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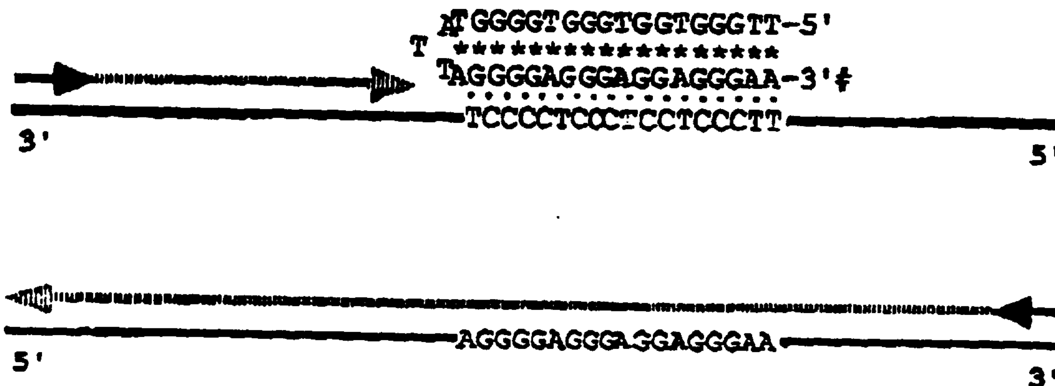

(57) Abstract

Specifically designed oligodeoxyribonucleotides form triplexes in single- or double-strand DNA at homopurine-homopyrimidine targets. These triplexes block *in vitro* DNA synthesis by all DNA polymerases studied, including Sequenase®, Taq, Vent, and Pol I. A similar phenomenon occurs when DNA polymerases are supplemented with accessory replication proteins, including SSB protein. Replication blockage is highly sequence-specific and even one or two point substitutions within either the target sequence or the oligonucleotide abolish the effect. Sequence-specific blocking of DNA replication *in vivo* is facilitated by the methods and compositions of the present invention.

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SEQUENCE-SPECIFIC INHIBITION OF DNA SYNTHESIS BY
5 TRIPLEX- FORMING OLIGONUCLEOTIDES

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BACKGROUND

Inhibition of DNA replication is effected by use of specific
10 oligonucleotides that form DNA or DNA-RNA triplexes and block polymerase action. Production of DNA or DNA-RNA triplexes at specific DNA targets provides a method to control genetic effects more effectively and more extensively *in vivo* and *in vitro* than methods directed to control of gene products or gene transcription.

15 Many DNA polymerases, including *E.coli* Pol I, thermophilic Vent polymerase, DNA polymerases of phages T5 and f29, and chemically or genetically modified T7 DNA polymerase (Sequenase®), are able to displace the non-template DNA strand in the course of DNA synthesis without the assistance of energy-driven helicases and other proteins (reviewed in Kornberg
20 and Baker, 1992). The role of displaced strand overhangs in genetic recombination is discussed in Lundquist and Olivera (1982). An interesting structural consequence of strand displacement, that the displaced strand folds back, forming an intramolecular triplex downstream of the replication fork, has not been reported.

It is known that intramolecular DNA triplexes are formed by special sequences, specifically perfect or near perfect homopurine-homopyrimidine mirror repeat sequences, designated "H motifs" (Mirkin *et al.*, 1987; Dayn *et al.*, 1992). Under mild acidic conditions, triplexes consist of two pyrimidine and one purine strand (H-DNA) (Mirkin *et al.*, 1987; Voloshin *et al.*, 1988; Johnston, 1988; Htun and Dahlberg, 1988; Hanvey *et al.*, 1988). Under neutral pH, in the presence of bivalent cations, triplexes include two purine and one pyrimidine strand (*H-DNA) (Kohwi and Kohwi-Shegematsu, 1988; Bernues *et al.*, 1989, 1990). In both sets of conditions, the orientation of the two chemically similar strands is antiparallel, and free energy from DNA supercoiling is required for triplex extrusion. Conditions of DNA polymerization *in vitro* are close to optimal for the formation of *H-DNA. Therefore, when DNA polymerase encounters a potential triplex-forming DNA sequence, the displacement of the purine-rich strand leads to triplex formation. The strand orientation in this case will be antiparallel by definition, and DNA-polymerase driven strand overhang formation might efficiently substitute for DNA supercoiling.

Manor and his co-authors (Lapidot *et al.*, 1989; Baran *et al.*, 1991) found that DNA polymerase terminates in the middle of $d(G\text{-}A)_n$ and $d(T\text{-}C)_n$ stretches in single-stranded templates. It was hypothesized that when DNA polymerase synthesizes half of the homopolymer strand, the rest of the stretch folds back forming an H-like structure which serves as a trap for the enzyme. Different *H type triplexes present in double-helical templates prior to polymerization cause DNA polymerase to terminate at triplex

borders (Dayn *et al.*, 1992). If polymerase driven strand displacement promotes triplex formation, this would block further polymerization.

It was suggested in a few studies that H motifs may account for termination of replication *in vivo*. One example came from the analysis of
5 polyoma virus-transformed rat cells. Polyomavirus DNA integrates in a particular chromosomal site (Mendelsohn *et al.*, 1982), and treatment of the cells with mitomycin C leads to the amplification of virus DNA and adjacent cellular sequences (Baran *et al.*, 1987). The boundary of the amplified DNA segment lies within a homopurine-homopyrimidine stretch $d(G\text{-}A)_{27}/d(T\text{-}C)_{27}$.
10 It was suggested that this DNA motif could be a natural replication terminator. Supporting this hypothesis, this motif, when cloned into SV40 DNA adjacent to the origin of replication, paused a replication fork progression causing slower viral growth (Rao *et al.*, 1988).

The *dhfr*-locus is amplified up to 1000 times in methotrexate resistant
15 Chinese hamster cells due to the activity of the strong bidirectional replication origin (Milbrandt *et al.*, 1981). An unusual cluster of simple-repeats, including $d(A\text{-}C)_{18}$, $d(A\text{-}G)_{21}$, $d(G)_{8}$, $d(A\text{-}G)_{27}$, is located 2 kb 3′ to the origin of replication (Caddle *et al.*, 1990). This cluster was cloned in the replication vector pSV011, and the efficiency of episome replication
20 was studied by different approaches. It was shown that when cloned on either side of the SV40 origin, it reduced episomal replication two fold, while, when placed on both sides of the origin, it blocked replication almost completely. Due to the complex nature of the cloned DNA segment, the observed effect is difficult to link with a particular element within the cluster.

However, because it was found that several DNA polymerases were unable to read through $d(A\text{-}G)_n$ tracts, the authors speculated that these triplex-forming repeats may play the key role in the termination of DNA replication (Brinton *et al.*, 1991).

5 Triplex formation is a simple consequence of strand displacement and a high level of DNA supercoiling is not required. This makes it especially attractive for eukaryotic cells where an actual torsion tension in intracellular DNA is questionable (Sinden *et al.*, 1980; Petripryak and Lutter, 1987). Detailed studies of several pro- and eukaryotic replication systems 10 revealed remarkably similar steps including initial unwinding of the *ori*, synthesis of the leading strand accompanied by the displacement of a non-template strand, assembly of a complex responsible for lagging strand synthesis on a displaced strand, and, finally, coordinated synthesis of both DNA strands (reviewed in Kornberg and Baker, 1992). Polymerase driven 15 triplex formation could potentially impair leading and lagging strand synthesis. If it indeed occurs, leading strand synthesis should be prematurely terminated, while the template for lagging strand synthesis is folded into an unusual DNA conformation.

Triplex-forming oligonucleotides (TFOs) interact specifically with 20 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 oligonucleotides 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 oligonucleotides 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^{+} 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 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.

Studies on the use of oligonucleotide-directed triplex formation for targeting genetic processes have focused on control of transcription. Triplex-forming oligonucleotides have been reported as artificial repressors preventing the binding of transcriptional regulators. Transcription was

affected by triplex formation in the human *c-myc* promoter (Cooney *et al.*, 1988; Postel *et al.*, 1991) and for the methallothionein gene promoter. In the latter case, a homopyrimidine oligonucleotide formed a triplex with the upstream portion of the promoter preventing the binding of the transcriptional activator Sp1 (Maher *et al.*, 1992). This in turn drastically reduced the promoter's activation in a cell-free transcription system (Maher *et al.*, 1992).

A triplex-forming oligonucleotide-intercalator conjugate was shown to act as a transcriptional repressor of the interleukin-2 receptor gene both *in vitro* and *in vivo* (Grigoriev *et al.*, 1992, 1993). Repression was due to the fact that the formation of a triplex additionally stabilized by cross linking to target DNA efficiently prevented the binding of the transcriptional activator NFκB. Different mechanisms of transcriptional inhibition have been also revealed. Initiations of transcription of the pBR322 *bla*-gene which contains a 13-bp homopurine-homopyrimidine target just immediately downstream from the transcriptional start site was caused by a 13-mer homopyrimidine oligonucleotide forming an intermolecular triplex with this target (Duval-Valentin *et al.*, 1992). Young *et al.* (1991) found that the transcribed portion of DNA from the adenovirus major late promoter contained a 15-bp homopurine-homopyrimidine tract that formed an intermolecular triplex with a homopyrimidine oligonucleotide. When the oligonucleotide was added prior to RNA polymerase, a significant portion of the transcripts were truncated. Thus, triplex formation may inhibit all

stages of the transcriptional cycle: formation of active promoter complex, initiation, and elongation.

Surprisingly, analogous studies targeting DNA replication are practically absent. The only published data concern the inhibition of the SV 40 growth by an octathymidilate covalently linked to an acridine derivative (Birg *et al.*, 1990). *In vitro* this complex forms a triplex with the dA_8-stretch located in the minimal origin of replication adjacent to the T-antigen binding site. *In vivo* it inhibits the cytopathic effect of SV40 in CV1 cells presumably due to the inhibition of T antigen binding or unwinding activity. Inhibition of DNA replication would provide a permanent effect on genetic action which would have advantages over other forms of genetic controls.

