

OPINION

Non-coding-RNA regulators of RNA polymerase II transcription

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Abstract | Several non-coding RNAs (ncRNAs) that regulate eukaryotic mRNA transcription have recently been discovered. Their mechanisms of action and biological roles are extremely diverse, which indicates that, so far, we have only had a glimpse of this new class of regulatory factor. Many surprises are likely to be revealed as further ncRNA transcriptional regulators are identified and characterized.

A new paradigm has emerged in biology in which RNA molecules are active participants in regulating, catalysing and controlling many reactions that define fundamental processes in cells — roles that in the not-so-distant past had been reserved for proteins. In general, RNAs that have such regulatory functions do not encode a protein and are therefore referred to as non-coding RNAs (ncRNAs). Eukaryotic ncRNAs are transcribed from the genome by one of three nuclear, DNA-dependent RNA polymerases (Pol I, II or III). They then elicit their biological responses through one of three basic mechanisms: catalysing biological reactions, binding to and modulating the activity of a protein, or base-pairing with a target nucleic acid. For the first two mechanisms, ncRNAs fold into unique higher-order structures that are required for their function, much like a protein (FIG. 1). Many ncRNAs become integral parts of large complexes that contain proteins, and possibly other RNAs, and the components of the complex function together as a unit (for example, the ribosome). ncRNAs have been shown to participate actively in many of the diverse biological reactions that encompass gene expression, such as splicing, mRNA turnover, gene silencing and translation¹. Notably, several studies have recently revealed that ncRNAs also actively regulate eukaryotic mRNA transcription, which is a key point for the control of gene expression.

In eukaryotic cells, mRNA transcription is an intricate process that is tightly and

temporally regulated in response to numerous signals, and involves the orchestrated interplay of DNA, many protein components and the RNA transcript itself (FIG. 2). The transcription reaction is catalysed by the Pol II enzyme, which synthesizes an mRNA copy of each protein-coding gene. Eukaryotic DNA and histones are packaged into a highly condensed structure that is known as chromatin, and the structure of chromatin dynamically participates in regulating mRNA transcription². The DNA elements that control transcription are contained in promoters. Promoters are the regions of genes that contain the sequences that bind to transcriptional activators and repressors³, as well as to the Pol II general transcription factors that are thought to be required for the transcription of all genes⁴. A diverse set of co-regulators mediates activation and repression, often bridging

contacts between regulators and the general transcription machinery⁵. All of these components function together to set the level of transcription for each gene, which provides nearly limitless targets for regulation.

Tellingly, the ncRNA transcriptional regulators that have been identified so far target many different components of the transcription reaction and elicit their effects through unique mechanisms. We believe that ncRNAs carry out many wide-ranging roles in regulating eukaryotic transcription that are yet to be discovered. The following discussion highlights the natural ncRNAs that have been discovered so far that control Pol II transcription *in trans* by targeting transcriptional activators and repressors, general factors, Pol II itself or chromatin (BOX 1). Some of these ncRNAs are familiar friends with recently recognized functions, whereas others are new acquaintances. It is our view that many surprising revelations await as the budding field of ncRNA regulators of Pol II transcription further unfolds.

Targeting activators and repressors

Transcriptional control results from the actions of activator and repressor proteins that bind in a sequence-specific manner to the promoters of genes³. Achieving transcriptional regulation in cells requires that activators and repressors interact with other factors including co-activators, co-repressors, the general transcription factors and Pol II (REF. 5). Beyond these interactions, transcriptional regulation can also be influenced by cell type and the intracellular localization of activators and repressors,

