Chloride channelopathy in myotonic dystrophy resulting from loss of posttranscriptional regulation for CLCN1

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Transmembrane chloride ion conductance in skeletal muscle increases during early postnatal development. A transgenic mouse model of myotonic dystrophy type 1 (DM1) displays decreased sarcolemmal chloride conductance. Both effects result from modulation of chloride channel 1 (CLCN1) expression, but the respective contributions of transcriptional vs. posttranscriptional regulation are unknown. Here we show that alternative splicing of CLCN1 undergoes a physiological splicing transition during the first 3 wk of postnatal life in mice. During this interval, there is a switch to production of CLCN1 splice products having an intact reading frame, an upregulation of CLCN1 mRNA encoding full-length channel protein, and an increase of CLCN1 function, as determined by patch-clamp analysis of single muscle fibers. In a transgenic mouse model of DM1, however, the splicing transition does not occur; CLCN1 channel function remains low throughout the postnatal interval, and muscle fibers display myotonic discharges. Thus alternative splicing is a posttranscriptional mechanism regulating chloride conductance during muscle development, and the chloride channelopathy in a transgenic mouse model of DM1 results from a failure to execute a splicing transition for CLCN1.

chloride channel 1; nonsense-mediated decay; alternative splicing; CUG repeats; developmental regulation; muscular dystrophy; ion channel

SKELETAL MUSCLE UNDERGOES extensive remodeling during early postnatal development. For example, the transverse tubule system (TTS) is a rudimentary structure in neonatal rodents. The TTS develops rapidly after birth and establishes normal spatial relationships with the sarcomere and sarcoplasmic reticulum by the age of 3 wk (14, 26).

Development of the TTS is temporally coupled with upregulation of sarcolemmal chloride (Cl) conductance (11, 40). If this upregulation does not occur, as in individuals with hereditary myotonia due to null mutations in chloride channel 1 (CLCN1), then potassium accumulation in the TTS during muscle activation leads to membrane depolarization, triggering repetitive action potentials (reviewed by Cannon, Ref. 8). CLCN1 is the main carrier of Cl current in skeletal muscle (10). Postnatal upregulation of Cl conductance is associated with an increase of CLCN1 mRNA (37, 40), but it is not known whether this increase is regulated at a transcriptional or post-transcriptional level.

Myotonic dystrophy type 1 (DM1) is the most prevalent degenerative disease of skeletal muscle. DM1 is characterized by hereditary myotonia and reduced expression of CLCN1 (9, 28). However, the mutation is in a gene, DMPK, that encodes a serine/threonine protein kinase, and the effect on CLCN1 is indirect. The genetic lesion in DM1 is an expansion of CTG triplet repeats in the 3′-untranslated region of DMPK (6). This mutation gives rise to an unusual RNA-mediated disease mechanism in which DMPK transcripts containing an expanded CUG repeat (CUGexp) accumulate in muscle nuclei (12). These mutant transcripts interfere with mRNA biogenesis for a select group of genes, including CLCN1. However, data concerning the mechanism of this RNA-dominant effect on CLCN1 expression are conflicting. The mutant transcripts are retained in the nucleus in discrete foci (38). Nuclear proteins that interact with CUGexp RNA, such as splicing factors in the muscleblind (MBNL) family, are sequestered in the RNA foci (25, 30). This leads to misregulated alternative splicing for transcripts that depend on MBNL for normal splicing regulation (21). For example, DM1 patients and transgenic mouse models express CLCN1 splice products that include an additional exon cassette, exon 7a, or that retain intron 2 (9, 28). These variant splice products contain premature termination codons (PTCs), and the proteins they encode are devoid of channel activity (4). We and others have postulated that effects on CLCN1 alternative splicing are the fundamental cause of myotonia in DM1 (9, 28). Alternatively, Ebralidze et al. (13) have proposed that myotonia in DM1 results from reduced CLCN1 transcription due to sequestration of transcription factors that control CLCN1 expression.

Here we show a link between physiological regulation of CLCN1 during postnatal development and pathogenesis of myotonia in DM1. CLCN1 undergoes a physiological transition of alternative splicing during the first 3 wk of postnatal development in mice. During this interval, a switch from production of isoforms containing PTCs to those encoding full-length CLCN1 channels is associated with a rise in levels of CLCN1 mRNA and an increase of CLCN1 current density in the muscle fiber membrane. In the HSA1LR transgenic mouse model of DM1, however, abnormal persistence of the neonatal splice isoforms is associated with marked reduction of CLCN1 function and development of myotonia.

