Stimulation of RecA-Mediated D-Loop Formation by Oligonucleotide-Directed Triple-Helix Formation: Guided Homologous Recombination (GOREC)†

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ABSTRACT: Oligonucleotide-directed triple helix formation provides an elegant rational basis for gene-specific DNA targeting and has been widely used to interfere with gene expression ("antigene" strategies) and as a molecular tool for biological studies. Various strategies have been developed to introduce sequence modifications in genomes. However, the low efficiency of the overall process in eucaryotic cells impairs efficient recovery of recombinant genomes. Since one limiting step in homologous recombination is the targeting to the homologous sequence, we have tested the contribution of an oligonucleotide-directed triple helix formation on the RecA-dependent association of an oligonucleotide and its homologous target on duplex DNA (D-loop formation). For this study, the recombinant ssDNA fragment was noncovalently associated to a triple helix-forming oligonucleotide. The physicochemical and biochemical characteristics of the triple helix and D-loop structures formed by the complex molecules in the presence or in the absence of RecA protein were determined. We have demonstrated that the triple helix-forming oligonucleotide increases the efficiency of D-loop formation and the RecA protein speeds up also the triple helix formation. The so-called "GOREC" (for guided homologous recombination) approach can be developed as a novel tool to improve the efficiency of directed mutagenesis and gene alteration in living organisms.

Since 1987, oligonucleotide-directed triple helix formation (*1*, *2*) has raised considerable interest because it provides an elegant rational basis for gene-specific DNA targeting, for interfering with gene expression at the transcriptional level and other biological processes (*3*−*5*). In the past decade, intense research in this field has provided a physicochemical and biochemical basis for developing high affinity and nuclease-resistant triple helix-forming oligonucleotides (TFOs)[1] (*6*). It has been shown that transcription can be effectively inhibited by forming stable triple helices that compete either with the binding of transcriptional factors in gene regulatory regions (promoter/enhancer) or with the elongation of RNA transcripts by the transcription machinery (*7*, *8*). Furthermore, it has been demonstrated that, at least, some of the target DNA sequences in their chromatin environment are accessible to TFOs (*9*, *10*).

In parallel with the development of the so-called "antigene strategy", sequence-specific targeting of Watson−Crick double-stranded DNA (dsDNA) has been exploited to create new tools for molecular and cellular biology (*4*−*6*). TFOs have been successfully converted to artificial nucleases: (i) by covalent attachment of a DNA cleaving reagent (including metal chelating complexes, alkylating or photoactivable agents and nucleases) (*2*, *11*−*14*); (ii) by preventing methylation at a specific site by a methylase and cleaving by the associated restriction endonuclease (known as the *Achilles heel* approach) (*15*); (iii) by recruiting topoisomerase I in vicinity of the triple helix site (*16*, *17*). It has also been used to bend DNA (*18*) and to form closed rings around dsDNA ("padlock" oligonucleotide) (*19*, *20*). Interstrand cross-linking of DNA by TFO−psoralen conjugates has been shown to trigger DNA repair events that lead to site-specific mutagenesis (*21*−*24*).

Many known diseases and pathologies originate from mutations in DNA sequences. In general, the correct repair of mutated genes either by directed mutagenesis or by homologous recombination might help to cure these diseases. However, the lack of efficient tools for directed mutagenesis in mammalian cells has so far hampered significant progress in this field, in part, due to very low frequency of homologous recombination (10^{-8} − 10^{-5}) (*25*) and comparatively high background of random integration of transfected DNA (*26*). Recent progress has been made using the chimeric RNA/DNA oligonucleotides in mammalian cells (*27*−*29*). However, the frequency of targeted gene correction by oligonucleotides remains very variable according to the cell type and the gene location. Indirect evidences suggest that recombination activity may be rate limiting in gene

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[1] Abbreviations: TFO(s), triple helix-forming oligonucleotide(s); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA, bp, base pair; nt, nucleotide; T_m, melting temperature; K_D, equilibrium dissociation constant; k_{ass}, association rate constant; k_{diss}, dissociation rate constant; GOREC, guided homologous recombination.

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conversion by oligonucleotides in cells with competent mismatch repair activities (*30*). In an effort to stimulate the early steps of the homologous recombination, we designed a novel system called GOREC (for guided homologous recombination). We thought that the low efficiency of recombinogenic events can be ascribed to the two limiting steps: (1) search of sequence homology, and (2) the stability of the intermediate RecA-mediated complex (called joint molecule). We reasoned that (1) if the recombinant DNA fragment containing the desired sequence were brought to the vicinity of the homologous sequence by a TFO which can bind to a nearby oligopyrimidine·oligopurine sequence, the speed of homology search could be greatly enhanced, and (2) the binding of TFO could also enhance, to some extent, the stability of the joint molecule. These considerations were supported by a recent work carried out by Meyer and co-workers. They showed that triple helix formation promoted D-loop formation between a dsDNA sequence located adjacent to a triplex site and a single strand homologous DNA covalently tethered to the triplex-forming oligonucleotide in a plasmid, but only under high negative supercoiling (*31*).

