J. Biochem. 119, 805-810 (1996)

Formation of a Triple-Stranded DNA between d(GGA:TCC) Repeats and d(GGA) Repeat Oligonucleotides[1]

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Received for publication, November 28, 1995

Incubation of the 162-bp duplex containing d(GGA:TCC)$_{11}$ repeats with d(GGA)$_{11}$ and d(GGT)$_{11}$ oligonucleotides, but not with d(TCC)$_{11}$, gave a mobility-shifted band in polyacrylamide gel electrophoresis, suggestive of the triplex formation. The two conformers, however, showed difference in binding affinity and structure. The complex formed with d(GGT)$_{11}$ exhibited patterns protected from DNase I digestion and from modifications with osmium tetroxide and dimethyl sulfate. The patterns were conformed to a proposed triplex model. In contrast, the complex with d(GGA)$_{11}$ was sensitive to DNase I and to osmium tetroxide, which is reactive to pyrimidine bases of single-stranded DNA. These results suggest that the d(GGA:TCC)$_{11}$ and d(GGA)$_{11}$ triple-stranded complex forms a D-loop-like structure. By considering the ability of d(GGA)$_{11}$ oligonucleotides to form a parallel homoduplex, the structure may be one in which the GGA-strand of the duplex pairs with d(GGA)$_{11}$ in parallel, with the TCC-strand being looped out.

Key words: D-loop-like structure, DNase I footprinting, osmium tetroxide, polypurine: polypyrimidine stretches, triplex DNA.

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Polypurine:polypyrimidine (Pu:Py) stretches are abundant in eukaryotic DNA. They constitute up to 1% of the mammalian genome and some of them are highly conserved (*1, 2*). The sequences exhibit a hypersensitivity to single-strand-specific nucleases, indicative of a non-B-DNA conformation (*3, 4*), and hence may provide candidate motifs for triple-stranded complexes in chromosomes. The first triplex of nucleic acids was reported nearly four decades ago and several triple-stranded structures were reported in the 1960s (*5*). They were rediscovered in the late 1980s and 1990s by *in vitro* experiments that show the formation of intermolecular triplexes between duplex DNA and synthetic oligonucleotides (*6-9*) and intramolecular triplexes in supercoiled DNA (*10-14*).

There are triplexes of two types, Pu/Pu:Py and Py/Pu:Py; the former has the purine-rich sequence as a third strand and the latter has pyrimidine-rich sequence instead. The Py/Pu:Py triplex was first observed in acidic conditions (*7, 8, 11, 12*). A low pH favors such structures since the C^+/G:C triplet is stabilized by the protonation of a C residue at N_3 position. More recently, the formation of Pu/Pu:Py triplexes has been demonstrated (*15-18*). Triplexes of this type can occur spontaneously at neutral pH and in the presence of Mg^{2+}. Their two purine strands are antiparallel, due to the formation of G/G:C and A/A:T triplets. Instead of A/A:T, the T/A:T base triplet also can participate in Pu/Pu:Py triplexes in which thymine in the third strand binds the A:T base pair in a reverse-Hoogsteen manner (*17*). The two triplet structures differ in the conformation about the glycosyl bond (*syn* or *anti*) and the location of the phosphate-deoxyribose backbone in the major groove of DNA (*17*).

We previously demonstrated that oligonucleotides consisting of the d(GGA) repeats associate intermolecularly (*19*). Interestingly, the conformer exhibits a parallel-oriented homoduplex under physiological conditions (*20*). This raises the possibility of formation of triplex with the third purine-strand parallel to the purine strand of the Watson-Crick duplex containing d(GGA:TCC) repeats. Here this possibility has been tested. The present paper demonstrates that the repeat oligonucleotide is able to form a triple-stranded complex with the duplex. Analyses of the complex with enzymatic and chemical probing reveal sensitivities to those reagents, and therefore the complex has a structure distinct from the triplexes previously reported. Our structural interpretation is discussed.

