3 Development of Gene-Profile-Responsive Antisense Agents

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3.1 INTRODUCTION: MAKING SENSE OF PHARMACOGENOMICS THROUGH ANTISENSE TECHNOLOGIES

The immediate aim of pharmacogenomics is to identify genes that determine differences in individual responses to particular drugs. The longer-term goal of this emerging discipline is to advance beyond the current approach to drug therapy to more individualized approaches. Drugs that are more suited to the molecular characteristics of individual patients should have greater efficacy and reduced toxicity [1–3]. Although individual human genomes are 99.9% identical, the 0.1% difference predicts as many as 3 million polymorphisms, including substitutions, deletions, and insertions [4]. Some of these polymorphisms affect protein expression or function, and may lead to disease or altered drug response. Newly available compilations of human genome sequence polymorphisms, particularly single-nucleotide polymorphisms (SNPs), can provide markers associated with characteristic genotypes and potentially identify genes directly responsible for the individual drug responses [5–10]. Initial optimism about the ability of pharmacogenomics to speed the

development of personalized medicine has been tempered by slower-than-expected progress, especially in drug target discovery and validation. One of the reasons is that drugs very rarely interact with only a single target, and even when they do, they usually affect several pathways [11, 12]. Even fewer drugs can distinguish between protein variants that differ as a result of minor polymorphisms such as SNPs. Many drug development programs still employ the traditional approach of identifying a single target and using combinatorial chemistry and high-throughput assays to identify drug leads. However, companies are increasingly employing a more directed approach using information provided by pharmacogenomics and structural biology.

Nucleic-acid-based antisense technologies are particularly amenable to rational design because of the straightforward pairing relationship between the sequences of the antisense agent and its RNA target. Moreover, the availability of the complete human genome sequence has given the antisense approach a new and powerful resource. While antisense technologies are conceptually elegant and straightforward, in practice their specificity and potency *in vivo* are unpredictable. In this chapter, we focus on the ability of various antisense agents to distinguish sequence polymorphisms and to access their intended target sites. These capabilities are central to their utility as gene-profile-responsive therapeutics. Other important issues include the efficient delivery of antisense agents into the appropriate cellular compartment, and their biostability (not covered by this chapter). Small interfering RNA (siRNA) has been extensively reviewed elsewhere, so the focus of this chapter is primarily on other antisense approaches.

3.2 ANTISENSE APPROACHES TO GENE SILENCING

Although antisense technologies have been under development for more than 25 years [13–22], antisense-mediated gene regulation has more recently been found to occur in a variety of natural systems, in the forms of antisense RNA [23–26], ribozymes [27–29], and RNA interference [30–33]. From a mechanistic point of view, there are five classes of nucleic-acid-based agents that can mediate gene knockdown via Watson-Crick pairing, which is the defining feature of antisense recognition. The mechanistically simplest class is synthetic antisense oligonucleotides that rely on covalent or strong noncovalent binding to the target RNA, resulting in steric blockage of translation without cleaving the target. Noncovalent blockers include nucleic acid analogs such as phosphorodiamidate morpholino oligomers (PMOs), N3′→P5′ phosphoramidates, 2′-O-methoxyethyl RNAs, locked nucleic acids (LNA), and peptide nucleic acids (PNA) [34–37]. Covalent-bond blocking agents comprise antisense oligonucleotides with reactive groups, such as alkylating, platinum, and photoactive derivatives [38–40], that are capable of covalent cross-linking with their RNA targets after hybridization.

A second class includes synthetic antisense oligodeoxynucleotides (ODNs), as well as certain nuclease-resistant analogs such as phosphorothioate or 2′-fluoroarabino derivatives that can recruit the endogenous ribonuclease RNase H to cleave a target RNA upon hybridizing with it [41–42]. Although partially phosphothioate-modified oligonucleotides were the basis for a wide variety of "first-generation" antisense drug candidates, only one has been approved by the FDA: Vitravene

(fomivirsen), which is used against cytomegalovirus infections of the eye, primarily in AIDS patients. In general, first-generation chemistries are less potent and have worse side-effects than subsequent designs [43]. More successful have been so-called second-generation chemistries, which combine phosphorothioate modifications, allowing RNase H cleavage, with 2′-ribose modifications of residues near the ends of the oligonucleotide that provide increased helix stability, such as 2′-O-methyl and 2′-O-methoxyethyl [44]. The stringency requirements of RNase H are low, and as little as a 5–7 bp of complementarity between such oligonucleotides and an intracellular RNA molecule may be sufficient to cleave the RNA, leading to unintended effects [41, 45, 46]. Strategies using alternative endogenous ribonucleases that avoid this issue include conjugation with 2′,5′-oligoadenylate, which recruits RNase L [47, 48], or mimicking the 3′-end of tRNA to recruit RNase P [49–51].

A third class is made up of catalytic nucleic acids, ribozymes, and deoxyribozymes that can hybridize to and cleave target RNAs without the need for any protein cofactor such as RNase H [17, 52–54]. A variant of this class, a group sometimes called artificial ribonucleases, consists of ODNs bearing catalytic groups that can cleave RNA either directly or with the assistance of metal ions [55–58].

A fourth class includes antisense RNAs expressed intracellularly from appropriate vectors [59–68]. Although such RNAs inherently lack chemical stabilization against nuclease degradation, they can be highly potent inhibitors of gene expression [69–72]. Antisense RNAs pair with RNA targets with a binding strength similar to that of the steric blockers (N3′→P5′ phosphoramidates, morpholino phosphorodiamidates, and 2′-O-methoxyethyl modifications) [36, 37, 73]. Antisense RNA can inactivate its target mRNA by the physical blocking or disruption of functionally active structures (for example, preventing splicing, export from nucleus to cytoplasm, or initiation of translation), or by induction of target RNA degradation by cellular nucleases such as RNAse III [23, 74–80].

The final group is small interfering RNA, which, unlike other antisense classes, are duplex rather than single-stranded oligonucleotides. Three subgroups can be distinguished, each typically 19–29 bp in length: small interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), and the natural cousins of shRNAs, microRNAs (miRNAs) [81–83]. In the case of siRNAs, the RNA duplex becomes incorporated into an RNA-induced silencing complex (RISC), one of the two strands (the sense strand) is displaced, and subsequent pairing of the antisense strand with the target mRNA leads to cleavage of the latter at the binding site, catalyzed by the Argonaute 2 component of RISC. Moreover, recent results concerning the complexes formed between antisense RNA and target RNA provide direct evidence for mechanistic links between antisense-mediated gene silencing and posttranscriptional gene silencing through RNA interference and suggest that their mechanisms of action could share steps in common at the double-stranded RNA stage [84–87].

siRNAs (including shRNAs), which were first developed only a few years ago, are generally more potent than other classes of antisense agents [88, 89]. However, there are currently several challenges to the use of siRNAs. Some RNAs appear to be poor targets for siRNAs. These may include highly structured RNAs [90–93], short-lived transcripts, and viral RNAs having high mutation rates [88, 94]. Many RNA viruses, whose hosts include plants as well as mammals, have found ways to

escape from the inhibitory effects of siRNAs by antagonizing RNA interference pathways [95–105]. siRNAs can also have significant "off-target" effects, knocking down unintended mRNAs that have similar sequences [106–114]. In addition to affecting specific "off-targets," several recent reports indicate that when siRNAs are introduced into cells, they can induce a nonspecific interferon response [115–120]. Finally, high-level expression of shRNA can be toxic, perhaps due to saturation of the cellular RNAi pathways that are needed for miRNA function [121].

