3′ UTR of NDUFB7 Regulates the Expression of Firefly Luciferase under Oxidative Stress

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Abstract—*Under adverse environmental conditions, like heat shock, oxidative stress, virus infection, nutrient limitation, the protein expression changes in order to maintain the cellular homeostasis for the survival of the cell and to facilitate its recovery from the stress. Translational arrest is a common and an instant cellular response to stress. During the cellular stress, selection of certain mRNAs for translation plays a vital role in cell survival and stress-recovery, which is mediated by RNA-Binding proteins.*

NDUFB7, is a mitochondrial complex I protein, which is involved in oxidative phosphorylation (OXPHOS), which is a means of generating energy for the cell. Mitochondrial response of cellular stress has been widely studied and is of great importance in understanding the mechanism of several diseases including cancer.

NDUFB7 endogenous expression at the translational level under stress was studied. It is found that NDUFB7 endogenous protein expression is significantly downregulated under oxidative stress condition caused by sodium arsenite. A reporter assay was designed to study the role of 5′untranslated region (UTR), Coding Region (CDS) and 3′ untranslated region (UTR) of NDUFB7 in regulating the expression of the reporter firefly luciferase under stress. From the reporter assay, it is found that 3′UTR plays a vital role in regulating the expression of firefly luciferase at the translational level under stress conditions.

1. INTRODUCTION

During adverse environmental conditions, regulation of gene expression at the translational level provides an immediate mechanism to control cellular protein content in order to maintain the cellular homeostasis and therefore, facilitating the cell survival and its recovery from the stress [1]. Initiation of translation is considered as the primary stage of regulation of gene expression under stress conditions. The process of translation requires a lot of cellular energy and therefore, under stress conditions translation process immediately gets affected and gets reprogrammed [2, 3]. However, there remains a continuous synthesis of proteins from selective mRNAs, which could be essential for cell survival and recovery from stress. This selective expression of mRNAs could vary with different cell types and also with the kind of stress. This mechanism of translational reprogramming could be central to the cellular stress response, where selective mRNAs continue synthesizing stress responsive proteins in order to overcome the adverse environmental conditions [4].

Untranslated regions (UTRs) and Coding regions of an mRNA play an important role in the regulation of an mRNA expression [5]. This process of gene expression could be mediated by the RNA Binding Proteins (RBPs), which bind to certain target regions in their target ligands and regulate their expression [6-8].

Mitochondrial Complex I is considered as a prime target which respond during environmental stress which are a characteristic feature of many diseases, including cancer [9]. Regulation in the expression of certain mitochondrial genes like β-F1-ATPase is considered as a hallmark of cancer progression [10, 11]. In this article, NDUFB7, which is a nuclear encoded mitochondrial complex I gene, expression was studied under oxidative stress induced by sodium arsenite [12]. NDUFB7, is a component of mitochondrial complex I which is involved in generating cellular energy via Oxidative Phosphorylation (OXPHOS).

NDUFB7 endogenous expression was found to be downregulated under cellular stress induced by sodium arsenite. Through the reporter assay, the involvement of 5′ or 3′UTR or coding region in regulating the expression of luciferase was studied and found that the cloned 3'UTR of NDUFB7, down regulates the expression of firefly luciferase under stress as compared to the control conditions.

2. MATERIALS AND METHODS

2.1. Cell culture

MCF7-wt, mammalian breast cancer cells were used for all the experiments described here. The cells were cultured in DMEM F12, supplemented with 10% fetal calf serum (GIBCO), 1% Pen-Strep cocktail (GIBCO). Cells were incubated at 37°C with 5% CO_2.

2.2. Stress condition

Sodium arsenite was used to induce oxidative stress, which caused the formation of stress granules. Titration of sodium arsenite concentration was performed from 7.5μM to 1mM and each concentration was trialled from 1 to 48 hrs. Incubation at 15μM $NaAsO_2$ for 24 hrs was found to be the

optimum treatment with minimal effects on cell viability and cell morphology (data not shown).

2.3. Antibodies

Primary antibodies used were as follows; Mouse anti-NDUFB7 (Abcam, ab55531) and Rabbit anti-beta-Actin (Sigma, A5060). Secondary antibodies used were as follows; Goat anti-rabbit HRP conjugated (Invitrogen, G21234) and Goat anti-mouse HRP conjugated (Invitrogen, G21040).

