Leading Edge
Review
Cell

The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones

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http://dx.doi.org/10.1016/j.cell.2014.03.008

Noncoding RNAs (ncRNAs) accomplish a remarkable variety of biological functions. They regulate gene expression at the levels of transcription, RNA processing, and translation. They protect genomes from foreign nucleic acids. They can guide DNA synthesis or genome rearrangement. For ribozymes and riboswitches, the RNA structure itself provides the biological function, but most ncRNAs operate as RNA-protein complexes, including ribosomes, snRNPs, snoRNPs, telomerase, microRNAs, and long ncRNAs. Many, though not all, ncRNAs exploit the power of base pairing to selectively bind and act on other nucleic acids. Here, we describe the pathway of ncRNA research, where every established "rule" seems destined to be overturned.

Introduction

The seeds of a revolution are invariably sown decades before it erupts. And so it is with the revolution in noncoding (nc)RNAs. The principal RNA participants in gene expression, the ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), were discovered in the 1950s and their central roles as scions of protein synthesis firmly established. (See Table 1 for definitions.) It was not until the early 1980s that the first renegade ncRNAs, the small nuclear (sn)RNAs, emerged as possible players in the excision of introns (see Table 1 for definitions). After their acceptance as building blocks of the spliceosome, other abundant classes such as small nucleolar (sno)RNAs joined the ranks of ncRNAs. The revolution gained huge momentum in the early 2000s with the discovery of micro (mi)RNAs and their many relatives, underscoring the importance of posttranscriptional events in gene expression particularly in eukaryotic organisms. Today, the ncRNA revolution has engulfed all living organisms, as deep sequencing has uncovered the existence of thousands of long (l)ncRNAs with a breathtaking variety of roles in both gene expression and remodeling of the eukaryotic genome.

Here, we review the genesis and recent explosion in our appreciation of the critical contributions of ncRNAs to gene expression and genome maintenance. These versatile cellular molecules regulate a remarkably broad spectrum of cellular processes (Figure 1). But this new knowledge was not uncovered and accepted in a steady, orderly progression. Instead, old rules that had provided a reasonable framework for thinking about RNA biology were overthrown, in some cases precipitously, and replaced by new rules. We are not referring to the rare exceptions, such as "the genetic code is not universal," that occur in a small fraction of extant genetic systems on the planet. To the contrary, these new rules were often established by thousands of representatives. Thousands of ribozymes in thousands of organisms refuted the rule that all enzymes are proteins. Two hundred thousand introns in the human genome alone refuted the rule that RNA processing occurs at the ends of RNAs. The same can be said of microRNAs, riboswitches, and lncRNAs; these are not rare creatures, but major classes of RNAs. They had completely escaped detection and then, suddenly, were found to be widespread and abundant. Here, we describe nine major events in the last 30 years of RNA research, occasions where old rules were trashed to make way for new ones.

Rule 1. Enzymes Are Proteins—*Then Came Ribozymes*

Although James B. Sumner was originally ridiculed for having the audacity to claim that the urease enzyme was a protein, the concept that enzyme = protein soon became sacrosanct and persisted through the 1970s. Certainly a few visionaries—chief among them Carl Woese, Leslie Orgel, and Francis Crick—understood that catalysis by RNA or another polynucleotide would provide an attractive solution to the origin-of-the-ribosome problem and the origin-of-life problem more generally. Yet it was difficult to conceptualize RNA catalysis in the absence of any experimental evidence. After all, "In biological systems we know that catalytic functions are performed by proteins and never by polynucleotides" (Orgel, 1968).

The first example of RNA catalysis was found, as often happens, when the researchers were looking for something else. The Cech research group was working to understand the splicing mechanism of an intron-containing rRNA precursor in the ciliated protozoan *Tetrahymena*, expecting the reaction to be protein catalyzed. They found instead that the RNA structure formed by the intron was necessary and sufficient to accomplish splicing (Kruger et al., 1982). The RNA active site performed a series of three reactions without being destroyed in the process,

Table 1. Classes of RNA and Their Sizes and Functions

	Definition	Function	Size
Airn RNA	antisense Igf2r RNA	lncRNA that induces imprinting of a mouse gene cluster including *Igf2r*	118 kb
B2 RNA	mouse RNA transcribed from a short interspersed element (SINE)	RNA that inhibits RNA polymerase II upon heat shock	180 nt
CRISPR RNA	clusters of regularly interspersed short palindromic repeat RNA	targets Cas nuclease to cleave a specific DNA, such as a phage DNA, in bacteria or archaea	24–48 nt
CsrB RNA	carbon storage regulator RNA	RNA acts as a sponge to sequester CsrA protein in *E. coli*	350 nt
ecCEBPA RNA	extracoding RNA from the *CEBPA* (CCAAT/enhancer binding protein alpha) gene	lncRNA that directly binds DNA methyltransferase 1 to regulate epigenetic CpG methylation	4.5 kb
eRNA	transcriptional enhancer element RNA	binds Mediator to enhance transcription	200–500 nt (some larger)
Gas5 ncRNA	growth arrest-specific transcript	binds and inhibits glucocorticoid receptor	600 nt
gRNA	guide RNA	base pairs with an RNA target, orienting bound proteins to carry out a site-specific cleavage, ligation or modification reaction	40–80 nt
Group I intron	a structural class of self-splicing RNAs	ribozyme that binds guanosine and uses it as nucleophile to catalyze RNA splicing	250–400 nt
Group II intron	a structural class of self-splicing RNAs	ribozyme that catalyzes splicing via formation of a lariat intron	600 nt
hairpin, hammerhead, and hepatitis delta virus ribozymes	three structural classes of self-cleaving RNAs	ribozymes that induce RNA cleavage to form 2′,3′-cyclic phosphate and 5′-OH termini; they also catalyze the reverse reaction, RNA ligation	30–80 nt
hnRNA	heterogeneous nuclear RNA	intron-containing pre-mRNA	2–40 kb
HOTAIR RNA	HOX antisense intergenic RNA	lncRNA that silences the HoxD locus and many other sites by recruitment of PRC2 and LSD1/CoREST/REST repressive chromatin modifying complexes	2.2 kb
HOTTIP RNA	HOXA transcript at the distal tip	lncRNA transcribed from the 5′ end of the HoxA cluster; controls HOX mRNA transcription; low abundance (~0.3 copies/cell)	3.8 kb
IRES	internal ribosome entry site	structured RNA element in a viral or (occasionally) cellular mRNA that binds factors to allow internal initiation of translation	200–300 nt
lncRNA	long noncoding RNA	autonomously transcribed RNA that does not encode a protein; often capped and polyadenylated; can be nuclear, cytoplasmic or both	>200 nt
MEN ε/β ncRNA	multiple endocrine neoplasia ε/β ncRNA; also known as NEAT1 (nuclear enriched abundant transcript 1)	abundant RNAs that nucleate formation of paraspeckles at their transcription site	3.7 kb, MEN epsilon; 23 kb, MEN beta
miRNA	microRNA	RNA that, in complex with AGO protein, uses seed sequences near its 5′ end to base pair with a target mRNA to induce deadenylation and decay or translational regulation	22 nt
mRNA	messenger RNA	contains a coding region that directs synthesis of a protein product; typically has both 5′- and 3′-untranslated sequences	2–5 kb
ncRNA	noncoding RNA	an RNA that does not encode a protein, but has other cellular functions	–
PCGEM1 lncRNA	prostate-specific transcript 1	lncRNA that promotes chromatin looping to enhance transcription of androgen receptor-responsive genes	1,643 nt