EXPERIMENTAL PROCEDURES

Probe DNA—Oligonucleotides used in this study were synthesized on an Applied Biosystems model 380B DNA synthesizer. HPLC-purified M4 (5′-GTTTTCCCAGTCACGAC-3′) and RV (5′-CAGGAAACAGCTATGAC-3′) primers complementary to the pUC118 sequence were purchased from Takara Syuzo (Kyoto). ^{32}P-labeled 162-bp target DNA containing the d(GGA:TCC)$_{11}$ repeat array was synthesized with PCR using this primer set and [α-^{32}P]-dCTP (DuPon-New England Nuclear) (*21*); the template used was pUC118 plasmid DNA that had an insert of synthetic d(GGA:TCC)$_{11}$ sequence between *Bam*HI and

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Abbrebiations: pu, polypurine; py, polypyrimidine; :, Watson-Crick base paring; /, non-Watson-Crick base pairing; bp, base pairs; DMS, dimethyl sulfate; ODN, oligodeoxyribonucleotide.

XbaI cloning sites. PCR products were purified by gel electrophoresis and used for association assay.

Association Reactions and Gel Mobility Shift Assay—Association was carried out in a 10-μl reaction volume containing 20 mM Hepes-NaOH, pH 8.0, 0.1 M NaCl, and 10 mM $MgCl_2$ at 37°C for 1 h except where otherwise indicated. The target ^{32}P-labeled 162-bp DNA and oligonucleotides shown in Fig. 1 were at concentations of 1 nM and 1 μM, respectively, except where noted otherwise. Products were separated through 5% polyacrylamide gels (acrylamide:bisacrylamide 19:1) in a buffer containing 90 mM Tris-borate, pH 8.0, 10 mM $MgCl_2$. Gels were dried and autoradiographed. In the analyses of enzymatically or chemically modified DNA described below, association reactions were the same as descibed above except that 25 nM (0.5 μg) pUC118 plasmid DNA containing d(GGA:TCC)$_{11}$ repeat was used instead of ^{32}P-labeled 162-bp DNA.

DNase I Digestions—After the association reaction, DNase I (0.1-0.5 unit as indicated) was added to the association mixture, followed by incubation at 18°C for 30 s. The reaction was terminated by the addition of 0.3 ml of a buffer containing 10 mM Tris-HCl, pH 7.4, 0.3 M NaCl, and 0.3% SDS (*15, 22, 23*). The digested DNA was treated with phenol and recovered by precipitation with ethanol.

Osmiun Tetroxide Reactions—One microliter of 10 μM OsO_4 was added to 100 μl of association mixture containing 2% pyridine that had been 10-fold diluted with the association buffer. After incubation at 25°C for 20 min, the reaction was stopped with 2 volumes of ethanol, then the DNA was subjected to piperidine treatment at 90°C for 30 min (*22, 23*). The piperidine was removed by lyophilization.

DMS Reactions—One microliter of 10% DMS was added to 100 μl of association mixture that had been 10-fold diluted with the association buffer. After incubation at 20°C for 3 min (*14, 20, 22, 23*), samples were processed in the same manner as in the OsO_4 modification.

Assay for the Level of Digestion and Modification—The levels of DNase I digestion and piperidine cleavage of modified bases were analyzed by the primer extension method. Enzymatically or chemically modified DNA was used as template for the primer extension method using a CircumVent thermal cycle dideoxy DNA sequencing system (New England BioLabs), except that 0.2 mM concentrations of dNTPs were used. The M4 and RV primers, shown in Fig. 1, were 5′ terminally labeled with [γ-^{32}P]-ATP and polynucleotide kinase (*22*) and used for the analysis of TCC- and GGA-strand, respectively. The location of the modified sites was determined by electrophoresis on a 6% polyacrylamide-8 M urea gel in parallel with the sequencing ladder. The bands were visualized by autoradiography.