The work herein describes the physicochemical and biochemical characterizations of the stimulation of the RecA mediated D-loop formation by triple helix formation. The binding of various TFOs to the oligopyrimidine·oligopurine target sequence was investigated by UV absorption melting experiments, gel retardation, and restriction enzyme assays. Since the lifetime of the triplex could also be a relevant factor for the search of sequence homology and the stabilization of RecA-mediated intermediate complex (joint molecule), the kinetics of triple helix formation and dissociation was studied by surface plasmon resonance (BIAcore2000). Similarly, the kinetics of dissociation between the DNA fragments was also studied in order to control the release of the TFO guide from the recombinogenic part, if necessary. This knowledge could be of importance in the exchange step of recombination. Finally, comigration assays of GOREC oligonucleotide(s) with the cognate plasmid Y0Gorec were carried out to provide evidence for triplex-enhanced homology search by RecA-mediated D-loop formation in vitro.

MATERIALS AND METHODS

Oligonucleotide. Oligonucleotides were purchased from Eurogentec (Sering, Belgium) and purified using quick-spin sephadex columns G-25 fine (Boehringer, Mannheim). Concentrations were determined spectrophotometrically at 25 °C using molar extinction coefficients at 260 nm calculated from a nearest-neighbor model (*32*), or an extinction coefficient of 8845 M^{-1}·cm^{-1} for acridine which was attached at the 5′-end of modified oligonucleotides (TFO1 and TFO1-S/L) (*33*), respectively

Plasmid. The plasmid Y0Gorec (6922 bp) was used to prepare duplex DNA. It was constructed by introducing a 42-bp sequence, containing the 18-bp oligopyrimidine·oligopurine sequence, which is a target for TFOs, five codons after the ATG initiation codon of the *URA3* gene in plasmid YEpURA3.22 (a gift from Dr. Barre). Supercoiled duplex DNA was purified on a cesium chloride gradient.

DNA Melting Experiments. Thermal denaturation and renaturation studies of duplexes and triplexes were carried out on a Kontron Uvikon 940 spectrophotometer with 1 cm optical path-length quartz cuvettes. The cell holder was thermoregulated by a circulating liquid composed of 80% water/20% ethylene glycol (v/v). Samples were cooled from 80 to 0 °C and heated back to 80 °C at 0.1−0.15 °C/min with absorption readings at 260 and 400 nm taken every 1 °C. Samples were kept for an additional 30 min at the lowest and highest temperatures. All samples were prepared in a buffer containing 20 mM sodium cacodylate, pH 7.2, 50 mM NaCl and 5 mM $MgCl_2$. For melting temperature (T_m) analysis, the baseline drift was corrected by subtracting absorption at 400 nm from that at 260 nm. The melting curve was obtained by plotting the corrected absorbance at 260 nm versus temperature (°C). The maximum of the first derivative of the melting curve ($\partial A/\partial T$) was taken as an estimation of T_m value. A hairpin duplex containing the 18-bp oligopyrimidine·oligopurine target sequence (5′-CGTCTA-GAAAAGAAAAGGGGGGA TACGC-T_4-GCGTATCCCC-CCTTTTCTTTTCTAGACG-3′, designated as 18YR) was used.

Kinetic Measurements. Kinetic studies were performed on a BIAcore2000 apparatus (BIAcore AB, Uppsala, Sweden). The fluidic system consists of four detection surfaces located in separate flow cells that can be addressed either individually or serially in a multichannel mode. The dsDNA or ssDNA target (see text) was immobilized on a dextran CM5 sensor chip via a biotin tag, which was captured by a streptavidin previously immobilized on the same chip according to the instructions provided by the manufacturer. The experiments were carried out at 37 °C in 20 mM cacodylate buffer (pH 7.2) containing 50 mM NaCl and 5 mM $MgCl_2$. The flow rate was set at 10 or 50 μL/min for triplex or duplex measurements, respectively. The regeneration was done by injection of a mixture containing 2 M urea, 20% ethanol, and 0.05% SDS for 30 s. One flow cell with nonspecific dsDNA or ssDNA was used as a reference to correct for either bulk refractive index contributions which are related to differences in the composition of injected samples as compared to running buffer or baseline drift during the course of data collection. The corrected sensorgrams were analyzed with the BIAevaluation software (version 2.0). The validity of rate constants was confirmed by comparing the calculated K_D (k_{diss}/k_{ass}) at T_m with that determined in DNA thermal denaturation experiments.

DNA Labeling. The short target duplex (18YR) or the oligopyrimidine triplex-forming oligonucleotide, as well as the recombinant (R) and control (M) fragments (see Figure 1) were 5′ end-labeled with [γ-^{32}P]ATP (Amersham Arlington Heights, IL) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). TFO1-S or TFO1-L was 3′-labeled by calf thymus terminal transferase (Boehringer Mannhein). Unincorporated radioactivity was eliminated using the Centri Spin-20 columns from Princeton Separation Inc. Specific activity was estimated by precipitation of aliquots with ice-cold 10% TCA and counting the radioactivity. When needed, the labeled oligonucleotides where diluted with unlabeled oligonucleotides to adjust the specific activity.

