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Antisense RNA Sequences Modulating the Ataxin-1 Message: Molecular Model of Gene Therapy for Spinocerebellar Ataxia Type 1, a Dominant-Acting Unstable Trinucleotide Repeat Disease

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Spinocerebellar ataxia type 1 (SCA1) is a dominant inherited disease caused by expanded trinucleotide repeats resulting in an increased polyglutamine tract in the gene product. As a potential therapeutic approach for SCA1, we tested antisense RNAs targeting two regions of the ataxin-1 message. Single-stranded regions around the translational initiation site and the intron 8 splice donor site of the ataxin-1 message were identified by computer-assisted RNA secondary structure prediction. Plasmids were generated to contain a 254-bp antisense sequence spanning the translation initiation site (pLasBDini) or a 317-bp sequence spanning the intron 8 splice donor site (pLasBDei) of the ataxin-1 message. These plasmids were transfected into Chinese hamster ovary cells engineered to express either expanded or unexpanded ataxin-1 message and protein. Reduced levels of mutant ataxin-1 message (82 CAG repeats), wild-type ataxin-1 message (30 CAG repeats), and ataxin-1 protein were observed by Northern and Western blot analyses in pLasBDini-transfected clones. pLasBDei-transfected 293 cells exhibited a shift in ataxin-1 message to a size several kilobases longer than that of the natural message. Reverse transcriptase/polymerase chain reaction assays demonstrated the retention of message spanning the intron 8 splice acceptor and the inability to amplify sequences between exons 8 and 9, implying that normal splicing of intron 8 had been interrupted. We conclude that antisense RNAs were effective in reducing or modifying ataxin-1 messages in transfected cells, and may be an effective genetic strategy for therapy of SCA1 and similar dominant-acting neurological disorders.

Key words: Cerebellum; Ataxia; Spinocerebellar ataxia type 1; Gene therapy

INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant inherited neurodegenerative disorder in which symptoms generally emerge in the fourth decade of life. Affected individuals develop cerebellar ataxia characterized by progressively uncoordinated voluntary movement, impaired balance, and difficulty in speaking, swallowing, and breathing (17,53). Spinal manifestations of the disorder can also bring about spasticity (17). Patients eventually succumb about 10 to 15 years after the onset of disease (19,54). Neuropathological examination of SCA1 patients reveals the loss of Purkinje cells in the cerebellum, degeneration of the dentatorubral and olivocerebellar pathways, and atrophy of cranial nerve nuclei (41).

Molecular studies over the last decade have elucidated the genetic basis of SCA1 and provided insight into the pathogenesis of SCA1 at the biochemical level (35,53). SCA1 is caused by an expansion of CAG repeats in the gene encoding ataxin-1 (36), resulting in a protein with an expanded polyglutamine tract (1). This CAG expansion is characterized by intergenerational instability, and increased size of the CAG expansion is related to a younger age of disease onset (a phenomenon termed anticipation) (36). The role of the expanded CAG repeat region in conferring ataxia was further confirmed by the generation of transgenic mice that express expanded ataxin-1 message and become ataxic in about the fourth week of life (5). Transgenic mice have subsequently been used for molecular modification of the ataxin-1 protein and inquiry into the role of specific motifs, intracellular signaling, and molecular sorting in the disease process, including nuclear-cytoplasmic transport

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(28), protein misfolding and aggregation (6), and degradation via the ubiquitin/proteasome pathway (7). Evidence for the dominant nature of the ataxic phenotype was provided in SCA1 knockout studies in mice, where ataxin-1-deficient animals exhibited a much milder learning deficit and a hippocampal paired pulse defect but no ataxia (31).

Elucidation of the molecular genetic basis of SCA1 makes possible the formulation of new therapeutic strategies for this and other polyglutamine diseases (22). Additionally, the establishment of an animal model for SCA1 provides an excellent setting for the testing of new therapeutic approaches. Neuronal transplantation studies have been proposed for the treatment of ataxias (38,46). At the molecular level, one therapeutic approach for SCA1 would be to counteract the effect of the expanded message, either by destabilizing the message or by inhibiting its translation. Here we describe the generation of antisense RNAs targeting the ataxin-1 message, with the subsequent effect of destabilizing steady-state message levels. Both expanded and unexpanded messages were vulnerable to antisense RNA-mediated targeting, indicating that expression of antisense RNA may be an effective means of reducing ataxin-1 message in the SCA1 animal model setting and, ultimately, in humans.

MATERIALS AND METHODS

Plasmid Construction

Regions of predicted single-stranded structure in the ataxin-1 message near the translational initiation site and near the intron 8 splice donor site were identified using the RNA LOOP-STEM program (Genetics Computer Group, Wisconsin). A 254-bp sequence spanning the translational initiation site and containing the identified single-stranded region was recovered from total RNA extracts of human 293 cells by using a coupled reverse transcriptase/polymerase chain reaction (see below). The 254-bp antisense fragment was generated using primers SCAP71405-5′ and SCAP71630-3′ (Table 1). A 317-bp sequence spanning the intron 8 splice donor site and containing the identified single-stranded region (Fig. 1A) was amplified from genomic HEK 293 cell DNA using primers SCAP73314-5′ and SCAP73630-3′ (Table 1). The PCR conditions used were: 94°C for 3 min, then 30 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 90 s. The recovered antisense fragments were gel purified, cloned into pGEM-easy (Promega), recovered as *Eco*RI fragments, and then cloned into the *Eco*RI site upstream of the β-actin promoter in pLBD (52) (Fig. 2) to generate pLasBDini and pLasBDei. Nucleotide sequencing was conducted to verify the identity of the antisense fragments recovered in the expression constructs.

Mammalian Cell Culture and Gene Transfer

DHFR-deficient Chinese hamster ovary cells (CHO/dhfr$^-$; ATCC, Rockville, MD), derivatives engineered to express human SCA1 (CHO-At) under induction of the TET-off system (16,55), and low passage human embryonic kidney cell line 293 were routinely cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum, 1× nonessential amino acids, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Ataxin-1 expression in CHO-At cells was suppressed by including 1 μg/ml doxycycline in the medium.

