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A phosphorothioate oligonucleotide blocks reverse transcription via an antisense mechanism

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Abstract

We have studied the inhibition by a phosphorothioate oligodeoxynucleotide (17PScap) of cDNA synthesis performed by either avian or murine reverse transcriptase. Three different mechanisms of inhibition were identified: at low concentrations (<100 nM), the cleavage of the RNA template by the retroviral RNase H at the level of the RNA/17PScap duplex accounted for most of the effect, whereas hybrid-arrested cDNA synthesis by an RNase H-independent mechanism marginally contributed to the inhibition. Both mechanisms were sequence-specific. Above 100 nM, the overall cDNA synthesis was reduced in a non-specific manner.

Key words: Antisense oligonucleotide; Inhibition mechanism; Retrovirus; RNase H

1. Introduction

Many studies have been devoted to the effect of antisense oligonucleotides on the expression of a gene [1]. Targets for these oligonucleotides can be of different origin: endogenous mRNAs or pre-RNAs of eukaryotes, microinjected mRNAs, or viral RNAs. Consequently, antisense oligonucleotides are potential inhibitors of viruses, by interfering with one or more of their developmental steps (splicing, translation or reverse transcription). Due to the urgent need for active drugs against AIDS, particular attention has been paid to HIV. Phosphorothioate (PS) oligonucleotides have been widely studied, as they can inhibit HIV multiplication in cultured cells with a higher efficacy than unmodified phosphodiester (PO) ones [2–7]. This difference is partly due to their increased resistance to nucleases in culture media [8], and more critically to their mechanism of inhibition: in contrast to PO oligonucleotides which display essentially specific effects, both sequence-dependent (antisense) and -independent activities have been reported for PS derivatives. In chronically infected cells, the inhibition is length- and sequence-dependent: PS oligonucleotides complementary to different regions of HIV RNA block viral replication following binding to their target sequence. Non-complementary (mismatched or random) oligonucleotides delay but do not completely inhibit HIV replication [9,10]. In these conditions, PS oligonucleotides seem to interfere with viral mRNA translation by an antisense mechanism [7]. In contrast, the inhibition of HIV in de novo infected cells by PS oligonucleotides is related more to their length than to their sequence. A homocytidine 28-mer, S-dC28, was demonstrated to interfere with the binding of gp120 to CD4 [11]; in addition, PS oligonucleotides have a great affinity for HIV reverse transcriptase (RT) [12].

Reverse transcription, which is an early event in retroviral infection, is an attractive step to target with anti-HIV drugs. The inhibition of cDNA synthesis would prevent the pro-viral DNA integration in the host genome. The antisense strategy is worth considering for this purpose. Several studies have already been published on the in vitro inhibition of reverse transcription by antisense oligonucleotides. On the one hand, an RNase H-mediated cleavage, generating a truncated RNA matrix, was demonstrated to be involved in the inhibition by unmodified oligonucleotides [13,14]. On the other hand, physical blocking was observed with oligonucleotides leading to a covalent linkage to the target [15], or with unreactive oligonucleotides [13,16,17]. The wide use of PS oligonucleotides to control HIV development prompted us to study the inhibition of reverse transcription of an RNA template by a PS oligonucleotide. In a previous report, we have shown that antisense PS oligonucleotides could be used as selective inhibitors of cDNA synthesis [18]. The results described in this paper

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Abbreviations: RT, reverse transcriptase; RNase H, ribonuclease H; AMV, avian myeloblastosis virus; MMLV, Moloney murine leukemia virus; MMLV H⁻ RT, MMLV RT devoid of RNase H activity; HIV, human immunodeficiency virus; PS, phosphorothioate; PO, phosphodiester; nt, nucleotide.

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extend this study and shed some light on the mechanism and on the efficacy of this process.

