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# Protonated pyrimidine-purine-purine triplex

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## ABSTRACT

**We have studied a protonated pyrimidine-purine-purine (Py-Pu-Pu) triplex, which is formed between the $d(C)_n d(G)_n$ duplex and the $d(AG)_m$ oligonucleotide as the third strand and carries the CG*A$^+$ protonated base-triads. We have observed such an intermolecular complex between a plasmid carrying the $d(C)_{18}$ $d(G)_{18}$ insert and the $d(AG)_5$ oligonucleotide without bivalent cations in 200 mM of Na$^+$ at pH4.0. Bivalent cations additionally stabilize the complex. We propose the structures for nearly isomorphous base-triads TA*A, CG*G and CG*A$^+$. To identify the H-DNA-like structure, which includes the triplex between $d(C)_n$ $d(G)_n$ duplex and the AG-strand, we have cloned in a superhelical plasmid the insert: $G_{10}$TTAA$(AG)_5$. The data on photofootprinting and chemical modification with diethyl pyrocarbonate, potassium permanganate and dimethyl sulfate demonstrate that the H-like structure with triplex carrying CG*G and CG*A$^+$ base triads is actually formed under acid conditions. In the course of this study we have come across unexpected results on probing of Py-Pu-Pu triplexes by dimethyl sulfate (DMS): the protection effect is observed not only for guanines entering the duplex but also for guanines in the third strand lying in the major groove. We have demonstrated this effect not only for the case the novel protonated Py-Pu-Pu triplex but also for the traditional non-protonated Py-Pu-Pu intramolecular triplex (H*-DNA) formed by the $d(C)_{37}$ $d(G)_{37}$ insert in supercoiled plasmid in the presence of Mg$^{2+}$ ions.**

## INTRODUCTION

The triplex mode of recognition of duplex DNA attracts increasing attention because of its potential application for mapping and cutting long chromosomal DNAs (1,2), in gene therapy (3), for sequence-specific extraction and purification of duplex DNA via triplex formation (4,5).

There are two major types of the triplexes: pyrimidine-purine-pyrimidine (Py-Pu-Py) and pyrimidine-purine-purine (Py-Pu-Pu). In the Py-Pu-Py triplex, the two pyrimidine strands are antiparallel and the complex is stabilized mainly due to TA*T and protonated CG*C$^+$ canonical base-triads (6–9). As a result, the Py-Pu-Py triplex is essentially stabilized under acidic conditions (10). Intramolecular Py-Pu-Py triplex (H-DNA) is formed in homopurine-homopyrimidine mirror repeats under negative superhelical stress and/or acid pH (10–14). In H-DNA, the pyrimidine strand forms the Py-Pu-Py triplex with one half of the purine strand, the other half of the purine strand remains unstructured (10–14).

Studies of the Py-Pu-Pu triplex lagged behind the corresponding studies of the Py-Pu-Py triplex and the Py-Pu-Pu triplex has attracted serious attention only recently (15–20). Most researchers agree that such triplexes may take place due to formation of nearly isomorphous base triads TA*A and CG*G (Fig. 1). Both triads do not carry sites protonated or deprotonated in the vicinity of neutral pH and as a result the formation of the Py-Pu-Pu triplex is believed to be pH-independent. Instead, Py-Pu-Pu triplexes are greatly stabilized by bivalent cations, especially $Mn^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ (16,18,19).

Two purine strands in the Py-Pu-Pu triplex are antiparallel, as the recent studies indicate (17,18), in variance to the early claim by Cooney *et al.* (15). This opens the possibility of formation of a Py-Pu-Pu analog of H-DNA, in which the purine strand forms triplex with a half of the pyrimidine strand while the other half of the pyrimidine strand remains unstructured. This structure, often labeled as H*-DNA, is actually formed in the presence of bivalent cations and its stability does not depend on pH (20–23).

Here we show the formation of a pH-dependent Py-Pu-Pu triplex between $d(C)_n$ $d(G)_n$ and $d(AG)_m$. In such a triplex the protonated CG*A$^+$ triad is formed. We show that this triplex is formed both inter- and intramolecularly.