Thus, traditional antisense methods may still provide useful alternative or complementary approaches for gene knockdown when siRNAs are insufficiently effective or specific [17, 122–126]. Indeed, "morpholino" oligonucleotides are as potent as siRNAs in inhibiting the translation of certain mRNA targets [73, 127, 128]. The same is true of some other types of antisense oligonucleotides whose sequences have been carefully optimized [15, 16, 36, 43, 129–134]. It is also to be noted that treatment of certain conditions requires calibrated modulation of a target mRNA rather than its complete inhibition [135].

Besides preventing translation, some antisense agents (those that block rather than cleave their targets) can be used for regulation of alternative splicing [136–143] and intracellular imaging of gene expressions [144–148]. It is important to emphasize that siRNAs are not suitable for these purposes. Yet another application, one that is currently generating particular excitement, and for which antisense oligonucleotides are uniquely suited, is the knockdown of miRNAs through the formation of stable, inactive antisense-miRNA duplexes [149–155]. Since some miRNAs are implicated in cancer [9, 156–158] and viral infection [159–161], antisense agents directed against these miRNAs are potential drug candidates.

3.3 MOLECULAR BASIS OF SEQUENCE SPECIFICITY OF ANTISENSE AGENTS

Antisense-based drugs and hybridization probes (we use the term *antisense agents* to describe both) share a very important feature: They are all designed to sequence-specifically bind their polynucleotide targets through complementary (usually Watson-Crick) base-pairing. They can also bind to imperfectly complementary (mismatched) sequences, but with a reduced affinity compared with perfectly matched partners. The differences in thermostability between a perfect duplex and a mismatched duplex depend on length, GC content and sequence, as well as the type and position of mismatches. In the presence of a limited number of different targets *in vitro*, shorter antisense agents are generally more selective than longer ones [40, 162–165]. However, when many different sequences are simultaneously present (such as in microarrays or cells), the probability of finding short, perfectly matching sequences in both target and nontarget polynucleotides increases, limiting the overall selectivity of short antisense sequences.

Assuming a distribution of nucleotides in the human genome, whose estimated length is 3×10^9 bp [166], antisense sequences longer than 18 nt would be required to ensure complete uniqueness of the perfectly matched complexes with the genome sequences. However, only an estimated 2–3% (~1×10^8 nt) of the genome encodes mature into mRNA sequences [167]. Because only about 25% of these mRNAs are

thought to be expressed in any given cell, the actual number of targets is closer to 2×10^7 nt [168]. Of this number, less than 20% are estimated to be accessible because of formation of stable secondary structures and RNA-protein complexes [40, 168, 169]. Thus, only about 4×10^6 nt are targetable, and hence an antisense molecule of as few as 11 nt ($4^{11} = 4.2 \times 10^6$ nt) could be long enough to uniquely match a specific mRNA target sequence. (Note that 11 bp is about the length of one turn of the A-form helix formed by both RNA-RNA and DNA-RNA duplexes.) Interestingly, several studies report that oligonucleotides only 7–11 nt in length can selectively inhibit translation of certain genes through targeting their mRNAs [170–175], although long oligonucleotides are usually far more potent inhibitors of gene expression than shorter ones [22, 40].

In general, RNA-RNA hybrids are more stable than the corresponding DNA-RNA and DNA-DNA duplexes [176–179]. As indicated above, the efficiency of probe-target hybridization can be hindered by the formation of secondary structures that reduce target site accessibility [180, 181]. For structured targets, longer probes (>20 nt) usually are more effective and have higher affinity to RNA targets than shorter probes because they have multiple opportunities for base-pairing. Because of the generally higher duplex stability of RNA-RNA pairing over DNA-RNA, antisense RNA probes have faster hybridization kinetics and a better ability to bind structured polynucleotide targets than corresponding DNA probes [165, 182]. It appears that high affinity for their target sites and fast hybridization kinetics [183–187] are the most important determinants of efficacy in the case of antisense agents that act through noncleaving, target-blocking mechanisms.

The trade-offs between high affinity for the target and low sequence specificity of binding have implications for the design of allele-specific antisense agents [40, 188]. Increasing the affinity of antisense agents to their intended polynucleotide targets, either by lengthening antisense sequences or by using appropriate chemical modifications of antisense oligonucleotides, will simultaneously decrease their selectivity, thereby enhancing off-target effects [40]. This limitation is also one of the major hurdles in the development of antisense agents that can discriminate SNPs and hence distinguish individual gene profiles. This is true even for *in vitro* assays that can otherwise be optimized for maximum selectivity (e.g., through variation of temperature, incubation time, salt and formamide concentration of the hybridization, and washing conditions). Antisense drugs lack this option because the intracellular environment provides fixed conditions, including constant physiological temperature and solutes.

There are several ways to design SNP-sensitive antisense agents. The first approach is to use antisense chemistries that provide tight binding even for short pairing regions. In this way, a single mismatch has a large impact on the helical stability, yet a short sequence is stable at body temperature. In the case of LNA, each substitution of an LNA residue for a DNA increases the melting temperature (T_m) by 2°C–10°C per LNA monomer (depending on sequence content) when hybridized to RNA targets [189, 190]. LNAs have been successfully used for this purpose [191], as have morpholinos [128].

Another approach uses side-by-side hybridizing oligonucleotides that can cooperatively bind adjacent sites in RNA targets [192, 193] (Figure 3.1).

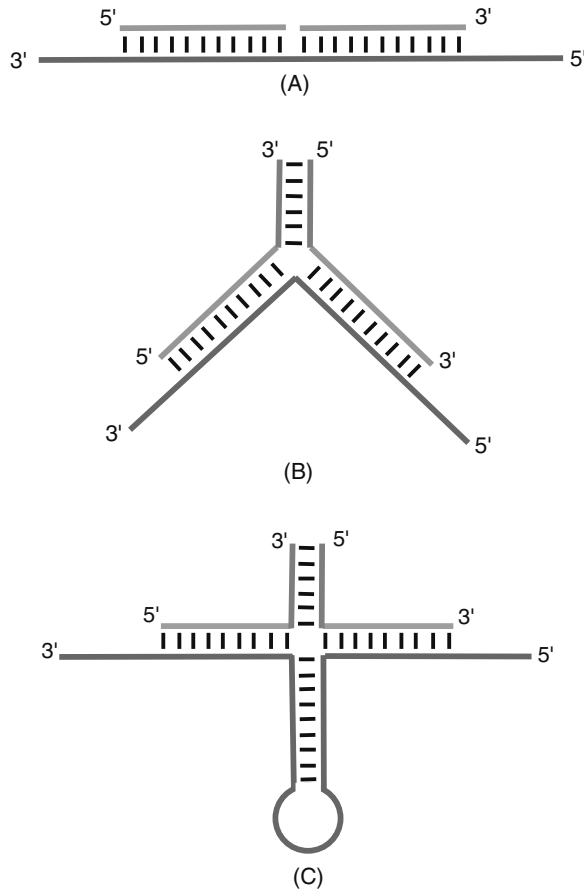

FIGURE 3.1 (See color insert following page 56.) Cooperative binding of two short oligonucleotides to RNA targets. (A) Side-by-side binding of two oligonucleotides to adjacent target sequences. The complex is stabilized through stacking interactions at the interface between the oligonucleotides. (B) Side-by-side binding of two partially complementary oligonucleotides to adjacent target sequences. The complex is stabilized through base-pairing between the oligonucleotide dimerization segments. (C) Binding of two partially complementary oligonucleotides to nonadjacent target sequences that are brought together in space by a secondary structure in the target. This complex is also stabilized through base-pairing between the oligonucleotide dimerization segments. RNA targets are shown in blue, antisense in red, and the dimerization segments in green.

