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Optimization of the efficiency of cross-linking Pt^{II} oligonucleotide phosphorothioate complexes to complementary oligonucleotides

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ABSTRACT

We have investigated the efficiency with which Pt^{II} complexes cross-link phosphorothioates of oligonucleotides to complementary DNA targets. The A and G residues 2–5 bases downstream from the 5′-phosphorothioate group are preferred sites for cross-linking. Replacement of residues in this part of the target by T residues results in greatly decreased cross-linking when cis platinum diammine dichloride (cisPt^{II}) or potassium platinous chloride (K_2PtCl_4) are used. Trans platinum diammine dichloride (transPt^{II}) forms cross-links with T residues if A and G residues are absent from the susceptible region of the target. Oligomers containing an internal phosphorothioate group can also be linked to their templates with transPt^{II}, but not with cisPt^{II} or K_2PtCl_4. Cross-linking via an internal phosphorothioate group tends to be less efficient than cross-linking via a 5′-terminal phosphorothioate. The S_p isomers of internal phosphorothioates are cross-linked more efficiently than the R_p isomers. Preliminary experiments suggest that the efficiency of cross-linking to RNA targets will prove similar to that found for DNA targets.

INTRODUCTION

The use of 'antisense' oligonucleotides to block the activity of specific genes has received a great deal of attention, since it provides a new approach to the pharmacology of viral diseases and cancer (1,2). It is well-recognized that oligonucleotides carrying a 'warhead' that could either cleave a target messenger RNA (3–6) or form an irreversible cross-link with it (7–11) might prove more effective than the unmodified oligonucleotide. In such an approach, it is essential to employ 'warheads' that will attack RNA within the intracellular environment. Here, work on the chemotherapy of cancer provides a useful guide—the classes of compounds that attack DNA in the nucleus are promising sources of intracellular cross-linking agents. In this paper we explore in detail the formation of cross-links between oligonucleotide phosphorothioates and complementary targets in the presence of Pt^{II} complexes.

The specific attachment of Pt^{II} complexes to oligonucleotides containing a phosphorothioate group was first described by Strothkamp and Lippard (12). In earlier papers we have reported that cis platinum diammine dichloride (cisPt^{II}), trans platinum diammine dichloride (transPt^{II}) and potassium platinous chloride (K_2PtCl_4) efficiently cross-link target DNAs to complementary oligonucleotide-5′-phosphorothioates (13) or to cysteamine adducts of oligonucleotides (14). We now explore the factors that lead to effective cross-linking and begin to extend our work from DNA to RNA targets.

MATERIALS AND METHODS

Materials

The following were obtained from commercial sources: cisPt^{II} and transPt^{II}, Sigma; K_2PtCl_4, Pfaltz and Bauer; adenosine-5′-[γ-^{35}S]-thiotriphosphate (650 mCi/μmole), adenosine 5′-[γ-^{32}P]-triphosphate (3 Ci/μmole) and guanosine-5′-[γ-^{32}P]-triphosphate (30 mCi/μmole), Amersham; adenosine 5′-γ-thiotriphosphate, Boehringer; DNA polymerase I (Klenow fragment) and polynucleotide kinase, New England Biolabs; T7 RNA polymerase, U.S. Biochemical Corp; P1 nuclease, Bethesda Research Laboratories.

The deoxy-oligomer sequences listed in Table 1 were synthesized on an automated DNA synthesizer. Three 37mer templates were used in the cross-linking experiments. T1 corresponds to residues 6164–6200 of the M13mp18 phage (+) strand. T2 differs from T1 in that the C residue at position 25 is changed to A. T3 is derived from T1 by replacing A, G and C residues in positions 28–37 by T residues. Two 50mer templates were used in primer-extension experiments with the Klenow fragment. T4 corresponds to residues 6164–6213 of the M13mp18 phage (+) strand; T5 is identical to T4 except that residues 28–37 are all T residues. The primer P is complementary to residues 48–38 of T4 and T5. The 16mers A1, A2, B, C, D, E, ϵ, F, G and H are complementary to subsequences of the templates. The 26mer ribo template T6 was synthesized as described below. The purification of oligonucleotides and their conversion to the 5′-phosphates, 5′-[^{32}P]-phosphates (s.a. 1–3 μC/pmole) or 5′-[^{35}S]-phosphoro-

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thioates (s.a. 0.0065 μC/pmole) were carried out by methods that have been described (13).

Methods

High performance liquid chromatography (HPLC) of oligonucleotides was performed on RPC-5 at pH 12 or pH 8.5 using a perchlorate gradient as previously described (13). They were then purified on a Du Pont Nensorb nucleic acid purification cartridge. Electrophoresis was carried out on 0.5 mm − 1 mm thick 20% polyacrylamide gels, cast and run in 90 mM Tris borate buffer (pH 8.0), 1 mM EDTA with 7 M urea. Non-denaturing gels were run on 7.5 cm long, 1 mm thick, 20% polyacrylamide at 25 v. (7 ma) for ~4 hours. Autoradiographs of gels were obtained by exposure to Kodak X-Omat AR film at −80°C with or without a Du Pont Cronex Lightning Plus intensifying screen. Radioactivity on gels was determined by slicing the appropriate zones and counting in a Beckman scintillation counter.

Table 1. Synthetic oligomers.

Templates	
T1	5'-TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTT-3'
T2	5'-TCGTATGTTGTGTGGAATTGTGAGAGGATAACAATTT-3'
T3	5'-TCGTATGTTGTGTGGAATTGTGAGCGGTTTTTTTTTT-3'
T4	5'-TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACA-3'
T5	5'-TCGTATGTTGTGTGGAATTGTGAGCGGTTTTTTTTTTCACACAGGAAACA-3'
Ribo T6	5'-GGTGTGGAATTGTGAGCGGATAACAA-3'
5'-Phosphorothioate Oligomers	
A1	5'-ACAATTCCACACAACA-3'
A2	5'-CACAATTCCACACAAC-3'
B	5'-TCACAATTCCACACAA-3'
C	5'-CTCACAATTCCACACA-3'
D	5'-GCTCACAATTCCACAC-3'
E	5'-CGCTCACAATTCCACA-3'
Ɛ	5'-CTCTCACAATTCCACA-3'
F	5'-CCGCTCACAATTCCAC-3'
G	5'-TATCCGCTCACAATTC-3'
H	5'-AAACCGCTCACAATTC-3'
Internal Phosphorothioate Oligomers	
A2 (s)	5'-CACAATTC(S)CACACAAC-3'
A2 (s; mismatch)	5'-CACAATTC(S)GACACAAC-3'
G (s)	5'-TATCCGCTCACAATT(S)C-3'
Primer	
P	5'-TTTCCTGTGTG-3'

Purification and identification of isomers of phosphorothioate oligodeoxynucleotides that contain an internal phosphorothioate residue

