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BNA^{NC} Gapmers Revert Splicing and Reduce RNA Foci with Low Toxicity in Myotonic Dystrophy Cells

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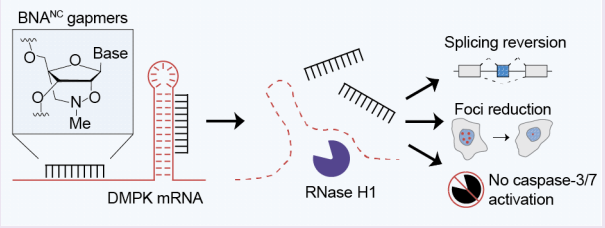
Supporting Information

ABSTRACT: Myotonic dystrophy type 1 (DM1) is a multisystemic disease caused by an expanded CTG repeat in the 3′ UTR of the *dystrophia myotonica protein kinase* (*DMPK*) gene. Short, DNA-based antisense oligonucleotides termed gapmers are a promising strategy to degrade toxic CUG expanded repeat (CUG_{exp}) RNA. Nucleoside analogs are incorporated to increase gapmer affinity and stability; however, some analogs also exhibit toxicity. In this study, we demonstrate that the 2′,4′-BNA^{NC}[NMe] (BNA^{NC}) modification is a promising nucleoside analog with high potency similar to 2′,4′-LNA (LNA). BNA^{NC} gapmers targeting a nonrepetitive region of the DMPK 3′ UTR show allele-specific knockdown of CUG_{exp} RNA and revert characteristic DM1 molecular defects including mis-splicing and accumulation of RNA foci. Notably, the BNA^{NC} gapmers tested in this study did not induce caspase activation, in contrast to a sequence matched LNA gapmer. This study indicates that BNA^{NC} gapmers warrant further study as a promising RNA targeting therapeutic.



Myotonic dystrophy is a multisystemic disease affecting approximately 1 in 8000 individuals.[1] Myotonic dystrophy type I (DM1) is caused by a CTG repeat expansion within the 3′ UTR of the *DMPK* gene.[2,3] Expanded CUG repeat (CUG_{exp}) RNA is retained in the nucleus and forms RNA foci that sequester the MBNL family of splicing factors and induces upregulation of CELF1 through PKC-mediated phosphorylation and altered microRNA regulation.[4−6] Altered MBNL and CELF1 activity in DM1 leads to defects in developmentally regulated alternative splicing.[1]

Antisense oligonucleotides (ASOs) are a promising therapeutic approach for diseases caused by an RNA gain of function, including DM1. The addition of chemical modifications greatly stabilizes the affinity and stability of ASOs. These modifications include altered backbone chemistry, such as phosphorothioate (PS), and altered ribose chemistry, such as the 2′-*O*-methoxyethyl (MOE) and 2′-*O*,4′-*C*-methylene-bridged nucleic acid (LNA).[7] However, the addition of 2′ ribose modifications abolishes RNase H1-mediated target degradation.[8] Chimeric ASOs called gapmers contain 2′ ribose modifications at the 5′ and 3′ ends, leaving an internal 6−10 nucleotide gap that maintains RNase H1 activity.[8−10] This is in contrast to mixmers, which contain modified analogs throughout the ASO and are unable to recruit RNase H1 for target cleavage (Figure S1A).[8]

A recent clinical trial using a MOE gapmer (IONIS-DMPKRx) for the treatment of myotonic dystrophy demonstrated promise with the approach with current work focused on improving tissue delivery (Ionis Pharmaceuticals and Biogen press release, January 2017). Comparison of LNA and MOE gapmers indicates that LNA gapmers are 5−10 fold more potent, eliciting initial interest as a more potent alternative to MOE gapmers.[11,12] However, multiple studies have indicated that LNA gapmers induce RNase H1-dependent hepatotoxicity and apoptosis in both mice and cell models, reducing suitability for translational studies.[12−15]

The 2′4′-BNA^{NC}[NMe] (BNA^{NC}) modification was developed following similar principles to those of the LNA modification, with a bridged structure that greatly increases its affinity (Figure S1B).[16,17] BNA^{NC} ASOs are more stable than LNA ASOs and have been shown to be well tolerated in mice.[16,18] However, BNA^{NC} gapmers have not been systematically tested in repeat expansion models, and it is unknown whether BNA^{NC} gapmers have similar toxicity concerns as LNA gapmers. We demonstrate that BNA^{NC} gapmers have comparable potency to LNA gapmers, display potentially lower propensity to induce caspase activity, and functionally rescue characteristic DM1 defects, making BNA^{NC} gapmers a promising alternative chemistry for therapeutic development.

