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Regulating Gene Expression through RNA Nuclear Retention

Kannanganattu V. Prasanth,[1] Supriya G. Prasanth,[1] Zhenyu Xuan,[1] Stephen Hearn,[1] Susan M. Freier,[2] C. Frank Bennett,[2] Michael Q. Zhang,[1] and David L. Spector[1,*]
[1]Cold Spring Harbor Laboratory
1 Bungtown Road
Cold Spring Harbor, New York 11724
[2]ISIS Pharmaceuticals
2280 Faraday Avenue
Carlsbad, California 92008

Summary

Multiple mechanisms have evolved to regulate the eukaryotic genome. We have identified CTN-RNA, a mouse tissue-specific ∼8 kb nuclear-retained poly(A)+ RNA that regulates the level of its protein-coding partner. CTN-RNA is transcribed from the protein-coding *m*ouse *c*ationic *a*mino acid *t*ransporter 2 (*mCAT2*) gene through alternative promoter and poly(A) site usage. CTN-RNA is diffusely distributed in nuclei and is also localized to paraspeckles. The 3′UTR of CTN-RNA contains elements for adenosine-to-inosine editing, involved in its nuclear retention. Interestingly, knockdown of CTN-RNA also downregulates *mCAT2* mRNA. Under stress, CTN-RNA is posttranscriptionally cleaved to produce protein-coding *mCAT2* mRNA. Our findings reveal a role of the cell nucleus in harboring RNA molecules that are not immediately needed to produce proteins but whose cytoplasmic presence is rapidly required upon physiologic stress. This mechanism of action highlights an important paradigm for the role of a nuclear-retained stable RNA transcript in regulating gene expression.

Introduction

In addition to harboring the cell's genome, the nucleus contains distinct substructures in the form of specialized domains/compartments that are characterized by the absence of delineating membranes but that contain defining sets of proteins (reviewed, Spector, 2001). A dynamic interplay between these domains and/or their constituents and the genome is thought to foster the efficient progression of gene expression. Gene expression in eukaryotes is carried out through a series of complex processes that includes transcription, pre-mRNA processing, and export of mature mRNA to the cytoplasm for translation. Recent evidence has indicated that these processes are coordinated and can be regulated at each level (reviewed, Maniatis and Reed, 2002). Changes in chromatin structure as well as interactions between *cis*-DNA sequences and *trans*-acting factors are required for the activation or silencing of genes. In addition to constitutive gene expression, transcription of specific genes can be induced in response to cell-cycle stage as well as several stimuli, including stress and hormonal induction. Cotranscriptional gene regulation can occur at the levels of 5′ capping; constitutive and alternative pre-mRNA splicing; polyadenylation; and, in some cases, RNA editing (reviewed, Bass, 2002; Orphanides and Reinberg, 2002). Furthermore, translation can be tightly regulated at either a global level or the level of specific mRNAs (i.e, via miRNAs) (reviewed, Gebauer and Hentze, 2004; He and Hannon, 2004).

The central dogma of molecular biology holds that genetic information normally flows from DNA to RNA to protein. As a consequence, it has been assumed that genes generally encode for proteins and that proteins fulfill not only structural and catalytic but also most regulatory functions in cells. However, a subset of RNAs execute functions without being translated into protein. Such RNAs include housekeeping RNAs (ribosomal RNA, transfer RNA, and uridine-rich small nuclear and nucleolar RNAs) and nuclear-retained RNAs that execute regulatory roles in various aspects of gene expression (*Xist*, *Tsix*, and *Rian* in mammals and *hsr*-ω-*n* and *roXs* in *Drosophila*) (reviewed, Szymanski and Barciszewski, 2003). Interestingly, the percentage of noncoding DNA in prokaryotes is <25% of the total genome compared to ∼98% in humans, most of which contains active transcription units (Mattick, 2004). The increased number of noncoding genes in multicellular organisms is likely to provide more complexity to the transcriptome.

An earlier study indicated that, in many cell types, a considerable fraction of the poly(A)+ RNA (as much as 30%) is nuclear retained and undetectable in the cytoplasm (Herman et al., 1976). More recently, RNA fluorescence in situ hybridization (RNA-FISH) analysis using an oligo dT probe in mammalian cells has shown that a population of poly(A)+ RNA is enriched in nuclear speckles, also known as interchromatin granule clusters (IGCs) (Carter et al., 1991; Huang et al., 1994; Visa et al., 1993). However, IGCs are not transcription sites; rather, they are thought to be involved in the assembly/modification and/or storage of the pre-mRNA processing machinery (reviewed, Lamond and Spector, 2003). The poly(A)+ RNA in the IGCs does not appear to be transported to the cytoplasm (Huang et al., 1994), as would be the case if they represented protein-coding mRNAs, suggesting that this RNA or these RNAs constitute new members of the nuclear regulatory RNA (nrRNA) family.

In the present study, we biochemically purified IGCs and associated nuclear domains from mouse liver nuclei and constructed a cDNA library from the poly(A)+ RNA. One of the cDNA clones localized in a micropunctate nuclear distribution, and, in a population of cells, it also localized to a subnuclear domain, paraspeckles (Fox et al., 2002). Northern blot and genomic sequence analysis revealed that this clone encodes a ∼8 kb nuclear-retained poly(A)+ RNA, transcribed from the *m*ouse *c*ationic *a*mino acid *t*ransporter 2 (*mCAT2*) gene, hence named *C*AT2 *t*ranscribed *n*uclear *RNA* (CTN-

*Correspondence: spector@cshl.org

RNA). The *mCAT2* gene also encodes for the protein-coding *mCAT2* mRNA. However, each transcript predominantly utilizes a different promoter and has unique untranslated regions (UTRs). The 3′UTR of CTN-RNA is essential for its nuclear retention. Interestingly, CTN-RNA is adenosine-to-inosine edited (A-to-I) in its 3′UTR. Depleting CTN-RNA from cells using antisense oligonucleotides resulted in downregulation of both CTN-RNA and *mCAT2* mRNA. Most interestingly, our results demonstrated that, under stress conditions, CTN-RNA gets posttranscriptionally cleaved at its 3′UTR, producing an mRNA containing the mCAT2 coding region and a unique 5′UTR, suggesting that CTN-RNA regulates the levels of its protein-coding partner. This mechanism of action brings to light an important paradigm for the role of a nuclear-retained stable RNA transcript in regulating gene expression.

Results

Characterization of a Nuclear-Retained poly(A)$^+$ RNA

In order to characterize the population of poly(A)$^+$ RNA enriched in nuclear IGCs and associated nuclear domains, we biochemically purified the IGC fraction from mouse liver nuclei (Mintz et al., 1999; Saitoh et al., 2004), isolated poly(A)$^+$ RNA, and constructed a plasmid-based cDNA library. Of the 2×10^3 clones screened, over 1500 clones were sequenced and analyzed by a blast similarity search of a nonredundant database (http://www.ncbi.nlm.nih.gov). Clones which did not show significant similarity to protein-coding genes were further characterized by RNA-FISH in various mouse cell lines. One of the clones, named CTN-RNA (~700 bp in length), showed a diffuse micropunctate nuclear distribution in mouse NIH 3T3 cells (Figure 1A) and was excluded from the nucleoli (see Figures S5B–S5B″ in the Supplemental Data available with this article online). In addition, in about 25%–30% of the cells, CTN-RNA also localized in 10–20 distinct nuclear foci (arrows in Figure 1A). A similar localization pattern of CTN-RNA was also observed in mouse liver tissue sections (Figure 1Ba) and primary mouse embryonic fibroblasts (wt MEFs; Figure S1). Specificity of the RNA-FISH was confirmed by either hybridizing with vector plasmid alone or RNase A pretreatment prior to hybridization (Figure S2). This restricted nucleoplasmic localization of CTN-RNA was reminiscent of the distribution of nuclear regulatory RNAs (reviewed, Szymanski and Barciszewski, 2003), as protein-coding mRNAs generally localize as one to two intranuclear dots (the sites of transcription) and a diffuse nuclear and cytoplasmic distribution.

Northern blot analysis using the partial CTN-RNA cDNA probe (~700 bp) revealed that CTN-RNA encodes a >8 kb transcript that is significantly enriched in mouse liver compared to primary and transformed mouse fibroblast cell lines (Figure 1C and Figure S3). To corroborate the nuclear enrichment of CTN-RNA observed by RNA-FISH, we isolated total RNA from nuclear and cytoplasmic fractions of mouse mammary tumor cells (C127I), which showed elevated levels of expression (Figure S3), and carried out Northern hybridization with the CTN-RNA probe. Such analysis confirmed that CTN-RNA was predominantly present in the nuclear fraction (Figure 1D). Furthermore, mouse tissue Northern analysis revealed that CTN-RNA was highly expressed in mouse liver, brain, and lung and was not detectable in kidney and spleen (Figure 1E). These results clearly indicated that CTN-RNA is a nuclear-localized poly(A)$^+$ transcript with tissue-specific expression.

CTN-RNA Is Transcribed from a Protein-Coding Gene

The complete CTN-RNA cDNA was characterized by RT-PCR using specific RT primers from the 700 bp 3′ end of the partial cDNA initially cloned, as well as by mapping EST clones from the region. The overlapping RT-PCR amplicons spanning the entire CTN-RNA were cloned and sequenced. Complete sequence alignment revealed that CTN-RNA is an ~8 kb transcript and is transcribed from the *mCAT2* gene (*Slc7a2*; chromosome 8A4) (reviewed, MacLeod, 1996). The *mCAT2* gene also encodes for ~4.2 kb protein-coding cytoplasmic *mCAT2* mRNA (Nicholson et al., 2001), whose protein product is involved in the cellular uptake of cationic amino acids (reviewed, MacLeod, 1996). The protein-coding exons of the *mCAT2* mRNA overlap with CTN-RNA, and, in addition, all of the introns are completely spliced from both transcripts (Figure 2A). Northern hybridization using probes from various exons that overlap between CTN-RNA and *mCAT2* mRNA revealed that they hybridize to both CTN-RNA and *mCAT2* mRNA (Figure 2B). However, sequence analysis revealed that *mCAT2* mRNA utilizes the first poly(A) site (AATAAA) in exon 12, whereas CTN-RNA uses the distal poly(A) site ~4.5 kb downstream of the first poly(A) site, thereby resulting in the production of a longer transcript.

