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# Initiation of DNA replication by DNA polymerases from primers forming a triple helix

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## ABSTRACT

**Despite extensive studies on oligonucleotide-forming triple helices, which were discovered in 1957, their possible relevance in the initiation of DNA replication remains unknown. Using sequences forming triple helices, we have developed a DNA polymerisation assay by using hairpin DNA templates with a 3′ dideoxynucleotide end and an unpaired 5′-end extension to be replicated. The T7 DNA polymerase successfully elongated nucleotides to the expected size of the template from the primers forming triple helices composed of 9–14 deoxyguanosine-rich residues. The triple helix-forming primer required for this reaction has to be oriented parallel to the homologous sequence of the hairpin DNA template. Substitution of the deoxyguanosine residues by N7 deazadeoxyguanosines in the hairpin of the template prevented primer elongation, suggesting that the formation of a triple helix is a prerequisite for primer elongation. Furthermore, DNA sequencing could be achieved with the hairpin template through partial elongation of the third DNA strand forming primer. The T4 DNA polymerase and the Klenow fragment of DNA polymerase I provided similar DNA elongation to the T7 polymerase–thioredoxin complex. On the basis of published crystallographic data, we show that the third DNA strand primer fits within the catalytic centre of the T7 DNA polymerase, thus underlying this new property of several DNA polymerases which may be relevant to genome rearrangements and to the evolution of the genetic apparatus, namely the DNA structure and replication processes.**

## INTRODUCTION

One essential and critical step of DNA replication is the initiation of replication, which requires the precise positioning of the oligonucleotide primer with an available hydroxyl residue (1). Due to their binding efficiency, several base pairs are generally required for primer–single stranded DNA pairing and initiation of DNA replication. X-ray diffraction studies of the *Escherichia coli* polymerase I Klenow fragment (2), *Taq* (3), human β (4), T7 (5) and *Bacillus stearothermophilus* DNA polymerases (6) complexed with their primer-template and nucleotide triphosphate have made it possible to characterise the main features in their active centre and the importance of metal ions in the catalytic process. Since the discovery of the association between three oligonucleotide strands (7), the configuration of this basic feature has been extended (8,9) notably to the parallel or antiparallel orientation for the polypurine third strand, which binds to the base pairs of the major groove of DNA through Hoogsteen bonds (10).

The intramolecular triplex from single-stranded DNA may be detected *in vivo* (11), as well as intermolecular structures that may result from DNA polymerisation on triple helix-forming structures (12), leading to the arrest of DNA synthesis *in vivo* (13) and *in vitro* (14). Triple helix-forming oligonucleotides (TFOs) have therefore been widely used in attempts to modulate gene expression (15–17) or to induce gene mutations (18).

Another implication of TFOs could be their involvement in genome rearrangement. Indeed, we previously observed that the direct repeats (DR) or homologous sequences present in the mitochondrial genome at potential mitochondrial DNA rearrangements are polypyrimidine-rich in the template sequence, suggesting a plausible basis for slipped mispairing of the replication complex from one DR to the other base-paired DR (19,20). This implies that DNA replication may start from oligonucleotides forming a triple helix, which has not been reported previously. In this article, we demonstrate that various DNA polymerases may indeed start replication from a DNA triple helix, thus suggesting a new basis for genome rearrangements through the DNA polymerase distributivity.

## MATERIALS AND METHODS

Oligonucleotides and *Taq* DNA polymerase were from Eurogentec (Seraing, Belgium). T7, T4 DNA polymerases, Sequenase and the T7 sequencing kit were purchased from Pharmacia Biotech (Orsay, France). DNA polymerase I (Klenow fragment) was from Roche Diagnostics (Meylan, France). [γ-$^{32}$P]ATP was from ICN (Orsay, France).

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A single-stranded control sequence (Dupl 50) complementary at its 3′ end (bold) to the primer TFO Dupl was used. Dupl 50, 5′-AGCTGTATCACGTTCAGTCCTCGCTCTTCTACTAT-GAACC**CCCCTCCCCA**-3′.

