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INHIBITION OF REPLICATION INITIATION BY TRIPLE HELIX - FORMING OLIGONUCLEOTIDES

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SUMMARY: Oligonucleotide-directed triple helix formation constitutes a new approach to block gene expression via transcription inhibition. In addition triple helices might inhibit replication. We have examined the capacity of triple helix-forming oligonucleotides to inhibit the initiation of replication on a single-stranded DNA template using T7 DNA polymerase (Sequenase). We show that triple helix formation at the primer initiation site efficiently inhibits DNA polymerization, by preventing binding of the polymerase. The effect is dependent on the distance between the 3'-end of the primer and the triple helix boundary. Inhibition becomes ineffective when this distance is greater than 3 nucleotides. The presence of three base-pairs outside the triple-helical region on the 3'-side of the primer is therefore sufficient to allow for initiatiation of DNA replication.

Oligonucleotides can bind to the major groove of oligopurine-oligopyrimidine sequences of double-stranded DNA to form a local triple helix (1, 2, 3). When the third strand oligonucleotide contains pyrimidines it binds in a parallel orientation to the purine strand of the duplex via Hoogsteen hydrogen bonding leading to the formation of T.A*T and C.G*C$^+$ base triplets. Triple helix formation requires cytosine protonation and is pH dependent. Purine oligonucleotides bind to duplex DNA under physiological pH conditions through the formation of C.G*G and T.A*A base triplets in an antiparallel orientation with respect to the oligopurine target sequence (4, 5). Oligonucleotides containing guanines and thymines can also form triple helices with duplex oligopurine.oligopyrimidine sequences with an orientation that is sequence-dependent (6, 7).

Oligonucleotide-directed triple helix formation has been used to block gene expression at the transcriptional level (8-16). Studies on replication are less abundant. The progression of DNA polymerases on a single-stranded template

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was inhibited by clamp oligonucleotides which form a triple-helical complex (17, 18). On a double-stranded plasmidic template, an arrest of polymerization near an intermolecular triple helix was described by Hacia et al. (19). More recently, Samadashwily and Mirkin (20) showed that an intermolecular triple helix formed on a single-stranded template induced a stop of polymerization by several DNA polymerases. They also showed that triplex formation on a plasmidic template could inhibit initiation of replication when the primer was involved in triplex formation. Previous studies have shown that an oligonucleotide-intercalator conjugate targeted to the origin of replication of SV40 inhibited viral replication in cell cultures (21).

In this study we report the efficient and specific inhibition of initiation of DNA replication by T7 DNA polymerase (Sequenase) through intermolecular triple helix formation on a DNA template. Triple helix-forming oligonucleotides of length varying from 17 to 29 nucleotides were tested for their ability to inhibit DNA synthesis when bound to the double helix formed by a primer oligonucleotide with the DNA template.

MATERIALS AND METHODS

Oligonucleotides and DNA polymerase

Oligonucleotides were purchased from Eurogentec (Belgium). They were purified on a 20% denaturing polyacrylamide gel. Sequences of the oligonucleotides used in this study are shown on figure 1. Sequenase (T7 DNA polymerase) was purchased from USBiochemicals.

Gel mobility shift analysis of triplex formation

The 55-mer DNA template (55-mer CT or 55-mer GA in figure 1) was incubated with 5'-^{32}P end-labeled 33-mer or 34-mer (see sequence on figure 1). Triple helix was pre-formed by incubating 25nM of duplex and 5μM of third strand oligonucleotide for 2 hours at 20°C in 1X sequenase reaction buffer (20mM Tris-HCl pH7.5, 25mM NaCl, 10mM $MgCl_2$). Samples were analyzed by electrophoresis at room temperature on a 10% polyacrylamide gel containing 50mM HEPES pH7.2, 10mM $MgCl_2$ as running buffer, and analyzed with a Molecular Dynamics phosphorimager.

