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Premature Termination Codon in the Aggrecan Gene of Nanomelia and Its Influence on mRNA Transport and Stability

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Aim. To analyze the influence of the premature termination codon on mRNA transport and stability.

Methods. Chondrocyte mRNA was isolated from homozygous and heterozygous nanomelic 17-days old embryos and examined by RT-PCR analysis. To analyze aggrecan mRNA stability, mRNA synthesis was inhibited with DRB [5,6 dichloro-1-(-D-ribofuranosyl benzimidazole)], a specific inhibitor of RNA polymerase II. Visualization of the aggrecan alleles was performed by *in situ* hybridization.

Results. The level of mutant aggrecan mRNA within the nucleus was equal to that of the control, but no mutant mRNA was observed in the cytoplasm. RT-PCR revealed that the mutant transcript was only detectable in the nucleus, compared with house-keeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene or collagen type II. A restriction site induced by premature termination codon TAA allowed the distinction of normal and mutant transcripts in chondrocytes derived from embryos heterozygous for the nanomelic mutation. After the treatment with DRB, identical decay rates were demonstrated for both transcripts within the heterozygous nucleus. *In situ* hybridization showed no abnormal mRNA accumulation.

Conclusion. This is the first evidence suggesting that the transcript of the mRNA with the premature termination codon within an exon does exit the nucleus.

Key words: *chondrocytes; codon, stop; codon, terminator; collagen type II; mRNA; peptide chain termination; RNA, messenger; translation, genetic*

Nanomelia is an example of chondrodistrophy in chickens, phenotipically characterized by limb deformities, brachyochephalic head, and a parrot-like beak (1). Most of the malformed embryos die between days 18 and 21. Genetically, this disease is due to a recessively inherited premature stop codon (TAA) of the aggrecan mRNA at codon 1513 which is located in the unusually large exon ten (2). The large aggregating chondroitin sulphate proteoglycan of the cartilage-aggrecan is the predominant proteoglycan in cartilage, which has numerous interactions with different components of the extracellular matrix. Therefore, a mutation in the aggrecan gene may cause exacerbated damage to the developing extracellular matrix, which results in such a severe phenotype.

The premature termination codon in the aggre- can gene presumably is directly responsible for this disorder, although it has not yet been shown how it affects the stability or transport of the aggrecan mRNA. Recent experiments confirm that the stop mutation in the aggrecan gene directly lowers the level of the type II collagen, whereas type I collagen and glyceraldehyde-3-phosphate dehydrogen- ase (GAPDH) remain at normal levels (3).

Although a variety of mechanisms have been put forth to explain how a nonsense codon can affect mRNA abundance and transport, it is still unclear how it happens. Some of the studies underline the idea that nonsense codons reduce the amount of mRNA in the nuclear compartment (4,5), i.e., that location of the nonsense mutation on certain genes may play an important role in its mRNA stability. It has been shown that the location of the mutation within the first three quarters of the triosephosphate isomerase gene reduces the half-life of mRNA within the nuclei by more than 20% (6). Recent work with yeast showed that a termination codon can be recognized and the nonsense mediated mRNA pathway can be activated if a specific sequence, called down-stream element, is present 3' of the premature ter-

mination codon (7). Most of the models available today suggest that a mutation that eventually generates premature termination codon results in a reduction of its representative mRNA. However, most authors propose that the reduction of mRNA happens in the nuclear compartment rather than in the cytoplasm. Here we present data which indicate that a mutation in the aggrecan gene resulting in nanomelia that generates a premature termination codon is handled distinctly from a mutation which causes a null allele due to a retained intron.

Material and Methods

Cell Culture

Fertile eggs were obtained from normal White Leghorn breeding stocks and Nanomelic breeding stocks maintained at the Storrs, Agricultural Experimental Station (CT, USA). Eggs were incubated for 17 days, at which time sterna from affected and normal embryos were dissected free of perichondrium and incubated in Ham's F12 medium containing 0.1% collagenase CLS2 (Worthington Biochemical, Lakewood, NJ, USA) for 4 hours. Liberated cells were plated at a density of 10^6 cells/100 mm^2 Petri plate in 9 mL of Ham's F12 medium containing 20% fetal calf serum and incubated at 37°C in humidified atmosphere with 7.5% CO_2.

