Under the Tucson sun: A meeting in the desert on mRNA decay

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INTRODUCTION

Scientists from all over the world gathered this past June in Tucson, Arizona, for a FASEB-sponsored meeting organized by George Mackie (Univ. of British Columbia) and Roy Parker (Univ. of Arizona) highlighting new developments in the area of posttranscriptional gene regulation by mRNA degradation. The researchers in attendance represented two biological realms, eubacteria and eukaryotes, and while the molecular players, pathways, and regulation of mRNA turnover in these two phyla are clearly distinct, recurrent themes were unmistakable. Regardless of the organism, data were presented reinforcing our understanding that mRNA degradation in all systems is modulated by *trans*-acting factors, ribonucleolytic or not, in response to *cis*-acting RNA sequences. Furthermore, the adjustment of *trans*-effector activity and the accessibility of the *cis*-acting regulatory sequences is used by both prokaryotes and eukaryotes to provide additional layers of complexity to the process. A role for RNA polyadenylation in destabilizing eukaryotic RNA, reminiscent of the decay of stable RNAs in bacteria, was recently uncovered, and in both organisms, alternative fates for mRNAs engaged in translation were presented. Structural information for ribonucleases and accessory RNA decay proteins is proving to offer insight into catalytic function and possible regulatory mechanisms governing mRNA turnover. Moreover, the characterization of mRNA degradation in new model organisms, both bacterial and eukaryotic, is leading to both the confirmation of existing decay pathways and the discovery of new ones.

mRNA STABILIZATION BY *CIS*-ACTING ELEMENTS: PARALLELS BETWEEN BACTERIA AND EUKARYOTES

The influence of the extreme 5' terminus on protecting mRNA against degradation is well documented for both

bacteria and eukaryotes. In *Escherichia coli*, the 5'-terminal triphosphate acts to stabilize the mRNA from a decay-initiating RNA cleavage event catalyzed by the endoribonuclease, RNase E (Mackie 1998, 2000). RNase E, in fact, displays a strong preference for monophosphorylated RNA substrates, ensuring that once decay of an mRNA is initiated, continued degradation of the 3' cleavage product is preferred over an intact pppRNA. The sensitivity of RNase E to the phosphorylation status of the RNA 5' terminus was presumed to be correlated simply to the relative affinity of the enzyme for monophosphorylated versus triphosphorylated mRNA. Work presented by Joel Belasco (NYU) suggests, rather, that 5' monophosphates serve a more participatory role in mRNA stability by increasing the catalytic efficiency and cleavage site specificity of RNase E and its homolog, RNase G (Jiang and Belasco 2004). Interestingly, both RNases must assemble into their multimeric state for this effect on catalysis, suggesting a model whereby 5'-end binding by one RNase E subunit, for example, can induce conformational changes that, depending on the status of the 5' end of the mRNA, differentially enhance RNA cleavage by a second subunit. The analysis of kinetic parameters for RNase E was greatly facilitated by the development of a continuous, real-time assay using synthetic fluorogenic RNA substrates (Jiang and Belasco 2004). The use of fluorescence resonance energy transfer (FRET) methodology allows for initial rate measurements that occur on a timescale that could not be accessed with conventional biochemical methods.

In eukaryotes, the 7-methyl guanosine cap structure present on the mRNA 5' terminus is inhibitory to 5'-3' exonucleolytic decay catalyzed in yeast by Xrn1p, and, moreover, its removal from the mRNA by the decapping complex, Dcp1/2, often represents the rate-limiting step in mRNA turnover (see Collier and Parker 2004). While the activity of Dcp1/2 is coupled to prior deadenylation of the mRNA, the scavenger decapping enzyme, DcpS, preferentially hydrolyzes cap structures from terminal oligoribonucleotides generated by the exosome-mediated 3'-5' pathway of decay (Liu et al. 2002). Findings presented by Mike Kiledjian (Rutgers) provide an explanation for the observa-

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tion that intact mRNAs are not substrates for DcpS. Kiledjian showed that the affinity and subsequent activity of mammalian DcpS for 5' cap structures is sensitive to the length of the RNA in which the cap is attached (Gu et al. 2004; Liu et al. 2004). DcpS demonstrates 2500-fold greater activity on a capped dinucleotide versus a full-length capped mRNA substrate, and, moreover, the robust activity of DcpS on capped decay intermediates effectively competes for, and hydrolyzes, cap structure in the presence of the cap-binding protein, eIF4E. These findings imply that DcpS activity will rarely act upon and initiate the decay of full-length capped mRNAs. Furthermore, DcpS activity can alleviate the potential titration of eIF4E on mRNA oligoribonucleotides unsuitable for translation. Kiledjian also showed that in HeLa cells, mammalian DcpS is predominantly nuclear, but is also present in the cytoplasm distinct from the foci harboring human Dcp2 (Liu et al. 2004). These data suggest that DcpS may play a catalytic and/or regulatory role in the nucleus, and that the two decapping pathways are likely to occur in different subcellular locations in the cytoplasm.

As important as the 5' end of an mRNA is to its stability, the 3' terminus also plays a vital role in regulating decay through the presence or absence of polyadenylate residues. Marv Wickens (Univ. of Wisconsin, Madison) introduced the *Caenorhabditis elegans* GLD2 protein as a divergent member of the DNA polymerase β nucleotidyl transferase superfamily that demonstrates poly(A)-addition activity (Wang et al. 2002b). In contrast to nuclear poly(A) polymerases, GLD2 is cytoplasmic and heterodimeric, comprised of one catalytic subunit and one RNA-binding protein subunit. In the absence of the KH protein, GLD3, for example, GLD2 demonstrates little poly(A) polymerase activity, suggesting that the modular nature of the complex serves to both stimulate polymerase activity and provide substrate specificity through interaction with various subsets of mRNAs. GLD2 represents a founding member of this new class of poly(A) polymerases that also contains, among others, the yeast Trf4 protein (Kwak et al. 2004, and references within).

