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Review

Hairpin RNA: a secondary structure of primary importance

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Abstract. An RNA hairpin is an essential secondary structure of RNA. It can guide RNA folding, determine interactions in a ribozyme, protect messenger RNA (mRNA) from degradation, serve as a recognition motif for RNA binding proteins or act as a substrate for enzymatic reactions. In this review, we have focused on *cis*-acting RNA hairpins in metazoa, which regulate histone gene expression, mRNA localization and translation. We also review evolution, mechanism of action and experimental use of *trans*-acting microRNAs, which are coded by short RNA hairpins. Finally, we discuss the existence and effects of long RNA hairpin in animals. We show that several proteins previously recognized to play a role in a specific RNA stem-loop function in *cis* were also linked to RNA silencing pathways where a different type of hairpin acts in *trans*. Such overlaps indicate that the relationship between certain mechanisms that recognize different types of RNA hairpins is closer than previously thought.

Keywords. dsRNA, miRNA, RNAi, stem-loop, hairpin, localization, translation.

Introduction

An RNA hairpin consists of a double-stranded RNA (dsRNA) stem, often containing mismatches and bulges (i.e. unpaired sequences within the stem), and a terminal loop. It is the most common secondary structure found in almost every RNA folding prediction. RNA hairpins originate by two mechanisms: (i) transcription by DNA-dependent RNA polymerase of an inverted repeat DNA resulting in the RNA folding into a stem-loop structure, and (ii) an RNA molecule formed as a folded-back template for RNA-dependent RNA polymerase, which synthesizes the second strand of the stem. This review will focus exclusively on the first case – RNA hairpins folded within RNA transcripts. The second mechanism, which produces perfect long dsRNA hairpins, is not widespread in nature and is probably restricted to a 'copy-back' mechanism of replication in certain viruses [1]. It is difficult to classify RNA hairpins into distinct categories because these structures easily arise and differ in many aspects. Structurally, RNA hairpins can occur in different positions within different types of RNAs; they differ in the length of the stem, the size of the loop, the number and size of bulges, and in the actual nucleotide sequence (Fig. 1a). These parameters provide an extreme variability allowing specific interactions with proteins (discussed in detail in [2]). Functionally, RNA hairpins can regulate gene expression in *cis* or *trans*, i.e. an RNA hairpin within an RNA molecule can regulate just that molecule (*cis*) or it can induce effects on other RNAs or pathways (*trans*). Hairpins serve as binding sites for a variety of proteins, act as substrates for enzymatic reactions as well as display intrinsic enzymatic activities.

Many of the pathways utilizing and/or responding to RNA hairpins have evolved independently and are not linked to others. It is not possible to provide a systematic and complete review of such a broad topic as the biology of hairpin RNAs in a single article. We have therefore decided to provide an insight into the specific roles of RNA hairpins in animals. The rationale for this combination is that we want to highlight some common factors for both

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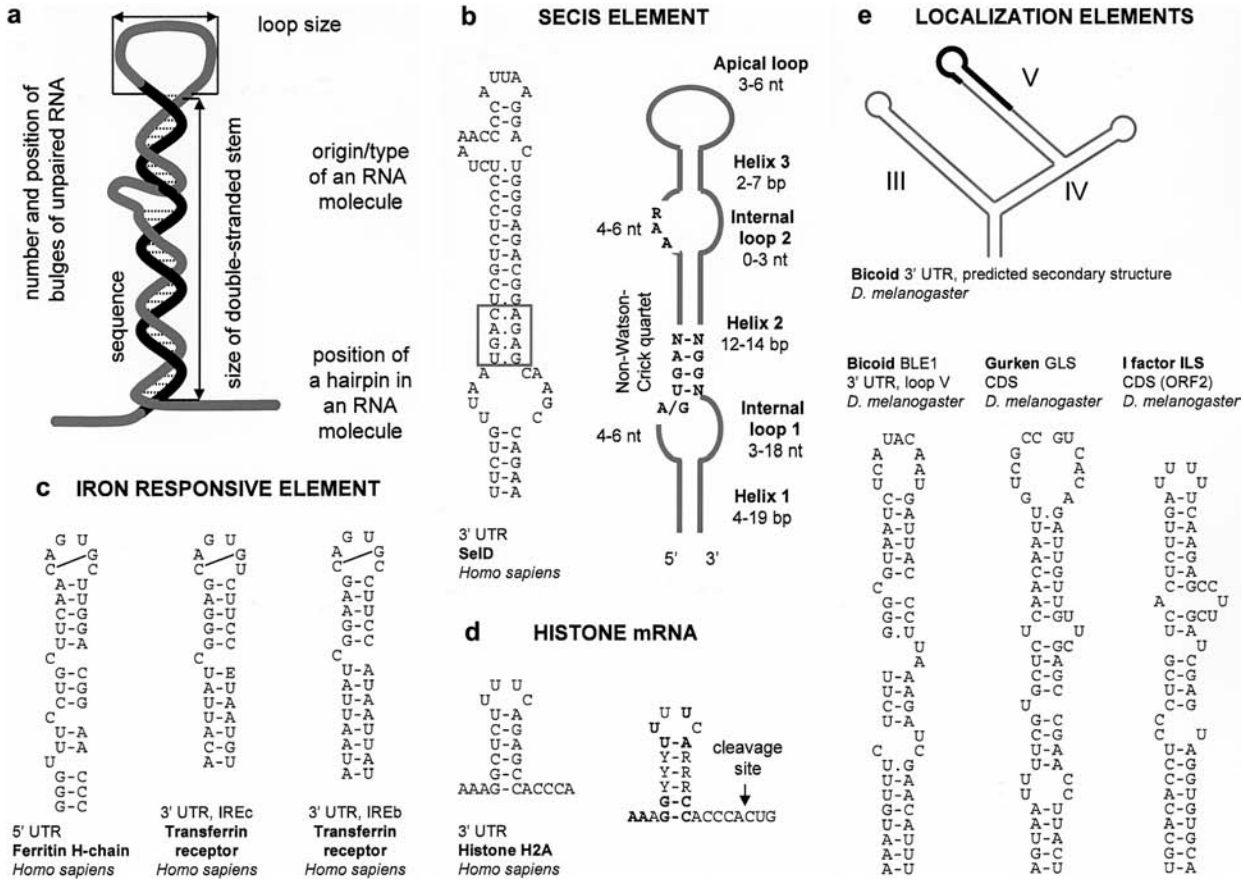

Figure 1. RNA stem-loops. (*a*) A schematic overview of an RNA stem-loop depicting the important parameters for the role of such a hairpin RNA. (*b*) The SECIS stem-loop structure element controlling selenoprotein synthesis. Right: A consensus of a secondary structure of a SECIS element [5]. Left: A specific example of the SECIS element in *Homo sapiens* [143]. (*c*) Examples of iron-responsive elements (IRE) stem-loop structures regulating iron metabolism in humans [144, 145]. (*d*) A histone mRNA stem-loop, which controls processing and expression of replication-dependent histone genes. Right: Consensus sequence. Absolutely conserved nucleotides are shown in boldface [8]. Left: A specific example of a histone stem-loop in *Homo sapiens*. (*e*) RNA hairpins in *bicoid*, *gurken* and *I*-factor mRNAs regulating mRNA localization in *Drosophila* oocytes [24, 35]. Top: Schematic view of modular architecture of *bicoid* localization signal consisting of three stem-loops (designated III, IV and V) implicated in the *bicoid* localization element (BLE). Region labeled in black is necessary and sufficient for transport of *bicoid* mRNA and its initial accumulation in the anterior part of *Drosophila* oocytes [24].

cis- and *trans*-acting RNA hairpins among the wide spectrum of possible biological effects.

This review is organized into three main sections discussing (i) short RNA hairpins acting in *cis*, (ii) short RNA hairpins acting in *trans* (microRNA pathway) and (iii) long RNA hairpins (stem >100 bp).