Inhibition of DNA synthesis

In order to obtain radiolabelled replication products, the 55-mer template (55-mer CT or 55-mer GA in figure 1) was incubated with either 5'-^{32}P end-labeled 33-mer or 34-mer (figure 1). The duplex (25nM final) was pre-formed in 2X sequenase reaction buffer by heating for 1 min. at 90°C and slowly cooling to room temperature. Triplex-forming oligonucleotides were then added to the duplex at the indicated concentration and incubated for 2 hours at 20°C to pre-form the triple-helix. The polymerization was initiated by adding 225μM of each dNTP and 1.3 Units of Sequenase (USBiochemichals) followed by incubation for the specified time at 30°C. The final volume was 10μl. The reaction was stopped by addition of 5μl of stop solution (95% Formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene Cyanol) and products

were analyzed on a 12% denaturing polyacrylamide gel. Data were quantitated with a Molecular Dynamics phosphorimager.

RESULTS AND DISCUSSION

Replication experiments used a 55-mer oligonucleotide template corresponding to a fragment of the murine c-Ki-ras promoter (position -332, -278) (Hoffman et al., 1990). This sequence includes a 29 base pair polypurine.polypyrimidine stretch which is a target for triplex formation. Polymerization was initiated by a 5'-end labeled 33 or 34-mer primer complementary to one of the 55-mer single strands of the template (see sequences in figure 1). The expected length of the product of polymerization with sequenase was 45 or 43 nucleotides depending on the 55-mer single strand used as template. We tested the ability of several oligonucleotides containing guanines and thymines to inhibit the initiation of polymerization *via* triple helix formation at the primer site.

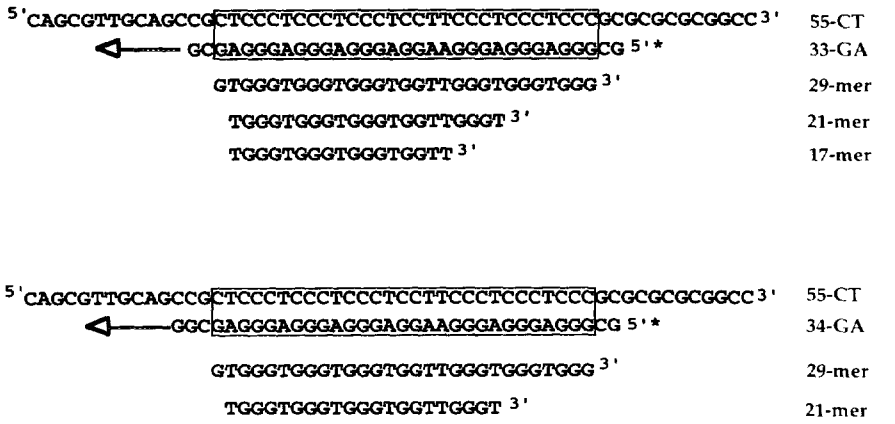
Triplex stability

DNase I footprinting and gel shift experiments allowed us to show that the GT-containing oligonucleotides were able to bind efficiently to the 55-bp duplex target and to form a stable triple helix at 37°C and pH7.2 (data not shown). They bind in an antiparallel orientation with respect to the purine-rich strand of the duplex . We then investigated the binding of the same oligonucleotides to the duplexes formed by each of the 55-mer single strands with the 33-mer or 34-mer, respectively (see sequences on figure 1). The triplex was preformed in the polymerization buffer and then analyzed by the gel shift method (Figure 2). In the presence of 5μM of 29-mer or 21-mer all the double-stranded target was shifted to a position assigned to the triple helix indicating that the triplex remained stable throughout the duration of migration at pH 7.2.

Kinetics and concentration dependence of replication inhibition

To study the ability of triplex-forming oligonucleotides to inhibit the initiation of polymerization, elongation experiments were performed in the presence of 5μM of various third strand oligonucleotides (figure 3). Under these conditions all the primer-template duplex was complexed to the third strand as shown by gel shift experiments. We studied the activity of T7 DNA polymerase (Sequenase) using a 55-mer single strand as a template. Depending on the 55-mer used as template, the polymerization was initiated by a purine or a pyrimidine-rich ^{32}P-labelled primer 33-mer (figure 1). The radiolabelled

