Locked Nucleic Acid: High-Affinity Targeting of Complementary RNA for RNomics

S. Kauppinen[1] · B. Vester[2] · J. Wengel[3] (✉)

[1]Department of Functional Genomics, Exiqon, Bygstubben 9, 2950 Vedbaek, Denmark

[2]Nucleic Acid Center, Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark

[3]Nucleic Acid Center, Department of Chemistry, University of Southern Denmark, 5230 Odense M, Denmark
jwe@chem.sdu.dk

Abstract Locked nucleic acid (LNA) is a nucleic acid analog containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA-mimicking sugar conformation. This conformational restriction is translated into unprecedented hybridization affinity towards complementary single-stranded RNA molecules. That makes fully modified LNAs, LNA/DNA mixmers, or LNA/RNA mixmers uniquely suited for mimicking RNA structures and for RNA targeting in vitro or in vivo. The focus of this chapter is on LNA antisense, LNA-modified DNAzymes (LNAzymes), LNA-modified small interfering (si)RNA (siLNA), LNA-enhanced expression profiling by real-time RT-PCR and detection and analysis of microRNAs by LNA-modified probes.

Keywords LNA · Locked nucleic acid · RNA targeting · LNA antisense · MicroRNA targeting

1
Introduction

The expanding inventory of sequence databases and the concomitant sequencing of more than 200 genomes representing all three domains of life—bacteria, archaea, and eukaryotes—have been the primary drivers in the process of deconstructing living organisms into comprehensive molecular catalogs of genes, transcripts, and proteins. The importance of the genetic variation within a single species has become apparent, extending beyond the completion of genetic blueprints of several important genomes, and a worldwide effort culminated in the publication of the human genome sequence in 2001 (Lander et al. 2001; Venter et al. 2001; Sachidanandam et al. 2001). Also, the increasing number of detailed, large-scale molecular analyses of transcription originating from the human and mouse genomes along with the recent identification of several types of non-protein-coding (nc)RNAs, such as small nucleolar RNAs, small interfering (si)RNAs, microRNAs (miRNAs), and antisense RNAs, indicates that the transcriptomes of higher eukaryotes are much more complex than originally anticipated (Wong et al. 2001; Kampa et al. 2004).

As a result of the central dogma—DNA makes RNA and RNA makes protein—RNAs have been considered as simple molecules that just translate the genetic information into protein. Recently, it has been estimated that although most of the genome is transcribed, almost 97% of the genome does not encode proteins in higher eukaryotes, but putative, non-coding RNAs (Wong et al. 2001). The ncRNAs appear to be particularly well suited for regulatory roles that require highly specific nucleic acid recognition. Therefore, the view of RNA is rapidly changing from a merely informational molecule to one that comprises a wide variety of structural, informational, and catalytic molecules in the cell.

The challenges of establishing genome function and understanding the layers of information hidden in the complex transcriptomes of higher eukaryotes call for novel, improved technologies for detection, quantification, and functional analysis of RNA molecules in complex nucleic acid samples. Locked nucleic acid (LNA) constitutes a new class of bicyclic high-affinity RNA analogs in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation by the introduction of a O2′,C4′-methylene bridge (Koshkin et al. 1998b; Obika et al. 1998). Several studies have demonstrated that LNA-modified oligonucleotides exhibit unprecedented thermal stability when hybridized with their DNA and RNA target molecules (Koshkin et al. 1998b; Obika et al. 1998; Braasch and Corey 2001; Jacobsen et al. 2004; Petersen and Wengel 2003). Consequently, an increase in melting temperature (T_m value) of +1 to +8 °C per introduced LNA monomer against complementary DNA and of +2 to +10 °C per LNA monomer against complementary RNA compared to unmodified duplexes has been reported. The first sections of this chapter describe some basic properties of LNA, whereas the latter sections are focused on

recent contributions in LNA-mediated RNA targeting in vitro and in vivo. For more general descriptions on the overall properties and biotechnological applications of LNA, including DNA diagnostics and double-stranded (ds)DNA targeting by triple helix formation, the reader is referred to recent reviews on LNA (Petersen and Wengel 2003; Jepsen and Wengel 2004; Vester and Wengel 2004).

