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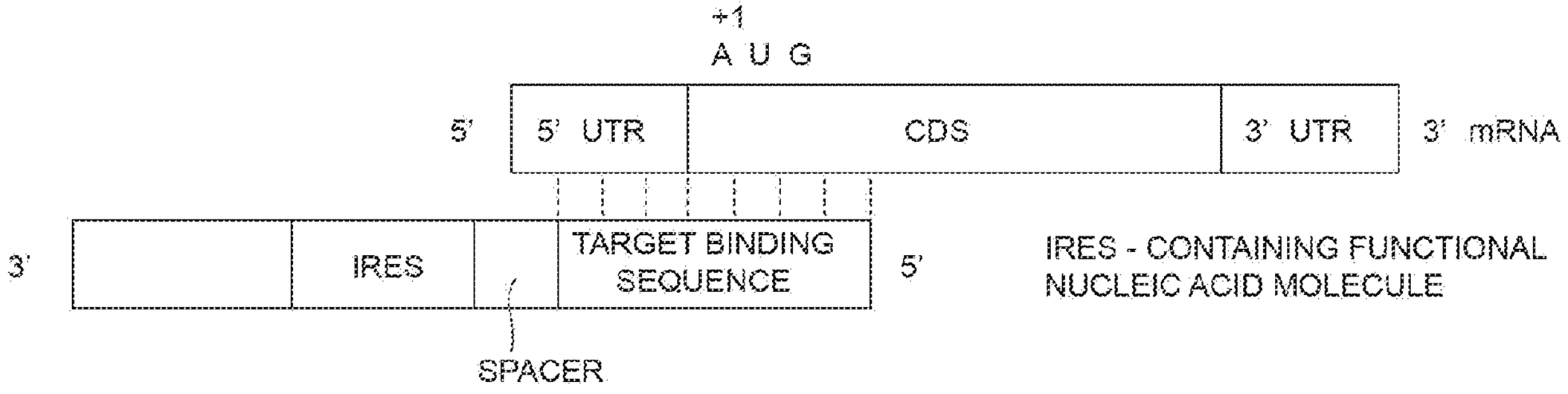

FIG. 1

(57) Abrégé/Abstract:
There is disclosed a trans-acting functional nucleic acid molecule comprising a eukaryotic target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced, and a regulatory sequence comprising an internal ribosome entry site (IRES) sequence or an internal ribosome entry site (IRES) derived sequence and enhancing translation of the target mRNA sequence, wherein the regulatory sequence is located 3' of the target binding sequence.

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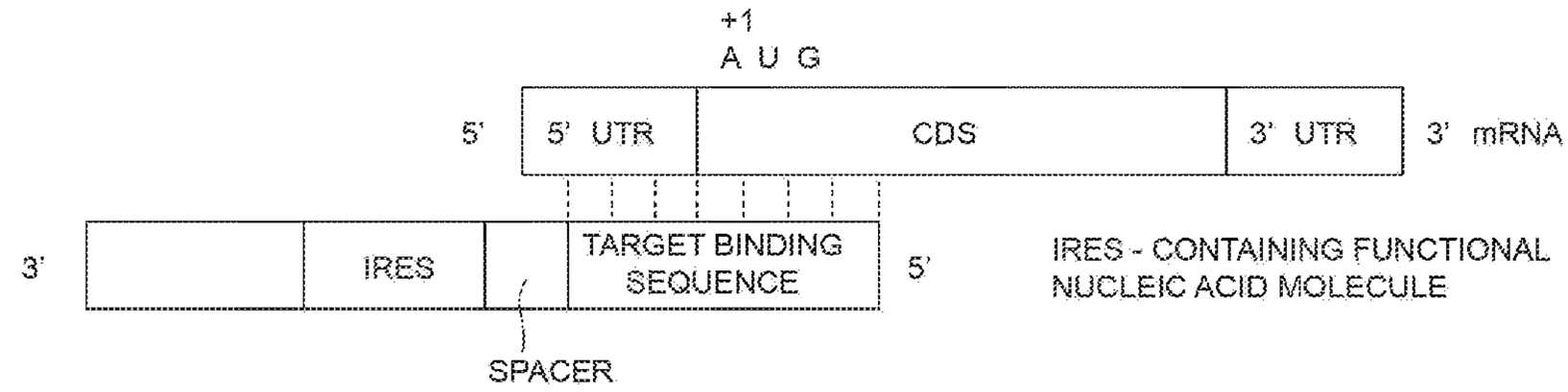

FIG. 1

(57) Abstract: There is disclosed a trans-acting functional nucleic acid molecule comprising a eukaryotic target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced, and a regulatory sequence comprising an internal ribosome entry site (IRES) sequence or an internal ribosome entry site (IRES) derived sequence and enhancing translation of the target mRNA sequence, wherein the regulatory sequence is located 3' of the target binding sequence.

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"FUNCTIONAL NUCLEIC ACID MOLECULE AND USE THEREOF"

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from Italian Patent Application No. 102017000015372 filed on 20/09/2017, the disclosure of which is incorporated by reference.

Technical Field of the Invention

The present invention relates to trans-acting functional nucleic acid molecules having the function of enhancing protein translation of specific target mRNAs in eukaryotes, to DNA molecules encoding such molecules, to uses of such molecules and to methods for enhancing protein translation.

Prior Art

In eukaryotes, mRNAs are primarily translated through a cap-dependent mechanism whereby initiation factors recruit the 40S ribosomal subunit to a cap structure at the 5' end of the mRNA. However, some viral and cellular messages initiate protein synthesis without a cap (Thompson SR, Trends Microbiol 2012; Jackson RJ, Cold Spring Harb Perspect Biol. 2013). In these cases, a structured RNA element termed Internal Ribosome Entry Site (IRES) recruits the 40S ribosomal subunit. IRESs were discovered over 20 years ago in Picornaviruses. In cells, IRES sequences promote cap-independent translation of a subset of protein coding mRNAs

to overcome the general inhibition of cap-dependent translation that occurs under stress conditions. IRES sequences are generally found in the 5′ untranslated region of cellular mRNAs coding for stress-response genes, thus stimulating their translation in *cis*.

Recent high-throughput screening systems have expanded the list of validated IRES sequences within cellular mRNAs (Weingarten-Gabbay S, et al., Science, 2016).

Gene-specific translation up-regulation can be achieved by the modification of protein coding mRNAs to include a modified 5′ sequence that contains internal ribosome entry (IRES) sequences or translation enhancer sequences. In such systems, IRES or translation enhancer sequences are placed in *cis* at the 5′ of cDNAs encoding for the specific gene of interest. This method has been applied for the construction of vectors to express two cistrons and for enhancing translation of overexpressed genes. However, *cis*-regulation of translation enhancement cannot be used when the goal is to induce translation up-regulation of endogenously expressed mRNAs. There is therefore the need to identify *trans*-regulatory elements that promote gene-specific translation up-regulation and act on endogenous mRNAs. There is also the need for translation up-regulation *trans*-regulatory elements that act as independent RNA domains.

Manipulating gene expression *in vivo* using nucleic acid

molecules has been of great interest in recent years for potential applications in clinics. Most efforts have focused so far on the ability to down-regulate toxic proteins, using siRNA, miRNA and antisense oligonucleotides. However, a large number of diseases are caused by reduced gene dosage, thus requiring an increase in protein product. While a number of studies have approached the problem at the transcriptional level, only one example exists that uses functional antisense RNA molecules (SINEUPs) to increase translation (Carrieri C., et al., Nature, 2012). SINEUPs are antisense long non-coding RNAs that are able to promote translation of partially overlapping protein-coding mRNAs with no effect on mRNA levels. SINEUP activity depends on two functional domains: the overlapping region, or "Binding Doman", dictates SINEUP specificity, while the embedded inverted SINEB2 element acts as "Effector Domain" and controls enhancement of mRNA translation (Zucchelli S., et al., Front Cell Neurosci 2015; Zucchelli S., et al, RNA Biol, 2015). By taking advantage of their modular structure, synthetic SINEUPs can be designed to specifically enhance translation of virtually any target gene of interest (Zucchelli S., et al., Front Cell Neurosci 2015; Zucchelli S., et al, RNA Biol, 2015; Indrieri A., et al., Scientific Reports, 2016; Gustincich S., et al., Prog Neurobiol, 2016; Zucchelli S., et al., Comput Struct Biotechnol J, 2016).

EP2691522 discloses functional nucleic acid molecules including SINEUPs.

Despite their potentials, SINEUPs rely on the translation enhancer activity of the embedded SINE element, a sequence derived from the mouse genome and with the potential to retrotranspose (move from one genomic location to another) in recipient cells. This would be detrimental for any therapeutical use which involves translation up-regulation for the correction of insufficient gene dosage. There is therefore a need for *trans*-regulatory elements that promote gene-specific translation up-regulation and act on endogenous mRNAs that are not derived from mouse sequences. There is also a need for *trans*-regulatory elements that promote gene-specific translation up-regulation and are not derived from transposable elements.

Most functional nucleic acid molecules of EP2691522 have rather long lengths. There is a need for the identification of shorter *trans*-regulatory elements that promote gene-specific translation up-regulation and act on endogenous mRNA, in order to render the delivery of the RNA molecules to the recipient cells more efficient.

The translation enhancement effect of the functional nucleic acid molecules of EP2691522 is typically 1.5-2.0 fold depending on the cell type. This level of protein increase may be insufficient if the goal is to induce

translation up-regulation in human for the correction of insufficient gene dosage. There is therefore the need to identify *trans*-regulatory elements that promote higher levels of gene-specific translation up-regulation and act on endogenous mRNAs.

Summary of the Invention

It is therefore an object of the present invention to provide a functional nucleic acid molecule that overcomes the above mentioned issues and, possibly, also has an enhanced function.

This object is achieved by means of the *trans*-acting functional nucleic acid molecule as defined in claim 1.

Other objects of the present invention are to provide a DNA molecule as defined in claim 10, an expression vector as defined in claim 11, a method for enhancing protein translation as defined in claim 12, a composition as defined in claim 13 and uses of the trans-acting functional nucleic acid molecule as defined in claims 14 and 15.

Brief Description of the Drawings

Figure 1 shows a schematic diagram of the *trans*-acting functional nucleic acid molecule according to the present invention.

Figure 2A shows a schematic diagram of a functional nucleic acid molecule according to the prior art (SINEUP).

Figure 2B shows the results of a Western blot carried

out on lysates of human embryonic kidney 293T/17 cells (hereinafter also referred to as HEK 293T/17 cells) transfected respectively with empty control plasmid (-), full-length SINEUP-DJ-1 (FL) and its deletion mutants (ΔED = mutant with deleted effector domain, ΔBD = mutant with deleted binding domain).

Figure 2C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and SINEUP RNA (bottom panel) carried out on samples as in Figure 2B.

Figure 2D shows a graphical representation of full-length SINEUP-DJ-1, ΔED and ΔBD translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=5). $p<0.05$

Figure 3 shows a schematic diagram of a generic *trans*-acting functional nucleic acid molecule according to the present invention (IRUP) targeting DJ-1 mRNA.

Figure 4 shows a schematic diagram of the experimental procedure for testing translation up-regulation activity of IRES-containing functional antisense nucleic acid molecules.

Figure 5A shows a schematic diagram of a functional nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including an HCV IRES.

Figure 5B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP

including an HCV IRES in direct (HCV(d)) and inverted orientation (HCV(i)).

Figure 5C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 5B.

Figure 6A shows a schematic diagram of a functional nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including a Poliovirus IRES.

Figure 6B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP including a Poliovirus IRES in direct (Polio(d)) and inverted orientation (Polio(i)).

Figure 6C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 6B.

Figure 7A shows a schematic diagram of a functional nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including an encephalomyocarditis virus (EMCV) IRES and a cricket paralysis virus (CrPV) IRES.

Figure 7B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP

including an EMCV IRES in direct (EMCV(d)) and inverted orientation (EMCV(i)), and a CrPV IRES in direct (CrPV(d)) and inverted orientation (CrPV(i)).

Figure 7C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 7B.

Figures 8A and 8B show graphs summarising the increase in quantities of DJ-1 protein in HEK 293T/17 cells transfected with HCV(d) and HCV(i) IRUPs, Polio(d) and Polio(i) IRUPs and EMCV(d), EMCV(i), CrPV(d) and CrPV(i) IRUPs of the present invention relative to empty vector transfected 293T/17 cells.

Figure 9A shows a schematic diagram of a functional nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including a human Apoptotic Peptidase Activating Factor 1 (Apaf-1) mRNA IRES.

Figure 9B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP including an Apaf-1 IRES in direct (Apaf-1(d)) and inverted orientation (Apaf-1(i)).

Figure 9C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 9B.

Figure 10A shows a schematic diagram of a functional

nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including a human Enhanced Level of Genomic instability 1 (ELG-1) mRNA IRES.

Figure 10B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP including an ELG-1 IRES in direct (ELG-1(d)) and inverted orientation (ELG-1(i)).

Figure 10C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 10B.

Figure 11A shows a schematic diagram of a functional nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including a human V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (cMYC) mRNA IRES.

Figure 11B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP including an cMYC IRES in direct (cMYC(d)) and inverted orientation (cMYC(i)).

Figure 11C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 11B.

Figure 12A shows a schematic diagram of a functional

nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including a shorter version of the human V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (cMYC) mRNA IRES of Figures 11A to 11C.

Figure 12B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP including an cMYC IRES (short variant) in direct (cMYC(d)) and inverted orientation (cMYC(i)).

Figure 12C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 12B.

Figure 13A shows a schematic diagram of a functional nucleic acid molecule according to the present invention (IRUP) targeting the DJ-1 gene and including a human Dystrophin (DMD) mRNA IRES.

Figure 13B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1, and an IRUP including an DMD IRES in direct (DMD(d)) and inverted orientation (DMD(i)).

Figure 13C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 13B.

Figures 14A and 14B show graphs summarising the increase

in quantities of DJ-1 protein in HEK 293T/17 cells transfected with Apaf-1(d) and Apaf-1(i) IRUPs, ELG-1(d) and ELG-1(i) IRUPs, cMYC(d) short version and cMYC(i) short version IRUPs, DMD(d) and DMD(i) IRUPs, and cMYC(d) long version and cMYC(i) long version IRUPs of the present invention relative to empty vector transfected 293T/17 cells.

Figure 15A-15D show the results of Western blots carried out on lysates of human hepatocellular carcinoma (HepG2) cells transfected respectively with empty control plasmid, SINEUP-DJ-1, an IRUP including an HCV IRES sequence in direct (d) or inverted (i) orientation, a Polio and cMYC IRES sequence in direct (d) or inverted (i) orientation, an Apaf-1 and ELG-1 IRES sequence in direct (d) or inverted (i) orientation, and a DMD IRES sequence in direct (d) or inverted (i) orientation.

Figures 16A and 16B show graphs summarising the increase in quantities of DJ-1 protein in HepG2 cells transfected with HCV(d) and HCV(i) IRUPs, Polio(d) and Polio(i) IRUPs, cMYC(d) short version and cMYC(i) short version IRUPs, Apaf-1(d) and Apaf-1(i) IRUPs, ELG-1(d) and ELG-1(i) IRUPs, and DMD(d) and DMD(i) IRUPs of the present invention relative to empty vector transfected HepG2 cells.

Figure 17A shows a schematic diagram of a miniaturised version of functional nucleic acid molecules according to

the present invention (miniIRUP) targeting the DJ-1 gene.

Figure 17B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1 and miniIRUPs including an HCV IRES in direct orientation (HCV(d)), a Poliovirus IRES in direct (Polio(d)) and inverted orientation (Polio(i)) and a cMYC short version IRES in direct orientation (cMYC short(d)).

Figure 17C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 17B.

Figure 17D shows a graphical representation of SINEUP-DJ-1 and mini-IRUP translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=6).

Figure 18A shows a schematic diagram of a miniaturised version of functional nucleic acid molecules according to the present invention (miniIRUP) targeting the DJ-1 gene. With respect to the miniIRUPs of Figures 17A-17D, the IRES-containing functional nucleic acid sequences are cloned under the control of a different promoter.

Figure 18B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1 and miniIRUPs including an HCV IRES in direct orientation (HCV(d)), a Poliovirus IRES in direct (Polio(d)) and inverted

orientation (Polio(i)) and a cMYC short version IRES in direct orientation.

Figure 18C shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 18B.

Figure 18D shows a graphical representation of SINEUP-DJ-1 and mini-IRUP translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=6).

Figure 19A shows a schematic diagram of pDUAL-GFP plasmids for the concomitant expression of functional nucleic acid molecule and genes of interest in mammalian cells.

Figure 19B shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINE-containing (SINE) or IRES-containing (IRES) mini-functional nucleic acid molecules shown in Figure 19A.

Figure 19C shows the results of a qRT-PCR to quantify expression of overexpressed GFP mRNA in HEK 293T/17 cells transfected with the constructs of Figure 19A.

Figure 19D shows the results of a qRT-PCR to quantify expression of functional nucleic acid SINE or IRES RNA.

Figure 20A shows a schematic diagram of pDUAL-GFP plasmids with GFP targeting functional nucleic acid molecules in which the Effector Domain is represented by the

SINE or HCV IRES sequence.

Figure 20B shows the results of a Western blot carried out on lysates of human neuroblastoma Neuro2a cells transfected respectively with empty control plasmid, SINE-containing (SINE) or IRES-containing (IRES) mini-functional nucleic acid molecules shown in Figure 20A.

Figure 21 shows a schematic representation of HCV IRES RNA secondary structure.

Figure 22A shows the results of a Western blot carried out on lysates of HEK 293T/17 cells transfected respectively with empty control plasmid, SINEUP-DJ-1 and IRUPs including a WT HCV IRES and the M2 and M5 mutated HCV IRESs.

Figure 22B shows the results of a qRT-PCR to quantify expression of endogenous DJ-1 mRNA (top panel) and IRUP RNA (bottom panel) carried out on samples as in Figure 22A.

Figure 22C shows a graphical representation of SINEUP-DJ-1 and IRUP translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=7).

Figure 23A shows a schematic representation of how the sequence at the 5′ of an IRES sequence and within an IRES-containing cellular mRNA can be considered as Binding Domain.

Figure 23B shows functional nucleic acid sequence elements within cMYC mRNA reference sequence (NM_002467). The Binding Domain (black), IRES sequence (dark grey), the coding sequence or CDS (light grey) and 3′ untranslated

region (white) are indicated.

Figure 23C shows the results of the bioinformatics analysis (BLAST) using the c-MYC mRNA as query sequence to identify partially overlapping, in antisense orientation, target mRNA protein-coding sequences.

Figure 24A shows a schematic representation of a mammalian expression plasmid encoding for full-length mRNA of MYC (cMYC-FL).

Figure 24B shows Western blots carried out on lysates of mammalian SAOS cells with anti-JAG2, anti-DYRK2, anti-LYS, anti-UBE3A, anti-NRF1 antibodies.

Figures 24C and 24D show results of qRT-PCR to quantify expression of endogenous JAG2, DYRK2, LIS1, UBE3A, NRF1 and cMYC mRNAs.

Figure 24E shows average cMyc full-length mRNA levels.

Figure 25A shows a schematic representation of a mammalian expression plasmid encoding for full-length mRNA of MYC (cMYC-FL) or variants that lack cMYC DNA binding domain (deltaC) and are comprised of the 5'UTR only (5'UTR) or of the IRES only (IRES) domains.

Figure 25B shows Western blots carried out on lysates of mammalian SAOS cells with anti-JAG2, anti-DYRK2, anti-LYS, anti-UBE3A, anti-NRF1 antibodies.

Figure 25C shows results of qRT-PCR to quantify expression of endogenous JAG2, DYRK2, LIS1, UBE3A, NRF1 and

cMYC mRNAs.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although many methods and materials similar or equivalent to those described herein may be used in the practice or testing of the present invention, preferred methods and materials are described below. Unless mentioned otherwise, the techniques described herein for use with the invention are standard methodologies well known to persons of ordinary skill in the art.

By the term "internal ribosome entry site (IRES) derived sequence" there is intended a sequence of nucleic acid with a homology to an internal ribosome entry site (IRES) sequence such as to retain the functional activity thereof, i.e. a translation enhancing activity. In particular, the internal ribosome entry site (IRES) derived sequence can be obtained from a naturally occurring IRES sequence by genetic engineering or chemical modification, e.g. by isolating a specific sequence of the IRES sequence which remains functional, or mutating/deleting/introducing one or more nucleotides in the IRES sequence, or replacing one or more nucleotides in the IRES sequence with structurally modified

nucleotides or analogs. More in particular, the skilled in the art would know that an internal ribosome entry site (IRES) derived sequence is a nucleotide sequence capable of promoting translation of a second cistron in a bicistronic construct. Typically, a dual luciferase (Firefly luciferase, Renilla Luciferase) encoding plasmid is used for experimental tests. A large-scale screening based on a dual reporter or bicistronic plasmid has been recently employed to survey sequences from the human genome for their ability to act as IRES (Weingarten-Gabbay S, et al., Science. 2016, 351:6270). A major database exists, namely IRESite, for the annotation of nucleotide sequences that have been experimentally validated as IRES, using dual reporter or bicistronic assays (http://iresite.org/IRESite_web.php). Within the IRESite, a web-based tool is available to search for sequence-based and structure-based similarities between a query sequence of interest and the entirety of annotated and experimentally validated IRES sequences within the database (http://iresite.org/IRESite_web.php?page=search). The output of the program is a probability score for any nucleotide sequence to be able to act as IRES in a validation experiment with bicistronic constructs. Additional sequence-based and structure-based web-based browsing tools are

available to suggest, with a numerical predicting value, the IRES activity potentials of any given nucleotide sequence (http://rna.informatik.uni-freiburg.de/; http://regrna.mbc.nctu.edu.tw/index1.php).

Detailed Description of the Invention

With reference to Figure 1, the *trans*-acting functional nucleic acid molecule of the invention (also referred to in the following as "IRUP") comprises a target binding sequence (also referred to as "binding domain") and a regulatory sequence (also referred to as "effector domain").

The target binding sequence comprises a sequence reverse complementary to a eukaryotic target mRNA sequence for which protein translation is to be enhanced.

The eukaryotic target mRNA sequence is preferably an animal or human target mRNA sequence, more preferably a human target mRNA sequence.

The regulatory sequence comprises an internal ribosome entry site (IRES) sequence or an internal ribosome entry site (IRES) derived sequence and enhances translation of the target mRNA sequence.

The regulatory sequence is located 3′ of the target binding sequence.

The *trans*-acting functional nucleic acid molecule hybridises to the target mRNA sequence through the target binding sequence and the IRES or IRES derived sequence

enhances the translation of the target mRNA sequence.

The functional nucleic acid molecule of the invention allows to exploit IRES sequences as *trans*-regulatory elements for gene-specific increase of translation of virtually any cellular endogenous or overexpressed protein-coding mRNA.

Preferably, the target binding sequence consists, from 3′ to 5′, of a sequence reverse complementary to 1 to 50 nucleotides of the 5′ untranslated region (5′ UTR) and 1 to 40 nucleotides of the coding sequence (CDS) of the target mRNA sequence. Specific non-limiting examples include target binding sequences consisting of:

- a sequence reverse complementary to 40 nucleotides of the 5′ untranslated region (5′ UTR) and 4 nucleotides of the coding sequence (CDS) of the target mRNA sequence (referred to the initiation methionine codon or to internal in-frame methionine codons);

- a sequence reverse complementary to 40 nucleotides of the 5′ untranslated region (5′ UTR) and 32 nucleotides of the coding sequence (CDS) of the target mRNA sequence;

- a sequence reverse complementary to 14 nucleotides of the 5′ untranslated region (5′ UTR) and 4 nucleotides of the coding sequence (CDS) of the target mRNA sequence (referred to the initiation methionine codon).

The regulatory sequence comprises an internal ribosome

entry site (IRES) sequence preferably derived from human viruses or human protein-coding genes. Several IRESs having sequences ranging from 48 to 576 nucleotides have been tested with success, e.g. human Hepatitis C Virus (HCV) IRESs (SEQ ID NO:36 and SEQ ID NO:37), human poliovirus IRESs (SEQ ID NO:38 and SEQ ID NO:39), human encephalomyocarditis (EMCV) virus (SEQ ID NO:40 and SEQ ID NO:41), human cricket paralysis (CrPV) virus (SEQ ID NO:42 and SEQ ID NO:43), human Apaf-1 (SEQ ID NO:44 and SEQ ID NO:45), human ELG-1 (SEQ ID NO:46 and SEQ ID NO:47), human c-MYC (SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO: 50, and SEQ ID NO:51), human dystrophin (DMD) (SEQ ID NO:52 and SEQ ID NO:53). More detail can be found in the example section. Sequences coding for structural elements of the IRESs, fundamental for the translation enhancing activity, have been identified, isolated and used as regulatory sequences of the *trans*-acting functional nucleic acid molecule.

As already mentioned in the definitions, internal ribosome entry site (IRES) derived sequences can include genetically engineered or chemically modified IRESs.

Chemical modifications include, but are not limited to, the following:

Base modifications: pseudouridine; 5′-Bromo-uridine; 5′-methylcytidine.

Sugar modifications (2′ modifications): 2′-O-methyl-

(2′-*O*-Me); 2′-*O*-methoxyethyl (2′-MOE); locked nucleic acid (LNA).

Backbone modifications (phosphate backbone modifications): Phosphorothioate (PS); phosphotriester.

Others (cell-type specific targeting domains): GalNAc linkage (hepatocytes).

Although the IRES sequence or IRES derived sequence is functional whether inserted - in the *trans*-acting functional nucleic acid molecule - in direct or inverted orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule, it is preferably oriented in direct orientation. In other words, by "direct" there is intended the situation in which the IRES sequence is embedded (inserted) with the same 5′ to 3′ orientation as the functional nucleic acid molecule. Instead, by "inverted" there is intended the situation in which a reverse complement of the IRES sequence is inserted in the nucleic acid molecule (the IRES sequence is 3′ to 5′ oriented relative to the functional nucleic acid molecule).

Preferably, the IRES sequence or IRES derived sequence is a sequence with 75% homology to a sequence selected from the group consisting of SEQ ID NO:36 to SEQ ID NO:65, more preferably a sequence with 90% homology to a sequence selected from the group consisting of SEQ ID NO:36 to SEQ ID NO:65, even more preferably a sequence selected from the

group consisting of SEQ ID NO:36 to SEQ ID NO:65.

The *trans*-acting functional nucleic acid molecule is preferably an RNA molecule or a modified RNA molecule. Examples of modifications are:

Base modifications: pseudouridine; 5′-Bromo-uridine; 5′-methylcytidine.

Sugar modifications (2′ modifications): 2′-O-methyl-(2′-*O*-Me); 2′-*O*-methoxyethyl (2′-MOE); locked nucleic acid (LNA).

Backbone modifications (phosphate backbone modifications): Phosphorothioate (PS); phosphotriester.

Others (cell-type specific targeting domains): GalNAc linkage (hepatocytes).

The *trans*-acting functional nucleic acid molecule preferably further comprises a spacer sequence between the target binding sequence and the regulatory sequence.

In addition, the *trans*-acting functional nucleic acid molecule optionally comprises a non-coding 3′ tail sequence, which e.g. includes restriction sites useful for cloning the molecule in appropriate plasmids.

Several *trans*-acting functional nucleic acid molecule have been generated according to the invention.