## MATERIALS AND METHODS

### DMS probing of intermolecular triplex

The pG18 plasmid DNA carrying the $d(G)_{18}$ $d(C)_{18}$ insert was linearized by the HindIII and BglI restriction enzymes and $^{32}$P-end-labeled with the Klenow fragment of DNA polymerase and [$\alpha^{32}$P] dCTP. The short fragment of DNA was isolated from

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0.8% agarose gel, eluted from the gel, phenol extracted and precipitated twice in ethanol. The $d(AG)_5$ oligonucleotide (3μM) was mixed with 0.2 pmole of labeled fragment and incubated for 2 hrs in 40 μl of appropriate buffer. To modify the samples, 2μl of 5% dimethyl sulfate (DMS) was added and incubated for 3 min. The reaction was terminated with 10μl DMS-STOP solution (1.5M NaOAc, pH5.2, 1.0M mercaptoethanol, 250μg/ml of tRNA). After double precipitation in ethanol, the samples were treated with 100μl of 10% piperidine at 90°C for 30 min and the products of cleavage were run in 15% polyacrylamide denaturing gel. The 3'-ends of the fragment are at the bottom.

### Plasmids

Oligonucleotide $AGCTTA_5G_{10}TTAA(AG)_5CTGCA$ was synthesized on the 391 DNA synthesizer (Applied Biosystems) by cyanoethyl phosphoamidite method and purified in 12% PAAG.

The pCGA63 plasmid was obtained by ligation of the phosphorylated oligonucleotide with the vector pBluescriptIIKS+ plasmid digested by the HindIII and Pst I restriction enzymes. The plasmid was used to transform the JM109 *E. Coli* cells. The clones carried the inserts of correct length were sequenced using the Sequence Version 2.0 Kit (USB).

The pPG37 plasmid, carrying $dG_{37}$ $dC_{37}$ tract incorporated in Pst I site of the polylinker of the pUC18 plasmid, was kindly provided by Dr. I.Panyutin.

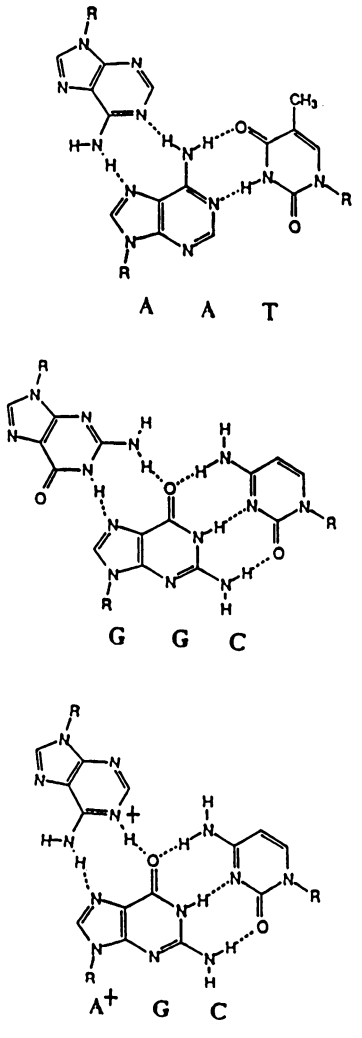


**Figure 1.** Three possible nearly isomorphous pyrimidine-purine-purine base-triads. The third strand is anti-parallel with respect to the purine strand in the duplex. All bases are in *anti* conformation.

### Chemical modifications of intramolecular triplex

Chemical modification under neutral conditions was performed in 50 mM Tris HCl, pH7.0; 200 mM NaCl; 1 mM EDTA and under acidic conditions in 200 mM NaOAc, pH4.0; 1 mM EDTA. Reactions were performed in 50 μl at room temperature (19–21°C). The reaction mixture contained 10–15 μg of supercoiled or 5 μg of linear DNA (linearized by the XhoI restriction enzyme). The final concentration of potassium permanganate (PP) was 20 μM, incubation time 5 min. The final concentration of dimethyl sulfate (DMS) was 0.08%, incubation time 7 min at pH7.0 and 15 min at pH4.0. The final concentration of diethyl pyrocarbonate (DEP) was 2%, incubation time 30 min. The reactions were stopped by ethanol precipitation.

After cutting with the XhoI restriction enzyme, the modified sample was separated into two test tubes and labeled by the 5'-end (the Pu-rich strand) or by the 3'-end (the Py-rich strand). After digestion with the XbaI restriction enzyme, the fragments were extracted from 6% PAAG. The samples were subjected to 1M pyrrolidine in 1mM EDTA for 20 min at 90°C and then were run in denaturing 8% PAAG.

