REPORT

Competency for Nonsense-Mediated Reduction in Collagen X mRNA Is Specified by the 3′ UTR and Corresponds to the Position of Mutations in Schmid Metaphyseal Chondrodysplasia

Jacqueline T. Tan,[1] Friederike Kremer,[1] Susanna Freddi,[1] Katrina M. Bell,[1] Naomi L. Baker,[1] Shireen R. Lamandé,[1,2] and John F. Bateman[1,2,]*

Nonsense-mediated decay (NMD) is a eukaryotic cellular RNA surveillance and quality-control mechanism that degrades mRNA containing premature stop codons (nonsense mutations) that otherwise may exert a deleterious effect by the production of dysfunctional truncated proteins. Collagen X (COL10A1) nonsense mutations in Schmid-type metaphyseal chondrodysplasia are localized in a region toward the 3′ end of the last exon (exon 3) and result in mRNA decay, in contrast to most other genes in which terminal-exon nonsense mutations are resistant to NMD. We introduce nonsense mutations into the mouse Col10a1 gene and express these in a hypertrophic-chondrocyte cell line to explore the mechanism of last-exon mRNA decay of Col10a1 and demonstrate that mRNA decay is spatially restricted to mutations occurring in a 3′ region of the exon 3 coding sequence; this region corresponds to where human mutations have been described. This localization of mRNA-decay competency suggested that a downstream region, such as the 3′ UTR, may play a role in specifying decay of mutant Col10a1 mRNA containing nonsense mutations. We found that deleting any of the three conserved sequence regions within the 3′ UTR (region I, 23 bp; region II, 170 bp; and region III, 76 bp) prevented mutant mRNA decay, but a smaller 13 bp deletion within region III was permissive for decay. These data suggest that the 3′ UTR participates in collagen X last-exon mRNA decay and that overall 3′ UTR configuration, rather than specific linear-sequence motifs, may be important in specifying decay of Col10a1 mRNA containing nonsense mutations.

Because mutations that introduce premature stop codons have been estimated to account for approximately one-third of inherited genetic disorders,[1,2] RNA surveillance and quality control imposed by the NMD process is of fundamental importance in the molecular pathology of many diseases. The molecular basis of NMD has thus been the subject of intense interest, and many components have been identified. In the classical model, the mammalian RNA surveillance machinery is an integrated component of the processes of RNA splicing, transport from the nucleus, and translation in the cytoplasm.[3–11] During pre-mRNA splicing, a complex of proteins is deposited ~20 nucleotides upstream of exon-exon junctions. This exon-junction complex (EJC) marks the site of intron excision and is composed of a range of proteins involved in splicing, mRNA transport, and NMD. During a proposed initial "pioneer" round of translation, the ribosome displaces these EJC proteins as it encounters them, continuing along the mRNA until a stop codon is recognized, triggering the recruitment of another RNA-binding-protein assembly including the translation release factors. If there is no EJC downstream of this surveillance-protein complex, such as occurs in the last exon of genes, the stop codon is recognized as the correct stop, and normal termination proceeds. However, if the stop codon is upstream of an EJC, and thus located prior to the terminal exon, it is identified as "premature." In this situation, communication between the surveillance complex and the EJC targets the mRNA containing the premature stop codon for NMD. In our previous studies on nonsense mutations in the human collagen X gene,

COL10A1, we found preliminary evidence that nonsense-containing collagen X mRNA is degraded. Collagen X is expressed only in hypertrophic cartilage, and mutations cause Schmid-type metaphyseal chondrodysplasia (MCDS; MIM #156500).[12,13] Two heterozygous nonsense mutations, COL10A1 W611X and Y632X, which are 210 bp and 147 bp, respectively, upstream of the normal stop codon led to decay of the mutant mRNA in the affected cartilage tissue.[14,15] Other recent studies have demonstrated that a premature termination at codon 663 (54 bp upstream of the normal stop) also triggers COL10A1 mRNA decay.[16] These findings were surprising in light of the fact that these mutations reside within the terminal exon, exon 3, and thus would not be expected to result in NMD because of the absence of a downstream EJC. Our studies also demonstrated that mRNA decay can be cell specific because decay of nonsense-containing human COL10A1 mRNA only occurred in cartilage cells and not in lymphoblasts and bone cells from the patients.[15] These two unusual aspects of the nonsense-mediated reduction in abundance of COL10A1 mRNA fall outside the parameters of the classical NMD process and raise critically important questions about the nature of COL10A1 mRNA decay. Because similar processes may also play a role in the decay of nonsense mRNA produced by other genes, a better understanding of collagen X mRNA decay should provide a more comprehensive understanding of the mechanistic complexities of mRNA-surveillance pathways.

