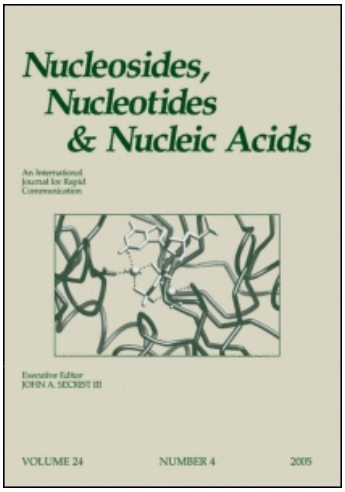


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

OLIGONUCLEOTIDE ANALOGUE INTERFERENCE WITH THE HIV-1 TAT PROTEIN-TAR RNA INTERACTION

Andrey Arzumanov[a]; Andrew P. Walsh[a]; Xiaohai Liu[b]; Vivek K. Rajwanshi[c]; Jesper Wengel[d]; Michael J. Gait[a]
[a] Laboratory of Molecular Biology, Medical Research Council, Cambridge, U.K. [b] Department of Chemistry, University of Cambridge, Cambridge, U.K. [c] Department of Chemistry, University of Copenhagen, Copenhagen, Denmark [d] Department of Chemistry, University of Southern Denmark, Odense M, Denmark

Online publication date: 31 March 2001

To cite this Article Arzumanov, Andrey , Walsh, Andrew P. , Liu, Xiaohai , Rajwanshi, Vivek K. , Wengel, Jesper and Gait, Michael J.(2001) 'OLIGONUCLEOTIDE ANALOGUE INTERFERENCE WITH THE HIV-1 TAT PROTEIN-TAR RNA INTERACTION', Nucleosides, Nucleotides and Nucleic Acids, 20: 4, 471 — 480
To link to this Article: DOI: 10.1081/NCN-100002321
URL: http://dx.doi.org/10.1081/NCN-100002321

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(4–7), 471–480 (2001)

OLIGONUCLEOTIDE ANALOGUE INTERFERENCE WITH THE HIV-1 TAT PROTEIN-TAR RNA INTERACTION

Andrey Arzumanov,[1] Andrew P. Walsh,[1] Xiaohai Liu,[2] Vivek K. Rajwanshi,[3] Jesper Wengel,[3,*] and Michael J. Gait[1,†]

[1]Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, U.K.
[2]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, U.K.
[3]Department of Chemistry, University of Copenhagen, Universiteitsparken 5, 2100 Copenhagen, Denmark

ABSTRACT

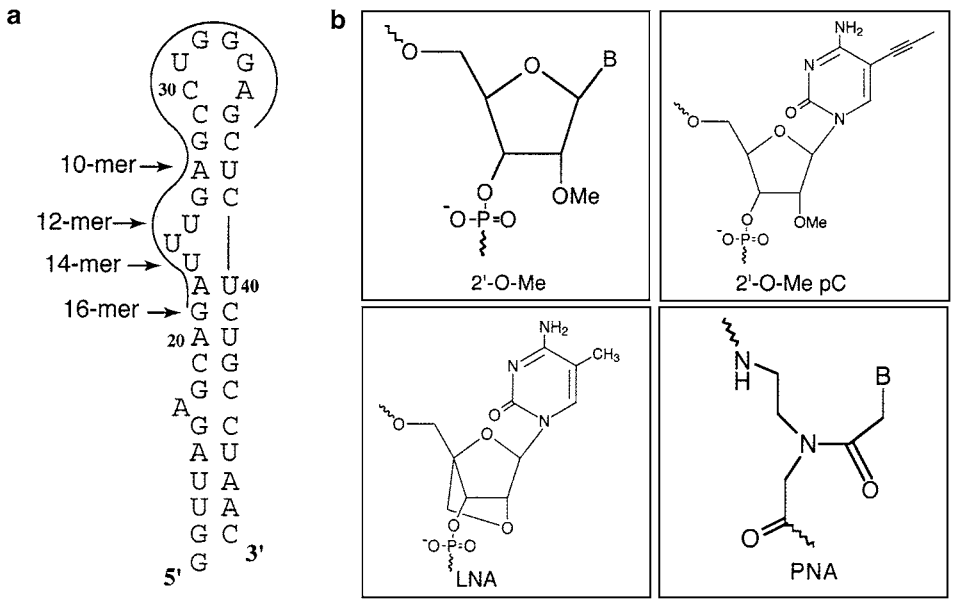
The HIV-1 Tat protein interaction with its RNA recognition sequence TAR is an important drug target and model system for the development of specific RNA-protein inhibitors. 2′-*O*-methyl oligoribonucleotides complementary to the TAR apical stem-loop effectively block Tat binding *in vitro*. Substitution by 5-propynylC or 5-methylC LNA monomeric units into a 12-mer 2′-*O*-methyl oligoribonucleotide leads to stronger inhibition, as does a 12-mer PNA. 10–16 mer 2′-*O*-methyl oligoribonucleotides give sequence- and dose-dependent inhibition of Tat-dependent transcription of an HIV DNA template in HeLa cell nuclear extract. Inhibition is maintained for the substituted 12-mer analogues but is poorer for PNA and is not correlated with TAR binding strength.