Deoxynucleotides containing a single internal phosphorothioate residue, A2 (s), A2 (s; mismatch) and G (s) (Table 1), were synthesized by Midland Certified Reagent Co., Midland, Texas. They were purified by HPLC on RPC-5, using a 0.01M-0.1M perchlorate gradient at pH 8.5. Each sequence gave rise to 3 peaks (Fig. 1). In each case, peak 1 had a mobility identical to that of the same deoxyoligonucleotide with a normal phosphodiester linkage in place of the anticipated phosphorothioate diester linkage. When solutions containing all 3 compounds were desulfurized by oxidation with a solution of 5 mg I_2 per ml of aqueous pyridine (75% pyridine) for 45 minutes (15), HPLC analysis showed conversion of peaks 2 and 3 to peak 1 (Fig. 1, dotted peak). We concluded that, in each case, peaks 2 and 3 corresponded to the isomers of the phosphorothioate oligomer and that peak 1 corresponds to the normal phosphate linked oligomer. When the phosphorothioate oligomers were purified by HPLC and desulfurized by oxidation with I_2, each isomer separately was converted to the oligomer containing a normal phosphodiester linkage.

The purified phosphorothioate isomers were analyzed on a C18 column, using a 6−24% acetonitrile gradient in 0.05M KH_2PO_4 buffer at pH 6. The isomers that moved faster on RPC-5 (peak 2) also moved faster on C18. It has previously been shown that the R_p isomers of a number of oligonucleotides containing one internal phosphorothioate residue elute on C18 before the S_p isomers (16), suggesting that the faster moving isomer

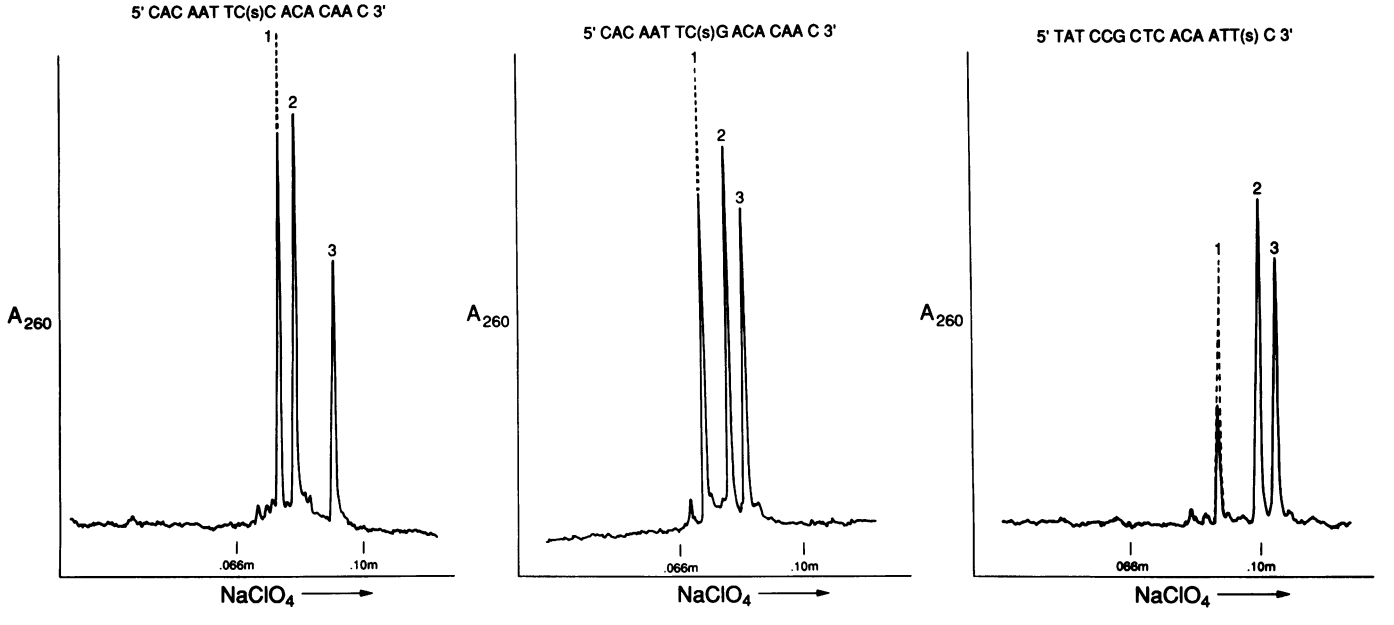

Figure 1. HPLC elution profiles, on RPC-5, of 3 commercially synthesized oligomers containing an internal phosphorothioate residue. Peaks are identified in order of elution as 1) the normal phosphodiester-linked oligomer; 2) the R_p isomer and 3) the S_p isomer of the phosphorothioate. (See text.) The dotted peaks indicate the products obtained when the R_p and S_p isomers are desulfurized with I_2.

corresponds to the R_p isomer in each of our phosphorothioate oligomers. To confirm this, the two isomers of each of the three oligomers were digested with P1 nuclease. This enzyme digests the phosphorothioate linkage of S_p isomers to give 5′-phosphorothioate mononucleotides, leaving the R_p isomers un-digested. Using the procedure described in reference 15, we found that the slower moving isomer in each case was hydrolyzed by P1 nuclease to mononucleotides, while the faster moving isomer in each case gave mononucleotides and a slower moving phosphorothioate-containing dinucleotide. This confirms that the faster moving compounds are the R_p isomers. The separated isomers of A2 (s), A2 (s; mismatch) and G (s) were purified by HPLC on RPC-5 at pH 8.5, and desalted and concentrated on a Du Pont Nensorb cartridge.

Synthesis of an oligoribonucleotide template

The ribo sequence 5′-[γ-^{32}P]-pppGGTGTGGAATTGTGAGC-GGATAACAA-3′ was synthesized using T7 RNA polymerase following a published procedure (17). 10 pmoles of the DNA template 5′-TTGTTATCCGCTCACAATTCCACACCTAT-AGTGAGTCGTATTA-3′ together with 20 pmoles of the (−) strand of the T7 promoter 5′-TAATACGACTCACTATAG-3′ in 8 μl of H_2O, were heated at 65°C for 3 minutes. 2 μl of buffer containing 0.2 M Tris (pH 8.1), 5 mM spermidine, 25 mM DTT, 250 μg/ml BSA, 10 mM $MgCl_2$ and 0.05% Triton X-100 (5×polymerase buffer) were then added. After 5 minutes at room temperature, 29.5 μl of a solution of nucleoside triphosphates, 8 μl of 5×polymerase buffer, and 2.5 μL (525 U) of T7 RNA polymerase were added to give a final 50 μl reaction mixture that contained 40 mM Tris (pH 8.1), 1 mM spermidine, 5 mM DTT, 50 μg/ml BSA, 2 mM $MgCl_2$ and 0.01% Triton X-100, 0.4 mM ATP, CTP, UTP and 5′-[γ^{32}P]-GTP (30 Ci/mmole). After 2 hours at 37°C, 4 μl of 0.5 M EDTA was added. The solution was heated at 65°C for 3 minutes and the RNA purified by gel electrophoresis on 20% polyacrylamide. The product consisted of a major component and a minor component of slightly lower molecular weight. Non-denaturing gel electrophoresis showed that the RNA product hybridized with oligomers A2, B, C, D, E, F and G, but not with oligomer A1 which, unlike the other oligomers, overlapped the template at only 11 positions.