We first sought to compare the efficiency of BNA^{NC} and LNA gapmers at targeting DMPK transcripts containing CUG_{exp}. COSM6 cells were transfected with two plasmids: an rtTA expression plasmid and the pBitetDT700ctgGFP (Bi700CTG) plasmid, which contains a bidirectional tet-inducible promoter to express GFP and exons 11−15 of DMPK, including ∼300 CUG repeats (Figure 1A). Twenty-four hours after plasmid

Received: May 16, 2017
Accepted: August 30, 2017
Published: August 30, 2017

DOI: 10.1021/acschembio.7b00416
ACS Chem. Biol. XXXX, XXX, XXX−XXX

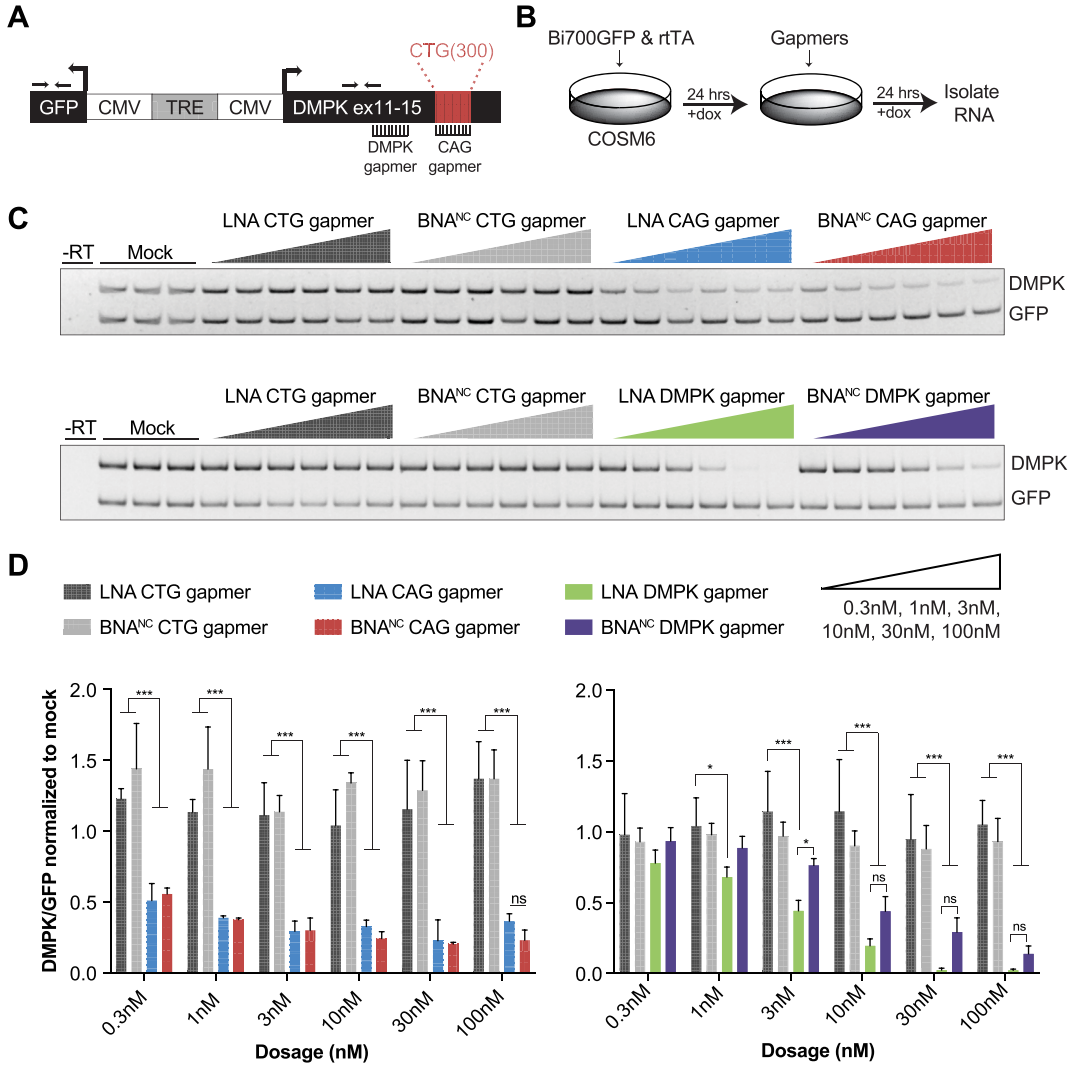


Figure 1. LNA and BNA^{NC} gapmers knockdown of CUG$_{exp}$ RNA. (A) A bidirectional, tetracycline inducible CMV promoter drives expression of both DMPK RNA containing 300 CUG repeats and GFP in the Bi700GFP minigene. RT-PCR primers and gapmer ASO positions are indicated. (B) Transient transfection strategy in COSM6 cells. rtTA = plasmid expressing reverse tetracycline-controlled trans activator. (C) RT-PCR demonstrates DMPK RNA knockdown after treatment with increasing concentrations of LNA and BNA^{NC} gapmers. Separate PCR reactions with different cycle numbers were performed for DMPK and GFP using the same cDNA. (D) Quantification of DMPK mRNA knockdown by LNA and BNA^{NC} gapmers; n = 3 (CAG gapmers) and n = 4 (DMPK gapmers), Tukey's multiple comparisons test.

transfection, cells were treated with increasing concentrations of LNA or BNA^{NC} gapmers targeting either within the CUG repeat (CAG gapmers) or in a nonrepetitive region of DMPK (DMPK gapmers; Figure 1; Table S1).