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Methods

Delivery of the 135 kb human frataxin genomic DNA locus gives rise to different frataxin isoforms

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ABSTRACT

Friedreich's ataxia (FRDA) is the most common form of hereditary ataxia caused by recessive mutations in the *FXN* gene. Recent results have indicated the presence of different frataxin isoforms due to alternative gene expression mechanisms. Our previous studies demonstrated the advantages of using high-capacity herpes simplex virus type 1 (HSV-1) amplicon vectors containing the entire *FXN* genomic locus (iBAC-*FXN*) as a gene-delivery vehicle capable of ensuring physiologically-regulated and long-term persistence. Here we describe how expression from the 135 kb human *FXN* genomic locus produces the three frataxin isoforms both in cultured neuronal cells and also in vivo. Moreover, we also observed the correct expression of these frataxin isoforms in patient-derived cells after delivery of the iBAC-*FXN*. These results lend further support to the potential use of HSV-1 vectors containing entire genomic loci whose expression is mediated by complex transcriptional and posttranscriptional mechanisms for gene therapy applications.

1. Introduction

Friedreich's ataxia (FRDA, [OMIM 229300]), the most common form of hereditary ataxia, is a predominantly neurodegenerative disease affecting certain neurons at the dorsal root ganglia, the spinal cord, the brainstem and the cerebellum. Neurodegeneration occurs first in the dorsal root ganglia, with loss of large proprioceptive neurons, followed by degeneration of posterior columns and corticospinal tracts of the spinal cord and the dentate nucleus in the cerebellum [17]. The onset of the disease usually occurs before adolescence, although in some cases it can start in adulthood or even later [29]. Symptoms include progressive limb and gait ataxia, as well as dysarthria, tendon areflexia, sensory loss, and pyramidal signs. The most important extra-neurological manifestations which may appear are hypertrophic cardiomyopathy, followed by glucose intolerance and diabetes mellitus [29].

From a genetic point of view, FRDA is an autosomal recessive disease caused by mutations in the *FXN* gene [8]. This gene [MIM 606829], located at chromosome 9q21.11, encodes a protein known as frataxin, which is highly conserved and ubiquitous with its highest expression in mitochondria-rich tissues including the nervous system, heart and muscle [19]. In most patients, the mutation responsible for the disease involves the expansion of a trinucleotide GAA repeat located in the first intron of the gene [5] leading to a reduction in the expression of frataxin [4,45].

Frataxin is mainly localised in the mitochondria [19]. The protein is synthesised as a precursor form which is subsequently imported to the mitochondria, where it undergoes consecutive proteolytic cleavages by the mitochondrial processing peptidase (MPP) to produce the mature form [11]. There are different biological functions in which frataxin appears to play an important role including iron homeostasis, iron–sulphur cluster biosynthesis, modulation of oxidative phosphorylation and regulation of the response to oxidative stress, but we still do not know how frataxin deficiency triggers the neurodegenerative process associated with the disease [for a review of frataxin function, see [3,40]].

The frataxin gene is known to generate several transcripts [5,33]. Interestingly, a recent report has highlighted the existence of two novel frataxin isoforms (*FXN* II and *FXN* III) which lack the mitochondrial targeting sequence and may also play a role in the pathogenesis of FRDA [44]. Thus, in addition to the canonical frataxin (*FXN* I) form which is found within mitochondria, *FXN* III, generated by alternative splicing, is localised to cell nuclei and *FXN* II, generated by a different transcription start site, is present in the cytosol [44]. Interestingly the expression of *FXN* II is particularly significant in the nervous tissue including the cerebellum whereas *FXN* III seems more enriched in the heart [44].

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As Friedreich's ataxia is a monogenic disease produced by a shortage of the frataxin protein, gene therapy appears to be a promising alternative treatment [9,20]. A pioneering study in FRDA demonstrated that both adeno-associated viral (AAV) and lentiviral (LV) vectors coding for frataxin cDNA were able to partially rescue the phenotype of fibroblasts derived from FRDA patients [10]. However, total restoration of the normal phenotype was not achieved, and it was suggested that this might be due to the fact that frataxin expression over physiological levels may be toxic [10]. Later, our group demonstrated that a herpes simplex virus type 1 (HSV-1) amplicon vector coding for frataxin cDNA was able to reverse the neurological defects observed in a FRDA mouse model [21]. In this case, the level of frataxin protein expressed by the HSV vector was similar to the physiological levels observed in wild-type neurons [21]. More recently, Perdomini and colleagues reported the prevention and reversal of severe cardiomyopathy by gene therapy using an AAV vector coding for frataxin cDNA in a mouse model with complete frataxin deletion in cardiac and skeletal muscle [30]. This study highlighted the rescue of the cardiomyocyte phenotype even after the onset of cardiac failure, which is extremely encouraging and establishes preclinical proof of concept for the potential of gene therapy to treat FRDA cardiomyopathy. Interestingly, no deleterious effects due to frataxin overexpression were observed. Whether this is due to cell type or species differences remains to be established. In all these studies, the vectors used contained the cDNA coding for the conventional mitochondrial frataxin (*FXN* I), suggesting that this isoform is crucial for both the development and the recovery of the pathology. However the possibility of a significant contribution of the other isoforms to the pathology of FRDA cannot be ruled out.