TFOs were 5′-end-labelled by 10 U T4 polynucleotide kinase and 50 µCi [γ-$^{32}$P]ATP. Non-incorporated ATP was separated by Sephadex G50 spin filtration.

### Gel shift assays

Aliquots (1–2 pmol) of $^{32}$P-5′-end-labelled TFOs were incubated for 2 min at room temperature in 10 µl of 100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 15 mM DTT with 70 pmol unlabelled oligonucleotide. The sample products were then separated by electrophoresis on a 20% polyacrylamide gel in 50 mM HEPES buffer pH 7.2 containing 10 mM $MgCl_2$ and subjected to autoradiography for 1 h at –80°C.

### Replication assay

Aliquots (1–2 pmol) of $^{32}$P-5′-end-labelled TFOs were incubated in 10 µl of 100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 15 mM DTT, 0.2 mM dNTP and 70 pmol template. Reactions were started with the addition of 2 U T7 DNA polymerase, 5 U T4 DNA polymerase, 1 U DNA polymerase I Klenow fragment, 4 U Sequenase or 2.5 U *Taq* DNA polymerase. After 2 min incubation at room temperature, samples were heat-denatured then subjected to electrophoresis on a 20% polyacrylamide gel containing 8 M urea in 50 mM Tris–borate, 2 mM EDTA buffer pH 7.5 and subjected to autoradiography.

### DNA sequencing

Replication reactions were carried out as above in the presence of dideoxynucleotide triphosphates for 5 min at room temperature using the T7 sequencing kit.

### Triplex modelling and fitting in the bacteriophage T7 DNA polymerase

The crystal structure of a bacteriophage T7 DNA replication complex refined at 2.2 Å resolution by Doublié *et al*. (5) was used as the construct framework. The complex structure was obtained in three successive and very straightforward steps.

The first step consisted of substituting the first 10 bases of the crystal structure of the dodecamer d(CGCGAATTCGCG) (21) in order to build the double strand 5′-CCCCTCCCCA-3′, 3′-GGGGAGGGGT-5′. Substitution was performed by superimposing the respective glycosidic bonds and keeping the base mean-plane unchanged.

The second step of the procedure was to build a third strand parallel to the purine-rich strand. For this purpose the triplex-containing crystal structure of d(GGCCAATTGG) (10) was used as the starting point. The two duplexes were superimposed and then the first two bases of the third strand of the triplex were positioned. The triplex was then completed by an iterative process described by Vlieghe *et al*. (10).

The last step was achieved by a least square fitting of the single strand d(CCCCTCCCCA) with the corresponding strand of the template observed in the crystal structure of the bacteriophage T7 DNA complex.


**Figure 1.** Representation of the oligonucleotides used in this study.The templates used in this study were Dupl 44, representing the sequence of the second DR found in a duplication–triplication with the hairpin structure used for *in vitro* replication with a 3′-dideoxy end and a 5′-end template. The 10 nucleotides of the DR are shown in bold. Del 50 is similar except that it represents the sequence found at the level of the second repeat found in the common deletion 13 nt long. G# and G° designate 2′3′-dideoxynucleotides and 7-deaza-2′-deoxyguanosine, respectively.

## RESULTS

### Formation of DNA triple helix at the level of DRs involved in DNA rearrangements

For this purpose, we designed gel shift assays with the third strand named TFO, which corresponds to the neo-synthesised H strand of the DR, and oligonucleotides forming hairpins containing the DRs. The target oligonucleotides were either fully double-stranded (Dupl 64), or single-stranded for further replication assays with a 5′-end extension of 20 nt and a 3′-end substituted by a 2′3′-dideoxynucleotide (Dupl 44) for the sequence involved in the duplication–triplication (20) and Del 50 for the sequence involved in the deletion (22). TFOs were synthesised homologous to the 3′-end of the hairpin forming the DR, but differing in their length by 1 nt at their 3′-end, with the TFO Dupl 1 containing 1 nt less than the DR and TFO Dupl 3 containing 1 nt more (Fig. 1).