[19] DMPK mRNA levels were quantified by RT-PCR relative to the GFP mRNA to standardize for transfection efficiency. Compared to the CTG negative control gapmers, both LNA and BNA^{NC} CAG gapmers gave significant knockdown of CUG$_{exp}$ RNA at the lowest gapmer dose (0.3 nM) and maximum knockdown to 65−80% of baseline at doses above 10 nM (Figure 1C,D). We next tested the efficiency of LNA and BNA^{NC} DMPK gapmers that target upstream of the expanded repeat. While neither DMPK gapmer showed significant knockdown at the lowest dose, they both induced over 70% knockdown at 30 nM. Interestingly, the DMPK gapmers displayed more potent knockdown than the CAG gapmers at the highest dose (100 nM), with only 2−14% of the target remaining (Figure 1C,D). The LNA DMPK gapmer was also more effective than the BNA^{NC} DMPK gapmer at the low dosage levels. It is likely that the CAG gapmer is more effective than the DMPK gapmer at low concentrations because there are more binding sites per molecule. At higher concentrations, the residual amount of DMPK RNA that cannot be degraded by the CAG gapmer suggests the CUG repeat is partially protected, potentially by MBNL proteins or RNA structure. Taken together, these results indicate that both LNA and BNA^{NC} gapmers are potent and effective for knockdown of CUG$_{exp}$ RNA.

After establishing comparable potency between LNA and BNA^{NC} gapmers, we compared the toxicity of BNA^{NC} and LNA gapmers. Non-DM1 TeloMyoD fibroblasts were treated with 30 nM of LNA or BNA^{NC} gapmers or mixmers and were assayed for membrane integrity, viability, and caspase-3/7 activity 32 h after transfection. We show a sequence-, chemistry-, and RNase H1-dependent increased caspase response specifically caused by the LNA CAG gapmer and not the BNA^{NC} CAG gapmer or the LNA CAG mixmer (Figure 2A, Figure S2A). Increased caspase was not due to differences in cell number between samples because only the staurosporine

