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ScienceDirect
Biochemical and Biophysical Research Communications 365 (2008) 232–238
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The polyglutamine-expanded protein ataxin-3 decreases *bcl-2* mRNA stability

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Received 18 October 2007
Available online 5 November 2007

Abstract

Machado-Joseph disease/Spinocerebellar ataxia type 3 is an autosomal dominant neurodegenerative disease caused by polyglutamine-expanded ataxin-3. In this study, COS7-MJD26-GFP and COS7-MJD78-GFP cells, which were stably transfected with GFP-tagged full-length MJD gene with either 26 or 78 glutamine repeat, were used to demonstrate that both protein and mRNA levels of *bcl-2* are decreased in the presence of expanded ataxin-3. However, the promoter activity in COS7-MJD78-GFP cells is much higher than that in COS7-MJD26-GFP, suggesting that the decrease of *bcl-2* expression may be due to defects in mRNA stability. Therefore, 5,6-dichloro-benzimidazole 1-β-D-ribofuranoside, an adenosine analogue to inhibit mRNA synthesis, was used to estimate the *bcl-2* mRNA degradation rate. Our results demonstrated that *bcl-2* mRNA decay in COS7-MJD78-GFP cells is about 3.5-fold faster than that in COS7-MJD26-GFP. Our study provides evidence, for the first time, that dysfunction of mRNA stability resulted from the presence of mutant ataxin-3.

Keywords: Spinocerebellar ataxia type 3; Polyglutamine-expanded ataxin-3; Bcl-2; mRNA stability

Among the heterogeneous group of autosomal dominantly inherited ataxias, Spinocerebellar ataxia type 3 (SCA3) is the most common type. This disease is caused by CAG trinucleotide repeat expansion and the abnormal CAG repeats are transcribed and translated into a polyglutamine (polyQ) stretch in the ataxin-3 protein. Ataxin-3 is an intracellular protein and is widely distributed in the central nervous system and peripheral tissues [1]. Ataxin-3 consists of a structured N-terminus with deubiquitinating activity, termed the joseph domain, two ubiquitin-interacting motifs (UIMs) and a polyQ tract in the C-terminal region [2–4]. Even though the physiological function of ataxin-3 is not completely clear, it was suggested that ataxin-3 is a component of the ubiquitin proteasome system [3]. Recent studies also suggested that ataxin-3 may be a regulatory component of aggresome formation [5,6], may have a neuroprotective function [7], or may be involved in gene regulation [8–13]. In addition, autocatalytic activity was demonstrated for ataxin-3 [14]. However, the causal relation between these cellular events and the pathogenesis has not been fully elucidated.

Expanded polyQ aggregates, both *in vitro* and *in vivo*, form characteristic inclusion bodies. Neuronal intranuclear inclusions have become the neuropathological sign at the late stage of the triplet diseases, but the relationship between aggregation and cytotoxicity remains controversial [15–17]. Overexpression of Bcl-2 has been shown to inhibit apoptosis by preventing the activation of caspase-9 [18]. In addition, overexpression of Bcl-2 in neuronal cells was shown to prevent programmed cell death both *in vitro* and *in vivo* [19,20] and the overexpression or inactivation of one component of the Bcl-2 protein family could drastically modify the sensitivity to apoptosis and greatly alter other parameters involved in cell physiology [21,22]. We

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.162

previously showed a significant decrease of Bcl-2 protein expression in SK-N-SH cells containing expanded ataxin-3 when compared with those of the parental cells. The same reduction of Bcl-2 was further confirmed in MJD fetal fibroblast cells [8].

In this study, COS7-MJD26-GFP and COS7-MJD78-GFP cells, which were COS7 cells stably transfected with GFP-tagged full-length MJD genes with either 26 or 78 glutamine repeats, were established in our laboratory to further understand the mechanism leading to the decrease of Bcl-2 in the presence of mutant ataxin-3. Our study provided a first study to demonstrate a dysfunction of mRNA stability associated with polyglutamine-expanded ataxin-3.