Formation of platinum-linked cross-products between 5′-phosphorothioates of 16mers and complementary [^{32}P]-37mer DNA templates or a [^{32}P]-26mer RNA template

Solutions (0.5 mM) of the Pt^{II} compounds were made up freshly in 1 mM phosphate buffer (pH 7) containing 0.1 mM EDTA. Dilutions were made into the same buffer. About 0.01 pmole

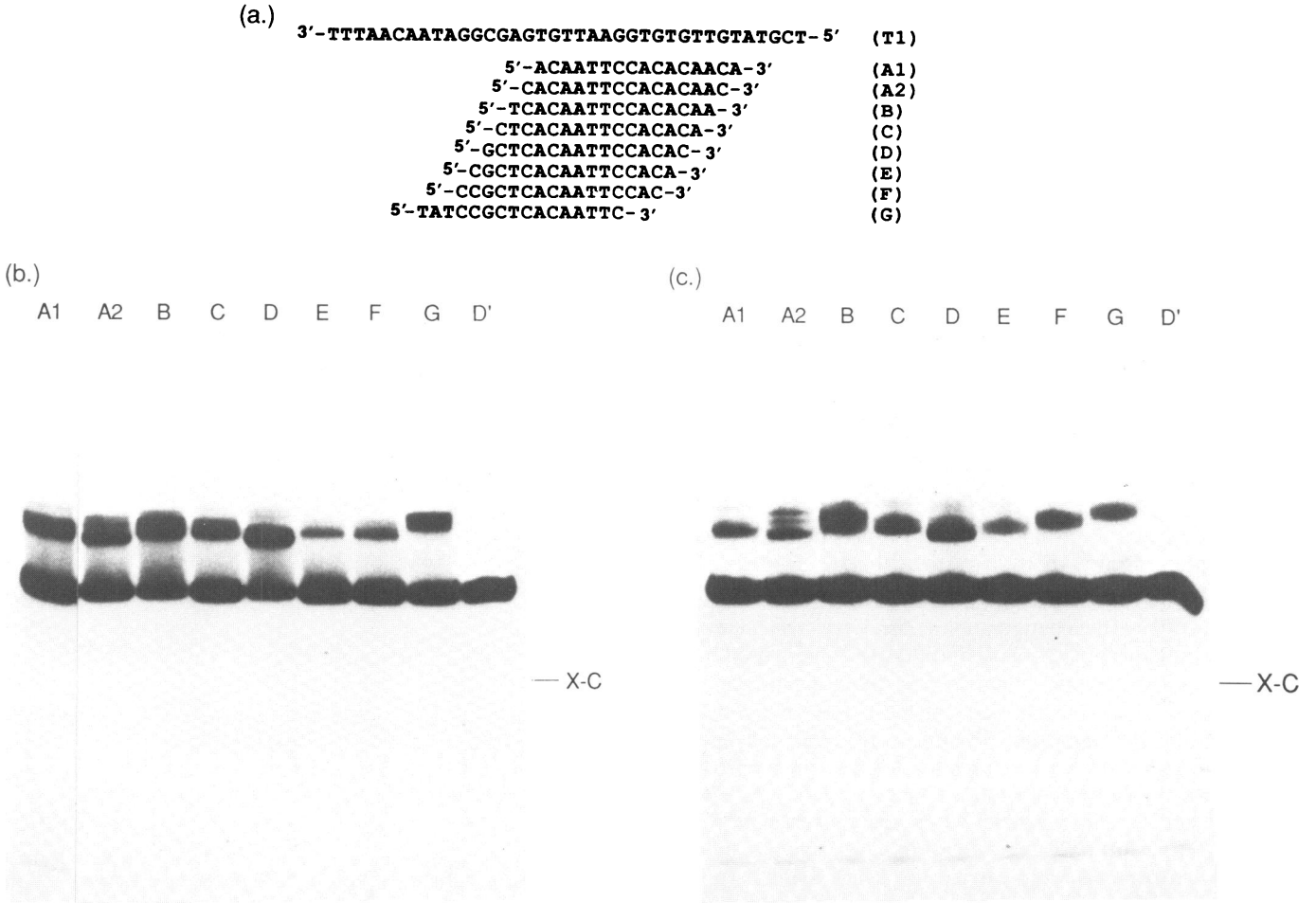

Figure 2. (a) Configuration of a 5′-[^{32}P]-37mer template T1 and cross-linking complementary 5′-phosphorothioate oligomers. (b) (c) Autoradiograms of 20% denaturing gels showing the formation of cross-linked products between T1 and the indicated 5′-phosphorothioate oligomers in the presence of (b) 5 μM transPt^{II}; (c) 5 μM cisPt^{II}. Lane D′ illustrates the products from a control experiment in which a corresponding non-phosphorylated oligomer replaced the phosphorothioate D.

of 5′-[^{32}P]-37mer (sp. act. 1−3 μCi/pmole) in 2.5 μl of 0.02 M phosphate buffer pH 7.7 was heated at 95−100°C for 1 minute and cooled on ice. The following were then added sequentially: 1 μl of buffer containing 1 mM phosphate (pH 7) and 0.1 mM EDTA, 1 μl of a solution containing 0.1 pmole of the phosphorothioate-16mer or the unphosphorylated 16mer, 0.5 μl of 0.5 M $NaClO_4$, and 0.55 μl of a solution containing the required amount of cisPt^{II}, transPt^{II} or K_2PtCl_4. The final volume was 5.55 μl. After incubation overnight at room temperature, the reaction solutions were made up to 10 μl with buffer and the cross-product separated from starting 5′-[^{32}P]-37mer by electrophoresis on a 20% denaturing gel.

A very similar procedure was used with the 26mer RNA template. The concentrations of RNA template and phosphorothioate 16mer were increased to 0.05 and 0.5 pmoles, respectively. The buffer contained 1 mM phosphate and 0.1 mM EDTA. The RNA template was not heated before hybridization.