DOI: 10.1021/acschembio.7b00416
ACS Chem. Biol. XXXX, XXX, XXX−XXX

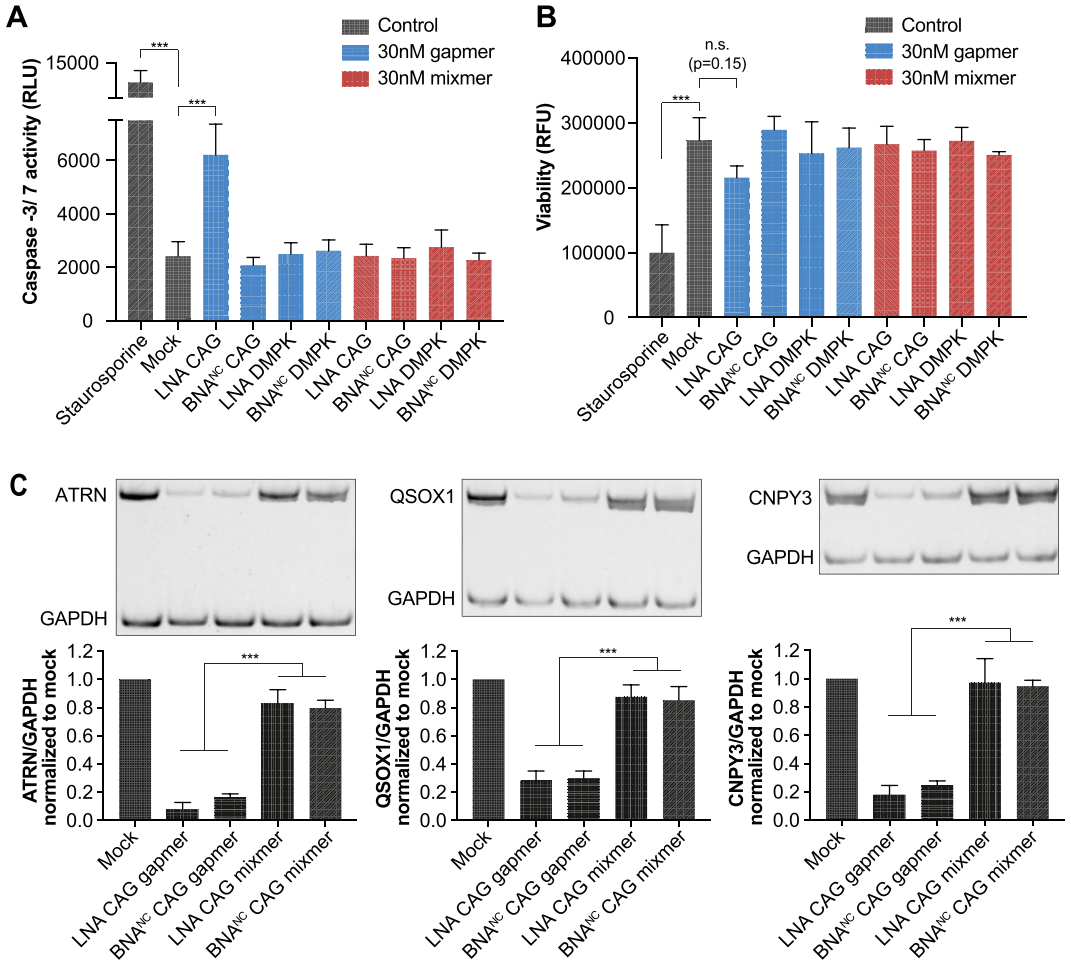


Figure 2. LNA but not BNA^{NC} CAG gapmers induce caspase activity. (A,B) Non-DM1 TeloMyoD fibroblasts were electroporated with 30 nM of LNA or BNA^{NC} gapmers or mixmers and plated into differentiation media. Caspase activity (A) and viability (B) were measured 32 h later; n = 3, Dunnett's multiple comparisons test. (C) RT-PCR of on-target but nondesired knockdown of mRNAs containing CUG_{6+} normalized to GAPDH in non-DM1 cells; n = 3, Tukey's multiple comparisons test.

treated cells showed a statistically significant decrease in viability (Figure 2B). Cytotoxicity was only elevated in cells treated with the positive control digitonin, indicating the ASOs tested do not display cytotoxic effects at 30 nM (Figure S2B).

Given that the LNA CAG mixmer did not elicit caspase activity, we hypothesized that LNA CAG gapmers induce caspase activity through on-target but nondesirable knockdown of non-DMPK transcripts that contain CUG_{6+}. However, we demonstrate that both LNA and BNA^{NC} CAG gapmers induce RNase H1-mediated knockdown of all 11 tested CUG_{6+}-containing mRNAs (Figure 2C, Figure S3). The question remains why LNA CAG gapmers induce caspase activity but the sequence-matched BNA^{NC} CAG gapmers do not. Gapmer-mediated RNase H1 activity includes off-target effects within intronic or exonic regions of long pre-mRNA transcripts.[14,20] Additionally, a variety of proteins bind ASOs and modulate their activity, and ASO chemistry can either promote or inhibit protein binding.[21,22] Therefore, differences in off-target effects or in the ASO-protein interactome could explain the differential caspase induction seen between the LNA and BNA^{NC} CAG gapmers.

