Human Molecular Genetics, 2009, Vol. 18, No. 8 ***1471–1481***
doi:10.1093/hmg/ddp058
Advance Access published on February 17, 2009

Transcriptional and post-transcriptional impact of toxic RNA in myotonic dystrophy

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Received December 1, 2008; Revised and Accepted January 29, 2009

Myotonic dystrophy type 1 (DM1) is an RNA dominant disease in which mutant transcripts containing an expanded CUG repeat (CUG^{exp}) cause muscle dysfunction by interfering with biogenesis of other mRNAs. The toxic effects of mutant RNA are mediated partly through sequestration of splicing regulator Muscleblind-like 1 (Mbnl1), a protein that binds to CUG^{exp} RNA. A gene that is prominently affected encodes chloride channel 1 (Clcn1), resulting in hyperexcitability of muscle (myotonia). To identify DM1-affected genes and study mechanisms for dysregulation, we performed global mRNA profiling in transgenic mice that express CUG^{exp} RNA, when compared with *Mbnl1* knockout and *Clcn1* null mice. We found that the majority of changes induced by CUG^{exp} RNA in skeletal muscle can be explained by reduced activity of Mbnl1, including many changes that are secondary to myotonia. The pathway most affected comprises genes involved in calcium signaling and homeostasis. Some effects of CUG^{exp} RNA on gene expression are caused by abnormal alternative splicing or downregulation of Mbnl1-interacting mRNAs. However, several of the most highly dysregulated genes showed altered transcription, as indicated by parallel changes of the corresponding pre-mRNAs. These results support the idea that *trans*-dominant effects of CUG^{exp} RNA on gene expression in this transgenic model may occur at the level of transcription, RNA processing and mRNA decay, and are mediated mainly but not entirely through sequestration of Mbnl1.

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is the most prevalent form of muscular dystrophy. This disorder is caused by expansion of a CTG repeat in the 3′ untranslated region (UTR) of *DMPK*, a gene encoding a protein kinase (1). The mutant *DMPK* mRNA contains a highly expanded CUG repeat (CUG^{exp}) and is retained in nuclear foci (2–4). Accumulation of this transcript in the nucleus leads to RNA dominant disease by interfering with the regulated expression of other genes (reviewed in 5). Expression of CUG^{exp} RNA in transgenic mice, or RNA containing an interrupted CUG repeat, reproduces features of DM1, such as, repetitive action potentials (myotonia) and degenerative changes in skeletal muscle (6,7).

The mutant *DMPK* mRNA is believed to affect gene expression through several distinct mechanisms. The first indication of *trans*-dominant effects on RNA metabolism came from studies suggesting an effect of the mutant *DMPK* mRNA on polyadenylation (8). Splicing regulators in the Muscleblind-like (MBNL) family, such as MBNL1, were shown to bind to CUG^{exp} RNA with high affinity and become sequestered in nuclear foci (9–11). Reduced MBNL1 activity leads to abnormal regulation of alternative splicing for several genes expressed in skeletal muscle and heart (10,12). MBNL proteins may also regulate mRNA localization (13), but it is unclear whether this activity is affected in DM1. In addition to effects on MBNL proteins, DM1 is associated with upregulation of two additional RNA binding proteins, CUGBP1 and hnRNP H (14–16). These proteins regulate alternative splicing, stability and translation for a group of muscle-expressed transcripts, some of which are

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also regulated by Mbnl1 (17–19). Moreover, CUG^{exp} hairpins are processed by Dicer, resulting in short poly(CUG) RNAs that may induce posttranscriptional silencing of genes that contain CAG repeats (20). Finally, CUG^{exp} RNA may influence transcription by activating signaling proteins, such as protein kinase C or the dsRNA-dependent protein kinase PKR, or by leaching transcription factors from chromatin (15,21,22).

A *trans*-effect of CUG^{exp} RNA on RNA processing that is linked to functional impairment in DM1 involves the muscle-specific chloride channel, Clcn1. Expression of CUG^{exp} RNA or ablation of Mbnl1 causes inclusion of an additional exon in the *Clcn1* mRNA. This leads to a shift of the reading frame, truncation of the Clcn1 protein, loss of channel function, repetitive action potentials and delay of muscle relaxation (myotonia) (23–26). A similar derangement exists in human DM1 (23,27). Myotonic discharges are known to influence gene expression in skeletal muscle (28–31), but the full spectrum of genes responding to muscle hyperactivity has not been determined.

To identify genes and pathways impacted by CUG^{exp} RNA, we used oligonucleotide microarrays to examine gene expression in *human skeletal actin long repeat* (HSA^{LR}) transgenic mice. These mice express skeletal actin mRNA containing ~250 CUG repeats in the 3′-UTR (6). The CUG^{exp}-containing transcript is only expressed in skeletal muscle, and the mice display characteristic features of DM1, such as sequestration of Mbnl1 protein in nuclear foci, structural changes in muscle and myotonia. To determine which changes in CUG^{exp}-expressing mice may result from repetitive action potentials, results were compared with *Clcn1* null mice that have severe myotonia. To determine which changes in CUG^{exp}-expressing mice may result from Mbnl1 sequestration, results were compared with *Mbnl1* knockout mice (12). We also used RNA immunoprecipitation to identify Mbnl1-interacting mRNAs whose expression may be affected by Mbnl1 sequestration. Our results indicate that gene expression abnormalities in DM1 may result from effects on transcription, processing and stability of RNA, and point to pathways impacted by this disease.