RNA Isolation

Confluent chondrocytes were scraped from the Petri dishes with a rubber policeman into 10 mL of cold phosphate-buffered saline (PBS) and collected by centrifugation for 3 minutes at 2,000 G in 15 mL of lysis buffer (10 mmol/L Tris pH 7.5, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.25% Triton X-100). The pellet was disrupted by dounce homogenization with a tight-fitting pestle. The suspension was centrifuged for 10 min at 2,200 G and the supernatant was transferred to a 30 mL corex tube (cytoplasmatic extract) containing 2.0 mL of a 10X extraction buffer (10% SDS, 0.10 mol/L Tris pH 7.5, 0.05 mol/L EDTA, 500 µg/mL of proteinase K). The nuclear pellet was re-extracted in 5 mL of lysis buffer with dounce homogenization. Subsequent steps of RNA isolation followed the SDS proteinase K protocol (3).

RT PCR

Two µg of nuclear or cytoplasmic RNA were transcribed by reverse transcription to cDNA using 2 µg of a core protein-specific primer that extended from the codon 2100 (5'GGGTGGGTCTGTGCACGACACCGGGT3') in 50 mmol/L Tris, pH 8.3, 7.0 mmol/L KCl, 3 mmol/L $MgCl_2$ and 10 mmol/L DTT. The mixture was heated at 65°C for 3 min, cooled, and adjusted to contain 500 mmol/L dNTPs and 500 U M-MLV RT (Gibco BRL, Rockville, MD, USA). The incubation lasted for 1 h at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. The sample was dissolved in 20 µL water and 1 µL was used in the PCR reaction. The region encompassing the CS2 repetitive domain (codons 1363-1762) was PCR amplified using oligonucleotides that flank the region (codon 1327: 5'GCCAACAGTTTCACAGGAG; and codon 1735: 5'GCTAACAGCAGGCAGCCCAG).

BsaB1 Restriction

The BsaB1 restriction fragment polymorphism that resulted from the nanomelia mutation was detected in PCR-amplified genomic DNA using the same primers that flanked the CS2 repetitive domain. The product was cut with the BsaB1 restriction enzyme for one hr at 60°C, electro- phoresed in 5% acrylamide, and visualized with ethidium bromide staining.

Determining the Half Life of the Normal and Mutant mRNA

Between 24 to 30 100-mm^2 plates were grown to visual confluence for both control and nanomelic cells. One day prior to harvesting the cells, they were placed in fresh media containing 50 µg/mL of ascorbic acid. Inhibition of mRNA transcription was begun by adjusting the media to 25 mmol/L 5,6 dichloro-1-(-D-ribofuranosyl benzimidazole) (DRB) which blocks Pol II elongation and/or initiation. To assess decay of the two populations of core protein mRNA transcripts from the nucleus of heterozygous nanomelic chondrocytes, 12 sister plates were removed after exposure to DRB for 0, 4.5, and 9 hours. RNA was prepared from the nucleus and cytoplasm, transcribed to cDNA and cut with the BsaB1 restriction enzyme, electrophoresed in 5% acrylamide and visualized with ethidium bromide staining. The densitometry of the normal and the mutant transcripts were performed by Alphalmager™ documentation and analysis system (Alpha Innotec Corporation, San Leandro, CA, USA).

Fluorescent In Situ Hybridization and Immunofluorescence Staining

Cells were prepared for FISH by washing in cytoskeletal buffer (CSK) (100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L $MgCl_2$, 10 mmol/L Pipes, pH 6.8) and incubating for 5 minutes on ice in CSK containing 5% Triton X-100 and 2 mmol/L vanadyl ribonucleoside complex (VRC, Gibco) prior to fixation (8,9). A fixation step in 4% paraformaldehide 1X PBS (pH 7.4) for 8 minutes immediately followed the extraction. Cells were stored in 70% ethanol at 4°C. A probe covering exon 10 was nick translated using biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN, USA). Cells were incubated with the probe mix at 37°C for 3 hours to overnight. The probes were detected by FITC-conjugated avidin (Boehringer Mannheim). A Zeiss Axioplan microscope equipped with a triple-bandpass epifluorescence filter (Chroma Technology, Inc., Brattleboro, VT, USA) was used for 2D sample analysis by both standard and digital imaging microscopy. Digital images were captured with a Photometrics series 200 CCD camera (Photometrics, Ltd., Tucson, AZ, USA) equipped with a custom-made color filter wheel, such that three colors can be captured and viewed simultaneously with no optical shift.