2. Materials and Methods

2.1 Oligonucleotides

Unmodified and phosphorothioate oligodeoxynucleotides (see Table 1 for sequences) were synthesized on a Millipore 7500 automated synthesizer, using the conventional phosphoramidite method, except that, for the phosphorothioate oligomer, the oxidation step was substituted by a sulfurization procedure using home-made Beaucage's reagent [19]. Oligodeoxynucleotides were usually purified in one step by HPLC on a reverse phase column eluted by an acetonitrile gradient (0–48%) in a 100 mM ammonium acetate (pH 7.0) buffer. For analysis, unmodified and phosphorothioate oligomers were 5′ end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase according to standard procedures [20]. The purity of the oligomers was evaluated by running ^{32}P-labelled samples on a 20% polyacrylamide/7 M urea gel.

21RNAsense (Table 1) was synthesized by transcription with T7 RNA polymerase as described by Milligan et al. [21], and purified by preparative gel electrophoresis [22]. Some oligodeoxynucleotides were purified similarly.

2.2. RNA template and enzymes

T4 polynucleotide kinase was from Boehringer, *E. coli* RNase H from Promega, and AMV RT from Appligène. MMLV and MMLV H$^-$ RTs, as well as rabbit globin mRNAs were purchased from Gibco BRL. T7 RNA polymerase was prepared from an over-producing strain (BL21/pAR1219) according to a previously published procedure [23].

β-Globin mRNA was used as a template for reverse transcription without any treatment. An RNA fragment, about 160 nucleotides long, prepared for Northern blot analysis, was produced by oligonucleotide-directed cleavage of rabbit β-globin mRNA by RNase H: 1 μg of globin mRNA was incubated with 10 U of *E. coli* RNase H, in the presence of 100 pmol of 15-mer'cleavage' complementary to nucleotides 147–161 of the β-globin message (Table 1). The reaction was performed for 2 h at 37°C, in 20 μl of a 20 mM Tris-HCl, pH 7.5, buffer containing 10 mM $MgCl_2$, 100 mM KCl and 0.1 mM dithiothreitol. At the end of the incubation, RNA was phenol-extracted, ethanol-precipitated and dissolved in 25 μl of sterile water. For reverse transcription, one tenth of the RNA solution (corresponding to 0.25 pmol β-globin mRNA) was used in each reaction as described below.

2.3. Reverse transcription

The standard reaction for cDNA synthesis was performed as follows: either 100 ng globin mRNAs corresponding to 0.25 pmol of β-globin mRNA (for cDNA analysis) or 0.25 pmol of the 160 nt RNA fragment (for RNA template analysis), 10 pmol of primer (17-mer'primer' except where indicated) and the desired amount of antisense oligodeoxynucleotide were pre-incubated for 20 min at 39°C, in 8 μl of a solution containing 100 mM Tris-HCl, pH 8.3, 72 mM KCl, 10 mM $MgCl_2$ and 10 mM dithiothreitol. After addition of 2 μl of the same solution containing 1 nmol of each of the four dXTPs and 2 pmol of [α-^{32}P]dCTP (111 TBq/mmol; Du Pont de Nemours) and 1 U AMV RT, 50 U MMLV RT or 50 U MMLV H$^-$ RT (final concentrations of about 17, 180 and 600 nM, respectively), the reaction was performed for either 15 min (RNA analysis) or 1 h (cDNA analysis) at 39°C. The products of the reverse transcription were chloroform-extracted, ethanol-precipitated according to standard procedures [20] and loaded on a 10% polyacrylamide/7 M urea gel. The yield of cDNA synthesis was determined by counting the radioactivity of gel slices in a β-scintillator counter.

2.4. Northern blot analysis of the RNA template

After reverse transcription (15 min), the RNA was chloroform-extracted and ethanol-precipitated. It was then loaded on a 10% polyacrylamide gel containing 7 M urea and electroblotted on to a nylon membrane (Pall) according to the supplier's instructions. Blots were revealed with the ^{32}P 5′ end-labelled 17-mer'probe'. Quantitative evaluation of cleavage was performed by densitometric analysis.