Although it is generally accepted that polyadenylation of eukaryotic mRNA serves to stabilize mRNA, the role of polyadenylation in the stability of stable, structured RNA has been relatively unexplored. James Anderson (Marquette) discussed the finding that polyadenylation of aberrant tRNA in yeast is destabilizing, akin to the surveillance pathway for defective tRNA and other stable RNAs in *E. coli* (Li et al. 1998, 2002; Deutscher 2003). Anderson showed that polyadenylation of hypomodified initiator tRNA^{met} in the nucleus by Trf4p generates a substrate for exosome-mediated degradation of the aberrant species (Kadaba et al. 2004). Interestingly, Trf4p-mediated destabilization is specific for aberrant over wild-type tRNA substrates. Although the nature of this specificity is undetermined, abnormal tRNA structure and/or retention of the aberrant species in

the nucleus might offer an explanation for this observation. Phil Mitchell (David Tollervey's laboratory, Wellcome Trust Centre, Univ. of Edinburgh) reported that a complex containing Trf4p stimulated the in vitro activity of the exosome on structured RNA substrates. Importantly, multiple rounds of polyadenylation appeared to be required for complete substrate decay, reminiscent of the action of *E. coli* poly(A) polymerase I (PAP I) in the degradation of structured RNA substrates by the exonucleases, RNase II, and polynucleotide phosphorylase (PNPase) (Coburn and Mackie 1996, 1998).

In bacteria, the destabilizing effect of polyadenylation on structured RNA is due to the creation of a single-stranded RNA "toehold" that provokes exonucleolytic digestion of the substrate by various 3'–5' exonucleases that are otherwise resistant to RNA secondary structure (see Dreyfus and Régner 2002). It has been unclear, however, how PAP I is targeted to its RNA substrate, prior to poly(A) addition. Work described by Sidney Kushner (Univ. of Georgia) and Philippe Régner (IBPC, Paris) suggests that in *E. coli*, the rho-independent transcriptional terminators that exist at the 3' termini of many mRNAs serve as specific polyadenylation signals for PAP I, and that binding of the Sm-related protein, Hfq, to the terminator modifies this substrate specificity. In support of this concept, Kushner showed that in an *hfq-1* strain, poly(A) tails are not detectable after rho-independent terminators (Mohanty and Kushner 2004). Under these conditions, PNPase represents the primary poly(A)/polynucleotide-adding activity in the cell, producing short heteropolymeric additions on RNA decay intermediates. Rho-independent terminators, conversely, are poor substrates for polyadenylation by PNPase. Therefore, in a manner resembling the action of GLD3 in *C. elegans*, Hfq binding provides substrate specificity and enhanced activity for polyadenylation catalyzed by PAP I in *E. coli*. Results described by Régner also suggests that Hfq alters the recognition of RNA by PAP I and activates poly(A) synthesis. However, he concludes that stimulation of poly(A) addition to mRNAs devoid of 3' terminal stable secondary structures by Hfq explains why the fraction of *rpsO* mRNA polyadenylated downstream of the terminator hairpin is more abundant when Hfq is inactive (Le Derout et al. 2003). Interestingly, Régner also demonstrated that, in vitro, Hfq binding to poly(A) tails protects the 3' terminus from exonucleolytic degradation by RNase II and PNPase (Folichon et al. 2003). Together, these findings present a paradoxical function for Hfq in the degradation of mRNA 3' ends that requires further analysis.

To further illustrate the bifunctional nature of a poly(A) tail on RNA stability, Laurie Read (SUNY, Buffalo) described opposing effects for poly(A) tail addition on mitochondrial RNA stability in the parasitic protozoan *Trypanosoma brucei* (Ryan et al. 2003). Read showed that, in vitro, polyadenylation serves to stabilize mitochondrial RNAs that have undergone partial or full editing, while, in contrast, a

poly(A) tail on an unedited RNA is a signal for rapid turnover. These findings suggest multiple levels of control of mitochondrial gene expression by polyadenylation in trypanosomes.

RIBONUCLEASES AND HELICASES GOVERN mRNA STABILITY

Ribonucleases represent the most direct *trans*-acting factors governing mRNA stability in both eukaryotes and prokaryotes. In work presented by Murray Deutscher (Univ. of Miami), the 3' exonuclease, ribonuclease R, can now be added to the growing list of enzymatic activities that aid in the decay of both structured mRNAs (e.g., repeated extragenic palindrome [REP] sequences) and aberrant stable RNA (e.g., defective tRNA precursors) in *E. coli*. In contrast to PNPase or RNase II, RNase R efficiently digests through stable, double-stranded RNA helices *in vitro*, once provided with a single-stranded toehold. Furthermore, this activity does not require ATP or additional factors, such as an RNA helicase, suggesting that RNase R might have the capacity to use energy derived from phosphodiester bond hydrolysis to break intermolecular hydrogen bonds of RNA helices. *In vivo*, deletion of *rnr*, coupled with a conditional mutant allele for the 3' exonuclease PNPase, causes cell lethality at nonpermissive temperatures and results in the stabilization of small rRNA fragments (Cheng and Deutscher 2003). Titration of ribosomal proteins by the stabilized rRNA fragments could be envisioned to compromise ribosome biogenesis and contribute to the observed cell lethality. RNase R was also implicated by A.J. Carpousis (CNRS, Toulouse) in a PAP I-dependent pathway of decay for REP sequences in *E. coli*, and, consistent with its function in degrading structured RNA, Philippe Régnier described a role for RNase R in the poly(A)-dependent degradation of *rpsO* mRNA protected by the stable hairpin of its rho-independent terminator.