Studies of intermolecular interactions were performed by gel retardation assays with 5′-end-labelled TFO and the DNA targets. Figure 2 shows that Dupl 2 and Dupl 3 bound to the hairpin Dupl 44 (lanes 5 and 8) and to the duplex Dupl 64 (lanes 6 and 9). In contrast, TFO Dupl 1 in which one nucleotide was eliminated did not demonstrate immediate binding but

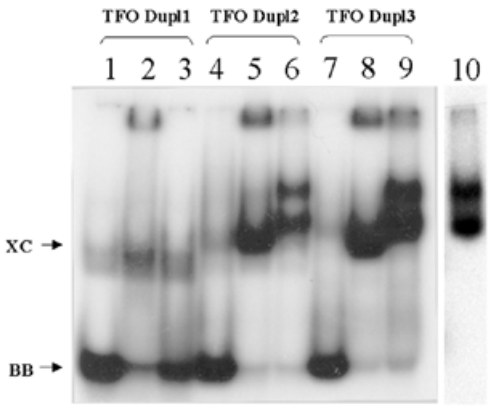


**Figure 2.** Association between TFO and Dupl 44 and Dupl 64 by gel retardation assays. Gel shift assays between $^{32}$P-5′-end-labelled TFO Dupl 1, Dupl 2 and Dupl 3 and various target unlabelled DNA. Upon mixing, the reagents were loaded on native polyacrylamide gel in the presence of 10 mM $MgCl_2$ and subjected to electrophoresis at room temperature, then to autoradiography at –80°C for 1 h. Lanes 1, 4 and 7, control of specificity with Del 50 as target; lanes 2, 5 and 8, association with Dupl 44; lanes 3, 6 and 9, association with Dupl 64. Lane 10, 0.5 pmol $^{32}$P-5′-end-labelled Dupl 64. BB and XC mark the positions of bromophenol blue and xylene cyanol [12 and 45 nt, respectively (31)].

required an incubation period of 16 h. No binding could be detected between oligonucleotides corresponding to the duplication and the target deletion, even for longer incubation times (lanes 1, 4 and 7), thus suggesting specific retardation of the TFO Dupl by Dupl 44 and Dupl 64. The surprising upper band viewed in lanes 6 and 9 with Dupl 64 was further explored by studying its migration rate in the absence of TFO. Lane 10 of Figure 2 shows that this $^{32}$P-5′-end-labelled oligonucleotide still migrated as two distinct species on a native polyacrylamide gel. The extraction, heat denaturation and remigration of the upper band led to the same migration rate as in the lower band.

These data show that the complete double-stranded Dupl 64 displays either two different conformations or is composed of a dimer by head-to-tail pairing of complementary monomers. The experiments in the presence of the $^{32}$P-5′-end-labelled TFO Dupl, as compared to the $^{32}$P-5′-end-labelled Dupl 64, thus show that the TFO Dupl binds to the double helix of the Dupl 64 and thereby forms a triple helix. As the TFO Dupl binds both the duplex Dupl 64 and the hairpin Dupl 44, they probably do not associate with its 5′-end single-stranded extension. Instead, they probably bind the common double-stranded sequence of the DR, thus forming a triple helix.

## Oligonucleotides forming triple helix primers can be elongated by T7 DNA polymerase

Due to the lack of inherent DNA melting activity of DNA polymerases, we had to provide a hairpin single-stranded DNA as a template to the DNA polymerase elongation assay. Additionally, its 3′ hydroxyl end was substitued by a 2′3′-dideoxynucleotide in order to prevent its *in vitro* elongation from its intramolecular 3′ end. In addition, we provided the system with a $^{32}$P-5′-end-labelled TFO (10 nt), which might be elongated by the DNA polymerase in the presence of deoxynucleotide triphosphates, due to their 5′ extension of 20 nt from the Dupl 44 template, thus leading to an expected product of 30 nt.