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Table 1. ***Continued***

	Definition	Function	Size
piRNA	PIWI-associated RNA	RNA that directs the modification of chromatin to repress transcription; best characterized in the male germline	27 nt
pre-miRNA	precursor miRNA	product of pri-microRNA processing by Drosha; typically an imperfect hairpin structure, which exits the nucleus and is then cleaved by Dicer to generate two mature miRNAs	60 nt
pre-mRNA	precursor mRNA	primary transcript of a protein-coding gene that contains intron(s)	2–40 kb
pre-rRNA	precursor rRNA	primary rRNA gene transcript or a processing intermediate that contains mature ribosomal RNAs separated by spacer sequences and/or 5′ and 3′ extensions	13.7 kb, human; 6.6 kb, yeast; 5 kb, *E. coli*
pre-tRNA	precursor transfer RNA	primary tRNA gene transcript or a processing intermediate that contains one or more mature transfer RNAs separated by spacer sequences and/or 5′ and 3′ extensions	>100 nt
pri-miRNA	primary microRNA transcript	can contain one or more pre-miRNAs and often comprises the intron of a protein-coding gene; requires processing by Drosha and then Dicer to generate mature miRNAs	>80 nt
RepA RNA	repeat element within Xist RNA; also independently transcribed	ncRNA expressed prior to and required for X-inactivation	1.6 kb
riboswitch	RNA element within a mRNA that toggles between two conformations upon exposure to a small-molecule ligand or other stimulus	inhibits or promotes gene expression at the level of transcription, translation, or RNA splicing	40–140 nt
RNase P RNA	RNA component of ribonuclease P (processing)	catalytic subunit of the enzyme that removes 5′-leaders from pre-tRNAs	400 nt
roX1 and *roX2* RNAs	RNA on the X, 1 and 2	male-specific nuclear RNAs that form RNPs to upregulate transcription from the single male X chromosome, achieving dosage compensation in *Drosophila*	3.7 kb, *roX1*; 0.5 kb, *roX2*
rRNA	ribosomal RNA	RNA component of the small or large ribosomal subunit; the largest is a ribozyme	120, 160, 1,868, 5,025 nt, human; 120, 1,541, 2,904 nt, *E. coli*
scan RNA	small conjugation-specific RNA (also known as scnRNA)	dsRNAs produced by an RNAi-related mechanism that recognize genomic internal eliminated sequences in the developing macronucleus of ciliates and target them for destruction	28 nt
scaRNA	small Cajal body-associated RNA	biogenesis and function similar to snoRNAs, but located in the Cajal body to guide modification of snRNAs	200–300 nt
siRNA	small interfering RNA	product of Dicer cleavage of dsRNA; when complexed with an AGO protein, induces cleavage of a perfectly-complementary target RNA	22 nt
sisRNA	stable intronic sequence RNA	as yet unknown	–
snoRNA	small nucleolar RNA; in vertebrates, most snoRNAs are processed intron fragments	essential for pre-rRNA processing or modification by serving as a guide RNA to direct a bound enzyme to either 2′-*O*-methylate or pseudouridylate a complementary sequence in rRNA	70 nt
snRNA	small nuclear RNA	RNA localized in the eukaryotic cell nucleus	100–300 nt

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Table 1. *Continued*

	Definition	Function	Size
sRNA	small RNA regulator	bacterial ncRNAs that base pair to mRNAs and regulate gene expression	<300 nt
T box	tRNA-binding leader element	structured RNA element of bacterial mRNAs that binds uncharged tRNA causing antitermination of transcription of genes that encode translation proteins	200 nt
telomerase RNA (TR, TER or TERC)	telomerase RNA	provides template for telomeric DNA synthesis and scaffolds protein assembly	450 nt, human; 1.2 kb, yeast; 160 nt, *Tetrahymena*
tRNA	transfer RNA	RNA adaptor connecting an mRNA codon and the activated form of the cognate amino acid during protein synthesis on the ribosome	70–90 nt
U snRNA	U-rich small nuclear RNA	subclass of snRNAs; many are building blocks of the major or minor spliceosome, serving to recognize intron boundaries and perhaps to catalyze intron removal; several abundant snoRNAs (U3, U8 and U13) are also U-rich	100–300 nt
U1/U11 snRNA	snRNA of the major/minor spliceosome	identifies by base pairing the 5′-splice site of introns	164/131 nt
U2/U12 snRNA	snRNA of the major/minor spliceosome	uses base pairing to identify branch-point sequences upstream of the 3′-splice site	187/150 nt
U4/U4atac snRNA	snRNA of the major/minor spliceosome	pairs with and delivers U6/U6atac snRNA to the spliceosome, inhibiting its function until the second step of mRNA splicing	145/132 nt
U5 snRNA	snRNA present in both the major and minor (U12-dependent) spliceosome	aligns the 5′ and 3′ exons for ligation during the second step of mRNA splicing	116 nt
U6/U6atac snRNA	snRNA of the major/minor spliceosome	after release of U4/U4atac, U6/U6atac base pairs with U2/U12 to form an active splicing complex	107/125 nt
U7 snRNA	nonspliceosomal U snRNA essential for the 3′-end maturation of histone mRNAs in metazoa	uses base pairing to designate the site of endonucleolytic cleavage by an associated 3′-end processing factor	62 nt
VS ribozyme	Varkud satellite ribozyme	mitochondrial RNA element in *Neurospora* that self-cleaves	160 nt
Xist RNA	X-inactive-specific transcript RNA	coats one X chromosome in female mammals, triggering heterochromatization and transcriptional repression	17 kb
6S RNA	an abundant *E. coli* ncRNA	binds and inhibits RNA polymerase during stationary phase	184 nt, *E. coli*
7SK RNA	RNA component of a nuclear complex containing Hexim1, LARP7, and P-TEFb subunits CycT1 and Cdk9	highly abundant RNA polymerase III transcript that controls RNA polymerase II transcription by scaffolding formation of an RNP complex that inhibits P-TEFb elongation factor	330 nt
7SL RNA	RNA component of the signal recognition particle (SRP)	scaffolds formation of a cytoplasmic RNP that enables transit of nascent proteins through the translocon and into the endoplasmic reticulum	300 nt

Sizes of ncRNAs are typical values or ranges; most are approximate.

justifying its characterization as an enzyme or ribozyme (RNA enzyme). Subsequent modification of the system produced a truly enzymatic RNA that could catalyze limitless cycles of RNA binding, cleavage, and product release. Soon dozens of structurally related so-called Group I introns from diverse organisms were demonstrated to be self-splicing (Garriga and Lambowitz, 1984; Gott et al., 1986), all utilizing the same biochemical mechanism: they bind a small molecule, guanosine, and use it as a nucleophile to cleave the RNA 5′-splice site (Bass and Cech, 1984). Magnesium ions bound in the active site contribute

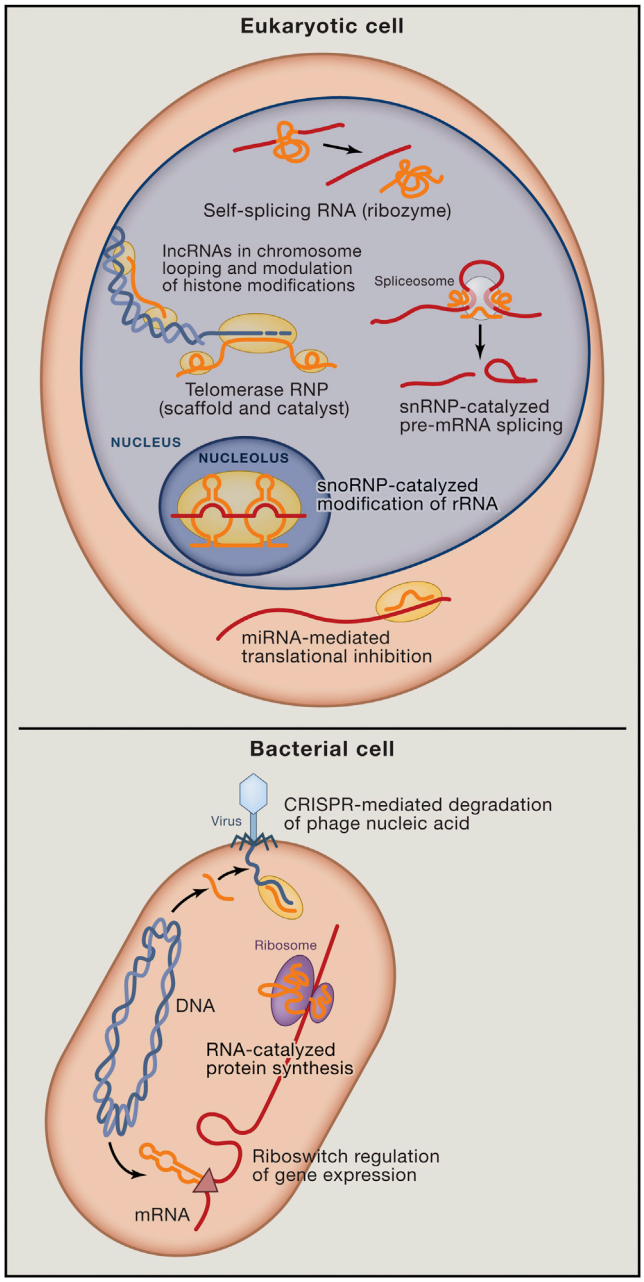

Figure 1. Noncoding RNAs Function in Diverse Contexts

Noncoding RNAs function in all domains of life, regulating gene expression from transcription to splicing to translation and contributing to genome organization and stability. Self-splicing RNAs, ribosomes, and riboswitches function in both eukaryotes and bacteria. Archaea (not shown) also utilize ncRNA systems including ribosomes, riboswitches, snoRNPs, and CRISPR. Orange strands, ncRNA performing the action indicated; red strands, the RNA acted upon by the ncRNA. Blue strands, DNA. Triangle, small-molecule metabolite bound by a riboswitch. Ovals indicate protein components of an RNP, such as the spliceosome (white oval), ribosome (two purple subunits), or other RNPs (yellow ovals). Because of the importance of RNA structure in these ncRNAs, some structures are shown but they are not meant to be realistic.

to catalysis, a strategy similar to that used by protein polymerases and phosphatases (Steitz and Steitz, 1993).