Primer extension on platinum cross-linked 50mer templates

Approximately 0.01 pmole of a 50mer template in 2.5 μl of 0.02 M phosphate buffer (pH 7.7) was heated at 95−100°C for 1 minute and cooled on ice. The following were then added sequentially: 0.5 μl of buffer containing 1 mM phosphate (pH 7) and 0.1 mM EDTA, 1 μl of a solution containing 0.1 pmole of 5′-[^{32}P]-11mer/primer P (s. a. 1−3 μCi/pmole), 0.5 μl of a solution containing 0.1 pmole of a complementary 5′-[^{35}S]-phosphorothioate-16mer (0.0065 μCi/pmole) or the unphosphorylated 16mer, 0.5 μl of 0.5 M $NaClO_4$, and 0.55 μl of a solution containing an appropriate concentration of cisPt^{II}, transPt^{II} or K_2PtCl_4, to give a final volume of 5.55 μl. After overnight incubation at room temperature, the following were added to the cross-product mixture: 2.4 μl H_2O, 1.1 μl of buffer containing 500 mM Tris (pH 7), 100 mM $MgCl_2$ and 1 mM DTT; 1 μl of a solution 1.25 mM in each of the 4 nucleoside triphosphates, and 1 μl of a solution containing 2.5 U of the Klenow fragment, to give a total volume of 11 μl. After 45 minutes at room temperature the enzyme reaction was stopped by addition of 1 μl of 0.5 M EDTA. The reaction mixture was then heated at 100°C for 1 minute in the presence of 7 M urea, and the products of the enzyme reaction were analyzed by denaturing gel electrophoresis on 20% polyacrylamide.

RESULTS

Formation of platinum-linked cross-products between a series of complementary 5′-phosphorothioate 16mers and 5′-[^{32}P]-37mer templates

Figure 2 (a) shows the configuration of the 37mer DNA template T1 and complementary 5′-phosphorothioate 16mers used in the platinum cross-linking experiments. Non-denaturing gel electrophoresis showed that each of these 16mers hybridized efficiently to the template. Figure 2 (b) and (c) present autoradiograms showing the formation of cross-linked products between the 5′-phosphorothioates of oligomers A1, A2, B, C, D, E, F and G and template T1 in the presence of 5 μM transPt^{II} (Fig. 2 (b)) or 5 μM cisPt^{II} (Fig. 2 (c)). Similar but less complete experiments were carried out using K_2PtCl_4 (results not shown). Each of the 5′-phosphorothioate oligomers is cross-linked to the template by any of the three Pt^{II} complexes, but no detectable cross-products are formed with the unphosphorylated oligomers (lane D′ shows that no cross-link is formed with the unphosphorylated oligomer corresponding to D; similar results obtained with the other unphosphorylated oligomers are not shown).

In most cases, transPt^{II} and K_2PtCl_4 form cross-links with comparable efficiency, while cross-linking with cisPt^{II} is less efficient. Excellent yields of cross-products are obtained when transPt^{II} is used to cross-link oligomers B (~50%) and D (~55%); similar yields are obtained with these oligomers and K_2PtCl_4. Oligomers A1, A2, C and G cross-linked with intermediate efficiency (28−43%) with transPt^{II} and K_2PtCl_4. Oligomers E and F behaved somewhat differently. Oligomer E gave about 35% of cross-linked product with K_2PtCl_4, but only about 18% with cisPt^{II} or transPt^{II}; oligomer F cross-linked to about the same extent (~25%) with each of the Pt^{II} compounds.

We suspected that the low-efficiency of cross-linking achieved with E might be due to a 'suicide' reaction of the activated Pt^{II} complex with the penultimate G residue of E 97 no other oligomer had a G residue in this position. Then the higher yield obtained with K_2PtCl_4 could be understood, since even after an intramolecular reaction, the product would still be activated and could form an intermolecular cross-link. To test this possibility, we prepared the 5′-phosphorothioate oligomer ε, in which the penultimate G residue of E is replaced by T, and a template T2 complementary to ε, in which the C residue at position 25 is replaced by A (Fig. 3 (a)).

Figure 3 (b) presents an autoradiogram comparing the yield of cross-products obtained when E is cross-linked to T1 or ε is

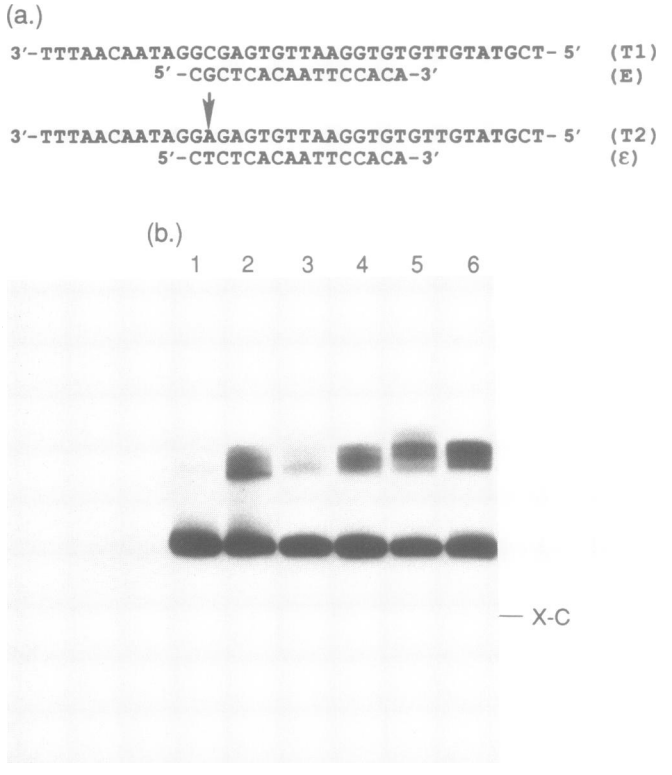

Figure 3. (a) Configurations of a 5′-[^{32}P]-37mer template T1 and a complementary oligomer E, and of a 5′-[^{32}P]-37mer template T2 and a complementary oligomer ε. The arrow marks the position at which a C:G base pair has been replaced by A:T. (b) Autoradiogram of a 20% denaturing gel showing formation of cross-products between the 5′-phosphorothioates of: lane 1) E and T1 using 5 μM transPt^{II}; lane 2) ε and T2 using 5 μM transPt^{II}; lane 3) E and T1 using 5 μM cisPt^{II}; lane 4) ε and T2 using 5 μM cisPt^{II}; lane 5) E and T1 using 5 μM K_2PtCl_4; lane 6) ε and T2 using 5 μM K_2PtCl_4.

cross-linked to T2 with transPt^{II}, cisPt^{II} and K_2PtCl_4. In lanes 1 and 2 we compare the efficiency of cross-linking E to T1 (lane 1) with the cross-linking of ϵ to T2 (lane 2) in the presence of 5 μM transPt^{II}. In lanes 3 and 4 the same comparison is made with the cis isomer and in lanes 5 and 6 with K_2PtCl_4. The efficiencies were ~16% and ~38% with transPt^{II}, ~17% and ~25% with cisPt^{II}, and ~31% and ~38% with K_2PtCl_4. Thus the cross-linking efficiency with transPt^{II} improved considerably when E was replaced by ϵ, but to a lesser extent with cisPt^{II} or K_2PtCl_4.