The features of some of these molecules are summarised in the following. (BD= Binding Domain; numbering in parenthesis is with respect to AUG triplet A=+1)

SEQ ID NO:1

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV, 383 nucleotides, direct orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES HCV (383 ntds) (SEQ ID NO:36)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 2

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV, 383 nucleotides, inverted orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES HCV (383 ntds) (SEQ ID NO:37)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 3

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Polio Virus, 312 nucleotides, direct orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES poliovirus (312 ntds) (SEQ ID NO:38)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 4

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Polio Virus, 312 nucleotides, inverted orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES poliovirus (312 ntds) (SEQ ID NO:39)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 5

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Encephalomyocarditis virus, EMCV-R, 576 nucleotides, direct orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES EMCV-R (576 ntds) (SEQ ID NO:40)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 6

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Encephalomyocarditis virus, EMCV-R, 576 nucleotides, inverted orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES EMCV-R (576 ntds) (SEQ ID NO:41)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 7

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Cricket Paralysis Virus, CrPV, 192 nucleotides, direct orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES CrPV (192 ntds) (SEQ ID NO:42)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 8

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Cricket Paralysis Virus, CrPV, 192 nucleotides, inverted orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES CrPV (192 ntds) (SEQ ID NO:43)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 9

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human Apaf-1, 231 nucleotides, direct orientation

(Ensembl: ENSG00000120868; MIM:602233)

Features: BD= DJ-1s (-40/+4)

ED= IRES Apaf-1 (231 ntds) (SEQ ID NO:44)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 10

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human Apaf-1, 231 nucleotides, inverted orientation

(Ensembl: ENSG00000120868; MIM:602233)

Features: BD= DJ-1s (-40/+4)

ED= IRES Apaf-1 (231 ntds) (SEQ ID NO:45)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 11

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human ELG-1, 460 nucleotides, direct orientation

(Ensembl: ENSG00000176208; MIM:609534)

Features: BD= DJ-1s (-40/+4)

ED= IRES ELG-1 (460 ntds) (SEQ ID NO:46)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID No: 12

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human ELG-1, 460 nucleotides, inverted orientation

(Ensembl: ENSG00000176208; MIM:609534)

Features: BD= DJ-1s (-40/+4)

ED= IRES ELG-1 (460 ntds) (SEQ ID NO:47)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 13

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human c-MYC, 395 nucleotides, direct orientation

(Ensembl: ENSG00000136997; MIM:190080)

Features: BD= DJ-1s (-40/+4)

ED= IRES c-MYC full-length (395 ntds) (SEQ ID NO:48)

Backbone= Delta5'ASUchl1

IRES orientation: direct

Others: includes the 48nt minimal sequence (SEQ ID NO:50) included in SEQ ID NO:15.

SEQ ID NO: 14

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human c-MYC, 395 nucleotides, inverted

orientation

(Ensembl: ENSG00000136997; MIM:190080)

Features: BD= DJ-1s (-40/+4)

ED= IRES c-MYC full-length (395 ntds) (SEQ ID NO:49)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

Others: includes the 48nt minimal sequence (SEQ ID NO:51) included in SEQ ID NO:16.

SEQ ID NO: 15

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human c-MYC, 48 nucleotides, direct orientation

(Ensembl: ENSG00000136997; MIM:190080)

Features: BD= DJ-1s (-40/+4)

ED= IRES c-MYC (48 ntds) (SEQ ID NO:50)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 16

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human c-MYC, 48 nucleotides, inverted orientation

(Ensembl: ENSG00000136997; MIM:190080)

Features: BD= DJ-1s (-40/+4)

ED= IRES c-MYC (48 ntds) (SEQ ID NO:51)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 17

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human dystrophin (DMD), 71 nucleotides, direct orientation

(Ensembl: ENSG00000198947; MIM:300377)

Features: BD= DJ-1s (-40/+4)

ED= IRES DMD (71 ntds) (SEQ ID NO:52)

Backbone= Delta5'ASUchl1

IRES orientation: direct

SEQ ID NO: 18

Definition: IRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human dystrophin (DMD), 71 nucleotides, inverted orientation

(Ensembl: ENSG00000198947; MIM:300377)

Features: BD= DJ-1s (-40/+4)

ED= IRES DMD (71 ntds) (SEQ ID NO:53)

Backbone= Delta5'ASUchl1

IRES orientation: inverted (reverse complement)

SEQ ID NO: 19

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#1, 303 nucleotides, delta II (40-119), interaction with ribosomal proteins

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, deltaII (SEQ ID NO:54)

Backbone= Delta5'ASUchl1

Mutant#1: delta II (40-119), interaction with ribosomal proteins

SEQ ID NO: 20

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#2, 367 nucleotides, delta IIIa (156-171), eIF3 binding site

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, deltaIIIa (SEQ ID NO:55)

Backbone= Delta5'ASUchl1

Mutant#2: delta IIIa (156-171), eIF3 binding site

SEQ ID NO: 21

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#3, 356 nucleotides, delta IIId (253-279), 18S rRNA binding region

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, deltaIIId (SEQ ID NO:56)

Backbone= Delta5'ASUchl1

Mutant#3: delta IIId (253-279), 18S rRNA binding region

SEQ ID NO: 22

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#4, 330 nucleotides, delta IV (331-383), AUG-containing terminal sequence

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, deltaIV (SEQ ID NO:57)

Backbone= Delta5'ASUchl1

Mutant#4: delta IV (331-383), AUG-containing terminal sequence

SEQ ID NO: 23

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#5, 383 nucleotides, G266->C; single point mutation, contact with 18S rRNA

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, G266->C (SEQ ID NO:58)

Backbone= Delta5'ASUchl1

Mutant#5: G266->C; single point mutation, contact with 18S rRNA

SEQ ID NO: 24

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#6, 383 nucleotides, U228->C; control single point mutation in another site of HCV IRES, decreases IRES activity without disrupting formation of pre-initiation complex

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, U228->C (SEQ ID NO:59)

Backbone= Delta5'ASUch11

Mutant#6: U228->C; control single point mutation in another site of HCV IRES, decreases IRES activity without disrupting formation of pre-initiation complex. Mutated version has reduced affinity for eIF3.

SEQ ID NO: 25

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#7, 383 nucleotides, G267->C; IIId loop, single point mutation, contact with 18S rRNA

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, G267->C (SEQ ID NO:60)

Backbone= Delta5'ASUch11

Mutant#7: G267->C; IIId loop, single point mutation, contact with 18S rRNA

SEQ ID NO: 26

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#8, 383 nucleotides, G268->C; IIId loop, single point mutation, contact with 18S rRNA

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, G268->C (SEQ ID NO:61)

Backbone= Delta5'ASUchl1

Mutant#8: G268->C; IIId loop, single point mutation, contact with 18S rRNA

SEQ ID NO: 27

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#9, 383 nucleotides, $G_{266}G_{267}G_{268}$-> $C_{266}C_{267}C_{268}$; IIId loop, triple point mutation, contact with 18S rRNA

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, $G_{266}G_{267}G_{268}$-> $C_{266}C_{267}C_{268}$ (SEQ ID NO:62)

Backbone= Delta5'ASUchl1

Mutant#9: $G_{266}G_{267}G_{268}$-> $C_{266}C_{267}C_{268}$; IIId loop, triple point mutation, contact with 18S rRNA

SEQ ID NO: 28

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#10,

383 nucleotides, G266->A/G268->T; double point mutant; HCV 5a isolate with poor infectivity

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, G266->A/G268->T (SEQ ID NO:63)

Backbone= Delta5'ASUchl1

Mutant#10: G266->A/G268->T; double point mutant; HCV 5a isolate with poor infectivity

SEQ ID NO: 29

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#11, 383 nucleotides, IIIa->IIIa-comp; AGTA->TCAT

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, IIIa->IIIa-comp; AGTA->TCAT (SEQ ID NO:64)

Backbone= Delta5'ASUchl1

Mutant#11: HCV IRES direct, IIIa->IIIa-comp; AGTA->TCAT)

SEQ ID NO: 30

Definition: IRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV mutant#12, 383 nucleotides, IIe->IIIe-comp; TGATAG->ACTATC

Features: BD= DJ-1s (-40/+4)

ED= HCV IRES direct, IIIe->IIIe-comp; TGATAG->ACTATC (SEQ ID NO:65)

Backbone= Delta5'ASUchl1

Mutant#12: HCV IRES direct, IIIe->IIIe-comp; TGATAG->ACTATC

SEQ ID NO: 31

Definition: miniIRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV, 383 nucleotides, direct orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES HCV (383 ntds) (SEQ ID NO:36)

IRES orientation: direct

SEQ ID NO: 32

Definition: miniIRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Polio Virus, 312 nucleotides, direct orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES poliovirus (312 ntds) (SEQ ID NO:38)

IRES orientation: direct

SEQ ID NO: 33

Definition: miniIRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Polio Virus, 312 nucleotides, inverted orientation

Features: BD= DJ-1s (-40/+4)

ED= IRES poliovirus (312 ntds) (SEQ ID NO:39)

IRES orientation: inverted (reverse complement)

SEQ ID NO: 34

Definition: miniIRUP Functional Nucleic Acid Molecule

IRES: cellular IRES, Human c-MYC, 48 nucleotides, direct orientation)(Ensembl: ENSG00000136997; MIM:190080)

Features: BD= DJ-1s (-40/+4)

ED= IRES c-MYC (48 ntds) (SEQ ID NO:50)

IRES orientation: direct

SEQ ID NO: 35

Definition: miniIRUP Functional Nucleic Acid Molecule

IRES: viral IRES, Human Hepatitis C Virus, HCV, 383 nucleotides, direct orientation

Features: BD= GFP (-40/+32)

ED= IRES HCV (383 ntds) (SEQ ID NO:36)

IRES orientation: direct

A DNA molecule according to the present invention encodes any of the above disclosed *trans*-acting functional nucleic acid molecules.

An expression vector according to the present invention comprises the above said DNA molecule. In particular, the following plasmids have been used for efficient expression of functional nucleic acid molecules.

Mammalian expression plasmids:

Plasmid Name: **pCS2+**

Expression: CMVie92 promoter

SV40polyA terminator

Plasmid Name: **pCDN3.1 (-)**

Expression: CMV promoter

BGH terminator

Plasmid Name: **pDUAL-eGFPΔ(modified from peGFP-C1)**

Expression: H1 promoter; CMV promoter

BGH terminator; SV40 terminator

Viral vectors:

Vector Name: **pAAV**

Virus: Adeno-Associated Virus

Expression: CAG promoter / CMV enhancer

SV40polyA terminator

Vector Name: **pLVX-TetOne-Puro**

Virus: Lentivirus

Expression: TRE3G promoter (inducible expression)

SV40polyA terminator

It should be noted that the experiments carried out have highlighted that the function of the *trans*-acting functional nucleic acid molecule is not influenced by the plasmid used.

A method for enhancing protein translation according to the invention comprises transfecting into a cell the above disclosed *trans*-acting functional nucleic acid molecule or DNA molecule or expression vector.

A composition according to the present invention comprises the above disclosed *trans*-acting functional nucleic acid molecule or DNA molecule or expression vector. The *trans*-acting functional nucleic acid molecule can be delivered as naked RNA, the RNA optionally including modifications adapted to increase RNA stability. As an alternative the *trans*-acting functional nucleic acid molecule can be an *in vitro* transcribed RNA encapsulated in an array of lipid-based nanoparticles or an *in vitro* transcribed RNA encapsulated in exosome-based particles.

The above disclosed *trans*-acting functional nucleic acid molecule or DNA molecule or expression vector can be used for enhancing translation of a target mRNA sequence. The examples show enhancement of translation of two different target mRNA sequences, PARK7/DJ-1 (also referred to as DJ-1) and GFP, but any other mRNA sequence could be targeted with success without influencing mRNA levels.

It should be noted that IRUPs can enhance translation of the gene of interest with no effects on its own mRNA quantities.

The above disclosed *trans*-acting functional nucleic acid molecule or DNA molecule or expression vector can therefore be successfully used as molecular tools to validate gene function in cells as well as to implement the pipelines of recombinant protein production.

The above disclosed *trans*-acting functional nucleic acid molecule or DNA molecule or expression vector can be used for treating a genetic disease caused by down-regulation of a protein-coding mRNA or a sporadic disease where reduced levels of a certain protein-coding mRNA is detrimental. The following are examples of such diseases. Haploinsufficiency is a condition that arises when the normal phenotype requires the protein product of both alleles, and reduction to 50% or less of gene function results in an abnormal phenotype. This is the cause of a wide spectrum of diseases including specific types of cancers, ataxias and those due to failures of developmental programs. A large number of Rare Diseases are caused by mutations or microdeletions that lead to reduced gene dosage. Transcription factors, synaptic proteins and chromatin remodeling enzymes seem to be particularly sensitive to gene dosage. Reduced gene expression can be also observed during aging.

Examples

Example 1

Figure 2 shows that the embedded Effector Domain (ED) is required for translation up-regulation function of an antisense long non-coding RNA (lncRNA) targeting endogenous human DJ-1 mRNA.

Figure 2A shows a schematic diagram of a functional antisense lncRNA molecule that up-regulates translation of

target-specific mRNAs. The original molecule that uses an embedded murine transposable element of the SINE B2 family is indicated (SINEUP). SINEUP functional domains are highlighted: SINEUP Binding Domain (BD): SINEUP sequence that overlaps, in antisense orientation, to the sense protein-coding mRNA; SINEUP Effector Domain (ED): the inverted SINEB2 element (SINE) in the non-overlapping portion of SINEUPs, that confers activation of protein synthesis. 5′ to 3′ orientation of sense and antisense RNA molecules is indicated. Target-specific mRNA includes 5′ untranslated region (5′UTR), coding sequence (CDS) and 3′ untranslated region (3′UTR). Translation initiating AUG codon is also shown.

A synthetic SINEUP was designed to target endogenous human DJ-1 mRNA (SINEUP-DJ-1). SINEUP-DJ-1 mutants were generated lacking the Binding Domain (ΔBD) or the Effector Domain (ΔED). Human embryonic kidney (HEK) 293T/17 cells were transfected with plasmids encoding for SINEUP-DJ-1 full length (FL) or its deletion mutants (ΔED = mutant with deleted effector domain, ΔBD = mutant with deleted binding domain). Control cells were transfected with an empty control plasmid (-). 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 2B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western

blot images normalized to β-actin and relative to empty control samples.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and SINEUP RNA was monitored by qRT-PCR using specific primers (Figure 2C). Data indicate mean ± st. dev. Data are representative of N=5 independent replicas.

Figure 2D shows a graphical representation of SINEUP-DJ-1 FL, ΔBD and ΔED translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=5). $p<0.05$

Example 2

Synthetic IRUPs were designed as follows to target endogenous human DJ-1 mRNA. As shows in Figure 3, IRES-containing functional nucleic acid molecules were generated by swapping the original SINE B2 sequence with IRES sequences derived from human viruses or human mRNAs having IRES activity.

Table 1 includes the list of some of the IRES sequences used in the *trans*-acting functional nucleic acid molecule according to the present invention.

IRES name, IRES origin, cloning orientation and IRES length are indicated.

Table 1

IRES	Origin	Orientation	Length (nt)
HCV	Human Virus	Direct	383
HCV	Human Virus	Inverted	383
Polio	Human Virus	Direct	312
Polio	Human Virus	Inverted	312
EMCV	Human Virus	Direct	576
EMCV	Human Virus	Inverted	576
CrPV	Human Virus	Direct	192
CrPV	Human Virus	Inverted	192
Apaf-1	Human mRNA	Direct	231
Apaf-1	Human mRNA	Inverted	231
ELG-1	Human mRNA	Direct	460
ELG-1	Human mRNA	Inverted	460
cMYC (long)	Human mRNA	Direct	395
cMYC (long)	Human mRNA	Inverted	395
cMYC (short)	Human mRNA	Direct	48
cMYC (short)	Human mRNA	Inverted	48
DMD	Human mRNA	Direct	71
DMD	Human mRNA	Inverted	71

Figure 4 shows a schematic diagram of the experimental procedure for testing translation up-regulation activity of IRES-containing functional antisense nucleic acid molecules. Antisense functional nucleic acid molecules for translation

activation were generated with human DJ-1 overlapping sequence (Binding Domain) and IRES sequences (Effector Domain). IRES-containing functional nucleic acid molecules were cloned into mammalian expression vectors for expression in mammalian cells in culture *in vitro*. Cells that express endogenous quantities of human DJ-1 mRNA were used. Timing for cell seeding, cell transfection and harvesting are shown. Cells were harvested to purify RNA (for quantitative real-time PCR, qRT-PCR) and proteins (for Western blot, WB).

Figure 5A shows a schematic diagram of a *trans*-acting functional nucleic acid molecule in which the effector domain is an IRES sequence from human Hepatitis C virus (HCV). IRES-containing functional nucleic acid molecules were generated with the HCV IRES sequence cloned in direct (HCV(d) - SEQ ID NO:1) or inverted (HCV(i) - SEQ ID NO:2) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with HCV IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for

protein quantities. Western blot (Figure 5B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher or similar with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 5C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 3

Figure 6A shows a schematic diagram of a *trans*-acting functional nucleic acid molecule in which the effector domain is an IRES sequence from human Poliovirus. IRES-containing functional nucleic acid molecules were generated with the Polio IRES sequence cloned in direct (Polio(d) - SEQ ID NO:3) or inverted (Polio(i) - SEQ ID NO:4) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid

molecules with Polio IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 6B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher than SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 6C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 4

Figure 7A shows a schematic diagram of a *trans*-acting functional nucleic acid molecules in which the effector domain is respectively an IRES sequence from human Encephalomyocarditis virus (EMCV) and an IRES sequence from

Cricket Paralysis virus (CrPV). IRES-containing functional nucleic acid molecules were generated with the EMCV IRES sequence cloned in direct (EMCV(d) - SEQ ID NO:5) or inverted (EMCV(i) - SEQ ID NO:6) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule and with the CrPV IRES sequence cloned in direct (CrPV(d) - SEQ ID NO:7) or inverted (CrPV(i) - SEQ ID NO:8) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with EMCV and CrPV IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 7B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher than SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of

endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 7C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 5

The increase in quantities of endogenous DJ-1 protein in HEK 293T/17 cells transfected with HCV(d) and HCV(i) IRUPs of example 2, Polio(d) and Polio(i) IRUPs of example 3 and EMCV(d), EMCV(i), CrPV(d) and CrPV(i) IRUPs of example 4 relative to empty vector transfected HEK 293T/17 cells was measured by Western blot.

The results are summarised in Figures 8A and 8B. As evident, IRES-containing functional nucleic acid molecules activate translation in *trans* and are more active than SINE-containing molecules. Data represents average and stdev of N>5 biological replicas. Single asterisks (*) indicate IRES sequences that have statistically significant translation enhancement activity in *trans* relative to empty control cells; double asterisks (**) indicate IRES sequences that show statistically significant increased potency relative to SINE-containing functional nucleic acid molecules.

Example 6

Figure 9A shows a schematic diagram of a *trans*-acting

functional nucleic acid molecule in which the effector domain is an IRES sequence from human Apoptotic Peptidase Activating Factor 1 (Apaf-1) mRNA. IRES-containing functional nucleic acid molecules were generated with the Apaf-1 IRES sequence cloned in direct (Apaf-1(d) - SEQ ID NO:9) or inverted (Apaf-1(i) - SEQ ID NO:10) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with Apaf-1 IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 9B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher or similar with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 9C). To compare RNA quantities

across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 7

Figure 10A shows a schematic diagram of a *trans*-acting functional nucleic acid molecule in which the effector domain is an IRES sequence from human Enhanced Level of Genomic instability 1 (ELG-1) mRNA. IRES-containing functional nucleic acid molecules were generated with the ELG-1 IRES sequence cloned in direct (ELG-1(d) - SEQ ID NO:11) or inverted (ELG-1(i) - SEQ ID NO:12) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with ELG-1 IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 10B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images

normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 10C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 8

Figure 11A shows a schematic diagram of a *trans*-acting functional nucleic acid molecule in which the effector domain is an IRES sequence from human V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (cMYC) mRNA. IRES-containing functional nucleic acid molecules were generated with the cMYC IRES sequence (long variant) cloned in direct (cMYC full length(d) - SEQ ID NO:13) or inverted (cMYC full length(i) - SEQ ID NO:14) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with cMYC IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were

transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 11B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher or similar with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 11C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 9

Figure 12A shows a schematic diagram of a *trans*-acting functional nucleic acid molecule in which the effector domain is a shorter version of the IRES sequence from human V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (cMYC) mRNA of Example 8. IRES-containing functional nucleic acid molecules were generated with the cMYC IRES sequence (short variant)

cloned in direct (cMYC short variant(d) - SEQ ID NO:15) or inverted (cMYC short variant(i) - SEQ ID NO:16) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with cMYC IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 11B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher or similar with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 12C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev.

Data are representative of N>5 independent replicas.

Example 10

Figure 13A shows a schematic diagram of a *trans*-acting functional nucleic acid molecule in which the effector domain is an IRES sequence from human Dystrophin (DMD) mRNA. IRES-containing functional nucleic acid molecules were generated with the DMD IRES sequence cloned in direct (DMD(d)- SEQ ID NO:17) or inverted (DMD(i) - SEQ ID NO:18) orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

HEK 293T/17 cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with DMD IRES sequence in direct (d) or inverted (i) orientation, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 13B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was higher with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and IRUP RNA was monitored by qRT-PCR using specific primers (Figure 13C). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev. Data are representative of N>5 independent replicas.

Example 11

The increase in quantities of endogenous DJ-1 protein in HEK 293T/17 cells transfected with Apaf-1(d) and Apaf-1(i) IRUPs of Example 6, ELG-1(d) and ELG-1(i) IRUPs of Example 7, cMYC full length(d) and cMYC full length(i) IRUPs of Example 8, cMYC short variant(d) and cMYC short variant(i) IRUPs of Example 9, and DMD(d) and DMD(i) IRUPs of Example 10 relative to empty vector transfected HEK 293T/17 cells was measured by Western blot.

The results are summarised in Figures 14A and 14B. As evident, IRES-containing functional nucleic acid molecules activate translation in *trans* and are more active than SINE-containing molecules. Data represents average and stdev of N>5 biological replicas. Single asterisks (*) indicate IRES sequences that have statistically significant translation enhancement activity in *trans* relative to empty control cells; double asterisks (**) indicate IRES sequences that show statistically significant increased potency relative to

SINE-containing functional nucleic acid molecules.

Example 12

Human hepatocellular carcinoma (HepG2) cells were transfected with plasmids encoding for IRES-containing functional nucleic acid molecules with HCV (Figure 15A) IRES sequence in direct (d) or inverted (i) orientation (SEQ ID NO:1 or SEQ ID NO:2), with Polio and cMYC (Figure 15B) IRES sequence in direct (d) or inverted (i) orientation (SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:13 or SEQ ID NO:14), with Apaf-1 and ELG-1 (Figure 15C) IRES sequence in direct (d) or inverted (i) orientation (SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 or SEQ ID NO:12), and with DMD (Figure 15D) IRES sequence in direct (d) or inverted (i) orientation (SEQ ID NO:17 or SEQ ID NO:18), as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of IRES-containing functional nucleic acid molecules was almost always higher than SINE-containing functional nucleic acid molecules.

Example 13

The increase in quantities of endogenous DJ-1 protein in HepG2 cells transfected with HCV(d) and HCV(i) IRUPs, Polio(d) and Polio(i) IRUPs and cMYC short variant(d) and cMYC short variant(i) IRUPs, Apaf-1(d) and Apaf-1(i) IRUPs, ELG-1(d) and ELG-1(i) IRUPs, DMD(d) and DMD(i) IRUPs relative to empty vector transfected HepG2 cells was measured by Western blot.

The results are summarised in Figures 16A-16B. IRES-containing functional nucleic acid molecules activate translation in *trans* and are more active than SINE-containing molecules. Data represents average and stdev of N>2 biological replicas.

Example 14

Figure 17A shows a schematic diagram of a miniaturised version of a *trans*-acting functional nucleic acid molecule (miniIRUP) in which the effector domain is an IRES sequence from HCV, Poliovirus or cMYC short version. IRES-containing functional nucleic acid molecules were generated with the HCV IRES sequence cloned in direct (HCV(d) - SEQ ID NO:31) orientation, with the Polio IRES sequence cloned in direct (Polio(d) - SEQ ID NO:32) and inverted (Polio(i) - SEQ ID NO:33) orientation and with the cMYC short IRES sequence cloned in direct (cMYC short variant(d) - SEQ ID NO:34) orientation, under the control of an SV40-based promoter for

expression by RNA polymerase II in mammalian cells.

HEK 293T/17 cells were transfected with plasmids encoding the above said mini IRES-containing functional nucleic acid molecules, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 17B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of mini IRES-containing functional nucleic acid molecules was higher than SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and mini-IRUP RNA was monitored by qRT-PCR using specific primers (Figure 17C).

Figure 17D shows a graphical representation of SINEUP-DJ-1 and mini-IRUP translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=6).

Example 15

Figure 18A shows a schematic diagram of a miniaturised version of a *trans*-acting functional nucleic acid molecule (miniIRUP) in which the effector domain is an IRES sequence

from HCV, Poliovirus or cMYC short version. IRES-containing functional nucleic acid molecules were generated with the HCV IRES sequence cloned in direct (HCV(d) - SEQ ID NO:31) orientation, with the Polio IRES sequence cloned in direct (Polio(d)- SEQ ID NO:32) and inverted (Polio(i) - SEQ ID NO:32) orientation and with the cMYC short IRES sequence cloned in direct (cMYC short version(d) - SEQ ID NO:34) orientation, under the control of an H1-based promoter for expression by RNA polymerase III in mammalian cells.

HEK 293T/17 cells were transfected with plasmids encoding the above said mini IRES-containing functional nucleic acid molecules, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 18B) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of mini IRES-containing functional nucleic acid molecules was higher (similar in one case) with respect to SINE-containing functional nucleic acid molecules.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and mini-IRUP RNA was monitored by qRT-

PCR using specific primers (Figure 18C).

Figure 18D shows a graphical representation of SINEUP-DJ-1 and mini-IRUP translation enhancement activity on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=6).

Example 16

Figure 19A shows a schematic diagram of pDUAL-GFP plasmids for the concomitant expression of two genes of interest in mammalian cells. pDUAL-GFP plasmids contain a CMV promoter element for the expression of GFP mRNA by RNA Polymerase II and an H1 promoter (in opposite orientation) for the expression of GFP-targeting miniaturized version of functional nucleic acid molecules for GFP translation enhancement. A control plasmid is produced that lacks the translation enhancer functional nucleic acid molecule and expressed basal levels of GFP protein. pDUAL-GFP/miniGFP plasmids were created in which the effector domain is represented by a SINE B2 sequence or by the HCV IRES, as indicated.

HEK 293T/17 cells were transfected with pDUAL plasmids encoding for GFP alone or GFP in combination with SINE-containing (SINE) or IRES-containing (IRES) mini-functional nucleic acid molecules. Cells transfected with pDUAL-GFP alone were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western

blot (Figure 19B) was performed with anti-GFP antibody. β-actin was used as loading control.

RNA was purified from transfected cells. Expression of overexpressed GFP mRNA (Figure 19C) and functional nucleic acid SINE or IRES RNA (Figure 19D) was monitored by qRT-PCR using specific primers.

The results show that IRES-containing functional nucleic acid molecules bearing a Binding Domain antisense to GFP mRNA enhance translation of GFP mRNA when GFP is overexpressed in HEK 293T/17 cells.

Example 17

Figure 20A shows a schematic diagram of pDUAL-GFP plasmids with GFP targeting functional nucleic acid molecules in which the Effector Domain is represented by the SINE or HCV IRES sequence.

Human neuroblastoma Neuro2a cells were transfected with the pDUAL-GFP plasmids shown in Figure 20A. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 20B) was performed with anti-GFP antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of mini IRES-containing functional nucleic acid molecules was higher than SINE-containing functional nucleic acid molecules.