Gel Retardation Assay. At 20 nM, radiolabeled duplex 18YR* was titrated by TFOs in the concentration range of 0.1 to 3 μM and incubated in 50 mM HEPES buffer (pH 7.2) containing 50 mM NaCl and 5 mM $MgCl_2$ at 37 °C for 1 h, then loaded on a nondenaturing 10% acrylamide gel

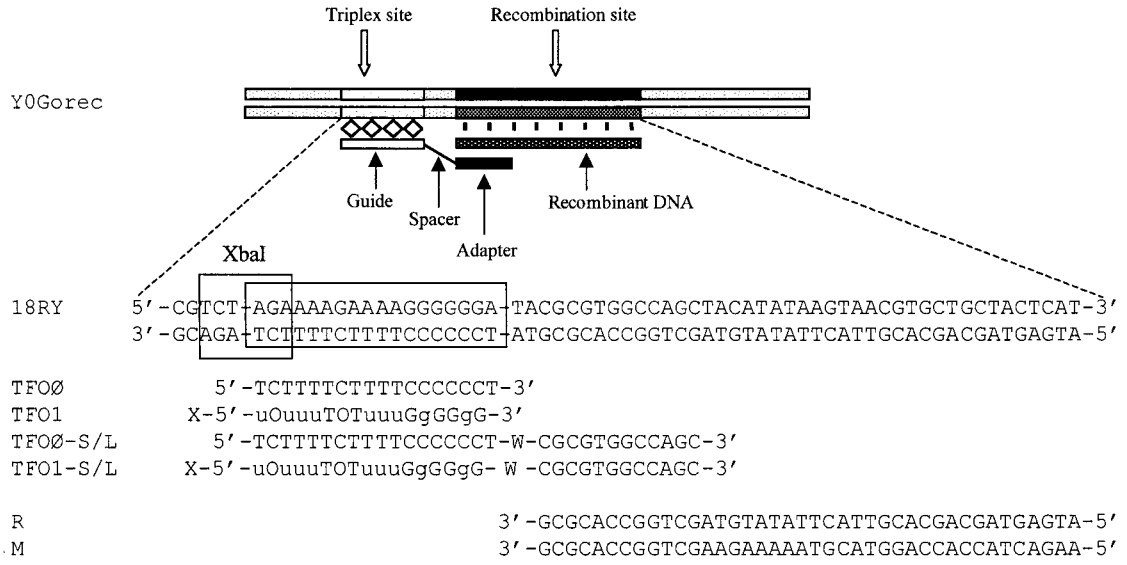

FIGURE 1: Schematic presentation of GOREC system and the sequences of Y0Gorec plasmid used in this study as well as their notations. The 18-bp oligopyrimidine•oligopurine sequence (18YR) for triplex formation is highlighted by a boxe. Hoogsteen hydrogen bonds between TFO and the target sequence are illustrated as diamonds and Watson−Crick interactions involved in D-loop between the recombinant fragment and the homologous duplex are indicated as vertical bars. TFO1 was composed of 5-propynyl-deoxyuracils (u), 5-methyl-deoxycytosines (O), deoxyguanines (G), and 7-deazadeoxyguanines (g) as well as 5′-tethered acridine derivative (X). W indicates either a triethyleneglycol (S) or a hexaethylenglycol (L) spacer. R and M stand for the homologous recombinant ssDNA and the non homologous fragments, respectively. The *Xba*I site which is overlapping the 5′-side of the triplex site is shown.

(19/1 mono/bisacrylamide). Electrophoresis was performed at 4 W for 3 h in 50 mM HEPES buffer (pH 7.2) in the presence of 5 mM $MgCl_2$ at 4 °C. The gel was then dried and quantified with a phosphorimager. The quantification of each band intensity (triplex, duplex, and TFO) allowed us to calculate the apparent triple helix dissociation constant K_D according to a simple two-state model.

Joint Molecule Assays. Joint molecules were formed by 10 min preincubation at 37 °C, using 0.5 μM labeled ssDNA in a reaction mixture containing 20 mM tris-HCl and 12.5 mM $MgCl_2$ (D-loop buffer) in the absence or in the presence of 15 μM RecA protein, 0.3 mM γ-ATPs, and 1.1 mM ADP. Y0Gorec plasmid (0.05 μM) was added at time zero. The final reaction volume was 10 μL. At the indicated time, one volume of stop buffer, containing 1% SDS and 20 mM EDTA, was added to the reaction mixture. The samples were separated at 4 °C by a 2 h electrophoresis (150 mA) on a 0.8% agarose gel using tris-borate buffer (pH 8) supplemented with 5 mM $MgCl_2$. The gel was dried and quantified with a phosphorimager.

Enzyme Assays. Various concentrations of TFOs were incubated for 1 h with 0.25 μg of the Pflm I linearized Y0Gorec plasmid to form complex at 37 °C. Cleavage reaction was triggered by addition of 20 units of *Xba*I and allowed to proceed for 15 min, and then stopped by addition of 0.1 M EDTA. The digested products were loaded on an ethidium bromide stained 1% agarose gel in 1 × TBE buffer.

Joint molecules were directly treated by the enzymes when RecA was absent. In reactions catalyzed by RecA protein, the protein was eliminated by adding EDTA and filtrating on a centrifugal concentrator Nanosep 100 K from Pall Filtron with washing in D-loop buffer. Joint molecules were either treated with different amounts of nuclease P1 (Boehringer Mannhein) for 15 min at 37 °C, in a buffer containing 20 mM Tris-HCl, 12.5 mM $MgCl_2$, and 5 mM $ZnCl_2$ or incubated with 10 units of *Xba*I. The extent of enzyme digestion was measured at 4 °C on a 0.8% agarose gel by a 2 h electrophoresis at 150 mA in tris-borate buffer (pH 8) supplemented with 5 mM $MgCl_2$. The gel was dried and quantified with a phosphorimager.