RESULTS

The 162-bp double-stranded DNA containing d(GGA:TCC)$_{11}$ repeat sequence in the middle was synthesized with PCR and used for an association assay (Fig. 1). The probe DNA was incubated with either of two complementary oligonucleotides, d(GGA)$_{11}$ and d(TCC)$_{11}$, or a sequence,

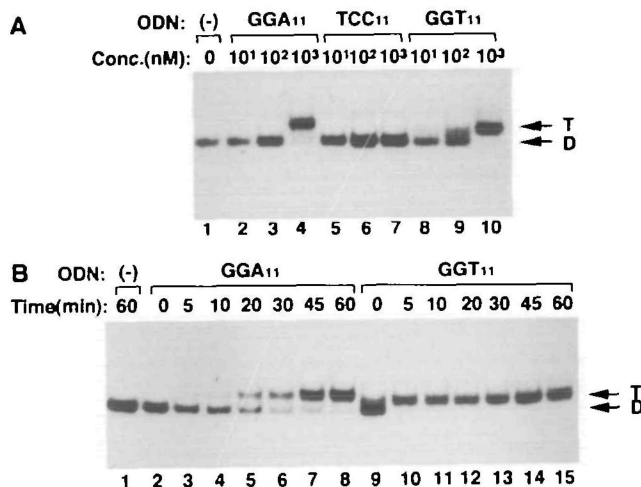

Fig. 2. **Gel mobility shift assay for triplex formation.** (A) Effect of ODN concentration on triplex formation. 1 nM ^{32}P-labeled 162-bp target duplex DNA containing d(GGA:TCC)$_{11}$ was incubated at 37°C for 1 h with the indicated concentrations of d(GGA)$_{11}$ (lanes 2-4), d(TCC)$_{11}$ (lanes 5-7), and d(GGT)$_{11}$ (lanes 8-10) as described in "EXPERIMENTAL PROCEDURES." Lane 1 shows the target DNA itself. The reaction mixtures were separated on a 5% polyacrylamide gel, dried and autoradiographed. (B) Time course of the complex formation. ^{32}P-labeled target DNA was incubated with 1 mM d(GGA)$_{11}$ (lanes 2-8) or 1 mM d(GGT)$_{11}$ (lanes 9-15) at 37°C for various periods of the time (min) indicated above the lanes. Lane 1 shows the target DNA alone. Arrows marked T and D indicate triplex and duplex, respectively.

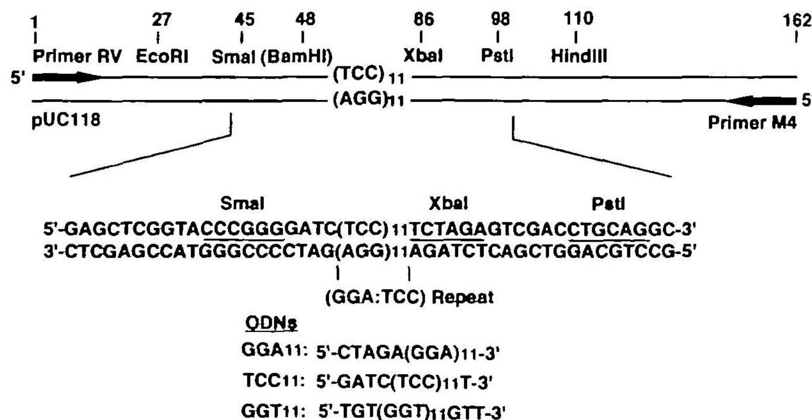

Fig. 1. **The target DNA containing d(GGA:TCC)$_{11}$ and triplex-forming oligodeoxyribonucleotides.** Map and sequence surrounding d(GGA:TCC)$_{11}$ repeats, which were inserted between *BamHI* and *XbaI* sites of plasmid pUC118, are shown at the top. Primers RV and U4 shown in the map were used for PCR reactions and primer extension assays. The first C residue of primer RV is designated as position 1. Sequences of oligodeoxyribonucleotide (ODN) termed (GGA)$_{11}$, (TCC)$_{11}$, and (GGT)$_{11}$ are also shown. (GGT)$_{11}$ was designed to bind in an antiparallel orientation relative to the GGA-strand of the duplex (*16, 17*).