Example 18

Functional nucleic acid molecules containing DJ-1 targeting Binding Domain and HCV IRES Effector Domain harboring specific mutations in structural regions important for HCV IRES activity in *cis* were designed.

Figure 21 shows a schematic representation of HCV IRES RNA secondary structure. HCV IRES structural domains (IIa, IIb, IIIa, IIIb, IIIc, IIId, IIIe and IIIf) are indicated in the different squares. The functionality of each structural domain for the Internal Ribosome Entry activity of the sequence is also shown, with contacts to ribosomal RNA and/or ribosomal proteins. A functional nucleic acid molecule in which the Effector Domain is represented by the IRES sequence derived from HCV and containing DJ-1 targeting Binding Domain was used as template for mutagenesis. An M2 mutant was produced by deletion of the IIIa stem loop (aminoacids 156-171 of HCV IRES sequence - IRES = SEQ ID NO:55; IRUP = SEQ ID NO:20), involved in contacts with the eukaryotic translation initiation factor eIF3 and the ribosomal protein eS27. An M5 mutant was produced by site-directed nucleotide substitution of nucleotide G266 (G266->C - IRES = SEQ ID NO:58; IRUP = SEQ ID NO:23), fundamental for base-pairing to 18S rRNA and for HCV IRES activity in *cis*.

Example 19

HEK 293T/17 cells were transfected with a mammalian expression plasmid encoding for IRES-containing functional nucleic acid molecule with HCV IRES sequence in direct orientation (WT) or with HCV IRES DIIIa (M2) or G266->C (M5) mutants, as indicated. Control cells were transfected with an empty control plasmid (-). Cells transfected with SINEUP-DJ-1 were used as reference for testing the potency of IRES-containing molecules. 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 22A) was performed with anti-DJ-1 antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Potency of WT and mutated IRES-containing functional nucleic acid molecule was higher or similar with respect to SINE-containing functional nucleic acid molecule.

RNA was purified from transfected cells. Expression of endogenous DJ-1 mRNA and functional nucleic acid RNA was monitored by qRT-PCR using specific primers (Figure 22B). To compare RNA quantities across SINE- and IRES-containing functional nucleic acid molecules, primers were positioned at the 3′ end of the Effector Domain. Data indicate mean ± st. dev.

Figure 22C shows a graphical representation of

translation enhancement activity of functional nucleic acid molecules with SINE- or HCV IRES-embedded sequences on endogenous DJ-1 mRNA in HEK 293T/17 cells (N=7). Double asterisks (**) indicate mutant IRES sequences that show statistically significant reduction in translation enhancement relative to WT HCV IRES-containing functional nucleic acid molecule.

This example shows that structural elements required for HCV IRES activity in *cis* contribute to the increased translation enhancement activity of HCV IRES in *trans* as embedded Effector Domain (ED) in a functional nucleic acid molecule containing DJ-1 targeting Binding Domain.

Example 20

This example shows that any nucleic acid sequence in the target mRNA can be recognised by the Binding Domain of an IRES-derived sequence containing a functional nucleic acid molecule.

Figure 23A shows a schematic representation of how the sequence at the 5′ of an IRES sequence and within an IRES-containing cellular mRNA can be considered as Binding Domain and Figure 23B shows functional nucleic acid sequence elements within cMYC mRNA reference sequence (NM_002467).

Figure 23C shows the results of the bioinformatics analysis (BLAST) using the c-MYC mRNA as query sequence to identify partially overlapping, in antisense orientation,

target mRNA protein-coding sequences. In the table, annotated gene ENSEMBL nomenclature, Gene Name and Gene pairing region are indicated. The IRES-derived Binding Domain can overlap, in antisense orientation, to target mRNA in the 5′ UTR, first exon, internal exon and with different pairing length, ranging from 18 to 198.

Mammalian SAOS cells were transfected with a mammalian expression plasmid encoding for full-length mRNA of MYC (cMYC-FL) (Figure 24A). Control cells were transfected with an empty vector plasmid (control). 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 24B) was performed with anti-JAG2, anti-DYRK2, anti-LYS, anti-UBE3A, anti-NRF1 antibodies as indicated. β-actin was used as loading control. Anti-cMYC antibody was used as additional control to verify expression of cMYC protein after transfection. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples (C). Potency of cMYC IRES-containing functional nucleic acid molecules was evident for all tested target mRNAs, with binding domains ranging from 144 (JAG2) to 20 (UBE3A, NRF1) nucleotides in length.

RNA was purified from transfected cells. Expression of endogenous JAG2, DYRK2, LIS1, UBE3A, NRF1 and cMYC mRNAs was monitored by qRT-PCR using specific primers (Figure 24C and

Figure 24D).

This example shows that the pairing region between the Binding Domain of an IRES-containing functional nucleic acid molecule and the target protein-coding mRNA can vary in position and in length, retaining its full translation enhancement activity.

Example 21

This example shows that the protein-coding CDS portion and the DNA-binding domain of cMYC are not required for the IRES-containing functional nucleic acid molecule to increase translation of partially-overlapping protein-coding mRNAs.

Mammalian SAOS cells were transfected with a mammalian expression plasmid encoding for full-length mRNA of MYC (cMYC-FL) or variants that lack cMYC DNA binding domain (deltaC) and are comprised of the 5'UTR only (5'UTR) or of the IRES only (IRES) domains. (Figure 25A). Control cells were transfected with an empty vector plasmid (control). 48 hours after transfection, cells were lysed and processed for protein quantities. Western blot (Figure 25B) was performed with anti-JAG2, anti-DYRK2, anti-LYS, anti-UBE3A, anti-NRF1 antibodies as indicated. β-actin was used as loading control. Anti-cMYC antibody was used as additional control to verify expression of cMYC protein after transfection. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Fold-

changes of protein quantities relative to control cells are indicated at the bottom of the western blot images.

RNA was purified from transfected cells. Expression of endogenous JAG2, DYRK2, LIS1, UBE3A, NRF1 and cMYC mRNAs was monitored by qRT-PCR using specific primers (Figure 25C), proving the post-transcriptional mechanism of the IRES-containing functional nucleic acid molecule.

Advantages

The *trans*-acting functional nucleic acid molecule of the present invention allows to enhance the translation of virtually any target mRNA sequence without affecting target mRNA levels.

With respect to the functional nucleic acid molecules disclosed in EP 2691522, those of the present invention avoid the risk of retrotransposition due to mouse SINE sequences and promote higher levels of enhancement of protein translation, as shown in Examples 2 to 11 in HEK 293T/17 and in Example 12 in HepG2 cells. In particular, examples of the more potent IRES sequences are given in Fig. 5 (HCV IRES, direct), in Fig. 6 (Polio IRES, direct and inverted), Fig. 7 (ECMV IRES, direct and inverted; CrPV IRES, direct and inverted), Fig. 9 (Apaf1 IRES, direct), Fig. 10 (ELG-1 IRES, direct and inverted), Fig. 11 (cMYC IRES, direct), Fig. 12 (short cMYC IRES, direct), Fig. 12 (DMD IRES, direct and inverted) in HEK cells. In addition, examples of more potent

IRES sequences are given also in HepG2 cells (Fig. 15).

Some IRES sequences are as short as 40 to 50 nucleotides. This allows the engineering of very short *trans*-acting functional nucleic acid molecules.

In addition, the *trans*-acting functional nucleic acid molecules of the invention can include a particularly short target binding sequence, in particular as compared to functional nucleic acid molecules disclosed in EP2691522.

Both the limited length of the regulatory sequence and the target binding sequence contribute to keeping the length of the molecule short while allowing an optimal targeting and protein synthesis enhancement. One of the main advantages of having a short molecule, is to overcome the difficulty in synthesizing RNAs longer than 100 nucleotides.

Another advantage of the *trans*-acting functional nucleic acid molecules of the invention is they have a modular structure, i.e. have an independent target binding domain and an independent effector domain.

CLAIMS

1. A *trans*-acting functional nucleic acid molecule comprising:

- a target binding sequence comprising a sequence reverse complementary to a eukaryotic target mRNA sequence for which protein translation is to be enhanced; and

- a regulatory sequence comprising an internal ribosome entry site (IRES) sequence or an internal ribosome entry site (IRES) derived sequence and enhancing translation of the target mRNA sequence,

wherein the regulatory sequence is located 3′ of the target binding sequence.

2. The *trans*-acting functional nucleic acid molecule according to claim 1, wherein the target binding sequence consists, from 3′ to 5′, of a sequence reverse complementary to 1 to 50 nucleotides of the 5′ untranslated region (5′ UTR) and 1 to 40 nucleotides of the coding sequence (CDS) of the target mRNA sequence.

3. The *trans*-acting functional nucleic acid molecule according to claim 2, wherein the target binding sequence consists, from 3′ to 5′, of a sequence reverse complementary to 10 to 45 nucleotides of the 5′ untranslated region (5′ UTR) and 2 to 6 nucleotides of the coding sequence (CDS) of the target mRNA sequence.

4. The *trans*-acting functional nucleic acid molecule

according to any of the preceding claims, wherein the IRES sequence or IRES derived sequence is oriented, in the *trans*-acting functional nucleic acid molecule, in direct orientation relative to the 5′ to 3′ orientation of the functional nucleic acid molecule.

5. The *trans*-acting functional nucleic acid molecule according to any of the preceding claims, wherein the IRES sequence or IRES derived sequence is a sequence with 75% homology to a sequence selected from the group consisting of SEQ ID NO:36 to SEQ ID NO:65.

6. The *trans*-acting functional nucleic acid molecule according to claim 5, wherein the IRES sequence or IRES derived sequence is a sequence with 90% homology to a sequence selected from the group consisting of SEQ ID NO:36 to SEQ ID NO:65.

7. The *trans*-acting functional nucleic acid molecule according to claim 6, wherein the IRES sequence or IRES derived sequence is a sequence selected from the group consisting of SEQ ID NO:36 to SEQ ID NO:65.

8. The *trans*-acting functional nucleic acid molecule according to any of the preceding claims, wherein the *trans*-acting functional nucleic acid molecule is an RNA molecule or a modified RNA molecule.

9. The *trans*-acting functional nucleic acid molecule according to any of the preceding claims, further comprising

a spacer sequence between the target binding sequence and the regulatory sequence.

10. A DNA molecule encoding the *trans*-acting functional nucleic acid molecule according to any of claims 1 to 9.

11. An expression vector comprising the DNA molecule according to claim 10.

12. A method for enhancing protein translation comprising transfecting into a cell the *trans*-acting functional nucleic acid molecule according to claims 1 to 9 or the DNA molecule according to claim 10 or the expression vector according to claim 11.

13. A composition comprising the *trans*-acting functional nucleic acid molecule according to any of claims 1 to 9 or the DNA molecule according to claim 10 or the expression vector according to claim 11.

14. Use of the *trans*-acting functional nucleic acid molecule according to any of claims 1 to 9 or the DNA molecule according to claim 10 or the expression vector according to claim 11 for enhancing translation of a target mRNA sequence.

15. A *trans*-acting functional nucleic acid molecule according to any of claims 1 to 9 or a DNA molecule according to claim 10 or an expression vector according to claim 11 for use in treating a genetic disease caused by down-regulation of a protein-coding mRNA or in treating a genetic or sporadic disease where reduced gene dosage is detrimental.

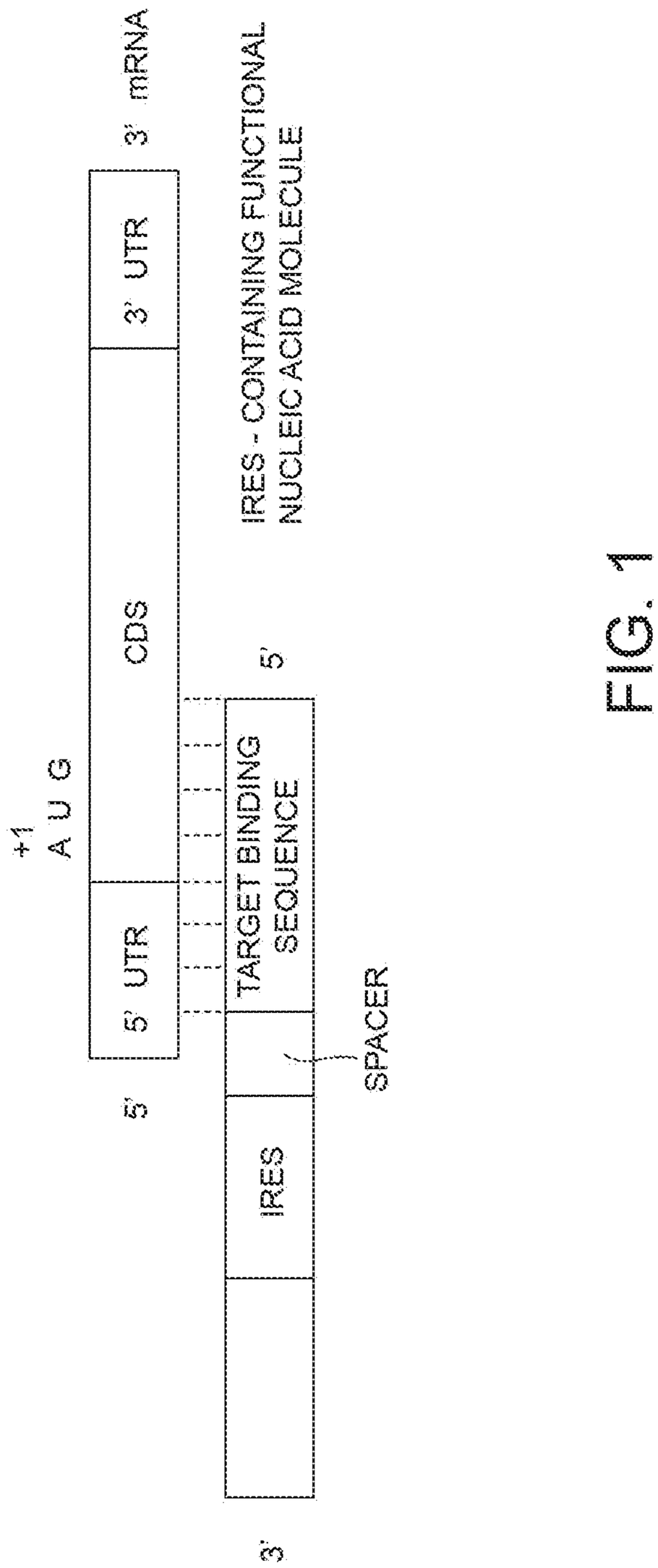
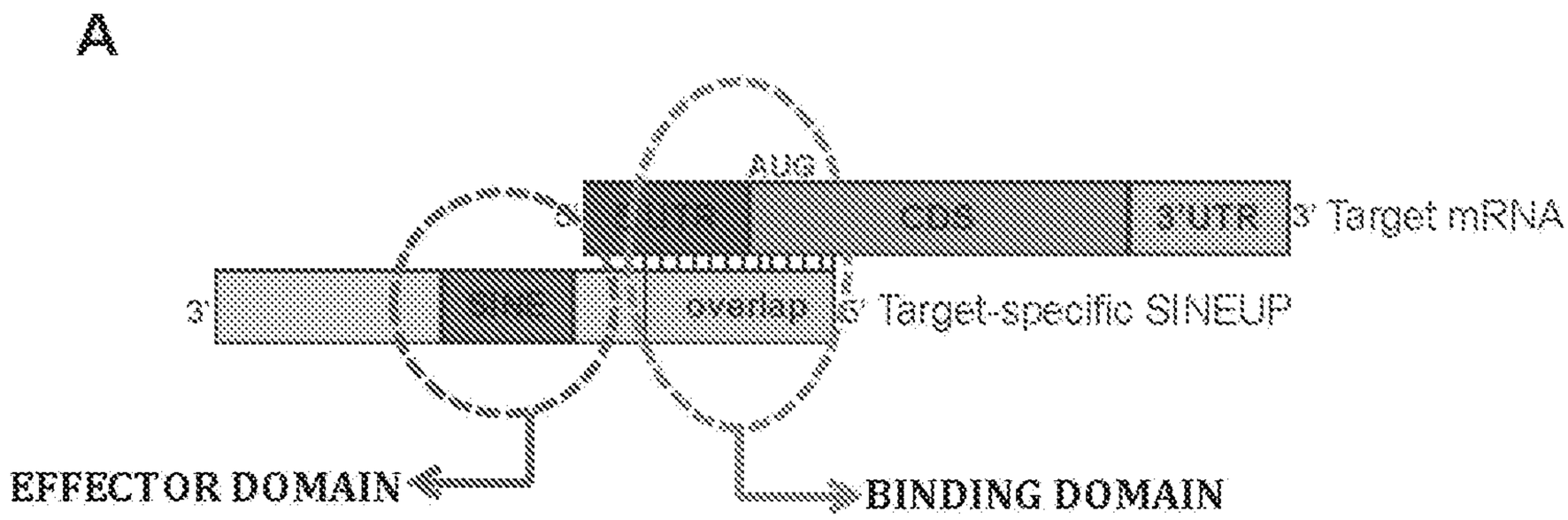

IRES - CONTAINING FUNCTIONAL NUCLEIC ACID MOLECULE
5'
IRES
SPACER
5' UTR
+1
A U G
CDS
3' UTR
5'
TARGET BINDING SEQUENCE
3'
mRNA

FIG. 1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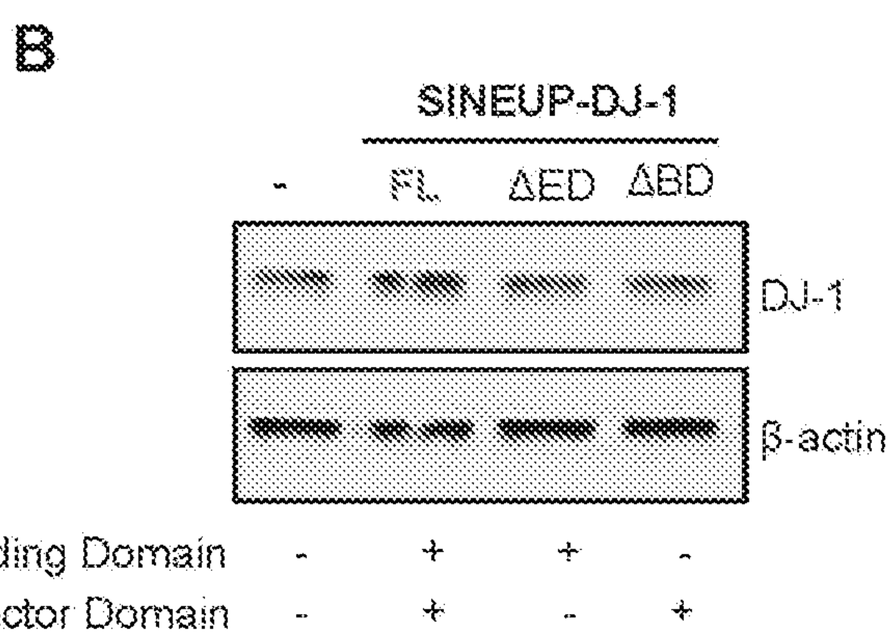
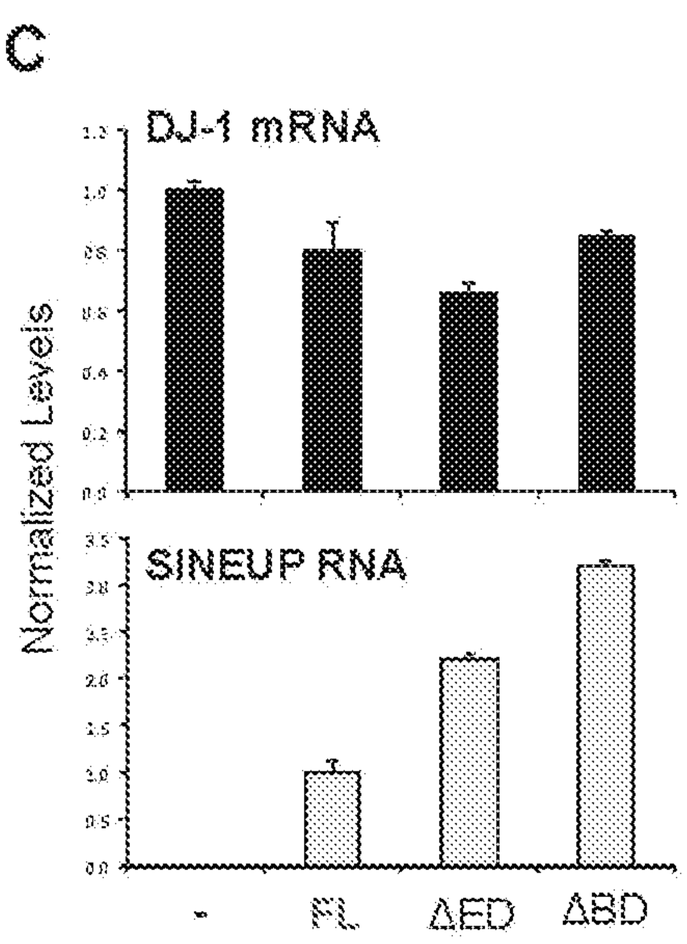
CDS
3'UTR
3' Target mRNA
overlap
5' Target-specific SINEUP
EFFECTOR DOMAIN
BINDING DOMAIN


B
SINEUP-DJ-1
-
FL
ΔED
ΔBD
DJ-1
β-actin
Binding Domain - + + -
Effector Domain - + - +


C
DJ-1 mRNA
Normalized Levels
SINEUP RNA
-
FL
ΔED
ΔBD

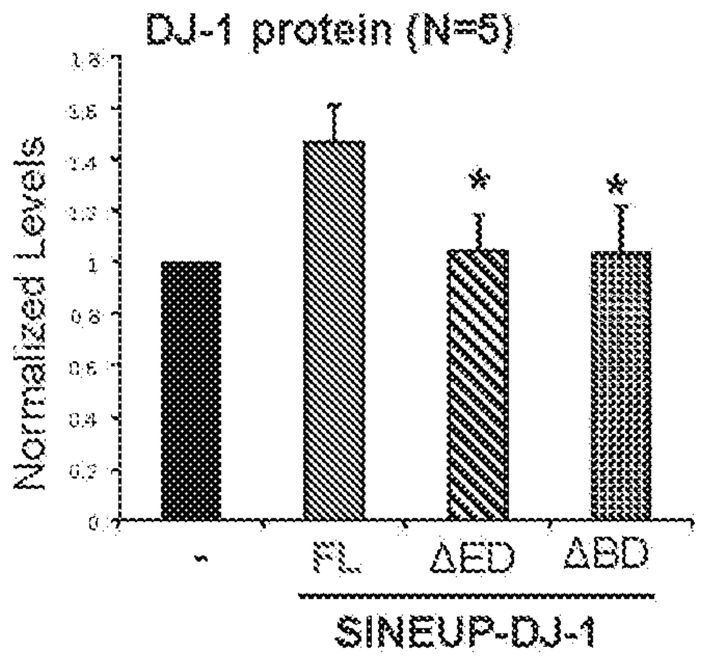

D
DJ-1 protein (N=5)
Normalized Levels
-
FL
ΔED
ΔBD
SINEUP-DJ-1

FIG. 2

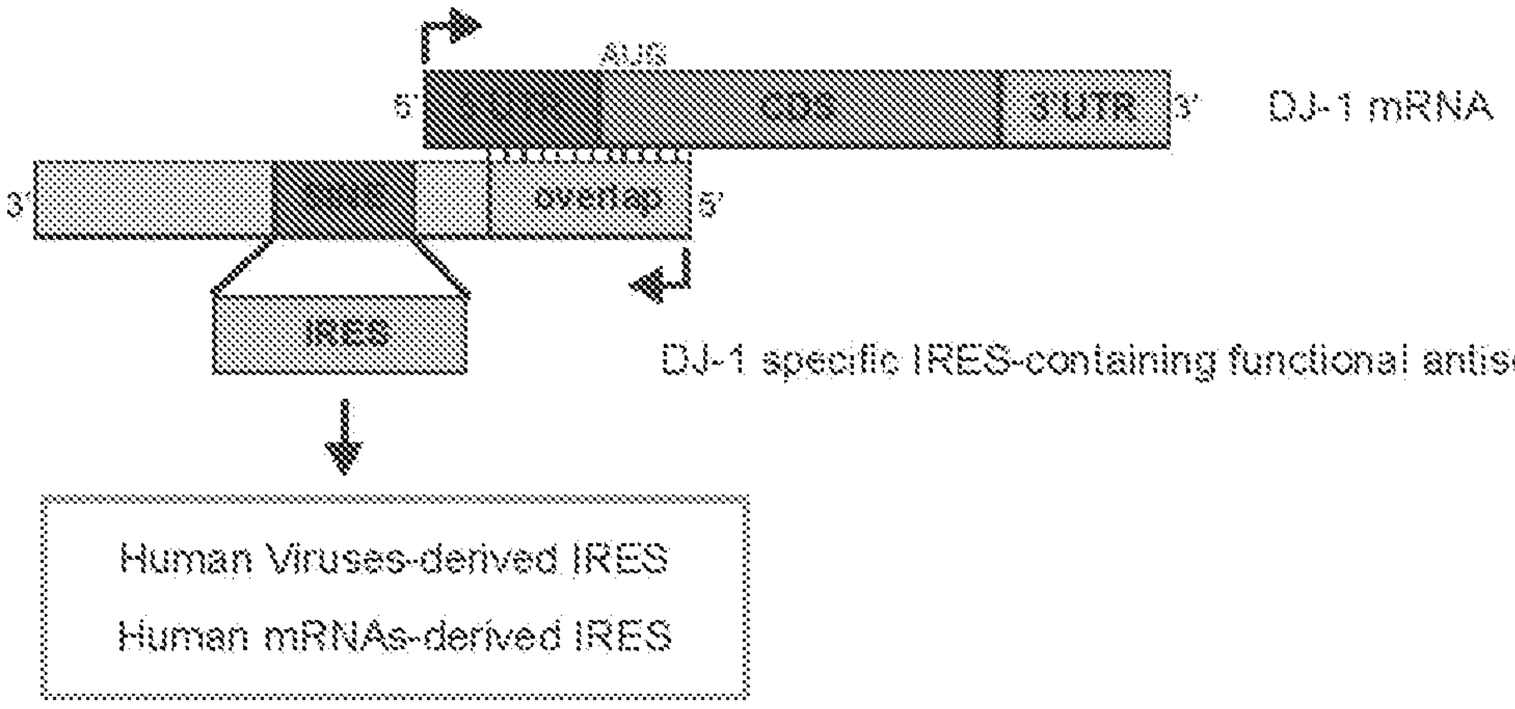

AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
3'
overlap
5'
IRES
DJ-1 specific IRES-containing functional antisense RNA
Human Viruses-derived IRES
Human mRNAs-derived IRES