1.1
LNA: Hybridization Properties and Structural Features

We have defined LNA as an oligonucleotide containing at least one LNA monomer, i.e., one 2′-O,4′-C-methylene-β-D-ribofuranosyl nucleotide (see Fig. 1; Singh et al. 1998; Koshkin et al. 1998b). The LNA monomers adopt N-type sugar puckers, also termed C3′-endo conformations (Singh et al. 1998; Obika et al. 1997). The vast majority of reports on LNA-mediated RNA targeting have dealt with mixmer LNA oligonucleotides, i.e., LNAs containing a limited number of LNA monomers in combination with other types of monomers, such as DNA, RNA, and 2′-O-Me-RNA monomers. We predict that this approach will also be important for future uses of LNA due to the following characteristics of LNA/DNA, LNA/RNA, and LNA/2′-O-Me-RNA mixmers: (1) synthetic convenience—standard methods for DNA oligomerization can be used for commercially available LNA, DNA and 2′-O-Me-RNA phosphoramidite building blocks (Pfundheller et al. 2005); (2) easy access—LNAs (fully modified or mixmers) are commercially available; (3) high-affinity and sequence-selective targeting of RNA molecules in vitro or in vivo (Singh et al. 1998; Koshkin et al. 1998b; Obika et al. 1998; Wengel 1999; Kumar et al. 1998; Braasch and Corey 2001); and (4) compatibility with standard modifiers and modifications, e.g., phosphorothioate linkages (Kumar et al. 1998).

A key feature of oligonucleotides containing LNA nucleotides—i.e., fully modified LNAs, LNA/DNA mixmers, LNA/RNA mixmers, etc.—is the very high thermal stability of duplexes towards complementary RNA or DNA (Singh et al. 1998; Koshkin et al. 1998b; Obika et al. 1998; Wengel 1999; Kumar et al. 1998; Braasch and Corey 2001). Notably, this increase in affinity goes hand in hand with preserved, or even improved, Watson-Crick base
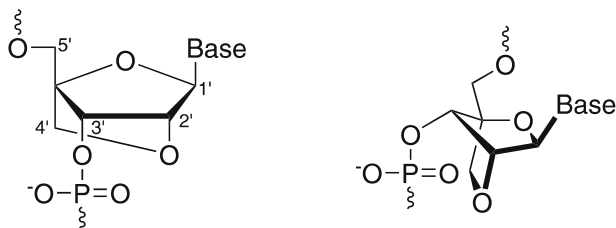
Fig. 1 The structure of locked nucleic acid (LNA) monomers

Duplexes	Melting temperature (°C)
5'-TTTTTT : *AAAAAA*	<10
5'-**TTTTT**T : *AAAAAA*	40
5'-GTGATATGC : 3'-*CACUAUACG*	28
5'-G**T**GA**T**A**T**GC : 3'-*CACUAUACG*	58
5'-**GTGATATGC** : 3'-*CACUAUACG*	74
5'-Biotin-T_{20} (DNA oligo) : A_{20}	40
5'-Biotin-(**T**T)$_{10}$ (LNA_2.T): A_{20}	71
5'-Biotin-$\mathbf{T}_{20}$ (LNA full) : A_{20}	>95

Fig. 2 Some examples of melting temperatures (T_m values) for hybridization of LNA and DNA oligonucleotides to complementary RNA sequences (Singh et al. 1998, Jacobsen et al. 2004). LNA monomers are shown in *capital boldface underlined letters*, DNA monomers in *capital letters*, and RNA monomers in *capital italics letters*

pairing selectivity. Examples of melting temperatures for LNAs complexed with RNA are shown in Fig. 2. LNA:LNA base pairing is also very strong (Koshkin et al. 1998a) and should be considered in relation to the risk of self-complementarity when designing LNAs for biophysical or biological experiments (see www.exiqon.com for LNA design tools). In general, the largest affinity increase per LNA monomer, and the optimum mismatch discrimination, is achieved for short oligonucleotides with a single or with several dispersed centrally positioned LNA monomers.

