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Silencing the Transcriptome's Dark Matter: Mechanisms for Suppressing Translation of Intergenic Transcripts

Review

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Large portions of the genomes of higher eukaryotes are transcribed into RNA molecules that are never destined for translation into proteins. Although some of these transcripts have clearly defined biological roles other than protein coding, most arise from genomic regions devoid of functional genes and many are antisense to regions containing annotated genes. A variety of mechanisms exist to prevent adventitious production of proteins from these transcripts, ranging from degradation within the nucleus to translational silencing in the cytosol.

Evidence is converging from several different directions that leads inescapably to the conclusion that transcripts are produced from at least half of the typical higher eukaryotic genome. A significant portion of this transcriptional "dark matter" (Yamada et al., 2003) arises from genomic sequences that have not been annotated either as protein-coding genes or as genes encoding otherwise functional RNAs. It seems clearly in the best interests of an organism to prevent adventitious translation of open reading frames that may occur randomly in these transcripts. This article summarizes the various mechanisms that are available, both within the nucleus and in the cytosol, to prevent the appearance of unwanted peptides in a cell (Figure 1). If nonsense peptides are produced, they can be degraded (Glickman and Ciechanover, 2002; Goldberg, 2003), but mechanisms of peptide degradation are beyond the scope of the present discussion.

Existence of Dark Matter in the Transcriptome

Prehistory: Heterogeneous Nuclear RNA

Only 1%–2% of the human genome comprises coding sequences, with the remainder consisting of introns and intergenic (i.e., unannotated) regions (Lander et al., 2001; Venter et al., 2001). It has been recognized for over 40 years that a major fraction of transcriptional activity within the nucleus yields a polydisperse array of nuclear RNA molecules (heterogenous nuclear RNA, or hnRNA), much of which never reaches the cytosol to become associated with polyribosomes (Harris, 1963; Hough et al., 1975; Houssais and Attardi, 1966; Penman, 1966; Warner et al., 1966). With the discovery of introns, and in particular the realization that intron sequences can comprise 95% or more of a primary transcript, it seemed that the mystery of hnRNA had been solved. By equating hnRNA with "pre-mRNA," much of the difference in size and complexity between hnRNA and cytoplasmic mRNA apparently could be accounted for by intron sequence, which was spliced out and degraded within the nucleus before export of mature mRNA. More recently, however, high-throughput analysis of the mammalian transcriptome has identified additional components in the compartment that had been experimentally defined as hnRNA.

The Transcriptome after the Millennium

In a major tour de force, the RIKEN cDNA project has provided full-length sequences of 102,801 mouse transcripts (Carninci et al., 2005). Within this set of cDNA clones, one-third (34,030) showed no evidence of protein-coding capacity. These nontranslated transcripts (referred to here as ntRNA) occurred at widely varying abundances (see below), arose from contiguous sequence in the mouse genome, showed evidence of tissue-specific expression, and sometimes resulted from splicing of primary transcripts (Carninci et al., 2005; Numata et al., 2003). Strikingly, well over half of the transcriptional units defined by the RIKEN study, whether within annotated genes or not, showed evidence of opposite strand transcription (Katayama et al., 2005). Similar studies of transcribed sequences from the human genome have led to conclusions comparable to those of the RIKEN study (Chen et al., 2004; Imanishi et al., 2004; Yelin et al., 2003).

A similar picture of the human transcriptome—one of wide-spread transcriptional activity by RNA polymerase II (Pol II) outside annotated genes and frequently in antisense directions within annotated regions—was drawn from high-resolution transcript mapping using tiled microarrays (Bertone et al., 2004; Kampa et al., 2004; Kapranov et al., 2002; Rinn et al., 2003; Schadt et al., 2004). Array technology was taken to five-base pair resolution by a research group at Affymetrix, mapping the locations of poly(A)$^+$ and poly(A)$^-$ transcripts on the two human sex chromosomes and eight autosomes (Cheng et al., 2005). Overall, 31.8% of the detected transcript sequences originated from outside annotated regions, and two-thirds of these were located in the poly(A)$^-$ fraction. From this study, a picture of diversity has emerged—some species of ntRNA are localized exclusively in the nucleus, some are exclusively in the cytoplasm, and some are distributed between both compartments (Cheng et al., 2005). Likewise, some ntRNAs are polyadenylated and some are not.