The *mCAT2* gene utilizes multiple promoters (A to E, with each having unique exon 1 variants: exons 1A to 1E, comprising the 5′UTRs) in a tissue-specific manner (Finley et al., 1995). RT-PCR analysis revealed that CTN-RNA was exclusively transcribed by the distalmost promoter A (Figure S4; for experimental details, see Supplemental Experimental Procedures), whereas *mCAT2* mRNA predominantly utilized promoter E in liver, C127I, and primary and transformed fibroblasts and promoter D in macrophage cells (Finley et al., 1995 and data not shown). Promoter A, utilized by CTN-RNA, is a TATA-less, CAAT-negative promoter with several high-affinity SP1 and AP1 binding sites and CAC boxes, and, most interestingly, it is located within a CpG island (Figure 2A; see also Finley et al., 1995). Sequence analysis revealed that both promoters A and E contain seven canonical interferon response elements (IREs), consistent with these promoters' being induced as a result of interferon activation (Nicholson et al., 2001 and data not shown). Because of specific promoter usage and poly(A) site selection, the nuclear-retained CTN-RNA has a unique 5′UTR (~145 bp) and 3′UTR (~4.5 kb) and the protein-coding *mCAT2* mRNA has a unique 5′UTR (~483 bp). Therefore, in the case of the *mCAT2* gene, promoter usage is linked to poly(A) site selection.

In order to study the cellular turnover of both CTN-RNA and *mCAT2* mRNA, total RNA was isolated from untreated C127I cells; cells that were incubated with α-amanitin, an RNA polymerase II inhibitor (irreversible

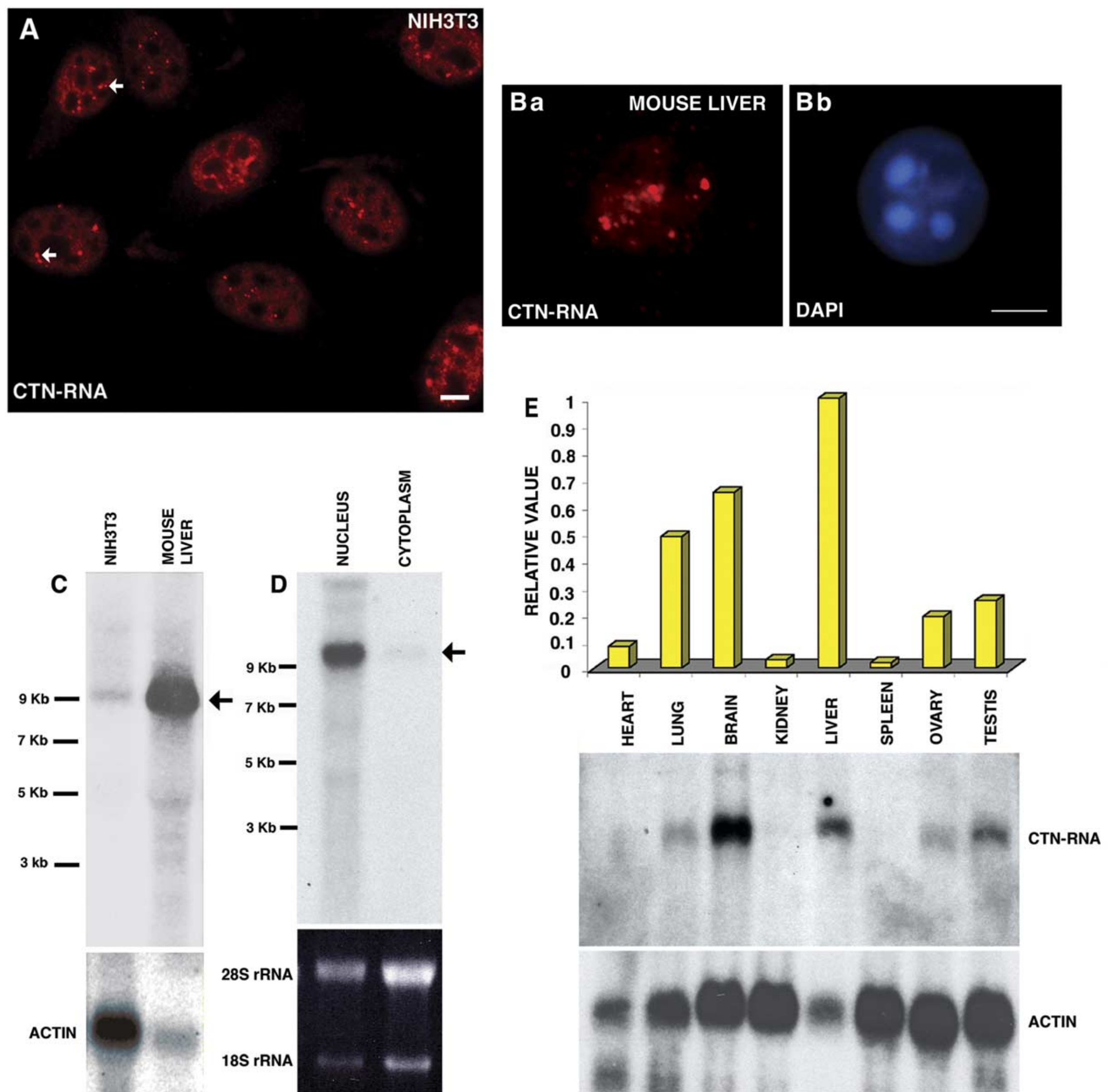


Figure 1. Characterization of CTN-RNA

(A and B) RNA-FISH to NIH 3T3 cells and mouse liver sections showing the diffuse nuclear localization of CTN-RNA and its distribution in 10–20 distinct foci (arrows). Scale bars equal 5 μm.

(C) Northern blot analysis using a CTN-RNA cDNA probe to NIH 3T3 and mouse liver RNA shows that CTN-RNA encodes for a $\sim$8 kb transcript. β-actin was used as loading control.

(D) Northern blot analysis of CTN-RNA in nuclear and cytoplasmic fractions from mouse mammary tumor cells (C127I) revealed that it is enriched in nuclei. Ethidium-bromide-stained gel showing the levels of 28S and 18S rRNA as loading control.

(E) Tissue-specific expression profile of CTN-RNA by Northern blot analysis of various mouse tissues. RNA levels are depicted as a histogram showing the highest level of expression of CTN-RNA in liver, brain, and lung. β-actin was used as loading control.

in tissue culture cells at 50 μg/ml), for 6 hr; and also from cells that were incubated for 3 and 9 hr in culture medium post α-amanitin washout. RT-PCR using transcript-specific primer sets for CTN-RNA and *mCAT2* mRNA (primer sets from the 5′UTR) revealed that CTN-RNA was present in the cells even after prolonged transcription inhibition, suggesting a low turnover (Figure 2C, upper panel). However, the *mCAT2* mRNA transcribed from promoter E completely turned over within 6 hr of transcription inhibition (Figure 2C, lower panel; see also below), demonstrating the differential stability of CTN-RNA and protein-coding *mCAT2* mRNA.

CTN-RNA Localizes to Paraspeckles and Is in a Complex with PSP1α and p54^{nrb}

In order to determine whether CTN-RNA localizes to a known nuclear domain, we performed simultaneous RNA-FISH and immunolocalization using antibodies

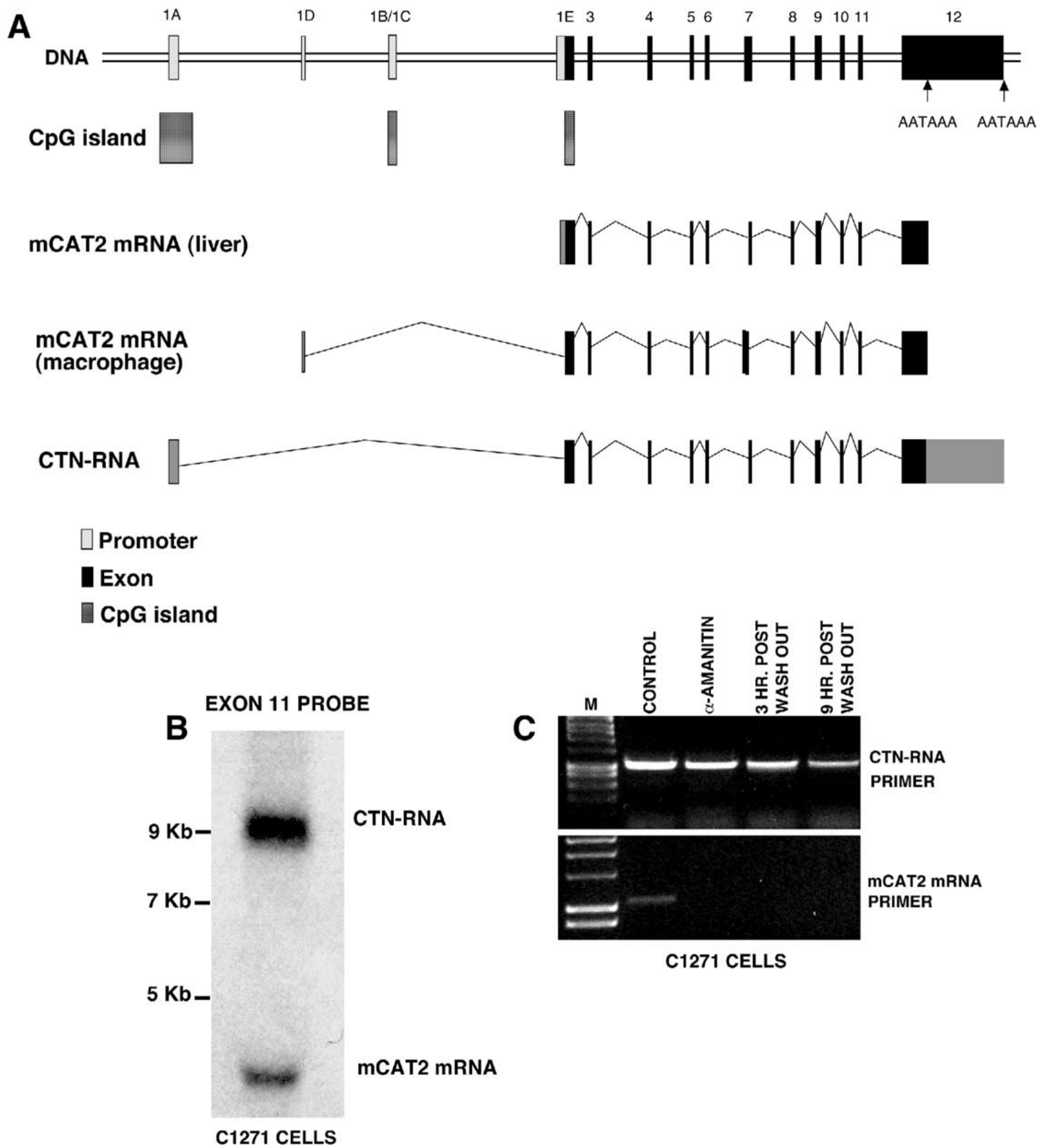


Figure 2. CTN-RNA and *mCAT2* mRNA Are Transcribed from the *mCAT2* Gene

(A) Diagram of the genomic region of the *mCAT2* gene on mouse chromosome 8A4. CTN-RNA is transcribed from the *mCAT2* gene, which also encodes the protein-coding *mCAT2* mRNA. The light gray boxes (1A to 1E) represent the multiple promoters of the *mCAT2* gene, each containing a unique 5′UTR (exon 1). CTN-RNA is transcribed from promoter A, has unique 5′ and 3′UTRs, and utilizes the distalmost poly(A) site (AATAAA). *mCAT2* mRNA is predominantly transcribed from promoter D in macrophages and E in mouse liver and utilizes the proximal poly(A) site. The black boxes represent exons, and the lines between the black boxes depict intronic regions. The hatched boxes represent CpG islands. The dark gray boxes in each of the RNAs represent the unique 5′ and 3′UTRs.