RESULTS

Effects of CUG^{exp} RNA on gene expression in skeletal muscle

To determine global effects of CUG^{exp} RNA on gene expression, we used Affymetrix Mouse Genome 430 microarrays to compare two founder lines of HSA^{LR} transgenic mice with WT controls ($n = 6$ per group). Of ~45 000 probe sets on the expression arrays, ~23 000 showed detectable gene expression in muscle (see Materials and Methods). Among the muscle-expressed transcripts, 269 were dysregulated either in transgenic line HSA^{LR}20b, transgenic line HSA^{LR}41, or both, based on criteria of a fold-change >2, nominal P-value <0.001 according to t-tests, and false discovery rate (FDR) <1% according to significance analysis of microarrays (SAM) (32) (Supplementary Material, Table S1). The number of differentially expressed transcripts was higher in line HSA^{LR}20b ($n = 252$) than in line HSA^{LR}41 ($n = 75$), and the fold-change was generally greater ($P < 0.001$ for paired t-test among transcripts differentially expressed in both lines). These results were consistent with our previous observations that the level of CUG^{exp} expression, severity of myotonia and extent of muscle histopathology were greater in line HSA^{LR}20b than in line HSA^{LR}41 (6). Out of 75 transcripts that were differentially expressed in line HSA^{LR}41, 73 (97%) showed a similar change in line HSA^{LR}20b (change in the same direction with a nominal P-value <0.01, Supplementary Material, Table S1). Out of 252 transcripts that were differentially expressed in line HSA^{LR}20b, 175 transcripts (69%) showed a similar change in line HSA^{LR}41 (change in the same direction with a nominal P-value <0.01). These results indicated that effects of CUG^{exp} RNA on gene expression were broadly similar in both HSA^{LR} founder lines, but the effect size was greater in line HSA^{LR}20b. We therefore focused our subsequent analyses on line HSA^{LR}20b. However, to eliminate changes that may depend on the site of transgene integration or extreme levels of CUG^{exp} accumulation, we report only those transcripts dysregulated in line HSA^{LR}20b that also showed a similar, if less profound change in line HSA^{LR}41 (i.e. transcripts showing a fold-change >2 with nominal P-value <0.001 and FDR <1% in line HSA^{LR}20b, and a change in the same direction with nominal $P < 0.01$ in line HSA^{LR}41). These transcripts constitute the 'dysregulated in HSA^{LR}' set.

By these criteria, 175 transcripts were dysregulated in HSA^{LR} mice, comprising 110 transcripts that were upregulated and 65 that were downregulated (Supplementary Material, Table S2). The 10 most-upregulated and downregulated transcripts are shown in Table 1. Quantitative RT–PCR analysis confirmed the change in mRNA level for each of the seven transcripts that we tested (Supplementary Material, Table S3). Moreover, for two genes, *Cpne2* encoding a Ca^{2+} binding protein involved in membrane trafficking, and *Uchl1* encoding ubiquitin carboxyl-terminal hydrolase L1, we confirmed a marked upregulation of protein expression by immunoblot (Fig. 1A–B).

Comparisons with Clcn1 and Mbnl1 null mice

Gene expression in skeletal muscle is modulated by patterns of electrical activity (33). To identify changes induced by CUG^{exp} RNA that may result from myotonia, results in HSA^{LR} mice were compared with *Clcn1* null mice that have generalized myotonia. Out of 175 transcripts dysregulated in HSA^{LR} mice, 42 showed a similar effect in the $Clcn1^{-/-}$ versus $Clcn1^{+/+}$ comparison, defined as a change in the same direction with a nominal P-value <0.01 (Supplementary Material, Table S4, Section A). In terms of function, many genes that were dysregulated in HSA^{LR} and $Clcn1^{-/-}$ mice pertained to cell signaling. These results suggest that a substantial fraction (~24%) of the major changes in gene expression in HSA^{LR} mice may result from increased electrical activity due to misregulated alternative splicing of a single Mbnl1 splicing target, *Clcn1*.

The set of 175 HSA^{LR}-dysregulated transcripts showed little overlap with a previous dataset from mice with muscular dystrophy due to dystrophin loss (34). Only four transcripts were

Table 1. Genes most highly dysregulated in *HSA*^{LR} mice

Gene	Functional category	HSALR20b Fold-change	*P*(*t*)	HSALR41 Fold-change	*P*(*t*)	Clcn1 null Fold-change	*P*(*t*)	Mbnl1 knockout Fold-change	*P*(*t*)
Sarcolipin	Ca2+ homeostasis	98.3	2.8E−06	72.8	6.0E−07	15.6	0.010	42.6	0.176
Ubiquitin carboxy terminal hydrolase L1	Proteolysis, neuronal	20.0	4.6E−12	25.3	0.003	1.2	0.102	16.1	0.002
Myosin IA	Vesicle trafficking	17.9	1.2E−06	28.7	7.0E−05	−1.1	0.362	1.7	0.007
Cartilage intermediate layer protein	ECM, IGF-1 signaling	16.9	3.7E−06	7.8	2.8E−04	3.6	0.008	5.1	4.4E−05
Myelin protein zero-like 3	Cell adhesion	16.7	1.0E−06	7.7	1.3E−05	−1.5	0.204	1.6	0.053
Copine II	Ca-dependent membrane binding	14.0	3.4E−06	10.1	7.2E−07	1.4	0.124	11.0	0.001
Plekho1	PI3-kinase signaling	11.0	4.5E−06	6.3	5.5E−06	1.4	0.004	6.6	0.001
Ectodysplasin A2 isoform receptor	Transmembrane receptor	9.8	2.8E−06	5.2	1.7E−05	−1.0	0.943	14.0	2.0E−04
Poliovirus receptor-related 3	Cell membrane and adhesion	8.2	1.6E−04	2.5	4.3E−05	1.0	0.968	2.5	0.010
Contactin associated protein-like 2	Cell adhesion, neuronal	7.9	1.4E−07	3.7	0.001	−8.2	0.002	3.7	1.1E−04
HtrA serine peptidase 1	IGF signaling	−4.3	1.8E−05	−2.2	4.7E−04	−4.3	0.001	−1.8	0.001
Caspase 12	Apoptosis	−5.3	5.8E−05	−2.2	0.010	−2.3	0.081	−3.3	6.0E−05
Solute carrier family 38, member 4	Amino acid transporter	−5.6	2.6E−06	−1.8	0.001	−15.5	6.4E−05	−5.4	0.001
Dickkopf homolog 3	Wnt signaling	−5.9	5.0E−05	−3.6	1.6E−04	−18.8	1.6E−04	−1.8	0.093
Mindbomb homolog 1	Notch signaling	−7.4	5.6E−07	−3.4	2.8E−05	−6.0	0.001	−5.0	0.018
Gdap1	Mitochondrial fission	−7.6	5.5E−05	−2.1	0.007	−123.1	0.021	−3.7	2.5E−04
Gremlin 2 homolog	BMP signaling	−7.6	3.2E−04	−4.3	9.1E−04	−2.6	0.006	−3.1	4.9E−05
Phospholipase A2, group VII	Inflammatory response	−9.3	1.5E−06	−3.3	3.6E−05	−28.5	0.039	−2.8	0.006
Tiam1	Receptor signaling, microtubule function	−10.0	5.3E−05	−3.8	3.4E−04	−10.3	0.009	−3.9	9.7E−05
Kcnab1 potassium channel	Ion channel	−24.7	3.4E−07	−3.6	2.3E−05	−4.1	3.4E−04	−21.6	8.4E−05

