

# Partial Correction of Sensitivity to Oxidant Stress in Friedreich Ataxia Patient Fibroblasts by Frataxin-Encoding Adeno-associated Virus and Lentivirus Vectors

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## ABSTRACT

Peripheral nervous system (PNS) sensory neurons are directly involved in the pathophysiology of a number of debilitating inherited and acquired neurological conditions. The lack of effective treatments for many such conditions provides a strong rationale for exploring novel therapeutic approaches, including gene therapy. Friedreich ataxia (FRDA), a sensory neuropathy, is a progressive neurodegenerative disease associated with a loss of large sensory neurons from the dorsal root ganglia. Because a mouse model for this well-characterized disease has been generated, we elected to use FRDA as a model disease. In previous studies we achieved efficient and sustained delivery of a reporter gene to PNS sensory neurons, using recombinant adeno-associated viral (AAV) and lentiviral (LV) vectors. In the current study, AAV and LV vectors encoding the human frataxin cDNA were constructed and assessed for frataxin expression and function in primary FRDA patient fibroblast cell lines. FRDA fibroblasts have been shown to exhibit subtle biochemical changes, including increased mitochondrial iron and sensitivity to oxidant stress. Despite the inherent difficulty in working with primary cells, transduction of patient fibroblasts with either vector resulted in the expression of appropriately localized frataxin and partial reversal of phenotype.

## OVERVIEW SUMMARY

We have previously demonstrated efficient and sustained transduction of primary human and murine dorsal root ganglia sensory neurons by recombinant adeno-associated virus type 2 (AAV2) and vesicular stomatitis virus glycoprotein-pseudotyped human immunodeficiency virus type 1-derived lentiviral (LV) vectors. To further explore the exciting potential of these vectors for gene therapy applications in the peripheral nervous system (PNS), we have chosen Friedreich ataxia (FRDA, a sensory neuropathy) as a model disease. In this initial study, AAV and LV vectors encoding the human frataxin cDNA were constructed and evaluated for their ability to direct frataxin to the mitochondrial compartment and to correct the phenotype in FRDA patient fibroblasts. Transduction of patient fibroblasts resulted in appropriate subcellular localization of

frataxin and partial phenotype correction, as measured by increased resistance to oxidant stress. The availability of mouse models of FRDA now provides the opportunity to test the therapeutic potential of these vectors in frataxin-deficient PNS sensory neurons in culture and *in vivo*.

## INTRODUCTION

PERIPHERAL NERVOUS SYSTEM (PNS) sensory neurons play a clinically significant role in the pathophysiology of a number of inherited and acquired neurological conditions. These include sensory neuropathies such as Friedreich ataxia (FRDA) (Campuzano *et al.*, 1996), neuropathic pain syndromes (Woolf and Mannion, 1999), and the spasticity associated with upper motor nerve damage (Young, 1994). Despite a growing understanding of the molecular basis of the underlying neuropath-

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ology, treatment options remain unsatisfactory. Accordingly, there is a pressing need to explore novel therapeutic options potentially capable of curing or favorably modifying the natural history of these debilitating conditions. One exciting, but challenging, possibility is the targeted delivery of therapeutic genes to sensory neurons. Although efficient and stable gene delivery will inevitably be required, the modest number (no more than 100,000) (Fitzgerald, 1985) and anatomically discrete clustering of sensory neuron cell bodies within individual dorsal root ganglia (DRG) places conditions affecting these cells within theoretical reach of contemporary gene transfer technology.

Several viral vector systems have the capacity to transduce neuronal cells, albeit with differing efficiencies and duration of transgene expression. The majority of more recent studies have concentrated on the use of lentivirus (LV) and adeno-associated virus (AAV) vectors to transduce neurons within the central nervous system (CNS) for the treatment of diseases such as Parkinson's disease (Bjorklund *et al.*, 2000; Kordower *et al.*, 2000; Tenenbaum *et al.*, 2004), mucopolysaccharidosis type VII (Bosch *et al.*, 2000; Daly *et al.*, 2001), and amyotrophic lateral sclerosis (ALS) (Azzouz *et al.*, 2004). In the CNS, both vectors are capable of efficient and sustained transduction of multiple neuronal cell types, with transgene expression persisting for at least 8 months (Haberman *et al.*, 1998; Mandel *et al.*, 1998; Kordower *et al.*, 2000; Consiglio *et al.*, 2001; Mazarakis *et al.*, 2001; Tenenbaum *et al.*, 2004). Neurons within the PNS have been less well studied, despite being more anatomically accessible, arranged in simpler neurological networks, and having a capacity for regeneration. In a previous study we demonstrated efficient transduction of both murine and human DRG sensory neurons established in primary culture. In dissociated and microinjected explant cultures, the majority of sensory neurons were transduced with either LV or AAV vectors at low multiplicities of infection (MOIs) (Fleming *et al.*, 2001).

