot region of the array, with gene expression in the *Crx⁻/⁻* retina represented by green, in the *Crx⁺/⁺* retina by red and genes expressed in both tissues by yellow. **(a,b)** Two independent hybridizations, standard view of two color image. **(c,d)** Ratio view of the hybridizations shown in (a,b), illustrating the Cy5/Cy3 ratio value for each spot. Arrows in all panels indicate genes as labelled in (a). Images are displayed such that the red and green colors for each pixel of each spot were mixed according to the single pixel Cy5/Cy3 ratio.

Figure 2

cDNA amplification and microarray hybridization are highly reproducible. **(a–d)** Histograms of the distribution of gene expression (Cy5/Cy3) ratios for all array elements for hybridizations comparing gene expression. (a) A brain RNA sample (blue tube) was reverse transcribed and amplified (RT, PCR) to generate a single cDNA pool (grey tube). This was then split into two tubes and labeled with Cy3 (green) or Cy5 (red) in order to assess the intrinsic variation introduced by the labeling and hybridization. (b) One stock of brain RNA was split in two; each tube of RNA was then subjected to reverse transcription and PCR amplification. These two tubes of cDNA were then labeled with Cy3 or Cy5 and hybridized to the same microarray in order to assess the reproducibility of the amplification procedure. (c,d) Repeat hybridizations of the single cDNA samples prepared from P10 *Crx⁺/⁺* (Cy5) and *Crx⁻/⁻* (Cy3) neural retina. **(e–h)** Scatter plots (on logarithmic axes) of the measured Cy5 and Cy3 fluorescence for each array element in each of the hybridizations in (a–d), respectively, including linear regression lines fitted to the data. R^2 indicates the co-efficient of determination for each plot. (b,f) Note the high values of R^2 for the hybridizations comparing independent preparations of brain cDNA, indicating that the samples are extremely similar, thus demonstrating that independent cDNA syntheses and amplifications are highly reproducible. (g,h) Note also that for two different samples, in this case cDNA prepared from P10 *Crx⁺/⁺* (Cy5) and *Crx⁻/⁻* (Cy3) neural retina, R^2 is considerably lower and there are many array elements/spots that lie off the regression line, corresponding to differentially expressed genes.

revealed that two of these spots encode the retina-specific gene phosducin [35], while another encodes the photoreceptor-specific sodium/potassium, calcium exchanger [36]. Given that we identified phosducin from a single array twice, this gene was included in the set for further analysis. Among those spots whose expressions do not change in the mutant retina are GAPDH, β-actin and the synapse-associated proteins SNAP-25b and α-adaptin.

Confirmation of differential expression of candidate genes

Northern blotting of nine genes identified as differentially expressed on the array was carried out in order to determine if the gene expression differences calculated using microarrays were significant. These genes covered the range of array-calculated gene expression ratios and included the single gene up-regulated in the mutant retina (T-type calcium channel), five phototransduction genes, XLRS-1 and two novel transcripts (spot 54 and spot 265/433, an EST found twice on each array). Northern blot analysis confirmed that all of these nine genes are differentially expressed between P10 *Crx⁺/⁺* and *Crx⁻/⁻* retinae (Figure 3a; Table 1), demonstrating that spots with gene expression ratios of 1.6 and higher identified on two arrays are accurate indicators of gene expression differences.

Table 1

Identity of genes differentially expressed between *Crx*$^{+/+}$ and *Crx*$^{-/-}$ developing retina.

Gene	No. of spots	Array	Northern	Cell type	Function	CBE	Crx motif
Rod transducin, alpha	5	5.9×	20×	Rod	Phototransduction	Yes	Yes
Spots 265,433	2	5.6×	10×			n.a.	
Recoverin	1	4.0×	5×	PhR, bipolar	Phototransduction	n.a.	n.a.
XLRS1	2	3.5×	10×	PhR	Intercellular signalling	Yes	n.a.
Spot 678	1	3.5×				n.a.	
Rhodopsin	13	3.4×	12×	Rod	Phototransduction	Yes	Yes
Spot 501	1	2.7×		PhR/INL		n.a.	
Spot 54	1	2.6×	4×	INL		n.a.	
AIP	1	2.3×		INL/GC	Signal transduction	n.d.	
PDE gamma	1	2.3×		Rod	Phototransduction	Yes	Yes
Spot 77	1	2.0×				n.a.	
Spot 491	1	1.9×				n.a.	
Spot 140	1	1.8×		Rod		n.a.	
Arrestin	3	1.8×	1.4×	Rod	Phototransduction	Yes	Yes
Peripherin	2	1.7×	1.4×	PhR	Photoreceptor disc integrity	n.a.	n.a.
Phosducin	2 (one array)	1.7×		Rod	Phototransduction	Yes	Yes
Retinitis pigmentosa-1	1	1.7×		PhR	Unknown	Yes	n.a
T-type calcium channel	1	0.5×	0.2×	Bipolar		n.d.	