Transcription of HIV-1 is regulated by a virally encoded *trans*-activator protein Tat. Tat interacts with a stem-loop RNA structure, the *trans*-activation responsive region TAR, that occurs at the 5′-end of all HIV RNA transcripts (see recent

*Current address: University of Southern Denmark, Department of Chemistry, Odense M, DK 5230 Denmark.
†Corresponding author.

reviews (1–3). Tat acts together with a Tat-associated kinase (TAK), which includes host cellular factors cyclin T1 and the kinase cdk9, to form a ternary complex with TAR RNA. Tat-dependent *trans*-activation involves cdk9-mediated hyperphosphorylation of the C-terminal domain of RNA polymerase II and resultant stabilisation of the transcription complex leading to full-length transcription (4).

The recognition site of Tat interation on TAR has been localised to a U-rich bulge (Fig. 1a) (5,6). Compounds that bind to TAR in the region of the U-rich bulge and which block the ability of the Tat protein to recognise TAR would be expected to lead to loss of HIV *trans*-activation and hence the ability of HIV to replicate. Thus the TAR stem-loop is an important anti-HIV drug target. A number of small molecules and peptidomimetics have been shown to bind TAR and block interaction with Tat, and in some cases such molecules have shown anti-HIV activity (7).



c

Name	Sequence 5′-3′
12 BRU OMe (25-36)	CUC CCA GGC UCA
12 BRU OMe/6xpC (25-36)	**C**U**C** **C**C**C**A GG**C** U**C**A
12 BRU 7xOMe/5xLNA (25-36)	**C**U**C** C**C**A GG**C** U**C**A
12 BRU PNA (25-36)	H_2N-CTC CCA GGC TCA-Lys-Lys-Lys-COOH
12 BRU PNA mismatched	H_2N-CTC-C**GC A**GC TCA-Lys-Lys-Lys-COOH

Figure 1. a) 39-mer TAR RNA and location of the complementary oligonucleotides; b) structures of the four nucleotide analogues studied, c) names and sequences of the oligonucleotide analogues. Underlined and bold residues refer to sites of substitution by propynyl or LNA analogues or the sites of mismatch in case of PNA.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

An alternative strategy for inhibition of a protein-RNA interaction is by sequence-specific binding of a complementary oligonucleotide to the part of the RNA involved in protein recognition. Ecker *et al* showed that oligonucleotides and their analogues can strand invade the apical stem-loop of TAR RNA and interfere with Tat binding *in vitro* (8). Strand invasion was poor for short phosphodiester oligonucleotides and phosphorothioates, but longer phosphorothioates of 26–28 residues were able to induce a partially sequence-specific antisense effect in a *trans*-activation reporter assay when transfected into cultured cells (9). By contrast, short oligoribonucleotides and 2′-*O*-methyl oligoribonucleotides were found to bind much more strongly to the TAR loop, allowing the formation of a half pseudo knot structure (8).