We previously reported that a 135 kb BAC containing approximately 38 kb of intact promoter region, the 80 kb frataxin gene *locus*, and 17 kb of downstream sequence can be efficiently packaged into HSV-1 particles (iBAC-*FXN*) [13] using a helper virus-free packaging system [36]. This genomic region contains all the regulatory elements of frataxin gene expression identified so far [35]. Furthermore, FRDA patient fibroblasts transduced with this iBAC-*FXN* vector showed a physiological level of frataxin expression and functional restoration of the wild-type cellular phenotype in response to oxidative stress [13]. The same construction was used in an assay to demonstrate the long-term persistence of gene expression after the injection of the iBAC-*FXN* vector into the mouse cerebellum in vivo [12].

In this work, we have used this HSV-1 amplicon vector (iBAC-*FXN*) to deliver the entire *FXN* genomic locus to test for the expression of the different frataxin isoforms both in vitro in cultured cells and in vivo after the intracranial injection of HSV vectors into the adult mouse cerebellum.

2. Materials and methods

2.1. BACs and HSV-1 amplicons used

The fully sequenced BAC RP11-265B8 obtained from the BACPAC Resources Center (http://bacpac.chori.irg), modified as described elsewhere [13] was used. For the transduction experiments, we used pHG-FRDA [13], which comprises the BAC containing the whole frataxin genomic locus supplied with the different elements needed for packaging into herpes viral particles.

2.2. Cell culture

Neuro-2A (N2A) mouse neuroblastoma cells (ATCC CCL131, American Type Culture Collection, Rockville, MD) were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FBS (Invitrogen, Life Technologies, Barcelona, Spain) containing 2 mM L-glutamine and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively), in a humidified incubator at 37 °C in 5% CO_2.

Human olfactory mucosa stem cells (OMSCs) were obtained from biopsies of healthy subjects and from patients with FRDA [26]. Cells were cultured in 2% CSC medium (DMEM/F12 (Invitrogen)) supplemented with 0.5% (w/v) Albumax I (Invitrogen), 0.5% HEPES 1 M (Invitrogen), 0.6% (w/v) glucose (Sigma, Madrid, Spain), 2% foetal bovine serum (Sigma), 1% N2 (Invitrogen), 1% non-essential amino acids, 0.1% penicillin/streptomycin and freshly added 8 ng/ml recombinant human fibroblast growth factor-2 (rhFGF-2, PeproTech, Rocky Hill, NJ, USA) and 50 ng/ml nerve growth factor (NGF, Sigma) at 37 °C in 5% CO_2. The detailed characterisation of human OMSC lines will be described in another paper (manuscript in preparation). Experimentation on human subjects to obtain the olfactory mucosa biopsies were performed according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) after approval by the Ethics Committee and informed consent from all the donors.

2.3. Production and transduction of HSV-1 amplicons

The pHG-FRDA vector (referred to here as the infectious bacterial artificial chromosome, or iBAC-*FXN*, vector) was packaged into HSV-1 particles using an improved HSV-1 helper virus-free system as described previously [36,42]. Typically, the supernatant from three 6-cm dishes was concentrated and re-suspended in 250 ml DMEM supplemented with 10% FCS to give a stock of 1–3 × 10^7 transducing units ml^{-1} when tittered for GFP expression on a confluent G16-9 cell layer. G16-9 cells, a derivative of the human Gli-36 glioma cell line expressing the HSV-1 VP16 protein to enhance expression from the pIE4/5-GFP promoter cassette, were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal calf serum 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and hygromycin-B (200 μg ml^{-1}).

For amplicon infection, 1–2 × 10^6 cells were plated onto a p100 and 24 h later, transduced with the HSV-1 amplicon for an additional 24 h in 8 ml medium. After transduction, the amplicon mixture was removed, and 8 ml of fresh media was added to the cells. The infection was prolonged for an additional 24 h and the cells were then harvested to perform either RT-PCR or western blotting.