Transfections were conducted by using a liposome transfection kit (Life Tech., Inc., USA). CHO-At cells or HEK 293 cells were subcultured into 35-mm dishes 1 day prior to transfection, and when 30–50% confluent the cells were exposed to DNA/liposomal reagent according to the manufacturer's instructions. Briefly, plasmid DNA was added to 100 μl OpTiMEM to a final concentration of 0.01 μg/ml, and then mixed with 100 μl OpTiMEM containing 10 μl liposome reagent. After 30 min the CHO-At or 293 cells were overlaid with the DNA–liposome mixture, incubated at 37°C for 2 h, and then the medium was replaced with fresh DMEM containing 10% fetal bovine serum. After overnight culture, the cells were subcultured into selective medium containing 0.3 μM methotrexate. Drug-resistant, stable transfectants were isolated after 2–3 weeks and expanded in culture for further analysis.

Northern Blot Hybridization

Stable, transfectant clones were expanded in culture and incubated under appropriate induction conditions (i.e., absence of doxycycline for CHO-At cells). Cells were harvested from four confluent 10-cm dishes. Total cellular RNA was extracted in guanidinum isothiocyanate solution and purified by ultracentrifugation in cesium chloride (13). Total RNA samples were treated with 1 unit DNase for 30 min at room temperature followed by inactivation at 80°C for 10 min. Total RNA (15 μg) was electrophoresed through 1% agarose-formaldehyde gels under denaturing conditions (2.2 M formaldehyde, 50% formamide, 1× MOPS buffer) (43), transferred to Zetabind membrane (CUNO Inc., Meriden, CT) using 20× SSC, and hybridized at 42°C overnight in 6× SSC containing 50% formamide, 5× Denhardt's solution, 0.1% SDS, and 200 μg/ml salmon sperm DNA. DNA probes were radiolabeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL; 3000 Ci/mmol) using the multiprime DNA labeling system (Amersham) to a specific activity of 4×10^8 cpm/μg. DHFR probe was generated from a 600-bp DHFR cDNA fragment isolated from pGEM-DHFR (see above). To generate a probe for ataxin-1 message, a 1-kb RT-PCR prod-

Table 1. PCR Primers

Name*	Location	Sequence (5′ to 3′)	Use‡
SCAP71405-5′	5′ UTR	CATCCAGAGCTGCTGTTGGCG	pLasBDini construction
SCAP71630-3′	Coding sequence	AGGACCGGCTGGTGGCGGGGA	pLasBDini construction
SCAP73314-5′	Exon 8	AACGGGGAGCTAAAGAAG	pLasBDei construction
SCAP73630-3′	Intron 8	GAGAACGCAGTTGGGAAA	pLasBDei construction
SCAP73037-5′	Exon 8	TCATCCCGGTCGGCAGCACT	Test for intron 8 splicing
SCAP93587-3′	Exon 9	ATGATAAGGGAGAGCCACGT	Test for intron 8 splicing
SCAP92651-5′	Intron 8	CACCTGCAGAGCCTAGCT	Test for intron 8 sequences
SCAP93045-3′	Exon 9	GGATACTCTCCAAAAC	Test for intron 8 sequences
SCAP72301-5′	Coding sequence	AGTACGTCCACATTTCCAGT	Ataxin-1 probe
SCAP73301-3′	Coding sequence	ATGGAGCCTTTCATGAAGTA	Ataxin-1 probe

*The number in the name corresponds to the position of the first nucleotide in GenBank accession number AL009031. 5′, sense primer; 3′, antisense primer.
†Uses: pLasBDini, pLasBDei construction (see Fig. 2). Testing for intron 8 splicing, sequences (see Fig. 7B). Ataxin-1 probe (see Figs. 4, 5, and 7A).

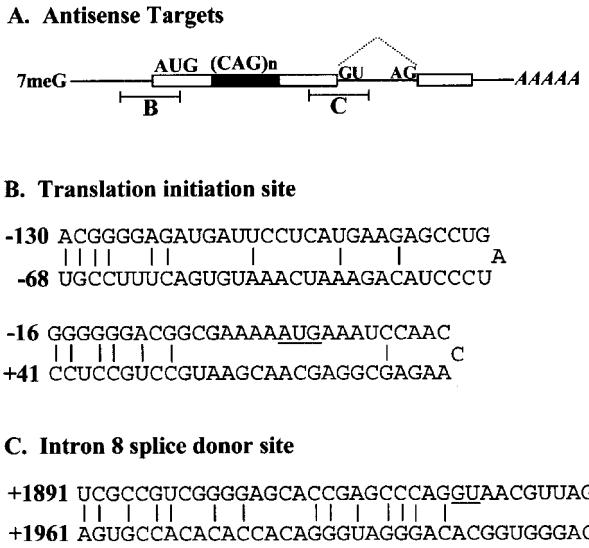

Figure 1. Antisense RNA targets in the ataxin-1 message. (A) Map of ataxin-1 messenger RNA, depicting the 5′ cap, translational initiation site (AUG), trinucleotide repeat region, (CAG)n, intron-8 5′ and 3′ splice sites (GU and AG), and poly-A tail. This depiction is idealized and omits the first 7 introns in order to highlight the regions targeted for antisense RNA binding around the translational initiation site (region B) and around the intron 8 splice donor site (region C). (B) To identify single-stranded regions for antisense RNA binding, the ataxin-1 mRNA sequence was analyzed for secondary structure using the RNA STEM-LOOP program. Two loop regions defining significant lengths of single-stranded structure were identified at −126 to −72 and from −7 to +32, including the translational initiation site (AUG, underlined). (C) A single-stranded loop region was identified at +1917 to +1937, including the intron 8 splice donor site (invariant GU underlined). Sequence numbering is relative to the translational start site.