Previous work indicates that gapmers targeting within the repetitive region of DMPK preferentially degrade the mutant allele, and this region is an attractive target given the higher number of potential binding sites across the repeat tract.[23] However, given our results that non-DMPK transcripts containing short CUG repeat tracts were degraded by CAG gapmers, we analyzed endogenous allele-specific RNA expression in DM1 cells after treatment with BNA^{NC} gapmers. To distinguish between the expanded and nonexpanded alleles, we utilized a polymorphic site within DMPK and confirmed that RNA transcripts from the expanded allele are retained in the nucleus in these DM1 cells (Figure 3A–C).[24] Treatment with 30 nM BNA^{NC} DMPK gapmers induces preferential knockdown of the nuclear-retained CUG_{exp} RNA without affecting the nuclear or cytoplasmic expression of the nonexpanded allele (Figure 3D,E). Unexpectedly, this preferential knockdown was not seen with the BNA^{NC} CAG gapmer, which produced over 60% knockdown of total DMPK, but at the expense of targeting both the expanded and nonexpanded alleles (Figure 3E). In contrast, the BNA^{NC} DMPK gapmer produced only 20% knockdown of total DMPK, but this was largely accounted for by knockdown of the nuclear-retained, expanded allele. It is possible that the high potency of the BNA^{NC} gapmer saturated knockdown of the expanded allele, leading to secondary degradation of the nonexpanded DMPK mRNA. A recent study demonstrated that *DMPK* knockout mice do not have muscle or cardiac dysfunctions, making it possible that the CAG gapmer would still be viable for clinical translation.[25] However, the relative low specificity of this

