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Selective binding of looped oligonucleotides to a single-stranded DNA and its influence on replication *in vitro*

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ABSTRACT

Complexing of looped and circular oligonucleotides, composed of either 2′-deoxyribo- or 2′-*O*-methylribonucleoside units, with completely matching or partially mismatching complementary DNA sequences was studied. Melting experiments revealed considerable differences among the stabilities of these hybrid complexes. Maximum stability and selectivity was displayed by oligomers 2 and 5. It was concluded that a linear stretch, attached to 1′-*O*- of 3′-deoxypsicothymidine unit (Z) increases the selectivity of hybridisation and stability of the complex as a whole. This allows one to aim the target DNA very precisely at its polyadenine part as well as at adjacent sequence simultaneously. Experiments on termination of primer extension catalysed by different DNA-polymerases—Sequenase, Klenow fragment and *Tth*—have demonstrated that looped oligomer 5, composed of 2′-*O*-methylribonucleosides appears to be a highly selective and potent inhibitor of replication *in vitro*. Features of looped oligonucleotides, composed of 2′-*O*-methylribonucleosides seem to be useful for design of highly specific antigene oligonucleotides.

INTRODUCTION

The antigene strategy reviewed by Thuong and Hélène (1) has been intensively investigated during past few years, and in the course of this research the triple-helix forming oligonucleotides have received very serious attention (2–12). The stability of pyrpurpyr triplexes and selectivity of their formation have been studied in detail (1,13–26). A number of recent investigations have also been devoted to *in vitro* inhibition of important biochemical processes, such as replication (27,28), transcription (29,30) and reverse transcription (31) by triplex formation. We have recently reported a new strategy for DNA recognition based on a bimolecular hybrid complex (double/triple helix) formation (32). In that study we found that looped oligonucleotides undergo simultaneous binding to a polyadenine region and an adjacent sequence of a single-stranded DNA target. We now report on the selectivity of binding of looped oligonucleotides composed of 2′-deoxyribo- or 2′-*O*-methylribonucleosides to a single-stranded complementary DNA sequence. Since a strong and selective association with target may not necessarily make an oligonucleotide a potent antigene agent, owing to poor inhibitory effect on the targeted biochemical process, we felt it important to investigate the correlation between the thermodynamics of hybridisation and the inhibitory effect on certain biochemical reactions. We describe here the results on the ability of looped oligonucleotides to terminate replication *in vitro*, catalysed by three different DNA-polymerases.

MATERIALS AND METHODS

Oligonucleotides

All the oligodeoxyribonucleotides used in the present study were synthesised and characterised as reported (32). Oligo-2′-*O*-methylribonucleotides were assembled using the ABI standard RNA coupling cycle and monomers from Glen Research. The earlier reported (32) non-nucleosidic monomers, 3′-deoxypsicothymidine monomer, solid supports and cross-linking technique were employed to prepare circular and looped oligomers [5,7], composed of 2′-*O*-methylribonucleosides. Overall yields of 5 and 7 were 38% and 27%, respectively.

Melting experiments

Melting experiments were performed on a Lambda 2 UV/VIS spectrophotometer equipped with two PTP-1 temperature programmers, comprising two electronic Control Units and two Peltier Cell Holders for sample and reference cells (Perkin-Elmer). The temperature was increased at a rate of 0.5–1 °C/min. The melting curves were recorded at 260 nm in a buffer containing 10 mM Tris–HCl, pH 6.8; 0.1 M NaCl; 10 mM $MgCl_2$, the oligomer concentrations being in the micromolar range. All melting curves were recorded three times and they proved to be well reproducible. The T_m values reported here were obtained by averaging the results of these three recordings. The uncertainty in T_m was estimated at less than ±0.5 °C, based on repetition of measurements. In several cases the curves for dissociation were compared with those for association. Very small or no hysteresis was observed in each case, showing that the hybridisation kinetics did not affect the shape of the melting curves.