RESULTS

Design and Description of the GOREC System. To separate the respective contributions of the TFO and the recombinant oligonucleotide, we have designed an assembly of two functional modules, a "guide" and a "recombinant DNA" fragment (Figure 1). Guide module is made of three covalently linked elements: a triple helix-forming oligonucleotide (TFO); a synthetic spacer (W); and an oligonucleotide adapter. In this study, the single-stranded recombinant DNA fragment (R) shares sequence homology with a target DNA sequence in the vicinity of the TFO binding site except at the mutation site. The two modules are linked together through Watson−Crick base pairing between the 12-nt adapter and the complementary sequence at the 5′-end of recombinant DNA (R).

Sequence-specific dsDNA recognition by TFO via stable triple helix formation under physiological conditions at neutral pH and 37 °C is a prerequisite in the GOREC approach. As depicted in Figure 1, an 18-bp oligopyrimidine•oligopurine sequence that had been chosen as a target was cloned into a DNA plasmid (Y0Gorec) in the promoter region of yeast *URA3* gene. A pyrimidine motif of triplex can be formed upon binding an 18-nt oligopyrimidine (TFOØ) under acidic condition. Such a triplex is not expected to be stable at neutral pH. As an alternative, a mixed motif, where a 17-nt TFO (TFO1) was composed of 5-propynyl-deoxyuracils (u), 5-methyl-deoxycytosines (O), deoxyguanines, and 7-deazadeoxyguanines (g) as well as a 5′-tethered acridine derivative, was chosen to form a stable triplex at neutral pH.

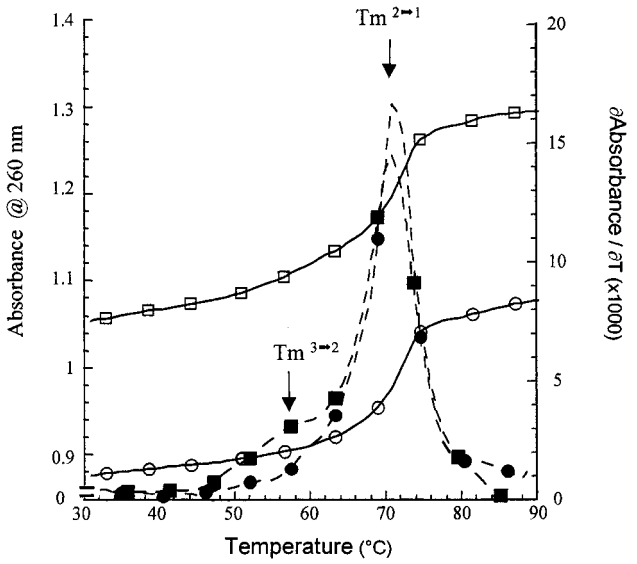

FIGURE 2: Melting curve of 18YR + TFO1 (solid line with open squares) and its first derivative (dashed line with filled squares). The melting curve of duplex 18YR alone (solid line with open circles) and its first derivative (dashed line with filled circles) is also shown. The T_m values of the triplex (18YR + TFO1, $T_m^{3\rightarrow2}$) and of the duplex (18YR, $T_m^{2\rightarrow1}$) are indicated by arrows.

It has been shown that (reviewed in ref *34*): (1) the replacement of six consecutive cytosines by guanines relieves the pH dependence of the triplex formation; (2) the partial substitution of guanines by 7-deaza-guanines prevents intermolecular G4-quadruplex formation between oligomers with G_6 tracts; (3) the 5-propynyl-uracils, 5-methylcytosines confer enhanced binding of TFO; and (4) the attachment of a dsDNA intercalator, such as acridine, at the 5′-end of the TFO further stabilizes the triplex. The TFOs were covalently attached to the linker which is made of a tri- or hexaethyleneglycol spacer (S or L, respectively) and a 12-nt oligonucleotide (adapter).

Thermal Stability and Binding Affinity. DNA melting experiments by UV spectrophotometry were first carried out to assess the thermal stability of triple helix formation by TFOs. A hairpin duplex containing the 18-bp oligopyrimidine•oligopurine target sequence (5′-CGTCTAGAAAAGAAAA-GGGGGGATACGC-T_4-GCGTA TCCCCCCTTTTCTTTT-CTAGACG-3′, designated as 18YR) was used. Figure 2 shows the melting curves of the TFO with the target double helix in 20 mM cacodylate buffer (pH 7.2) containing 50 mM NaCl and 5 mM $MgCl_2$. A biphasic melting curve was observed with the sample containing 1 μM duplex 18YR and 1.5 μM TFO1. The transition occurring at high temperature (70 °C) was identical to that observed for the duplex alone; therefore, it was attributed to duplex ↔ single strand transition. The transition at lower temperature was assigned to triplex ↔ duplex + TFO transition, since the TFO1 alone did not exhibit any melting profile. The melting temperature ($T_m^{3\rightarrow2}$) of the triplex formed by TFO1 and 18YR was about 56 °C as measured by the first derivative of the melting curve. Though, as anticipated, the nonmodified oligopyrimidine TFOØ did not form a stable triple helix under these conditions. However, triplex formation by TFOØ was observed at pH 6.2 ($T_m^{3\rightarrow2}$ = 28 °C, data not shown). When the adapter was linked to the TFO through a triethyleneglycol (S) spacer and a 40-nt ssDNA fragment (R) were appended to the guide module (TFO1-S or TFO1-S + R, respectively), the $T_m^{3\rightarrow2}$ value was slightly decreased by 4−6 °C (data not shown). It is known that a dangling end on the third strand destabilizes triple helix, probably due to charge repulsion (*35*). The melting temperature ($T_m^{2\rightarrow1}$) of the 12-bp duplex formed between the 12-nt adapter and the R fragment was about 64 °C under the same conditions (data not shown).