SUMMARY OF THE INVENTION

An aspect of the invention is a method for blocking replication of a target DNA by a specifically designed oligonucleotide from a template DNA strand. The method comprises bringing an oligonucleotide into contact with said DNA, thereby forming a triplex structure that precludes replication of the DNA. The triplex structure prevents the replication fork formed by a polymerase from proceeding past a sequence in the DNA target. The method blocks polymerization of a DNA strand by contacting an oligonucleotide with a template to form a triplex DNA structure at a specific target nucleotide sequence. The triplex structure blocks polymerization downstream of the sequence.

A suitable oligonucleotide forms a triplex strand configuration of pyrimidine/purine/purine. Replication of a target DNA strand occurs *in vitro* at a neutral pH and a high concentration of Mg^{2+} and is effected by a polymerase which is selected from the group that includes Sequenase® T7 DNA polymerase, Taq polymerase, Vent polymerase, and *E.coli* Pol I. The invention also relates a triplex-forming oligonucleotide that has a nucleotide sequence having mirror symmetry of G clusters, and is at least 12 bases in length The mirror symmetry of G clusters is selected from a mirror image of a homopurine strand with guanine (G) and adenine (A) residues. Also suitable is an oligonucleotide wherein the G residues are mirror images of guanine (G) in the homopurine strand, and intervening regions have thymines (Ts) which reflect As in the homopurine strand. Different from the art, the target DNA may be in either a non-transcribed or a transcribed region. Because transcribed regions tend to be relatively small, blockage of non-transcribed regions is an advantage of the present invention. Alternate strand oligonucleotides and those incorporating non-natural bases may be used to block replication.

A use for the oligonucleotide composition of the present invention is in a method for detecting a mutation in a DNA template for an oncogene. The method comprises:

(a) providing conditions for a polymerase chain reaction to take place using primers specific for the oncogene DNA template;

(b) denaturing the DNA template of the oncogene;

(c) adding to the denatured template a triplex-forming oligonucleotide at a high molar excess of about 10^4, said oligonucleotides having 3′ - OH groups substituted for amino groups to prevent the oligonucleotides from serving as PCR
5 primers; and

(d) determining whether there is a product of the polymerase chain reaction, said product indicating the presence of a mutation.

In this method for detecting a mutation, the polymerase chain reaction conditions include:

10 (a) use of the Stoffel fragment of Taq polymerase which lacks exonuclease activity and is active at high magnesium concentrations;

(b) a high (10 mM) magnesium concentration which makes DNA triplexes stable at elevated temperatures; and

15 (c) an optimal temperature for the reaction which depends on the DNA template.

The method is suitable for detection of an oncogene which is a member of the *ras* oncogene family. In particular mutations in codon 12 or 13 of a *ras* oncogene are sought.

20 Important therapeutic aspects of the invention include a method for blocking elongation of a target DNA strand by polymerization of a DNA template *in vivo* by providing an oligonucleotide capable of forming a triplex with the target DNA strand under polymerizing conditions and providing the conditions for polymerization of the template. The DNA includes mammalian

DNA, for example, human DNA. With regard to attack by the oligonucleotide on infectious microorganisms, the invention is suitable for use against the Herpes Simplex Virus 1 and the Hepatitis B Virus. Specific oligonucleotides are related herein for blocking the replication of these viruses.

5 Preferably oligonucleotides used for human therapy are: (1) easily synthesized and can be prepared in bulk; (2) may be made stable *in vivo*; (3) are capable of entering a target cell; (4) are retained by the target cell long enough to inhibit replication, thereby precluding cell division; and (5) show specificity of action. In general, oligonucleotides may be synthesized 10 by methods known to those of skill in the art or produced within a cell, for example, by recombinant technology. These intracellular oligonucleotides can inhibit DNA replication within the cell, or can be extracted for use elsewhere.

Delivery of oligonucleotides to a target site within an organism is accomplished by one of the following or an equivalent method; direct 15 intravenous injection of free oligonucleotides or oligonucleotides delivered by use of liposomes, and other means of delivering oligonucleotides to a target in an organism.

Another aspect of the invention is a method for treating a microbial infection in an organism. The method includes providing a composition 20 comprising triplex-forming oligonucleotides directed toward a specific sequence in the agent causing the infection, said oligonucleotides in a pharmacologically acceptable carrier and delivering an effective amount of the composition to the organism.

Methods and compositions designed to halt DNA replication at defined chromosomal sequences by means of triplex-forming oligonucleotides are aspects of the invention. Elongation of DNA polymerization is blocked at specific sequences by triplex-forming oligonucleotides that provide highly sequence specific recognition of target DNA. Such artificial terminators have therapeutic potential because they can prevent deleterious genes from replicating. An advantage of this method is a permanent inhibition of the propagation of specific gene segments. A consequence of this inhibition is repression of the growth of specific cell types, including cancer cells and infectious agents. Regulation of DNA replication may also be elucidated using these methods and compositions.

Specifically designed oligonucleotides form triplexes of single-or double-strand DNA at homopurine-homopyrimidine targets. A number of targets are homopurine-homopyridine sequences in eukaryotic gene promoters as exemplified below:

Human c-*myc* gene	CCTTCCCCACCCTCCCACCCTCCCC
Human c-*ets*-2 gene	TCCTCCTCCCTCCTCCCTCCTCCT
Human EGF-R gene	TTCTCCTCCCTCCTCCTC
Human IgA switch region	(AGAGG)^n
Human U1 RNA gene 3′-flank	(CT)^n
Chicken β-globin gene	GGGGAAGAGGAGGGG
Chicken β-globin 5′-end	(G)^{16}
Chicken α2 (1) collagen promoter	TCCCTCCCCTTCCTCCCTCCCT
Rabbit β1 globin gene	(TC)^{24}
Mouse c-Ki-*ras*	CTCCCTCCCTCCCTCCTTCCCTCCCTCCC
Rat rRNA genes	(CT)^n and (CCCT)^n
*Drosophila hsp*2 6	AGAGAGAGAAGAGAAGAGAGAGA

These complexes almost completely block in vitro DNA synthesis by all DNA polymerases studied, including Sequenase®, Taq, Vent, PoI and the like. A similar phenomenon occurs when DNA polymerases are supplemented with accessory replication proteins, including SSB protein. Replication blockage
5 is highly sequence-specific and even one or two point substitutions within either the target sequence or the oligonucleotide abolish the effect. This presents an opportunity for sequence-specific blocking of DNA replication *in vivo*.

In vitro Escherichia coli SSB protein helps DNA polymerase overcome
10 the triplex barrier, but with an efficiency dramatically dependant on the triplex configuration. Strong sequence-specific effects of TFOs were observed on both initiation and elongation during DNA polymerization. SSB protein can only partially reverse the TFO-driven termination of DNA synthesis. Therefore, TFOs are agents for blocking DNA replication *in vivo* in a
15 site-specific manner. There are many potential targets for TFOs within eukaryotic genes, specifically in introns and promoter regions. These targets may be utilized to block elongation of replication. In addition, several eukaryotic origins of replication located in the human c-*myc* and histone H4 genes, hamster *dhfr* gene, yeast chromosome III, *Stylonihia* tubulin gene, and
20 the like contain the consensus Pur element: GGNNGAGGGAGAPuPuPu (Bergemann and Johnson, 1992). These sequences are good targets for TFOs to block replication.

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. *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. DNA 5 polymerization is blocked when the primer strand is incorporated into a triplex. This effect does not depend on which strand is intended as the primer.

DEFINITIONS

Triplex: Three strands of DNA or a DNA-RNA combination linked by 10 hydrogen bonding: Watson-Crick and Hoogsteen bonding.

H-Motifs: Perfect or near perfect homopurine - homopyrimidine mirror repeats.

H-DNA: An intramolecular triplex formed by two pyrimidine and one purine DNA strand (formed under mild acidic conditions).

15 *H-DNA: An intramolecular triplex formed by two purine and one pyrimidine strand (formed under neutral pH in the presence of bivalent cations).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Conformational suicide within H motifs during DNA 20 polymerization.

A. Strand displacement during DNA polymerization may cause triplex formation. The diamond shows the original nick in the

double-helical template providing a 3'-OH end for DNA polymerase; the black boxes represent two halves of a hypothetical homopurine-homopyrimidine mirror repeat involved in the formation of an intramolecular triplex; the striated arrow shows the newly synthesized DNA chain.

5 **B.** Four sequences are shown with varying triplex-forming potential. Vertical lines show a pseudosymmetry axis. Broken arrows illustrate mirror symmetry within G-clusters. Diamonds indicate point substitutions.

FIG. 2. Construction and sequencing strategy of open circular
10 DNA templates. The black box represents an H motif, the striated arrow shows newly synthesized labeled DNA.

FIG. 3. Short triplexes within single stranded templates. The 3' Amine-ON group is shown by a # mark. Arrow corresponds to the reverse primer.

15 **FIG. 4.** DNA templates containing short double- and triple-helical stretches. Hoogsteen pairs are shown by asterisks (.), 3' amino groups are shown by pound symbols (#). Arrows correspond to the "reverse" primer.

FIG. 5. DNA templates containing polymerization primers within triple-helical stretches.

20 Hoogsteen pairs are shown by asterisks (.), 3' amino groups are shown by pound symbols (#). Either homopyrimidine (A-C) or

homopurine (D-F) oligonucleotides served as primers. These primers were added to the templates either alone to form Watson-Crick 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
5 lacking triplex-forming ability due to G-to-T substitutions (C, F). The primer is a component of a triplex in both B and E; in B it is involved only in Watson-Crick base pairing, while in E it participates in Watson-Crick and Hoogs*een base pairing.