RNase P is a tRNA processing enzyme that cleaves off a leader sequence to produce the mature 5′ ends of all tRNAs. Sidney Altman's group had characterized *E. coli* RNase P as requiring both an RNA and a protein subunit; with Norman Pace, they showed that the RNA subunit contained the catalytic center and was able to act as a true enzyme to cleave tRNA precursors with multiple turnover without being consumed in the reaction (Guerrier-Takada et al., 1983). In addition to its importance as an RNA enzyme, RNase P provided a paradigm for one class of RNPs, in which the protein subunit allows the intrinsically catalytic RNA to fold into an active conformation under physiological conditions. The large subunit of the ribosome is another such RNP catalyst (see below), and many self-splicing introns have specific protein partners and function as RNPs in vivo.

Group II introns, structurally distinct from Group I (Michel et al., 1982), undergo self-splicing by a distinct mechanism. They use the 2′-OH group of an internal adenosine to cleave the 5′-splice site, forming a branched "lariat" intermediate and intron product (Peebles et al., 1986; van der Veen et al., 1986). The spliceosome uses the same biochemistry when acting on nuclear mRNA precursors in eukaryotes, raising suspicions of an ancient connection between the two systems. This proposal, exciting but at first highly speculative, has been bolstered by recent findings: structural studies including determination of the crystal structure of a Group II intron revealed similarities with snRNA structures (Toor et al., 2008), and a U6-U2 snRNA complex was shown to catalyze an RNA splicing reaction in the complete absence of proteins (Valadkhan et al., 2009).

The Group I and II and RNase P RNAs are >250 nt (Table 1). How small can an RNA that has substantial catalytic function be? Other naturally occurring ribozymes (the hammerhead, hairpin, hepatitis delta virus, VS, and twister ribozymes; see Roth et al., 2014, and references therein) are in the range of 30–80 nt. In nature, they undergo self-cleavage by catalyzing the attack of a specific 2′-OH group on the adjacent phosphate, forming a 2′,3′-cyclic phosphate product analogous to protein ribonucleases (Uhlenbeck, 1987). Rate accelerations are in the range of 10^5-fold over the uncatalyzed rate. Their catalytic mechanisms include acid-base catalysis, a strategy common for protein enzymes (Perrotta et al., 1999).

Extant natural ribozymes comprise a limited catalytic repertoire, so with the invention of in vitro evolution (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990), scientists turned their attention to fishing for fresh RNA catalysts from complex sequence pools. Small ribozymes have been identified that can catalyze reactions as diverse as Diels-Alder carbon-carbon bond formation, insertion of a metal into a porphyrin ring, aminoacylation of RNA, and RNA polymerization (Illangasekare et al., 1995; Shechner et al., 2009).

Because RNA catalysis, like protein catalysis, requires a specific well-folded structure, catalytic RNAs ignited a revolution in RNA structural biology. There had been little success in deciphering structures of large, biological RNAs since tRNA was solved in 1974. Today, RNA structure prediction has advanced considerably (Michel and Westhof, 1990), and high-resolution structures of ribozymes both small and large have

been solved by X-ray crystallography (Ferré-D'Amaré et al., 1998; Golden et al., 1998; Toor et al., 2008).

A robust current and future direction for ribozyme research is in synthetic biology. Synthetic biologists aim to engineer cells to carry out useful processes, such as detecting pollutants or warfare agents, cleaning the water supply, or producing biofuels. To do so, they design molecular circuits with toggle switches, tunable oscillators, and logic gates. Ribozymes can be engineered to cleave RNA and trigger changes in gene expression if and only if they bind specific small molecules, making them valuable components for constructing synthetic biological circuits.

Rule 2. RNA Processing Occurs at Ends—*Then Introns and RNA Editing*

During the 1960s it became apparent that stable RNA species (tRNAs and rRNAs) in both bacteria and eukaryotic cells were not the direct products of transcription (Burdon, 1971). For tRNAs, at least 10% of the nucleotides, coming from both ends of pre-tRNAs, were found to be discarded. For pre-rRNAs, the wastage was up to 50% in vertebrates; internal cleavages first separated the functional 18S, 5.8S, and 28S rRNA molecules and the ends were further trimmed. In that era, no one even dreamed that internal bits might be removed from RNA transcripts and the flanking pieces religated to form functional tRNAs and rRNAs, as well as mRNAs.

Findings of the early 1970s set the stage for the discovery of mRNA splicing (Darnell, 1976). First, polyadenylate tails were identified on mRNA 3′ ends. Then the 5′ ends of mRNAs were shown to be capped with backward G residues. Subsequent examination of the termini of the huge transcripts in eukaryotic cell nuclei dubbed heterogeneous nuclear (hn)RNA—the apparent precursors of cytoplasmic mRNAs—unexpectedly revealed the same modifications. How could these precursors have mature termini yet be so much longer than mature mRNAs?

At the annual Cold Spring Harbor Symposium in 1977, results from several laboratories coalesced into the realization that regions of considerable length (introns) are indeed excised from the interior of nascent RNA molecules. The labs of Phil Sharp (Berget et al., 1977) and of Louise Chow, Tom Broker, and Rich Roberts (Chow et al., 1977) provided direct evidence from studies of adenoviral early mRNAs, showing that stretches of transcript arising from distinct and distant portions of the viral genome (exons) were pieced together to form the final mRNAs. Could jumping RNA polymerases be responsible? No, apparently the hnRNAs were full-length transcripts with the excision and discard of intron sequences explaining the huge wastage of newly synthesized RNA documented for mammalian cell nuclei. But these remarkable findings just shifted the question to a new one. What cellular machinery could be responsible for these RNA acrobatics?

NcRNAs and base pairing came to the rescue. Earlier studies had uncovered the presence of small (100–300 nt) highly abundant U-rich RNAs ($\sim 10^6$ copies; Table 1) in the nuclei of vertebrate cells (Busch et al., 1982; Weinberg and Penman, 1968). These snRNAs were discovered to associate tightly with a set of proteins that are targets of autoantibodies (anti-Sm) found in patients with lupus, forming so-called Sm snRNPs (Lerner and Steitz, 1979). The Sm proteins are now known to be related to Hfq, which binds multiple ncRNAs in bacteria (Vogel and Luisi, 2011). Failure to assemble snRNAs with Sm proteins due to a deficit in the cellular assembly factor SMN (survival of motor neurons) leads to a devastating disease spinal muscular atrophy (Wirth et al., 2006). The conserved 5′-end sequence of U1 snRNA remarkably exhibited perfect complementarity to the consensus sequence that emerged for the 5′ ends of introns (Lerner et al., 1980). Subsequent investigations showed that the 5′-splice site base pairs not only with U1, but later with U6, that the branch site of the intron pairs with U2 (extruding the branch-site A residue for nucleophilic attack on the 5′-splice site during the first step of splicing), and that the ends of the exons are aligned for the second step of splicing by U5 (Nilsen, 1998). U4 snRNA uses complementarity to bind and deliver U6 to the spliceosome. The base-pairing interactions with the pre-mRNA substrate involve the most highly conserved sequences in each of the snRNAs.

