Interference of Crx-dependent transcription by ataxin-7 involves interaction between the glutamine regions and requires the ataxin-7 carboxy-terminal region for nuclear localization

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Spinocerebellar ataxia type 7 (SCA7) is an inherited neurodegenerative disorder caused by expansion of a polyglutamine tract in the ataxin-7 protein. A unique feature of SCA7 is degeneration of photoreceptor cells in the retina, resulting in cone–rod dystrophy. In an SCA7 transgenic mouse model that we developed, it was found that the cone–rod dystrophy involves altered photoreceptor gene expression due to interference with Crx, a homeodomain transcription factor containing a glutamine-rich region. To determine the basis of the Crx–ataxin-7 interaction, Crx and ataxin-7 truncation and point mutants were generated, and the ability of mutant versions of either protein to co-immunoprecipitate the normal version of the other protein was tested. Thus Crx’s ataxin-7 interaction domain was localized to its glutamine-rich region and ataxin-7’s Crx binding domain was mapped to its glutamine tract. The importance of each protein’s respective glutamine region for a productive interaction was confirmed by performing Crx transactivation assays in HEK293 cells and correlating the extent of Crx transcription interference with the intactness of each protein’s glutamine region. It was also established that ataxin-7 must localize to the nucleus to repress Crx transactivation, and the likely nuclear localization signals were mapped to ataxin-7’s carboxy-terminal region. Finally, using chromatin immunoprecipitation, it was demonstrated that Crx and ataxin-7 engage in a functionally significant interaction by co-occupying the promoter and enhancer regions of Crx-regulated retinal genes in vivo. The results suggest that one mechanism of SCA7 disease pathogenesis is transcription dysregulation, and that Crx transcription interference is a predominant factor in SCA7 cone–rod dystrophy retinal degeneration.

INTRODUCTION

Spinocerebellar ataxia type 7 (SCA7) is one of nine inherited neurodegenerative disorders caused by the expansion of an unstable CAG trinucleotide repeat in the coding region of the associated gene. Included in this disease category are Huntington’s disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubral pallidoluysian atrophy (DRPLA) and five other forms of spinocerebellar ataxia (SCA1, 2, 3, 6 and 17). A unique feature that distinguishes SCA7 from the eight other polyglutamine diseases and from other cerebellar ataxias is the degeneration of cone and rod photoreceptor neurons in the retina (1,2). Interestingly, loss of cone function proceeds that of rod function, making SCA7 one of the few examples of...
RESULTS

The glutamine-rich region of Crx is required for its interaction with ataxin-7

To determine which domain(s) of Crx mediates interaction with ataxin-7, we generated Crx expression constructs containing various truncations, deletions and point mutations (Fig. 1A). Using in vitro co-ip assays, we then tested the ability of these Crx mutants to bind to ataxin-7 with 10 glutamines. Of the various Crx truncation mutants tested, only Crx:1–87 failed to co-ip with ataxin-7 (Fig. 1B; Table 1). As Crx:1–98 does bind with ataxin-7 (see lane 3), the ataxin-7 interaction domain in Crx probably includes amino acid residues 88–97. The importance of this Crx domain for interaction with ataxin-7 was confirmed by co-ip analysis of three amino-terminal deletion mutants, Crx:34–299, Crx:88–299 and Crx:111–299 (Fig. 1C; lanes 20–22), as this experiment revealed that only the Crx:111–299 mutant is incapable of co-ip with ataxin-7. This result, taken together with the co-ip data generated with the Crx carboxy-terminal deletion mutants, mapped the ataxin-7 interaction domain in Crx to amino acid residues 88–110 (Table 1). As a short Crx peptide, Crx:34–107, is sufficient for mediating an interaction between Crx and ataxin-7 in an in vitro co-ip (Fig. 1C; lane 23), the ataxin-7 interaction domain in Crx may be further delineated to amino acid residues 88–107. To confirm that amino acids 88–107 are critical for interaction with ataxin-7, we had generated an internal deletion mutant, Crx:Δ88–107, and tested its ability to interact with ataxin-7 using in vitro co-ip. Crx:Δ88–107 was unable to co-ip ataxin-7 (Fig. 1B; lane 9), verifying that the 88–107 amino acid region of Crx is required for interaction with ataxin-7.

The Crx 88–107 amino acid region consists of the carboxy-terminal one-fifth of Crx’s homeodomain followed by a glutamine rich region. The amino acid sequence at the homeodomain–activation domain border consists of 11 residues of which nine are glutamines: 96-QQRQQQKQQQ-106...
Since the glutamine-rich regions of several transcription factors and co-factors have been reported to mediate interaction with other polyglutamine disease proteins (20,21), it was reasoned that the glutamine-rich region might be critical for mediating Crx’s interaction with ataxin-7. To test this hypothesis, a Crx expression construct was generated lacking the last seven glutamines but retaining the final two glutamines from the homeodomain, and this construct was called Crx:DQ7. The glutamines at positions 96 and 97 were also changed to tryptophans, and thereby a Crx:Q96/97W construct was created. Finally, these alterations were combined to yield a construct lacking all nine glutamine residues, the so-called Crx:Q96/97W-DQ7 construct. We then tested the ability of these Crx mutants to co-ip ataxin-7. While neither deletion of the seven glutamine stretch nor the Q96/97W double mutation alone could abolish the ability of Crx to interact with ataxin-7, removal of all nine glutamines in the Crx:Q96/97W-DQ7 construct completely abolished the ability of Crx to co-ip ataxin-7 (Fig. 1D; Table 1). Interestingly, a single mutation of either homeodomain glutamine (i.e. Q96W...
or Q97W) in combination with the ΔQ7 background still permitted a Crx–ataxin-7 interaction in vitro (data not shown).