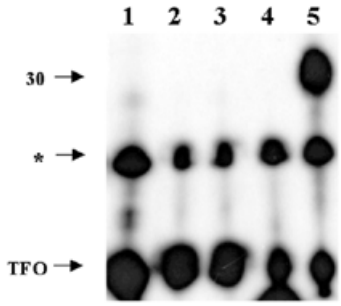


**Figure 3.** Conditions for the elongation of 5′-end-labelled TFO Dupl 2 by T7 DNA polymerase. Autoradiogram of $^{32}$P-5′-end-labelled TFO Dupl 2 upon migration in a 20% polyacrylamide, 8 M urea gel, following incubation under various conditions. Lane 1, TFO Dupl 2 + Dupl 44 + 10 mM $MgCl_2$; lane 2, TFO Dupl 2 + 0.2 mM dNTP + T7 DNA polymerase; lane 3, TFO Dupl 2 + Dupl 44 + dNTP + T7 DNA polymerase; lane 4, TFO Dupl 2 + T7 buffer + dNTP + T7 DNA polymerase; lane 5, TFO Dupl 2 + Dupl 44 + dNTP + T7 buffer + T7 DNA polymerase. The abnormal spot is shown by an asterisk.

The fate of the $^{32}$P-5′-end-labelled TFO Dupl 2 was then checked under various incubation conditions in a T7 DNA polymerisation assay directed by Dupl 44. Figure 3 shows the autoradiogram of the elongation of TFO Dupl 2 to the expected size of 30 nt (lane 5), which requires all of the common molecules for DNA replication, namely the Dupl 44 template, dNTP, $MgCl_2$ and the T7 DNA polymerase. The abnormal spot present in all the lanes probably corresponds to dimers of TFO Dupl 2 due to their high deoxyguanosine content (23).

We conclude that T7 DNA polymerase may elongate the oligonucleotide, which corresponds to the neo-synthesised sequence of the direct repeat. This result therefore indicates that a third DNA strand can be used as a primer, which may enable its further elongation on a single-stranded template.

## Generalisation

We then tested the various TFOs and their corresponding hairpin structures to be elongated by the T7 DNA polymerase. Figure 4A, lane 3, shows the elongation of TFO Dupl 1 to the expected size of 30 nt; the lack of this band in lane 2 is a result of the omission of DNA polymerase. Similar elongation results were obtained using TFO Dupl 3 and Dupl 44 as template (Fig. 4A, lane 7). These results were extended by using Del 50 as a template, a system mimicking the formation of the common deletion (Fig. 4B) and by using the $^{32}$P-5′-end-labelled TFOs Del 1, Del 2 and Del 3 as primers.

These different experiments using various templates and TFOs therefore provide general evidence that a third DNA strand may prime DNA replication using the T7 DNA polymerase.

## Specificity of the elongation

Because the primers display the same sequence and orientation as the 3′-end nucleotides of the hairpin templates, they might displace this sequence and base-pair through Watson–Crick bonds to the complementary strand of the template, even with TFO concentration 30–70-fold lower than that of the template in the binding and the replication assays. This possibility was indirectly tested by substituting the 2′-deoxyguanosines by 7-deaza-2′-deoxyguanosines in Dupl 44 (Deaza Dupl 44) and Del 50 (Deaza Del 50) at the level of the homologous sequence