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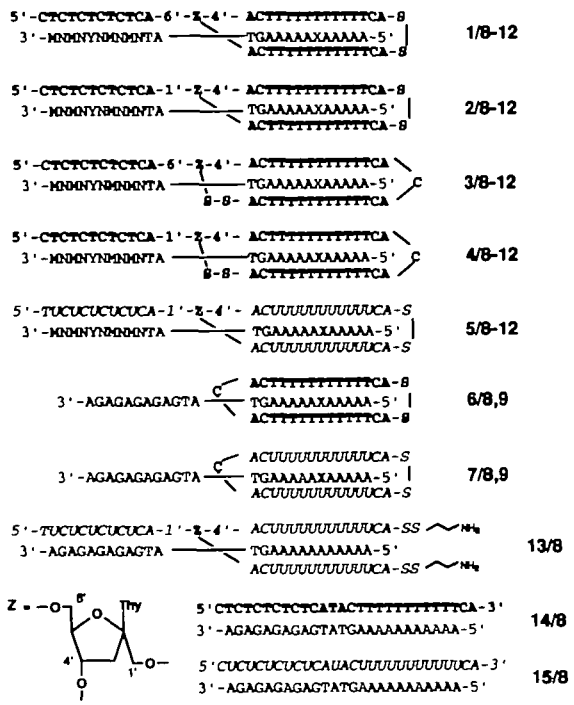

Figure 1. Structures of complexes studied. 2′-Deoxyribonucleoside units are given in plain and bold and 2′-*O*-methylribonucleoside units are in italics. **8**: X=M=Y=A, N=G; **9**: X=T, M=Y=A, N=G; **10**: X=M=A, Y=T, N=G; **11**: X=Y=T, M=A, N=G; **12**: X=A, Y=M=T, N=C.

Thermodynamic analysis

The thermodynamic parameters for the dissociation melting curves of the hybrid complex to a looped (or triplex to circular) oligonucleotide and a single-stranded DNA were calculated by applying a two-state model and the methods described by Breslauer (33). From the experimental curves the fractions of hybrid complex (or triplex) dissociation, α, as a function of temperature were obtained. These were transformed to equilibrium constants, K, by equation $K = \alpha/0.5C_t(1-\alpha)^2$ (eq. 1), where C_t equals the total strand concentration of both associated oligomers present in equal concentration, namely, $0.5C_t$. Equilibrium constants at 37°C, $K_{37°C}$, were then calculated by linear van't Hoff equation $\ln K = -\Delta H°/RT+\Delta S°/R$ (eq. 2), and transformed to Gibbs free energy of dissociation at 37°C, $-\Delta G°_{37°}$, via equation $\Delta G°_{37°} = -RT\ln K_{37°C}$ (eq. 3). Free energy of dissociation at 55°C, $-\Delta G°_{55°}$, was calculated analogously. The correlation coefficients of the van't Hoff plots fell within the range 0.997 to 0.999, lending support to the validity of the two-state model applied. In four cases, van't Hoff's parameters were additionally determined by measuring T_m as a function of concentration. The enthalpies obtained by equation $1/T_m = R/\Delta H° \times \ln(C/C°) +$ constant (eq. 4) agreed within 7% with those extracted from the melting curve, again suggesting that the two-state approximation may be considered a reasonable one for our hybrid complexes. Uncertainty in the $\Delta G°_{37°}$ values was estimated to be less than ±10%.

Replication termination experiments

Replication termination experiments were conducted on synthetic 75mer single-stranded DNA templates. These templates contained completely matching (**M0**) or mismatching (**M1**, **M2**, **M3**, **M4**) stretches for the recognition by looped oligonucleotides and a stretch for primer recognition. The target sequences for looped oligonucleotides were identical with oligomers **8–12**, employed in the melting experiments (Fig. 2). Prior to the enzymatic reaction, a mixture of 10 pmol of template and 100–200 pmol of the inhibitory oligomer in Sequenase Buffer (40 mM Tris–HCl pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$) was heated to 80°C and cooled slowly to room temperature. 5′-^{32}P-phosphorylated primer was added, the mixture was kept for additional 15 min at room temperature and the primer extension reaction was started by adding the DNA polymerase (1–3 U) in Sequenase Buffer and a mixture of 2′-deoxynucleoside 5′-triphosphates (75 μM each) in an equal amount of Sequenase Buffer. The total volume of the reaction mixture was 16 μl. Polymerisation reactions were performed for 15 min at 37°C with Sequenase Version 2.0 (Amersham) and *E.coli* Klenow fragment (Pharmacia), and at 55°C with thermostable *Tth*-polymerase (Hytest OY, Finland). The enzymatic reactions were quenched by adding the equal volume of formamide-dyes containing stop-solution. Samples were then heated to 95°C for 5–10 min, chilled in ice and applied to a 7M urea–8% polyacrylamide gel (24 × 60 cm). Electrophoresis was run at 65°C.