The snRNPs assemble on an intron along with a host of proteins to form the spliceosome, which undergoes a dynamic series of ordered (and reversible) conformational alterations and protein exchanges (Hoskins and Moore, 2012; Wahl et al., 2009), leading to the first and second steps of splicing and ultimate release of the lariat intron and spliced RNA. ATP-utilizing helicases contribute importantly to these structural transitions, as well as to fidelity (Staley and Guthrie, 1998). A highly conserved splicing factor Prp8 located in the spliceosomal active site contains an RNase H-like domain that appears to toggle between structural states at multiple steps in the spliceosome cycle (Schellenberg et al., 2013). Not only has splicing-related catalysis by snRNAs alone been reported (Valadkhan and Manley, 2001), but mutational and metal-rescue strategies argue that the U6 snRNA catalyzes both splicing steps (Fabrizio and Abelson, 1992; Fica et al., 2013) via a two-metal ion mechanism (Steitz and Steitz, 1993), similar to the Group II self-splicing intron (Toor et al., 2008). What is needed to resolve the question of whether splicing is RNA catalyzed are high-resolution structures of spliceosome assemblies. So far, we have structures of only the U1 and U4 snRNPs at 5.5 and 3.6 Å, respectively (Leung et al., 2011; Pomeranz Krummel et al., 2009).

Only in 1996 was the existence of a second spliceosome, present in mammals and most other metazoans, established (Tarn and Steitz, 1997).The snRNAs of the minor spliceosome—U11, U12, U4atac and U6atac—are low-abundance homologs ($\sim 10^4$ copies/cell) of those in the major spliceosome; the U5 snRNP and a number of protein components are shared (Will and Lührmann, 2005). The U11, U12, and U6atac snRNPs base pair with the distinctive 5′-splice site and branch-point sequences of minor-class (or U12-type) introns, which represent <1/100 of the introns in mammalian pre-mRNAs. Appealing speculations on how the minor and major spliceosomes evolved from a common ancestor leave open the question of why the minor spliceosome has been retained (Burge et al., 1998). In humans, developmental disorders can be traced to changes in the single genes specifying U snRNAs of the minor spliceosome (Pessa and Frilander, 2011); in contrast, multiple genes contribute to the pool of each major-class spliceosomal snRNA in vertebrate cells.

The versatility of Sm snRNPs was underscored when the U7 snRNP was assigned an essential role in the 3′-end processing of the major histone mRNAs in metazoans. U7 snRNA base pairs

with a sequence downstream of the cleavage site in histone pre-mRNAs to assemble a nucleolytic protein complex that shares many components with the mRNA 3′-end processing machinery (Dominski and Marzluff, 2007; Kolev and Steitz, 2005; Schümperli and Pillai, 2004). In organisms that carry out *trans*-splicing (such as nematodes and trypanosomes), a spliced leader (SL) RNP (also of the Sm class) appends a common 5′-leader sequence to a 3′-splice site of many protein-coding pre-mRNAs (Lasda and Blumenthal, 2011). An additional role for the U1 snRNP in vertebrates explains its previously mysterious overabundance relative to other spliceosomal snRNPs: it binds the many cryptic 5′-splice sites in introns, shielding the nascent pre-mRNA from premature cleavage and polyadenylation (Kaida et al., 2010). Some herpesviruses have acquired Sm snRNPs from the host cell, but use them quite differently—to bind and target a host miRNA for degradation (Cazalla et al., 2010).

NcRNAs also participate in RNA editing events involving nucleotide exchanges or very small (1–3 nt) deletions or insertions within an RNA transcript. Wholesale editing of mRNAs in the mitochondria of kinetoplastid protozoa can alter as many as 50% of the coding nucleotides! The molecular machinery again relies on short (40–80 nt) guide (g)RNAs, which base pair with the editing sites to direct the action of endonucleases and U-specific exonucleases or TUTases (terminal uridylyl transferases) that execute the deletion or insertion of U residues (Hajduk and Ochsenreiter, 2010).

Rule 3. Splicing Removes Intronic Junk—*Then Alternative Splicing and snoRNAs*

The discovery of introns sparked a lively debate about the evolutionary nature of noncoding (then considered "junk") DNA (Gilbert, 1985). Did junk come first, with the protein-coding modules taking advantage of the junk sequences separating them to recombine and generate new proteins by exon shuffling? Or did introns jump in later in evolution by some transposition-like process? Perhaps both occurred.

Relegating introns to the junk pile turned out to be premature. A clear-cut "use" of intronic sequences that redefines them as not-junk is in alternative splicing (Black, 2003; Nilsen and Graveley, 2010), whereby sequences that are sometimes eliminated from the mRNA appear instead as exonic (coding) regions. This occurs through the selection of alternative 5′- or 3′-splice sites or by cassette exons being included (or not) during the splicing process. Alternative splicing is pervasive with the latest estimates from deep-sequencing data assigning detectable alternatively-spliced transcripts to 95% of human genes. To accomplish alternative splicing, general factors like SR proteins and hnRNPs recognize intronic and exonic silencer and enhancer sequences (Zhang et al., 2008), and splicing factors bind specific sites to alter the pattern of spliceosome formation on a pre-mRNA (Licatalosi et al., 2008). Alternative splicing sometimes goes hand in hand with alternative mRNA 3′-end formation, which can also contribute importantly to the expression of the encoded protein (Mayr and Bartel, 2009).

Most small nucleolar (sno)RNAs are pieces of intron (~70 nt) that lead a second life after their release from excised introns through exonucleolytic processing (Liu and Maxwell, 1990; Watkins and Bohnsack, 2012). There are several hundred different snoRNAs in vertebrates. SnoRNAs are conserved even to archaea (Terns and Terns, 2002), but are processed from independent (nonintronic) transcripts in lower organisms. SnoRNPs use intermolecular base pairing to direct the modification of ribose 2′-hydroxyl groups or the isomerization of uridines to pseudouridines within pre-rRNAs. The reactions are catalyzed by an RNP protein, either fibrillarin for 2′-*O*-methylation or dyskerin for pseudouridylation, at generally conserved sites in nascent rRNAs (Kiss-László et al., 1996; Ni et al., 1997). Structurally and functionally similar to snoRNAs, small Cajal body-associated (sca)RNAs guide comparable nucleotide modifications of spliceosomal snRNAs, but reside in a nuclear compartment transited by snRNPs before they participate in splicing (Richard et al., 2003). What is stunning is the high abundance (~10^4 and 10^3/cell) of individual sno- and scaRNAs, especially as we do not yet truly understand the functions of the nucleotide modifications they introduce.

In addition to catalyzing nucleotide modification, snoRNP association with pre-rRNAs may also serve to chaperone the correct RNA fold for rRNA processing and ribosome assembly (Steitz and Tycowski, 1995). This idea is in accord with findings that a few nonintronic snoRNAs like U3 and U8 do not appear to guide modification but instead enable (apparently indirectly) important cleavages within pre-rRNA. SnoRNA structures are sometimes built into lncRNAs to stabilize their termini against intracellular degradation (Yin et al., 2012). There are also many "orphan" snoRNAs that lack apparent complementarity to rRNAs or snRNAs and may not guide nucleotide modification. Assigning functions is highly challenging, and roles as divergent as mediating metabolic stress (Michel et al., 2011) have been reported.

A recent revelation concerning intronic "junk" is the discovery that entire introns or portions thereof, called stable intronic sequence (sis)RNAs, can sometimes accumulate to significant levels, rather than undergo rapid turnover. In the *Xenopus* oocyte, such sequences dominate the nuclear transcriptome (Gardner et al., 2012). Some sisRNAs are selectively nuclear and others cytoplasmic, hinting at special functions in early development. Some sisRNAs correspond to viral introns (Kulesza and Shenk, 2006), perhaps because viruses are masters at squeezing the maximum information out of their limited genomes.

Rule 4. Ribosomal RNA Is a Scaffold—*Then a Catalyst*

The discovery in 1961 that the ribosome, already known to be the cellular agent of protein synthesis, contains multiple different polypeptide components (Waller and Harris, 1961) focused efforts to assign their presumed catalytic roles in translation. Beforehand, it had been believed that ribosomal subunits resembled viruses with copies of identical proteins coating an RNA core. Ribosomal proteins occupied the limelight through the 1970s with the amino acid sequence determination of the complete roster of *E. coli* ribosomal proteins (Wittmann-Liebold et al., 1984) and the development of new methodologies to locate them within the subunits (Engelman and Moore, 1975; Lake, 1976). The rRNAs were viewed as mere racks on which to hang proteins, serving to orient catalytic polypeptides in three dimensions to execute the steps of protein synthesis.