Gene, gene identifier; No. of spots, number of spots representing each gene found on the array; Array, average gene expression ratio (*Crx*$^{+/+}$/*Crx*$^{-/-}$) of each gene calculated by array hybridization; Northern, gene expression ratio (*Crx*$^{+/+}$/*Crx*$^{-/-}$) of each gene calculated by northern 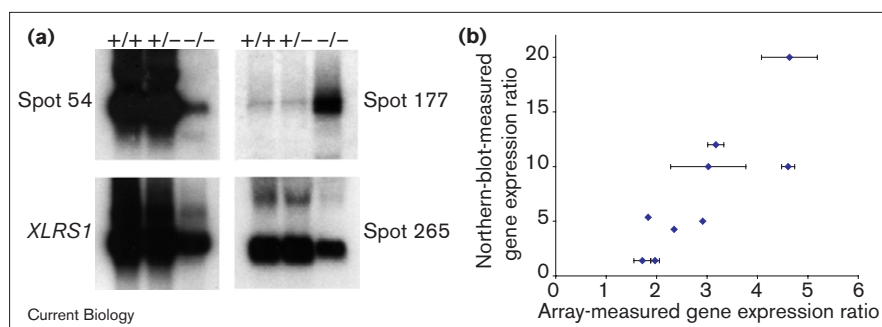blot; Cell type, cell type expressing the gene in the adult retina; Rod, rod photoreceptors; PhR, rod and cone photoreceptors; bipolars, bipolar neurons; Function, putative function of gene product; CBE, presence of consensus Crx-binding element in promoter, where available; Crx motif, presence of novel 11 base motif in promoter, where available; n.a., not available; n.d., not done.

To correlate the fold change detected by microarray analysis with that seen by northern blot, the ratio of expression between wild-type and mutant for each of the nine genes was measured by densitometry. Gene expression ratio values calculated from northern blots were plotted against fold-change calculated by array analysis (Figure 3b). Although independent in execution, there is a strong linear relationship between the two methods (for two independent array hybridizations, $r = 0.84$ and $r = 0.72$), particularly given the relatively small sample numbers. It is also noteworthy that for those genes represented more than once on the array there is significantly lower variability in the measurements of the gene expression ratio on independent spots on a single array than in the ratio between arrays (Table 2).

Genes identified as expressed at a low level in the *Crx*$^{-/-}$ retina are enriched in the adult retina

To further characterize the set of array elements identified as possible Crx targets, the expression of those elements in the adult retina and adult brain, and in the developing retina compared to adult retina, was studied using the same retina cDNA microarrays. As rod photoreceptors differentiate postnatally in rodents, such an approach should identify those genes that fulfill criteria for being Crx target genes: down-regulated in the *Crx* mutant, retina-enriched and expressed postnatally. The comparison of gene expression between the adult retina and retina from embryonic days 15 and 17 identified many array elements that were significantly enriched in either the adult or developing retina (Figure 4a and data not

Figure 3

Correlation of gene expression differences calculated by microarray hybridization and northern blot. **(a)** Northern blots of total RNA comparing gene expression in P10 wild-type (+/+), heterozygous (+/−) and homozygous (−/−) *Crx* mutant retina for two EST transcripts, the alpha-1H T-type calcium channel (spot 177) and *XLRS1*. **(b)** Linear correlation between gene expression ratios calculated by microarray hybridization and by northern blot analysis. Error bars indicate standard errors of the mean values for those genes for which there were multiple spots on the array.

Table 2

Variation in gene expression ratios within and between microarray hybridizations.

