Effect of P-chirality of oligo(deoxyribonucleoside phosphorothioate)s on the activity of terminal deoxyribonucleotidyl transferase

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Abstract Phosphorothioate analogues of oligonucleotides (PS-oligos) of predetermined chirality at the phosphorus atom at each internucleotide linkage have been used as primers for terminal deoxyribonucleotidyl transferase (*TdT*, EC 2.7.7.31). The enzyme catalyzes efficient elongation of PS primers in which all phosphorothioate internucleotide linkages are uniformly of the [R_P] configuration, while the presence of the linkage(s) of the [S_P] configuration significantly decreases or completely inhibits the primer extension. Our results indicate that for the elongation of phosphorothioate oligomers the most important is the internucleotide bond located between the second and the third nucleoside from the 3′-end. The presence of [S_P] linkage at this position strongly reduces the enzyme activity while the [R_P] bond allows for effective elongation of the primer. The activity of the enzyme is also influenced by base composition and sequence of phosphorothioate primer as well as the dNTP used for elongation process.

Key words: Stereoselectivity; Phosphorothioate; Terminal deoxyribonucleotidyl transferase

1. Introduction

In the course of our studies on the influence of the absolute configuration at the phosphorus atoms of internucleotide phosphorothioate functions of PS-oligonucleotides upon the action of enzymes associated with DNA metabolism [1–3], we turned our attention towards terminal deoxyribonucleotidyl transferase (*TdT*, EC 2.7.7.31). *TdT* catalyzes the addition of deoxyribonucleoside phosphates to the 3′-ends of oligo- and polynucleotide primers. As distinct from all the other known polymerases, *TdT*-catalyzed DNA synthesis is not template-directed. This enzyme requires an oligodeoxyribonucleotide primer containing at least three phosphate groups and a free 3′-hydroxyl [4,5]. *TdT* activity has been found only in the nuclei of pre-Y and pre-B lymphocytes and its biological function, even after 25 years of intensive research, is not known [6].

It was established that *TdT* accepts as substrates 5′-*O*-deoxyribonucleoside triphosphates (dNTP) or 5′-*O*-deoxyribonucleoside-α-thiotriphosphates (dNTPαS) and this property of the enzyme has been utilized for the 3′-end labeling of single stranded DNA with ^{32}P-phosphates or ^{35}S-phosphorothioates. Recently Grosse and Manns reported the use of *TdT* for the elongation of a natural oligonucleotide such as $p(dC)_{10}$ with dCTPαS and the tailing of restriction fragments or linearized plasmid DNA with dNTPαS [6]. However, to the best of our knowledge, the stereochemical requirements for *TdT*-catalyzed elongation with dNTPαS have not been studied so far with respect to the absolute configuration of α-phosphorus of dNTPαS, or the configuration at internucleotide phosphorus atoms of the phosphorothioate primers. It is known that all polymerases studied so far accept for template-dependent polymerization only S_P-dNTPαS giving oligo(deoxyribonucleoside phosphorothioate)s of an [all-R_P] configuration [7,8]. However, distinct properties of the terminal deoxyribonucleotidyl transferase prompted us to study in detail its possible diastereoselectivity. We have used as the primers phosphorothioate oligodeoxyribonucleotides (PS-oligos) of predetermined chirality (absolute configuration) at the phosphorus atom at each internucleotide phosphorothioate function. Because phosphorothioate oligonucleotides are evaluated as potential therapeutic agents, their influence on functions of different biomolecules is of special interest [9]. It is well documented that PS-oligos can change the activity of many enzymes involved in DNA or RNA metabolism [2,10–12]. Also their influence on *TdT* activity cannot be excluded. The studies on the involvement of PS-oligos in *TdT*-catalyzed synthesis of DNA can enrich our knowledge about their mechanisms of action.

2. Materials and methods

2.1. Materials

All oligo(deoxyribonucleoside phosphorothioate)s were synthesized according to the oxathiaphospholane methodology elaborated by Stec et al. [13]. The oligonucleotides **1**–**6** obtained as 5′-DMT derivatives were purified by two-step HPLC [13]. Then their purity was analyzed by electrophoresis in 20% denaturing polyacrylamide gel (PAGE). Tris-HCl, bovine serum albumin (BSA) and dNTP were purchased from Sigma, β-mercaptoethanol from Serva, [γ-^{32}P]ATP (specific activity 1500 Ci/mmol) from Radioisotop, spermidine from Fluka, Biogel A 1.5m from Bio-Rad, calf thymus *TdT* and phage T4-polynucleotide kinase from Amersham. Nuclease P1 (nP1, EC 3.1.30.1) and dGTPαS were obtained from Pharmacia. Snake venom phosphodiesterase (svPDE, EC 3.1.15.1) was purchased from Boehringer.