(B) Northern blot analysis of total RNA from C127I cells using an exon 11 probe detects both the ~8 kb CTN-RNA and the ~4.2 kb *mCAT2* mRNA.

(C) CTN-RNA is a stable RNA. cDNA was prepared from RNA of untreated (control) and α-amanitin-treated cells (α-amanitin, 3 hr post washout, and 9 hr post washout; see Experimental Procedures for details). RT-PCR was carried out with specific primers from the 5′UTRs of CTN-RNA or *mCAT2* mRNA.

against protein members of known nuclear domains in wt MEFs (Figure S5). Dual labeling of CTN-RNA (red; Figure S5E) and the nuclear speckle protein SF2/ASF (green; Figure S5E′) revealed that some of the CTN-RNA foci were located at the periphery of the SF2/ASF-positive speckles and showed partial overlap (Figure S5E″; see yellow signal in the higher-magnification inset in Figure S5E‴). This finding is consistent with the fact that our cDNA library was generated from the RNAs of IGCs and associated nuclear compartments.

Recently, Lamond and colleagues identified a nuclear domain, paraspeckles, localized adjacent to nuclear

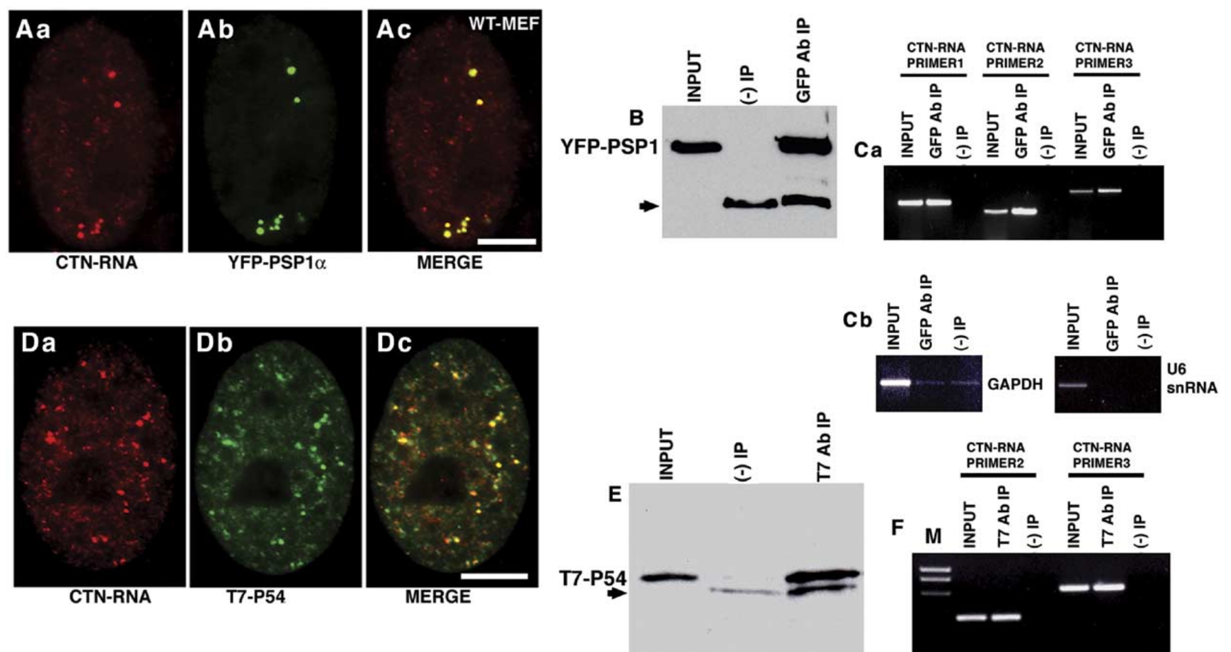


Figure 3. CTN-RNA Localizes to Paraspeckles

(A) RNA-FISH using CTN-RNA probe (red, Aa) in wt MEF cells transiently transfected with YFP-PSP1α (Ab) showed complete colocalization of CTN-RNA and PSP1α in paraspeckles (Ac). Scale bar equals 5 μm.

(B) Immunoprecipitation (IP) from YFP-PSP1α stably expressing NIH 3T3 cells using anti-GFP antibody or anti-HA ((–) IP) antibody. Arrow in (B) and (E) denotes IgG.

(C) RT-PCR from the IP using CTN-RNA-specific primers showed amplification only in the GFP-IP sample and not (–) IP, revealing a specific interaction of CTN-RNA and YFP-PSP1α protein (Ca). RT-PCR using *GAPDH* mRNA-or *U6* snRNA-specific primers from the above IP sample showed no amplification (Cb).

(D) RNA-FISH using CTN-RNA probe (red, Da) in wt MEF cells transiently transfected with T7-p54^{nrb} (green, Db) showed complete colocalization of CTN-RNA and T7-p54^{nrb} in paraspeckles (Dc). Scale bar equals 5 μm.

(E) Immunoprecipitation from NIH 3T3 cells transiently transfected with T7-p54^{nrb} using anti-T7 antibody or anti-HA ((–) IP).

(F) RT-PCR from the IP using CTN-RNA-specific primers showed amplification only in the T7-IP sample and not (–) IP.

speckles (Fox et al., 2002). Paraspeckles range in number from 10 to 20 per nucleus, are present in primary and transformed cells, and contain paraspeckle protein 1 (PSP1α and β), PSP2, p54^{nrb}, and cleavage factor 1m 68 (CF 1m68) (Dettwiler et al., 2004; Fox et al., 2002). All of these proteins possess characteristic *R*NA *r*ecognition *m*otifs (RRMs). Detailed analysis of CTN-RNA localization by RNA-FISH in wt MEF cells expressing YFP-PSP1α showed complete colocalization at paraspeckles (Figures 3Aa–3Ac). Furthermore, by using anti-GFP antibodies, we immunoprecipitated the YFP-PSP1α-containing RNP complex from NIH 3T3 cells stably expressing YFP-PSP1α (Figure 3B). Total RNA was isolated from the immunoprecipitate (IP), and RT-PCR was carried out using CTN-RNA-specific primers (Figure 3Ca). However, YFP-PSP1α did not immunoprecipitate *GAPDH* mRNA or *U6* snRNA, ruling out nonspecific interactions (Figure 3Cb). These results revealed that PSP1α interacts with CTN-RNA. Similarly, p54^{nrb}/NonO also colocalized with CTN-RNA at paraspeckles, as evident by T7 antibody labeling in T7-p54^{nrb} transiently transfected wt MEF cells (Figures 3Da–3Dc). In addition, immunoprecipitation using a T7 antibody from NIH 3T3 cells transiently transfected with T7-p54^{nrb} (Figure 3E) followed by RT-PCR with CTN-RNA-specific primers showed specific interaction of p54^{nrb} and CTN-RNA (Figure 3F). The colocalization, as well as the IP results of PSP1α and p54^{nrb} with CTN-RNA, confirms the interaction of paraspeckle proteins and CTN-RNA. Furthermore, double-label immunogold electron-microscopic analysis of NIH 3T3 cells stably expressing YFP-PSP1α showed that paraspeckles are highly enriched with RNA molecules (Figure S6). In addition, paraspeckles were sensitive to RNase A but not DNase I pretreatment, suggesting a role for RNA in maintaining the structural integrity of paraspeckles (Figures S7 and S8).

CTN-RNA Is A-to-I Edited in Its 3′UTR

RNAs such as *Xist* and *hsr*-ω-*n* as well as the newly identified CTN-RNA are retained in the nucleus even after they are properly processed (reviewed, Szymanski and Barciszewski, 2003 and the present study). However, the mechanism or mechanisms by which these RNAs are retained in the nucleus are not clear. Post-transcriptional modification of adenosine (A) residues to inosine (I) within RNAs, known as A-to-I editing (reviewed, Bass, 2002), has been suggested to be one of the mechanisms of nuclear retention of RNAs. Multiple I residues in the edited RNA have been shown to in-

teract with a protein complex comprised of p54^{nrb}, PSF, and matrin 3, resulting in the retention of this RNP complex in the nucleus (Zhang and Carmichael, 2001). Adenosine deaminase acting on RNA (ADAR) enzymes catalyze the A-to-I editing of RNA and recognize the double-stranded regions of the RNA for their activity (reviewed, Bass, 2002). The finding that p54^{nrb} is present in paraspeckles and is involved in retaining A-to-I edited RNAs in the nucleus and our finding that CTN-RNA interacts with p54^{nrb} raised the possibility that CTN-RNA might be a target for RNA editing. Consistent with this possibility, immunolocalization studies in wt MEFs using an antibody against PSF revealed that it colocalized with YFP-PSP1α in paraspeckles, further strengthening the link between RNA editing and paraspeckles (Figure S9).

Sequence analysis of the entire ~8 kb CTN-RNA transcript revealed that there is a ~100 nucleotide region (forward repeat) in the 3′UTR that is present as three inverted repeats of SINE origin further downstream (Figure 4A). Each of these inverted repeats can form an imperfect stem loop with the forward repeat, which is a characteristic target site for recognition by the ADAR enzymes. Sequence comparison of several mouse liver CTN-RNA cDNA clones with the genomic DNA sequence revealed that inverted repeat 2 and its immediate 3′ region contained 3 A residues that exhibited A-to-I editing. Quite strikingly, the forward repeat, which is present in both *mCAT2* mRNA and CTN-RNA, showed hyperediting only in CTN-RNA (4–9 A residues in various cDNAs; Figure 4B). A-to-I editing in the CTN-RNA 3′UTR and its interaction with p54^{nrb} suggest that editing could be a possible mechanism of nuclear retention of CTN-RNA.