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MATERIALS AND METHODS

Mouse strains and muscle samples. HSA\(^{14-R}\) transgenic mice express human skeletal actin mRNA with \(-250\) CUG repeats inserted in the 3' untranslated region (27). HSA\(^{SR}\) transgenic mice express skeletal actin with insertion of 5 CUG repeats at the same position. These mice were maintained on an inbred FVB strain background. The HSA\(^{1-R}\) mice in these experiments were homozygotes from line HSA\(^{1-R20b}\). adr-mto2J mice (CLCNI null mutant mice) were obtained from Jackson Laboratories (Bar Harbor, MA). Gastrocnemius muscle from mdx mice and wild-type (WT) controls was obtained from Dr. Paula Clemens (University of Pittsburgh, PA). Gastrocnemius muscle from mice with sodium channel myotonia due to a targeted mutation in SCN4a (SCN4a\(^{M1950V}\)) (19) and WT littersmates was obtained from Dr. Stephen Cannon (University of Texas, Southwestern, Dallas, TX). Total cellular RNA was isolated from hindlimb muscle using TRI Reagent (Molecular Research, Cincinnati, OH). Surgical denervation of hindlimb muscle was performed under general anesthesia by removing a 4-mm section of the sciatic nerve exposed in the sciatic notch. General anesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg). Mice were euthanized 4 and 8 days after the procedure. RNA was isolated from gastrocnemius muscle of the denervated and non-denervated hindlimb from each animal. All procedures were approved by the University of Rochester Committee on Animal Research.

CLCNI splicing and expression analysis. To test for alternative splicing, mouse CLCNI cDNAs extending from the first to the final exon (exons 1–23, nt 1–2671, accession no. AY046403) were amplified by RT-PCR, cloned in pBSK, and sequenced as described (28). Assays for alternative splicing of CLCNI exon 7a were performed by RT-PCR as described (28). In brief, CLCNI cDNA was amplified using primers in exons 5 and 8. PCR was performed within the exponential range of amplification. RT-PCR products were resolved on agarose gels, stained with SybrGreen I, scanned on a fluorimager, and quantified using ImageQuant software. Results were normalized for increased binding of fluorochrome to larger PCR products. Levels of CLCNI pre-mRNA were also determined by RT-PCR, cloned in pBSK, and sequenced as described (28). In these assays, all RNA samples were reverse transcribed on the same day with the same RT reaction mixture. Total cell RNA was isolated from hindlimb muscle of the denervated and non-denervated hindlimb from each animal. All procedures were approved by the University of Rochester Committee on Animal Research.

RESULTS

As a first step to examine the role of posttranscriptional controls in regulating CLCNI during development, we cloned and sequenced CLCNI cDNAs from hindlimb muscle of WT mice. Results from neonatal mice were compared with those obtained from mature WT mice by the same method (28). Among 23 cDNA clones obtained at postnatal day 2 (P2), 10 (43%) showed alternative splicing. Retention of intron 2 (6 instances, leading to a PTC in the retained intron) and inclusion of exon 7a (5 instances, leading to a PTC in exon 7) were the most frequent events. By comparison, these splicing events were stored at room temperature in Ringer solution and remained experimentally viable for several hours.

Macroscopic chloride current recordings. The whole cell variant of the patch clamp technique (18) was used to monitor the magnitude and voltage dependence of macroscopic chloride currents in individual 9- to 20-day-old FDB fibers. Only healthy FDB fibers exhibiting clear striations and clean surfaces were chosen for electrophysiological recordings. The external recording solution contained (in mM): 145 tetraethylammonium (TEA)-Cl, 10 CaCl\(_2\), 10 HEPES, and 0.003 nifedipine (pH \(7.4\)). The internal solution contained (in mM): 30 CsCl, 110 CsAsp, 5 MgCl\(_2\), 10 Cs\(_2\)EGTA, and 10 HEPES (pH \(7.4\)). The Nernst potential for chloride (\(E_{Cl}\)) for these solutions was \(-35.8\) mV. Recording conditions provided adequate blockade of sodium (0 Na\(^+\)/depolarized holding potential of \(-40\) mV), calcium (nifedipine), and potassium (TEA/Cs) channel currents. Muscle fibers were whole cell patch clamped using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled using a Flaming Brown micropipette puller (P97; Sutter Instrument, Novato, CA) and fire polished to obtain electrode resistances of 0.4–1 M\(\Omega\). In cell-attached configuration, seal resistances were \(\approx 1\) G\(\Omega\). After achieving whole cell configuration, the internal solution was diazylized for 5–10 min before all electrophysiological recordings.