2.4. Stereotaxic injection

Eight-week-old male C57BL/6 mice (Charles River breeding Laboratories, Barcelona, Spain) were used in this study. The mice were housed in a temperature-controlled room under a 12-h light/12-h dark cycle. Briefly, the mice were anesthetised with isoflurane and placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) for injection of 3 ml of the vector iBAC-*FXN* (7 × 10^6 transducing units ml^{-1}) into the cerebellum (AP: 6.5, lateral: 2.0; V: 2.0) over 3 min using a 5 ml Hamilton syringe. At the end of the experiment, the skin was sutured and the anaesthesia was discontinued. The mice were kept in individual cages with water and food ad libitum, and observed periodically in the following days.

Mice were anesthetised at 0, 2, and 7 days with CO_2, decapitated and the cerebellum was dissected and collected in RNAse-free polypropylene tubes, immediately frozen in liquid nitrogen and stored at −80 °C until further use. The animal study and protocol were approved by the Institutional Animal Care and Use Committee at the Centro de Biología Molecular and followed European Directive 2010/63/EU. All efforts were made to minimize suffering and the numbers of animals used.

2.5. RT-PCR

RT-PCR analysis of *FXN* expression was performed in the mouse N2A cells, the patient-derived olfactory stem cells, and the mouse cerebellum.

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's protocol, with additional on-column DNAse digestion (Qiagen). The RNA concentration was

determined with a Nanodrop ND-1000 spectrophotometer and its integrity checked by electrophoresis in 1% agarose gel.

First strand cDNA was synthesised from 3 μg of total RNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. For each sample we perform a cDNA synthesis with and without retrotranscriptase to ensure non-genomic DNA was being amplified.

Isoform amplifications were performed in a 25-μl PCR using 1 μl of the resulting cDNA using the same primers as described by Xia and colleagues [44], except for the amplification of isoform III, for which a specific forward primer was designed spanning exon1AΔ141/exon 2 junction (5′-GGCGCCGCGCAGTTCGAACC-3′). Fig. 1A is a schematic representation of the three frataxin isoforms showing the localisation of the primers used. As internal controls we used, GAPDH-Fw (5′-TGAAGGTC GGAGTCAACGGATTTGGT-3′) and GAPDH-Rv (5′-CATGTGGGCCATGA GGTCCACCAC) to amplify a fragment of 900 bp of murine GAPDH or βActin-Fw (5′-CCACACTGTGCCCATCTACGAGGGGT-3′) and βActin-Rv (5′-AGGGCAGTGATCTCCTTCTGCATCCT-3′) to amplify a fragment of 479 bp of human actin. All the pictures presented are representatives of three independent experiments.

2.6. Cell lysis, subcellular fractionation and western blotting

The cells were washed twice with phosphate-buffered saline (PBS), harvested, placed on ice and then homogenised in a buffer containing 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) [pH 7.4], 100 mM sodium chloride (NaCl), 100 mM sodium fluoride (NaF), 1% Triton X-100, 1 Mm sodium orthovanadate (Na_3VO_4), 5 mM ethylenediaminetetraacetic acid and the COMPLETE™ protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain). The soluble fraction