Superhelical or linear (linearized by the BglI restriction enzyme) pPG37 plasmid DNA was modified by 0.05% DMS in the buffer, 20mM Tris HCl pH7.5, 25mM NaCl, 2mM $MgCl_2$, at 15°C for 7 min. Reaction was stopped by 1M mercaptoethanol and DNA was precipitated with ethanol. The plasmid was digested by the EcoR1 restriction enzyme, radiolabeled and after cutting with the HindIII restriction enzyme, the smaller fragment was extracted from 8% PAAG. Then it was subjected to piperidine treatment (10% piperidine, 30 min, 90°C) and the product was run in 8% PAAG.

### Photofootprinting

The pCGA63 plasmid was irradiated as described in (16) at pH4.0 and 7.0 in corresponding solution (see above, the 'chemical modifications of intramolecular triplexes' section). The autoradiograph was scanned on Phosphoimager (Molecular Dynamics).

## RESULTS

### Intermolecular protonated pyrimidine-purine-purine triplex

To study formation of intermolecular triplex containing both CG*G and $CG^*A^+$ base triads, we used the plasmid pG18 carrying the $d(G)_{18}$ $d(C)_{18}$ insert and oligonucleotide $d(AG)_5$.

In the present study we used dimethyl sulfate (DMS) to detect triplex formation. Since the triplex formation shelters the N7 position of guanines in the duplex (see Fig. 1), the triplex zone remains unmodified by DMS while all guanines outside the zone are modified, as usual. As a result, a characteristic DMS footprinting pattern emerges first demonstrated for intramolecular Py-Pu-Py triplexes (H-DNA) by Voloshin *et al.* (14).

Fig. 2A, lane 2 shows that in 200 mM of $Na^+$ at pH4.0 the $d(AG)_5$ oligonucleotide protects the $d(C)_{18}$ $d(G)_{18}$ insert against

modification by DMS. The protection effect strongly depends on pH and is not observed at pH5.5 (lane 4). We actually could not observe the protection effect at pH4.6 (data not shown).

Note that in the control experiment (without oligonucleotide, Fig.2A, lane 1) the 3′-half of the G-tract is modified significantly heavier than the 5′-half. This is, most probably, due to the H-form extrusion. The fact that the H-y5 isoform, which carries the pyrimidine-purine-pyrimidine triplex at the 5′-half of the purine strand, is observed agrees with the data in (12−13) indicating that this isoform prevails in the absence of supercoiling.

Panels B and C in Fig. 2 show that bivalent cations additionally stabilize the protonated triplex permitting its observation at higher pH. In the presence of $Mg^{2+}$ ions the triplex is stable up to pH5.0. The $Zn^{2+}$ ions seem to stabilize the triplex even stronger.

Thus, our data indicate that at acid pH a strong complex is formed between the $d(C)_n$ $d(G)_n$ insert and the $d(AG)_m$ oligonucleotide. We therefore assume that the triplex is formed consisting of the alternating CG*G and protonated CG*$A^+$ base triads (see Fig. 1). All triads in Fig. 1 correspond to the antiparallel polarity of the third purine strand with respect to the purine strand in the duplex and to the *anti* conformation of all nucleotides.

### Intramolecular protonated Py-Pu-Pu triplex

Since the third strand, $d(AG)_m$, in the protonated triplex is most probably antiparallel to the purine strand in the duplex, one could expect that the $G_{10}TTAA(AG)_5$ insert within a supercoiled plasmid at acid pH would form an H-like structure presented in Fig. 3. To check this prediction we cloned the above insert within the pBluescriptIIKS+ plasmid and subjected it to chemical and photofootprinting probing. We used diethyl pyrocarbonate (DEP), which reacts with single-stranded adenines, reacts very weakly with adenines in B-DNA and does not react with adenine within the triplex (14), potassium permanganate (PP), which react with single-stranded thymines, and dimethyl sulfate, which modifies guanines in duplex and single-stranded DNA but cannot react with duplex guanines within the triplex because their N7 positions