2.2. Labeling of oligonucleotides

An assay mixture (volume 10 μl) contained 30 pmol of the oligonucleotide, 10 μCi of [γ-^{32}P]ATP, 10 units of polynucleotide kinase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 15 mM β-mercaptoethanol, 1 mM spermidine, 100 mM NaCl, and 10 μg/ml BSA. The reaction was carried out for 3 h at 37°C and was terminated by heating the mixture at 70°C for 10 min. The labeled oligonucleotides **1**–**4** were purified by gel filtration on Biogel A 1.5m, while labeled tetradeoxyribonucleotides **5** and **6** were purified on Sephadex G-10.

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Table 1
Activity of *TdT* in elongation of oligonucleotides **1–4**

Compound	Sequence	Oligonucleotide elongated in the presence of dNTP substrate (%)	
		dGTP	dTTP
1a	[all-R_P]-d[$(A_{PS})_{11}$A]	95	95
1b	[all-S_P]-d[$(A_{PS})_{11}$A]	<5	<5
1c	[mix]-d[$(A_{PS})_{11}$A]	65	55
2a	[all-R_P]-d[GCT ATA ATG G]	>95	>95
2b	[all-S_P]-d[GCT ATA ATG G]	<5	<5
2c	[mix]-d[GCT ATA ATG G]	80	55
3a	[all-R_P]-d[$(T_{PS})_{11}$T]	>95	>95
3b	[all-S_P]-d[$(T_{PS})_{11}$T]	0	0
3c	[$(R_P)_{10}S_P$]-d[$(T_{PS})_{11}$T]	70	40[a]
3d	[mix]-d[$(T_{PS})_{11}$T]	60	40[b]
4	d[CCC ACT CAC GAC GT]	95	>95

[a]The main product of the elongation is a tetradecamer d[$(T_{PS})_{11}T_{PO}T_{PO}$T].
[b]The main products of the elongation are two oligonucleotides: 13-mer and 14-mer.

2.3. Terminal deoxyribonucleotidyl transferase assays[1]

An assay mixture (volume 5 μl) contained 10 μM 5′-labeled primer, 3 units of *TdT*, dNTP (concentrations as indicated in Figure legends), 100 mM sodium cacodylate, pH 7.2, 10 mM $MgCl_2$, and 1 mM $CoCl_2$. The reaction was performed by adding the enzyme and incubation for 30 min at 37°C, and was terminated by adding 3 μl of deionized formamide containing 50 mM EDTA, 0.1% bromophenol blue and xylene cyanol. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gels, and these gels were autoradiographed using Kodak XRP-5 film.

An assay mixture for inhibition experiments contained the 10 μM radioactively labeled tetradecadeoxyribonucleotide primer ([^{32}P]d(CCCAGTCACGACGT) and all other components as shown above. Additionally, specified quantities of unlabeled oligonucleotides **1a** or **1b** were added. Efficiency of elongation was measured by scanning spots in the tracks on autoradiograms.

2.4. Primer elongation with dGTPαS

The enzymatic assay was carried out under the same conditions as above with 5 mM dGTPαS instead of dNTP and with oligodeoxyribonucleotides **3a–d** and **5a** as the primers. The digestion of products resulting from this reaction was carried out in the presence of nuclease P1 or svPDE under conditions described elsewhere [13].

3. Results and discussion

In the first set of experiments 5′-^{32}P-labeled phosphorothioate decamers and dodecamers **1a–c**, **2a–c** and **3a–d** were used as the primers. In each series of oligonucleotides we used so called random mixtures of diastereoisomers [mix] and diastereomerically pure ones, with a defined sense of chirality at each internucleotide phosphorothioate. The oligonucleotides **1–3** were elongated in the presence of *TdT* using dGTP and dTTP as substrates. The efficiency of elongation was determined by the densitometry of bands corresponding to unreacted primers. We have observed that the efficiency of the elongation process depends to some extent upon the dNTP substrate; the highest efficiency was observed for dGTP while it was much lower for dTTP. A similar correlation was earlier described for unmodified primers [4].

