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## Trapping DNA polymerases using triplex-forming oligodeoxyribonucleotides\*

(DNA polymerization; termination; triplex)

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### SUMMARY

Triplexes (triple helices) formed within DNA templates prior to or during DNA synthesis cause DNA polymerase to terminate [Samadashwily et al., EMBO J. 13 (1993) 4975–4983]. Here, we show that triplex-forming oligodeoxyribonucleotides (oligos) efficiently trap DNA polymerases at target DNA sequences within single-stranded (ss) templates. This was observed for all studied DNA polymerases, including Sequenase and the thermophilic *Taq* and Vent polymerases. The termination rate depends on the fine structure of a triplex, as well as on ambient conditions such as temperature and the concentration of magnesium ions. Inhibition of DNA synthesis was observed not only when triplexes blocked the path of DNA polymerase, but also when a polymerization primer was involved in triplex formation. *Escherichia coli* ss-binding (SSB) protein helps DNA polymerase overcome the triplex barrier, but with an efficiency dramatically dependent on the triplex configuration. These results describe a novel method for blocking DNA replication at target homopurine-homopyrimidine sequences by means of triplex-forming oligos in direct analogy with similar results during transcription.

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### INTRODUCTION

Triplex-forming oligos (TFOs) interact specifically with homopurine-homopyrimidine sequences in genomic DNA (Le-Doan et al., 1987; Moser and Dervan, 1987; Lyamichev et al., 1988). TFOs are usually homopurine or homopyrimidine sequences representing mirror

images of chemically similar strands in target DNAs (Mirkin et al., 1987; Beal and Dervan, 1991). Homopyrimidine oligos form stable triplexes under acidic pH (Lyamichev et al., 1988), a requirement which may be somewhat overcome by methylation of cytosines or by the presence of polyamines (Maher et al., 1989; Hampel et al., 1991). Homopurine oligos form triplexes under physiological pH in the presence of bivalent cations (Malkov et al., 1993, and references therein). Canonical components of the pyrimidine/purine/pyrimidine (YR\*Y) triplexes are CG\*C<sup>+</sup> and TA\*T base triads (Felsenfeld et al., 1957; Morgan and Wells, 1968), while orthodox pyrimidine/purine/purine (YR\*R) triplexes consist of CG\*G and TA\*A triads (Kohwi and Kohwi-Shigematsu, 1988; Bernues et al., 1989). In the latter case, however, thymines may also be incorporated in the otherwise homopurine strand of the TFO opposite adenines in the target sequence, thus forming TA\*T triads (Beal and Dervan, 1991). Studies of non-canonical triads showed

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Abbreviations: bp, base pair(s); dd, dideoxy; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ss, single strand(ed); SSB, ss DNA-binding (protein); TFO, triplex-forming oligo; triplex, triplex helix.

that mismatches could be somewhat tolerated, though each significantly disfavored triplex formation (Belotserkovskii et al., 1990; Mergny et al., 1991; Beal and Dervan, 1992). Mismatch energies were within the range of 3–6 kcal/mol, i.e., similar to the cost of B-DNA mismatches. As a result, TFOs bind to DNA in a highly sequence specific manner recognizing, for example, unique sites in the yeast (Strobel and Dervan, 1990) and human (Strobel et al., 1991) chromosomes.

The sequence-specificity of TFOs is the basis for the anti-gene strategy reviewed by Hélène (1991) where selective binding of a TFO to a target gene prevents its normal functioning. Most such studies concerned the inhibition of transcription. This is due to the existence of functionally important homopurine-homopyrimidine stretches in many eukaryotic promoters which are appropriate targets for TFOs (reviewed in Mirkin and Frank-Kamenetskii, 1994). The inhibitory effects of TFOs on the transcription of the *c-myc* (Cooney et al., 1988), methallothionein (Maher et al., 1989; 1992) and interleukin-2 receptor (Grigoriev et al., 1992) genes were observed in vitro. The likely mechanism of transcriptional inhibition in these cases is competition between the TFO and the transcriptional activators for a target promoter sequence. TFOs also arrest initiation and elongation of transcription in vitro (Young et al., 1991; Duval-Valentin et al., 1992). Recently it was found that TFOs inhibit transcription from the *c-myc* and interleukin-2 receptor genes in cell cultures (Postel et al., 1991; Grigoriev et al., 1993).

The influence of TFOs on DNA replication is less studied. An octathymidilate bound with an acridine derivative recognizes a d(A)<sub>8</sub> stretch in SV40 DNA adjacent to the T-antigen binding site. In vivo it inhibits SV40 replication presumably by interfering with the DNA-binding or unwinding activities of the T-antigen (Birg et al., 1990). We have recently shown the inhibitory effect of TFOs on the elongation of DNA synthesis in vitro (Samadashwily et al., 1993). TFOs completely blocked purified T7 DNA polymerase at a target site within a ss template.

Here we describe that the same is true for different DNA polymerases including such highly processive enzymes as *Taq* and Vent polymerases. *E. coli* SSB protein partially ameliorate the termination of polymerization caused by TFOs, but some triplexes escape the action of SSB. Finally, we found that TFOs may also prevent the initiation of DNA polymerization. These results raise hopes that TFOs could be further used for impeding DNA replication in vivo.