These promising data provided the impetus for further developing these vectors in the context of FRDA, a slowly progressive neurodegenerative disease in which early morbidity is thought to result from loss of DRG sensory neurons (Hughes *et al.*, 1968). Subsequently the posterior columns, pyramidal and corticospinal tracts, and Clarke's column of the spinal cord become involved with less severe degeneration occurring in the cerebellum, medulla, and pons (Delatycki *et al.*, 2000). The disease is inherited in an autosomal recessive manner, affects approximately 1 in 30,000 live births in white populations, and is the most common inherited ataxia (Cossee *et al.*, 1997). Clinical manifestations include gait ataxia, loss of deep tendon reflexes, pes cavus and dysarthria. Late developing anomalies include cardiac hypertrophy, diabetes mellitus, blindness, and deafness (Harding, 1981). Most patients become wheelchair-bound by their early twenties and die in late middle age from cardiorespiratory complications. The mutation responsible for FRDA is most commonly a GAA expansion in intron 1 of the frataxin gene (Campuzano *et al.*, 1996), resulting in reduced levels of frataxin mRNA and protein (Campuzano *et al.*, 1997). The 210-amino acid frataxin protein carries an amino-terminal sequence that targets the molecule to the inner mitochondrial membrane (Campuzano *et al.*, 1997; Priller *et al.*, 1997). Studies in yeast and mammalian cells have shown that reduced frataxin levels lead to deficiencies in respiratory chain enzyme and aconitase activities (Bradley *et al.*, 2000), accumulation of

mitochondrial iron (Delatycki *et al.*, 1999; Wong *et al.*, 1999) and mitochondrial damage (Karthikeyan *et al.*, 2003), and defective oxidative phosphorylation and production of reactive oxygen species (Lodi *et al.*, 1999; Ristow *et al.*, 2000). These free radicals are then hypothesized to cause death in sensitive cell types, via oxidative damage (Priller *et al.*, 1997; Lodi *et al.*, 1999; Cossee *et al.*, 2000; Karthikeyan *et al.*, 2003). Studies in yeast support a primary role for frataxin in the biogenesis of cellular iron/sulfur proteins (Muhlenhoff *et al.*, 2002; Tan *et al.*, 2003).

In the current study, we describe the construction and functional evaluation of AAV and LV vectors encoding the human frataxin cDNA. Both vector systems conferred frataxin expression on target cells, correct subcellular localization of the protein, and partial reversal of increased sensitivity to oxidant stress in FRDA patient fibroblasts. These results provide support for a vector-mediated gene delivery approach to the treatment of diseases of the PNS.

## MATERIALS AND METHODS

### *Cell lines and culture conditions*

The HEK 293 human epithelial cell line, the HeLa cell line, and four FRDA patient and age-matched control fibroblast cell lines have been described previously (Gey *et al.*, 1952; Graham *et al.*, 1977; Delatycki *et al.*, 1999; Jauslin *et al.*, 2002). Four of the FRDA patient fibroblast and age-matched control cell lines were obtained at passage 9 from M. Delatycki (Murdoch Childrens Research Institute, Melbourne, Australia), and one patient cell line (designated F2) and one control cell line (designated C2) were obtained at passage 3 from the Coriell Cell Repositories (Camden, NJ). FRDA patient fibroblast cell lines were examined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) (as described below) and were demonstrated to exhibit reduced frataxin mRNA levels compared with control cell lines (data not shown). Fibroblast lines were split 1:3 approximately once per week and experiments were performed within a further 11 passages. HEK 293 and HeLa cells were cultured in Dulbecco's modified essential medium (DMEM; GIBCO-BRL, Gaithersburg, MD), 10% calf serum (Starrate, Bethungra, New South Wales, Australia), or fetal bovine serum (CSL, Parkville, Victoria, Australia) and 2 mM glutamine (GIBCO-BRL). Except where indicated, fibroblasts were cultured in DMEM with 10% calf serum, 2 mM glutamine, 10  $\mu$ M uridine (Sigma, St. Louis, MO), and 2 mM sodium pyruvate (Sigma). All cultures were grown at 37°C in a humidified 5% CO<sub>2</sub>-95% air atmosphere.

### *Construction, production, and titration of frataxin-encoding AAV and lentiviral vectors*

The human frataxin cDNA (designated X25 + 5a) (Campuzano *et al.*, 1996) was excised from plasmid pT7T3 (Stratagene, La Jolla, CA) as a *Hind*II fragment, A-tailed with *Taq* DNA polymerase, and ligated into the TA cloning vectors pGEM-T Easy and pTarget (Promega, Madison, WI). The frataxin cDNA was then excised as either a *Not*I fragment (by partial digestion) from pGEM-T Easy or as a *Bam*HI-*Sal*I fragment from pTarget. These fragments were then subcloned into

the *NotI* sites of the AAV vector plasmid pTRUF2 (Muzyczka, 1992) or into the *BamHI* and *SalI* sites of the late-generation human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vector plasmid pRRL.sin.cPPT.CMV.Wpre (Follenzi *et al.*, 2000), respectively. The correct orientation and integrity of the frataxin open reading frame in each construct were confirmed by restriction fragment analysis and sequencing. The resultant AAV and lentiviral vector constructs were designated AAVFrat and LVFrat, respectively (Fig. 1).