FIG. 3

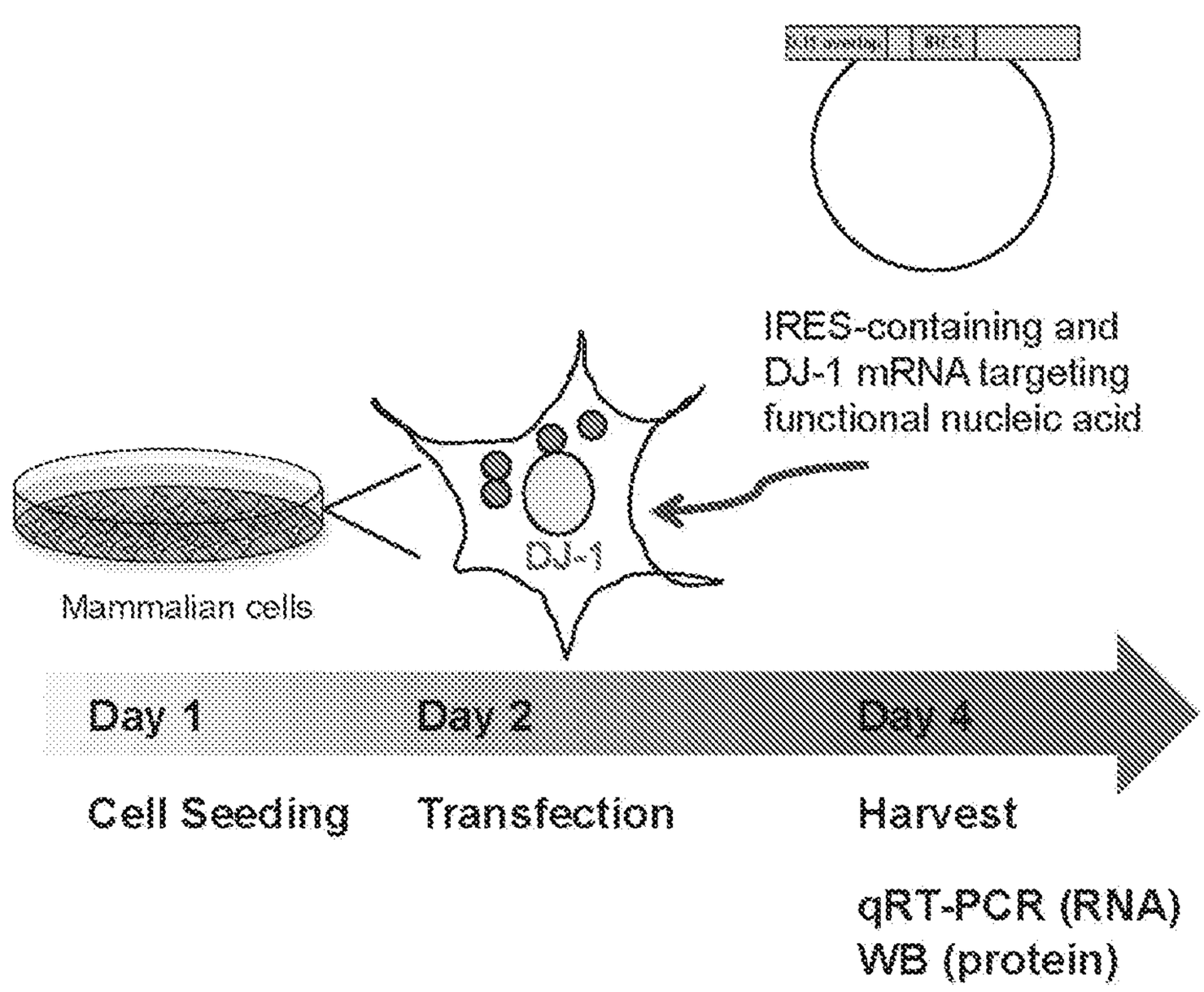

IRES-containing and
DJ-1 mRNA targeting
functional nucleic acid
DJ-1
Mammalian cells
Day 1
Day 2
Day 4
Cell Seeding
Transfection
Harvest
qRT-PCR (RNA)
WB (protein)

FIG. 4

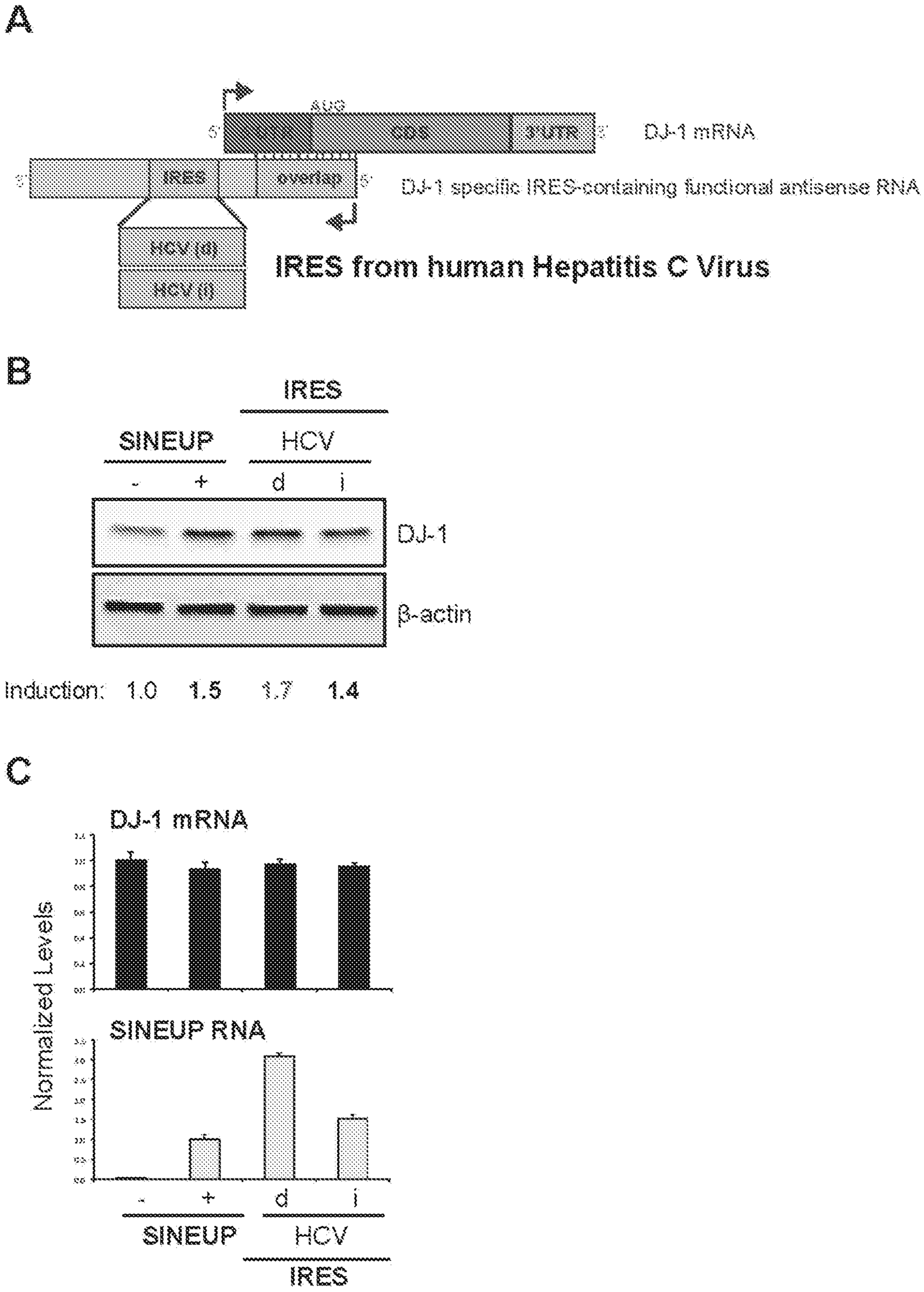

A
AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
3'
IRES
overlap
5'
DJ-1 specific IRES-containing functional antisense RNA
HCV (d)
HCV (i)
IRES from human Hepatitis C Virus
B
IRES
SINEUP
HCV
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.5 1.7 1.4
C
DJ-1 mRNA
Normalized Levels
SINEUP RNA
-
+
d
i
SINEUP
HCV
IRES

FIG. 5

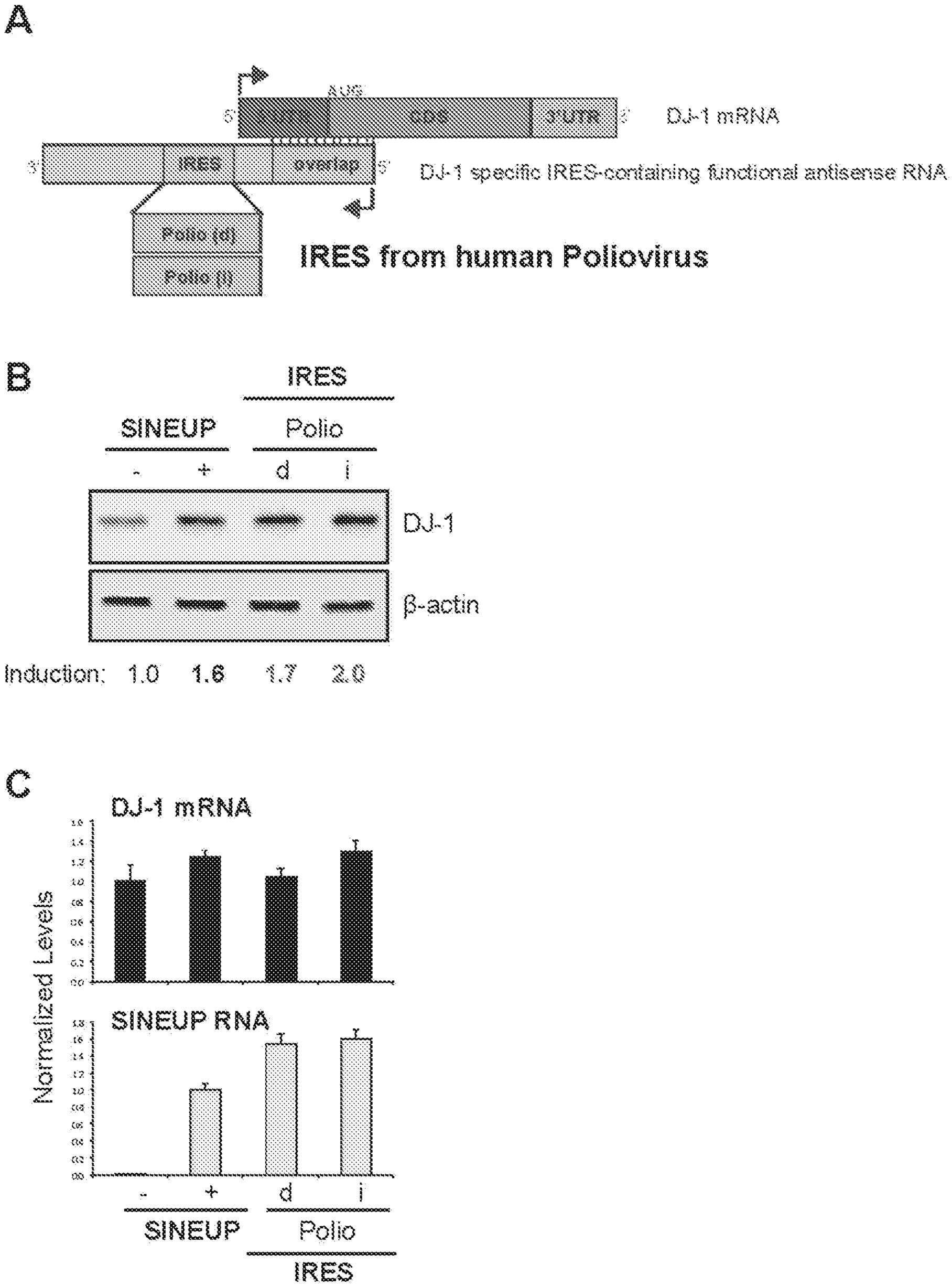
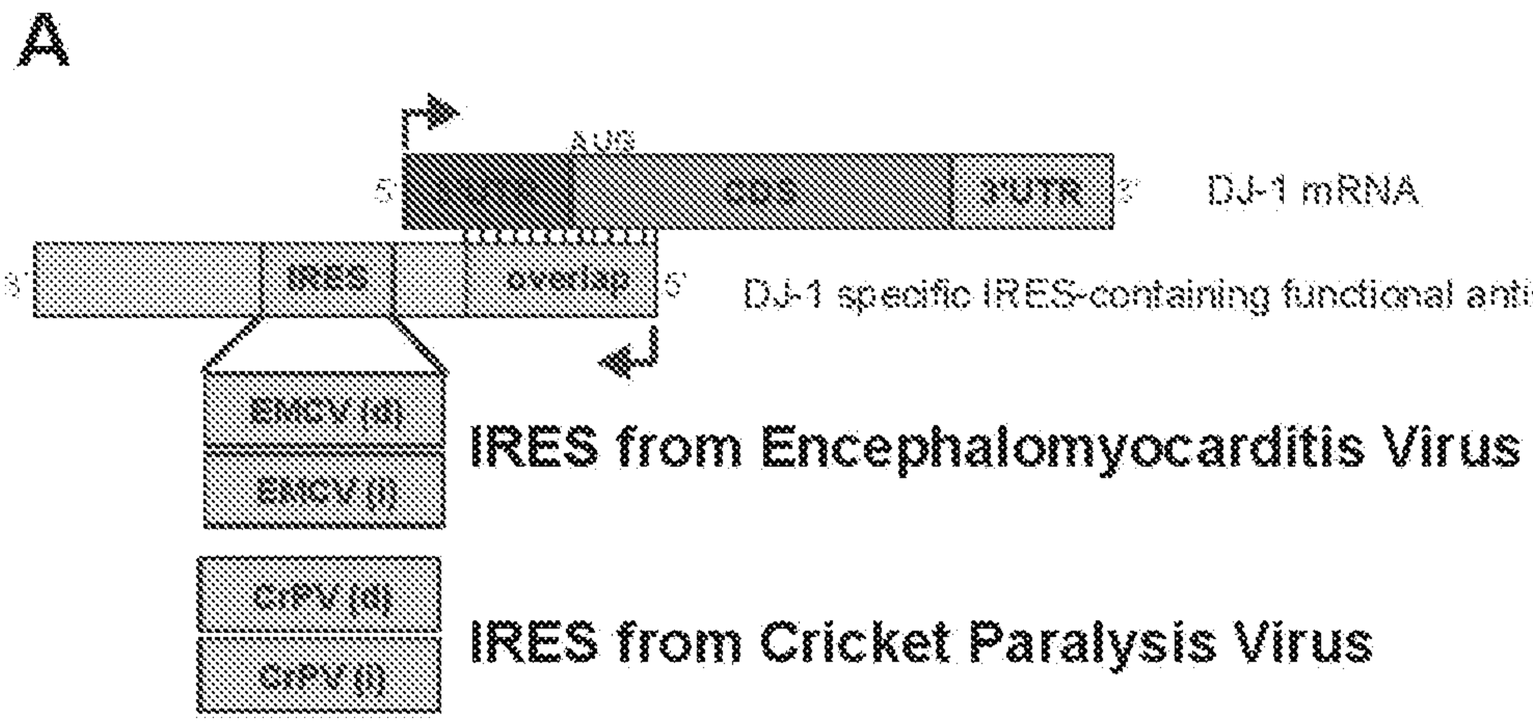
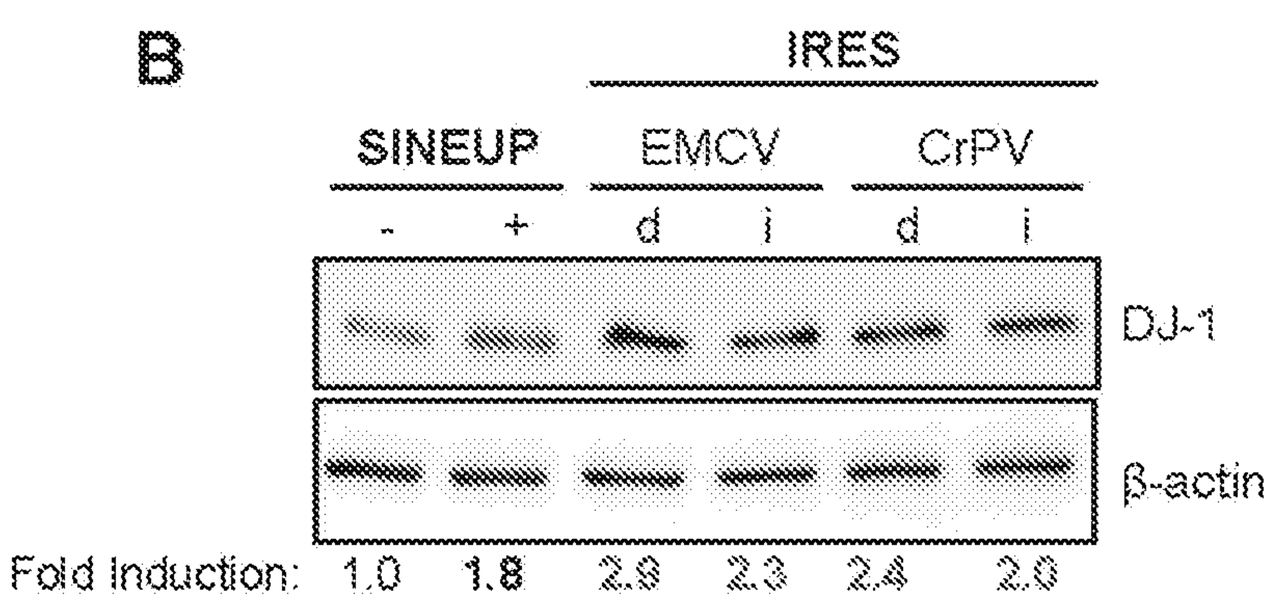

A
AUG
5'UTR
CDS
3'UTR
DJ-1 mRNA
IRES
overlap
DJ-1 specific IRES-containing functional antisense RNA
Polio (d)
Polio (i)
IRES from human Poliovirus
B
IRES
SINEUP
Polio
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.6 1.7 2.0
C
DJ-1 mRNA
Normalized Levels
SINEUP RNA
-
+
d
i
SINEUP
Polio
IRES

FIG. 6


A
AUG
5'
3'
DJ-1 mRNA
IRES
overlap
DJ-1 specific IRES-containing functional antisense RNA
EMCV (d)
EMCV (i)
IRES from Encephalomyocarditis Virus
CrPV (d)
CrPV (i)
IRES from Cricket Paralysis Virus


B
IRES
SINEUP
EMCV
CrPV
-
+
d
i
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.8 2.8 2.3 2.4 2.0

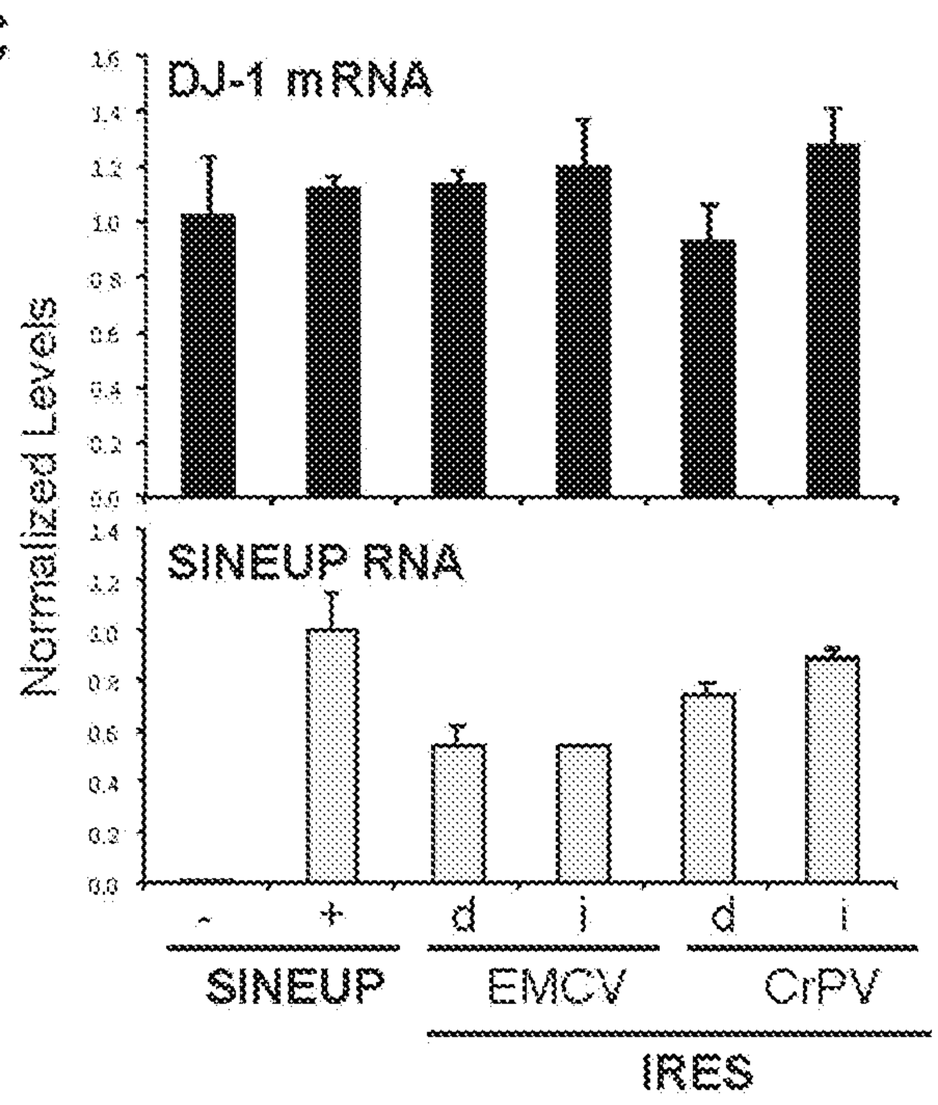
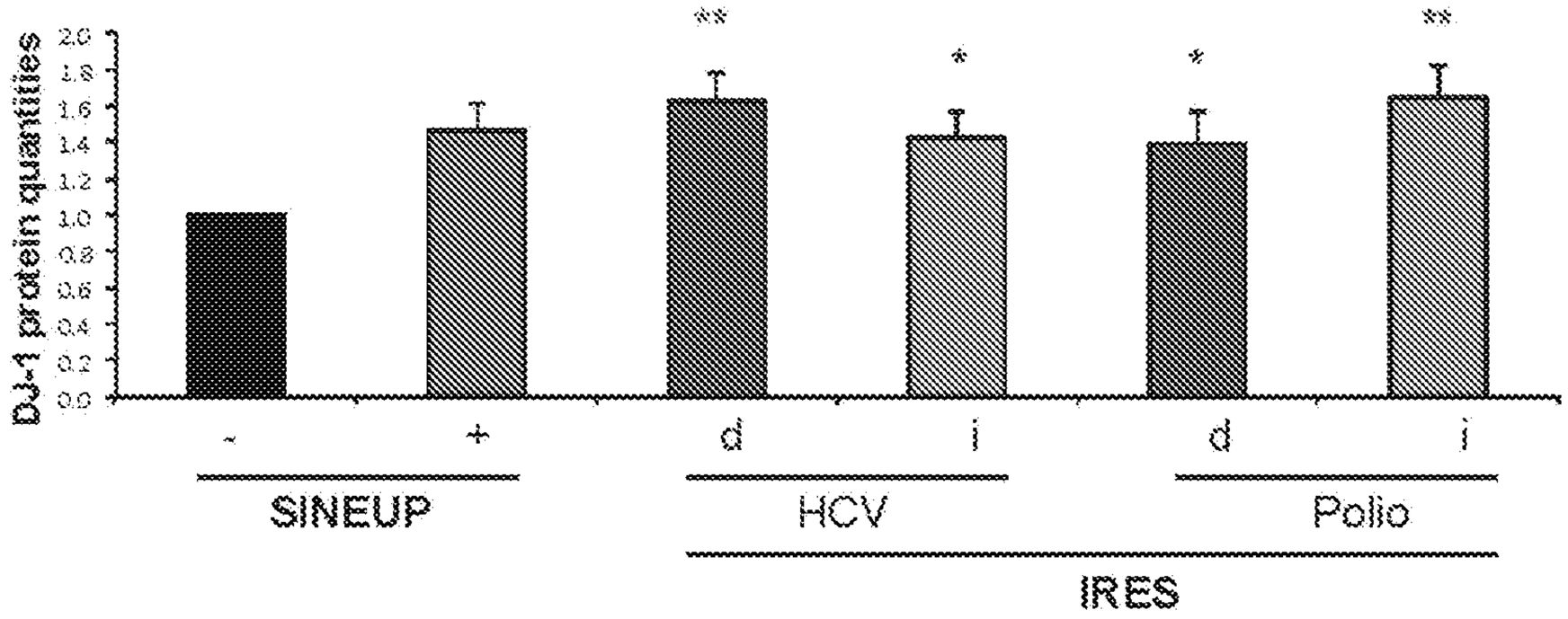

C
DJ-1 mRNA
SINEUP RNA
Normalized Levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-
+
d
i
d
i
SINEUP
EMCV
CrPV
IRES

FIG. 7

A


DJ-1 protein quantities
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-
+
d
i
d
i
SINEUP
HCV
Polio
IRES
**
*
*
**

B

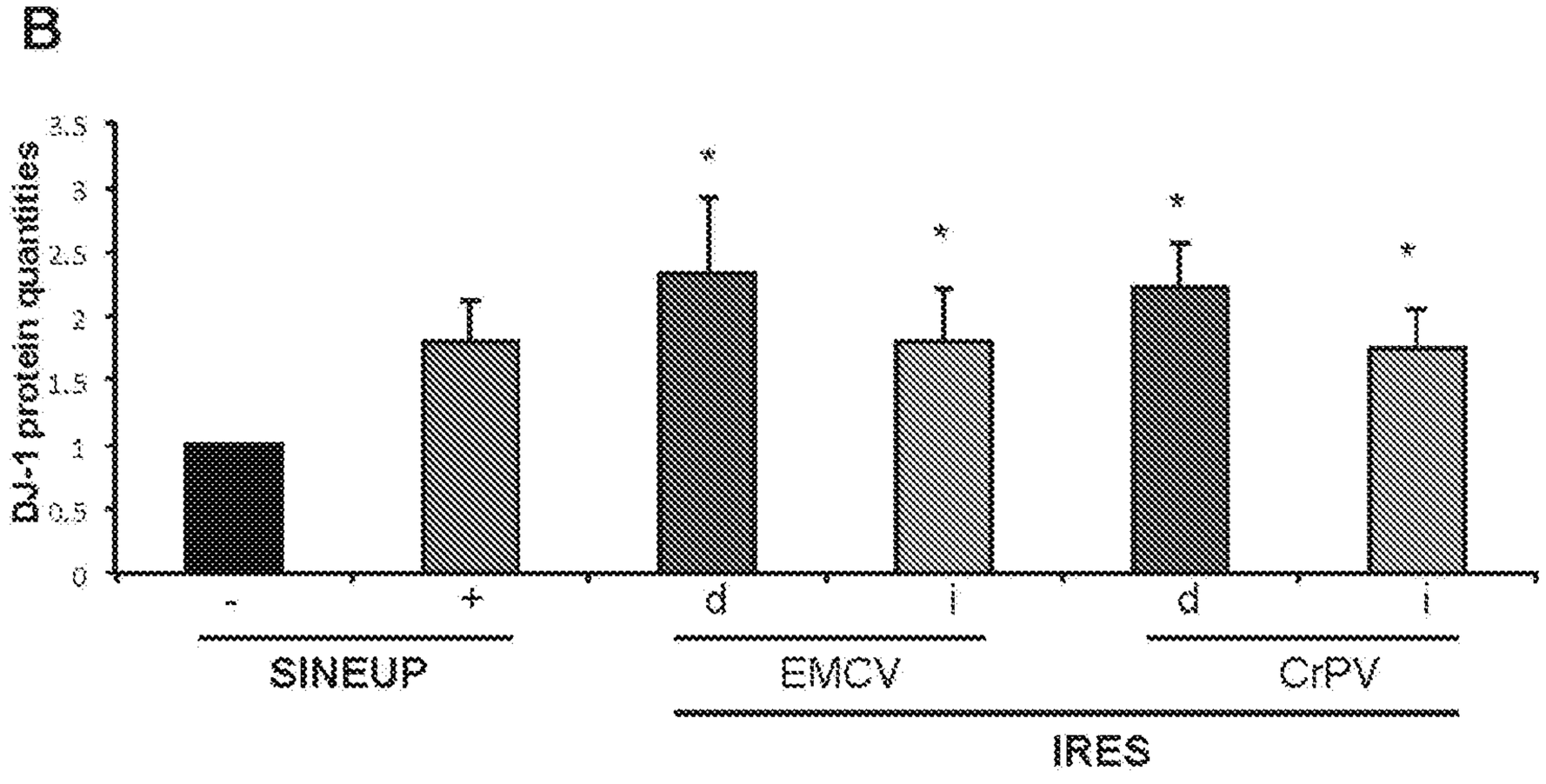

DJ-1 protein quantities
3.5
3
2.5
2
1.5
1
0.5
0
-
+
d
i
d
i
SINEUP
EMCV
CrPV
IRES
*
*
*
*