Materials and methods

Plasmids. Full-length MJD constructs containing 26 or 78 CAG repeats in pEGFP-N1 were amplified from pCMX-MJD26 (a generous gift from Dr. Akira Kakizuka at Kyoto University) or pcDNA3-HA-MJD78 (a generous gift from Dr. Henry Paulson at the University of Iowa) and then subcloned into the expression vector pEGFP-N1. *Bcl-2* promoter–reporter constructs (LB124, LB335, and LB322) containing different regions of *bcl-2* promoter (P1, P2, P1 + P2) [23] were kindly provided by Dr. Linda Boxer at Stanford University.

Generation of stably transfected MJD cells. COS7 cells were transfected with empty pEGFP-N1 vector or pEGFP-N1 containing full-length MJD with either 26 or 78 glutamine repeats. After transfection, cells were selected in a culture medium supplemented with G418. Selected G418-resistant cells were subcloned and maintained in the same medium until each cell line contained homogenous transfected cells. After 3 months of selection and subcloning, we obtained stable cells expressing GFP alone (COS7-GFP), MJD26-GFP (COS7-MJD26-GFP) or MJD78-GFP (COS7-MJD78-GFP). For all experiments, cells were placed in a medium lacking G418. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% nonessential amino acids.

Isolation of total RNA and semi-quantitative reverse-transcriptase PCR. Total RNA was extracted from cells by Trizol reagent (Invitrogen, USA). Procedures were performed according to the manufacturer's protocol. For each sample, 5 μg RNA was reverse-transcribed into cDNA in a final volume of 20 μl with 40 pmole of oligod(T) and 40 U MMLv reverse transcriptase (Perkin-Elmer, USA) for 15 min at 42 °C. *Bcl-2* and G3PDH mRNA were measured by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). The primers are: *bcl-2* upstream 5′-CG ACGACTTCTCCCGCCGCTACCGC-3′, *bcl-2* downstream 5′-CCGCA TGCTGGGGCCGTACAGTTCC-3′; and G3PDH upstream 5′-CCATG TTCGTCATGGGTGTGAACCA-3′, downstream 5′-GCCAGTAGAG GCAGGGATGATGTTC-3′. PCR was performed in a final volume of 25 μl using 10 μl (*bcl-2*) or 3 μl (G3PDH) cDNA amplified from the RT reaction. Conditions of PCR were as follows: an initial denaturing for 5 min at 94 °C, followed by 30 s at 94 °C, 1 min at 72 °C, for 30 (*bcl-2*) or 20 (G3PDH) cycles. Ethidium bromide-stained PCR products were separated on 3% agarose gels and quantified by Image Gauge V3.46 software (Fuji film, Japan). For every oligonucleotide pair, a preliminary analysis was conducted to define the appropriate range of cycles consistent with an exponential increase in the amount of the product.

Determination of bcl-2 mRNA decay in cells. Cells were treated with the transcription blocker 5,6-dichloro benzimidazole 1-β-D-ribofuranoside (DRB) (Sigma, St. Louis, MO, USA) at a final concentration of 75 mM for 1, 2, 4, and 8 h. Total RNA was isolated at indicated times by Trizol and semi-quantitative RT-PCR was performed. The *bcl-2* mRNA half-life was shown in a semilogarithmic transformation of the quantified data plotted against the time of incubation with DRB.

Western blot analysis. Proteins were separated by 10% SDS–PAGE and then transferred onto nitrocellulose membranes. The following primary antibodies were used: monoclonal anti-Bcl-2 (BD Transduction Laboratories), polyclonal AUF-1 (Santa Cruz), monoclonal anti-β-actin (Sigma), monoclonal anti-GFP and anti-Nucleolin (Santa Cruz). The signal was detected by the enhanced chemiluminescence Western blot system (Pierce).

Transient transfection and Luciferase reporter assay. Transient-transfection was performed in COS7-MJD26-GFP and COS7-MJD78-GFP cells. For each experiment, a β-galactosidase expression plasmid was used as a control. Cells were transfected with reporter plasmid and β-galactosidase expression plasmid by lipofectamine (Invitrogen). An optimum DNA mixture was replaced with a complete medium for 6 h and cells were harvested after 48 h. Assays of luciferase activity were carried out according to the Luciferase Assay System manufacturer's protocol (Promega, USA). Luciferase activities were determined by luminometer (TURNER TD-20/20) and were normalized to β-galactosidase activity.