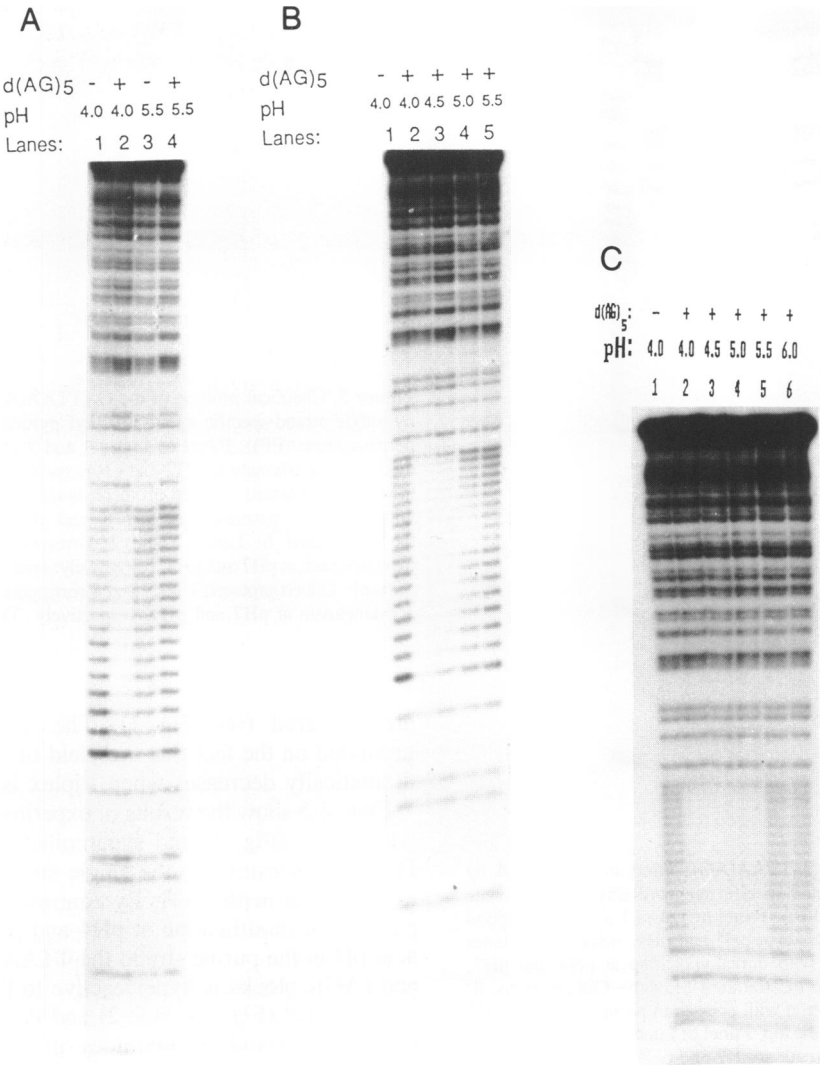


**Figure 2.** The results of footprinting experiments using dimethyl sulfate (DMS) for the $d(G)_{18}$ $d(C)_{18}$ insert without (lanes 1) and in the presence of the $d(AG)_5$ oligonucleotide at different pH. **A)** the samples were in a 200mM $Na^+$ buffer (50mM NaOAc, 150mM NaCl); **B)** 50mM NaOAc buffer supplemented with 10mM $MgSO_4$; **C)** 50mM NaOAc with 10mM $ZnSO_4$ (except pH6.0) or 50mM NaMES plus 10mM $ZnSO_4$ for pH6.0.

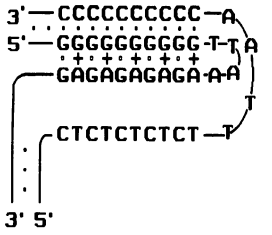


**Figure 3.** The proposed scheme of an H-DNA-like structure containing the protonated Py-Pu-Pu triplex. Filled squares show the Watson–Crick pairs. Open squares and plus symbols show Hoogsteen pairing (respectively, non-protonated and protonated). The $(CT)_5TTAA$ sequence is unstructured.