FIG. 8

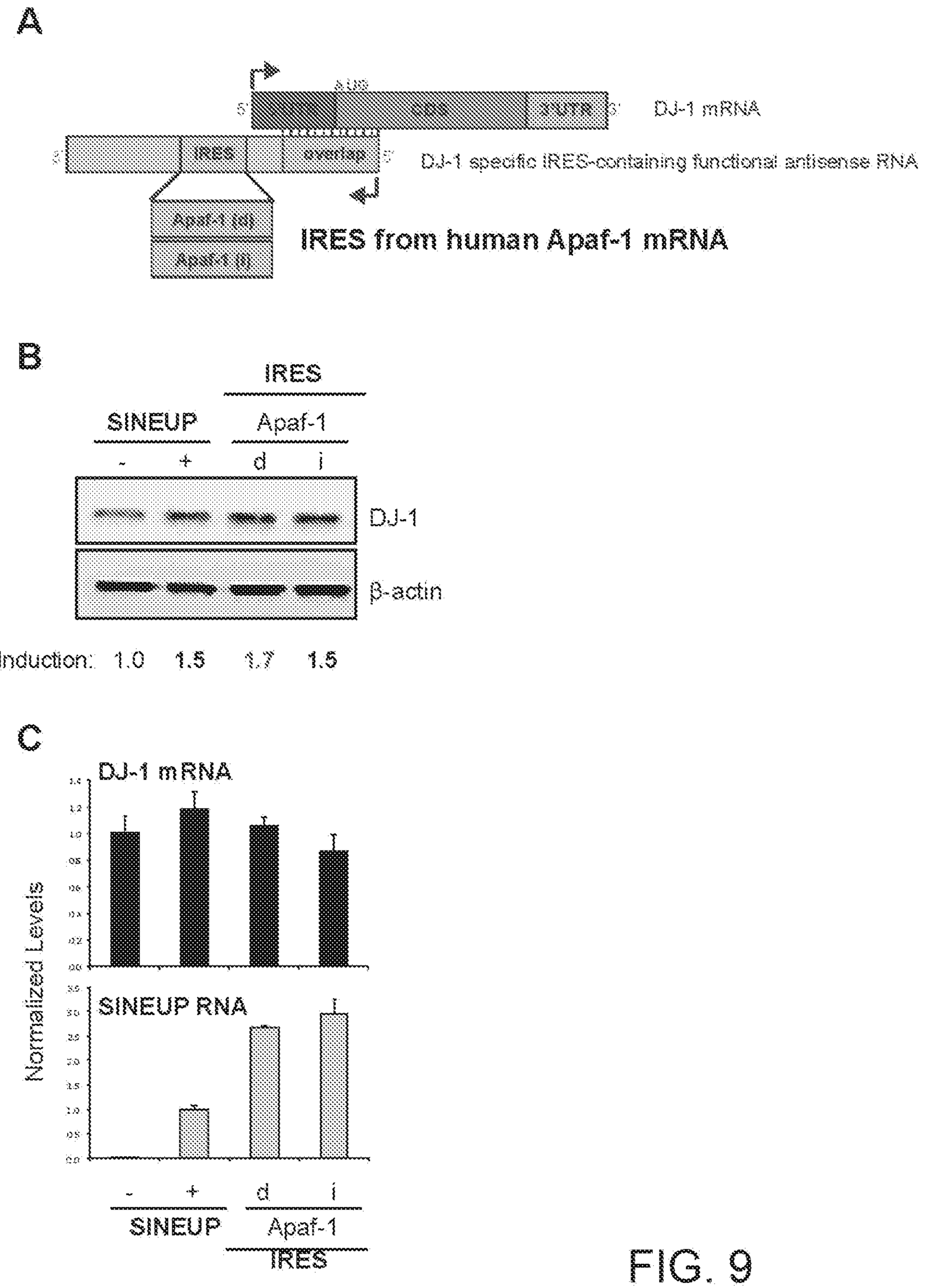
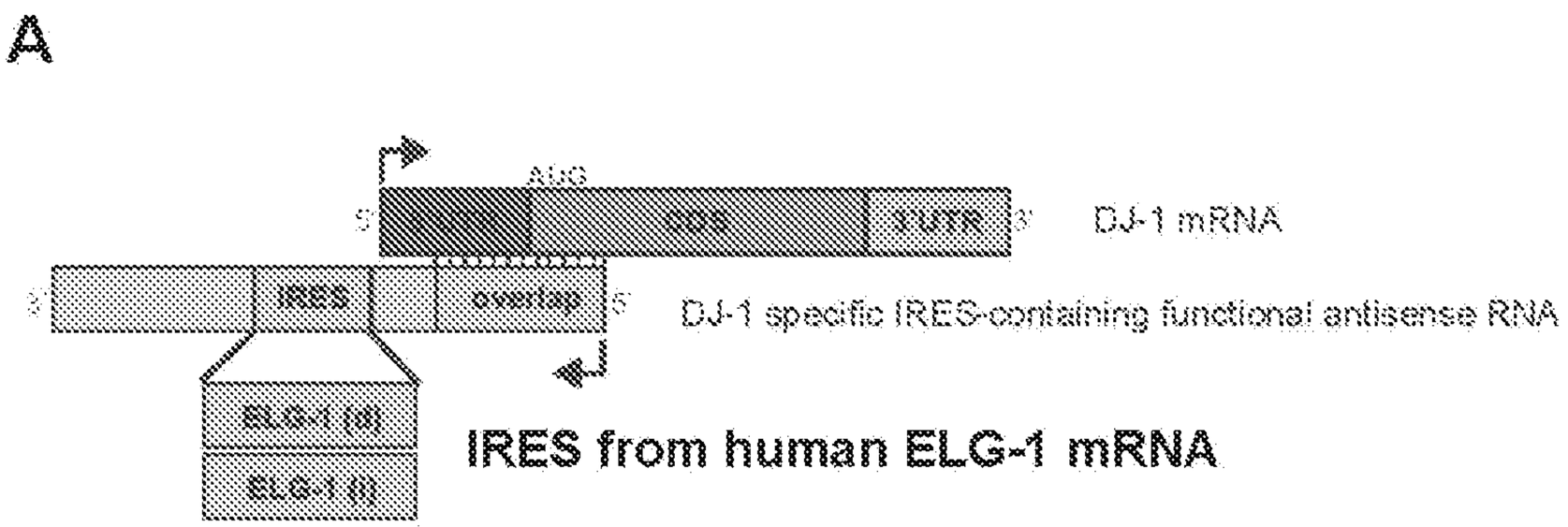
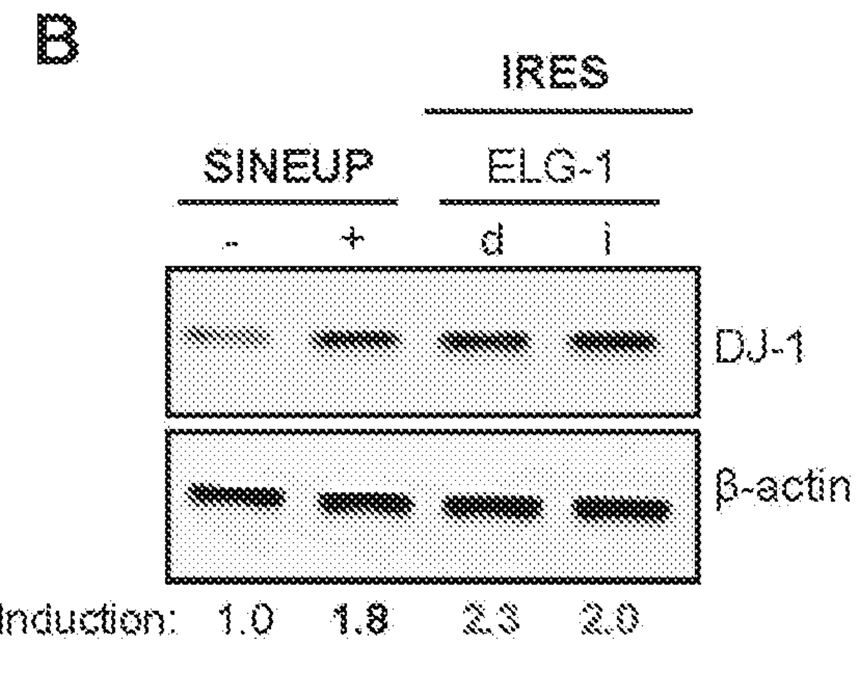

A
AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
5'
IRES
overlap
3'
DJ-1 specific IRES-containing functional antisense RNA
Apaf-1 (d)
Apaf-1 (i)
IRES from human Apaf-1 mRNA
B
IRES
SINEUP
Apaf-1
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.5 1.7 1.5
C
DJ-1 mRNA
SINEUP RNA
Normalized Levels
- + d i
SINEUP Apaf-1
IRES

FIG. 9


A
AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
IRES
overlap
5'
DJ-1 specific IRES-containing functional antisense RNA
ELG-1 (d)
ELG-1 (i)
IRES from human ELG-1 mRNA


B
IRES
SINEUP
ELG-1
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.8 2.3 2.0

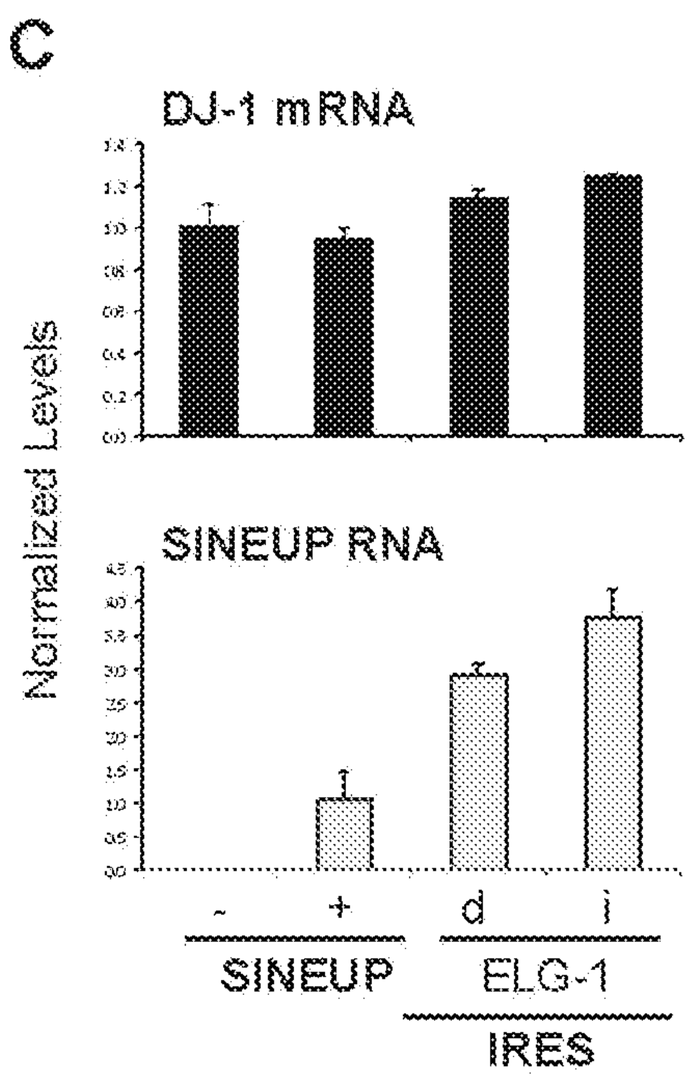
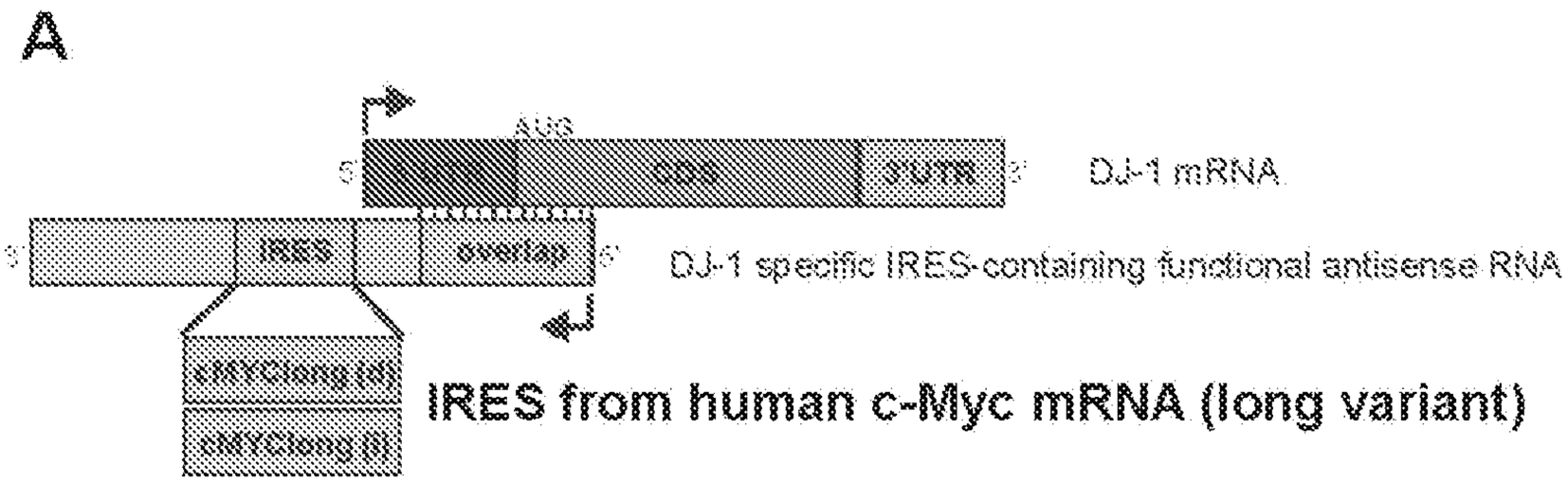
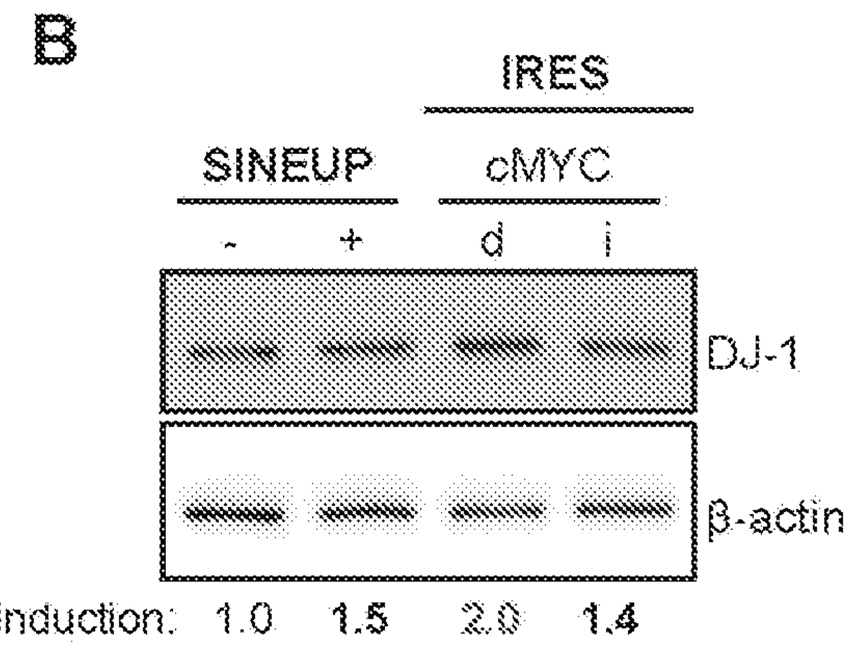

C
DJ-1 mRNA
Normalized Levels
SINEUP RNA
-
+
d
i
SINEUP
ELG-1
IRES

FIG. 10


A
AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
IRES
overlap
5'
DJ-1 specific IRES-containing functional antisense RNA
cMYClong (i)
cMYClong (d)
IRES from human c-Myc mRNA (long variant)


B
IRES
SINEUP
cMYC
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.5 2.0 1.4

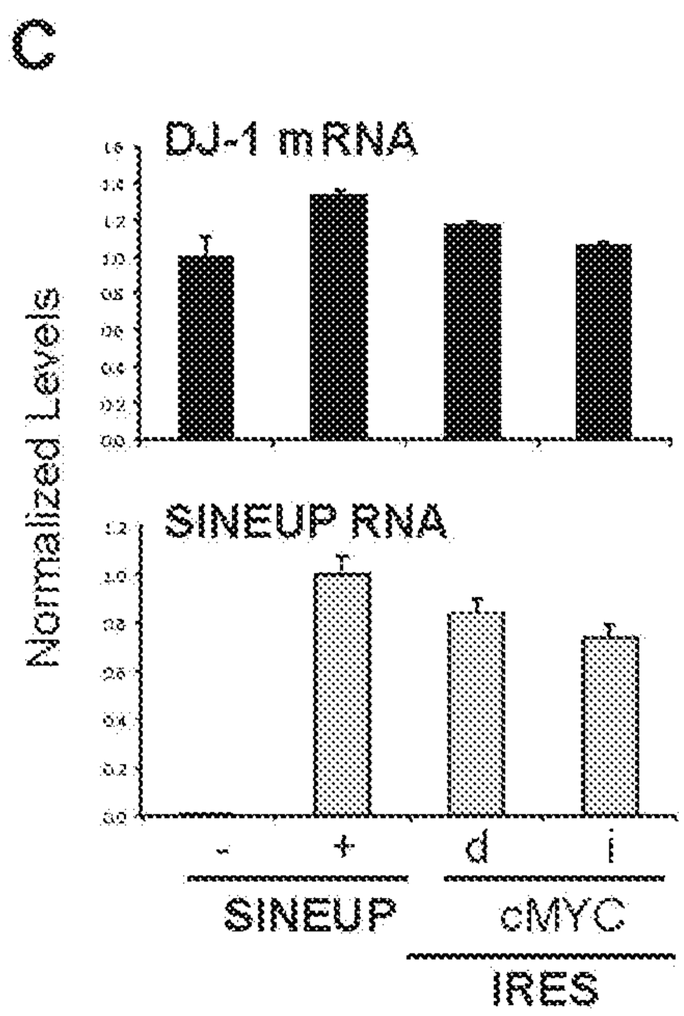

C
DJ-1 mRNA
Normalized Levels
SINEUP RNA
-
+
d
i
SINEUP
cMYC
IRES

FIG. 11

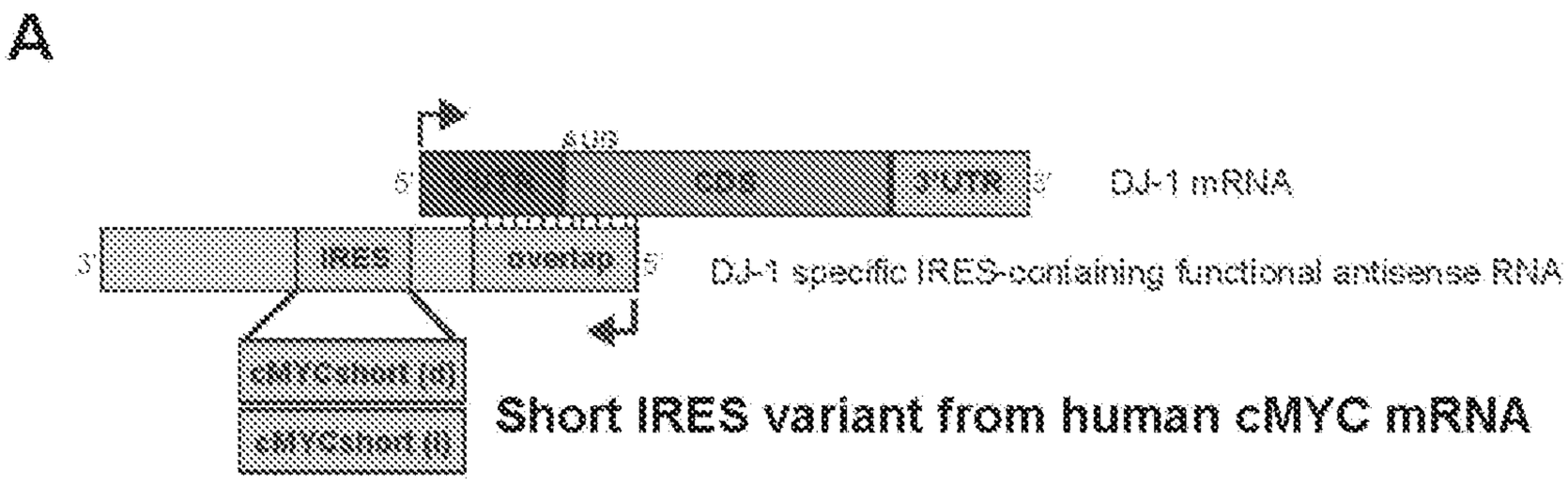
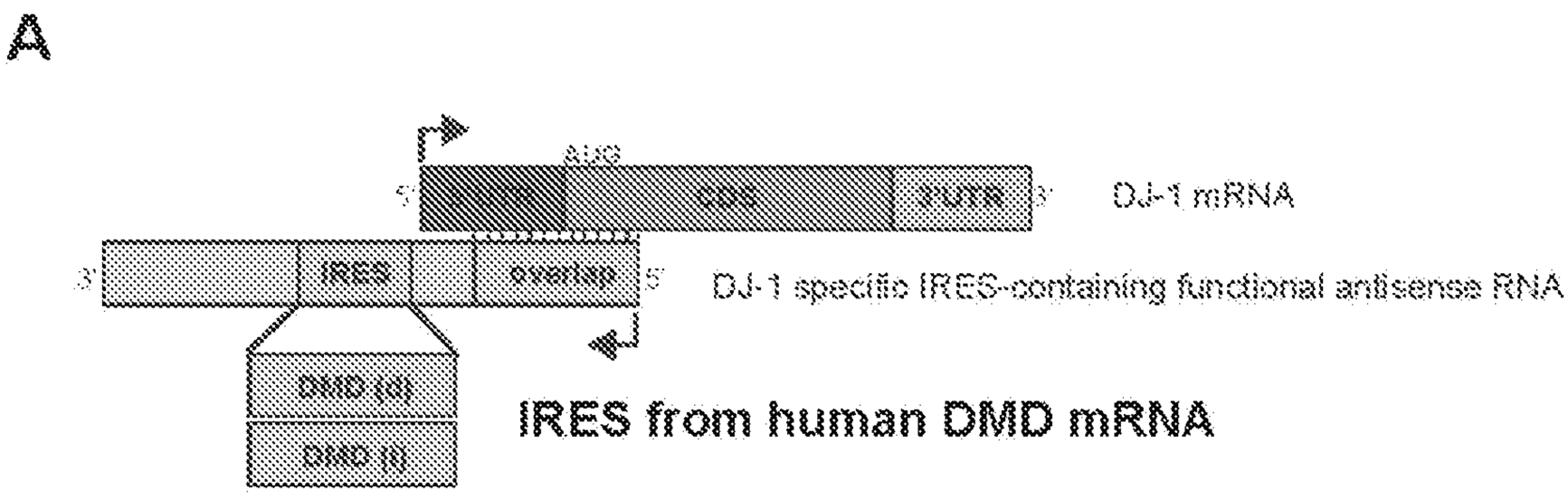
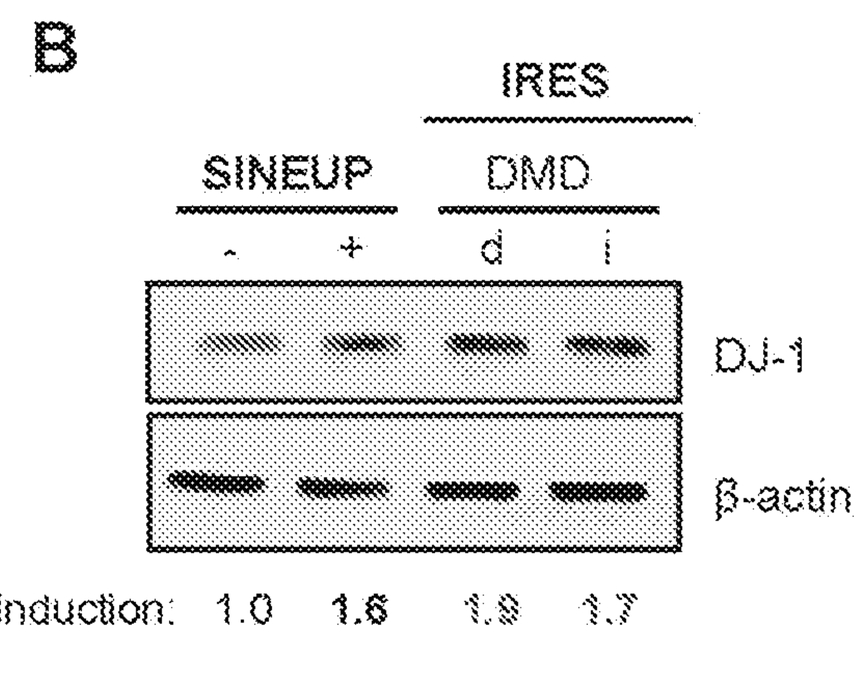

A
5'UTR
CDS
3'UTR
5'
3'
DJ-1 mRNA
IRES
overlap
DJ-1 specific IRES-containing functional antisense RNA
Short IRES variant from human cMYC mRNA
B
IRES
SINEUP
cMYC
-
+
d
i
DJ-1
β-actin
Fold induction: 1.0 1.6 2.0 1.6
C
DJ-1 mRNA
Normalized Levels
SINEUP RNA
SINEUP
cMYC
IRES

FIG. 12


A
AUG
5'
3'
DJ-1 mRNA
IRES
overlap
DJ-1 specific IRES-containing functional antisense RNA
DMD (d)
DMD (i)
IRES from human DMD mRNA


B
IRES
SINEUP
DMD
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.6 1.9 1.7