Results

Nanomelic embryos, analyzed 17 days after incubation, showed gross abnormalities of the limbs, legs, head, and beak in comparison to normal embryos (Fig. 1).

In our previous work we demonstrated by RNAse protection assays the decreased level of the aggrecan mRNA in the cytoplasmic compartment in nanomelic chondrocytes. At the same time, collagen type II (mRNA) was present in equal amounts in both the nuclear and cytoplasmic compartments (1). To extend our findings, we performed a more sensitive analysis of the nuclear and cytoplasmic mRNA using RT-PCR. As a control gene in this experiment we used GAPDH since it is not known that any interaction exists between aggrecan and this particular house-keeping gene. Finally, we compared levels of the nuclear and cytoplasmic aggrecan mRNA from normal chondrocytes to that of nanomelic chondrocytes.

As shown in Fig. 2, it is apparent that the aggrecan mRNA is not detectable within the cytoplasmic compartment of the mutant chondrocytes but is present in the nuclear compartment (lanes 4 and 3, respectively). As expected, aggrecan mRNA was present in both nuclear and cytoplasmic compartments of normal chondrocytes

Figure 1. Three 17-day old chicken embryos. The normal embryo is at center flanked by homozygous nanomelic embryos. The body of the nanomelic embryo is extremely short compared with normal littermates, with malformed limbs, wings, and head with a parrot-like beak.

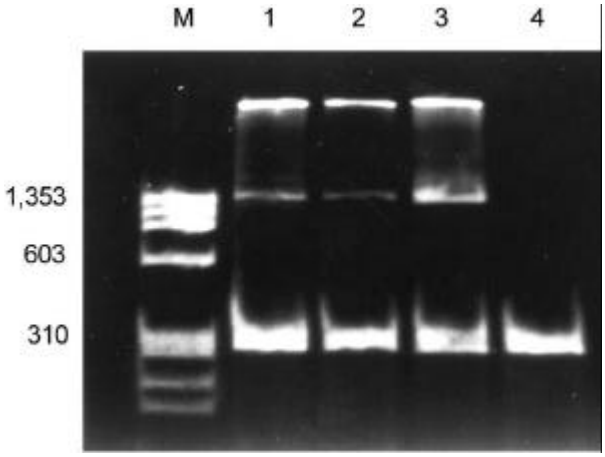

Figure 2. RT-PCR analysis of the mutant nanomelic and normal transcript for the aggrecan and GAPDH gene. M – DNA marker; lanes 1 and 2 – normal nuclear and cytoplasmic mRNA; lanes 3 and 4 – nanomelic nuclear and cytoplasmic mRNA. Aggrecan band runs at about 1.3 kb, and GAPDH runs at 300 bp.

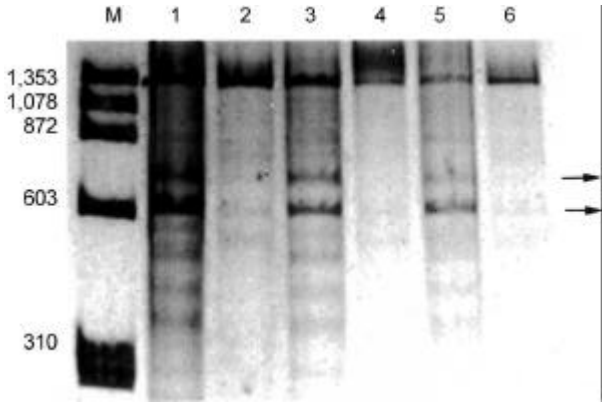

Figure 3. RNA half-life determination. BasB1 restriction digestion of the CS2 fragment obtained by RT-PCR amplification of heterozygous embryous 0 hours after incubation with DRB (lanes 1 and 2), 4.5 hours after incubation with DRB (lines 3 and 4), and 9 hours after incubation with DRB (lanes 5 and 6). Lines 1, 3, and 5 are the mRNA isolated from the nuclear compartment while lines 2, 4, and 6 represent mRNA isolated from the cytoplasmic compartment. Arrows indicate two fragmets that represent mutant mRNA.