Results

Non-expanded and expanded ataxin-3 differentially affected Bcl-2 expression

COS7-GFP, COS7-MJD26-GFP, and COS7-MJD78-GFP cells, which were COS7 cells stably transfected with GFP or GFP-tagged full-length MJD genes with either 26 or 78 glutamine repeats (Fig. 1A), were established. To confirm the expression of non-expanded or expanded ataxin-3-GFP fusion proteins in these stable cell lines, Western blot analysis was performed and antibody against GFP was applied to detect GFP fusion proteins. Fig. 1B shows similar expression levels of non-expanded MJD26-GFP and expanded MJD78-GFP fusion proteins in COS7-MJD26-GFP and COS7-MJD78-GFP cells. Meanwhile, monoclonal anti-MJD also confirmed the expression of MJD-GFP fusion proteins in these stable cell lines (data not shown). Therefore, COS7-MJD26-GFP and COS7-MJD78-GFP cells were used hereafter in the following analysis.

Consistent with our previous report [8], Western blot analysis demonstrated a significant decrease of Bcl-2 protein expression in stable cells containing expanded ataxin-3, SK-N-SH-MJD78 cells (lane 1 of Fig. 1C), when compared with that of the parental SK-N-SH cells (lane 2 of Fig. 1C). However, due to lack of SK-N-SH cells over-expressing MJD26, Bcl-2 expression was further examined by comparing protein levels of Bcl-2 in COS7-GFP, COS7-MJD26-GFP or COS7-MJD78-GFP cells. As shown in Fig. 1C, the protein level of Bcl-2 in COS7-MJD78-GFP cells (lane 3) significantly decreased as compared to that in COS7-MJD26-GFP cells (lane 4), with β-actin as an internal control. It was noted that the Bcl-2 protein level in COS7-MJD26-GFP cells was higher than that in COS7-GFP cells (lane 5). A quantitative assessment of the percentage of protein expression revealed that Bcl-2 in mutant cells retained less than 30% of that in cells with normal ataxin-3 (Fig. 1D). Our results demonstrated that the protein level of Bcl-2 is significantly decreased in the presence of expanded ataxin-3 in the COS7 MJD model.