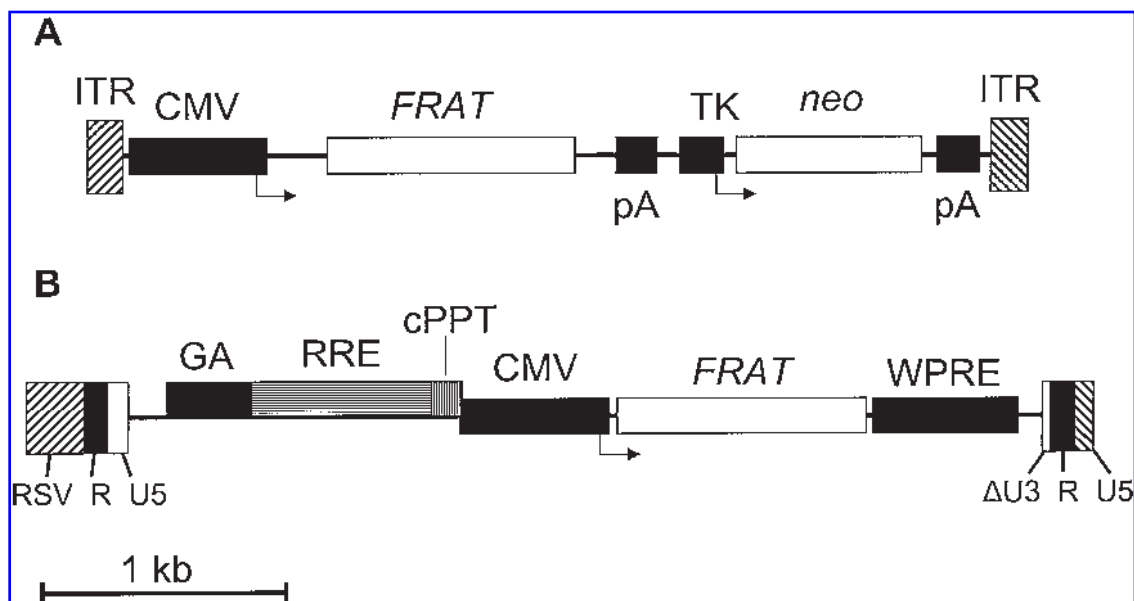
Recombinant AAVFrat vector stocks were produced with Ad helper plasmid (pXX-6) and AAV2 helper plasmid (pXX-2) and pAAVFrat, using a three-plasmid transfection protocol in HEK 293 cells as previously described (Xiao *et al.*, 1998; Fleming *et al.*, 2001). Recombinant LVFrat vector stocks pseudotyped with the vesicular stomatitis virus glycoprotein (VSVg) envelope were prepared by a four-plasmid transfection protocol in HEK 293 cells, as previously described (Dull *et al.*, 1998; Barry *et al.*, 2001; Ginn *et al.*, 2003). For AAVFrat vector stocks functional transduction titers were assigned on HeLa cells by two independent methods, colony formation in G418 (600  $\mu\text{g}/\text{ml}$ , active; Roche, Basel, Switzerland) and immunohistochemical staining for frataxin-expressing cells (Campuzano *et al.*, 1997) after exposure to serial dilutions of vector stocks. Transduction titers for LVFrat vector stocks were assigned on HeLa and HEK 293 cells by immunohistochemical staining for frataxin-expressing cells. All LVFrat vector stocks were tested for replication-competent lentivirus, using the HIV-1 p24 Gag enzyme-linked immunosorbent assay (ELISA) (PerkinElmer Life and Analytical Sciences, Boston, MA) as previously described (Ginn *et al.*, 2003).

#### Immunohistochemical analysis of frataxin expression

To investigate frataxin expression, naive and transduced cells were plated in two-well glass chamber slides (Lab-Tek; Nalge Nunc International, Naperville, IL). Cells were then fixed and permeabilized in 4% (w/v) paraformaldehyde (pH 7.4) and then methanol, and stained with frataxin-specific mouse monoclonal antibody 1G2 (diluted 1:250; Chemicon International, Temecula, CA) (Campuzano *et al.*, 1997) and secondary goat anti-mouse rhodamine red-conjugated antibody (diluted 1:250; Jackson ImmunoResearch Laboratories, West Grove, PA). Mitochondria were labeled with the mitochondrial dye Mito-Tracker Green, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Images were captured with a SPOT camera attached to a fluorescence microscope with the appropriate Leitz or Chroma excitation and emission filters (Leica Microsystems, Bensheim, Germany; and Chroma Division of Waldeck, Rockingham, VT) and colocalization of frataxin expression (red) with mitochondrial staining (green) was observed by merging the two images (orange). To estimate transduction efficiency, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.1  $\mu\text{g}/\text{ml}$ ; Sigma) and the number of transgene-positive cells was determined.

#### PCR analyses

Semiquantitative RT-PCR was performed to determine the relative levels of frataxin-encoding mRNA (both endogenous and proviral) in control, naive, and transduced FRDA patient fibroblasts. Total RNA was extracted from approximately  $1 \times 10^7$  cells per treatment group, using TRI reagent (Sigma).



**FIG. 1.** Frataxin-encoding AAV and lentiviral vector genomes. Diagrammatic representation of AAVFrat (A) and LVFrat (B) constructs. Promoters are indicated by arrows and coding regions by open boxes. Abbreviations: ITR, inverted terminal repeat; CMV, human immediate-early cytomegalovirus promoter; *FRAT*, human frataxin cDNA; pA, polyadenylation signal; TK, thymidine kinase promoter; *neo*, neomycin resistance gene; RSV-R-U5, hybrid Rous sarcoma virus promoter; GA, truncated *gag* sequence; RRE, Rev-responsive element; cPPT, central polypurine tract; WPRE, woodchuck posttranscriptional regulatory element;  $\Delta\text{U3-R-U5}$ , U3-deleted HIV-1 terminal repeat.