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$d(GGT)_{11}$, under conditions of 20 mM Hepes buffer, pH 8.0, 0.1 M NaCl, and 10 mM $MgCl_2$. Products in the mixtures were analyzed by polyacrylamide gel electrophoresis (Fig. 2A). A mobility-shifted band was observed for the incubations with $d(GGA)_{11}$ and $d(GGT)_{11}$, but not with $d(TCC)_{11}$. This indicated that the former two oligonucleotides were able to associate with the duplex. Formation of the two complexes, however, showed different time courses (Fig. 2B). Complex formation with $d(GGA)_{11}$ required 30-60 min, whereas that with $d(GGT)_{11}$ took less than 5 min. This demonstrated a difference in association kinetics between the two conformers, probably reflecting their distinct structures.

Structural analyses of triplexes consisting of a polypurine:polypyrimidine stretch and a polypurine strand were performed previously, providing a triplex model (*17, 24*). The third purine strand is positioned within the major helix groove and the two purine strands run in antiparallel through G/G:C and A/A:T pairing; here, / indicates non-Watson-Crick pairing; : denotes Watson-Crick pairing. Triplexes also occur with a third strand substituting thymidines for adenines; they are formed by G/G:C and T/A:T triplets (*17, 24*). DNase I hardly cleaves such triplexes of DNA, whereas it easily digests both the double-stranded and single-stranded DNA (*15, 16, 18*). The two complexes formed between DNA containing $d(GGA:TCC)_{11}$ and either $d(GGA)_{11}$ or $d(GGT)_{11}$ were partially digested with DNase I, and the sensitivity was measured by the extension method using ^{32}P-labeled primers (shown in Fig. 1) as described in "EXPERIMENTAL PROCEDURES." Figure 3 shows gel electrophoretic patterns of the two PCR products. In the triple-stranded complex with $d(GGT)_{11}$, both strands of the duplex showed decreased sensitivity to DNase I in the repeat region relative to the control duplex. Of the GGA-strand, intense bands in the middle are presumed to be due to stalling of Taq DNA polymerase. This is consistent with a prediction from the Pu/Pu:Py triplex model. In contrast, the triple helix with $d(GGA)_{11}$ exhibited increased sensitivity for both strands of the duplex and also showed a hypersensitive site in the vicinity of the repeat region of the purine strand. This pattern demonstrated a clear difference in structure between the two conformers.

Osmium tetroxide (OsO_4) is known to oxidize the C_5=C_6 bond of pyrimidine bases of single-stranded DNA, particularly that of thymidine residues. On the other hand, the DNA region forming a triplex or duplex is protected from modification with OsO_4 (*14, 23*). Figure 4 shows modification patterns of the two triple-stranded complexes, together with those of the duplex and the duplex incubated with $d(TCC)_{11}$. The TCC-strand of the GGT/GGA:TCC complex did not show much difference from that of the duplex, which was consistent with the triplex model. A hypersensitive pattern at the repeat end was observed in the presence of $d(TCC)_{11}$. This is an unexpected result,