2.4. Immunoblotting

Cells were cultured in T75 cm^2 flasks until 80-85% confluency was reached and subsequently treated with sodium arsenite (15µM) to induce oxidative stress and incubated further for 24 hrs. Cells were then harvested and resuspended in RIPA (SIGMA) buffer supplemented with a cocktail of protease inhibitors (SIGMA). To promote cell lysis, the crude lysates were passed through a 25 G needle and incubated at -80°C for 15 minutes. Lysates were cleared by a 20 min centrifugation at 14000 x g at 4°C. The protein concentrations of the cleared total cell lysates were determined using the DC protein assay kit (BioRad Laboratories). Proteins were separated on a 10% SDS-PAGE and transferred to a PVDF (Millipore) membrane. Equal loading of protein samples and proper transfer to the membrane was assessed by Ponceau S staining, followed by blocking with 5% Milk Powder in TBST (25 mM Tris-base, 250 mM glycine, 0.1% SDS, 0.1% Tween-20). The membranes were incubated overnight at 4°C with primary antibodies detecting NDUFB7 (1:150) and beta-Actin (1:1000). Incubation with horse radish peroxidase-conjugated secondary antibodies for 1 hr at room temperature was followed by visualisation of the enhanced chemiluminescence (Millipore, Immobilon chemiluminescence substrate) signal on a VersaDoc (BioRad) imaging station. Densitometry of the protein bands was performed using the QuantityOne software (BioRad) and signals were normalised to the loading control beta-Actin.

2.5. Plasmid construction

A reporter construct was designed to identify the role of 5′UTR, Coding Region (CDS) and 3′UTR of NDUFB7 mRNA as the target region responsible for the transcriptional and the translational response to stress. A PCR amplicon harbouring the 5′UTR, Coding Region (CDS) and 3′UTR of the NDUFB7 mRNA was generated using specific primers (Table 1).

Table 1: List of Primers used to perform the cloning of NDUFB7 reporter vector

GENE NAME	REGION AND DIRECTION OF PRIMER	PRIMER SEQUENCE
NDUFB7	5′UTR Forward	gatctagaCTGACTGAGGGG TCAGTGGTTC
NDUFB7	5′UTR Reverse	gatctagaGGCTGCAGTCGCT GCAGATCC
NDUFB7	CDS Forward	gatctagaATGGGGGCCCACCT GGTCCG
NDUFB7	CDS Reverse	catctagaCTACAGGGCCACCT TGGGGTC
NDUFB7	3′UTR Forward	cttctagaGGGGTGCACCCCCCA CCCTATGGACCAGTCAAATAA AAGCCTTCAGGCCCCTCtctagatc
NDUFB7	3′UTR Reverse	gatctagaGAGGGGCCTGAAGGCT TTTATTTGACTGGTCCATAGGG TGGGGGGTGCACCCCtctagaag

Lower case plus underline text represents restriction sites added to the primer sequences, tctaga – XbaI; Lower case alphabets, represents the additional nucleotides added to the primers for the ease of restriction digestion.

The primers incorporated Xba1 sites at the 5′ end to facilitate cloning into the pGL3 vector (Promega, E176A). The XbaI site in the pGL3 vector is downstream of the Luciferase gene but 5′ to the poly adenylation site. The chimeric transcript from the construct contains the Luciferase coding sequence and the 5′UTR, Coding Region (CDS) or 3′UTR of NDUFB7 respectively and is referred to as the chimeric luciferase-NDUFB7-5′/CDS/3′UTR reporter construct. Changes in the expression levels of the Luciferase protein from the chimeric transcript can be attributed to the introduced 5′/CDS/3′UTR of NDUFB7 region. The construct was amplified in E.Coli strain JM109 and plasmids from positive clones were purified, sequenced and used to transfect MCF7-wt cells to perform luciferase reporter assay and the transcript stability assay.

2.6. Transient transfection and Luciferase assays

$2.4x10^5$ cells were seeded into each well of a 6-well plate, and incubated until they reached 75% confluency. Cells were transiently transfected with equimolecular amounts of all the constructs using the Lipofectamine 2000 reagent (Invitrogen). Additionally, cells were treated with 15µM $NaAsO_2$ for 24 hrs starting 4 hrs post transfection. Cells were then lysed using 1X Reporter Lysis Buffer (Promega) and lysates were collected into tubes. Luciferase Reporter kit (Promega) was used to assess the expression level of the luciferase following the manufacturer’s instructions. The Luciferase values were normalized to the total protein concentrations of the sample.