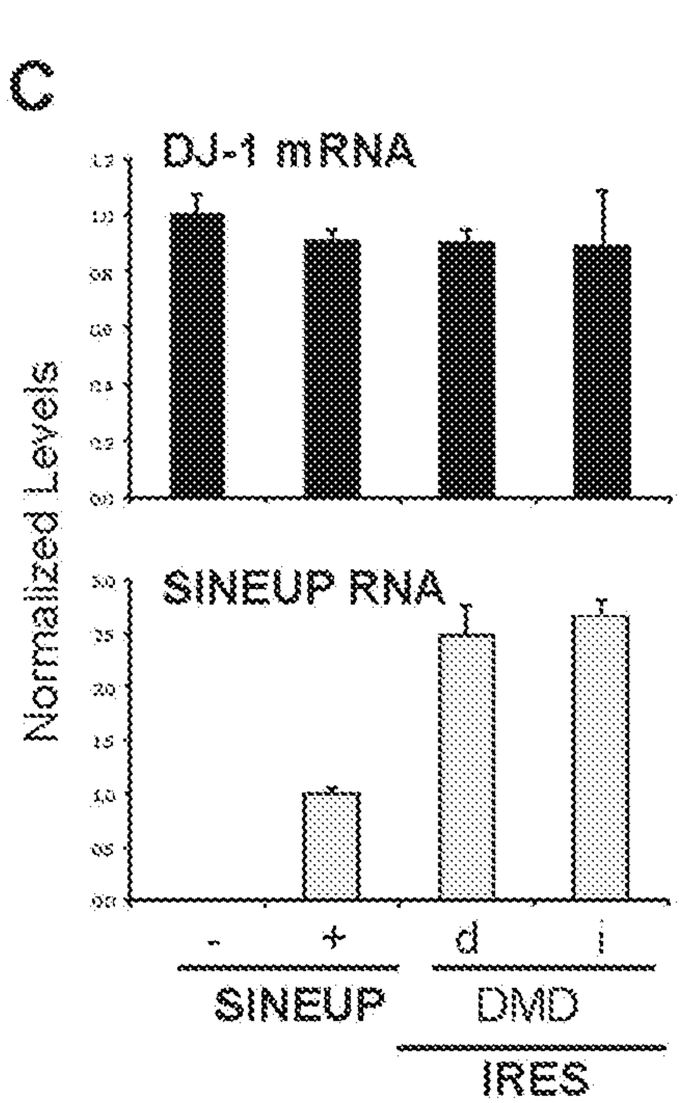
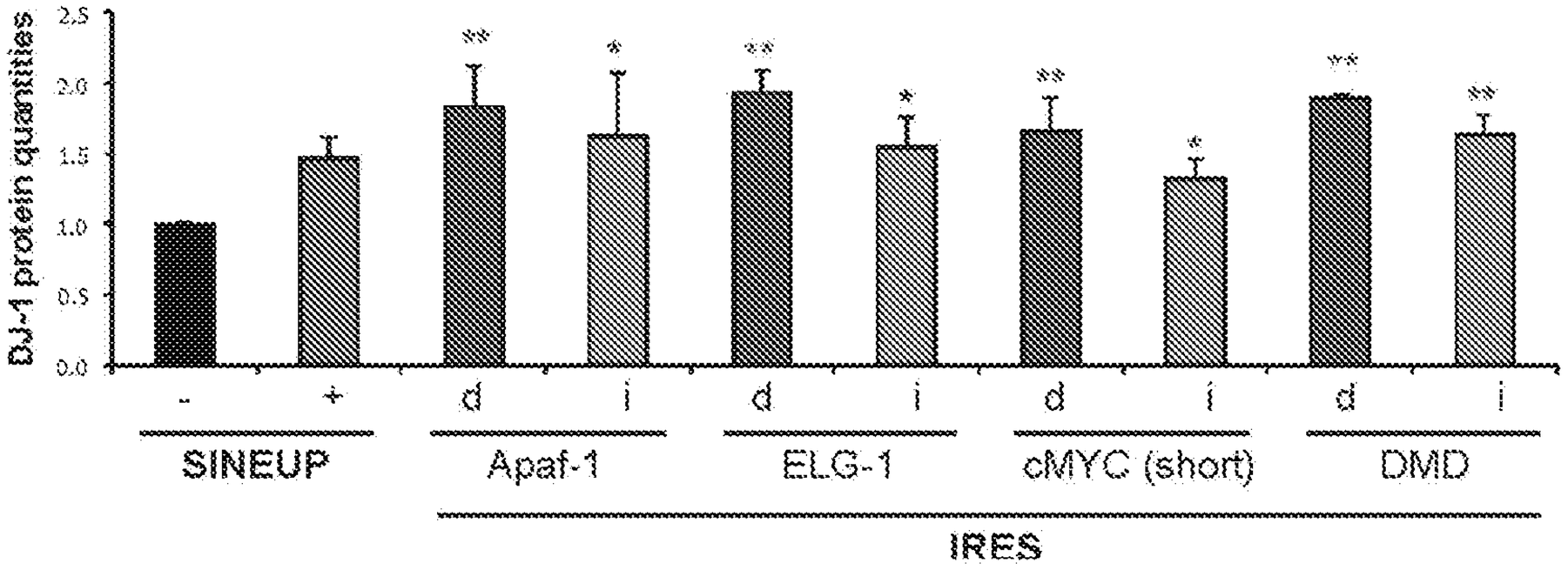

C
DJ-1 mRNA
SINEUP RNA
Normalized Levels
-
+
d
i
SINEUP
DMD
IRES

FIG. 13

A


DJ-1 protein quantities
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
*
**
*
**
**
-
+
d
i
d
i
d
i
d
i
SINEUP
Apaf-1
ELG-1
cMYC (short)
DMD
IRES

B

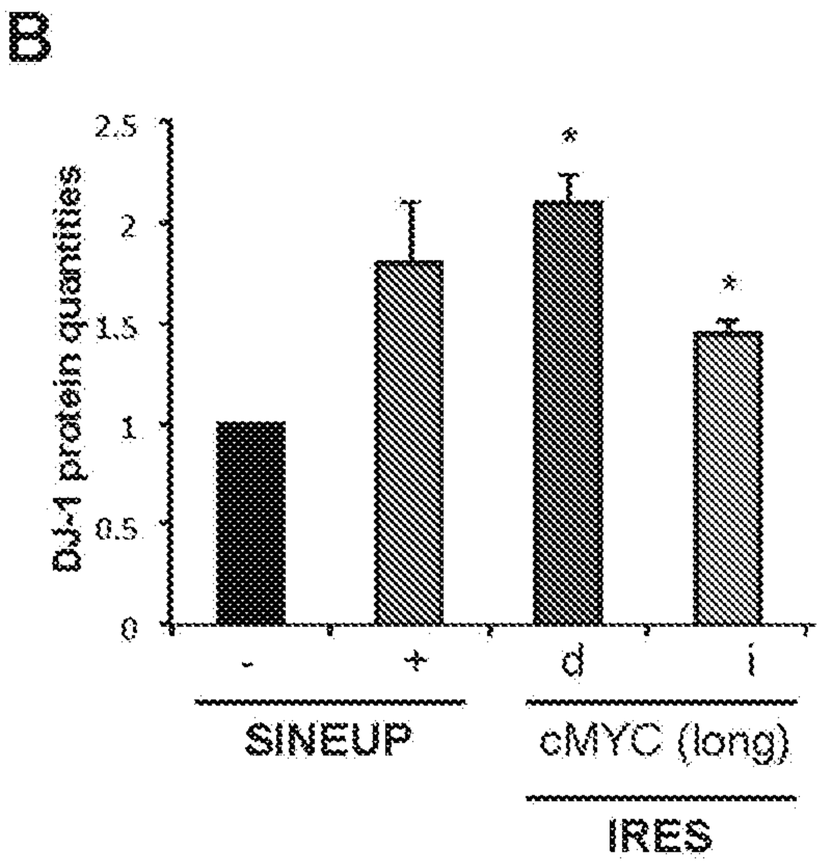

DJ-1 protein quantities
2.5
2
1.5
1
0.5
0
*
*
-
+
d
i
SINEUP
cMYC (long)
IRES

FIG. 14

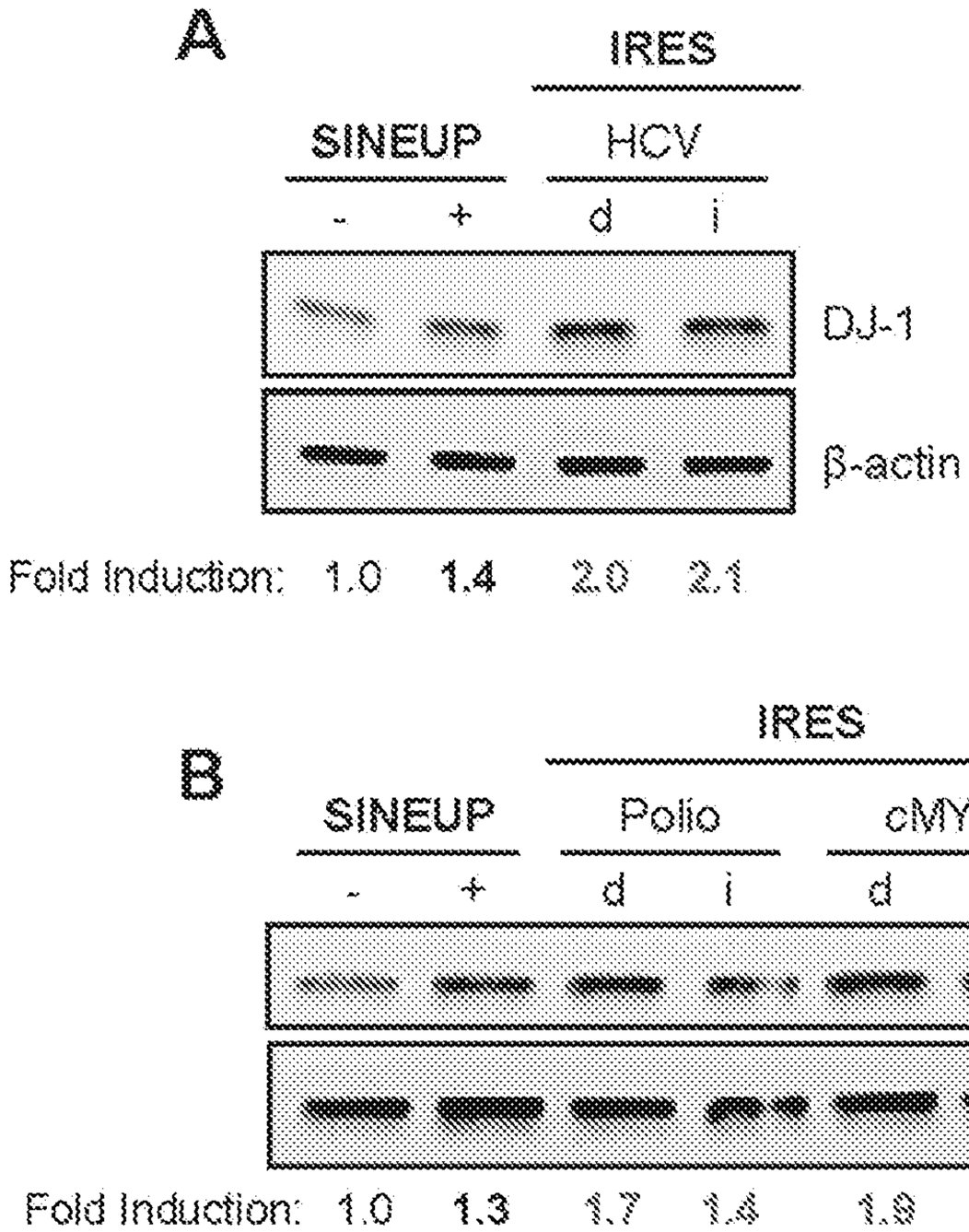
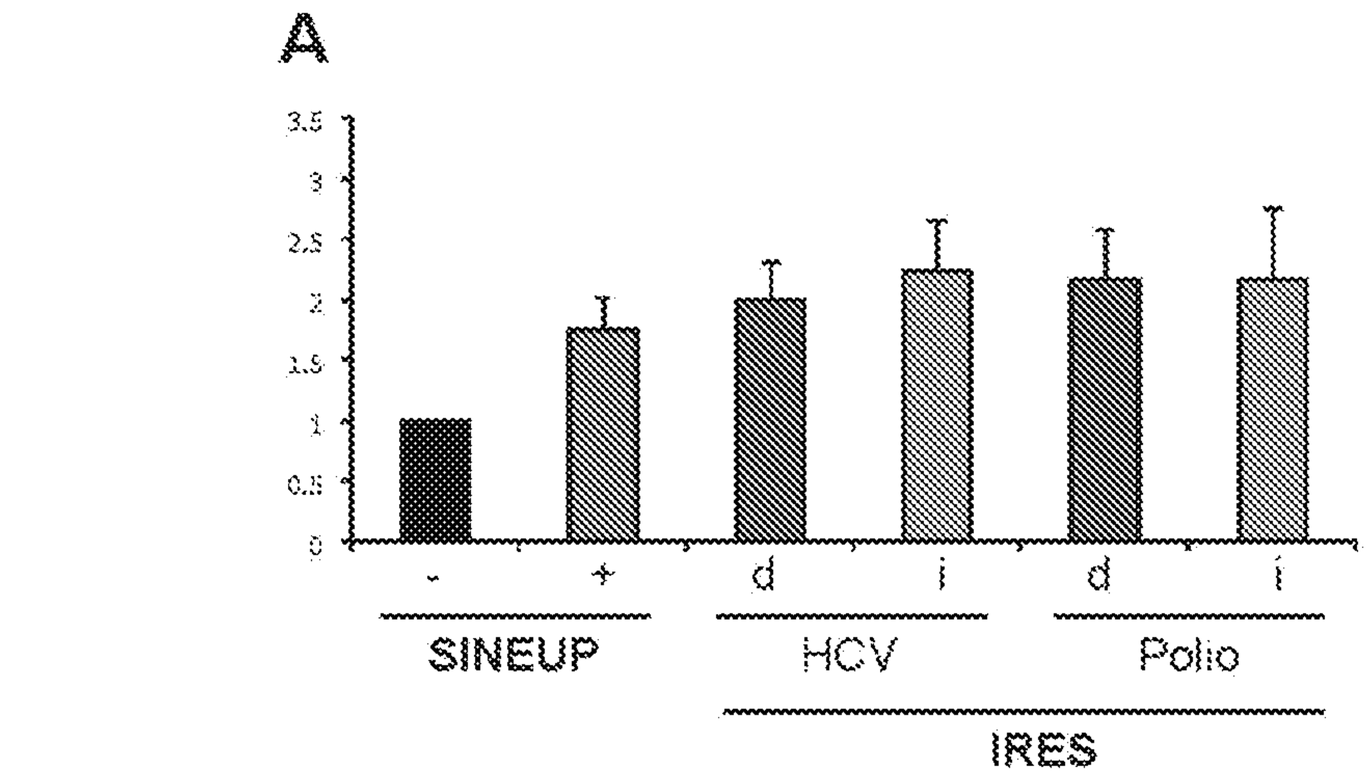

A
IRES
SINEUP
HCV
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.4 2.0 2.1
B
IRES
SINEUP
Polio
cMYC
-
+
d
i
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.3 1.7 1.4 1.9 1.6
C
IRES
SINEUP
Apaf-1
ELG-1
-
+
d
i
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.8 1.9 1.8 1.9 1.6
D
IRES
SINEUP
DMD
-
+
d
i
DJ-1
β-actin
Fold Induction: 1.0 1.2 1.4 1.8

FIG. 15


A
3.5
3
2.5
2
1.5
1
0.5
0
-
+
d
i
d
i
SINEUP
HCV
Polio
IRES

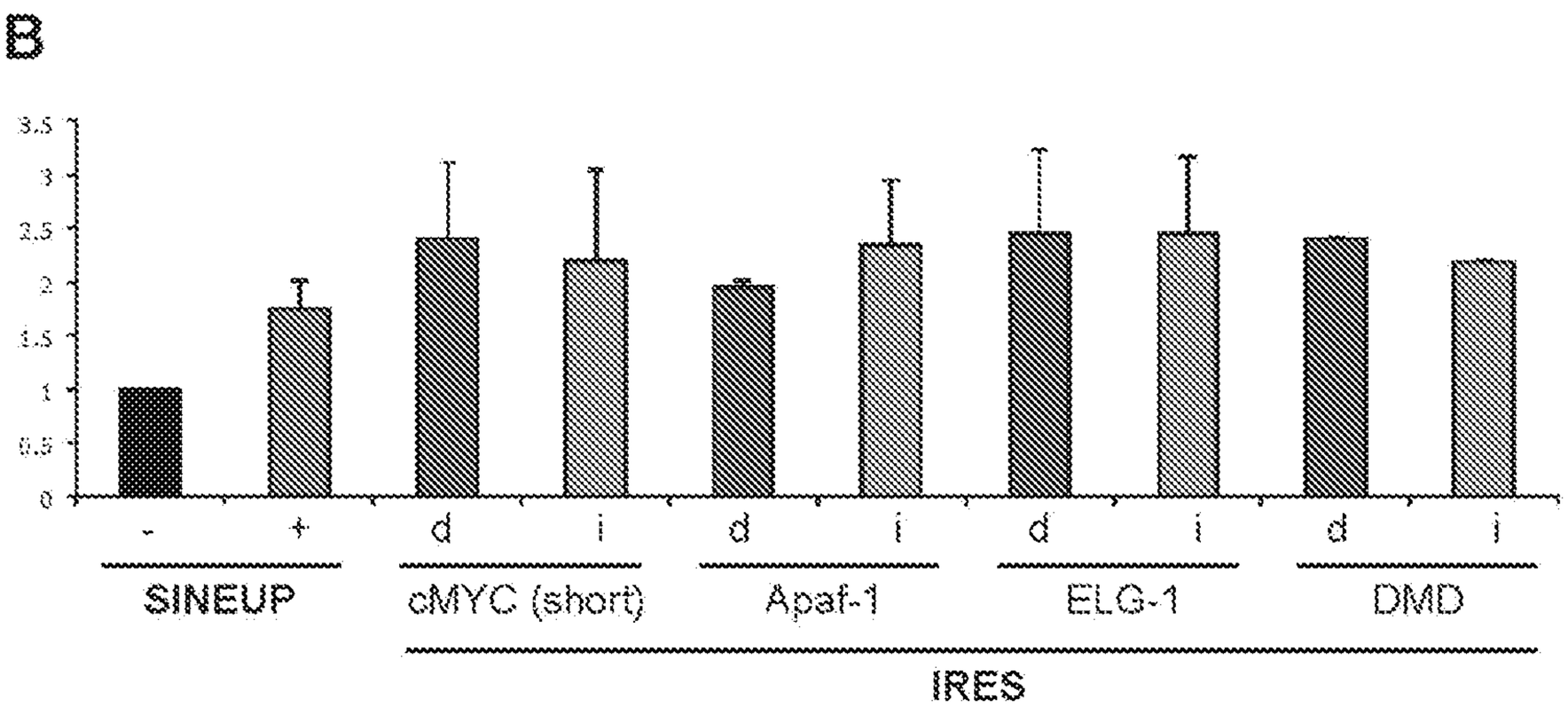

B
3.5
3
2.5
2
1.5
1
0.5
0
-
+
d
i
d
i
d
i
d
i
SINEUP
cMYC (short)
Apaf-1
ELG-1
DMD
IRES

FIG. 16

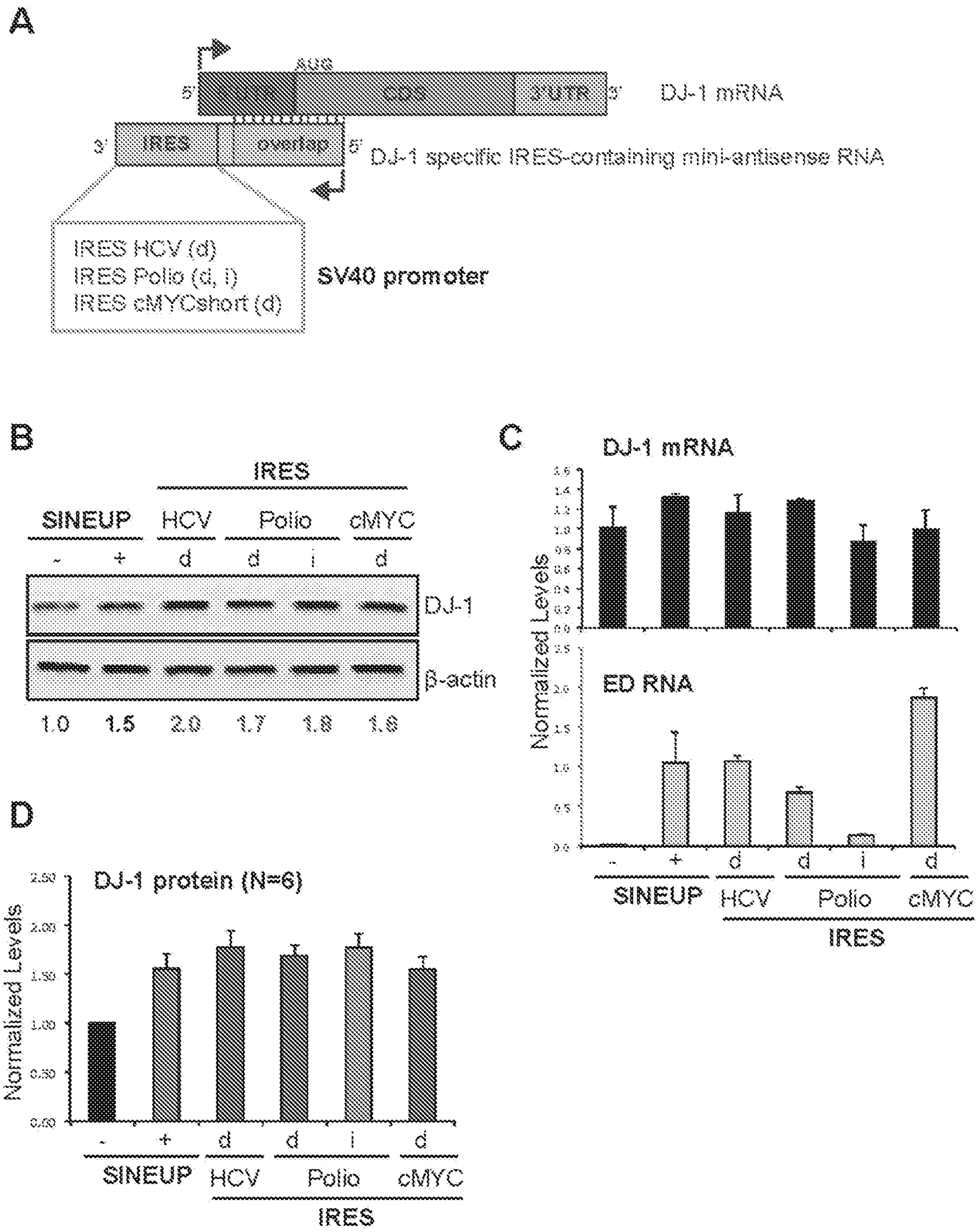

A
AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
3'
IRES
overlap
5'
DJ-1 specific IRES-containing mini-antisense RNA
IRES HCV (d)
IRES Polio (d, i)
IRES cMYCshort (d)
SV40 promoter
B
IRES
SINEUP
HCV
Polio
cMYC
-
+
d
d
i
d
DJ-1
β-actin
1.0
1.5
2.0
1.7
1.8
1.6
C
DJ-1 mRNA
Normalized Levels
ED RNA
SINEUP
HCV
Polio
cMYC
IRES
D
DJ-1 protein (N=6)
Normalized Levels
SINEUP
HCV
Polio
cMYC
IRES

FIG. 17

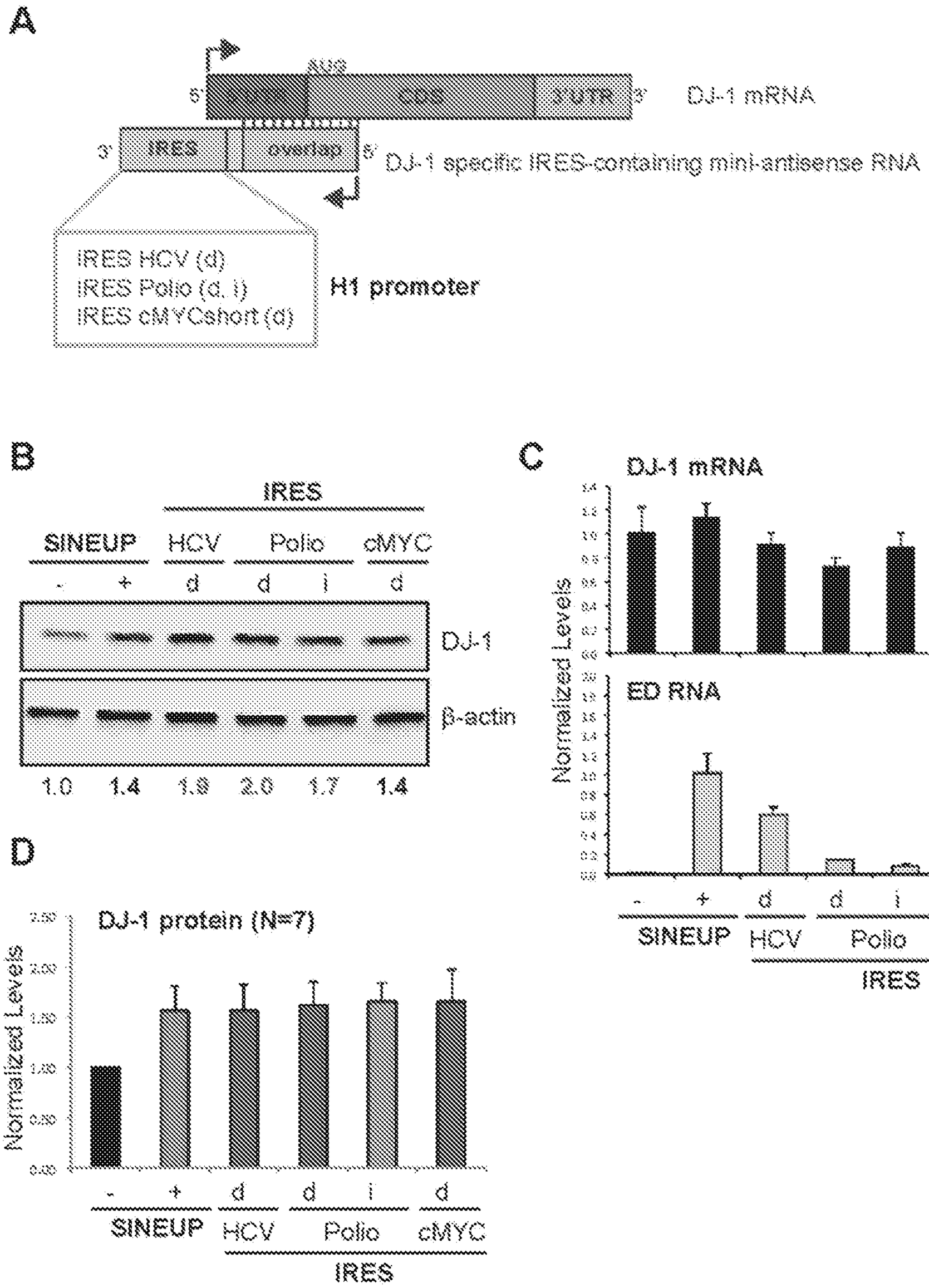
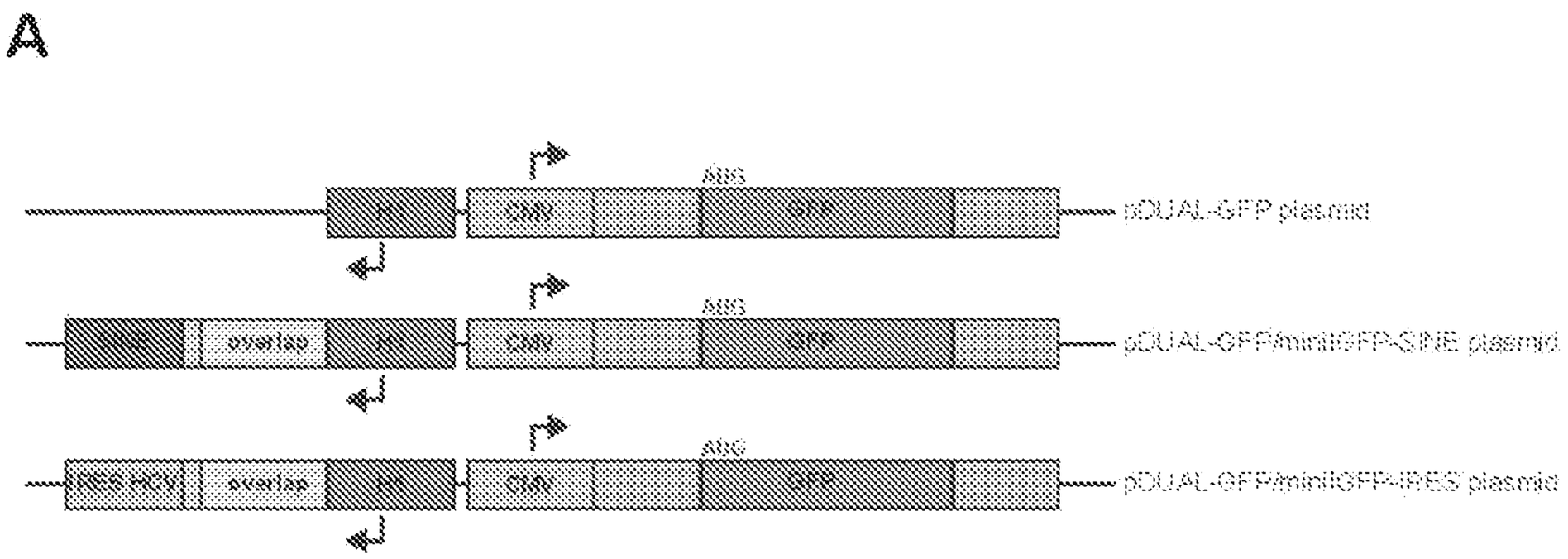