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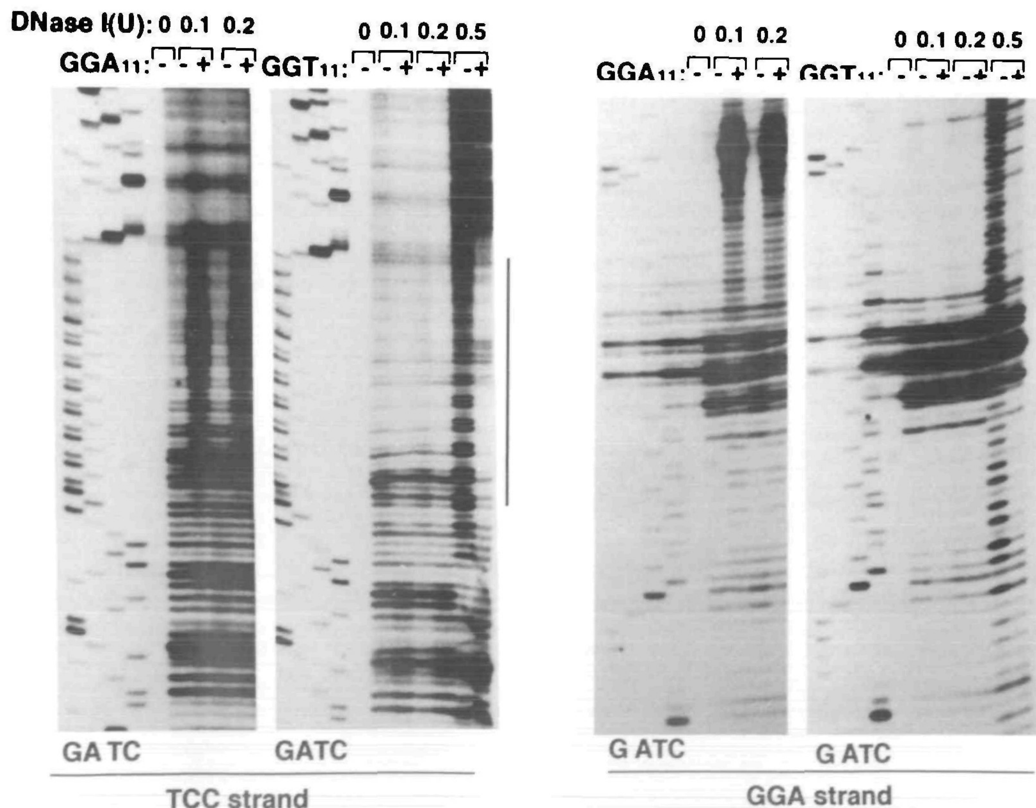

Fig. 3. **DNase I footprinting.** Circular plasmid DNA containing $d(GGA:TCC)_{11}$ repeats was incubated with either $d(GGA)_{11}$ or $d(GGT)_{11}$, then digested with the indicated concentrations (0.1-0.5 unit) of DNase I. Cleavage sites of the products were determined by primer extension assay. The left and right panels show the cleavage patterns of the TCC- and GGA-strand, respectively. Note that the fragment patterns shown here correspond to the opposite strand extended from the ^{32}P-labeled primer. Vertical lines at right of the panels indicate the repeat regions. High intensity bands were seen in the repeat region of GGA-strand in all reactions, which is presumed to be due to stalling of Taq DNA polymerase.

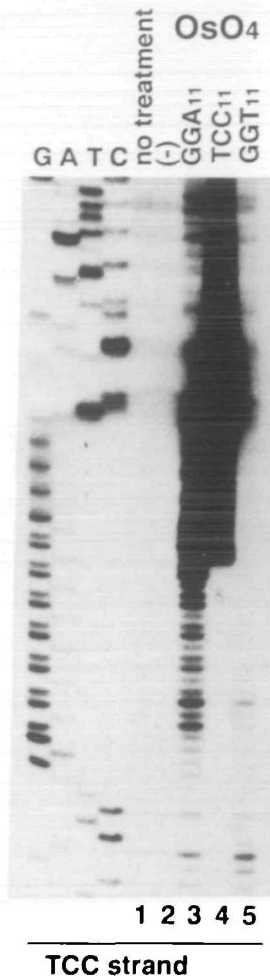

Fig 4. **Patterns of OsO_4 modification.** Circular plasmid DNA containing d(GGA:TCC)$_{11}$ repeats was incubated with d(GGA)$_{11}$ (lane 3), d(TCC)$_{11}$ (lane 4), d(GGT)$_{11}$ (lane 5), or without ODN (lanes 1 and 2), followed by OsO_4 modification (except for lane 1) and piperidine treatment. Modification sites of TCC-strand were determined by extension from the ^{32}P-labeled primer M4 Vertical line at right of the panel indicates the TCC repeat region.