## RESULTS AND DISCUSSION

### (a) General strategy

We studied the influence of purine-rich TFOs on the activity of several different DNA polymerases. These

TFOs were chosen because YR\*R triplexes are favorable under polymerization conditions (Baran et al., 1991; Dayn et al., 1992). Our templates were circular pBluescript ss DNAs carrying a 18-nt long homopyrimidine (Fig. 1A–C) or homopurine (Fig. 1D–F) targets. Different oligos were added to form short Watson-Crick duplexes (Fig. 1A,D), hypothetical Hoogsteen duplexes (Fig. 1E), orthodox intermolecular triplexes (Fig. 1B,F), or H-like triplexes formed by a purine-rich hairpin and its homopyrimidine target (Fig. 1C). All triplexes were built from CG\*G and TA\*T base triads (Samadashwily et al., 1993). This triad composition was chosen because such triplexes appear to be more stable than orthodox YR\*R triplexes that consist of CG\*G and TA\*A triads (Beal and Dervan, 1991). To prevent these oligos from serving as primers for DNA polymerization, their 3'-ends were blocked by a 3' amino group (Nelson et al., 1989). A standard 'reverse' primer was annealed to all these templates and the pattern of DNA polymerization was analyzed by DNA sequencing or primer extension.

### (b) DNA triplexes block elongation of different DNA polymerases

We have previously found that T7 DNA polymerase (Sequenase) was completely blocked by short triplexes within ss templates (Samadashwily et al., 1993). In that study we used the DNA templates presented in Fig. 1A–C. No apparent differences were observed between intermolecular and H-like triplexes. Since only homopyrimidine sequences served as templates for the polymerase in those cases, it was not clear if termination would be the same for homopyrimidine templates. Neither is it clear that the purine-rich TFO alone causes termination of DNA synthesis by forming either a hypothetical Hoogsteen-type duplex, or complex quadruple structures (reviewed in Williamson, 1994) with the template. To address these questions, we studied DNA polymerization on the templates presented in Fig. 1D–F where the homopurine-strand is replicated. The results presented in Fig. 2 show that neither short Watson-Crick duplexes, nor the G-rich strand alone cause prominent termination of polymerization, but triplex formation unconditionally blocks Sequenase at the triplex border. Taken together with our previous work, these results show that triplexes prevent Sequenase elongation regardless the chemical nature of the template DNA.

To see if the TFO-caused termination of polymerization was general to other polymerases, we repeated our experiments on the DNA templates presented in Fig. 1A–C but with *Taq* polymerase. The results of DNA sequencing are shown in Fig. 3. Similarly to Sequenase, short duplex does not block *Taq* polymerase. Triplexes do lead to the premature termination of polymerization