DOI: 10.1021/acschembio.7b00416
ACS Chem. Biol. XXXX, XXX, XXX−XXX

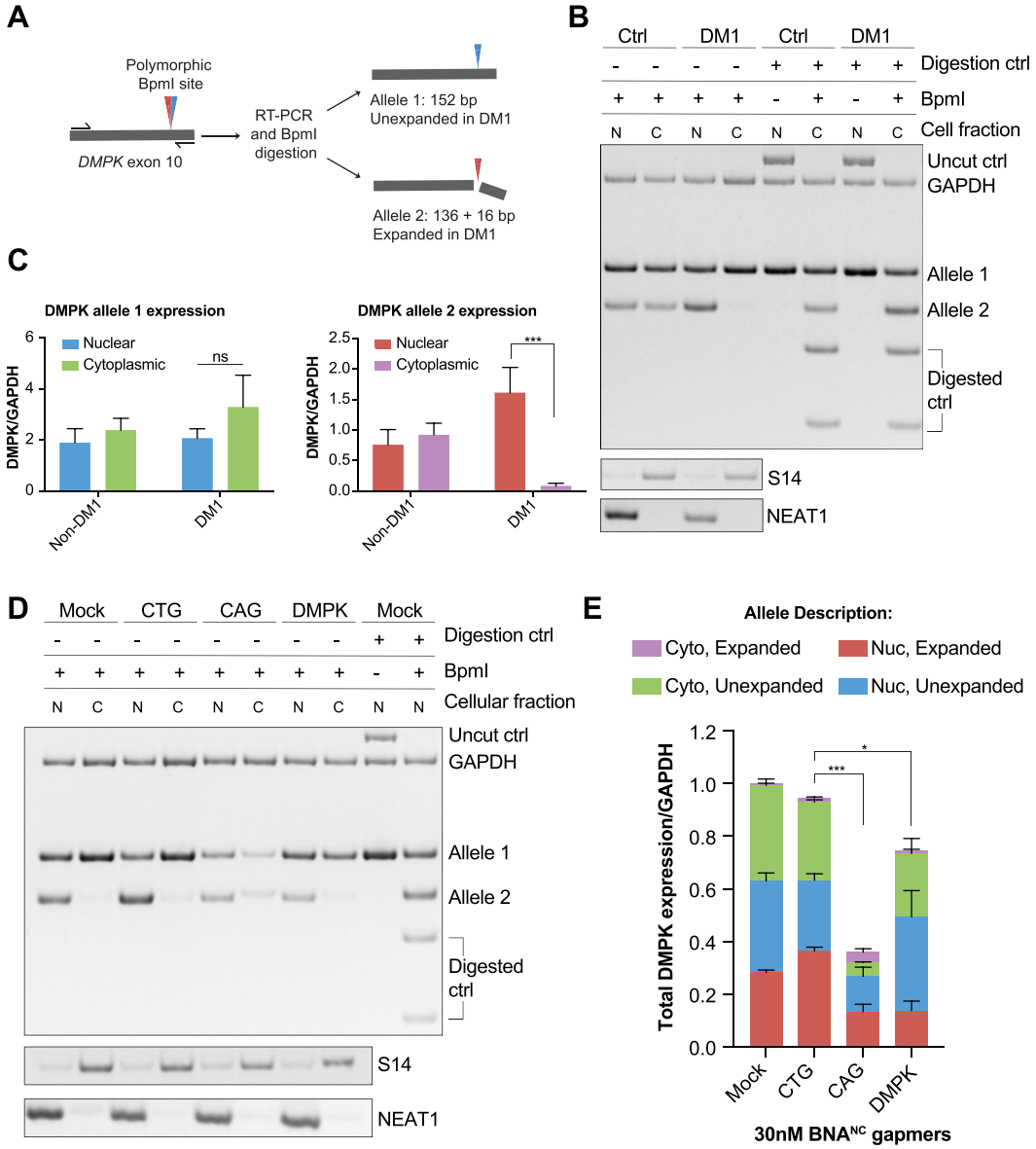


Figure 3. Allele-specific analysis of DMPK expression in DM1 and non-DM1 TeloMyoD fibroblasts before and after treatment with BNA^{NC} gapmers. (A) Diagram of the RT-PCR product containing the BpmI polymorphism. (B,C) RT-PCR and quantification of DMPK allele 1 and allele 2 mRNA levels in the nucleus and cytoplasm from DM1 and non-DM1 TeloMyoD fibroblasts. A control PCR product containing the BpmI site was added to BpmI digestions in lanes 5−8 to confirm complete BpmI digestion. Efficiency of nuclear and cytoplasmic separation is indicated by RT-PCR for NEAT1 and S14, respectively. N = nuclear, C = cytoplasmic; $n = 3$, Sidak's multiple comparisons test. (D,E) Allele-specific RT-PCR and quantification of DMPK RNA from DM1 fibroblasts after treatment with 30 nM BNA^{NC} gapmers. Expression of each allele in either the nucleus or cytoplasm was normalized to GAPDH and displayed relative to the total DMPK expression in mock treated cells. Statistically significant differences in total DMPK mRNA relative to mock are shown for the comparison of CTG control gapmers to CAG and DMPK gapmers; $n = 3$, Dunnett's multiple comparisons test.

gapmer and higher toxicity seen for the LNA gapmer of this sequence indicate that targeting this repetitive region confers higher risk.

We next sought to determine whether BNA^{NC} gapmers could functionally rescue characteristic defects in DM1 cells. First, we used fluorescent *in situ* hybridization (FISH) and immunofluorescence (IF)-FISH to determine whether BNA^{NC} gapmers can reduce foci load and release sequestered MBNL1. DM1 and non-DM1 TeloMyoD fibroblasts were treated with 30 nM of BNA^{NC} CAG or DMPK gapmers and differentiated for 6 days to induce the myogenic program.[26,27] CUG_{exp} RNA foci were visualized by RNA FISH (Figure S4A), and the number of foci per nucleus was quantified across three biological replicates (Figure 4A). Nearly all (97% ± 2.3%) mock treated DM1 cells had nuclear RNA foci (Figure 4A, Figure S4C), while none of the 150 analyzed non-DM1 cells had nuclear foci. The total number of foci in 150 cells decreased from 738 in DM1 mock treated cells, to 334 and 383 after treatment with BNA^{NC} CAG or DMPK gapmers, respectively. This is reflected as a shift in the distribution of foci number per cell (Figure 4A). While over 30% of mock treated cells had more than six foci, only 5−7% of cells treated with the BNA^{NC} CAG or DMPK gapmers had more than six foci. This effect was even more pronounced in undifferentiated cells assayed 24 h after gapmer treatment (Figure S4B−D). Partial foci knockdown, despite significant transcript knockdown by RT-PCR, may be a result of foci