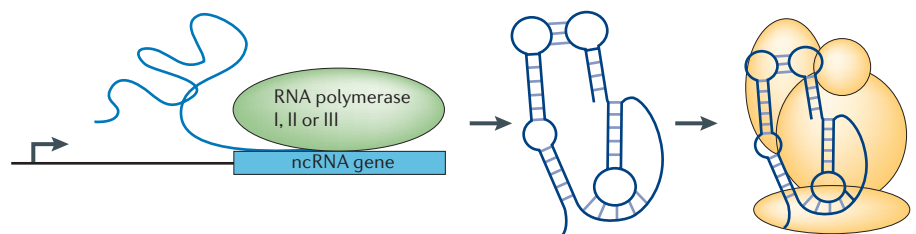


Figure 1 | The structure of a non-coding RNA is crucial for its function. Non-coding RNA (ncRNA) genes can be transcribed by either RNA polymerase I, II or III, depending on the individual ncRNA. ncRNAs fold into specific structures that impart a function to the molecule. Often these RNAs are incorporated into large complexes (yellow) that contain proteins and sometimes other nucleic acids. These complexes then regulate biological reactions, and their function is strictly dependent on the presence of the ncRNA.

which is ultimately controlled by extracellular stimuli and signalling pathways. As described below, the four eukaryotic ncRNAs that are known to target activators and repressors elicit transcriptional control using distinct mechanisms: by functioning as a co-activator, by changing the regulatory properties of a repressor, by regulating the oligomerization and activity of an activator, and by controlling the intracellular localization of an activator. The ncRNAs that are discussed are also diverse with respect to their size, tissue-specific expression pattern and biological function. The identification of these ncRNAs has both answered important biological questions about transcriptional control and stimulated more questions about the potential breadth of ncRNAs in targeting the activators and repressors of mRNA transcription.

SRA functions as a co-activator. The steroid receptor RNA activator (SRA) is a natural ncRNA that functions as a transcriptional co-activator for several steroid-hormone receptors⁶. The ~700-nucleotide SRA was identified in a yeast two-hybrid screen for factors that would interact with a domain of the human progesterone receptor and activate transcription. Interestingly, the initial characterization of SRA presumed that the factor was a protein. However, experiments ultimately showed that the factor functioned as an ncRNA. SRA operates as part of a ribonucleoprotein complex. Importantly, the RNA itself is required for the complex to co-activate transcription by various nuclear hormone receptors⁶ (FIG. 2). More recent structural-modelling and deletion studies have indicated that distinct sequence motifs that are spread throughout SRA are required for it to co-activate transcription⁷. The mechanism by which this occurs is not fully understood. If SRA truly functions as an ncRNA transcriptional co-activator, it probably makes direct contacts with components of the general transcription machinery, thereby connecting nuclear hormone receptors to the core promoter.

NRSE RNA changes a repressor. A small ncRNA — neuron-restrictive silencer element double-stranded RNA (NRSE dsRNA) — has been found to activate neuronal gene expression in stem cells. It modulates the activity of a transcriptional repressor and thereby stimulates stem cells to differentiate into neurons⁸. NRSE dsRNA is an ~20-base-pair dsRNA. It was identified because of its sequence complementarity to a promoter element that