Gene	Spots	Array 1	Array 2
Rhodopsin	13	3.17 ± 0.16	3.62 ± 0.18
Transducin	5	4.63 ± 0.55	7.22 ± 0.92
Arrestin	3	1.97 ± 0.09	1.72 ± 0.05
Spot 265;433	2	4.61 ± 0.13	6.65 ± 0.89
Peripherin	2	1.72 ± 0.17	1.75 ± 0.01
XLRS1	2	3.02 ± 0.74	3.98 ± 1.73

Gene, gene identifier; Spots, number of spots representing gene on array; Array 1,2, mean gene expression ratio (wild-type/mutant) ± s.e.m. for each gene from each of two independent hybridizations.
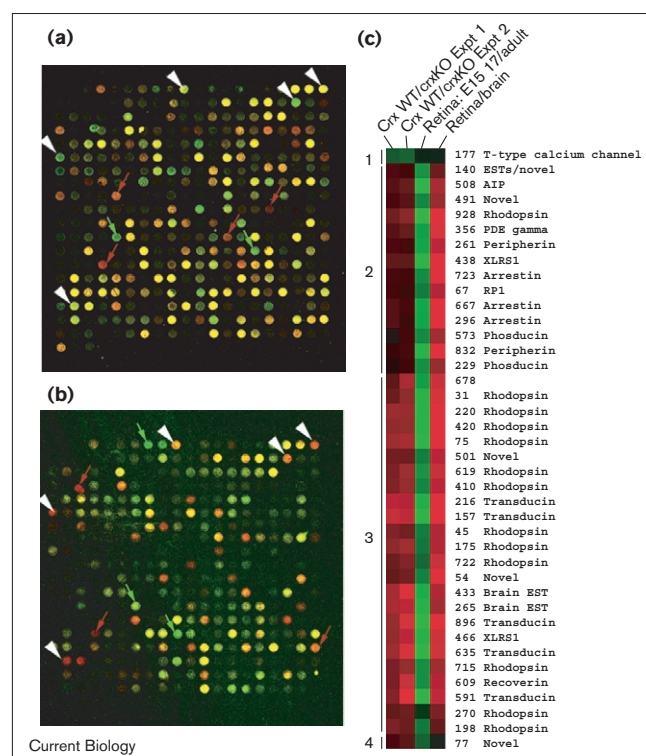
shown). Similarly, a comparison of gene expression from adult retina and whole adult brain identified many retina-specific genes, many more than were identified as possibly Crx-regulated (Figure 4b and data not shown).

Figure 4

Genes identified as differentially expressed between *Crx*+/+ and *Crx*−/− neural retina fulfil the criteria for Crx targets. **(a)** Microarray hybridization, showing the same region of the array as in Figure 1, comparing gene expression in the adult mouse retina (green) with gene expression in the prenatal developing mouse retina (red). Note that, in addition to the labelled spots corresponding to genes identified as differentially expressed between *Crx*+/+ and *Crx*−/− retina (indicated by arrowheads), there are many other spots representing genes enriched in either the developing retina (red arrows) or adult retina (green arrows). **(b)** Microarray hybridization comparing gene expression in the adult mouse retina with gene expression in the adult mouse brain. Note that, in addition to the labelled spots corresponding to genes identified as differentially expressed between *Crx*+/+ and *Crx*−/− retina (arrowheads), there are several other spots representing genes enriched in the adult retina (red arrows) or adult brain (green arrows). **(c)** Cluster analysis of the set of 40 spots identified as differentially expressed between *Crx*+/+ (WT) and *Crx*−/− (KO) retina, including data on their expression in the developing and adult retina and adult brain. Clustering was calculated by a hierarchical method based on Euclidean distance measurements and the data represented using the method of Eisen *et al.* [13]. Color intensity reflects the magnitude of the gene expression ratio for each spot, red indicates genes expressed at a higher level in sample 1 (compared to sample 2 of each pair), green indicates genes expressed at a higher level in sample 2 and black indicates gene expression ratios near 1. Each column represents gene expression ratios from a separate experiment: column 1, P10 *Crx* wild-type retina/*Crx* mutant retina, experiment (expt) 1; column 2, P10 *Crx* wild-type retina/*Crx* mutant retina, experiment 2; column 3, embryonic day 15 and 17 neural retina/adult neural retina; column 4, adult retina/adult whole brain. Two major and two minor groups were identified: 1, the single gene (a T-type calcium channel) upregulated in the mutant retina; 2, spots whose expressions differ by a lesser degree between *Crx*+/+ and *Crx*−/− retina, are enriched in retina as compared to brain and are enriched in adult as compared to prenatal retina; 3, spots whose expressions differ by a large degree between *Crx*+/+ and *Crx*−/− retina, are enriched in retina as compared to brain and are enriched in adult as compared to prenatal retina; 4, the single spot (representing an EST sequence) differentially expressed between *Crx*+/+ and *Crx*−/− retina that is equally expressed in retina and brain, although expressed at a lower level in the developing retina.