Reporter Analysis of the CTN-RNA 3′UTR

The 3′UTRs of RNAs have been shown to contain sequence elements that regulate RNA localization and stability (Mignone et al., 2002). In order to address the role of the 3′UTR of CTN-RNA in its nuclear retention, we developed an in vivo reporter assay. A mammalian vector containing a plasma-membrane targeting signal fused to cyan fluorescent protein (MEM-CFP) was fused to either the 3′UTR of the *mCAT2* mRNA (MEM-CFP-mCAT2 3′UTR; Figure 5Aa) or the entire 3′UTR of CTN-RNA, which includes the 4.5 kb sequence downstream of the mCAT2 3′UTR (MEM-CFP-CTN-RNA 3′UTR; Figure 5Ab). In the latter construct, we deleted the first poly(A) site so that only the distal poly(A) site of CTN-RNA was utilized. Mouse NIH 3T3 cells were transiently transfected with either the vector alone (MEM-CFP-vector), MEM-CFP-mCAT2 3′UTR, or MEM-CFP-CTN-RNA 3′UTR, and the localization of CFP was monitored 24 hr posttransfection. In the vector control as well as in cells transfected with MEM-CFP-mCAT2 3′UTR, the reporter CFP properly expressed and localized to the plasma membrane (Figures 5B, 5C, and 5E). However, cells transfected with MEM-CFP-CTN-RNA 3′UTR expressed very low levels of CFP protein (Figures 5D and 5E), although CFP mRNA was expressed (Figure 5F), demonstrating that the CTN-RNA 3′UTR contains elements that restrict the translation of CTN-RNA. Surprisingly, in a population of transfected cells, the reporter RNA showed a homogenous nuclear and cytoplasmic localization (data not shown). One of the possibilities is that the overexpression of the reporter construct, driven by the CMV promoter, limits the quantities of factors involved in nuclear RNA retention/stability. Alternatively, the 3′UTR of CTN-RNA is essential but not sufficient for nuclear retention. To substantiate this possibility, the entire ~8 kb CTN-RNA was cloned into a mammalian expression vector and transfected into human IMR-90 cells, and RNA-FISH was carried out to detect CTN-RNA in this heterologous system, taking advantage of the fact that the CTN-RNA probe does not crosshybridize to human cells. RNA-FISH revealed that the entire ~8 kb transcript showed nuclear localization, whereas the construct lacking the CTN-RNA-specific 3′UTR shows entirely cytoplasmic distribution (data not shown). These results suggest that the nuclear retention of CTN-RNA is a consequence of specific folding of the entire RNA as well as A-to-I editing in its 3′UTR.

CTN-RNA Regulates Its Protein-Coding Partner, *mCAT2* mRNA

To address the functional significance of CTN-RNA, we used antisense oligonucleotides to knock down CTN-RNA in mouse mammary tumor (C127I) and macrophage (RAW264.7) cell lines. Several antisense oligonucleotides were designed from the unique CTN-RNA-specific 5′UTR (exon 1A) and 3′UTR (nonoverlapping region with *mCAT2* mRNA 3′UTR) in order to specifically target CTN-RNA. Two independent oligonucleotides, AS109 and AS113 from the CTN-RNA 3′UTR, showed >80% knockdown of CTN-RNA as quantified by Q-PCR using CTN-RNA-specific primers from the 5′UTR (Figure 6A and Figure S10). Control oligonucleotides (sense or scrambled) did not show any knockdown of CTN-RNA (Figure 6A). BrdU incorporation studies did not show any difference between cells exposed to sense or antisense molecules, suggesting a normal cell-cycle progression (Figures S11C and S11D). Although RNase A treatment disrupted paraspeckles, NIH 3T3 cells stably expressing YFP-PSP1α and treated with CTN-RNA antisense oligonucleotides did not show any disorganization of YFP-PSP1α-containing paraspeckles (Figures S11E and S11E′). This result suggests either that the low levels of CTN-RNA retained in the antisense-oligonucleotide-treated cells are sufficient to provide a structural framework to paraspeckles or that CTN-RNA is not a structural RNA and localizes in paraspeckles due to its modification.

Surprisingly, C127I cells that were treated with CTN-RNA-specific antisense oligonucleotides also exhibited knockdown of *mCAT2* mRNA (>50%) as observed by Q-PCR using specific primer pairs from the *mCAT2* mRNA-specific exon 1E 5′UTR (Figure 6B). Similarly, two additional antisense oligonucleotides that were designed from the 5′UTR of CTN-RNA (exon 1A; AS713 and AS716) resulted in the knockdown of CTN-RNA and in addition elicited knockdown of *mCAT2* mRNA (Figure 6C). However, general transcription in cells treated with antisense oligonucleotides to CTN-RNA, as observed by BrUTP incorporation studies (Figures S11A and S11B) as well as by RT-PCR (actin and NOS2; Figure

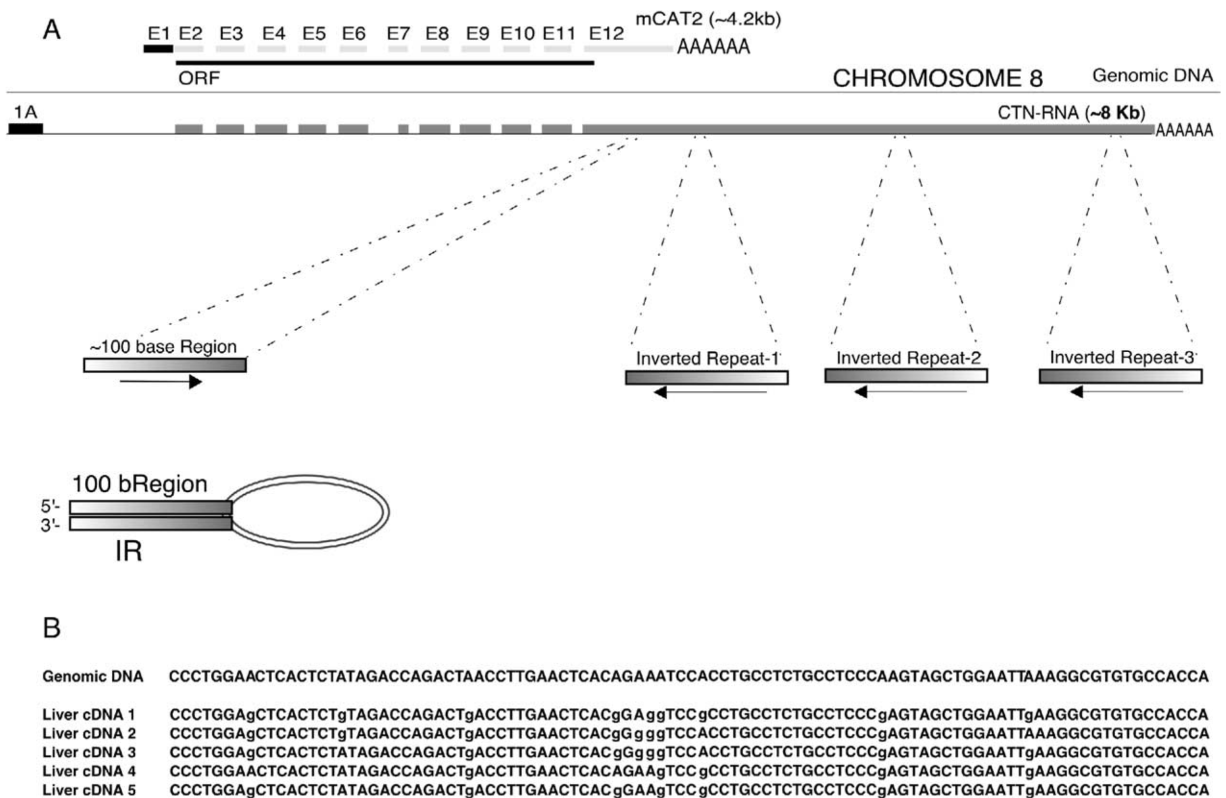


Figure 4. CTN-RNA Is A-to-I Edited

(A) Schematic representation of CTN-RNA and *mCAT2* mRNA. Sequence analysis of CTN-RNA revealed a ∼100 nt fragment in the 3′UTR overlapping the *mCAT2* mRNA and the CTN-RNA, which is present as three inverted repeats of SINE origin further downstream in the 3′UTR unique to CTN-RNA (Inverted Repeats 1 to 3). These inverted repeats can form imperfect stem loops with the ∼100 nt region.

(B) Sequence blast analysis of several cDNA clones of CTN-RNA from mouse liver and their comparison with the genomic DNA sequence revealed multiple residues showing A-to-I editing in the forward repeat. The adenosine (A) in the pre-mRNA is converted to inosine (I), which is structurally similar to guanosine (G), by the ADAR enzyme and, during the first-strand cDNA synthesis, is incorporated as cytosine (C). Subsequently, in the second-strand synthesis, this C complements to form G, which is what is observed in the cDNA instead of A. The edited residues are denoted in lowercase.

S10), was unaffected as compared to cells treated with the sense oligonucleotide. Therefore, the knockdown of CTN-RNA specifically correlates with the loss of the protein-coding *mCAT2* mRNA, suggesting a positive role for CTN-RNA in regulating the stability and/or expression of *mCAT2* mRNA.

To define the specific region of CTN-RNA that regulates the stability of the *mCAT2* mRNA, we have generated several stable cell lines with reporter constructs (MEM-CFP) containing different regions of CTN-RNA under the control of the endogenous CTN-RNA promoter (Figure 6D). In these cells, endogenous CTN-RNA was knocked down using specific antisense oligonucleotides, and the levels of *mCAT2* mRNA were determined by Q-PCR using *mCAT2* mRNA-specific primers. The results showed that cell lines expressing reporter constructs containing the common mCAT2 3′UTR (Figures 6D and 6E; constructs 2, 3, and 4) rescued the *mCAT2* mRNA downregulation, though the endogenous CTN-RNA was efficiently knocked down by the antisense oligonucleotides. These results clearly demonstrate that the ∼1.5 kb 3′UTR region (common to mCAT2 and CTN-RNA) is critical for the stability of the *mCAT2* mRNA.