uct between positions 736 and 1736 (with respect to the translation initiation site) in the ataxin-1 message (Table 1) was cloned into plasmid pGEM to generate pGEM-736. This 1-kb fragment was subsequently isolated from the pGEM-736 plasmid for preparation of ataxin-1 probe. GAPDH probe was prepared as a 500-bp *Hin*dIII fragment isolated from plasmid pUC-GAPDH9 (5). Single-stranded sense RNA probe to detect the antisense ataxin-1 message in pLasBDini-transfected cells was prepared from pGEMini by in vitro transcription in the presence of [α-^{32}P]UTP using the T7/SP6 Transcription kit from Boehringer Mannheim (Germany). Hybridization reactions contained 2×10^7 cpm/ml radiolabeled probe. After hybridization, membranes were washed twice in 2× SSC/0.1% SDS at 42°C for 30 min, washed twice in 0.1× SSC/0.1% SDS at 60°C for 30 min, and then exposed to X-ray film (X-Omat, Eastman Kodak, Rochester, NY). Hybridization signals were quantified either by densitometric scanning or by using a phosphorimager (Molecular Dynamics).

Reverse Transcriptase/Polymerase Chain Reaction Analysis

First-strand cDNA was synthesized from 1 μg total RNA in a 20-μl reaction mixture containing 4 μl of 5× RT buffer, 200 μM dNTP, 0.5 μg oligo dT(15) primer, and 5 units SuperTranscrip II™ (Promega) incubated at 42°C for 60 min. Reverse transcriptase reactions were inactivated at 75°C for 5 min and then put on ice. PCR was subsequently carried out (see Table 1 for sequences of forward and reverse primers) using 0.25 μM forward primer and reverse primer, 4 μl 10× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2 μl first-strand cDNA, and 2 units Taq polymerase. Cycling conditions were

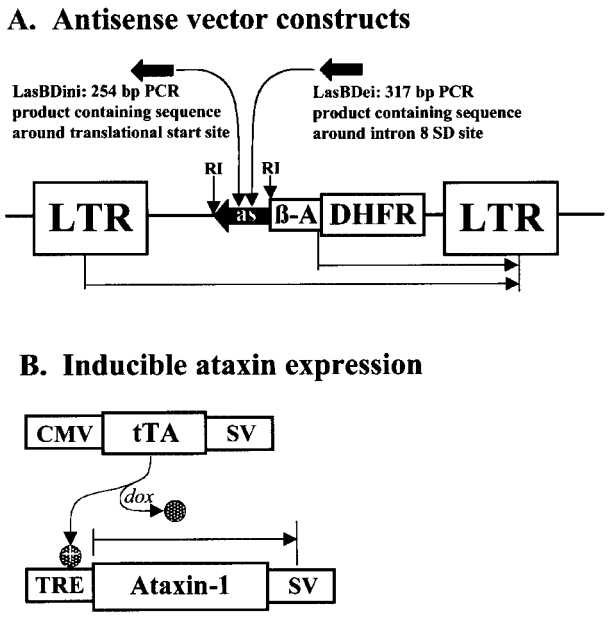

Figure 2. Antisense and ataxin-1 expression constructs. (A) Retroviral plasmid constructs. PCR products targeting the translational initiation site (254 bp) and the intron 8 splice donor site (317 bp) were generated as described in the text, cloned into pGEM-easy, and then inserted into the *Eco*RI site of retroviral vector plasmid pLBD to generate pLasBDini and pLasBDei, respectively. LTR, retroviral 5′ and 3′ long terminal repeats; DHFR, L22Y murine drug-resistant DHFR coding sequence; β-A, 250-bp chicken β-actin promoter. Full-length and internally initiated mRNA transcripts are depicted by the arrows underneath the vector diagram. (B) Inducible expression of ataxin-1. Top, cytomegalovirus early promoter-regulated expression of tetracycline transactivator protein tTA, with SV40 polyadenylation signal. Bottom, TRE (tetracycline response element) regulated expression of ataxin-1, with SV40 polyadenylation signal. Incubation of CHO-At cell transfectants with doxycycline (dox) inhibits binding of the tTA transactivator to TRE, preventing activation of ataxin-1 expression. Withdrawal of doxycycline from the medium allows binding of tTA to TRE and induction of ataxin-1 expression.

94°C for 60 s, 55°C for 60 s, and 72°C for 30 s for 40 cycles. PCR products were subsequently analyzed by agarose gel electrophoresis with ethidium bromide staining.

Western Immunoblotting

Test cells were washed twice with PBS and then total protein was extracted using lysis buffer consisting of 1% Triton X-100, 1% NP-40, 0.1% SDS, 1 mM PMSF, and 50 mM leupeptin (50 mM Tris, pH 8.0). Lysates were sonicated at 80 mA output for 15 times (2 s each time) and centrifuged for 10 min, collecting supernatants for Western blot analysis. Protein extracts were loaded and resolved on two mini 10% polyacrylamide SDS gels. One gel was used for Coomassee blue R250 staining, and proteins were blotted from the other gel onto a NP45 nitrocellulose membrane by electrotransfer. The membrane was sealed with 5% milk/Tween 20/PBS buffer and exposed to polyclonal antibody 11750, prepared against the N-terminal domain of ataxin-1 (44) (working concentration, 1:4000) at 30°C for 2 h. Membranes were washed three times with PBS and then incubated with appropriate goat anti-mouse IgG heavy chain second monoclonal antibody conjugated with horseradish peroxidase (NEB, working concentration, 1:2500) at 30°C for 1 h. After washing, the blot was developed using an illuminochemical detection system (Amersham Life Sciences).