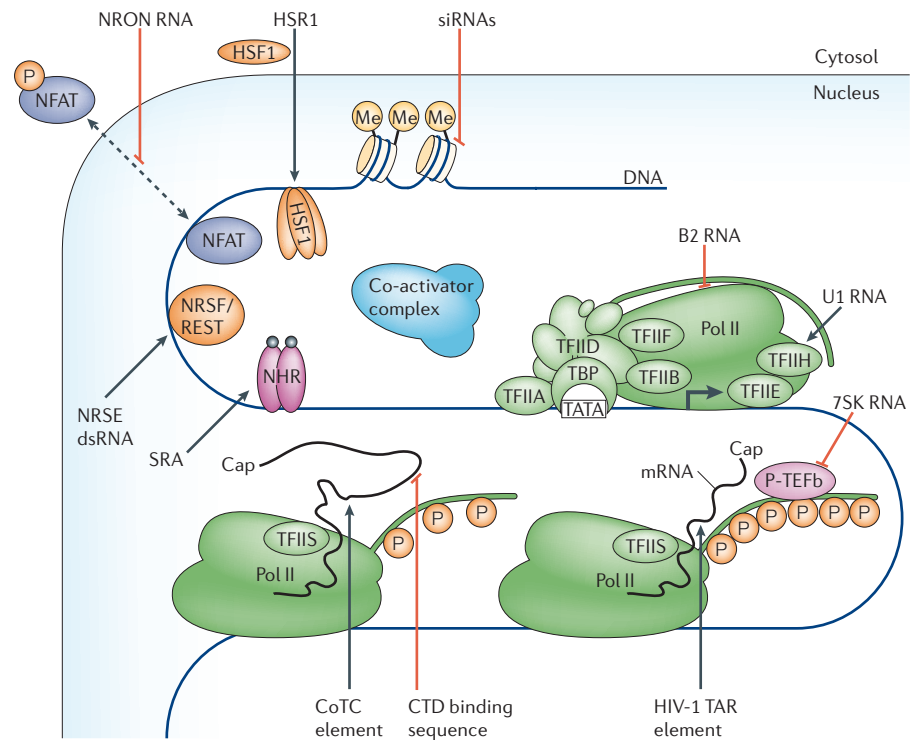


Figure 2 | Eukaryotic RNA polymerase II transcription is a complex process that can be regulated at many points. This schematic figure illustrates how chromatin structure, many protein regulatory factors, RNA polymerase II (Pol II) and the mRNA transcript itself all work together to set the level of transcription for a given gene. The Pol II transcription reaction involves many sequential steps, some of which are depicted by the polymerase binding to the promoter (represented by TATA), moving away from the transcriptional start site, and continuing through the elongation phase of the reaction. Natural non-coding RNAs (ncRNAs) have been shown to target many different participants in the reaction. The black arrows and red blocking arrows highlight the natural ncRNAs that are known to regulate Pol II transcription, the factors they target and whether the effect of the ncRNA on transcription is stimulatory or inhibitory, respectively. CoTC, co-transcriptional cleavage; CTD, C-terminal domain; HIV-1 TAR, human immunodeficiency virus-1 transactivation response; HSF1, heat-shock-factor protein-1; HSR1, heat-shock RNA-1; Me, methyl group; NFAT, nuclear factor of activated T cells; NHR, nuclear hormone receptor; NRON, non-coding repressor of NFAT; NRSE dsRNA, neuron-restrictive silencer element double-stranded RNA; NRSF/REST, neuron-restrictive silencing factor/RE-1-silencing transcription factor; P, phosphoryl group; P-TEFb, positive transcription-elongation factor-b; siRNA, short interfering RNA; SRA, steroid receptor RNA activator; TATA, TATA box; TBP, TATA-binding protein; TF, transcription factor.

binds to the repressor protein NRSF/REST (neuron-restrictive silencing factor/RE-1-silencing transcription factor) in order to silence the transcription of neuron-specific genes in non-neuronal cells. NRSE dsRNA does not seem to function by base pairing with the promoter element with which it shares sequence homology, but, rather, it functions through a direct RNA–protein interaction between itself and the repressor NRSF/REST⁸ (FIG. 2). This interaction activates the neuron-specific genes that still have NRSF/REST bound to their promoter, and effectively converts the repressor into a transcriptional activator. It has been proposed that NRSE dsRNA prevents NRSF/REST from associating with its known co-repressor proteins⁸.