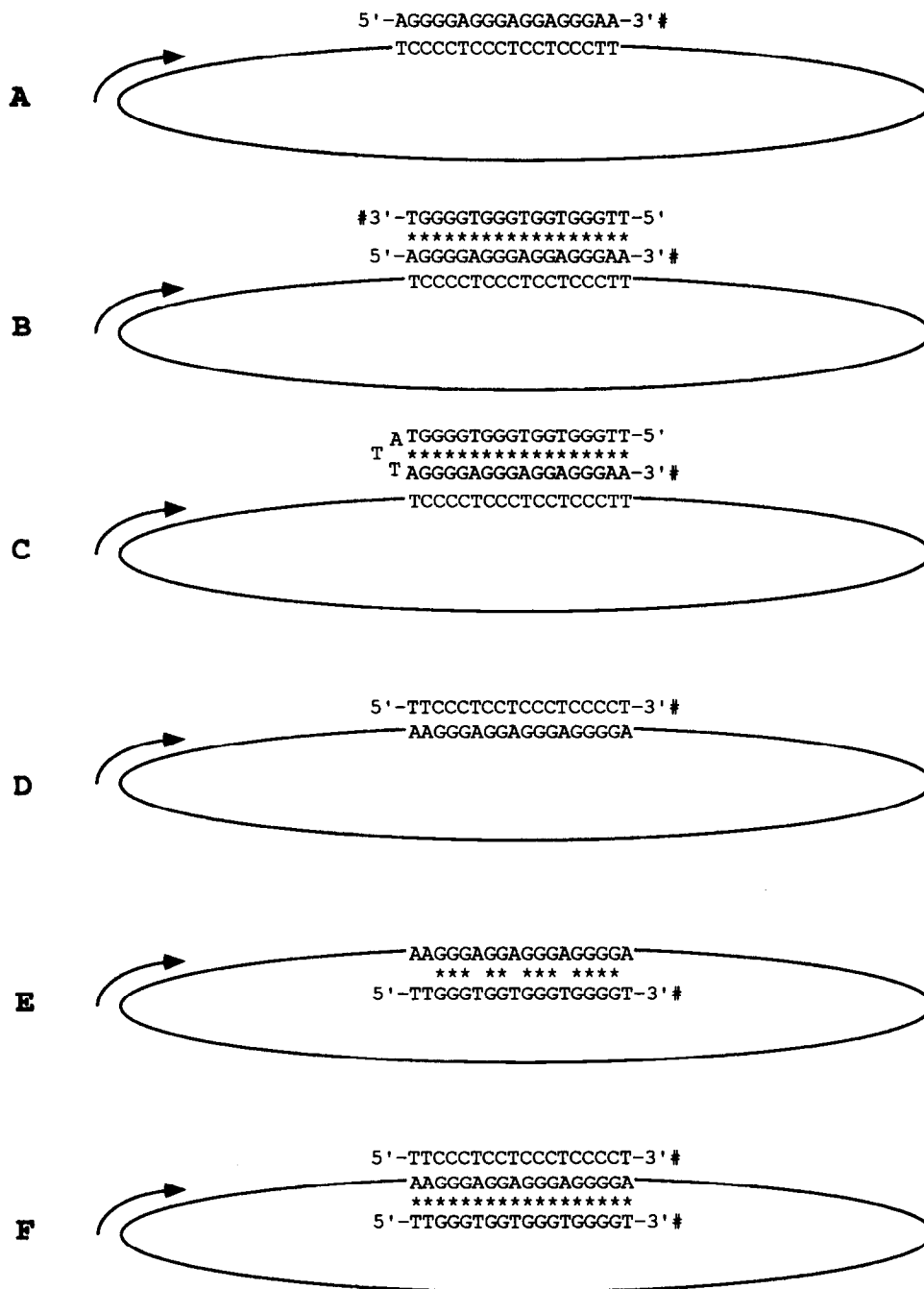


Fig. 1. DNA templates containing short double- and triple-helical stretches. Hoogsteen pairs are shown by asterisks (\*), 3' amino groups are shown by symbols (#). Arrows correspond to the 'reverse' primer. Circular pBluescript ss DNAs carry homopyrimidine (A-C) or homopurine (D-F) targets. Different oligos were added to form short Watson-Crick (W-C) duplexes (A,D), canonical intermolecular triplexes (B,F) or H-like triplexes formed by a purine-rich hairpin and its homopyrimidine target (C). All triplexes were built from CG\*G and TA\*T base triads. E is a control where a purine-rich oligo alone was added to form a hypothetical Hoogsteen duplex. In this case AT base pairing may occur in either Hoogsteen or W-C way.

which is reflected by strong stop signals in all four sequencing ladders. However, this termination is incomplete, and it is much stronger for the H-like triplexes (shown in Fig. 1C) than for the intermolecular triplexes (shown in Fig. 1B). These differences from the Sequenase case may have several causes. First, the lower  $Mg^{2+}$  concentration and higher temperatures optimal to *Taq* polymerase (5 mM  $Mg^{2+}$  at 72°C as opposed to 10 mM  $Mg^{2+}$

at 37°C for Sequenase) could significantly destabilize YR\*R triplexes. Second, *Taq* polymerase has a 5'-exonuclease activity on the non-template strand (Longley et al., 1990), which may contribute to triplex disruption and consequent cessation of termination.

To distinguish between these possibilities, we compared two DNA polymerases, *Taq* and Vent. While both enzymes work under wide range of temperatures and