P(*t*) denotes nominal *P*-value for *t*-test.

dysregulated in common (Supplementary Material, Table S2). Furthermore, whereas *Clcn1*$^{-/-}$ mice showed upregulation of embryonic myosin and vimentin, two markers for muscle regeneration (35,36), the regenerative changes in *HSA*^{LR}20b mice were less pronounced (Supplementary Material, Table S4, Section B). These results suggest that relatively few of the changes in the *HSA*^{LR}-dysregulated transcript set result from a non-specific response of muscle to chronic disease or regeneration.

To identify changes induced by CUG^{exp} RNA that depend on Mbnl1 sequestration, we compared gene expression changes in *HSA*^{LR} mice with *Mbnl1* knockouts. We first backcrossed a disrupted allele of *Mbnl1* (12) onto the same genetic background as *HSA*^{LR} mice (FVB strain), and then compared gene expression in *Mbnl1* knockouts with their WT littermates ($n = 5$ per group). Among 175 transcripts in the *HSA*^{LR}-dysregulated set, 127 (73%) were similarly dysregulated in *Mbnl1* knockout mice (fold-change >1.5, $P < 0.01$), and most of these were not affected in *Clcn1*$^{-/-}$ mice (70 transcripts, Supplementary Material, Table S4, Section C). However, a group of 17 *HSA*^{LR}-dysregulated transcripts showed no trend for altered expression either in *Mbnl1* knockout or *Clcn1*$^{-/-}$ mice (nominal *P*-value >0.05 for both comparisons, Supplementary Material, Table S4, Section D). These results indicate that most of the major changes in gene expression in *HSA*^{LR} mice result directly or indirectly (via myotonia) from sequestration of Mbnl1, but that loss of function for this protein cannot account for all effects of CUG^{exp} RNA.

Pathways dysregulated in *HSA*^{LR}, *Mbnl1* knockout, or *Clcn1*$^{-/-}$ mice

The results presented here were based on stringent criteria for differential gene expression. However, more subtle differences in gene expression may have important consequences, particularly if several genes involved in the same pathway are affected. We used ingenuity pathway analysis (37) (IPA), gene set enrichment analysis (38) (GSEA) and expression analysis systematic explorer (39) (EASE) to test for effects CUG^{exp} RNA, Mbnl1 loss or myotonia on functionally related genes, including effects that might not be obvious based on the largest fold differences or lowest *P*-values. Results of pathway analysis are summarized in Table 2, and specific findings from GSEA are shown in Supplementary Material, Table S5.

By IPA, the pathway most prominently affected in *HSA*^{LR} and *Mbnl1* knockout mice comprised genes involved in Ca^{2+} signaling and homeostasis (Supplementary Material, Table S6). These changes were complex, including upregulation of some

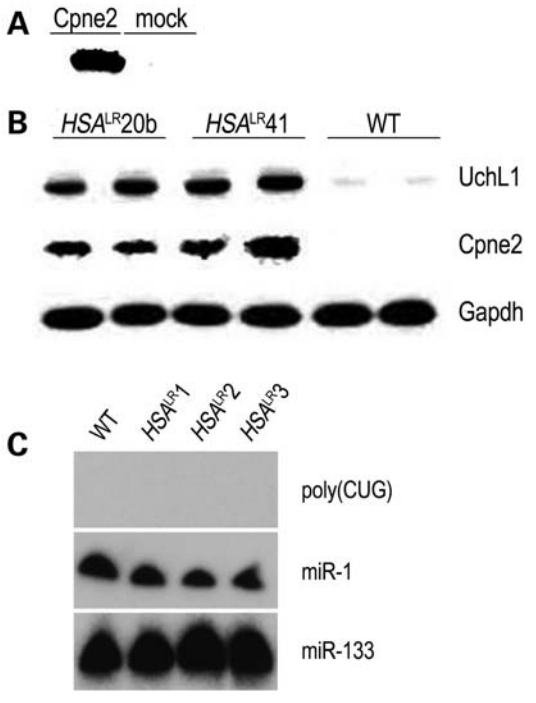

Figure 1. Upregulation of Cpne2 and UchL1 protein, and absence of short poly(CUG) RNA, in mice that express CUG^{exp} RNA. (**A**) Specificity of anti-Cpne2 antibody. Immunoblot shows detection of appropriate 60 kDa protein in COS cells transfected with Cpne2 expression construct, but not in mock transfected cells. (**B**) Cpne2 protein is detected by immunoblot in muscle homogenate from HSA^{LR}20b and HSA^{LR}41 mice, but not in muscle from wild-type (WT) mice of the same strain background (FVB). Gapdh serves as loading control. A parallel blot was probed with the anti-UchL1 antibody. (**C**) RNA blot analysis was performed under conditions designed to detect short CUG repeats. Shown are RNAs isolated from quadriceps muscle of three HSA^{LR} transgenic mice and WT control (FVB). Probes for miR-1 and miR-133 RNAs serve as controls for RNA loading and miRNA detection. Note that short poly(CUG) RNA was undetectable even after a 20-fold longer film exposure (not shown).

transcripts and downregulation of others. The transcript most highly upregulated in HSA^{LR} mice encoded sarcolipin, a protein that modulates activity of the Ca^{2+} reuptake pump of the sarcoplasmic reticulum (Serca) (40,41). Upregulation of sarcolipin mRNA ranged from 16-fold in $Clcn1^{-/-}$ mice to 98-fold in HSA^{LR}20b mice by microarray, and was confirmed as 60-fold increased in both lines of HSA^{LR} mice by qRT–PCR (Supplementary Material, Table S3). Also common to *Clcn1* null, *Mnbl1* knockout and HSA^{LR} transgenic mice were downregulation of *CaM kinase 2 alpha*, *CaM kinase 2 gamma*, *calcineurin-B* and all three of the calmodulin genes expressed in skeletal muscle. However, other effects on Ca^{2+} signaling and homeostasis differed between *Clcn1* null mice and the DM1 models (Supplementary Material, Table S6).