Effect of replacement of template bases by T on Pt^{II} cross-linking efficiencies

It is known that reactive Pt^{II} complexes react primarily with G and A, to a lesser extent with C and least with T (18). We carried out a series of experiments to determine whether the low reactivity of T with free Pt^{II} complexes would extend to reactions of Pt^{II} complexes attached to oligonucleotide phosphorothioates.

Selected phosphorothioate 16mers were cross-linked to a template T3 in which bases 28–34 of T1 were replaced by T residues (Fig. 4 (a)). The 5′ end of oligo F, when hybridized to T3, is opposite to base 27, that is, adjacent to a string of T residues (residues 28–34). H hybridizes with T3 forming 3 A-T base pairs at its 5′ end and having further T residues adjacent to its 5′-terminus (Fig. 4 (a)). Figure 4 (b) presents an autoradiogram that shows that, as expected, the cross-linking of oligo F with cisPt^{II} is significantly reduced from ~25% to ~10% when T3 replaces T1 (lanes 3 and 4), but that, surprisingly, transPt^{II} cross-links F to the two templates with comparable efficiency (lanes 1 and 2). We also found transPt^{II} to be much more efficient (Fig. 4 (c)) (lane 1) than cisPt^{II} (lane 2)) and K_2PtCl_4 (lane 3) in cross-linking oligomer H to template T3. Apparently efficient cross-linking to T residues is possible with transPt^{II}, but not with cisPt^{II} or K_2PtCl_4.

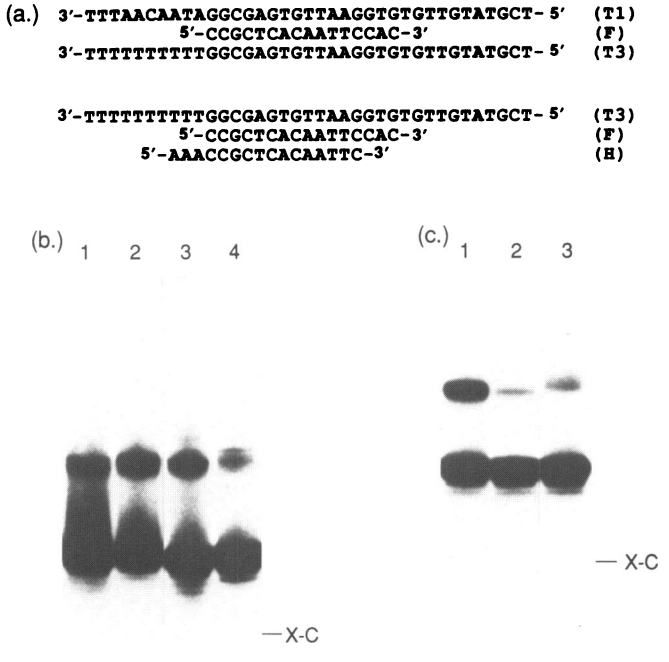

Figure 4. (a) Configurations of 5′-[^{32}P]-37mer templates T1 and T3 and complementary oligomers F and H. (b) Autoradiogram of a 20% denaturing gel showing the formation of cross-linked products between the 5′-phosphorothioates of lane 1) F and T1 using 5 μM transPt^{II}; lane 2) F and T3 using 5 μM transPt^{II}; lane 3) F and T1 using 5 μM cisPt^{II}; lane 4) F and T3 using 5 μM cisPt^{II}. (c) Autoradiogram of a 20% denaturing gel showing the formation of cross-linked products between T3 and the 5′-phosphorothioate of H using lane 1) 5 μM transPt^{II}; lane 2) 5 μM cisPt^{II}; lane 3) 5 μM K_2PtCl_4.

Formation of platinum-linked cross-products between a [^{32}P]-37mer template T1 and 16mers containing an internal phosphorothioate residue

We prepared and purified the R_p and S_p isomers of three oligomers containing a single internal phosphorothioate residue, and used them to study cross-linking with cisPt^{II}, transPt^{II} and K_2PtCl_4. A2 (s) and G (s) are oligomers complementary to the template T1, and having phosphorothioate groups near the middle and at the 3′-terminus of the sequence, respectively. A2 (s; mismatch) is derived from A2 by changing a single base. It is complementary to the template except at a residue next to the phosphorothioate group, where a G:G mismatch occurs (Fig. 5 (a)).

We found that cisPt^{II} and K_2PtCl_4 did not bring about detectable cross-linking in any case (results not shown). The results shown in Fig. 5 (b) indicate that transPt^{II} brings about cross-linking for each of the oligomers, but that cross-linking tends to be less efficient than that achieved with oligomers carrying a terminal phosphorothioate group, particularly for G

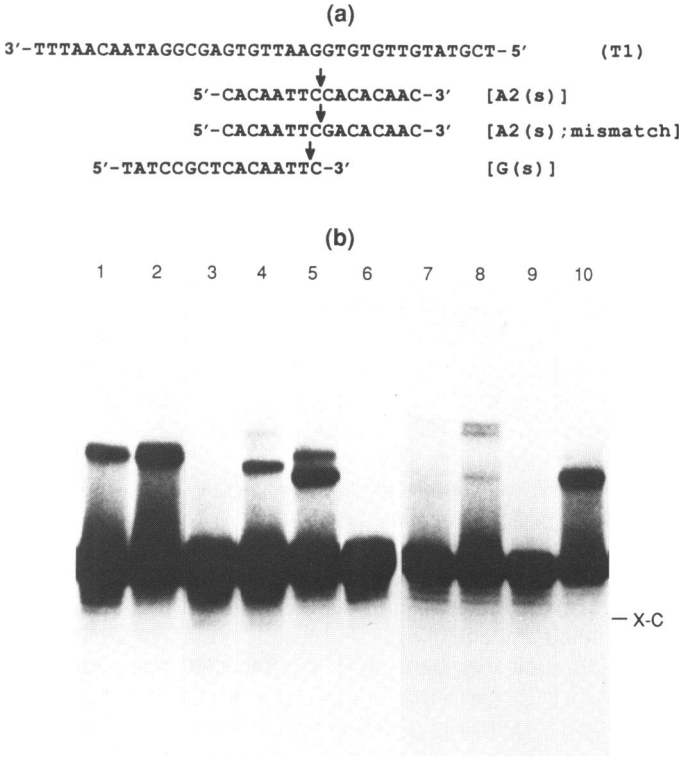

Figure 5. (a) Configuration of 5′-[^{32}P]-37mer T1 and internal phosphorothioate-containing oligomers A2 (s), A2 (s; mismatch) and G (s). The arrows indicate the position of the phosphorothioate groups. (b) Autoradiogram of a 20% denaturing gel showing formation of cross-products in the presence of 5 μM transPt^{II} between 5′-[^{32}P]-37mer T1 and lane 1) the R_p isomer of A2 (s); lane 2) the S_p isomer of A2 (s); lane 4) the R_p isomer of A2 (s; mismatch); lane 5) the S_p isomer of A2 (s; mismatch); lane 7) the R_p isomer of G (s); lane 8) the S_p isomer of G (s); lane 10) the 5′-phosphorothioate G. Lanes 3, 6 and 9 illustrate control experiments in which the corresponding normal phosphate-linked oligomers are used in place of the phosphorothioate oligomers.