For exploration of the molecular determinants of collagen X mRNA decay in detail, a range of stop-codon
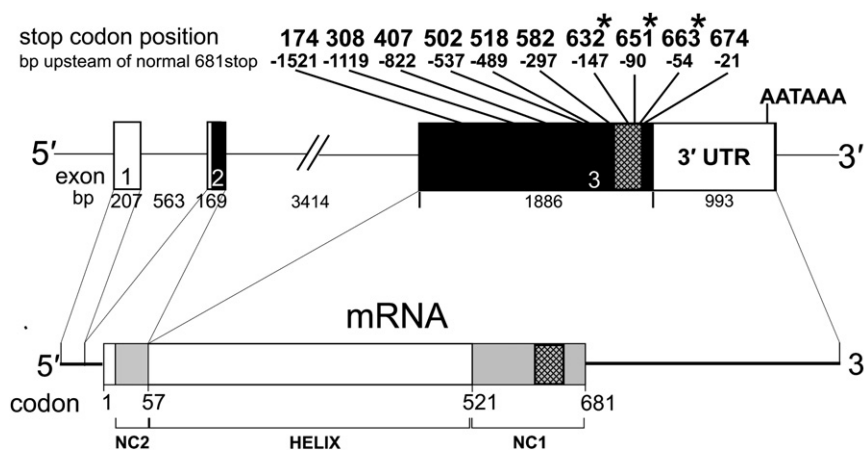
Figure 1. *Col10a1* Gene Organization

The three exons of *Col10a1* are drawn as rectangles, whereas the introns are denoted by lines. The poly(A) signal in the 3′ UTR is AATAAA. The sizes of the introns and exons are shown in bp under the gene. The normal termination is codon 681, and the location of the engineered mutations is shown above the gene. The distance upstream to the normal stop codon is shown in bp below the engineered premature stop codons. The coding region arising from exon 2 and 3 is denoted by the filled regions of the exons. The region where MCDS patient mutations are clustered (hatched) corresponds to the location of the engineered mutations 632X, 651X, and 663X (asterisks). In the lower part of the diagram, the corresponding parts of the mRNA are shown with the regions coding for the protein NC2, triple helical, and NC1 trimerization domain indicated.

mutations were engineered into a mouse *Col10a1* gene construct.[17] This construct contained all three exons, 2 kb of 5′ promoter sequence, and 1.3 kb of 3′ flanking sequence including the entire 3′ UTR (Figure 1). Specific mutations in *Col10a1* were produced by overlap extension PCR as previously described.[18] We sequenced constructs to ensure that the correct mutations were introduced and that there were no PCR errors. The finding that *COL10A1* mRNA decay was cell specific, only occurring in cartilage cells expressing collagen X,[15] necessitated the use of a mouse hypertrophic cartilage cell line for our expression studies. We used the mouse MCT hypertrophic-chondrocyte cell line (SV40 temperature-sensitive large T antigen transformed)[19] because it expresses endogenous collagen X and thus should have the capacity to direct collagen X nonsense mRNA decay when the cell line is grown at the nonpermissive temperature (37°C). MCT cells maintained in Dulbecco's modified Eagle's Medium containing 10% fetal-bovine serum were grown to ~70% confluence and cotransfected with *Col10a1* premature-stop-codon mutant constructs and pGK-Hygro with FuGENE 6 transfection reagent (Roche) as previously described.[20] Cells were selected and maintained with 150 μg/ml hygromycin B (Roche), and individual clones were isolated and expanded into cell lines. To assess mRNA decay, we grew cells for 7 days at 37°C and added ascorbic acid to a final concentration of 0.25 mM on days 6 and 7. Cells were then preincubated for 6 hr with and without 100 μg/ml cycloheximide (Sigma), an inhibitor of NMD,[15,21] prior to RNA isolation (RNeasy total RNA kit, QIAGEN). Because of the low levels of collagen X expression, the ratio of mutant to endogenous collagen X mRNA was measured by an allele-specific primer extension assay (Table 1) as previously described.[15] A protection of mutant mRNA produced by the transfected *Col10a1* gene relative to the endogenous wild-type collagen X mRNA in the cycloheximide-treated cells was taken as a measure of the extent of decay of the mutant mRNA.[15] To overcome clonal variability in expression levels of the endogenous and transfected *Col10a1*, and to ensure that a significant proportion of the clones analyzed for mRNA-decay competency were expressing the appropriate hypertrophic-chondrocyte phenotype, we analyzed a large number of individual clonal lines for each construct.