Pyrimidine-rich template oligonucleotide



Purine-rich template oligonucleotide

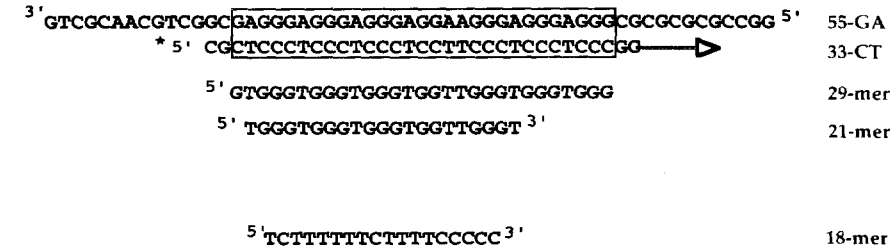


Figure 1. Schematic representation of the oligonucleotides used in this study.
The templates are 55 nucleotide long and contain a 29 nucleotide-long polypyrimidine or polypurine stretch (boxed) (55-mer CT and 55-mer GA, respectively). The oligonucleotides used as primers for polymerization are 33- or 34-mer and are complementary either to the 55-mer CT or to the 55-mer GA. The arrow indicates the direction of polymerization. The full-length products are 43 or 45 nucleotides when 33-mer CT or 33-mer GA are used as primers, respectively.
Triple helix-forming oligonucleotides (29, 21 and 17-mers) are composed of thymines and guanines and bind in a anti-parallel orientation with respect to the purine-rich strand of the duplex. The sequence of the 18-mer control is indicated.

elongation product was analyzed on a 10% denaturing polyacrylamide gel. In the absence of triple helix-forming oligonucleotide, the expected length of 45 nucleotides was obtained with the purine-rich 33-mer primer on the 55-mer CT template. As shown in figure 3, in the presence of 5μM of the 29-mer oligonucleotide the polymerization using 55-mer CT as a template was strongly inhibited as compared to a 18-mer oligonucleotide control that could not form a triple helix. A 29-mer GT rich oligonucleotide control gave no inhibition (data

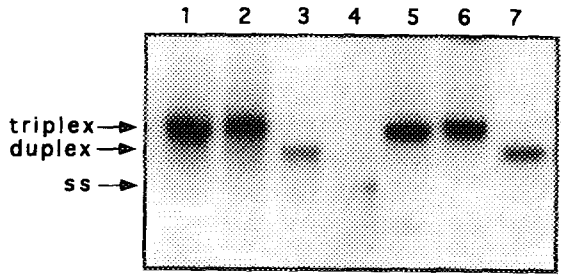


Figure 2. Gel shift experiments.
The 33-mer GA (or 33-mer CT) was end-labeled and incubated with the unlabeled 55-mer CT (lane 3) (or 55-mer GA, lane 7). The double helix was then incubated at pH 7.5, as described under materials and methods, with 5µM of 29-mer (lanes 2 and 6) or 21-mer (lanes 1 and 5) (see figure 1 for sequences). lane 4 : end-labeled 33-mer CT alone; ss: single-strand.

not shown). Inhibition with triplex-forming 21-mer and 17-mer oligonucleotides was less efficient, these shorter oligonucleotides giving termination efficiencies of 40% and 30%, respectively, after 5min of incubation whereas the 29-mer gave 90% inhibiton. When the purine-rich 55-mer GA strand was used as a template, 5µM of 29-mer inhibited replication to the same extent as with the 55-mer CT template, whereas the 21-mer and 17-mer were inefficient at inhibiting elongation of the purine strand (data not shown).

The concentration dependence of replication inhibition is shown on figure 4 for the 55-mer GA template. The 29-mer inhibited replication in a concentration-dependent manner whereas the 21-mer was inefficient. These results indicated that formation of the 29-mer oligonucleotide triplex strongly inhibited polymerization. The absence of truncated product showed that the 29-mer oligonucleotide blocked the initiation of polymerization when it was bound to the primer duplex.

Role of the primer-template structure in replication inhibition

The differences observed when comparing the three oligonucleotides,29, 21 and 17-mer (figure 3), were not correlated to their relative triplex stability as determined by gel shift experiments. Indeed, the Kd values showed that more than 99% of the duplex target was in the triplex form under our experimental conditions (unpublished data).