RESULTS

Strategies of Ataxin-1 Antisense RNA Sequence Design and Target Site Analysis

As strategies for downregulating expression of expanded ataxin-1 messenger RNA and/or protein, two different target sites were investigated: (i) the translation initiation site of the ataxin-1 message (Fig. 1A), as a means of either preventing translation of ataxin-1 messenger RNA into protein or promoting the degradation of ataxin-1 messenger RNA through the formation of regions of double-stranded RNA; (ii) the intron 8 splice donor site (Fig. 1A), as a means of preventing ataxin-1 message splicing, with subsequent inhibition of SCA1 mRNA maturation. Effective binding of antisense RNAs requires that the target messenger RNA exists to some extent in single-stranded form. We therefore searched the sequence of the ataxin-1 message around the translational start site and the intron 8 splice donor site for regions of single strandedness, as predicted using the RNA STEM-LOOP algorithm (GCG). As shown in Figure 1B, we predicted two regions of single-stranded character near the translational start site of the ataxin-1 message, one 54 bases in length at position −126 to −72 and one 39 bases in length at position −7 to +32. We also identified a 21-base single-stranded region at position 1917 to 1937 near the intron 8 splice donor site (Fig. 1C). This extent of single strandedness in both target regions was deemed sufficient for generation of antisense messages for binding and interruption of message function and stability.

Recombinant Ataxin-1 Antisense RNA Expression Plasmids

Two antisense sequences were recovered by using the polymerase chain reaction and inserted into a Moloney murine leukemia virus-based retroviral plasmid pLBD (52): (i) a 254-bp sequence generated by RT-PCR from the ataxin-1 message, from bases −160 to +65 with respect to the translation initiation site of the ataxin-1 message (see Table 1), including that site as well as the single-stranded regions depicted in Figure 1B, and (ii) a

317-bp sequence generated by PCR from 293 cell DNA, extending from bases −168 to +149 with respect to the exon 8 splice donor site, including that site as well as the single-stranded region depicted in Figure 1C. These PCR products were generated and recovered as *Eco*RI fragments (see Materials and Methods) and ligated into the *Eco*RI site of pLBD (Fig. 2A), screening for clones containing inserts in the reverse (antisense) orientation with respect to the direction of transcription from the retroviral long terminal repeat promoter. This position was chosen based on previous results demonstrating effective downregulation of a target sequence (the BCR/ABL message) by insertion of an antisense sequence at this site (52). The resultant plasmids, designated pLasB Dini and pLasBDei, also contain a β-actin-regulated DHFR coding sequence conferring resistance to methotrexate for selection of stable transfected clones (see below).

Transfection and Expression of Antisense RNA in Ataxin-1-Expressing Mammalian Cells

To evaluate the effect of antisense RNAs targeting the translation initiation site of the ataxin-1 message, we used cell lines established on a Chinese hamster ovary (CHO) cell background. These cell lines were engineered to contain; (i) an SCA1 gene including a normal [30] or expanded [82] number of CAG repeats under transcriptional regulation of the tetracycline response element (TRE) (16), and (ii) a constitutively expressed tetracycline transactivator (tTA) sequence (16), which in the absence of tetracycline or tetracycline derivatives binds to TRE and activates expression of SCA1 (Fig. 2B). CHO-At-30 and CHO-At-82 clonal cell lines were established by screening a large number of cotransfectants for the highest level of induced ataxin-1 expression by Western blot analysis (55).

CHO-At-30 cells (expressing unexpanded ataxin-1) were transfected with pLBD control plasmid or with pLasBDini, selecting positive transfectants by incubating in medium containing methotrexate. Drug-resistant clones were isolated and expanded in culture for subsequent analysis of antisense RNA expression and the effect of that expression on the level of ataxin-1 message. To verify expression of antisense RNA in the transfected cells, total RNA was extracted from several of the pLas BDini-transfected CHO-At-30 clones for Northern blot analysis. The blot shown in Figure 3Awas probed with a 600-bp DHFR cDNA sequence that hybridizes to the full-length message containing the antisense sequence and also to the smaller, internally initiated message regulated by a β-actin promoter (see Fig. 2). All of the tested clones expressed the shorter, β-actin-regulated message and varying amounts of the longer, LTR-regulated transcript (Fig. 3A). We were also concerned about the stability of antisense message expression in cells continuously cultured after induction of ataxin-1 expression by removal of doxycyline from the medium. However, we found that RNA samples extracted from one of the pLasBDini-transfected CHO-At-30 clones at several times following induction maintained a high level of the LTR-regulated antisense-encoding message, indicating that expression of the antisense message is stable in these clones for up to 42 days.

Antisense RNA Targeting the Translation Initiation Site Downregulates Ataxin-1 Message and Protein

We next examined the efficiency of pLasBDini-mediated downregulation of SCA1 gene expression, defined here as a net reduction in the steady-state level of ataxin-1 message and protein in pLasBDini-transfected CHO-At cells. pLasBDini-transfected CHO-At-30 clones were maintained in continuous culture after removal of doxycycline from the medium to induce ataxin-1 expression. Ten to 20 days after withdrawal of doxycyline, RNA was extracted for Northern blot analysis using a 1-kb probe specific for the ataxin-1 message. As seen in Figrue 4, the ataxin-1 message level induced in clones A1 and D1 was considerably reduced (fourfold and 10-fold, respectively, as determined by densitometric scanning) in comparison with the pLBD-transfected control samples. These results demonstrate the ability of antisense RNA targeting the translational initiation site to achieve a significant reduction in the steady-state level of unexpanded ataxin-1 message.

We also tested the effect of antisense message targeting the translation intitiation site on expanded ataxin-1 message. CHO-At-82 cells were transfected with pLasB Dini, selecting stable transfectants in medium containing methotrexate. Clones were isolated and expanded in culture for Northern analysis of ataxin-1 message induced 20 days after withdrawal of doxycycline from the culture medium. As seen in Figure 5, ataxin-1 message in pLasBDini-transfected CHO-At-82 clones C1, D2, F1, and E1 was undetectable, in contrast with the presence of ataxin-1 message in control pLBD-transfected cell lines. These results demonstrate that antisense RNA targeting the translation initiation site achieved effective reduction in the steady-state level of expanded as well as unexpanded ataxin-1 message.