(s) in which the phosphorothioate group is 3'-terminal (lanes 7 and 8). In every case the S_p isomer (lanes 2, 5 and 8) cross-linked more efficiently than the R_p isomer (lanes 1, 4 and 7). The mismatched S_p oligomer cross-linked somewhat more efficiently (lane 5) than the corresponding matched S_p oligomer (lane 2).

Formation of platinum-linked cross-products between a 5'-phosphorothioate 16mer and a [^{32}P]-labelled 26mer RNA template

The DNA/RNA hybrid whose structure is shown in Fig. 6 (a) was used in cross-linking experiments. The 26mer RNA template corresponds to residues 10–34 of T1 with an extra G at the 5' end. Figure 6 (b) presents an autoradiogram showing the formation of cross-linked products between the 5'-phosphorothioate of oligomer D and the RNA template in the presence of 2 μM transPt^{II} (lane 2). No detectable cross-product is seen in lane 3, where the corresponding unphosphorylated oligomer D was used in place of the phosphorothioate under the same conditions. The yield of cross-product was ~55%, similar to the yield of cross-product when a DNA template is used. These results suggest that when RNA templates are used, a pattern of cross-linking similar to those obtained with a DNA template can be expected.

Determination of the sites of cross-linking

DNA synthesis by DNA polymerase I (Klenow fragment) terminates before a template site cross-linked to a complementary oligomer (13). This enables us to determine the site of cross-linking. However, since the 37mer targets that we have used are too short to accommodate a suitable primer, we had to replace them by 50mers that are identical to the target oligomers over the first 37 residues.

Extension of the primer on template T4 was observed in the presence of oligomers D, E, F and G but not in the presence of the oligomers A1, A2, B and C or in the absence of an oligomer. The primer could be extended on template T5 in the presence of any of the oligomers. We believe that template T4 forms a self-structure that prevents the hybridization of the primer or the 16mer, and that oligomers D, E, F and G, but not oligomers A1, A2, B and C, destabilize that self-structure, thus enabling the primer to bind.

The configuration of template, primer and cross-linker used in these experiments is illustrated in Fig. 7 (a). A1, A2, B and C were cross-linked to T5 and D, E, F and G to T4. The templates, for convenience, have been numbered from the 5' end of the primer, rather than in the conventional fashion from the 5' terminus of the template. Full-length extension of the primer by the Klenow fragment gives a product 48 bases long. The formation of a truncated product at position n on the diagram indicates cross-linking at position n + 1. If, for example, the length of a truncated product is 23, then cross-linking must have occurred 24 bases from the 5' end of the primer.

Figure 7 (b) presents an autoradiogram of the products formed in these reactions with transPt^{II}. The lanes are numbered A1, A2, B, C, etc. indicating the particular phosphorothioate used in the experiment. The unnumbered lanes beside each numbered lane are the control experiments where the unphosphorylated oligomer was used in place of the phosphorothioate under identical cross-linking conditions. The lengths of the truncated products are determined by comparison with a T sequence ladder.

The positions of the bands in the different lanes of Fig. 7 (b)

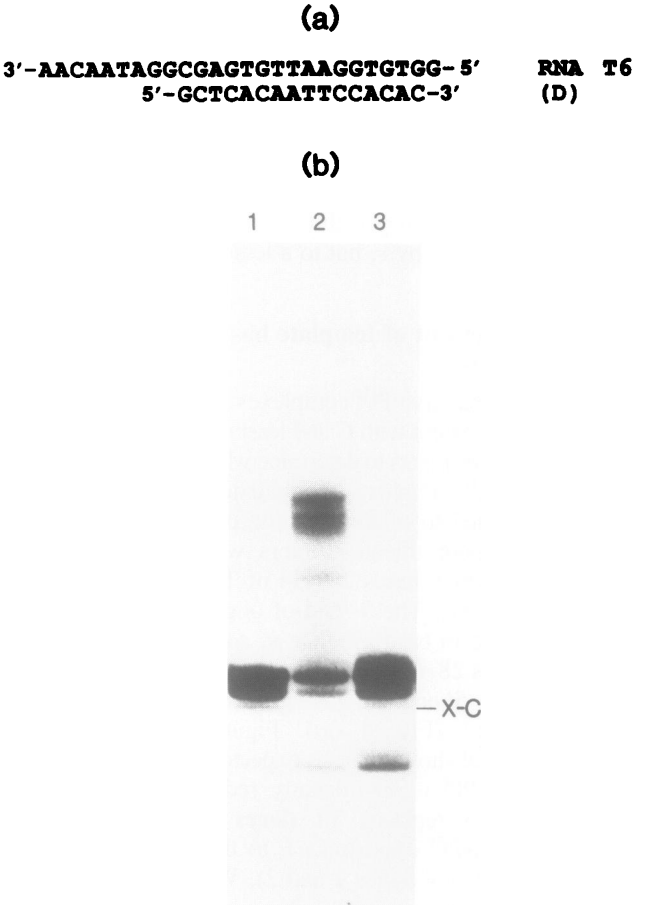

Figure 6. (a) Configuration of 5'-[γ-^{32}P]-ppp26mer ribo T6 and D. (b) Autoradiogram of a 20% denaturing gel showing: lane 1) ribo T6; lane 2) the reaction products of ribo T6 with 5'-phosphorothioate D in the presence of 2 μM transPt^{II}; lane 3) same as lane 2, except with the corresponding unphosphorylated oligomer in the place of the phosphorothioate D.

clearly indicate that the truncated products get shorter as the 5'-end of the cross-linker approaches the primer. Oligomers A1, A2, B, C for example, give truncated products 21–24 bases long while oligomer G gives products only 15–18 bases long. By comparing the position of the phosphorothioate group of the oligomer with the length of the truncated products, one finds that the preferred positions of cross-linkage with cisPt^{II} and transPt^{II} on the template are usually 2–5 bases downstream from the 5' end of the cross-linker.