In summary, a current picture of the mammalian genome depicts a complex, interdigitating network of transcription units that often overlap on opposite strands. This general view would appear to be of broad biological applicability, because similar conclusions have emerged from tiled array studies of the *Arabidopsis* and *Drosophila* genomes (Stolc et al., 2004; Yamada et al., 2003), supplementing the extensive evidence from human and mouse.

Functions of ntRNA

Conflicting Views of ntRNA

One thought is that individual ntRNA species arise as "transcriptional noise" from aberrant firing of weak Pol II promoters scattered throughout the genome. According to this interpretation, there is no obvious reason

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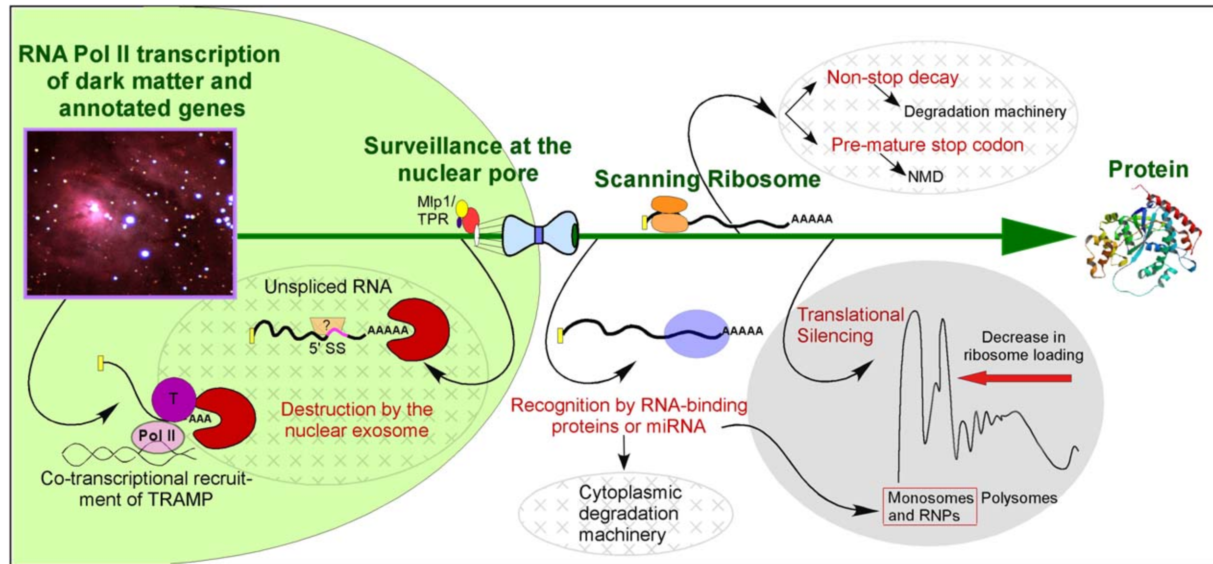

Figure 1. Like the Dark Matter of the Universe, the Dark Matter of the Eukaryotic Transcriptome Is Becoming Better Defined
Several safeguards exist within eukaryotic cells that prevent adventitious expression of protein from transcripts resulting from spurious RNA Pol II initiation while simultaneously providing cells with valuable potential to evolve new functional genomic loci. In this figure, areas marked with X's highlight mechanisms that result in transcript degradation and the area with light gray designates translational silencing.