The structures of LNA:RNA duplexes have been studied by NMR spectroscopy showing similarities with the native nucleic acid duplexes, i.e., Watson-Crick base pairing, *anti* orientation of the nucleobases, base stacking, and a right-handed double helix conformation. A study including three 9-mer LNA/DNA:RNA hybrids, in which the LNA/DNA mixmer strand contained one, three, and nine LNA nucleotides, shows an increasing A-like character of the hybrids upon an increase in the LNA content of the LNA strand (Petersen et al. 2002; Nielsen et al. 2004). Thus, both the fully modified LNA:RNA duplex and the duplex with three LNA modifications were shown to adopt near canonical A-type duplex geometry, indicating that the LNA nucleotides tune the DNA nucleotides in the LNA strand conformationally to attain *N*-type sugar puckers (Petersen et al. 2002). An analogous, but localized, effect was observed in the LNA/DNA:RNA duplex with one LNA modification, where the LNA nucleotide was shown to perturb the sugar puckers of the neighboring DNA nucleotides, predominantly in the 3'-direction of the LNA nucleotide (Petersen et al. 2002).

1.2
Susceptibility of LNA to Nucleases

The basic biophysical properties of LNA make it attractive for diagnostic and therapeutic applications. This implies contact with various media containing nucleases such as serum or cellular media. In general, it is considered an ad-

vantage if modified oligonucleotides are resistant to degradation by nucleases. However, one exception is RNase H-mediated cleavage of the RNA target strand of a heteroduplex formed with an antisense oligonucleotide. As only very few fully modified oligonucleotides support RNase H-mediated cleavage, the so-called gapmer strategy using oligonucleotides composed of modified segments flanking a central DNA (or phosphorothioate DNA) segment that is compatible with RNase H activity is often used. In an early study, Wahlestedt et al. (2000) found that both an LNA/DNA/LNA gapmer, with a 6-nt DNA gap, and an LNA/DNA mixmer, with 6 DNA and 9 LNA nucleotides interspersed, elicited RNase H activity. However, no RNase H-mediated cleavage was observed with a fully modified 11-mer LNA or with an 11-mer LNA/DNA mixmer (Sørensen et al. 2002). Kurreck et al. (2002) investigated various LNA/DNA mixmers and gapmers and found that a gap of six DNA nucleotides is necessary for noteworthy RNase H activity, and that a gap of seven DNA nucleotides allows complete RNase H activity. Also Elmén et al. (2004) demonstrated that LNA/DNA mixmers activate RNase H when the mixmer has a DNA stretch of 6 nt. In accordance, Frieden et al. (2003a) concluded that a DNA gap size between 7 and 10 nt is optimal for LNA/DNA/LNA antisense gapmers. The overall conclusion is that antisense LNA oligonucleotides can be designed to elicit RNase H activity while still containing LNA monomers for improved binding and target accessibility (Jepsen and Wengel 2004).

With respect to other nucleases the situation is reversed, as resistance towards cleavage is advantageous. Complete stability against the 3′-exonuclease snake venom phosphodiesterase (SVPD) was reported for a fully modified LNA (Frieden et al. 2003b) while a significant increase in 3′-exonucleolytic stability was observed by blocking the 3′-end with two LNA monomers. However, with only one penultimate LNA nucleotide or with a single LNA monomer in the middle of a sequence, no protection—or only a very minor protection—is induced (Morita et al. 2002; Lauritsen et al. 2003). S1 endonuclease susceptibility was also investigated, and fully modified LNA is very stable against S1 endonuclease, which is not the case for phosphodiester LNA/DNA/LNA gapmers. In a study of oligonucleotide stability in serum, LNA mixmers were found to be very stable, and LNA/DNA/LNA gapmers to be significantly more stable than DNA alone (Wahlestedt et al. 2000). Furthermore, Kurreck et al. (2002) reported that oligonucleotides with LNA nucleotides at the ends are more stable in human serum than the corresponding oligonucleotides composed of a phosphothioate DNA gaps with 2′-O-methyl-RNA flanks. DNase I endonuclease degradation of end-modified 30-mer dsDNA showed that incorporation of one or two terminal LNA nucleotides ensures markedly increased stability (Crinelli et al. 2002). Similarly, Bal-31 exonucleolytic degradation of the same oligonucleotides showed that two terminal LNA nucleotides provide significant protection (Crinelli et al. 2002). The presence of a single 3′-terminal LNA nucleotide significantly slowed degradation by the 3′-5′ proofreading exonuclease of DNA polymerases Pfu and Vent (Di Giusto et al. 2004). From