After extraction, cDNA was generated from 1  $\mu\text{g}$  of total RNA, using 0.5  $\mu\text{g}$  of oligo(dT)<sub>12-18</sub> primers (Invitrogen, Carlsbad, CA) and 200 units of SuperScript II RNase H-reverse transcriptase (Invitrogen) in 20- $\mu\text{l}$  reactions according to the manufacturer's instructions. Forward and reverse primers for frataxin and  $\beta$ -actin were, respectively, 5'-AAGCAGACGC-CAAACAAGC-3' and 5'-CCGACAATCCAAAAAATCTTCC-3', and 5'-TCATGAAGTGTGACGTCGACATCCG-3' and 5'-CCTAGAAGCATTGCGGTGGACGATG-3', giving amplification products of 395 and 285 bp. Amplification conditions for frataxin were 94°C for 5 min, followed by 28 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min followed by 72°C for 10 min; for  $\beta$ -actin the amplification conditions were 94°C for 5 min, followed by 27 cycles of 94°C for 30 sec, 50°C for 1 min, and 72°C for 30 sec followed by 72°C for 10 min. Products were run on 2% (w/v) agarose gels in Tris–borate–EDTA (TBE) buffer.

Quantitative real-time PCR (Q-PCR) was used to examine vector genome copy numbers in transduced cells as a function of time after vector exposure. DNA was extracted from between 10<sup>5</sup> and 10<sup>7</sup> cells per time point, using microkit DNA columns (Qiagen, Valencia, CA), and included treatment with DNase-free RNase (Roche) as described by the manufacturer. Quantitation of AAVFrat and LVFrat genomes was then performed under previously described conditions, primers, and probes (Sastry *et al.*, 2002; Veldwijk *et al.*, 2002) on a Corbett real-time PCR machine (Corbett Research, Mortlake, NSW, Australia).

#### *Analysis of sensitivity to oxidative stress*

Fibroblasts were plated at a density of 3  $\times$  10<sup>3</sup> cells per well in a 96-well plate (BD Biosciences Discovery Labware, Bedford, MA) in medium (Jauslin *et al.*, 2002) consisting of phenol red-free 64% minimal essential medium (MEM) with Earl's balanced salt solution (GIBCO-BRL), 25% M199 (GIBCO-BRL), 10% fetal bovine serum (Invitrogen), 2 mM glutamine (GIBCO-BRL), human insulin (10  $\mu\text{g}/\text{ml}$ ; Eli Lilly, Indianapolis, IN), epidermal growth factor (EGF, 10 ng/ml; Sigma), and fibroblast growth factor (FGF, 10 ng/ml; Sigma), and after 24 hr were treated with 0.1 to 1 mM L-buthionine-(S,R)-sulfoxamine (BSO; Sigma) for 24–48 hr, until signs of cell death were observed. Cell viability was then measured with calcein AM (0.4  $\mu\text{M}$ ; Molecular Probes) as described previously (Jauslin *et al.*, 2002).

#### *Quantitation of mitochondrial iron*

Accumulation of mitochondrial iron was measured by atomic absorption spectroscopy as previously described (Delatycki *et al.*, 1999). Mitochondrial protein measurements were performed using the Bio-Rad D/C protein assay (Bio Rad, Hercules, CA).

#### *Statistical analyses*

Oxidative stress and mitochondrial iron assays were analyzed by one-tailed nonparametric Wilcoxon tests. One-tailed tests were employed because differences in cell viability and mitochondrial iron were predicted to change in one direction only.