After establishing a role for Crx’s glutamine-rich region in ataxin-7 binding by co-ip, we chose to test whether deletion and/or alteration of Crx’s glutamine rich region might protect it from transcription interference by polyglutamine-expanded ataxin-7 (8). To do this, Crx transactivation assays were performed using a rhodopsin promoter–reporter construct in HEK293 cell transfections (8,9). This was begun by validation of the Crx mutants (i.e. Crx:ΔQ7, Crx:Q96/97W and Crx:Q96/97W-ΔQ7) being tested in a series of four experiments. First, electrophoretic mobility shift assays (EMSAs) were performed with a high-affinity Crx DNA binding site (BAT-1) as the probe, and it was observed that the Crx mutants display DNA binding activity comparable to wild-type Crx (Fig. 2A). Second, HEK293 cell transfections were performed and it was noted that all three Crx mutants localize to the nucleus (Fig. 2B). Third, transactivation assays were performed and it was observed that these mutants activate the rhodopsin promoter–reporter in similar degrees (3- to 6-fold) to the wild-type Crx (data not shown). Finally, protein expression levels were surveyed from the various Crx constructs by western blot analysis, and similar amounts of protein expression products noted (Fig. 2C). After completing these validation experiments, the ability of ataxin-7 with either 10 or 92 glutamines to repress transcription activation by wild-type Crx as well as that of Crx mutants at ataxin-7 92Q plasmid:Crx plasmid ratios of 1:1 or 2:1 was tested. Consistent with previous results (8), polyglutamine-expanded ataxin-7 markedly repressed wild-type Crx-mediated transactivation in a concentration- and polyglutamine-length-dependent manner (Fig. 2D). Interestingly, ataxin-7 92Q at a high concentration was significantly less effective in interfering with Crx-mediated transactivation for all the mutants, Crx:ΔQ7, Crx:Q96/97W and Crx:Q96/97W-ΔQ7 (P < 0.05, P < 0.005 and P < 0.0001 respectively). The difference in transcription interference was most notable for Crx:Q96/97W-ΔQ7, as polyglutamine-expanded ataxin-7 at a 2:1 ratio yielded a mere 35% reduction in transactivation competence, compared with a 76% reduction for wild-type Crx (Table 1). To assess if the reduction in Crx transcription activation is specific to an altered interaction involving the glutamine-rich region, we tested a Crx mutant with an amino acid substitution at amino acid position 80, Crx:E80A (12), in the transactivation assay and observed marked repression by ataxin-7 92Q, comparable to that of wild-type Crx (data not shown). These results indicated to us that Crx must possess an intact glutamine-rich region to be fully susceptible to ataxin-7 92Q transcription interference.

**Identification of the Crx interaction domain in ataxin-7, and the effect of glutamine tract length**

To determine which region of ataxin-7 interacts with Crx, yeast two-hybrid assays were carried out using truncated forms of ataxin-7 as the bait and full-length Crx as the prey. We generated three ataxin-7 truncation mutants, SCA7-T1 (1–66), SCA7-T3 (289–584) and SCA7-T5 (1–584) (Fig. 3A), and a productive interaction was observed between Crx and either the SCA7-T1 or SCA7-T5 truncation mutant (Fig. 3B), suggesting that only the amino-terminal is required for ataxin-7 to interact with Crx. The inability of the amino-terminal truncated SCA7-T5 mutant to interact with Crx in the yeast two-hybrid assay was consistent with this conclusion (Fig. 3B). An important question regarding the ataxin-7–Crx interaction is the role of glutamine tract length in modulating the strength of the interaction. To address this, quantitative yeast two-hybrid assays were carried out by measuring the β-galactosidase (β-gal) activity driven off the lacZ reporter in Crx–ataxin-7 10Q or 62Q co-transformations, as previous studies have shown that β-gal activity correlates in most cases with bait-prey binding affinity determined using biochemical means (22,23). This comparison indicated that the ability of ataxin-7 62Q to bind Crx is approximately 50% stronger than that of ataxin-7 10Q in this system (Fig. 3C; 2.25 units versus 1.56 units; P < 4.31 × 10^-6).

To confirm the yeast two-hybrid assay results and further delineate the ataxin-7–Crx interaction domain, it was decided to perform another round of co-ip assays, this time using in vitro-translated Crx and a series of ataxin-7 truncation deletion mutants. First, a large number of ataxin-7 deletion constructs was generated and one ataxin-7 point mutant construct, all fused in-frame to an amino-terminal 5x myc tag (Fig. 4A). As expected, ataxin-7 truncation mutants lacking various lengths of the carboxy-terminal region are able to physically interact with Crx in vitro (Fig. 4B; Table 2). In contrast, ataxin-7 amino-terminal truncation mutants lacking as few as the first 65 amino acids (which includes the polyglutamine tract), could not physically interact with Crx (Fig. 4B; Table 2). Interestingly, deletion of the first 26 amino acids of the ataxin-7 protein, just upstream of the glutamine tract which starts at residue 30, yielded an ataxin-7 truncation mutant (27–892) that could be pulled down with Crx just as well as full-length ataxin-7 (Fig. 4B). To confirm these observations in a more physiological system, various ataxin-7 constructs were co-transfected into HEK293 cells along with a human CRX expression construct.
with an amino-terminal FLAG tag. After obtaining the co-IP material by affinity purification with anti-FLAG M2 agarose beads (see Materials and Methods), each pull-down lysate was probed with an anti-myc antibody. It was observed that both ataxin-7 full-length and ataxin-7 1–65 were present in the FLAG co-IP elutes if the HEK293 cells had been transfected with the FLAG-CRX construct (Fig. 4C). These ataxin-7 proteins were pulled down with CRX regardless of the length of the construct.

Figure 2. Crx glutamine region mutants are resistant to ataxin-7-mediated repression. (A) The Crx glutamine-region mutants bind to DNA normally. Electrophoretic mobility shift assays using a 32P-labeled BAT-1 oligomer as a probe were performed as previously described (9). In lane 2, we added 1 μl of the anti-Crx antibody p261, and arrows indicate the resulting supershift, confirming our detection of a Crx-dependent band shift. Lane 7 did not receive any protein, and an arrow at the bottom of the gel indicates the position of free probe. (B) The Crx glutamine-region mutants localize to the nucleus based upon immunofluorescence localization studies. HEK293 cells were transfected with the indicated Crx mutant constructs, and immunocytochemistry was performed using a monoclonal antibody to Xpress as the primary antibody and a goat anti-mouse Alexa Fluor® 488 (green) as the secondary antibody. Nuclei were counterstained with Hoechst. Fifty Xpress-positive cells derived from the wild-type and each Crx mutant were counted randomly, and all showed nuclear localization. (C) Crx and the Crx glutamine region mutants are expressed at comparable levels. Western blot analysis with an anti-Crx antibody was performed on immunoblots of equivalent amounts of protein extracts from HEK293 cells transfected with the indicated construct. The arrow indicates Crx protein. (D) Transient transfection assays in HEK293 cells with a bovine rhodopsin promoter–luciferase construct (BR-225-luc) as reporter. A 100 ng sample of the indicated Crx expression constructs was co-transfected into HEK293 cells in the presence of the SCA7 10Q construct (hatched) or the SCA7-92Q construct (solid) at 100 or 200 ng as indicated. Fold activation for each sample was calculated based on the luciferase activity relative to that of the sample receiving only the reporter (0-fold). The results are presented as percentage transactivation relative to empty vector samples that were arbitrarily set at 100% (data not shown). Each experiment was performed in duplicate, and all measurements were repeated four times independently. The error bar represents the standard error of mean (n = 4). While all Crx glutamine region mutants exhibited resistance to ataxin-7 92Q repression of transactivation competence, the extent of the resistance differed from least to greatest as follows: Crx:ΔQ7 < Crx:Q96/97W < Crx:Q96/97W-ΔQ7.
The carboxy-terminal region of ataxin-7 is essential for its nuclear localization