A third approach is to use ribozymes that employ two comparatively short antisense sequences (binding arms) acting in concert, such as hammerhead (7 + 7 nt) [194, 195] (Figure 3.2A) and hairpin ribozymes (6 + 4 nt) [194, 196] (Figure 3.2B). Indeed, SNP specificity of RNA cleavage by hammerhead ribozymes has been demonstrated *in vitro* [197, 198]. However, other results indicate that hammerhead ribozymes with longer arms, which are less sensitive to mismatches, are substantially more potent in cells than those with short arms [199–201]. Short antisense RNAs

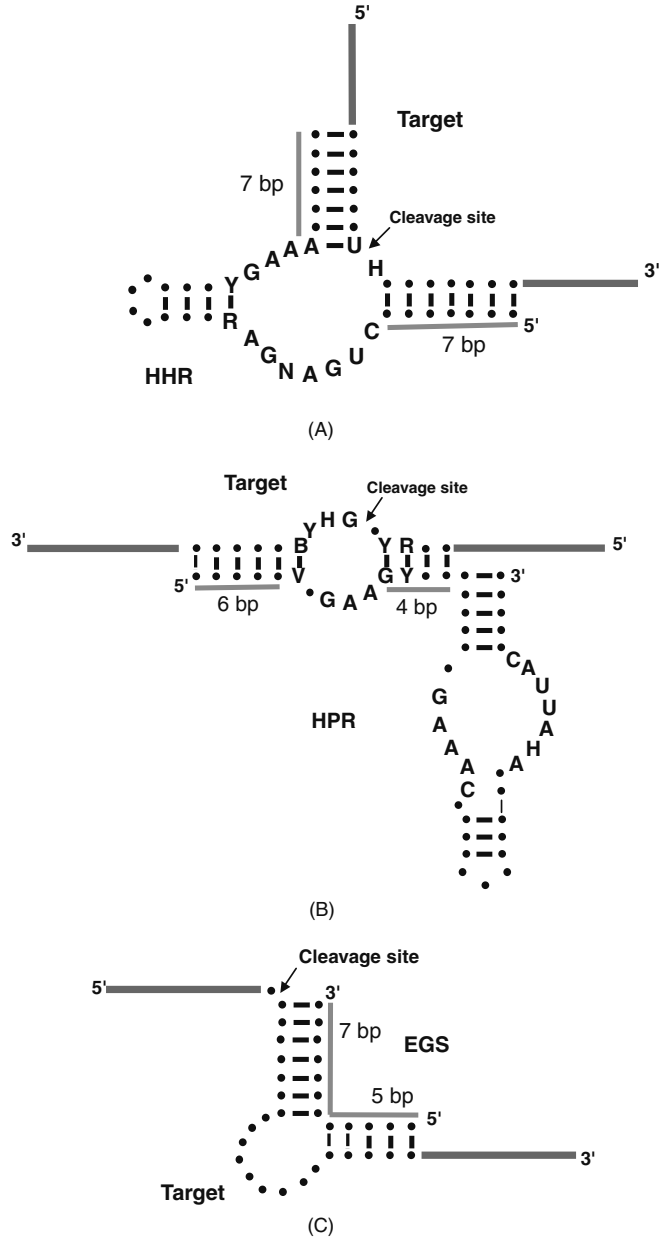

FIGURE 3.2 (See color insert following page 56.) Secondary structures and consensus sequences of representative ribozymes cleaving their RNA targets in bimolecular reactions (*in trans*). (A) Hammerhead ribozyme (HHR). (B) Hairpin ribozyme (HPR). (C) External guide sequence (EGS) directing cleavage of target RNA by the human RNase P ribozyme. Dots represent any nucleotide (A, U, G, or C); dashes represent required pairings; V is "not U" (A, C, or G); Y is a pyrimidine (U or C); R is a purine (A or G); B is "not A" (U, C, or G); and H is "not G" (A, C, or U) [275]. RNA targets are shown in blue, and antisense ribozyme arms are in red.

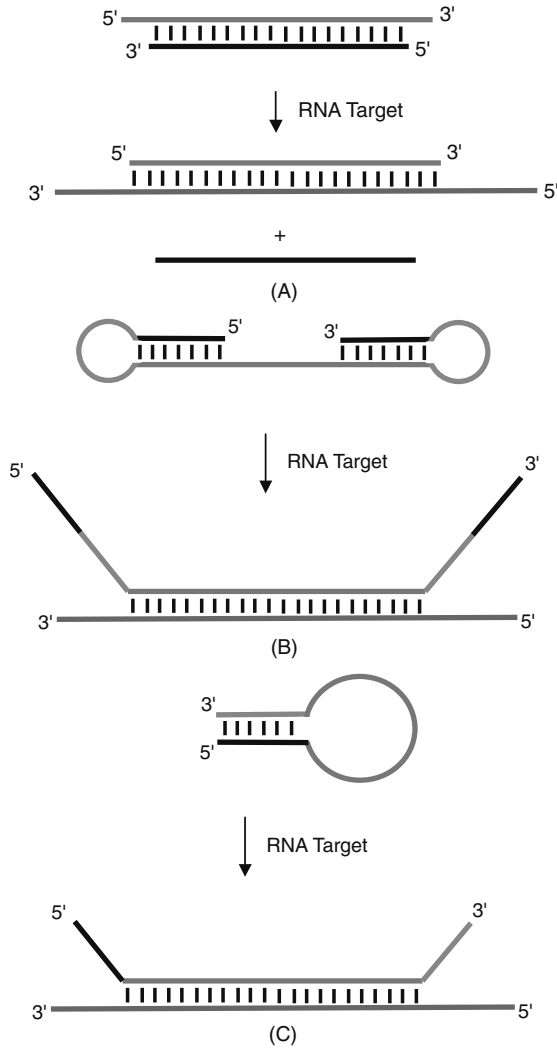

FIGURE 3.3 (See color insert following page 56.) Antisense oligonucleotides equipped with various types of stringency elements. (A) Antisense oligonucleotide prehybridized with a complementary masking oligonucleotide that covers the target site but is shorter by a few nucleotides at one or both ends. As a result of the competitive hybridization, the antisense sequence forms a perfect duplex with the target, and the masking oligonucleotide gets displaced. (B) Antisense sequence extended at either one or both ends (two-end extension is shown) by sequences forming terminal hairpin structures. As a result of the competitive hybridization with the target, the antisense sequence forms a perfect duplex, whereas the terminal masking sequences gets displaced. (C) Antisense sequence extended at both ends by short complementary sequences that form a stem-and-loop structure known as a "molecular beacon." When the antisense sequence in the loop anneals to a complementary target sequence, the longer and stronger probe-target duplex overcomes the internal secondary structure, leading to opening of this structure. Antisense oligonucleotides having all these stringency elements form stable, perfect duplexes with normal target sequences, whereas targets containing mismatches form either unstable duplexes, or no duplexes. RNA targets are in blue, antisense in red, and the stringency elements are in black and green as shown.

encoding external guide sequences for human RNase P, consisting of two short (7 + 5 nt) arms [202] (Figure 3.2C), can induce cleavage of the target RNA in cells while providing true SNP sensitivity [203].