Methods: 0.5 μg of single-stranded DNAs were mixed with 10 pmol of
10 polymerization primers and 100 pmol of TFOs and incubated in Sequenase® Buffer (40 mM Tris·HCl pH 7.5/ 50 mM NaCl/ 20 mM $MgCl_2$) for 15 min at 37°C. DNA sequencing was performed as described in FIG. 2.

FIG. 6. Inhibition of PCR by TFO. Solid lines represent template strands; solid arrows represent PCR primers; dashed arrows
15 represent newly synthesized DNA chains, points represent Watson - Crick hydrogen bonds; asterisks represent Hoogsteen hydrogen bonds; the ponds symbol represents the 3'amino group.

FIG. 7. Partial nucleotide sequences for three members of the *ras* gene family. Codons 12 and 13 where most of the tumorigenic
20 mutations occurs are outlined.

FIG. 8. Scheme of TFO-caused PCR inhibition of the wild-type human N-*ras* gene. Mutations within GGT repeats that cause oncogene activation make triplex formation unfavorable and reverse PCR inhibition. For details see FIG. 1.

DESCRIPTION OF THE PREFERRED EMBODIMENT

A series of results described below led up to the claimed invention. First, results showed that *H-DNA is a template that blocks DNA polymerization. Second, results showed that *H-DNA may appear in the course of DNA polymerization and block it, a mechanism termed "conformational suicide." From these two discoveries, the invention developed for use of specific triplex-forming oligonucleotides to block DNA replication at specific targets. The examples presented herein illustrate methods and compositions of the present invention.

Triplexes in DNA are formed in several different ways, and have several types of structures. Structural consequences of DNA-strand displacement as a result of polymerization is diagrammed in FIG. 1A. Certain sequences in double-stranded DNA block DNA replication.

Strand displacement during DNA polymerization of double-helical templates brings three DNA strands (duplex DNA downstream of the polymerase plus a displaced DNA strand overhand) into close proximity, provoking triplex formation, which in turn prevents further DNA synthesis. By inducing triplex formation at specific sequences in front of the replication fork, DNA polymerase causes self-replication termination. This mechanism, termed "conformational suicide," provides a novel way to target DNA polymerases at specific sequences using triplex-forming oligonucleotides. Conversely, mutations which destroy the triplex potential of triplex-forming sequences prevent termination of polymerases such as T7 DNA

polymerase (Sequenase) on open circular DNAs, whereas compensatory mutations which restored triplex potential also restored termination.

Direct evidence that the triplexes formed by strand displacement cause termination, was provided by DNA polymerization studies on
5 otherwise single-stranded templates containing triple-helical stretches. DNA polymerase could not overcome a triplex barrier. Sequenase® is unable to propagate through short triple-helical stretches within single-stranded DNA templates. Remarkably, triplexes of different configurations with or without purine-rich hairpins show similar effects on
10 polymerization. Therefore, the triplex itself rather than the complex *H configuration is necessary and sufficient for polymerase blockage. DNA polymerase can be stopped at homopurine-homopyrimidine sequences using triplex-forming oligonucleotides (TFO). Therefore, these oligonucleotides are useful for therapeutic strategies to counter the action
15 and propagation of deleterious genes. Assays to detect gene mutations also use the oligonucleotides.

Triplex-forming oligonucleotides (TFOs) efficiently trap DNA polymerases at target DNA sequences within single-stranded templates. This was observed for all DNA polymerases studied, including Sequenase®
20 and thermophilic *Taq* and Vent polymerases. The termination rate depends on the fine structure of a triplex, as well as on ambient conditions such as the 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.

Comparing the activity of modified T7 DNA polymerase on nicked double-helical templates containing sequences with different *H forming
5 potential showed that when the purine-rich strand is displaced during polymerization, Sequenase® is almost completely blocked at the middle of these sequences as expected in the case of encountering triplex formation. Conversely, when the pyrimidine-rich strand is displaced, no termination occurred. Therefore, a perfect correlation exists between
10 triplex-forming ability of a given sequence and its termination efficiency.

H forming repeats in double stranded templates serve as efficient terminators for modified T7 DNA polymerase. Several lines of evidence indicate that this effect is due to triplex formation rather than sequence specificity. First, a remarkable correlation exists between the
15 triplex potential of a sequence and its terminator strength. Mutations destroying triplex formation release polymerase blockage, while compensatory mutations restoring triplex formation restore termination. Second, characteristic strand asymmetry is observed, i.e., termination occurs only when the purine-rich strand of an H motif is displaced during
20 polymerization. Because only purine/purine/pyrimidine triplexes are stable under polymerization conditions, the displacement of the purine-rich strand may provoke their formation, preventing further elongation. Finally, when intermolecular triplexes having the same sequence and configuration as ones presumably formed by strand displacement occur within

single-stranded templates, a complete termination of DNA polymerase occurs at the junction between single- and triple-stranded DNA. Strand displacement in the course of polymerization promotes *H-DNA formation, causing self termination.

5 ***H-forming potential of different DNA sequences***

Several sequences with varying *H forming potential were synthesized and cloned into the pBLUESCRIPT plasmid (FIG. 1B). In the "wild type" sequence, guanine clusters in the purine-rich strand are mirror repeated, while the intervening thymines in one half of the sequence are 10 reflected by adenines in the other half. As a result, when the 5′-half of the purine-rich strand folds back, GGC and TAT base triads are formed. This sequence adopts a corresponding isoform of *H-DNA in supercoiled plasmids (Dayn *et al.*, 1992). Double mutant I has two G-to-T substitutions in the 5′-half of the purine-rich strand, while double mutant II has two 15 symmetrical G-to-A substitutions in its 3′-half. In both cases, the mirror symmetry of the G clusters is destroyed, preventing triplex formation. Finally, the quadruple mutant combines the point substitutions of both double mutants, restoring the original pseudosymmetry and consequent triplex-forming ability.

20 The efficiency of *H formation in all four cases was investigated by use of chemical footprinting of supercoiled DNAs. Triplex formation in the wild type sequence causes hyperreactivity of the 3′-half of the pyrimidine-rich strand to CAA (Dayn *et al.*, 1992). Therefore, CAA was

used to test triplex formation in all four cases. Plasmids were CAA-modified in the presence of bivalent cations (Mg^{2+} or Zn^{2+}) followed by restriction digestion and end-labeling. Samples were then treated with either formic acid (purine reaction) or hydrazine in high salt (cytosine 5 reaction) followed by piperidine cleavage and sequencing gel-electrophoresis. CAA modification of cytosines leads to the enhancement of corresponding bands on the cytosine ladder and appearance of new bands on a purine ladder, while the adenine modification enhances corresponding bands in a purine ladder and leads 10 to the appearance of new bands in the cytosine ladder (Kohwi and Kohwi-Shigematsu, 1988). Only for the wild type sequence is the 3′-part of the pyrimidine-rich strand CAA-reactive in the presence of magnesium ions. Thus, under these conditions only this sequence adopts an *H conformation. In the presence of zinc ions, however, both the wild type 15 and the quadruple mutant are chemically hyperreactive. The double mutants show no CAA-reactivity under any conditions studied.

Thus, both wild type and quadruple mutant sequences may adopt the *H-conformation in the presence of bivalent cations, while the double mutants are incapable of this. Clearly, the wild type sequence has a better 20 *H forming ability because it forms a triplex under all conditions tested and demonstrates a more prominent modification pattern than the quadruple mutant in the presence of zinc ions. The difference between the two sequences in cation requirements is most probably due to the difference in their AT-content. The quadruple mutant was derived from the wild type

by four GC-to-AT substitutions and is, therefore, significantly more AT-rich. *H-DNA formed by $d(G)_n{\cdot}d(C)_n$ sequences is stabilized by Mg^{2+}, but the same structure formed by $d(G\text{-}A)_n{\cdot}d(T\text{-}C)_n$ stretches is stable only in the presence of Zn^{2+}. Similar effects are observed for intermolecular triplexes as well (reviewed in Malkov *et al.*, 1992). Thus, changes in GC content from 100% to 50% cause a switch in cation requirements. Even moderate alterations in GC content (from 75% to 63%) leads to the same cation switch for a particular sequence to form *H-DNA.

Blockage of DNA polymerization by H motifs

To analyze the influence of H motifs on DNA polymerization, the activity of modified T7 DNA polymerase (Sequenase®) on circular, double-helical DNA templates containing a single-stranded nick (serving as a primer) was studied. The strategy for obtaining such templates is outlined in FIG. 2. Single-stranded circular phage DNA containing either the purine-rich or the pyrimidine-rich strand of inserts from *E.coli* cells carrying pBLUESCRIPT plasmids as described in Materials and Methods was recovered. Then RecA-mediated strand transfer to these single-stranded molecules from corresponding linear double-stranded DNAs was carried out (Soltis and Lehman, 1983). The resultant open circle contained a unique 3′-OH end available for DNA polymerase. Depending on the orientation of the triplex-forming sequence relative to the nick, either the purine- or pyrimidine-rich strand served as a template. In open circular templates, the formation of *H-DNA prior to polymerization is forbidden,

because triplexes extrusion depends highly upon DNA supercoiling (Dayn *et al.*, 1992).

To analyze the fine pattern of DNA polymerization DNA sequencing reactions were conducted on open circular templates as described in the Materials and Methods. To provide a unique reference end for newly synthesized fragments, DNA samples were digested with Sst I restriction enzyme after polymerization (the Sst I site could only be restored in the course of DNA synthesis). The most striking results were obtained for the "wild type sequence," an arbitrary designation for the synthetic sequence from which "mutations" were generated. When the pyrimidine-rich strand served as a template, almost complete termination of polymerization at the middle of this sequence was observed. Conversely, when the purine-rich strand of the same sequence served as a template, no termination was observed. This remarkable difference between the two strands may be explained through triplex formation. Indeed, for the pyrimidine-rich template, the purine-rich strand is displaced and may form a triplex under polymerization conditions, while a displaced pyrimidine-rich strand cannot form a triplex, because pyrimidine-purine-pyrimidine triplexes are stable only under acidic pH. In the above experiments, a DNA sequencing protocol was used to locate termination sites at a nucleotide base level. Control experiments showed that termination is not specific for dideoxyNTP substrates. The same extent of termination was observed when "normal" dNTP substrates were used in the experiment.