3. RESULTS

3.1. Sodium arsenite induced oxidative stress down-regulates the endogenous NDUFB7 protein expression

Sodium arsenite induces oxidative stress which reprogrammed the translation of NDUFB7. Fig. 1, panel A, shows the endogenous expression of NDUFB7 under stress and control through western blot. Fig. 1, panel B, bar diagram represent the densitometry performed on the western blot, which shows a significant down regulation in the expression of NDUFB7 under stress condition as compared to the control.

3.2. 5´UTR and Coding region (CDS) does not regulate the expression of firefly luciferase at the translational level

The chimeric construct with cloned 5´UTR/3´UTR or coding region of NDUFB7 in the Firefly luciferase reporter vector were generated and transiently transfected in MCF7 cells. After 4 hours post transfection, stress was induced by replacing the media with 15µM $NaAsO_2$ supplemented media and incubated further for 24 hours. After 24 hours cells were harvested and lysed using reporter lysis buffer and firefly luminescence was measured and normalized with the luminescence value of Renilla. Fig. 2, panel A and panel B, represent the data that 5´UTR and Coding Region does not regulate the expression of firefly luciferase under stress as compared with the control respectively.

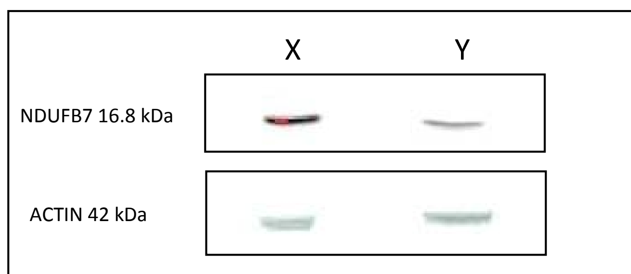


Fig. 1 (A): Regulation of endogenous NDUFB7 expression after treatment with 15µM sodium arsenite for 24 hours. Whole cell lysate from MCF7 cells after treatment with 15µM sodium arsenite. Lane X shows the expression of endogenous NDUFB7 (unstress) sample and Lane Y, 24 hours post stress treatment. Actin was used as a loading control. N=3, student t-test, p≤ 0.001.

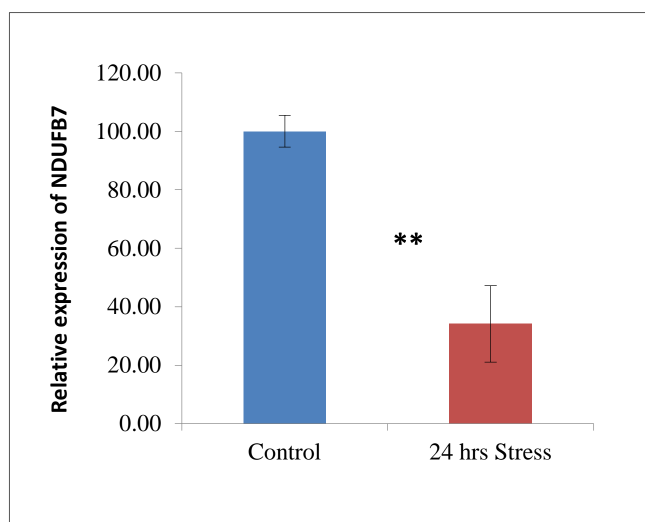
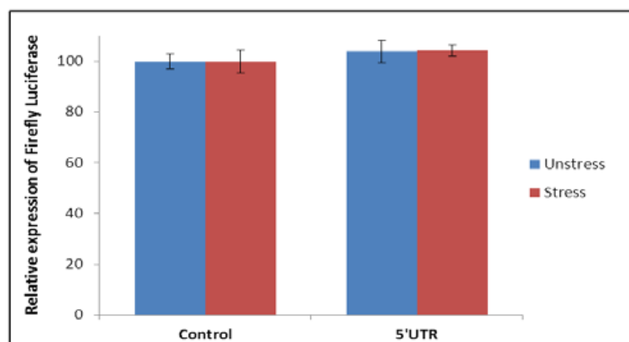


Fig. 1. (B): Densitometry representation of regulation of NDUFB7 after treatment with 15µM sodium arsenite for 24 hours.