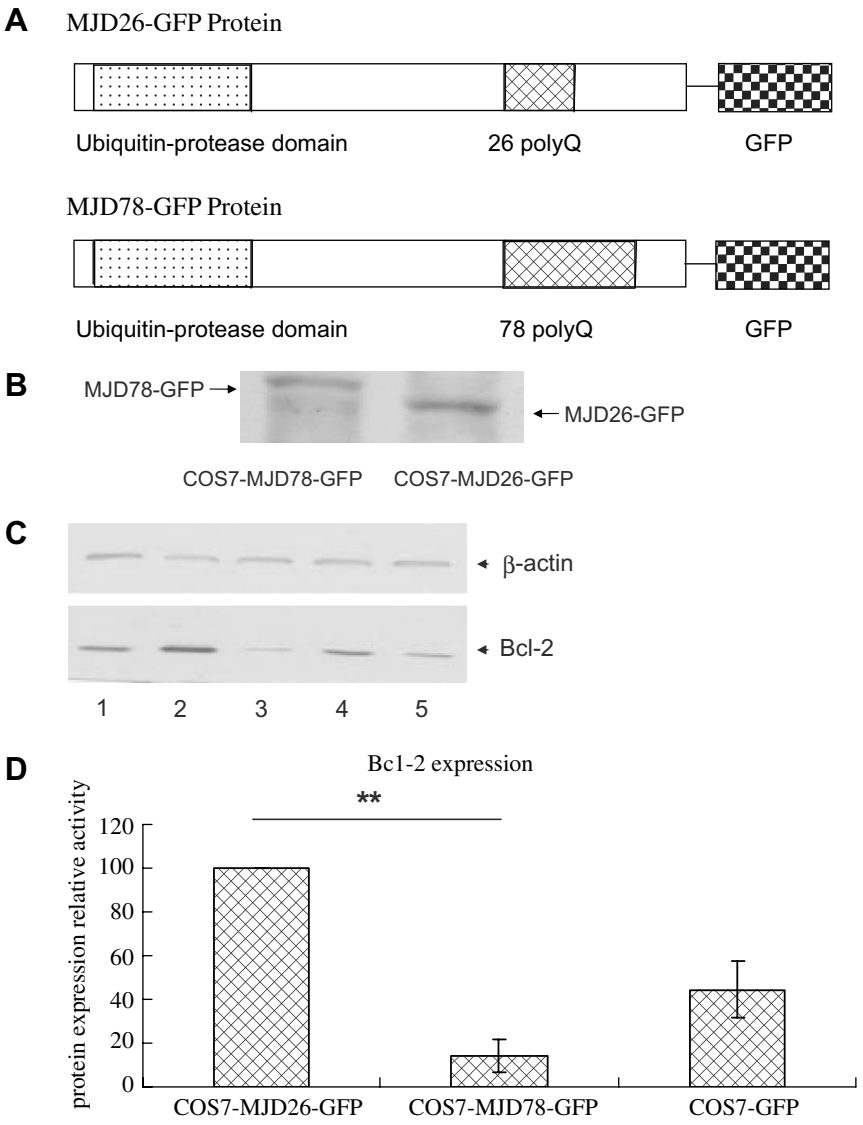

Fig. 1. (A) Structure of the MJD-GFP constructs used in this study. (B) Detection of expressed MJD26-GFP and MJD78-GFP in stable cell lines. Western blot analysis of protein extracts from COS7-MJD26-GFP cells and COS7-MJD78-GFP cells. Monoclonal anti-GFP was used in the blotting. (C) Western blot analysis of Bcl-2 in COS7 and SK-N-SH stable cell lines. Proteins extracted from SK-N-SH-MJD78 (lane1), SK-N-SH (lane 2), COS7-MJD78-GFP (lane 3), COS7-MJD26-GFP (lane 4), and COS7-GFP (lane 5) cells were analyzed. The transferred blot was double labeled with Bcl-2 and β-actin monoclonal antibodies to show different levels of the protein expression. (D) Quantitative assessment of Bcl-2 expression in COS7-GFP, COS7-MJD26-GFP, and COS7-MJD78-GFP cells. The band intensity was assessed by an image analysis program (LAS-1000 plus). Compared to the level of a β-actin internal control, the mean relative levels of Bcl-2 protein were plotted with error bars representing standard error mean (SEM). $n = 3$, $^{**}P < 0.005$ compared with that of COS7-MJD26-GFP cells.

Decreased expression of Bcl-2 due to defects on mRNA levels

Next, we tried to determine whether this reduction in Bcl-2 protein expression is due to defects at the transcriptional level. Semi-quantitative RT-PCR was performed to analyze *bcl-2* mRNA levels, with G3PDH being an internal control (Fig. 2A). Our results revealed a 70% decrease of *bcl-2* mRNA in cells expressing mutant ataxin-3 as compared to that of cells expressing non-expanded ataxin-3 (Fig. 2B), suggesting defects on the mRNA levels. Because transcription regulation plays important roles in regulating steady-state levels of transcripts in cells, we then investigated *bcl-2* promoter activity. It is well known that the 5'-flanking sequence of the human *bcl-2* gene contains two distinct promoters (P1 and P2) that are required for initiation of transcription [24]. To assay promoter activity of the *bcl-2* gene, Luciferase reporter constructs containing the full-length *bcl-2* promoter region (that containing both P1 and P2 regions), and the isolated P1 or P2 region [23,24] were co-transfected with a β-galactosidase expression plasmid into COS7-MJD78-GFP and COS7-MJD26-GFP cells. Luciferase activity was calibrated to β-galactosidase activity for transfection efficiency control. This experiment was repeated at least three times and results are summarized in Fig. 2C. Our results demonstrated that promoter activities from three different promoter constructs of *bcl-2*