As transcriptional interference of Crx by polyglutamine-expanded ataxin-7 takes place in the nucleus, an important issue in understanding ataxin-7 biology and SCA7 pathogenesis is the regulation of ataxin-7 subcellular localization. To determine which domain(s) of ataxin-7 might be responsible for its entry into the nucleus, the pattern of subcellular localization of the ataxin-7 truncation mutants used in the above experiments was evaluated. To do this study, these ataxin-7–92Q constructs were transfected into HEK293 cells along with wild-type Crx to facilitate detection of nuclear-localized ataxin-7. While full-length ataxin-7 localizes completely to the nucleus regardless of glutamine tract length, it was noted that progressive truncation of the carboxy-terminal region initially decreased then ultimately eliminated the ability of ataxin-7 to localize to the nucleus (Fig. 5A; Table 2). Partial nuclear localization of ataxin-7:1–645 followed by the nearly complete loss of nuclear localization by ataxin-7:1–239 therefore indicated that one or more nuclear localization signals may reside in the carboxy-terminal one-half of the ataxin-7 protein. The ability of all the amino-terminal truncated ataxin-7 expression constructs, including ataxin-7:646–892, to nearly completely localize to the nucleus was entirely consistent with this interpretation, and suggested that ataxin-7’s nuclear localization signal (NLS) must reside downstream of amino acid residue 646. In the case of ataxin-7–92Q:1–65, nuclear localization was observed despite the absence of the carboxy-terminal region (Fig. 5A). It is unlikely that the nuclear localization of this severely truncated version of ataxin-7 can be attributed to simple diffusion secondary to its small size, since ataxin-7–10Q:1–65 remains mostly cytosolic (data not shown). Rather, the presence of the polyglutamine tract expansion probably somehow facilitates binding of this severely truncated ataxin-7 with proteins that move into the nucleus together, possibly as part of a complex.

Analysis of ataxin-7’s primary amino acid sequence revealed two likely NLS motifs, a KKKR sequence at position 705 and a KKKR sequence at position 835. While our studies of ataxin-7 subcellular localization in HEK293 cells argued for the existence of one or more NLS’s in the carboxy-terminal region of ataxin-7, our results did not substantiate the need for the same alteration shown to abolish the function of this NLS in a previously published study in Cos-1 cells (24), and its localization pattern was assessed. Complete nuclear localization of ataxin-7:K380T in HEK293 cell co-transfections with Crx was observed (Fig. 5A; bottom panel). To verify the physiological relevance of the putative carboxy-terminal NLS’s to SCA7, evaluation of ataxin-7 subcellular localization by transfecting primary cultures of cerebellar granule cell neurons with a subset of the constructs that we had analyzed in HEK293 cells was carried out. For each transfection, subcellular localization was scored in at least 60 granule cell neurons as nuclear, cytoplasmic or nuclear and cytoplasmic. As expected, truncated versions of ataxin-7 lacking the carboxy-terminus (i.e. ataxin-7:1–239 and ataxin-7:1–460) mostly localized to the perinuclear cytosol, while both full-length ataxin-7 and ataxin-7 lacking the amino-terminus predominantly displayed nuclear localization (Fig. 5B and C). The results of the cerebellar granule cell transfections thus corroborated our finding of carboxy-terminal NLS domains in ataxin-7.
The glutamine tract and nuclear localization of ataxin-7 are required for full Crx repression

To confirm the importance of ataxin-7’s NLS sequences and its Crx-interacting domain (i.e. the glutamine tract) for inhibition of Crx-mediated transactivation, another series of Crx transactivation assays was performed, this time evaluating the ability of various ataxin-7 truncation mutants to repress Crx-dependent transactivation (Fig. 6A). First, the ability of full-length ataxin-7 92Q to interfere with Crx-mediated transactivation was tested, and, as expected, a marked decrease was observed. Ataxin-7 92Q: 1–830, ataxin-7 92Q: 1–645, and ataxin-7 92Q: 1–460 also showed marked interference with Crx transactivation. As carboxy-terminal truncation beyond amino acid residue 239 of ataxin-7 (ataxin-7 92Q: 1–239) had eliminated its nuclear localization (Fig. 5A–C), this truncation relieved the Crx transactivation repression (Table 2). Repression by ataxin-7 92Q: 1–65 was significant, however, as this protein moves into the nucleus and interacts with Crx. Ataxin-7 10Q versions of these constructs were also evaluated and decreases of just 14–27% were noted (data not shown), consistent with polyglutamine-length dependent repression of Crx-mediated transactivation. Lastly, the ability of ataxin-7 92Q: K380T to block Crx transactivation was tested, and marked interference was observed at levels comparable to wild-type ataxin-7 92Q (Fig. 6A), further confirming that the K380T mutation does not prevent ataxin-7 nuclear localization.

In the second half of the Crx transactivation repression study, the interference potency of the amino-terminal truncated ataxin-7 constructs was evaluated, and it was found that all ataxin-7 constructs lacking the glutamine tract were unable to prevent Crx from fully driving gene expression (Fig. 6A). Interestingly, although ataxin-7: 27–892 had been shown to completely localize to the nucleus in the HEK293 cell IHC studies (Fig. 5) and to interact with Crx in co-ip assays (Fig. 4B), Crx transcription interference by ataxin-7 92Q: 27–892, although quite significant, did not match that of full-length ataxin-7 92Q (Fig. 6A). To insure that the differences in transactivation repression observed for the 27–892 truncation mutant and for any other truncation mutant could not be attributed to differences in protein expression levels, repeated western blot analysis was performed. For all of the carboxy-terminal and amino-terminal ataxin-7 truncation mutants, protein expression levels that were equivalent to or slightly greater than wild-type full-length ataxin-7 92Q were always observed (Fig. 6B).
Ataxin-7 and Crx co-occupy the promoter/enhancer regions of photoreceptor genes in vivo

To determine if the ataxin-7-Crx interaction occurs in vivo, we examined whether these proteins bind to transcription regulatory regions in Crx target genes by performing chromatin immunoprecipitation assays (ChIP) on retinas and livers obtained from SCA7 transgenic mice as well as from wild-type controls (25). In the retina, either Crx antibody p261 or ataxin-7 antibody K could be used to successfully immunoprecipitate the promoter and enhancer regions of four known Crx target genes: rhodopsin, L/M-cone opsin, S-cone opsin, and β-PDE (Fig. 7A). PCR analysis of the retinal ChIP DNA samples, however, was negative for amplicons 300 to these target genes (Fig. 7B), as was PCR analysis for Crx target promoter/enhancer regions performed upon ChIP DNA samples obtained from the liver (data not shown). Isolation of DNAs corresponding to the promoter and enhancer regions of Crx target genes from retinas of mice by ChIP with an ataxin-7 specific antibody thus confirms the in vivo nature of the ataxin-7–Crx interaction.