$Clcn1^{-/-}$ mice showed upregulation of genes involved in fat metabolism and oxidative phosphorylation, with a concurrent decrease in genes involved in carbohydrate catabolism, consistent with known effects of increased muscle activity on these pathways (28–30). However, these effects were generally absent in *Mbnl1* knockout or HSA^{LR} mice, despite the presence of myotonia (Supplementary Material, Table S5). In contrast, a set of genes upregulated in *Mbnl1* knockout and HSA^{LR} mice but not in *Clcn1* null mice comprised transcripts that encode ribosomal proteins. While the statistical significance of this change was high (false detection rate $<1\%$ by GSEA and $P < 10^{-10}$ by EASE), owing to coordinate upregulation of many different transcripts in this pathway, the magnitude of the effect on individual transcripts was small (1.1- to 1.3-fold increase for most transcripts).

The computer algorithms did not identify pathways that differed between HSA^{LR} and *Mbnl1* knockout mice. However, inspection of gene lists did show a functional connection. Among transcripts strongly affected by inactivation of *Mbnl1* (3-fold up- or downregulated in $Mbnl1^{-/-}$ mice with a nominal $P < 0.0001$, $n = 39$ transcripts), most were similarly dysregulated in line HSA^{LR}20b or HSA^{LR}41 ($n = 31$ or 79%), but eight showed no parallel change in either HSA^{LR} transgenic line. Six of these eight transcripts encoded contractile proteins of slow-twitch muscle fibers, and were strongly downregulated in *Mbnl1* knockout and $Clcn1^{-/-}$ mice but not in HSA^{LR} mice (Supplementary Material, Table S4, Section E, see Discussion).

Mechanism of gene dysregulation in response to CUG^{exp} RNA

Krol *et al*. (20) proposed that CUG^{exp} hairpins are processed by Dicer to short CUG fragments that induce post-transcriptional silencing of transcripts that contain CAG repeats. To examine this mechanism, we performed Northern blots to detect short CUG repeat RNAs. Under conditions that readily detected miR-1 and miR-133 microRNAs, we were unable to demonstrate short poly(CUG) RNAs in skeletal muscle of HSA^{LR} mice (Fig. 1C). We also carried out BLAST analysis of mouse RefSeq mRNAs to identify potential targets for degradation by this mechanism. Out of 89 muscle-expressed transcripts that contained 19 or more nucleotides of CAG repeats, 11 showed a trend for differential expression in HSA^{LR} transgenic mice (nominal $P < 0.05$) that did not occur in *Mbnl1* knockout or $Clcn1^{-/-}$ mice. Among these 11 transcripts, 8 were downregulated (-1.2- to -2.0-fold) whereas 3 were upregulated (1.3- to 2.7-fold) (Supplementary Material, Table S4, Section F). However, only one of the downregulated transcripts, *Med12*, satisfied stringent criteria for differential expression (see Discussion).

Changes in RNA decay may also result from misregulated alternative splicing. For example, this occurs when variant splice products contain premature termination codons (PTCs) that induce nonsense-mediated decay (NMD). In DM1 patients and mouse models, inclusion of *Clcn1* exon 7a is increased, which creates a PTC that triggers NMD of the *Clcn1* mRNA (23,42). Consistent with this effect, the microarray data showed reduction of *Clcn1* mRNA in HSA^{LR}20b (-1.9-fold-change, $P < 0.0001$) and *Mbnl1* knockout mice (-2.1-fold-change, $P = 0.001$). However, cross-referencing of other HSA^{LR}- and *Mbnl1* knockout-dysregulated transcripts with an alternative splicing database (43) yielded only a single additional candidate, *ninein*, for dysregulation by this mechanism, and RT–PCR analysis failed to confirm a splicing change (data not shown). We also cloned and sequenced cDNAs from two genes that were highly dysregulated in HSA^{LR}20b and *Mbnl1* knockout mice, *ectodysplasin A2 isoform receptor*

Table 2. Pathway analysis of gene expression profiles in mouse models of DM1 and Clcn1 null mice

	HSALR20b			HSALR41			Clcn1 null			Mbnl1 knockout		
	IPA	GSEA	EASE	IPA	GSEA	EASE	IPA	GSEA	EASE	IPA	GSEA	EASE
Signaling												
Calcium signaling and homeostasis	↑↓			↑↓			↑↓	↓		↑↓		
Downregulated by TNF alpha in endothelial cells		↓			↓							
Metabolism												
Glycerolipid and fatty acid metabolism		↓					↑	↑	↑			
Mitochondrial oxidative phosph./TCA cycle			↑				↑	↑	↑			
Amino acid degradation/metabolism							↑	↑				
Glutathione metabolism							↑					
Glycolysis/glycogen metabolism			↓				↓		↓			
Other												
Ribosomal proteins		↑	↑		↑	↑					↑	↑
Translation/translation elongation			↑			↑						
MyoD targets					↑							
Microtubule binding						↑						
Caveola						↓						
Calcium-dependent cell adhesion molecule activity			↓									
Purine nucleotide/GTP binding			↓									

'↑↓' denotes pathway in which some genes are upregulated (↑) and others are downregulated (↑).

(*Xedar*, 39 cDNA clones sequenced) and *copine 2* (*Cpne2*, 35 clones sequenced), and found splicing patterns that were similar in WT and HSA^{LR}20b mice.

RNA processing may also influence gene expression through use of alternative 3′ ends. Microarray data suggested that this occurred for *Asph/junctin/junctate*, a gene encoding three functionally distinct proteins, depending on which 3′ end is utilized (44). Among seven probe sets for *Asph/junctin/junctate* on the arrays, only those targeting the longer isoforms, utilizing downstream 3′ ends, were upregulated 2- to 4-fold in HSA^{LR}, $Mbnl1^{-/-}$, and $Clcn1^{-/-}$ mice. RT–PCR analysis confirmed that utilization of 3′ ends was altered (Supplementary Material, Fig. S1).