Binding of the GOREC system to target dsDNA was evaluated by gel retardation and by a restriction enzyme assay in solution. Triple helix formation can easily be detected by the altered mobility of radiolabeled duplex (18YR*) in nondenaturing polyacrylamide gel electrophoresis. As the concentration of TFOs increased, the migration of labeled duplex was retarded in a concentration-dependent manner, indicative of triple helix formation (Figure 3A). Quantification of gel retardation as a function of TFOs' concentration provided the dissociation equilibrium constant K_D of the various triplex systems (Table 2). It was observed that the binding constant (K_D) of TFO1 was about 0.3 μM. The covalent attachment of a 12-nt adapter to TFO1 did not significantly affect the binding affinity whether a short

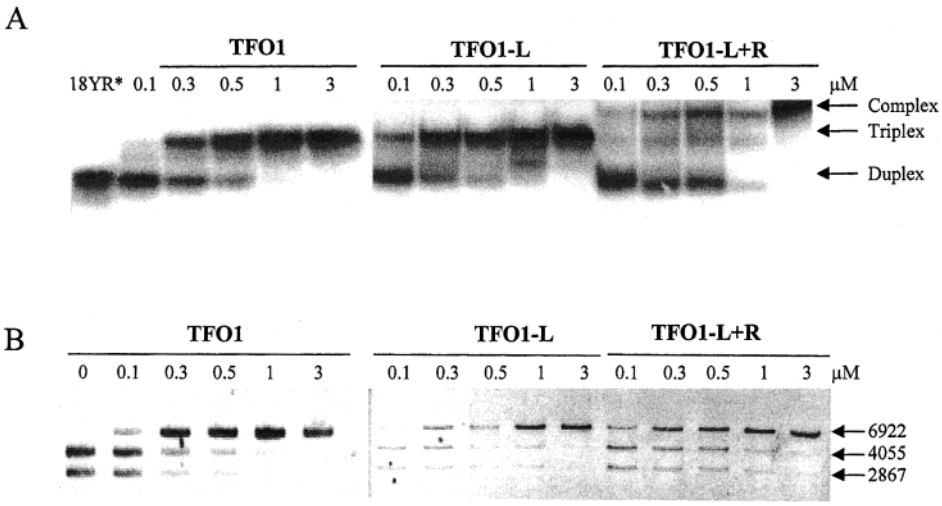

FIGURE 3: Increasing concentrations of the guide (TFO1), the guide-linker (TFO1-L) and the GOREC system (TFO1-L + R) were added to the 5′-labeled duplex (18YR*) (A) or to the Y0Gorec plasmid DNA (B). (A) Gel retardation of different triplex systems in 10% nondenaturing polyacrylamide gel. (B) *Xba*I restriction enzyme assays of the same systems.

Table 1: Composition and Nomenclature of Different GOREC Systems Used in This Study

name	guide	spacer	recombinant DNA
TFO1-S + R	TFO1	S	R
TFO1-L + R	TFO1	L	R
TFO1-S + M	TFO1	S	M
TFOØ-S + R	TFOØ	S	R
TFOØ-S + M	TFOØ	S	M

Table 2: Summary of Binding Studies of Different Triplex-Forming Oligonucleotides and GOREC Systems as Measured by Gel Retardation (K_D) and by Restriction Enzyme Assay (IC_{50}) as Shown in Figure 3[a]

	K_D (μM)[a]	IC_{50} (μM)[b]
TFO1	0.3	0.2
TFOØ−S	NB	NI
TFO1-S	0.3	0.5
TFO1-S + R	0.7	0.8
TFO1-L	0.4	0.2
TFO1-L + R	0.5	0.3

[a] NB and NI stand for no binding and no inhibition, respectively. The accuracy of the measurements is about 20%.

(triethyleneglycol) or a long (hexaethyleneglycol) spacer was used (TFO1-S or TFO1-L, respectively). However, the attachment of a 40-nt ssDNA fragment (R) caused a 2-fold decrease of binding affinity for the GOREC system with a short spacer (TFO1-S + R) whereas the loss was less pronounced with a long spacer (TFO1-L + R) (Table 2). It pointed out again that the presence of a dangling oligonucleotide end was, to some extent, detrimental to the binding of TFO, especially when the length of the spacer was short.

In parallel with gel retardation experiments, another assay based on the inhibition of restriction enzyme activity in solution was carried out. The binding site of *Xba*I nuclease overlaps that of TFO; therefore, triple helix formation prevents the binding of the enzyme, and thus inhibits its DNA cleaving activity. The 6922-bp plasmid Y0Gorec was first linearized by the restriction enzyme *Pfl*mI. The subsequent digestion by *Xba*I generates two DNA fragments of 2867 bp and 4055 bp. Figure 3B shows that the cleavage of the linearized plasmid by *Xba*I was inhibited when the concentration of TFOs was increased. The concentration required to achieve 50% inhibition of *Xba*I digestion (IC_{50}) was deduced from the gel quantification (Table 2). It reflects the binding affinity of TFOs, provided the enzyme cutting was not a limiting step. It is interesting to note that, in general, the IC_{50} values correlated quite well with the K_D values obtained by gel retardation experiments. A similar trend of binding affinity for various GOREC systems as regard to the effect of linker length and of dangling end was observed using both gel retardation and enzyme inhibition assays (Table 2).