The data for constructs containing premature termination mutations at codons 632, 651, and 663 (147 to 54 bp upstream of the normal termination codon; Figure 1) corresponding to characterized MCDS mutations[13] are shown in Figures 2A–2C. The analysis of multiple transfected clones demonstrated that cycloheximide pretreatment resulted in a significant protection of the mutant mRNA relative to the wild-type (mean protection 200%–300%), clearly demonstrating that mRNA decay occurred for these three MCDS mutations. Because previous studies have directly shown that the 632 and 651 nonsense mutations result in mRNA decay in MCDS cartilage in vivo,[14–16] our data confirmed that mRNA decay also occurs for nonsense mutations in this region of the mouse gene and validated the use of this system for detailed studies of the mRNA-decay competency of *Col10a1* premature-termination-codon mutations. The 3′ boundary of this decay-competent region was assessed by transfection of MCT chondrocytes with a construct containing a nonsense mutation at codon 674, which is 21 bp upstream from the normal 681 stop. With this construct, none of the clones showed a protection with cycloheximide, demonstrating that mRNA decay did not occur for premature termination codons immediately upstream of the normal stop (Figure 2D). The presence of this 3′ mRNA-decay immune region was confirmed by recent studies in a transgenic mouse in which *Col10a1* with a 13 bp deletion resulting in premature stop at codon 672 was not subject to mRNA decay, and the mutant protein with a short C-terminal truncation was expressed in mouse hypertrophic cartilage.[22] This immunity to nonsense mutant RNA decay may be due to the spatial relationship of this region's premature stop codons proximal to the normal stop with putative downstream elements that may be involved with regulating type X collagen mRNA decay.

Table 1. Primers Used in RT-PCR and Allele-Specific Primer Extension

Mutation	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Extension Primer (5′ to 3′)
G174X	TAAGAGGAGAACAAGGC	TTGTGCACCCTTCTCTC	TTGTGCACCCTTCTCTC
G308X	TTCCAGGCCCTCAAGGTCCC	ACCAGCAGGTCCCCTTTGTC	ACCAGCAGGTCCCCTTTGTC
G407X	AAGGTGAGATAGGTCTAGTTGG	ACTCCAGGATCACCTTTTTGT	ACTCCAGGATCACCTTTTTGT
G502X	GGCAGGTCCAAGAGG	CTGGGAGACCAGGTTCTC	CTGGGAGACCAGGTTCTC
Q518X	TTGGCCCTGTAGGAGCTAAAG	CATCAGGCATGACTGCTT	CATCAGGCATGACTGCTT
Y582X	GTACAATAGGCAGCAGCATTA	TAGAAGTGAGGAAACTTGGTC	GTACAATAGGCAGCAGCATTA
Y632X	GGCAGGTCCAAGAGG	AATGTCAGCCTTTAAGGGTG	TGAGTACAGCAAAGGCTA
W651X	GGCAGGTCCAAGAGG	AATGTCAGCCTTTAAGGGTG	ACAGAAAATGACCAGGTATG
Y663X	GGCAGGTCCAAGAGG	AATGTCAGCCTTTAAGGGTG	AGAATCAAACGGCCTCTA
G674X	GGCAGGTCCAAGAGG	AATGTCAGCCTTTAAGGGTG	TGGGAGCCACTAGGAATC
Y632X-ΔI	TGAGTACAGCAAAGGCTA	AATGTCAGCCTTTAAGGGTG	TGAGTACAGCAAAGGCTA
Y632X-ΔII	TGAGTACAGCAAAGGCTA	AATGTCAGCCTTTAAGGGTG	TGAGTACAGCAAAGGCTA
Y632X-ΔIII	TGAGTACAGCAAAGGCTA	AATGTCAGCCTTTAAGGGTG	TGAGTACAGCAAAGGCTA
Y632X-ΔIII[AU]	TGAGTACAGCAAAGGCTA	AATGTCAGCCTTTAAGGGTG	TGAGTACAGCAAAGGCTA

Total RNA (200 ng) from MCT cells transfected with mutant *Col10a1* constructs was used as a template for RT-PCR with the primers shown above. The cDNA fragment was gel purified, and 5 ng used as a template for allele-specific primer extension. Primers used in the primer-extension reactions were designed to anneal one base 5′ to the mutation so that extension with specific α-^{33}P-dNTPs would thus discriminate between the mutant and endogenous mRNAs. The extension reactions were carried out at 95°C for 1 min, 50°C (G407X and Q518X), 55°C (G174X), 56°C (W651X), and 60°C (G308X, G502X, Y582X, Y632X, Y663X, G674X, Y632X-ΔI, Y632X-ΔII, Y632X-ΔIII, and Y632X-ΔIII[AU]) for 2 min, and 72°C for 1 min in 10 μl of 10 mM Tris/HCl (pH 8.3) containing 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% (w/v) gelatin, 1 μM primer, and 1 μCi of [α-^{33}P]-dCTP, dGTP, or dATP (Perkin Elmer) in the absence of nonradioactive dNTPs. The products were analyzed on 15% (w/v) denaturing polyacrylamide gels containing 7 M urea, and the radioactivity incorporated into the extended products was quantified with a phosphor imager (Molecular Dynamics) with the program ImageQuant TL (Amersham Biosciences).