Whole cell currents were measured at room temperature (sampled at 10 kHz and filtered at 2 kHz) in response to 250-ms voltage steps from +60 to \(-140\) mV (in 10-mV increments) immediately following a 200-ms prepulse to +60 mV (to fully activate CLCNI channels). Individual test pulses were separated by a 10-s interpulse period at a holding potential of \(-40\) mV. For all experiments, this protocol was delivered first in the absence and then in the presence of 1 mM 9-anthracene carboxylic acid (9-AC), a blocker of CLCNI channels (7). Offline subtraction of currents recorded in the presence of 9-AC from those recorded in its absence resulted in residual leak and capacitative currents and resulted in 9-AC-sensitive currents that reflect CLCNI channel activity. Following block of CLCNI currents, total cell capacitance (\(C_m\)), membrane time constant (\(\tau_m\)), and uncompensated access resistance (\(R_a\)) were determined by integration of the capacity transient resulting from a 10-mV depolarizing pulse applied from the holding potential. The estimated maximal voltage error (peak Cl current at \(-140\) mV \(\times R_a\)) for all experiments averaged \(-4\) mV and was \(<10\) mV even for the largest currents (Table 1). Current density (pA/pF) was then calculated for each record to enable comparison of data obtained from cells of different sizes. Total cell capacitance increased from postnatal day 9 to day 20 to a similar degree in both WT and HSA\(^{1-R}\) mice (see Table 1). Only experiments in which seal resistance following addition of 9-AC was \(\approx 100\) M\(\Omega\) were analyzed. Instantaneous current vs. voltage relationship (I-V) plots were fitted according to the following modified Boltzmann function:

\[
I(V) = (I_{max} - I_0)/(1 + \exp[(V/V_{1/2} - V_0)/k_v]) \times I_0
\]

where \(I_{max}\) is the maximum current at the test potential (\(V_n\)), \(I_0\) is a constant offset, \(V_{1/2}\) is the half-maximal activation voltage, and \(k_v\) is a slope factor.
were not observed in 25 clones from 6-mo-old WT mice of the same (FVB) inbred strain. Thus CLCN1 undergoes a transition of alternative splicing during postnatal development.

We used RT-PCR splicing assays to assess the frequency of exon 7a inclusion and determine the time course of the postnatal splicing transition. Similar to previous observations in adult HSA<sup>LR</sup> transgenic mice (28), in the region from CLCN1 exons 5–8, we found three different alternative splice products in neonatal WT mice, of which the exon 7a inclusion isoform was most abundant (Supplemental Fig. 1; supplemental data are available at the online version of this article). Other variant splice products also included an additional exon cassette, exon 8a, or retention of a portion of intron 6. Because all variant splice products included exon 7a, and they all contained a PTC, they were quantified together as “ex7a<sup>+</sup> isoforms.” The fraction of CLCN1 mRNA that included exon 7a was 45 ± 0.7% at embryonic day 18 (E18) and subsequently declined, reaching levels at P20 (4 ± 1%) similar to adult muscle (5% ± 2%) (Fig. 1). Levels of intron 2 retention could not be quantified by RT-PCR because of the length of this intron, but qualitative analysis showed a similar decline in the interval between P2 and P20 (data not shown).

To determine whether the CUG<sup>exp</sup> RNA interfered with developmental regulation of CLCN1 splicing, we assessed inclusion of exon 7a in the postnatal interval in HSA<sup>LR</sup> transgenic mice. Skeletal actin is expressed by E13.5 in mice (5), and nuclear foci of CUG<sup>exp</sup> RNA are already prominent in HSA<sup>LR</sup> mice by P2 (25). The results showed a higher fraction of ex7a<sup>+</sup> isoforms in HSA<sup>LR</sup> than WT mice at all postnatal time points, ranging from 45–59% (Fig. 1). By comparison, levels of exon 7a inclusion were not increased in disease controls having dystrophin deficiency (10 ± 5% in adult mdx mice vs. 7 ± 2% in WT controls) or sodium channel myotonia (5% ± 2% in SCN4a<sup>M1592V</sup> knockin mice vs. 7 ± 1% in WT littermates) or in mice that overexpress skeletal actin transcripts with a nonexonspliced (CUG<sub>S</sub>) repeat (5 ± 1% in HSA<sup>SR</sup> transgenic mice). Also, section of the sciatic nerve produced only a slight increase in the frequency of exon 7a inclusion in denervated muscle (10 ± 1% in gastrocnemius on day 4 following section and 10 ± 0.5% on day 8 following section vs. 7 ± 3% in the contralateral nondenervated muscle). These results indicate that HSA<sup>LR</sup> mice fail to execute the postnatal splicing transition for CLCN1. The effect is a specific response to CUG<sup>exp</sup> RNA rather than a nonspecific consequence of muscular dystrophy, muscle hyperexcitability, loss of nerve-muscle trophic support, or overexpression of actin.