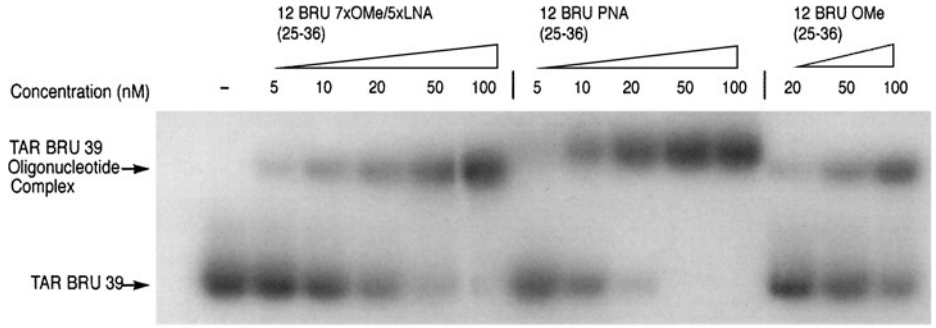
Recently, we showed by use of improved polyacrylamide gel mobility shift assays that certain 2′-*O*-methyl oligoribonucleotides of 12–16 residues bound TAR RNA *in vitro* with affinities in the 20–100 nM range and blocked Tat binding very efficiently (10,11). We have now synthesised 12-mer 2′-*O*-methyl oligoribonucleotides containing 5-propynyl-2′-*O*-methyl C or by 5-methylC LNA monomeric units as well as a PNA 12-mer. We show that the ability to displace Tat correlates well with TAR binding strength. We also show that 2′-*O*-methyl oligoribonucleotides and their substituted analogues as well as PNA inhibit sequence-specifically Tat-dependent transcription *in vitro* directed by HeLa cell nuclear extract but that the magnitude of inhibition is not correlated with their ability to block *in vitro* Tat binding.

RESULTS

We found previously that a 12-mer 2′-*O*-methyloligonucleotide complementary to residues 25–36 (Fig 1c, 12 BRU OMe (25–36)) bound to TAR RNA sequence specifically with apparent K_d of 50–70 nM and inhibited 50% Tat interaction also at about 50 nM as judged by gel mobility shift analysis (10,11). We wished to establish whether the ability to inhibit Tat could be enhanced by the incorporation of other nucleotide analogues known to bind strongly to RNA. Phosphorothioate or 2′-*O*-allyl oligonucleotides substituted with a 5-propynyl uridine or cytidine have been shown to enhance interaction with RNA *in vitro* and within cells (12,13). Similarly, LNA oligonucleotides containing at least one LNA monomeric unit (a 2′-*O*, 4′-C-methylene-β-D-ribofuranosyl nucleotide) have shown remarkable RNA binding properties either in the form of an all LNA oligonucleotide or when combined with standard 2′-deoxynucleotides (14). Combined LNA/DNA oligonucleotides have also shown potent antisense activity *in vivo* (15). An entirely different analogue PNA, which has a *N*-(aminoethyl)glycine backbone but normal heterocyclic bases, also binds strongly to RNA and has found wide utility as a sequence specific antisense agent (16).

We have therefore synthesised chimeric 12-mer 2′-*O*-methyl oligoribonucleotides containing either a) six 5-propynyl-2′-*O*-methylC (pC) units or b) five

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016



Analogue	50% shift conc. (nM)
12 BRU OMe	50-70
12 BRU OMe/6xpC	15
12 BRU 7xOMe/5xLNA	15-25
12 BRU PNA	10
12 BRU PNA mismatched	>100