Short RNA hairpins acting in *cis*

Hairpin structures differ in size and localization within a messenger RNA (mRNA) sequence. Several examples of well-studied stem-loop structures and their functions in metazoa are shown in Figure 1. Short hairpins in mRNA are involved in diverse processes such as specific subcellular localization of specific mRNAs, regulation of translation and mRNA stability. Stem-loops also function in initiation of translation via internal ribosomal entry sites [3], and are involved in viral replication [4]. The SECIS element found in selenoprotein 3′ mRNA (Fig. 1b), which controls synthesis of selenoproteins (reviewed in [5]), represents another specific stem-loop structure. It serves as a binding site for the SECIS-binding protein 2 (SBP2) involved in mediating UGA redefinition from a stop codon to selenocysteine. Iron-responsive element (IRE, Fig. 1c) is another example of a stem-loop structure acting in *cis* (reviewed in [6]). Cellular iron homeostasis is maintained by IRE-dependent posttranscritpional regulation of mRNA of the transferrin receptor, ferritin and other mRNAs that mediate iron uptake and storage. Post-transcriptional regulation by IRE involves interaction with two iron regulatory proteins, IRP1 and IRP2. During iron starvation, IRPs stabilize the transferrin receptor and inhibit translation of ferritin mRNAs by binding to IREs within untranslated regions. IRE is found either in the 5′ or 3′ UTR [6].

Among *cis*-acting hairpins, we have selected the following three subjects for a more detailed discussion: (i) histone mRNA metabolism, (ii) mRNA localization dependent on 3′ UTR stem-loops and (iii) TRBP-mediated regulation of protamine and human immunodeficiency virus (HIV) trans-activation responsive (TAR) mRNAs.

3′ stem-loop in the mRNA of replication-dependent histones

Metazoan replication-dependent histone genes have several features that differentiate them from other eukaryotic genes. They are physically linked in clusters, do not have introns, express the only known non-polyadenylated mRNAs, and they require a distinct set of factors for expression and regulation (reviewed in [7, 8]). Expression of histone genes is associated with formation of Cajal (coiled) bodies (subnuclear domains involved in nuclear RNA metabolism) [9]. Replication-dependent histone mRNA contains a conserved 3′ end 26-nucleotide (nt) sequence harboring a 16-nt stem-loop (6-bp stem and a 4-nt loop, Fig. 1d) [10]. Mutations within the stem-loop structure or flanking sequences impair the correct processing of the 3′ end of histone mRNA. However, processing is not absolutely dependent on the stem-loop since a small number of processed transcripts can be detected even in its absence [11]. The histone 3′ stem-loop is bound by the stem-loop binding protein (SLPB, also named HBP – hairpin-binding protein), which is involved in several aspects of the histone mRNA metabolism. The stem-loop/SLPB complex is the key factor in cell-cycle regulation of histone mRNA expression, including processing of the nascent transcript by the U7 small nuclear ribonucleoprotein (snRNP) complex, control of translation and regulation of the half-life of mRNA. SLBP has a unique RNA binding domain that binds the 3′ hairpin with a high affinity (Kd = 1.5 nM) when specific nucleotides are present in the stem-loop [12]. SLPB and U7 snRNP are found in Cajal bodies located in proximity to histone gene clusters, suggesting that coiled bodies play a role in histone mRNA expression and processing [9, 13]. SLBP/U7 snRNP complex recruits a cleavage factor, which releases mature histone mRNA cleaving five nucleotides downstream of the histone stem-loop [8]. A recent study implicates polyadenylation factor CPSF-73 in the histone mRNA cleavage, suggesting a link between 3′ end histone mRNA processing and polyadenylation [14]. Processed histone mRNA remains bound to SLBP, which is required for efficient translation and cell-cycle dependent regulation but not for nuclear export of histone mRNA [15].

Regulated degradation of histone mRNAs requires interaction of the stem-loop/SLPB complex with Upf1, a key regulator of the nonsense-mediated decay (NMD) pathway, and ATR, a key regulator of the DNA damage checkpoint pathway activated during replication stress [16]. Notably, the stem-loop region of human replication-dependent histone mRNA can also interact with a conserved 3′-5′ exonuclease, 3'hExo, which is a candidate for the exonuclease that initiates rapid decay of histone mRNA upon completion and/or inhibition of DNA replication [17]. The 3'hExo orthologue in *Caenorhabditis* is known as ERI-1 [18]. ERI-1 is an exonuclease that degrades small interfering RNAs (siRNAs), thus negatively regulating RNA interference (RNAi) in *Caenorhabditis* and mammals [18, 19]. ERI-1 is unlikely to function as a general exonuclease because *eri-1*-null mutants are viable and show limited pleiotropic phenotypes, which may be a consequence of a defect in the siRNase (or possibly microRNase) function of ERI-1 [18]. It is still unknown, however, whether ERI-1 can also recognize and degrade small hairpin precursors of miRNAs. Replication-dependent histone mRNA thus provides an example of how a hairpin structure in *cis* can serve as a highly specific docking point providing a specific regulation of expression in a unique family of mRNAs.

mRNA localization regulated by stem-loop structures

mRNA localization is a means for protein localization via spatially restricted translation. Numerous specifically localized mRNAs have already been identified, mostly in oocytes or early embryos of *Drosophila* and *Xenopus* (reviewed in [20]). There are three main mechanisms for localized mRNA distribution: (i) active, directed transport of an mRNA to its destination where the mRNA is anchored, (ii) local stabilization/spatially selective degradation of mRNA and (iii) diffusion of mRNA combined with local anchoring at the target site (reviewed in detail in [21]). Individual mRNAs use different mechanisms for localization at their destination sites. The destination is encoded within a 'zip code', which acts as an mRNA localization signal. The term 'zip code' is used in this review for a sequence essential for mRNA localization – its removal impairs localization, and its fusion to non-localized mRNA is sufficient to localize it. Zip codes are diverse in terms of primary or secondary structure; however, they are usually localized in the 3′UTR, and their function is mediated by binding proteins [21].

Specific mRNA localization in *Drosophila* oocytes and embryos

Zip codes can be represented by stem-loop structures, which are found for example in *bicoid*, *gurken*, *I*-factor (Fig. 1e), and several other mRNAs localized in *Drosophila* oocytes or early embryos. The *bicoid* mRNA is a famous example of a localized cytoplasmic determinant that produces a morphogen gradient upon translation [22, 23]. The *bicoid* localization element (BLE) is a well-studied example of an element with a modular architecture

(Fig. 1e). Signals required for the *bicoid* mRNA localization to the anterior of the oocyte are located within the first 720 nucleotides of the bicoid 3′ UTR (reviewed in [24]). Several sequential steps in transport and localization are linked to three predicted stem-loops in the BLE (Fig. 1e). Stem V carries a 50-nucleotide element called BLE1 (*bicoid* localization element 1), which is required and sufficient for transporting the mRNA into oocytes and its initial accumulation in the anterior end. Stems V and VI regulate accumulation of the transcript in the oocyte and its anterior end. Finally, anchoring of the localized mRNA is controlled by the stem-loop III. Tthe *bicoid* gene is found only in *Drosophila* and closely related species [25]. The determinants of secondary structure appear to be conserved amongst them [26].

Several trans-acting factors regulate the bicoid mRNA localization. The current model proposes that mRNA is bound by the *Drosophila*-specific adaptor protein Swallow, which interacts with a dynein motor component, resulting in mRNA transport along microtubules [27]. *Swallow* and *bicoid* mRNAs are found in a multiprotein complex implicated in *bicoid* mRNA localization. This complex consists of Swallow, RNA binding proteins Modulo, PABP and Smooth, and the kinesin family member Nod [28]. The anterior anchoring of the transcript requires the Staufen protein [29], a conserved protein containing four to five dsRNA binding domains (dsRBDs). Staufen is one of several proteins which contain multiple dsRBD domains and play multiple roles in mRNA regulation. In addition to anchoring *bicoid* mRNA, *Drosophila* Staufen has been implicated in microtubule-dependent transport of *osk* mRNA, actin dependent localization of *pros* mRNA [30] and translational activation of *osk* mRNA [31]. Mammalian Staufen1 has been also implicated in recruiting the nonsense-mediated mRNA decay factor Upf1 to the Arf1 mRNA, inducing its decay [32]. Since Staufen and its homologues are ubiquitously expressed, there are many other functions which probably remain to be discovered.

Gurken (*grk*) and *I*-factor are localized in a crescent on one side of the nucleus in *Drosophila* oocytes. The *grk* mRNA is also localized by dynein-mediated transport along microtubules [33]. Localization and translational regulation of *grk* play key roles in establishment of primary embryonic axes in the oocyte [34]. Interestingly, the *I*-factor is a non-LTR retrotransposon, which appears to utilize (and interfere with) the *grk* localization pathway when it is mobilized in the female germline. Unlike other mRNAs, the minimal localization signals of *grk* and *I*-factor map to protein coding sequences. They are predicted to form similar stem-loop structures (Fig. 1e) with little, if any, sequence similarity [35] (Fig. 1e).