To explore the 5′ range of mRNA-decay competency, we examined nonsense codons at 174, 308, 407, 502, 518, and 582 (1521 bp to 297 bp upstream of the normal stop; see Figure 1). These are 5′ of the region in exon 3 where MCDS mutations have been characterized.[13] For all these mutations, cycloheximide did not cause protection of the mutant mRNA, indicating that mRNA decay did not occur for these stop-codon mutations, which are more than ~300 bp upstream of the normal stop (Figures 3A–3F).

Our data demonstrated that the region of the *Col10a1* mRNA competent for nonsense mutant mRNA decay was restricted to a relatively small region of exon 3 that corresponds to the region of the gene containing the known MCDS nonsense mutations (Figure 4). Nonsense mutations beyond this region in *Col10a1*, either immediately downstream at codon 674 or upstream from codon 582 to at least codon 174, did not undergo mRNA decay (Figure 4). Although there are some examples of polarity in NMD susceptibility along genes,[23–25] the complete restriction of nonsense-mRNA-decay competency to a very localized region of the last exon of *Col10a1* is unique and further suggests previously unrecognized mechanisms of mRNA-decay specification.

A possible mechanism of *Col10a1* mRNA destabilization, consistent with such a localized region of decay competence, could involve the disturbance of a putative mRNA stability element in this region of exon 3. Although there is no evidence suggesting the presence of such an element in *Col10a1* mRNA, there are several examples, such as elastin[26] and *Cited-2*,[27] in which sequence elements within the mRNA coding sequence are involved in controlling mRNA decay in response to TGFβ. If such an element existed in *Col10a1*, it would be expected that missense and other mutations in this critical region would also disturb such regulatory sequences and cause mRNA decay. However, recent studies on a MCDS mouse model in which a heterozygous N617K missense mutation has been knocked into *Col10a1* showed equal abundance of the wild-type and N617K allele mRNAs (H. Rajpar and R. Boot-Handford, personal communication). Furthermore, studies on MCDS patient cartilage mRNA containing a heterozygous S600P mutation shows no mRNA decay of the mutant mRNA.[28] These data demonstrate that the presence of a mutation in this region, per se, does not cause mRNA decay and that the mutant mRNA decay is nonsense mediated.

The most likely reason for the inability of mutations in these upstream regions to elicit mRNA decay is that they are unable to be recognized by the RNA surveillance machinery. In yeast, there is a 150–200 bp limit in the distance from a downstream element and the stop codon for functional NMD.[29] In mammals, little is known about the maximum distance from the EJC to the premature stop codon. Data showing that the limit for *TPI* is 559 bp,[30] whereas in *BRCA1* stop codons as far as 3395 bp upstream result in NMD,[31] suggest that there may not be a global maximum limit. The out-of-range limit may be gene specific and controlled by other regulatory elements or by the ability of the local RNA structure to allow juxtaposition of the critical surveillance-machinery components. In the intron-less genes in yeast, it has been shown that 3′ downstream sequence elements substitute for the EJC to define premature termination codons, target the transcript for NMD,[29] and define the out-of-range limit, and it is tempting to suggest that a downstream element may thus be involved in *Col10a1* mRNA decay. The location of the mRNA-decay-competent region of exon 3,
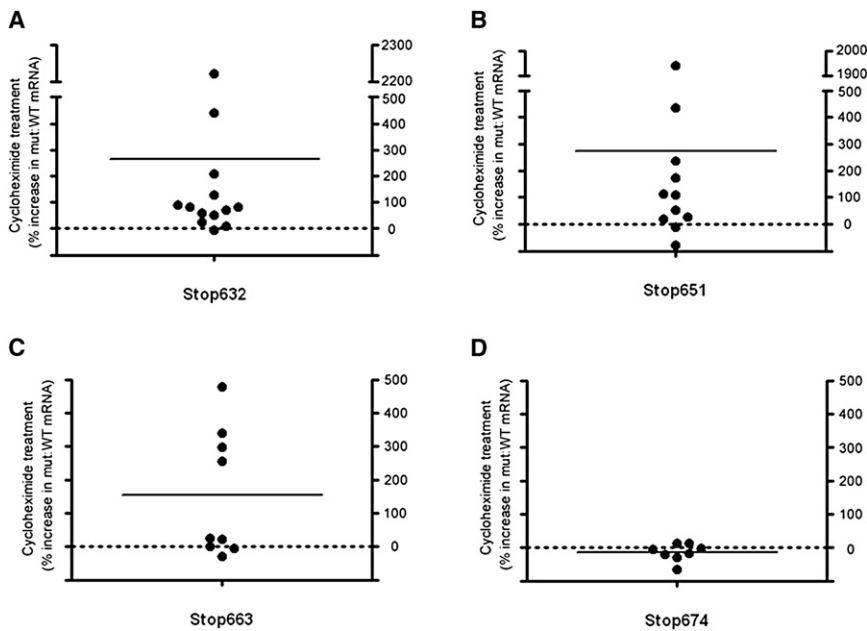
Figure 2. Premature Stop Codons Located in the MCDS Mutation Region Cause mRNA Decay
Each data point represents the determination of the change in the ratio of the mRNA of transfected mutant construct (mut) to the endogenous normal collagen X mRNA (WT) caused by cycloheximide treatment of an individual stably transfected MCT clone expressing Y632X (A), W651X (B), Y663X (C), and YG674X (D). The dotted horizontal line denotes no change in the mut:WT ratio resulting from treatment of the cells with the NMD inhibitor cycloheximide. An increase in the mut:WT mRNA ratio after cycloheximide treatment indicates that the transfected construct undergoes mRNA decay. The mean of the percentage change in the mut:WT ratio caused by cycloheximide treatment is denoted by the solid horizontal line. Y632X, W651X, and Y663X nonsense codons all lead to *Col10a1* mRNA decay, whereas G674X does not cause mRNA decay.