Proteins that bind to pre-mRNA to regulate splicing may also bind to mRNA to regulate stability or translation. To address this possibility for Mbnl1, we prepared muscle homogenates for immunoprecipation of ribonucleoprotein complexes using anti-Mbnl1 antibodies (Supplementary Methods). We first showed that Mbnl1 protein was highly enriched in the immunoprecipitate (IP) from WT and HSA^{LR} muscle (Fig. 2A). The HSA^{LR} (CUG^{exp}-containing) transcript was also enriched in the IP from HSA^{LR} muscle, confirming that a known Mbnl1-interacting transcript was pulled down by this method (Fig. 2B). We then used microarrays to analyze transcripts that co-immunoprecipitated with Mbnl1 from WT muscle. A parallel IP from *Mbnl1* knockout muscle served as a control for non-specific pull down of mRNA. Whereas the number of transcripts expressed in WT and *Mbnl1* knockout muscle was similar (22 917 versus 23 174 probe sets detected in total cellular RNA), the number of transcripts in the IP was much higher in WT than $Mbnl1^{-/-}$ muscle (9144 versus 2609 probe sets detected). These findings indicate that many transcripts have direct or indirect association with Mbnl1 protein, suggesting that functions of this protein are broader than originally suspected. Notably, the spectrum of RNAs in the control ($Mbnl1^{-/-}$) IP was limited, mainly comprising transcripts above the 90th percentile for expression level (Supplementary Material, Fig. S2, right panels), consistent with non-specific pull down of high abundance mRNAs. In contrast, the IP from WT mice represented a broad spectrum of expression levels, including many mRNAs of low or intermediate abundance (Supplementary Material, Fig. S2, left panels).

To test for effects of Mbnl1 ablation on expression of Mbnl1-interacting mRNAs, we next defined a set of transcripts that were misregulated in *Mbnl1* knockout mice in a myotonia-independent fashion (fold-change >1.5 with nominal $P < 0.01$ in $Mbnl1^{-/-}$ versus $Mbnl1^{+/+}$ mice, $P > 0.05$ in $Clcn1^{-/-}$ versus $Clcn1^{+/+}$ mice). This set included 733 probe sets, of which 339 (46%) were downregulated and 394 (54%) were upregulated. We compared this transcript set with transcripts that were highly enriched in the anti-Mbnl1 IP ($>$50-fold higher signal in the WT than $Mbnl1^{-/-}$-IP, $n = 847$, see Supplementary Methods). The intersection comprised 52 transcripts, of which 48 (92%) were downregulated in $Mbnl1^{-/-}$ muscle, whereas only 4 (8%) were upregulated (Fig. 3, Supplementary Material, Table S7). The overrepresentation of downregulated transcripts ($P < 0.001$) among Mbn1-interacting mRNAs suggests that one mechanism for gene dysregulation in Mbnl1-deficient muscle involves destabilization of transcripts that normally interact with Mbnl1 protein. However, it appears that relatively few of the major changes induced by CUG^{exp} RNA can be accounted for by this mechanism. Only five transcripts in the HSA^{LR}-dysregulated set were highly enriched in the anti-Mbnl1 IP, all of which were downregulated (Supplementary Material, Table S7).

Altered transcription is another putative mechanism for gene deregulation in DM1. Previously, we found that CUG^{exp} RNA binds to and activates the double-stranded-RNA-dependent protein kinase, PKR, *in vitro* (21). Activation of this kinase *in vivo* would be expected to induce genes involved in the interferon response. However, HSA^{LR} mice did not show upregulation of interferon-responsive genes (data not shown).

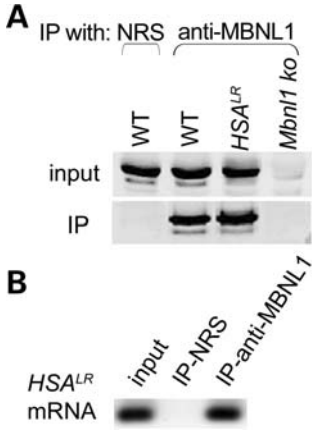

Figure 2. Immunoprecipitation (IP) of ribonucleoprotein complexes containing Mbnl1 protein and Mbnl1-interacting mRNA. (**A**) Immunoblot shows Mbnl1 protein in homogenate prepared from WT or HSA^{LR} muscle ('input') but not in homogenate prepared from *Mbnl1* knockout (ko) mice. Immunoblot in lower panel shows that Mbnl1 protein is Immunoprecipitated by affinity-purified anti-Mbnl1 antibody but not by normal rabbit serum (NRS). (**B**) RT–PCR assay showing that a known Mbnl1-interacting mRNA (HSA^{LR} mRNA) is co-Immunoprecipitated by anti-Mbnl1 antibodies by not by non-immune serum.

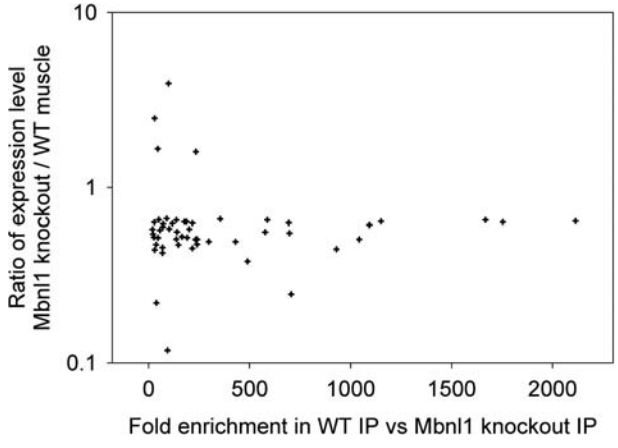

Figure 3. Overrepresentation of downregulated transcripts among Mbnl1-interacting mRNAs that are dysregulated in Mbnl1 knockout mice. Vertical axis shows fold-change in expression level in Mbnl1 knockout mice, expressed as a ratio with WT mice.