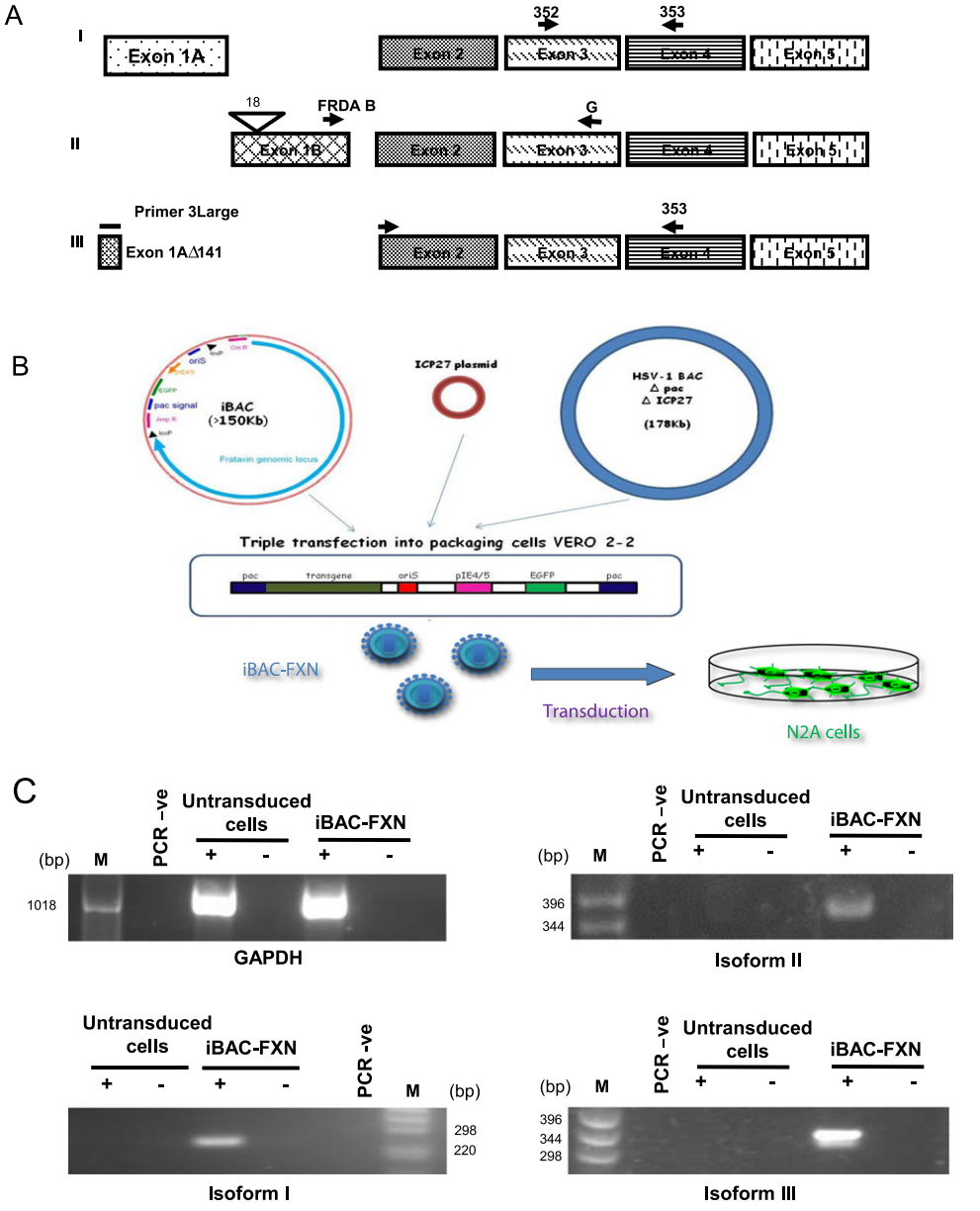

Fig. 1. Delivery of the frataxin gene to mouse neuroblastoma cells in vitro. Mouse neuro-2A (N2A) cells were transduced with a HSV-1 amplicon harbouring the complete frataxin genomic locus (iBAC-*FXN*) and RNA was prepared for the analysis of *FXN* isoform expression. (A) Schematic representation of the different frataxin isoforms showing the localisation of the primers used for their amplification. (B) Diagram showing the *FXN*-containing BAC used and its packaging into herpes viral particles using the helper-free method described elsewhere. (C) RT-PCR with specific primers for human frataxin showing the expression of all frataxin isoforms in N2A cells transduced with iBAC-*FXN*. M: 1 kb ladder (Invitrogen); PCR −ve: negative control for PCR reactions; (+): cDNA used as template for PCR amplified with RT enzyme; (−): cDNA used as template for PCR amplified without RT enzyme. Numbers to the right indicate the size (in base pairs) of marker bands.

was obtained by centrifugation at 16,000 *g* for 10 min at 4 °C. Subsequently, samples were mixed with electrophoresis buffer containing sodium dodecyl sulphate (SDS), boiled for 5 min and separated by gel electrophoresis in the presence of SDS on 8–15% acrylamide–bisacrylamide gels. The proteins were then electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) following standard procedures, and the membranes were subsequently blocked with 10% non-fat dried milk in PBS plus 0.2% Tween-20 (PBST). The blocked membranes were incubated overnight with primary antibodies diluted in blocking solution at 4 °C and the filters were then rinsed in PBST at least three times. After incubation with the corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature, antibody binding was visualized using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, United Kingdom).

Cytosolic, mitochondrial and nuclear fractions were obtained using the Cell Fractionation Kit (MS861, MitoSciences, Abcam, Cambridge, UK) following the manufacturer's recommendations. Briefly, control cells and patients' cells were grown to confluence in a 100 mm tissue culture plate and then trypsinized and collected by centrifugation. After being washed with Buffer A, the cells were resuspended to a concentration of 6.6×10^6 cells ml^{-1} and cellular extractions obtained after two sequential detergent-extraction steps. The purity of the fractions was assessed by western blotting against Lamin B1 (nuclear), β-tubulin (cytoplasmic) and COX-V (mitochondrial) proteins. All the western-blotting showed are representative of at least three different experiments.