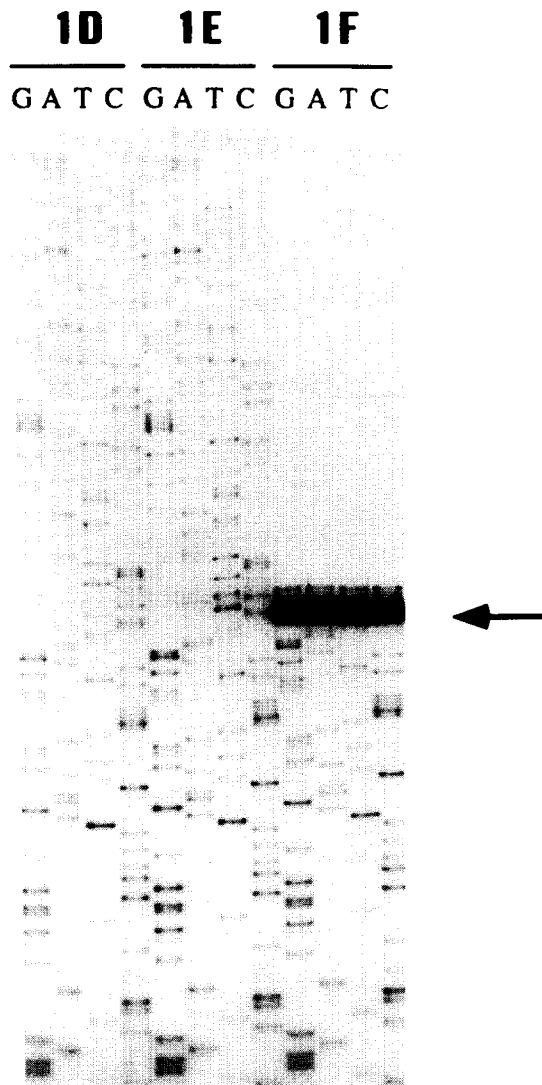


Fig. 2. Sequenase is blocked by TFOs that interact with ss DNA templates. Panels **1D**, **1E** and **1F** correspond to templates presented in Fig. 1. Lanes **G**, **A**, **T** and **C** correspond to ddGTP, ddATP, ddTTP and ddCTP sequencing reactions, respectively. The arrow shows the termination sites which are represented by strong bands in all four sequencing ladders. **Methods:** 0.5  $\mu$ g of ss DNAs was mixed with 100–200 pmol of TFOs and incubated in Sequenase Buffer (40 mM Tris-HCl pH 7.5/50 mM NaCl/20 mM  $MgCl_2$ ) for 15 min at 37°C. 15 pmol of the 'reverse' primer was used for DNA sequencing according to the Sequenase Version 2.0 sequencing protocol (US Biochemical, Cleveland, OH, USA) with the following modifications. Labeling was done in the presence of 230 nM dNTP (N = G, C or T) supplemented by 5  $\mu$ Ci of [ $\alpha$ - $^{32}P$ ]dATP (Amersham) for 2–5 min at room temperature. Then dNTPs were added up to 33.3  $\mu$ M and ddNTP up to 3.3  $\mu$ M, followed by polymerization for 10 min at 37°C. Sequencing electrophoresis was performed on a 7 M urea-8% polyacrylamide gel.

ionic conditions, Vent lacks the 5'-exonuclease activity (Kong et al., 1993). Fig. 4 compares the pattern of primer extension by *Taq* and Vent polymerases on a template with intermolecular triplexes (shown in Fig. 1B) under different  $Mg^{2+}$  concentrations. Triplexes within ss templates were preformed at low (2.5 mM)  $Mg^{2+}$ , followed by DNA labeling under the same conditions, while chain

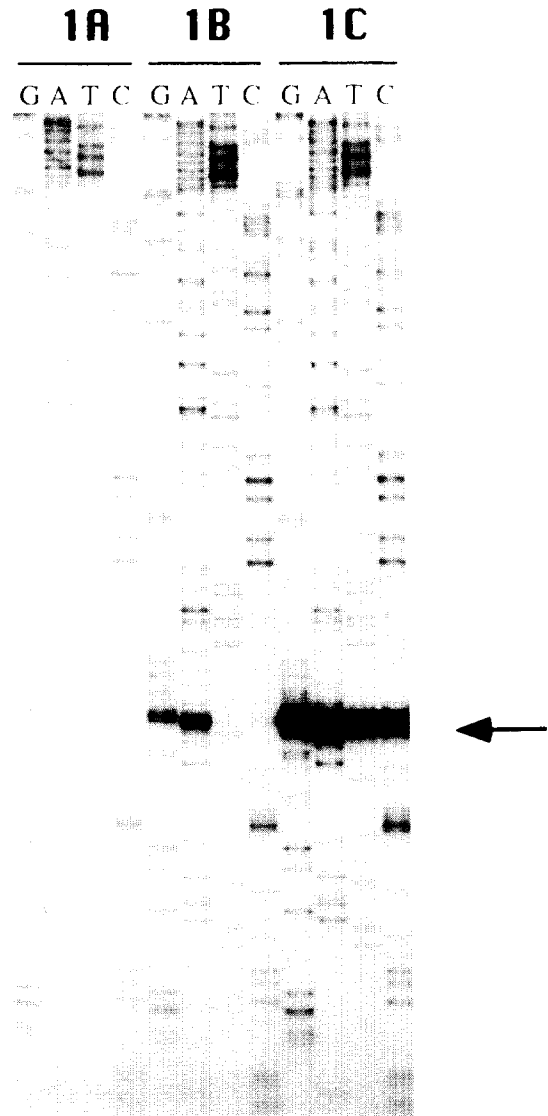


Fig. 3. *Taq* polymerization in the presence of TFOs added to ss DNA templates. Panels **1A**, **1B** and **1C** correspond to the templates presented in Fig. 1. The arrow and lanes are as in Fig. 2. **Methods:** 0.5  $\mu$ g of ss DNAs were mixed with 10 pmol of the 'reverse' primer and 100 pmol of TFOs and incubated in *Taq* polymerase sequencing buffer (40 mM Tris-HCl pH 8.0/4 mM  $MgCl_2$ ) for 15 min at 37°C. Labeling was done as in Fig. 2, but at 37°C. Then dNTPs were added up to 7.5  $\mu$ M and ddNTPs up to 600  $\mu$ M, followed by polymerization for 10 min at 72°C. Gel electrophoresis was performed as in Fig. 2.

elongation was carried out at different  $Mg^{2+}$  concentrations. The most prominent termination was observed at 10 mM of  $Mg^{2+}$ . At 5 mM  $Mg^{2+}$ , termination was less prominent for Vent polymerase and almost invisible for *Taq* polymerase, while at 2 mM  $Mg^{2+}$  there was no termination in either case. As a control, we studied the influence of magnesium ions on Sequenase activity with the same template at 37°C. As with the thermophilic polymerases, triplex-caused termination was most pronounced at 10 mM  $Mg^{2+}$  (the high background at lower  $Mg^{2+}$  concentrations is due to the sharp decrease in the

Sequenase processivity under these conditions). Since the stability of YR\*R triplexes increases with magnesium concentration (Malkov et al., 1993), it is not surprising that strongest termination in all cases occurred at 10 mM  $Mg^{2+}$ .