RESULTS AND DISCUSSION

Binding of looped oligonucleotides to single-stranded complementary targets

Figure 1 shows the structures of the complexes of looped [**1–5**] and circular [**6,7**] oligonucleotides with a completely matching [**8**] and partially mismatching [**9–12**] complementary DNA sequences. To elucidate the effect of mismatching on the efficiency of hybridisation the following five sets of melting experiments were performed: (i) the completely matching oligomer **8** with **1–7**; (ii) oligomer **9** (one mismatch in the triplex) with **1–7**; (iii) oligomer **10** (one mismatch in the duplex) with **1–5**; (iv) oligomer **11** (one mismatch in the duplex and one in the triplex) with **1–5**; (v) oligomer **12** (completely mismatching duplex) with **1–5**. Table 1 summarises T_m and $\Delta G°_{37°}$ ($\Delta G°_{55°}$) values obtained. One can see that all looped oligonucleotides are capable of forming stable complexes with a single-stranded complementary DNA (set 1), while experiments with mismatching targets (sets 2–5) reveal considerable differences. Thus among structures composed of 2′-deoxyribonucleosides **1–4**, the highest stability as well as selectivity is displayed by loop **2** (T_m = 61.8°C; $-\Delta G°_{37°}$ = 27.0 kcal/mol), where the circular part consists of a normal sugar–phosphate backbone crosslinked with an -S–S- bond (analogously to the circular oligonucleotide **6**) and the linear part is linked to the circle via 1′-*O*- of the 3′-deoxypsicothymidine unit Z. Complete mismatch in the linear part brings about a considerable destabilisation compared to matching complex ($sel_{37°}$ = 9.5 kcal/mol). Substantial destabilisation is also caused by a single mismatch in the triplex-forming part ($sel_{37°}$ = 5.8 kcal/mol), and even more so by a single mismatch in both the triplex and duplex parts ($sel_{37°}$ = 8.5 kcal/mol). On the other hand one mismatch in the duplex leads to the insignificant loss in the selectivity ($sel_{37°}$ = 3.6 kcal/mol). One may conclude

Figure 2. Structures of synthetic templates **M0**, **M1**, **M2**, **M3**, **M4**, control oligonucleotides **C1**, **C2** and primer employed in replication termination experiments. The looped oligonucleotide, associated with a matching (**M0**) or mismatching (**M1–M4**) template is drawn schematically. Stretches for inhibitor recognition are identical with oligomers **8–12** used in physico-chemical experiments. Mismatches are given in bold.

that the linear stretch when attached to 1′-*O*- of Z helps to increase the selectivity of the ligand and stability of complex as a whole. This allows one to aim the target DNA very precisely at its polyadenine part as well as at adjacent sequence simultaneously.

Table 1 also includes the results obtained with looped **5** and circular **7** oligonucleotides composed of 2′-*O*-methylribonucleoside units. These compounds display an even stronger binding (T_m = 66.2°C; $-\Delta G°_{37°}$ = 29.9 kcal/mol for **5/8** and T_m = 61.9°C; $-\Delta G°_{37°}$ = 23.0 kcal/mol for **7/8**) than their 2′-deoxyribonucleoside analogues **2** (T_m = 61.8°C; $-\Delta G°_{37°}$ = 27.0 kcal/mol) and **6** (T_m = 54.1°C; $-\Delta G°_{37°}$ = 18.4 kcal/mol), the selectivity of binding being comparable. The ability of branched oligonucleotide **13**, precursor of loop **5**, to form a stable hybrid with **8** is also noteworthy. Complex **13/8** melts in a co-operative manner, showing only one transition at 62°C ($-\Delta G°_{37°}$ = 26.1 kcal/mol). This result is surprisingly different from the two-transition melting curve, displayed by a similar branched oligonucleotide composed of 2′-deoxyribonucleosides (32).

Influence of looped oligonucleotides on replication *in vitro*

The looped oligomers used as inhibitors were able to form a short triplex (~10 bases) with the template near its 5′-terminus, and a short duplex (~10 bases) towards the 3′-end from the triplex forming site (Fig. 2). The primer employed was a 15mer that hybridised with the complementary 3′-terminal stretch of the template. Accordingly, in our experiments the DNA polymerase starts the primer extension on a single-stranded template. Then at the point where the enzyme meets the hybrid complex the situation changes: the polymerase encounters a double-stranded DNA. Finally, after overcoming the duplex moiety the enzyme comes to contact with the triplex. It is known that while some polymerases posses helicase activity, others are taking advantage of 'breathing of double-stranded DNA ends' (27). It remains obscure whether the mechanism of double-stranded DNA unfolding should be taken into account while studying the inhibition of replication by hybrid complexes.