Only [all-R_P]-d[$(A_{PS})_{11}$A] (**1a**) was elongated with high efficiency. The [all-S_P] isomer (**1b**) was not elongated at all, while the [mix] form of this PS-oligo (**1c**) exhibited rather low priming efficiency (Table 1). The low activity of the [mix] form is probably caused by the presence of a fraction of molecules containing one or two [S_P] linkages at the 3′-end. Efficient elongation of [all-R_P]-d[$(A_{PS})_{11}$A] prompted us to compare the priming activity of this PS-oligo and the unmodified tetradecadeoxyribonucleotide d[CCCACTCACGACGT] (**4**) using different concentrations of dGTP and dTTP as substrates. The dodecamer [all-R_P]-d[$(A_{PS})_{11}$A] (**1a**) was found to be almost as efficient as the natural oligonucleotide **4** in priming DNA synthesis. Because [all-S_P] isomers of PS-oligos are not accepted as primers in the elongation process, it was interesting to check if [S_P] isomers could compete with unmodified primers. We found that the unlabeled PS-oligo **1a**, when present in the reaction mixture containing 5′-^{32}P-labeled unmodified PO-oligo **4**, considerably decreased the efficiency of elongation of **4**, while oligomer **1b** had a very low inhibitory effect. Fig. 1 demonstrates that the [all-R_P] isomer (**1a**), present at the same concentration as oligomer **4**, inhibits its elongation ca. 50% while the [all-S_P] isomer (**1b**) shows a comparable inhibitory effect only at a 10-fold higher concentration. This result indicates that the [all-S_P] isomer poorly binds to *TdT*, thus explaining its poor priming activity.

Similar results were obtained with oligonucleotides **2**. We found that [all-R_P]-**2a** was elongated very efficiently while its

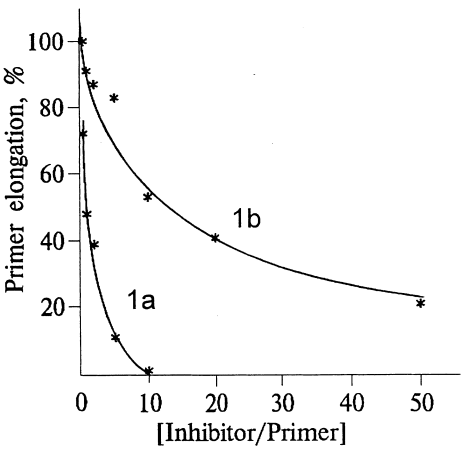

Fig. 1. Inhibition of primer **4** elongation as a function of [all-R_P]-d[$(A_{PS})_{11}$A] (**1a**) and [all-S_P]-d[$(A_{PS})_{11}$A] (**1b**) concentration.

[1] In all experiments we used the terminal transferase from Amersham. A comparison of two commercially available *TdT* preparations (from Promega and Amersham) showed that in our hands the Promega enzyme catalyzes the formation of very short products: only $n+1$ and $n+2$ oligomers were observed (n is the length of primer). Under the same conditions the Amersham enzyme is much more active and a primer is elongated with the formation of mixture of much longer products.

[all-S_P] counterpart was totally inactive. However, unexpectedly, the [mix] form of this oligonucleotide was more active in the elongation reaction than [mix]-d[$(A_{PS})_{11}$A]. We suppose that the presence of two dG residues at the 3′-end of this oligomer increases its affinity for the enzyme and the efficiency of elongation of the [mix] form. The kinetic parameters derived from the studies with a series of oligodeoxynucleotide primers differing in base composition show that the affinity of the terminal transferase for primers decreases in order dI > dG > dA > dT > dC [5,6]. Therefore, despite the presence of the otherwise unpreferred and poorly recognized [S_P] linkages at the 3′-end of [mix]-**2c**, the enzyme is able to catalyze its elongation in a quite efficient manner.

Interesting results were obtained for oligonucleotides **3a–d** (Table 1, Fig. 2). The oligomer **3c** contains 10 consecutive [R_P] internucleotide bonds followed by a single [S_P] linkage located between the penultimate and the final nucleoside at the 3′-end. Again, the [all-R_P] isomer of **3** was very efficiently elongated by the enzyme, while the [all-S_P] isomer was inactive. The [$(R_P)_{10}S_P$] isomer (**3c**) appeared to be a worse primer than the [all-R_P], especially in the reaction with dTTP as a substrate. Moreover, in the reaction with dTTP, the main product of elongation was found to be tetradecamer d[$(T_{PS})_{11}T_{PO}T_{PO}T$] and longer products were present only in trace amounts (Fig. 2). This result indicates that the presence of a single linkage of the [S_P] configuration at the 3′-end of a phosphorothioate primer significantly decreases the enzyme activity.

