Enhanced Inhibition of Transcription Start by Targeting with 2′-OMe Pentaribonucleotides Comprising Locked Nucleic Acids and Intercalating Nucleic Acids

Vyacheslav V. Filichev,[a] Birte Vester,[b] Lykke H. Hansen,[b] Mohammed T. Abdel Aal,[a, c] B. Ravindra Babu,[a] Jesper Wengel,[a] and Erik B. Pedersen*[a]

Inhibition of gene expression has been achieved by targeting the open complex formed during transcription initiation with short, pentameric, modified oligonucleotides.[1] This approach is an alternative to double-stranded (ds) DNA targeting by using triplex-forming oligonucleotides, in which longer probes (>10 nucleotides) are required for high-affinity binding and specificity.[2] A local opening of the dsDNA by RNA polymerase at the start of transcription is the key to the sequence-selective

[a] *Dr. V. V. Filichev, Dr. M. T. Abdel Aal, Dr. B. R. Babu, Prof. J. Wengel, Prof. E. B. Pedersen*
Nucleic Acid Center, Department of Chemistry
University of Southern Denmark
Campusvej 55, 5230 Odense M (Denmark)
Fax (+45) 6615-8780
E-mail: ebp@chem.sdu.dk

[b] *Prof. B. Vester, L. H. Hansen*
Nucleic Acid Center, Department of Biochemistry and Molecular Biology
University of Southern Denmark
Campusvej 55, 5230 Odense M (Denmark)

[c] *Dr. M. T. Abdel Aal*
Present address:
Chemistry Department, Faculty of Science, Menoufia University
32511 Shebin El-Koam (Egypt)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 DOI: 10.1002/cbic.200400457

targeting and inhibition. It has been shown that oligoribonucleotides possessing a nonextendable terminal 3′-deoxyribonucleotide can hybridize to sequence −5 to +2 of the template strand formed by *Escherichia coli* RNA polymerase with the *lac* UV-5 and *trp* EDCBA promoters, and inhibit transcription.[1] It is expected that the hybridization affinity for such short polynucleotides to the target is crucial for efficient inhibition at physiological temperatures. However, pentameric oligodeoxynucleotides, peptide nucleic acids, 2′-methoxyethoxy RNA, phosphoramidate ($^{3'}$N—$^{5'}$P) and morpholino oligonucleotide analogues did not show any substantial inhibition.[1d] An improved inhibition of transcription of the *lac* UV5 open complex was achieved at 20 μM with a tethered pyrene attached to 2′-amino uridine in 5′-$G^{2'OMe}U^{2'OMe}G^{2'OMe}G^{2'OMe}A^{3'deoxy}$ (−4 to +1) instead of 2′-OMe uridine.[1e] It has been shown that the ribonucleotide pentamer does not bind to the dsDNA without the influence of RNA polymerase.[1a] The hybridization affinity of the pentameric inhibitors to the ssDNA template is expected to be too low for binding at 37 °C. Therefore, unspecific interactions between the pentanucleotide and the open complex consisting of DNA and RNA polymerase might be considered for enhanced inhibition. In order to identify the most appropriate design of various inhibitors, combinations of RNA-like oligonucleotides with intercalating units should be screened.

Previously, we have reported the synthesis and hybridization properties of locked nucleic acids (LNA) and intercalating nucleic acids (INA®). LNA is a class of oligonucleotide analogues containing one or more conformationally locked nucleotide monomers with a 2′-*O*,4′-*C*-methylene linkage (Figure 1).[3] INA is another class with bulge insertions of (*R*)-1-*O*-(pyren-1-ylmethyl)glycerol (monomer **P**, Figure 1) into the oligonucleotide.[4] Both of these analogues display stronger binding affinity towards complementary ssDNA than 2′-OMe-RNA, and a very good mismatch discrimination.[3b,4b] The commercial availability of LNA and INA oligonucleotides is a competitive advantage of these analogues for the screening of modified oligonucleotides in biological assays. In this report, we show that the use of LNA and INA monomers in combination with 2′-OMe-RNA monomers furnishes pentanucleotidic inhibitors of *E. coli* RNA polymerase with more than 95% inhibitory efficiency at 16 μM compared to 60% as earlier reported for 5′-$G^{2'OMe}U^{2'OMe}G^{2'OMe}G^{2'OMe}A^{3'deoxy}$ (**1**).[1e]