Studies of the mutated sequences provided additional support for this interpretation. For both double mutants, no termination of DNA polymerase was observed even when the pyrimidine-rich strand served as a template. With the quadruple mutant prominent termination was detected, starting from the middle of the pyrimidine-rich template. The termination is not complete in the quadruple mutant, where stop signals tend to slide downstream from the center of this sequence, showing that DNA polymerase may partially overcome termination. Overall, a perfect correlation was observed between the ability of a sequence to form *H conformation and its termination ability: the wild type sequence causes a complete termination, the quadruple mutant has a lower triplex potential and weaker termination, and the double mutants are unable to form triplexes and show no signs of termination.

The quadruple mutant requirement for zinc, rather than magnesium, to adopt *H conformation was intriguing because DNA was polymerized in the presence of magnesium. A possible explanation is that during DNA polymerization, the displacement of the purine-rich strand provides a sufficiently high local concentration of the third strand at the double-helical stretch to favor triplex formation even in the presence of magnesium ions. To check this hypothesis, *inter*molecular triplexes reflecting triple-helical portions of *H-like structures formed by strand displacement were studied. Efficiency of triplex formation when the third strand was present at a high molar excess was estimated. For this purpose two oligodeoxynucleotides corresponding to the 5′-part of the purine-rich strand of the wild-type

(dGGGTGGTGGGTGGGG) or double mutant I (dGGGTGGTTGGTGTGG) were synthesized. Each of these oligonucleotides was then incubated in Sequenase® buffer with linear double-stranded DNAs containing either the wild type or the quadruple mutant sequences. Four oligonucleotide-to-
5 template combinations corresponding to all the triplexes formed by all our sequences were available.

To detect triplex formation a photofootprinting approach originally described by Lyamichev *et al.* (1990) was used. Formation of *inter-* and *intra*molecular triplexes prevents <6-4> dipyrimidine accumulation in DNA
10 after UV irradiation (Lyamichev *et al.*, 1990; Tang *et al.*, 1991). These photo products are easily detected at a sequence level because they are cleavable by piperidine. Therefore, photo reactivity of the pyrimidine-rich strand in all oligonucleotide-to-template combinations described above was studied. DNA mixtures were UV irradiated and treated with piperidine
15 followed by sequencing gel-electrophoresis (in all the cases the pyrimidine-rich strands of double-stranded DNAs were end-labeled). Photo protection appeared in the pyrimidine strand in two cases: (i) both oligonucleotide and template are of the wild type, and (ii) mutant oligonucleotide mixed with the mutant template. In the other two cases no
20 triplex formation was detected. The former cases correspond to triplexes formed by the wild type and quadruple-mutant sequences, while the latter cases reflect potential triplexes in both double mutants. These data show that high molar excess (100x) of the third strand allows triplex formation for

both wild type and quadruple mutant sequences under polymerization conditions.

Blockage of DNA polymerization by triplexes within single-stranded templates

5 A hairpin loop configuration of the purine-rich strand is not essential for polymerase blockage. This was shown by use of DNA templates containing artificially designed triple-helical stretches reflecting triplexes *per se* as well as the complex *H-like configuration triplexes. FIG. 3 illustrates that a circular single-stranded DNA template containing the 10 5'-half of the pyrimidine-rich strand of the wild type sequence was obtained. Different triplex-forming oligonucleotides were annealed to this template. In the case of A, only the complementary duplex forming oligonucleotide was added. In B two purine-rich oligonucleotides that allow triplexes with antiparallel orientations of purine-rich strands to form were 15 annealed. This intermolecular triplex does not contain any hairpin-loop structures. C represents the case when the above two purine-rich oligonucleotides are covalently bound via an ATT loop. This configuration is identical to an intramolecular triplex formed by the wild type sequence in supercoiled DNA and during strand displacement. D is another 20 hairpin-looped triplex with the loop distal from DNA polymerase. Finally, E is similar to C but contains two G-to-T point substitutions disrupting triplex ability. This configuration reflects an intramolecular triplex that could be formed by the double mutant I and which is unfavorable. The formation of triplexes in cases B through D was confirmed by footprinting assays.

To study the pattern of DNA polymerization a reverse primer was annealed to all these templates and sequencing reactions were conducted (Materials and Methods). To prevent triplex-forming oligonucleotides from serving as primers for DNA polymerase, their 3'-ends were blocked by 3' amine-ON group (Nelson *et al.*, 1989). This was done using an ABI oligonucleotide synthesizer and 3' Amine-ON CPG (Cruachem). Results showed that Sequenase® easily reads through the duplex area which is due to its strand displacement activity; in cases B through D almost complete termination of polymerization was observed exactly at the junction between single and triple-stranded DNA. Finally, destabilization of a triplex by two point substitutions allows DNA polymerase to elongate normally.

These data provided the first direct evidence that DNA polymerase cannot overcome a triplex barrier. Termination appears to be qualitatively the same for a putative intermolecular triplex and the hairpin-looped triplexes. Therefore, a triple-helix itself rather than a complex *H type configuration is responsible for polymerization blockage.

Triplex formation generally inhibits polymerase activity

The influence of purine-rich TFOs on the activity of several different DNA polymerases showed that DNA triplexes block elongation of different DNA polymerases. YR.R triplexes are favored under polymerization conditions (Baran *et al.*, 1991; Dayn *et al.*, 1992). Templates were circular pBluescript single-stranded DNAs carrying 18 nt-long homopyrimidine (FIG. 4A-C) or homopurine (FIG. 4D-F) targets. Different oligonucleotides were added to form short Watson-Crick duplexes (FIG. 4A,D), hypothetical

Hoogsteen duplexes (FIG. 4E), orthodox intermolecular triplexes (FIG. 4B,F), or H-like triplexes formed by a purine-rich hairpin and its homopyrimidine target (FIG. 4C). All triplexes were built from CG.G and TA.T base triads (Samadashwily *et al.*, 1993). 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 oligonucleotides 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.

T7 DNA polymerase (Sequenase®) was completely blocked by short triplexes within single-stranded templates (Samadashwily *et al.*, 1993). Methods: 0.5 μg of single-stranded DNAs were 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 (U.S. Biochemical) with the following modifications. Labeling was done in the presence of 230 nM dNTP (N=G,C,T) supplemented by 5 mCi of [a-^{32}P]dATP (Amersham) for 2-5 min at room temperature. Then dNTPs were added up to 33.3 mM and ddNTP up to 3.3 mM, followed by polymerization for 10 min at 37°C. Sequencing electrophoresis was performed on 8% polyacrylamide gel with 7M urea.

No apparent differences in effect were observed between intermolecular and H-like triplexes. DNA polymerization on the templates

presented in FIG. 4D-F where the homopurine-strand is replicated showed that neither short Watson-Crick duplexes, nor the G-rich strand alone cause prominent termination of polymerization, whereas triplex formation unconditionally blocks Sequenase® at the triplex border. These results
5 show that triplexes prevent Sequenase® elongation regardless of the chemical nature of the template DNA.

Similar to Sequenase®, short duplexes do not block *Taq* polymerase. Methods: 0.5 μg of single-stranded DNAs were mixed with 10 pmol of the "reverse" primer and 100 pmol of TFOs and incubated in
10 *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 mM and ddNTPs up to 600 mM, followed by polymerization for 10 min at 72°C. Gel electrophoresis was performed.

Triplexes do lead to the premature termination of polymerization
15 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. 4C) than for the intermolecular triplexes (shown in FIG. 4B). These differences from the Sequenase® case may have several causes. First, the lower Mg^{2+} concentration and higher
20 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, two DNA polymerases, *Taq* and Vent were compared. While both enzymes work under wide range of temperatures and ionic conditions, Vent lacks the 5'-exonuclease activity (Kong *et al.*, 1993). The pattern of primer extension by *Taq* and Vent
5 polymerases was compared on a template with intermolecular triplexes (shown in FIG. 4B) under different Mg^{2+} concentrations. Methods: Triplexes were formed in either Taq polymerase 2xPCR buffer (20 mM Tris·HCl pH 9.0/ 100 mM KCl/ 5 mM $MgCl_2$/0.2% Triton X100) for 15 min at 37°C for thermophilic polymerases, or in modified Sequenase® buffer (5
10 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 mM and polymerization was carried out at 2.5, 5 and 10 mM $MgCl_2$ for 10 min at
15 65°C (Taq and Vent) or 37°C (Sequenase®). Gel electrophoresis was performed.

Triplexes within single-stranded templates were preformed at low (2.5 mM) Mg^{2+}, followed by DNA labeling under the same conditions, while chain elongation was carried out at different Mg^{2+} concentrations. The
20 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, the influence of magnesium ions on Sequenase® activity with the same template at 37°C was observed. 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 unexpected that strongest termination in all cases occurred at 10 mM Mg^{2+}.

Temperature dependence of DNA polymerization on the same templates with intermolecular triplexes was observed (where primer extension was carried out at 5 mM Mg^{2+}). 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.

Termination efficiency was compared while increasing the polymerization time from the standard 3-5 min. period up to 30 min. Even a 10 fold increase in polymerization time didn't change the termination pattern for the wild type sequence, suggesting that DNA polymerization through the triplex portions of a template is thermodynamically forbidden.

The influence of triplexes of different configurations on the activity of both thermophilic enzymes at 65°C was compared. While intermolecular triplexes at this temperature are unable to stop polymerization, H-like structures cause prominent termination. A possible explanation is that dissociation of the DNA triplex proceeds in two steps: (1) triplex-to-duplex and (2) duplex-to-single strands. Because short duplexes do not influence DNA polymerization, only the first transition with regard to termination need

be considered, 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
5 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).