why these regions should be under selection pressure. However, comparisons of intergenic sequences between *Drosophila* species (Andolfatto, 2005) and between rodents and humans (Bejerano et al., 2004), as well as clear patterns of exclusion of transposable elements from intergenic regions of the human genome (Semon and Duret, 2004), suggest that many of these transcripts may be subject to positive selection and not just neutral, functionless "noise." A hypothesis at the other extreme is that ntRNAs form a network of regulatory molecules (Mattick, 2004). The varied properties of these molecules suggest that they could have multiple biological roles. Many ntRNAs are antisense to annotated genes, and they can be polyadenylated or not, and spliced or not, depending on the particular transcription unit. Some ntRNAs are under developmental regulation, and some are present at levels high enough to be detected on Northern blots.

In considering potential biological roles for intergenic transcription, two classes of ntRNAs can clearly be distinguished. The first class is composed of those ntRNAs whose functions are mediated by the structures of the transcripts themselves. Well-defined examples of this class of transcriptional units include the Pol I and Pol III transcripts as well as the microRNAs, the snRNAs, and the snoRNAs. In contrast, it seems that there may be transcriptional units that exert their biological roles through the physical act of transcription, including assembly of the initiation complex and movement of Pol II along the chromatin. In the latter instance, the act of transcription may alter chromatin conformation or transcription factor binding, with the resulting ntRNA molecule being simply a byproduct of the regulatory process. Examples of these contrasting mechanisms of ntRNA action are discussed in this section.

ntRNAs and Chromatin Structure

One suggested role for the act of intergenic transcription is to provide continued, low-level transcription activity for maintenance of an open chromatin state. Intergenic transcription has been found in the locus control regions of the mammalian β-globin and MHC class II loci (Masternak et al., 2003; Routledge and Proudfoot, 2002), in the promoter regions of the IL-4 and IL-13 genes (Rogan et al., 2004), in the V(D)J region of the mouse immunoglobulin heavy chain locus (Bolland et al., 2004), and within the *Drosophila* bithorax complex (Schmitt et al., 2005). Pol II is found upstream of many genes that seem not to be expressed in stationary-phase *S. cerevisiae* (Radonjic et al., 2005). In addition to perhaps influencing the structure of surrounding chromatin, there are established instances where transcription from intergenic regions is involved in repressing transcriptional initiation at nearby genes, either by local competition between promoters (Hirschman et al., 1988) or through interference by Pol II elongating from an upstream promoter (Martens et al., 2005). With this group of ntRNAs, it is the process of synthesis of the molecule, and not the ntRNA itself, that performs the key regulatory act. Therefore, ntRNAs that are produced in this case are byproducts that must be prevented from spurious association with the translational machinery of the cell. The recent discovery of the accumulation of short transcripts originating from the regions of known promoters in the absence of a nuclear exosome subunit is likely related to this class of ntRNAs (Davis and Ares, 2006).

Some ntRNAs Are Antisense

Perhaps a clue to another function of some ntRNAs is their antisense nature, providing the potential of forming double-stranded RNA (dsRNA) intermediates. The vast majority of bimolecular dsRNA found in cells is formed within the nucleus, and there is no direct evidence that these molecules are exported. The fates and biological functions of these molecules have recently been highlighted in the literature (for review see Wang and Carmichael [2004] and Werner and Berdal [2005]).

Regulatory roles for some dsRNAs in gene silencing and centromere assembly have been suggested to be mediated by the RNAi machinery in the nucleus. Nuclear-localized Dicer, an RNase III endoribonuclease family member, cleaves long dsRNA into short 21–23 nt siRNAs (Bernstein et al., 2001; Haussecker and Proudfoot, 2005) that, together with other components of the RNAi machinery, guide changes in histone and DNA methylation that silence sites of dsRNA transcription, contribute to heterochromatin formation, and participate in sister chromatid separation ([Fukagawa et al., 2004; Morris et al., 2004; Pal-Bhadra et al., 2004; Volpe et al., 2002]; Wang and Carmichael [2004] and references therein).