HSR1 triggers activator trimerization. Heat-shock RNA-1 (HSR1) is a human ncRNA that mobilizes the transcriptional activator heat-shock-factor protein-1 (HSF1) in response to heat shock⁹. HSF1 exists as a monomer until cells are stressed, at which point the protein trimerizes. Trimeric HSF1, but not monomeric HSF1, binds to the promoters of heat-shock-induced genes and activates transcription. HSR1 significantly stimulates HSF1 trimerization and DNA binding⁹ (FIG. 2). HSR1 does not work alone, but, instead, partners with a well-known translation factor, the protein eEF1A (eukaryotic translation-elongation factor-1A). The fate of HSR1 after HSF1 trimerization is not yet known, but one intriguing possibility is that

Box 1 | Non-coding RNAs that target chromatin to silence mRNA transcription

Non-coding RNAs (ncRNAs) can trigger transcriptional silencing by regulating chromatin structure. The earliest characterized example of ncRNA-mediated transcriptional silencing in eukaryotes was the transcriptional inactivation of the second X chromosome in females (dosage compensation). This involves a rather large ncRNA known as X-inactive-specific transcript (Xist). The presence of Xist on the X chromosome is crucial for maintaining the inactive state, but its precise mechanism of action is unclear²⁵.

More recently, short ncRNAs have been shown to target the structure of chromatin to silence gene expression, in addition to triggering mRNA degradation and blocking translation²⁶. The ncRNAs that are involved are typically 21–28 nucleotides in length, are collectively referred to as microRNAs (miRNAs) and short interfering RNAs (siRNAs), and function by targeting homologous sequences in genes²⁶. RNA-mediated transcriptional silencing was initially characterized in *Arabidopsis thaliana*, was later shown to occur in *Schizosaccharomyces pombe* and *Tetrahymena thermophila*, and was most recently found in human cells, which shows that this mechanism of controlling mRNA transcription is conserved²⁶. Transcription is blocked when siRNAs, as part of a larger protein complex, are directed to homologous sequences in genes, although the precise ways in which siRNAs are guided to, and gain access to, genomic DNA remain unknown. This results in the recruitment of factors such as DNA methyltransferase and histone methyltransferase that methylate DNA and histone proteins, respectively, which results in the silencing of transcription from the targeted regions of the genome²⁶. siRNAs can therefore direct the gene-specific regulation of chromatin modifications, and thereby control transcription (FIG. 2).

this ncRNA associates with HSF1 trimers at the promoters of genes and has a direct role in transcriptional activation. These recent findings have revealed a novel functional convergence of a translation factor, an ncRNA and a transcription factor.

NRON RNA regulates nuclear trafficking.

A cell-based screen for ncRNAs that could modulate the activity of the transcriptional activator NFAT (nuclear factor of activated T cells) identified NRON (non-coding repressor of NFAT)¹⁰. NRON is much larger than most regulatory ncRNAs. Northern-blot analysis revealed transcripts that ranged from 2–4 kilobases in length, probably due to alternative splicing. Expression of NRON is highly tissue specific and it is enriched in lymphoid tissues, which is consistent with its role in modulating NFAT¹⁰. Uniquely, NRON does not target the transcriptional-activation properties of NFAT, but, instead, seems to regulate its nuclear localization. In response to extracellular signals, NFAT is dephosphorylated and localized to the nucleus. NRON, probably through interactions with various nuclear transport factors, disrupts the nuclear localization of NFAT, thereby preventing it from activating transcription¹⁰ (FIG. 2). Further experiments are now required to understand the mechanism by which NRON functions and to reveal the diversity of ncRNAs that might control the nuclear localization of transcription factors.

Targeting general factors

General factors are thought to be required for the transcription of all genes. These

include the transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, which facilitate the formation of functional initiation complexes with Pol II at promoters¹¹. It also includes factors such as TFIIS and P-TEFb (positive transcription-elongation factor-b), which facilitate transcript elongation¹². One conundrum regarding an ncRNA that targets a general factor is whether it affects the transcription of all genes or functions in a gene-specific fashion by targeting a general factor at only selected promoters. Two natural ncRNAs that target general factors have been identified so far. Furthermore, non-coding

regions of the primary transcript itself can regulate elongation (BOX 2).