these studies it is clear that LNA nucleotides impose significant protection against nucleolytic degradation, especially if more than one LNA nucleotide is incorporated into an oligonucleotide.

2
RNA Targeting by LNAzymes

DNAzymes are catalytically active DNA molecules that are able to cleave RNA in a sequence-specific manner after binding to complementary sequences. The so-called 10–23 DNAzyme (Fig. 3) was found by in vitro selection (Santoro and Joyce 1997). It consists of a 15-nt catalytic core flanked by two binding arms that lead to the sequence selectivity of a given DNAzyme. LNAzymes are LNA-modified DNAzymes, and incorporation of two LNA nucleotides in each of the binding arms yielded an LNAzyme with a highly enhanced efficiency of RNA cleavage (Vester et al. 2002). Interestingly, cleavage of highly structured targets (a 58-nt RNA with known secondary structure and a 23S ribosomal RNA of 2904 nt) was shown to be significantly improved for LNAzymes compared to the corresponding unmodified DNAzymes, and multiple turnover cleavage reactions were observed both with a 17-nt minimal substrate and with a structured 58-nt substrate. These features, especially the improved RNA target accessibility, may be very significant for future uses. A similar approach was used by Schubert et al. (2003), who incorporated 3–4 LNA monomers at the ends of the binding arms and also observed a highly enhanced efficiency of RNA cleavage. Recently, it has been demonstrated that LNAzymes containing 3–4 LNA monomers at the ends of the binding arms cleave viral RNA structures that are resistant to hydrolysis by the corresponding unmodified DNAzyme, i.e., that efficient cleavage is correlated with improved binding affinity towards the target (Schubert et al. 2004).
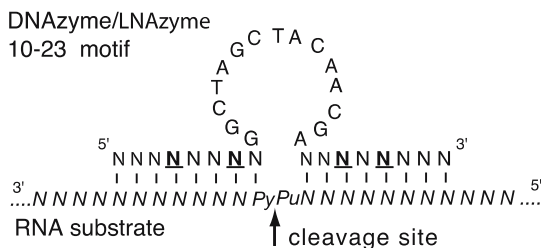
Fig. 3 Structure of 10–23 motif DNA- and LNAzymes. *Lines* indicate Watson-Crick base pairing with the RNA target sequence. LNA monomers are shown in *capital boldface underlined letters*, DNA monomers in *capital letters*, and RNA monomers in *capital italics letters*. The LNA monomers are introduced in the binding arms for enhanced RNA targeting and cleavage

One report on cellular activity of LNAzymes has been published. Fahmy and Khachigian (2004) used an LNAzyme design with two LNA monomers dispersed in each binding arm and they also included an 3′-3′ inverted thymidine monomer at the 3′-end to ensure nuclease stability. Based on a previous report (Khachigian et al. 2002) on inhibition of expression of EGR-1 by DNAzyme-mediated cleavage, Fahmy and Khachigian (2004) showed that serum-inducible smooth muscle cell proliferation was inhibited by greater than 50% at 20 nM LNAzyme, while no inhibition was evident by the corresponding DNAzyme at that concentration. Based on these data, it appears that the LNAzyme is superior at cleaving the EGR-1 transcript and inhibiting endogenous EGR-1 protein expression, SMC proliferation, and re-growth after injury.

LNAzymes containing LNA nucleotides in the binding arms display enhanced RNA affinity, allowing them to access RNA structures that cannot be targeted by the corresponding DNAzymes. As they furthermore show enhanced nuclease stability, and as efficient gene silencing in a cellular system has been reported, LNAzymes are promising novel agents for use in cells and for in vivo applications.