A previous study suggested that transcription of CLCN1 is reduced in DM1 cells (13). However, these studies were performed in cultured myotubes, in which expression of CLCN1 is 100-fold lower than in mature muscle fibers (2). To address this question in mature muscle fibers, we used quantitative RT-PCR to examine levels of mRNA and pre-mRNA for CLCN1 in HSA<sup>LR</sup> transgenic mice. Barring an effect on kinetics of RNA processing, the steady-state levels of CLCN1 pre-mRNA should reflect relative rates of CLCN1 transcription. The level of CLCN1 mRNA in HSA<sup>LR</sup> mice was 44 ± 8% of WT controls (Fig. 2A), similar to the value of 43 ± 15% of WT controls that we previously observed in the same transgenic line by quantitative real-time RT-PCR (28). By contrast, quantitative RT-PCR analysis of the pre-mRNA showed no difference in HSA<sup>LR</sup> transgenic mice compared with WT controls (Fig. 2A). These results argue that the effects of CUG<sup>exp</sup> RNA on CLCN1 expression are predominantly posttranscriptional.

Relative to their fractional synthetic rate, ex7a<sup>+</sup> isoforms would be underrepresented at steady state if they undergo accelerated degradation through the nonsense-mediated decay (NMD) pathway. However, the efficiency of NMD varies among genes (reviewed by Maquat, Ref. 29), and some transcripts containing PTCs may escape this mechanism of surveillance. To examine the effects of PTCs on metabolism of CLCN1 mRNA, we identified mice having a PTC in the CLCN1 gene. <i>ad<sup>mdx2f</sup></i> mice display severe recessive myotonia resulting from a spontaneous mutation in the BALB inbred strain. Previously, we found a complete absence of immunoreactive CLCN1 protein in these mice using antibodies directed

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**Table 1. Linear properties and estimated maximal voltage error**

<table>
<thead>
<tr>
<th></th>
<th>&lt;i&gt;n&lt;/i&gt;</th>
<th>&lt;i&gt;C&lt;sub&gt;m&lt;/sub&gt;&lt;/i&gt; pF</th>
<th>&lt;i&gt;τ&lt;sub&gt;m&lt;/sub&gt;&lt;/i&gt; μs</th>
<th>&lt;i&gt;R&lt;sub&gt;m&lt;/sub&gt;&lt;/i&gt; MΩ</th>
<th>Peak &lt;i&gt;I&lt;sub&gt;C&lt;/sub&gt;&lt;/i&gt;, pA</th>
<th>Estimated Maximal Voltage Error, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT day 9</td>
<td>9</td>
<td>320 ± 33</td>
<td>77 ± 13</td>
<td>0.27 ± 0.07</td>
<td>−8.459 ± 1.142</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>HSA&lt;sup&gt;LR&lt;/sup&gt; day 9</td>
<td>6</td>
<td>267 ± 20</td>
<td>48 ± 5</td>
<td>0.18 ± 0.02</td>
<td>−915 ± 262</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>WT day 13</td>
<td>4</td>
<td>368 ± 15</td>
<td>255 ± 53</td>
<td>0.68 ± 0.12</td>
<td>−9.355 ± 1.064</td>
<td>6.3 ± 1.3</td>
</tr>
<tr>
<td>HSA&lt;sup&gt;LR&lt;/sup&gt; day 13</td>
<td>11</td>
<td>332 ± 23</td>
<td>117 ± 18</td>
<td>0.37 ± 0.06</td>
<td>−2.099 ± 0.556</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>WT days 18 and 19</td>
<td>9</td>
<td>660 ± 77</td>
<td>199 ± 41</td>
<td>0.34 ± 0.08</td>
<td>−30.951 ± 3.956</td>
<td>9.3 ± 1.8</td>
</tr>
<tr>
<td>HSA&lt;sup&gt;LR&lt;/sup&gt; days 18 and 19</td>
<td>29</td>
<td>665 ± 19</td>
<td>302 ± 19</td>
<td>0.46 ± 0.03</td>
<td>−11.975 ± 5.129</td>
<td>4.1 ± 0.7</td>
</tr>
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Values are means ± SE. Average total cell capacitance (<i>C<sub>m</sub></i>), membrane time constant (<i>τ<sub>m</sub></i>), uncompensated access resistance (<i>R<sub>m</sub></i>), peak chloride current magnitude (peak <i>I<sub>C</sub></i>, at −140 mV), and estimated maximum voltage error (peak current × <i>R<sub>m</sub></i>) for the no. (<i>n</i>) of experiments shown.