U1 RNA stimulates initiation. In a search for human ncRNAs that associate with general transcription factors, an ~160-nucleotide RNA was found to co-purify with the multisubunit transcription factor TFIIH¹³ (FIG. 2). Sequencing revealed that this band was U1 RNA, a small nuclear ncRNA that is transcribed by Pol II and has a prototypical function in splicing. Further biochemical experiments showed that U1 RNA binds directly and specifically to the cyclin-H subunit of TFIIH¹³. Crosslinking followed by tryptic digestion and mass spectrometry were used to map the regions of U1 RNA and cyclin H that interact¹⁴. U1 RNA can stimulate both transcription initiation by Pol II *in vitro* and the kinase activity of TFIIH, which phosphorylates the C-terminal domain (CTD) of the large subunit of Pol II (REF. 13). However, exactly how U1 RNA regulates transcription in cells remains to be determined. It is possible that U1 RNA functions as a general regulator of TFIIH function at all promoters, perhaps by linking transcription and splicing.

7SK RNA modulates elongation. 7SK RNA is an abundant 331-nucleotide ncRNA that is transcribed by Pol III. It co-purifies with the elongation factor P-TEFb and represses transcript elongation by Pol II (REFS 15,16) (FIG. 2). There are two classes of P-TEFb in human cells. 7SK-free P-TEFb functions as

Box 2 | Regulation of RNA polymerase II transcription by the mRNA transcript

It has long been known that some mRNA transcripts can regulate their own transcription in bacteria, often by forming distinct structures (for example, stem-loops) that bind to protein regulators to control elongation and termination²⁷. This phenomenon, which can be thought of as regulation in *cis*, has not yet been widely observed in eukaryotic transcription. However, in the few characterized examples, the non-coding regions of primary transcripts regulate the process of RNA polymerase II (Pol II) transcription by acting in *cis*.

Pol II transcription of the human immunodeficiency virus-1 (HIV-1) genome after viral infection is regulated by a transactivation response (TAR) element — a 59-nucleotide sequence in the 5' untranslated region of HIV-1 transcripts that forms a stem-loop structure^{28,29} (FIG. 2). The viral transactivator (Tat) protein is a transcriptional activator that recognizes and binds to the structured TAR element, and this protein–RNA interaction is necessary for inducing the high levels of viral transcription that are required for infection^{30,31}.

Two more recent examples of the mRNA transcript regulating Pol II transcription in *cis* involve sequences at the 3' end of the RNA. In the first example, the 3' end of the human β -globin pre-mRNA contains the co-transcriptional cleavage (CoTC) element, a sequence that folds into an autocatalytic structure and cleaves itself, which is crucial for efficient transcription termination *in vitro* and *in vivo*³² (FIG. 2). In the second example, the C-terminal domain (CTD) of Pol II was found to bind sequence specifically to the mRNA transcript downstream of a polyadenylation signal to suppress 3'-end formation and transcription termination³³ (FIG. 2). Similar to the non-coding RNAs that regulate Pol II transcription in *trans*, it is likely that these examples of *cis* regulation by the mRNA transcript provide glimpses of a broader and more diverse way of controlling mRNA transcription.

a general and HIV-1-specific transcription-elongation factor, whereas 7SK-bound P-TEFb is inactive. This is because 7SK RNA represses the CTD kinase activity of P-TEFb, which blocks its ability to stimulate elongation¹⁵. This repression involves another protein known as HEXIM1 (hexamethylene bisacetamide-induced protein-1) that interacts with 7SK-bound P-TEFb¹⁷. Various cell-stress conditions, such as treatment with actinomycin D and DRB (5,6-dichloro-1- β -D-ribofuranosylbenzimidazole), lead to the dissociation of 7SK and HEXIM1 from P-TEFb. This results in the derepression of the P-TEFb kinase and the stimulation of Pol II elongation¹⁷.