A
AUG
5'
CDS
3'UTR
3'
DJ-1 mRNA
IRES
overlap
5'
DJ-1 specific IRES-containing mini-antisense RNA
IRES HCV (d)
IRES Polio (d, i)
IRES cMYCshort (d)
H1 promoter
B
IRES
SINEUP
HCV
Polio
cMYC
-
+
d
d
i
d
DJ-1
β-actin
1.0
1.4
1.9
2.0
1.7
1.4
C
DJ-1 mRNA
Normalized Levels
ED RNA
SINEUP
HCV
Polio
cMYC
IRES
D
DJ-1 protein (N=7)
Normalized Levels
SINEUP
HCV
Polio
cMYC
IRES

FIG. 18


A
pDUAL-GFP plasmid
pDUAL-GFP/miniIGFP-SINE plasmid
pDUAL-GFP/miniIGFP-IRES plasmid
overlap
CMV
ATG
GFP

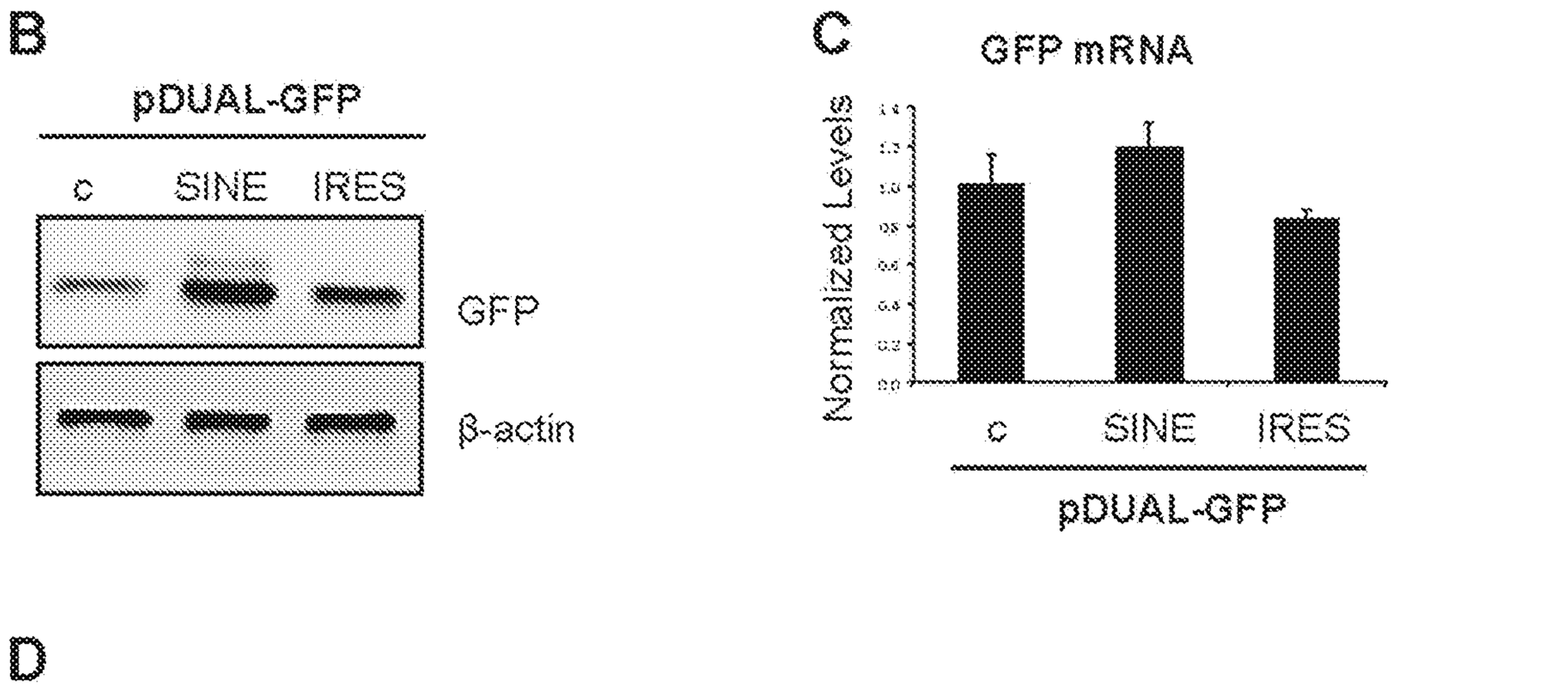
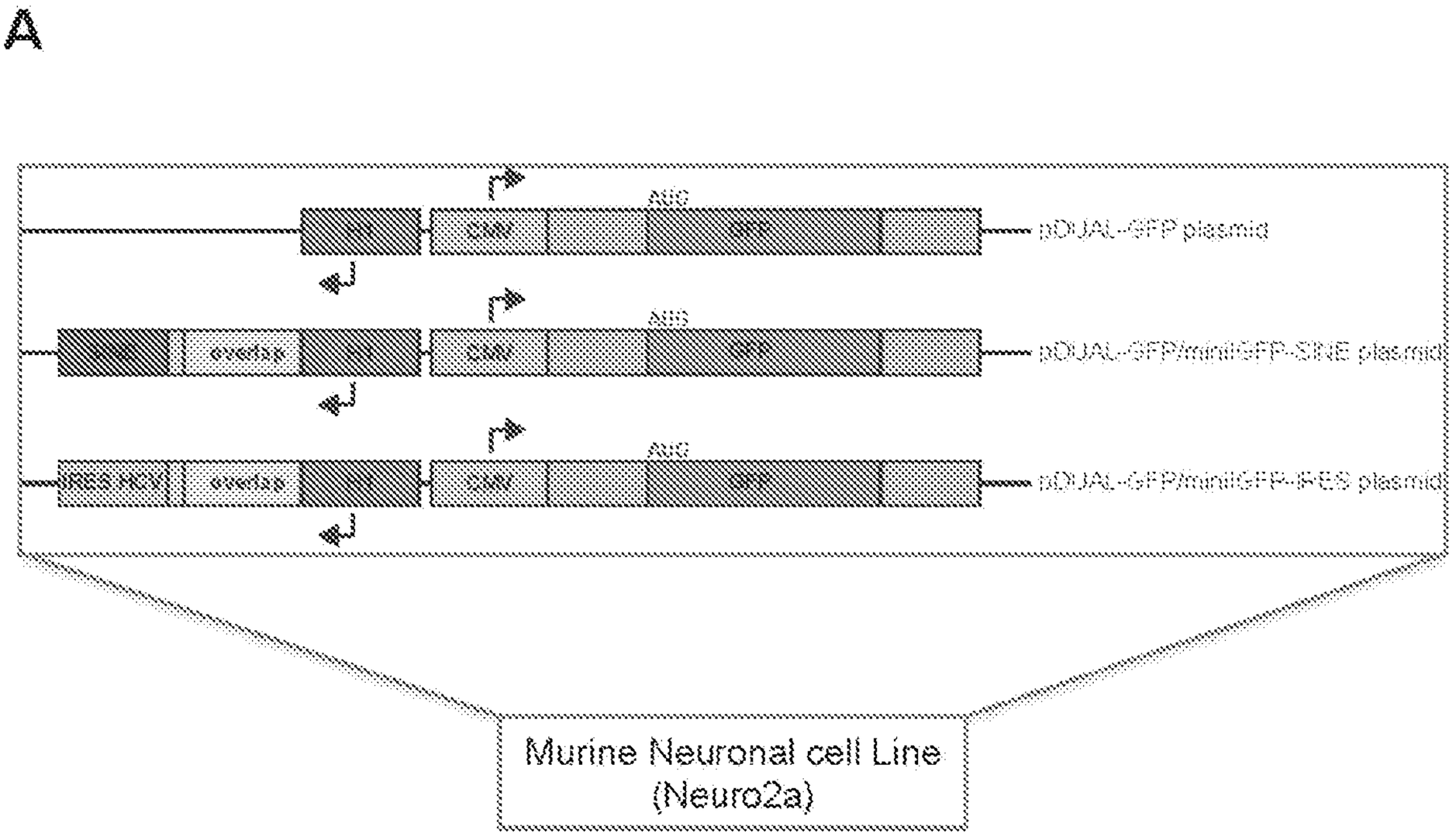

B
pDUAL-GFP
c
SINE
IRES
GFP
β-actin
C
GFP mRNA
Normalized Levels
pDUAL-GFP
D
SINE RNA
IRES RNA
Normalized Levels
c
SINE
IRES
pDUAL-GFP

FIG. 19

A


AUG
pDUAL-GFP plasmid
AUG
pDUAL-GFP/miniGFP-SINE plasmid
AUG
pDUAL-GFP/miniGFP-IRES plasmid
Murine Neuronal cell Line
(Neuro2a)

B

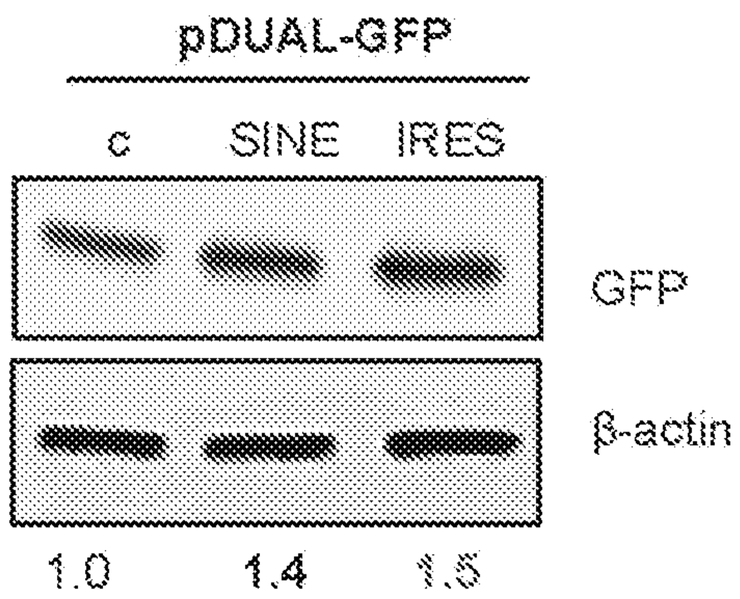

pDUAL-GFP
c
SINE
IRES
GFP
β-actin
1.0
1.4
1.5

FIG. 20

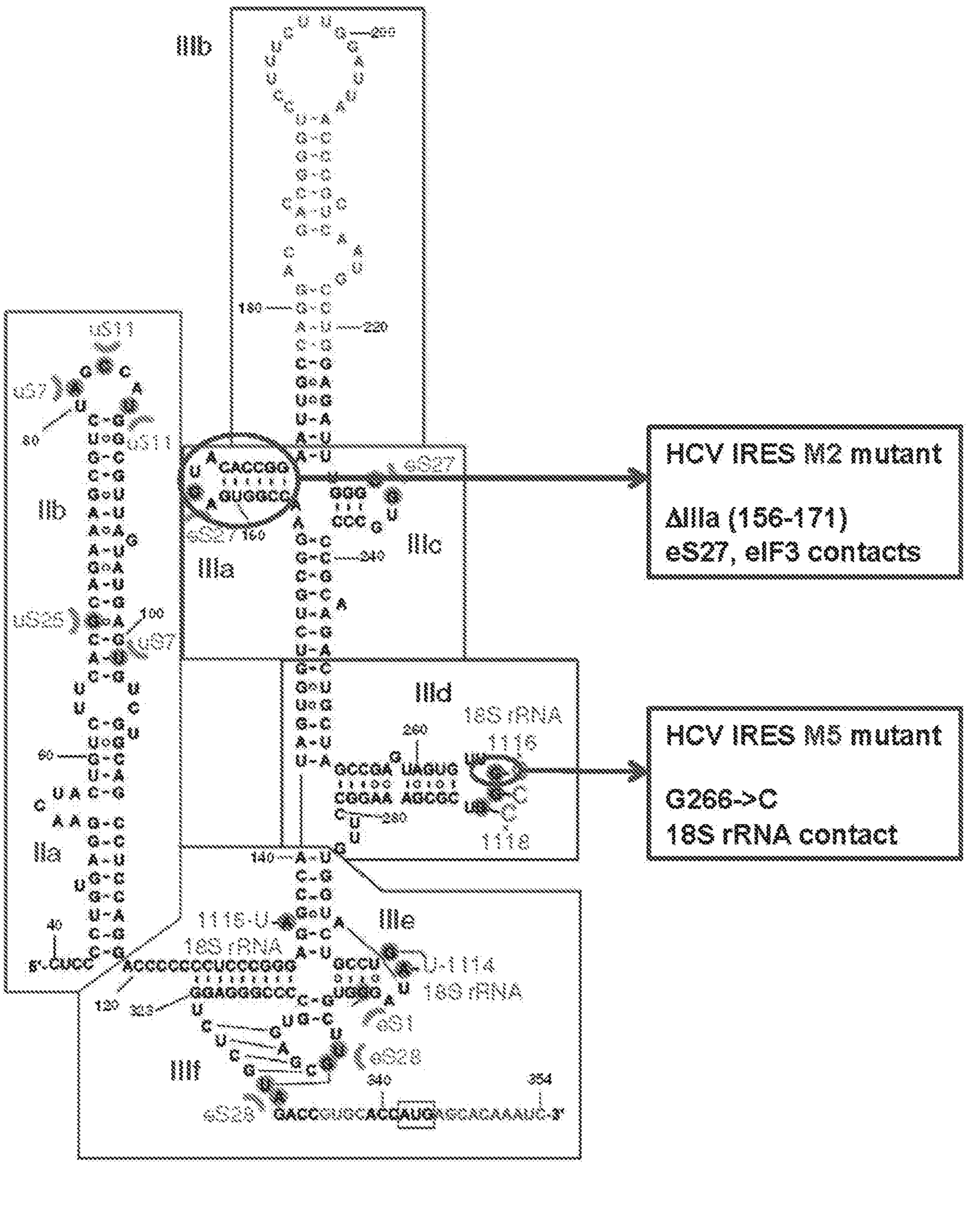

IIIb
IIIa
IIIc
IIIe
IIIf
IIIb
IIIa
IIIc
IIId
IIIe
IIIf
eS11
eS7
eS25
eS27
eS1
eS26
18S rRNA
HCV IRES M2 mutant
ΔIIIa (156-171)
eS27, eIF3 contacts
HCV IRES M5 mutant
G266->C
18S rRNA contact

FIG. 21

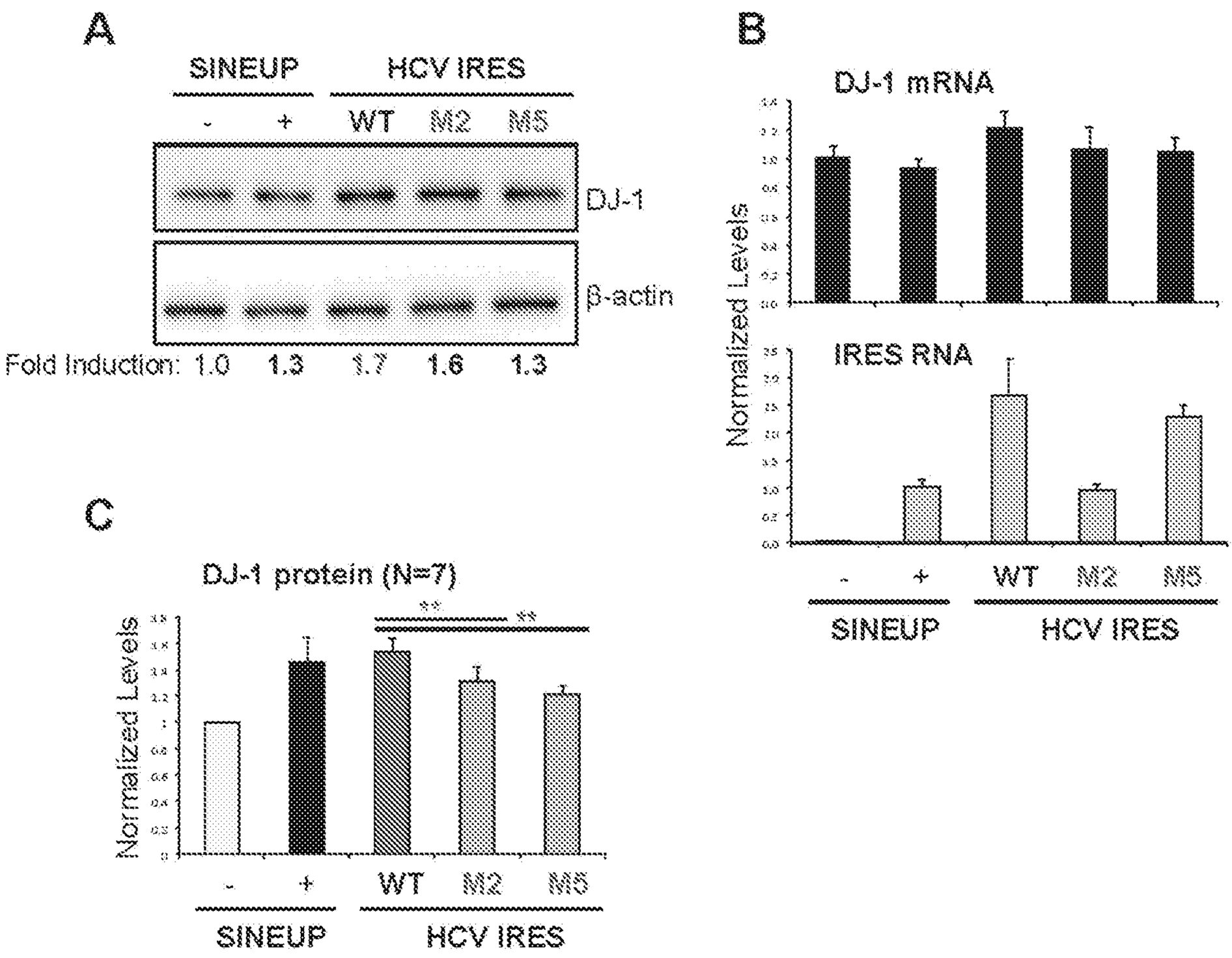

A
SINEUP
HCV IRES
-
+
WT
M2
M5
DJ-1
β-actin
Fold Induction: 1.0 1.3 1.7 1.6 1.3
B
DJ-1 mRNA
IRES RNA
Normalized Levels
C
DJ-1 protein (N=7)
Normalized Levels
**
**
SINEUP
HCV IRES

FIG. 22

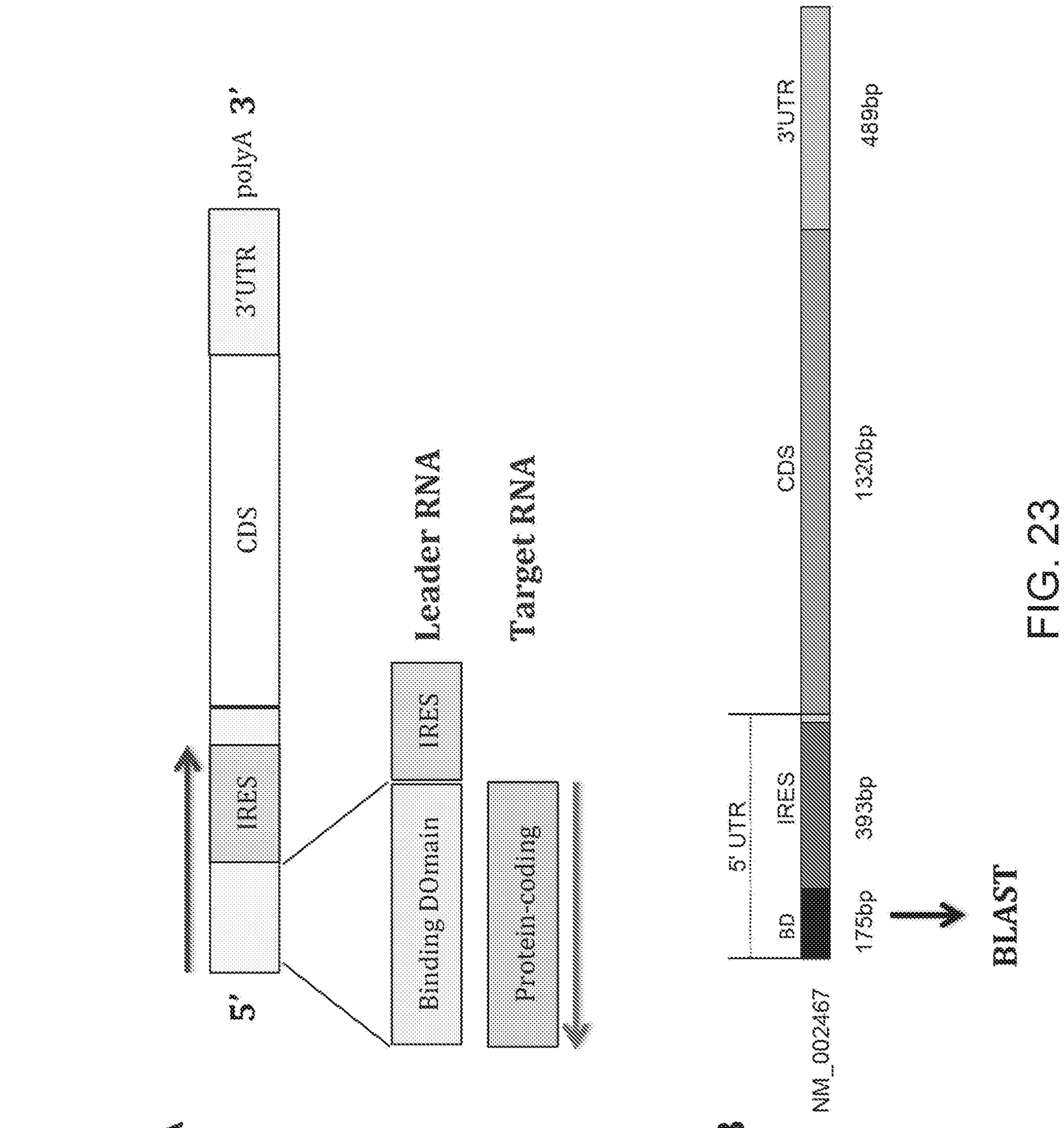

A
5'
IRES
CDS
3'UTR
polyA 3'
IRES
Binding DOmain
Protein-coding
Leader RNA
Target RNA
B
NM_002467
5'UTR
BD
IRES
CDS
3'UTR
175bp
393bp
1320bp
489bp
BLAST

FIG. 23

C

Annotated Gene	Gene Name	Gene pairing region	Transcript type	Position	Genomic location	Orientation	Alignment length	% Alignment	E-value	c-Myc pairing region
ENST00000347004	IAG2	5'UTR	protein_coding	157-397	14:105168528-105168669	Reverse	144	65.97	0.014	IRES
ENST00000460863	KAB6B	5'UTR	protein_coding	240-311	9:133895526-133895597	Reverse	76	72.37	0.014	IRES
ENST00000433688	C11orf95	1st exon	protein_coding	120-183	11:63768496-63768559	Reverse	66	72.73	0.1	IRES
ENST00000358935	CABLES1	5'UTR	protein_coding	3760-3787	18:23359225-23359252	Reverse	28	92.86	0.087	IRES
ENST00000399816	UBE2QL1	5'UTR	protein_coding	184-201	5:6448806-6448823	Reverse	18	100	0.57	BD
ENST00000399816	UBE2QL1	5'UTR	protein_coding	166-205	5:6448806-6448823	Reverse	40	85	0.005	BD
ENST00000519361	MYRF	internal exon (nc)	retained_intron	1764-1785	11:61783950-61783971	Reverse	22	100	0.002	BD
ENST00000564062	ARIH1	1st coding exon	protein_coding	217-235	15:72474861-72474879	Reverse	19	100	0.14	BD
ENST00000575477	PAFAH1B1	1st exon (nc)	processed_transcript	83-178	17:2593901-2593992	Reverse	101	68.32	0.0002	BD
ENST00000397195	PAFAH1B1	5'UTR	protein_coding	156-347	17:2593901-2593992	Reverse	101	68.32	0.0002	BD
ENST00000391533	DYRK2	5'UTR	protein_coding	313-360	12:67849033-67849080	Reverse	48	79.17	0.034	BD
ENST00000408291	ZIC2	last exon (nc)	processed_transcript	378-455	13:99985415-99985492	Reverse	83	68.29	0.25	BD
ENST00000630342	ZIC2	last exon	protein_coding	1329-1407	13:99985415-99985492	Reverse	83	68.67	0.092	BD
ENST00000446283	ODC1	1st exon (nc)	processed_transcript	73-114	2:10448196-10448237	Reverse	43	79.07	0.67	BD
ENST00000234111	ODC1	5'UTR	protein_coding	268-309	2:10448196-10448237	Reverse	43	79.07	0.67	BD
ENST00000493910	NRF1	5'UTR	protein_coding	6056-6075	17:31252125-31252144	Reverse	20	100	0.1	BD
ENST00000220496	DNAJC17	5'UTR	protein_coding	1000-1020	15:40767866-40767886	Reverse	21	100	0.028	5'UTR
ENST00000309539	OLR1	5'UTR	protein_coding	1954-2063	12:10158700-10158809	Reverse	110	66.36	0.018	5'UTR
ENST00000533834	RNPC3	5'UTR	protein_coding	3260-3359	1:103552380-103552479	Reverse	100	66.04	0.018	5'UTR
ENST00000410980	IBTK	5'UTR	protein_coding	4749-4819	6:82170615-82170685	Reverse	71	73.61	0.018	5'UTR
ENST00000395213	SPATA18	5'UTR	protein_coding	3644-3703	4:52096540-52096598	Reverse	60	78.33	0.018	5'UTR
ENST00000398559	TMF1	5'UTR	protein_coding	3818-3930	3:49023046-49023080	Reverse	113	68.7	0.0001	5'UTR
ENST00000318497	VPS4B	5'UTR	protein_coding	1873-1990	18:61390519-61390641	Reverse	138	65.22	0.0003	5'UTR
ENST00000396126	PSMD12	5'UTR	protein_coding	2542-3636	17:67339896-67339780	Reverse	87	72.41	0.001	5'UTR
ENST00000528335	C11orf54	5'UTR	protein_coding	28-117	11:93746912-93746601	Forward	92	69.57	0.002	5'UTR
ENST00000357917	RHOBTB1	5'UTR	protein_coding	2533-2613	10:60871165-60871240	Reverse	82	70.73	0.002	5'UTR
ENST00000392519	TMEM121	5'UTR	protein_coding	620-696	14:105529290-105529366	Reverse	80	70	0.033	CDS
ENST00000325468	GYLTL1B	5'UTR	protein_coding	128-187	11:45922905-45922964	Reverse	60	76.67	0.053	CDS
ENST00000363754	KAT2B	5'UTR	protein_coding	168-282	3:20040239-20040354	Reverse	117	63.25	0.14	CDS
ENST00000543491	PCDH7	5'UTR	protein_coding	404-595	4:30721346-30721522	Reverse	193	61.62	0.38	CDS