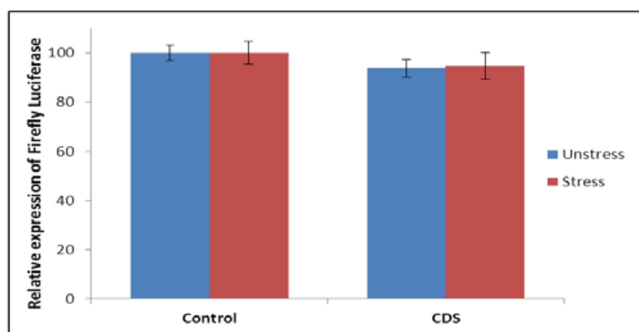
Significant down regulation in the endogenous expression of NDUFB7 in the whole cell lysate from MCF7-wt cells was observed after treatment with 15µM sodium arsenite for 24 hours as compared to the control (No stress). n=3, student t-test, **p≤ 0.001

3.3. 3´UTR of NDUFB7 down-regulates the expression of firefly luciferase at the translational level.

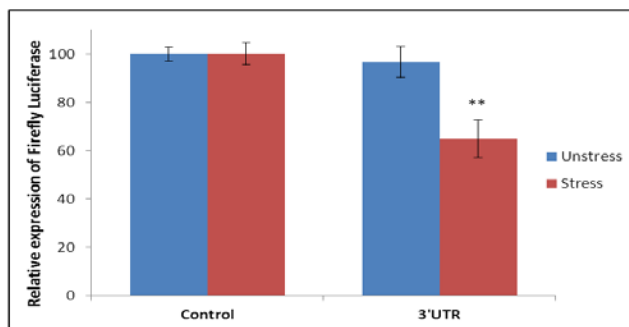
3´UTR of NDUFB7 was cloned into the XbaI restriction site of the reporter vector in order to study the role of 3´UTR of NDUFB7 in regulating the expression of firefly luciferase. After the transient transfection of the specific chimeric construct reporter assay was performed. Fig. 2, panel C represent the relative expression of firefly luciferase for the control reporter vector (intact reporter vector) under stress and control conditions and also for the chimeric construct bearing the 3´UTR of NDUFB7 under similar conditions like the control construct. The data suggest that there was a significant down regulation in the expression of firefly luciferase under stress condition as compared to control condition.



(a)



(b)



(c)

Fig. 2: Reporter assay for different chimeric constructs of NDUFB7 under stress condition compared to the unstress condition.

Panel A, represent the relative expression of firefly luciferase for control (intact reporter vector) under stress and control condition compared to the chimeric construct bearing 5′UTR of NDUFB7. There was no significant change in the expression of firefly luciferase irrespective of the treatment condition. Panel B, represent the relative expression of control reporter vector and the chimeric construct bearing the coding region (CDS) of NDUFB7. There was no significant change in the expression of firefly luciferase irrespective of the treatment condition. Panel C, represent the relative expression of control reporter vector and the chimeric construct bearing the 3′UTR of NDUFB7. There was significant down regulation in the expression of firefly luciferase for the construct bearing the 3′UTR of NDUFB7 under stress condition as compared to the control (unstress) condition. n=3, student t-test, $^{**}p \leq 0.001$.

4. DISCUSSION

Global repression of protein synthesis is considered as a common cellular effect due to adverse environmental conditions. Regulation of mitochondrial gene expression is considered as a characteristic feature of several stress responsive disorders and diseases including cancer. NDUFB7 is a nuclear encoded mitochondrial complex I protein which is involved in energy production via OXPHOS. NDUFB7 endogenous protein expression was downregulated due to oxidative stress induced by sodium arsenite.

The data from reporter assay implicates that 5´UTR and the coding region of NDUFB7, does not have any influence on the expression of firefly luciferase irrespective of the treatment condition. However, the reporter assay data for 3´UTR of NDUFB7 suggest that 3´UTR might have some specific sequences which might interact with the specific RNA Binding Protein (RBP) and regulate the expression of firefly luciferase under stress conditions.

This information suggest that 3´UTR of NDUFB7 might possess some regulatory sequences which facilitate its interaction with the target specific RBP or any stress responsive protein, which regulates its expression under stress conditions as compared with the control.

5. AUTHORS CONTRIBUTION

AN carried out all the experiments and performed the data analysis and drafted the manuscript.

6. ACKNOWLEDGEMENTS

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