CTN-RNA Is Posttranscriptionally Cleaved upon Cellular Stress

The mCAT2 protein is a cell-surface receptor involved in the cellular uptake of L-arginine, which is a precursor for the synthesis of nitric oxide (NO). The NO pathway is induced in cells under various stress conditions, including viral infection and wound healing, as a part of a cellular defense mechanism (reviewed, Lowenstein and Padalko, 2004). The relative abundance of nuclear-retained CTN-RNA over *mCAT2* mRNA in unstressed cells suggests a critical role for CTN-RNA in possibly providing a quick cellular response during stress. One model posits that CTN-RNA could serve as an intermediary for the rapid production of mCAT2 protein via posttranscriptional cleavage of the 3′UTR of CTN-RNA. This would release the protein-coding mRNA for transport to the cytoplasm. In situ hybridization using a probe that recognizes both CTN-RNA and *mCAT2* mRNA showed predominant nuclear labeling (Figure 7Aa). When cells were stressed by inhibiting RNA polymerase II transcription with α-amanitin or DRB (data not shown), decreased nuclear and increased cytoplasmic labeling was observed as compared to untreated cells (compare Figures 7Aa and 7Ba). In addition, para-

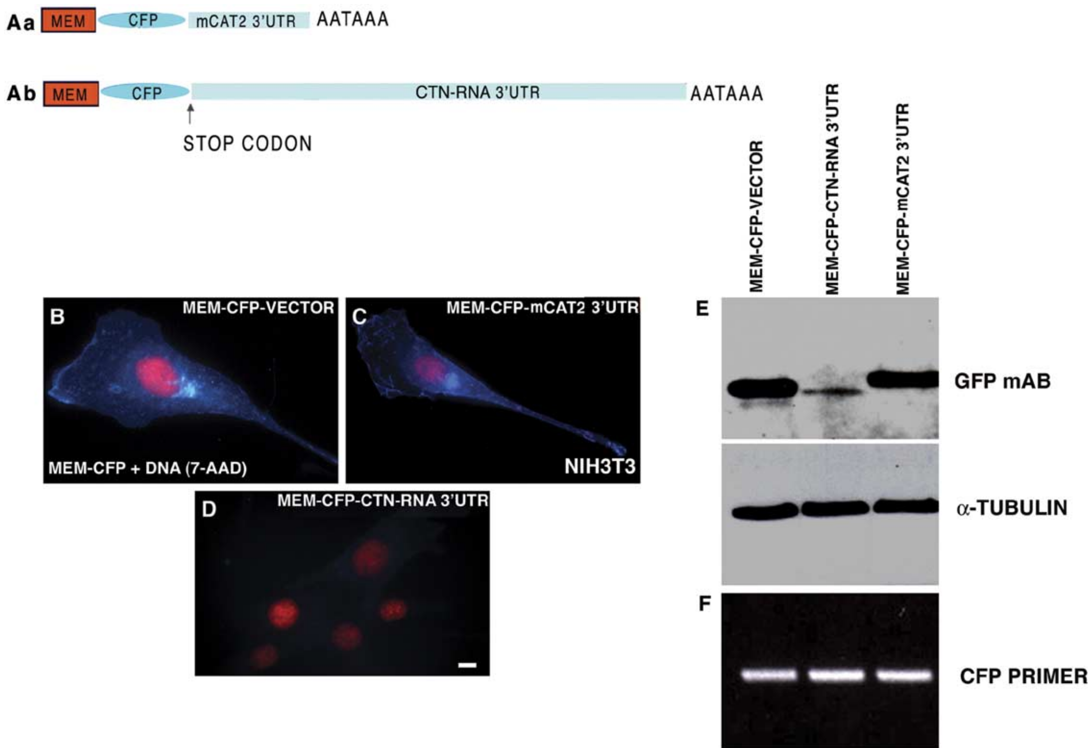


Figure 5. Reporter Analysis Using the CTN-RNA 3′UTR

(A) A mammalian expression vector encoding a membrane targeting signal at the amino terminus of CFP was fused to either the 3′UTR of the *mCAT2* mRNA (Aa) or the 3′UTR of the CTN-RNA (Ab), which includes a ∼4.5 kb sequence downstream of the mCAT2 3′UTR.

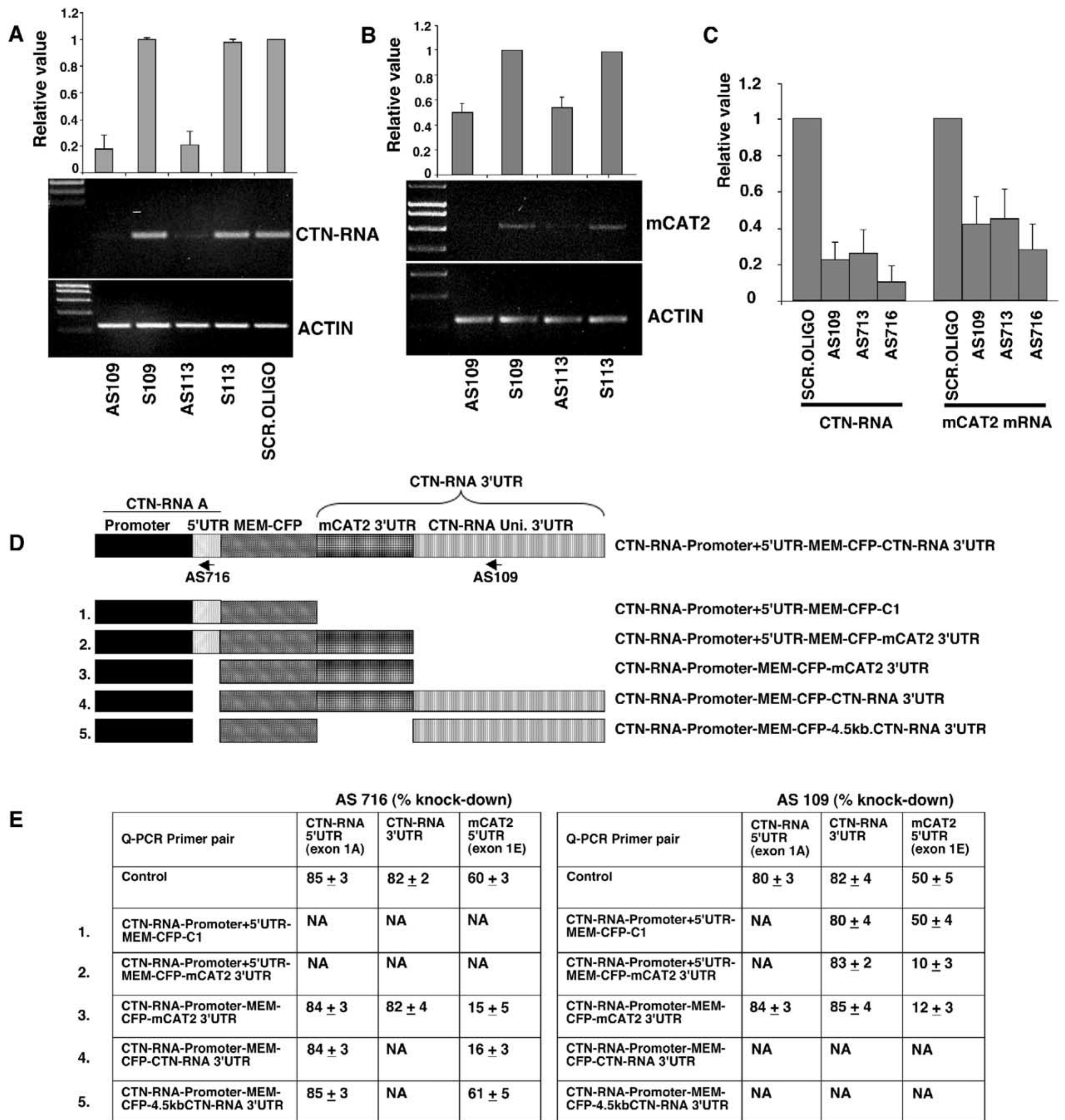
(B–D) Mouse NIH 3T3 cells were transiently transfected with either the MEM-CFP-vector alone (B), MEM-CFP-mCAT2 3′UTR (C), or MEM-CFP-CTN-RNA 3′UTR (D). Expression and localization were monitored 24 hr posttransfection. DNA is stained with 7-AAD. Scale bar equals 5 μm.

(E) Immunoblot analysis using anti-GFP antibody in NIH 3T3 cells transiently transfected (24 hr) with the above constructs. The vector alone and mCAT2 3′UTR-containing constructs showed expression of the GFP reporter, whereas the construct containing the CTN-RNA 3′UTR showed extremely low levels of GFP protein. α-tubulin was used as loading control.

(F) Total RNA was isolated from NIH 3T3 cells transiently transfected with the above plasmid constructs and RT-PCR carried out using CFP primers.

speckle labeling of CTN-RNA was abolished. Addition of IFNγ + LPS post α-amanitin treatment further increased the cytoplasmic labeling, concomitant with reduced nuclear labeling (Figure 7Ca). Northern analysis using an exon probe (hybridizing to both CTN-RNA and *mCAT2* mRNA) further demonstrated that in untreated cells there is ∼2-fold more CTN-RNA than *mCAT2* mRNA (Figure 7D, lane 2). Furthermore, cells treated with α-amanitin followed by IFNγ + LPS showed a significant decrease in the level of ∼8 kb CTN-RNA, with a concomitant increase in the ∼4.2 kb mRNA (Figure 7D, lane 3). Similarly, Northern blot hybridization using a CTN-RNA 5′UTR oligonucleotide probe predominantly hybridized to the ∼8 kb band in unstressed cells (Figure 7E, lane 2). In α-amanitin-treated cells as well as in cells treated with α-amanitin followed by IFNγ + LPS, the CTN-RNA 5′UTR probe now showed an increased hybridization signal at ∼4.2 kb, with a concomitant decrease in ∼8 kb signal (Figure 7E, lanes 1 and 3). Furthermore, the mCAT2-specific 5′UTR probe hybridized to a ∼4.2 kb band only in untreated cells and not in either α-amanitin-treated cells or cells treated with α-amanitin followed by IFNγ + LPS, corroborating the Q-PCR results (Figures 7F and 7G). Finally, Q-PCR analysis to evaluate the levels of CTN-RNA in cells treated with α-amanitin or α-amanitin followed by IFNγ + LPS, using two independent primer pairs from CTN-RNA (5′UTR exon 1A and unique 3′UTR), detected fewer transcripts containing the 3′UTR, corroborating the cleavage of CTN-RNA to release the protein-coding mRNA (Figure 7H). These observations demonstrate that, under stress conditions, the ∼8 kb CTN-RNA is posttranscriptionally cleaved at its 3′UTR, releasing a protein-coding message for transport to the cytoplasm.