For more detailed studies on the influence of absolute configuration at internucleotide phosphorus atoms of PS-oligos upon the *TdT* activity, two series of phosphorothioate tetramers with a nucleotide sequence d[$(A_{PS})_3$A) (**5a–f**) and d[$(T_{PS})_3$T] (**6a–b**) were synthesized (Table 2). Using primers **5a–f** and dTTP as the substrate we found that the tetramer [$R_PR_PR_P$]-d[$(A_{PS})_3$A] is elongated very efficiently resulting in a mixture of products up to 15-mers. Prolonged incubation time or 2-fold increase of the enzyme concentration gave much longer products (up to 30-mers). Under the same conditions, the tetramer **5d** containing one linkage of the [S_P] configuration at the 3′-end was elongated only by one or two dT residues resulting in the oligomers d[$A_{Rp}A_{Rp}A_{Sp}A_{PO}T$] and d[$A_{Rp}A_{Rp}A_{Sp}A_{PO}T_{PO}T$], respectively (Fig. 3).

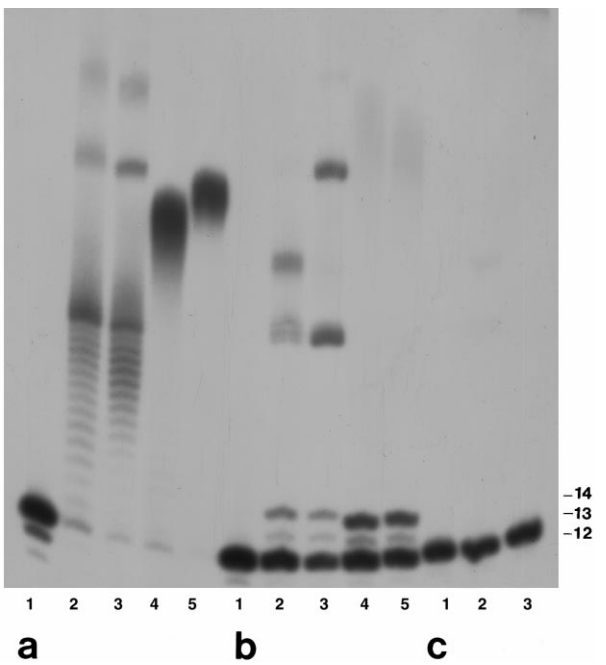

Fig. 2. Extension of dodecamers **3a–c** by *TdT* in the presence of dGTP or dTTP. a: [all-R_P] isomer: (1) primer+enzyme; (2, 3) as in 1+dGTP: 0.5 mM and 5 mM, respectively; (4, 5) as in 1+dTTP: 0.5 mM and 5 mM, respectively. b: [$(R_P)_{10}S_P$] isomer: (1) primer+enzyme; (2, 3) as in 1+dGTP: 0.5 mM and 5 mM, respectively; (4, 5) as in 1+dTTP: 0.5 mM and 5 mM, respectively. c: [all-S_P] isomer: (1) primer+enzyme; (2) as in 1+dGTP (5 mM); (3) as in 1+dTTP (5 mM). The numbers on the right indicate the length of oligonucleotides.

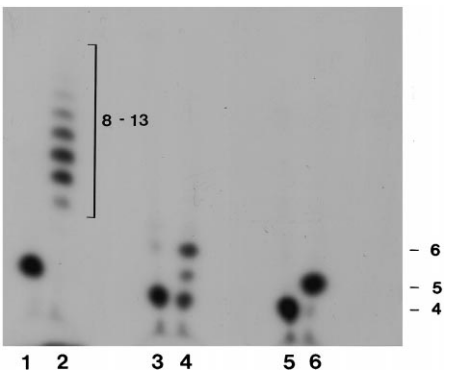

Fig. 3. Extension of tetramers **5** by *TdT* in the presence of 5 mM dTTP. Lanes 1, 3 and 5: the tetramers with no *TdT* added: **5a**, **5d** and **5c**, respectively. Lanes 2, 4 and 6: the tetramers **5** elongated by the enzyme: **5a**, **5d** and **5c**, respectively. The numbers on the right indicate the length of oligonucleotides.