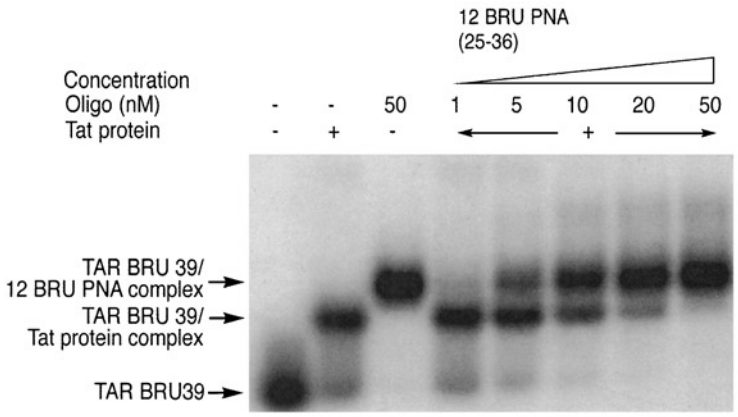
Figure 2. Autoradiograph and results of gel mobility shift analysis of direct binding of oligonucleotide analogues to ^{32}P-labelled TAR BRU 39 RNA.

5-methylC LNA units. We have also synthesised a PNA 12 mer of the same sequence, as well as a 3-base mismatched control (Fig. 1b and c). The PNA oligomers were prepared with a tri-lysine tail in order to enhance solubility and binding kinetics (17). These oligonucleotides were compared to the standard 12-mer 2′-*O*-methyl oligoribonucleotide in a gel mobility shift assay (Fig. 2) for binding to ^{32}P-labelled TAR RNA. The results showed that both the 5-propynyl-2′-*O*-methylC and LNA substituted oligonucleotides bound TAR 2–3 fold more strongly than the standard, whereas the PNA was 5–7 fold more strongly bound. By contrast a 3-residue mismatched PNA was at least 10 fold poorer in binding TAR than the correctly matched PNA.

The same oligonucleotide analogues were tested for their ability to inhibit Tat protein interaction with TAR RNA, again by use of a gel mobility shift assay as previously described (10–11). Figure 3 shows that the inhibitory power of the oligonucleotides followed the same order as the direct TAR RNA binding strength. Thus the PNA 12-mer showed the strongest inhibition, 5–10 fold better than the standard 12-mer 2′-*O*-methyloligoribonucleotide. The LNA and PNA chimeric analogues were 2–3 fold more inhibitory.

The next question was whether 2′-*O*-methyl oligonucleotides and their chimeric analogues could inhibit the action of Tat under the conditions of active Tat-dependent transcription. An *in vitro* transcription system has been described previously that is Tat-dependent and based on a HeLa cell nuclear extract (18). The template DNA contains the HIV LTR and a synthetic terminator sequence (τ)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016



Analogue	50% Tat-TAR displacement conc. (nM)
12 BRU OMe	50
12 BRU OMe/6xpC	40
12 BRU 7xOMe/5xLNA	20-40
12 BRU PNA	5-10

Figure 3. Autoradiograph and results of gel mobility shift analysis of oligonucleotide inhibition of binding of Tat protein to TAR BRU 39 RNA.

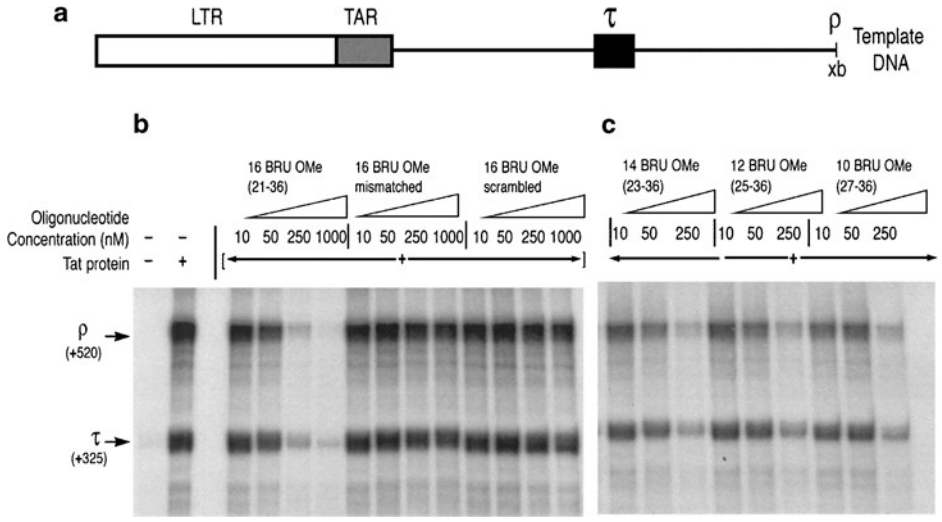


Figure 4. a) Scheme of HIV template used for *in vitro* transcription studies; b) autoradiograph of PAGE of transcription products in the presence and absence of Tat protein and in the presence of increasing concentrations of 16-mer 2′-*O*-methyl oligonucleotides (sequences of scrambled and mismatched as previously described (10,11), c) autoradiograph as b) but with 14, 12 and 10-mer 2′-*O*-methyl oligoribonucleotides.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