3
LNA Antisense

The term "antisense" is generally used for nucleic acid-based approaches that inhibit, in a sequence selective way, the processing of RNA from its transcription via messenger (m)RNA to protein, or the function of other forms of RNA. This includes, e.g., inhibition or alteration of splicing, translational arrest, or degradation of mRNA. By virtue of their intrinsic properties, oligonucleotides containing LNA nucleotides are obvious candidates for antisense-based gene silencing, and many previous LNA antisense studies are already reviewed (Petersen and Wengel 2003; Jepsen and Wengel 2004). Therefore, only selected examples are included herein supplemented with results from very recent studies.

The first in vivo antisense experiment with two different LNA sequences targeting DOR mRNA (coding for a receptor) in the central nervous system of living rats gave promising results (Wahlestedt et al. 2000). Upon direct injection of the antisense LNA oligonucleotides into the brain of living rats no tissue damage was observed, and a dose-dependent and highly efficacious knockdown of DOR was observed with both an LNA/DNA mixmer and an LNA/DNA/LNA gapmer. In another in vivo study, a fully modified LNA, targeting the RNA polymerase II gene product, inhibited tumor growth in mice and appeared non-toxic at doses less than 5 mg/kg per day (Fluiter et al. 2003). This study indicates LNA to be a much more potent class of antisense agents than the corresponding phosphothioate-DNA oligomers, which underlines the importance of differentiating between the variety of antisense chemistries available.

Several studies in vitro or in cells support the usefulness of LNA antisense for gene silencing (Obika et al. 2001; Hansen et al. 2003). One example of inhibition by LNA oligonucleotides involves telomerase (Elayadi et al. 2002), which is responsible for maintaining the telomere length from one generation to the next. Telomerase is a ribonucleoprotein that contains a protein domain and RNA with an 11-base sequence that binds telomeric DNA thereby guiding the addition of telomeric repeats. As telomerase is expressed in cancer cells but not in adjoining normal tissue, inhibition of telomerase will reduce tumor growth. LNA antisense constructs targeting the RNA moiety of telomerase are potent and selective inhibitors (Elayadi et al. 2002) with IC_{50} values of 10 nM for a 13-mer fully modified LNA, and a 13-mer LNA-DNA mixmer with 3 DNA monomers. By the introduction of two terminal phosphorothioate linkages, the potency was increased further by tenfold, but this was accompanied by a decreased match versus mismatch discrimination. Notably, even very short 8-mer fully modified LNAs are potent inhibitors (IC_{50} values of 2 nM and 25 nM with and without terminal phosphorothioate linkages, respectively). Also, in cells upon employing transfection 13-mer LNA oligomers induce inhibition of more than 80% of the telomerase activity (Elayadi et al. 2002). Short LNAs could be expected to cause problems due to binding to non-target nucleic acids, but no alteration of cell morphology was observed 7 days after transfection of 8-mer LNAs. Importantly, these results suggest that short LNAs may provide adequate affinity and sufficient selectivity for biologically relevant RNA targeting in cells.

Another important target for LNA antisense-based silencing is viral RNA. Effects of different LNAs on the interactions of the human immunodeficiency virus (HIV)-1 *trans*-activation responsive element (TAR) have recently been published (Arzumanov et al. 2003). Binding of oligonucleotides to TAR can inhibit Tat-dependent transcription thereby blocking full-length HIV transcription and hence viral replication, and chimeric sequences composed of LNA and 2′-*O*-methyl RNA nucleotides were shown to inhibit transcription in vitro (Arzumanov et al. 2003). Various LNA oligonucleotides were transfected into HeLa cells and derivatives with a minimum length of 12 residues showed 50% inhibition using nanomolar concentration of LNAs (Arzumanov et al. 2003), revealing the potential of LNA antisense oligonucleotides for in vivo targeting of RNA using non-RNase H dependent approaches. In another study, Elmén et al. (2004) demonstrated that LNA/DNA mixmers enhance the inhibition of HIV-1 genome dimerization and activate RNase H, show good uptake of the LNA/DNA mixmers in a T cell line, and inhibit replication of a clinical HIV-1 isolate.