The extent of cross-linking at different residues is indicated by the intensity of the bands on the gel. A1, for example, cross-links strongly to positions 23 (G) and 25 (G) but only weakly to position 24 (C). The strong cross-links are found 3 and 5 bases from the 5' terminus of the cross-linker. With D, the main cross-links are at positions 19 (A), 21 (A) and 22 (G) with very little cross-linking at position 20 (T). The strong cross-links are again 2, 3 and 5 residues from the 5'-terminus of the cross-linker. A survey of all of our data suggests that G and A are most susceptible to attack by Pt^{II} complexes, C has intermediate reactivity, and that T reacts least well.

We carried out a set of primer extension experiments completely equivalent to those described above, but using cisPt^{II}

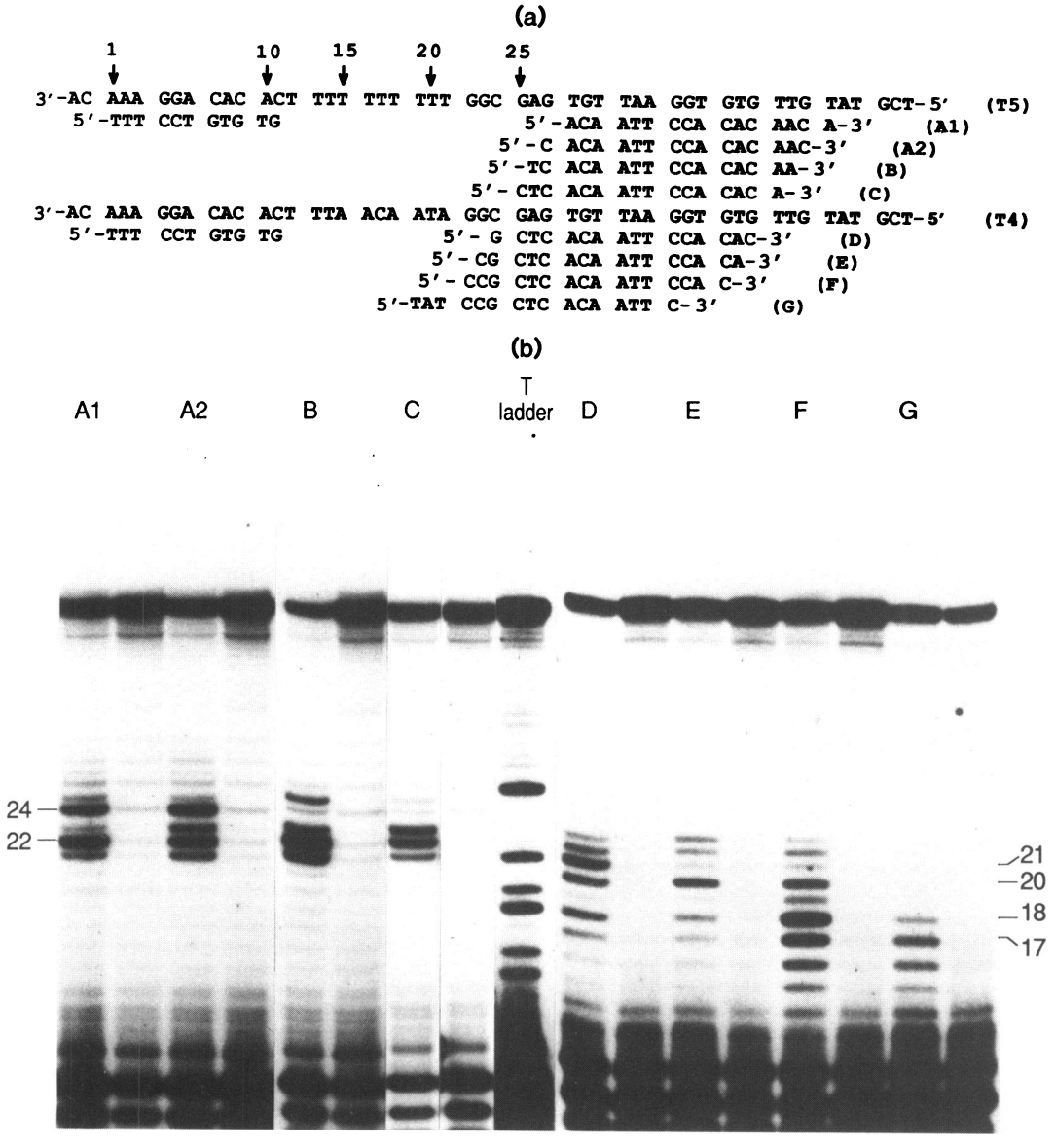

Figure 7. (a) Configurations of primers and oligomers used in template elongation experiments. The numbers above the template indicate the distance from the 5' end of the primer. A truncated product of length n indicates cross-linking at position n + 1. (b) Autoradiogram of a 20% denaturing gel showing 5'-[^{32}P]-primer extension by the Klenow fragment on template T5 cross-linked to 5'-phosphorothioates A1, A2, B and C, or on template T4 cross-linked to 5'-phosphorothioates D, E, F and G in the presence of 2 μM transPt^{II}. The designations of the lanes correspond to the oligomer used. The unmarked lane to the right of each labelled lane illustrate control experiments in which unphosphorylated oligomers were used in place of phosphorothioates. The center lane is the T sequence ladder of template T4.

as cross-linker, and a less complete set of experiments using K_2PtCl_4. We found that the region of the target to which cross-linking occurred was the same with the three reagents, but that there were reproducible changes in the relative intensities of the bands when the Pt^{II} complex was changed. This indicates that cross-linking efficiency depends not only on the nature of the base in the target, but also on the detailed orientation of the Pt^{II} complex relative to the target base.

Our direct experiments on cross-linking suggested that transPt^{II} will cross-link to T residues. To explore this reaction further, we compared the pattern of products obtained when selected oligomers were cross-linked to template T4 or to T5 in which A, G and C residues in the central region of the target are replaced by T residues (Fig. 8 (a)). When D is reacted with T4, cross-links are formed at residues 19 (A), 21 (A), 22 (G) and to a lesser extent at 23 (G) (Fig. 8 (b)) (lane 1). When D is cross-linked to T5 more efficient cross-linking occurs at 22 (G) and 23 (G) but no cross-linked products are formed with residues 19 (T), 20 (T) or 21 (T) (lane 2). Thus, if good target residues (A, G) are available, T is not attacked. The situation is different when H is cross-linked to T5. Now there are no target residues other than T residues. It is seen from lane 4 that truncated products of length 15–18 are formed, corresponding to cross-links at T residues in positions 16, 17, 18 and 19.

In the course of this work we found that the Klenow enzyme can be blocked on template T5 by certain complementary oligomers even in the absence of cross-linking. Termination occurs at residue 21, when T5 is hybridized to oligomer F or H (lane 4), but not when hybridized to oligomer D (lane 3). The termination site follows immediately after the long sequence of Ts in the template T5; the phenomenon may be related to 'pausing' sites observed in transcription reactions (19).