For the purposes of the experiments reported here, the expression values of the set of 40 elements that were identified as differentially expressed between the *Crx*+/+ and *Crx*−/− retina (38 spots identified from two hybridizations plus the two phosducin spots) were selected from these other data sets. These values and the expression values for those 40 elements in the two *Crx* hybridizations were grouped using a cluster analysis algorithm (based on Euclidean distance measurements) to identify patterns of gene expression within the larger set of genes. With the exception of one, all of the genes expressed at a higher level in the wild-type retina are retina-enriched and expressed at a higher level postnatally than prenatally. Two major clusters of genes were identified, defined by their degree of down-regulation in the *Crx* mutant (supported by northern blot analysis): both clusters contained genes that were enriched in adult retina compared to both prenatal retina and whole brain. However, the two clusters differed in the scale of the gene expression differences, such that the majority of genes in cluster 2 (Figure 4) had wild-type/mutant gene expression ratios less than 2.5, whereas the majority of the genes in cluster 3 (Figure 4) had ratios of approximately 2.5 and over. In addition to those groups, two single genes were not grouped into either cluster: the single gene up-regulated in the mutant and a single gene down-regulated in the mutant but expressed at the same level in adult brain and adult retina (i.e., not retina enriched).

The cell types expressing several of the EST transcripts in the normal adult retina were studied by *in situ*
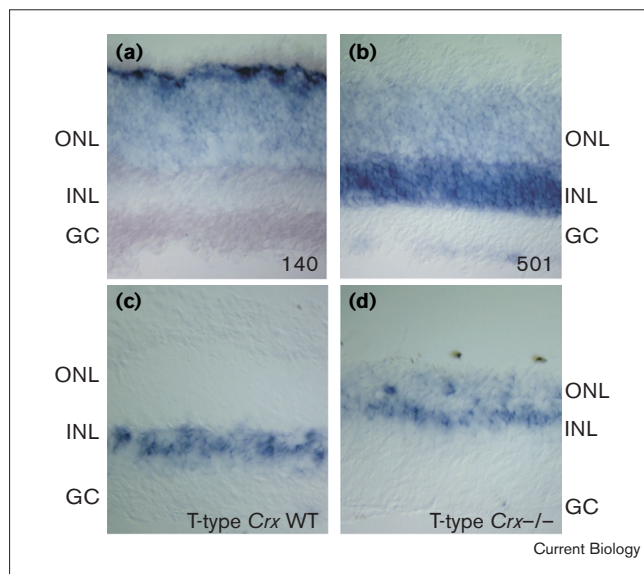

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hybridization. One of the novel transcripts was found to be expressed only in photoreceptors in the adult retina (Figure 5a), while another is expressed in photoreceptors in the outer nuclear layer (ONL) and all inner nuclear layer neurons (INL; Figure 5b). A third is expressed by INL neurons but not by photoreceptors, as is AIP; data not shown). The T-type calcium channel up-regulated in the mutant retina is expressed in the outer half of the inner nuclear layer in the normal adult retina, most likely in bipolar neurons (Figure 5c), and is also expressed only in those cells in the adult *Crx* mutant retina (Figure 5d).

Analysis of the upstream sequences of putative Crx target genes

Consensus Crx-binding elements (CBEs; C/TTAATC/T) have been noted in the promoter regions of several of the putative Crx target genes, including rhodopsin, arrestin, *Xlrs1* and *Rp1* [4,5,31,37]. To confirm this, we searched 750 bp of all of the available sequences in all available species for CBEs: human, mouse and chick rhodopsin, human rod α-transducin, human and mouse phosducin, mouse and human arrestin and human rod γ-phosphodi-esterase. In all genes in all species studied, one or more CBEs were found.

Figure 5

Cell types in the mouse retina expressing sequences identified as differentially expressed between developing *Crx+/+* and *Crx–/–* retina. *In situ* hybridization for three differentially expressed genes in the adult mouse retina. **(a)** Spot 140, expressed by photoreceptors in the ONL. **(b)** Spot 501, expressed by photoreceptors and cells in the INL (bipolar cells, amacrine cells and potentially horizontal cells and Müller glia). **(c)** Expression of the α-1H T-type calcium channel subunit in bipolar neurons in the normal adult retina. **(d)** Expression of the α-1H T-type calcium channel subunit in bipolar neurons in the adult *Crx–/–* retina. Note the markedly thinner ONL compared to wild type, due to photoreceptor degeneration. GC, ganglion cell layer.

