LNA-Modified Primers Drastically Improve Hybridization to Target RNA and Reverse Transcription†

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ABSTRACT: Knowledge about the structure of RNA is crucial to understanding its biological activities. Very often, the presence of unusually thermodynamically stable structural fragments in RNAs, such as hairpins, makes it impossible to apply primer extension to visualize the results of chemical mapping experiments. However, replacement of DNA primers with LNA-modified primers overcomes this limitation. This approach was tested successfully on regulatory OxyS RNA and DsrA RNA from *Escherichia coli*.

Determining the secondary and tertiary structures of RNAs is essential for improving our understanding of their biological functions. Moreover, any usage of RNA as a potential target or agent for therapeutic treatments is correlated with the structure−function relations of RNA (*1*). Chemical mapping and enzymatic mapping are classic methods for studying the secondary structure of RNA. In these procedures, specific chemical reagents or enzymes are used to distinguish single- and double-stranded fragments within the target RNA (*2*). Recently, a new approach to determining the secondary structure of RNA, based on isoenergetic RNA microarrays, was introduced (*3, 4*). In this method, information about single- and double-stranded fragments of target RNA is obtained directly from hybridization. For chemical mapping of RNA, visualization of modifications is achieved indirectly by primer extension with DNA primers. In most cases, DNA primers are sufficient for successful reverse transcription. However, when some fragments of the target RNA (for example, hairpins with long GC rich stem) form thermodynamically stable structures, primer extension often fails. In this case, the annealing of primer to target RNA results in the restoration of the more thermodynamically favorable RNA hairpin instead formation of the RNA/DNA primer duplex necessary for primer extension. Herein, we demonstrate that using a chimeric DNA−LNA-modified primer instead of a DNA primer results in a thermodynamically stable duplex with target RNA and prime reverse transcription.

In addition to the large thermodynamic stability of LNA-modified duplexes, oligonucleotides containing LNA have enhanced chemical stability and resistance to enzymatic degradation (*5–10*). Such properties have led to their widespread applications as antisense oligonucleotides (*11*),

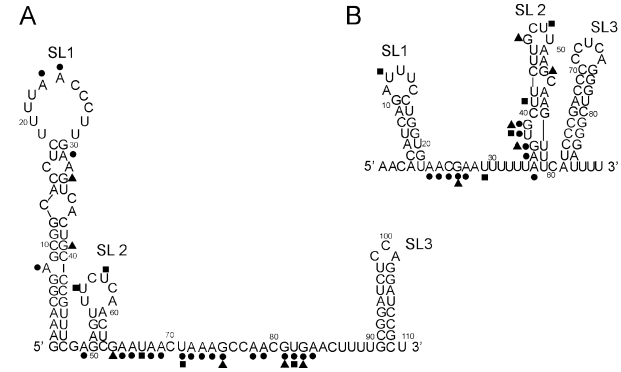

FIGURE 1: Secondary structures of (A) OxyS RNA and (B) DsrA RNA. Strong chemical modification data are superimposed: kethoxal (▲), CMCT (■), and NMIA (●).

as ribozymes (*12*), in miRNA detection (*13*), in miRNA regulation (*14*), and as probes in isoenergetic RNA microarrays to study the structure and interactions of RNA (*3, 4*). It was also reported that application of LNA-containing primers to PCR results in increased sensitivity and performance (*15, 16*). Moreover, LNA nucleotide triphosphates were used with good results as substrates for DNA and RNA polymerases (*17*).

The results described here demonstrate the application of chimeric DNA−LNA primers for reverse transcription of highly structured RNA, such as regulatory OxyS RNA (*18*) and DsrA RNA (*19*) from *Escherichia coli*. Both RNAs have very stable hairpins on their 3′-ends (Figure 1), and during the annealing of the DNA primer to these regions, RNA hairpins can not refold into the duplex necessary for reverse transcription because they are thermodynamically more favorable. As a result, primer extension fails and information about the structure of significant fragments of RNA is not obtained. This has affected structural analyses of RNA; for example, Altuvia et al. (*18*) examined the structure of OxyS RNA by DMS modification followed by primer extension and were unable to obtain structural information on 40 nucleotides at the 3′-end. Thus, overcoming this limitation and disrupting highly structured elements may be beneficial in many cases where hybridization of an oligonucleotide to thermodynamically stable RNA fragments is required.