Long dsRNAs could be subjects of A to I "hyperediting" by specific adenosine deaminases (ADARs), causing partial or complete disruption of the RNA-RNA double helix (Bass and Weintraub, 1988; Polson and Bass, 1994). A conserved protein complex composed of a highly specific I-RNA (RNA containing inosines) binding protein, p54nrb, along with the PSF splicing factor and nuclear matrix protein, matrin3, has been shown to prevent export of these transcripts to the cytoplasm, possibly by facilitating attachment to the nuclear matrix (Kumar and Carmichael, 1997; Zhang and Carmichael, 2001). Interestingly, Wang et al. propose a model that connects dsRNA editing with chromatin silencing pathways through an ADAR interaction with nuclear Vigilin, an abundant RNA binding protein with high affinity for inosines (Huertas et al., 2004; Wang et al., 2005). In an RNA-dependent manner, Vigilin, along with the RHA dsRNA helicase and a Ku70/86 heterodimer involved in DNA break repair, will further recruit and activate DNA-dependent protein kinase, DNA-PKcs, which phosphorylates proteins involved in heterochromatin formation and gene silencing at sites of DNA-damage (Wang et al., 2005).

These two dsRNA metabolizing pathways are most likely in competition with each other (Wang and Carmichael, 2004). Both serve to regulate gene expression and, importantly, both prevent adventitious expression by either nuclear retention of hyperedited strands or dsRNA cleavage by RNAi machinery.

A Regulatory ntRNA

A function for one ntRNA has been identified in an elegant study using RNA interference to interrogate, in cell-based assays, a set of ntRNAs that were chosen because of strong sequence conservation between human and mouse (Willingham et al., 2005). These workers identified eight ntRNAs that elicit phenotypes when eliminated from cells by siRNA. One of these ntRNAs, NRON, is an inhibitor of the regulated transcription factor NFAT. NRON occurs at low abundance, is composed of three exons, is polyadenylated, and shows no evidence of protein-coding capacity. Expression of NRON is tissue specific, both in level and in patterns of alternative splicing. Three proteins, which are involved in the nucleocytoplasmic transport of NFAT, are specifically bound by NRON, suggesting a role for this ntRNA in intracellular trafficking of this regulated transcription factor. These ground-breaking results clearly point to NRON as a model for at least one mode of action of the ntRNAs.

Physical and Functional Silencing of ntRNAs

As mentioned above, many species of ntRNA arise from Pol II transcription from intergenic regions and do not encode known functional proteins. These ntRNAs likely contain randomly occurring open reading frames with potential to compete for translation capacity in the cytosol and be expressed as possibly toxic peptides. However, some ntRNAs possess a biological function (e.g., NRON or micro-RNAs), and therefore, mechanisms must be present to prevent their degradation while allowing delivery to their sites of action. Here, we bring together a consortium of mechanisms that have evolved to operate at multiple levels by either physically degrading inherently nonfunctional transcripts or preventing their translation by means other than degradation. Depending on the transcriptional origin and inherent structure of an ntRNA, one or more of these mechanisms could be responsible for its elimination or translational repression.

Cotranscriptional Recognition and Degradation of ntRNA in the Nucleus

One class of ntRNAs, termed cryptic unstable transcripts (CUTs), was recently discovered to arise from intergenic regions and was found to decay rapidly at the sites of transcription (Wyers et al., 2005). Degradation of CUTs requires the exosome complex, which is composed of an array of 3′ to 5′ exonucleases and is a key contributor to RNA degradation in both the nucleus and the cytoplasm (Hilleren et al., 2001; Mitchell et al., 1997; Mitchell and Tollervey, 2000; Vasudevan and Peltz, 2003). The nuclear exosome contains two nucleus-specific proteins, Rrp6p and Lrp1p, responsible for surveillance in this compartment (reviewed in Vasudevan and Peltz [2005]), and a deletion in *RRP6* resulted in CUT stabilization (Wyers et al., 2005). Nuclear exosome-dependent degradation of CUTs is stimulated by a conserved complex, TRAMP, which is composed of an alternative poly(A) polymerase, Trf4p, and an RNA binding protein, Air1p or Air2p (Kadaba et al., 2004, 2006; LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). The RNA helicase Mtr4p is speculated to link the TRAMP complex with the nuclear exosome through its interactions with both complexes (Houseley and Tollervey, 2006; LaCava et al., 2005).