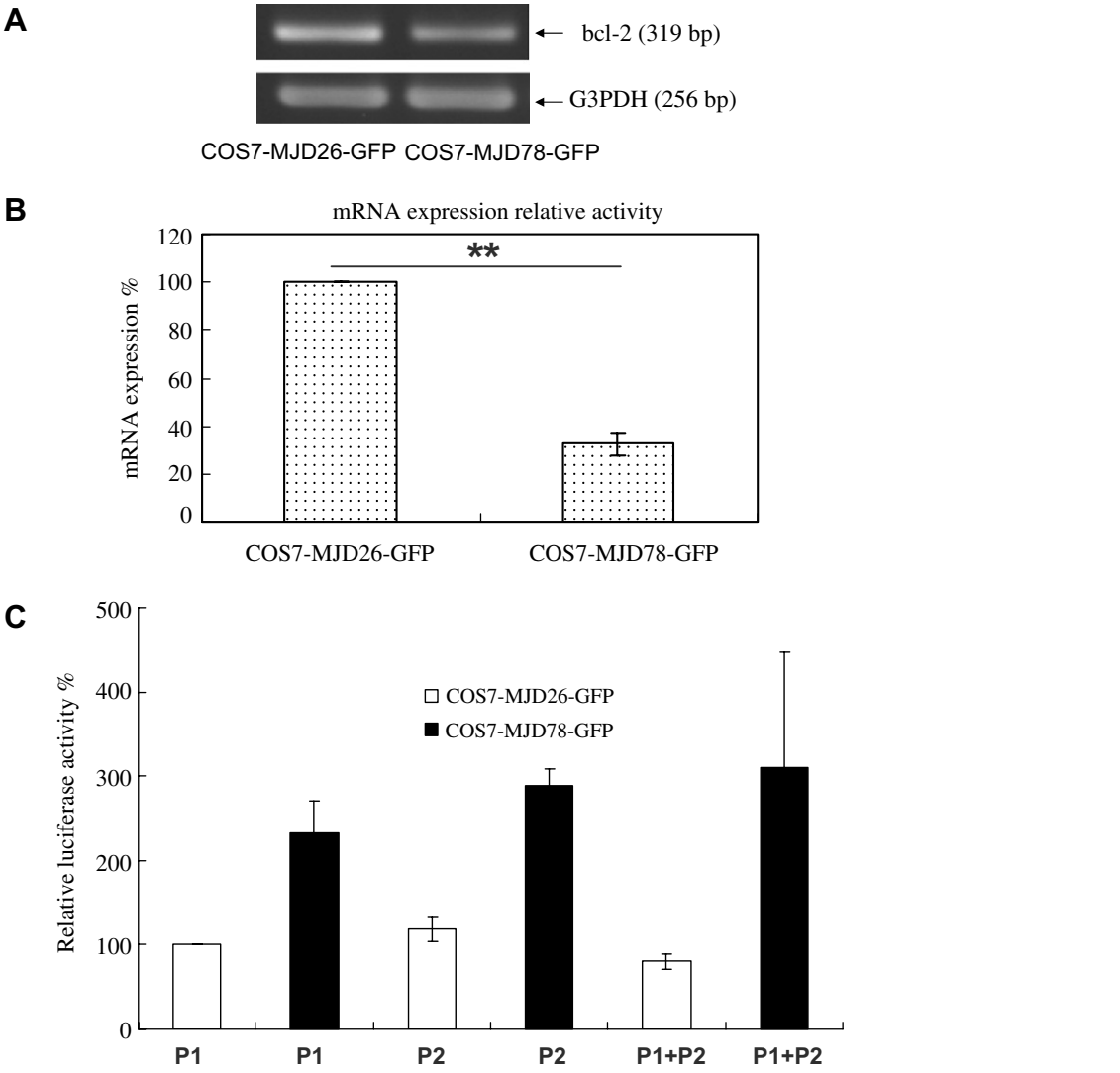

Fig. 2. (A) Semi-quantitative RT-PCR of *bcl-2* gene expression. Total RNA from COS7-MJD26-GFP and COS7-MJD78-GFP cells were analyzed. (B) Quantitative assessment of *bcl-2* mRNA expression in COS7-MJD26-GFP and COS7-MJD78-GFP cells. The band intensity was assessed by an image analysis program (LAS-1000 plus). Compared to the level of a G3PDH internal control, the mean relative levels of *bcl-2* mRNA were plotted with error bars representing standard error mean (SEM). $n = 3$, $^{**}P < 0.005$ compared with that of COS7-MJD26-GFP cells. (C) Activation of various *bcl-2* promoter constructs in the COS7 MJD model. In all cases, promoter activity is compared to the P1 construct in COS7-MJD26-GFP cells (set at 100%). Values are the mean of three experiments with standard error mean (SEM) shown by the bars. $n = 3$.

gene in COS7-MJD78-GFP cells were all markedly higher than those in COS-7-MJD26-GFP cells, indicating that the decreased expression of *bcl-2* transcript is not due to a decreased promoter activity in the presence of expanded ataxin-3.