encompassing ~50 to <300 bp upstream of the normal stop, suggests that the adjacent 3′ UTR could contain the downstream element(s) regulating *Col10a1* mRNA decay. Furthermore, recent studies have shown that a properly configured 3′ UTR is important for correct translation termination and mRNA stability.[32]
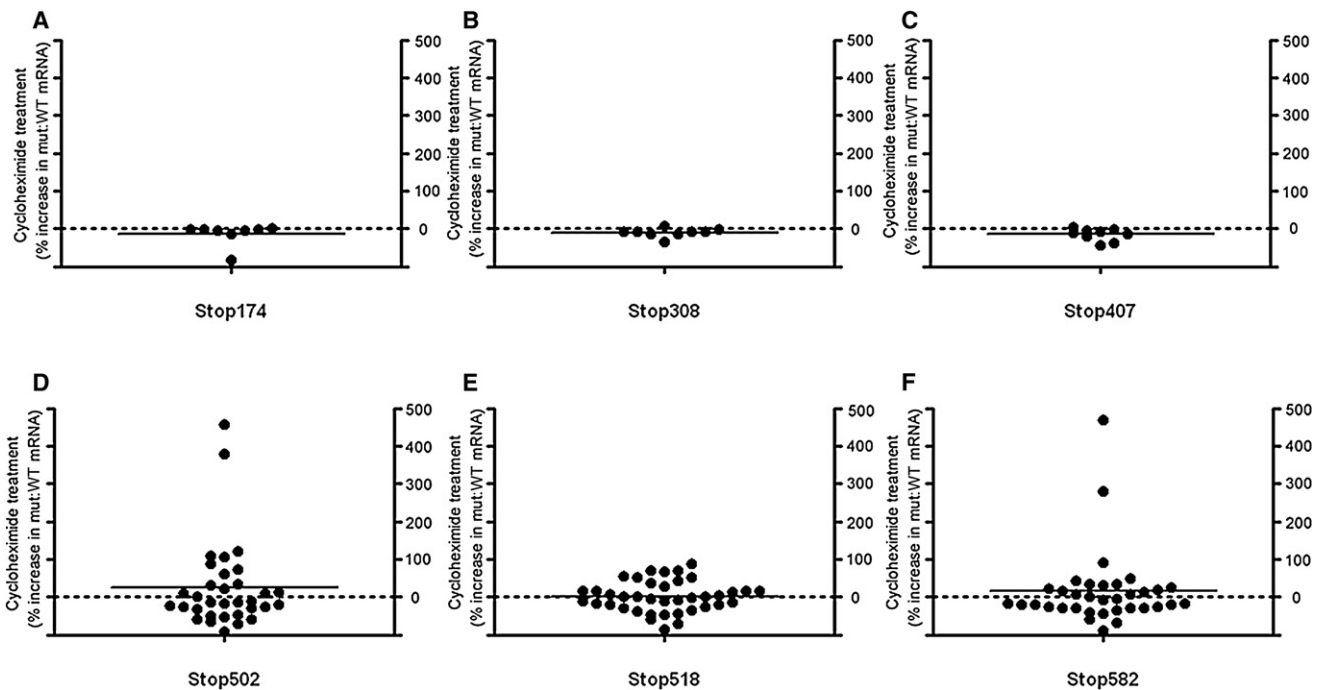