In order to address whether the cleaved CTN-RNA product is translation competent, we carried out reporter analysis since there is no mCAT2 antibody available. The CTN-RNA or *mCAT2* mRNA 3′UTRs were cloned downstream of a MEM-CFP reporter, which is under the control of the SV2 promoter. In unstressed cells, the CTN-RNA 3′UTR reporter did not get translated even though it is efficiently transcribed (Figure 8A, lane 3 and Figure S12E). However, upon addition of IFNγ + LPS, reporter protein was detected (Figure 8A, lane 4) and localized to the plasma membrane (Figure S12F). The



	AS 716 (% knock-down)				AS 109 (% knock-down)		
Q-PCR Primer pair	CTN-RNA 5'UTR (exon 1A)	CTN-RNA 3'UTR	mCAT2 5'UTR (exon 1E)	Q-PCR Primer pair	CTN-RNA 5'UTR (exon 1A)	CTN-RNA 3'UTR	mCAT2 5'UTR (exon 1E)
Control	85 ± 3	82 ± 2	60 ± 3	Control	80 ± 3	82 ± 4	50 ± 5
1. CTN-RNA-Promoter+5'UTR-MEM-CFP-C1	NA	NA	NA	CTN-RNA-Promoter+5'UTR-MEM-CFP-C1	NA	80 ± 4	50 ± 4
2. CTN-RNA-Promoter+5'UTR-MEM-CFP-mCAT2 3'UTR	NA	NA	NA	CTN-RNA-Promoter+5'UTR-MEM-CFP-mCAT2 3'UTR	NA	83 ± 2	10 ± 3
3. CTN-RNA-Promoter-MEM-CFP-mCAT2 3'UTR	84 ± 3	82 ± 4	15 ± 5	CTN-RNA-Promoter-MEM-CFP-mCAT2 3'UTR	84 ± 3	85 ± 4	12 ± 3
4. CTN-RNA-Promoter-MEM-CFP-CTN-RNA 3'UTR	84 ± 3	NA	16 ± 3	CTN-RNA-Promoter-MEM-CFP-CTN-RNA 3'UTR	NA	NA	NA
5. CTN-RNA-Promoter-MEM-CFP-4.5kbCTN-RNA 3'UTR	85 ± 3	NA	61 ± 5	CTN-RNA-Promoter-MEM-CFP-4.5kbCTN-RNA 3'UTR	NA	NA	NA

Figure 6. CTN-RNA Regulates Its Protein-Coding Partner, *mCAT2* mRNA

(A) Antisense oligonucleotides designed from the unique 3′UTR of CTN-RNA (AS109 and AS113) were used to knock down CTN-RNA in C127I cells. 80% knockdown of CTN-RNA was achieved as assessed by Q-PCR (see histogram). Control sense oligonucleotides (S109 and S113) or scrambled oligonucleotides (SCR.OLIGO) had no effect. β-actin was used as loading control. CTN-RNA or *mCAT2* mRNA levels were normalized to β-actin mRNA and are presented relative to RNA levels in mock-transfected cells (histograms [A], [B], and [C]). The data in (A)–(C) and (E) are shown as mean and standard deviation values of three measurements per data point.

(B) Antisense oligonucleotide (AS109 and AS113) treatment, specific only to CTN-RNA, showed significant knockdown of *mCAT2* mRNA (~50%) when analyzed by Q-PCR using primers from the unique *mCAT2* mRNA region (exon 1E of the 5′UTR).

(C) Antisense oligonucleotide (AS713 and AS716) treatment specific only to CTN-RNA (5′UTR exon 1A) showed significant knockdown of *mCAT2* mRNA (~50%) when analyzed by Q-PCR.

(D) To define the specific region of CTN-RNA that is important for the stability of the *mCAT2* mRNA, several stable cell lines expressing different regions of CTN-RNA with reporter constructs (MEM-CFP) under the control of the endogenous CTN-RNA promoter were generated (constructs 1–5).

(E) *mCAT2* mRNA downregulation is rescued by the common *mCAT2* mRNA and CTN-RNA 3′UTR. Cell lines containing constructs 1–5 were treated with CTN-RNA-specific antisense oligonucleotides from either 5′UTR (AS716) or 3′UTR (AS109) individually, and the levels of endogenous CTN-RNA and *mCAT2* mRNA were monitored by Q-PCR (see table). Note: AS oligos targeting only the endogenous CTN-RNA were used in each cell line. NA, not applicable.

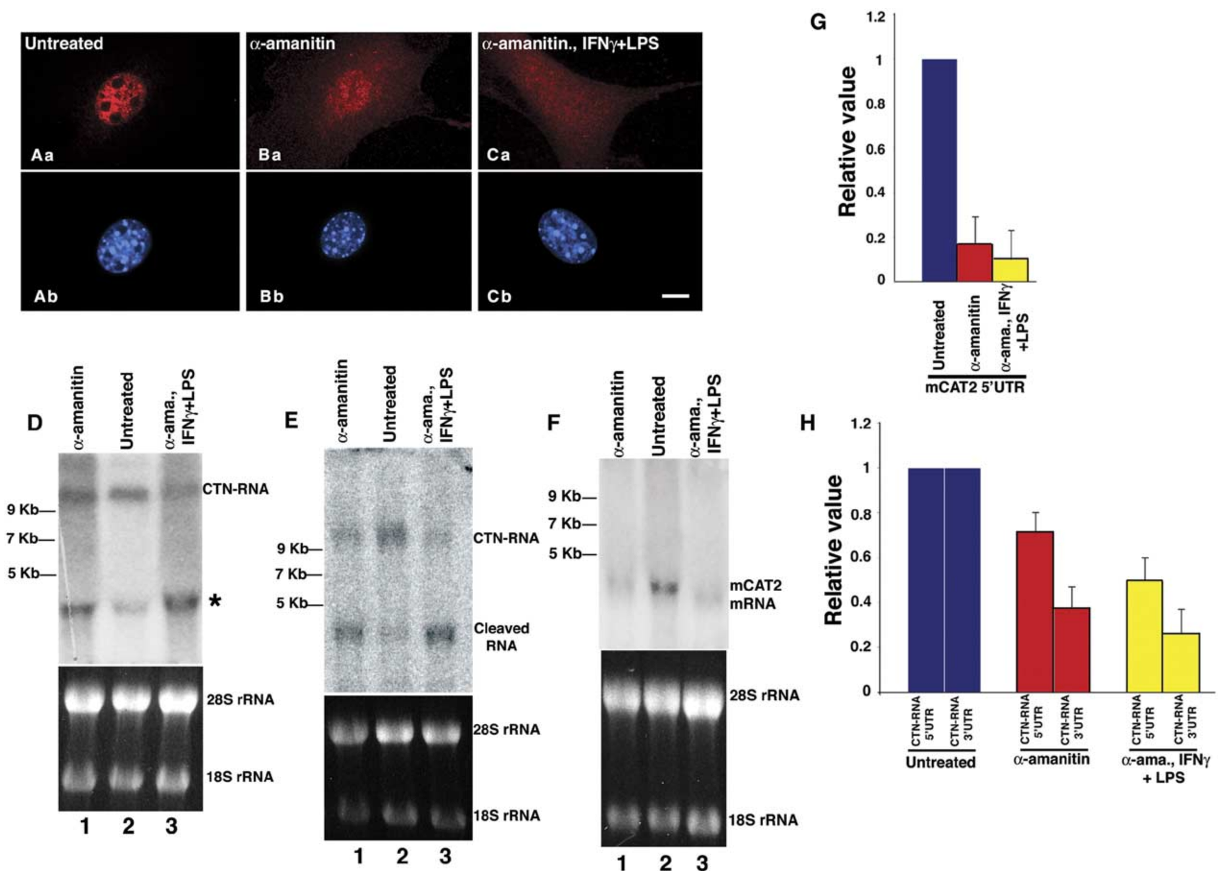


Figure 7. CTN-RNA Is Posttranscriptionally Cleaved to Produce Protein-Coding mRNA in Response to Cellular Stress

(A–C) RNA-FISH to wt MEFs using an exon probe common to CTN-RNA and *mCAT2* mRNA. In untreated cells (A), the RNA signal is predominantly nuclear with some cytoplasmic localization. Upon transcriptional inhibition, the nuclear labeling decreases, concomitant with an increase in the cytoplasmic labeling (compare [A] and [B]). Transcriptional inhibition followed by IFNγ + LPS treatment further decreases the nuclear labeling (C). DNA was stained with DAPI (Ab, Bb, and Cb). Scale bar equals 5 μm.

(D) Northern blot analysis using the common-exon-specific probe revealed that, in untreated cells, the ~8 kb CTN-RNA transcript is in 2-fold excess of the ~4.2 kb *mCAT2* mRNA. Treatment with α-amanitin followed by IFNγ + LPS showed a decrease in CTN-RNA, concomitant with an increase in the ~4.2 kb transcript, the latter being in 2-fold excess as compared to former. Asterisk denotes *mCAT2* mRNA in lane 2 and cleaved ~4.2 kb RNA in lanes 1 and 3. Ethidium-bromide-stained gel showing the levels of 28S and 18S rRNAs as loading control ([D], [E], and [F]).

(E) Northern blot analysis using CTN-RNA 5′UTR-specific oligonucleotide probe (exon 1A) showed that it predominantly hybridized to ~8 kb CTN-RNA in untreated cells (lane 2). Treatment with α-amanitin and α-amanitin followed by IFNγ + LPS showed a dramatic increase in signal at ~4.2 kb, with a concomitant decrease in ~8 kb (compare lanes 1 and 3 with lane 2).

(F) Hybridization using mCAT2 5′UTR-specific probe (exon 1E) showed the presence of *mCAT2* mRNA only in untreated cells (lane 2).

(G) *mCAT2* mRNA levels transcribed from promoter E were analyzed using primers from exon 1E of its 5′UTR and showed high turnover after α-amanitin treatment. The data in (G) and (H) are shown as mean and standard deviation values of three measurements per data point.

(H) CTN-RNA levels were monitored by Q-PCR using primers designed from exon 1A (representing the unique 5′UTR) and the 3′UTR. Fewer CTN-RNA transcripts with an intact 3′UTR were present in cells treated with α-amanitin or α-amanitin followed by IFNγ + LPS.

percentage of cells positive for membrane localization of the reporter also increased after IFNγ + LPS treatment (Figure 8B). These results demonstrate that the reporter RNA containing the CTN-RNA 3′UTR is cleaved in response to IFNγ + LPS and becomes translation competent. Furthermore, since all the reporter constructs are driven by the SV2 promoter, which does not contain any IRE, and since there is no observed increase in translation in constructs containing either MEM-CFP alone (Figure 8A, lanes 1 and 2 and Figures S12A and S12B) or mCAT2 3′UTR (Figure 8A, lanes 5 and 6 and Figures S12C and S12D), the increased translation of the reporter RNA containing the CTN-RNA 3′UTR upon IFNγ + LPS treatment is clearly due to posttranscriptional cleavage.