downstream of the TAR region (Fig. 4a) (19,20). Addition of Tat protein resulted in a substantial stimulation of transcription as witnessed by increases in the intensity of bands corresponding to ^{32}P-labelled terminator (τ) and run off (ρ) transcription products. Addition of increasing concentrations of a 16-mer 2′-*O*-methyl oligoribonucleotide complementary to residues 21–36 of TAR showed dose-dependent inhibition of *in vitro* transcription as seen in a reduction of the intensities of the transcription products (Fig. 4b). By contrast, addition of mismatched and scrambled 16-mer sequences did not give rise to any reduction in amounts of transcription products even up to 1000 nM dosage. 14-mer, 12-mer and 10-mer complementary 2′-*O*-methyl oligoribonucleotides also showed dose-dependent transcription inhibition (Fig. 4c).

We tested also the three oligonucleotide analogues for their ability to inhibit Tat-dependent transcription. Both the propynylC and LNA containing 12-mers showed dosedependent inhibition of transcription at the same level as that found for the 2′-*O*-methyl 12-mer (50% inhibition at *ca*. 70–150 nM) (Fig. 5). By contrast, the PNA 12-mer was poorer and showed 50% inhibition of transcription at *ca*. 150–300 nM.

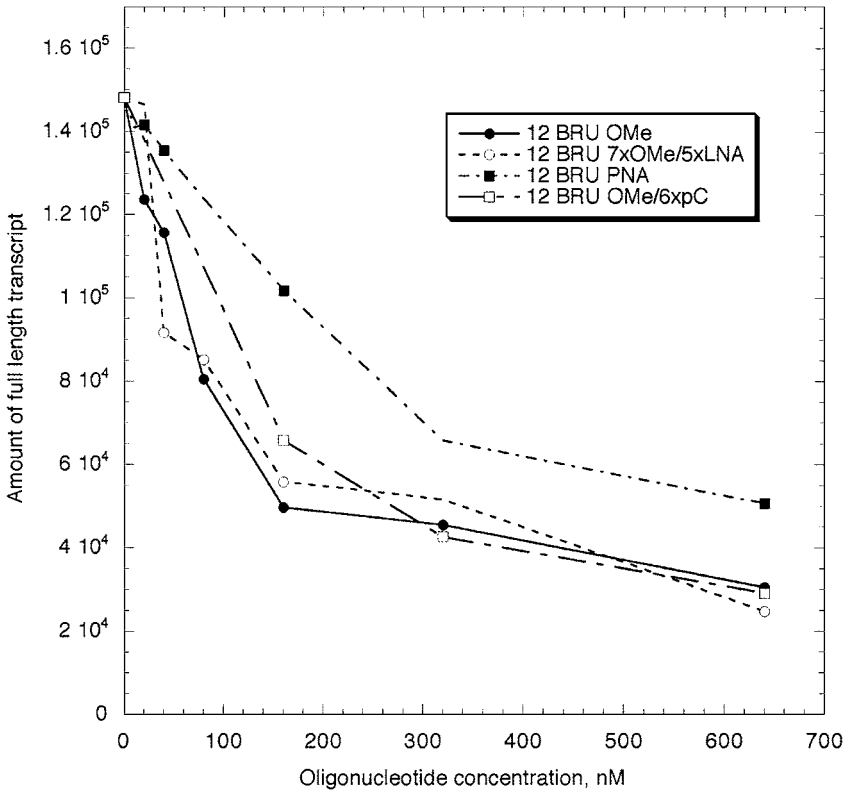


Figure 5. Comparative plot of amounts of full length transcripts as a function of concentrations of oligonucleotide analogues.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