As an alternative approach, the proximal promoter regions of all of the putative Crx-regulated genes for which promoter sequence is available were searched for novel, shared sequence motifs. To do this, the proximal 250 bp of upstream sequence of all of the five available mouse and human genes were aligned using the Align-ACE program [38]: mouse and human rhodopsin, mouse and human arrestin, human γ-phosphodiesterase, mouse and human phosducin and human rod α-transducin. The highest scoring motif identified by this analysis was an 11 base motif that contained a conserved head-to-tail arrangement of one strong CBE-like sequence with a second weaker CBE-like sequence (Figure 6a). This motif was found twice in the human rhodopsin, human rod transducin alpha and human γ-phosphodiesterase promoters, and three times in the mouse arrestin pro-moter. This motif was not identified in the human arrestin promoter.

To test the specificity of this sequence for photoreceptor-enriched genes, we searched for the presence of this motif (using ScanACE [38]) in approximately 750 bp of the 5′ region of a set of 42 mouse and human retinal and non-retinal sequences, and also chick and bovine rhodopsin. This set included 6 kb of the promoters of the genes encoding human red visual pigment and mouse red-green pigment (to include the locus control regions), the 5′ regions of the genes encoding photoreceptor ABCR, cone α-transducin, the human and mouse blue visual pigment, and the genes encoding mouse and human interphotoreceptor retinoid binding protein, human ROM1, the neural retina leucine zipper (NRL) gene, metabotropic glutamate receptor-6 (mGluR6; expressed in bipolar neurons) and a retina pigment epithelium-specific protein.

Sites highly similar to this motif were found in all of the set of sequences used in the original alignment and also in the chick and bovine rhodopsin, cone α-transducin and human blue opsin promoters (Figure 6b,c). The bovine motif is within the BAT-1 motif that is protected by Crx in a DNase footprinting assay [5]. Two similar motifs were also found in the human red opsin promoter, 3.5 and 3.9 kb from the transcriptional start site, that is within and close to the region of the locus control region for the human red and green opsin genes [39]. Of the randomly selected genes, a weakly similar site (similarity score almost two standard deviations from the mean score for the motif found by AlignACE) was only found in the promoter of the human growth hormone-releasing hormone recep-tor. Weaker sites (similarity scores greater than two stan-dard deviations from the mean score for the motif found by AlignACE) were found in a number of other promoters, including the mouse red-green opsin promoter, but also in the promoters for tyrosine hydroxylase and pyruvate kinase, indicating that these weaker sites are not specific

to photoreceptor genes. Very weakly similar sites (almost three standard deviations from the mean motif score) were found in the mouse and human IRBP promoters.

Discussion

By combining studies of gene expression in wild-type and mutant animals with expression analyses between developmental stages and tissue types, we were able to quickly identify a subset of genes that fulfill the criteria for being transcriptional targets. We identified 17 genes that are differentially expressed between the wild-type and mutant *Crx* retina, 10 of which are photoreceptor-specific or photoreceptor-enriched genes. Analysis of the available promoter sequences of these Crx target genes identified a novel motif containing a head-to-tail arrangement of consensus Crx binding elements (CBEs) that is very similar to the consensus binding site for dimers of homeodomain proteins.
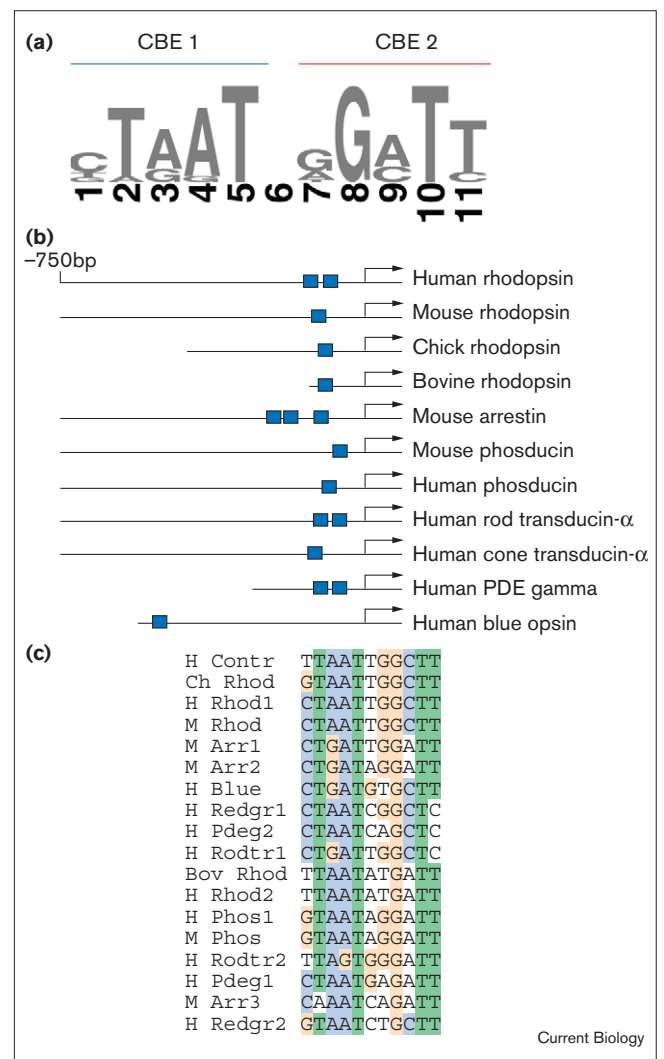
We also demonstrate that such studies can be carried out with limiting amounts of tissue. We used 1 μg of total RNA as the starting material and generated enough material for over ten hybridizations. As 1 μg of total RNA is the equivalent of 1–5ng of poly-A⁺ mRNA, we used 100–1,000-fold less starting RNA than is currently recommended for a single microarray hybridization. We also confirmed that the amplification procedure generates highly reproducible populations of cDNA.