Mutant *DMPK* mRNA also is reported to cause leaching of transcription factors RARγ and Sp1 from chromatin, resulting in downregulation of several transcripts, including *Clcn1*, *Sp1*, *Sp3* and *RARγ* in myogenic cells (22). Among these target transcripts, however, only the expression of *Clcn1* was reduced in HSA^{LR} lines, an effect that is not caused by reduced transcription (42). To test for effects on transcription more directly, we used competitive RT–PCR to quantify pre-mRNA levels for several genes that showed major dysregulation in HSA^{LR} and *Mbnl1* knockout mice. There was marked upregulation of *Cpne2* mRNA (14-fold and 11-fold increase for HSA^{LR} and *Mbnl1* knockout mice, respectively, $P < 0.0001$) and protein (Fig. 1) in DM1 models, but the level of pre-mRNA was not different from WT controls, supporting a post-transcriptional mechanism for dysregulation of this gene. However, levels of pre-mRNA for *Uchl1*, *ectodysplasin A2 isoform receptor* (*Xedar*), *voltage-gated K+ channel β subunit 1* (*Kcnab1*), *myosin 1A* (*Myo1A*) and *sarcolipin* (*Sln*) all showed changes in HSA^{LR} and *Mbnl1* knockout mice that paralleled the change in mRNA (Table 3), supporting a transcriptional mechanism. These results indicate that altered transcription is an important disease mechanism in mouse models of DM1.

Table 3. Competitive RT–PCR analysis of pre-mRNA

Gene	HSA^{LR}41 versus WT		HSA^{LR}20b versus WT		$Mbnl1^{-/-}$ versus $Mbnl1^{+/+}$	
	FC	$P(t)$	FC	$P(t)$	FC	$P(t)$
Sarcolipin	16	0.02	10	0.001	4.9	0.01
Ubiquitin carboxy-terminal hydrolase L1	3.8	0.02	2.9	0.04	2.2	0.09
Copine II	0.77	0.17	1.1	0.27	1.0	1.0
Ectodysplasin A2 isoform receptor	2.5	0.06	3.1	0.003	2.0	0.04
Kcnab1	−1.5	0.03	−4.0	0.002	−2.0	0.13
Myosin IA	5.7	0.13	1.7	0.27	5.0	0.03

$P(t)$ denotes P-value for t-test; FC, fold-change.

DISCUSSION

Studies of RNA dominance in DM1 have focused mainly on misregulated alternative splicing in cardiac and skeletal muscle. The present study is the first to examine global effects of CUG^{exp} RNA on gene expression. The major findings are that CUG^{exp} transcripts induce specific changes in gene expression, and that similar but non-identical changes occur in *Mbnl1* knockout mice. Together with previous observations that splicing abnormalities in HSA^{LR} transgenic and *Mbnl1* knockout mice are quite similar (10,12), these findings suggest that sequestration of Mbnl1 is the predominant mechanism for CUG^{exp}-induced re-modeling of the muscle transcriptome in this transgenic model. While many of the expression changes can be attributed to hyperexcitability, as evidenced by similar changes in $Clcn1^{-/-}$ mice, it appears that most of the effects of CUG^{exp} RNA or Mbnl1 loss are independent of myotonia. Furthermore, gene expression changes in HSA^{LR} mice show little overlap with *dystrophin* null mice (34), suggesting that changes in these DM1 models mainly reflect a specific response to CUG^{exp} RNA rather than a non-specific consequence of muscle injury or repair. Taken together, our results support the hypothesis that CUG^{exp}-Mbnl1 interaction is a critical event in DM1 pathogenesis, and strengthen the rationale for targeting this interaction as a therapeutic strategy.

A subset of changes (at least 10%) induced by CUG^{exp} RNA did not occur in *Mbnl1* knockout or *Clcn1* null mice. As Mbnl2 and Mbnl3 proteins are also sequestered by CUG^{exp} RNA (45), it is possible that these changes may result from combinatorial loss of several proteins in the muscleblind-like family. Alternatively, these changes may result from overexpression of CUG-BP1 or NKX-2.5, or other CUG^{exp}-binding proteins that have not yet been identified.

Mouse models of DM1 used in this study have recognized limitations. Most importantly, the circumstances of RNA toxicity that exist in patients with DM1 are not fully recapitulated in HSA^{LR} mice. The $(CTG)_{250}$ repeat in HSA^{LR} mice is ~10-fold shorter than repeat tracts in muscle tissue of DM1 patients (46–48). Unlike DM1, the mutant transcript in HSA^{LR} mice is not expressed in myogenic precursor cells or subsynaptic nuclei (49), and therefore cannot replicate problems with muscle regeneration or synaptic function that may exist in DM1. Furthermore, the CUG^{exp} element is expressed in the context of a different 3′-UTR and does not contain any other sequence from the *DMPK* gene. Presently, it is unclear exactly how these differences may relate to the failure of HSA^{LR} mice to replicate certain biochemical [upregulation of CUGBP1 (14,18) and NKX2-5 protein (50)] or histologic (progressive muscle wasting) features of DM1, or whether humans and mice may differ in their response to CUG^{exp} RNA. These considerations suggest that the changes in gene expression observed in this study could reflect a subset of all changes that may occur in human DM1 muscle.

Our results point to calcium signaling and homeostasis as an aspect of muscle biology especially prone to derangements in DM1. A structure that may be particularly impacted is the sarcoplasmic reticulum (SR). The transcript showing the most extreme splicing change in DM1 is *Serca1* (10,51), encoding the calcium reuptake pump of the SR. This splicing switch results in the expression of Serca1 protein with a different carboxyl terminus, but the functional impact has not been determined. Intriguingly, the gene most highly upregulated in DM1 models encodes sarcolipin, a modulator of Serca1 function. We have not determined the extent to which sarcolipin protein is overexpressed, because we were unable to generate antibodies to this protein, but the levels of sarcolipin mRNA were increased 60-fold in both founder lines of HSA^{LR} mice. In cardiac myocytes, sarcolipin overexpression caused reductions of Ca^{2+} re-uptake, Ca^{2+} transient amplitude and contractility (40,52,53), suggesting that sarcolipin overexpression may alter the function of striated muscle. In addition to *Serca1* and *sarcolipin*, other sarcoplasmic reticulum-associated genes whose expression or splicing is altered in DM1 models include *Asph*, *triadin* and *ryanodine receptor*.