A fourth approach is the use of stringency elements that can improve mismatch discrimination upon hybridization, including displacement hybridization [204, 205] (Figure 3.3A), hairpins [206–208] (Figure 3.3B), and molecular beacons [209, 210] (Figure 3.3C).

A fifth approach is the introduction of artificial mismatches in antisense sequences [211, 212] (Figure 3.4). Because two mismatches a certain distance apart are especially destabilizing to a duplex, introducing an artificial mismatch that distance from an SNP is an effective way to increase the discrimination between the SNP variants. This last approach is similar to what is often found in natural antisense mechanisms [23–24, 212–214]. Most naturally occurring regulatory antisense RNAs, which are typically 60–100 nt in length and transcribed from a locus different from that encoding the target RNA, do not perfectly match their RNA targets [23, 212]. Complexes of natural ribozymes and aptamers with their targets also frequently contain mismatches and noncanonical base-pairing [52, 54, 215, 216), suggesting that such structural elements may function to enhance sequence specificity.

3.4 RNA LASSOS®

An ideal antisense agent, capable of SNP discrimination, would combine the excellent hybridization efficacy and high target affinity of long antisense sequences with the ability of short antisense sequences to discriminate closely related gene sequences. To avoid the usual trade-off between these two desirable features, one might try to use short recognition sequences to assure high specificity while stabilizing the antisense-target duplex by other means once it is formed. One manifestation of this approach is the RNA Lasso [217–221] (Figure 3.5). RNA Lassos contain an internal hairpin ribozyme (HPR) moiety (Figure 3.5A) that has both self-cleavage and self-ligating capabilities [196]. This HPR moiety exists as a dynamic equilibrium between linear and circular forms that can be regulated by features of the ribozyme sequence [196]. The size and sequence of the three loops that connect the helical segments of the HPR can be modified without substantial effects on catalytic activity if the loop sequences do not interfere with the proper folding of the ribozyme [222–224]. The principle of the Lasso is that insertion of an antisense sequence into one of these loops (typically loop 2) allows the Lasso to pair with a target mRNA, intertwining the two RNAs if it is in the linear form. The Lasso can then self-circularize, creating a "topologically linked" complex with a linear target mRNA. (If the target were itself circular, the complex would have a true topological linkage; in the case of a linear target, the complex could dissociate only if all pairing with the antisense sequence were disrupted and the Lasso slipped off the end, or if the Lasso were to become linear through cleavage of its backbone.) In contrast to the conventional application of ribozymes as sequence-specific nucleases, Lassos do not cleave their targets, but by linking to them, they create complexes that are thermodynamically more stable than ordinary RNA-RNA duplexes. Another advantage of circularity is that it makes Lassos resistant to exonucleases.

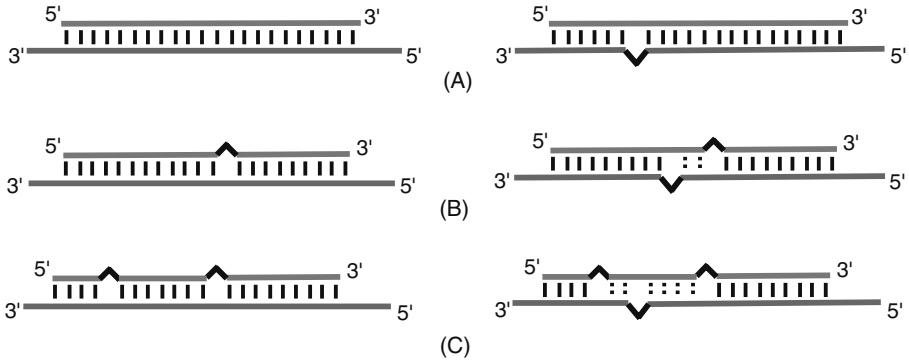

FIGURE 3.4 (See color insert following page 56.) Perfect and mismatched duplexes between an antisense oligonucleotide and an RNA target. (A) Conventional allele-specific hybridization of a "perfect" antisense with either a normal target (left) or one with a single-nucleotide substitution due to a mutation or SNP (right). (B) Hybridization of a single-base mismatched oligonucleotide with the normal (left) and mutated targets (right). (C) Hybridization of doubly mismatched oligonucleotide with normal (left) and mutated targets (right). In all cases the oligonucleotide forms a more stable duplex with the normal target than the mutated target; however, because mismatches spaced a certain distance apart are especially destabilizing, the presence of two or three mismatches between antisense oligonucleotide and the target may provide better discrimination between the two targets. RNA targets are shown in blue, antisense in red, and mismatches in black. The interactions between complementary bases that are weakened by the nearby mismatches are shown as dotted lines.

Lassos can be highly sequence-specific [221], presumably due at least in part to their circularity. It has been shown that antisense DNA circles show higher sequence selectivity (more destabilization by the presence of mismatches) than do linear ODNs of the same sequence [225, 226]. Because stable binding requires disruption of some secondary structure (Loop A and Helix 2 in Figure 3.5A), the latter may act as a stringency element (see above) by competing with the target for binding and thereby reducing the net energy gain upon binding. When binding is weaker at a given temperature, the complex is more prone to destabilization by mismatches. In this respect, Lassos are reminiscent of molecular beacons (see Figure 3.3C). In some cases, where a given Lasso is not optimally specific, it can be made fully SNP-sensitive by incorporating an additional stringency element (see above) into its antisense domain [221].

In their ability to topologically link around a target, RNA Lassos are also reminiscent of the Padlock probes [227–229], although they differ in several respects (Figure 3.6). Lassos are 120–130-nt RNAs that can be either transcribed *in vitro* or expressed from DNA vectors *in situ*. In contrast, Padlock probes are 70–100-nt synthetic DNAs that allow target-dependent ligation of their ends by a DNA ligase. The need for an exogenous protein ligase largely restricts the use of Padlock probes to diagnostic assays [228, 230], whereas RNA Lassos can function autonomously inside cells and are therefore of therapeutic as well as diagnostic interest.

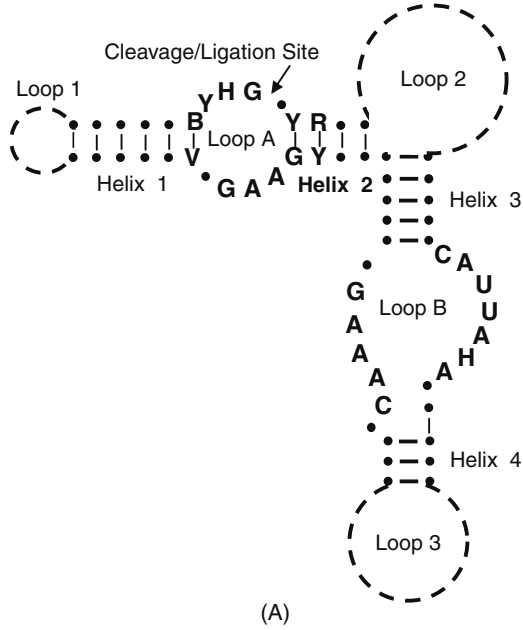

FIGURE 3.5 Structure and self-processing properties of an RNA Lasso. (A) Consensus structure of the hairpin ribozyme (HPR), derived from sequences in the minus strand of tobacco ringspot virus satellite RNA. Self-cleavage at the site shown produces 2′,3′-cyclic phosphate and 5′-OH termini. The reverse reaction can efficiently ligate those ends, and the molecule shown can exist as a dynamic equilibrium between linear and circular forms. The position of the equilibrium depends on the relative stability of the cleaved and ligated forms. Dots represent any nucleotide (A, U, G, or C); dashes represent base pairings; V is "not U" (A, C, or G); Y is a pyrimidine (U or C); R is a purine (A or G); B is "not A" (U, C, or G); and H is "not G" (A, C, or U) [275]. (B) Scheme of Lasso self-processing. Trimming the ends of a longer RNA Lasso precursor (typically made by *in vitro* transcription) through self-cleavage generates semiprocessed intermediates and the fully processed linear form, which can then convert into the circular form through self-ligation.