Fig. 5A shows the temperature dependence of DNA polymerization on the same templates with intermolecu-

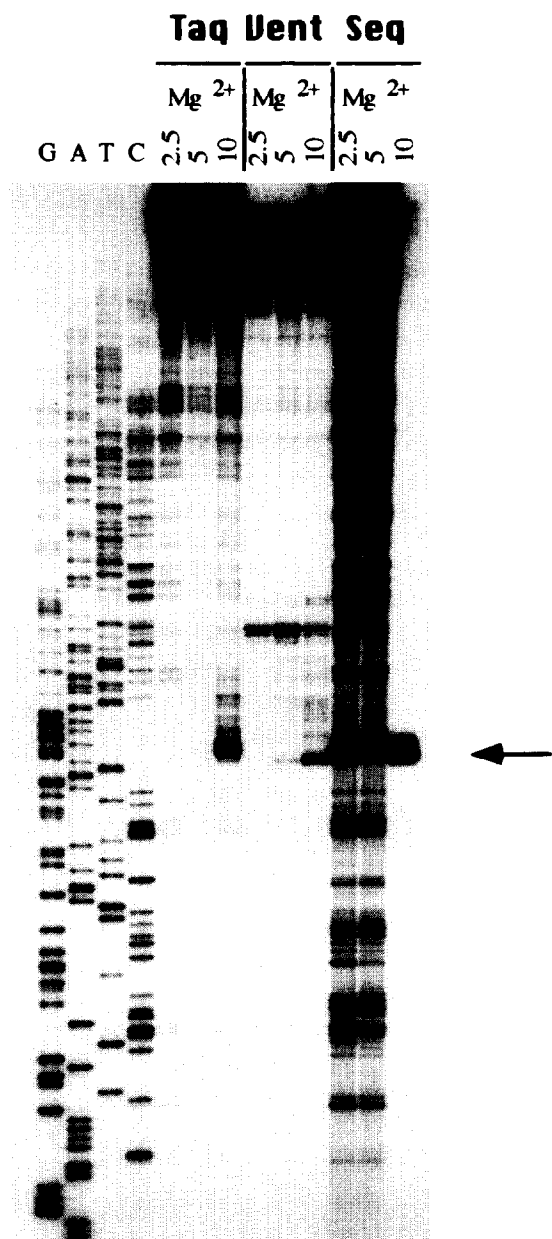


Fig. 4. Influence of  $Mg^{2+}$  on termination by triplex structures. For details, see Fig. 2. **Methods:** Triplexes were formed in either *Taq* polymerase 2× PCR buffer (20 mM Tris·HCl pH 9.0/100 mM KCl/5 mM  $MgCl_2$ /0.2% Triton X-100) for 15 min at 37°C for thermophilic polymerases, or in modified Sequenase buffer (5 mM  $MgCl_2$ /40 mM Tris·HCl pH 7.5/50 mM NaCl) for 15 min at room temperature for Sequenase. Primer extensions were performed as follows: labeling was done as in Fig. 3 for *Taq* and Vent polymerases or as in Fig. 2 for Sequenase, then dNTPs were added up to 100  $\mu$ M and polymerization was carried out at 2.5, 5 and 10 mM  $MgCl_2$  for 10 min at 65°C (*Taq* and Vent) or 37°C (*Seq*). Gel electrophoresis was performed as in Fig. 2.

lar triplexes (where primer extension was carried out at 5 mM  $Mg^{2+}$ ). One can see that the termination pattern is similar for both polymerases, being most pronounced at 37°C, rather prominent at 53°C and insignificant at 65°C. It is likely, therefore, that under these conditions a 18-mer intermolecular triplex is unstable beyond 53°C.

We also compared the influence of triplexes of different configurations on the activity of both thermophilic enzymes at 65°C. Fig. 5B shows that while intermolecular triplexes at this temperature are unable to stop polymerization, H-like structures cause prominent termination. To explain this, recall that dissociation of the DNA triplex proceeds in two steps: (1) triplex-to-duplex and (2) duplex-to-single strands. Since short duplexes do not