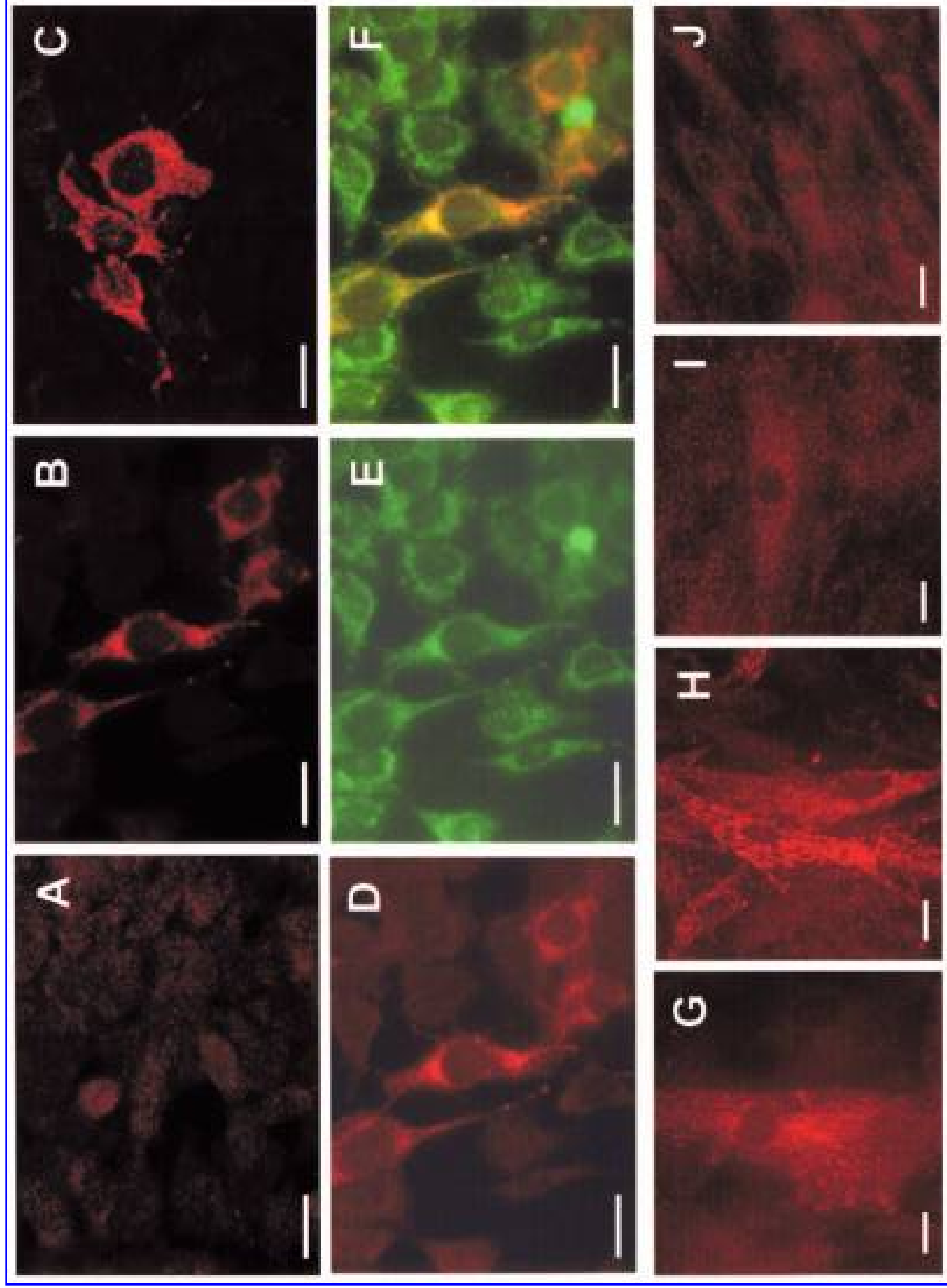
## RESULTS

### *Vector-mediated frataxin expression in HeLa cells and FRDA patient fibroblasts*

After construction and production of AAVFrat and LVFrat vectors, as described in Materials and Methods, transduction titers were assigned on HeLa cells by immunohistochemical staining with an anti-frataxin antibody. Before vector exposure, endogenous frataxin expression could not be detected (Fig. 2A). Within 72 hr of exposure to either the AAVFrat or LVFrat vector, however, a distinctive cytoplasmic pattern of frataxin expression became evident (Fig. 2B and C), with the level of frataxin expression in individual cells varying widely for both vectors. Maximal apparent transduction titers obtained by frataxin antibody staining were 5  $\times$  10<sup>4</sup> and 2  $\times$  10<sup>5</sup> transducing units (TU)/ml for AAVFrat and LVFrat, respectively. Titers obtained for equivalent AAVFrat vector stocks by colony formation under G418 selection were up to three orders of magnitude higher. The distinctive pattern of antibody staining in transduced HeLa cells was further shown to be consistent with correct subcellular localization of frataxin in the mitochondrial compartment by counterstaining with a mitochondrial-specific dye (Fig. 2D–F).

Both vectors were also shown to transduce five independent primary FRDA patient fibroblast cell lines (Delatycki *et al.*, 1999; Jauslin *et al.*, 2002), albeit with markedly reduced efficacies relative to HeLa cells, but with essentially the same pattern of frataxin subcellular localization (Fig. 2G and H). Interestingly, anti-frataxin antibody staining was not sufficiently sensitive to detect the putatively reduced levels of frataxin expression present in naive FRDA patient fibroblasts (Fig. 2I) or in age-matched control fibroblasts (Fig. 2J) expressing endogenous frataxin at physiological levels. This insensitivity in the antibody detection of physiological levels of frataxin expression makes it likely that the transduction titers assigned to the AAVFrat and LVFrat vectors underestimate the true titers, and rendered it difficult to reliably evaluate the frataxin expression status of individual cells in vector-treated cultures.