In addition to self-ligation, a convenient feature of the HPR is its ability to excise itself from a primary transcript, cleaving off all irrelevant flanking sequences at both the 5′ and 3′ ends, prior to circularizing (Figure 3.5B). This allows the Lasso to be independent of any sequences that may be introduced for convenience, e.g., to aid in expression. Thus, RNA Lassos can be either transcribed *in vitro* from DNA vectors by T7 RNA polymerase and delivered to cellular targets directly (e.g., in liposomal complexes), or expressed *in vivo* by RNA pol II or pol III, using appropriate plasmid, PCR-amplicon, or viral vectors.

Like antisense RNA, Lassos can potentially disable a target RNA either by physically blocking its function, causing misfolding of functionally active structures, or inducing its degradation by cellular nucleases. Which mechanism predominates can be controlled by the design features of the Lasso.

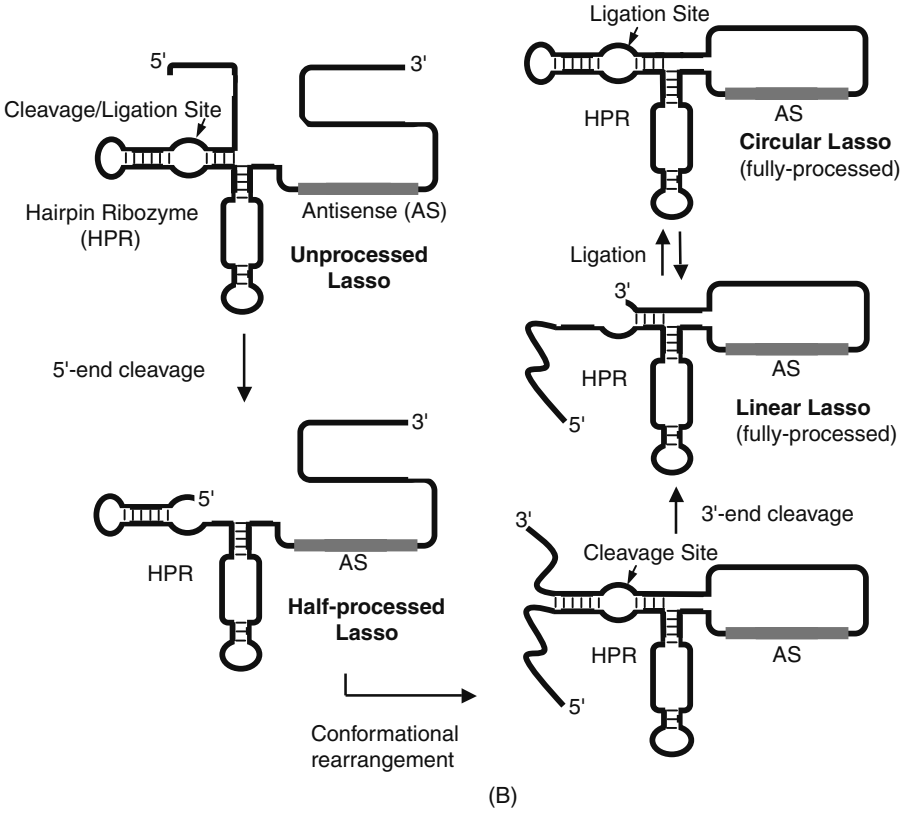

FIGURE 3.5 (Continued.)

3.5 IDENTIFYING POTENT AND SPECIFIC ANTISENSE TARGET SEQUENCES

The original concept of antisense-based drug design embodied the notion that a gene of interest can be specifically targeted as long as its sequence is known. However, several facts argue that this concept is oversimplified [133, 231–235]. First, not all sites of target RNA are equally accessible for hybridization under physiological conditions. Second, some antisense sequences may regulate multiple related genes. Third, antisense molecules can frequently form imperfect (mismatched) complexes with unintended sequences (off-target effects). Fourth, certain antisense sequences can nonspecifically alter the expression of unrelated genes, resulting in toxic effects. All of these problems make the targeting of certain sites (selected, for example, based on pharmacogenomics considerations) difficult.

There are a few approaches that in some cases can make poorly accessible sites more accessible for antisense oligonucleotides and ribozymes/deoxyribozymes, including use of longer antisense sequences (also discussed above), use of chemical modifications that increase thermostability of complementary complexes (see above), and use of helper/facilitator oligonucleotides hybridized to sequences

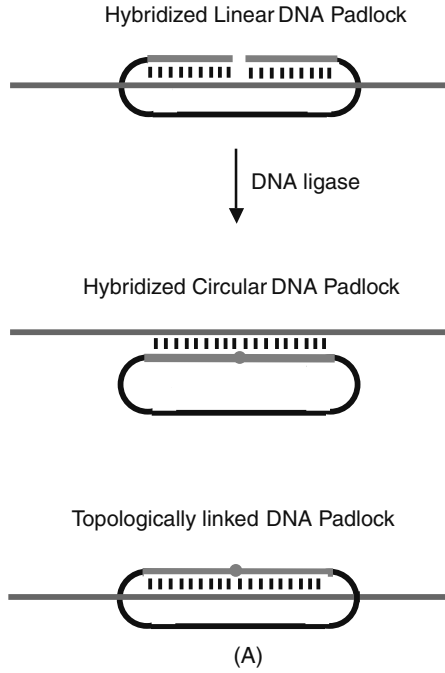

FIGURE 3.6 (See color insert following page 56.) Circularizable nucleic acid agents. (A) Padlock Probe (DNA). (B) RNA Lasso. These agents are linear polynucleotides that can hybridize by their antisense segments (shown in red) to an RNA target (blue). Their terminal sequences are joined by either DNA ligase (Padlock Probe) or self-ligated by the encoded ribozyme (RNA Lasso). Note that the ligation site (dot) for the Padlock Probe lies within the antisense-target duplex, whereas for the RNA Lasso it is outside this duplex. Circularization of linear forms of these agents prebound to their targets results in the formation of topologically linked complexes.

flanking the target site [236–239]. However, even where accessibility is not a problem, the function of a sequence within an mRNA can also influence its effectiveness as an antisense target. MicroRNAs mainly target 3′-UTRs, while most effective antisense drugs target 5′-UTRs [128]. Noncleaving antisense agents that target the coding mRNA regions are in general less efficient, presumably because the strong helicase activity of ribosome complexes can displace even strongly bound antisense agents such as morpholino oligonucleotides [175]. This displacement activity represents another challenge for the use of antisense agents to target SNPs within coding regions.