The transcriptional network controlled by Crx

Crx controls expression of a diverse group of photoreceptor-specific and enriched genes. These genes fall into two categories based on their degree of down-regulation in the absence of *Crx*: genes that are down-regulated more than 2.5-fold by array measurement (approximately 5-fold and higher by northern blot); and genes that are down-regulated less than 2.5-fold by array (approximately 1.5–4-fold by northern blot). The difference between the groups in the degree of down-regulation suggests that the transcription of genes in the latter group can be compensated for by other factors. Given the regulation of the photoreceptor-specific gene IRBP by Otx2 [40,41] and that the consensus binding sequences for Otx2 and Crx are very similar [4,5], it is possible that these genes are co-regulated by Otx2. Alternatively, regulation through the Crx site does not contribute significantly to the transcription of this set of genes.

This study did not identify several photoreceptor-specific genes predicted to be Crx targets based on their lower expression in the retina of the *Crx* mutant mouse. For example, the β-subunit of rod phosphodiesterase and the various cone opsins were found to be down-regulated 1.3-fold and over 25-fold, respectively, by northern blot analysis [10]. The most likely reason for the lack of detection of these genes in the current study is the size

Figure 6

Identification of a conserved arrangement of CBE-like sequences in the 5′ regulatory regions of putative Crx target genes that is conserved in sequence and position among genes. **(a)** Consensus sequence of highest scoring motif from alignment of available sequences of the 5′ regulatory regions of putative Crx targets. The motif is composed of a head-to-tail arrangement of one strong Crx-binding element-like sequence (CBE 1) and a second, weaker CBE-like sequence (CBE 2). The size of each letter is proportional to the percentage of sites containing the respective nucleotide. **(b)** Location of motif shown in (a) in the proximal 750 bp (or maximum available) of putative Crx target gene 5′ regulatory regions. Blue boxes indicate the position of the motif. Arrows indicate approximate transcriptional start sites. **(c)** ClustalW pairwise alignment of the Crx motif sequences found in the 5′ regulatory regions of Crx target genes using ScanACE. Abbreviations: m, mouse gene; h, human; ch, chick; bov, bovine; rhod, rhodopsin; contr, cone transducin; redgr, human red opsin; rodtr, rod transducin; arr, arrestin; blue, blue opsin; phos, phosducin; pdeg, γ-phosphodiesterase; motifs found multiple times in a given sequence are numbered in descending order of similarity score relative to the original motif identified (shown in (a)).

of the array used (960 library clones), that is, the array would not have contained all of the target genes.

Crx target genes contain a novel, specific regulatory sequence motif

In addition to searching for consensus CBEs by sequence homology in the 5′ regulatory regions of putative Crx target genes, we have also applied an approach successfully used in yeast genomics of searching for sequence motifs conserved in the promoter regions of genes regulated in a similar fashion [38]. Using this approach, we identified a novel motif in the promoter regions of putative Crx target sites. This motif contains a head-to-tail arrangement of one strong CBE-like sequence (CTAAT) followed by a second, complementary partial CBE-like sequence (GATTN), separated by two bases. This site was found within 50–150 bp of the transcriptional or translational start site in all of the available 5′ regulatory regions of genes down-regulated in the *Crx* mutant.