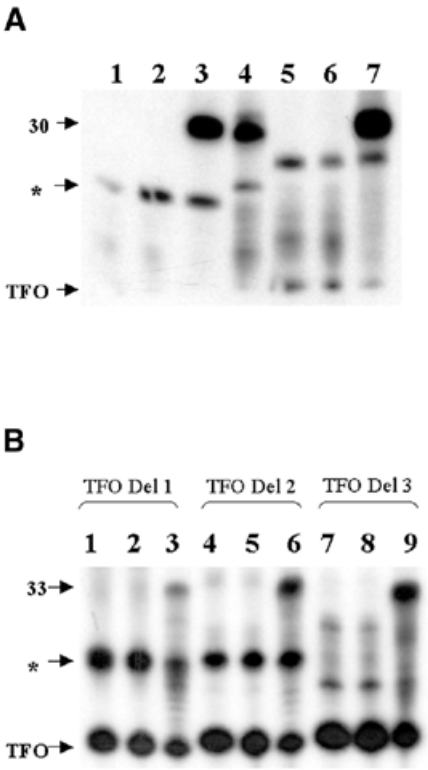


**Figure 4.** Elongation of different TFOs with various templates by T7 DNA polymerase. (**A**) The template is Dupl 44. Lane 1, TFO Dupl 1 alone; lane 2, TFO Dupl 1 in the replication assay without T7 DNA polymerase; lane 3, Dupl 1 in the replication assay with T7 DNA polymerase; lane 4, complete system with TFO Dupl 2; lane 5, TFO Dupl 3 alone; lane 6, TFO Dupl 3 in the replication assay without T7 DNA polymerase; lane 7, TFO Dupl 3 in the replication assay with T7 DNA polymerase. (**B**) The template is Del 50. Lanes 1, 4 and 7, TFO Del alone; lanes 2, 5 and 8, TFO Del in the replication assay without T7 DNA polymerase; lanes 3, 6 and 9, TFO Del in the replication assay with T7 DNA polymerase.

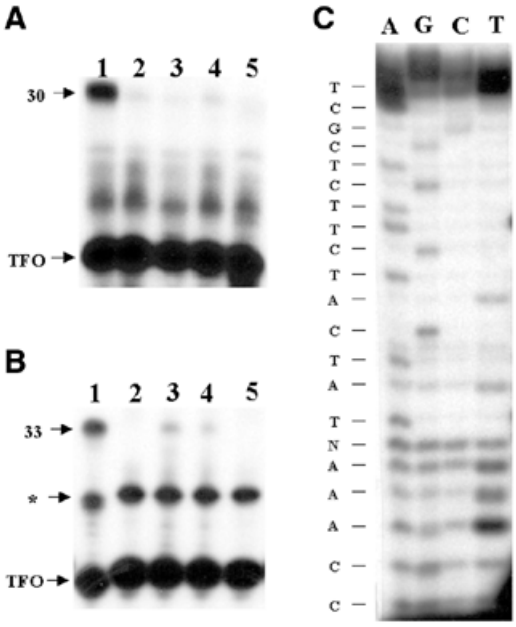


**Figure 5.** Specificity of the primer and identification of the replication product. (**A**) Elongation of TFO Dupl 2 using Dupl 44 or Deaza Dupl 44 as template. DNA elongations were with either template Dupl 44 (lanes 1 and 2) or with N7 Deaza Dupl 44 (lanes 3 and 4) with T7 DNA polymerase (lanes 1 and 3) or without T7 DNA polymerase (lanes 2 and 4). Lane 5 is the control TFO Dupl 2. (**B**) Elongation of TFO Del 2 using Del 50 or Deaza Del 50 as template. Lane 1, complete system with T7 DNA polymerase; lane 2, without T7 DNA polymerase; lane 3, complete system directed by Deaza template; lane 4, without T7 DNA polymerase; lane 5, TFO alone. (**C**) Sequence of the TFO Dupl 1 DNA extension using Dupl 44 as template and T7 DNA polymerase. The sequence of the template strand (3′→5′, bottom to top) is indicated on the left. Note the A N which should be G.

of the TFOs. This modification prevents bonds between the N7 of the deoxyguanosines of the DNA in double helix and the N2 of the deoxyguanosines of the third strand (24). Using these templates, we were unable to detect significant DNA elongation as compared with the Dupl 44 and Del 50 (Fig. 5A and B, lanes 1 and 3). Therefore, the deoxyguanosines of the TFOs require the N7 bond from the deoxyguanosines of the direct repeats for their elongation. These experiments led us to conclude that the TFO indeed needs the bonds with the homologous sequence of the DR to enable its DNA elongation.