(lanes 1 and 2, respectively). The level of the GAPDH remained equal in all samples (lanes 1-4). Failure of the mutant aggrecan transcript to appear in the cytoplasm may result from impaired export to the cytoplasm or to rapid nuclear or cytoplasmic degradation. In order to investigate the mechanism and location of the mutant degradation, we performed half-life experiments by using a specific inhibitor of RNA Polymerase II, DRB (Fig. 3). DRB treatment prevents new mRNA synthesis and permits the monitoring of residue mRNA for both normal and mutant alleles within the nuclear compartment. To analyze both mutant and normal mRNA we performed this study on heterozygous chondrocytes; mRNA was separately isolated from the nuclear and cytoplasmic compartments, cDNA was synthesized and amplified by RT-PCR. Since the TAA mutation generates a unique BasB1 restriction site, it is relatively easy to distinguish the fragments obtained after digestion (the normal uncut fragment is 1.3 Kb; the mutant transcript is cleaved to 700 and 600 bp fragments). By measuring the intensity of the fragments obtained after digestion with BasB1 enzyme, we noticed that the rate of degradation within nuclei for normal and mutant mRNA (shown by the two arrows) was the same even after different lengths of transcription inhibition with DRB (0, 4.5, and 9 hours; lanes 1, 3, and 5, respectively). However, no mutant mRNA was observed in the cytoplasmic compartment 0, 4.5, or 9 hours after incubation with DRB (lanes 2, 4, and 6, respectively). When we plotted the data from the DRB experiment as a graph, no difference in decay rate between the mutant and normal transcripts within nuclear compartment was apparent (Fig. 4). To be sure that the cytoplasmic area of the mutant nanomelic cells completely lacked aggrecan mRNA, we performed *in situ* hybridization, which confirmed its absence in chondrocytes isolated from nanomelic cells (Fig. 5, left).

Discussion

Even though a premature termination codon may result in numerous genetic diseases, it is still not clear how such events are mediated. The mechanisms involved in the recognition and destruction of the mutant mRNA are still unknown. Some suggest the importance of a stabilizer sequence as exists in *Saccharomyces cerevisiae*, which can inactivate nonsense-mediated mRNA decay (10); also emphasized is the importance of a downstream element that is located 3' of the premature stop codon, and whose function is to recognize mutant transcripts as aberrant and promote their rapid decay. Thermann et al (11) have suggested that complications arising from the synthesis of C-terminally truncated polypeptides may be avoided by the activation of "nonsense mediated" decay pathway. They maintain that to destroy a mutant transcript two separate steps are necessary, one of which involves defining the stop codon by the presence of a 3' splice tag, after which the mRNA travels to the cytoplasmic compartment where, during translation, the 3' splice tag is recognized and a mechanism of nonsense-mediated decay is activated (11). In contrast, Gersappe and Pintel (12) described the recognition of the premature termination codon in the nuclear compartment in a parvovirus system.

Nanomelia may be a model system to evaluate the effect of premature termination codon on the transport and stability of the mRNA. It has been shown that a premature termination codon (TAA) at position 1513 on the aggrecan gene appears to be responsible for this lethal disease, where embryos die around day seventeen. Studies previously performed have shown that nanomelic chondrocytes demonstrate the presence of a mutant mRNA in their nucleus but not in the cytoplasm (3). The same study confirmed that the aggrecan transcript shows no alternative splicing. These observations may be of importance in discovering human homology to nanomelia since this syndrome has not yet been recognized.