We anticipated that the hybridization affinities of LNA and INA would significantly increase the stability of the complexes of pentanucleotides with the template ssDNA, thus leading to better inhibition. LNA is an RNA mimic,[3] therefore 2′-OMe pentaribonucleotides possessing LNA monomers are expected to be recognized by RNA polymerase and bind more strongly to the open complex. Furthermore, due to the unnatural structure of INA and LNA, these modified nucleotides are expected to block in situ elongation if they are incorporated at the 3′-termini of the pentanucleotides.

To evaluate the above-mentioned hypotheses, we synthesized a number of 2′-OMe-pentamers (**ON 2**–**10**, Table 1) that encompass LNA and INA in different positions of the sequence complementary to the template strand (−4 to +1) of the open complex formed by *E. coli* RNA polymerase with the *lac* UV-5 promoter. The incorporation of LNA and INA monomers was fully compatible with the automated synthesis of 2′-OMe-RNA by using 4,5-dicyanoimidazole as an activator (see Supporting Information). To evaluate transcriptional inhibition, all the synthesized pentanucleotides were checked in an in vitro transcription assay at two different concentrations, 2 and 10 μM (Figure 2).

Table 1. Inhibition efficiency[a] and hybridization affinity[b] of pentaribonucleotides synthesized.

ON	Sequence	2 μM	10 μM	T_m [°C]
1	5′-$G^{2'OMe}U^{2'OMe}G^{2'OMe}G^{2'OMe}A^{3'deoxy}$	15%	50%	19.0
2	5′-$G^{2'OMe}U^{2'OMe}G^{2'OMe}G^{2'OMe}\mathbf{A^L}$	10%	55%	23.0
3	5′-$\mathbf{G^LT^LG^LG^L}A^{3'deoxy}$	0%	50%	44.0
4	5′-$G^{2'OMe}\mathbf{P}U^{2'OMe}G^{2'OMe}G^{2'OMe}A^{3'deoxy}$	0%	0%	10.5
5	5′-$G^{2'OMe}U^{2'OMe}G^{2'OMe}G^{2'OMe}A^{2'OMe}\mathbf{P}$	5%	75%	29.5
6	5′-$G^{2'OMe}U^{2'OMe}\mathbf{P}G^{2'OMe}G^{2'OMe}A^{3'deoxy}$	0%	0%	< 5.0
7	5′-$G^{2'OMe}\mathbf{P}U^{2'OMe}G^{2'OMe}G^{2'OMe}\mathbf{P}A^{3'deoxy}$	30%	80%	13.5
8	5′-$\mathbf{P}G^{2'OMe}U^{2'OMe}G^{2'OMe}G^{2'OMe}A^{3'deoxy}$	30%	40%	27.5
9	5′-$G^{2'OMe}\mathbf{T^L}G^{2'OMe}\mathbf{G^LA^L}$	25%	95%	44.5
10	5′-$G^{2'OMe}U^{2'OMe}U^{2'OMe}G^{2'OMe}A^{2'OMe}\mathbf{P}$	0%	5%	< 5.0

[a] Inhibition efficiency is defined as percentage decrease of transcription relative to noninhibited RNA transcription; [b] T_m [°C] was determined at 260 nm as the maximum of the first derivative plots of the melting curves obtained by measuring absorbance at 260 nm against increasing temperature (1.0 °C min^{-1}) on equimolar mixtures (1.0 μM in each strand) of **ON 1**–**10** and the complementary DNA strand (3′-CACACCTT) in a hybridization buffer (40 mM Tris·HCl, 100 mM KCl, 10 mM $MgCl_2$, pH 7.9).