For all of these reasons, there is a need for the development of convenient and reliable methods for identifying the most "sensitive" target sequences. Most approaches to addressing this need have involved either computer prediction or *in vitro* selection/mapping using combinatorial libraries [240–254]. Although *in vitro* screening of libraries of antisense agents by methods such as mapping mRNA targets by RNAse H cleavage or scanning target sequences by microarrays have had some success in predicting targetable sequences, many other studies show little or no correlation

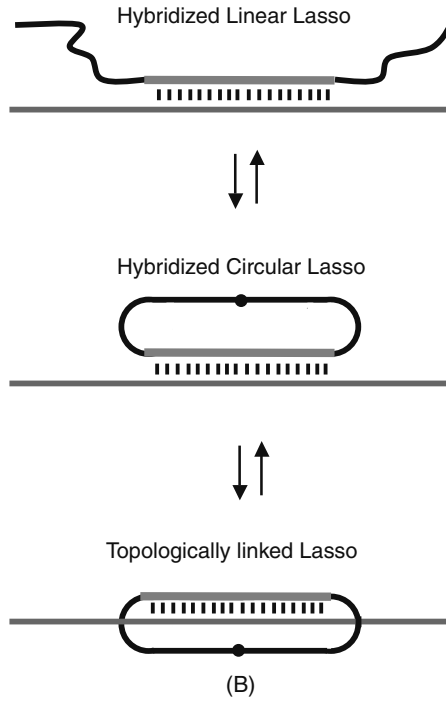

FIGURE 3.6 (Continued.)

between the *in vitro* data and the intracellular efficacy of the same antisense agents [135, 255–259]. This incongruity may reflect different folding of the RNAs within the microenvironments of the living cell versus in the test tube, or result from the presence of RNA-binding proteins in cells. Ideally, all possible target-specific RNA sequences within an appropriate range of lengths should be prepared and individually tested in cells to ensure finding the best inhibitors for a given mRNA. However, such a "brute force" approach is expensive and time consuming.

As an alternative, screening for target sites can be performed by using random (degenerate) libraries of antisense sequences directly in cells [260–262]. However, this approach has several major problems. First, the high complexity of random libraries (e.g., 4^{20} or $\approx 10^{12}$ molecules for 20-nt antisense sequences represented only about once in the human genome) [166] may make this approach prohibitively expensive for cell-based assays. This is because cell-based selection requires either approximately one construct per cell, or if there are multiple constructs per cell, each must be potent enough to provide the basis for selection even though it is diluted by the presence of inactive members of the library. In the latter case, subsequent rounds of selection must be made to identify the active species from among the inactive ones present in the selected cells [262]. Second, since each sequence in a degenerate library has its complement also represented, the two can form stable duplexes, thus reducing their availability for interaction with accessible target sites [263]. Third, experiments have shown that degenerate libraries are highly toxic to cells because

they can block the functioning of unintended cellular RNAs as well as the intended target [262, 264, 265].

Directed, or gene-specific, oligonucleotide libraries composed of all 15–25-nt long sequences represented within the target gene(s) of interest offer a superior alternative to screening completely random libraries. The use of directed libraries prepared *in vitro* significantly simplifies cell-based screening, since comparatively small libraries need to be assayed. For example, a 20-nt directed library targeting a 2000-nt mRNA consists of only 1981 distinct molecules. Moreover, unintended knockdown of nontargeted genes is reduced, allowing more efficient cell-based assays with the directed libraries cloned into appropriate vectors. Several methods for preparing directed libraries that can be cloned, amplified, and inserted into appropriate antisense, ribozyme, or siRNA expression vectors have been described [246, 264–270].

Preparation of high-quality libraries is important, but represents the simpler part of the selection procedure. The functional screening of such libraries in cell-based environments is more complicated and problematic. The development of adequate cellular screening methods is critical to identifying the most potent and least toxic antisense agents. This subject has been reviewed elsewhere [135, 270–274].

3.6 CONCLUSION

Because antisense agents obtain their specificity from Watson-Crick pairing rules, they are natural candidates for exploiting the often-subtle DNA sequence differences among individuals that are the basis of pharmacogenomics. However, because the RNA sequences affected by those polymorphisms may have a secondary structure or be bound to proteins, not all the polymorphic sites are readily accessible to antisense agents. The inability to pick and choose accessible sites is why the use of antisense for pharmacogenomics is a bigger challenge than its use for ordinary therapeutic goals, where there is more choice of target sites. Inroads have been made on this problem by two types of advances: more potent antisense technologies, including strong RNA blockers and siRNAs, and better methods for identifying good target sites so that the likelihood of finding an "antisensitive" site that overlaps a relevant polymorphism is greater. Ultimately, the role of antisense agents in this area may be as sequence-sensitive adjuncts to less specific but more potent small-molecule drugs, consistent with current trends toward the use of nucleic acids in combination therapies.

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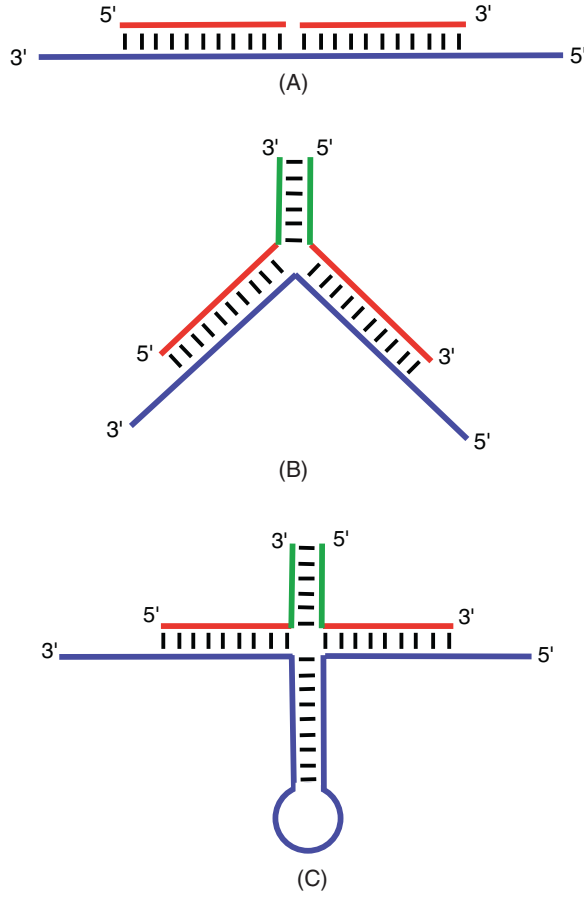

FIGURE 3.1 Cooperative binding of two short oligonucleotides to RNA targets. (A) Side-by-side binding of two oligonucleotides to adjacent target sequences. The complex is stabilized through stacking interactions at the interface between the oligonucleotides. (B) Side-by-side binding of two partially complementary oligonucleotides to adjacent target sequences. The complex is stabilized through base-pairing between the oligonucleotide dimerization segments. (C) Binding of two partially complementary oligonucleotides to nonadjacent target sequences that are brought together in space by a secondary structure in the target. This complex is also stabilized through base-pairing between the oligonucleotide dimerization segments. RNA targets are shown in blue, antisense in red, and the dimerization segments in green.