The first demonstration of a direct role for rRNA in protein synthesis was the finding that the 3′ end of 16S rRNA in the small subunit of bacterial ribosomes base pairs with a sequence just 5′ to initiator codons in mRNAs, fixing the start site for translation (Steitz and Jakes, 1975). Appreciation of the importance of rRNA mounted as the results of laborious RNA sequencing efforts accumulated, culminating in the early 1980s when the advent of DNA sequencing made it possible for Harry Noller (Noller, 1984) to complete the elucidation of the *E. coli* rRNA sequences. The resulting secondary structure maps and identification of compensatory phylogenetic changes then revealed astounding conservation of the core rRNA structures (Noller, 1984; Woese et al., 1983). Biochemical mapping of functional sites, including bound tRNAs (Moazed and Noller, 1989), next confirmed that rRNA is always present where there is action in the ribosome. Clearly, rRNA was very important, but was it a direct or indirect player in the chemistry of peptide bond formation?

In 1992, Noller bit the bullet by asking whether ribosomes extensively digested with proteases could still stimulate peptide bond formation (Noller et al., 1992). The qualified ''yes'' was bolstered by concurrent elegant demonstrations of additional direct roles for rRNA: the CCA ends of tRNAs bound to the A and P sites of the ribosome are held in place by base-pairing interactions with specific 23S rRNA nucleotides (Samaha et al., 1995).

Meanwhile, ribosome crystals obtained by Ada Yonath (Yonath et al., 1982) inspired intense crystallographic efforts. These ultimately yielded high-resolution views of the large and small ribosomal subunits from bacteria, published by the T. Steitz (Ban et al., 2000) and Ramakrishnan (Wimberly et al., 2000) labs, as well as a picture of the complete bacterial ribosome (Yusupov et al., 2001). These structures, and subsequent biochemical work, established that rRNA is indeed the catalytic moiety of the large ribosomal subunit. Not only is the active site distant from any protein, but the peptide chain elongation mechanism (10^6-fold rate enhancement) involving only RNA functional groups can now be understood in atomic detail (Voorhees and Ramakrishnan, 2013). The catalytic power of the rRNA derives, as for protein and other RNA enzymes, from substrate orientation (achieved when the A site substrate induces conformation changes in the rRNA) and specific chemical catalysis (here involving an extensive ''proton shuttle'') (Schmeing et al., 2005). In the small subunit, rRNA is responsible for ensuring the fidelity of codon-anticodon pairing, using an ingenious mechanism whereby certain conserved rRNA bases inspect the minor groove of the tRNA-mRNA helix, demanding a precise fit in order to proceed (Ogle et al., 2001). A look at the bacterial world only confirms the centrality of rRNA. The many ribosome-directed antibiotics that microorganisms have fashioned to war against one another selectively bind to rRNA rather than protein, detecting nucleotide differences that allow them to discriminate against the invader (Blaha et al., 2012).

Structural evidence for a ratchet-like large-scale rotation between the large and small ribosomal subunits occurring during the coupled translocation of mRNA and tRNA came first from cryo-EM (Frank and Agrawal, 2000) and is now being refined by X-ray studies (Zhang et al., 2009). Although we lack enough structures of intermediate states for a detailed picture, it is important to remember that translation can occur in the absence of GTP-hydrolyzing protein factors (Pestka, 1968). Moreover, since the interface between the two subunits is largely RNA (albeit solvated), the intersubunit bridges that are preserved, rearranged or newly formed during translocation and chain termination reflect RNA-RNA interactions (Schuwirth et al., 2005). High-resolution structures of eukaryotic ribosomes have revealed that the expansion segments (not present in the conserved rRNA core) serve to bind eukaryote-specific proteins and build eukaryote-specific intersubunit bridges (Klinge et al., 2012).

Many questions concerning the roles of RNA in translation remain. Despite recent insights into how internal ribosome entry site (IRES) elements upstream of start codons in mRNAs can orchestrate translation initiation without a full complement of factors (Berry et al., 2011), we still lack a detailed picture of the relative roles of rRNA and many protein factors in eukaryotic initiation (Voigts-Hoffmann et al., 2012). Why are so many proteins (greater than the number of ribosomal proteins) necessary (Dragon et al., 2002) to assemble each eukaryotic ribosomal subunit? How extensively does the rRNA contribute to ribosome interactions with the signal recognition particle (SRP), a complex scaffolded by the 7SL RNA, to enable translocation of a nascent polypeptide across a membrane (Estrozi et al., 2011)? Does rRNA play a role in the use of free energy derived from GTP hydrolysis by translation factors (Moore, 2012)? Will we ever reproduce early evolutionary states of the ribosome in the lab using rRNA alone?

Rule 5. So, Noncoding RNAs Are Not Scaffolds—*Then Telomerase, HOTAIR, 7SK, roX*

Although the idea that RNA might provide a scaffold to bring together multiple proteins into an active complex turned out not to apply to the ribosome, other ncRNAs have been found to function in this manner. One example is telomerase, the RNP enzyme that maintains the ends of linear eukaryotic chromosomes. Telomerase contains an RNA subunit, a portion of which serves as a template for synthesis of the telomeric DNA repeats (Greider and Blackburn, 1989) by the telomerase reverse transcriptase (TERT) subunit. But the template accounts for only a small portion of the RNA, and sequences that bind the TERT protein account for a bit more. What is the remainder of the RNA doing?

The 1,200 nt *Saccharomyces cerevisiae* telomerase RNA folds into a three-armed structure, with the template and TERT-binding regions forming a central core. At the end of each of the three arms is a binding site for a different accessory protein: the ever-shorter telomeres 1 protein, which recruits telomerase to chromosome ends; the Ku heterodimer, involved in nuclear localization of the RNP; and the seven-protein Sm ring, necessary for RNP stability. This arrangement provides a ''flexible scaffold'' in the sense that the sequence, the length, and the relative location of the RNA arms can vary as long as they maintain their protein-binding sites (Zappulla and Cech, 2004). Human telomerase RNA is also a scaffold, with the 5′-terminal domain containing the template and the TERT-binding elements, while two stem-loops in the 3′ half bind the dyskerin complex (for

nuclear localization) and TCAB1 (for Cajal body localization) (Tycowski et al., 2009; Venteicher et al., 2009; Egan and Collins, 2010).The much shorter *Tetrahymena* telomerase RNA (159 nt) forms a central core with TERT and p65, but the RNA is not known to provide binding sites for the accessory protein subunits; perhaps they are brought into the complex through protein-protein interactions (Jiang et al., 2013).

7SK is another example of a scaffold RNA. The trigger for RNA polymerase II to enter into productive elongation involves sequential phosphorylation of its carboxy-terminal domain, first at Ser5 and then at Ser2 of its multiple YSPTSPS repeats. One of the Ser2 kinases, P-TEFb, binds to the 331 nt 7SK snRNA. Other components of this RNP are a La-related protein that binds the RNA directly (LARP7), the methylphosphate capping enzyme (MePCE), which adds a monomethyl cap to the 5′ end of the RNA, and dimers of the HEXIM protein. Binding of HEXIM to 7SK RNA causes a conformational change, revealing P-TEFb-binding domains and inhibiting P-TEFb kinase activity (Yik et al., 2003). Release of P-TEFb by the Brd4 protein or by the HIV viral protein Tat allows P-TEFb to activate the expression of cellular and viral genes. Thus, the 7SK RNA scaffold is dynamic with respect to both its protein components and its allosteric effects.

Two ncRNAs, *roX1* (3.7 kb) and *roX2* (0.5 kb), are essential (but functionally redundant) for gene dosage compensation in *Drosophila* (Kageyama et al., 2001). These RNAs bind five proteins (MSL1, MSL2, MSL3, MLE, and the histone H4 acetyltransferase MOF) to form the MSL complex, which binds to hundreds of sites on the male X chromosome and increases transcription from X-linked genes. By providing a scaffold for arrangement of the MSL proteins, the *roX* RNA changes the conformation and/or activity of the complex (Deng and Meller, 2006).