Kinetic Studies. A hairpin dsDNA fragment containing the 18-bp oligopyrimidine·oligopurine target sequence (5′-CGTCTAGAAAAGAAAAGGGGGGA TACGC-T_4-GCG-TATCCCCCCTTTTCTTTTCTAGACG-T_7-3′-biotin) was anchored via a biotin tag which was captured by a streptavidin previously immobilized on the sensor chip. The real-time association and dissociation of TFO were monitored and recorded by BIAcore. As expected, the association process was concentration-dependent whereas the dissociation was concentration independent. All sensorgrams could be analyzed according to a simple two-state model. Table 3 shows the rate constants of triple helix formation at 37 °C.

Table 3: Kinetics of Triple Helix Formation Using BIAcore (see Materials and Methods for details)[a]

	k_{diss} (10^{-4} s^{-1})	k_{ass} (M^{-1}·s^{-1})	K_D (μM)
TFO1	2.5 ± 0.2	1100 ± 26	0.2
TFO1-L	2.1 ± 0.4	812 ± 60	0.3
TFO1-L + R	3.1 ± 0.2	627 ± 31	0.5

[a] Eight concentrations (0.75, 1, 1.5, 2, 3, 4, 6, and 8 μM) were used to evaluate the apparent association constant.

It is noted that the dissociation rate constant (k_{diss}) was slightly increased, whereas the association rate constant (k_{ass}) was also decreased in the presence of a tethered 40-nt recombinant oligonucleotide (TFO1-L + R). This observation can again be explained by charge repulsion between the target dsDNA and the dangling tail of the third strand oligonucleotide. Under these conditions, the lifetime ($\ln 2/k_{diss}$) of triplexes was about 40 min. It should be noted that the dissociation equilibrium constants (K_D) calculated as the ratio of k_{diss} over k_{ass} were consistent with those obtained in binding assays (see above).

Under the same conditions, kinetic experiments were performed by BIAcore to measure the rate constants of the 12-bp duplex (5′-CGCGTGGCCAGC-3′/3′-GCGCACCG-GTCG-5′) formed between the 12-nt adapter and the 5′-end of R fragment. As usual, a biotinylated oligonucleotide (biotin-3′-T_7-GCGCACCGGTCG-5′) was captured on the sensor chip to reach 700 RU. The sensorgrams of the binding of the 12-nt oligonucleotide with complementary sequence was analyzed according to a two-state model. The association rate constant of this 12-bp duplex was about 4×10^4 M^{-1}·s^{-1} (36-fold faster than that of triplex), whereas the duplex dissociation rate constant was about 1.7×10^{-4} s^{-1} and was slightly lower than that of the triplex. The lifetime of the duplex was about 1.1 h.

Joint Molecules Formation Is Stimulated by RecA Protein and Triplex Formation. The ability of various GOREC systems to form stable joints with the cognate plasmid Y0Gorec was assessed. The plasmid Y0Gorec carries a sequence homologous to the R oligonucleotide in the vicinity of the oligopyrimidine·oligopurine sequence where the guide TFO forms an intermolecular triple helix (Figure 1). Depending on the incubation conditions and the presence or the absence of the RecA protein, oligonucleotides can interact with the duplex plasmid DNA and form two kinds of structures: a D-loop and/or an intermolecular triple helix. The joint molecules were detected as labeled oligonucleotides comigrating with the circular plasmid (both open circle and supercoiled) and the nature of their junctions was further analyzed by nuclease digestion. Molecules with TFOØ-S and M sequences were used as controls as they did not form triplex at neutral pH and had no homology with the plasmid, respectively.

The reactions were first performed in the absence of RecA protein (Figure 4). The joint molecule formation was not detected when the guide TFOØ-S was associated to either M or R oligonucleotides. In contrast, they were efficiently formed when the guide TFO1-S was associated with either M or R fragment. These results indicate that the presence of a DNA fragment with an homologous or heterologous

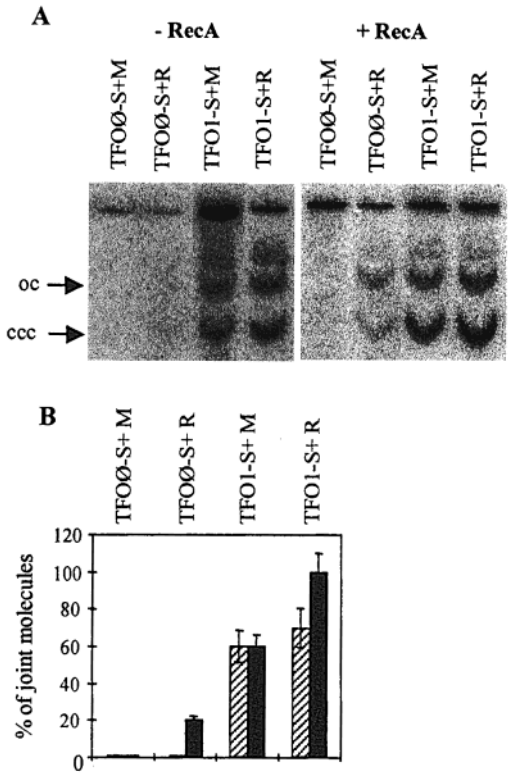

FIGURE 4: Comigration gel analysis (A) and quantification (B) of joint molecules. DNAs were incubated 45 min in absence (hatched boxes) or presence (black boxes) of RecA protein. oc, open circle; ccc, supercoiled plasmid.