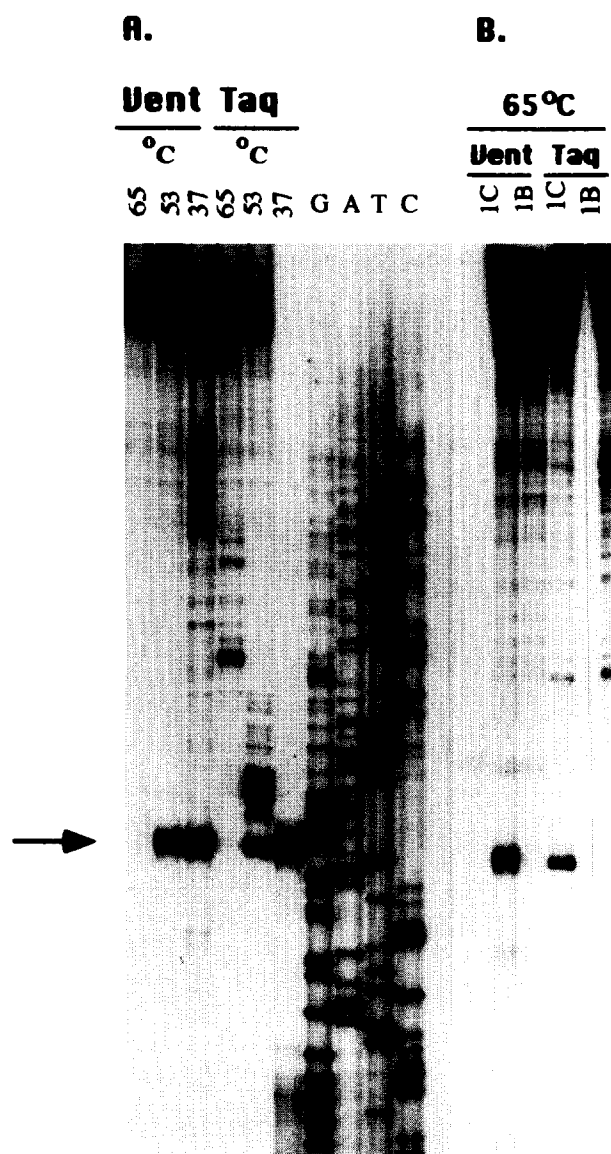


Fig. 5. Temperature dependence of termination by triplex structures. For details, see Fig. 2. **Methods:** Triplexes were preformed and labeling was done as described in the legend to Fig. 4. Primer extension was carried out at 37°C, 53°C or 65°C for 30 min.

influence DNA polymerization, we need to consider only the first transition with regard to termination, and the triplex-duplex equilibrium is clearly different between intermolecular and H-like triplexes. In the H-like structure, the third strand is covalently linked to a duplex, so that triplex dissociation is not irreversible as it is for intermolecular complexes. This leads to the higher kinetic stability of H-like triplexes than intermolecular triplexes at high temperatures (for a detailed description of the kinetic stability of DNA, see Anshelevich et al., 1984).

Altogether our results show that there is a direct link between triplex stability and the arrest of DNA polymerization. The lack of differences between *Taq* and Vent

polymerases strictly indicates that the 5'-exonuclease activity does not affect triplex-caused termination (Fig. 5).

### (c) The influence of SSB protein on TFO-caused termination

Strong inhibition of DNA polymerases by TFOs make them promising candidates for blocking DNA replication in vivo. However, the actual replication fork contains not only DNA polymerase but a complex of replication proteins, including helicases, SSB proteins, primases, topoisomerases, etc. (reviewed in Kornberg and Baker, 1992). The influence of accessory proteins on triplex-caused termination are yet to be studied. The only published data concerned the inhibition of the DNA-helicase activity of the SV40 T-antigen (Peleg and Manor, 1993). Here we studied the influence of the *E. coli* SSB protein on DNA synthesis of triplex-containing templates.

We compared DNA templates with the intermolecular and H-like triplexes shown in Fig. 1B and C. We used Sequenase T7 DNA polymerase for primer extension in



Fig. 6. Influence of the *E. coli* SSB protein on triplex-caused termination for Sequenase. Templates **1C** and **1B** are presented in Fig. 1. Triplexes were formed as in Fig. 2. Primer extensions were performed as described in the legend to Fig. 4 with the addition of the *E. coli* SSB protein (US Biochemical) to 0.25 or 0.4 mg/ml.

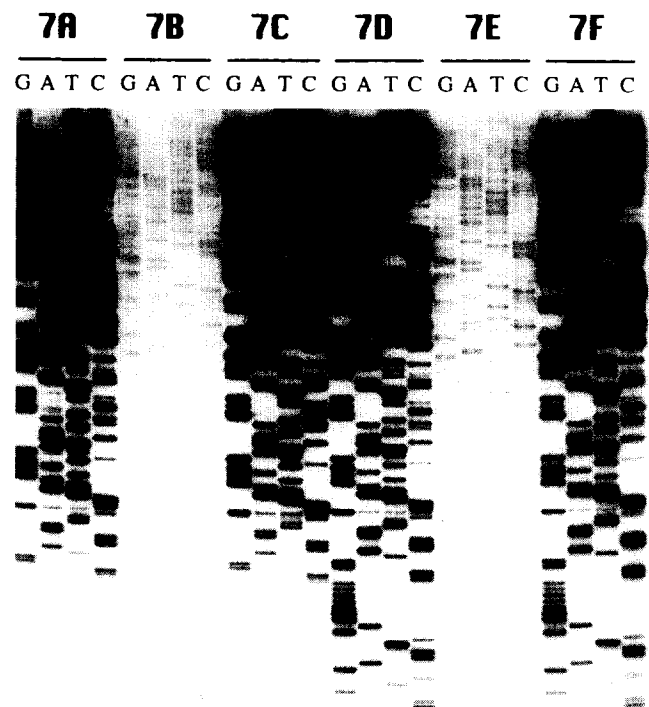


Fig. 8. Influence of TFOs on the initiation of DNA polymerization. Panels **7A** through **7F** correspond to templates presented in Fig. 7. Lanes **G**, **A**, **T** and **C** correspond to standard sequencing reactions. The efficiency of DNA synthesis is reflected by the intensity of sequencing ladders. **Methods:** 0.5  $\mu$ g of ss DNAs were mixed with 10 pmol of polymerization primers and 100 pmol of TFOs and incubated in Sequenase buffer (40 mM Tris-HCl pH 7.5/50 mM NaCl/20 mM MgCl<sub>2</sub>) for 15 min at 37°C. DNA sequencing was performed as described in the legend to Fig. 2.