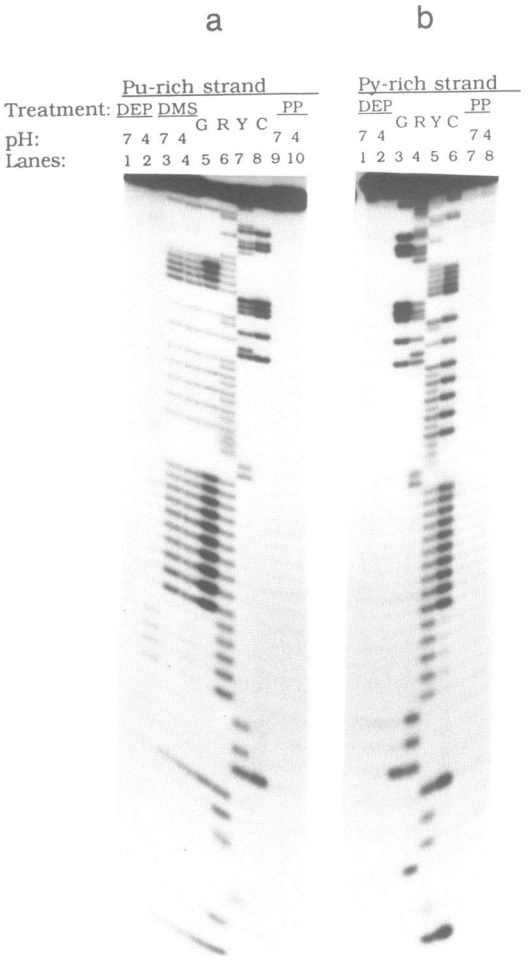


**Figure 4.** Chemical probing of the $G_{10}TTAA(AG)_5$ insert in linear DNA by dimethyl sulfate (DMS) and single-strand-specific agents (diethyl pyrocarbonate (DEP) and potassium permanganate (PP)). **Panel a:** Lanes 1 and 2 correspond to modification with diethyl pyrocarbonate at pH7 and pH4, respectively; lanes 3 and 4 correspond to modification with dimethyl sulfate at pH4 and pH7, respectively; lanes 5–8 correspond to the standard Maxam–Gilbert protocol; lanes 9 and 10 correspond to modification with potassium permanganate at pH7 and pH4, respectively. The 3′-end is at the top. **Panel b:** Lanes 1 and 2 correspond to modification with diethyl pyrocarbonate at pH7 and pH4, respectively; lanes 3–6 correspond to the standard Maxam–Gilbert protocol; lanes 7 and 8 correspond to modification with potassium permanganate at pH7 and pH4, respectively. The 3′-end is at the bottom.