DISCUSSION

SCA7 is unique among the polyglutamine repeat diseases as well as among the dominant SCAs because SCA7 patients suffer visual impairment and ultimately go blind due to a cone–rod dystrophy-type retinal degeneration (1,2). Our interest in SCA7 retinal degeneration led us to model this disease in transgenic mice, and our studies revealed that the SCA7 cone–rod dystrophy phenotype successfully recapitulated in our transgenic mice involves interference with the transactivation function of Crx (8). In that work, we attributed Crx transcription interference by the polyglutamine-expanded ataxin-7 to a direct physical binding interaction. The goal of the current study was...
A full-length 92Q construct was calculated using student constructs co-transfected to assess interference with Crx transactivation. The HEK293 cells were performed with 200 ng of the indicated SCA7 expression mutant constructs. A nitrocellulose membrane, and probed with an anti-myc antibody (9E10). Transfected HEK293 cells were loaded and run on SDS–PAGE gels, transferred to the ataxin-7 mutants. Equal quantities of protein extract derived from transected HEK293 cells were tested. Analysis of Crx localized the ataxin-7 protein was tested. Analysis of Crx localized the ataxin-7 protein product are observed for the various mutant constructs.

to determine the nature of the Crx–ataxin-7 physical interaction and to evaluate the factors that modulate it.

To map the interaction domains that permit Crx and ataxin-7 to bind one another, a parallel series of experiments was pursued in which various truncation and point mutants of each protein were generated, and then the ability of the different mutants to respectively co-ip the wild-type version of the other protein was tested. Analysis of Crx localized the ataxin-7 interaction domain to the carboxy-terminal region of its homeodomain and the adjacent amino-terminal region of its activation domain, and in particular to an 11 amino acid stretch containing nine glutamines. Further directed analysis of the Crx glutamine-rich region confirmed that the glutamine rich region mediates Crx’s interaction with ataxin-7, but that Crx's glutamine-rich region can tolerate significant alteration before the binding interaction with ataxin-7 is abrogated. In the case of ataxin-7, the in vitro co-ip studies similarly led to the glutamine tract as the key site of interaction with Crx.

To validate the interaction domain identifications that were shown by the in vitro co-translation co-ip experiments, functional transcription interference assays in living cells were performed. In our previous study and as again demonstrated herein, co-transfection of polyglutamine-expanded ataxin-7 and wild-type Crx results in a marked decrease in Crx transactivation competence (8). After confirming that various Crx mutants (i.e. Crx:Q7, Crx:Q96/97W and Crx:Q96/97W–ΔQ7) possess normal transactivation competence, attain adequate expression levels and localize to the nucleus, we tested whether ataxin-7 92Q could block their ability to drive transcription off the luciferase reporter containing Crx responsive elements (CRE). In each case, a significant increase was seen in Crx transactivation activity, meaning that ataxin-7 92Q was less able to repress each Crx glutamine-region mutant. These results strongly suggested that the Crx glutamine-rich region is indeed the key domain for the inhibitory interaction with ataxin-7 92Q.

The ability of various amino-terminal ataxin-7 92Q truncation mutants to interfere with reporter transactivation by wild-type Crx in HEK293 cells was similarly tested. With the glutamine tract deleted, ataxin-7 could no longer repress Crx transactivation function, confirming the co-ip findings that had implicated ataxin-7’s glutamine tract region as the key site of interaction with Crx.

Interestingly, however, deletion of the first 26 amino acids of the ataxin-7 92Q protein yielded a mutant with an intact 92 glutamine tract but with a diminished ability to interfere with Crx transactivation of its reporter in HEK293 cell co-transfections. This finding suggested that amino acid information adjacent to the glutamine tract in ataxin-7 is probably necessary for a functionally significant interaction with Crx, despite our observation that Crx can co-immunoprecipitate the ataxin-7:27–892 truncation mutant just as well as wild-type ataxin-7 (Fig. 4B). Review of all the Crx transactivation assay results using the rhodopsin-promoter–reporter construct in HEK293 cell co-transfections with ataxin-7 may thus be summarized as follows: (1) the transactivation assays confirm that an intact glutamine-rich region of Crx and the glutamine tract region of ataxin-7 are necessary for the Crx–ataxin-7

| Table 2. Summary of functional analysis results for ataxin-7 truncation and point mutants |
|------------------------------|------------------|------------------|------------------|------------------|
| SCA7 constructs | Interact with Crx| Repress Crx | Subcellular localization |
| 1–892 (full-length) | + | ++++ | Nuc | Nuc + Cyto | Cyto |
| 1–830 | + | ++ | 43 | 5 | 2 |
| 1–645 | + | ++ | 0 | 33 | 17 |
| 1–460 | + | ++ | 0 | 13 | 37 |
| 1–239 | + | – | 0 | 2 | 48 |
| 1–65 | + | ++++ | 50 | 0 | 0 |
| 27–892 | + | + | ND | ND | ND |
| 66–645 | – | – | 0 | 43 | 7 |
| 240–892 | – | – | 50 | 0 | 0 |
| 461–892 | – | – | 49 | 0 | 0 |
| 646–892 | – | – | 48 | 2 | 0 |
| 1–892: K380T | + | +++ | 42 | 5 | 3 |

*Based upon the data presented in Figure 6.
*Based upon the data presented in Figure 4B and C.
*Numerical results for the Figure 5A subcellular localization experiments in which a total of 50 double-stained cells were counted for each construct. Nuc, nuclear; Cyto, cytoplasmic.
*Data not shown.
ND = not determined.

Figure 6. Both the amino- and carboxy-terminal regions of ataxin-7 are required for its potency to repress Crx. (A) Transient transfection assays in HEK293 cells were performed with 200 ng of the indicated SCA7 expression constructs co-transfected to assess interference with Crx transactivation. The significance of the result for each 92Q deletion/mutation construct versus the full-length 92Q construct was calculated using student t-test and presented as a P-value (9). (B) Western blot analysis confirms equivalent expression of the ataxin-7 mutants. Equal quantities of protein extract derived from transfected HEK293 cells were loaded and run on SDS–PAGE gels, transferred to a nitrocellulose membrane, and probed with an anti-myc antibody (9E10). Comparable amounts of ataxin-7 protein product. To map the interaction domains that permit Crx and ataxin-7 to bind one another, a parallel series of experiments was pursued in which various truncation and point mutants of each protein were generated, and then the ability of the different mutants to respectively co-ip the wild-type version of the other protein was tested. Analysis of Crx localized the ataxin-7 interaction domain to the carboxy-terminal region of its homeodomain and the adjacent amino-terminal region of its activation domain, and in particular to an 11 amino acid stretch containing nine glutamines. Further directed analysis of the Crx glutamine-rich region confirmed that the glutamine rich region mediates Crx’s interaction with ataxin-7, but that Crx's glutamine-rich region can tolerate significant alteration before the binding interaction with ataxin-7 is abrogated. In the case of ataxin-7, the in vitro co-ip studies similarly led to the glutamine tract as the key site of interaction with Crx.
interaction; and (2) the transactivation assays are more stringent than in vitro co-ip analysis for characterizing the Crx–ataxin-7 interaction, as they reveal that mutant versions of Crx and of ataxin-7 that co-ip successfully show differences in transactivation repression.