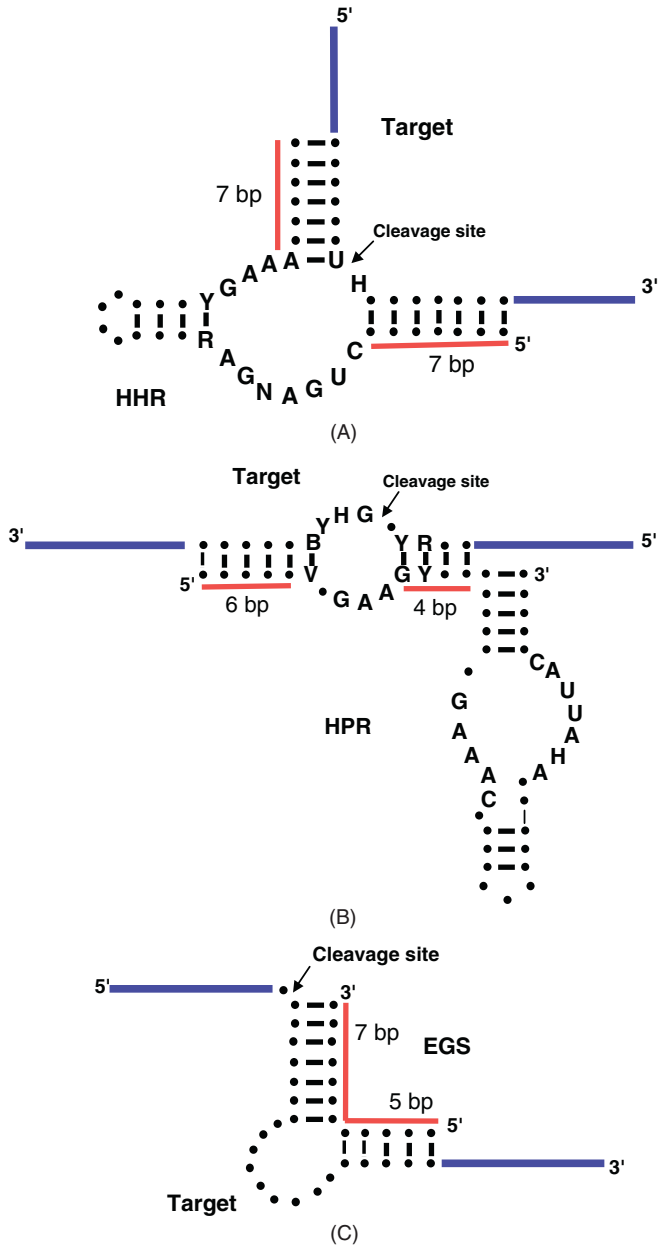

FIGURE 3.2 Secondary structures and consensus sequences of representative ribozymes cleaving their RNA targets in bimolecular reactions (*in trans*). (A) Hammerhead ribozyme (HHR). (B) Hairpin ribozyme (HPR). (C) External guide sequence (EGS) directing cleavage of target RNA by the human RNase P ribozyme. Dots represent any nucleotide (A, U, G, or C); dashes represent required pairings; V is "not U" (A, C, or G); Y is a pyrimidine (U or C); R is a purine (A or G); B is "not A" (U, C, or G); and H is "not G" (A, C, or U) [275]. RNA targets are shown in blue, and antisense ribozyme arms are in red.

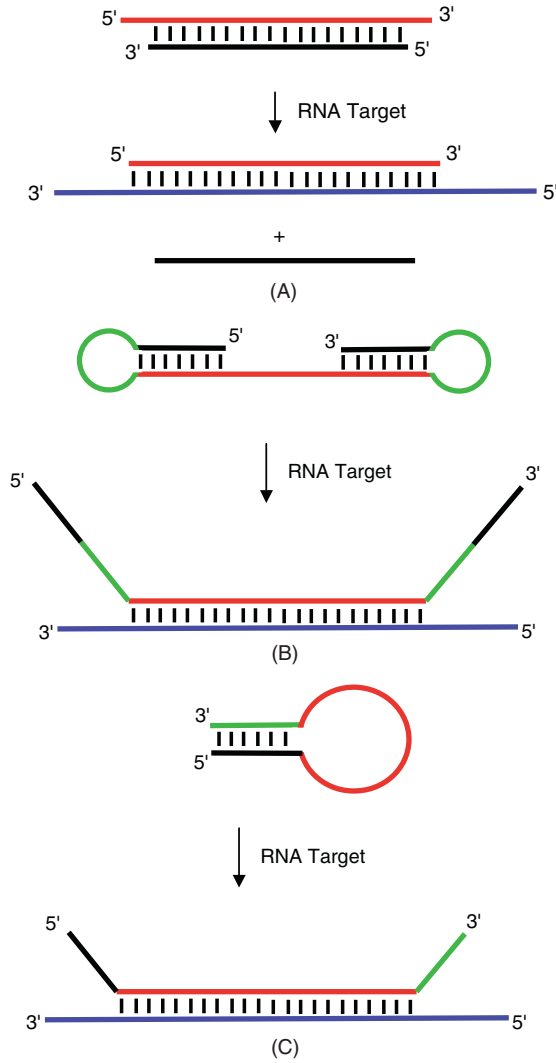

FIGURE 3.3 Antisense oligonucleotides equipped with various types of stringency elements. (A) Antisense oligonucleotide prehybridized with a complementary masking oligonucleotide that covers the target site but is shorter by a few nucleotides at one or both ends. As a result of the competitive hybridization, the antisense sequence forms a perfect duplex with the target, and the masking oligonucleotide gets displaced. (B) Antisense sequence extended at either one or both ends (two-end extension is shown) by sequences forming terminal hairpin structures. As a result of the competitive hybridization with the target, the antisense sequence forms a perfect duplex, whereas the terminal masking sequences gets displaced. (C) Antisense sequence extended at both ends by short complementary sequences that form a stem-and-loop structure known as a "molecular beacon." When the antisense sequence in the loop anneals to a complementary target sequence, the longer and stronger probe-target duplex overcomes the internal secondary structure, leading to opening of this structure. Antisense oligonucleotides having all these stringency elements form stable, perfect duplexes with normal target sequences, whereas targets containing mismatches form either unstable duplexes, or no duplexes. RNA targets are in blue, antisense in red, and the stringency elements are in black and green as shown.

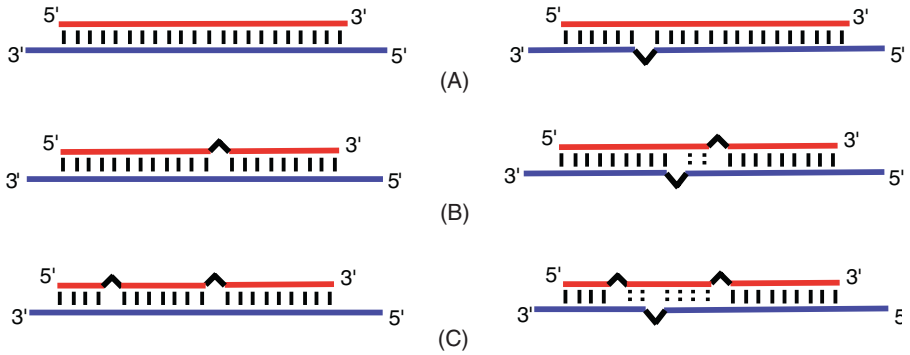

FIGURE 3.4 Perfect and mismatched duplexes between an antisense oligonucleotide and an RNA target. (A) Conventional allele-specific hybridization of a "perfect" antisense with either a normal target (left) or one with a single-nucleotide substitution due to a mutation or SNP (right). (B) Hybridization of a single-base mismatched oligonucleotide with the normal (left) and mutated targets (right). (C) Hybridization of doubly mismatched oligonucleotide with normal (left) and mutated targets (right). In all cases the oligonucleotide forms a more stable duplex with the normal target than the mutated target; however, because mismatches spaced a certain distance apart are especially destabilizing, the presence of two or three mismatches between antisense oligonucleotide and the target may provide better discrimination between the two targets. RNA targets are shown in blue, antisense in red, and mismatches in black. The interactions between complementary bases that are weakened by the nearby mismatches are shown as dotted lines.