Furthermore, the tetramer **5c**, containing a phosphorothioate linkage of the [S_P] configuration between the second and the third nucleosides from the 3′-end, was elongated by only one dT residue giving the pentamer d[$A_{Rp}A_{Sp}A_{Rp}A_{PO}T$] (Fig. 3). The tetramer d[$A_{Rp}A_{Sp}A_{Sp}A$] (**5e**) was elongated in the same way giving only small amounts of the pentamer d[$A_{Rp}A_{Sp}A_{Sp}A_{PO}T$] as the final product (data not shown). These results can be explained in terms of the model of Chang and Bollum, who postulated that *TdT*-metal ion complex binds to the 3′-hydroxyl and the phosphoryl group of the third nucleotide from the 3′-end of the primer [14]. For the sake of simplicity we will use α, β, γ and δ descriptions for the first, the second and the following internucleotide phosphates from the 3′-end, respectively. After binding and the addition of another nucleotide the complex dissociates from the primer and again binds to the 3′-hydroxyl and γ phosphoryl group of the growing oligonucleotide chain (non-processive mecha-

Table 2
Stereoregular phosphorothioate tetramers used in studies of *TdT* activity

Compound	Sequence
5a	[$R_PR_PR_P$]-d[$(A_{PS})_3$A]
5b	[$S_PR_PR_P$]-d[$(A_{PS})_3$A]
5c	[$R_PS_PR_P$]-d[$(A_{PS})_3$A]
5d	[$R_PR_PS_P$]-d[$(A_{PS})_3$A]
5e	[$R_PS_PS_P$]-d[$(A_{PS})_3$A]
5f	[$S_PS_PS_P$]-d[$(A_{PS})_3$A]
6a	[$R_PR_PR_P$]-d[$(T_{PS})_3$T]
6b	[$R_PR_PS_P$]-d[$(T_{PS})_3$T]

nism) [14,15]. Our results indicate that the enzyme is able to recognize and elongate the tetramer d[$A_{Rp}A_{Rp}A_{Sp}A$] and the pentamer d[$A_{Rp}A_{Rp}A_{Sp}A_{PO}T$] but it does not bind to the hexamer d[$A_{Rp}A_{Rp}A_{Sp}A_{PO}T_{PO}T$] which is not elongated and accumulates as the final product. Even 2-fold higher concentration of the *TdT* and prolonged incubation of the tetramer **5d** with the enzyme did not change the length of the final product. This hexamer as well as the pentamer d[$A_{Rp}A_{Sp}A_{Rp}A_{PO}T$] are not accepted as primers because in both cases the γ phosphorothioate internucleotide function is of the [S_P] configuration. Summarizing, for binding to a phosphorothioate primer, terminal deoxyribonucleotidyl transferase requires the [R_P] configuration at the phosphorus atom of the γ internucleotide bond of a primer. For this reason, the [$(R_P)_{10}S_P$] isomer of d[$(T_{PS})_{11}T$] is elongated only by two dT residues to give the tetradecamer d[$(T_{PS})_{11}T_{PO}T_{PO}T$] which contains at this key position phosphorothioate internucleotide linkage of [S_P] configuration and, therefore, is not elongated by the enzyme.

The elongation of oligonucleotide primers with dAMP or dGMP requires the presence of a divalent cation with an order of efficiency of $Mg^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$. On the other hand, the polymerization of pyrimidines is most efficient in Co^{2+}-containing buffers [6]. For many enzymes a replacement of the internucleotide phosphate by a phosphorothioate can dramatically diminish an enzyme reactivity under normal conditions, i.e. when Mg^{2+} ions are present in a reaction mixture. However, an addition of Mn^{2+} or Zn^{2+} can restore enzyme activity. It is caused by different ability of metal ions to coordinate sulfur: Mn^{2+} readily accepts sulfur as a ligand whereas Mg^{2+} does not [16]. Trying to restore the *TdT* activity toward the phosphorothioate primer **5d** containing an [S_P] linkage at the γ position, we used the cacodylate buffer with Mn^{2+} or other cations (Mg^{2+}, Co^{2+}, Zn^{2+}). The corresponding incubation mixtures were analyzed by PAGE. However, the replacement of Mg^{2+} or Co^{2+} by manganese cations did not restore the enzyme activity toward this primer (data not shown). These experiments suggest that the metal ion required for this reaction does not bind directly to the sulfur atom. Mg^{2+} or Co^{2+} cations bind rather to the *TdT* protein than to internucleotide bonds of primers.