Despite the compelling nature of the in vitro co-ip data and the results of the transcription interference study, it was felt that documentation of a functionally significant in vivo interaction between Crx and ataxin-7 was needed. To accomplish this, we turned to the ChIP method as we hypothesized that ataxin-7 and Crx would co-occupy the CRE-containing regions of Crx target genes in the retina. Using an antibody directed against the amino-terminal region of ataxin-7, DNA regions were successfully pulled down from the promoters and enhancers of Crx-regulated genes, but not from irrelevant DNA regions downstream of these genes or from the albumin gene that is not a Crx target (Fig. 7A and B). Interestingly, PCR amplification of the promoter–enhancer regions of the Crx-regulated genes was positive for ChIP material obtained from both ataxin-7 transgenic mice and non-transgenic mice, indicating that anti-ataxin-7 antibody K cross-reacts with human and mouse ataxin-7. Detection of Crx target gene sequences containing Crx binding sites in the retinas of mice using the ChIP approach with an anti-ataxin-7 antibody provides strong evidence in support of an in vivo interaction between Crx and ataxin-7.

Given the importance of the ataxin-7 glutamine tract in mediating Crx transcription interference, it was decided to assess the effect of ataxin-7 glutamine tract length upon the strength of the binding interaction with Crx. To do this, we used the yeast two-hybrid assay and measured the amount of β-galactosidase activity generated by co-transformation of Crx with either ataxin-7 10Q or ataxin-7 62Q. We did observe significantly higher β-galactosidase activity when Crx was combined with ataxin-7 62Q; however, the difference was only 50% greater. This difference alone seems unlikely to account for the down-regulation of Crx activity reported in our SCA7 transgenic mouse model to produce the cone–rod dystrophy phenotype (8). Rather, we suspect that the marked accumulation of polyglutamine-expanded ataxin-7 in SCA7 patients and in our transgenic mouse model is the key determinant of mutant ataxin-7 toxicity. Numerous studies have shown that
proteins containing polyglutamine expansions are resistant to proteasomal degradation, and therefore accumulate instead of being normally turned over (26). Immunostaining of our SCA7 transgenic mouse model indicated that the accumulation of polyglutamine-expanded ataxin-7 correlates best with the onset of a neurological phenotype, and that ataxin-7 immunoreactivity increases over time in neuronal nuclei (8,27). Therefore, in our view, it is the dramatic accumulation of ataxin-7 92Q in photoreceptor nuclei that is largely responsible for Crx transcription interference and SCA7 retinal degeneration.

The interaction of polyglutamine-expanded ataxin-7 with Crx takes place in the nucleus, as Crx is a nuclear transcription factor. For these reasons, we hypothesized that nuclear localization of ataxin-7 would be required for Crx transcription interference and sought to map the domain(s) responsible for ataxin-7 nuclear localization. To assess ataxin-7 subcellular localization, we transfected HEK293 cells with amino-terminal and carboxy-terminal ataxin-7 truncation mutants and analyzed where these expression constructs localized. We determined that the carboxy-terminal region of ataxin-7 is absolutely required for complete nuclear localization in HEK293 cells, and the Crx transactivation assays agreed with this observation. Inspection of the ataxin-7 amino acid sequence suggests that two potential NLSs, both of the KKKR motif (28), reside at amino acid positions 705–708 and 835–838. Our studies of ataxin-7 subcellular localization in HEK293 cells could not confirm that a previously reported putative NLS at amino acid position 378–393 is functional (24). Indeed, to evaluate this NLS, a K380T mutant version of ataxin-7 was generated and its subcellular localization pattern in HEK293 cells analyzed. It was found that the K380T mutant moved into the nucleus just as well as the wild-type version of ataxin-7 did. This observation contrasted with the loss of nuclear localization of a protein fused to the K380T mutant version of this NLS in the previous study (24). While there are a number of possible reasons for this discrepancy, an important difference in the previous study was the evaluation of the NLS by fusion to an unrelated, normally cytosolic protein (24).

To clarify the physiological relevance of the various putative NLSs to SCA7 disease pathogenesis, it was decided to study the effect of different truncations upon subcellular localization in primary neuron cultures. Cerbellar granule cells have been shown to highly express ataxin-7 and may play a role in the cerebellar degeneration (27,29,30), so we surmised that analysis of ataxin-7 subcellular localization in cerebellar granule cells would be meaningful. We transfected a subset of ataxin-7 expression constructs into primary cultures of cerebellar granule neurons and observed predominant nuclear localization for full-length ataxin-7 and ataxin-7 lacking its first 459 amino acids (Fig. 5B and C). On the other hand, ataxin-7 1–239 and ataxin-7 1–460 showed mostly cytoplasmic localization in individually transfected cerebellar granule cells. These results were completely consistent with our HEK293 cell data, and independently confirm that one or more NLS sequences reside in the carboxy-terminal region of ataxin-7. The cerebellar granule cell transfection results similarly failed to reveal a role in subcellular localization for the NLS at position 378–393.