Perinuclear mRNA localization in mammalian cells

Several mammalian mRNAs, such as slow troponin C, metallothionein-1, vimentin and c-myc exhibit perinuclear localization. This localization is dependent on the 3′ UTR, and necessary sequences are found in predicted hairpin structures [36–39]. Putative perinuclear localization signals do not share apparent sequence or structural similarity (Fig. 2), and molecular mechanisms controlling perinuclear localization appear different in the aforementioned genes. Tissue-specific slow troponin C mRNA localization signal (Fig. 2) is bound by a myotube-specific 42-kDa polypeptide, and perinuclear localization is observed in differentiated myocytes but not in proliferating myoblasts or HeLa cells. Mutations within the stem region of the localization signal reduce perinuclear mRNA localization [39]. The perinuclear localization of rat metallothionein-1 mRNA is also dependent on the 3′ UTR. In particular, nucleotides 66–76 containing a CACC motif are required for localization (Fig. 2). Mutations that are predicted to alter the secondary structure of this region impair localization [37]. A possible candidate protein that binds to the localization signal is eukaryote elongation factor 1 alpha (eEF1-α) [40].

Vimentin mRNA, coding for an intermediate microfilament protein, also exhibits perinuclear localization. Mislocalized vimentin mRNA alters cellular morphology and motility [41]. The deduced vimentin perinuclear localization signal sequence is also predicted to form a stem-loop structure (Fig. 2) [36]. Several candidate proteins (HAX-1, eEF1-γ, hRIP) have been identified as binding this sequence, including HAX-1 protein, the best candidate for vimentin mRNA retention in the perinuclear space via a simple diffusion-entrapment model [42]. Interestingly, the vimentin sequence necessary for perinuclear localization carries a putative binding site for human microRNA (miRNA) hsa-miR-17_3p [43]. This raises the question whether hsa-miR-17_3p plays any role in vimentin mRNA localization and expression, for example by targeting vimentin mRNA mislocalized outside of the perinuclear space.

Perinuclear localization of c-myc mRNA is regulated by an AU-rich 3′ UTR hairpin structure reminiscent of two AU-rich stem-loop structures found in the 3′ UTR of *K-10* and *Orb* (Fig. 2), two mRNAs localized to the oocyte anterior cortex in *Drosophila* [38]. This is an interesting observation connecting a hairpin structure containing the AU-rich element (ARE) AUUUA to mRNA localization. The c-myc (and likely c-fos) AUUUA sequence is embedded in a stem-loop structure, which is different from the ARE-containing hairpin structure regulating tumor necrosis factor α (TNF-α) mRNA stability [44] (Fig. 2). In any case, the c-myc localization signal is likely a specific example of an ARE hairpin structure possibly bound by Annexin A2, thus leading to association with the cytoskeleton and perinuclear localization [45].

There are thousands of mRNAs containing ARE elements [46], which can be classified into several groups (reviewed in [47]). AREs are known to bind several

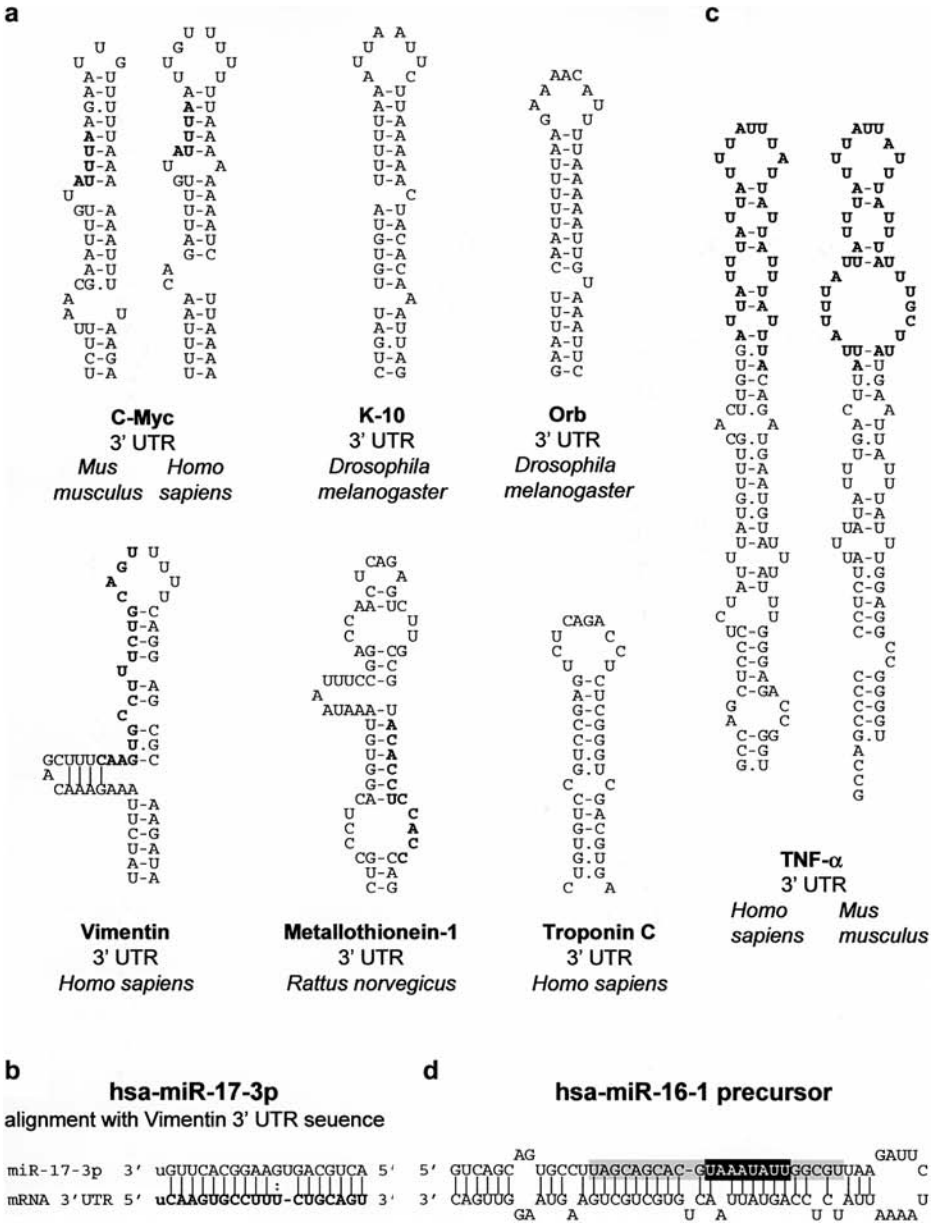

Figure 2. Sequences and secondary structures implicated in perinuclear mRNA localization in mammalian cells. (*a*) Various mRNA secondary structures found in the 3′ UTR of perinuclear mRNAs [36–40]. Boldface usage: vimentin, putative hsa-miR-17–3p binding site; metallothionein, minimal localization signal; c-myc, an ARE element. (*b*) Alignment of hsa-miR-17–3p and vimentin mRNA (boldface). (*c*) ARE sequences (boldface) within a stem-loop structure of human and murine TNF-α. (*d*) hsa-miR-16-1 precursor. The mature miRNA sequence is shaded in gray; the ARE-homologous sequence is shown in white letters.

trans-acting factors known to regulate translation or mRNA stability, such as TIA-1, HuR, HuB, AUF and TINO [48–50]. The functionality of AREs is determined by their sequence accessibility and binding proteins; however, mechanistic features that promote preferential binding of one *trans*-factor over another are not well understood. In this context, a specific stem-loop structure would be a determinant of the function of specific AREs. Interestingly, one of the *trans*-factors regulating the instability of mRNA containing AREs could be miRNA miR-16 [51]. Mature miR-16 contains a UAAAUAUU sequence which is complementary to an ARE (Fig. 2). The work of Jing et al. [51] showed that components of RNA silencing – Dicer1, Argonaute 1 and Argonaute 2 – are required for rapid decay of mRNAs containing ARE of TNF-α in *Drosophila* cells. Similarly, Dicer is required for ARE-mediated RNA turnover in mammalian cells. The role of miR-16 is sequence specific and requires the ARE binding protein TTP, which interacts with Argonaute family proteins, providing another ex-