In yeast, RNA cleavage by the RNase III homolog, Rnt1p, requires a double-stranded RNA stem-loop structure capped by an AGNN-type tetraloop (Wu et al. 2001). Using whole genome theoretical folding and gene expression profiling in *rnt1Δ* mutant cells, Guillaume Chanfreau (UCLA) described the identification of numerous mRNA substrates for Rnt1p. In addition to playing a role in the regulation and degradation of unspliced pre-mRNAs coding for ribosomal proteins (Danin-Kreiselman et al. 2003), Rnt1p was shown to control the decay of several genes involved in the response to iron starvation. Consistent with a role for Rnt1p in anti-iron toxicity, *rnt1Δ* strains exhibit sensitivity to high concentrations of iron. Further characterization of Rnt1p by Sherif Abou-Elela (Univ. of Sherbrook) demonstrated that, *in vitro*, Rnt1p can bind not only to its characterized dsRNA substrate, but also to dsDNA (Lamontagne et al. 2004). Although cleavage of dsDNA by Rnt1p was not observed *in vitro*, Rnt1p was shown to cleave

DNA/RNA hybrids in yeast extracts, independent of ATP. These data suggest Rnt1p can exhibit RNA-dependent deoxyribonuclease activity, a finding that might spur new antiviral strategies.

In *E. coli* the decay of many, if not most, mRNAs is initiated by an endonucleolytic cleavage catalyzed by RNase E (Kushner 2002). In vertebrates, however, few endonucleases have been identified and, in most cases, their role in initiating RNA decay is unknown (Schoenberg and Chernokalskaya 1997). Notwithstanding, the best characterized protein within this class of enzymes is PMR1, a polysome-associated endonuclease that selectively degrades serum protein mRNAs in *Xenopus* liver following estrogen stimulation (Pastori et al. 1991a,b). It has remained unclear, however, how PMR1 is targeted and restricted to specific mRNA substrates. To facilitate the characterization of PMR1 targeting, Daniel Schoenberg (Ohio State Univ.) described a Cos-1 cell system that accurately recapitulates PMR1-mediated mRNA decay. Using this system, protein domains important for targeting of PMR1 to polysomes were shown to be necessary for the association of PMR1 with its substrate mRNA (i.e., albumin mRNA), and, in agreement with this, albumin mRNA is stabilized in PMR1 polysome targeting mutants. Furthermore, target mRNA blocked for translation does not become associated with PMR1 and is prevented from being rapidly degraded. These findings suggest a model whereby PMR1 is recruited to its polysome-associated target through interaction with an RNP specific to the mRNA (Yang and Schoenberg 2004). Consistent with an association between PMR1 and actively translating mRNA, PMR1 is found distributed throughout the cytoplasm and does not colocalize with human Dcp1 protein in Cos-1 cells (Yang and Schoenberg 2004).

Of significant interest to those in the field of RNA interference (RNAi) is the isolation and characterization of the enzymatic activity that mediates siRNA interaction with its cognate target and subsequent mRNA cleavage. Eric Sontheimer (Northwestern) described the use of native gel electrophoresis to analyze complexes from *Drosophila* that mediate RNAi. Exogenous short interfering RNAs (siRNAs) were shown to initially associate with the RNase III homolog, Dicer-2, in an RNAi initiator complex that cofractionates with dsRNA processing activity. The initiator complex acts as a precursor to a very large (~80S) RISC effector complex that also contains Dicer-2 and siRNA, indicating that Dicer-2 does not pass newly processed siRNAs to a distinct complex, but rather plays a role beyond the initiation phase of RNAi. The 80S complex can bind and cleave targeted mRNAs in a cognate siRNA-dependent manner, and is being considered a holoRISC/RNAi effector complex (Pham et al. 2004). Frédéric Souret (Pamela Green's laboratory, Univ. of Delaware) showed that in *Arabidopsis*, 3' products of miRNA-mediated mRNA cleavage can be substrates for XRN4, one of three plant homologs of the yeast Xrn1 protein (Souret et al. 2004). The cytoplasmically lo-

calized AtXRN4 plays a clear role in the stability of 3' cleavage products generated by the miRNA-directed cleavage of several mRNA targets, but, curiously, it manifests no growth phenotype when deleted in plants. This work implicates an Xrn1 homolog in mRNA degradation in a multicellular eukaryote, and denotes a new role for the 5'-3' exonuclease in the miRNA-mediated pathway of mRNA decay.

The role for RNA helicases as *trans*-acting regulators in multiple RNA-related processes, including mRNA turnover, is critical. For example, A.J. Carpousis provided the first evidence that the nonessential *E. coli* helicase, RhlB, is required for mRNA decay in vivo (Khemici and Carpousis 2004). RhlB was shown to enable the degradation of highly structured REP sequences by PNPase. Interestingly, this activity requires the association of both proteins with RNase E as components of the RNA degradosome. Therefore, in addition to initiating mRNA turnover through endonucleolytic cleavage, the multienzyme degradosome complex is also involved in the 3'-5' decay of structured mRNA fragments. Work was also presented by Carpousis suggesting that RhlB plays a role in initiating mRNA degradation through facilitating endonucleolytic cleavage by RNase E, presumably by unwinding RNA structure to expose cleavage sites. Two additional *E. coli* DEAD-box RNA helicases, SrmB and CsdA, were shown by Marc Dreyfus (CNRS, Paris) to be involved in assembly of the 50S ribosomal subunit (Charollais et al. 2003, 2004). In vitro experiments assessing the substrate specificity and mechanisms of the helicases indicate that they unwind duplexes with either 5' or 3' single-stranded RNA extensions, but that they demonstrate low processivity. An interpretation of this work is that helicases work in a highly ATP-consuming, direct pulling apart of the RNA strands, and not in an inch-worm type mechanism as previously hypothesized (Bizebard et al. 2004).