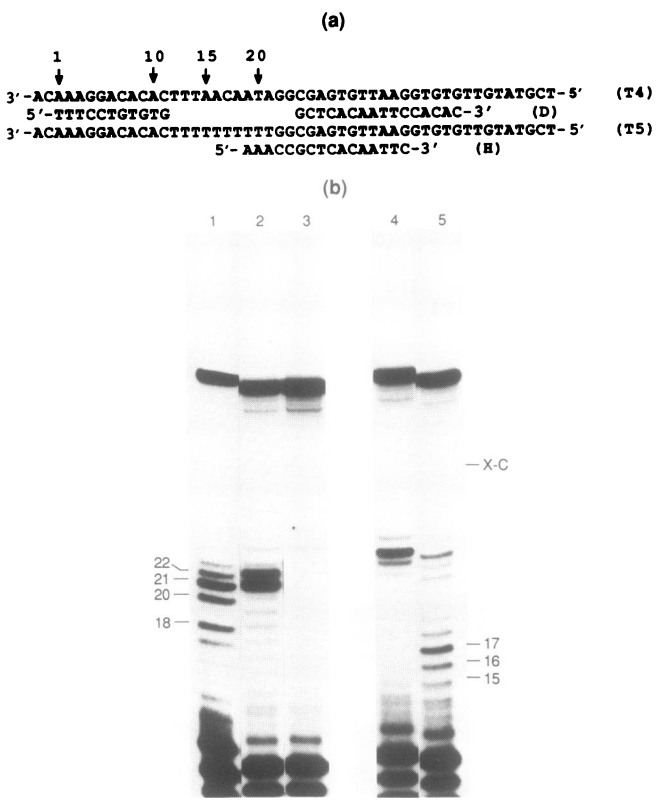

Figure 8. (a) Configuration of templates T4 and T5 in which residues 28–34 of T4 are replaced by T residues, and complementary oligomers D and H used in template elongation experiment. The numbers above the template indicate the distance from the 5′ end of the primer. A truncated product of length n indicates cross-linking at position n + 1. (b) Autoradiogram of a 20% denaturing gel showing 5′-[^{32}P]-primer extension by the Klenow fragment in the presence of 2 μM transPt^{II} on: lane 1) T4 cross-linked to D; lane 2) T5 cross-linked to D; lane 3) T5 hybridized to an unphosphorylated oligomer corresponding to D; lane 5) T5 cross-linked to H; lane 4) T5 hybridized to an unphosphorylated oligomer corresponding to H.

DISCUSSION

Oligodeoxynucleotides containing phosphorothioate residues in selected positions are now available commercially. Our results show that chromatography on RPC-5 results in an excellent separation of the R_p and S_p isomers from each other and from the desulfurized oligomer. The samples that we purchased contained comparable amounts of the phosphorothioate isomers and up to 40% of the desulfurized product. We suspect that desulfurization occurred when iodine was used to oxidize the phosphite esters to phosphate esters (2).

Cross-linking of 16mer phosphorothioates to 37mer templates was carried out under conditions in which hybridization of the template was essentially complete, as demonstrated by non-denaturing gel electrophoresis. Differences in efficiency of cross-linking, therefore, principally reflect differences in the availability of target bases in the template and differences of reactivity of bases in the 16mer that, by reacting with the 5′-terminal Pt^{II}, can lead to 'suicide' intramolecular reactions. A penultimate G residue seems to favor intramolecular 'suicide'. Much more experimental work will be needed to develop a detailed picture of the competition between cross-linking and 'suicide'.

The phosphate groups of one chain of a double helix are far removed from the bases of the other. Cross-linking of 5′-terminal phosphorothioates must occur when the single-strand tail of the template bends around as illustrated in Fig. 9. Our results suggest that bases 2–5 residues from the 5′-phosphorothioate group are best placed to react. However, the details of the cross-linking depend on the sequence of the unpaired region of the template and the nature of the Pt^{II} complex as well as on the position of base relative to the 5′-end of the phosphorothioate oligomers. G and A residues and, to a lesser extent C residues, 2–5 bases from the phosphorothioate are preferred sites of cross-linking but, in the absence of these residues in reactive positions, cross-linking with transPt^{II} occurs at T residues. It is not clear why transPt^{II} cross-links to T, but cisPt^{II} and K_2PtCl_4 do not.

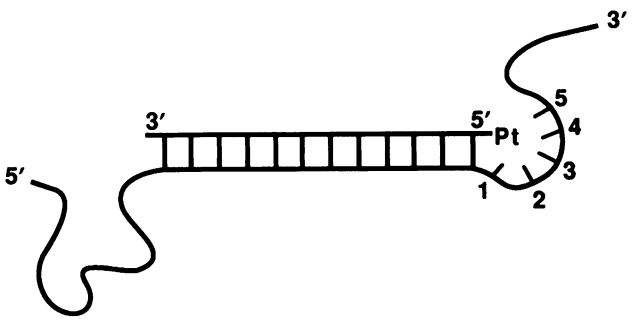

Figure 9. Diagrammatic structure of template cross-linker hybrid. The platinum atom at the 5′ end of the cross-linker can attack bases a few residues downstream on the template.

Internal phosphorothioate groups in double-helical structures are so far away from the bases of the complementary chain, that cross-linking is unlikely, except when the normal hydrogen bonding scheme is disrupted and the double-helix extensively distorted. It is not surprising, therefore, that oligonucleotides with internal phosphorothioate groups do not cross-link with cisPt^{II} or K_2PtCl_4. However, cross-linking is relatively efficient with transPt^{II}, perhaps because transPt^{II} stretches over long distances more easily than does the cis isomer and lacks the negative charge that is present on adducts formed by the $[PtCl_4]^{2-}$ ion. Models of DNA show that the sulfur atoms in S_p isomers point away from the double helix while sulfur atoms in R_p isomers point in the general direction of the major groove, so it is not clear why S_p isomers are cross-linked to the template more efficiently than R_p isomers by transPt^{II}. The improved cross-linking observed when there is a mismatch between template and phosphorothioate oligomer may be due to the easier deformation of the mismatched helix.

The results obtained in our experiments suggest that target sequences rich in purine residues will usually cross-link efficiently with all three Pt compounds, but that it will be very difficult to predict quantitatively from the sequence of the target the positions and extent of cross-linking. Preliminary experiments with an RNA template suggest that RNA templates are as efficiently cross-linked as DNA templates. If, as seems likely, the regularities observed for cross-linking RNA turn out to be similar to those that we have found for DNA, our results show that oligomer sequences that are complementary to purine-rich regions of the target RNA that lack self-structure are promising candidates as cross-linking antisense oligomers. The rules for triple-strand cross-linking (20, 21) must be determined independently and may well turn out to be different from those for cross-linking in a double helix.

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