Experiments presented here demonstrate that DNA−LNA primers can compete with hairpins and form stable RNA/DNA−LNA duplexes. For the OxyS RNA, 5′AGCGGATCCTGGAGATCCGCAA was used as a DNA primer and 5′AGcGgATCcTgGaGaTCCG was used as a DNA−LNA primer (where uppercase letters represent DNA nucleotides and lowercase letters LNA nucleotides). This modified primer

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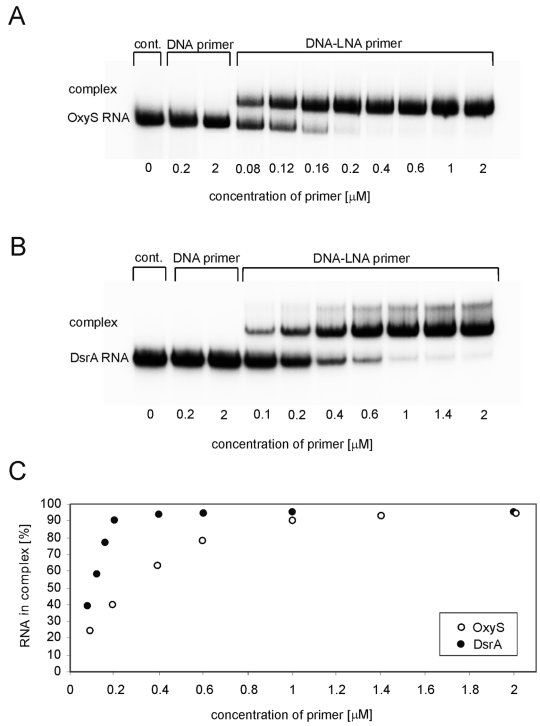

FIGURE 2: Formation of (A) OxyS RNA/DNA−LNA primer and (B) DsrA RNA/DNA−LNA primer complexes. (C) Quantification of equilibrium constants of formation of both RNA/primer complexes. ^{32}P-labeled (10000 cpm) and unlabeled (0.2 μM) RNAs were incubated with increasing concentration of DNA or DNA−LNA primer and then analyzed by nondenaturing gel electrophoresis as described in the Supporting Information.

contains six LNA nucleotides and is shortened by three nucleotides relative to the DNA primer.

For DsrA RNA, the length and sequence of DNA and LNA-modified primers are the same; however, the last primer contained five LNA nucleotides (5′AAATcCcGAcCcTgAGG). The listed number of LNA substitutions was used to ensure that the free energy of the duplex is more favorable than that of the original RNA hairpin (*9*; www.ibch.poznan.pl/kierzek). Measurements of dissociation constants of DNA and DNA−LNA primers unambiguously demonstrated the advantage of modified primers. During those measurements, the labeled OxyS RNA and DsrA RNA were incubated with increasing concentrations of DNA or DNA−LNA primers. In the case of DNA primers, even a 10-fold molar excess did not result in formation of any duplex (Figure 2). In contrast, both DNA−LNA primers bind to appropriate RNA very efficiently with K_d values of 66 nM for OxyS RNA and 234 nM for DsrA RNA. Furthermore, gel mobility profiles show that even less than 1 equiv of DNA−LNA primer is sufficient for formation of a duplex, especially for OxyS RNA.

These results are consistent with the UV melting experiments. The chemically synthesized 3′-end hairpin fragments of both RNAs, 5′GCGGAUCUCCAGGAUCCGC (OxyS $SL3_{91-109}$) and 5′AUCCCGACCCCCUCAGGGUCGGGAU (DsrA $SL3_{61-85}$), were used for UV melting in the absence and presence of corresponding DNA−LNA primers. The low concentration of NaCl (5 mM) was used to keep melting temperature (T_m) in a measurable range. The OxyS $SL3_{91-109}$ hairpin melted at 78.4 °C, reflecting the high stability of the hairpin and explaining its inaccessibility to DNA primer. The