A CUT-type ntRNA may become accessible to the nuclear exosome as the result of inefficient polyadenylation during 3′ processing of the transcript. For normal pre-mRNA processing, the Pap1p complex cleaves and polyadenylates mRNAs in a highly processive manner, partly due to rapid interaction with the Pab1p poly(A) binding protein, providing resultant protection from the exosome (Brown and Sachs, 1998; Dunn et al., 2005; Wahle, 1995). In contrast, Trf4p (or Trf5p), the inefficient poly(A) polymerase associated with TRAMP, is thought to go through rounds of dissociation and reassociation while polyadenylating its substrates, most likely preventing the length of these poly(A) tails from reaching a length appropriate for Pab1p binding (LaCava et al., 2005; Wyers et al., 2005; Vanacova et al., 2005). It has been proposed, however, that TRAMP may initially recruit and stimulate the nuclear exosome at CUT sites of transcription in a manner that is not dependent on its polyadenylation activity (Wyers et al., 2005; LaCava et al., 2005).

It is not known how TRAMP identifies CUTs. Some evidence suggests target specificity may come from the RNA binding proteins Air1p and Air2p, which are

required by TRAMP (LaCava et al., 2005, Vanacova et al., 2005, Wyers et al., 2005, Houseley and Tollervey, 2006). Although it is known that CUT promoters are not important for their recognition, TRAMP may recognize differences in 3′ processing factors or RNP (ribonucleoprotein) complexes that are formed cotranscriptionally (Wyers et al., 2005). Alternatively, recognition by TRAMP may be through structural features in the RNA molecules, as has been suggested for targets other than CUTs (Vanacova et al., 2005; Kadaba et al., 2006).

Promoter-associated ntRNAs, which are byproducts of transcriptional interference on downstream genes, were recently found to be targets of TRAMP and are therefore classified as CUTs (Davis and Ares, 2006). Interestingly, the TRAMP/nuclear exosome degradation pathway is also utilized in the destruction of Pol II transcripts with defective splicing and lariat introns after RNase III endonucleolytic cleavage, demonstrating this pathway's target versatility (Danin-Kreiselman et al., 2003; Egecioglu et al., 2006).

Surveillance at the Nuclear Pore

If an ntRNA is released from its site of transcription and associates with Pab1p and other factors necessary for the nuclear export process, export is allowed (Hieronymus et al., 2004; Saguez et al., 2005). If the necessary export factors are not recruited to an ntRNA nascent transcript, nuclear degradation most likely occurs in an Rrp6 and nuclear exosome-dependent fashion (Burkard and Butler, 2000; Hilleren et al., 2001; Vasudevan and Peltz, 2003). In addition to the necessity for correct RNP formation, the route and mechanism of nuclear export are dependent on RNA length, suggesting that the nucleus provides an additional obstacle for export of short ntRNAs (Darzacq et al., 2005; Masuyama et al., 2004). If an ntRNA interacts with the splicing machinery, but in a defective manner, it may be retained in the nucleus and degraded. A complex called RES was recently discovered to be important for efficient splicing, and one of the components, Pml1p, promotes retention of unspliced RNAs in the nucleus (Dziembowski et al., 2004). Recent evidence suggests that quality control at the nuclear pore complex is exerted through mRNP interaction with Mlp1p (Tpr in humans), a protein that can detect unspliced transcripts with a wild-type 5′ splice site and prevent export (Casolari and Silver, 2004; Galy et al., 2004). Mlp1p may directly recognize the unprocessed 5′ splice site (5′ SS) or perhaps indirectly through an unknown associated protein (Galy et al., 2004). Surveillance at the nuclear pore may not be stringent, however, as some Pol II transcripts that are not able to assemble a spliceosome, due to a weak or unrecognizable 5′ SS, are exported and degraded by cytoplasmic degradation enzymes (Hilleren and Parker, 2003).