There is a direct link between triplex stability and the arrest of DNA polymerization. The lack of differences between *Taq* and Vent polymerases
10 strictly indicates that the 5′-exonuclease activity does not affect triplex-caused termination.

SSB protein does not interfere with TFO-caused termination. Strong inhibition of DNA polymerases by TFOs *in vitro* suggest TFOs are effective in blocking DNA replication *in vivo* also. However, the actual replication
15 fork contains not only DNA polymerase but a complex of replication proteins, including helicases, SSB proteins, primases, topoisomerases, and the like (reviewed in Kornberg and Baker, 1992). Inhibition of the DNA-helicase activity of the SV40 T-antigen by TFOs were reported by Peleg and Manor (1993). The influence of the *E.coli* SSB protein on DNA
20 synthesis of triplex-containing templates was investigated by comparing DNA templates with the intermolecular and H-like triplexes shown in FIG. 1 B and C. Sequenase® T7 DNA polymerase was used for primer extension in these experiments, because 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^{2+}) at 37°C. A primer was labeled with Sequenase® and then elongation was carried out at different concentrations of SSB. SSB helps DNA polymerase to overcome a triplex barrier, but its efficiency
5 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/ml of SSB almost completely abolishes it. In contrast, intermolecular triplexes are relatively resistant towards SSB, and prominent termination is still observed at 0.4 mg/ml of SSB.

10 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. 4C), that is nearly the minimal binding site of *E.coli* SSB protein which is 4 nt (Ruyechan and Wetmur, 1976). The loop
15 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 single-stranded regions.

Although H-like TFOs are the strongest inhibitors of DNA
20 polymerization *in vitro*, a possibility existed that 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.

Influence of triplexes on the initiation of DNA polymerization

TFOs sometimes stop polymerization not only prior to the target sequence, but also immediately after it. A triplex may be formed behind the DNA polymerase just as it passes a target sequence.

5 DNA templates used to analyze the efficiency of DNA synthesis are presented in FIG. 5. Either homopyrimidine (5 A-C) or homopurine (5 D-F) oligonucleotides complementary to the target sequences served as primers. These primers were annealed to the templates alone (5 A,D), in the presence of a purine-rich TFO (5 B,E), or in the presence of a mutant 10 TFO (5 C,F) which lacks triplex-forming ability due to two G-to-T transversion (Samadashwily *et al.*, 1993). Templates 5B and 5E are similar in that the primer is a component of the triplex. However, the homopyrimidine primer is involved in only Watson-Crick (Watson-Crick) base pairing, while the homopurine primer participates in both Watson-15 Crick and Hoogsteen base pairing.

Profiles of DNA sequencing on these templates showed the wild-type TFO dramatically (at least ten fold) 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 20 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. 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.

5 ***Construction of oligonucleotides from alternate DNA strands or non-natural bases***

Target DNA choices are enlarged by use of alternate or non-natural oligonucleotides. Alternate strand oligonucleotides are constructed by choosing bases that match alternate strands of DNA, e.g.:

```
             GTGTGT____ oligo A
10      1.   GAGAGACCCT
        2.   CTCTCTGGGA
                   GGGT  oligo B
```

and joining A and B into one oligonucleotide (see Johnson W O 93/2230). Non-natural bases that form Hoogsteen's bonding with C and T include

15 pseudocytosine. In the case of RNA oligonucleotides, uracil replaces thymine (U←T).

Example 1: Inhibition of the Polymerase Chain Reaction (PCR) By Triplex-Forming Oligonucleotides For Detection of Oncogene Mutations in Tumors.

20 Detection of oncogene mutations in DNA isolated from tumor tissues is invaluable for both diagnostic and therapeutic purposes. Currently available direct methods include cloning and sequencing of oncogenes of interest, or PCR amplification of corresponding oncogenes with subsequent DNA sequencing. Although such approaches give the most relevant

25 information, they are technically difficult, expensive, time-consuming and

generally cannot be performed in a clinical laboratory. Thus, it is important to develop less sophisticated approaches that can be routinely and quickly used for large numbers of biological samples. A novel and powerful approach to detecting oncogene mutations utilizes the inhibition of PCR by
5 triplex-forming oligonucleotides (TFOs).

Short triple-helical stretches within single- or double-stranded DNA templates arrest DNA polymerization (Dayn, 1992; Samadashwily, *et al.*, 1993). Py·Pu.Pu triplexes that are stable under conditions optimal for DNA polymerization *in vitro*, i.e. neutral pH and high concentration of Mg^{2+} ions
10 almost completely block elongation of DNA polymerases, including such highly processive enzymes as Sequenase® T7 DNA polymerase, thermophilic Taq and Vent polymerases and *E.coli* Pol I. Severe inhibition of polymerization was observed at ≥10 mM of Mg^{2+} even at high temperatures (65°-70°C) (Samadashwily, 1994).

15 FIG. 6 illustrates the approach. If a DNA template contains an appropriate target for triplex formation, i.e., a purine-rich or pyrimidine-rich cluster, a stable triplex forms upon denaturation and addition of TFOs, and DNA polymerization is blocked. Because PCR includes subsequent rounds of DNA denaturing and primer annealing, the presence of TFOs continually
20 blocks the amplification of this DNA segment. To optimize the blockage of PCR on DNA templates containing homopurine-homopyrimidine stretches in the presence of TFOs, the protocol uses:

(i) the Stoffel fragment of Taq polymerase which lacks exonuclease activity affecting triplex stability and works at high magnesium concentrations;

(ii) high (10 mM) magnesium concentration in the PCR mixture
5 which makes triplexes stable under elevated temperatures;

(iii) a specific temperature regime for the PCR amplification which depends on the template being copied;

(iv) TFOs with their 3'-OH groups substituted for the amino groups to prevent them serving as primers for DNA polymerase;

10 (v) high (10^4) molar excess of TFOs over their targets in template DNAs. Point mutations were found within a target sequence which make triplex formation unfavorable, reversing the inhibition of PCR amplification.

The approach is suitable for inhibition of natural oncogenes, e.g., the *ras* oncogenes. The products of the highly conserved *ras* gene family play
15 a pivotal role in signal transduction and regulation of cellular proliferation. Mutations within these genes occur in many tumor cells obtained *in vitro* or from natural tumors. Mutations within N-*ras*, c-Ki-*ras* and Ha-*ras* were detected in as many as 30% of human tumors (Bos, 1988). Remarkably, the vast majority of these mutations are clustered within a short segment
20 of the protein coding sequence containing several GGT repeats. These mutations cause oncogene activation and subsequent tumor development.

Sequences of this area for three members of the *ras* family are presented in FIG. 7. They contain numerous G-clusters which are a prerequisite for triplex formation. The scheme for the PCR inhibition for

these sequences and the design of TFOs are presented in FIG. 8. TFOs are generated to prevent PCR amplification of *ras* genes. Mutations that are known to cause oncogene activation make triplex formation unfavorable. Therefore, PCR of an activated *ras* gene should proceed normally. The lack of the PCR product of a *ras* gene in the presence of TFOs would indicate that the gene is normal, while the appearance of such a product leads to the inference of the existence of mutations within oncogenes.

GGT repeats from different *ras* oncogenes are cloned by techniques known to those of skill in the art (presented in FIG. 7) into the pBluescript plasmid. The efficiency of PCR amplification of those sequences is determined in the absence (control) or presence of TFOs. Optimal conditions, i.e., ionic strength, temperature, TFOs concentrations and the like are determined based on complete blockage of amplification. Mutated versions of *ras* genes are synthesized and cloned using the known mutations observed in human tumors (reviewed in Bos, 1988). These mutants are amplified in the same conditions in the presence of TFOs to determine that PCR will proceed efficiently.

PCR of human DNAs isolated from normal cells or from human tumors with known *ras* mutations are employed. The optimal conditions for PCR amplification of *ras* genes in the absence and presence of TFOs, as well as the conditions where the difference in amplification efficiency between wild-type and mutated oncogenes are most notable, are determined by doing PCR and determining conditions that are most

appropriate for production of a product from a "wild type" *ras* and, ideally, a known mutation.

As reviewed in Mirkin and Frank-Kamenestkii (1994), formation of triplexes is restricted to purine/pyrimidine based sequences. *Ras* genes
5 are a very convenient target for triplex formation because important mutations causing tumors are located within such sequences.

Example 2: Inhibition of Replication of the Herpes Simplex Virus 1.

HSV-1 is responsible for a variety of skin lesions and other infections. Herpes virus infections represent a serious health problem in
10 the U.S. and worldwide. The genome of this virus has a number of homopurine-homopyrimidine sequences that are targeted by TFOs to block viral replication.

Target 1 is in exon 3 of IR110 at the position 122558-122593:

5′-TTCCTCCCTCCCCCCCCCTCCTCCTCCTCCCCC-3′

15 3′-AAGGAGGGAGGGGGGGGGAGGAGGAGGAGGGGG-5′

Two **TFOs** for targeting:

(i) 5′-AAGGAGGGAGGGGGGGGGAGGAGGAGGAGGAGGGGG-3′
(GA-containing)

(ii) 5′-TTGGTGGGTGGGGGGGGGTGGTGGTGGTGG%GGGGG-5′
20 (GT-containing)

Target 2 is a 185-bp-long stretch located within the inverted repeat (IR-L) at the positions 117156-117341.