Figure 3. Nonsense Mutations Upstream of the MCDS Region Do Not Lead to mRNA Decay
Each data point represents the determination of the change in the ratio of the mRNA of transfected mutant construct (mut) to the endogenous normal collagen X mRNA (WT) caused by cycloheximide treatment of cells stably transfected with G174X (A), G308X (B), G407X (C), G502X (D), Q518X (E), and Y582X (F). The dotted horizontal line denotes no change in the mut:WT ratio resulting from treatment of the cells with the NMD inhibitor cycloheximide. The mean of the percentage change in the mut:WT ratio caused by cycloheximide treatment is denoted by the solid horizontal line. All constructs show no effect of cycloheximide treatment on the mean mut:WT ratio, demonstrating that the G174X, G308X, G407X, G502X, Q518X, and Y582X *Col10a1* constructs do not undergo mRNA decay.
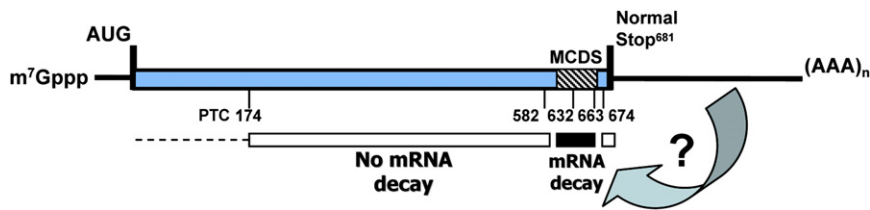
Figure 4. *Col10a1* **Nonsense mRNA Decay**

The localization of the premature termination codons that cause mRNA decay are shown by the filled rectangle below the diagram of the *Col10a1* mRNA. Regions that are resistant to nonsense mRNA decay are designated with open rectangles. The corresponding region of the mRNA that contains the human MCDS mutations is designated by the hatching. The arrow connecting the 3′ UTR to the mRNA-decay-competent domain represents these studies' conclusion that implicates the 3′ UTR in specifying mRNA decay for premature termination mutations localized in this region of *COL10A1*.

Terminal exon nonsense-mediated reduction in collagen X abundance could also, in principle, be explained by the classical model of NMD if exon 3 was not the terminal exon and an unrecognized spliceable intron lay downstream of the normal stop codon. If this was the case, the putative intron would have to be within 50 bp of the normal stop to conform to the "50–55 nt-rule,"[33] so that the wild-type transcript would not be targeted for NMD. There is no bioinformatic evidence for a downstream intron in the type X collagen gene, and direct RT-PCR amplification of the 3′ UTR demonstrated only the expected single 3′ UTR amplicon (data not shown) ruling out the presence of a downstream spliceable intron.[34]

Our data suggest that the 3′ UTR of *Col10a1* could contain the molecular trigger, other than a downstream EJC, to provide the crucial additional signal for decay of nonsense-containing mRNA. *Col10a1* has a long 3′ UTR (993 bp), and bioinformatic analysis revealed that it contains three regions of homology strongly conserved across species, suggesting that these may have important biological roles (regions I, II, and III). Region I (23 bp) has 91.3% homology between human and mouse *Col10a1*, region II (170 bp) has 87% homology, and region III (76 bp) has 86% homology. Region III was found to contain a 13 bp AU-rich region, which have been shown in other genes to play a role in regulating mRNA stability.[35] To determine whether these conserved 3′ UTR regions were involved in specifying NMD, we deleted them individually from the *Col10a1* construct containing the mRNA-decay-competent 632X stop mutation (Figure 5). Deletions of region I (23 bp; 632X-ΔI), region II (170bp; 632X-ΔII), or region III (76 bp; 632X-ΔIII) were produced along with a further region III deletion in which only the 13 bp AU-rich region (632X-ΔIII[AU]) was removed. Transfection of these into MCT chondrocytes resulted in the abrogation of the mRNA-decay competency of the 632X mutant construct when any of the three homologous domains (I, II, or III) were removed (Figures 6A–6C), clearly demonstrating that the 3′ UTR is critical in specification of *Col10a1* nonsense mRNA decay (Figure 4). Interestingly, the deletion of only the AU-rich region (13 bp) within region III did not abolish the ability of the 632X mutant mRNA to undergo decay (Figure 6D). The mechanism of how the 3′ UTR directs mRNA decay is not known and is the subject of current detailed study, but our data suggest that because all three deletions (regions I, II and II; Figure 5)

in the 3′ UTR lead to mRNA-decay incompetency, it is likely that disturbance to the structure or configuration of the 3′ UTR,[32] rather than the deletion of a specific linear-sequence motif, is the underlying cause of the inhibition of NMD. Recently, a mechanism for nonsense-mutation-containing mRNA degradation, specified by the 3′UTR, called Staufen 1-mediated mRNA decay (SMD) has been described.[36,37] SMD occurs for the cohort of genes that bind Stau-1 in their 3′ UTR, suggested to be ~1% of the HeLa cell transcriptome.[36] Stau-1 binding to double-stranded RNA structures in the 3′ UTR recruits the NMD factor Upf1 and initiates SMD of transcripts that have termination codons more than ~25 bp upstream of the Stau-1-binding region. Whether such a Stau-1 mechanism is involved in *Col10a1* mRNA decay is not yet known; however, current evidence indicates that SMD is not limited to premature termination codons in only restricted regions of the gene,[37] as we observed for *Col10a1* (Figure 4), making it unlikely that SMD is involved in the decay of nonsense-containing *Col10a1* transcripts. Further detailed studies will test this and explore the nature of the specification of *Col10a1* mRNA by the 3′ UTR.