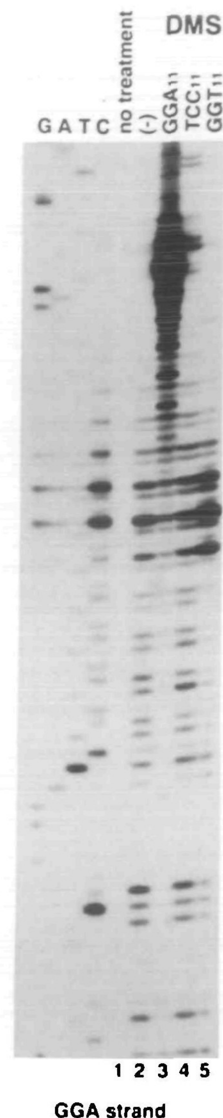

Fig. 5. **Patterns of DMS modification.** Circular plasmid DNA containing d(GGA TCC)$_{11}$ repeats was incubated with d(GGA)$_{11}$ (lane 3), d(TCC)$_{11}$ (lane 4), d(GGT)$_{11}$ (lane 5), or without ODN (lanes 1 and 2) followed by DMS modification (except for lane 1) and piperidine treatment Modification sites of GGA-strand were determined by extension from the ^{32}P-labeled primer RV. Vertical line at right of the panel indicates the GGA repeat region.

because d(TCC)$_{11}$ did not associate with d(GGA:TCC)$_{11}$ repeat under the conditions used (see Fig. 2A). On the other hand, the TCC-strand of the GGA/GGA:TCC complex was cleaved remarkably at the repeat region relative to that of control duplex. This suggests that the TCC-strand of the probe duplex forms a single-stranded loop by the binding of GGA-repeat oligonucleotide.

It is known that Hoogsteen hydrogen bonds play a prominent structural role in the formation of triplexes observed in polypurine:polypyrimidine sequences (*14, 23*). This base pairing involves the N_7 position of guanines and hence gives protection from modification with dimethyl sulfate (DMS). The complexes and the control duplex were treated with DMS and subjected to gel electrophoresis after piperidine cleavage (Fig. 5). The GGA-strand of the GGT/GGA:TCC complex showed a slightly protected pattern relative to that of the duplex. This pattern can be conformed to the triplex model. The GGA-strand of GGA/GGA: TCC exhibited a peculiar pattern, however. One flanking region 3′ to the repeats was resistant to DMS and the opposite site showed a hypersensitivity, which was similar to that observed in the DNase I-cleavage pattern (Fig. 3). The repeat region was composed of a resistant 3′ half and a hypersensitive 5′ half. This pattern was unexpected and did not provide information on the involvement of N_7 of guanines in this triplex formation. Diethyl pyrocarbonate was also used as probe. It gave a protection pattern similar to that of the DMS treatment (data not shown).

DISCUSSION

The present study demonstrated that the DNA containing the d(GGA:TCC)$_{11}$ repeats associates with d(GGA)$_{11}$ oligonucleotide and forms a triple-stranded complex under conditions of neutral pH and 10 mM Mg^{2+}. The conformer

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has a unique structure, distinct from the triplex. It exhibited cleavage patterns that were sensitive to DNase I and OsO_4 (Figs. 3 and 4). Such patterns are diffrent from patterns predicted from the triplex model (*15-18, 25*). The Pu/Pu:Py triplex was first reported for the promoter region of the human *c-myc* gene. AG-rich oligonucleotide binds to the promoter region site-specifically and represses transcription of the gene *in vitro* (*15*). Structural analysis with affinity cleavage experiments revealed that a third purine-rich oligonucleotide binds in the major groove of the duplex, being antiparallel to the Watson-Crick purine strand (*17*). The G/G:C triplet and the A/A:T or T/A:T triplet participate in the triplex formation (*17, 26*). Indeed, the complex formed between the d(GGA:TCC)$_{11}$ and d(GGT)$_{11}$ exhibited patterns predicted from the triplex model, *i.e.*, patterns protected from DNase I and OsO_4.