In addition to the genes identified in this study, there are two others that are known to be down-regulated in the *Crx* mutant retina, encoding cone α-transducin and blue opsin [10], and the human promoters of both of these genes contain this motif. The motif is also conserved in position in the chick and bovine rhodopsin promoters, and lies within the BAT-1 site of the bovine promoter that is protected by Crx in a footprinting assay [5]. This motif was also found within and adjacent to the human red-green opsin locus control region [39], suggesting that it may also be required for Crx-mediated expression of human cone opsins. However, it is noteworthy that only a weakly similar motif was found in the mouse red-green opsin promoters and no such motif was found in the mouse blue opsin, genes down-regulated over 20-fold in the developing *Crx* mutant retina [10].

The Crx motif was specific to those photoreceptor-enriched genes that down-regulate in the absence of Crx function. A very weakly similar motif was found in the photoreceptor gene IRBP, a gene that is not down-regulated in the absence of Crx function, while no motifs were found in the ROM1 promoter, a gene whose expression does not change in the *Crx* mutant retina [10]. Although the proximal 123 bp of the IRBP promoter can be transactivated by Crx in cell lines [4,5], a recent yeast one-hybrid study using this promoter fragment found that Otx2 bound specifically to the promoter but did not identify Crx [41], supporting the proposal that IRBP expression may not be regulated by Crx *in vivo*. Several lines of evidence indicate that this motif is likely to be the Crx binding site *in vivo*: the demonstration by a number of different approaches, including electrophoretic mobility shift assays, transactivation assays and promoter footprinting, that Crx binds and transactivates from sites containing the consensus CBE sequence T/CTAATC/T [4,5]; the presence of at least one strong CBE sequence within this motif; the specificity of this site for the 5′ regions of genes down-regulated in the absence of Crx function;

and the conservation of the Crx motif in sequence and position between mouse and human genes.

The arrangement of CBEs within the motif suggests that Crx may bind to the promoter regions of its target genes as a homodimer, or as a heterodimer with another protein, possibly a homeodomain protein such as Otx2 [42]. Binding of homeodomain transcription factors to DNA as dimers is a common feature of these transcription factors, including the related protein Otx2 [43]. The binding site for homeodomain dimers typically contains a palindromic arrangement of the sequence TAAT, separated by a fixed gap of three bases where the protein contains a lysine residue at position 50 of the homeodomain [44]. This is very similar to the consensus sequence identified by promoter sequence alignment here (see Figure 6). Although this sequence is asymmetrical, homodimers of homeodomain proteins have been shown to commonly bind asymmetrical sequences in the promoters of their target genes [45].

The biology of the *Crx* transcriptional network

Mutations in many of the genes identified as Crx targets each result in photoreceptor degeneration. For example, mutations in rhodopsin or in the RP-1 gene result in retinitis pigmentosa [31,32,46,47]. It is likely that the combination of reduced expression of all of the Crx target genes makes it impossible to construct a morphologically differentiated photoreceptor. However, the possibility that reduced or absent expression of one major protein, such as RP1 or rhodopsin, is solely responsible for failure of outer segment formation cannot be excluded.

Conclusions

We demonstrate here that combining cDNA microarray analysis of gene expression in mutant and wild-type animals with further array analyses to highlight genes fulfilling specific criteria can be successfully used to identify transcription factor targets *in vivo*. In this case, we show that Crx controls expression of a core set of photoreceptor genes involved in a number of different functions characteristic of the differentiated photoreceptor. Those genes that fulfill all of the criteria for being Crx targets share an 11 base motif that is similar to the standard binding site for homeodomain dimers. This motif is conserved in position between mouse and human promoters and appears to be specific to photoreceptor genes that down-regulate in the absence of Crx function. We conclude that genomics approaches developed for simpler organisms, such as yeast, can be successfully applied to studying gene expression networks in vertebrates *in vivo*.

Materials and methods

Microarray construction

To construct a mouse retina cDNA microarray, the inserts of 960 random clones from an adult mouse retina cDNA library were amplified by PCR using T3 and T7 primers. The oligo-dT primed cDNA library was constructed in the lambda-ZAP phage vector (Stratagene) using RNA

extracted from adult C57Bl/6 retinae. PCR products were ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in $2 \times SSC$ for printing. Microarrays were printed on poly-L-lysine coated glass slides (Poly-prep, Sigma) with a piezo-electric array printing system.