Unexpectedly, the phosphorothioate tetramer d[$A_{Sp}A_{Rp}A_{Rp}A$] (**5b**) containing the third internucleotide phosphorothioate bond of the [S_P] configuration was elongated with a formation of a mixture of oligonucleotides of different length, although the efficiency of this reaction was lower (ca. 20%). We assume that the elongation of this primer resulted from the presence of phosphate group at the 5′-end of the tetramer, which was introduced in ^{32}P-labeled form to enable the analysis of *TdT*-catalyzed reactions by autoradiography of electrophoretic gels. Because of the presence of the unpreferred [S_P] linkage at the γ position, the enzyme cannot bind the primer using this linkage, but it may interact with the phosphate group located at the 5′-end of the primer. This interaction is relatively weak and allows for kinetically slow elongation, which yields the pentamer d[$_{PO}A_{Sp}A_{Rp}A_{Rp}A_{PO}T$]. This product fulfills the enzyme's requirements much better (a phosphate group at the α position and two [R_P] phosphorothioates at the β and γ positions) and is elongated very efficiently. The enzyme's affinity for this pentanucleotide is much higher than the affinity for the tetramer **5b** and therefore the pentamer becomes the only compound which is elongated in the next step of the reaction. To prove this hypothesis, unlabeled (i.e. non-phosphorylated at the 5′-end) tetramer d[$A_{Sp}A_{Rp}A_{Rp}A$] (**5b**) was incubated with *TdT* in the presence of dTTP, followed by 5′-radiolabeling of products with the polynucleotide kinase and [γ-^{32}P]ATP. PAGE analysis showed that the tetramer **5b** was not elongated at all (Fig. 4). The same experiment carried out with primer d[$A_{Rp}A_{Rp}A_{Rp}A$] (**5a**) showed its efficient elongation. This experiment confirmed our hypothesis and showed that the phosphate group in the δ position also plays an important role in activation of the enzyme. This additional contact between the enzyme and the phosphate or [R_P] phosphorothioate in the δ position is not sufficient for elongation of **5f**, but makes it possible to explain the presence of small amounts of longer products generated by the *TdT* during the elongation of oligomers **3c** and **6b** (Figs. 2 and 5B).

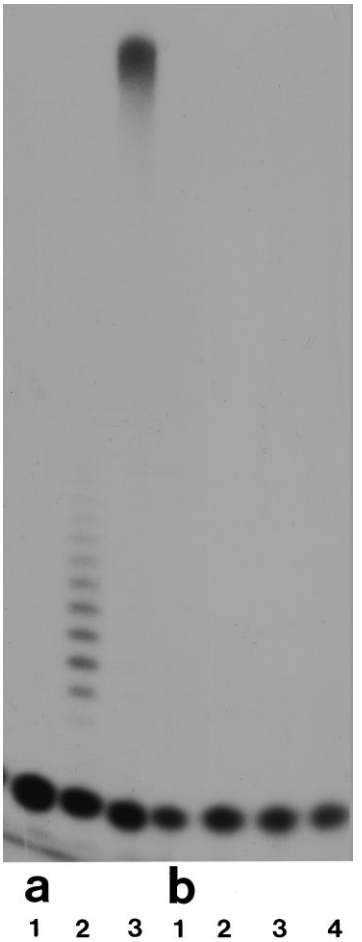

Fig. 4. a: Extension of 5′-^{32}P-labeled [$S_PR_PR_P$]-d[$(A_{PS})3A$] (**5b**) in the presence of 0.5 mM dTTP. Lane 1: primer+enzyme; lanes 2, 3: *TdT*-catalyzed elongation after 15 and 30 min of incubation, respectively. b: Results of incubation of non-phosphorylated **5b** with *TdT* and dTTP followed by 5′-labeling with polynucleotide kinase carried out after heat denaturation of *TdT*. Lane 1: primer+*TdT*; lanes 2–4: as in lane 1+0.5 mM dTTP after 5, 15 and 30 min of incubation with *TdT*.

Our results show that *TdT* is a [R_P] diastereoselective enzyme, however, this effect is strongly influenced by the dNTP used and/or the sequence of a primer. It was reported that for purine triphosphates (dATP and dGTP) V_{max} values are higher than those obtained with corresponding pyrimidine sub-