In an attempt to further quantitate the levels of exogenous frataxin in transduced fibroblast populations, semiquantitative RT-PCR was performed. Early-passage patient FRDA fibroblasts (F2 line) (Jauslin *et al.*, 2002) were transduced with either AAVFrat or LVFrat at MOIs of approximately 20, and analyzed for frataxin protein expression and frataxin mRNA levels. The results obtained appeared discordant, further supporting insensitivity of the anti-frataxin antibody in the detection of physiological and subphysiological levels of frataxin expression. Maximal levels of transduction, as determined by antibody staining, were observed at early time points after vector exposure (up to two passages), when approximately 1 and 20% of fibroblasts expressed visibly detectable anti-frataxin antibody staining after exposure to the AAVFrat and LVFrat vectors, respectively. After a further four to six passages frataxin expression dropped to levels undetectable by antibody staining in cultures exposed to the AAVFrat vector (despite selection and maintenance in G418 at 300  $\mu\text{g}/\text{ml}$ ) and to less than 1% in cultures exposed to LVFrat vector (data not shown). Concurrent analysis of frataxin mRNA levels revealed that the mean levels of frataxin expression in vector-treated FRDA patient fi-

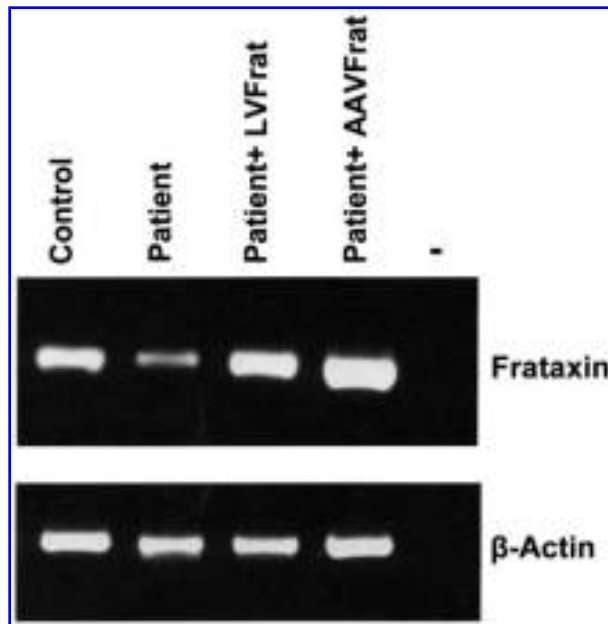


**FIG. 2.** Transduction of HeLa cells and frataxin-deficient patient fibroblasts. HeLa cells were stained for frataxin expression with the antibody 1G2 before (A) and after transduction with either AAVFrat (B) or LVFrat (C). AAVFrat-transduced HeLa cells were double-stained for frataxin expression (D) and for mitochondria with MitoTracker Green dye (E) [merged images in (F)], demonstrating correct localization of frataxin in the mitochondrial compartment. (G–J) Patient fibroblasts transduced with either AAVFrat (G) or LVFrat (H), and untransduced FRDA patient fibroblasts and age-matched control cells (I and J) antibody stained for frataxin expression. Data shown are representative of five independent FRDA patient fibroblast lines studied. Scale bars: 20  $\mu$ m.

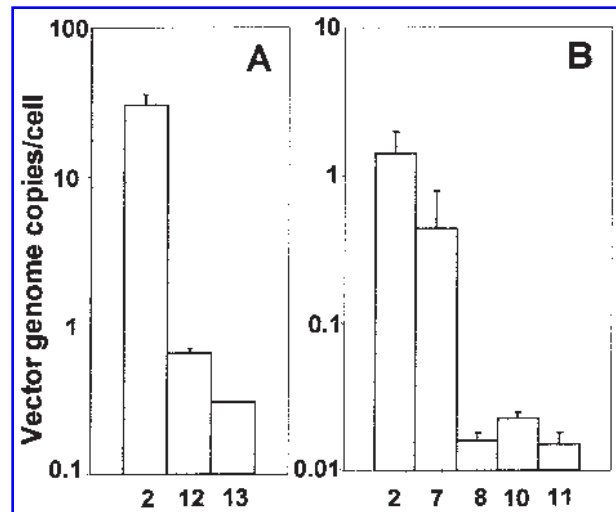
broblast cultures remained at or above physiological levels observed in control fibroblast cultures and markedly above the pathologically low levels expressed in naive FRDA patient fibroblast cultures (Fig. 3).

#### *Loss of vector genomes in transduced fibroblast populations over time*

Because transgene expression fell rapidly over time, transduced fibroblast populations were examined by quantitative real-time PCR (Q-PCR) to establish whether this phenomenon might correlate with a decline in the number of vector genomes present, or alternatively might be due to transcriptional silencing of the frataxin expression cassette. After transduction of FRDA patient fibroblasts with either the AAVFrat or LVFrat vector there was a rapid decline in the number of vector genomes per cell as a function of time (Fig. 4A and B). In cultures exposed to the AAVFrat vector there was an initial rapid decline in the number of vector genomes present per cell, a result consistent with the mainly episomal nature of recombinant AAV vectors, but levels did not fall below approximately one vector genome copy per cell, a result consistent with the maintenance of AAVFrat-transduced cells under G418 selection pressure. In cultures exposed to the LVFrat vector this decline was progressive, falling greater than 100-fold to approximately 1 vector genome per 100 cells within 8 to 10 passages. Given



**FIG. 3.** Frataxin transcript abundance in FRDA patient fibroblasts before and after transduction. *Top:* Semiquantitative RT-PCR analysis of frataxin transcript abundance in age-matched control fibroblasts (C2 line, lane 1) and FRDA patient fibroblasts (F2 line) before (lane 2) and after transduction with either LVFrat (lane 3) or AAVFrat (lane 4), each at an MOI of 20. Lane 5, no-template control. *Bottom:* Quantitation controls obtained by subjecting the same RNA samples to RT-PCR with  $\beta$ -actin-specific primers are shown. RNA samples analyzed were harvested from LVFrat- and AAVFrat-transduced cells six and eight passages after transduction, respectively.