2.5. Analysis of hybrid stability

Melting curves of RNA/oligonucleotide duplexes were obtained as follows: the 21RNAsense and either 17POcap or 17PScap (1 μM each) were mixed in a 10 mM sodium cacodylate, pH 7.0, buffer containing 50 mM NaCl and 1 mM magnesium acetate. UV absorbance was continuously recorded at 260 nm on a Uvikon 930 spectrometer, in a 1 cm quartz cuvette, while the temperature was increased from 20°C to 95°C, at 30°C/h. The melting temperatures (T_m) were deduced from the curves.

3. Results

3.1. A phosphorothioate oligonucleotide blocks reverse transcription via an antisense mechanism

β-Globin mRNA was used as a template to direct reverse transcription by AMV or MMLV RTs, primed by a 17-mer complementary to nt 113–129 of mRNA (Table 1). As shown in Fig. 1, this produced the expected 129 nt long cDNA product. Addition of different concentrations of the antisense phosphorothioate oligonucleotide, 17PScap, or of the phosphodiester homologous sequence, 17POcap, reduced the amount of the full-length cDNA and generated shorter fragments, the length of which (about 110 nt) corresponded to an arrest of polymerization at, or close to, the 5′ end of the RNA-bound antisense oligonucleotide. The use of 15-mer'primer' complementary to nt 34–48 of the RNA template (see Table 1), which generated a shorter cDNA, allowed a more precise mapping of the 3′ end of the cDNA products in the presence of antisense oligonucleotides. The stop sites were located at the same positions for 17POcap and 17PScap, and corresponded to the first and third base pairs of the hybrid (from the 5′ end of the oligonucleotide) with the AMV RT, and to the first and second base pairs with the MMLV RT (not shown).

Table 1
The sequence of oligonucleotides used throughout this study

Name	Sequence	Target
DNA		
17POcap/17PScap	TTGTGTCAAAAGCAAGT	3–19
17-mer'primer'	CACCAACTTCTTCCACA	113–129
15-mer'primer'	CTGTTTTGGGGGATT	34–48
17-mer'probe'	GACAGATGCACCATTCT	51–67
15-mer'cleavage'	GTAGACAACCAGCAG	147–161
RNA		
21RNAsense	GGCACUUGCUUUUGACACAAC	–

The sequences are given in the 5′ (left) to the 3′ (right) direction. The corresponding abbreviation is given to the left, and the location of the target on rabbit β-globin mRNA to the right (+1 is the first nt after the 7-methylguanosine residue [27]). The sequence of 21RNAsense is similar to the sense 2–20 sequence, except that two extra G's were added at the 5′ end to improve the transcription efficacy.

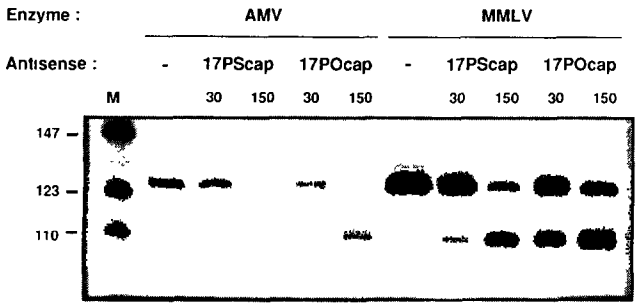

Fig. 1. Effect of antisense oligonucleotides on reverse transcription. cDNA synthesis performed either by AMV (left) or MMLV (right) RTs, in the absence (−) or presence of 30 and 150 nM 17PScap or 17POcap, was initiated by 17-mer'primer'. M is the size marker in nt.

3.2. *The antisense oligonucleotides 17PScap and 17POcap inhibit reverse transcription with similar efficiencies*

We quantified the relative cDNA synthesis of the truncated 110 nt long fragments. Results obtained with the AMV (Fig. 2a) or the MMLV RT (Fig. 2b) showed that 17PScap was slightly less efficient than 17POcap: concentrations yielding 50% truncated fragments ($C_{1/2}$) were about 2- to 4-fold higher for the phosphorothioate than for the phosphodiester oligonucleotide with both enzymes (Table 2). cDNA synthesis was 50–80% abolished by a ratio of 4 oligonucleotides per RNA template (25 nM).