Before starting experiments on replication inhibition we checked whether looped oligonucleotides are capable of forming hybrid complexes with the 75mer template **M0** exactly in the way they do with the shorter complementary sequence **8**. For this purpose we recorded melting curves for the complexes of loops **1**, **2** or **5** with oligomer **M0** in Sequenase Buffer at pH 7.5. In each case a single step transition was observed, the melting points being 59.5°C, 60.9°C and 65.8°C respectively. In other words, the T_m values are only 0.4–1.3°C lower than those of complexes **1/8**, **2/8** and **5/8**. This strongly suggests that one is really dealing with hybrid complexes on attempting to inhibit the DNA polymerase reaction.

Termination of replication catalysed by Sequenase version 2. Figure 3 shows the PAGE of the primer extension reactions on templates **M0–M4** catalysed by Sequenase Version 2 in the absence and presence of looped oligonucleotides **1** and **2**. As seen, both oligomers exhibit a weak termination effect on the reaction performed on template **M0**, i.e. when the matching between the template and the looped oligonucleotide is complete. The termination appears to take place near the 3′-end of the A_{11} sequence engaged in the triple helix formation with **1** and **2**. The product migrates slightly further than a 54meric control oligonucleotide (**C2**), complementary to **M0** from the 3′-end to the beginning of the A_{11} sequence. Evidently, two to three 3′-terminal nucleotides of the A_{11} sequence are also replicated. Introduction of a mismatch in any part of the hybrid complex was sufficient to practically remove this termination effect under the experimental conditions, as shown by the results obtained with templates

M1–M4. Circular oligonucleotide **6**, composed of 2′-deoxyribonucleosides analogously to **1** and **2**, was also observed to exhibit a weak inhibitory influence on replication catalysed by Sequenase (data not shown).

Table 1. Melting points (T_m), free energies of association (ΔG°) and Selectivity (Sel) of the complexes studied in five sets of experiments

Set No	Complex	T_m (°C)	$-\Delta G°_{37°}$ (kcal/mol)	$-\Delta G°_{55°}$ (kcal/mol)	$Sel_{37°}$ (kcal/mol)
1	**1/8**	60.8	23.3	–	–
1	**2/8**	61.8	27.0	15.1	–
1	**3/8**	60.0	20.0	–	–
1	**4/8**	60.9	23.0	–	–
1	**5/8**	66.2	29.9	18.5	–
1	**6/8**	54.1	18.4	–	–
1	**7/8**	61.9	23.0	–	–
2	**1/9**	55.4	20.8	–	2.5
2	**2/9**	55.9	21.2	11.0	5.8
2	**3/9**	54.8	18.5	–	1.5
2	**4/9**	55.6	19.1	–	3.9
2	**5/9**	61.4	24.4	15.1	5.5
2	**6/9**	45.0	12.8	–	5.6
2	**7/9**	58.0	17.7	–	5.3
3	**1/10**	57.5	21.6	–	1.7
3	**2/10**	58.7	23.4	12.8	3.6
3	**3/10**	56.8	19.9	–	0.1
3	**4/10**	57.3	20.1	–	2.9
3	**5/10**	54.4	27.2	16.1	2.7
4	**1/11**	51.1	18.3	–	5.0
4	**2/11**	51.7	18.5	8.6	8.5
4	**3/11**	51.0	16.8	–	3.2
4	**4/11**	51.5	17.8	–	5.2
4	**5/11**	60.2	22.3	12.9	7.6
5	**1/12**	52.0	15.7	–	7.6
5	**2/12**	53.8	17.5	10.0	9.5
5	**3/12**	52.7	16.4	–	3.6
5	**4/12**	52.9	16.9	–	6.1
5	**5/12**	61.6	20.4	13.0	9.5
	13/8	62.3	26.1	–	–
	14/8	59.1	22.2	–	–
	15/8	56.8	18.8	–	–