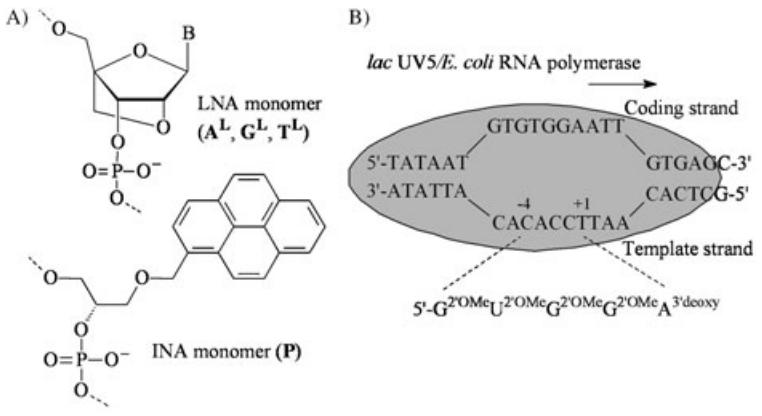

Figure 1. A) Structures of LNA ($\mathbf{A^L}$, $\mathbf{G^L}$, $\mathbf{T^L}$) and INA (**P**) monomers. B) A representation of an open complex formed by dsDNA and RNA polymerase. The 2′-OMe-pentaribonucleotides designed to target the template strand at transcription start site (+1) and inhibit in vitro transcription are shown. $A^{3'deoxy}$ denotes 3′-deoxyadenosine; $G^{2'OMe}$, $U^{2'OMe}$, $A^{2'OMe}$ denote 2′-*O*-methylribonucleotides.

The polymerase reaction was quenched 4 min after transcription initiation because a longer reaction time resulted in some degradation of the RNA product, as observed in transcription experiments without any inhibitor. To compare the inhibition effect of LNA- and INA-containing pentanucleotides with previously published results, we included the

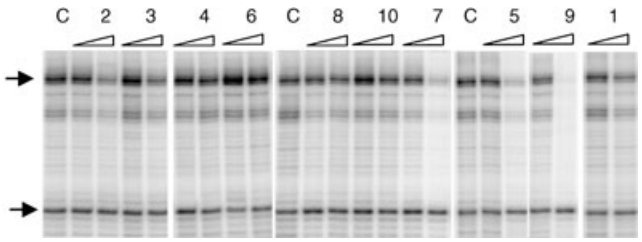

Figure 2. Denaturing gel electrophoresis of RNA transcription by using *E. coli* RNA polymerase with the *lac* UV-5 promoter in the presence of **ON 1**–**10** and in the absence of inhibitors (**C**; see Supporting Information). The upper arrow points to the 61-mer RNA transcript, and the lower arrow to a 30-mer DNA loading control. Concentrations of inhibitors **1**–**10**: 2 μM (left) and 10 μM (right).

sequence **1** investigated by Hwang et al.[1e] The pentamer/template ratio was 40:1 at 2 μM pentamer. Quantification of the inhibition effect was performed with Phorphor Imager analysis in which radioactivities in the different bands are compared and related to transcription without inhibitor added (see Supporting Information).

All experiments were repeated several times, and average inhibition efficiencies are presented in Table 1. To establish the relationship between the binding affinity of pentamers towards ssDNA and inhibition, we checked the thermal stability of their corresponding duplexes (Table 1). When LNA or INA monomers were placed at the 3′-end of 2′-OMe-pentaribonucleotides, a good inhibition and no elongated products were observed, despite the presence of secondary OH-groups in the carbohydrate moieties of locked and intercalating nucleic acids. This gives an advantage of using these monomers instead of 3′-deoxynucleotides because it simplifies the synthesis of inhibitors and increases hybridization affinity as compared to **ON 1**. Intercalating moieties were inserted at different positions of the pentameric sequence. Placing **P** at the 3′- and 5′-ends (**ON 5** and **ON 8**, Table 1) was more effective than inserting it in the middle of the sequence (**ON 6**); this was also correlated with low hybridization affinity as T_m of **ON 6** was $< 5.0\,^{\circ}C$. The insertion of a single **P** near the 5′-end in **ON 4** gave no inhibition effect, but addition of a second **P**, as in sequence **7**, resulted in considerable inhibition. This could hardly be a result of the increased binding affinity ($T_{m(ON7)}$-$T_{m(ON4)} = 3.0\,^{\circ}C$), which is considered to be small. Interestingly, pentamer **7** had a lower value of T_m than the reference **1**, but considerable enhancement of inhibition efficiency was observed. This supports an alternative explanation that stacking interactions of **P** with aromatic amino acids in the RNA polymerase could enhance inhibition for oligonucleotides with **P** insertions. To ensure the sequence specificity of pentanucleotides possessing INA monomers, the oligonucleotide **10** was synthesized as a single mismatched sequence of one of the inhibitors (**ON 5**). Marginal inhibition was observed with **10** at 10 μM. The thermal stability of pentamers with INA insertions towards ssDNA increased only when **P** was a dangling end (**ON 5** and **ON 8**). Low affinity of 2′-OMe-pentaribonucleotides with bulged **P** towards ssDNA is very similar to the previously observed thermal stability of INA-containing oligodeoxynucleotides towards ssRNA.[4] This means that incorporation of INA as a bulge either in DNA or in RNA strands decreases the stability of DNA/RNA duplexes.