Further evidence confirming the downregulation of ataxin-1 expression was obtained in the analysis of total and specific protein present in extracts of pLasBDini-transfected CHO-At-82 clonal isolates. Protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis, first evaluating total proteins by staining with Coomassee blue. A band corresponding to the correct size for ataxin-1 protein was identified in control CHO-At samples and in control pLBD-transfectant samples (Fig.

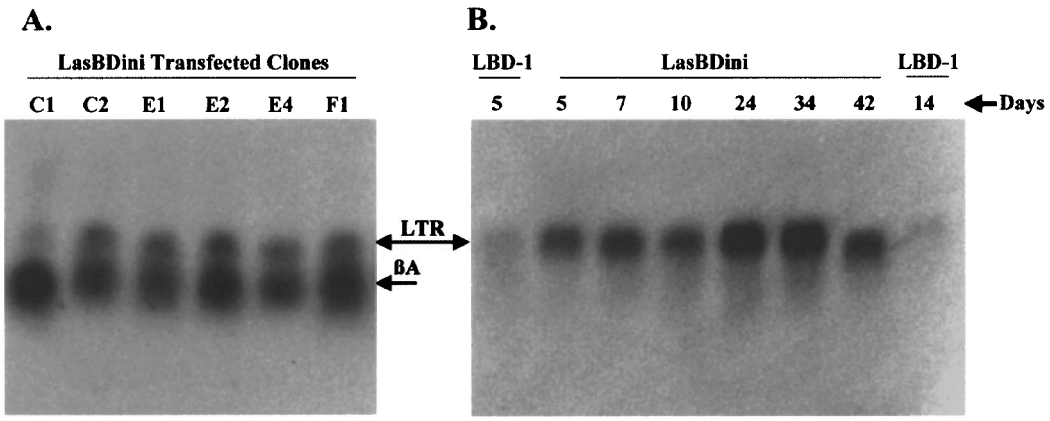

Figure 3. Northern blot analysis of DHFR and antiataxin-1 messages in pLasBDini-transfected CHO-At-30 cells. (A) Double-stranded DHFR probe. Total RNA was extracted from the pLasBDini-transfected CHO-At-30 clones indicated, electrophoresed, blotted, and probed using a radiolabeled DHFR cDNA sequence (see Fig. 2). The DHFR probe identifies both the full-length LTR-regulated message (LTR), including the antisense sequence, as well as the internally initiated DHFR message (βA). (B) Single-stranded antisense probe. Total RNA was extracted from pLBD-transfected CHO-At-30 clone #1, or from pLasBDini-transfected CHO-At-30 clone #D2 after induction of ataxin-1 expression for the indicated number of days by withdrawal of doxycycline from the growth medium. The RNA was electrophoresed, blotted, and probed for the LTR-regulated message using a single-stranded antisense RNA probe prepared against the 254-bp antisense ataxin region as described in Materials and Methods. This probe identifies only the LTR-regulated antisense message.

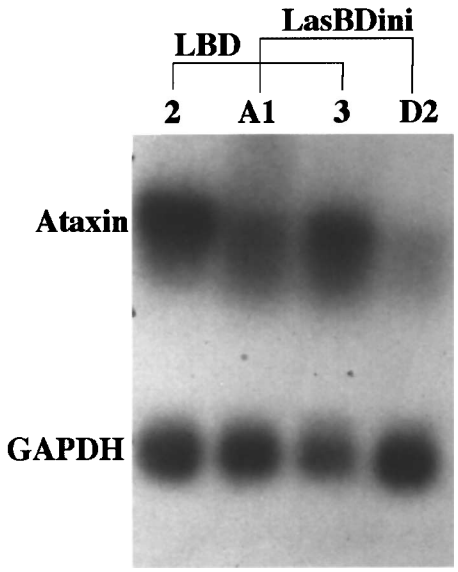

Figure 4. Northern hybridization analysis of ataxin-1 message in pLasBDini-transfected cells. Total RNA was extracted from two pLasBDini-transfected CHO-At-30 cell clones (A1 and D2) and two pLBD-transfected control clones (2 and 3), electrophoresed, blotted, and probed for both ataxin-1 and for GAPDH message as a loading control. The locations of the ataxin-1 message and GAPDH loading control are indicated.

6A) but not in pLasBDini-transfected samples. An identical gel blotted and probed with an anti-ataxin-1 antibody verified this protein as ataxin-1 and confirmed that this protein is present in control CHO-At samples and pLBD-transfectant samples but absent in pLasBDini-transfected samples. These results are consistent with the reduction in ataxin-1 message available for translation into ataxin-1 protein when ataxin-1-expressing cells are modified to express antisense RNA targeting the region around the translation initiation site.

Antisense-Mediated Inhibition of Ataxin-1 Pre-mRNA Splicing

The SCA1 gene consists of a 10660 base coding sequence plus extensive 5′ and 3′ untranslated regions distributed across 450 kb of human chromosome 6q32. The gene contains eight introns, the first seven of which are located in the 5′ untranslating region while the last one is located in the protein coding sequence. To evaluate the effectiveness of antisense RNA to modulate message processing as a strategy for inhibiting the pathologic effect of expanded ataxin-1 message, plasmid pLasBDei, containing an LTR-regulated antisense sequence targeting the intron 8 splice donor site, was transfected into human 293 cells, selecting stable transfectants in medium containing methotrexate. Drug-resistant clones were isolated and expanded in culture to determine the effect of pLasBDei transfection on the endogenous ataxin-1 message expressed in these cells. Northern blot