CTCCCCTCTCCCCCCCTCTCCCCTCTCCCCCCCTCTCCCCTCTCCCCCCCTCTCCCCTCTC
CCCCC

GAGGGGAGAGGGGGGGAGAGGGGAGAGGGGGGGAGAGGGGAGAGGGGGGGAGAGGG
GAGAGGGGGG

CTCTCCCCTCTCCCCCCCTCTCCCCTCTCCCCCCCTCTCCCCTCTCCCCCCCTCTCCCCTC
TCCCC

GAGAGGGGAGAGGGGGGGAGAGGGGAGAGGGGGGGAGAGGGGAGAGGGGGGGAGAG
GGGAGAGGGG

CCCTCTCCCCTCTCCCCCCCTCTCCCCTCTCCCCCCCTCTCCCCTCTCCCCTCT

GGGAGAGGGGAGAGGGGGGGAGAGGGGAGAGGGGGGGAGAGGGGAGAGGGGAGA

Several TFOs that will target this sequence are described below:

(i) 5'-GAGAGGGGAGAGGGGGGGAGA-3' or

5'GTGTGGGGTGTGGGGGGGTGT-3'

(ii) 5'-AGAGGGGAGAGGGGGGGAGAG-3' or

5'-TGTGGGGTGTGGGGGGGTGTG-3'

Because target 2 is a very long target, the combination of TFO (i) and (ii) is preferred to achieve higher specificity.

Example 3. Inhibition of Replication of the Hepatitis B Virus.

HBV is a serious health problem worldwide. Chronic carriers of HBV reach almost 10% of some populations, and chronic hepatitis and cirrhosis are major causes of mortalities in those cases. It is also commonly accepted that HBV is involved in the development of hepatocellular carcinoma, which is one of the most common cancers in the world. Thus TFOs against HBV show promise as powerful therapeutics.

Target 1 is within the P-gene of the virus (at the position 2670-2681):

5'-GAGAAAAGAAGA-3'

3'-CTCTTTTCTTCT-5'

TFOS:

5'-AGAAGAAAAGAG-3' or 5'-TGTTGTTTTGTG-3'

Target 2 is within the C-gene of the virus (at the position 2037-2048):

5'-CTTCTTTCCTTC-3'

3'-GAAGAAAGGAAG-5'

TFOS:

5'-GAAGAAAGGAAG-3' or 5'-GTTGTTTGGTTG-3'

Target 3 is within the regulatory area of the virus responsible for the activation of gene expression and reverse transcription (at the position 1809-1829).

5'-GGGAGGAGTT-GGGGGAGGAGA-3'

3'-CCCTCCTCAACCCCCTCCTCT-5'

This target is not a perfect homopurine-homopyrimidine sequence and contains an interruption (underlined). Suitable TFOs are:

5'-AGAGGAGGGGG--GAGGAGGG-3' or

5'-TGTGGTGGGGG--GTGGTGGG-3',

where dashes correspond to abasic linkers incorporated into the TFO opposite thymines in the homopurine strand of the target.

Example 4. Inhibition of the Papilloma virus.

Papilloma virus is the major cause of cervical cancer, the most common malignancy in the developing world and in some populations in the U.S. Different strains of the human papilloma virus contain the same

homopurine-homopyrimdine sequence which could be targeted by TFOs to prevent viral replication and cancer development.

Target 1 is located within the protein-coding sequence ORF-Ec (at the position 436-452 in HPV57 and 438-452 in HPV2).

5 5'-GGGAGGAGAAGGAGG-3'

3'-CCCTCCTCTTCCTCC-5'

TFOS:

5'-GGAGGAAGAGGAGGG-3' or 5'-GGTGGTTGTGGTGGG-3'

MATERIALS AND METHODS

10 ***Enzymes***

Sequenase® Version 2.0 T7 DNA polymerase (E.C. 2.7.7.7), *E.coli* RecA protein, and *E.coli* single-strand DNA binding protein were obtained from the United States Biochemical. Restriction enzymes and enzymes for end-labeling of DNA were obtained from the Bethesda Research

15 Laboratories.

Oligonucleotides

Oligonucleotides were synthesized on ABI High Throughput DNA/RNA synthesizer Model 394 as described in User's Manual (Applied Biosystems). They were deprotected by incubation in a concentrated

20 ammonium hydroxide for 15 hours at 55°C, followed by concentrating in a SpeedVac and precipitation by 2 volumes of 2M solution of $LiClO_4$ in

acetone. Dried pellets were dissolved in 0.5 ml of TE buffer and additionally purified on NAP-5 columns (Pharmacia).

To obtain oligonucleotides that cannot serve as substrates for DNA polymerases (FIG.6), a primary aliphatic amine was introduced at their 3′ terminal nucleotides. This was done by an automatic conventional solid phase DNA synthesis using a 3′ Amine-ON CPG prepacked column (Cruachem) which transfers a primary amine to the 3′ terminus of a synthesized oligonucleotide without changing any chemistry or adding extra steps.

Oligonucleotides may also be produced within a cell, for example, by recombinant technology. These intracellular oligonucleotides can then act on DNA replicating within the cell, or be extracted from the cell for use elsewhere.

Plasmid construction

Oligonucleotides corresponding to H forming sequences presented in FIG.1B were cloned in the pUC19 polylinker between the BamH1 and EcoR1 sites. In order to obtain single-stranded DNA, these sequences were further recloned in two orientations in the phagemid pBLUESCRIPT SK(-) (Stratagene). Either the purine-rich or the pyrimidine-rich strand could be rescued in a single-stranded state after the isolation of phage DNA. Supercoiled plasmid DNA was isolated by standard alkali lysis followed by twice repeated equilibrium centrifugation in a cesium chloride - ethidium bromide gradient.

Isolation of single-stranded DNA

Insertion derivatives of the phagemid pBLUESCRIPT SK(-) were transformed into the *E.coli* XL-1 strain. Fresh transformants were inoculated into 50 ml of 2xYT media containing 10^{8}-10^{9} pfu/ml of VCS helper phage. After 2 hours of incubation at 37°C, kanamycin was added up to 70mg/ml and cultures were subsequently incubated for 16 hours with vigorous aeration. Phage particles were separated from cells by centrifugation at 17,000 g for 10 min. followed by precipitation with 4% PEG in 0.7 M NH_4CH_3COO. Phage particles were resuspended in 300 ml H_2O, and DNA was isolated by phenol-chloroform extraction followed by ethanol precipitation. DNA was rinsed with 70% ethanol and diluted in 60 ml of H_2O.

Chemical footprinting of DNA

10 mg of supercoiled DNA samples were modified in 25 mM Na Cacodylate, pH 7.1, in the presence of 4 mM $MgCl_2$ or 1 mM $ZnCl_2$ by chloroacetaldehyde as described by Dayn *et al* (1992). Control DNA samples were incubated under the same conditions without CAA. DNAs were then digested with the restriction enzyme HindIII, end labeled, incubated with either formic acid or hydrazine, piperidine treated and loaded on a sequencing gel.

Photo footprinting of DNA

10 mg of plasmid DNA were linearized with HindIII and end-labeled by the Klenow fragment of DNA polymerase I and a-^{32}P-dCTP, followed by spin chromatography on Sephadex G50. Sequenase® buffer was added
5 up to 1x concentration, triplex-forming oligonucleotides were added up to 5 mM, and samples were irradiated in the UV Stratalinker 2400 at 3000 mkW/cm^2 for 2 min. DNA samples were then digested with BglI. 250-bp end-laheled fragments were eluted from an agarose gel, treated with piperidine, and loaded on a sequencing gel.

10 ***Construction of open circular templates***

Single-stranded DNAs containing the purine- or pyrimidine-rich strand of the original and mutated sequences were isolated as described above. 0.8 mg of single-stranded DNA was then mixed with 1.2 mg of the corresponding double-stranded DNA, linearized by SstI. A strand transfer
15 reaction was carried out by 4.4 mg of RecA protein in 480 ml of 5% glycerol, 1mM DTT, 25 mM tris-HCl (pH 7.2), 10 mM $MgCl_2$ for 10 min. at 37°C. *E. coli* SSB protein and ATP were added up to 61 mM and 4mM, respectively, and incubation continued for another 30 min. The reaction was terminated by 0.5% SDS, 20 mM EDTA, followed by proteinase K
20 treatment and phenol extraction. Open circular DNAs were then isolated from an agarose gel by elution on an ion-exchange membrane NA45 (Schleicher and Schuell).

Sequencing of open circular DNA

0.1 mg of nicked DNA was dissolved in 10 ml of 1xSequenase® buffer (U.S. Biochem.) and labeled in the presence of 1 ml 0.1M DTT, 1 ml a-^{32}P-dATP (3000 Ci/mmol, Amersham), 2 ml labeling mix (U.S. Biochem.)
5 and 13 units of Sequenase® version 2 for 3 min. at room temperature. Termination was then carried out for 5 min. at 37°C according to the manufacturer protocol (U.S. Biochem), followed by 15 min. of polymerase inactivation at 65°C. To provide a unique reference end for all synthesized fragments, DNA samples were digested with Sst I (note that the Sst I site
10 was restored in the course of DNA synthesis) and loaded on a sequencing gel.

Sequencing of single-stranded DNA with short triplexes

0.5 μg of single-stranded DNAs containing pyrimidine-rich targets were mixed with 100-200 pmol of duplex or triplex-forming oligonucleotides
15 (FIG. 6) 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 (U.S. Biochemical) with the following modifications. Labeling was done in the presence of 230 nM a-dNTP
20 (N=G,C,T) supplemented by 5 mCi of a-^{32}P-dATP (Amersham) for 2-3 min at room temperature. Then dNTP were added up to 33.3 mM and ddNTP up to 3.3 mM, followed by polymerization for 10 min. at 37°C.

In vivo methods of delivering oligonucleotides

There are many methods of delivery (administration) of oligonucleotides into an organism. These methods include direct intravenous injection of oligonucleotides and delivery via liposome
5 encapsulation.