Scaffolding is also an attractive function for HOTAIR RNA and lncRNAs more generally (see Rule 8 below). The multiple examples of RNA scaffolds lead to the question of "why use an RNA scaffold"? After all, cells contain protein scaffolds such as those that bind and organize the three sequentially-acting protein kinases involved in mitogen-activated protein kinase (MAPK) signaling. The answer to "why RNA"? might be "frozen accident," but more interesting possibilities come to mind. First, RNA tethers can be very long. A typical RNA "arm" of 50 base pairs (with bulges and internal loops) extends for 13 nm, whereas a 50-amino-acid alpha helix extends for 7.5 nm. Second, an RNA arm of 50 interrupted base pairs (100 nt) could easily bind multiple proteins, whereas a 50- or 100-amino-acid domain might bind a single protein partner. Thus, an RNA scaffold may have a selectable advantage over protein for many applications.

Rule 6. Gene Repressors Are Proteins—*Then MicroRNAs*

In their classic 1961 paper on the regulation of protein synthesis, Jacob and Monod ventured the notion that the Lac repressor might be "an RNA fraction" (Jacob and Monod, 1961). Instead, it turned out to be a polypeptide, and subsequently-discovered gene regulators fell into lockstep as the roster of protein regulators of transcription and translation expanded over the next decades. Real cracks in the armor first appeared in 1993 when the Ambros and Ruvkun labs announced the discovery of a short RNA that controls the timing of developmental transitions in *C. elegans* by base pairing to partially complementary sequences in the 3′-UTR of its target mRNA (Lee et al., 1993; Wightman et al., 1993). This RNA was considered an oddity until conservation of "heterochronic regulatory RNAs" and then hundreds of similar ~22 nt RNAs, now called miRNAs, in *Drosophila*, worm and human cells were reported (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

Meanwhile, adding to earlier indications of RNA-directed silencing activities in plants, the phenomenon of RNA interference (RNAi) was discovered in worms and trypanosomes (Fire et al., 1998; Ngô et al., 1998). Hints rapidly emerged that the cellular machinery for RNAi might be the same as that used in the miRNA pathway. Hamilton and Baulcombe (1999) made the connection by observing 20 to 25 nt pieces of RNA corresponding to plant genes undergoing posttranscriptional gene silencing after viral infection or upon introduction of exogenous gene copies. Next, an RNase III-like enzyme called Dicer was shown to generate RNAs of this size from long double-stranded RNAs (Hammond et al., 2000; Knight and Bass, 2001; Zamore et al., 2000) and then from miRNA precursors (Hutvágner et al., 2001). After assembling with an Argonaute (AGO) protein (Hutvagner and Simard, 2008) and other polypeptides to form an RNA-induced silencing complex (RISC), such short RNAs direct AGO to endonucleolytically cleave a perfectly complementary mRNA molecule, thereby silencing expression.

Cellularly encoded miRNAs, in contrast, are imperfectly complementary to their target mRNAs with sites most frequently within the 3′-UTR. Their action does not usually involve cleavage, but translational repression followed by decay of the mRNA (Bazzini et al., 2012; Béthune et al., 2012; Djuranovic et al., 2012). The precise molecular mechanisms of these processes have been surprisingly difficult to decipher. Both translational repression and deadenylation (Braun et al., 2011; Chekulaeva et al., 2011; Fabian and Sonenberg, 2012), which precede decapping and 5′- to 3-exonucleolytic decay of miRNA-targeted mRNAs, are orchestrated by the GW182 component (Braun et al., 2013) of RISC. Virtually every stage of translation has been reported to be miRNA-repressed. Indeed, a recent paper argues that RISC deposits a roadblock consisting of eIF4AII to inhibit the ribosome-scanning step of initiation (Meijer et al., 2013). Conversely, miRNA-induced translational activation has been reported in quiescent cells (Vasudevan et al., 2007) where a plausible scenario is that the repressive GW182 in RISC is conditionally replaced by a stimulatory factor.

The rules of engagement between a RISC-associated miRNA and its target mRNAs are still not fully defined. Most important are good base-pairing interactions with the 5′-most eight nucleotides of the miRNA (the seed sequence; Bartel, 2009), but even strongly predicted target sites require functional validation. With as many as a thousand different miRNAs encoded by mammalian genomes, each miRNA targets multiple mRNAs and most mRNAs are targeted by multiple miRNAs (Chi et al., 2009). Thus, collaborations between miRNAs bound to the same mRNA—like transcription factors—contribute importantly to

gene regulatory networks (Gurtan and Sharp, 2013). Much of the regulation by miRNAs may be fine-tuning (about a two-fold effect on protein production from a targeted mRNA), but some miRNAs have large switch-like effects under conditions of stress or disease (van Rooij et al., 2007).

The impact of an miRNA on gene expression clearly correlates with its abundance (from <1 to 50,000 molecules per cell), and there are seemingly endless ways to regulate miRNA abundance (Ameres and Zamore, 2013). All miRNAs are transcribed as longer primary (pri)-miRNAs, which can include other miRNAs and even protein-coding exons, in addition to spacer sequences. Most pri-miRNAs are cleaved in the nucleus by the Microprocessor complex (Gregory et al., 2004), which includes the RNase III enzyme Drosha (Lee et al., 2003) and its dsRNA-binding cofactor DGCR, to generate hairpin-shaped ~60 nt precursor (pre)-miRNAs that exit to the cytoplasm. There, they are processed by Dicer, creating two mature miRNAs that are usually differentially assembled into RISC. Not only is each of these steps in miRNA biogenesis subject to regulation (Kim et al., 2009), but there are multiple alternative miRNA processing pathways (Yang and Lai, 2011) perhaps designed to operate in different tissues or cell states. Some miRNAs are even reported to be processed from well-characterized ncRNAs, such as tRNAs or snoRNAs. Both precursors and mature miRNAs are subject to regulated degradation (Ameres and Zamore, 2013) often involving prior nucleotide addition or modification at the 3′ end.

Like protein regulators of gene expression, miRNAs contribute importantly to the control of developmental, differentiation and disease processes (Gurtan and Sharp, 2013). Not surprisingly, certain viruses have acquired and manipulated host miRNA genes to enhance infection (Skalsky and Cullen, 2010), while others harness specific host miRNAs for viral functions such as genome replication (Jopling et al., 2005). Novel therapeutics that alter miRNA levels or block function hold promise for combating a variety of disease states.

Another potential sphere of function for miRNAs is in transcriptional gene silencing, through DNA modification or deposition of repressive histone marks. The involvement of small RNAs in such phenomena is best characterized in plants and fission yeast (Volpe et al., 2002). In animals, another class of small RNAs (~27 nt) called piRNAs, which associate with AGO-related PIWI proteins, clearly act in this way not only to transcriptionally silence transposons in the male germline (Sabin et al., 2013) but also to regulate somatic development (Ross et al., 2014). Mammalian miRNAs do have a nuclear existence (Hwang et al., 2007) and could direct chromatin silencing at specific loci by base pairing to nascent transcripts. Reports of such activities (Benhamed et al., 2012) can be expected to increase.

Rule 7. At Least Bacterial Repressors Are Proteins—*Then Riboswitches, T Boxes, and sRNAs*

Countering their own proposal that genetic regulators might be RNA, Jacob and Monod reasoned that proteins might be better candidates for gene repressors because "the capacity to form stereospecific complexes with small molecules appears to be a privilege of proteins" (Jacob and Monod, 1961). Although elegant examples of RNA structure modulating bacterial transcription were subsequently described by Yanofsky, in the case of attenuation (Yanofsky, 1981), and Tomizawa, in the case of colicin E1 plasmid DNA replication (Tomizawa et al., 1981), these seemed exceptions rather than harbingers of a new "RNA rules" rule. Only in the last decade have we realized that RNA repressors and activators of bacterial transcription are extremely pervasive and highly varied.

Riboswitches, which directly bind small-molecule metabolites to regulate gene expression in *cis* (Gelfand et al., 1999; Nou and Kadner, 2000), rely on RNA's ability to form stereospecific complexes with small molecules, contradicting Jacob and Monod's reasonable skepticism. T-box riboswitches bind uncharged tRNAs to regulate gene expression in response to deficiency of particular amino acids. And finally sRNAs, antisense or small structured RNAs that regulate translation by intermolecular binding to mRNAs, illustrate additional models of regulation of RNA by RNA. More than in the previous ncRNA cases, bioinformatics and computational techniques have teamed up with genetics and biochemistry to blaze the trail of discovery of these riboregulators.