Decreased expression of Bcl-2 due to defects in mRNA stability

Having established that no decreased *bcl-2* promoter activity is associated with mutant ataxin-3, we next investigated the mRNA stability. The stability of *bcl-2* mRNA was examined *in vivo* by treating cells with DRB, a selective inhibitor of RNA polymerase II, which can prevent transcription initiation with little effect on mRNA stability [25]. A semi-quantitative RT-PCR was used to determine levels of *bcl-2* and G3PDH mRNAs versus time. The logarithmic phase of *bcl-2* and G3PDH cDNA amplification was determined by progressive numbers of PCR cycles. We confirmed that 20 amplification cycles for G3PDH and 30 cycles for *bcl-2* were in a linear range and not at a plateau (data not shown). Therefore, 20 cycles for G3PDH and 30 cycles for *bcl-2* were performed for quantification. The decay kinetics of *bcl-2* mRNA in COS7-MJD78-GFP and COS7-MJD26-GFP cells is shown in Fig. 3A, along with the data quantified by densitometry (Fig. 3B). Interestingly, the COS7-MJD78-GFP cells displayed the shorter *bcl-2* half-life (~4 h), while the COS7-MJD26-GFP cells displayed a half-life of about 14 h. It is noted that the *bcl-2* half-life in COS7 is similar to that in

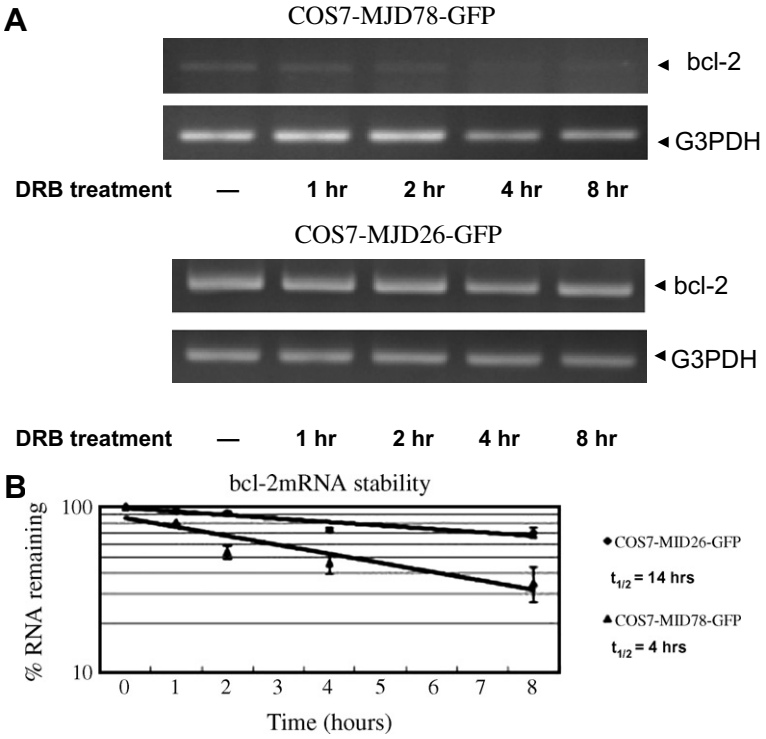

Fig. 3. Half-life of *bcl-2* mRNA in COS7-MJD26-GFP and COS7-MJD78-GFP cells. (A) Total RNA from COS7-MJD78-GFP or COS7-MJD26-GFP cells exposed to 20 μg/ml DRB was isolated at the indicated time points. RT-PCR was performed with *bcl-2* and G3PDH primers as control. (B) Gels from (A) were analyzed by densitometry and plotted in a semilogarithmic scale expressing the percentage of mRNA remaining (means ± SEM) versus time. The half-lives of *bcl-2* are indicated. The data were from three independent experiments.