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**Fig. 1. Regulation of chloride channel 1 (CLCN1) alternative splicing during postnatal development.** In wildtype (WT) mice, the fraction of CLCN1 mRNA containing exon 7a declines from embryonic day 18 (E18) to postnatal day 20 (P20). The transition of CLCN1 alternative splicing does not occur in HSA<sup>LR</sup> mice. Values for %ex7a<sup>+</sup> splice products in WT mice were as follows: E18, 45 ± 0.7%; P2, 29 ± 2%; P6, 23 ± 2%; P10, 16 ± 3%; P20, 4 ± 4%; and 6 mo, 5 ± 1%. Values in HSA<sup>LR</sup> mice were as follows: P2, 59 ± 1%; P6, 52 ± 1%; P10, 50 ± 1%; P20, 54 ± 1%; and 6 mo, 45 ± 2% (means ± SD of triplicate assays).
against the carboxyl terminus (28). To identify the mutation, we derived CLCN1 cDNA clones from homozygous \textit{adr}^{mto2J} mice. Sequence analysis showed a single nucleotide deletion in exon 18 at position 2269 (accession no. NM_013491), the only mutation in the coding region. The C2269del→6s 782X mutation, which caused a frame shift and premature termination codon in exon 19 (of 23 total exons), was confirmed by sequencing genomic DNA. Because this mutation had no effect on splicing of exon 18 or 19 (data not shown), it was unlikely to directly affect splicing at exon 7a.

Several effects on CLCN1 mRNA accumulation would be expected if CLCN1 transcripts containing a PTC are subject to NMD. First, the level of CLCN1 mRNA should be reduced in homozygous \textit{adr}^{mto2J} mice. Second, the fraction of ex7a transcripts should be increased in homozygous \textit{adr}^{mto2J} mice, because all CLCN1 transcripts in these mice contain a frame shift mutation and would be similarly subject to NMD. Third, because all CLCN1 transcripts in these mice contain a frame shift mutation in the coding region. The C2269del→6s 782X mutation, which caused a frame shift and premature termination codon in exon 19 (of 23 total exons), was confirmed by sequencing genomic DNA. Because this mutation had no effect on splicing of exon 18 or 19 (data not shown), it was unlikely to directly affect splicing at exon 7a.

To test these predictions, we used quantitative RT-PCR to compare CLCN1 RNA in homozygous \textit{adr}^{mto2J} mice and WT littermates. In accordance with the first prediction, the level of CLCN1 mRNA in \textit{adr}^{mto2J} mice was reduced to 30 ± 3% of WT littermates, indicating that a PTC in exon 19 elicits an ~70% reduction in the half-life of CLCN1 mRNA. This effect was comparable to that observed for other PTC-containing transcripts (29, 33). By comparison, the level of CLCN1 pre-mRNA in \textit{adr}^{mto2J} mice was unchanged (Fig. 2B), which indicates that reduction of the mRNA results from accelerated decay, and that loss of channel function did not induce compensatory upregulation of CLCN1 transcription.

Next, we examined the fraction of ex7a+ mRNA in homozygous \textit{adr}^{mto2J} mice. In accordance with the second prediction, the fraction of ex7a+ CLCN1 was increased throughout postnatal development in \textit{adr}^{mto2J} mice relative to WT littermates (Fig. 3; representative gel is shown in online Supplemental Fig. 2). This finding argues that ex7a+ isoforms are normally subject to NMD, so that placement of a second PTC in a downstream exon caused no additional shortening of their half-life. Because the PTC in ex7a+ mRNA is at codon 290, it is noteworthy that a point mutation in human CLCN1 causing premature termination at codon 289 also led to marked reduction of CLCN1 mRNA (31).

In accordance with the third prediction, the transition to ex7a-skipped CLCN1 during postnatal development was associated with a threefold rise of CLCN1 mRNA in WT mice (Fig. 4).

The data presented in Figs. 1–4 indicate that levels and splicing of CLCN1 mRNA are regulated during postnatal development and misregulated in response to CUG\textsuperscript{exp} RNA. However, alterations in relative mRNA levels may not accurately reflect channel function. In addition, although truncated CLCN1 proteins resulting from aberrantly spliced transcripts do not function alone as chloride channels, they could potentially interact with residual WT proteins to form dimers having altered function (4). To test these possibilities, we characterized CLCN1 function during postnatal maturation in WT and \textit{HSA}^{LR} mice (Fig. 5). For these experiments, whole cell patch clamp recordings were made from individual FDB skeletal muscle fibers dissociated from 9- to 20-day-old FVB and \textit{HSA}^{LR} mice. Figure 5A–C, shows representative macroscopic recordings of raw (Fig. 5A), 9-AC-insensitive (Fig. 5B), and 9-AC-sensitive (Fig. 5C) currents in a FDB fiber obtained from a 19-day-old WT mouse. 9-AC-sensitive CLCN1 currents in a FDB fiber of a 19-day-old \textit{HSA}^{LR} mouse are shown for comparison in Fig. 5D. For these experiments, identical voltage protocols were used for recording raw and 9-AC-blocked currents. Test potentials were applied to voltages between 60
obtained for the experiment shown in /H11002
a reversal potential (\(28.8 - I \cdot V\) independent at depolarized potentials. Third, the instantaneous test potentials, while current amplitude was relatively time
CLCN1 antagonist. Second, deactivation kinetics of 9-AC-blocked (\(I_{CLCN1}\) currents were completely blocked by 0.1–1 mM 9-AC, a potent
fibers exhibit several hallmarks of CLCN1 currents. First, these
ologously expressed CLCN1 channels (35), macroscopic
activate CLCN1 channels. Similar to that observed for heter-
uncompensated access resistance (\(R_a\))
480
Current decay [thick continuous line: total cell capacitance (\(C_m\))
for FDB fibers obtained from 18- to 20-day-old WT (solid circles, HSA
LR mice (open circles, \(HSA^{LR}\)) mice exhibit a profound reduction in CLCN1 current density at all voltages. Specifically, instantaneous CLCN1 current density measured at \(-140\) mV was reduced \(-71\%\) in 18- to 20-day-old \(HSA^{LR}\) fibers (WT, \(-48.50 \pm 3.59\) pA/pF; \(HSA^{LR}, -13.97 \pm 1.62\) pA/pF), and EMG recordings from hindlimb muscle at this age demonstrated robust myotonic discharges (not shown). \(HSA^{LR}\) fibers exhibited a similar relative reduction in CLCN1 current density compared with age-matched control WT fibers at each postnatal age studied (Fig. 5F). Moreover, relative CLCN1 current density actually increased in parallel for both WT and \(HSA^{LR}\) fibers during this developmental period.