In eucaryotic cells the most important steps in mRNA processing beside transcription are splicing and transport of mRNA to the cytoplasm where protein syn-

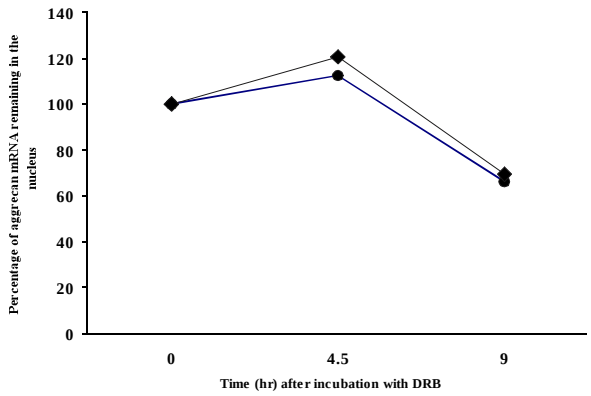

Figure 4. Decay of aggrecan mRNA from the nuclear compartment of the heterozygous nanomelic cells. Rhombs – normal mRNA; circles – mutant mRNA. DRB – 5,6 dichloro-1-(-D-ribofuranosyl benzimidazole), specific inhibitor of RNA polymerase II.

thesis occurs. Transport of the mRNA occurs through large multiprotein structures and that place may play an important role in recognizing a premature stop codon as well. Moreover, it has been shown that some viruses have proteins that are critical for protecting the life cycle of HIV-1 virus by allowing transport for the partially spliced mRNA that encode structural proteins necessary for viral packing (13). This could be a potential mechanism that explains how some not fully processed mRNA can exit the nucleus. Stover and al (14) showed that unspliced COL1A1 mRNA that contains a retained intron does not exit the nucleus at all. Here it was postulated that in the case of a donor mutation U1 snRNA may bind to the intron, preventing mRNA from exiting the nucleus (15). It was suggested that certain splicing factors interact with nuclear structures keeping the unspliced mRNA sequestered within nucleus.

It has been shown recently that nuclear transcripts of cells transfected with the human β-globine gene that are either normal or defective in splicing or 3'-end formation are detectable only intranuc- learly where the transcripts co-localize with the template gene locus (16). These data suggest that effective pre-mRNA splicing is the critical and rate limiting step for the release of the transcript from the site of transcription. Many studies have shown that nonsense or frame shift mutations that create nonsense codon within mRNA result in a reduction of total mRNA and that the reduction takes place within nucleus (17). We were not able to observe these events in our experiments.

Recently we reported on the failure of nano- melic cells to produce mRNA within the cytoplasmic compartment (18). We, therefore, inhibited mRNA synthesis with DRB, which blocks Pol II elongation and/or initiation (18). If the nuclear transport were normal and cytoplasmic degradation rapid we would see equal half lives of both mRNA for the mutant and normal transcript. Our observations were that after 4.5 and 9 hours equal degradation rates and kinetics were present for both normal and mutant alleles. Furthermore, visualization of the aggrecan alleles by in situ hybridization showed no abnormal retention of the mutant mRNA within the nuclear compartment. When similar experiments were performed with mRNA isolated from the patient with osteogenesis imperfecta type I due to a retained intron it was shown that mutant mRNA remains within the nuclear compartment even 9 hours after inhibition with DRB (15). These data suggest distinct mechanisms of degradation and transport of mRNA containing a premature stop codon from that containing a retained intron. Just what these mechanisms are remains to be elucidated.

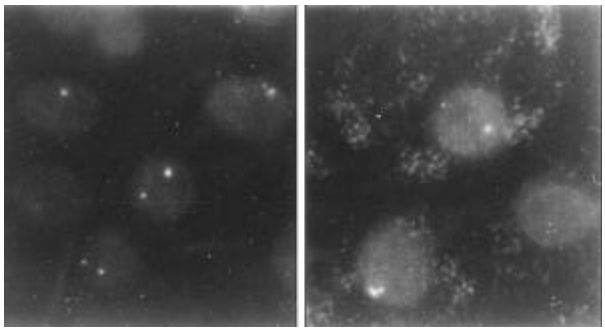

Figure 5. Direct visualization of the core protein mRNA of normal (right) and nanomelic chondrocytes (left) by in situ hybridization. Note the complete absence of aggrecan mRNA within the cytoplasmic compartment of the nanomelic chondrocytes.

Acknowledgments

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