At low concentration the effect was specific: below 100 nM, neither 17POcap nor 17PScap led to a decrease in the overall cDNA synthesis. However, this was no longer true for 17PScap concentrations above 100 nM (see Fig. 1). This was most likely due to a sequence-independent interaction between RT and the phosphorothioate oligomer, as previously described for the HIV RT [12].

Table 2
Characteristics of 17POcap/RNA and 17PScap/RNA hybrids

	$C_{1/2}$ AMV [a] (nM)	$C_{1/2}$ MMLV[a] (nM)	T_m[a] (°C)
17PScap	100	100	35
17POcap	25	60	43

The oligonucleotide concentration $C_{1/2}$ promoting 50% inhibition of cDNA synthesis (a), and the melting temperature (b) were determined as described in section 2, are indicated.

3.3. *Inhibition of cDNA synthesis results from an RNase H-mediated cleavage of the template*

We have previously shown that 17POcap leads to the cleavage of the RNA template by the RT-associated RNase H [13]. We therefore looked for a similar cleavage produced in the presence of 17PScap.

For this purpose, a 160 nt long fragment corresponding to the 5′ end of β-globin mRNA was prepared, and probed by Northern blot analysis after reverse transcription. In the presence of the primer alone, fragments of about 130 nt long resulting from cleavage at the site of hybridization of the primer, were detected in addition to the intact RNA (Fig. 3). Upon addition of 17PScap, part of the 160 nt long RNA template was cleaved, generating truncated fragments shortened by about 20 nt (Fig. 3). These fragments were very likely due to the cleavage of the template region bound to 17PScap by the retroviral RNase H. The same fragments were detected when 17POcap was used (Fig. 3). The efficiencies of reverse transcription inhibition by either 17POcap or 17PScap were roughly correlated with the cleavage efficiencies (±10%), both with AMV (Fig. 2a) and MMLV RTs (Fig. 2b). This result indicates that the cleavage of the RNA template by RNase H is the major mechanism of inhibition.

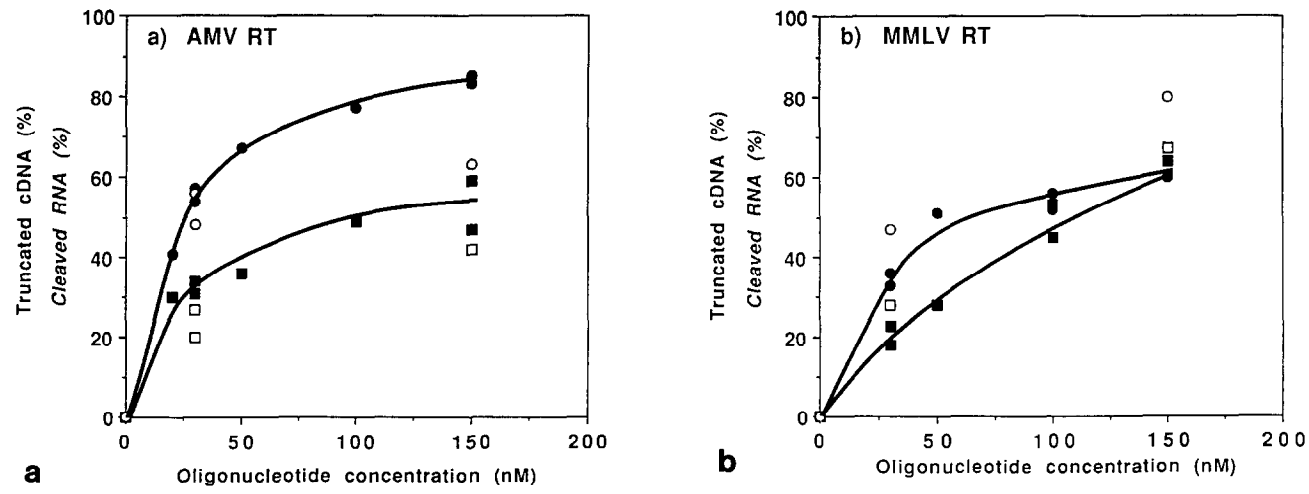

Fig. 2. Effect of antisense oligonucleotides on cDNA synthesis and template RNA. Reverse transcription by AMV (a) or MMLV (b) RTs was performed in the presence of either 17POcap (●,○) or 17PScap (■,□). Relative cDNA synthesis (●,■) and template RNA analysis (○,□) were carried out as indicated in section 2.