Sensitivities to both DNase I and OsO_4 suggest that the GGA/GGA:TCC complex consists of three loosely associated strands. We have recently shown that d(GGA)$_{11}$ oligonucleotides form a homoduplex and the strands run in parallel (*20*). It is therefore likely that the GGA-oligonucleotide associates with the GGA-strand of the duplex in parallel through G/G pairing. The resulting TCC-single-stranded DNA is probably looped out (Fig. 6). This is similar to the D-loop structure observed as an intermediate in the recombination process (*27, 28*).

It is not clear why the GGA:TCC trinucleotide repeats form a conformer distinct from triplexes observed in other polypurine/polypyrimidine DNA stretches. All such triplexes have guanine-rich, adenine-poor sequences in one strand and some contain thymines (*15-18, 29, 30*). The triplexes comprise some repeat structures but the repetition is not perfect. The perfect repetition of d(GGA)$_n$ and the repeat unit consisting of two G and one A might be factors responsible for the D-loop-like structure.

The d(GGA) repeats belong to the microsatellite group and show considerable genetic polymorphisms in mammalian genomes (*1, 2*). The polymorphisms reflect the genetic instability that is probably due to frequent recombination between the repeat units or DNA slippage at DNA replication (*31*). Repeats of GGA:TCC may, therefore, be located in recombination- or mutation-prone sites on chromosomes and involved in such DNA transactions. The properties of the d(GGA:TCC) repeats may provide a structural basis for the instability. We previously showed the existence of a protein specifically binding to TCC repeats in the mouse nuclear extracts (*19*), which also supports this idea.

The parallel-stranded complex may have another biological implication. The repeats have a property of adopting two single-stranded loops, as revealed by nuclease S1 digestion experiments (*3, 4, 19*). Hence the association may involve two double-stranded DNAs harboring such repeats. This would result in four-stranded complexes among many chromosomal regions because the d(GGA) repeats are abundant in chromosomes. These structures might be involved in the initiation of the pairing of homologues during meiosis. This idea has already been suggested for G-rich four-stranded DNA (*32-35*). The synapsis formation between homologues usually begins at telomeres but is sometimes initiated at intra-chromosomal loci.

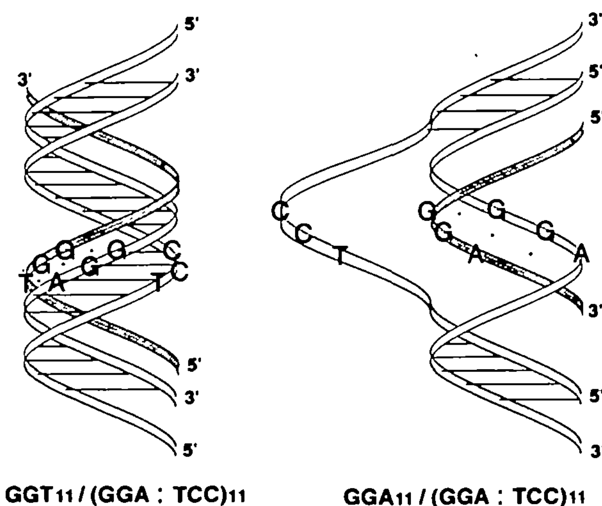

Fig. 6. **Simplified triplex models.** The pu/pu:py triplex model shown on the left is compatible with the complex formed between d(GGA:TCC)$_{11}$ and d(GGT)$_{11}$, in which d(GGT)$_{11}$ is bound antiparallel to the purine strand. Note that the sequence GGT shown in this figure corresponds to 5'-TGG-3'. D-loop-like triplex model shown on the right is consistent with the complex formed between d(GGA:TCC)$_{11}$ and d(GGA)$_{11}$, in which d(GGA)$_{11}$ may be bound parallel to the purine strand.

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