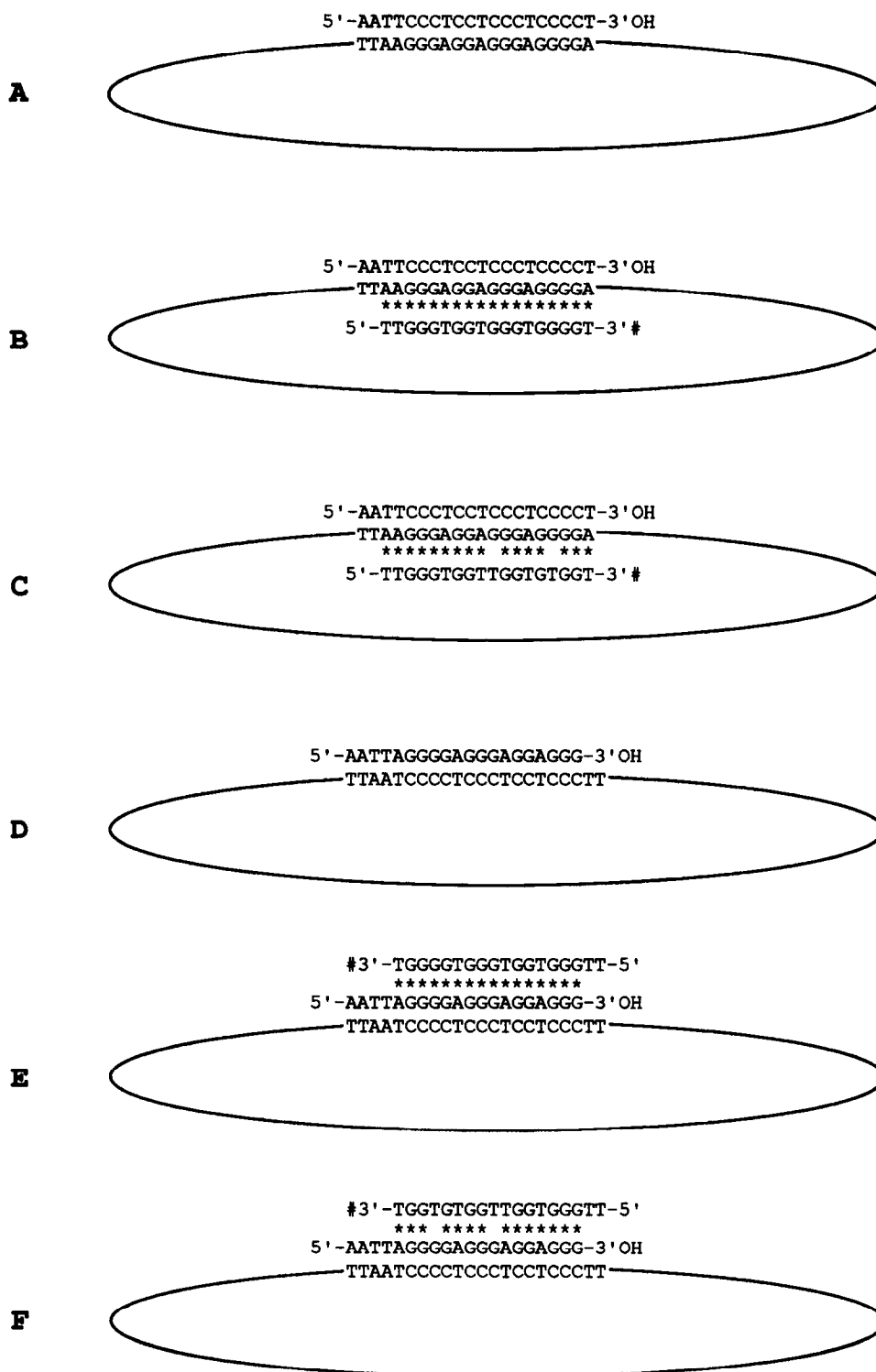


Fig. 7. DNA templates containing polymerization primers within triple-helical stretches. Hoogsteen pairs are shown by asterisks (\*), 3' amino groups are shown by symbols (#). Either homopyrimidine (A-C) or homopurine (D-F) oligos served as primers. These primers were added to the templates either alone to form W-C duplexes (A,D), or together with purine-rich TFOs (B,E) forming intermolecular triplexes consisting of CG\*G and TA\*T triads, or in the presence of mutant TFOs lacking triplex-forming ability due to G→T substitutions (C,F). The primer is a component of a triplex in both B and E; in B it is involved only in W-C base pairing, while in E it participates in W-C and Hoogsteen base pairing (see legend to Fig. 1).