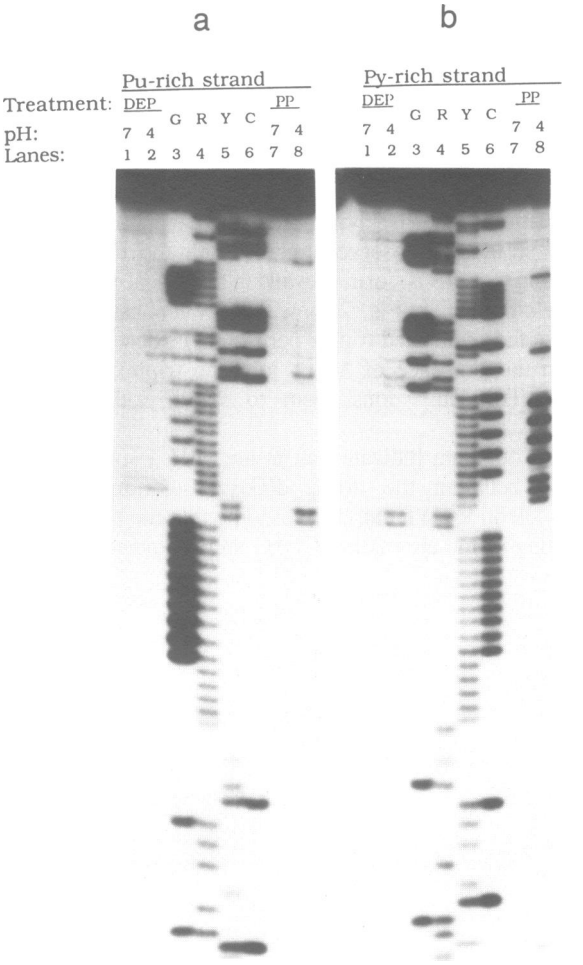


**Figure 5.** Chemical probing of the $G_{10}TTAA(AG)_5$ insert in supercoiled DNA by single strand-specific agents (diethyl pyrocarbonate (DEP) and potassium permanganate (PP)). **Panel a:** Lanes 1 and 2 correspond to modification with diethyl pyrocarbonate at pH7 and pH4, respectively; lanes 3–6 correspond to the standard Maxam–Gilbert protocol; lanes 7 and 8 correspond to modification with potassium permanganate at pH7 and pH4, respectively. The 3′-end is at the top. **Panel b:** Lanes 1 and 2 correspond to modification with diethyl pyrocarbonate at pH7 and pH4, respectively; lanes 3–6 correspond to the standard Maxam–Gilbert protocol; lanes 7 and 8 correspond to modification with potassium permanganate at pH7 and pH4, respectively. The 3′-end is at the bottom.

are sheltered (see Fig. 1). The photofootprinting assay is grounded on the fact that the yield of the [6–4] photoproducts dramatically decreases when triplex is formed (9,16,19).

Figs. 4,5 show the results of experiments on chemical probing with linear (Fig. 4) and supercoiled (Fig. 5) plasmid. Linear DNA is insensitive to the single strand-specific agents at acid as well as at neutral pH. By contrast, in supercoiled DNA the patterns of modification at pH4 and pH7 differ drastically. At acid pH in the purine strand the TTAA spacer between the $G_{10}$ and $(AG)_5$ blocks is hyperreactive to the single strand-specific agents, DEP (Fig. 5a, lane 2) and PP (Fig. 5a, lane 8). In the pyrimidine strand, all thymines of the $(CT)_5$ block, as well as adenines and thymines of the TTAA spacer, prove to be hyperreactive (Fig. 5b, lanes 2,8). The densitogram of photofootprinting experiment in Fig. 6 clearly indicates a

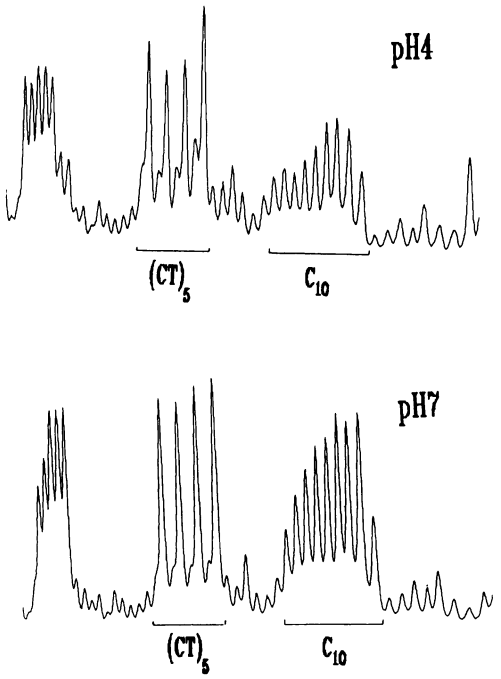


**Figure 6.** Densitogram of the yield of the [6−4] photoproducts in supercoiled DNA at pH4.0 and pH7.0.

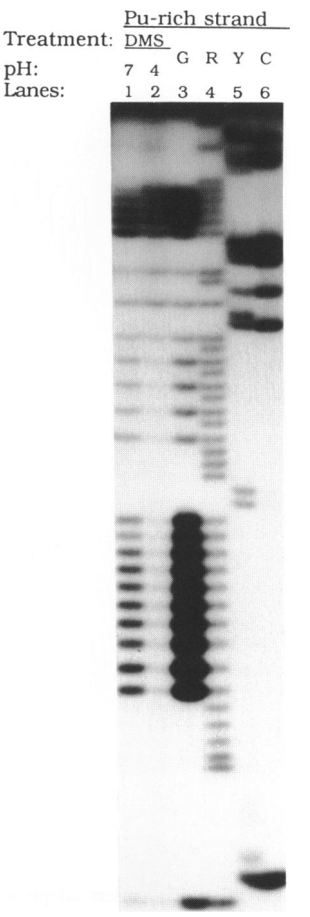


**Figure 7.** Probing of supercoiled DNA at acid and neutral pH with dimethyl sulfate (DMS). Lanes 1 and 2 correspond to modification with diethyl pyrocarbonate at pH7 and pH4, respectively; lanes 3−6 correspond to the standard Maxam−Gilbert protocol.

significant drop in the yield of [6−4] photoproducts in the $C_{10}$ tract at acid pH.