However, the observed spots attributed to elongation of the primers at the level of the DR might correspond to other unexpected binding or artefactual polymerisation events. We therefore sequenced the product of the Dupl 44 using 5′-end-labelled TFO Dupl 1 as primer according to F.Sanger, with a partial elongation by the T7 DNA polymerase in the presence of dideoxynucleotide triphosphates. The sequencing conditions were similar to those used for the gel shift assays and the replication reactions, except for the dideoxynucleotide inclusion. The autoradiogram in Figure 5C shows the partial replication sequence of the template strand. Similar results were obtained with TFO Dupl 2 and TFO Dupl 3 (data not shown).

These data therefore confirmed that the species evidenced at 30 nt with Dupl 44 as template and the replication assay corresponds to true replication products primed by oligonucleotides forming a triple helix.

Taken together, our experiments show first that oligonucleotides ~10 nt in length may specifically bind to duplex DNA presenting a homologous sequence, thus indicating the formation of a binary complex through Hoogsteen bonds, a feature commonly encountered in the interaction of triple helices (10). Secondly, this property enables the third DNA strand to be positioned within the catalytic centre of the T7 DNA polymerase in such a way that elongation may be evidenced under these conditions. This new property might be restricted to the T7 DNA polymerase, which could be a unique feature among the DNA polymerase family.

### Elongation of triple helices by other DNA polymerases

We therefore tested the possibility of elongating primers TFO Dupl using Dupl 44 as template and other DNA polymerases, namely *Taq* polymerase, T4 DNA polymerase, the Klenow fragment of *E.coli* DNA polymerase and Sequenase. As all DNA polymerases were tested in the T7 buffer, we controlled their activities (Fig. 6, lane 1 for each polymerase) under these conditions by using a linear template of length 50 nt including the single-stranded sequence complementary to the TFO Dupl (Dupl 50). Figure 6 shows that the *E.coli* DNA polymerase I

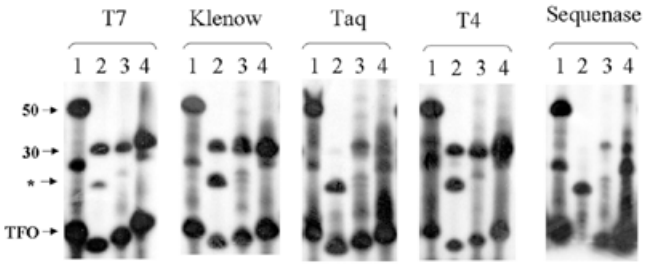


**Figure 6.** Elongation of various TFO Dupl by different DNA polymerases. Autoradiogram showing the replication products obtained upon incubation in the replication assay primed by TFO Dupl 2 directed either with a linear control DNA, Dupl 50 (lanes 1), or with the hairpin Dupl 44 template with TFO Dupl 1 (lanes 2), TFO Dupl 2 (lanes 3) or TFO Dupl 3 (lanes 4) as primers.

Klenow fragment and the phage T4 DNA polymerase behaved similarly to phage T7 DNA polymerase.

In contrast, neither *Taq* polymerase nor Sequenase could use TFO Dupl 1 as a primer and could hardly use TFO Dupl 2 and TFO Dupl 3 under the same assay conditions as for T7 DNA polymerase, although the control replication was effective (Fig. 6, lane 1 for each polymerase). These findings argue against a possible displacement of the homologous sequence of the hairpin template by the TFO, as all the results would be similar if the converse were true. On the other hand, they point to subtle differences in the catalytic centres of these various DNA polymerases, either in the accommodation of the third DNA strand, or in the suitability for the recognition and elongation of the primer triple strand due to their catalytic potency. It was therefore of interest to model the interaction between the triple helix-forming primer and the catalytic centre of a known DNA polymerase.