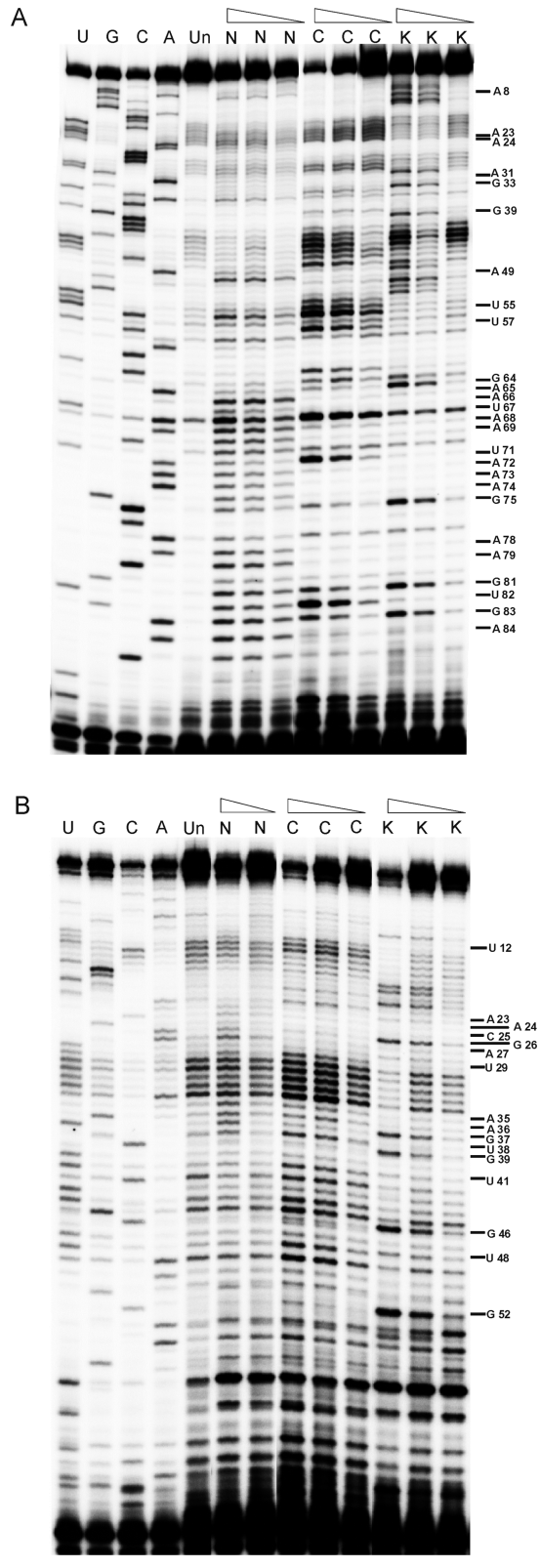

FIGURE 3: Chemical mapping results for (A) OxyS RNA and (B) DsrA RNA. Shown is the primer extension reaction carried out with a DNA−LNA primer. On the gel, U, G, C, and A are sequencing lanes, lane Un shows results for unmodified RNA (control), and lanes N, C, and K present modifications with NMIA, CMCT, and kethoxal, respectively.

calculated T_m for the undetectable duplex formed by the DNA primer (5′AGCGGATCCTGGAGATCCGCAA) and OxyS $SL3_{91-109}$ should be 52.1 °C (*20*). As expected, for the duplex formed by OxyS $SL3_{91-109}$ and the DNA−LNA primer

(5′AGcGgATCcTgGaGaTCCG), T_m was higher and equal to 84.9 °C. The DsrA $SL3_{61-85}$ hairpin is more stable than OxyS $SL3_{91-109}$, and its T_m was equal to 84.6 °C. The duplex formed by DsrA $SL3_{61-85}$ and the corresponding DNA−LNA primer (5′AAATcCcGAcCcTgAGG) was too stable to determine its T_m (>90 °C) even at 5 mM NaCl. For the sake of comparison, the calculated T_m for the undetectable duplex formed by the DNA primer (5′AAATCCCGACCCTGAGG) and DsrA $SL3_{61-85nt}$ should be 44.9 °C. Moreover, experimentally data demonstrate that RNA/DNA−LNA heteroduplexes melt in a cooperative and two-state manner. Representative UV melting curves are shown in the Supporting Information (Figure S1).

Encouraged by results indicating that DNA−LNA primers anneal efficiently to target RNAs, we performed primer extension experiments using SuperScript III reverse transcriptase to detect chemical mapping results. The chemical modifications of DsrA and OxyS RNAs were carried out with kethoxal, CMCT, and NMIA. Folding of DsrA and OxyS RNAs and chemical mapping reactions were performed as described in the Supporting Information. These experiments demonstrate that SuperScript III reverse transcriptase accepts the LNA-modified primer annealed to the RNA and extends it to the full-length product with good yield, allowing visualization of chemical mapping results, which was not possible with the DNA primer (data not shown). Representative examples of the chemical mapping experiments are shown in Figure 3, and results are summarized in Figure 1. Most of the modifications of both RNAs confirmed the structures proposed previously (*18, 21, 22*). In OxyS RNA, chemical modifications were detected for loop 2 and most of the linker region between stem loops 2 and 3. The modification pattern of DsrA RNA is consistent with previous suggestions that stem loop 2 is relatively conformationally dynamic (*22, 23*).