Targeting Pol II

The Pol II core enzyme contains 12 protein subunits. ncRNAs that directly target Pol II could either be general transcriptional regulators or gene-specific regulators that are recruited to selected promoters to modulate polymerase activity. The single natural ncRNA that is known to target Pol II is a general repressor of transcription, although its effect in cells is probably controlled in a gene-specific fashion. Synthetic RNAs that target Pol II and regulate its activity have also been identified. These synthetic RNAs have been used as tools to study the transcription reaction and the regulation of Pol II by ncRNAs that function *in trans* (BOX 3). Together the studies of these natural and synthetic RNAs have led us to propose a model in which many ncRNAs can bind directly to a common RNA-docking site on Pol II to regulate transcription at numerous steps.

B2 RNA represses transcript synthesis. We found that B2 RNA, an ~178-nucleotide mouse ncRNA, binds directly to core Pol II (FIG. 2) and represses transcription from specific genes after heat shock^{18,19}. B2 RNA is transcribed from a family of short interspersed elements (SINEs), which are retrotransposons that are abundantly scattered throughout the mouse genome. The transcription of B2 SINEs by Pol III is upregulated following various cellular stresses²⁰. During the heat-shock response, the newly transcribed B2 RNA represses the transcription of genes that encode, for example, actin and hexokinase II (REF. 18). *In vitro*, B2 RNA binds to Pol II, assembles into stable pre-initiation complexes at promoters and blocks RNA synthesis¹⁹. This work has provided a mechanism that explains the long-known observation that general Pol II transcription is repressed

BOX 3 | Using RNA aptamers to control RNA polymerase II transcription

With the goal of developing tools to probe the mechanisms of transcription, various synthetic RNA aptamers have been selected for their ability to bind to transcriptional regulators, general factors and RNA polymerase II. The most common targets for the selection of RNA aptamers have been transcriptional-regulatory proteins, such as the transactivator Tax from human T-cell leukaemia virus³⁴, the zinc-finger protein Wilms' tumour protein-1 (WT1)³⁵, the architectural protein T-cell factor (TCF)³⁶ and the activator nuclear factor- κ B³⁷. The selected RNA aptamers have proven to be powerful tools for probing the mechanism by which these regulatory proteins control transcription *in vitro* and *in vivo*, as well as for understanding how non-coding RNAs interact with proteins that control transcription. RNA aptamers that bind to the general transcription factor TATA-binding protein (TBP) have also been selected³⁸. TBP is involved in transcription by all three nuclear eukaryotic RNA polymerases (I, II and III), and experiments with these aptamers have shown that the DNA-binding surface of TBP is used differently in transcription by all three polymerases, particularly at the initiation and reinitiation stages of transcription³⁹. Uniquely, aptamers have also been selected for their ability to activate transcription when tethered to a promoter by a fusion protein that contains a DNA-binding domain and a bacteriophage coat protein (MS2) that binds to a specific RNA⁴⁰. Although aptamers have been used to a limited degree to study transcription in the past, we believe that their value as novel tools for understanding the mechanisms of transcriptional regulation will be appreciated now that non-coding RNAs are known to be involved in the control of eukaryotic transcription.

following heat shock. However, how heat-shock-induced genes are transcriptionally activated in the presence of the B2 RNA repressor remains to be determined. We believe these studies open the door for the discovery of other natural ncRNAs that bind to Pol II and regulate transcription in response to diverse biological stimuli. Moreover, ncRNA transcriptional regulators that function by binding directly to an RNA polymerase are not limited to eukaryotes. In bacteria, 6S RNA binds to RNA polymerase *in trans* to regulate gene expression as cells enter stationary phase²¹.

Structure of an aptamer–Pol-II complex.

