

# Regulation of TNF mRNA stability by an RNA-binding protein implicated in myotonic dystrophy

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## Summary

Myotonic dystrophy (DM1) is caused by expression of a toxic RNA containing an expanded CUG repeat. The RNA-binding protein CUG-BP is over-expressed and aberrantly confined to the nucleus in DM1 cells resulting in well-characterized changes in splicing of various clinically relevant genes.

Expression of the toxic expanded-repeat RNA in C2C12 mouse myoblast cells, which induces similar changes in CUG-BP expression as seen in DM1, results in stabilization of TNF RNA. We have also mimicked the cytoplasmic depletion of CUG-BP seen in DM1 by expressing shRNAs against CUG-BP in C2C12 cells. This also resulted in stabilization of the TNF mRNA. These data are consistent with the observation that TNF levels are routinely elevated in DM patients.

We find that CUG-BP binds to the 3'UTR of the TNF mRNA, recognizing both the AU-rich element and flanking UG-rich tetramers. Moreover, binding of CUG-BP results in rapid poly(A) shortening *in vitro*. Finally, we find that CUG-BP interacts directly with a deadenylase, PARN.

Taken together our results suggest that CUG-BP is required to modulate levels of TNF mRNA in muscle cells. Aberrant expression of TNF in DM1 appears to result directly from insufficient cytoplasmic CUG-BP levels. As excess TNF has been linked with muscle wasting, cardiac conduction defects and insulin resistance, it may well cause or exacerbate aspects of DM pathogenesis.

## Introduction

Myotonic dystrophy (DM1) is an autosomal dominant, late onset, inherited disease caused by a triplet repeat expansion in the 3'UTR of the dystrophin protein kinase (DMPK) gene. Symptoms include myotonia, cardiac problems and insulin resistance(1). DM1 is unusual in that the majority of the pathology is not caused by insufficiency of the affected gene, but rather by toxic effects of the mRNA expressed from it. The DMPK mRNA bearing the expanded repeat accumulates in nuclear foci where it sequesters various proteins including splicing factors(2), and transcription factors(3). Much of the pathogenesis has in fact been attributed to altered splicing patterns(4;5).

One protein whose expression is affected by the toxic RNA is CUG-BP. In DM1 patients, CUG-BP is over-expressed and confined to the nucleus resulting in an overall reduction in its cytoplasmic concentration(6). CUG-BP is an RNA-binding protein that has been previously implicated in regulation of splicing, translation and mRNA stability, thus alterations in its expression pattern have broad implications for gene expression.

Here we have examined the role of CUG-BP in regulating decay of TNF mRNA in muscle cells. TNF undergoes a large amount of post-transcriptional regulation, most of which is mediated by an AU-rich element (ARE) in its 3' UTR(7). TNF levels are elevated in DM1 patients

although it is presently unclear whether this is a direct cause of pathogenesis or merely reflects the disease state(8). In addition, TNF levels are regulated during muscle differentiation and aberrant levels of TNF can prevent this process(9).

## Results

In order to determine whether changes in CUG-BP expression seen in DM1 have effects on TNF mRNA stability, we expressed an RNA consisting of the last three exons of DMPK, including the 3'UTR, in C2C12 mouse myoblasts. Two constructs were used, one with the normal 3'UTR, the other containing 960 CUG repeats in the 3'UTR. Following transfection of the expression plasmids, transcription was inhibited by addition of actinomycin D and cells were harvested for analysis of RNA abundance at several time points. Levels of TNF and GAPDH mRNAs at each time were assessed by qRT-PCR.

The results showed that in cells expressing the normal DMPK 3'UTR, the TNF mRNA decayed very rapidly with a half life of 8 min. However, transfection of the expanded repeat 3'UTR, which is known to induce formation of nuclear foci and disrupt CUG-BP expression, resulted in stabilization of the TNF mRNA such that its half life increased to 20 min.

As the DMPK toxic RNA has a wide range of effects on cellular gene expression, we needed to ascertain whether the paucity of CUG-BP was

responsible for the changes in TNF decay. We used an shRNA against the 3'UTR of CUG-BP to generate a C2C12 cell line with a 90% reduction in CUG-BP expression. We measured the half life of TNF mRNA in this cell line and compared it to that in cells bearing an empty vector instead of the shRNA-expressing construct. We found that depletion of CUG-BP again resulted in stabilization of the TNF mRNA such that the half life increased from 9.5 min to 18 min.

Given that TNF mRNA decay is regulated through its 3'UTR, we wanted to determine whether CUG-BP binds directly to this region of the TNF mRNA. Using both gel-shift and cross-linking assays, we find that CUG-BP associates specifically with a 250 nt region of the TNF 3'UTR flanking the ARE(10). Gel shift experiments are consistent with co-operative binding of up to four CUG-BP monomers to this substrate. Interestingly, although the ARE increases CUG-BP binding affinity, flanking sequences, likely UG-rich tetramers, are also involved in recruiting CUG-BP to the RNA.

Next, we wanted to determine the functional significance of CUG-BP binding. The decay of most mRNAs initiates with removal of the poly(A) tail. We used an *in vitro* mRNA decay assay developed in our laboratory to examine the role of CUG-BP in regulating deadenylation of the TNF RNA. Briefly, capped and polyadenylated radio-labeled RNA substrates are incubated in HeLa cytoplasmic extracts and changes in poly(A) tail length are assessed by separation on a denaturing polyacrylamide gel. When the 250nt TNF mRNA substrate was incubated in HeLa extracts it underwent rapid poly(A) shortening, similar to that seen for a control RNA (Gem). However, immunodepletion of CUG-BP from the extracts resulted in a significant repression of deadenylation of the TNF RNA, with minimal effects on the control substrate(10). This suggested that binding of CUG-BP to the RNA substrate mediates rapid poly(A) shortening.

In our extracts the majority of deadenylation is carried out by the PARN deadenylase. When we used antibodies against CUG-BP to precipitate the CUG-BP protein from HeLa extracts we could readily detect PARN protein in the precipitate. This was not the case when pre-immune serum was used. This result suggests that CUG-BP functions by recruiting PARN to the RNA substrate.

We confirmed that there is a direct interaction between PARN and CUG-BP using a GST

pull-down assay with recombinant proteins. Importantly, interaction between CUG-BP and PARN was detected even in the absence of RNA, thus PARN and CUG-BP must bind each other directly.

## Discussion

Taken together our results demonstrate that in muscle cells CUG-BP binds the TNF mRNA and initiates its rapid decay by recruiting the PARN deadenylase. We propose that the aberrant expression of CUG-BP in DM1 results in stabilization of TNF mRNA and elevated expression of this potent cytokine. As excess TNF is known to cause muscle wasting, cardiac conduction defects and insulin resistance, it is very possible that this is contributing significantly to the disease state.

Future experiments will be directed at determining whether TNF mRNA stability is altered in mouse models of the disease, as well as in patient myoblasts. In addition we are interested in characterizing the interaction between PARN and CUG-BP in more detail.

## References

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