TRANS-ACTING FACTORS MODULATE THE STABILITY OF mRNA SUBPOPULATIONS

Some *trans*-acting factors demonstrate regulatory specificity in *cis* by interacting with and modulating the stability of a subset of mRNAs, while, conversely, others network directly with the ribonucleases that mediate mRNA degradation. In an example of the latter, the *E. coli* protein RraA was described by Stanley Cohen (Stanford) to interact with and inhibit RNase E endoribonuclease activity (Lee et al. 2003; Monzingo et al. 2003). RraA does not interact detectably with RNA substrates, but was shown by microarray profiling of *rraAΔ* cells to modulate the stability of numerous mRNAs (~80) at least twofold (Lee et al. 2003). Interestingly, inhibition of RNase E activity by RraA does not require the RNase E carboxy-terminal scaffold region important in formation of the RNA degradosome; however, RraA binding is enhanced by the carboxy-terminal half of RNase

E. Cohen also presented evidence for a second regulatory protein, RraB, which also modulates the catalytic function of RNase E.

In eukaryotes, PUF proteins interact directly with specific RNA targets through sequence motifs within the 3' UTR to regulate both translation status and mRNA stability (for review, see Wickens et al. 2002). Findings were presented by two speakers describing progress in characterizing the interaction between the conserved structural motifs of the PUF protein family and the specific RNA sequences found within target mRNAs. Marv Wickens discussed the elucidation of consensus sequences for the binding of several yeast PUF proteins. Additionally, the specificity of RNA binding was shown to be inherent in the structure of the conserved PUF repeat domains as demonstrated by the hybrid binding specificity of chimeric proteins generated by swapping of PUF repeat elements between two *C. elegans* PUF proteins. Wendy Olivas (Univ. of Missouri, St. Louis) furthered this discussion by detailing both the identification of Puf3p consensus binding sites on COX17 mRNA (Jackson et al. 2004) and, more appreciably, the identification and confirmation of mRNA targets for the previously uncharacterized Puf1 and 2 proteins of yeast. An exciting outcome of her work was the demonstration that Puf proteins in yeast can either stimulate mRNA decay (through accelerated deadenylation and/or decapping) or stabilize target mRNAs.

AU-rich element (ARE)-mediated decay represents a classic example of how *trans*-acting regulators influence degradation of a subset of mRNAs (for review, see Brennan and Steitz 2001). Perry Blackshear (NIEHS, North Carolina) described the deletion of the ARE-binding protein, tristetraprolin (TTP), in mice. TTP-deficient mice develop a systemic inflammatory syndrome as a consequence of enhanced secretion of TNF α and GM-CSF, the products of two ARE-containing mRNAs. TTP has previously been shown to stimulate the deadenylation of these messages (Lai et al. 2003), and consistent with this, it was shown that the enhanced secretion of TNF α in the mice was due to stabilization of its mRNA. Interestingly, deletion of a different TTP-family member (i.e., Zfp36L1/CMG-1) resulted in chorioallantoic fusion defects and embryonic lethality in the mice (Stumpo et al. 2004). The distinct phenotypes observed for the knock-out mice highlights that these two homologous proteins have distinct subsets of RNA substrates, and that in the case of Zfp36L1, at least one of these substrates is critical for proper embryonic development. Following up on this concept, Heidy Hau (Paul Bohjanen's laboratory, Univ. of Minnesota) showed that different ARE sequences exhibit different affinities for TTP and HuR, an ARE-binding protein whose interaction with mRNA results in stabilization rather than destabilization (Raghavan et al. 2001). Importantly, the relative binding affinities of these proteins to the AREs were shown to correlate with transcript fate. Since both HuR and TTP are induced upon

stimulation of T cells, these findings suggest that the RNA-binding affinities, relative abundances, and the opposing effects of these proteins on mRNA stability all contribute to differential degradation rates of ARE-containing mRNAs following T cell activation.

Paul Anderson (Harvard Medical School) added to the complexity of ARE-mediated mRNA decay by showing that phosphorylation of TTP by MAPKAP kinase-2 (MK2) upon T cell activation serves to abrogate TTP-dependent degradation of ARE-containing mRNA (Stoecklin et al. 2004). Moreover, inhibition of TTP activity upon phosphorylation was determined to result from binding of TTP to the regulatory adaptor protein 14-3-3. Interestingly, TTP:14-3-3 complex formation excludes TTP from stress granules (SGs), the dynamic cytoplasmic foci which harbor stalled translation initiation complexes in conditions of environmental stress (see Kedersha and Anderson 2002), suggesting that the decision to degrade ARE-containing RNA may occur, to some degree, in SGs (Anderson et al. 2004; Stoecklin et al. 2004). Of note is the observation that under conditions of stress, SGs juxtapose with P-bodies, the cytoplasmic foci where mRNA decay can occur (Sheth and Parker 2003; Cougot et al. 2004), and, moreover, overexpressed levels of TTP are found colocalized with P-bodies (Stoecklin et al. 2004). The relationship between TTP-mediated mRNA decay, the recycling of translational arrested mRNA complexes from SGs, and localized mRNA turnover in P-bodies is fascinating, and will certainly be the focus of further experimentation.

In a manner reminiscent of the ARE-binding proteins, the Sm-like eubacterial RNA chaperone, Hfq, plays a role in RNA stabilization through the binding of AU-rich, single-stranded RNA in bacteria. Udo Blasi (Univ. of Vienna) has previously shown that Hfq can mediate the rapid degradation of the *E. coli ompA* mRNA by binding to sequences in the 5' UTR, leading to structural rearrangements involving the ribosome binding site (Vytvytska et al. 2000). Here, Blasi showed that Hfq plays a protective role through binding of AU-rich sequences within the RyhB and DsrA small regulatory RNAs (sRNAs) which overlap with RNase E recognition sites (Moll et al. 2003). The stabilization of sRNAs by Hfq further influences mRNA stability through modulating sRNA availability to its cognate mRNA target. For example, stabilization of RyhB by Hfq leads to increased interaction of the sRNA with its target, *sodB* mRNA, that results in functional inactivation and enhanced decay of the mRNA (Vecerek et al. 2003). Interestingly, Hfq levels are growth-rate controlled and are in abundance in *E. coli* during slow growth (Vytvytska et al. 1998), suggesting that the regulation of mRNA levels of the primary and/or secondary RNA targets of Hfq is important for adaptation to limiting growth conditions.