these experiments, since the *E. coli* SSB protein can substitute for the T7 SSB in a reconstituted replication system of phage T7 (Nakai and Richardson, 1988). Triplexes were preformed in Sequenase buffer (10 mM

Mg<sup>2+</sup>) at 37°C. A primer was labeled with Sequenase and then elongation was carried out at different concentrations of SSB. Fig. 6 shows that SSB helps DNA polymerase to overcome a triplex barrier, but its efficiency

dramatically depends on the structure of the triplex. For H-like triplexes, even 0.25 mg/ml of SSB decreases termination, and 0.4 mg SSB/ml almost completely abolishes it. In contrast, intermolecular triplexes are relatively resistant towards SSB, and prominent termination is still observed at 0.4 mg SSB/ml.

These results are strikingly different from the data with individual polymerases, where H-like triplexes caused the strongest termination. This may be due to the fact that the H-like triplex contained a 3-nt long single-stranded loop (Fig. 1C), that is nearly the minimal binding site of the *E. coli* SSB protein, which is 4 nt (Ruyechan and Wetmur, 1976). We suggest that the loop may serve as a nucleus for SSB binding, and subsequent cooperative interaction between SSB molecules would cause complete dissociation of the complex. Intermolecular triplexes, in turn, are resistant to SSB because they do not contain ss regions.

Thus, though H-like TFOs are the strongest inhibitors of DNA polymerization *in vitro*, they may be inefficient as DNA-binding drugs *in vivo*, because the corresponding triplexes could be easy targets for SSB. Because SSB interacts with the sugar-phosphate backbone of DNA (Ruyechan and Wetmur, 1976), joining the two halves of the TFO with a non-sugar-phosphate linker may solve this problem.

#### (d) Influence of triplexes on the initiation of DNA polymerization

In the course of our studies, we noticed that TFOs sometimes stop polymerization not only prior to the target sequence but also immediately after it (see, for example, Fig. 5, lane Taq, 53°C). The same phenomenon was independently observed by others (C. Hélène, unpublished data). We suggest that in this case a triplex may be formed behind the DNA polymerase just as it passes a target sequence. We began to investigate this hypothesis by analyzing the efficiency of DNA synthesis from a primer incorporated into a triplex.

DNA templates used for this purpose are presented in Fig. 7. Either homopyrimidine (Fig. 7A–C) or homopurine (Fig. 7D–F) oligos complementary to our target sequences served as primers. These primers were annealed to the templates alone (Fig. 7A,D), in the presence of a purine-rich TFO (Fig. 7B,E), or in the presence of a mutant TFO (Fig. 7C,F) which lacks triplex-forming ability due to two G-to-T transversions (Samadashwily et al., 1993). Templates 7B and 7E are similar in that the primer is a component of the triplex. However, the homopyrimidine primer is involved in only Watson-Crick (W-C) base pairing, while the homopurine primer participates in both W-C and Hoogsteen base pairing.

Profiles of DNA sequencing on these templates are

shown in Fig. 8. One can see that the wild-type TFO dramatically (at least tenfold) reduces the efficiency of DNA synthesis. All polymerization signals are very weak, showing that the first labeling step was affected. The mutant TFO does not influence DNA synthesis. This clearly indicates that triplex formation is responsible for the indigent priming of polymerization. There are no apparent differences between the two primers used, so primers within a triplex are generally inaccessible for the DNA polymerase. We believe, therefore, that TFO prevents the DNA polymerase from recognizing a primer by forming a complex with it. This is most likely due to profound differences between double- and triple-helical DNA structures. In the cases described above, the 3'-ends of the primers were at the boundary of the triple-helical stretch. Experiments are in progress to study the efficiency of DNA polymerization when the 3'-end is at various distances from the triplex.

In summary, we observed strong and sequence-specific effects of TFOs on both initiation and elongation during DNA polymerization. SSB protein can only partially reverse the TFO-driven termination of DNA synthesis. This makes TFOs promising agents for blocking DNA replication *in vivo* in a site-specific manner. There are many potential targets for TFOs within eukaryotic genes, specifically in introns and promoter regions (reviewed in Wells et al., 1988). These targets may be utilized to block elongation of replication. At the same time, several eukaryotic origins of replication located in the human *c-myc* and histone H4 genes, hamster *dhfr* gene, yeast chromosome III, *Styloichia* tubulin gene, etc., contain the consensus Pur element: GGNNGAGGGAGAPuPuPu (Bergemann and Johnson, 1992). Targeting of such elements by TFOs could affect the initiation of replication.

#### (e) Conclusions

(1) Purine-rich TFOs block the elongation of all studied DNA polymerases (*Taq*, Vent and Sequenase) at target DNA sequences. There exists a clear correlation between the YR\*R triplex stability and the termination efficiency.

(2) *E. coli* SSB protein partially relieves this block. However, this capability depends on the triplex structure: SSB is relatively powerless with regard to intermolecular triplexes but easily disrupts H-like triplexes.

(3) DNA polymerization is blocked when the primer is incorporated in a triplex. This effect does not depend on the strand of the triplex intended as the primer.

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