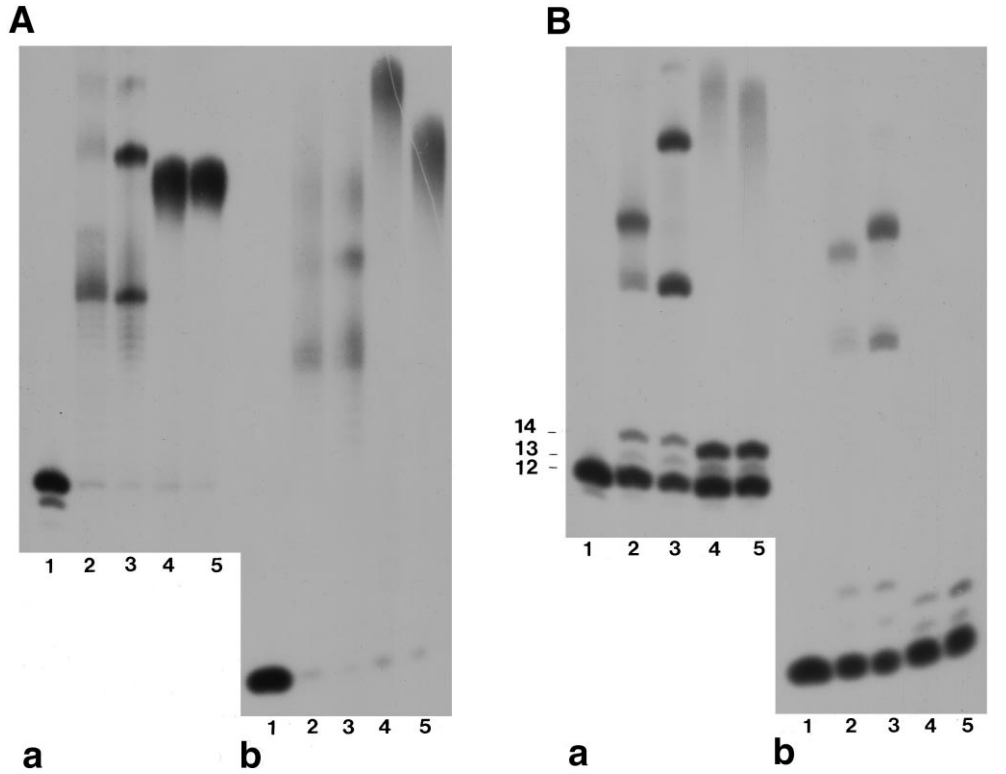

Fig. 5. A: Extension of primers **3a** and **6a** in the presence of dGTP or dTTP. a: [all-R_P]-d[$(T_{PS})_{11}$T] (**3a**): (1) primer+enzyme; (2, 3) as in 1+dGTP: 0.5 mM and 5 mM, respectively; (4, 5) as in 1+dTTP: 0.5 mM and 5 mM, respectively. b: [all-R_P]-d[$(T_{PS})_3$T] (**6a**): (1) primer+enzyme; (2, 3) as in 1+dGTP: 0.5 mM and 5 mM, respectively; (4, 5) as in 1+dTTP: 0.5 mM and 5 mM, respectively. B: Extension of primers **3c** and **6b** in the presence of dGTP or dTTP. a: [$(R_P)_{10}S_P$]-d[$(T_{PS})_{11}$T] (**3c**): (1) primer+enzyme; (2, 3) as in 1+dGTP: 0.5 mM and 5 mM, respectively; (4, 5) as in 1+dTTP: 0.5 mM and 5 mM, respectively. b: [$R_PR_PS_P$]-d[$(T_{PS})_3$T] (**6b**): (1) primer+enzyme; (2, 3) as in 1+dGTP: 0.5 mM and 5 mM, respectively; (4, 5) as in 1+dTTP: 0.5 mM and 5 mM, respectively.

strates [5]. Also the base composition of primers influences the rate of *TdT*-catalyzed reaction. Homooligonucleotides containing only dG or dA have much higher affinity for the enzyme than dT- or dC-rich oligomers [5]. Therefore, in the presence of dGTP, the dodecamer **3c** was elongated not only to the tetradecamer d[$(T_{PS})_{11}T_{PO}G_{PO}$G] but the formation of much longer products was also observed (Fig. 2). Due to differentiated activity of *TdT* towards primers of different base composition, two oligomers [$R_PR_PS_P$]-d[$(A_{PS})_3$A] (**5d**) and [$R_PR_PS_P$]-d[$(T_{PS})_3$T] (**6b**) are elongated with different efficiency although each of them contains at the α position the phosphorothioate linkage of the [S_P] configuration. Both are elongated only by two dT residues, however the tetramer **5d** is elongated more efficiently than **6b** for which we could observe only traces of the penta- and hexanucleotide products (compare lane 4 in Fig. 3 and lanes 4 and 5 in Fig. 5B).