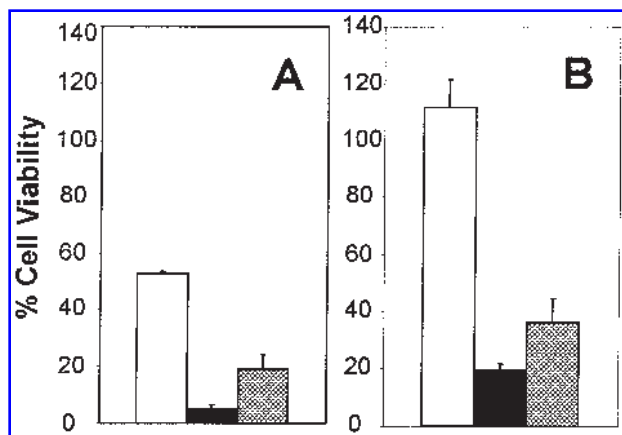


**FIG. 4.** Progressive loss of frataxin-encoding vector genomes from transduced FRDA patient fibroblasts. Real-time Q-PCR analysis of vector genome copy number in serial DNA samples isolated at intervals from transduced FRDA patient fibroblasts (F2 line) after transduction with AAVFrat (A) and LVFrat (B) at MOIs of approximately 10 and 1 TU/cell, respectively. The number of passages after transduction is depicted underneath each column. Columns and error bars represent means and SEM ( $n \geq 4$ ).

that lentiviral vectors transduce cells by stable genomic integration, this latter result is consistent with either loss of transduced cells from the culture and/or a reduced replication rate in transduced cells relative to naive cells. Both possibilities are consistent with overexpression of frataxin being toxic in fibroblasts.

#### *Partial correction of sensitivity to oxidant stress in FRDA patient fibroblasts*

Finally, both the AAVFrat and LVFrat vectors were assessed for their capacity to reverse the increased sensitivity of FRDA patient fibroblasts (F2 line) to oxidant stress induced by exposure to BSO, a pathological property previously shown to be a phenotypic feature of these frataxin-deficient cells (Jauslin *et al.*, 2002). Given the demonstrated loss of vector genomes from transduced cultures over time, analyses were performed at MOIs of approximately 20 for both the AAVFrat and LVFrat vectors, and within seven passages of transduction, when the vector genome copy number was known to approximate one copy per cell. Relative to naive FRDA patient fibroblasts, the same cells transduced with either the LVFrat vector (Fig. 5A) or AAVFrat vector (Fig. 5B) consistently exhibited increased resistance to BSO-induced oxidant stress, but not to levels approaching that seen in normal age-matched control cells. Although interexperimental variability was high, analysis of five independent experiments on LVFrat-transduced patient fibroblasts demonstrated a significant 2.9-fold mean increase (range, approximately 1- to 5-fold difference) in cell viability ( $p = 0.0215$ ) in transduced versus untransduced patient cells after oxidant stress. This effect of exogenous frataxin expression was confirmed in two additional experiments on AAVFrat-trans-



**FIG. 5.** Partial correction of sensitivity to oxidant stress in FRDA patient fibroblasts after transduction with frataxin-encoding vectors. Age-matched control fibroblasts (open columns), naive FRDA patient fibroblasts (F2 line) (solid columns), and transduced FRDA patient fibroblasts (F2 line) (shaded columns) were subjected to oxidant stress by exposure to BSO and the proportion of cells remaining viable after 24 hr was taken as a measure of resistance to oxidant stress. Results shown are from representative experiments for patient cells transduced with LVFrat (A) and AAVFrat (B). Columns and error bars represent means and SEM ( $n = 3$ ).

duced fibroblasts in which a similar 2.5-fold mean increase in cell viability was observed. Naive and transduced FRDA fibroblasts were also assayed for changes in mitochondrial iron content by atomic absorption spectroscopy, because mitochondrial iron has previously been shown to be increased in FRDA patient fibroblasts compared with age-matched control cells (Delatycki *et al.*, 1999; Wong *et al.*, 1999). Both AAVFrat- and LVFrat-transduced cells exhibited a small reduction in iron levels below those detected in naive FRDA patient cells (data not shown). The difference, however, did not achieve statistical significance ( $p = 0.058$ ), but was nevertheless consistent with the modest increase in resistance to oxidative stress observed. Collectively, these data confirm that the AAVFrat and LVFrat vectors described produce functionally active frataxin protein and are capable of at least partially correcting the phenotype of frataxin-deficient FRDA patient cells.

## DISCUSSION

Treatment options for FRDA and other inherited and acquired conditions involving pathology in PNS sensory neurons remain inadequate. Accordingly, there is a pressing need to explore novel therapeutic options, including gene therapy. In the case of FRDA, there are still no effective therapies for the treatment of the ataxic features of this disease, which are thought to correlate with loss of sensory neurons located within DRG (Hughes *et al.*, 1968). Iron chelators, such as desferrioxamine, have been associated with side effects in the clinic and have not been effective in reducing iron levels within the mitochondria (Voncken *et al.*, 2004). Newer, less toxic mitochondrially targeted chelators are now being investigated (Richardson,

2004). More recently, antioxidant therapy with idebenone (Jauslin *et al.*, 2003; Sarsero *et al.*, 2003; Seznec *et al.*, 2004) has been reported in a number of studies to reduce the cardiac hypertrophy observed in FRDA patients (Rustin *et al.*, 1999; Hausse *et al.*, 2002). Unfortunately, however, there are no reports of measurable improvement in the ataxic features of this disease (Hausse *et al.*, 2002).