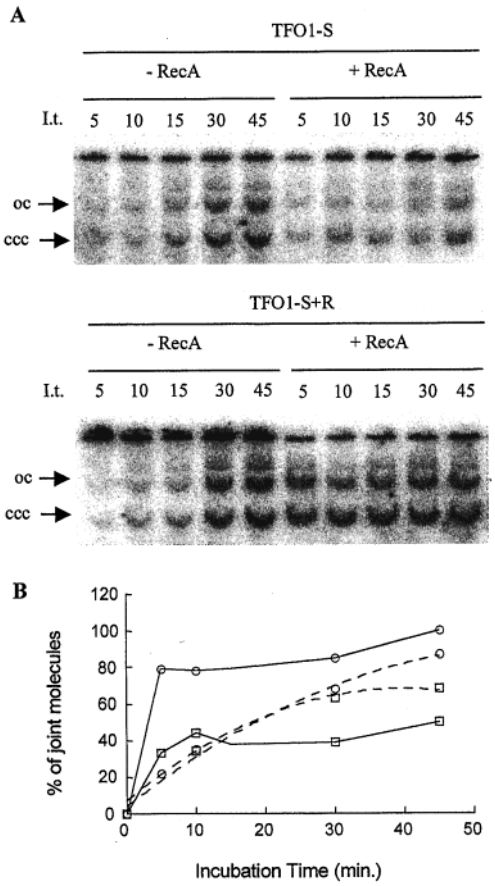

FIGURE 5: Comigration gel analysis (A) and quantification of kinetics of joint molecule formation (B). TFO1-S + R complexes (circles) and TFO1-S molecules (squares) were incubated for different times with duplex DNA in a reaction buffer containing (full lines) or not (dashed lines) the RecA protein. oc, open circle; ccc, supercoiled plasmid. I.t., Incubation time in minutes.

sequence did not affect triple helix formation on a plasmidic DNA. In addition, the absence of joint molecule formation with the TFOØ-S + R (which carries the recombinant DNA but cannot form a triplex) confirmed that the joint molecules formed with the plasmid under these conditions were only due to triplex formation.

In vitro, the RecA protein has been shown to promote formation of joint molecules between oligonucleotides and homologous duplexes (*36*). The efficiency of the reaction depends on the size of the oligonucleotide probably because RecA filaments are very unstable on short ssDNAs (*37*−*39*). Under our experimental conditions, few joint molecules were detected, after 45 min of incubation with the TFOØ-S + R oligonucleotide and the duplex DNA (Figure 4). These joint molecules were not formed in absence of RecA protein. The efficiency of the reaction was 5-fold stimulated when the R fragment was associated to the TFO1-S rather than the TFOØ-S oligonucleotide (Figure 4). The formation of joint molecules between TFO1-S + M complex and duplex DNA was not affected by the RecA protein. This result indicated that the binding of RecA protein did not significantly interfere with triplex formation. Moreover, RecA was able to enhance the formation of joint molecule between TFO1-S + R complex and duplex DNA: 100% of the complex were associated to the plasmid duplex in the reaction catalyzed by the RecA protein.

To assess the effect of the RecA protein on the formation of the joint molecule between the GOREC system (TFO1-S + R) and the Y0Gorec plasmid, the kinetics of the reactions with and without the RecA protein were compared (Figure 5). Similar kinetic measurements were performed with the guide TFO1-S alone, to estimate the contribution of the triplex formation during the reaction. The main effect of the RecA protein appears to be the acceleration of the reaction: 80% of the joint molecules was formed in 5 min with TFO1-S + R in the presence of RecA whereas only 22% was detected in the absence of the protein at the same time (Figure 5B). The slow rate of triple helix formation in the absence of RecA protein was not due to the R recombinant oligonucleotide since the TFO1-S alone also exhibited slow kinetics of triple helix formation. It is noted that the presence of RecA accelerated also the triple helix formation by TFO1-S alone, probably due to the rapid interaction between the RecA-coated TFO1-S oligonucleotide (data not shown) and the plasmid. All these data taken together suggest that both kinds of structures, the triplex and the RecA-mediated D-loop, were formed in this reaction

Nature of the Junctions within the Joint Molecules. Joint molecules formed by the TFO1-S + R in the reaction stimulated by RecA protein can result from (1) the formation of a triple-helical structure between the guide TFO1-S and its target, and/or (2) the formation of a D-loop structure between the R recombinant DNA and the homologous sequence on the plasmid. To determine the respective contribution of the two structures, the sensitivity of the joint molecules to different enzymes whose activity is specific of different DNA structures was measured. It was found that most of the plasmids in the joint molecule formed with TFO1-S + R were resistant to *Xba*I cleavage indicating the