Both LNA-containing sequences **3** and **9** had similar binding affinities towards ssDNA, but these were considerably higher than for **ON 1**, as deduced from melting temperatures. However, one of them, the pentanucleotide **9**, showed considerable inhibition both at 2 and 10 μM, meanwhile the LNA-rich pentamer **3** did not improve activity as compared to reference **1**. Therefore, the hybridization affinity of pentanucleotides seems not to be the only factor for inhibition efficiency, and other interactions inside the open complex have to be considered. The postulate of concurrent binding to both ssDNA and the enzyme becomes even clearer when considering the high efficiency of the inhibition of **ON 7**, although the melting temperature of its corresponding duplex was as low as 13.5 °C. However, very low or no binding to ssDNA resulted in a very low inhibition, as was seen for pentamers **4**, **6** and **10**.

The apparently most potent inhibitors **5**, **7** and **9** were chosen for further investigations of transcription inhibition at various concentrations, as shown in Figure 3. All of these oligonucleotides showed more than 95% transcription inhibition in comparison with 60% for the reference **1** at 16 μM. For LNA inhibitor **9**, this level of inhibition efficiency was still maintained at 10 μM, while INA analogues **5** and **7** exhibited 75–80% inhibition and sequence **1** showed 50% inhibition. This result is a significant improvement on previously published short 2′-OMe-oligoribonucleotides in a similar assay.[1e]

Gene inhibition by oligonucleotide targeting of dsDNA has been approached in various ways including triplex-forming

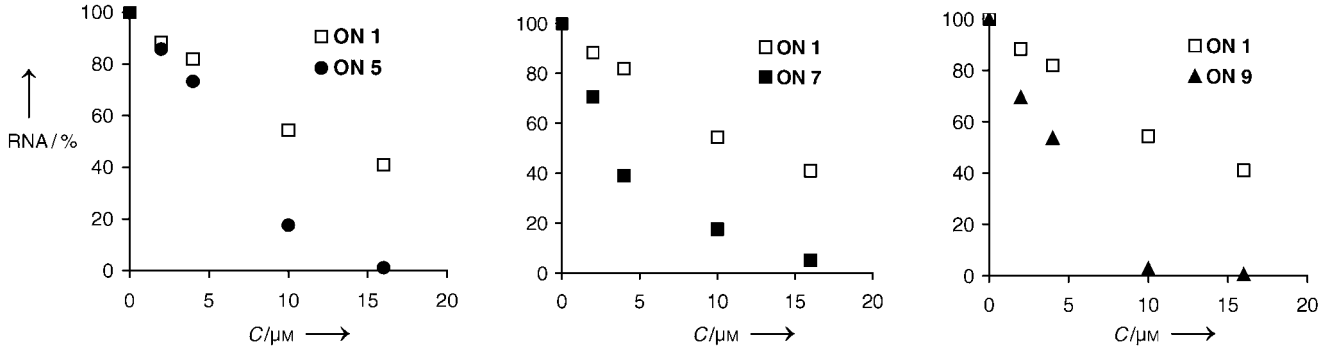

Figure 3. Curves showing concentration-dependent inhibition of RNA transcription in the presence of **ON 1**, **5**, **7** and **9**.