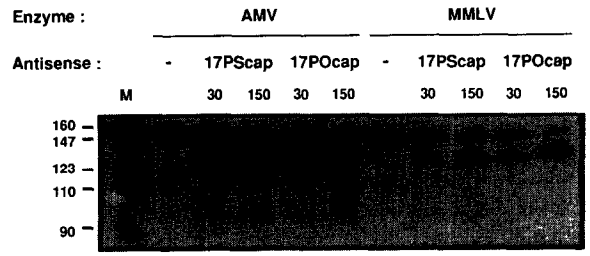

Fig. 3. RNA template analysis by Northern blot after reverse transcription by AMV (left) or MMLV (right) RTs, in the absence (-) or presence of antisense oligonucleotides 17PScap and 17POcap, at the concentration (in nM) indicated at the top of the lanes.

3.4. *Inhibition of reverse transcription performed by an enzyme devoid of RNase H activity*

Due to the key role of RNase H in inducing the inhibition of cDNA synthesis, we speculated that the use of an RT enzyme devoid of RNase H activity would abolish the antisense effect of 17PScap. Indeed, we had previously reported that 17POcap did not arrest DNA synthesis by such an enzyme [13]. We have used the MMLV RT devoid of RNase H activity (named MMLV H^-) to check for the properties of 17PScap compared to that of 17POcap. As illustrated in Fig. 4a, 17POcap did not lead to a detectable reduction in the amount of the 129 nt long cDNA fragment; in contrast, 17PScap significantly inhibited cDNA synthesis by 20% at 50 nM and 38% at 100 nM. A longer exposure of the autoradiography allowed the detection of fragments shorter than the full-length cDNA, the length of which (110 nt) indicated that MMLV H^- RT had been stopped by the hybrid (Fig. 4b). These short fragments represented about 15% of the total cDNA synthesis at 100 nM 17POcap or 17PScap. Thus, hybridization of either 17POcap or 17PScap by itself can block the progression of the enzyme along the template and contribute to the overall inhibition of reverse transcription.

3.5. *Stability of RNA/PO and RNA/PS hybrids*

A 21-mer RNA (21RNAsense) comprising the sequence complementary to 17cap was prepared by transcription and incubated with equimolar amounts of 17POcap or 17PScap. The stability of the resulting DNA/RNA hybrids was determined from melting curves as described in section 2. Melting temperatures (T_m) were significantly different for the two hybrids: 43°C and 35°C for RNA/17POcap and RNA/17PScap, respectively (Table 2).

4. Discussion

Phosphorothioate analogues, which are of high interest due to their combined nuclease resistance and capacity at eliciting RNase H activity, have been known for several years as inhibitors of reverse transcription, essentially by a mechanism involving their binding to the enzyme, i.e. by a process that is mostly sequence-independent. Indeed, a number of phosphorothioate oligomers displayed anti-HIV activity, one of the most potent being the sulfur analogue of $(dC)_{28}$ [6].

In a previous study, we described the inhibition of reverse transcription by an unmodified antisense oligonucleotide (17POcap) using the β-globin mRNA as a template [13]. We have clearly demonstrated here that the phosphorothioate oligonucleotide (17PScap) complementary to the same target was able to reduce cDNA synthesis by a true antisense effect, i.e. related to its hybridization to the complementary template sequence. The cleavage of the template strand by the RT-born RNase H activity accounted for most of the inhibition. However, a low level of hybrid-arrested cDNA synthesis was observed with both phosphorothioate and phosphodiester 17-mers when a recombinant RT devoid of RNase H activity (MMLV H^-) was used. This phenomenon has recently been observed for the inhibition of HIV RT-mediated reverse transcription by an unmodified oligomer [16], but had escaped our attention in a previous study [13] as it contributes only marginally to the antisense effect. The parameters driving the relative level of RNase H-dependent and -independent mechanisms of inhibition are unclear as yet. However, for oligonucleotides able to mediate RNase H cleavage, this seems related to sequence/structure peculiarities of the antisense binding site rather than to the retroviral enzyme. Indeed, results similar to those described here were obtained with 17PScap and 17POcap used against the rabbit β-globin mRNA reverse transcribed by the HIV enzyme (Boiziau et al., unpublished data).