In conclusion, the extraordinary thermodynamic stability of some structural elements of RNA, particularly hairpins, makes it difficult to perform experiments when hybridization of oligonucleotides to such fragments is required (e.g., primer extension). One place where this problem was encountered was the regulatory OxyS and DsrA RNAs from *E. coli*, where the presence of thermodynamically stable hairpins on 3′-ends inhibits reverse transcription within these regions. It was shown that DNA primers complementary to these stable hairpins cannot disrupt the hairpin to form duplexes and make reverse transcription impossible. To overcome this problem, DNA primers containing LNA nucleotides were used. The presence of LNA nucleotides within the primers dramatically enhances the thermodynamic stability of heteroduplexes formed with target RNA, and formation of that heteroduplex is favorable and occurs efficiently. Additionally, we demonstrated that the presence of LNA nucleotides within primers does not decrease the activity of SuperScript III reverse transcriptase. Previously, the advantage of using LNA-modified primers was demonstrated for PCR (*15, 16*). We expand application of LNA-modified primers for reverse transcription after chemical mapping of RNA.

SUPPORTING INFORMATION AVAILABLE

A description of the experimental procedures and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Pace, N. R., Thomas, B. C., and Woese, C. R. (1999) Probing RNA structure, function, and history by comparative analysis. In *The RNA World* (Gesteland, R. F., Cech, T. R., and Atkins, J. F., Eds.) pp 113−141, Cold Spring Harbor Laboratory Press, Plainview, NY.
2. Ziehler, W. A., and Engelke, D. R. (2000) *Curr. Protoc. Nucleic Acid Chem. 2*, 6.1.16.1.21.
3. Kierzek, E., Kierzek, R., Moss, W. N., Christensen, S. M., Eickbush, T. H., and Turner, D. H. (2008) *Nucleic Acids Res. 36*, 1770–1782.
4. Kierzek, E., Kierzek, R., Turner, D. H., and Catrina, I. E. (2006) *Biochemistry 45*, 581–593.
5. McTigue, P. M., Peterson, R. J., and Kahn, J. D. (2004) *Biochemistry 43*, 5388–5405.
6. Kierzek, E., Ciesielska, A., Pasternak, K., Mathews, D. H., Turner, D. H., and Kierzek, R. (2005) *Nucleic Acids Res. 33*, 5082–5093.
7. Pasternak, A., Kierzek, E., Pasternak, K., Turner, D. H., and Kierzek, R. (2007) *Nucleic Acids Res. 35*, 4055–4063.
8. Kaur, H., Wengel, J., and Maiti, S. (2007) *Biochem. Biophys. Res. Commun. 352*, 118–122.
9. Kaur, H., Wengel, J., and Maiti, S. (2008) *Biochemistry 47*, 1218–1227.
10. Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., Olsen, C. E., and Wengel, J. (1998) *Tetrahedron 54*, 3607–3630.
11. Wahlestedt, C., et al. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 5633–5638.
12. Christiansen, J. K., Lobedanz, S., Arar, K., Wengel, J., and Vester, B. (2007) *Bioorg. Med. Chem. 15*, 6135–6143.
13. Neely, L. A., et al. (2006) *Nat. Methods 3*, 41–46.
14. Elmen, J., et al. (2008) *Nature 452*, 896–910.
15. Levin, J. D., Fiala, D., Samala, M. F., Kahn, J. D., and Peterson, R. J. (2006) *Nucleic Acids Res. 34*, e142.
16. Ballantyne, K. N., van Oorschot, R. A. H., and Mitchell, R. J. (2008) *Genomics 91*, 301–305.
17. Veedu, R. N., Vester, B., and Wengel, J. (2008) *J. Am. Chem. Soc. 130*, 8124–8125.
18. Altuvia, S., WeinsteinFischer, D., Zhang, A. X., Postow, L., and Storz, G. (1997) *Cell 90*, 43–53.
19. Sledjeski, D. D., and Gotessman, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2003–2007.
20. Sugimoto, N., Nakano, S., Katoh, M., Matsumura, A., Nakamuta, H., Ohmichi, T., Yoneyama, M., and Sasaki, M. (1995) *Biochemistry 34*, 11211–11216.
21. Zhang, A. X., Wassarman, K. M., Ortega, J., Steven, A. C., and Storz, G. (2002) *Mol. Cell 9*, 11–22.
22. Lease, R. A., and Belfort, M. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 9919–9924.
23. Rolle, K., Zywicki, M., Wyszko, E., Barciszewska, M. Z., and Barciszewski, J. (2006) *J. Biochem. 139*, 431–438.

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