In *Arabidopsis*, microarray experiments indicate that select mRNAs containing the *cis*-acting "downstream" (DST) element (Perez-Amador et al. 2001) or mRNAs identified as

unstable (Gutierrez et al. 2002) are regulated temporally by the circadian clock. In her presentation, Pam Green (Univ. of Delaware) established that circadian regulation efficiently controls the differential stability of certain mRNAs harboring the DST instability determinant at different times throughout the day (Gutierrez et al. 2002). Consistent with this, *dst1* mutants are deficient in circadian clock-regulated decay of *CCL* mRNA (a unique subclass of DST-containing mRNA) and, interestingly, are also defective in circadian-regulated leaf movement.

In some situations, the RNA targets of *trans*-acting regulators of decay are not manifested as simple primary sequence recognition elements. Such is the case for mRNA substrates of the *trans*-acting regulator of nonsense-mediated decay (NMD), UPF1. In an effort to identify natural mRNA substrates of NMD, Hal Dietz (HHMI, Johns Hopkins) and Andreas Kulozik (Univ. of Heidelberg) presented microarray data from human cell lines depleted of UPF1 (hUPF1). Expression profiles from the Dietz laboratory revealed the upregulation of various subsets of RNAs, which, not unexpectedly, all manifest a spliced intron downstream of the termination codon. This architecture is consistent with the proposed role of an exon junction complex (EJC) in promoting NMD through an interaction with Upf proteins (see Singh and Lykke-Andersen 2003). These studies also identified an increase in expression of genes involved in amino acid metabolism and transport, suggesting that under conditions of amino acid starvation, inhibition of translation and, consequently, inhibition of NMD preferentially increases the expression of mRNAs that promote restoration of amino acid homeostasis. Dietz also discussed that while UPF1 is essential for embryonic viability in mice (Medghalchi et al. 2001), mice expressing a dominant-negative UPF1 allele are viable, and show impaired T-cell maturation, which correlates temporally with the stabilization of nonsense-containing TCR β mRNA generated by nonproductive rearrangements. Together these findings suggest that the NMD pathway may act as a molecular capacitor by restraining phenotypic variation under normal conditions and releasing this variation when functionally compromised.

The present model for NMD in mammalian cells suggests a linear recruitment of UPF3, UPF2, and UPF1 to the RNA by EJC components (see Singh and Lykke-Andersen 2003). It was, therefore, anticipated that RNA substrates which increased in abundance in hUPF1-depleted cells would also show increased expression in hUPF2- and hUPF3b-ablated cells. Kulozik presented data showing, in contrast, that only a subset of the hUPF1-modulated mRNAs respond to the depletion of hUPF2 (while the data gathered from the deletion of hUPF3b was inconclusive). These findings are in opposition to data presented by Dietz showing that transcripts regulated by UPF1 are also regulated by UPF2 and UPF3. It is noteworthy that both groups have analyzed only a small number of UPF1 targets and, therefore, further

studies will be required to understand the basis for this contradictory observation. Kulozic further discussed experiments in which various NMD factors were tethered to the 3' UTR of otherwise normal mRNAs in depleted cells. These studies indicate that hUPF1 represents a late step in the NMD pathway, but that NMD substrates exhibit differential requirements for hUPF2 and hUPF3b and components of the exon junction complex such as Y14/MAGOY, eIF4AIII, or RNPS1. These findings challenge the model of a single, linear NMD pathway as previously put forward; however, they should be interpreted with caution as they are based on a tethering assay that may bypass some essential steps of the NMD pathway.

ALTERNATIVE FATES FOR mRNAs HARBORING STALLED OR ABERRANTLY TERMINATING RIBOSOMES

A delicate relationship exists between mRNA translation and stability in both eubacteria and eukaryotes. Specifically, mRNAs that are functional and translated efficiently are generally stabilized, while mRNAs that are either aberrant or whose protein products are no longer required are rapidly degraded. In eukaryotes, this relationship can be exemplified by the nonsense-mediated mRNA decay pathway, in which mRNA harboring a premature termination codon (PTC) is subject to rapid mRNA degradation by deadenylation-independent mRNA decapping and 5'–3' exonucleolytic degradation, and/or the 3'–5' pathway of decay (for review, see Baker and Parker 2004; Maquat 2004). One important question in understanding the mechanism of NMD is the nature by which a PTC is recognized as aberrant. Allan Jacobson (Univ. of Massachusetts Medical School) began to address this question using a toeprint assay to demonstrate that, *in vitro*, translation termination at a PTC is distinct from termination at a normal stop codon. In contrast to normal termination, ribosomes at a PTC fail to efficiently release and have a propensity to migrate to upstream AUG sequences. The aberrant ribosome toeprint at a PTC was shown to require Upf1p, and, interestingly, was eliminated by either flanking the PTC with the native 3' UTR or tethering Pab1p downstream of the PTC. Thus, in yeast, activation of NMD appears to be directly linked to the intrinsically aberrant nature of premature termination. Moreover, the requirement for a “normal” relationship between the terminating ribosome and a properly configured mRNA 3' UTR supports the “faux UTR” model for NMD in yeast (Hilleren and Parker 1999; Jacobson and Peltz 2000).

In her presentation, Elisa Izaurralde (EMBL, Germany) showed that, in *Drosophila*, aberrant ribosome termination at a PTC leads to endonucleolytic cleavage of the NMD substrate in the vicinity of the termination event (Gatfield and Izaurralde 2004). Strikingly, initiation of decay of an NMD substrate by endonucleolytic cleavage has not been

previously described and has, to date, been observed only in *Drosophila*. This pathway prevails in flies despite the conservation of NMD machinery components between flies, worms, and humans, and mechanistic commonalities shared between flies and yeast (Gatfield et al. 2003). Although considerable effort has been given to identifying the activity responsible for the endonucleolytic cleavage event, the activity has yet to be defined. In observations similar to those previously made in yeast, NMD in flies exhibits a polarity effect whereby 5'-proximal PTCs elicit a stronger NMD response than those distal to the 5' end of the mRNA. In yeast, the length of 3' UTR elements is reasonably invariable, and the polarity effect has been considered in terms of the relative distance between the PTC and 3' UTR elements (Cao and Parker 2003). However, it was noted that in *Drosophila*, the 3' UTRs of mRNAs are significantly diverse in size such that a model simply hinging upon the relative distance between a terminating ribosome and elements within the 3' UTR to explain the strength of an NMD response is unlikely.