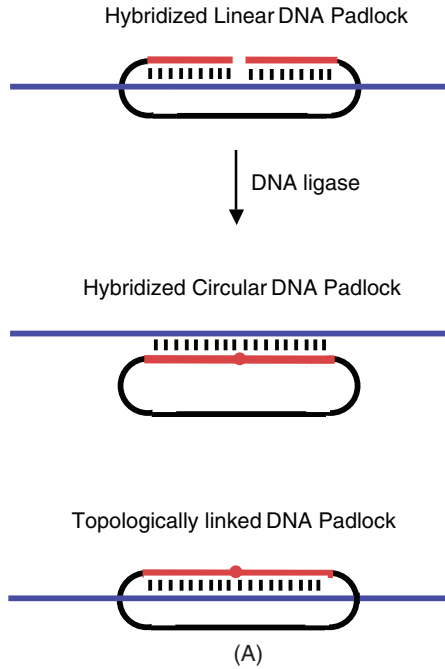

FIGURE 3.6 Circularizable nucleic acid agents. (A) Padlock Probe (DNA). (B) RNA Lasso. These agents are linear polynucleotides that can hybridize by their antisense segments (shown in red) to an RNA target (blue). Their terminal sequences are joined by either DNA ligase (Padlock Probe) or self-ligated by the encoded ribozyme (RNA Lasso). Note that the ligation site (dot) for the Padlock Probe lies within the antisense-target duplex, whereas for the RNA Lasso it is outside this duplex. Circularization of linear forms of these agents prebound to their targets results in the formation of topologically linked complexes. (Continued on next page.)

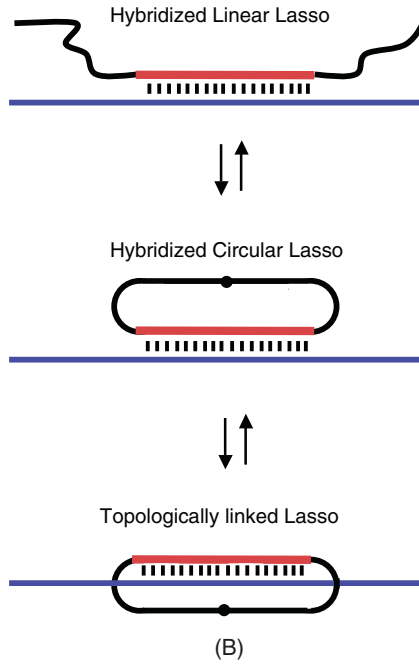

FIGURE 3.6 (Continued.)

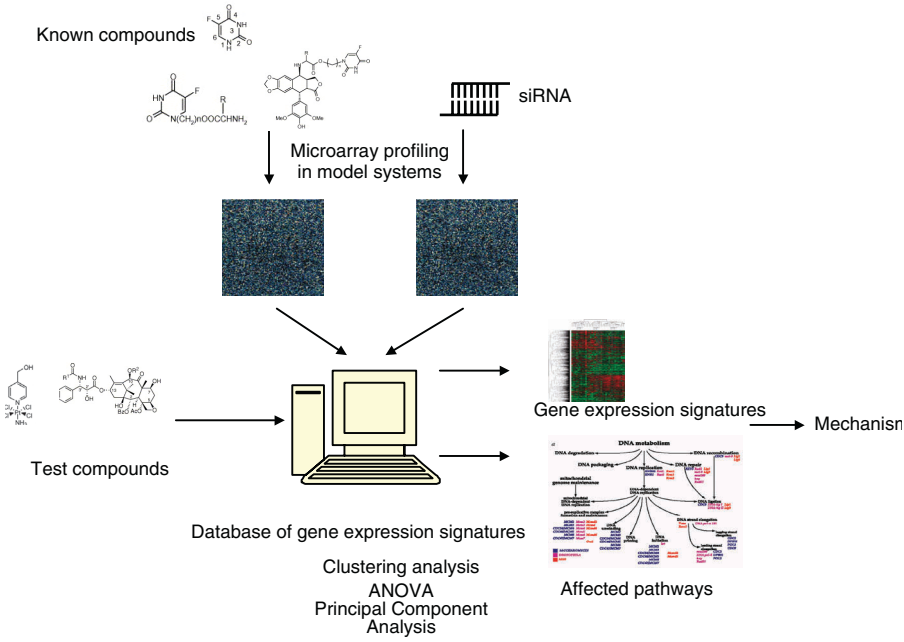

FIGURE 4.2 A genomics database for compound selection and optimization. The database is populated with gene profiles for compounds with known mechanisms of action as well as siRNA. The profiles for novel compounds are then clustered alongside the reference compounds and siRNA to make conclusions about their mechanism. (Reprinted with permission from *Preclinical Development Handbook–Toxicology*. D. Semizarov and E. A. G. Blomme. Shayne Cox Gad, ed. Genomics, 801–839. ©2008. Hoboken, NJ: John Wiley & Sons.)

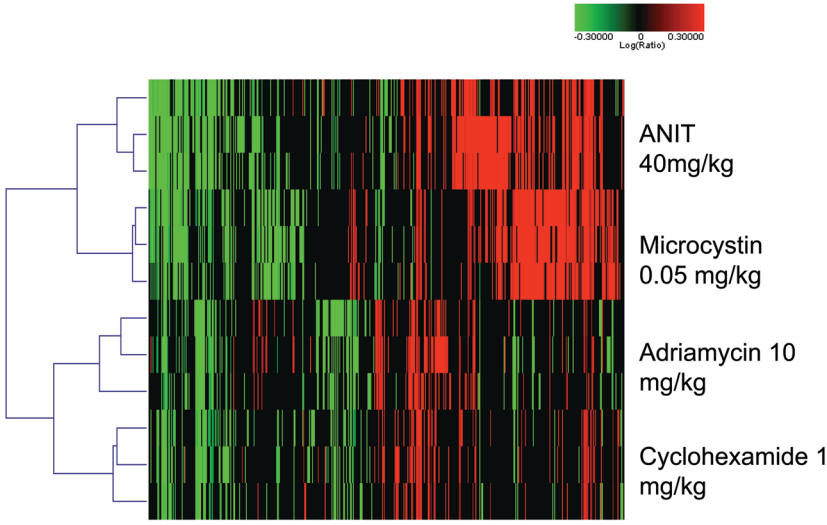

FIGURE 4.3 Hierarchical clustering of gene profiles from the livers of rats treated for 3 days with various hepatotoxicants at toxic doses. Included in the heatmap are genes that were up- or down-regulated by a factor of fl2 with a p value less than 0.01. Green indicates down-regulation, while red indicates up-regulation. Despite significant variability in responses observed with clinical pathology and histopathology, there is limited interindividual variability in gene expression profiles.