### Modelling of the triple helix initiation complex with DNA polymerases

The three-dimensional structure of the T7 DNA polymerase has been solved at 2.2 Å resolution with its template primer (5), as well as the DNA triple helix formed by a deoxyguanosine-rich sequence parallel to the homologous strand (10). We thus investigated the position of the primer-forming triple helix by replacing the previous coordinates of the template primer in the co-crystal complex by coordinates of the triple helix modified according to the sequence used for these studies. The model shown in Figure 7 indicates that the primer (red strand) lies within the major groove of the DNA duplex through Hoogsteen interactions. All the bases of the third strand are located on the opposite side of the protein, but the last 3′-end nucleotide is located in the vicinity of the main amino acids involved in the catalysis, namely Asp475, Asp654 and Glu655.

This model, which is probably a rough approximation of the reality and which will require co-crystallisation and X-ray diffraction studies, therefore provides further evidence that a third DNA strand could be involved in a new mechanism of DNA polymerisation.

## DISCUSSION

We undertook these studies in order to demonstrate the possible initiation of replication from primers forming a triple

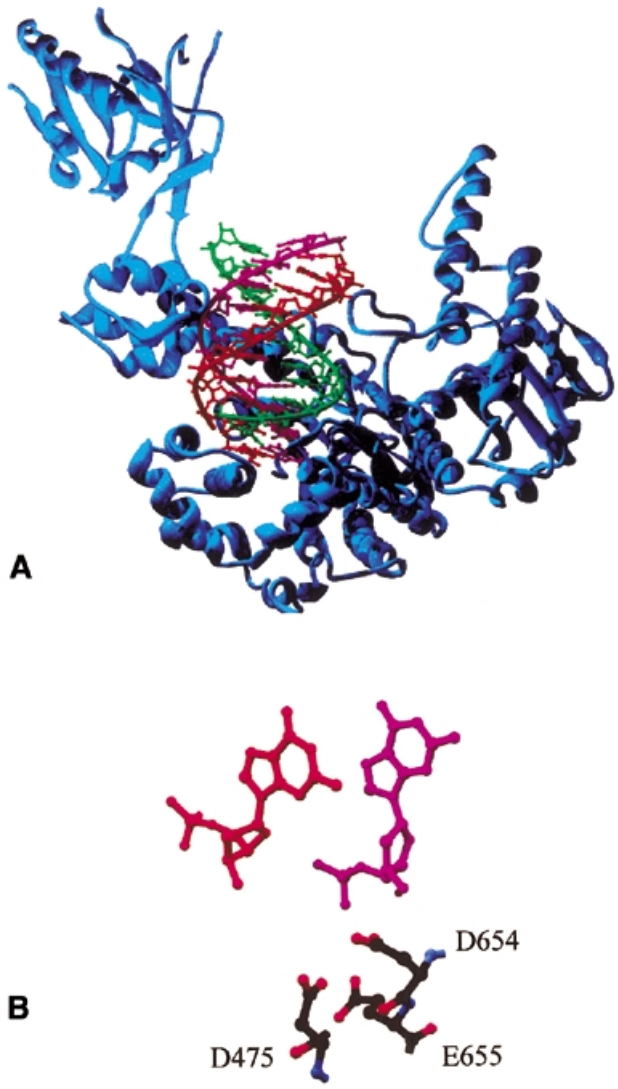


**Figure 7.** Three-dimensional model of the T7 DNA polymerase complexed with the triple helix primer and the direct repeat of Dupl 44. This Figure was produced with SPDBV (32) and rendered with POV-Ray. (**A**) The green strand represents the template DNA which is linked above (not represented) by the four thymidine residues to the magenta strand representing the hairpin Dupl 44, whose 5′ template extension probably lies towards the left of the Figure. The red strand represents TFO Dupl 2 of the same sequence and polarity as the magenta one. (**B**) Proposed position of the 3′-end deoxyguanosine of TFO Dupl 2 (red) together with the 2′3′-dideoxyguanosine of Dupl 44 (magenta) with the main amino acid residues involved in the catalytic process of T7 DNA polymerase (Asp475, Asp654 and Glu655).