FIG. 23

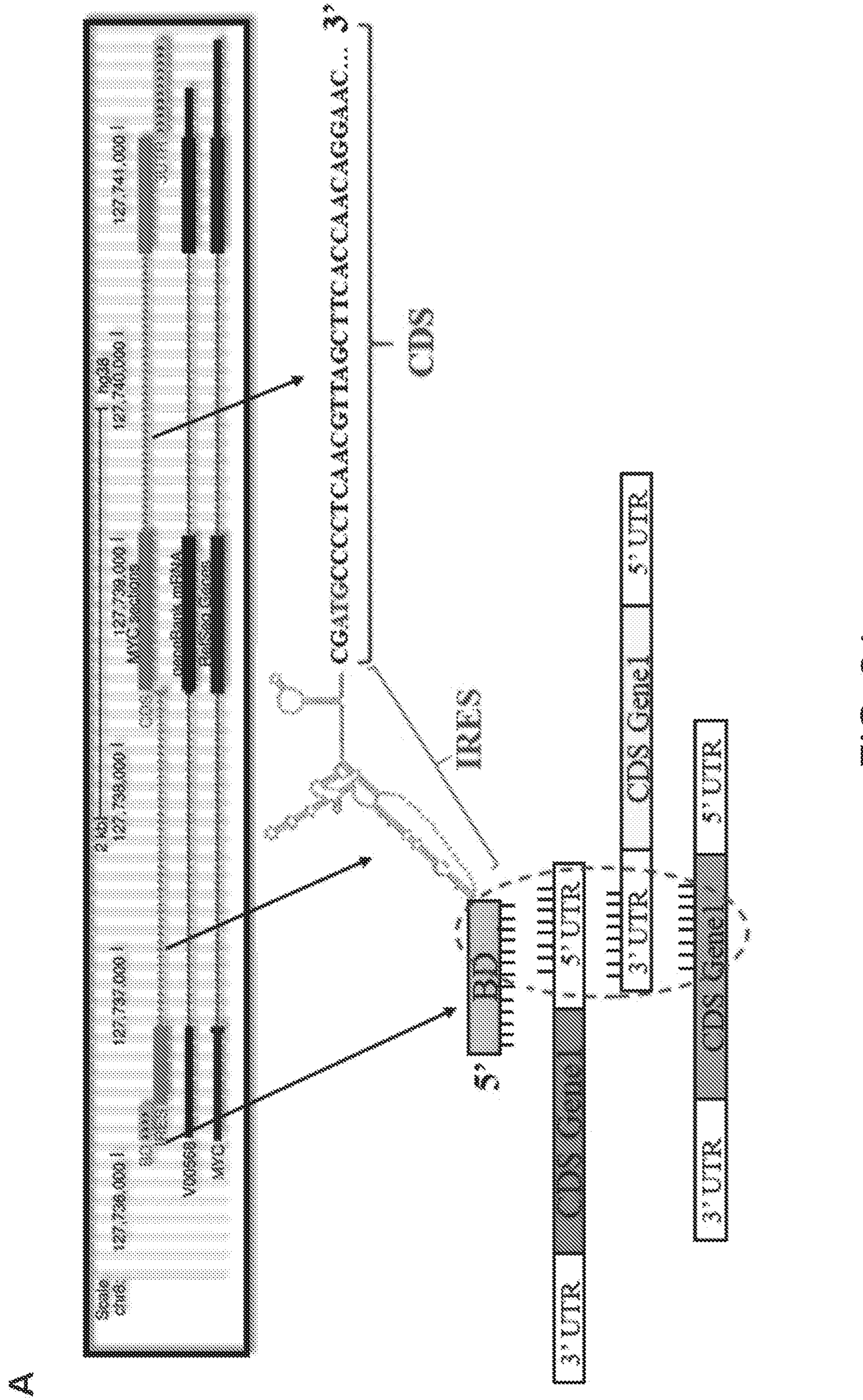

A
CGATGCCCCTCAACGTTAGCTTCACCAAGAGGAAC... 3'
CDS
IRES
5' UTR
3' UTR
Gene1
CDS Gene1
3' UTR
5' UTR
CDS Gene1
3' UTR

FIG. 24

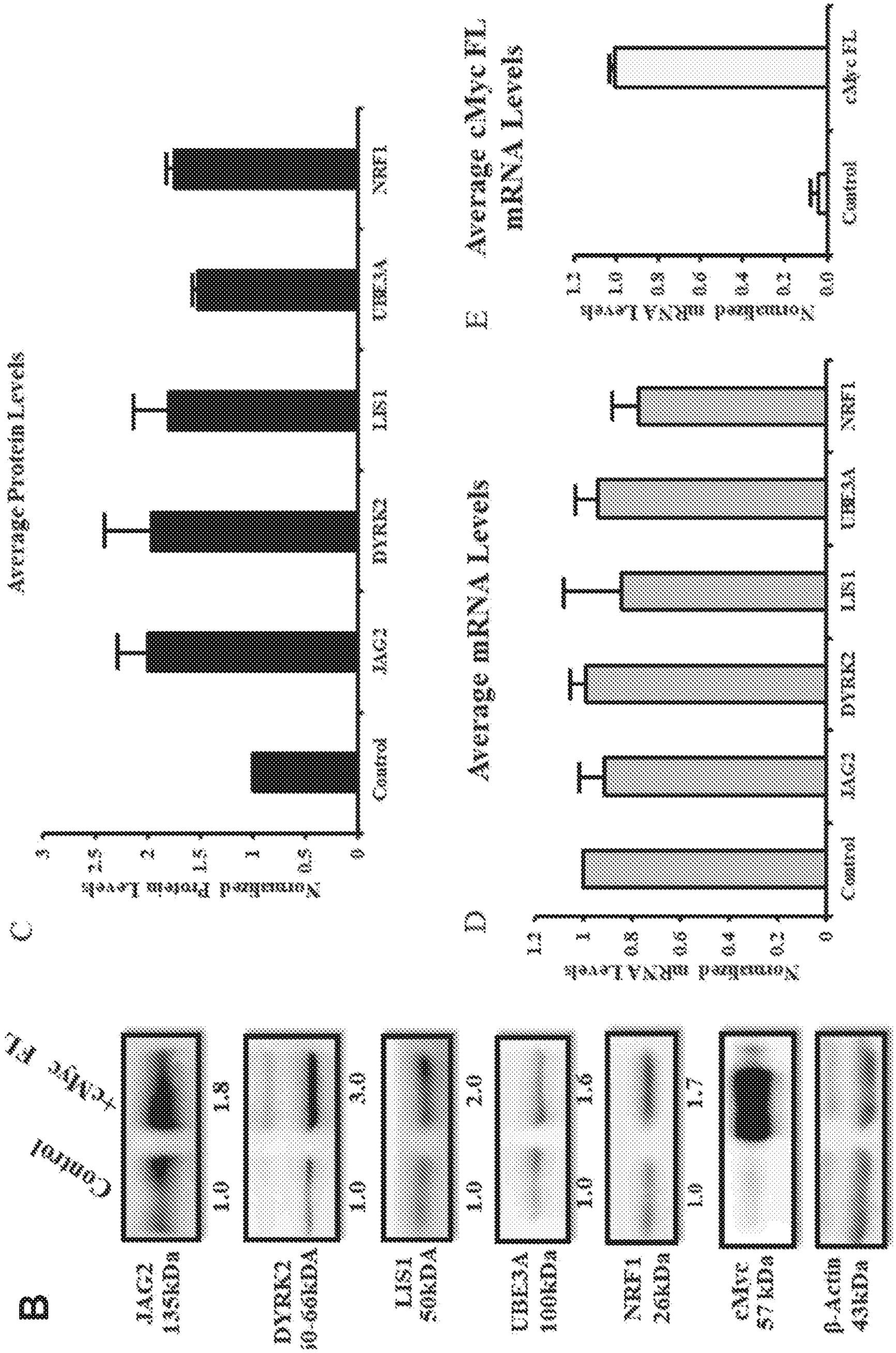

A
Average Protein Levels
Normalized Protein Levels
Control
IAG2
BYRK2
LIS1
UBE3A
XRF1
B
Control
IAG2
BYRK2
LIS1
UBE3A
XRF1
cMyc
β-Actin
C
Normalized Protein Levels
D
Average mRNA Levels
Normalized mRNA Levels
E
Average cMyc FL mRNA Levels
Normalized mRNA Levels
cMyc FL
Control

FIG. 24

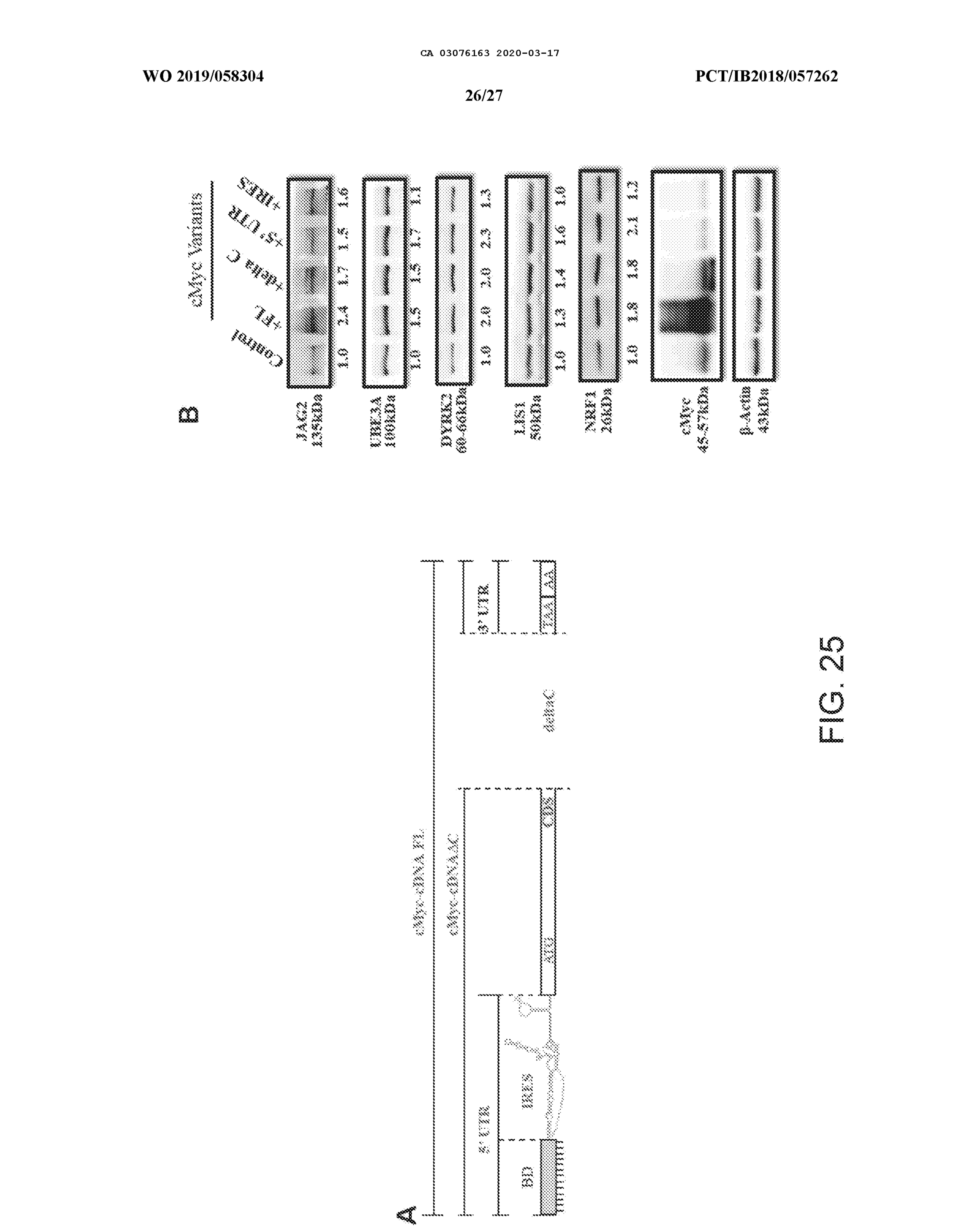
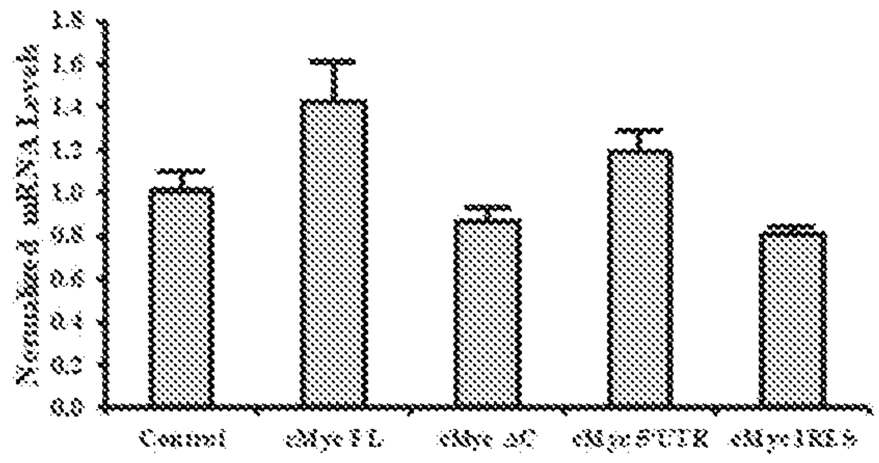
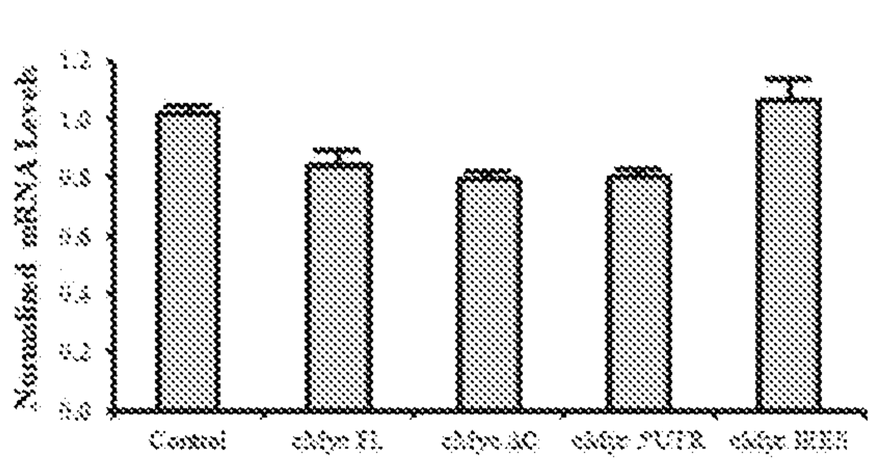
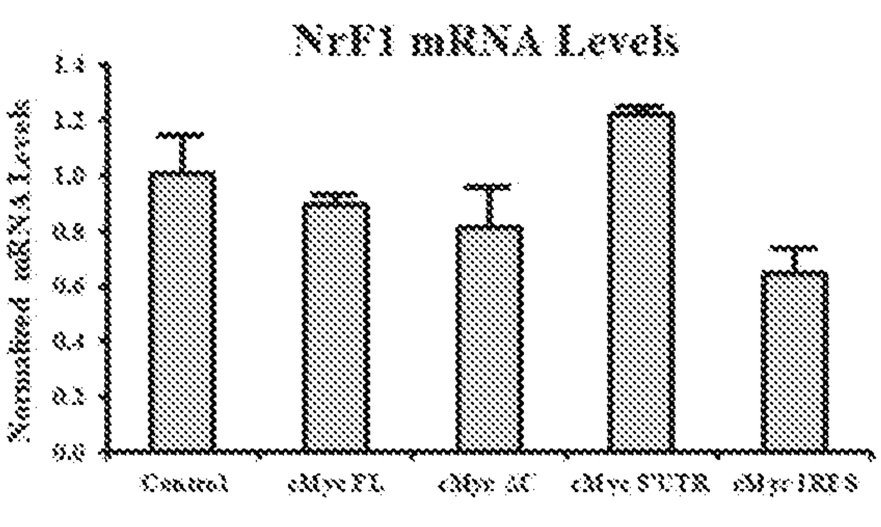

A
cMyc-DNA #1
cMyc-DNA #2
cMyc
ATG
TAG
BD
cMyc Variants
Control
TET
Dox
TET + Dox
Dox + Rapa
TET + Dox + Rapa
JAG2
134kDa
UBE3A
100kDa
DYRK2
60-66kDa
LIS1
50kDa
NRF1
26kDa
cMyc
45-67kDa
β-Actin
43kDa
B
5' UTR
TAG AA

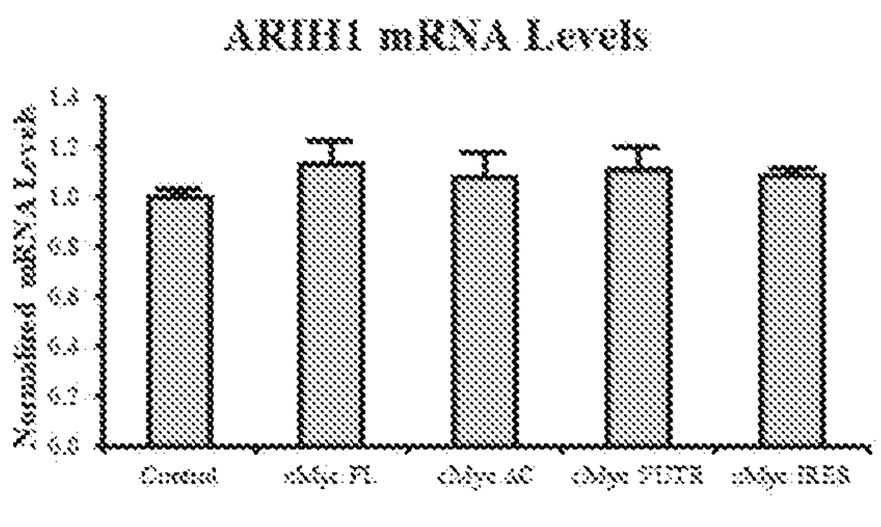
FIG. 25

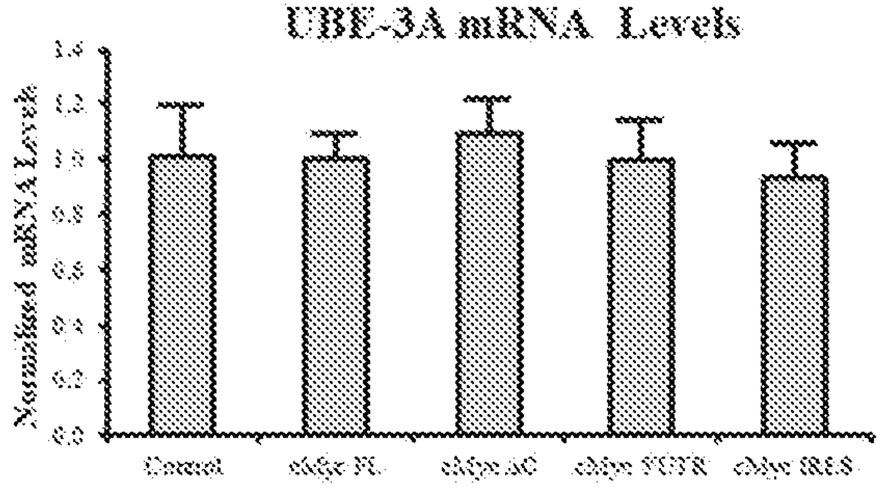
C

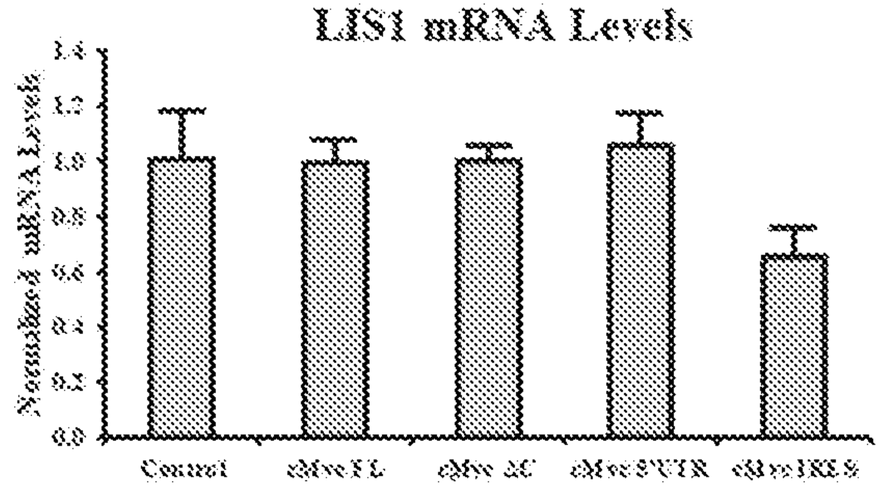

JAG2 mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES

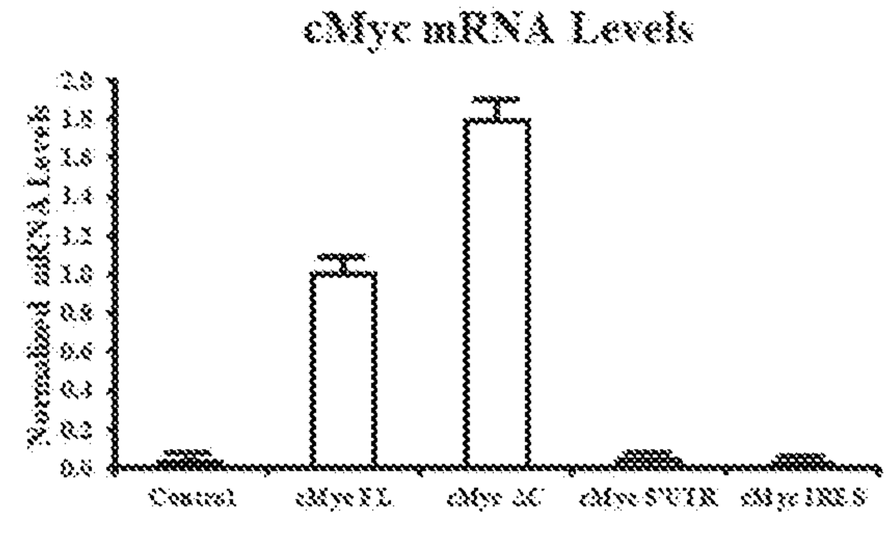

DYRK2 mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES


NrF1 mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES


ARIH1 mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES


UBE-3A mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES


LIS1 mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES


cMyc mRNA Levels
Normalized mRNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES

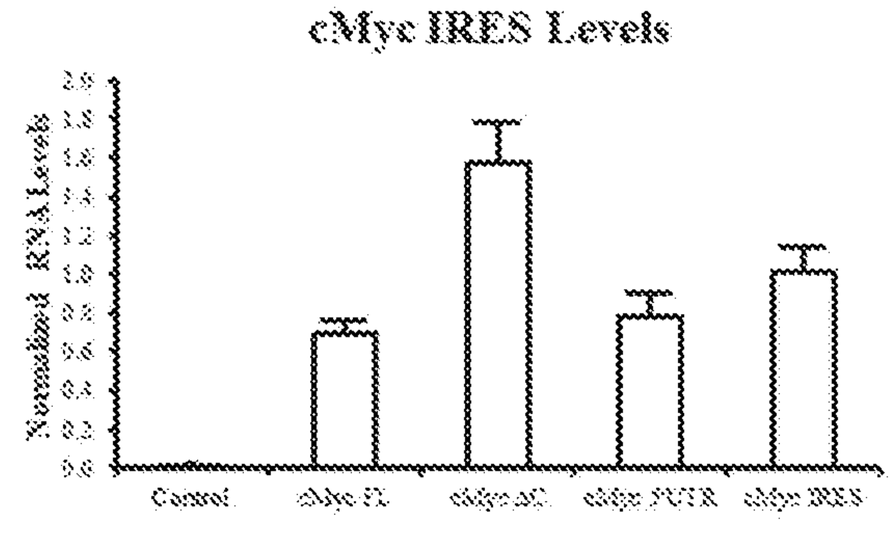

cMyc IRES Levels
Normalized RNA Levels
Control
cMyc FL
cMyc ΔC
cMyc 5'UTR
cMyc IRES

FIG. 25

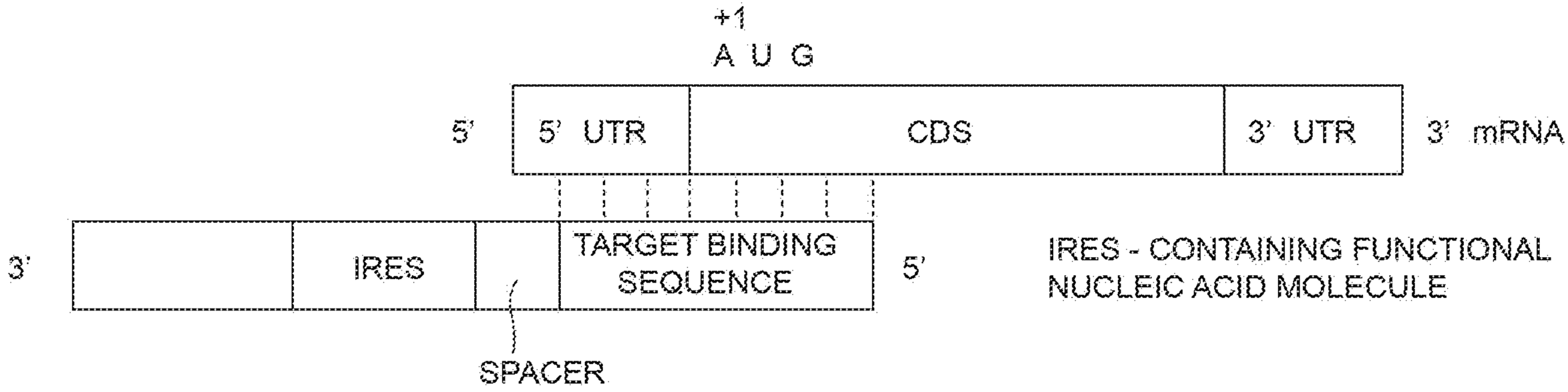

+1
A U G
5'
5' UTR
CDS
3' UTR
3' mRNA
3'
IRES
TARGET BINDING SEQUENCE
5'
IRES - CONTAINING FUNCTIONAL NUCLEIC ACID MOLECULE
SPACER

FIG. 1