Looped **5** and circular **7** oligonucleotides composed of 2′-*O*-methylribonucleosides display an even stronger termination of DNA replication than their deoxyribonucleoside counterparts. Figure 4 shows the results obtained on template **M0** at different concentration ratios inhibitor/template. It is clearly seen that while linear oligonucleotides, either 2′-deoxyribo- or 2′-*O*-methylribo oligomers, are unable to inhibit the replication on **M0**, the presence of **5** or **7** results in a clear-cut termination. The duplex

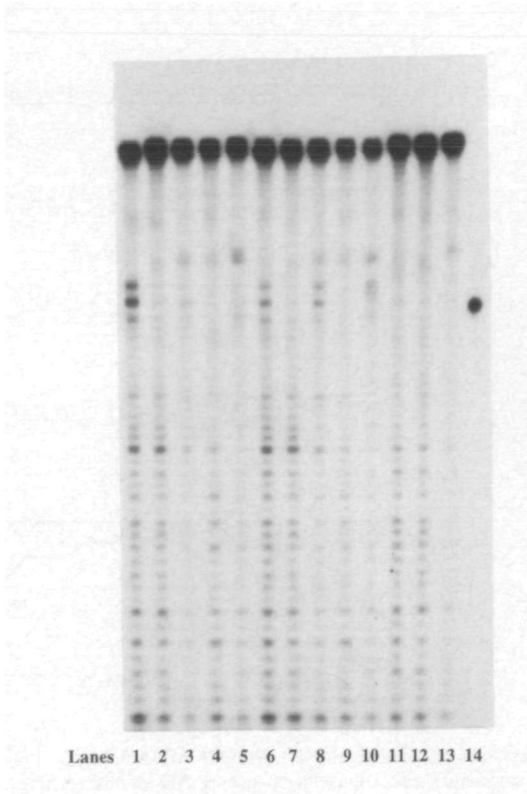

Figure 3. Influence of loops **1** and **2** on a Sequenase catalysed polymerisation on templates **M0–M4**. Lane 1, influence of **2** on polymerisation on **M0**; lane 2, influence of **2** on polymerisation on **M1**; lane 3, influence of **2** on polymensation on **M2**; lane 4, influence of **2** on polymerisation on **M3**; lane 5, influence of **2** on polymerisation on **M4**; lane 6, influence of **1** on polymerisation on **M0**; lane 7, influence of **1** on polymerisation on **M1**; lane 8, influence of **1** on polymerisation on **M2**; lane 9, influence of **1** on polymerisation on **M3**; lane 10, influence of **1** on polymerisation on **M4**; lanes 11, 12 and 13, polymerisation on **M0**, **M1** and **M2** in the absence of inhibitor; lane 14, control oligonucleotide [^{32}P]-**C2**.

part of hybrid complex again appears to be easily opened, and the triplex part allows permeation of the growing chain up to three bases inside the triplex. At this point the chain extension essentially stops. Figure 5 demonstrates the changes that mismatches in either the double or triple helix forming part of the template (**M1–M4**) cause in the terminating ability of **5**. Qualitatively, the termination efficiency appears to correlate with the affinity of **5** to its target sequence (Table 1). The overall yield of terminated products is decreased on increasing the number of mismatches. When the linear moiety of **5** completely mismatches with the template (**M4**), **5** essentially acts as the circular oligonucleotide **7**, exhibiting an equally strong inhibition on the replication. Interestingly, when a single mismatch is introduced in the triplex (**M1**), the primer extension is stopped two bases earlier than on the completely matching template **M0**, the overall yield of termination being lower. Possibly a mismatch in the relatively short triple helix is reflected as a change in geometry, and this in turn affects the enzyme/nucleic acid recognition. In summary, looped 2′-*O*-methylribo- oligomer **5** appears to be a selective and potent inhibitor of Sequenase Version 2 DNA-polymerase.