The difference in inhibition efficiencies observed with various third strands may depend upon the distance between the 3'-end of the primer and the triplex boundary. When the 29-mer was bound to its target, this distance was 2 base pairs. In the presence of the 21-mer, the length of the duplex

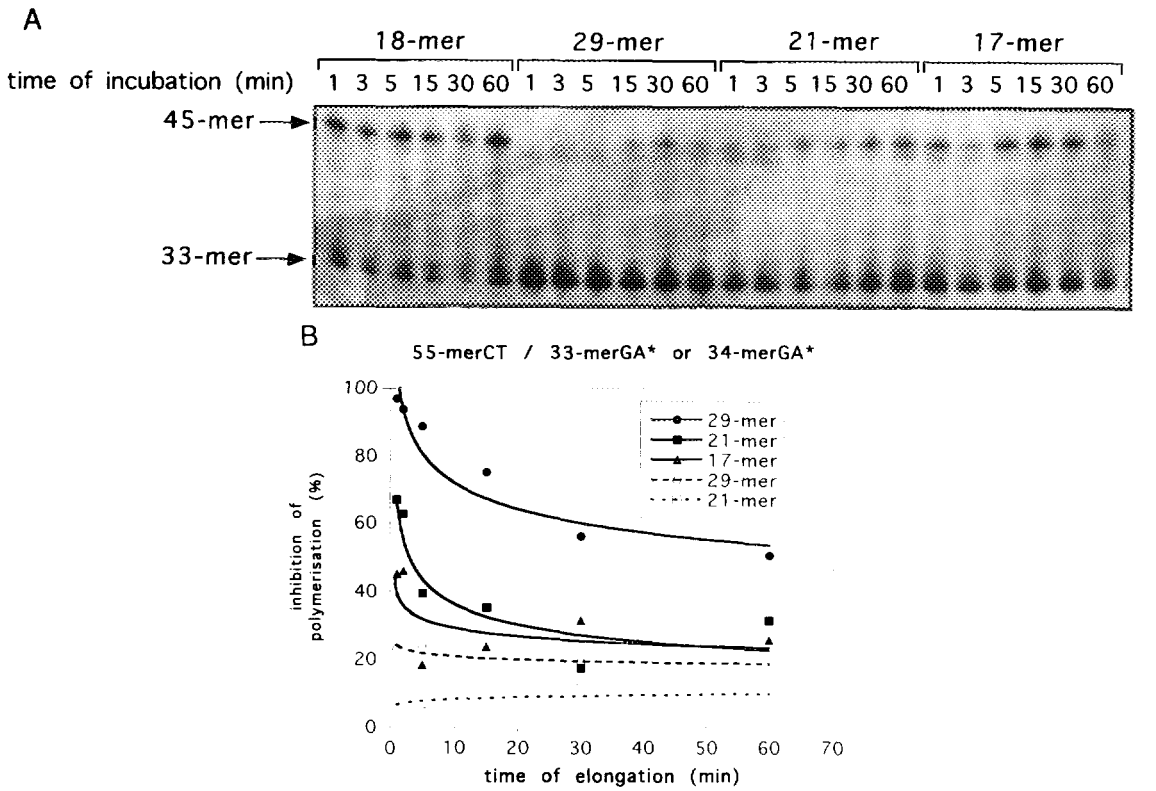


Figure 3 . Kinetics of triple helix-mediated inhibition of DNA synthesis by sequenase.

A - Polymerization experiments using the 5'-^{32}P 33-mer GA as a primer and the 55-mer CT as a template in the presence of various oligonucleotides. The pre-formed duplex (25nM) was incubated with 5μM of 29-mer, 21-mer, 17-mer or 18-mer control. The polymerization reaction was performed as described in Materials and Methods and stopped at the time indicated on top of the gel. The products of polymerization were analyzed by electrophoresis on a 10% denaturing polyacrylamide gel. The position of migration of the intact labeled 33-mer GA and the product of elongation (45 nucleotides) are indicated by the arrows on the left side of the gel.