For directed delivery to specific targets, antibody-targeted small liposomes may be used (Leserman, *et al.*, 1994). Advantages of liposome delivey compared to use of oligonucleotides free in solution include protection against degradation, reduction of toxicity (if any), improved
10 pharmacokinetics, and, possibly, increased intracellular transport. Liposome size and composition, the nature of the encapsulated molecule, the type of liquid used for targeting and its linkage to the liposome, as well as the cell type and target molecule will vary with a specific use of the oligonucleotides and may be determined by consideration of knowledge
15 in the art, some of which is cited herein. For example, cationic liposomes successfully delivered antisense oligonucleotides to cells infected by human papillomavirus (Lappalainen, *et al.*, 1994) and antisense oligonucleotides in antibody-targeted liposomes (immunoliposomes) bound to HIV-1 infected cell targets and inhibited HIV-1 proliferation (Zelphati, *et*
20 *al.*, 1994). Retroviral infection appears to stimulate cellular uptake of oligonucleotide liposomes (Robert, *et al.*, 1993).

If direct injection of free oligonucleotides is the method used, higher concentrations of oligonucleotides may be required as compared to delivery by liposomes (Leonetti, *et al.*, 1990). A suitable dose for systemic

administration of oligonucleotides is 0.01-50 mg/kg once or twice a day (Krawczyk, 1993).

Oligonucleotides may be incorporated into a carrier, e.g., a polyethylene oxide-polypropylene oxide copolymer (Dekeyser, *et al.*, 1993).

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WE CLAIM:

1. A method for blocking replication of a strand in a target DNA strand from a template DNA strand, said method comprising bringing an oligonucleotide into contact with said DNA thereby
5 forming a triplex structure that precludes replication.

2. A method of blocking polymerization by a DNA polymerase, said method comprising contacting an oligonucleotide with a template DNA to form a triplex structure which blocks polymerization downstream of the sequence.

10 3. The method of claim 1, wherein the oligonucleotide forms a triplex strand region in which one strand is pyrimidine-rich, and the other two strands are purine-rich.

4. The method of claim 1, wherein replication of a target DNA strand occurs *in vitro* at a neutral pH and a high concentration of Mg^{2+}.

15 5. The method of claim 4, wherein replication is effected by a polymerase selected from the group consisting of T7 DNA polymerase, Taq polymerase, Vent polymerase and *E.coli* Pol I DNA.

6. A triplex-forming oligonucleotide having a nucleotide sequence
20 having mirror symmetry of G clusters and at least at least 12 bases in length.

7. The triplex of claim 6 formed by self annealing of the oligonucleotides and bonding with a template DNA.

8. The oligonucleotide of claim 6, wherein the mirror asymmetry of G clusters is selected from a mirror image of a homopurine strand with G A residues, and the G residues are mirror images of G in the homopurine strand and intervening regions have T's which reflect A's in the homopurine strand.

9. A method for detecting a mutation in an oncogene, said method comprising contacting said oncogene with a triplex-forming oligonucleotide that binds to a DNA region of an oncogene, subjecting said complex to a polymerization reaction, and determining if a product is formed, wherein the presence of a product indicates a mutation.

10. The method of claim 9, wherein the polymerase is the Stoffel fragment of Taq polymerase and a triplex-forming oligonucleotide is added at a high molar excess 10^4, said oligonucleotide having a 3′ -OH groups substituted for amino groups.

11. The method of claim 9, wherein said polymerization takes place at a high magnesium concentration.

12. The method of claim 9, wherein the oncogene is a member of the *ras* oncogene family.

13. The method of claim 12, wherein a member of the *ras* oncogene family includes N-*ras*, c-Ki-*ras* and Ha-*ras*.

14. The method of claim 10, wherein the mutation is in codon 12 or 13 of a *ras* oncogene.

15. A method for blocking elongation of a strand DNA *in vivo*, said method comprising:

    (a) contacting an oligonucleotide capable of forming a triplex with the target DNA strand under polymerizing conditions;

5 and

    (b) providing the conditions for polymerization of the template.

16. The method of claim 15, wherein the DNA is mammalian DNA.

17. The method of claim 15, wherein the DNA template is in exon 3 of IR110 of HSV-1 at the position 122558-122593 and the triplex-

10 forming oligonucleotides are:

    (i) 5′-
AAGGAGGGAGGGGGGGGAGGAGGAGGAGGGG-
3′

    (GA-containing)

15 (ii) 5′-
TTGGTGGGTGGGGGGGGTGGTGGTGGTGG%GGGGG-5′

    (GT-containing).

18. The method of claim 15, wherein the DNA template is within the P-gene of the Hepatitis B virus at positions 2670-2681 and the triplex-

20 forming oligonucleotide is 5′-AGAAGAAAAGAG-3′ or 5′-
TGTTGTTTTGTG-3′.

19. The method of claim 15, wherein the DNA template is within the protein- coating sequence ORF-Ec of the Papillomavirus at positions 436-452 in HPV57 and 438-452 in HPV2α and the triplex-forming

oligonucleotides are 5'-GGAGGAAGAGGAGGG-3' or 5'-GGTGGTTGTGGTGGG-3'.

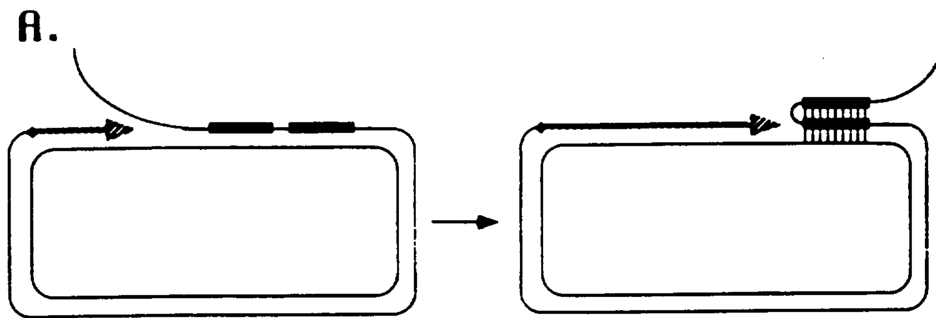
20. A method for treating a microbial infection in an organism, said method comprising:

5 (a) providing a composition comprising triplex-forming oligonucleotides directed toward a specific sequence in the agent causing the infection said oligonucleotides in a pharmacologically acceptable carrier; and

(b) delivering an effective amount of the composition to the

10 organism.

A.



B.