Termination of replication catalysed by Klenow fragment. The results obtained with Klenow fragment (Fig. 6) basically resemble those described above for Sequenase. The primer

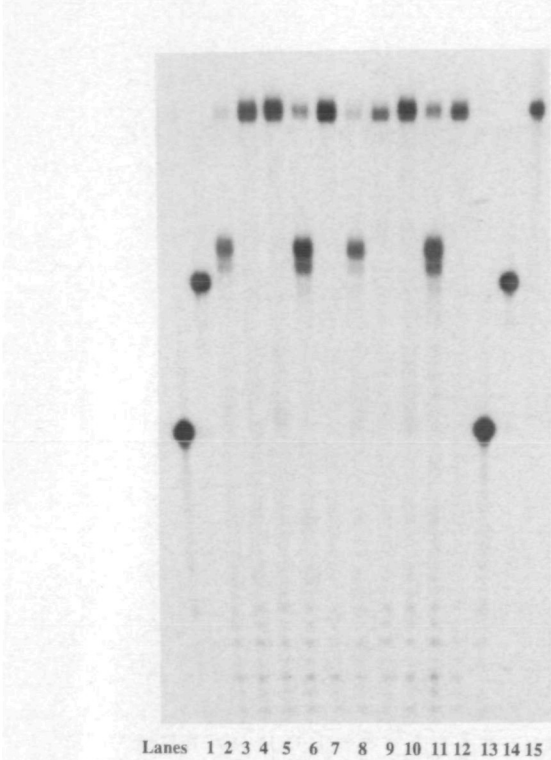

Figure 4. Influence of loop **5** and circle **7** on a Sequenase catalysed polymerisation on template **M0**. Lanes 1 and 13, control oligonucleotide [^{32}P]-**C1**; lanes 2 and 14, control oligonucleotide [^{32}P]-**C2**; lane 3, influence of **5** on polymerisation (**5**:**M0** = 20:1); lane 4, influence of linear oligomer **14** on polymerisation (**14**:**M0** = 20:1); lane 5, influence of linear oligomer **15** on polymerisation (**15**:**M0** = 20:1); lane 6, influence of **7** on polymerisation (**7**:**M0** = 20:1); lane 7 and 12, polymerisation in the absence of inhibitor; lane 8, influence of **5** on polymerisation (**5**:**M0** = 10:1); lane 9, influence of linear oligomer **14** on polymerisation (**14**:**M0** = 10:1); lane 10, influence of linear oligomer **15** on polymerisation (**15**:**M0** = 10:1); lane 11, influence of **7** on polymerisation (**7**:**M0** = 10:1); lane 15, [^{32}P]-**M0**.

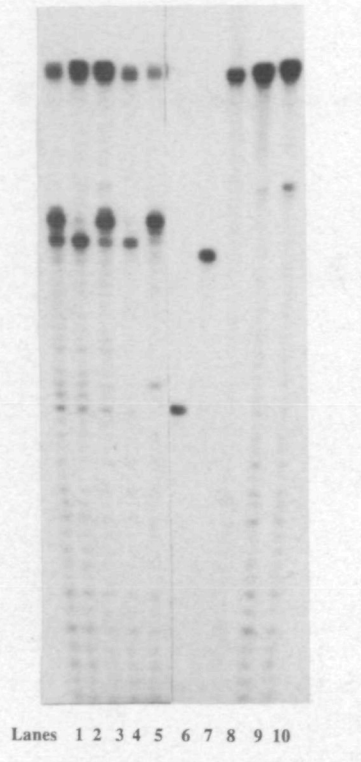

Figure 5. Influence of loop **5** on a Sequenase catalysed polymerisation on templates **M0**–**M4**. Lane 1, influence of **5** on polymerisation on **M0**; lane 2, influence of **5** on polymerisation on **M1**; lane 3, influence of **5** on polymerisation on **M2**; lane 4, influence of **5** on polymerisation on **M3**; lane 5, influence of **5** on polymerisation on **M4**; lane 6, control oligonucleotide [^{32}P]-**C1**; lane 7, control oligonucleotide [^{32}P]-**C2**; lane 8, [^{32}P]-**M0**; lane 9, polymerisation on **M3** in the absence of inhibitor; lane 10, polymerisation on **M4** in the absence of inhibitor.

extension reaction performed with completely matching loop **5** and template **M0** also results in a high yield of terminated products. This termination also occurs in the region occupied by the triplex. The overall yield of terminated products again drops considerably when a mismatch is brought inside the triplex. Analogously to Sequenase, the presence of a complete mismatch in the duplex forming region (**M4**) reduces the selectivity and strength of binding to the target (Table 1) but still brings about the same overall yield of termination products. With looped oligodeoxyribonucleotide **2**, no substantial termination of replication occurs. This result also agrees with the data obtained with Sequenase. Our experiments with these two enzymes also demonstrate that while the processivity of Klenow fragment is lower than that of Sequenase (34), this feature of the enzyme does not significantly influence on the ability of polymerase to overcome the inhibitory effect of the hybrid complex, at least under the experimental conditions employed in this work.