B - Polymerization reactions using the 5'-^{32}P 33-mer GA (dark lines) or the 5'-^{32}P 34-mer GA (dotted lines) as a primer on the 55-mer CT template. The extent of inhibition of polymerization was calculated from densitometric analysis of the products of polymerization after gel electrophoresis (see part A for incubation conditions). The reference used as 100% was the amount of full length product (45-mer) obtained in the presence of 5μM of 18-mer control oligonucleotide after the same incubation time.

adjacent to the triplex was 3 base-pairs when 55-mer CT was used as a template and 9 base-pairs when 55-mer GA was used as a template (see figure 1). The weak inhibitory effect might be due to the fact that 3 terminal base-pairs were sufficient to allow for DNA polymerase binding and initiation of polymerization. To test this hypothesis, we performed experiments using a

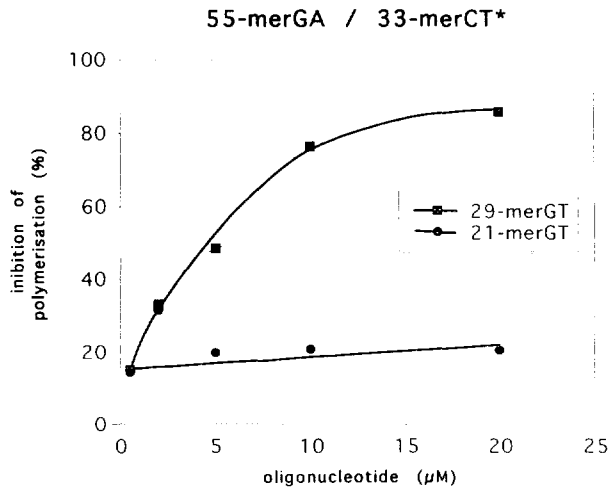


Figure 4 . Effect of increasing concentrations of oligonucleotides on primer extension reactions. Inhibition was determined as described in the legend to figure 3 using the synthesis in the presence of the 18-mer control as a reference.

The duplex was pre-formed with the 55-mer GA and 33-mer CT and pre-incubated with increasing concentrations of 29-mer or 21-mer third strand. The polymerization reaction was performed as described in Materials and Methods and was stopped after 3 minutes.

duplex formed with 55-mer CT and a 34-mer primer instead of the 33-mer (see sequence in figure 1). When the triple helix was formed with the 29-mer bound to this target, the duplex adjacent to the triplex was 3 base-pair long. As shown in figure 3B, when the 34-mer was used as a primer the 29-mer (5μM) was inefficient at inhibiting polymerization. We checked by gel shift experiments that the binding of the 29-mer oligonucleotide to the 34 base-pair duplex was not affected by the addition of one base pair to the target duplex (data not shown). Therefore, a distance of 3 base-pairs from the 3' end of the primer to the triplex boundary was enough to allow DNA polymerase to bind to the primer-template complex and thus to initiate polymerization in spite of the presence of the third-strand oligonucleotide. Once bound to the 3 base-pair duplex, the polymerase initiate elongation without beeing affected by the neighboring triplex or else it could induce a destabilization of the triple helix and dissociation of the triplex-forming oligonucleotide from its target. In addition the results obtained with the triple helix-forming 29-mer when polymerization was primed with the 34-mer demonstrated that the inhibition obtained with the 33- mer primer (figure 3) was not due to the sequestering of sequenase by the 29-mer used in excess in our experiments.

CONCLUSION

We have shown that triple helix-forming oligonucleotides could strongly inhibit initiation of primer extension by T7 DNA polymerase. Elongation

experiments with various primer-template complexes showed that the 3' end of the primer in the duplex part emerging from the triplex, should not exceed 2 nucleotides in order to obtain efficient inhibition . The triple helix formed at the primer site inhibited DNA polymerization probably by preventing binding of the polymerase to the primer. The same mechanism was proposed for triple helix-mediated inhibition of transcription by T7 RNA polymerase (22). More recently, Samadashwily and Mirkin (20) proposed a similar mechanism for inhibition of DNA synthesis on a single-stranded plasmidic template, when the initiation primer was involved in triplex formation. In addition we have shown that a 3 base pair duplex outside the triple-helical region is sufficient to allow T7-DNA polymerase to initiate replication despite the presence of a triplex structure involving the rest of the primer.

In conclusion, our results show that triple helix-forming oligonucleotides can efficiently inhibit initiation of replication when they are bound to the primer site, provided the distance from the 3'-end of the primer in the primer-template duplex to the triplex end is not longer than 2 nucleotides. This property could be used to inhibit DNA replication by targeting origins of replication and, in particular, could be exploited to prevent viral replication.

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