DISCUSSION

Results of the present study are consistent with a developmental mechanism in which \(CLCN1\) is regulated by transcriptional and posttranscriptional controls. Levels of CLCN1 mRNA increase \(-10^3\)-fold in the transition from myoblasts to mature muscle (2). However, \(CLCN1\) transcripts in late fetal and neonatal muscle are mainly regulated by alternative splicing to a “discard” pathway. These splice products contain PTCs, they undergo accelerated decay, and they encode truncated proteins that do not form functional chloride channels. During postnatal development, the transition to splice isoforms encoding full-length channel protein is associated with greater stability and accumulation of CLCN1 mRNA and increased Cl channel activity. Developmental regulation of \(CLCN1\), therefore, is an example of regulated unproductive splicing and translation, or RUST (24). Bioinformatic analysis suggested that RUST was a common mechanism for regulating mammalian gene expression and that >1,000 human genes were candidates for posttranscriptional regulation in this manner (17, 24). However, global analysis using exon junction microarrays failed to confirm widespread coupling of alternative splicing to NMD (34), and physiological regulation by RUST has been clearly shown for only a small number of genes (23, 41). Our study is the first to show developmental regulation of a mammalian ion channel by this mechanism.

On the basis of observations in muscle cell cultures, Ebralidze et al. (13) proposed that decreased \(CLCN1\) expression in DM1 resulted from sequestration of transcription factors Sp1 and retinoic acid receptor-\(\gamma\) (RAR\(\gamma\)) in nuclear foci of CUG\textsuperscript{exp} RNA, leading to reduced transcription of the \(CLCN1\) gene. However, our findings argue against this mechanism for CUG\textsuperscript{exp}-induced chloride channelopathy in muscle fibers. First, the level of \(CLCN1\) pre-mRNA in the \(HSA^{LR}\) transgenic mouse model was not reduced (Fig. 2A), indicating that expression of CUG\textsuperscript{exp} RNA did not inhibit \(CLCN1\) transcription. Second, by combining fluorescence in situ hybridization for CUG\textsuperscript{exp} RNA with immunofluorescence for nuclear proteins, we saw no recruitment of Sp1 or RAR\(\gamma\) into nuclear foci in DM1 cortical neurons (20) or muscle fibers (H. Jiang and C. A. Thornton, unpublished observation). Third, ablation of MBNL1, a factor that regulates splicing but that is not known to regulate transcription, repro-

Figure 5E depicts the mean instantaneous \(I-V\) relationship for 9-AC-sensitive CLCN1 currents observed for FDB fibers obtained from 18- to 20-day-old WT (\(n = 12\)) and \(HSA^{LR}\) (\(n = 29\)) mice. These data demonstrate that FDB fibers from \(HSA^{LR}\) mice exhibit a profound reduction in CLCN1 current density at all voltages. Specifically, instantaneous CLCN1 current density measured at \(-140\) mV was reduced \(-71\%\) in 18- to 20-day-old \(HSA^{LR}\) fibers (WT, \(-48.50 \pm 3.59\) pA/pF; \(HSA^{LR}, -13.97 \pm 1.62\) pA/pF), and EMG recordings from hindlimb muscle at this age demonstrated robust myotonic discharges (not shown). \(HSA^{LR}\) fibers exhibited a similar relative reduction in CLCN1 current density compared with age-matched control WT fibers at each postnatal age studied (Fig. 5F). Moreover, relative CLCN1 current density actually increased in parallel for both WT and \(HSA^{LR}\) fibers during this developmental period.