Termination of replication catalysed by thermostable Tth-polymerase. Experiments with thermostable *Tth*-polymerase were conducted at an elevated temperature, 55°C. The results are shown in Figure 7. The inhibitory effects of the looped oligonucleotides on the replication catalysed by thermostable polymerase were not checked at higher temperatures, e.g. at 70°C as in (28), since most of the complexes melt well below this point. In a striking contrast to results obtained with Sequenase and Klenow Fragment polymerases, *Tth*-catalysed primer extension on matching template **M0** was terminated by loop **5** not only within the triple helical part of the complex, but also within the duplex part of the hybrid. The shorter product migrates on PAGE slightly slower than a 40meric control oligonucleotide (**C1**), complementary to **M0** from the 3′-end to the beginning of 3′-$(AG)_5$-5′ sequence. In principle any mismatch in the target sequence lowers the overall yield of termination. One mismatch within the triplex (**M1**) considerably lowers the yield of the longer termination product, one mismatch within the duplex (**M2**) reduces the yield of the shorter termination product, and double mismatch (**M3**) or complete mismatch within duplex (**M4**) brings about a complete disappearance of the inhibitory effect. One may conclude that in this case experiments on the relative stability of complexes of loop **5** with matching and mismatching target sequences (Table 1) are in a perfect agreement with the results obtained for inhibition of primer extension. Our results show that termination of *Tth*-catalysed replication occurs at two sites within the triplex and duplex of the hybrid complex. It is obvious that the presence of duplex part within the hybrid, that the polymerase encounters in the process of primer extension brings about an additional inhibitory effect [it was reported previously (28) that simple

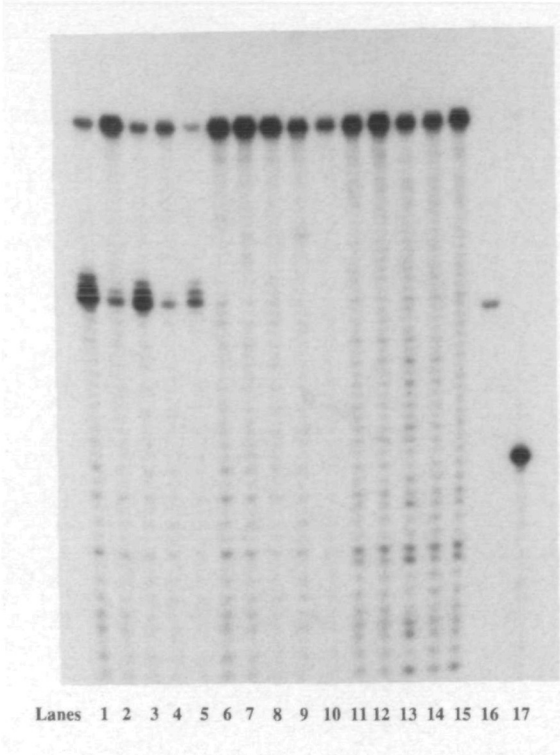

Figure 6. Influence of loops **2** and **5** on a Klenow fragment catalysed polymerisation on templates **M0–M4**. Lane 1, influence of **5** on polymerisation on **M0**; lane 2, influence of **5** on polymerisation on **M1**; lane 3, influence of **5** on polymerisation on **M2**; lane 4, influence of **5** on polymerisation on **M3**; lane 5, influence of **5** on polymerisation on **M4**; Lane 6, influence of **2** on polymerisation on **M0**; lane 7, influence of **2** on polymerisation on **M1**; lane 8, influence of **2** on polymerisation on **M2**; lane 9, influence of **2** on polymerisation on **M3**; lane 10, influence of **2** on polymerisation on **M4**; Lanes 11 and 15, polymerisation on **M0** and **M4** in the absence of inhibitor; lane 16, control oligonucleotide [^{32}P]-**C2**; lane 17, control oligonucleotide [^{32}P]-**C1**.