oligonucleotide targeting and so-called strand invasion. As none of the methods has so far made a big impact, we have explored another line of attack. At transcription start, the RNA polymerase opens up the dsDNA promoter region and exposes a small stretch of ssDNA. Inhibition of transcription has been achieved by using nonextendable short 2′-OMe-ribonucleotides that bind to the DNA template strand immediately upstream from the transcription start site.[1] We aimed at improving this interaction by incorporating locked nucleic acids (LNAs) and intercalating nucleic acids (INAs) into the structure of 2′-OMe pentaribonucleotides, and obtained considerably stronger inhibition (80–95% at 10 μM) compared to the previously published results. As hybridization affinity of the pentamers to ssDNA seems too low to account for the total binding, the interaction must be influenced by contacts other than the base pairing. We therefore believe there is further room for improvement with a view to creating a promoter-specific transcription inhibitor with efficient in vivo activity. The next steps in our study will be to investigate the effect of various pentamers on different RNA polymerases and see the effect of our present oligonucleotides in vivo in *E. coli* cells.

Acknowledgements

Kirsten Østergaard is thanked for oligonucleotide synthesis and HPLC purification. We are grateful to Dr. Michael Meldgaard, Exiqon A/S and to Dr. Kenneth B. Jensen for mass-spectrometric analysis of oligonucleotides. The Nucleic Acid Center is funded by The Danish National Research Foundation for studies on nucleic acid chemical biology.

Keywords: DNA · *E. coli* · intercalation · nucleic acids · transcription

[1] a) D. M. Perrin, A. Mazumder, F. Sadeghi, D. S. Sigman, *Biochemistry* **1994**, *33*, 3848–3854; b) D. M. Perrin, C.-H. B. Chen, Y. Xu, L. Pearson, D. S. Sigman, *J. Am. Chem. Soc.* **1997**, *119*, 5746–5747; c) L. Milne, Y. Xu, D. M. Perrin, D. S. Sigman, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 3136–3141; d) L. Milne, D. M. Perrin, D. S. Sigman, *Methods* **2001**, *23*, 160–168; e) J.-T. Hwang, F. E. Baltasar, D. L. Cole, L. Milne, Y. Xu, D. M. Perrin, D. S. Sigman, C.-H. B. Chen, M. M. Greenberg, *Bioorg. Med. Chem.* **2003**, *11*, 2321–2328.

[2] a) M. P. Knauert, P. M. Glazer, *Hum. Mol. Genet.* **2001**, *10*, 2243–2251; b) S. Buchini, C. J. Leumann, *Curr. Opin. Chem. Biol.* **2003**, *7*, 717–726; c) T. G. Uil, H. J. Haisma, M. G. Rots, *Nucleic Acids Res.* **2003**, *31*, 6064–6078.

[3] a) S. K. Singh, P. Nielsen, A. A. Koshkin, J. Wengel, *Chem. Commun.* **1998**, *4*, 455–456; b) A. A. Koshkin, S. K. Singh, P. Nielsen, V. K. Rajwanshi, R. Kumar, M. Meldgaard, C. E. Olsen, J. Wengel, *Tetrahedron* **1998**, *54*, 3607–3630; c) S. Obika, D. Nanbu, Y. Hari, J. Andoh, K. Morio, T. Doi, T. Imanishi, *Tetrahedron Lett.* **1998**, *39*, 5401–5404.

[4] a) U. B. Christensen, E. B. Pedersen, *Nucleic Acids Res.* **2002**, *30*, 4918–4925; b) U. B. Christensen, E. B. Pedersen, *Helv. Chim. Acta* **2003**, *86*, 2090–2097; c) V. V. Filichev, E. B. Pedersen, *Org. Biomol. Chem.* **2003**, *1*, 100–103; d) V. V. Filichev, K. M. H. Hilmy, U. B. Christensen, E. B. Pedersen, *Tetrahedron Lett.* **2004**, *45*, 4907–4910.

Received: December 29, 2004
Published online on May 24, 2005