The following antibodies were used for western blotting: a polyclonal antisera against human frataxin (R6.3s, 1:1000) raised against the peptide TLGHPGSLDETTYERLAEETLC (Protein Tools, Madrid, Spain), previously described by Palomo and colleagues [28], polyclonal antibodies against Lamin B1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-OxPhos complex V subunit β (1:1000; Molecular Probes, Eugene, OR, USA), and monoclonal antibodies against β-tubulin (1:1000; Sigma).

3. Results

In this work, we have checked whether a BAC containing the whole frataxin genomic locus is able to produce the three frataxin isoforms which have been recently described [44]. We first assessed the expression of these isoforms in the mouse neuroblastoma N2A cell line as target cells. The iBAC-*FXN* used in this study (derived from the RP11-265B8 BAC clone) contains the 80 kb frataxin genomic locus plus 38 kb upstream and 17 kb downstream and is supplied with the elements needed to be packaged into herpes viral particles [13] (Fig. 1B). For the packaging of the HSV-1 virions, we used a helper-free packaging system consisting in triple transfection into Vero 2-2 packaging cells [36]. Our group has already demonstrated that HSV-1 amplicons expressing whole genomic loci provide sustained expression of the transgene either in vitro or in vivo [12,13], but we wished to test whether this construct contained all the regulatory elements needed to produce the different frataxin isoforms. For this experiment, cells were transduced with HSV-1 amplicons containing iBAC-*FXN*. Forty-eight hours post-transduction, we performed reverse transcription-polymerase chain reaction (RT-PCR) to detect amplification of the frataxin isoforms. As shown in Fig. 1C, all three frataxin isoforms were amplified in N2A cells upon transduction with the whole genomic locus, showing for the first time that it is possible to express all frataxin isoforms using the iBAC-*FXN* genomic locus vector.

Frataxin isoforms mainly differ in the first exon, being identical from exons 2 to 5. For the amplification of all of them we used the same primers already described [44], with the exception of isoform III (see Section 2.5.). Fig. 1A shows a schematic representation of three frataxin isoform and the localisation of the primers used for their amplification (Supplementary Fig. 1 shows a schematic representation of frataxin isoforms whereas the corresponding sequence alignments are shown in Supplementary Figs. 2 and 3). As the primers for amplifying the three isoforms are human-specific, no amplification of endogenous mouse frataxin isoforms was observed in N2A neuroblastoma cells.

We next checked whether the iBAC-*FXN* vector was also able to produce all frataxin isoforms in vivo. For this purpose, we used stereotaxic injection into the cerebellum of adult C57BL/6 mice to deliver the iBAC-*FXN*. As frataxin expression reaches its highest levels between 24 and 48 h post-injection [12], the mice were killed at day 2 and day 7, using a mouse that was killed just after injection as control (0 day). Expression of the three isoforms was detected by RT-PCR as soon as 2 days after injection, and expression persisted up to 7 days, the last day of our experiment (Fig. 2). Expression for isoforms I and III was observed to be qualitatively higher at day 7 than at day 2, providing evidence of increased expression over time, although for isoform II the increase was not so clear.

Our laboratory has previously isolated human olfactory mucosa stem cells (OMSCs) from FRDA patients and healthy donors [26]. Using these OMS cells, we next investigated the amount of all frataxin isoforms expressed by these cells in both control (C3c) and FRDA patient cells. Human FRDA patient cells expressed low levels of both FXN mRNA and protein, so we decided to characterise isoform gene expression in OMS cells from two different FRDA patients (AF1 and AF6) and compare them with their expression in OMS cells from a healthy donor (C3c). The analysis of frataxin isoform mRNA revealed the presence of isoforms I, II and III both in control and patient-derived cells (Fig. 3A), with isoform II almost undetectable in AF6 cells (Fig. 3A). As PCR data are not quantitative, we also examined the expression of frataxin protein isoforms by western blotting after performing cellular fractionation to obtain isolated cytosolic, mitochondrial, and nuclear fractions (Fig. 3B). We observed that C3c cells presented all frataxin isoforms in the different compartments whereas AF1 and AF6 contained significantly reduced levels of all them. The predominantly expressed frataxin isoform was the FXN I-derived mature form in the mitochondrial fraction. The level of this mitochondrial frataxin was much higher in control C3c cells than in FRDA patient-derived AF1 and AF6 cells. Curiously AF6 cells expressed much lower levels of FXN I-derived precursor protein at the cytosol than either C3c or AF1 cells. A low level of mature frataxin (which is presumably derived from FXN II mRNA) was detected in the cytosol of control cells. Cytosolic mature frataxin was hardly detectable in FRDA-derived patient cells. Nuclear frataxin (which presumably comes from FXN III mRNA) consists of several bands of an apparent molecular mass larger than that corresponding to mature mitochondrial frataxin. The occurrence of several bands probably reflects the presence of degradation products which may arise from iron-mediated self-cleavage as previously suggested [44]. Again nuclear frataxin was hardly detectable in FRDA-derived patient cells.