A different antisense effect has been obtained by using LNA oligonucleotides to inhibit intron splicing. In an in vitro study, an 8-mer fully modified LNA and a 12-mer LNA/DNA chimera were shown to display a 50% inhibition at 150 nM and 30 nM, respectively, of a group I intron splicing in a transcription mixture from *Candida albicans* (Childs et al. 2002). The LNAs introduce misfolding of the RNA and consequently inhibition of the splicing process. In

another study (Ittig et al. 2004), the antisense effects of tricyclo-DNA and LNA oligonucleotides on exon skipping were compared. Nuclear antisense effects of cyclophilin A pre-mRNA splicing by 9- to 15-mers fully modified oligonucleotides were investigated, and significant inhibition was observed for 11- to 15-mers tricyclo-DNAs and 13- to 15-mers LNAs with tricyclo-DNA being most potent.

It is cleat that LNA antisense—either LNA/DNA/LNA gapmers for RNase H activation or LNA mixmers for RNase H-independent activity—represents a favorable approach for gene silencing in vitro and in vivo, and reports indicate that LNA antisense rivals siRNA as the method of choice.

4
LNA-Modified siRNA

The discovery of the phenomenon of RNA interference (RNAi), in which dsRNA leads to the degradation of RNA that is homologous (Fire et al. 1998), has drawn much attention, as it mediates potent gene silencing in a number of different organisms and in mammalian cells. RNAi relies on a complex and ancient cellular mechanism that has probably evolved for protection against viral attack and mobile genetic elements.

A crucial step in the RNAi mechanism is the generation of siRNAs—dsRNAs that are about 22 nt each. The siRNAs lead to the degradation of homologous target RNA and might even lead to the production of more siRNAs against the same target RNA (Lipardi et al. 2001). In a recent review by Jepsen and Wengel (2004), LNA oligonucleotides in antisense experiments were compared with siRNA, leading to the conclusion that LNA antisense oligonucleotides combined with other modifications such as phosphothioate linkages might rival the currently very popular siRNA approach for gene silencing in vitro and in vivo. There is one study directly comparing the inhibitory effect of siRNA, LNA/DNA/LNA gapmers, phosphothioate-DNA, and 2′-O-methyl-RNA on the expression of vanilloid receptor subtype 1 in cells (Grünweller et al. 2003). Both siRNA and LNA/DNA/LNA gapmers were found to be very efficient, and siRNA to be slightly more potent than the LNA/DNA/LNA gapmers. The siRNAs composed of dsRNA are themselves an obvious choice for incorporation of modified nucleotides for improved biostability and RNA targeting. Although the mechanisms of RNAi are not fully elucidated, a clearer picture of si/microRNA function and the usefulness of modified nucleotides in siRNA applications is emerging.

LNA monomers have been shown to be tolerated by the RNAi machinery and to provide thermal stability (Braasch et al. 2003). A new finding indicates that weak base pairing at the 5′-end of the antisense strand is an important selection criterion for determining which siRNA strand will be used as template for mRNA degradation (Schwarz et al. 2003). Therefore the exact positioning

and the overall number of LNA monomers will be very crucial for optimizing LNA-containing siRNA (siLNA). To address these issues, Elmén et al. (2005) systematically modified siRNA duplexes with LNA monomers and showed that siLNAs have substantially enhanced serum half-life compared to the corresponding siRNAs. Moreover, they provided evidence that the use of siLNAs reduces sequence-related off-target effects. Furthermore, they reported on improved efficacy of siLNAs on certain RNA motifs targeted against the severe acute respiratory syndrome coronavirus (SARS-CoV). These results emphasize siLNA's promise in converting siRNA from a functional genomics technology to a therapeutic platform.