COS7-MJD26-GFP cells (data not shown). Therefore, we concluded that the decreased *bcl-2* mRNA in the presence of expanded ataxin-3 is due to decreased mRNA stability.

Nucleolin and AUF-1 protein levels are not affected by mutant ataxin-3

It is known that many of the elements that regulate mRNA stability are located in the 3'-untranslated region (3'-UTR) of the mRNA [26], involving a cis-acting AU-rich element (ARE) that binds to a number of ARE-binding proteins (AUBPs). Nucleolin was previously identified as an AU-rich element binding protein involved in *bcl-2* mRNA stability [27]. It was shown that when cells are treated with okadaic acid or taxol, nucleolin is phosphorylated and subsequently cleaved into fragments that no longer bind *bcl-2* mRNA [27]. Therefore, nucleolin levels were examined by Western blots. As shown in Fig. 4A, both cell lines contain a major band corresponding to full-length nucleolin and similar minor bands. Thus, there was no increase in proteolytic fragments of nucleolin in the presence of expanded ataxin-3. Meanwhile, AUF-1, another *bcl-2* AU-rich element binding protein involving *bcl-2* mRNA destabilization during apoptosis [28], was also examined by Western blot analysis. Nevertheless, our results indicated that all AUF-1 isoforms retain similar expression levels in cells with non-expanded or expanded ataxin-3 (Fig. 4B). Therefore, we concluded that both Nucleolin and AUF-1 protein levels are not affected by expanded ataxin-3.

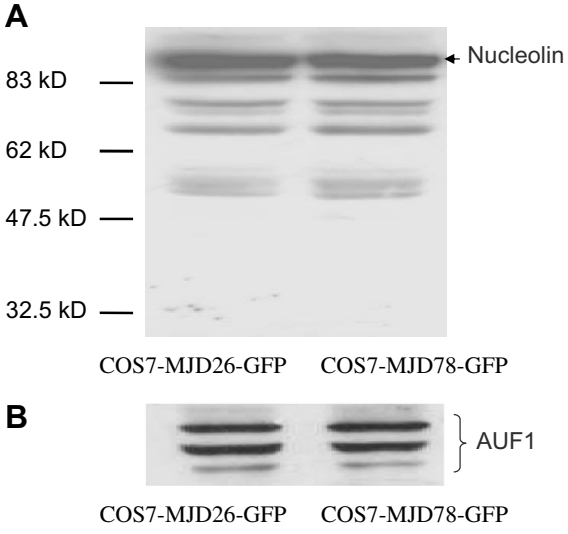

Fig. 4. Western blot analysis of nucleolin and AUF-1 in the COS7 MJD cellular model. Protein extracts from cells with non-expanded ataxin-3 (COS7-MJD26-GFP) or expanded ataxin-3 (COS7-MJD78-GFP) were analyzed. The transferred blot was labeled with anti-nucleolin (A) or anti-AUF-1 (B) antibodies.

Discussion

It is well known that the *bcl-2* gene plays important roles in cell growth, differentiation control, prevention of apoptosis, and oncogenesis [29–31]. Down-regulation of *bcl-2* expression is a general response of the cell to apoptotic

stimuli [32]. In addition, several lines of evidence suggest that expanded ataxin-3 sensitizes cells to apoptosis [8,33]. Therefore, we are interested in the mechanism leading to *bcl-2* down-regulation in the presence of expanded ataxin-3. In the present study, we utilized COS7 cells stably expressing non-expanded or expanded ataxin-3 to analyze the regulation of *bcl-2*. Western blot analysis demonstrated that COS7-MJD78-GFP cells showed a significantly decreased Bcl-2 level when compared with COS7-MJD26-GFP cells (Fig. 1B). To understand whether the down-regulation of Bcl-2 protein expression is due to defects in mRNA transcripts, semi-quantitative RT-PCR was performed and an over 70% decrease of *bcl-2* mRNA was observed in COS7-MJD78-GFP cells when compared with COS7-MJD26-GFP cells. Based on the abovementioned, results from the COS7 MJD model are consistent with that in the human neuronal SK-N-SH MJD model [8]. Compared to the SK-N-SH MJD model, the COS7 MJD model, with relatively high transfection efficiency, serves as a better cellular model because cells containing either non-expanded or expanded ataxin-3 are available for comparison. Therefore, the COS7 MJD model was chosen for further mechanism study.