The above data leave few doubts, if any, that the H* structure presented in Fig. 3 is formed, which includes the protonated Py-Pu-Pu triplex consisting of CG*G and CG*$A^+$ base triads and unstructured single-stranded loop $(CT)_5$ TTAA.

The data in Fig.5 indicate that at pH4 both DEP and PP modify some bases separated from the insert $G_{10}$TTAA$(AG)_5$ by as many as 16 base pairs. This modification is observed only at the 3′-flanking region of the purine strand (Fig.5a, lanes 2,8) and the 5′-flanking region of the pyrimidine strand (Fig.5b, lanes 2, 8). Such an effect of extended modification of the duplex region adjacent the single-stranded region of H-DNA was previously observed in numerous studies (14,20). The bases are modified due to stable or transient opening of the duplex from its open end under superhelical stress. The observed effect of modification of the flanking nucleotides provides an additional strong support for our model in Fig.3.

### DMS reaction with Py-Pu-Pu triplexes

We also studied the reactivity of the protonated H* structure with respect to DMS (Fig. 7). This agent had provided with very convincing data supporting usual H-DNA: modification by DMS of guanines in a half of the purine strand dramatically decreased in the H form due to protection of guanines by triplex formation (14).

In full accordance with our expectation, at pH4.0 we observed virtually complete protection against methylation by DMS of the $G_{10}$ block (Fig. 7, lane 2). However, quite unexpectedly, similar protection effect was observed for the $(AG)_5$ block (Fig. 7, lane 2). This was really strange because the N7 positions of guanines in this block did not participate in hydrogen bonding (see Fig. 1). To check whether such non-trivial inaccessibility of the N7 position of guanines in the third strand of the Py-Pu-Pu triplex is a general rule, we have studied reaction with DMS of the well-characterized intramolecular Py-Pu-Pu triplex, which is formed under superhelical stress and neutral pH in the presence of $Mg^{2+}$ ions by polyG polyC inserts (20,21).

This H*-structure for the $d(G)_n$ $d(C)_n$ insert is well known to adopt only one isoform (20), in which the 3′-half of the G-strand forms the Hoogsteen pairs with guanines of the 5′-half of the insert. Therefore, the N7 position of guanines of the 3′-half of the insert do not participate in the hydrogen bonding. Fig. 8 demonstrates, however, that the protection against DMS modification is observed throughout the whole insert with the exception of the two central guanines, which enter the loop in the H* form. We conclude that inaccessibility of guanines in the third strand is the general feature of the Py-Pu-Pu triplexes.

## DISCUSSION

Lyamichev *et al.* (9) were the first to observe some indications to the effect that the $d(C)_n$ $d(G)_n$ duplex may form complex with the $d(AG)_m$ oligonucleotide at acid pH. More recently, complex