The comparison of the antisense efficacy of 17POcap and 17PScap showed that the former was a slightly better inhibitor. On the one hand, considering the RNA/antisense oligonucleotide duplexes, this could be related to the higher affinity of 17POcap for its RNA target, compared with 17PScap (Table 2), or to a greater sensitivity of RNA/PO- vs. RNA/PS-oligomer duplexes to RNase H- mediated cleavage. Indeed, such a difference was previously described with human RNase H [24]. On the other hand, we still do not know what the active species

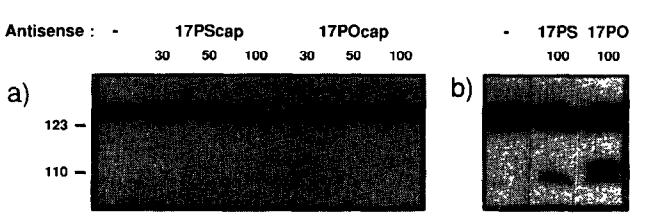

Fig. 4. Reverse transcription performed by MMLV H^- RT, in absence (-) or presence of antisense oligonucleotides 17POcap or 17PScap, at the concentration indicated (in nM). The right part (b) is an overexposure of the corresponding lanes given to the left (a). Size marker lengths (in nt) are indicated to the left.

inhibiting reverse transcription is: it may be a ternary hybrid formed by a non-polymerizing RT molecule sitting on the antisense/RNA duplex. The sites of elongation arrest (and of RNase H-mediated cleavage) support this hypothesis. It is known that the polymerase site is operating about 15 nt ahead of the RNase H catalytic site [25]. It is therefore unlikely that the molecule elongating the cDNA strand is responsible for the cleavage of the template bound to the antisense oligomer. Consequently, in addition to the stability of RNA/antisense duplexes and to the capacity of the antisense to elicit an RNase H- mediated cleavage of the RNA template, the antisense efficacy would also be related to the affinity of RT for the duplex.

In a recent paper, Hatta and co-workers [26] used exactly the same combination of RNA template, primer and antisense oligonucleotides as the one used in our previous paper [13] and in this one, to investigate the effect of 17PScap on reverse transcription. In contrast to our results, they concluded that inhibition occured only via the binding of the oligomer to the AMV RT. Despite the poor quality of their autoradiographs, it seems that a shortened cDNA species, indicative of a sequence-dependent effect, can be detected (see Fig. 2a in [26]). However, the high concentrations of oligonucleotide they used (>1 μM) prevented them from demonstrating antisense effects.

In conclusion we have demonstrated that phosphorothioate oligonucleotides can inhibit the reverse transcription by hybridization to the RNA template. This could in part be responsible for the inhibition of HIV development either in de novo or in chronically infected cultured cells.