An emerging theme in the polyglutamine disease field is the propensity of polyglutamine-expanded proteins to interact with proteins containing glutamine tracts and/or glutamine-rich regions. Indeed, CREB-binding protein (CBP), TAFII-130, and Sp1 are among the glutamine-containing proteins implicated in polyglutamine disease pathogenesis (31–33). As glutamine-rich regions are important for transcriptional activation, all of these proteins have been shown to serve as transcription activators or co-activators involved in driving gene expression in the nucleus. Our finding that ataxin-7 interacts with Crx, a nuclear transcription factor with a glutamine-rich region, and that an aberrant interaction between polyglutamine-expanded ataxin-7 and Crx in photoreceptor nuclei may be a key event in SCA7 retinal degeneration is in agreement with this concept. Indeed, one of us recently coined the term ‘transcriptionopathy’ to describe this possible pathway, pointing out that many of the polyglutamine disease proteins are transcription factors or have been hypothesized to be transcription regulators (7). Our findings in the current study strengthen the view that SCA7 retinal degeneration is due to a transcriptionopathy involving Crx. However, while our data advocates the involvement of Crx in SCA7 retinal degeneration, it by no means excludes the possibility that polyglutamine-expanded ataxin-7 could also be interfering with other transcription factors and co-regulators. Indeed, we have shown that CBP is present in NIs in the retinal photoreceptors of the SCA7 transgenic mice, and have suggested that polyglutamine-expanded ataxin-7 inhibition of the neural retinal leucine zipper protein (NRL), observed in rhodopsin promoter reporter transactivation assays, may involve CBP interference (8). Additional work done on Otx-2, a closely related family member of Crx that is also expressed in the retina and contains a glutamine-rich region (34), suggests that Otx-2 can interact with ataxin-7 based upon in vitro co-IP assays (S. Chen et al., unpublished data). Given these considerations, we favor a model of SCA7 retinal degeneration that envisions polyglutamine-expanded ataxin-7 transcription interference of various factors or co-activator proteins in addition to Crx (Fig. 8). As SCA7 patients and transgenic mice develop a cone–rod dystrophy phenotype, we propose that inhibition of Crx transactivation is a predominant aspect of the mutant ataxin-7 transcriptionopathy, especially in light of the fact that autosomal dominant Crx mutations are sufficient to cause cone–rod dystrophy in humans (13,15). This model for SCA7 retinal degeneration is also consistent with the recent report of a cone–rod dystrophy phenotype in SCA7 knock-in mice expressing ataxin-7 with 266 glutamines (35). This knock-in mouse model developed a retinal phenotype similar to the phenotype seen in our own SCA7 transgenic mice in terms of histological nature, timing of onset and rate of progression. While changes in the expression levels of gene products not regulated by Crx were observed in SCA7 knock-in mice, importantly, down-regulation of Crx-regulated gene products was observed. Thus, numerous similarities exist between our SCA7 transgenic mouse model and the knock-in model, and studies of both models report retinal gene expression changes as a likely initiating event in disease pathogenesis, supporting the transcriptionopathy model for SCA7 retinal degeneration.

With this study, our understanding of how mutant ataxin-7 may produce SCA7 retinal degeneration has been advanced. The goal of future studies will be to determine if rescue of Crx function in our SCA7 transgenic mice is sufficient to prevent or even reverse the retinal degeneration phenotype. Importantly,
Figure 8. Model for interference of Crx-dependent photoreceptor gene transcription by polyglutamine-expanded ataxin-7. Once the glutamine tract of ataxin-7 exceeds the normal range, polyglutamine-expanded ataxin-7 moves into photoreceptor nuclei and inhibits gene expression. Among the possible mechanisms of transcription interference is a direct repression of Crx bound to CRE at distal regulatory (enhancer) or proximal regulatory (promoter) sites. In addition, polyglutamine-expanded ataxin-7 may directly interfere with other specific transcription factors, or more likely, may disrupt the basal transcriptional machinery by an aberrant interaction with a co-activator complex. (+) represents activation; (−) represents repression effect.

the findings reported herein argue that transactivation competent versions of Crx with alterations in the glutamine-rich region may be immune to inhibition by mutant ataxin-7, and thus even more likely to rescue SCA7 retinal phenotype. If delivery of Crx to the retinas of our transgenic mice is successful in treating their visual defects, then such an approach could have therapeutic implications for humans, given the accessibility of the retina and the development of highly effective adeno-associated virus gene therapy vectors for photoreceptor transduction (36). If the transcriptionopathy of SCA7 is more global, then agents such as histone deacetylase inhibitors (HDAC Is) may be a useful option. Indeed, HDAC Is have shown promise in reversing polyglutamine neurotoxicity in transgenic flies and useful option. Indeed, HDAC Is have shown promise in such as histone deacetylase inhibitors (HDAC Is) may be a therapy, if transcriptional alterations turn out to be the crux of the problem. if transcriptional alterations turn out to be the crux of the problem.

MATERIALS AND METHODS

Construction of SCA7 expression vectors and oligonucleotide mutagenesis

The coding sequences of full-length ataxin-7-10Q and -92Q were cloned as EcoRI–SmaI fragments into the EcoRI/EcoRV sites of pSecTag-A (Invitrogen) that had been modified by replacing the 5′ myc epitope tag with a NheI–EcoRI fragment (encoding the Ig κ chain leader sequence). Approximately 1 kb of vector backbone sequences between SphI- and SmaI were deleted from this construct, and the resulting vectors were designated as CMV–ataxin-7-10Q and -92Q.

C-terminal deletions in SCA7 were generated by first inserting a translation termination sequence (TCTAGATAAGTAGC) between the XbaI site at the 3′ end of the ataxin-7 cDNA and the Pmel site at the 3′ end of the multiple cloning site. These vectors (CMV–ataxin-7-10Q/92Q:1–830) terminate translation after the XbaI cleavage site, and thus encode only the first 830 amino acids of ataxin-7. These vectors were cut with XbaI and a series of second enzymes (PshAI, BpiI, NsiI and Apal); the resulting overhangs were then filled in and chewed back with Klenow, and the vector DNA was cloned. The cloned DNA yielded a series of vectors (CMV–ataxin-7-10Q/92Q:1–645; CMV–ataxin-7-10Q/92Q:1–460; CMV–ataxin-7-10Q/92Q:1–239; and CMV–ataxin-7-10Q/92Q:1–65) which terminate translation at amino acids 645, 460, 239 and 65, respectively.

N-terminal deletions between the 5′ myc and ataxin-7 were generated by digesting CMV–ataxin-7-10Q/92Q:1–892 with EcoRI (which cuts between the 5′ myc tag and the SCA7 cDNA) and two second enzymes (NsiI + BpiI); the resulting overhangs were filled in and chewed back with Klenow, and the vector DNA was cloned. The cloned DNA yielded two new deletion vectors (CMV–ataxin-7-10Q/92Q:240–892 and CMV–ataxin-7-10Q/92Q:461–892) which fuse the myc tag to amino acids 240 and 461 of ataxin-7, respectively. CMV–ataxin-7-10Q/92Q:1–645 was used to generate an N-terminal deletion at the N-terminal Apal site because it lacks the second C-terminal Apal site located between the PshAI site and the C-terminus of ataxin-7. CMV–ataxin-7-10Q/92Q:1–645 was cut with EcoRI + Apal; the resulting overhangs were filled in and chewed back, and the vector DNA was subcloned. The cloned DNA yielded a new vector (CMV–ataxin-7-10Q/92Q:66–645) which fuses the myc tag to amino acid 66 of ataxin-7. The N-terminal 645 amino acids of SCA7 were deleted by first inserting a Smal restriction site (Gaa-ttc-gcc- cgg-ggG-GCC-GC) between the EcoRI site positioned at the 5′ end of ataxin-7 and the NolI site positioned just 5′ of the CAG repeat. (The sequence corresponding to the coding strand oligo used to generate the deletion is shown in lower-case and the flanking EcoRI/NolI vector sequence is shown in upper-case text.) This newly derived vector (ataxin-7-10Q/92Q:27–892) was cut with XmaI + PshAI; the resulting overhangs were filled in with Klenow and the vector DNA was subcloned. The cloned DNA yielded a new vector (ataxin-7-10Q/92Q:646–892) which lacks the first 645 amino acids of ataxin-7. The DNA sequences flanking the ligation sites were PCR amplified and sequenced for all truncation vectors described above.