Nonsense-mediated mRNA decay is a fundamentally important process in inherited genetic diseases and in the regulation of normal protein expression. Our studies have revealed surprising mechanistic features of the decay
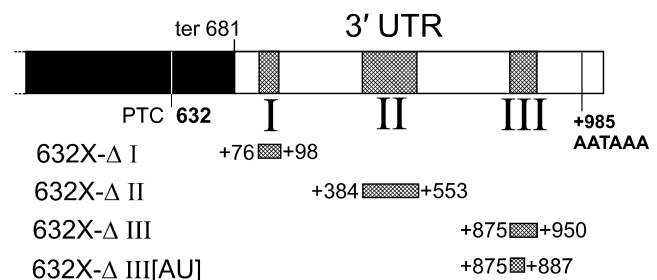

Figure 5. Diagrammatic Representation of the 3′ UTR Deletions

Three highly homologous regions (I, II, and III) were individually deleted in the Y632X construct. The cross-hatched rectangles show the corresponding deletions made in Y632X-ΔI, Y632X-ΔII, and Y632X-ΔIII. Numbers under the rectangles indicate the distance (bp) downstream of the normal stop codon. A further subdeletion of an AU-rich region found in region III was also created (Y632X-ΔIII[AU]).
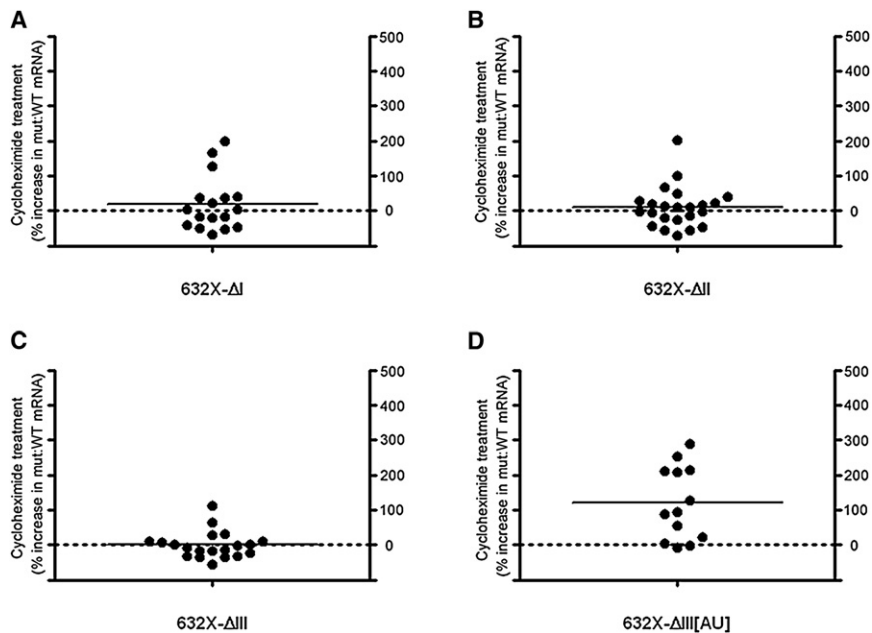
Figure 6. The 3′ UTR Is Important for Decay of *Col10a1* Nonsense mRNA

Each data point represents the determination of the change in the ratio of the mRNA of transfected mutant construct (mut) to the endogenous normal collagen X mRNA (WT) caused by cycloheximide treatment of cells stably transfected with an mRNA-decay-competent Y632X construct (see Figure 2A) with further mutations in the 3′ UTR. Y632X-ΔI (A), Y632X-ΔII (B), and Y632X-ΔIII (C) abolished Y632X mRNA decay because cycloheximide treatment now did not affect the ratio of mut:WT collagen X mRNA. Deletion of a 13 bp AU-rich region Y632X-ΔIII[AU] (D) does not affect mRNA decay of Y632X. The dotted horizontal line denotes no change in the mut:WT ratio resulting from treatment of the cells with the NMD inhibitor cycloheximide. The mean of the percentage change in the mut:WT ratio caused by cycloheximide treatment is denoted by the solid horizontal line.

of *Col10a1* mRNA containing nonsense mutations in the terminal exon such as chondrocyte specificity[15] and the involvement of the 3′ UTR in mRNA-decay specification. It is possible that this 3′ UTR-directed last-exon mRNA decay could represent a crucial "failsafe-RNA surveillance" mechanism for genes, such as *Col10a1*, which have large terminal coding exons. Further definition of the mechanistic features of *Col10a1* nonsense mRNA decay should be informative in defining the molecular complexity of this important quality-control process.