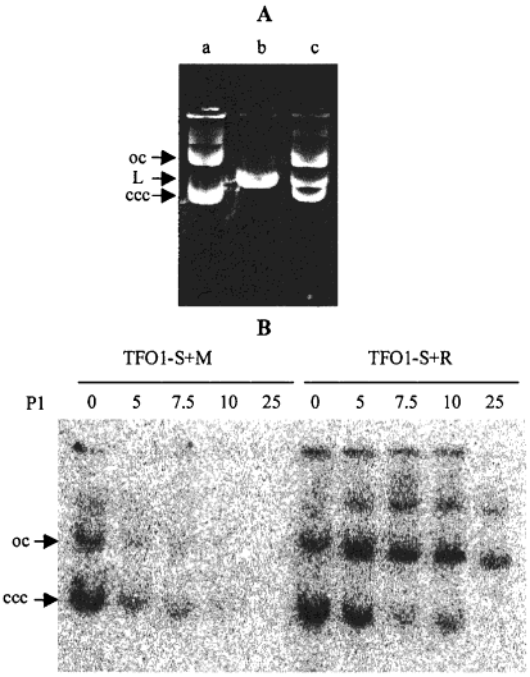

FIGURE 6: Analysis of the enzyme digestion protection of Y0Gorec plasmid or recombinant ssDNA fragment by joint molecule formation. (A) *Xba*I restriction enzyme digestion. Joint molecules formed with TFOØ-S + R (lane b) or TFO1-S + R (lane c) were treated by *Xba*I enzyme as described in Materials and Methods. The undigested plasmid was run on the same gel (lane a). (B) Analysis of P1 nuclease digestion protection of the homologous R or heterologous M oligonucleotides in joint molecules. Joint molecules were formed by incubating DNAs with RecA protein for 45 min and digested by increasing amount of P1 nuclease ($\times 10^{-4}$ units) as described in Materials and Methods. oc, open circle; ccc, supercoiled plasmid. L, linear plasmid.

formation of the triplex structure (Figure 6A). As expected, joint molecules formed with TFOØ-S + R were sensitive to *Xba*I restriction enzyme. In D-loop structures, the ssDNA which had invaded the homologous duplex DNA to form a duplex structure became resistant to degradation by ssDNA nucleases. The R recombinant fragment was resistant to P1 exonuclease in the joint molecules formed by TFO1-S + R in the presence of RecA protein. In contrast, the non homologous M sequence was sensitive to the ssDNA nuclease in the joint molecule formed by TFO1-S + M. Digestion of M fragment indicates that the protection observed in the TFO1-S + R joint molecule was not due to remaining RecA binding but rather to homology-dependent interaction with the plasmid. The TFO1-S + R joint molecule formed in absence of the RecA protein as sensitive to P1 digestion as the TFO1-S + M (data not shown). This observation indicated that triplex formation was not able to promote D-loop formation in absence of RecA protein. This observation was consistent with the previous work that triple helix formation was not able to promote a D-loop formation between a dsDNA sequence located adjacent to a triplex site and a single strand homologous DNA covalently tethered to the triplex-forming oligonucleotide in a plasmid without highly negative supercoiling and in the absence of RecA protein (*31*). The two analysis of the sensitivity of joint molecules to enzymatic digestion showed that most of the joint molecules formed by TFO1-S + R in the presence of RecA protein, contained both triplex and D-loop junctions.

DISCUSSIONS

The affinities of the TFO of various GOREC systems (TFO1-S or TFO1-L + R) as measured by gel retardation experiments and restriction enzyme assays, or calculated from kinetic rate constants, are mutually consistent and are in the submicromolar concentration range under near physiological conditions at 37 °C (Tables 2 and 3). The attachment of a recombinant fragment slightly weakens the binding constant of the TFO (guide). The use of a long spacer (hexaethyleneglycol) allowed us to limit the loss to a factor of 2. On the other hand, the rate constants measured by BIAcore experiments gave an apparent association rate constant (k_{ass}-[TFO] + k_{diss}) of about 10 min which is in reasonable agreement with that measured in D-loop assay (Figure 5) in the absence of the RecA protein. It is noted that the lifetime ($\ln 2/k_{diss}$) of the duplex formed by the recombinant ssDNA and the adapter was about 30 min longer than that of the triplex formed by the TFO and the target sequence. It implies that, on average, the GOREC complex (TFO1-L + R) has a longer lifetime than that of the triplex. In addition, the lifetime of the triplex is about 40 min under the conditions of recombination at 37 °C. It remains to determine whether these kinetic characteristics provide enough time for homology search and for stabilization of the joint molecules formed by the recombinant ssDNA with the homologous dsDNA sequence in vivo. Kinetic studies showed that different steps in the GOREC approach can be kinetically controlled, and subsequent fine-tuning should be possible by adjusting the length or/and the sequence of either the TFO or the adapter. Such a kinetic control might be important for in vivo applications.

This modular design of the GOREC system was motivated by two reasons: (1) the noncovalent attachment of recombinant DNA to the guide sequence might facilitate the exchange step of recombination, and most importantly, it should reduce the risk of unexpected mutation due to the TFO (which is not a homologous DNA fragment), as compared to a covalent attachment; (2) the 12-nt adapter can be used as one of the primers to obtain recombinant DNA by PCR amplification. In the present study, the recombinant fragment was a 40-nt long oligonucleotide involved in a short 12-bp duplex. A fully double-stranded recombinant fragment can also be carried out by the GOREC system. This might provide an increased stability since the dsDNA fragment is expected to be more resistant to nuclease degradation in vivo than ssDNA.

While this work was in progress, Glazer's group has reported an enhanced triplex-directed mutagenesis in mammalian cell using a 30-nt GA-motif TFO covalently linked to a recombinant ssDNA or dsDNA (*40*). A similar work has also been reported by Culver et al. (*41*). However, triplex formation was not demonstrated in the last study. Our GOREC approach mainly distinguishes from the previous works by the modular concept which, in principle, could have advantages to prevent unexpected mutagenesis due to the TFO and to be flexible and versatile since the GOREC approach is, in principle, not limited by the length of the recombinant DNA fragments (ssDNA or dsDNA) that can be easily obtained by PCR. In the present study, the 17-nt TFO is by itself too short to be a substrate for recombination. Work is in progress to provide evidence that GOREC can

work within cells to direct mutations not only at proximal but also at distal sites from the target site in an error-free manner.

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