It is becoming clear that there is a competition between degradation mechanisms and those mechanisms for processing functional RNAs (Bousquet-Antonelli et al., 2000; Fasken and Corbett, 2005; Saguez et al., 2005). Some ntRNAs, along with mature mRNAs, need to be exported to the cytoplasm. Within these processing and export pathways is the potential of exporting ntRNAs that could produce harmful proteins, and therefore, degradation pathways in the cytosol are critical for elimination of these transcripts.

Cytoplasmic Degradation Pathways

In the cytosol, several mechanisms are known to be involved in degradation of RNA molecules. RNAs in the cytosolic compartment are degraded via two major processes, usually with deadenylation at the 3′ terminus as the common initiating step (Parker and Song, 2004) (reviewed in Parker and Song [2004]). In one process, deadenylation is followed by removal of the 5′ cap, which in turn allows 5′ to 3′ exonucleolytic decay (Dunckley and Parker, 1999; Hsu and Stevens, 1993; LaGrandeur and Parker, 1998; Wang et al., 2002). These catabolizing enzymes are often found colocalized in foci called processing bodies (P bodies) (Sheth and Parker, 2003), and the transcripts held within P body compartments cannot concurrently associate with translating ribosomes (Brengues et al., 2005; Coller and Parker, 2005; Teixeira et al., 2005). The second major process after deadenylation is 3′ to 5′ decay through action of the cytoplasmic exosome (Araki et al., 2001).

How might an ntRNA become targeted for degradation by one of these cytoplasmic processes? An RNP status that allows access to the ntRNA by degradation machinery could be achieved through transcript features associated with either inappropriate translation or interaction with *trans*-acting factors (Kim et al., 2005; Maquat, 2004; Parker and Song, 2004; Jing et al., 2005; Valencia-Sanchez et al., 2006). Mechanisms known to be utilized by the cell to recognize and target mRNAs for degradation are available to cull out ntRNAs.

Some mechanisms require engagement of the translational machinery, and ntRNAs that harbor a stop codon that is recognized as premature (PTC) could be a target of nonsense-mediated decay (NMD) (Maquat, 2004; Moore, 2005). In mammals, transcripts containing PTCs followed by exon-junction complexes, which are detected in a pioneering round of translation, trigger NMD (Conti and Izaurralde, 2005; Gaba et al., 2005; Maquat, 2004). One would expect, therefore, that only ntRNAs that have been spliced would be detected by this mechanism. Likewise, ntRNAs with a functional initiation codon, but lacking a stop codon, could be detected during the first round of translation by the cytoplasmic nonstop decay pathway (Frischmeyer et al., 2002; van-Hoof et al., 2002). ntRNAs that contain sequence or structural features that cause elongating ribosomes to stall could be subject to no-go decay in which endonucleolytic cleavage is followed by exonucleolytic degradation of both the 5′ and 3′ cleavage products (Doma and Parker, 2006).

Sequence or structural-specific motifs found in ntRNAs might target them for decay or translational repression through interaction with factors such as RNA binding proteins and miRNAs ([Chen et al., 2001; Jing et al., 2005; Kim et al., 2005; Lai et al., 2003; Lykke-Andersen and Wagner, 2005; Stoecklin et al., 2002]; and Valencia-Sanchez et al. [2006] and references therein). These interactions are usually specific; however, computational methods and microarray analysis demonstrate prevalence of these *cis* elements in the genome (Lewis et al., 2005; Lim et al., 2005; Shalgi et al., 2005; Xie et al., 2005), suggesting potential for ntRNA targeting.