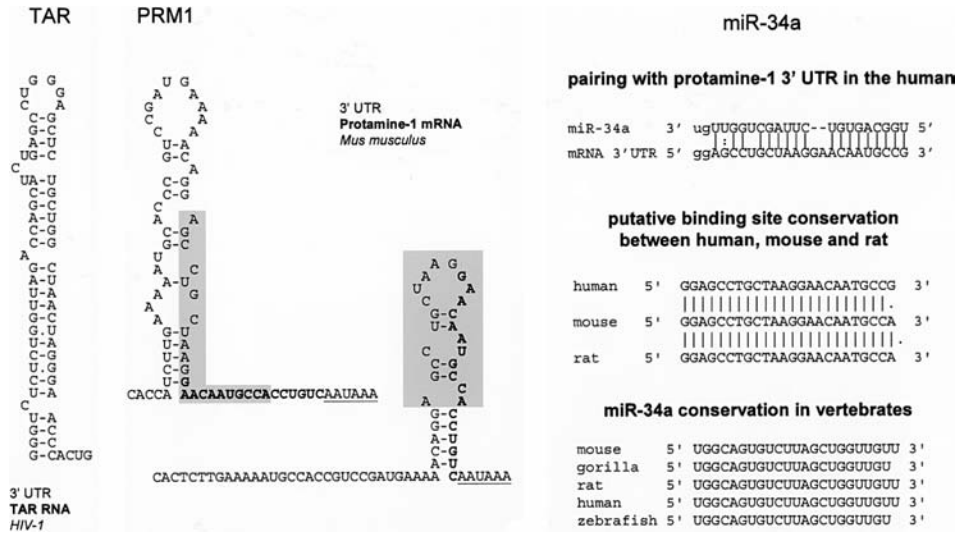

Figure 3. Predicted secondary structures of TAR and murine Prm1, which are recognized by TRBP. There are two predicted foldings of the same Prm1sequence [62]. A conserved repetitive motif found in the Prm1 3′ UTR is labeled in boldface [62]. Gray shading depicts the predicted binding region of miR-34a. The panel to the right shows predicted binding of miR-34a to the Prm1 mRNA and conservation of miRNA and its putative binding site.

ample of overlap between *cis* mechanisms acting on the 3′ UTR and the miRNA pathway.

Regulation of translation of TAR and PRM by TRBP2

The last reviewed examples of *cis*-acting hairpins are found in the 3′ UTR of HIV-Tar RNA and protamine mRNA (Fig. 3). We include this regulation because a protein binding these hairpins has recently been implicated in RNA silencing, raising the recurring question of how much RNA silencing is interconnected with other pathways capable of recognizing and responding to stem-loop structures.

TAR RNA is the HIV RNA sequence required for transactivation of the TAT protein, which activates gene expression of HIV-1 [52, 53]. The TAR sequence is located in the R region of the long terminal repeat (LTR) and is a binding site for the viral protein TAT and several cellular partners that positively and negatively regulate HIV translation. These cellular proteins include TAR RNA binding protein (TRBP) [54] and dsRNA-dependent protein kinase R (PKR) [55]. PKR is a part of the interferon response and blocks translation by phosphorylation of eIF2α (reviewed in [56]). TRBP is an approximately 45-kDa protein containing three dsRBDs that may localize in the nucleus or the cytoplasm, where it is associated with ribosomes and endoplasmic reticulum [57]. TRBP has a dual role in the regulation of TAR RNA – it stimulates translation of TAR RNA [58] and inhibits PKR [59]. Notably, the stimulation of TAR RNA by TRBP is independent of its ability to inhibit PKR [58]. TRBP can also directly interact with PKR [55]. PKR itself can be induced by TAR stem-loop binding and block translation [60]. Thus, recruitment of TRBP by HIV can be seen as a viral strategy to evade the cellular defense response.

PRBP, the mouse homologue of TRBP, was identified to act in translational regulation during spermatogenesis. PRBP is required for activation of translation of protamine mRNAs [61]. The mouse protamine mRNAs, Prm-1 and Prm-2, contain putative *cis*-acting stem-loop structures within the 3′ UTR and are translationally repressed for several days during male germ cell differentiation [62]. TRBP was observed interacting with protamine 3′ UTR initially, implicating it as a candidate for translational repression [62]. However, subsequent analysis revealed that TRBP is required for translational activation of protamine mRNA [61]. TRBP is predicted to bind to the upper part of the stem-loop via a '2-G hook' [63]. This could explain why stem-loop prediction of Prm-1 mRNA generates stem-loops with much shorter stems than in TAR RNA. Interestingly, human miRNA mir-34a has a predicted binding site [43] within the predicted stem-loop in the protamine 3′ UTR (Fig. 3). Furthermore, this binding site overlaps with a short conserved sequence, which is found twice in the protamine-1 3′ UTR [62]. This is the third example we present here where a stem-loop sequence is a putative miRNA binding site. However, it is unclear whether this observation could have a functional consequence. Protamine-1 expression is restricted to the testis, while so far miR-34 has been isolated only from embryonic stem cells and neural tissues [64–66].

Recently, two groups made the surprising discovery that mammalian TRBP is not only involved in regulation of mRNAs via *cis*-acting stem-loops but is also a component of the RNA silencing pathway [67, 68] (reviewed in

more detail in the next section). TRBP is an example of a protein that regulates specific mRNAs via *cis*-acting elements, which was also recruited by RNA silencing. This should not be surprising. If proteins and pathways existing in the cell are the major source for molecular evolution, then one would expect evolving regulatory mechanisms to acquire components from existing ones. Therefore, it is likely that RNA silencing interacts with other mechanisms regulating mRNA expression via a 3′ UTR (including those not involving 3′ stem-loops). It will be of interest to discover how many established pathways involving mRNA instability and/or translational regulation include previously unidentified miRNA pathway components.

Short RNA hairpins with *trans* effects – the miRNA pathway

The first glimpses of the existence of RNA hairpins inducing sequence-specific *trans* effects on gene expression came from experiments in plants and *Caenorhabditis*. Identification of *lin-4*, a small temporal RNA (miRNA, in fact) that controls developmental timing of gene expression in *Caenorhabditis*, was one of the milestones in exposing the existence of RNA silencing [69, 70]. *Lin-4* was found to be a small, untranslated RNA existing either as a 61-nt hairpin or 22-nt single strand, which negatively regulated translation by binding the 3′ UTR of *lin-14* [69, 70]. It was nearly a decade before the significance of RNA silencing by miRNAs was truly recognized. Small temporal RNAs were considered an interesting 'curiosity' in *Caenorhabditis* until the discovery of sequence-specific mRNA degradation by RNAi [71] and the initial biochemical dissection of that pathway [72]. By then it emerged that the size of a mature miRNA corresponds to siRNA intermediates of RNAi and that RNAi and miRNA pathways are very similar. In this context, RNA hairpins are just one of several types of RNA molecules that can induce RNA silencing pathways. What makes miRNA-coding short hairpins unique is that they control the expression of endogenous genes and they are likely the most common RNA silencing inducer in mammals.

miRNAs and their maturation from short hairpin precursors

Some researchers view RNA silencing as a single biochemical pathway in which short RNAs target homologous transcripts through similar (if not identical) effector complexes. This notion is supported by idea that a single ancestral RNA silencing pathway gave rise to RNAi, miRNA and other silencing pathways. However, this view is likely oversimplified, because in some cases (e.g. *Drosophila*) the miRNA pathway has genetically diverged from RNAi.

A miRNA is defined as a single-stranded RNA of 21–22 nt in length that typically induces inhibition of translation of its cognate mRNA and which is released by RNAse III-like enzymes from a local hairpin structure within an endogenous transcript [73]. miRNAs are transcribed as long primary transcripts (pri-miRNA), which are processed into hairpin intermediates (pre-miRNA) in the nucleus. Pre-miRNA is subsequently transported to the cytoplasm and cleaved to release a miRNA duplex of a miRNA and a passenger strand. Finally, the miRNA is loaded onto an effector ribonucleoprotein (RNP) complex capable of recognizing cognate mRNAs and inhibiting their expression (see also Fig. 4). The functional definition of miRNAs as inhibitors of translation is arbitrary because the process of mRNA cleavage versus translational inhibition strongly depends on perfect or imperfect pairing of miRNA with its target rather than on the origin of short RNA. In other words, inhibition of translation can be achieved by imperfectly bound siRNA [74], while miRNAs can cleave their substrates if their pairing with cognate mRNAs is perfect [75]. Pairing of animal miRNAs with cognate RNA typically contains bulges and results in inhibition of translation (an exception is Hoxb8 mRNA cleavage induced by miR-196 [76]), while in plants miRNAs [77] usually perfectly pair with their targets, inducing cleavage. The latest data suggest that the mechanism of translational inhibition may involve mRNA decapping complex [78]. Therefore, the miRNA effect of imperfect binding, where direct cleavage is prevented, may be viewed as a commitment of targeted RNA to degradation.