Our new data defining the mouse *Col10a1* regions that are nonsense mRNA decay competent also provoke speculation about the molecular pathology of MCDS caused by nonsense mutations. It is clear that if nonsense mutations were upstream of the mRNA-decay-susceptible region in *Col10a1* (Figure 4), that is, if they were more than ~300 bp upstream of the normal stop codon, they would not be subject to surveillance and would produce truncated collagen X with little or no NC1 domain. The carboxyl-terminal NC1 domain of collagen X has been shown in numerous studies to be essential for the interaction of the subunits and trimerization of the collagen X molecule.[20,38–40] Furthermore, our previous studies with in vitro expression of collagen X constructs with sequential C-terminal truncations have shown that a protein region corresponding to codons 589–601 is absolutely necessary for the initiation of collagen X trimer assembly, and without this region, the truncated collagen X does not trimerize and, most importantly, does not interfere with the efficiency of normal allele collagen X protein trimerization.[39] These data suggest that truncated proteins that do not contain this region, that is, those that are produced by stop mutations upstream of approximately codon 589, would not associate and would probably be rapidly degraded intracellularly,[20] resulting in

haploinsufficiency and no dominant interference of assembly of the normal allele product. Because no MCDS nonsense mutations have been found in these upstream regions, it seems likely that such mutant collagen X is innocuous and tolerated by the cartilage cells and does not result in a clinical phenotype. Although it is also formally possible that such severely truncated proteins result in a more severe cartilage phenotype via an unexpected intracellular gain-of-function activity, no mutations in these upstream regions of *Col10a1* have been found in patients with more severe growth-plate cartilage phenotypes (J.F.B., unpublished data).

The upstream cutoff for the stop codons that would cause dominant interference[39] corresponds to the 5′ limit of the region in which MCDS nonsense mutations have been characterized[13] (Figure 4), and stop codons downstream of this region, corresponding to the MCDS mutation domain, would result in the production of truncated collagen X that could interact with normal collagen X and exert a dominant-negative effect if nonsense mRNA decay was not complete. Although we have shown complete decay of mutant collagen X mRNA in cartilage from two patients,[14,15] other studies on a patient mutation at codon 663 indicate that significant amounts of mutant mRNA escape decay and produce abnormal truncated collagen X. Recent studies have shown in a mouse model of MCDS that the truncated protein produced as a result of a frameshift mutation generating a premature stop at codon 621 was retained within the hypertrophic chondrocytes and activated the unfolded protein response.[16] Such an ER-stress response has been previously described for MCDS missense mutations in which structural mutations in the NC1 domain prevent normal protein folding.[22,40] The unfolded protein response resulting from collagen X structural

mutations has significant downstream consequences on gene expression in the hypertrophic chondrocytes, disrupting cellular differentiation and the coordination of the endochondral ossification program, and thus contributes to the chondrodysplasia phenotype.[22] The unfolded protein response recently described for the truncated protein produced by the premature stop at codon 621[16] also disrupts hypertrophic-chondrocyte differentiation. These data clearly suggest that although nonsense mutations result in mRNA decay and reduced collagen X, the mutant truncated protein produced from mRNA that escapes the mRNA-surveillance-quality process can exert a significant gain-of-function effect, by unleashing the unfolded protein response with deleterious downstream effects on cellular expression and function.

The relative contribution of mRNA-decay-induced haploinsufficiency[14,15] and dominant interference[16] to the MCDS clinical phenotype is currently unknown and will require the analysis of further patient and mouse model cartilage-tissue samples. It is rare for NMD to completely remove the mutant mRNA, and residual levels of 10%–20% mutant mRNA are common for nonsense mutations in other genes.[1] In addition, recent studies on collagen VI nonsense mutations[41,42] have shown that haploinsufficiency of collagen VI can either be asymptomatic or cause Bethlem myopathy, suggesting that different genetic backgrounds, or polymorphisms, can influence the clinical consequences of haploinsufficiency. We suggest that it is likely that the most common outcome of MCDS nonsense mutations is not haploinsufficiency from complete mRNA decay but reduced collagen X resulting from partial decay in combination with the production of some mutant truncated collagen X. This truncated collagen has aberrant folding properties that can dominantly interfere with normal collagen X assembly as well as induce a deleterious cellular unfolded protein response, contributing further to the chondrodysplasia phenotype.

Acknowledgments

Web Resources

The URLs for data presented herein are as follows:

Align, http://www.ebi.ac.uk/emboss/align/
Blast2, http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
ClustalW, http://www.ebi.ac.uk/clustalw/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
University of California Santa Cruz, http://genome.ucsc.edu/

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