DOI: 10.1021/acschembio.7b00416
ACS Chem. Biol. XXXX, XXX, XXX−XXX

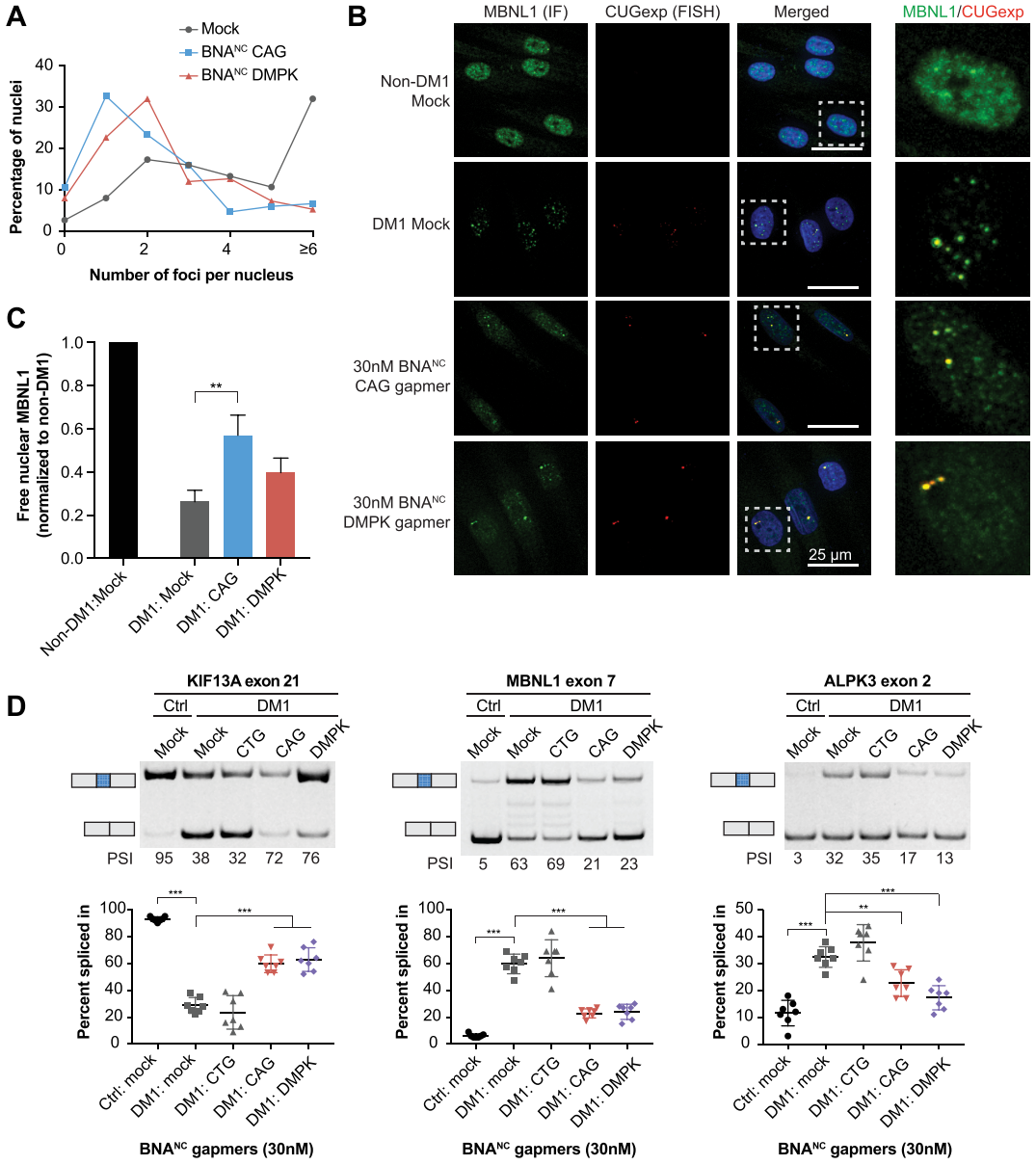


Figure 4. Rescue of characteristic DM1 defects by BNA^{NC} gapmers. (A) The distribution of foci number per nucleus from 150 DM1 TeloMyoD differentiated fibroblasts after treatment with 30 nM BNA^{NC} gapmers. (B,C) Quantification and visualization of diffuse nuclear MBNL1 (60 cells per replicate); $n = 3$, Dunnett's multiple comparisons test. (D) RT-PCR gels and quantification of splicing rescue for three representative splicing events. Up to two additional PCR cycles were used for ALPK3 in non-DM1 cells compared to DM1 cells, to account for variance in baseline expression levels. PSI = percent spliced in $n = 7$, Dunnett's multiple comparisons test.

nucleation by a low absolute number of transcripts.[28] It is also possible that the MBNL-bound CUG$_{exp}$ RNA region is partially protected from exonuclease activity following RNase H1 cleavage.

Given that the majority of cells still had at least one RNA focus after gapmer treatment, we were interested to determine the degree to which MBNL1 was released in cells that had decreased levels of RNA foci. We demonstrated that diffuse MBNL1 levels increased in the nucleoplasm for cells treated with BNA^{NC} CAG gapmers; however, this result was not significant in the BNA^{NC} DMPK gapmers (Figure 4B,C and Figure S5). The increase in free MBNL1 is visible even in cells that contain foci, indicating that the presence of RNA foci is not necessarily a direct measure of MBNL1 availability. Our results in both differentiated and undifferentiated DM1 cells indicate that RNA foci are reduced, and MBNL1 activity may be increased after treatment with 30 nM of BNA^{NC} gapmers.