Discussion

Recent studies from many organisms have indicated that the vast majority of the transcriptional output of the respective genomes does not encode for protein products. These RNA are speculated to play regulatory roles (reviewed, Mattick, 2003; Suzuki and Hayashizaki, 2004), though it has been difficult to determine their mechanism of action (reviewed, Mattick, 2003; Szymanski and Barciszewski, 2003). In the present study,

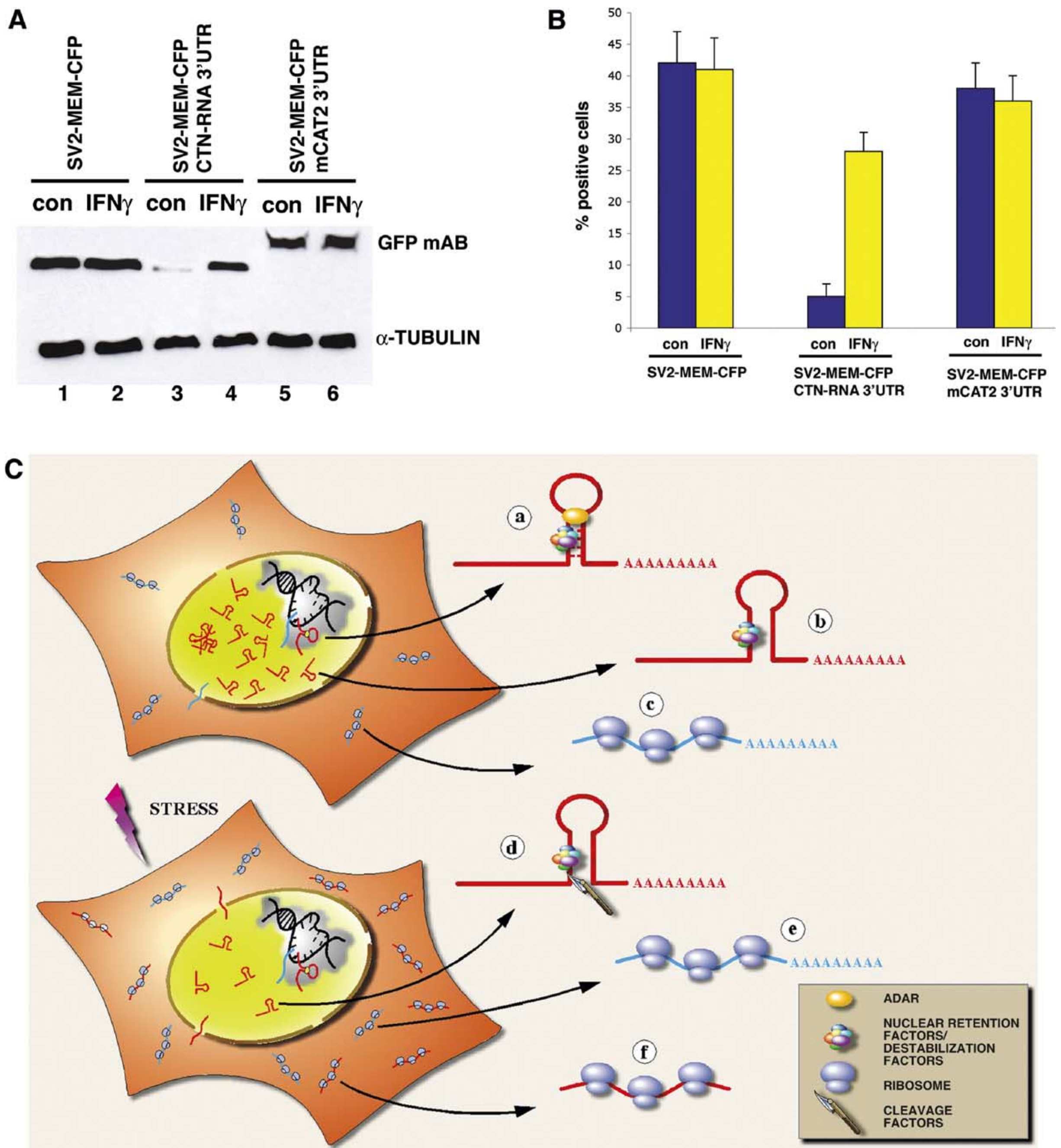


Figure 8. Posttranscriptionally Cleaved CTN-RNA Is Translation Competent

NIH 3T3 cells were transiently transfected with SV2-MEM-CFP, SV2-MEM-CFP-CTN-RNA 3′UTR, and SV2-MEM-CFP-mCAT2 3′UTR. Expression and localization were monitored 24 hr posttransfection in untreated cells and cells treated with IFNγ + LPS for 8 hr.

(A) Immunoblot analysis using anti-GFP antibody in the above assay. The vector alone (lanes 1 and 2) and mCAT2 3′UTR-containing constructs (lanes 5 and 6) showed similar expression levels of the GFP reporter in untreated and IFNγ + LPS-treated cells. The construct containing the CTN-RNA 3′UTR showed extremely low levels of GFP protein in untreated cells, but a significant increase of GFP was observed after IFNγ + LPS treatment (lanes 3 and 4). α-tubulin was used as loading control.

(B) Histogram depicting the percentage of cells positive for membrane-CFP localization in the above assay. Error bars indicate standard errors.

(C) Model of *mCAT2* mRNA regulation by CTN-RNA. In unstressed cells (upper cell), the *mCAT2* gene transcribes CTN-RNA (a and b) and *mCAT2* mRNA (c, blue) through alternative promoter and poly(A) site usage. CTN-RNA is nuclear retained and regulates the level of *mCAT2* mRNA. Upon stress (lower cell), CTN-RNA is posttranscriptionally cleaved in its 3′UTR (d), and the released mRNA (f, red), similar to *mCAT2* mRNA (e, blue), is transported to the cytoplasm and translated into mCAT2 protein. This mechanism provides a rapid response for the production of mCAT2 protein to modulate the cellular uptake of L-arginine for the NO pathway.

we identified CTN-RNA, a nuclear-retained poly(A)+ RNA that is transcribed from the protein-coding *mCAT2* gene. The 3′UTR of CTN-RNA contains elements that retain this RNA in the nucleus, in part through A-to-I editing, thereby inhibiting its translation. Knockdown of CTN-RNA results in the downregulation of both CTN-RNA and *mCAT2* mRNA. Rescue experiments demonstrated that a common region of the 3′UTR of CTN-RNA and *mCAT2* mRNA contains an element or elements that govern *mCAT2* mRNA stability. Most interestingly, upon cellular stress, CTN-RNA is posttranscriptionally cleaved to release a translation-competent mRNA, thereby revealing an important cellular mechanism for the rapid production of protein-coding mRNAs.

CTN-RNA Is a Member of the Nuclear Regulatory RNA (nrRNA) Family

Typically, nascent pre-mRNAs of protein-coding genes are processed at the site of transcription and are transported to the cytoplasm for translation. However, several studies have shown that a significant population of RNAs are retained within the nucleus and are suggested to play structural roles or act as riboregulators (Herman et al., 1976; Huang et al., 1994). The X chromosome-encoded nuclear-retained RNA, *Xist* (reviewed, Plath et al., 2002), and its anti-sense transcript, *Tsix* (Lee et al., 1999), are among the best studied nuclear regulatory RNAs (nrRNAs) in mammalian cells.

Similar to the *mCAT2* locus, the stress-inducible and developmentally regulated *hsr*-ω locus in *Drosophila* is an example of a gene that transcribes two independent transcripts from the same DNA strand (Hogan et al., 1994). A longer transcript, *hsr*-ω-*n* RNA (∼10–14 kb), is an unspliced, polyadenylated nuclear-retained transcript suggested to regulate the intranuclear trafficking of various hnRNPs (Prasanth et al., 2000). A shorter 1.2 kb transcript, *hsr*-ω-*c* RNA, is transcribed from the same promoter as *hsr*-ω-*n* RNA but uses the proximal poly(A) site. The *hsr*-ω-*c* RNA is spliced and polyadenylated and, although it localizes in the cytoplasm, does not encode for a protein (Fini et al., 1989). The nuclear distribution of CTN-RNA suggests that it is a previously unidentified member of the nrRNA family.

The 3′UTR of CTN-RNA Is Essential for Nuclear Retention

The mechanisms underlying nuclear retention of RNA are not well understood. However, A-to-I editing of RNA is postulated to be one such mechanism (Zhang and Carmichael, 2001). Biochemical purification studies using in vitro-transcribed RNAs (containing viral sequences) that are A-to-I edited revealed that they copurify with p54^{nrb}, PSF, and matrin 3 as a complex, and it has been suggested that this p54^{nrb} complex is involved in the nuclear retention of hyperedited RNAs (Zhang and Carmichael, 2001). Sequence comparison of CTN-RNA cDNAs from mouse liver against genomic DNA showed A-to-I editing at multiple residues in the CTN-RNA 3′UTR. The interaction of CTN-RNA with a p54^{nrb}-containing RNP complex further suggests that A-to-I editing of CTN-RNA facilitates its nuclear retention. Our finding that p54^{nrb} and PSF localize to paraspeckles suggests that paraspeckles may serve as a depot for sequestering A-to-I-edited nuclear RNAs. The intense labeling of paraspeckles by colloidal-gold-conjugated RNase T1 (RNase T1 cleaves RNA 5′ to G or I with maximum efficiency; Morse and Bass, 1997) is consistent with the possibility of edited RNAs being present in paraspeckles.

Recently, several studies using computational as well as experimental approaches have shown that more than ∼1700 new candidate human RNAs are potentially A-to-I edited (Athanasiadis et al., 2004; Kim et al., 2004; Levanon et al., 2004; Morse et al., 2002). In most cases, the editing was confined to the noncoding region of the mRNA (5′UTR, 3′UTR, or introns) and the edited structures were moderately repetitive sequences, inverted repeats predominantly of Alu origin. Our description of A-to-I editing in the Alu-like SINE repeats in the 3′UTR of CTN-RNA demonstrates that like in humans, the editing machinery shows a similar preference for repeats in the UTRs of mice. Our results elucidate CTN-RNA as the first endogenous nuclear-retained RNA that is hyperedited in its 3′UTR. This nuclear retention is involved in the regulation of *mCAT2* gene expression, in contrast to previous studies suggesting that hyperedited viral RNAs are retained in the nucleus for degradation (Kumar and Carmichael, 1997; Zhang and Carmichael, 2001). It would be critical to evaluate whether, like CTN-RNA, the above-mentioned edited RNAs are nuclear retained and whether paraspeckles serve as a depot for such RNAs.

Regulation of *mCAT2* mRNA by CTN-RNA

Interestingly, knockdown of CTN-RNA using antisense oligonucleotides designed from the unique 5′ or 3′UTR of CTN-RNA also resulted in the downregulation of mCAT2 transcripts, suggesting that the nuclear-retained CTN-RNA positively regulates the levels of the protein-coding *mCAT2* mRNA. Furthermore, rescue experiments revealed that the 3′UTR sequence common to *mCAT2* mRNA and CTN-RNA was regulating the stability of *mCAT2* mRNA. This regulation may occur through the sequestration of a certain factor or factors that are involved in the stability of *mCAT2* mRNA by this 1.5 kb region of the CTN-RNA 3′UTR. Since *mCAT2* mRNA and CTN-RNA share this region, they could compete for binding of such a factor or factors. Also, it is important to note in this context that CTN-RNA is more abundant than *mCAT2* mRNA in unstressed cells and thus may more efficiently sequester such a factor or factors. Thereby, the knockdown of CTN-RNA may allow access of this factor or these factors to the mCAT2 transcripts, resulting in their degradation. An example of such regulation has been reported for the mouse *Makorin1* gene (Hirotsune et al., 2003). *Makorin1* pseudogene transcripts were proposed to be essential for the stability of *Makorin1* mRNA by providing a means of sequestering destabilizing factors through direct competition. Similar to the *mCAT2* locus, studies using high-density oligonucleotide arrays of human chromosomes 21 and 22 revealed the existence of subpopulations of coordinately regulated coding and noncoding transcripts from the same genomic region (Cawley et al., 2004). Future studies will determine whether these RNAs are regulated in a similar manner and will

elucidate the factor or factors that are involved in the coordinate regulation of these transcripts.