Riboswitches are RNA domains that regulate gene expression by switching from one structure to another upon binding a specific metabolite (McDaniel et al., 2003; Mironov et al., 2002; Winkler et al., 2002). To achieve gene repression, the metabolite-bound RNA forms a terminator stem-loop that causes transcriptional termination or sequesters the AUG start codon and/or Shine-Dalgarno sequence to inhibit translational initiation. To activate a gene, the metabolite-bound RNA forms a structure that ties up an intrinsic terminator stem-loop, preventing early termination of transcription, or frees up the sequences required for translational initiation. The RNA binds the specific metabolite whose concentration reports the need to regulate the gene; for example, binding of flavins (FMN and FAD) to the *rfn* box promotes transcriptional termination, repressing the expression of five genes that encode enzymes for riboflavin synthesis in *B. subtilis* and other Gram-positive bacteria.

Structural biology of riboswitches has revealed general features of ligand recognition (Batey et al., 2004; Serganov et al., 2009). First, the RNA folds into a very specific structure that forms a pocket for the metabolite. Yet, in the bound complex, the RNA completely encapsulates the metabolite, so the RNA must to some extent fold around the ligand instead of providing a rigid preformed binding pocket. Finally, the ligand in the binding pocket is recognized by an extensive array of hydrogen bonds from RNA bases and ribose sugars. The ability of specific RNA structures to bind specific small molecules was presaged by guanosine binding to Group I introns (Bass and Cech, 1984) and in vitro selection of aptamers that bound many different small molecules (Ellington and Szostak, 1990).

Additional twists on the simple riboswitch paradigm are being uncovered. For example, riboswitches can act as thermosensors instead of metabolite-sensors, so folding around a ligand is not always required (Johansson et al., 2002). Riboswitches can control expression of antisense RNAs in the Listeria pathogen, an example of two different riboregulatory elements working in concert (Mellin et al., 2013). Finally, in eukaryotes riboswitches have been found to control mRNA splicing (Cheah et al., 2007; Wachter et al., 2007).

T boxes differ from other riboswitches in that the ligand is not a small-molecule metabolite but rather an entire uncharged tRNA (Grundy and Henkin, 2003). T-box riboswitches occur in 5′-untranslated leaders of mRNAs encoding aminoacyl-tRNA sythetases and other proteins. In this case, the unbound RNA structure serves as the repressor of gene expression, forming a stem-loop that terminates transcription. When uncharged tRNA accumulates, it binds the T box at two sites: the tRNA anticodon pairs with a trinucleotide in the T box to recognize the particular tRNA, while the CCA acceptor end and T/D-loops of the tRNA bind and stabilize an antiterminator element, thereby preventing formation of the terminator stem-loop. As the end result, if the concentration of a particular amino acid is low, genes involved in synthesis or utilization of that amino acid are upregulated. Recent cocrystal structures of T-box elements and cognate tRNAs have revealed how a relatively small RNA element can specifically recognize a tRNA (Grigg and Ke, 2013; Zhang and Ferré-D'Amaré, 2013).

Several classes of small RNA regulators, or sRNAs, have been identified. The simplest are antisense RNAs transcribed from the opposite DNA strand as the mRNA that they regulate. In some, but not all cases, binding of the sRNA to its mRNA target requires the RNA chaperone Hfq (Møller et al., 2002; Soper and Woodson, 2008; Zhang et al., 2002). Thus, in a very general sense the bacterial sRNA repressors are analogous to miRNA and siRNA inhibitors of eukaryotic gene expression, where the inhibitory RNA-RNA base pairing requires the Ago proteins and formation of a RISC complex. Repression by sRNAs occurs by a number of mechanisms, including binding at or near the mRNA ribosome-binding site to block translation (Altuvia et al., 1998) or forming a target for RNase III cleavage and mRNA degradation (Krinke and Wulff, 1987). Furthermore, just as riboswitches are sometimes activators rather than repressors, sRNAs can activate translation by competing with the formation of inhibitory secondary structure elements (Morfeldt et al., 1995). This dual potential is a recurring theme in ncRNA regulation: RNA-RNA base pairing can inhibit interactions required for gene expression, or can just as easily block the inhibitory interactions and thereby activate gene expression.

Rule 8. Most Human Genes Encode Proteins—*Then lncRNAs*

Early work on transcription in mammalian cells identified hnRNA, a heterogeneous population of huge nuclear RNAs that were short-lived. The discovery of introns explained some of this RNA "dark matter," but just a fraction (Salditt-Georgieff and Darnell, 1982). Only in the last decade has extensive cataloging of these lncRNAs been accomplished, enabled by next-generation deep sequencing. For example, the ENCODE project identified 9,600 lncRNAs (>200 nt). Although the pendulum of scientific opinion has now swung away from the idea that much of this RNA could be "transcriptional noise" or junk RNA transcribed from junk DNA, our view is that it will take a decade of analysis of specific lncRNAs before this question is fully answered.

Reviewing the biological functions and mechanisms of lncRNAs is a daunting task for several reasons. New lncRNA papers are published daily, and entire new categories and paradigms are proposed annually. And although our human penchant for categorization drives a desire to assign individual functions to individual lncRNAs, a single 1 kb lncRNA is long enough to carry out a large number of functions with perhaps different subsets of these functions being active in different tissues and at different stages of development.

Dueling Polymerases

Arguably the simplest function for a lncRNA occurs when the act of transcription, rather than the RNA product, serves a regulatory function. Transcription from an upstream promoter can interfere with transcription factor loading at a downstream promoter, thereby repressing the downstream gene (Martens et al., 2005). Extending this paradigm, a pair of *cis*-interfering lncRNAs transcribed in opposite directions can provide a toggle switch to give variegated gene expression in yeast (Bumgarner et al., 2009). In mammals, similar events can occur over enormous genomic distances. The 118 kb "macro" lncRNA *Airn* induces imprinted silencing of the gene for insulin-like growth factor 2 receptor (Igf2r) simply because the *Airn* transcripts overlap with the *Igf2r* promoter (Latos et al., 2012), while the same ncRNA silences the *Slc22a3* gene by recruiting an H3K9 histone methyltransferase (Nagano et al., 2008).

Antisense RNA Base Pairing

Given the powerful specificity of complementary RNA-RNA base pairing and the rampant antisense transcription in mammalian cells, lncRNAs would seem to have great potential to target mRNAs by forming intermolecular hybrids. Not surprisingly, there have been many such proposals. The "acid tests" for base pairing in RNA are as follows: pairing between two sequences is considered proven if (1) during evolution, base changes at several positions in one partner are accompanied by compensatory changes in the other partner; (2) the two strands can be reversibly crosslinked in vivo by psoralen, which intercalates into duplex RNA regions; or (3) mutations designed to disrupt base pairing in either proposed partner are deleterious to function, but combining two deleterious mutations to restore complementarity also restores function. Such tests would provide strong validation of proposals for lncRNA-mRNA pairing.

Recruiting Histone-Modifying Complexes in cis

Xist, the X-inactive-specific transcript, is the grandmother of lncRNAs, not just because it was one of the first to be discovered (Brockdorff et al., 1992; Brown et al., 1992) but also because its biological function is so important and so dramatic. This ~17 kb RNA is expressed from only one of the two X chromosomes, coats that same chromosome, and triggers transcriptional silencing, thereby providing gene dosage compensation between female and male mammals. Xist RNA is involved in the recruitment of the Polycomb repressive complex 2 (PRC2) histone methyltransferase, which deposits the H3K27me3 mark and leads to transcriptional repression. In one model, a two-hairpin RNA motif within the RepA transcript of *Xist* directly binds PRC2 (Zhao et al., 2008). However, the recruitment of PRC2 does not always involve specific protein-binding motifs on a lncRNA. Promiscuous binding of PRC2 to thousands of RNAs has been suggested to allow it to survey for genes that have escaped repression and then to restore the repressed state (Davidovich et al., 2013).

Many lncRNAs, especially those present at only one or a few copies per cell, are thought to act "in *cis*" (i.e., at their site of

transcription) rather than diffusing to other loci ("in *trans*"). For example, a lncRNA has been reported to recruit and allosterically activate Fused in Sarcoma (FUS/TLS), inhibiting histone acetyltransferase activity and repressing transcription in *cis* (Wang et al., 2008). The 3.7 kb HOTTIP RNA recruits a protein complex that trimethylates K4 of histone H3 to activate the HOXA gene cluster (Wang et al., 2011).