The length of the phosphorothioate primer seems to be a less important factor influencing enzyme activity although for unmodified oligomers a correlation between primer length and K_m value was reported [5]. The results of our experiments with elongation of **3a** and **6a**, and independently **3c** and **6b**, indicate that tetramers d[$(T_{PS})_3$T] are elongated with similar efficiency as dodecamers d[$(T_{PS})_{11}$T]. Despite their different lengths, both **3a** and **6a** were elongated with high efficiency

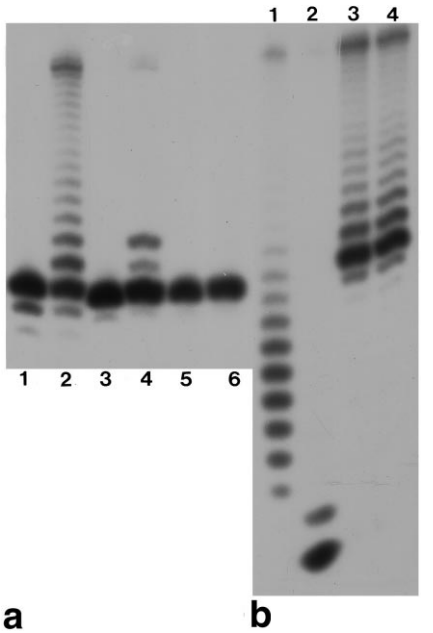

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Fig. 6. Extension of phosphorothioate oligonucleotides in the presence of dGTPαS. a: lane 1: [all-R_P]-d[$(T_{PS})_{11}$T] (**3a**) without *TdT*; lane 2: **3a** elongated by *TdT*; lane 3: [$(R_P)_{10}S_P$]-d[$(T_{PS})_{11}$T] (**3c**) without the enzyme; lane 4: **3c** after incubation with *TdT*; lane 5: [all-S_P]-d[$(T_{PS})_{11}$T] (**3b**) without the enzyme; lane 6: **3b** after incubation with *TdT*. b: lane 1: *TdT*-elongated **3a** after treatment with *sv*PDE (0.05 μg); lane 2: *TdT*-elongated **3a** after treatment with *sv*PDE (0.5 μg); lane 3: *TdT*-elongated **3a** after incubation with nP1 (0.1 μg); lane 4: *TdT*-elongated **3a** after incubation with nP1 (0.2 μg).

(~95%), while **3c** and **6b** containing a single [S_P] bond at their 3′-end were poor primers for *TdT* (Fig. 5A,B).

We have also studied the elongation of phosphorothioate oligomers **3** and **5** in the presence of dGTPαS. The tetramer [$R_PR_PR_P$]-d[$(A_{PS})_3$A] (**5a**) was elongated very efficiently resulting in a mixture of oligonucleotides up to 20-mers, while tetramer [$R_PR_PS_P$]-d[$(A_{PS})_3$A], due to the R_P diastereoselectivity of the enzyme, was elongated only by one or two dG residues. However, a 3- or 6-fold increase of enzyme concentration resulted in the accumulation of a hexamer d[$(A_{PS})_3A_{PS}G_{PS}$G] and the formation of small amounts of much longer products (up to 20 mers). The elongation of [all-R_P]-d[$(T_{PS})_{11}$T] in the presence of dGTPαS gave a mixture of oligomers (up to 30-mers). Incubation of this mixture with svPDE resulted in its complete digestion, which may be an indication that internucleotide phosphorothioates were of the [R_P] configuration. Incubation of the same mixture with [S_P] specific nuclease P1 showed that the products were resistant to the enzyme (Fig. 6). This result confirmed our conclusion that phosphorothioate products of *TdT*-catalyzed elongation were of the [R_P] configuration.

4. Conclusions

The terminal deoxynucleotidyl transferase has been used for elongation of phosphorothioate analogues of oligonucleotides. The efficiency of this process depends upon several different factors. The most important is the absolute configuration at the phosphorus of the internucleotide phosphorothioate functions of PS-oligos used as primers. [All-S_P]-PS-oligos are not accepted by the enzyme and are not elongated at all. On the other hand, the [all-R_P] isomers are very good primers for *TdT*. Their elongation is almost as efficient as the extension of natural oligonucleotides. The elongation of oligomers depends upon the absolute configuration at the phosphorus atom of the internucleotide bond located between the second and the third nucleosides from the 3′-end (so called γ position). The presence of [S_P] linkage in this position strongly reduces the enzyme activity while the γ-located [R_P] bond allows for effective and fast elongation. This phenomenon is observed for both short (4-mer) and long (up to 12-mer) primers. However, the [R_P] diastereoselectivity of the *TdT* is also influenced by the sequence of primer and the dNTP used in the reaction. For the purine-rich primers, and in the presence of dGTP as the substrate for the polymerization process, the enzyme is very efficient *and its [R_P] diastereoselectivity is, to some extent, suppressed.* Under these conditions even the primers containing [S_P] linkages at the γ position are elongated with a noticeable efficiency.

When dNTPαS were used as substrates for primer elongation, phosphorothioate oligonucleotide tails were found to contain exclusively internucleotide phosphorothioate functions of the [R_P] configuration.

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