DISCUSSION

Results of the present study are consistent with a developmental mechanism in which \(CLCN1\) is regulated by transcriptional and posttranscriptional controls. Levels of CLCN1 mRNA increase \(-10^3\)-fold in the transition from myoblasts to mature muscle (2). However, \(CLCN1\) transcripts in late fetal and neonatal muscle are mainly regulated by alternative splicing to a “discard” pathway. These splice products contain PTCs, they undergo accelerated decay, and they encode truncated proteins that do not form functional chloride channels. During postnatal development, the transition to splice isoforms encoding full-length channel protein is associated with greater stability and accumulation of CLCN1 mRNA and increased Cl channel activity. Developmental regulation of \(CLCN1\), therefore, is an example of regulated unproductive splicing and translation, or RUST (24). Bioinformatic analysis suggested that RUST was a common mechanism for regulating mammalian gene expression and that >1,000 human genes were candidates for posttranscriptional regulation in this manner (17, 24). However, global analysis using exon junction microarrays failed to confirm widespread coupling of alternative splicing to NMD (34), and physiological regulation by RUST has been clearly shown for only a small number of genes (23, 41). Our study is the first to show developmental regulation of a mammalian ion channel by this mechanism.

On the basis of observations in muscle cell cultures, Ebralidze et al. (13) proposed that decreased \(CLCN1\) expression in DM1 resulted from sequestration of transcription factors Sp1 and retinoic acid receptor-\(\gamma\) (RAR\(\gamma\)) in nuclear foci of CUG\textsuperscript{exp} RNA, leading to reduced transcription of the \(CLCN1\) gene. However, our findings argue against this mechanism for CUG\textsuperscript{exp}-induced chloride channelopathy in muscle fibers. First, the level of \(CLCN1\) pre-mRNA in the \(HSA^{LR}\) transgenic mouse model was not reduced (Fig. 2A), indicating that expression of CUG\textsuperscript{exp} RNA did not inhibit \(CLCN1\) transcription. Second, by combining fluorescence in situ hybridization for CUG\textsuperscript{exp} RNA with immunofluorescence for nuclear proteins, we saw no recruitment of Sp1 or RAR\(\gamma\) into nuclear foci in DM1 cortical neurons (20) or muscle fibers (H. Jiang and C. A. Thornton, unpublished observation). Third, ablation of MBNL1, a factor that regulates splicing but that is not known to regulate transcription, repro-

![Fig. 5. CLCN1 current density is reduced throughout postnatal development in flexor digitorum brevis (FDB) fibers of \(HSA^{LR}\) mice. Representative whole cell raw (A), 9-anthracene carboxylic acid (9-AC)-blocked (B), and 9-AC-sensitive (raw – 9-AC blocked) C CLCN1 currents recorded from a FDB fiber isolated from a 19-day-old WT mouse. D: representative macroscopic 9-AC-sensitive CLCN1 currents recorded from a FDB fiber isolated from a 19-day-old \(HSA^{LR}\) mouse. A–D: dotted lines represent the zero current level. C, inset: capacitative current recorded from the 19-day-old WT FDB fiber following blockade with 1 mM 9-AC (scale = 3 nA, 4 ms). The time constant of capacitative current relaxation (\(\tau_m = 435\) ms) is well described by a first-order exponential fit to the current decay [thick continuous line: total cell capacitance (\(C_m\) = 592 pF, uncompensated access resistance (\(R_a\)) = 0.64 Mf]). Similar values were obtained for the experiment shown in B (\(C_m = 592\) pF, \(R_a = 0.81\) Mf, \(\tau_m = 480\) ms). E: average (±SE) instantaneous CLCN1 current-voltage relationship for FDB fibers obtained from 18- to 20-day-old WT (solid circles, \(n = 12\)) and \(HSA^{LR}\) (open circles, \(n = 29\)). F: average (±SE) peak CLCN1 current density (measured at \(-140\) mV) for FDB fibers obtained from 9- to 20-day-old WT (solid circles) and \(HSA^{LR}\) (open circles). No. of experiments is shown in parentheses. \(V_m\), test potential; \(I_{\text{cl}}\), chloride current magnitude.