Several lines of evidence suggest that most (if not all) miRNA precursors are pol II transcripts: (i) miRNA transcription is sensitive to α-amanitin at concentrations that inhibit pol II but not pol I or pol III [79], (ii) miRNA hairpins are often localized within pol II transcription units [80], (iii) miRNA precursors have been shown to contain cap and polyA tails [79, 81], and (iv) chromatin immunoprecipitation experiments have identified the physical association of pol II with the promoter of the mir-23a-27a-24-2 cluster [79]. Approximately 70% of the miRNAs are located in different sequence contexts within known pol II transcripts. They are typically found in introns of protein coding genes and in both exons and introns of noncoding RNAs [80]. The transcribed pri-miRNA is cleaved by RNAse III Drosha to release a precursor of miRNA (pre-miRNA) [82]. The Drosha protein (~160 kDa) was initially discovered as a factor processing ribosomal RNA [83]. Drosha is a conserved metazoan protein containing two RNAse III domains and a dsRBD. It forms a large complex (~500 kDa *Drosophila*, ~650 kDa human) known as a Microprocessor complex, which also contains DGCR8 protein (known as Pasha in *Drosophila*). DGCR8/Pasha (~120 kDa) contains two dsRBDs and is believed to assist Drosha with substrate recognition [84, 85]. Drosha

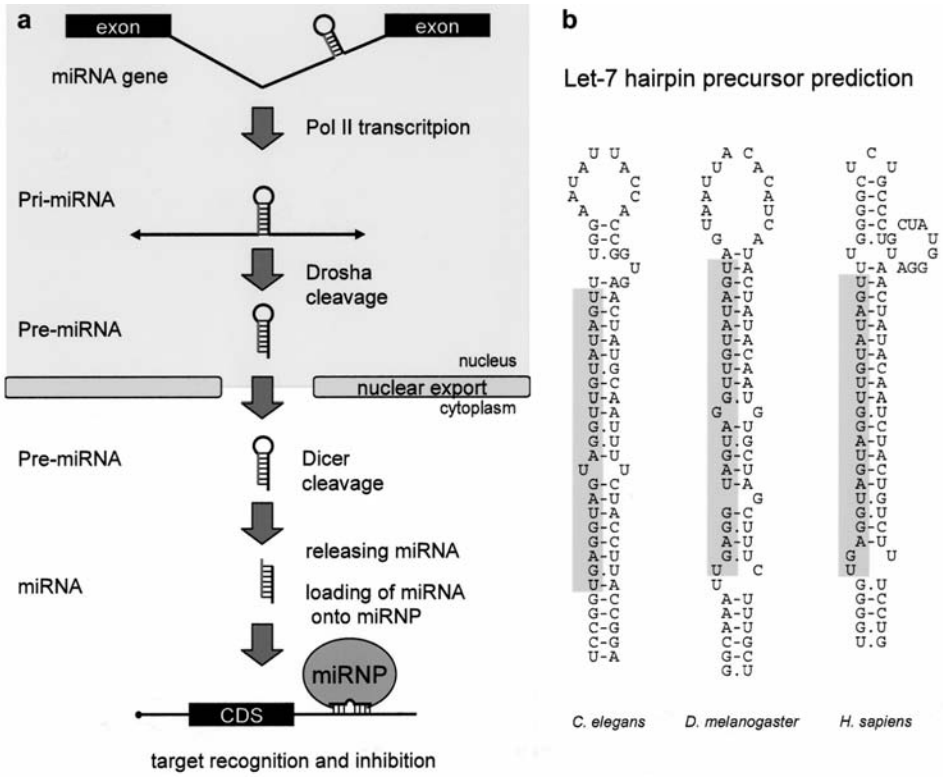

Figure 4. MicroRNA pathway. (*a*) Schematic overview of miRNA maturation in eukaryotic cells. (*b*) Examples of Let-7 hairpin precursors from different species. The mature miRNA strand is shown in the gray frame. Note the variability in the secondary structure prediction.

cleaves the pri-miRNA at the base of the stem, approximately two helical turns from the terminal loop. The cleavage produces a 2-nt, 3′ overhang [82]. The substrate determinants for Drosha are not fully understood. There are several hundred different animal pri-miRNAs that do not share common sequence motifs. Mutagenesis studies suggest that the stem structure around the cleavage site, the large terminal loop and the single-stranded flanking sequences are important for Drosha cleavage [82, 86, 87]. Drosha-processed pre-miRNAs are exported into the cytoplasm via Exportin 5 [88]. Mutagenic screens suggest that an RNA stem bigger than 16 bp and a short 3′ overhang are among the structural requirements for export [89].

Cytoplasmic pre-miRNAs are processed by Dicer (~220 kDa), an RNAse III, which is highly conserved amongst eucaryotes. It recognizes a 2-nt, 3′ overhang in pre-miRNA via its PAZ domain [90], and cleaves it 20-bp from the base of the stem removing the loop and leaving another 2-nt, 3′ overhang [91]. In mammals, only one Dicer acts in RNA silencing, while *Drosophila* utilizes two proteins – Dicer -1 (processing pre-miRNA) and Dicer-2 (generating siRNA) [92]. After cleavage, a proper miRNA strand is separated from the passenger strand and loaded onto the effector microRNP complex [RNA-induced silencing complex (RISC)-like complex] that contains an Argonaute family member mediating the effect on the cognate mRNA. This process appears to require the previously mentioned TRBP, which has been found to directly interact with Dicer. Although it may have other roles in miRNA processing, TRBP (and similarly its *Drosophila* homologue Loquacious [93, 94]) plays an important role in efficient assembly of the RNP-effector complex [67, 68]. The discovery of the role of TRBP in RNA silencing raises several interesting questions. TRBP can also inhibit PKR, which activates a sequence-independent response to dsRNA in mammalian cells. Could TRBP control a switch between RNA silencing and a nonspecific response to dsRNA? Based on recent evidence, the answer is probably yes. A robust PKR response was shown to correlate with lower levels of TRBP, while an increase of TRBP opposed this effect [95].

Regulation by miRNAs is likely widespread. The miRNA registry database [96] contained a total of 3424 entries, with 10 to a few hundred entries found in each species at the time of submission of this review (November 2005). The extent and complexity of miRNA regulations could be high, considering that one miRNA can target different (most likely unrelated) genes, and one gene may contain recognition sites for several miRNAs. It is proposed that there is a miRNA expression pattern unique to each cell type, which in turn alters the expression of thousands of mRNAs and provides a selective pressure for the evolution of all metazoa mRNA sequences [97]. Experiments with misexpression of tissue-specific human miRNAs showed

that miR-124 and miR-1 can possibly downregulate about hundred genes each and that miRNAs may be involved in defining tissue-specific gene expression [98]. It is not known how many genes are regulated by miRNAs, and estimations may range between 10 and 50%. Computer prediction of conserved miRNA binding sites has identified that about 10% of human transcripts contain putative binding sites for miRNAs within their 3′ UTRs [43]. How many of these putative targets are truly regulated and how many of these regulations are biologically significant is unknown. Therefore, it is remarkable that Dicer knockout in embryonic stem cells is not immediately lethal as these cell 'only' exhibit slower proliferation and differentiation defects [99, 100]. This suggests that miRNAs are possibly involved in buffering gene expression in a given cell type, and such buffering is probably more needed during developmental transitions. Differentiation defects could then be a result of a summation of small changes in many mRNAs and/or a result of the absence of a major regulatory miRNA silencing one or more critical genes. However, this problem can only by addressed by systematic studies of individual miRNAs. Technically, it may be extremely difficult to systematically knock out mammalian miRNAs; however, promising new technologies are emerging that can specifically block particular miRNAs (e.g. 'antagomirs' [101]).

Evolution of miRNAs: how to evolve short hairpin RNA homologous to another mRNA?