We are interested in the possibility of favorably modifying the natural history of FRDA by targeting gene therapy to PNS sensory neurons. In a previous study we demonstrated efficient and sustained transduction of mouse and human DRG sensory neurons by both AAV and lentiviral vectors (Fleming *et al.*, 2001). In the current study we report the development of AAV and lentiviral vectors encoding the human frataxin cDNA and initial phenotype correction studies in fibroblasts from FRDA patients. Although fibroblasts are not directly involved in the pathophysiology of FRDA, those obtained from FRDA patients have previously been shown to have reduced frataxin mRNA levels (Wong *et al.*, 1999), increased sensitivity to oxidant stress (Wong *et al.*, 1999; Jauslin *et al.*, 2002, 2003), and mitochondrial iron accumulation to levels approximately 1.5-fold that observed in age-matched control samples (Delatycki *et al.*, 1999). Fibroblasts, therefore, provide an excellent surrogate target in which to undertake initial phenotype correction studies. Furthermore, the capacity of the human frataxin cDNA to function in a murine context (Pook *et al.*, 2001) provides the opportunity for future evaluation of phenotype correction using the AAV and lentiviral vectors described in frataxin-deficient mouse DRG sensory neurons (Puccio *et al.*, 2001; Simon *et al.*, 2004). Both vectors conferred high levels of frataxin expression, with appropriate subcellular localization, in HeLa cells and primary FRDA patient fibroblasts. Importantly, transduced FRDA fibroblasts exhibited increased resistance to oxidant stress, although not to wild-type levels, and this was accompanied by a trend toward reduced mitochondrial iron levels that did not achieve statistical significance ( $p = 0.058$ ). This partial correction of phenotype has several possible explanations, but is most likely to reflect the inherent molecular pathology of FRDA. For example, the accumulation of mitochondrial iron, previously reported in FRDA fibroblasts (Delatycki *et al.*, 1999), is likely to be a time-dependent process that is not rapidly reversed after transduction. More robust model systems in which the time between transduction and phenotype analysis can be readily extended will be required to definitively resolve this issue. Failure to achieve physiologically appropriate levels of frataxin expression in a proportion of cells is also a plausible possibility. Investigation of this possibility was complicated by the relative insensitivity of the available anti-frataxin antibody, which could readily detect supraphysiological levels of expression but not the physiological levels present in normal control fibroblasts. It was, therefore, not possible on a cell-by-cell basis to definitively establish what proportion of vector-treated FRDA fibroblasts expressed exogenous frataxin, although semiquantitative RT-PCR analysis of frataxin transcript abundance confirmed, on a culture-wide basis, that the mean level of frataxin expression in transduced FRDA fibroblast cultures remained at or above the levels present in control fibroblast cultures at the time phenotype correction studies were performed. A final theoretical possibility is that frataxin overexpression in cultured primary fibroblasts might be toxic.

This latter possibility gains support from Q-PCR analyses that confirmed the presence of multiple copies of both AAV and LV vector genomes per cell at early time points after transduction, but progressive loss of vector genomes over subsequent passages. Although expected after AAV transduction (Russell *et al.*, 1994), this pattern after lentiviral transduction is less readily explained. Frataxin overexpression up to 6-fold above physiological levels has been reported to exert no observable toxicity in transgenic mice (Miranda *et al.*, 2004). In the current *in vitro* study it is possible that even higher levels of overexpression were achieved in a proportion of transduced cells, although the fold increases in frataxin expression achieved could not be quantitated as the available anti-frataxin antibody was insufficiently sensitive to allow detection of physiological levels of frataxin (baseline) in untransduced cells. Even low-level toxicity, insufficient to result in cell death, could confer a relative growth advantage to untransduced cells such that the proportion of cells containing vector genomes would fall over successive passages. Further evidence of frataxin toxicity comes from FRDA fibroblast cultures transduced with the AAV vector and maintained under G418 selection, thereby ensuring maintenance of at least one functional genome per cell. Even in this context cells expressing supraphysiological levels of frataxin, readily detected by antibody staining, disappeared over time.

In summary, we report the construction of AAV and lentiviral vectors encoding the human frataxin cDNA and initial functional characterization in phenotype correction studies using FRDA fibroblasts. The partial correction of phenotype observed functionally validates the vectors described, and raises a series of important questions. These include determining the extent to which phenotype reversal can be achieved in frataxin-deficient DRG sensory neurons, the time course required to achieve optimal reversal, and whether tight physiological control of frataxin expression levels will be required. The capacity of the human frataxin cDNA to rescue frataxin-deficient mice (Pook *et al.*, 2001), and the generation of viable mouse models of FRDA that recapitulate many of the features evident in the human disease (Puccio *et al.*, 2001; Simon *et al.*, 2004), including evidence of pathology in the sensory neurons of the DRG, provide the ideal context in which to further pursue these studies.

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