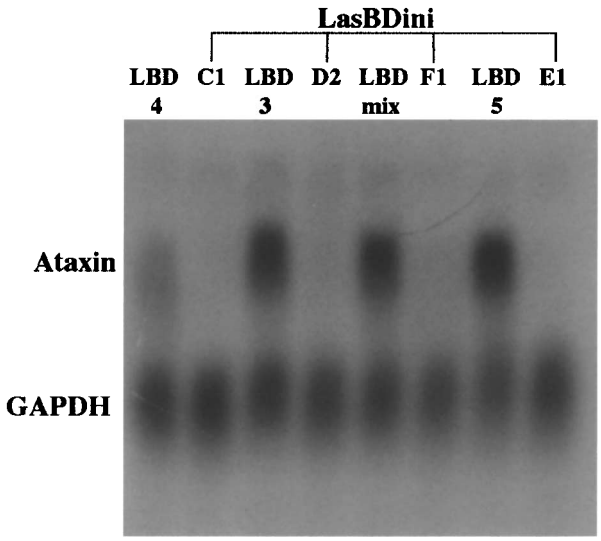

Figure 5. Downregulation of the expanded ataxin-1 message in CHO-At-82 cells transfected with pLasBDini. Total RNA was extracted from the indicated pLasBDini- or pLBD-transfected clones, electrophoresed, blotted, and then probed for ataxin sequences as described in Materials and Methods. GAPDH probe (indicated) was included as a loading control.

analysis demonstrated presence of the 10.6-kb ataxin-1 message in control pLBD-transfected 293 cells (Fig. 7A). pLasBDei-transfected cells contained ataxin-1 message of an increased size, consistent with inhibited processing of intron 8. The observed size increase is not consistent with complete retention of intron 8 (19 kb in length), which would generate a 29-kb message. Most likely, pLasBDei transfection and expression of antisense RNA targeting the splice donor site resulted in alternative splicing or recognition of cryptic splice signals in the ataxin-1 message and generation of a longer message.

To further characterize the effect of pLasBDei on ataxin-1 message splicing, RT-PCR reactions were carried out to assay for the presence or absence of intron 8 sequences in pLBD-transfected versus pLasBDei-transfected 293 cells (Fig. 7B). RNA extracted from pLBD-transfected 293 cells successfully generated a 1500-bp RT-PCR product between exons 8 and 9 (reaction A in Fig. 7B), indicating that intron 8 had been spliced away in these samples. No such RT-PCR product was generated using RNA extracted from pLasBDei-transfected 293 cells as template, indicating that intron 8 had not been correctly spliced from these samples and that a PCR reaction spanning a region retaining intron 8 was not possible under the cycling conditions used. RNA extracted from pLasBDei-transfected 293 cells was, however, capable of mediating an RT-PCR reaction between intron 8 and exon 9 sequences (reaction B in Fig. 7B), while RNA extracted from pLBD-transfected 293 control cells did not generate such an RT-PCR product. These results again are consistent with the absence of intron 8 sequences in the ataxin-1 message of pLBD-transfected control cells, while intron 8 sequences are retained in the ataxin-1 message extracted from pLasB Dei-transfected 293 cells. These results support the conclusion that antisense RNA targeting the splice donor region of intron 8 effectively inhibited splicing and prevented the complete processing of ataxin-1 message in these cells.

DISCUSSION

As a strategy for reducing the level of pathologic ataxin-1 expression in SCA1, antisense expression constructs were developed that target either the region around the translation initiation site or the region around the intron 8 splice donor site of the SCA1 message. The positioning of antisense/target sequence binding was optimized by computer-assisted secondary structure analysis, predicting single-stranded areas in the two target regions. Stable transfection and expression of pLasBDini, targeting the translation initiation site, conferred a substantial reduction in the steady-state level of either expanded or unexpanded ataxin-1 message detected in Northern blots, as well as a reduction in the level of ataxin-1 protein detected in Western blots. Stable transfection of pLasBDei, targeting the intron 8 splice donor site, inhibited splicing of intron 8 as determined by Northern blot and PCR analysis for intron 8 sequences in cellular RNA. We conclude that the expression of antisense RNA is a molecularly effective strategy for modulating ataxin-1 message, and may be applicable as a therapeutic approach for SCA1.

SCA1 is one of several dominant-acting neurologic disorders associated with expanded, unstable trinucleotide repeats (8), many of which (including SCA1) result in an increased polyglutamine tract in the protein product (35). The cytologic hallmark of SCA1 is the degeneration of cerebellar Purkinje cells, concomitant with progressive loss of motor function in the affected individual (17,42,53). Accumulation of expanded ataxin-1 results in the formation of aggregates of the protein, although the presence of such aggregates does not appear to be required for progression of the disease (28). Nuclear localization, however, is required in order for progression of ataxia to be observed in animals transgenic for engineered SCA1 sequences (28). These results implicate ataxin-1 protein in the pathologic mechanism underlying SCA1 disease, and also imply the potential feasibility of targeting either ataxin-1 message or ataxin-1 protein as therapeutic strategies for reducing expression of ataxin-1.