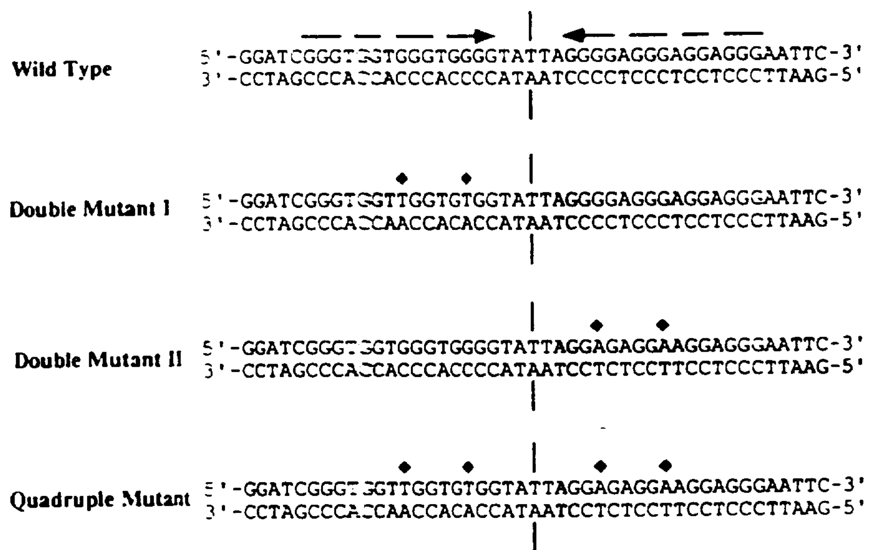


FIG. 1

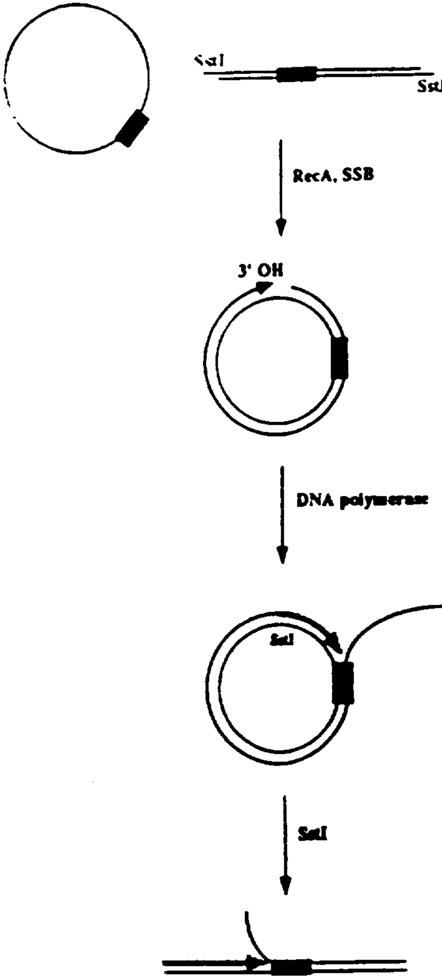


FIG. 2

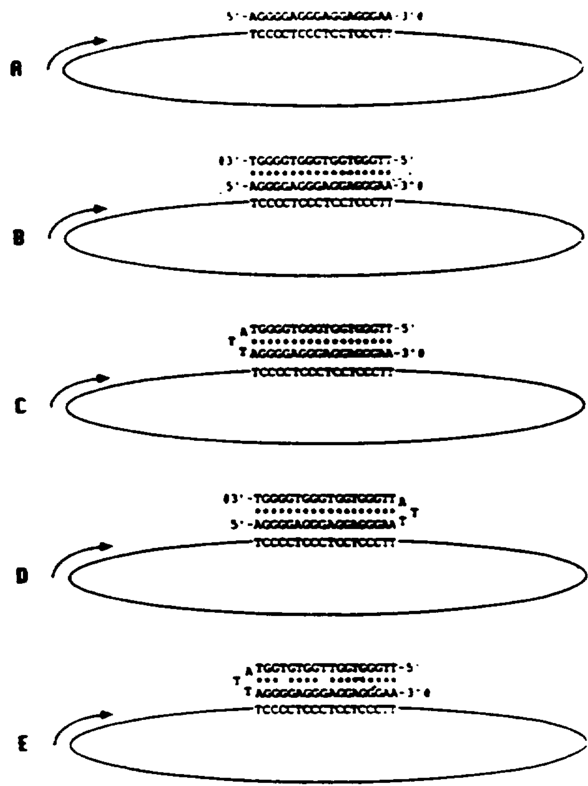


FIG. 3

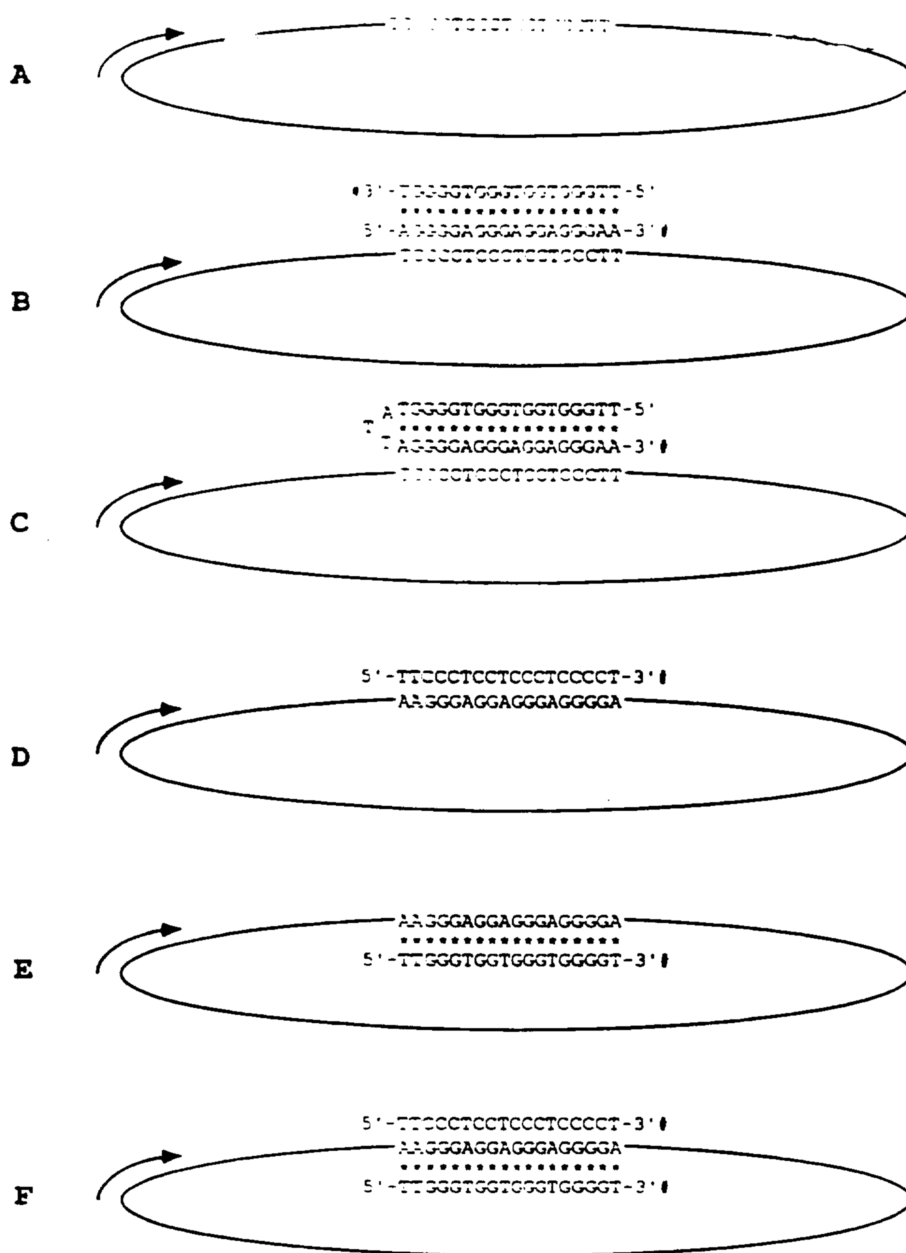


FIG. 4

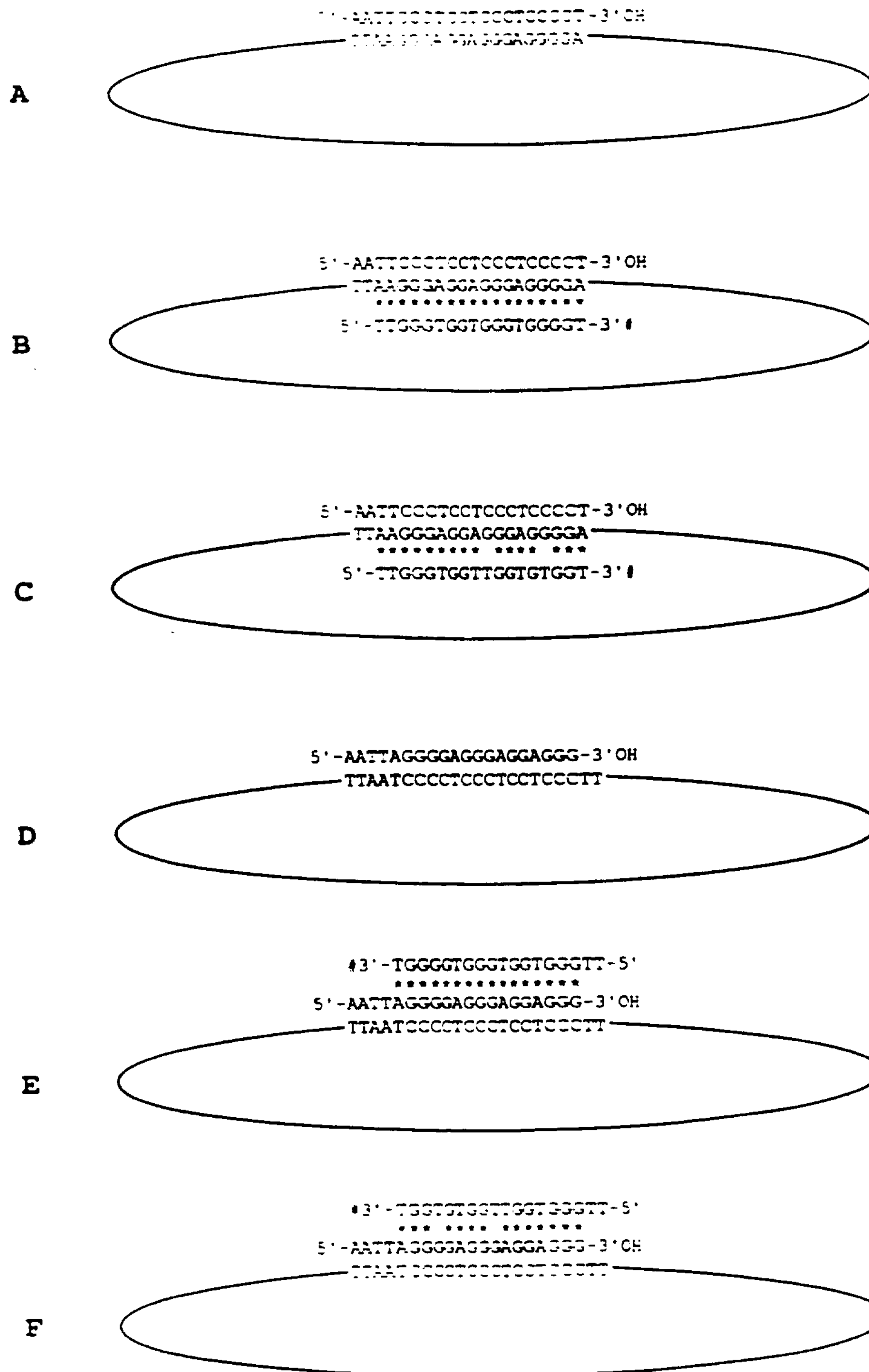
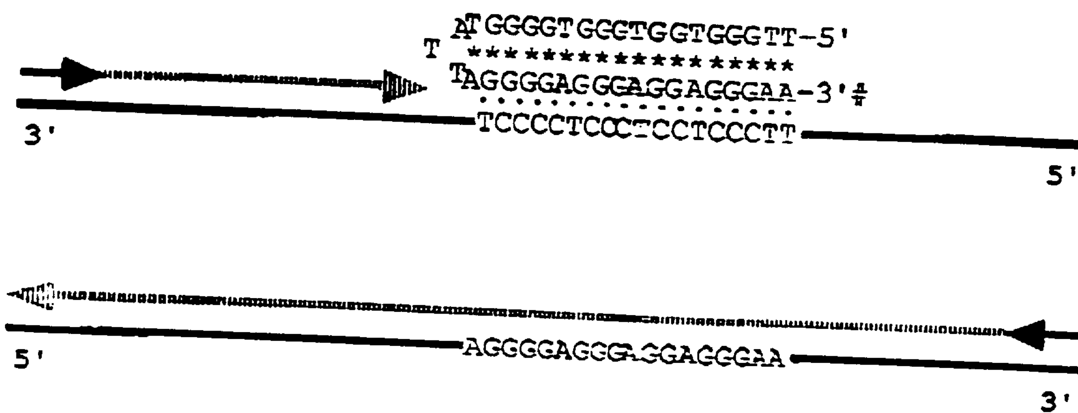


FIG. 5

FIG. 6



N-ras	GTT	GGA	GCA	GGT	GGT	GTT	GGG	AAA	AGC
Ki-ras	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGT
Ha-ras	GTG	GGC	GCC	GGC	GGT	GTG	GGC	AAG	AGT

FIG. 7

FIG. 8