The K380T point mutation was generated by changing the lysine to threonine at amino acid position 380 with the QuickChange™ Kit (Stratagene). The pBluescript KS++–SCA7-10Q (1–892) vector was amplified with Pfu Turbo polymerase (Stratagene) using sense (5′-GCTGTCCAGGGTAGAAGAAC-TCGATTGATGTGTTATTA-3′) and antisense (5′-TAATAAC- ACAACAAATCGAGTCTTACCTGACACG-3′) primers designed to introduce the threonine (ACT) mutation at position 380 as well as a diagnostic TaqI (TCGA) restriction site. PCR was performed according to the manufacturer’s recommendations (95°C × 2 min; 18 cycles of 95°C × 30 s, 55°C × 30 s, 68°C × 12 min). PCR products were digested with DpnI (to fragment the original vector template) and then used to transform E. coli. The cloned DNA was amplified using primers flanking the mutation and subjected to restriction enzyme (TaqI) and sequence analysis. The K380T mutation was transferred to the CMV–ataxin-7-10Q/92Q:1–892 vectors as part of a 663 bp NsiI-BpiI fragment. Transfer of the K380T mutation yielded two new vectors designated CMV–ataxin-7-10Q/92Q:K380T.
Mammalian expression constructs for Crx and its deletions and mutations

For immunocytochemistry studies, a bovine Crx cDNA with a hemagglutinin (HA) epitope tag fused to its N-terminus was PCR amplified using Pfu Turbo polymerase (Stratagene) and cloned into pCX. Oligonucleotides (5'-ggaggtctgatgtagc-caccatatgctacctag-3' and 5'-ggaggtctgatgtagc-gaactctcagcgc-3') were designed to introduce XbaI and SpeI sites at the 5' end of the PCR product and a XhoI site at the 3' end. The PCR product was gel-purified, cut with XbaI/ XhoI, and cloned into the NheI/XhoI sites in pCX. The insert of one clone was sequenced to confirm its identity. This expression vector is designated pCX-HA-Crx. For FLAG-CRX pull-down assays, a human CRX containing a 3× FLAG tag at the N-terminus (FLAG-CRX) in the p3xFLAG-CMV-10 vector (Sigma) was obtained from Donald J. Zack.

For transactivation assays and in vitro co-immunoprecipitation, the full-length bovine Crx and its deletion constructs were generated and described previously (12). Crx:ΔQ7, in which the coding region for amino acids 99–107 (QQKQOKQOPP) was replaced by a HindIII spacer (coding for amino acids S and F), was constructed by a three-way ligation. Briefly, two bovine Crx cDNA PCR fragments, one coding for amino acids 2–98 (with HindIII) and the other coding for 99–107 (with HindIII 5' and EcoRI 3'), were cloned into pcDNA3.1HisC vector cut with HindIII and EcoRI. Similarly, Crx:Δ88–107 was constructed by three way ligation as described above, except for using a shorter 5' PCR fragment corresponding to the coding DNA for 2–87 amino acids. These deletion constructs were identified by harboring a HindIII site in the insert and subsequently verified by sequencing. The Crx:1–98 and Crx:Q7 CRX pull-down assays, 5μg of mammalian FLAG-CRX expression vector, or its control vector p3xFLAG-CMV-10, and pCMV–ataxin-7-10Q/92Q constructs were co-transfected into human embryonic kidney (HEK) 293 cells cultured on 100 mm plates using the calcium phosphate method (9). Forty-eight hours post-transfection, cells were harvested and washed twice with phosphate-buffered saline (PBS) at pH 7.4 and lysed in 1 ml of Lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 0.1% NP40 supplemented with 10 μl of protease inhibitor cocktail (Sigma)] at 4°C for 20 min. The insoluble debris was removed by centrifugation for 10 min at 12,000 g. Aliquots of 950 μl of cell extracts containing FLAG-CRX or empty vector were incubated with 40 μl of anti-FLAG M2 affinity resin equilibrated with TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) at 4°C for 3 h with gentle agitation. After three washes with 1 ml TBS with 350–450 mM NaCl, bound proteins were eluted using 100 μl of 3× FLAG peptide elution buffer [150 ng/ml of 3× FLAG peptides (Sigma) in TBS], analyzed using SDS–PAGE with anti-Crx 9E10 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at 1:1000 dilution. Western blot proteins were detected using goat anti-mouse IgG-HRP (for anti-myc) or anti-rabbit IgG-HRP (for anti-Crx; Santa Cruz) at 1:10,000 dilution, ECL + western blotting detection reagents, and ECL Hyperfilm (Amersham).