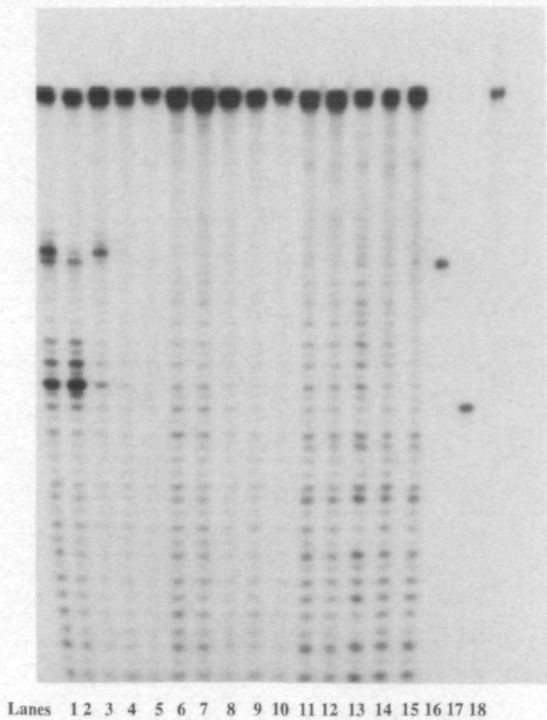

Figure 7. Influence of loops **2** and **5** on a *Tth* catalysed polymerisation on templates **M0–M4**. Lane 1, influence of **5** on polymerisation on **M0**; lane 2, influence of **5** on polymerisation on **M1**; lane 3, influence of **5** on polymerisation on **M2**; lane 4, influence of **5** on polymerisation on **M3**; lane 5, influence of **5** on polymerisation on **M4**; Lane 6, influence of **2** on polymerisation on **M0**; lane 7, influence of **2** on polymerisation on **M1**; lane 8, influence of **2** on polymerisation on **M2**; lane 9, influence of **2** on polymerisation on **M3**; lane 10, influence of **2** on polymerisation on **M4**; lane 11 and 15, polymerisation on **M0, M4** in the absence of inhibitor; lane 16, control oligonucleotide [^{32}P]-**C2**; lane 17, control oligonucleotide [^{32}P]-**C1**; lane 18, [^{32}P]-**M0**.

linear oligonucleotides, forming duplexes are not capable of terminating properties]. Thus in this case the linear part of loop serves not only to increase the selectivity of binding and stability of complex with the target but also considerably enhances the overall yield of terminated products. Lanes 6–10 in Figure 7 show analogous experiments with loop **2**. Unlike loop **5** composed of 2′-*O*-methylribosides, loop **2** composed of 2′-deoxyribosides display practically no inhibitory effects.

CONCLUSION

The present work demonstrates that several looped oligonucleotides are capable of forming very stable and selective hybrid complexes. Accordingly, loop **2** composed of 2′-deoxyribonucleosides and loop **5** composed of 2′-*O*-methylribonucleosides display very similar properties in terms of complex formation with a single-stranded DNA. The dramatic differences observed in the inhibitory effects of these two oligonucleotides upon replication *in vitro*, catalysed by three different DNA-polymerases, may by no means be explained by mere differences in the stability of their complexes with the target. Circular oligonucleotide **7**, for example, forms a weaker complex with the complementary sequence than **2** but inhibits Sequenase catalysed replication *in vitro* much more strongly. Oligonucleotide **5**, in turn, though forms a much more stable complex with the target than **7**, inhibits replication to a comparable extent. We can give no clear explanation for this phenomenon at this point. We feel that the geometry of the complex and its structure as a whole may play a much more important role in the inhibitory properties of oligonucleotide analogues than simply the relative stability. One can speculate that the differences described above may result from either substitution of thymines to uracils within the circular part of the loop, or differences in sugar puckering of 2′-deoxyribonucleotides compared to that of 2′-O-methylribonucleosides. Whatever influences the structure of looped oligonucleotides, it appears impossible at the present state of knowledge to predict how this factor alters the inhibitor–enzyme interaction and what properties of the enzyme are mostly affected by these changes. Nevertheless we strongly believe that on designing potential antigene agents, one should not only study details of complex formation with the target, but also investigate the potency of new compounds to inhibit key biochemical reactions *in vitro*, such as replication, transcription, reverse transcription etc. By combining these two approaches in the present work, we have demonstrated that a looped oligonucleotide composed of 2′-*O*-methylribonucleosides forms a very stable and selective hybrid complex with the complementary single-stranded DNA and displays a strong inhibitory effect on replication *in vitro* catalysed by different

DNA-polymerases. These features may appear to be useful for the design of highly specific antigene oligonucleotides.

ACKNOWLEDGEMENT

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