Finally, we transduced OMS cells with the frataxin genomic construct in order to test whether the BAC was able to reconstitute frataxin levels in FRDA patient cells. OMS cells from a FRDA patient (AF6) were harvested 48 h post-transduction with iBAC-*FXN* and tested for mRNA and protein level expression, using non-transduced OMS cells from AF6 as control. Frataxin-deficient OMS cells from patient AF6 had increased mRNA expression of all frataxin isoforms compared with non-transduced control cells after two days of transduction with the iBAC-*FXN* (Fig. 4A). As for the sub-cellular localisation of the isoforms, western blot analysis of the compartment lysates showed that there was a clear increase in frataxin isoforms in the mitochondrial and nuclear fractions (Fig. 4B). There is only a very faint signal corresponding to mature frataxin in the cytosol which may reflects the low level of expression of *FXN* II in these cells.

Overall, these results provide proof of principle that the iBAC-*FXN* vector contains all the regulatory elements needed for proper FXN gene expression including correct alternative splicing (for frataxin isoform III) and promoter usage (for frataxin isoform II).

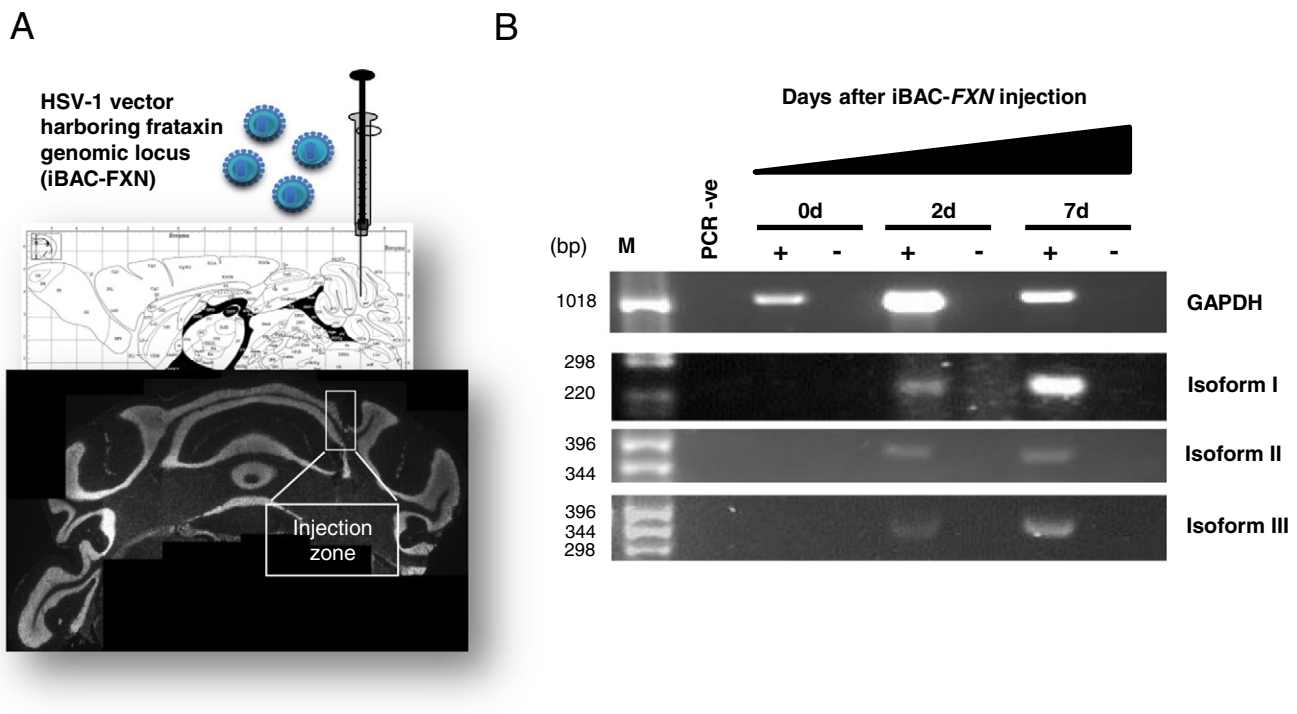

Fig. 2. Delivery of the frataxin gene into mouse cerebellum in vivo. A herpes viral amplicon harbouring the complete frataxin genomic *locus* (iBAC-*FXN*) was injected into the mouse cerebellar cortex and RNA was prepared for the analysis of *FXN* isoform expression. (A) Diagram showing the stereotaxic injection of the iBAC-*FXN* into the adult mouse cerebellum. (B) RT-PCR detection of frataxin isoforms at different times after injection. M: 1 kb ladder (Invitrogen); PCR −ve: negative control for PCR reactions; (+): cDNA used as template for PCR amplified with RT enzyme; (−): cDNA used as template for PCR amplified without RT enzyme. Numbers to the right indicate the size (in base pairs) of marker bands.