The evolution of a network of stem-loop hairpins homologous to their targets is puzzling because it requires the simultaneous evolution of at least three complementary sequences (both hairpin strands and the cognate sequence). The inverted duplication (ID) model proposes that some miRNA sequences and their targets originate from the same sequence [102]. This model assumes that some miRNAs have evolved from long inverted repeats formed from the gene sequence (Fig. 5). These long inverted repeats were subsequently eroded during evolution, and only a short stem – the miRNA precursor, remained as a functional remnant of the long RNA hairpin. This hypothesis was investigated in plants, and, indeed, some plant miRNAs appear to be located within what appears to be an eroded inverted repeat [102]. The inverted repeat duplication hypothesis provides an elegant explanation for the evolution of perfectly pairing miRNAs in plants. However, as we will discuss, long inverted repeats from single-copy sequences are rare in mammals. It is possible that human and *Caenorhabditis* genomes currently do not contain a single event of a miRNA evolving via inverted repeat duplication from a bona fide endogenous gene. In addition, this hypothesis cannot explain networks of miRNAs, where multiple miRNA can target multiple unrelated genes. Therefore, there must be many miRNA precursors that did not descend from large inverted repeats.

We present another hypothesis for miRNA evolution, which we call the random selection hypothesis. This hypothesis assumes sequential evolution of miRNA and 'target acquisition' (Fig. 5). Briefly, we propose that putative miRNAs are produced by random formation of Drosha/Dicer substrates. These putative miRNAs form a pool from which miRNAs acquire their target(s) at random. Could such putative miRNAs exist? A miRNA

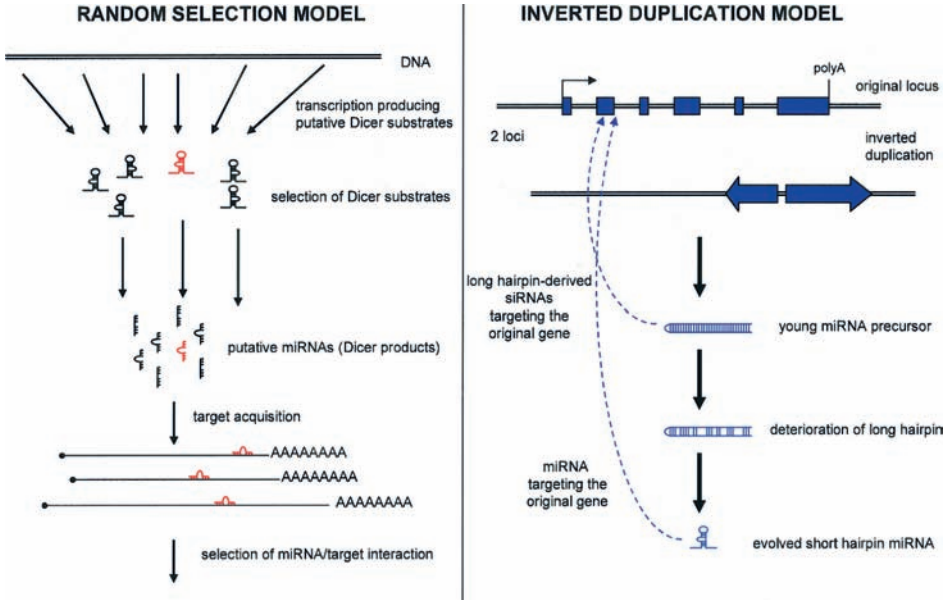

Figure 5. Two models for the evolution of miRNA regulation. Left: the random selection model presented in this review. An evolving functional, single miRNA is shown in red. Right: the inverted duplication model evaluated previously in plants [102]. Sequences from the same gene are shown in blue.

is defined as a short RNA molecule which is able to induce posttranscriptional gene silencing and originates from a cleavage of a precursor containing a short RNA hairpin. However, silencing effects are usually not considered when miRNAs are deposited in the miRNA registry, so the annotated miRNAs typically fit only the second half of the definition above – i.e. they originate from cleavage of a hairpin precursor [96]. Therefore, it is likely that the pool of biologically active miRNAs is smaller than the total number identified in the miRNA registry. We will argue here that such a situation actually should be expected if miRNAs are evolving. We predict that a random RNA can with a certain probability form a secondary structure, which can be recognized and processed by miRNA processing machinery. If the miRNA machinery were to cleave only perfect dsRNA hairpins, such probability would be so low as to rule out the idea of random evolution of a short hairpin precursor. However, there are a few arguments in our favor: First, Dicer can cleave precursors containing various bulges and mismatches, which dramatically increase the probability that such a structure can occur by chance. Moreover, our prediction can be tested using randomized sequences and miRNA precursor prediction software. Testing computationally predicted precursors in a Dicer assay would clarify how rare such an event is in a random sequence. Second, the genomic sequence is not absolutely random, as duplications and the shuffling of sequences may result in more imperfect short inverted repeats than in a random sequence. But, in contrast to the ID model, ours assumes that there is not a common origin of the miRNA sequence and its target. We propose that a target is acquired at random, based on partial complementarity between the putative miRNA and its putative target. If such a pairing results in positive selection, it is maintained and point mutations, either in the precursor or in the target, strengthen the interaction, increasing the specificity and efficiency of such a regulation. If we simplify the problem of target acquisition to the appearance of the seeding region for miRNA binding, then a specific minimal 6-nt seeding region occurs theoretically once in every 4 kb (7-nt seeding in every 16 kb). Therefore, a single seeding region can occur in thousands of different RNA molecules, and several of these seeding sites would likely exhibit thermodynamic properties similar to those of true miRNAs. So when a putative miRNA finds its target, it is decided through natural selection whether such an interaction would evolve as post-transcriptional regulation or not. This idea is consistent with recent data showing that mammalian mRNAs are under selective pressure to maintain and/or avoid specific 7-nt seeding regions [103].

Our hypothesis has several important implications and predictions.

1) It provides an explanation for the evolution of regulatory networks where one miRNA can target several mRNAs and one mRNA can be targeted by numerous miRNAs.
2) It explains the evolution of novel miRNAs in mammals, where long hairpin RNA expression induces the PKR-interferon response.
3) It views miRNAs cloned from a species as pools of evolving miRNAs which have a more or less defined function (or no function). It is possible that many of the identified miRNAs do not have any significance other than that their hairpin precursor just makes a good substrate for Drosha and Dicer.
4) A high incidence of false positives during miRNA precursor and miRNA target predictions may actually reflect reality more than we think. So-called false positive software predictions of miRNA precursors and their targets may occur in nature but demonstrate weak effects that are below usual experimental detection limits.
5) The number of miRNAs and their targets exhibiting strong biological effects in cells would be relatively small compared with the number of miRNAs and their putative targets in various databases.

Short RNA hairpins used for experimental gene silencing

RNA silencing can be used for highly selective, experimental interference with a gene function. Different model systems and types of experiments have dictated different vector designs, so that today there is a wide variety of hairpin-expressing RNA systems for gene silencing. In mammalian cells, induction of RNA silencing with expressed dsRNA is usually achieved with a short hairpin RNA system since short hairpins are considered too small to induce the interferon response. Short hairpin systems are also a method of choice for large-scale experiments because a large number of short RNAs can be easily synthesized and cloned into a vector. Long RNA hairpins can also be used to induce RNA silencing, but they are typically used in small-scale experiments in plants and invertebrates. The use of long RNA hairpins for RNA silencing in mammals is limited to only those few cell types that do not exhibit sequence-nonspecific responses to long dsRNAs (discussed in the last section).

Gene silencing with short hairpin RNAs (shRNAs) was introduced in 2002 with numerous reports of pol III-driven shRNA appearing over a few months [104–107]. Short hairpin expression from a plasmid remedied two concerns about RNA silencing: the transfection efficiency of siRNAs and duration of silencing. In terms of the structure of expressed shRNA, there is a great deal of variation amongst published data. Short hairpin systems can be divided into two classes: Class I hairpins are based on

covalent linking of strands carrying functional siRNA sequences. The minimal Class I hairpin contains a 19-bp dsRNA stem and 4–9 nt loop, and it is probably not processed like a classical miRNA [105–108]. Class II hairpins are directly modeled after miRNA hairpin precursors [104, 105].