and \(-140\) mV following a 60-mV prepulse used to fully activate CLCN1 channels. Similar to that observed for heterologously expressed CLCN1 channels (35), macroscopic 9-AC-sensitive currents recorded from individual WT FDB fibers exhibit several hallmarks of CLCN1 currents. First, these currents were completely blocked by 0.1–1 mM 9-AC, a potent CLCN1 antagonist. Second, deactivation kinetics of 9-AC-sensitive currents were progressively faster at more negative test potentials, while current amplitude was relatively time independent at depolarized potentials. Third, the instantaneous \(I-V\) relationships exhibited prominent inward rectification and a reversal potential (\(-28.8 \pm 6.6\) mV) that was close to the Nernst potential for chloride (\(-35.8\) mV).
duced the effects of DM1 on CLCN1 expression and muscle excitability (21, 25). Finally, the results of the present study indicate that effects on alternative splicing, when coupled with accelerated decay of ex7a+ splice products, can explain the modest reduction of CLCN1 mRNA observed in HSA^{LR} mice and DM1 patients, without needing to invoke an independent effect on transcription. Taken together, these results indicate that CUG^{exp} RNA evokes myotonia by compromising the posttranscriptional component of CLCN1 control. Moreover, the loss of postnatal splicing transition for CLCN1 is similar to that observed for other DM1 target transcripts (25), except that CLCN1 is currently the only such transition in which alternative splicing is coupled with NMD. At present, these effects on CLCN1 provide the clearest evidence that symptoms of DM1 can result directly from effects on splicing regulation.

CLCN1 accounts for >90% of the resting Cl conductance in skeletal muscle (10). Several lines of evidence, including experiments with Cl channel blockers, replacement of chloride by impermeant ions, observations in heterozygous carriers of null mutations in CLCN1, and mathematical modeling, have led to the conclusion that a 70% or greater reduction of chloride conductance is required to induce myotonia in muscle fibers (1, 7, 16, 22). Accordingly, we found that macroscopic CLCN1 channel activity is reduced by ~71% in FDB fibers from 9- to 20-day-old HSA^{LR} mice. These results confirm, in a different muscle and by different methods, that myotonia in HSA^{LR} mice is likely to result from loss of CLCN1 channel function (28). Notably, HSA^{LR} mice display a reduction of CLCN1 activity at each postnatal day studied from P9 to P20 (Fig. 5F). This finding is consistent with observations that levels of exon 7a inclusion in HSA^{LR} mice were higher than in WT mice from P2 to P20 (Fig. 1), and that nuclear foci of CUG^{exp} RNA, together with sequestered MBNL1 protein, were present in muscle nuclei throughout the postnatal interval (25).

As shown by the whole cell patch clamp studies on single WT muscle fibers, changes in CLCN1 splicing and mRNA accumulation were tightly correlated with functional chloride channel activity during different stages of postnatal maturation. The splicing switch and upregulation of CLCN1 channel activity occur during a period of rapid fiber hypertrophy and maturation of the TTS (14). These changes in fiber morphology are probably associated with an increased requirement for chloride current to stabilize the membrane potential. This may explain why HSA^{LR} mice manifest robust myotonia as early as P14, whereas neither HSA^{LR} nor WT mice exhibit myotonia at P2 (A. Mankodi and C. A. Thornton, unpublished observations). A similar age dependency for development of myotonia has been documented previously in mice homozygous for null mutations in CLCN1 (15). While the failure to execute the splicing switch appears to be the key event underlying myotonia in HSA^{LR} mice, in human DM1 the onset of myotonia is considerably delayed. It is possible that, in DM1 muscle, the splicing switch is executed properly during postnatal development but that later it reverts to the immature pattern of splicing. Alternatively, factors other than CLCN1 splicing may condition the onset of myotonia in human DM1 (32, 36).

The observed reduction in CLCN1 activity in FDB fibers of HSA^{LR} mice could arise from a reduction in the 1) number of functional CLCN1 channels (N), 2) unitary current (i), and/or 3) channel open probability (P_o). Reductions of CLCN1 current density in HSA^{LR} mice (~71% decrease from WT in 18- to 20-day-old HSA^{LR} fibers) are similar in magnitude to effects on mRNA (57% reduction of CLCN1 mRNA, of which at least 54% contains a PTC, resulting in 80% reduction of mRNA encoding full-length CLCN1). These findings suggest that the primary mechanism responsible for loss of CLCN1 function is a reduction in synthesis and number of functional CLCN1 channels. However, Cl conductance was preserved in heterozygous CLCN1 knockout mice (10), despite a 50% reduction of CLCN1 mRNA, suggesting that increased translation or channel stability may partially compensate for reduced CLCN1 mRNA. Thus our functional data cannot rule out possible dominant-negative effects of truncated CLCN1 splice isoforms combining with WT CLCN1 monomers to form dimers with altered channel function (e.g., deactivation kinetics, open probability, and/or single-channel conductance). Indeed, a recent report by Berg et al. (4) concluded that truncated CLCN1 proteins may exert dominant-negative effects on CLCN1 channel function. Thus definitive conclusions regarding mechanistic effects of CUG^{exp} RNA on CLCN1 function will require determination of open probability, single-channel conductance, and the number of functional membrane channels in FDB fibers of WT and HSA^{LR} mice using nonstationary noise analysis (35).

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