Previous studies demonstrated that *bcl-2* is regulated at both the transcriptional and post-transcriptional levels. Therefore, to investigate whether defects in transcriptional regulation of *bcl-2* result in a decreased mRNA level, *bcl-2* promoter activity was performed. However, in contrast to our expectation, luciferase assays from all three different promoter constructs of *bcl-2* in COS7-MJD78-GFP cells showed markedly higher activities than those in COS7-MJD26-GFP cells. Even though we do not know yet the reasons for the higher transcription activity in the presence of mutant ataxin-3, it seems that through unidentified post-transcriptional regulation, *bcl-2* transcripts from COS7-MJD78-GFP cells in the steady state become lower than those from COS7-MJD26-GFP cells. Therefore, our results pointed to the other possibility that the decreased expression of *bcl-2* transcripts in COS7-MJD78-GFP cells is due to post-transcriptional regulation.

One of the mechanisms of post-transcriptional regulation of *bcl-2* is the mechanism that modulates *bcl-2* mRNA stability. To determine *bcl-2* mRNA decay in cells, COS7 cells containing either non-expanded or expanded ataxin-3 were treated with the transcription blocker DRB for various time periods and assayed by semi-quantitative RT-PCR. Our results demonstrated that the COS7-MJD78-GFP cells displayed the shorter *bcl-2* half-life (~4 h), while the COS7-MJD26-GFP cells displayed a half-life of about 14 h. It is noted that the half-life of *bcl-2* in COS7 cells is around 15 h (data not shown) which is similar to that in COS7-MJD26-GFP cells, indicating a faster turnover of *bcl-2* mRNA in the presence of expanded ataxin-3. Therefore, we concluded that the drastically decreased mRNA level in the presence of mutant ataxin-3 is due to mRNA instability. However, the fast turnover of *bcl-2* mRNA in cells with expanded ataxin-3 requires further investigation. It is known that many of the elements that regulate mRNA stability are located in the 3'-untranslated region (3'-UTR) of the mRNA [31], involving a cis-acting AU-rich element (ARE) that binds to a number of ARE-binding proteins (AUBPs). The trans-acting RNA-binding proteins regulating *bcl-2* mRNA stability have been identified, including nucleolin and AUF1 [27,28]. Nucleolin has been identified as a *bcl-2*-stabilizing factor and its degradation leads to destabilization of *bcl-2* mRNA [27]. In addition, AUF1 is involved in *bcl-2* mRNA destabilization during apoptosis [28]. To investigate the potential role of nucleolin to stabilize or AUF-1 to destabilize *bcl-2* mRNA, Western blot analysis was performed (Fig. 4). As shown in Fig. 4, there was no alteration in nucleolin or AUF-1 expression in the presence of expanded ataxin-3 when compared with cells with non-expanded ataxin-3. However, we cannot exclude the possibility that altered protein activity or protein–protein interaction involving nucleolin or AUF-1 may be responsible for the decreased stability of *bcl-2* mRNA. It is also likely that there are unidentified factor(s) involved in *bcl-2* mRNA regulation in COS7-MJD78-GFP cells. Further investigations to identify the factor(s) involved in the fast turnover of *bcl-2* mRNA in the presence of expanded ataxin-3 will be required to complete the story.

In summary, this study is the first report to demonstrate that polyQ-expanded ataxin-3 is involved in mRNA destabilization. The findings discussed here may have implications for understanding molecular pathogenesis of other polyQ diseases.

Acknowledgments

We thank Dr. Linda Boxer for the gift of the bcl-2 promoter clones; Dr. Akira Kakizuka for pCMX-MJD26; Dr. Henry Paulson for pcDNA3-HA-MJD78. This work was supported by grants from the National Science Council of the Republic of China (NSC 95-2311-B-029-007; NSC94-2311-B-029-006).

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