Translational Silencing

For some ntRNAs that escape the multiple mechanisms for physical destruction described above, there is an

additional barrier to production of potentially harmful peptides. Each transcript within a transcriptome has its own inherent efficiency of translation (Arava et al., 2003; MacKay et al., 2004; Preiss et al., 2003), which is dictated by elements of primary and secondary structure that either positively or negatively influence translation (Gebauer and Hentze, 2004; Kozak, 2005; Morris and Geballe, 2000). It then follows that, in order to be translated, an ntRNA must possess qualities that allow efficient initiation, elongation, and termination of ribosomes. If instead, it contains impediments to any of these translational processes, a transcript would be, in effect, translationally silenced.

Within the transcriptome of normally growing yeast, there is a class of transcripts that is poorly loaded with ribosomes and implied to be translated weakly, if at all (Law et al., 2005; MacKay et al., 2004). A representative survey of these undertranslated transcripts, from three different regulatory networks, demonstrated that under conditions where transcription from known promoters is suppressed, these "nontranslated" transcripts originate as much as two kilobases upstream in unannotated intergenic regions (Law et al., 2005). The long 5′ leaders of these alternative transcripts are replete with small ORFs, which is at least one explanation for the strong inhibition of ribosome loading (Morris and Geballe, 2000). Depending on one's definition, 5′UTRs extending one kilobase or more into unannotated regions may be categorized as "intergenic transcripts" in experiments using tiled microarrays.

It seems likely that this mechanism for functionally silencing Pol II transcripts at the level of translation occurs more generally than just in yeast. Broad occurrence in nature of alternative 5′UTRs is suggested by estimates that 9%–18% of mammalian transcripts have multiple first exons, consistent with common utilization of multiple promoters (Landry et al., 2003; Tan et al., 2005). Two mammalian transcripts with alternative first exons that have been studied in detail are tissue inhibitor of metalloproteinases (TIMP) and oncogene *mdm2*. With both of these genes, translation of the long transcripts is greatly suppressed due to the presence of upstream open reading frames in the extended 5′ leaders (Jin et al., 2003; Waterhouse et al., 1990). Interestingly, inappropriate promoter use, which eliminates two upstream open reading frames in the long *mdm2* transcript, leads to translational derepression of oncoprotein production with resultant oncogenesis (Brown et al., 1999). It therefore seems possible that the process of "translational silencing" by extended inhibitory 5′UTRs is found commonly across nature, providing an additional mechanism through which undesirable translation of ntRNAs can be prevented.

Summary: The Substratum for Evolution of ntRNAs

Multiple lines of evidence point to the existence of ntRNAs. In light of their structural diversity, the roles of these molecules are probably many and varied. One speculation for the origin of some ntRNAs is that they are "mistakes," generated because the price of absolute accuracy is simply too high in an organism with billions of base pairs of genomic DNA. If this is indeed an accurate explanation for occurrence of some species of ntRNA, their evolution must have been molded by selection pressures to silence sequences whose expression would put the organism at a disadvantage. We have presented in this article a review of the available mechanisms—what we term the "substratum" for evolution of ntRNA sequences—that inhibit inappropriate expression of transcripts as peptides. In acquiring this substratum, cells have evolved efficient mechanisms for recognizing and preventing expression of ntRNAs, while at the same time sparing desirable mRNA transcripts and allowing their translation into functional proteins. This surveillance system exerts quality control both in the nucleus and the cytosol, and these mechanisms are capable of dealing with both poly(A)$^+$ and poly(A)$^-$ ntRNAs. These properties are consistent with what we know about the intracellular distribution and molecular characteristics of the dark matter of the transcriptome (Cheng et al., 2005). It is also of note the many species of silenced ntRNA provide a wealth of raw material for evolution of new functional genomic loci.

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