DISCUSSION

2′-*O*-methyl oligoribonucleotides are a good starting point for investigation of steric block mechanisms of inhibition of RNA-protein interactions because they are reasonably nuclease-resistant and they do not permit the action of RNase H when bound to RNA (21). 2′-*O*-methyl oligoribonucleotides are also known to hybridise with RNA much faster than DNA and to have a higher melting temperature (22). We have shown that 10–16-mer 2′-*O*-methyl oligoribonucleotides are effective in inhibiting Tat-dependent transcription sequence-specifically from a DNA template carrying the HIV promoter and TAR sequence. The results are consistent with this inhibition occurring at the RNA level by strand invasion of the TAR sequence that is formed during transcription and by blocking of *trans*-activation directed by Tat and its associated cellular factors.

We also investigated three types of oligonucleotide analogue known to bind strongly to RNA. The 5-propynyl-2′-*O*-methylC and 5-methylC LNA analogues were incorporated together with standard 2′-*O*-methyl nucleotide units to form mixed 12-mer oligonucleotides. Although both of these mixed oligonucleotide derivatives showed enhanced binding to TAR RNA and an improved ability to inhabit Tat binding *in vitro*, neither oligonucleotide showed improvements in inhibition of *in vitro* Tat-dependent transcription. Further, the inhibition of transcription by the PNA 12-mer was less than that for the other analogues containing phosphodiester linkages. The lack of correlation of activity in this transcription inhibition assay with TAR binding strength *in vitro* suggests that other parameters limit the activity of steric blocking oligonucleotides. Such parameters could include, for example, the kinetics of TAR association and dissociation, the displacement or sequestration of oligonucleotides by protein factors under transcription conditions etc. and these are currently under investigation. It should be noted that, compared to PNA, 2′-*O*-methyloligonucleotides were also found recently to have higher inhibitory properties against telomerase RNA in a cell lysate-directed TRAP assay, despite their lower binding strength (23). However, conjugation of basic peptides to PNA has been shown to improve telomerase RNA inhibition (17).

A further goal of this research is to investigate the ability of steric blocking oligonucleotides to inhibit Tat-directed *trans*-activation within cells that harbour HIV DNA. This requires the oligonucleotides to be delivered into the cell nucleus where transcription takes place. It should be noted that 2′-*O*-methyl oligonucleotides targeted to splice junctions have been shown to be ineffective in their ability to correct splicing of globin mRNA within the nuclei of HeLa cells when delivered either by scrape loading or by cationic lipids (24). In addition, in our preliminary studies of cellular uptake of 3′-fluorescein labelled 2′-*O*-methyloligoribonucleo tides delivered by cationic lipids into HeLa cells, we observed fluorescence in the cytosol but not in the nucleus (Walsh, A.; Arzumanov, A.; Gait, M. J., unpublished studies). Addition of phosphorothioate linkages incorporated into 2′-*O*-methyl oligoribonucleotides has been shown to lead to significant activities in cellular telomerase inhibition assays (23) and in splicing inhibition assays (24). We

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

are currently investigating phosphorothioate modifications of 2′-*O*-methyl oligoribonucleotides as well as the propynyl and LNA modifications described in this article for nuclear delivery by cationic lipids and for intra-cellular activity. Further, conjugates of oligonucleotide analogues with cell-penetrating peptides, prepared through a new native ligation method we have recently introduced (25), are also under investigation for their potential lipid-free delivery properties.