Recruiting Transcriptional Regulators in trans *and Scaffolding*

As described for telomerase RNA, RNA structure is well suited to organize protein-binding motifs along an extended scaffold. HOTAIR RNA binds the H3K27 methyltransferase PRC2 and the H3K4 demethylase LSD1, both causing transcriptional repression (Tsai et al., 2010). Separate regions of the PCGEM1 lncRNA bind methylated androgen receptor and the PHD-domain protein PYGO2, which promotes chromatin looping, enhancing transcription at perhaps 2,000 AR-responsive genes (Yang et al., 2013). Indeed, hundreds of lncRNAs bind chromatin-modifying complexes such as PRC2 and affect gene expression (Khalil et al., 2009; Zhao et al., 2010). Possible molecular interactions targeting these *trans*-acting lncRNAs include lncRNA-mRNA base pairing and lncRNA-DNA triplex formation, the latter being proposed for the promoter-associated pRNA that recruits DNMT3b to silence rRNA genes (Schmitz et al., 2010).

Decoys for Proteins

This function for lncRNAs differs from those above in that the role of the RNA is not to recruit or organize proteins, but to inhibit their action. Early bacterial examples presaged the discoveries of lncRNAs with the same sort of "sponge" function in eukaryotes. For example, in *E. coli* the CsrB RNA molecule has multiple sites for binding the CsrA protein and negatively regulates its activity (Liu et al., 1997), and the abundant 6S RNA binds to the active site of RNA polymerases containing the sigma70 subunit to regulate transcription (Wassarman and Storz, 2000). Similarly, the mouse B2 RNA, a 178 nt ncRNA, binds directly to RNA polymerase II to repress transcription in response to heat shock (Espinoza et al., 2004). The ~600 nt Gas5 ncRNA binds directly to the DNA-binding domain of the glucocorticoid receptor, thus acting as a decoy and inhibiting glucocorticoid-regulated transcription in growth-arrested cells (Kino et al., 2010). Elements within the ~4,500 nt ecCEBPA RNA bind directly to the catalytic domain of DNA methyltransferase DNMT1; this interaction is thought to block local DNA methylation (Di Ruscio et al., 2013). Recently, circular lncRNA molecules have been discovered to be avid intracellular sponges for miRNAs, an extraordinary example of hierarchical regulation of one ncRNA by another (Memczak et al., 2013; Hansen et al., 2013).

Organizing Chromatin Domains, Loops, Chromosomes, and Nuclear Structures

Cases in which lncRNAs have been implicated in chromosome looping or pairing are numerous (Wang et al., 2011; Yang et al., 2013) and cannot be adequately summarized in a few sentences. A few examples highlight the range of interactions that have been observed. The DNA-binding protein CTCF forms a complex with the DEAD-box RNA helicase p68 and steroid receptor RNA activator (SRA). While the complex is necessary for CTCF's transcriptional insulator function, the role of the SRA RNA remains to be determined (Yao et al., 2010). A meiosis-specific ncRNA mediates the pairing of homologous chromosomes during fission yeast meiosis (Ding et al., 2012). The assembly of nuclear bodies called paraspeckles is seeded by the multiple endocrine neoplasia (MEN) ε/β (and its overlapping NEAT1) ncRNAs (Sasaki et al., 2009; Chen and Carmichael, 2009; Clemson et al., 2009; Mao et al., 2011).

Enhancer RNAs

The recent discovery of ncRNAs transcribed directly from transcriptional enhancer elements (eRNAs) (Kim et al., 2010; Ørom et al., 2010) may rewrite our understanding of transcriptional regulation in higher organisms. The textbook view has been that each enhancer DNA element binds a specific protein that engages in protein-protein interactions with the core promoter, forming a large DNA loop that allows the "distant" enhancer-binding protein to contribute to enhanced transcription at the core promoter. The emerging new model is that ncRNA transcribed from the enhancer itself binds the Mediator complex to bridge to the core promoter, locking in a stable transcription initiation process.

Rule 9. At Least the Genome Is Safe from RNA Intervention—*Then Scan RNAs and CRISPR*

As revolutionary as many of the new discoveries about ncRNAs have been, they have fallen in line with the general rule that RNA is downstream from DNA. That is, even though an ncRNA may affect the physical organization or expression of DNA, it does not alter the DNA itself. Admittedly, there have been a few exceptions—telomerase RNA is directly involved in telomeric DNA synthesis (see Rule 5), and Group II intron insertion occurs when the intron RNA uses its ribozyme activity to reverse-splice into a new DNA site where it is then reverse-transcribed by an intron-encoded enzyme (Yang et al., 1996). But few would have guessed that large-scale DNA elimination, genome rearrangement and genome editing could be mediated by RNA. Now, like the "rules" preceding it, the rule that RNA does not remodel DNA has been overturned.

Ciliates are unicellular eukaryotes with two kinds of nuclei within the same cell. Diploid micronuclei maintain and transmit the germline genome, while polyploid macronuclei provide most of the gene expression. During macronuclear development in *Tetrahymena*, about 15% of the genome (comprising internal eliminated sequences [IESs]) is specifically deleted. Scan RNAs, small dsRNAs produced by an RNAi-related mechanism, recognize the IESs in the developing macronucleus and target them for destruction (Mochizuki et al., 2002). Macronuclear development in *Oxytricha* is even more dramatic, involving destruction of 95% of the germline DNA, chromosome fragmentation and massive DNA rearrangement. A complete RNA copy of the somatic genome has been proposed to provide the template for these precise DNA rearrangements (Nowacki et al., 2008).

CRISPRs (clusters of regularly interspersed short palindromic repeats) are bacterial DNA elements that provide resistance against invading viruses and plasmids, a bacterial adaptive immunity system (Barrangou et al., 2007). The sequences between the repeats are copies of genetic material from previous invaders. The CRISPR elements are transcribed and processed into unit-sized RNAs, which then recognize any invading DNA of

the same sequence and lead to its cleavage by the CRISPR-associated Cas9 nuclease (Brouns et al., 2008). The bacterial "guide RNAs" and Cas9 nuclease have robust activity in *Drosophila* and worms and in human and mouse cells, and can be engineered to knockout or edit designated sites within genomes (Cong et al., 2013; Mali et al., 2013; Jinek et al., 2013). This new-found ability of RNA to remodel the mammalian genome is quickly becoming an invaluable research tool, and its pharmaceutical potential is already being investigated.

Finally, All Functions of Noncoding RNAs Are Known? *Not a Chance!*

Notwithstanding the fact that there are definable classes of ncRNAs that work by similar principles (e.g., tRNAs, riboswitches, miRNAs), it could be argued that every ncRNA studied has a different function. Certainly no two mammalian lncRNAs appear to have the same function. Thus, with perhaps 10,000 lncRNAs yet to be studied in the human genome alone, it seems safe to predict that many new functions of ncRNAs will be identified—perhaps thousands of functions. It may only be a matter of time before someone finds a lncRNA that binds a small-molecule metabolite, triggering self-cleavage and release of a bound histone methyltransferase, thereby repressing further transcription! The combinations and permutations of imaginable functions are endless.

In order to really understand ncRNA function, the systems biology of ncRNPs will need to mature. The human proteome contains hundreds of different RNA-binding proteins, each binding a few RNA nucleotides, so even a 200 nt ncRNA is likely to engage multiple RNA-binding proteins. In some cases the proteins will bind cooperatively, in other cases they will compete for overlapping binding sites. Biochemistry is good at examining one-protein-one-RNA interactions and can even be stretched to examine several proteins at a time. Biochemistry cannot deal with 1,000 purified proteins. Transcriptome-wide approaches such as CLIP-seq, on the other hand, always interrogate interactions within the full complexity of the cell. The challenge here is that these technologies reveal what is happening, but not how and why. New experimental and computational approaches are needed to understand RNPs as dynamic systems. And who knows what rules will be overturned then?

ACKNOWLEDGMENTS

We thank colleagues who commented on various sections of this article, including R. Batey, E. Guo, E. Lund, P. Moore, T. Steitz, K. Tycowski, and M. Xie. Editorial assistance from A. Miccinello and L. Konyha is much appreciated. Limitation of the number of references precluded us from citing a great many important contributions, and we appreciate the understanding of colleagues whose work could not be cited. The authors are investigators of the Howard Hughes Medical Institute.

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