Co-immunoprecipitation and FLAG-CRX pull-down assays

Co-immunoprecipitation with in vitro translated ataxin-7 or Crx proteins was performed as described previously (8). For FLAG-CRX pull-down assays, 5μg of mammalian FLAG-CRX expression vector, or its control vector p3xFLAG-CMV-10, and pCMV–ataxin-7-10Q/92Q constructs were co-transfected into human embryonic kidney (HEK) 293 cells cultured on 100 mm plates using the calcium phosphate method (9). Forty-eight hours post-transfection, cells were harvested and washed twice with phosphate-buffered saline (PBS) at pH 7.4 and lysed in 1 ml of Lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 0.1% NP40 supplemented with 10 μl of protease inhibitor cocktail (Sigma)] at 4°C for 20 min. The insoluble debris was removed by centrifugation for 10 min at 12,000 g. Aliquots of 950 μl of cell extracts containing FLAG-CRX or empty vector were incubated with 40 μl of anti-FLAG M2 affinity resin equilibrated with TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) at 4°C for 3 h with gentle agitation. After three washes with 1 ml TBS with 350–450 mM NaCl, bound proteins were eluted using 100 μl of 3× FLAG peptide elution buffer [150 ng/ml of 3× FLAG peptides (Sigma) in TBS], analyzed using SDS–PAGE with anti-Crx 9E10 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at 1:1000 dilution. Western blot proteins were detected using goat anti-mouse IgG-HRP (for anti-myc) or anti-rabbit IgG-HRP (for anti-Crx; Santa Cruz) at 1:10,000 dilution, ECL + western blotting detection reagents, and ECL Hyperfilm (Amersham).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays with adult mouse retina and liver were performed as previously described (25), but with minor modifications. Six retinas or 30 mg of liver tissue from SCA7–92Q, SCA7–24Q or wild-type non-transgenic mice at 4 weeks of age were dissected and treated with formaldehyde at a final concentration of 1% in 1× PBS for 15 min at room temperature to cross-link chromatin binding proteins. After cell lysis, the retina or liver chromatin was fragmented to an average length of about 600 bp by sonication, and subjected to immunoprecipitation with a rabbit antibody specific to Crx (p261) or to ataxin-7 (antibody K) (8), in the presence of Protein-A beads (Pharmacia). No antibody and mock (no chromatin) samples were also included as negative controls. The immunoprecipitated chromatin was washed as previously described (39), and immunoprecipitated protein–DNA complexes were eluted from the beads with 300 μl of elution buffer (50 mM NaHCO3, 1% SDS) and heated at 67°C for 4–5 h to reverse the cross-links. The DNA in the complexes was purified by ethanol precipitation and analyzed using PCR with the following gene-specific primers: Rhodopsin, 5’–200+/64 (5’-gggggcaagc-aagataagagc-3’ + 5’-tctgagcagacagagac-3’); Rhodopsin, 3’ 195 bp (5’-tctgagc-gtaagtaggact-3’ + 5’-cctctgcttcgcttg-3’); Rhodopsin enhancer region, 5’–1569/–1430 (5’-cagcctc-gaagcatacaac-3’ + 5’-cagcctc-gaagcatacaac-3’); L/M-opsin, 5’–216/–23 (5’-tgagccttcagctttgacgg-3’+ 5’-ggaggtctgatgtagc-gaactctcagcgc-3’); L/M-opsin, 3’ 152 bp (5’-tctgagcagacagagac-3’ + 5’-attgggctttcagcgc-3’); locus control region of L/M-opsin,
5′−4084/−3730 (5′-ctcaggtttgcctgtct-3′ + 5′-ctcaggcttcctcactc-ccctaac-3′); S-opsin, 5′−396/−202 (5′-caacagctcctctctgccct-3′ + 5′-ggctca-gagtttagctgtctgtgc-3′); S-opsin, 3′ 165 bp (5′-tta- ctcctgtctctcctcactc-3′ + 5′-ctcactcactcagtttagctgt-3′); β-PDE, 5′−201/+56 (5′-actgccataactctgtaac-3′ + 5′-gtctctctctggtgc- cgg-3′); β-PDE, 3′ 187 bp (5′-taagcactcacaacctc-3′ + 5′- acaaacggagatgccgtggtc-3′); albumin, 5′−547/−82 (5′-ggacacaag- gcttcctcctccttcac-3′ + 5′-tactcctcctccttcacatc-3′) (40,41). The PCR was carried out for 35 cycles with JumpStart Taq DNA polymerase (Sigma), analyzed on 1.2% agarose gels and repeated at least twice per primer pair.

**Immunocytochemistry studies with transiently transfected HEK 293 cells**

HEK 293 cells were co-transfected with CMV-myc-ataxin-7, pCX-HA-Crx or CMV-Xpress-Crx (for truncated and mutated Crx) plasmids using Lipofectamine-2000 (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours post-transfection, cells were washed with PBS at pH 7.4 and fixed in 4% paraformaldehyde at room temperature (RT) for 10 min, washed twice with PBS, permeabilized for 15 min at RT with PBS containing 0.1% Triton X-100, and then blocked with Problock (SeyTek Laboratories) at RT for 30 min. Cells were incubated with the anti-myc antibody 9E10 coupled to FITC (Santa Cruz) and anti-hemagglutinin coupled to Alexa Fluor® 488 (Molecular Probes, Inc., Eugene, OR, USA), or anti-Xpress antibodies (Invitrogen). Antibodies were used at a final concentration of 1, 5 and 5 ng/ml respectively. The Xpress antibody was detected using a goat anti-mouse antibody coupled to Alexa Fluor® 488 (Molecular Probes) at a final concentration of 2 ng/ml.

Fluorescence microscopy and digital image collection were performed on a Olympus IX inverted fluorescence microscope and a Photometrix cooled CCD camera (CH350/LCCD) driven by DeltaVision software from Applied Precision (Seattle, WA, USA). Twenty 200 nm optical sections were taken through the depth of the cell using a 100× objective lens, and DeltaVision software (softWoRx) was used to deconvolve these images and construct 3-D volume views. DeltaVision softWoRx uses a Constrained Iterative Deconvolution algorithm to remove out of focus blur in fluorescence optical sections and was set for a minimum of 15 iterative cycles.

**Immunocytochemistry studies with transiently transfected cerebellar granule cell neurons**

Neurons were culture from 7-day-old (P7) mice as described previously (42,43). Briefly, cerebella were dissected from P7 mice and minced in cold Hanks buffered saline. Tissue was trypsinized for 25 min at 37°C, then dissociated by trituration with a P1000 pipette tip. Cells were counted and plated at a density of 3 × 10⁵ cells/ml in beta-mercaptoethanol with 10% FBS, 2 mM glutamine, 25 mM KCl, 9.5 mM glucose, and 100 units/ml penicillin/streptomycin. Twenty-four hours after plating, cytosine arabinoside was added (10 µM). This resulted in a culture of ~95% granule cell neurons. After 5 days in culture, neurons were transfected using LipoFectamine 2000 (Invitrogen). Cells were fixed at 18h post-transfection, and stained with a polyclonal c-myc antibody (1:1000, Molecular Probes) and monoclonal MAP2 antibody (1:500, Chemicon). Nuclei were visualized with Hoechst’s dye (1:25 000). Images were captured at 40× with an Axiovert 200M microscope (Zeiss).

**Western blot analysis**

Western blots using transfected HEK293 cell extracts were performed as described previously (8). The anti-myc monoclonal antibody 9E10 (Santa Cruz) at a 1:1000 dilution and rabbit anti-Crx p261 antibody (8) at a 1:250 dilution were used for detecting recombinant ataxin-7 and Crx, respectively. The primary antibodies were visualized with horseradish-peroxidase coupled anti-mouse (for myc) or anti-rabbit (for Crx) antibodies (Amersham) at a 1:5000 dilution and enhanced chemiluminescence (Amersham).

**Transient co-transfection and luciferase assays in HEK293 cells**

Transient transfection experiments with a bovine rhodopsin luciferase reporter (BR-225-luc), bCrx-pcDNA3.1/HisC and pCMV-SCA7–92Q (with 5′ myc tag) constructs and dual luciferase assays were performed as described previously (8). The results are presented by percent transactivation activity relative to those obtained from samples received the empty vector for SCA7. Error bars representing standard error of mean (SEM) generated from three independent experiments. The P-values were calculated by a Student’s t-test assuming two samples with equal variance.

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