EXPERIMENTAL SECTION

Oligonucleotides

2′-*O*-methyl oligoribonucleotides and TAR BRU 39 RNA were synthesised as described previously (10,11). 2′-*O*-methyl oligoribonucleotides containing 5-propynyl-2′-*O*-methylC residues were synthesised by standard phosphoramidite solid phase synthesis (10,11) with the modified phosphoramidite obtained from Glen Research (*via* Cambio Ltd). Mixed 2′-*O*-methyl/LNA oligoribonucleotides were prepared by standard phosphoramidite solid phase synthesis as described previously (14). PNA-triLys conjugates were synthesised by standard Fmoc chemistry manually on a Tentagel resin preloaded with one Fmoc-Lys(Boc) residue (5 μmole) (26). The other two lysine residues were assembled using Fmoc-Lys(Boc)-OH (5 eq), PyBOP® (5 eq), HOBt (5 eq) and disisopropylethylamine (DIPEA, 10 eq) in DMF (0.2 ml). Each lysine residue was double coupled and coupling steps were allowed to proceed for 90 min at room temperature. Individual PNA residue assembly was achieved in 60 min at room temperature using Fmoc monomer (4 eq), HATU (4 eq), HOAt (3.6 eq), DIPEA (6.25 eq), 2,4,6-collidine (3.75 eq) and NMP (0.2 ml). Fmoc deprotection was carried out with 20% piperidine in DMF (2×10 min). The final cleavage and concurrent deprotection were carried out with TFA/DCM/triisopropylsilane(TIS)/water (0.5 ml, 50:50:2.5:2.5) for 60 min at room temperature. The PNA conjugate was purified by HPLC and analysed by ESMS.

In Vitro TAR Binding and Tat Inhibition Assays

These were carried out as previously described (10,11).

Inhibition of Tat-dependent Transcription

Plasmid, p10SLT (19,20) carrying the wild type HIV-1 LTR was cut with Eco RV and Xba I (New England BioLabs) and the -346 to $+520$ fragment was isolated by 1% agarose gel electrophoresis followed by purification with QIAquick gel extraction kit (QIAGEN) according to the manufacturer's specifications. Cell-free transcription reactions were carried out essentially as described (19,20,26) with minor differences. Thus the transcription reaction mixture (40 μL) contained 15 μL

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

HeLa cell nuclear extract (27), 10 nM template DNA, 80 mM KCl, 2–4 mM $MgCl_2$ (depending on the HeLa nuclear extract), 20 mM HEPES (pH 7.9), 2 mM DTT, 10 μM $ZnSO_4$, 10 μM creatine phosphate (Boehringer Mannheim), 100 μg/mL creatine kinase (Boehringer Mannheim), 1 μg poly[d(I-C)] (Boehringer Mannheim), 50 μM ATP, GTP and CTP, 5 μM UTP, [α-^{32}P]UTP (10 μCi), 1 unit/μL RNasin (Promega), 200 ng recombinant Tat protein (27) and increasing concentrations of inhibitor oligonucleotide. The reactions were incubated at 30°C for 20 min and then stopped by addition of 50 μL of 150 mM sodium acetate solution, 0.5% SDS, 10 mM EDTA and 20 μg/mL tRNA, extracted with an equal volume of phenol/chloroform followed by precipitation with two volumes of ethanol. The reaction products were analysed by 6% PAGE containing 7 M urea followed by autoradiography. The autoradiographs were scanned densitometrically using a Personal Densitometer SI (Molecular Dynamics, Inc.) and the resulting digitized images were analyzed by *Geltrak* software (28).

ACKNOWLEDGMENTS

We thank Tony Lowe for HeLa cell nuclear extract, Jon Karn for Tat protein and Mark Churcher and Tom Barlow for advice on *in vitro* transcription. Ms Britta M. Dahl is thanked for LNA synthesis. VKR and JW thank the Danish Natural Science Research Council and the Danish Technical Research Council for financial support.