FC RNA was identified in a screen for synthetic RNA aptamers that bind to *Saccharomyces cerevisiae* Pol II (REF. 22). This aptamer was subsequently found to repress transcription by *S. cerevisiae* Pol II, but not by *Schizosaccharomyces pombe* or wheatgerm Pol II (REF. 22). Recently, a crystal structure of FC RNA bound to *S. cerevisiae* Pol II reveals how this aptamer inhibits transcription²³. The crystal structure shows that FC RNA forms a double stem-loop in the DNA-binding cleft of Pol II, which is normally occupied by promoter DNA. It was therefore proposed that FC RNA interferes with the formation of open complexes (that is, Pol II complexes containing DNA that is melted around the transcription start site)²³. Further biochemical studies will be required to confirm that open-complex formation is indeed inhibited. In this work, competition binding experiments with FC RNA and mouse B2 RNA indicated that the two ncRNAs can bind to overlapping

sites on *S. cerevisiae* Pol II, which led to the proposal that B2 RNA might inhibit transcription by a mechanism similar to that used by FC RNA²³.

An ncRNA-docking site on Pol II. The first RNA that was found to bind to a mammalian Pol II with high affinity and specificity was not B2 RNA but, rather, a synthetic RNA. We found that a 20-nucleotide RNA that was composed entirely of guanines (20G-oligo) bound tightly ($K_d \leq 1$ nM) and specifically to human Pol II and potently inhibited a specific step during early transcription²⁴. Surprisingly, when the 20G-oligo was shortened to 10 nucleotides, it still bound tightly to human Pol II but no longer inhibited transcription²⁴. In other words, high-affinity binding could be uncoupled from transcriptional inhibition. We believe that there is a high-affinity RNA-docking site (that is distinct from the RNA-exit channel) on human Pol II and that, following docking, the 20G-oligo represses transcription through interactions with a separate site on the polymerase.

This idea led to the prediction that natural ncRNAs would bind to the high-affinity RNA-docking site on the polymerase and inhibit transcription, and ultimately led to the identification of B2 RNA as such an inhibitor. Indeed, B2 RNA binds with high affinity ($K_d < 1$ nM) to human Pol II, evidently to the same site that the 20G-oligo binds, although, interestingly, these two ncRNAs repress transcription by targeting different steps of the reaction¹⁹. We propose that the binding of B2 RNA or the 20G-oligo to the high-affinity docking

site tethers the ncRNA to the polymerase. This subsequently allows a region(s) of the ncRNA that is not necessarily involved in the initial binding to interact with a distinct area of the polymerase to repress transcription. The inhibitory regions of B2 RNA and the 20G-oligo probably target two different areas of the polymerase and thereby repress transcription by two different mechanisms. Based on our observations, we speculate that other natural ncRNAs will bind to the high-affinity RNA-docking site on mammalian Pol II and either inhibit or activate transcription using distinct mechanisms in a regulated manner. Curiously, B2 RNA and FC RNA do not bind to *S. cerevisiae* Pol II with a particularly high affinity (~35 nM)²³, so it is our opinion that *S. cerevisiae* Pol II either does not contain a high-affinity RNA-docking site or that B2 RNA and FC RNA do not associate with this site. The interactions that dictate the high-affinity binding of B2 RNA and the 20G-oligo to human Pol II are therefore yet to be identified.

“...the complexity and breadth of ncRNA transcriptional regulators will begin to be revealed, which will create a new frontier in our understanding of the control of eukaryotic mRNA transcription.”

Conclusions

Although the number of ncRNA regulators of eukaryotic mRNA transcription that have been identified so far is small, their mechanisms of action are widely diverse. We believe that researchers have uncovered only the tip of the iceberg. Given the complexity of eukaryotic transcriptional regulation, the number of potential targets for ncRNAs is virtually limitless. It is likely that many more ncRNAs will prove to be active participants in controlling mRNA transcription, and will therefore affect many biological processes. For example, we wonder whether some of the hundreds of recently identified mammalian microRNAs and short interfering RNAs might associate with transcriptional activators, repressors and the general transcription machinery to regulate mRNA transcription. As more researchers consider the potential for ncRNAs to regulate transcription in many diverse systems, the complexity and breadth

of ncRNA transcriptional regulators will begin to be revealed, which will create a new frontier in our understanding of the control of eukaryotic mRNA transcription.

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Competing interests statement

The authors declare no competing financial interests.

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