4. Discussion

The correct expression of distinct frataxin isoforms may be important for the therapy of FRDA. There are still many uncertainties in our understanding of the pathophysiology of FRDA [23], which may be crucial for the design of an effective gene therapy approach. Little is known about the regulation of frataxin gene expression in response to distinct physiological and pathological conditions. The threshold level of frataxin which causes the pathology is not well established. It is still an open question whether the relevant level of frataxin is not that found in the basal state but rather in states in which frataxin is up-regulated in response to some stimuli. Furthermore, it is not clear why frataxin deficiency affects selected cell types far more than others [23]. The selectivity of expression of the novel frataxin isoforms [44] may possibly contribute to the cellular selectivity of FRDA. Furthermore, previous studies have shown the occurrence of a cytosolic pool of frataxin [1,6,7,22] which may correspond to the novel *FXN* II isoform [44]. Of interest in this regard is the demonstration of a role for extra-mitochondrial frataxin in the promotion of cell survival [7]. Whether the expression of extra-mitochondrial *FXN* II in the cerebellum is required for correct neuronal survival and function is still an open question.

Thus the use of the cDNA coding for the conventional mitochondrial frataxin (*FXN* I) under the control of a viral promoter may not be the optimal approach for the gene therapy of FRDA despite the promising results obtained so far [21,30]. Moreover there is a serious concern about the danger of frataxin overexpression, since this has been shown to be quite harmful in some experimental model organisms

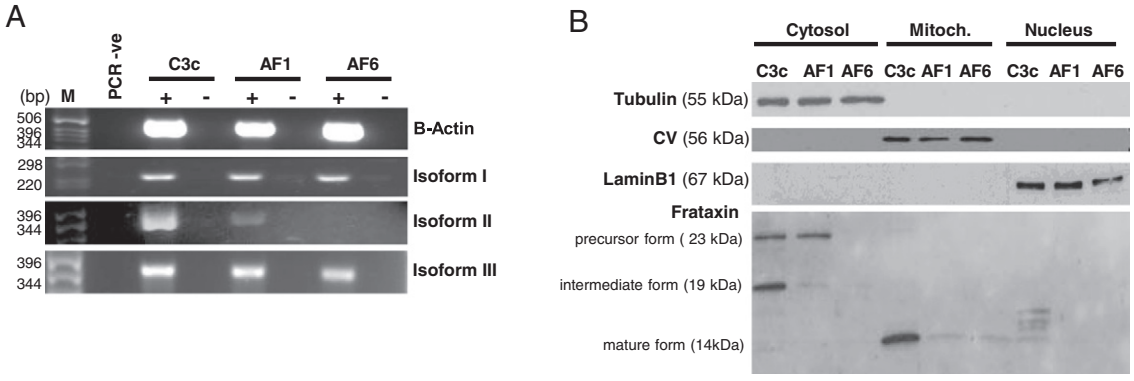

Fig. 3. Presence of frataxin isoforms in human OMS cells from healthy subjects and FRDA patients. A) Human olfactory mucosa stem cells obtained from healthy subjects and FRDA patients were cultured and RNA was isolated to test for *FXN* isoform expression. RT-PCR analysis demonstrated the expression of three different frataxin isoforms in these OMSCs. M: 1 kb ladder (Invitrogen); PCR −ve: negative control for PCR reactions; (+): cDNA used as template for PCR amplified with RT enzyme; (−): cDNA used as template for PCR amplified without RT enzyme. Numbers to the right indicate the size (in base pairs) of marker bands. B) Human olfactory mucosa stem cells obtained from healthy subjects and FRDA patients were cultured and then subjected to cell fractionation to test for the subcellular distribution of *FXN* isoforms. Western blot revealed the subcellular localisation of the three different frataxin isoforms.