REFERENCES

1. Taube, R.; Fujinaga, K.; Wimmer, J.; Barboric, M.; Peterlin, B. M. *Virology*, **1999**, *264*, 245–253.
2. Karn, J. *J. Mol. Biol*., **1999**, *293*, 235–254.
3. Rana, T. M.; Jeang, K.-T. *Arch. Biochem. Biophys*., **1999**, *365*, 175–185.
4. Isel, C.; Karn, J. *J. Mol. Biol*., **1999**, *290*, 929–941.
5. Roy, S.; Delling, U.; Chen, C.-H.; Rosen, C. A.; Sonenberg, N. *Genes Dev*., **1990**, *4*, 1365–1373.
6. Dingwall, C.; Ernberg, I.; Gait, M. J.; Green, S. M.; Heaphy, S.; Karn, J.; Lowe, A. D.; Singh, M.; Skinner, M. A. *EMBO J*., **1990**, *9*, 4145–4153.
7. Wilson, W. D.; Li, K. *Curr. Medicinal Chem*., **2000**, *7*, 73–98.
8. Ecker, D. J.; Vickers, T. A.; Bruice, T.W., Freier, S.M., Jenison, R.D., Manoharan, M.; Zounes, M. *Science*, **1992**, *257*, 958–961.
9. Vickers, T.; Baker, B. F.; Cook, P. D.; Zounes, M.; Buckheit, R. W.; Germany , J.; Ecker, D. J. *Nucl. Acids Res*., **1991**, *19*, 3359–3368.
10. Mestre, B.; Arzumanov, A.; Singh, M.; Boulmé, F.; Litvak, S.; Gait, M. J. *Biochim. Biophys. Acta*, **1999**, *1445*, 86–98.
11. Arzumanov, A.; Gait, M. J. *Coll. Symp. Series*, **1999**, *2*, 168–174.
12. Wagner, R. W.; Matteucci, M. D.; Lewis, J. G.; Guttierez, A. J.; Moulds, C.; Froehler, B. C. *Science*, **1993**, *260*, 1510–1513.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

13. Moulds, C.; Lewis, J. G.; Froehler, B. C.; Grant, D.; Huang, T.;, Milligan, J. F.; Matteucci, M. D.; Wagner, R. W. *Biochemistry*, **1995**, *34*, 5044–5053.
14. Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron*, **1998**, *54*, 3607–3630.
15. Waehlstadt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hökfelt, T.; Broberger, C.; Porreca, F.; Lai, J.; Ren, K.; Ossipov, M.; Koshkin, A.; Jakobsen, N.; Skouv, J.; Oerum, H.; Jacobsen, M. V.; Wengel, J. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 5633–5638.
16. Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature*, **1993**, *365*, 566–568.
17. Harrison, J. G.; Frier, C.; Laurant, R.; Dennis, R.; Raney, K. D.; Balasubramanian, S. *Bioorg. Med. Chem. Lett*., **1999**, *9*, 1273–1278.
18. Graeble, M. A.; Churcher, M. J.; Lowe, A. D.; Gait, M. J.; Karn, J. *Proc. Natl. Acad. Sci. USA*, **1993**, *90*, 6184–6188.
19. Keen, N. J.; Gait, M. J.; Karn, J. *Proc. Natl. Acad. Sci. USA*, **1996**, *93*, 2505–2510.
20. Keen, N. J.; Churcher, M. J.; Karn, J. *EMBO J*., **1997**, *16*, 5260–5272.
21. Lamond, A. I.; Sproat, B. S. *FEBS Lett*., **1993**, *325*, 123–127.
22. Majlessi, M.; Nelson, N. C.; Becker, M. M. *Nucl. Acids. Res*., **1998**, *26*, 2224–2229.
23. Pitts, A. E.; Corey, D. R. Proc. *Natl. Acad. Sci. USA*, **1998**, *95*, 11549–11554.
24. Schmajuk, G.; Sierakowska, H.; Kole, R. *J. Biol. Chem*., **1999**, *274*, 21783–21789.
25. Stetsenko, D.; Gait, M. J. *J. Org. Chem*., **2000**, *65*, 4900–4908.
26. Thomson, S. A.; Josey, J. A.; Cadilla, R.; Gaul, M. D.; Hassman, C. F.; Luzzio, M. J.; Pipe, A. J.; Reed, K. L.; Ricca, D. J.; Wiethe, R. W.; Noble, S. A. *Tetrahedron*, **1995**, *51*, 6179–6194.
27. Rittner, K.; Churcher, M. J.; Gait, M. J.; Karn, J. *J. Mol. Biol*., **1995**, *248*, 562–580.
28. Smith, J.; Singh, M. *Biotechniques*, **1996**, *20*, 1082–1087.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Request Permission or Order Reprints Instantly!