Such a relationship, in contrast, appears to be important in the cell-cycle-regulated stability of histone mRNA. In metazoa, histone mRNAs are not polyadenylated and bound by poly(A) binding protein, but, rather, are stabilized by a 3' terminal stem-loop structure that is bound by stem-loop binding protein (SLBP) (for review, see Marzluff and Duronio 2002). Bill Marzluff (Univ. of North Carolina, Chapel Hill) indicated that the position of the 3' stem-loop must be 25–50 nt from the stop codon to participate in regulated histone mRNA stability, highlighting the importance of a proper spatial relationship between a ribosome engaged in translational termination and the stem-loop/SLBP complex. Marzluff also demonstrated that regulated histone mRNA stability was affected by depletion of hUPF1, and, consistent with this, inhibition of hUPF1 phosphorylation by the Smg1 homolog, ATR, led to stabilization of histone mRNA. How UPF1 modulates the relationship between “normal” translation termination and histone mRNA 3' stem-loop elements in a cell-cycle-dependent manner is presently unknown, but may provide important insight into the mechanism of NMD in mammalian cells.

In bacteria, mRNAs harboring a stalled ribosome can undergo two unique fates. In the first case, Hiroji Aiba (Nagoya Univ., Japan) and Christopher Hayes (Bob Sauer's laboratory, MIT) reported that, dependent upon the codon context, ribosomes engaged in normal translation in *E. coli* can stall during elongation, and that pausing at either a sense or a stop codon results in mRNA cleavage that generates termini ending either within or immediately adjacent to the pause site (Hayes and Sauer 2003; Sunohara et al. 2004a,b). Significantly, the carboxy-terminal amino acids of the nascent polypeptide chain, frequently arginine and proline, were implicated in enhancing the ribosome pausing through their conformational and/or chemical properties (Sunohara et al. 2002; Hayes et al. 2002a,b). Interestingly,

the 5' product of the mRNA cleavage event was shown to be a substrate for *trans*-translation by the tmRNA quality control system (for review, see Withey and Friedman 2003), suggesting that the stalled ribosome remains bound to the 5' cleavage product, and its release is facilitated by tmRNA. It was proposed that the coupling of ribosome arrest with mRNA cleavage and *trans*-translation ensures quality control of protein synthesis and may represent a mechanism of regulating expression of particular genes. Analyses of the mRNA products generated after ribosome stalling indicate that they are created from an endonucleolytic cleavage event (Sunohara et al. 2004a,b). Markedly, cleavage does not require tmRNA, bacterial toxins such as RelE (see below), or any other known endoribonuclease in *E. coli*.

In the second case, mRNAs harboring stalled ribosomes are substrates for endonucleolytic cleavage in the ribosomal A-site promoted by the toxin RelE (Pedersen et al. 2003). Ken Gerdes (Univ. of Southern Denmark, Odense) explained that in *E. coli*, mRNA cleavage involving RelE occurs predominantly under conditions of nutritional stress and leads to global inhibition of protein synthesis. Gerdes also proposed that RelBE and similar antitoxin:toxin systems serve to facilitate recovery from limiting growth conditions and represent conserved stress response elements (Christensen et al. 2003). Consistent with this viewpoint, the toxic affect of RelE activity is counteracted by tmRNA, indicating that the 5' product of mRNA cleavage is also a substrate for *trans*-translation and that rapid ribosome recycling and amino acid salvage is critical for cell survival (Christensen and Gerdes 2003). Curiously, *in vitro*, RelE does not demonstrate ribonuclease activity on mRNA free of ribosomes, but, rather, cleaves only mRNA bound by stalled ribosomes (Pedersen et al. 2003). These observations suggest that RelE either acquires RNase activity upon ribosome binding or it triggers RNA cleavage by an as yet unidentified ribonuclease. In light of the fate of the mRNA in both this and the above situations, it was not overlooked at this meeting that, when stalled on an mRNA, the ribosome itself may catalyze the endonucleolytic cleavage event (Christensen and Gerdes 2003; Hayes and Sauer 2003; Sunohara et al. 2004a).

In eukaryotes, mRNAs devoid of an in-frame translational termination codon permit ribosome translocation to proceed to the extreme 3' terminus and are the targets for rapid degradation by the nonstop mRNA decay pathway (Frischmeyer et al. 2002; van Hoof et al. 2002). It is hypothesized that, similar to truncated mRNA in bacteria, nonstop mRNAs harbor ribosomes stalled at the 3' termini that are required to be removed prior to decay of the transcript. A model for nonstop decay was presented by Ambro van Hoof (Univ. of Texas, Houston) proposing that, in yeast, the Ski7 protein interacts with the A-site of a ribosome stalled at the end of a nonstop mRNA and facilitates its release from the mRNA. Furthermore, the interaction between Ski7p and the stalled ribosome was suggested to

promote decay of the mRNA through recruitment of the exosome complex. In support of this model, the carboxy-terminal domain of Ski7p, which shows homology to the GTPase eRF3, was shown to be required for nonstop decay, but not for normal mRNA turnover. Furthermore, the N terminus of Ski7p, which interacts indirectly with the exosome complex, is required to be in *cis* with the carboxy-terminal domain to mediate mRNA degradation. Therefore, in nonstop decay, Ski7p may act both as a release factor mimic to liberate stalled ribosomes and as a recruiter for the 3'-5' mRNA decay machinery.