Both RNA polymerases, pol II and III, can be used to produce functional silencing hairpin. The pol II strategy is based on placing siRNA sequence into a pri-miRNA-like transcript, which is then processed by the miRNA pathway [104]. This allows generation of constructs that harbor a shRNA within an intron of a reporter (such as EGFP), thus allowing for tissue-specific delivery and simple screening for the presence of an active transgene [109]. However, most of the shRNA vectors currently use pol III promoters, usually U6 or H1. The key feature of pol III systems is termination of transcription at a stretch of thymidines, resulting in 2–4 uridines at the 3′ terminus of the hairpin transcript, which makes it similar to pre-miRNAs. U6 and H1 promoters appear comparably efficient, although differences have occasionally been observed. For example, H1 promoter appeared superior to the U6 promoter when used for vector-based RNAi in cell culture [110]. In transgenic mice, one group concluded that H1 and U6 showed comparable activity in transgenic mice [111]. Another group reported that constructs containing the H1 promoter were significantly less effective in transgenic mice, while both promoters functioned equally well in cultured cells [112]. The U6 promoter has been reported to induce more strongly than H1 interferon–stimulated genes (ISGs) such as oligoadenylate synthetase (OAS) [113]. However, this does not necessarily make the U6 promoter inferior. More detailed evaluation of the effect showed that ISG induction is a consequence of the presence of an AA dinucleotide near the transcription start site and single-nucleotide deletion in the siRNA sequence abolished OAS1 induction. To avoid problems, the authors recommend preserving the wild-type sequence of U6 vectors around the transcription start site [114]. Pol III systems are efficiently used for constitutive expression in cell culture. Further development also produced Tet-inducible pol III systems [108]. However, one of the disadvantages of pol III systems is that there are no tissue-specific pol III promoters, which complicates experiments in transgenic animals. This problem is partially solved by using a loxP recombination activating pol III [115, 116]. Although it is functional and versatile in the sense that one targeting transgene can be combined with existing animals expressing Cre recombinase in different tissues, the loxP strategy may sometimes be too complicated due to the required crossing of both transgenes and screening for recombination. It is faster and easier to generate transgenic animals with pol II-driven, tissue-specific, short hairpin RNA-induced knockdown than to produce animals with a loxP transgene that needs to be crossed to Cre-expressing animals in order to reveal which founder line provides the best knockdown.

Long (>100 bp) RNA hairpins with variable effects

There are several possible effects of expression of a long RNA hairpin. Experimentally, long RNA hairpins introduced as transgenes into different species can induce RNA silencing [117–120]. However, experiments in mammalian cells also show nonspecific inhibitory effects, presumably due to induction of the interferon response, which includes activation of PKR and oligoadenylate synthetase [121]. Naturally occurring RNA hairpins were found in *Caenorhabditis* while searching for substrates of adenosine deaminases that act on RNA (ADARs) [122, 123].

ADARs are metazoan RNA-editing enzymes that convert adenosine to inosine (which is recognized as guanosine). RNA editing has been implicated in alternative splicing, RNA stability, codon change and other processes (reviewed for example in [124, 125]). ADAR-mediated editing appears important but nonessential in invertebrates, as *Drosophila* and *Caenorhabditis* strains lacking ADAR activity are viable but exhibit behavioral defects [126, 127]. Mice lacking ADAR die embryonically (*adar1 –/–*) or shortly after birth (*adar2 –/–*). Notably, *adar2 –/–* animals are completely rescued by the glutamate receptor-B (gluR-B) allele containing an edited sequence, suggesting that gluR-B is responsible for the *adar2 –/–* phenotype and thus is a critical substrate for ADAR2 [125]. The critical substrates of ADAR1 in mammals remain unknown.

Several mRNAs with long hairpin structures, typically in their 3′ UTR, are among ADAR substrates in *Caenorhabditis* [122, 123]. The purpose of adenosine deamination within noncoding regions is unclear. One possibility is that hyperediting by ADARs (and possibly nuclear retention) prevents RNAs with long hairpins from triggering RNAi. This idea is supported by experiments showing that chemotaxis defects in ADAR-deficient *Caenorhabditis* can be rescued by mutations in the RNAi pathway [128]. However, ADARs do not always prevent long hairpin-induced RNAi, as was shown by RNAi effects in worms expressing long RNA hairpins from a transgene [118]. Interestingly, while RNAi and ADAR pathways have an antagonistic relationship, they share one of the components – Tudor staphylococcal nuclease (Tudor-SN). Tudor-SN has been described as a component of unknown function in the RISC complex in *Drosophila*, *Caenorhabditis* and mammals [129]. Likewise, a recent paper from Scadden shows in *Xenopus*, that Tudor-SN specifically interacts with and promotes cleavage of hyperedited dsRNAs [130]. This raises the question of whether these two pathways intersect. In mammals, an ADAR substrate

harboring a longer inverted repeat was recently discovered [131]. CTN-RNA is an alternative transcript of the mouse cationic amino acid transporter 2 (mCAT2) and contains an 100-bp stem-loop structure, which is edited. The mCat hairpin does not seem to induce RNAi, as the induced knockdown of CTN-RNA (with antisense-DNA oligo) downregulates mCat mRNA. Edited RNA, on the other hand, is retained in the nucleus where, under stress, it is cleaved to produce protein-coding mCat2 mRNA.

Do long hairpin RNAs naturally occur in mammals?

Long RNA hairpins transcribed from inverted repeats should be rather a rare source of natural long dsRNA, because the accumulation of mutations within arms of inverted repeats creates bulges within a hairpin and erodes it over time. But how rare are natural long hairpins in animals? To understand this problem better, we searched for long inverted repeats within *Caenorhabditis*, mouse and human genomes. We aimed at obtaining better insight into two major problems. First, the inverted repeat duplication hypothesis (discussed above) assumes that some miRNAs evolved from longer inverted repeats. Knowing the number and sequence of inverted repeats would help us to understand how significant a source long inverted repeats could be for the evolution of miRNA genes in mammals. Second, transcription of an inverted repeat produces with high efficiency dsRNA capable of inducing distinct sequence-specific and sequence-independent effects. Knowing the frequency and sequence of potentially transcribed inverted repeats would allow for making better assumptions about the role of long dsRNA in mammalian cells.

To identify inverted repeats, we searched genomic sequences with a simple script that calculates a score of identity for a given minimal stem and maximal loop size. For example, for a 200-bp stem and 50-nt loop hairpin, a sequence of 450 nt is taken. The first 200 nucleotides are compared with last 200. If the identity reaches a given threshold, the sequence is recorded. Then the script moves one nucleotide forward and repeats the calculation. This search is crude because it does not contain any real alignment algorithm. In other words, it only compares identity with mismatches, and the output is strongly affected by insertions and deletions (indels) within the arms of an inverted repeat. On the other hand, it is simple, fast and sufficient for a brief survey of the most perfectly paired inverted repeats present in large genomes. We have searched mouse, human and *Caenorhabditis* genomes with several different parameters, and the results are summarized in Table 1. Our search did not identify any known miRNA precursors, probably because ignoring the indels

Table 1. Survey of long inverted repeats in genomes of *Homo sapiens*, *Mus musculus* and *Caenorhabditis elegans*.

Minimal stem	Maximal loop	Minimal identity	Species	Total	SINE	LINE	LTR	DNA	Unique	Simple repeats	Other
(bp)	(nt)	(%)			% Total	% Total	% Total	% Total	% Total	% Total	% Total
200	50	80	*H. sapiens*	501	75 377	4 22	1 3	1 3	2 10	16 80	1 6
			M. musculus	59	37 22	34 20	10 6	0 0	5 3	14 8	0 0
			C. elegans	502	0 0	0 1	0 0	78 391	17 84	4 20	1 6
100	10	90	*H. sapiens*	61	21 13	16 10	7 4	2 1	20 12	34 21	0 0
			M. musculus	23	13 3	30 7	9 2	0 0	17 4	30 7	0 0
			C. elegans	401	0 0	0 0	0 0	78 312	19 77	2 9	1 3
50	5	94	*H. sapiens*	207	1 3	2 5	1 3	0 1	23 48	71 147	0 0
			M. musculus	12	8 1	17 2	0 0	0 0	17 2	58 7	0 0
			C. elegans	22	0 0	0 0	0 0	27 6	55 12	14 3	5 1

'Unique' are sequences that do not match any class of known repetitive sequences. 'Other' sequences are repetitive sequences such as satellite repeats, which are not classified as SINE (short interspersed elements), LINE (long interspersed elements), LTR (LTR-retrotransposons), or DNA (transposons).