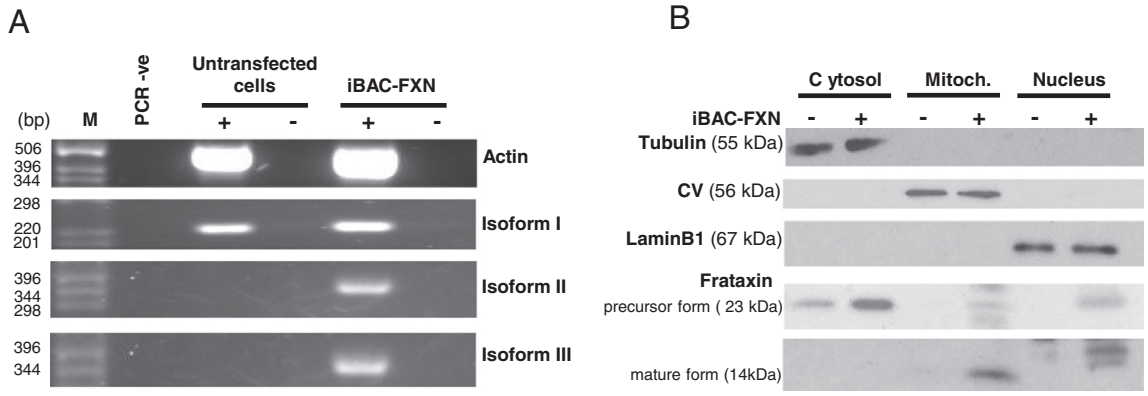

Fig. 4. Recovery of frataxin isoforms in human olfactory mucosa stem cells after transduction with the frataxin-containing BAC. Human olfactory mucosa stem cells obtained from FRDA patients were cultured and transduced with a herpes viral amplicon harbouring the complete frataxin genomic *locus* (iBAC-*FXN*). They were then subjected to either RNA isolation or subcellular fractionation. A) RT-PCR demonstrated the expression of the different frataxin isoforms in cells before (UT) and 48 h after transduction (iBAC-*FXN*). M: 1 kb ladder (Invitrogen); PCR −ve: negative control for PCR reactions; (+): cDNA used as template for PCR amplified with RT enzyme; (−): cDNA used as template for PCR amplified without RT enzyme. Numbers to the right indicate the size (in base pairs) of marker bands. B) Western blot showing the subcellular localization of the three different frataxin isoforms. Lanes 1, 3 and 5: untransduced cells, lanes 2, 4 and 6: iBAC-*FXN*-transduced cells.

[25,43]. It thus seems that an optimal gene therapy approach for FRDA should guarantee physiologically-regulated expression of the frataxin gene.

Indeed, the use of large DNA molecules containing whole genomic loci has recently received attention as an emerging alternative to the use of cDNAs within viral vectors for gene therapy [18,31]. Studies performed in transgenic mice have demonstrated the benefits of using large fragments of genomic DNA, which allow the delivery of intact mammalian genes with all their regulatory elements. In this way, a level and control of expression comparable to the endogenous expression of the gene can be achieved. In this regard, the experience gained from using yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs) has shown that large genomic fragments drive tissue specific expression at physiological levels [34,37–39]. Specifically, the use of a YAC or BAC carrying the entire *frataxin* gene *locus* has rescued frataxin *knock-out* mice from embryonic lethality [34,37,38].

Amplicon vectors based on *herpes simplex* virus 1 (HSV-1) are one of the most promising alternatives for the delivery of large DNA molecules in gene therapy protocols as they can accommodate DNA molecules as big as 150 kb [14,31]. This makes them ideal vehicles for delivering entire genomic *loci* with all their regulatory elements [41,42]. Interestingly, HSV vectors bearing genomic *loci* also exhibit the potential to drive the production of multiple transcripts arising from transcriptional and posttranscriptional mechanisms [15,32]. This may be particularly relevant for genes to be expressed in neuronal tissues, particularly in the cerebellum which is characterised by a predominant use of alternative transcription and splicing to generate transcriptome diversity [24,27]. Additional advantages of using HSV-1 amplicons as gene delivery vehicles include their ability to transduce both dividing and non-dividing cells, and their ability to persist as non-integrating episomes, thus eliminating the risk of insertional mutagenesis in vivo [16]. Furthermore, HSV-1 amplicon vectors are particularly well suited for gene therapy of neurological disorders due to their outstanding ability to deliver genes into neurons [2,16].

In the present study, we have demonstrated that the iBAC-*FXN* vector is capable of producing all the different frataxin isoforms which have recently been described [44] both in vitro in cultured cells and in vivo after its injection into the mouse cerebellum. We have also observed the correct expression of frataxin isoforms in patient-derived cells after the delivery of the 135 kb human *FXN* genomic locus. This vector is therefore a promising candidate for gene therapy as it likely contains all the regulatory elements needed for physiologically-controlled gene expression.

5. Conclusions

Our results are evidence for the great potential of the iBAC system for gene therapy involving the delivery of genomic loci whose expression is mediated by complex splicing and promoter usage, which may be particularly relevant for transcripts expressed in the cerebellum. Thus, the use of a gene locus which produces all frataxin isoforms may be crucial for full restoration of frataxin expression and the recovery of neuronal function in selected neuronal cell types.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

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