Recognition and aberrant translation termination at a PTC is conventionally believed to occur by ribosomes engaged in translation in the cytoplasm. However, some PTC-containing messages manifest reduced levels in the nuclear fraction of cells (e.g., Belgrader et al. 1993), and PTC recognition has been reported to cause alterations in events that clearly occur in the nucleus. For example, a PTC at various positions within the immunoglobulin μ (Ig- μ) gene results in the accumulation of its pre-mRNA in the nucleus near the site of transcription (Mühlemann et al. 2001). Likewise, PTCs upregulate the level of TCR β pre-mRNA at or near the site of transcription (Mühlemann et al. 2001). Miles Wilkinson (Univ. of Texas) provided additional evidence in support of this nonsense-mediated upregulation of pre-mRNA (NMUP) response. He demonstrated that the target of NMUP is not unspliced TCR β mRNA but rather a partially spliced species containing two introns. Furthermore, mapping experiments revealed that the TCR β VDJ exon and adjacent intron sequences are sufficient to elicit NMUP. Wilkinson also discussed an additional intranuclear response to a PTC referred to as nonsense-associated altered splicing (NAS), in which alternatively spliced mRNA void of the offending nonsense mutation is upregulated. While many cases of NAS are the result of nonsense mutations disrupting splicing enhancers (see Maquat 2002), in the case of the TCR β gene, nonsense and frameshift mutations increase alternatively spliced mRNA due to disruption of reading frame (Wang et al. 2002a). Wilkinson presented preliminary evidence that the TCR β -specific NAS response is the result of a feedback mechanism that increases the production of alternatively spliced mRNA in response to a PTC detected in constitutively spliced mRNA.

Oliver Mühlemann (Univ. of Bern, Switzerland) described yet another intranuclear response to a nonsense codon: nonsense-mediated transcriptional gene silencing (NMTGS). He reported that introduction of a PTC in an Ig- μ gene causes reduced transcription, as assessed by nuclear run-on analysis. While NMTGS is seemingly contradictory to the NMUP response, as the former decreases pre-mRNA levels and the latter increases pre-mRNA levels, the observation might be reconciled by the fact that they occur in different circumstances, NMTGS in transfected HeLa cells and NMUP in B-cell hybridomas. The mechanism of NMTGS is not yet clear; nonetheless, it appears to

involve the formation of heterochromatin (as determined by histone acetylation/methylation status), suggesting that chromatin remodeling may play a role in mediating this form of gene silencing. Accordingly, Mühlemann also presented preliminary evidence that small dsRNAs and the RNAi pathway may be involved in mediating NMTGS, similar to the heterochromatic silencing by RNAi examined in fission yeast (Volpe et al. 2002) and in plants (Zilberman et al. 2003).

Two models have been put forward to explain how a PTC, whose recognition requires ribosome scanning, might impact events in the nucleus (Wilkinson and Shyu 2002; Dahlberg et al. 2003; Dahlberg and Lund 2004). One involves an undefined “feedback mechanism” whereby the detection of aberrant mRNA by cytoplasmic ribosomes signals changes in gene expression patterns in the nucleus. The second model pivots around the controversial yet unresolved issue of nuclear translation. Only future experimentation will help distinguish which of the models, if either, contributes to PTC-induced intranuclear events.

STRUCTURAL DATA PROVIDE INSIGHT INTO mRNA TURNOVER

Protein structure elucidation is beginning to play an important role in understanding both the detailed catalytic events performed by ribonucleases and the protein–protein or protein–RNA interactions that act to regulate catalytic activity. Haiwei Song (Institute of Molecular and Cell Biology, Singapore) discussed the elucidation of the crystal structures of the yeast Dcp1 and Ski8 proteins and their implications for mRNA turnover (Cheng et al. 2004; She et al. 2004). In particular, mutation of conserved sequences determined to be on the molecular surface of Dcp1p has facilitated the identification of residues critical for mediating interactions with regulatory proteins involved in mRNA decapping (She et al. 2004). In separate presentations, the crystal structures of the *E. coli* DEDD exonucleases, RNase D and oligoribonuclease (Fiedler et al. 2004), were described by Yuhong Zuo from Arun Malhotra’s laboratory (Univ. of Miami). While these proteins share similar sequence motifs at the amino acid level, structural analysis indicates that they exhibit strikingly dissimilar structural domains. These two homologous proteins exhibit strong differences in both RNA substrate preference and catalytic function, and it is anticipated that comparison between these and other DEDD protein structures will assist in understanding these functional divergencies.

Kenneth McDowall (Univ. of Leeds) reported a collaboration with the group of Ben Luisi (Cambridge) in which biophysical techniques were being used to examine the role of metal in determining the structure and function of RNase E. It was shown that an intramolecular pairing of amino acid residues within a highly conserved motif serves to coordinate zinc as part of a dimerization interface. Importantly, mutation of conserved cysteine residues comprising the zinc coordination site results in disruption of the RNase

E tetramer into stable dimers, which maintain RNA binding capacity but show little catalytic activity. Moreover, tetrameric RNase E, formed by chemical crosslinking in the absence of zinc, was found to be catalytically active. These results suggest that metal ion binding by RNase E enhances enzyme activity by promoting tetramerization rather than increasing RNA binding or activating catalysis directly.

John McCarthy (Univ. of Manchester, UK) reported on NMR and cryo-EM studies aimed at understanding the mechanisms underlying stabilization of the eIF4E:cap interaction by eIF4G and ribosome recruitment to the mRNA 5′ end during cap-dependent translational initiation in eukaryotes (von der Haar et al. 2004). Significantly, eIF4G interaction with cap-bound eIF4E elicits a coupled folding transition in which eIF4G wraps around the loosely structured N-terminal domain of eIF4E, which, in turn, is coupled to conformational changes at distal locations of eIF4E that are presumably responsible for increased cap affinity (Gross et al. 2003). McCarthy also described a dramatic conformational change that befalls the 40S ribosomal subunit upon binding of the initiation factors that prepare this subunit for recruitment to the 5′ end of the mRNA. This structural alteration can be envisioned as a switch promoting an association between the 43S ribosome complex and the mRNA which renders the complex competent for scanning along the 5′ UTR. In light of the intimate relationship between translation initiation and mRNA decapping (see Collier and Parker 2004), a more detailed understanding of the steps involved in initiation factor and 43S ribosomal subunit binding to the mRNA 5′ cap will undoubtedly offer insight into the transition between a translationally competent mRNP and one destined for degradation.