Dominant-acting diseases present a conceptual chal-

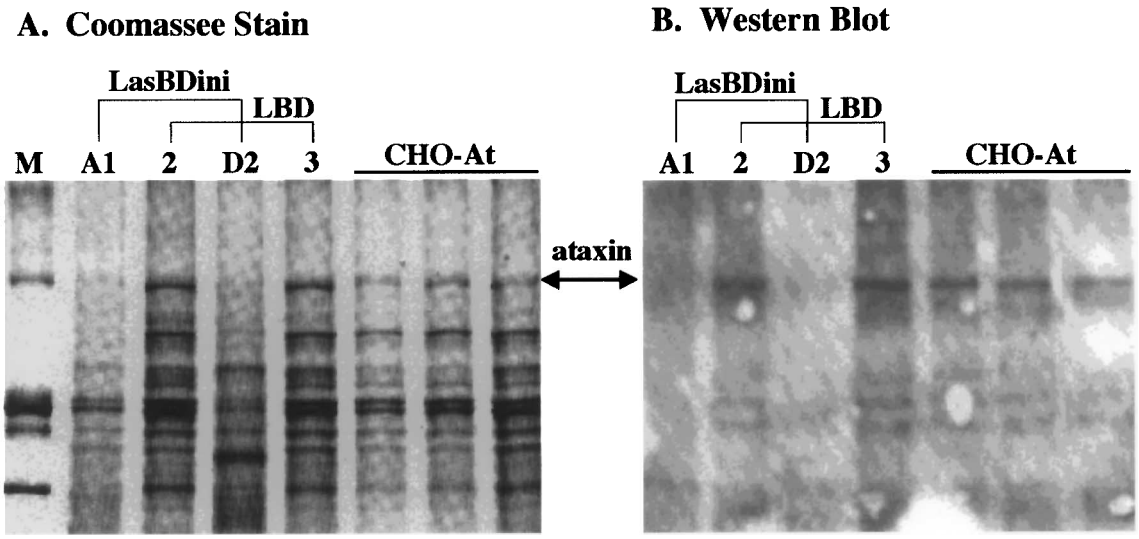

Figure 6. Analysis of ataxin-1 protein levels in pLasBDini-transfected CHO-At-82 cells. Protein was extracted from the indicated pLBD- or pLasBDini-transfected CHO-At-82 cell clones, electrophoresed on SDS polyacrylamide gel, and then either stained for total protein with Coomassie blue (A) or electroblotted and then probed using antibody prepared against an N-terminal peptide of the ataxin-1 protein (B). M, protein marker lane; CHO-At, extracts from CHO-At-82 cells as a control. Arrow: position of ataxin-1 protein.

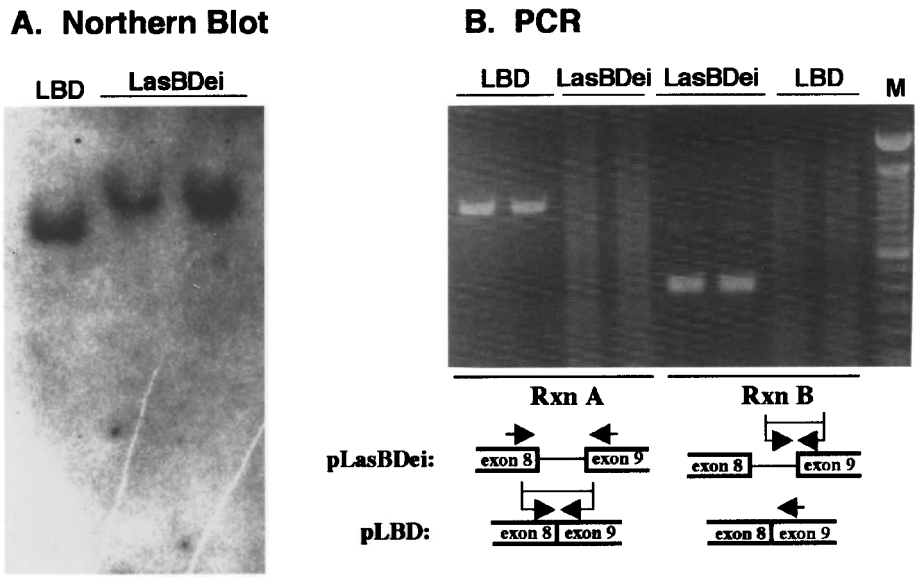

Figure 7. Analysis of ataxin-1 message splicing in 293 cells transfected with pLasBDei. (A) Northern hybridization analysis. Total RNA was extracted from 293 clones transfected with pLBD or with pLasBDei (in duplicate), electrophoresed, blotted, and then probed for ataxin sequences as described in Materials and Methods. (B) PCR analysis. RNA from pLBD- or pLasBDei-transfected clones (each in duplicate) was used as template as indicated. PCR reaction A extended from exon 8 to exon 9 (see Table 1), thus generating a 1500-bp PCR product from spliced message, but did not generate a PCR product from unspliced message. Reaction B extended from the 3′ end of intron 8 into exon 9, thus generating a 400-bp PCR product only from unspliced message. M, 100 bp ladder.

lenge that is fundamentally distinct from that of recessive disorders in the formulation of strategies for genetic therapy (34). Rather than restoring a deficient genetic function, one is faced rather with the necessity of reducing or inhibiting expression of a message or protein product that is responsible for the pathologic condition. The goals of gene transfer and expression in this case are complex in a number of ways. (i) The level of reduction in gene expression required for a beneficial effect is difficult to predict, and most likely varies depending on the target condition. (ii) The frequency of corrected cells that would be necessary for a beneficial effect may also be difficult to predict, and again this most likely depends on the characteristics of the target condition. (iii) For expanded trinucleotide repeat disorders such as SCA1, specific recognition of the expanded message (rather than the normal, unexpanded message) is complicated by nearly identical sequence content between the two messages. For treatment of dominant-acting trinucleotide repeat disorders such as SCA1, the ultimate goal would be complete inhibition of pathologic gene expression in all of the relevant cell types. However, because achievement of such a goal is unlikely in early studies, identification of the minimal requirements for a beneficial outcome is worth consideration and characterization. In the case of SCA1, some transgenic animals heterozygous for an expanded ataxin-1 allele exhibited a much less severe phenotype than animals homozygous for the same transgene (5). This implies that in some cases a 50% reduction in the level of ataxin-1 expression in the Purkinje cell population may reduce the severity of the disease. There is also evidence from experiments in SCA1 transgenic mice that limited grafts of normal Purkinje cells can modify the ataxic phenotype (24), implying that Purkinje cell function is cell autonomous. Genetic correction of a limited proportion of Purkinje cells in the setting of SCA1 may thus bring about a reduction in the severity of the disease.