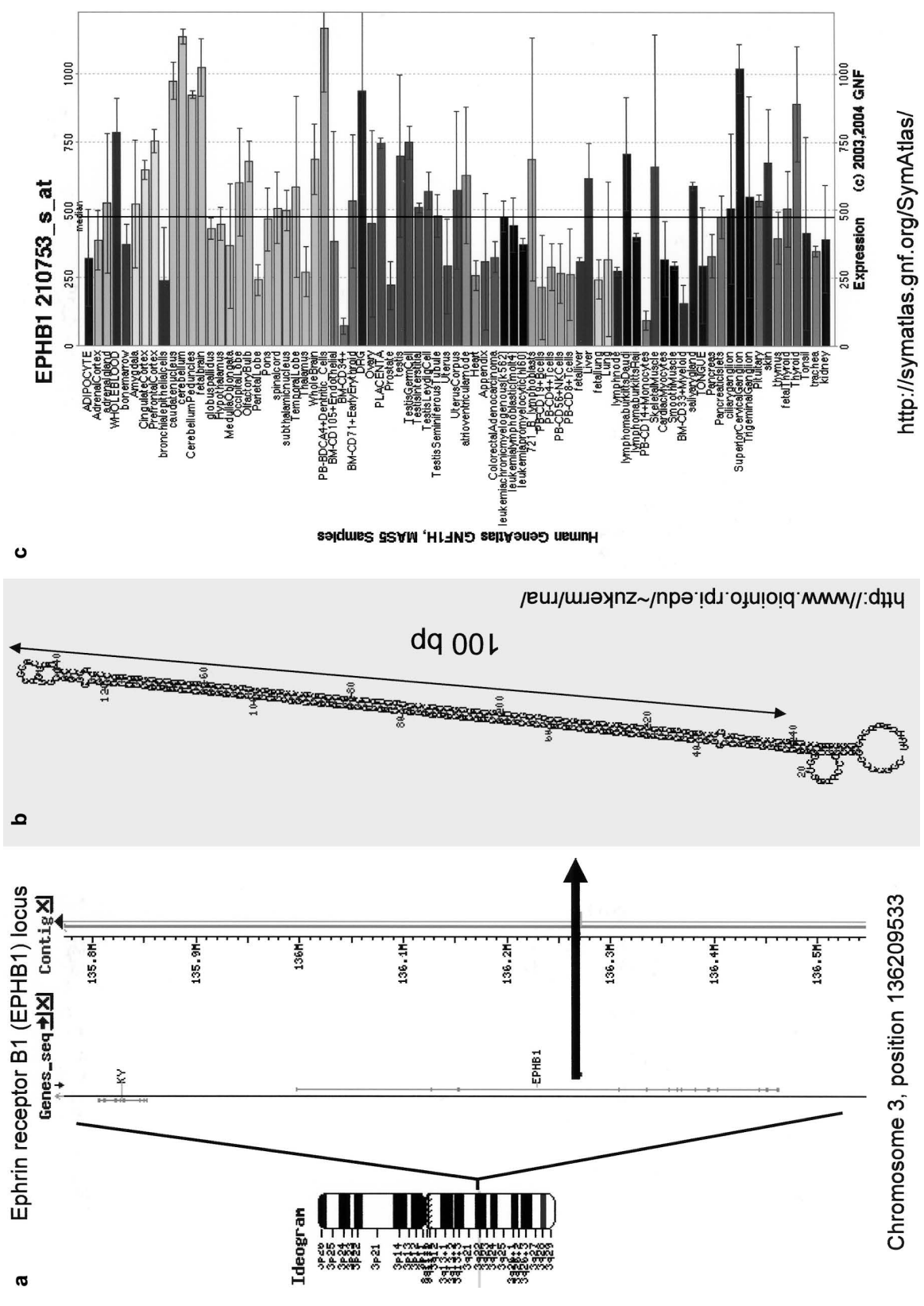

Figure 6. Example of an L1 inverted repeat likely generating a long RNA hairpin. (*a*) Schematic overview of the locus. The inverted repeat is localized in an intron of the Ephrin receptor B1 (EPHB1) gene on the human chromosome 3. (*b*) MFOLD-predicted structure of the L1 hairpin has an ~100-bp, almost perfect stem (http://www.bioinfo.rpi.edu/~zukerm/rna/ [146]). (*c*) GNF database entry showing ubiquitous expression of the host gene (http://symatlas.gnf.org/SymAtlas/ [147]).

increases the degree of stringency of the search. However, we found about 50 unique human sequences capable of forming a stem-loop structure with a 50-bp minimal stem containing a maximum of three mismatches and a 5-nt loop. It would be interesting to test whether any of these sequences can be processed by miRNA/RNAi machinery. Apart from low complexity repeats (e.g. TA_n), the majority of long inverted repeats are derived from repetitive sequences. In the human genome, the vast majority of long inverted repeats derive from SINE (Alu) sequences. The second most abundant source is L1 sequences. We have also found several long inverted repeats made of unique sequences (all sequences are available upon request). In the mouse, LINE/L1 inverted repeats are more abundant than SINEs presumably because mouse SINE sequences are not as uniform as human Alu sequences. A fraction of these inverted repeats is clearly transcribed as they reside within introns; however, we did not find inverted repeats among mammalian expressed sequence tags (ESTs) or mRNAs present in the GenBank database. Interestingly, the abundance of long inverted repeats in the human genome correlates with the classification of human targets for adenosine deamination [132] and the observation that RNA editing of Alu elements mostly targets intramolecular duplexes [133]. This likely reflects the fact that human Alu and L1 are so abundant that they have the highest probability of inverted repeat formation.

Our results also suggest that L1-derived inverted repeats could be a source of dsRNA, resulting in downregulation of L1 retrotransposition via RNAi. L1 retrotransposition occurs in germ cells or in early embryos [59, 134]. There is evidence for a role of RNAi during early mammalian development in silencing of the LTR retrotransposons IAP and MuERV-L, which potentially generate dsRNA [135]. At the same time, long hairpin expression from a transgene induces RNAi in mouse oocytes [120]. There are tens of L1 inverted repeats of various sizes and at least 10 of them reside within introns (Fig. 6). Since an inverted repeat ensures a high efficiency of dsRNA formation, it is tempting to speculate that this could be a source of dsRNA downregulating L1 retrotransposition in the germline. However, small RNA cloning from mammalian species does not provide solid evidence that L1 sequences are processed by the RNAi machinery [65, 136]. Therefore, whether RNA from L1 inverted repeats is a substrate for ADARs or RNAi (or other dsRNA-responding pathways) needs to be tested.

When we performed the same search on the *Caenorhabditis* genomic DNA, the frequency of inverted repeats in its genome surprisingly was about several hundred times higher than that in mammals (Table 1). More detailed analysis discovered that the difference is due to a high abundance of a special class of transposon-like DNA elements, which are structured as long inverted repeats (~100–800 nt/arm) with short loops (~7–57 nt) [137]. This observation is somewhat counterintuitive, considering that transposons are subjected to silencing by RNAi in *Caenorhabditis* [138]. In any case, our search did not identify SINE, LINE or LTR inverted repeats in *Caenorhabditis*; most of the inverted repeats originated from DNA elements and unique sequences. Although these differences are remarkable, their biological significance is unclear.

Long RNA hairpins used for experimental gene silencing

Expression of a long RNA hairpin was the first approach to stable induction of RNA silencing by transgenes [118]. Today, long RNA hairpins are shadowed by short hairpin systems, and they are used only in special cases where a short hairpin RNA system cannot be used efficiently. Long hairpin RNA was successfully used to block gene function in several types of mammalian cells, but aside from mouse oocytes, it never acquired wider attention (reviewed in [139]). Long dsRNA expression from a large inverted repeat remains a common solution for transgenic RNAi approach in invertebrates and plants. The advantage of a long hairpin RNA is that it delivers a population of different siRNAs, ensuring a robust RNAi effect. It can also be combined with tissue-specific pol II promoters. Working with inverted repeats may be complicated (reviewed in [139]), but despite all the possible pitfalls, transgenic RNAi in mouse oocytes has produced a functional knockdown in several instances [120, 140–142], and the list of successful knockdowns in *Caenorhabditis* and *Drosophila* is much longer.

Concluding remarks

In the present review we have attempted to provide a birds-eye view of the mechanisms that recognize and respond to RNA hairpin structures in metazoa. Naturally, our review cannot be comprehensive; however, sometimes it is useful to zoom out and take a more panoramic view. RNA silencing is not completely isolated from many other pathways, which operate with RNA hairpins. Hairpin structures and a combination of factors which interact with them will determine the final effect. It is difficult, if not impossible, to predict the effect of an RNA hairpin predicted from an RNA sequence. While we cannot provide a key to this problem, at least we can remind you of the possible options that exist.

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