Elena Conti (EMBL, Germany) presented the crystal structure of the amino-terminal domain of human SMG7. SMG7 is one of three related and essential NMD-associated proteins (i.e., SMG5, 6, and 7) which, in metazoans, targets phosphorylated UPF1 for dephosphorylation by protein phosphatase 2A (PP2A) (Page et al. 1999; Anders et al. 2003; Chiu et al. 2003). The structure reveals that hSMG7 displays a strong similarity with 14-3-3, a signal transduction protein that binds serine-phosphorylated proteins (Tzivion and Avruch 2002; Yaffe 2002). The phosphoserine-binding site of 14-3-3 is conserved in hSMG7, suggesting that SMG7 may bind serine-phosphorylated UPF1 via its N-terminal 14-3-3-like domain. Together with data from Elisa Izauralde showing that hSMG7 localizes to cytoplasmic mRNA decay foci, a picture is emerging where SMG7 provides a link between proteins of the NMD surveillance complex and those of mRNA turnover.

ADDITIONAL MODEL ORGANISMS FOR THE ANALYSIS OF mRNA DECAY

It is now generally accepted that *E. coli* will provide only a part of the whole eubacterial RNA decay puzzle. Studies in

Bacillus subtilis, *Streptomyces coelicolor*, and *Synechocystis* species, while preliminary, are offering alternative model organisms for the advancement of our understanding of RNA metabolism. Presently, identification of the major enzymatic activities and pathways for mRNA decay is the goal. For example, David Bechhofer (Mount Sinai, New York) reported that in *B. subtilis*, many of the decay activities characterized in *E. coli* (e.g., RNase E, RNase II, and oligoribonuclease), are absent, and that PNPase has emerged as one of the key enzymes in mRNA turnover. Expression profiling of a *pnp* mutant led to the discovery that PNPase is the primary enzyme required for the degradation of many small decay intermediates and that this property is critical both for recycling of the *B. subtilis* *trp* operon regulator, TRAP (Deikus et al. 2004), and for growth on ammonium as sole nitrogen source. Interestingly, PNPase was identified as the primary polyadenylation, or more accurately, polynucleotidylation activity in *Synechocystis*, *S. coelicolor*, and *B. subtilis* (Gadi Schuster [Israel Institute of Technology, Israel], Gabriela Cohen [Stanford], and Cinvestav-Unidad Olmedo-Alvarez [Irapuato, Mexico] laboratories, respectively). These discoveries provide a significant step forward toward understanding alternative pathways of mRNA decay in eubacteria.

While the enzymes and pathways of eukaryotic mRNA degradation are best characterized in the unicellular budding yeast, *Saccharomyces cerevisiae* (see Parker and Song 2004), it is now evident that additional eukaryotic organisms will contribute significantly to furthering our understanding of mRNA turnover. Excitingly, analysis of “alternative” models are both corroborating previous findings and exposing new players and mechanisms for mRNA decay. For example, Elmer Wahle (Univ. of Halle, Germany) reported that the CCR4-NOT complex, the major cytoplasmic mRNA deadenylase in yeast (Tucker et al. 2001, 2002), is conserved in *Drosophila*. Furthermore, the CCR4 and CAF1 protein homologs were shown to exhibit poly(A)-specific 3′ exoribonuclease activity in vitro, while *ccr4* mutant flies manifest elongated bulk poly(A) mRNA (Temme et al. 2004). Meanwhile, the nonsense-mediated pathway of decay in flies appears to be unique (see Izaurralde above). One obvious advantage for studying *Drosophila* as a model organism is the ability to use genetic techniques such as transfection and efficient RNAi-mediated factor depletion of cultured *Drosophila* cells coupled with the evaluation of complex physiological phenotypes as a consequence of genetic defects in whole animals.

Additional model organisms each offer unique advantages for the analysis of RNA metabolism in eukaryotes. For example, in *Arabidopsis*, multiple homologs of characterized decay factors are being identified that, at first glance, might be considered redundant nucleases. Yukako Chiba (Pamela Green’s laboratory, Univ. of Delaware) described seven homologs of the deadenylase complex protein CCR4p in *Arabidopsis*. Microarray analyses of plants mutant for two of

the members indicate that while the enzymes share some putative mRNA substrates in common, most substrates are unique to each particular enzyme. Based on these findings, analysis of mRNA decay in *Arabidopsis* may allow for an understanding of small subtleties in nuclease function and substrate specificity as well as uncover regulatory mechanisms that underlie the need for several proteins with similar catalytic activities. Finally, it is undeniable that the advent of RNAi as a reverse genetic technique has made mammalian primary and transformed cells suitable targets for extensive molecular analysis. It can be predicted that it is only a matter of time before this technology provides an important link between our ability to acquire knowledge about RNA metabolism and the implementation of therapeutic strategies that regulate gene expression at the level of RNA.

CONCLUDING REMARKS

This meeting highlighted the synergy and camaraderie that is openly shared between scientists in the field of eubacterial and eukaryotic mRNA decay. Although it is apparent that no canonical pathway for mRNA degradation exists, the collective advancement in both of these areas of research continues to uncover mechanisms in which RNA is targeted and fed into this important pathway for posttranscriptional regulation of gene expression. Interestingly, since the last gathering of this group of researchers, several descriptions of cytoplasmic foci which harbor various RNA decay proteins have been communicated (van Dijk et al. 2002; Ingelfinger et al. 2002; Sheth and Parker 2003; Cougot et al. 2004). These observations have noticeably impacted on how the field is “looking” at mRNA degradation; however, the full impact of subcellular localization of mRNA degradation remains to be unveiled.

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