The protein product of *mCAT2* mRNA is a member of the cationic amino acid transporter family of proteins involved in the uptake of extracellular arginine, which is essential for sustained NO production via nitric oxide synthase 2 (NOS2) (Nicholson et al., 2001; Stevens et al., 1996). Overproduction of NO has been shown to play a pivotal role in inflammatory responses (Hierholzer et al., 1998). Persistent activation of NOS2 can lead to the production of toxic levels of NO, resulting in several diseases, including multiple sclerosis, Huntington's disease, and Parkinson's disease (Lee et al., 2003). It was previously reported that interleukin 10, which is an anti-inflammatory cytokine, attenuated the induction of NO by inhibiting *mCAT2* expression (Huang et al., 2002). Furthermore, lack of L-arginine in cells results in the production of superoxide via the NOS2 pathway, resulting in cell death (Rosen et al., 2002). This highlights the importance of cellular uptake of L-arginine as a method of regulating inducible NO biosynthesis. In addition, the tight regulation of the cellular uptake of L-arginine is also governed by the *CAT1* or *Slc7a1* gene (another cationic amino acid transporter). *CAT1* translational control is governed by the activity of an internal ribosomal entry site (IRES) within the mRNA leader (Yaman et al., 2003). *mCAT1* is expressed in tissues in a manner mutually exclusive of *mCAT2*. In cells expressing *mCAT2*, the *mCAT1* transcript is degraded by miRNAs (miR-122), suggesting that a critical balance is required for maintaining cellular homeostasis (Chang et al., 2004). The present study has revealed a cell-autonomous level of regulation whereby CTN-RNA dictates the level of *mCAT2* mRNA, thereby controlling the cellular level of mCAT2 protein.

Posttranscriptional Cleavage of CTN-RNA: A Rapid Response for Gene Expression

We have shown by RNA-FISH, Northern, and Q-PCR analysis a precursor/product relationship between CTN-RNA and *mCAT2* mRNA when cells are subjected to cellular stress. Our data demonstrate that the large excess of the nuclear-retained ~8 kb CTN-RNA may act as a storage form of the transcript until the cell encounters stress, upon which it gets cleaved posttranscriptionally to release a protein-coding mRNA (Figure 8C). As the cleaved transcript would not contain the extended 3′UTR of CTN-RNA, which is involved in nuclear retention and translation inhibition, it can be exported to the cytoplasm and translated to produce the mCAT2 protein. If the response were to start with the initiation of transcription of *mCAT2* upon stress, it would take ~25 min for RNA polymerase II to transcribe the 24.5 kb *mCAT2* gene. The posttranscripional cleavage of CTN-RNA upon stress ensures that the cell can act rapidly by producing mCAT2 protein that is essential for NO production.

Although cleavage and polyadenylation of nascent pre-mRNA is generally believed to commence co-transcriptionally (reviewed, Colgan and Manley, 1997; Proudfoot, 2004), our results suggest that some transcripts such as CTN-RNA can be posttranscriptionally cleaved upon stress. Interestingly, a recent study showed that CF 1m68, involved in the early stages of pre-mRNA cleavage, localizes to paraspeckles, suggesting a possible involvement of paraspeckles in this process (Dettwiler et al., 2004). Though the exact site of cleavage is not defined, based upon the size of the cleaved transcript, it is likely that cleavage occurs in the common 3′UTR. Longer exposure of the Northern blot reveals a smeared signal below the 4.2 kb region in stressed cells, suggesting that there is not a single cleavage point and therefore that cleavage may occur at multiple closely positioned points. In light of our results from unstressed cells, we would predict that cleavage occurs upstream of the potential binding site of a destabilization factor or factors.

The cell nucleus is usually thought of as an active environment involved in processes such as transcription, RNA processing, and DNA replication. We have revealed a novel role of the cell nucleus in harboring RNA molecules that are not immediately needed to produce proteins but whose cytoplasmic presence is rapidly required upon physiologic stress or other cellular signals (Figure 8C). Such a role is analogous to the presence of some transcription factors in the cytoplasm, such as the glucocorticoid receptor, which is rapidly imported into the nucleus to initiate transcription upon a cellular signal (reviewed, Hager et al., 2004). The rapid response mechanism of nuclear RNA release for protein synthesis elucidated in our study may be a general paradigm for the production of some critical regulatory proteins.

Experimental Procedures

cDNA Constructs

Mouse liver IGCs were isolated (Mintz et al., 1999) and poly(A)+ RNA was purified by passing total IGC RNA (isolated using Tri reagent; Molecular Research Center, Inc., Cincinnati) through oligo dT columns (Stratagene, La Jolla, California). A plasmid-based cDNA library was constructed from poly(A)+ RNA using the pBluescript II XR cDNA library kit as per the manufacturer's instructions (Stratagene). The complete CTN-RNA cDNA was identified with the help of RT-PCR (RT primers designed from the original ~700 bp CTN-RNA 3′UTR) as well as by analyzing EST clones upstream of the 700 bp 3′UTR region. Finally, the complete CTN-RNA was cloned and sequenced (NCBI Accesion Number DQ086834). Reporter constructs containing various regions of CTN-RNA were designed as described in Supplemental Experimental Procedures.

Cell Culture and Drug Treatments

wt MEF, C127I, and RAW264.7 cells were grown in DMEM containing high glucose (Invitrogen, Carlsbad, California) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah). NIH 3T3 cells were grown in DMEM plus 10% calf serum. Cells were electroporated using 2 μg of plasmid DNA plus 40 μg of salmon-sperm DNA (Amresco, Solon, Ohio) and were seeded onto acid-washed coverslips and processed for immunofluorescence localization or RNA in situ hybridization (Spector et al., 1998).

To inhibit RNA polymerase II transcription, murine cells were incubated with α-amanitin (50 μg/ml; Sigma, St. Louis) for 6 hr at 37°C.

Antisense-Oligonucleotide Treatment

Synthesis and purification of phosphorothioate-modified oligodeoxynucleotides (AS109, GTTTGCTCGCTAGTCAAAT; AS113, TCT TGGTGATGTACTGCTC; AS713, CAGTGCCCTGGCCGCGCGAC; AS716, CGCAGACAGAAGCTCCCGCC) were performed using an Applied Biosystems 380B automated DNA synthesizer as described

previously (McKay et al., 1999). Oligonucleotides were administered to C127I and RAW264.7 cells using Lipofectamine 2000 reagent as per the manufacturer's instructions (Invitrogen). Optimal Lipofectamine 2000/oligonucleotide ratios were empirically determined. Cells were incubated with a mixture of Lipofectamine 2000 and oligonucleotide in OptiMEM medium (Invitrogen) at 37°C, 5% CO_2. After 5 hr, the transfection mixture was aspirated from the cells and replaced with fresh DMEM plus 10% FBS and incubated at 37°C, 5% CO_2 for 16–18 hr.

To induce *mCAT2* mRNA and CTN-RNA expression, untreated or oligonucleotide-treated cells were incubated with IFNγ (50 units/ml; Sigma) + LPS (100 ng/ml, *Escherichia coli* 055:B5; Sigma) for various time points prior to the harvest of cells for RNA analysis.

RNA Fluorescence In Situ Hybridization

To detect CTN-RNA, cells were rinsed briefly in PBS and then fixed in 2% formaldehyde in PBS (pH 7.4) for 15 min at RT. Cells were permeabilized in PBS containing 0.5% Triton X-100 and 5 mM VRC (New England Biolabs, Inc., Beverly, Massachusetts) on ice for 10 min. Cells were washed in PBS 3× 10 min and rinsed once in 2× SSC prior to hybridization. Hybridization was carried out using nick-translated cDNA probes (nick-translation kit; VYSIS Inc., Downers Grove, Illinois) in a moist chamber at 37°C for 12–16 hr as described earlier (Spector et al., 1998). For colocalization studies, after RNA-FISH, cells were again fixed for 5 min in 2% formaldehyde, and IF and imaging were performed as described elsewhere (Prasanth et al., 2003).

Cellular Fractionation and RNA-Protein-Complex Immunoprecipitation

Nuclear and cytoplasmic fractionation and RNA isolation were described previously (Topisirovic et al., 2003). NIH 3T3 cells transiently transfected with a T7-p54^{nrb} construct or stably expressing YFP-PSP1α were used for immunoprecipitation. Lysates were prepared as described previously (Platani et al., 2000). Following lysate centrifugation, the supernatant was precleared and used for IP using either HA, T7, or GFP antibodies. IP was carried out for 4 hr at 4°C. This was followed by 1 hr incubation with γ bind G Sepharose beads (Amersham Biosciences Corp., Piscataway, New Jersey). Beads were washed five times in buffer B (Platani et al., 2000). Half of the IP material was used for immunoblot analysis and the other half for extraction of RNA from the IP using Tri reagent (Molecular Research Center, Inc.). For RT-PCR, RNA isolated from the IPs was DNase I treated and reverse transcribed using random hexamers, and the resulting material was used for PCR amplification using CTN-RNA-specific primer pairs.

Northern blot hybridization using random-labeled probes and immunoblotting were performed according to previously published procedures (Janicki et al., 2004). Northern hybridization using oligonucleotide probes (exon 1A) was performed as per manufacturer's instructions (Ambion Inc., Austin, Texas).

RT-PCR and Q-PCR

RNA was isolated from cultured cells or mouse tissues using Tri reagent solution according to the manufacturer's instructions (Molecular Research Center, Inc.). For RNA analysis, equivalent amounts of total RNA served as template for cDNA synthesis using reverse transcriptase, followed by PCR using specific primers (Invitrogen).

For real-time quantitative PCR (Q-PCR), total RNA was isolated using Tri reagent, treated with RNase-free DNase I (Invitrogen), and converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, California). Gene-specific TaqMan primer sets were designed using Primer 3 software (sequences are available from the authors upon request). Q-PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 Sequence Detector (Applied Biosystems), and β-actin served as an endogenous normalization control. Sequence Detector software (version 1.7) was utilized for data analysis, and relative fold induction was determined by the comparative threshold cycle method. The data are shown as mean and standard deviation values of three measurements per data point.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and 12 figures and can be found with this article online at http://www.cell.com/cgi/content/full/123/2/249/DC1/.

Acknowledgments

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