Microarray hybridization and data gathering
Total RNA from postnatal day 10 retina of *Crx*-mutant and wild-type littermates (1 μg from each), adult retina (100 ng), adult brain (100 ng and 1 μg) and pooled embryonic day 15 and 17 mouse retina (100 ng total) was reverse transcribed and amplified using the SMART system (Clontech). To generate probes for array hybridization, 1−2 μg cDNA was labeled by incorporation of either Cy5 or Cy3-dCTP during oligo-dT-primed or random hexamer-primed primer extension in the presence of Klenow DNA polymerase (Roche). Arrays were pre-hybridized with poly-adenylic acid (Sigma) and mouse Cot-1 DNA (Life Technologies) before overnight hybridization with paired Cy3 and Cy5-labeled probes in the presence of poly-adenylic acid and mouse Cot-1 DNA [48]. Arrays were washed at room temperature in $0.1 \times SSC$, 0.1%SDS for 3 min, followed by 2 washes in $0.1 \times SSC$ for 2 min each. Hybridized arrays were scanned with the ScanArray 5000 (GSI Lumonics) and images imported into the ScanAlyze package (M. Eisen, Stanford).

Data analysis
Data were filtered to remove values from poorly hybridized spots using the cutoff that more than 60% of pixels had intensity levels greater than twofold above the background local to each spot. The Cy5/Cy3 ratio measurements for each remaining spot on each array were calculated using the ScanAlyze package (M. Eisen, Stanford). Data were scaled such that the median ratio value for each hybridization data set was 1. The distribution of ratio values was used to empirically determine which array spots/library clones to sequence and study further. Spots whose ratios differed significantly from the normal distribution of gene expression ratios from at least two hybridizations were chosen for further study. For cluster analysis, ratio measurements for the set of genes under study (see text) under each condition were imported into the Cluster package (M. Eisen, Stanford) [13] in order to assign elements/genes to clusters using Euclidean distance measurements. Results were displayed using the TreeView program [13].

Sequences of upstream regions of genes identified as putative Crx targets were analyzed for motifs by searching the proximal 250 bp of each 5′ regulatory region (where available; see text for details) for conserved regions using the AlignACE program [38]. Sequences were first searched for rodent or human repeat sequences using the RepeatMasker online service (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). To search for AlignACE motifs in mouse and human promoters, 750 bp (or less if that was all available) of the 5′ regulatory regions from a random selection of those genes in GenBank in which either the TATA box, transcriptional start site or the translational start sites were clearly indicated were assembled in a single file and searched using ScanACE [38]. Sequences that scored within two standard deviations of the average motif score were taken as significant matches. Promoters included were (human unless otherwise noted): rhodopsin (mouse, human, chick and bovine), human and mouse blue opsin, 6 kb of human and mouse red and red-green opsin 5′ regulatory region, peripherin (intermediate filament protein; mouse and human), phosducin (mouse and human), NRL, rod arrestin (mouse and human), IRBP (mouse and human), ROM1 (mouse and human), rod α-transducin, cone α-transducin, rod γ-PDE, ABCR (mouse and human), mouse γ-crystallin, mGluR6, mouse Pax-6 P1, sonic hedgehog, retinaldehyde dehydrogenase-2, Wnt-5A, glutamate decarboxylase, STAT1, fructose-6-bisphosphatase, SMN, heme oxygenase-1, RPE65, Smad7, caveolin, CD52, GHrhR, pyruvate kinase 2, Gli-1, tyrosine hydroxylase. All of the motif sequences found in the 5′ regions of photoreceptor genes were aligned using the online ClustalW server at the European Bioinformatics Institute (http://www2.ebi.ac.uk).

DNA sequencing, northern blotting and in situ hybridization
cDNA inserts from lambda phage clones of interest were amplified by PCR using T3 and T7 primers or harvested as plasmids by phagemid rescue. PCR products were purified (Qiagen) before dye-terminator fluorescent sequencing. Sequences were compared to GenBank using the BLASTN and BLASTX algorithms [49]. Northern blotting was used to confirm differences in gene expression between *Crx*-mutant and wild-type mice, using ^{32}P-labelled PCR products (Rediprime, Amersham) according to standard techniques [50]. Northern blot gene expression ratios were calculated by analyzing 16-bit TIFF images of autoradiograms using NIHimage. Blots were also probed for GAPDH expression or gels ethidium bromide stained and the intensity of the 28S and 18S bands measured to correct for RNA loading. Non-radioactive *in situ* hybridisation using probes synthesized from cDNA clones was carried out as described [51].

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