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References

[1] Hélène, C. and Toulmé, J.J. (1990) Biochim. Biophys. Acta 1049, 99–125.
[2] Cheng, Y.C., Gao, W.Y. and Han, F.S. (1991) Nucl. Nucl. 10, 155–166.
[3] Vickers, T., Baker, B.F., Cook, P.D., Zounes, M., Buckheit Jr., R.W., Germany, J. and Ecker, D.J. (1991) Nucleic Acids Res. 19, 3359–3368.
[4] Agrawal, S., Ikeuchi, T., Sun, D., Sarin, P.S., Konopka, A., Maizel, J. and Zamecnik, P.C. (1989) Proc Natl. Acad. Sci. USA 86, 7790–7794.
[5] Agrawal, S., Goodchild, J., Civeira, M.P., Thornton, A.H., Sarin, P.S. and Zamecnik, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 7079–7083.
[6] Matsukura, M., Shinokuza, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J.S. and Broder, S. (1987) Proc. Natl. Acad. Sci. USA 84, 7706–7710.
[7] Matsukura, M., Zon, G., Shinozuka, K., Robert-Guroff, M., Shimada, T., Stein, C.A., Mitsuya, H., Wong-Staal, F., Cohen, J.S. and Broder, S. (1989) Proc. Natl. Acad. Sci. USA 86, 4244–4248.
[8] Hoke, G.D., Draper, K., Freier, S.M., Gonzalez, C., Driver, V.B., Zounes, M.C. and Ecker, D.J. (1991) Nucleic Acids Res. 19, 5743–5748.
[9] Lisziewicz, J., Sun, D., Klotman, M., Agrawal, S., Zamecnik, P. and Gallo, R. (1992) Proc. Natl. Acad. Sci. USA 89, 11209–11213.
[10] Lisziewicz, J., Sun, D., Metelev, V., Zamecnik, P., Gallo, R.C. and Agrawal, S. (1993) Proc. Natl. Acad. Sci. USA 90, 3860–3864.
[11] Stein, C.A., Neckers, L.M., Nair, B.C., Mumbauer, S., Hoke, G. and Pal, R. (1991) J. AIDS 4, 686–693.
[12] Majumdar, C., Stein, C.A., Cohen, J.S., Broder, S. and Wilson, S.H. (1989) Biochemistry 28, 1340–1346.
[13] Boiziau, C., Thuong, N.T. and Toulmé, J.J. (1992) Proc. Natl. Acad. Sci. USA 89, 768–772.
[14] Loreau, N., Boiziau, C., Verspieren, P., Shire, D. and Toulmé, J.J. (1990) FEBS Lett. 252, 53–56.
[15] Chu, B.C.F. and Orgel, L.E. (1989) Nucleic Acids Res. 17, 4783–4798.
[16] Bordier, B., Hélène, C., Barr, P.J., Litvak, S. and Sarih-Cottin, L. (1992) Nucleic Acids Res. 20, 5999–6006.
[17] Hanvey, J.C., Peffer, N.J., Bisi, J.E., Thomson, S.A., Cadilla, R., Josey, J.A., Ricca, D.J., Hassman, C.F., Bonham, M.A., Au, K.G., Carter, S.G., Bruckenstein, D.A., Boyd, A.L., Noble, S.A. and Babiss, L.E. (1992) Science 258, 1481–1485.
[18] Boiziau, C., Larrouy, B., Moreau, S., Cazenave, C., Shire, D. and Toulmé, J.J. (1992) Biochem. Soc. Trans. 20, 764–767.
[19] Iyer, P.I., Lawrence, R.P., Egan, W., Regan, J.B. and Beaucage, S.L. (1990) J. Org. Chem. 55, 4693–4698.
[20] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Nolan, C., Eds. New York.
[21] Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) Nucleic Acids Res. 15, 8783.
[22] Fedor, M.J. and Uhlenbeck, O.C. (1990) Proc. Natl. Acad. Sci. USA 87, 1668–1672.
[23] Davanloo, P., Rosenberg, A.H., Dunn, J.J. and Studier, F.W. (1984) Proc. Natl. Acad. Sci. USA 81, 2035–2039.
[24] Agrawal, S., Mayrand, S.H., Zamecnik, P.C. and Pederson, T. (1990) Proc. Natl. Acad. Sci. USA 87, 1401–1405.
[25] Oyama, F., Kikuchi, R., Crouch, R.J. and Uchida, T. (1989) J. Biol. Chem. 264, 18808–18817.
[26] Hatta, T., Kim, S.G., Nakashima, H., Yamamoto, N., Sakamoto, K., Yokoyama, S. and Takaku, H. (1993) FEBS Lett. 330, 161–164.
[27] Efstratiadis, A., Kafatos, F.C. and Maniatis, T. (1977) Cell 10, 571–585.