5
Transcriptome Analysis Facilitated by LNA

Efficient selection of polyadenylated mRNA from eukaryotic cells and tissues is an essential step for a wide selection of functional genomics applications, including full-length complementary (c)DNA library construction and sequencing, Northern and dot blot analyses, gene expression profiling by microarrays, and quantitative RT-PCR. The key to successful selection of intact poly(A)$^+$RNA is a fast extraction of total RNA from cells and tissues using strong denaturing agents to disrupt the cells with the simultaneous denaturation of endogenous RNases followed by mRNA sample preparation from the extracted total RNA (Aviv and Leder 1972; Chirgwin et al. 1979; Chomczynski and Sacchi 1987). Since most eukaryotic mRNAs contain tracts of poly(A) tails at their 3′ termini, polyadenylated mRNA can be selected by oligo(dT)-cellulose chromatography. Jacobsen et al. (2004) have recently reported on efficient isolation of intact poly(A)$^+$RNA using an LNA-substituted oligo(dT) affinity ligand, based on LNA-T's increased affinity to complementary poly(A) tracts. Poly(A)$^+$RNA could be isolated directly from 4 M guanidinium thiocyanate (GuSCN)-lysed *Caenorhabditis elegans* worm extracts as well as from lysed human K562 and K562/VCR leukemia cells using the LNA_2.T oligonucleotide (see Fig. 2) as an affinity probe, in which every second thymidine was substituted with LNA thymidine. In accordance with the significantly increased stability of the LNA_2.T-A duplexes in 4 M GuSCN, Jacobsen et al. (2004) obtained a 30- to 50-fold mRNA yield increase by using the LNA-substituted oligo(T) affinity probe compared to the DNA oligo(dT)-selected mRNA samples. The LNA_2.T affinity probe was furthermore shown to be highly efficient in isolation of poly(A)$^+$RNA in a low salt concentration range of 50 to 100 mM NaCl in the poly(A) binding buffer. The utility of the LNA oligo(T)-selected mRNA in quantitative real-time PCR was also demonstrated by analyzing the expression of the human *mdr1* gene in the two K562 human cell lines employing pre-validated Taqman assays (Jacobsen et al. 2004).

Large-scale expression analysis is increasingly important in many areas of biological research aiming at deciphering complex biological systems, thereby greatly facilitating the understanding of basic biological processes as well as human disease. Quantitative real-time RT-PCR has become the method of choice for accurate expression profiling of selected genes and validation of microarray data, and is especially suitable for analysis of low abundant mRNAs and small samples (Bustin 2000; Liss 2002). However, real-time RT-PCR is often hampered by the labor-intensive assay design and validation, which significantly lowers its throughput compared to genome-wide expression profiling by DNA microarrays. In response to this, Mouritzen et al. (2004) have developed a novel concept for quantitative real-time RT-PCR based on a library of 90 pre-validated dual-labeled LNA-enhanced detection probes, designated as ProbeLibrary. The probes are shortened to only 8–9 nt by substitution with LNA nucleotides, ensuring adequate duplex stability and compatibility in standard real-time RT-PCR assays. The use of short recognition sequences for the detection probes enables targeting of the frequently recurring short sequence tags in the human transcriptome, and this facilitated the re-use of the same LNA-enhanced probe in detection and quantification of many different transcripts. By careful selection of the most common 8- and 9-mer sequences in the human transcriptome, Mouritzen et al. (2004) constructed a library of 90 detection probes that detects 98% of all human transcripts. On average, each mRNA contains target sites for 16 ProbeLibrary probes, whereas the recognition sequence of each LNA-modified detection probe was found in more than 7,000 of the 38,556 human transcripts in the RefSeq database at the National Center for Biotechnical Information (NCBI). Similar LNA-based ProbeLibraries for expression profiling by real-time RT-PCR have recently been developed for mouse, rat, *Arabidopsis thaliana*, *Drosophila melanogaster*, and *C. elegans*. The use of LNA in microarrays has also been exploited. An optimal design for LNAs as capture probes for gene-expression profiling, together with a microarray study of *C. elegans* cytochrome expression, has been published (Tolstrup et al. 2003). The oligo design program, which might be useful in many applications, is freely available at http://lnatools.com/.