helix. For these investigations, we used sequences of direct repeats of the mitochondrial genome involved in mitochondrial (mt)DNA rearrangements. We present the first evidence that deoxyguanosine oligonucleotides of ~10 residues may interact immediately and specifically with a double-stranded sequence having a homologous strand and might further enable DNA replication by DNA polymerases. These oligonucleotides thus forming triplexes can be faithfully elongated by the T4, T7 and Klenow fragment DNA polymerases by using a hairpin DNA template with a homologous strand; substitution by an N7 deazadeoxyguanosine prevents any elongation. This indicates

that elongation of the primers needs an association through hydrogen bonds between the homologous strands.

The sequencing experiments showed the correct elongation of the triple helix-forming primer by T7 DNA polymerase under similar experimental conditions to the gel shift assays and the replication reaction. We thus conclude that TFOs may prime replication, provided that they are oriented parallel to the homologous strand, thereby leaving a 3′-hydroxyl end available for elongation. Although we cannot totally rule out that a certain displacement of the homologous strands occurs within the enzyme's active site, we think that a real triple helix may be accommodated within the catalytic centre as TFO Dupl 1 may also be elongated. Unlike Dupl 2, the latter oligonucleotide lacked apparent physical interaction with the hairpin template Dupl 44 though elongation experiments were positive. Three additional hydrogen bonds in Dupl 2 (10) may account for this result. A similar difference has also been observed under other circumstances, by UV cross-linking various oligonucleotides 5′-substituted by a psoralen and a target DNA (P.Lestienne, unpublished data). Our replication assay suggests that DNA polymerases can interact with TFO Dupl 1 and position it at the level of the DR, thus enabling further elongation, at least for T7, T4 and *E.coli* DNA polymerase I. Other DNA polymerases such as Sequenase and *Taq* DNA polymerases did not show any significant elongation of this primer, thus providing a fifth argument against a simple displacement of the homologous strand by the primer, since results similar to those obtained with T7 DNA polymerase would have been found.

Taken together, our experiments provide initial evidence that TFOs may be elongated by various DNA polymerases. These results are also compatible with modelling crystallographic studies of *E.coli* DNA polymerase I and of the T7 DNA polymerase complexed with their template-TFO primer, as no specific bond between the major groove of the template primer and DNA polymerase could be detected (2,5). Furthermore, our modelling experiments are in agreement with our data suggesting a certain flexibility within their catalytic centres, because the TFO Dupl 1 may also serve as a primer, though its 3′ end is included in the double helix of the DR.

Our data also suggest a plausible basis for gene rearrangements. If eukaryotic DNA polymerase δ were to behave to some extent as the bacterial counterpart, then deletion of some sequences could occur from mispairing during the replication process from the first DR to the second through the distribution of DNA polymerase and of the replication complex, thus bypassing the intervening sequence. Furthermore, duplications may have emerged through replication errors by binding the guanosine-rich sequence of the 3′-end of replicated DNA to the polymerase and by binding to a second identical duplex sequence from which the replication might start again.

The structure of the triple helix proposed here, with an orientation parallel to the homologous strand, appears similar to recombination intermediates mediated through RecA but for shorter sequences (25). Moreover, triple helix DNA binding proteins have been identified in eukaryotes (26–28) and we speculate that they may induce the present mechanism by positioning the neo-synthesised DNA strand in the homologous duplex sequence.

Triple-stranded DNA intermediates have been suggested to play a role during the process of chemical ligation of preformed oligonucleotides (29). The vestige of triple helix oligonucleotide as a primer might function today in the priming system of the mammalian mtDNA. Indeed, the processed RNA transcript from the human mtDNA light strand promotor is used as a primer for mtDNA replication and binds to the closed circular DNA through the formation of a triple helix (30). Hence these present data may throw light on a mechanism for gene rearrangements and for the evolution of the duplication process through TFOs.

## ACKNOWLEDGEMENTS

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