Several genetic strategies have emerged to address the expression of dominant-acting disorders, including the expanded trinucleotide repeat diseases. Lewin et al. reported the expression of an engineered ribozyme, delivered by using an adeno-associated virus vector, in a strategy for treatment of dominant retinitis pigmentosa in a rat model (29). Phylactou et al. reported the application of a trans-splicing technology for correction of myotonic dystrophy, a trinucleotide repeat disorder (37). AAV vectors have been engineered for expression of inhibitory RNA for downregulation of ataxin message in animal models of SCA1 (51) and Huntington's disease (20). Antisense RNA expression has also been tested as a potential strategy for inhibition of tumor progression (47,50) and HIV replication (23,30,49,50). Here we demonstrate that expression of antisense RNA can be used either to reduce the steady state level or inhibit processing of SCA1 pathologic message or gene product. We delivered antisense RNA sequences targeting the ataxin-1 message in the context of a retroviral message previously demonstrated to be effective in reducing the level of BCR/ABL message in a murine model of chronic myeloid leukemia, another dominant-acting system (52). Antisense RNA may similarly be used to affect the expression of other pathologic genes.

Our initial strategy in targeting the translational start site was that antisense RNA may be capable of inhibiting translation of SCA1 message into protein by duplex formation and prevention of large ribosomal subunit coupling with the small ribosomal subunit after scanning from the mRNA cap structure. Indeed, our analysis of pLasBDini-transfected CHO-At-82 cells indicates a reduced level of ataxin-1 protein. However, transfection with pLasBDini resulted in reduced ataxin-1 message levels as well. pLasBDini transfection thus reduces the level of SCA1 message, thereby resulting in a reduced level of ataxin-1 protein. While the mechanism by which binding of antisense message around the translational start site reduces the level of ataxin-1 message has yet to be addressed experimentally, downregulation of ataxin message in pLasBDini-transfected cells may result from the targeting of messages by short lengths of interfering double-stranded RNA sequences (12) associated with induction of ribonuclease activity at the targeted site (26). Whether the translation initiation site serves as a generally effective target for antisense RNA binding with subsequent target message destabilization has yet to be determined.

We also hypothesized that targeting a splice donor site in the ataxin-1 pre-messenger RNA would inhibit splicing and maturation of the ataxin-1 message. Such modification of pre-mRNA splicing has been previously demonstrated by introduction of antisense oligonucleotides (9,11,45) or by expression of antisense RNA (15,18,40). Our strategy in these experiments was to inhibit the normal binding of U1 snRNA to the 5′ splice site (33) and impede its contribution to the subsequent splicing process (42). The results from our experiments are consistent with the inhibition of normal ataxin-1 pre-mRNA splicing (i.e., there was an increase in message size observed in Northern blots of RNA extracted from pLasBDei-transfected cells), and RT-PCR reactions were consistent with maintenance of the 3′ end of intron 8 and the absence of contiguous coding sequence. Northern blot analysis was not consistent (Fig. 7A) with the complete retention of intron 8 (19 kb in length), raising the possibility of alternative splicing or cryptic splice site recognition resulting from antisense RNA binding to the intron 8 splice donor site. Because intron 8 is positioned downstream from the CAG repeat region

in the SCA1 message, it is not optimally located for achieving a reduction in polyglutamine-containing SCA1 protein. As a therapeutic strategy, inhibition of normal intron 8 splicing could destabilize either the ataxin message or subsequently generated protein, although message destabilization was not apparent from Northern blot analysis. Alternatively, one of the upstream splice donor sites in the 5′ untranslated region could be targeted for antisense inhibition of ataxin pre-mRNA splicing.

The antisense targeting strategies described in this article are not designed to distinguish between expanded and unexpanded ataxin-1 message. While specific reduction of the expanded message would be a superior approach, the expanded ataxin-1 message differs from the normal message only in the number of CAG repeats contained in the expanded region, aside from the presence of two CAT interruptions in the normal message that are missing in the expanded message (1). This sequence similarity renders difficult the task of specifically targeting expanded ataxin-1 message in the presence of normal message using antisense, ribozyme, or inhibitory RNA strategies. As a therapeutic strategy, targeting both expanded and normal messages would be acceptable if detrimental effects associated with reduced levels of the normal message are not anticipated. In the case of SCA1, mice engineered to be genetically deficient in ataxin-1 expression exhibited relatively mild (in comparison with the ataxia exhibited in animals expressing expanded ataxin-1 message and protein) deficits in learning and hippocampal paired pulse facilitation (31). Generalized reduction in both normal and expanded ataxin-1 message might thus be considered a reasonable strategy, particularly if delivery and expression of the antisense RNA can be directed to cerebellar Purkinje cells (see below). Another strategy would be to repair the expanded ataxin-1 message by trans-splicing, using either a group 1 intron/ribozyme as an invading replacement message [as previously tested for myotonic dystrophy (37)], or a spliceosome-mediated trans-splicing approach (39). In this strategy, replacement of the CAG repeat region in either the expanded or the unexpanded message would retain normal message function, thus obviating the need for specific recognition of the expanded message. The efficiency of trans-splicing in this system, however, has yet to be determined.

An important part of any genetic strategy for treatment of a CNS disorder is the efficiency of gene delivery to the affected cell population. In the case of SCA1, the target cell population consists of cerebellar Purkinje cells, which degenerate as a part of the developing disease process. Numerous vector systems have been tested for gene transfer into the central nervous system (2,4, 14,21,48). Adeno-associated virus (AAV) in particular has emerged as a vector type that is remarkably effective for this purpose, due in part to its remarkable propensity for neuronal cell types when introduced into several different regions of the brain (3,10,27,32). We have found cerebellar Purkinje cells to be particularly susceptible to AAV-mediated gene transfer after stereotactic injection either into deep cerebellar nuclei or into the cerebellar cortex (25). Up to 3% of cerebellar Purkinje cells were transduced in one hemisphere after a single injection and more recent assessments have indicated that expression is stable for up to 1 year. AAV vectors engineered to express the antisense RNAs described in this article could thus be used for gene transfer into cerebellar Purkinje cells of SCA1 transgenic mice as an animal model for gene therapy of SCA1. These studies are currently in progress.

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