6
MicroRNA Detection Using LNA Probes

MicroRNAs (miRNAs) are 19- to 25-nt non-coding RNAs that are processed from longer endogenous hairpin transcripts by the enzyme Dicer (Ambros 2001; Ambros et al. 2003). To date, more than 1,500 microRNAs have been identified in invertebrates, vertebrates, and plants according to the miRNA registry database, and many miRNAs that correspond to putative genes have also been identified (Griffiths-Jones 2004). The high percentage of predicted miRNA targets acting as developmental regulators and the conservation of

target sites suggests that miRNAs exhibit a wide variety of regulatory functions and exert significant effects on cell growth, development, and differentiation (Ke et al. 2003; Bartel 2004), including human development and disease.

The current view that miRNAs represent a hidden layer of gene regulation has resulted in high interest among researchers in the discovery of miRNAs, their targets, their expression, and their mechanism of action. Most miRNA researchers use Northern blot analysis combined with polyacrylamide gels to examine the expression of both the mature and precursor miRNAs, since it allows both quantitation of the expression levels and miRNA size determination (Lagos-Quintana et al. 2001; Reinhart et al. 2000; Lee and Ambros 2001). A major drawback of this method is its poor sensitivity, especially when monitoring expression of low-abundant miRNAs. In a recent paper, Valoczi et al. (2004) have described a new method for highly efficient detection of miRNAs by Northern blot analysis using LNA-modified oligonucleotide probes and demonstrated its significantly improved sensitivity by designing several LNA-modified oligonucleotide probes for detection of different miRNAs in mouse, *A. thaliana*, and *Nicotiana benthamiana*. They used the LNA/DNA mixmer probes in Northern blot analysis employing standard end-labeling techniques and hybridization conditions, and the sensitivity in detecting mature miRNAs by Northern blots is increased by at least tenfold compared to DNA probes, while it is simultaneously highly specific as demonstrated by the use of different single and double mismatched LNA probes (Valoczi et al. 2004). Besides being efficient as Northern probes, LNA-modified oligonucleotides have proved highly useful for in situ localization of miRNAs in cells and tissues. Accordingly, the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs were determined in zebrafish embryos using LNA-modified oligonucleotide probes (Wienholds et al. 2005). Interestingly, most miRNAs were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development. These miRNAs may therefore play crucial roles in the maintenance of tissue identity or in differentiation (Wienholds et al. 2005).

7
Future Prospects

LNAs constitute an important addition to the tools available for biotechnology, as well as nucleic acid-based diagnostics and therapeutics. The remarkable hybridization properties of LNA, both with respect to its affinity and specificity, position it as an enabling molecule for molecular biology research and biotechnology innovation. The fact that LNA phosphoramidites and oligomers are commercially available, and that LNA nucleotides can be freely mixed with, e.g., DNA, RNA, and $2'$-O-Me-RNA monomers and standard probes, make LNA a highly flexible tool allowing fine-tuning of properties.

Short LNA oligonucleotides (fully modified) and short LNA/DNA and LNA/RNA mixmer oligonucleotides are uniquely suited for targeting of complementary RNA and DNA. LNA will likely have a significant impact especially with respect to in vitro and in vivo RNA analysis and interference with RNA-mediated processes. LNA antisense rivals siRNA for gene silencing, but a satisfactory answer to the demand for efficient cellular delivery in vivo remains to be developed for LNA antisense, as for any other therapeutic oligonucleotide that relies on hybridization to a nucleic acid target for biological activity. Simple molecular-scale autonomous programmable computers based on nucleic acid interactions have been demonstrated, and a distinct prospect is nucleic acid-based, autonomous biomolecular computers that logically analyze the levels of mRNAs or non-coding RNAs, such as miRNAs, and in response produce a molecule capable of affecting levels of gene expression (Benenson et al. 2004). The superb hybridization properties of LNA—allowing shortening of target sequences—and its nuclease stability could make LNA an important partner in such designs. LNA is a unique molecule, which is likely to make a significant impact on the future developments within many areas of biotechnology and medicine.

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