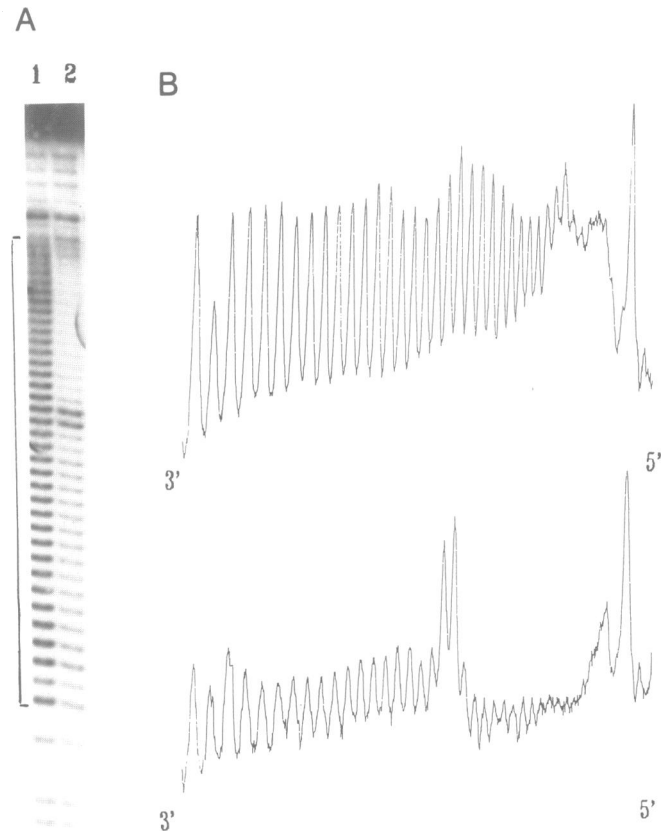


**Figure 8.** Probing of the $dG_{37}$ $dC_{37}$ insert adopting the H* structure in supercoiled and linear DNA. **Panel A:** autoradiograph. Lane 1 linear DNA, lane 2 supercoiled DNA. The 3'-end is at the bottom. **Panel B:** densitogram of the autoradiograph in panel A. At the top linear plasmid, at the bottom supercoiled.

between poly(rA) and the poly(rC) poly(rG) duplex was reported (24), though the authors did not vary pH in their studies.

In this paper we present conclusive evidence that a stable protonated triplex is formed between the $d(C)_n$ $d(G)_n$ duplex and the $d(AG)_m$ oligonucleotide. In contrast with the well-characterized non-protonated Py-Pu-Pu triplexes (15–19), our protonated triplex does not require bivalent cations though their presence additionally stabilizes the complex.

Using specially designed construction, $G_{10}TTAA(AG)_5$, we demonstrate that this type of triplex also forms intramolecularly resulting in an H-DNA-like structure. In sharp contrast with usual H-DNAs carrying either Py-Pu-Py or Py-Pu-Pu triplexes, this new type of triplex violates the major sequence requirement for H-DNA: the mirror symmetry (10,11,14). Previously only solitary violations of the mirror symmetry were studied and they resulted in significant destabilization of H-DNA (11,25).

Note that while designing the sequence capable to adopt the structure in Fig. 3, we deliberately chose short tracts $G_n$ and $(AG)_m$ because longer tracts could start adopting various H-like structures separately either stabilized by protons or bivalent cations or both (12,13,20–23,26–28). Because we studied a short insert we failed to observed the transition by 2-D gel electrophoresis (B.P.Belotserkovskii, unpublished).

To explain the triplex formation consisting of alternating CG*G and CG*$A^+$ base triads we assume that the protonated triad is isomorphous to the CG*G triad (see Fig. 1). Such Hoogsteen pairing between the CG base pair and non-protonated adenine has been postulated before (29). Another mode of pairing was demonstrated by NMR when the CG*$A^+$ triads appears as a mismatch in Py-Pu-Py triplex. Such a mismatched triad significantly destabilizes the Py-Pu-Py triplex (30).

By contrast, our data demonstrate that the Py-Pu-Pu triplex with half of triads being 'non-canonical' is quite stable. Most strikingly, the complex is stable even in the absence of any bivalent cation, which is never the case for known Py-Pu-Pu triplexes (16,18,19). The same is true for the H* structure we observe. Never before the H*-type structure (carrying the Py-Pu-Pu triplex) has been observed in the absence of bivalent cations (20–23). We conclude that what we observe is a new kind of stable protonated triplex, rather than usual Py-Pu-Pu triplex highly enriched with mismatched triads.

Our experiments on probing of the Py-Pu-Pu triplexes with DMS have led to an unexpected observation. We have found that DMS does not modify the N7 positions of guanine in the third strand. We believe that this finding may be very essential for structural studies of pyrimidine-purine-purine triplexes.

The discussion of possible biological implication of our findings is beyond the scope of the present paper. Note only that any search for potential H-DNA-forming regions has been limited to homopurine-homopyrimidine mirror repeats. Our present data provide with quite new H-DNA-forming motif: $G_{2n}N_x(AG)_n$. Although we have not shown this yet, it is tempting to assume that the $(AG)_n$ tract may be replaced by any or almost any sequence of adenines and guanines. This would dramatically increase the set of the potential H-DNA-forming sequences. As to the apparently non-physiological requirement for low pH, it could be overcome as we have recently done in case of usual H-DNA: by complexing with oligonucleotide complementary to the single-stranded region of the H form (31).

## ACKNOWLEDGEMENTS

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