As RNA foci can be indicative of MBNL1 sequestration in DM1, we next analyzed whether characteristic DM1 splicing abnormalities are rescued by BNA^{NC} gapmers. Differentiated DM1 TeloMyoD fibroblasts recapitulate splicing defects observed in DM1 skeletal muscle. Treatment of these cells with 30 nM of BNA^{NC} CAG or DMPK gapmers significantly reverted splicing of ALPK3, MBNL1, and KIF13A toward the pattern in non-DM1 cells (Figure 4D). The splicing rescue for MBNL1 and KIF13A was robust, while the rescue was more moderate for the less severely affected event ALPK3. The level of splicing rescue for these events is notable, especially given the residual number of about 1−3 foci per cell after gapmer treatment. This suggests that decreased foci load can be sufficient to revert splicing without complete degradation of all

DOI: 10.1021/acschembio.7b00416
ACS Chem. Biol. XXXX, XXX, XXX−XXX

RNA foci and demonstrates that BNA^{NC} gapmers potently revert splicing defects in DM1 cells.

In conclusion, we have shown that BNA^{NC} gapmers are a promising therapeutic option to target toxic RNA in myotonic dystrophy. The allele-specific knockdown achieved with the BNA^{NC} DMPK, but not CAG, gapmers indicates that targeting a nonrepetitive region improves preferential knockdown of the expanded allele. Determining the mechanism responsible for differences in caspase induction between LNA and BNA^{NC} CAG gapmers is a key area of interest and will aid in overcoming toxicity concerns for future gapmer-based therapies.

METHODS

Antisense Oligonucleotides. LNA and BNA^{NC} ASOs were purchased from Exiqon and Bio-Synthesis, respectively.

Cell Models and Assays. SB TeloMyoD (control, non-DM1) and KB TeloMyoD (DM1, 400 CTG repeats) immortalized human fibroblast cell lines express telomerase and contain a tetracycline-inducible MyoD to promote the myogenic program in response to growth for 6 days in low serum media (1% FBS) supplemented with 1 μg/mL doxycycline.[26,27] Toxicity analyses were conducted using the ApoTox-Glo Triplex Assay (Promega). Additional assay details and RT-PCR primers are listed in the Supporting Information and Table S2.

Microscopy. TeloMyoD fibroblasts were electroporated with 30 nM of gapmers, differentiated for 6 days, and visualized by RNA FISH using the $(CAG)_5$ TYE 563 LNA probe (CAGCAGCAGCAGCAG) or by IF/FISH using MBNL1 (Santa Cruz, sc-47740). For further details, see the Supporting Information.

Statistical Analysis. Data are expressed as mean ± standard deviation. All data shown are the summary of three or more biological replicates, and statistical analyses were completed in Prism 7. For data sets where three or more groups were analyzed simultaneously, one-way ANOVA was used with ungrouped data, and two-way ANOVA was used with grouped data, and they were corrected for multiple comparisons. Statistical values used: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00416.

Tables S1 and S2, Figures S1−S5, and supplemental methods (PDF)

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Funding

This work was funded by a National Science Foundation Graduate Research Fellowship [DGE1255980 to K.S.M.]; the National Institutes of Health [5T32 GM008231 to K.S.M.; R01AR045653, R01HL045565, and R01AR060733 to T.A.C.]; the Muscular Dystrophy Association [RG 276796 to T.A.C.]; and Baylor Research Advocates for Student Scientists. This work was also supported by the Integrated Microscopy Core at Baylor College of Medicine, with funding from the National Institutes of Health [DK56338, and CA125123], the Dan L. Duncan Cancer Center, and the John S. Dunn Gulf Coast Consortium for Chemical Genomics.

Notes

The authors declare the following competing financial interest(s): None for T.A.C., K.S.M., and A.N.R. M.C. is the CEO of Bio-Synthesis Inc.

ACKNOWLEDGMENTS

We are grateful to J. Dubrulle at the Integrated Microscopy Core at Baylor College of Medicine for providing computational image analysis. We thank D. Brook for the TeloMyoD cell lines and P. Sarkar for the expanded pure CTG repeats.

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DOI: 10.1021/acschembio.7b00416
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DOI: 10.1021/acschembio.7b00416
ACS Chem. Biol. XXXX, XXX, XXX−XXX