Increased muscle activity modulates gene expression in several pathways, but chiefly those involved in energy metabolism, contractility and calcium homeostasis. Experimentally, such effects have been induced by chronic low frequency electrical stimulation of muscle fibers, but similar changes are observed without external stimuli in *Clcn1* null mice, and are driven by myotonic discharges (28–30). Our results provide a more comprehensive picture of myotonia-induced alterations of gene expression than was previously available. Consistent with previous findings, $Clcn1^{-/-}$ mice showed altered expression of genes involved in energy metabolism (upregulation of genes involved in fat metabolism and oxidative phosphorylation, downregulation of carbohydrate catabolism), contractility (increase of myosin heavy chain 2A) and calcium signaling (downregulation of CaM kinases and calmodulins). While all mice that we examined have myotonia, the pattern of activity-dependent gene expression in DM1 models differed from $Clcn1^{-/-}$ mice in several respects. In terms of genes involved in energy metabolism, the changes in *Mbnl1* knockout and HSA^{LR} mice were much less apparent than in $Clcn1^{-/-}$ mice. Whereas changes in calcium signaling and homeostasis were conspicuous in all myotonic mice, the changes in DM1 models were qualitatively different (Supplementary Material, Table S6). These differences may reflect subtotal loss of Clcn1 channels and less severe myotonia in the DM1 models (25). Alternatively, it is possible that CUG^{exp} RNA may have modified the transcriptional response to muscle hyperexcitability, enhancing certain aspects while repressing others.

An unexpected finding in $Clcn1^{-/-}$ and *Mbnl1* knockout mice was the marked downregulation of genes encoding contractile proteins of type 1 muscle fibers (Supplementary Material, Table S4, section E). These data suggest that fiber-type transformations driven by muscle activity are not invariably characterized by a unidirectional shift from fast to slow, but in the particular case of myotonia there is a convergence from both ends of the spectrum (type 1 or type 2B) on an intermediate contractile phenotype. When compared with continuous low frequency stimulation, the electrical discharges in myotonic muscle are higher frequency (40–150 Hz) and intermittent, which may resemble natural firing patterns in type 2 muscle fibers. This pattern of electrical activity may repress the program of gene expression for type 1 fibers. Notably, these changes did not occur in HSA^{LR} mice. We suggest that this disparity results from decreased severity of chloride channelopathy in type 1 fibers of HSA^{LR} mice, which may reflect differences in the stoichiometry of CUG^{exp} RNA and Mbnl1 protein in these fibers.

Effects of CUG^{exp} RNA on the transcriptome must reflect changes in the balance of RNA synthesis and decay. Among several potential effects of CUG^{exp} RNA on mRNA decay, our findings suggest that destabilization of Mbnl1-interacting mRNAs may impact the greatest number of genes, but the magnitude of the expression change was typically modest (-1.5- to -3.1-fold). Further work will be needed to determine whether Mbnl1 protein has a direct or indirect interaction with these mRNAs, and how the interaction may affect stability. While misregulated alternative splicing can also impact mRNA decay, as shown for *Clcn1*, we could not identify other candidates for dysregulation by this mechanism. However, because PTC-containing transcripts are underrepresented in the EST/mRNA database, other examples of increased turnover via NMD may have been overlooked.

Processing of CUG^{exp} RNA to short poly(CUG) RNAs may induce posttranscriptional silencing of genes containing CAG repeats (20). Although we were not able to directly demonstrate short CUG repeat RNAs by Northern blot, our results indicate that *Med12* is a candidate for this effect. *Med12* encodes the transcriptional coactivator mediator, mutations of which are associated with infantile hypotonia and mental retardation (54), making it an interesting candidate for the involvement in congenital DM1. We suggest that this transcript is selectively vulnerable to short poly(CUG) because it contains five distinct CAG repeat tracts, each ≥ 15 nt in length. Notably, each of these CAG tracts is conserved in human *MED12*. However, few other CAG-repeat-containing transcripts showed downregulation. Our data indicate that posttranscriptional silencing of $(CAG)_n$-containing transcripts is not a prevalent mechanism for gene dysregulation in CUG^{exp}-expressing mice.

By examining six of the more extreme changes in gene expression, we found that most are accompanied by a parallel change in the level of pre-mRNA, supporting an effect of CUG^{exp} RNA on transcription. The mechanism for this effect is presently unknown. GSEA analysis of promoter regions in genes that are dysregulated, when compared with genes not affected, did not show enrichment for known transcription factor binding sites (data not shown). Mbnl1 is not known to have DNA binding or transcriptional-regulatory activity. While we cannot eliminate the possibility that this protein may directly regulate transcription, it seems more likely that effects of Mbnl1 loss on transcription are indirect, via post-transcriptional modulation of other genes that encode transcription factors. Of note, Ebralidze *et al*. (22) have postulated that transcription factors are 'leached' from chromatin by mutant *DMPK* transcripts. However, this mechanism cannot account for HSA^{LR}-dysregulated genes that show similar changes in *Mbnl1* knockout mice, unless Mbnl1 itself is a transcription factor.

MATERIALS AND METHODS

Mouse strains

HSA^{LR} transgenic mice in lines 20b and 41 were derived and maintained on the FVB inbred background (6). Clinical and electromyographic myotonia was verified in each mouse as previously described (42). *Mbnl1* knockout mice (12) were backcrossed six generations onto the FVB background. *Clcn1* null myotonic mice were obtained from Jackson Laboratory (strain *adr-mto2J*, BALB background). Mice were studied according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Experimental groups

Microarray analysis was carried out on 34 mice in seven experimental groups. Homozygous HSA^{LR} transgenic mice in line 20b were compared with WT controls (FVB background, $n = 6$ mice per group, age 6 months). Homozygous HSA^{LR} transgenic mice in line 41 ($n = 6$) were compared with the same WT FVB controls. *Mbnl1* knockout mice were compared with $Mbnl1^{+/+}$ littermates ($n = 5$ per group, age 3 months). $Clcn1^{-/-}$ mice were compared with $Clcn1^{+/+}$ littermates ($n = 3$ per group, age 3 months). *Clcn1* and *Mbnl1* knockouts were studied at an earlier age because they display increased mortality after age 5 months.

RNA isolation and microarray hybridization

Mice were euthanized and vastus (quadriceps) muscle was immediately dissected, flash frozen in liquid nitrogen and stored at -70°C. Total cellular RNA was extracted using Tri-reagent (Molecular Research Center). Biotinylated cRNA was generated and used to probe Affymetrix microarrays (Santa Clara, CA, USA) as previously described (55). HSA^{LR}20b, HSA^{LR}41, FVB WT, $Clcn1^{-/-}$ and $Clcn1^{+/+}$ samples were processed in parallel and analyzed on Mouse Genome 430 A/B GeneChip Arrays. Because additional time was required for backcrossing *Mbnl1* knockouts, the $Mbnl1^{-/-}$ and $Mbnl1^{+/+}$ samples were processed at a later date using 430 2.0 Arrays. 430 2.0 Arrays have the same probes as the 430 A/B Arrays, but all probes are included on a single chip. Arrays were hybridized with cRNA, washed, stained with streptavidin–phycoerythrin and scanned (before and after antibody enhancement) according to standard Affymetrix protocols.

Microarray data analysis

Array data were analyzed using the gcRMA algorithm (56). Comparisons of gene expression were limited to probe sets that showed detectable expression in skeletal muscle. Expression was defined according to the Affymetrix probability of detection ($P_{\text{detection}}$) algorithm as previously described (55). In brief, a probe set was considered non-expressed if the $P_{\text{detection}}$ was >0.1 for more than half the samples in whichever group showed the higher mean signal value. For between-group comparisons, we calculated a fold-change in mean signal intensity and a nominal P-value using an equal-variance two-tailed t-test. A nominal $P < 0.001$ in this study always corresponded to a FDR of $<1\%$ as defined by the SAM procedure (32). Probe sets showing off-target hybridization with CTG repeats were eliminated (see Supplementary Methods). The primary data (.CEL) files and primary analysis have been deposited in the gene expression omnibus (GEO) archive (http://www.ncbi.nlm.nih.gov/geo/).

IP of ribonucleoprotein complexes and microarray analysis

Homogenates were prepared from quadriceps muscle and then immunoprecipitated using anti-Mbnl1 antibodies (see Supplementary Methods). The co-precipitating RNA was extracted, amplified by isothermal linear amplification, converted to biotin-labeled cDNA and then analyzed on Affymetrix U430 2.0 arrays (Supplementary Methods).

Competitive RT–PCR

Microarray data were validated by RT–PCR with competitive internal standards as described (57), using $n = 4$ mice per each experimental group. The RT–PCR confirmations were carried out on different mice than those used in the microarray analysis. For RT–PCR analysis of pre-mRNA, RNA was treated with DNase (DNA-free Kit, Ambion) prior to synthesis of cDNA, and controls omitting reverse transcriptase were included. All primers are listed in Supplementary Methods.

Immunoblot

Mouse quadriceps muscle was pulverized under liquid nitrogen, homogenized in lysis buffer, resolved on SDS–PAGE gels and transferred to nitrocellulose (Bio-Rad Laboratories) as previously described (58). Equal protein loading was confirmed by Ponceau Red staining. After incubation with primary antibody at 4°C overnight, membranes were incubated with secondary antibody at room temperature for 1 h, washed and then imaged on the Odyssey Infrared Imaging System (Licor). Antibodies were anti-Mbnl1 polyclonal antibody

A2764 (1:10,000), anti-GAPDH mouse monoclonal (1:10,000, Biogenesis, Brentwood, NH, USA) and anti-UCHL1 monoclonal ZMD.339 (1:1,000, Zymed Laboratories, South San Francisco, CA, USA). Secondary antibodies were IRDye 800- or IRDye 700DX-conjugated anti-rabbit or anti-mouse IgG (Rockland Immunochemicals). To generate primary antibodies against copine 2 (Cpne2), rabbits were immunized with peptide QYFKHKNLPPTNSEPA, the C terminus of mouse Cpne2.

Search for alternative splice isoforms that are substrates for nonsense mediated decay

For *Xedar* and *Cpne2*, cDNAs extending from the first to the final exon were amplified from WT and HSA^{LR}20b RNA through 30–31 cycles of RT–PCR. Products from five to seven PCR reactions were combined, gel-purified, cloned and sequenced. The EMBL-EBI Alternative Splicing and Transcript Diversity 1.1 database (http://www.ebi.ac.uk/astd/main.html) (43) was used to survey HSA^{LR}-dysregulated genes for alternative splice products that contained PTCs.

Small RNA northern

Twenty microgam of total RNA was resolved on 10% urea/polyacrylamide gels and transferred to Hybond N^+ membranes (Amersham) at 200 mA for 3 h using a *Trans*-blot SD semi-dry transfer cell (Biorad). Blots were cross-linked with ultraviolet radiation using a Stratalinker. Blots were prehybridized at least 1 h at 37°C in ULTRAhyb^{TM}-Oligo hybridization buffer (Ambion) before overnight incubation at 37°C in hybridization buffer containing denatured [^{32}P]-end-labeled probe. Probes were DNA oligonucleotides consisting of $(CAG)_7$ or sequence complementary to the indicated miRNA. Blots were washed (2X SSC, 0.1% SDS) at 37°C for 30 min followed by two 30 min room temperature washes and exposed to Kodak BioMax film.

Analysis of repeat containing transcripts

To determine whether transcripts containing CAG repeats had abnormal expression, we used BLAST to identify 286 mouse RefSeq mRNAs having 19 nt of uninterrupted CAG repeats [i.e. $(CAG)_6C$, $(AGC)_6A$ or $(GCA)_6G$].

Pathway analysis

To identify functionally related genes whose expression was altered in DM1 models, we used ingenuity pathways analysis (37) (www.ingenuity.com), GSEA (38) (www.broad.mit.edu/gsea/) and EASE (39) (see Supplementary Methods).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

This authors wish to thank Linda Richardson and Kirti Bhatt for technical assistance. This work comes from the Wellstone Muscular Dystrophy Cooperative Research Center of the University of Rochester.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the National Institutes of Health (AR046806, U54NS48843, AR48143), the Run America Foundation and the Saunders Family Neuromuscular Research Fund.

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