Photofootprinting of DNA triplexes

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ABSTRACT

We have used a photofootprinting assay to study intermolecular and intramolecular DNA triplexes. The assay is based on the fact that the DNA duplex is protected against photodamage (specifically, against the formation of the (6–4) pyrimidine photoproducts) within a triplex structure. We have shown that this is the case for PyPuPu (YRR) as well as PyPuPy (YRY) triplexes. Using the photofootprinting assay, we have studied the triplex formation under a variety of experimentally defined conditions. At acid pH, $d(C)_n \cdot d(G)_n \cdot d(C)_n$ and $d(CT)_n \cdot d(GA)_n \cdot d(CT)_n$ triplexes are detected by this method. The $d(CT)_n \cdot d(GA)_n \cdot d(CT)_n$ triplexes are additionally stabilized by divalent cations and spermidine. PyPuPu triplexes are pH-independent and are stabilized by divalent cations, such as Mg^{++} and Zn^{++} . The effect depends on the type of cation and on the DNA sequence. The $d(CT)_n \cdot d(GA)_n \cdot d(GA)_n$ triplex is stabilized by Zn^{++} , but not by Mg^{++} , whereas the $d(C)_n \cdot d(G)_n \cdot d(G)_n$ triplex is stabilized by Mg^{++} . In H-DNA, virtually the entire pyrimidine chain is protected against photodimerization, whereas only half of the pyrimidine chain participating in a triplex is protected in the CGG intramolecular triplex.

INTRODUCTION

DNA triplexes have been attracting considerable attention in recent years. They are inherent in homopurine-homopyrimidine sequences. Two major classes of triplexes have been analyzed: (a) intermolecular ones, which include a duplex complexed with a single-stranded oligonucleotide (1–8), and (b) intramolecular ones (the H form, see (9) for review). H-DNA is believed to be of potential importance in gene regulation and recombination. Intermolecular triplexes are used for site-specific modification of DNA (1, 5, 6, 8) and for modulation of site-specificity of enzymes interacting with DNA (7, 10). The triplex formation is being suggested for sequence-specific labeling of duplex DNA in genome mapping (11). The possibility of 'gene drugs' coupled to oligonucleotides and delivered via triplex formation to specific sites of the genomes has even been considered.

A variety of techniques are used to detect triplexes: co-migration of oligonucleotides with DNA (2); cleavage of DNA

by chemical groups attached to oligonucleotides (1, 5, 6, 8); DNA footprinting using DNase I or chemicals (3, 4); and immunofluorescent staining with monoclonal antibodies to triplex DNA (12). Recently, we applied a novel method for the investigation of triplexes, a photofootprinting assay (13). The method relies on our observation that the yield of the photoproducts induced by UV-irradiation (pyrimidine dimers, basically [6–4]-photoproducts (14)) is greatly diminished within the triplexes. The great advantage of this method is that it may be used under arbitrary ambient conditions, including, in principle, even *in vivo* conditions (15). In the present paper we exploit the photofootprinting assay to study inter- and intramolecular triplex formation under various conditions.

MATERIALS AND METHODS

Plasmids. The plasmids pTC33.4, pG18 and pG31 carrying $d(T-C)_{16} \cdot d(GA)_{16}$, $d(G)_{18} \cdot d(C)_{18}$ and $d(G)_{31} \cdot d(C)_{31}$ inserts, respectively, were prepared as described (13). The inserts were incorporated into the polylinker of the pUC19 plasmid. The sequences of inserts, plus a flanking polylinker, between boundary EcoRI and HindIII sites, are listed. Only strands carrying the homopyrimidine tract of the inserts are shown:

pTC33.4: 5'-AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGATCC (TC)₁₆ GAATTGATCC(TC)₁₆GAATTGGGTACCGAGCTCGAATT -3'

pG18: 5'-AGCTTGCATGCCTGCA(C)₁₈TGCAGGTCGACTCTAGAGGATCCCC GGGTACCGAGCTCGAATT-3'

pG31:-AATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCA(C)₃₁ TGCAGG CATGCAAGC-3'

The pTC33.4 plasmid prepared by I.K. Chung (The Ohio State University) carries two copies of a $d(TC)_{16}$ track, tandemly orientated, and separated by 10 b.p. The pTC45 plasmid (16), which carries the 45 bp long $(TC)_x \cdot (GA)_x$ insert, was kindly provided by Dr. David Pulleyblank.

Oligonucleotides $d(CT)_5$, $d(C)_{10}$, $d(AG)_5$ and $d(G)_{10}$ were synthesized on an oligonucleotide synthesizer at The OSU Biochemical Instrument Center by Jane Tolley.

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Intermolecular triplex analysis. The plasmids were linearized with the EcoRI restriction enzyme, 3'-end labeled with the Klenow fragment of DNA polymerase I, and treated with a second restriction enzyme Hind III to release the insert. The latter was separated in a 6% polyacrylamide gel, and the shorter labeled fragments were isolated. About 0.05 pmoles of them were incubated with 3 to 6 pmoles of oligonucleotides in 20 μ l of appropriate buffer (see legends to figures) for 30 min at 25°C. The samples were irradiated by two germicidal lamps (15 W, 254 nm) for ~60 to 80 sec with an intensity of ~60 J·sec⁻¹·m⁻². Irradiated DNA was twice ethanol precipitated and dissolved in 80 μ l of 1M piperidine. The modified Maxam–Gilbert method of sequencing was used (13). Reaction with piperidine (1M piperidine, Fisher, 99.9% purity) was carried out at 90°C for 30 min, and samples were loaded onto sequencing gels to reveal the alkali-labile cleavages sites at the photoproducts (14).

Intramolecular triplex assay. Supercoiled DNA (1 μ g) of the pTC45, pTC33.4 or pG31 plasmids was incubated in 20 μ l of appropriate buffer (see figure legends) for 1 hr at 37°C. After UV-irradiation for 40 sec (performed as described in the previous Section), DNA was transferred to a restriction endonuclease buffer. The pTC45 and pTC33.4 plasmid DNAs were cut with

EcoRI, and pG31 plasmid DNA was cut with HindIII. DNA was 3'-end labelled with the Klenow fragment of DNA polymerase I, and cut with a supplementary restriction endonuclease to produce an EcoRI-HindIII short fragment with a radioactive homopyrimidine strand. Fragments were electrophoretically isolated and subjected to piperidine treatment as described above. Alkali-labile breaks were analyzed by sequencing gel electrophoresis.

RESULTS

Intermolecular PyPuPy-triplexes

The influence of pH, divalent ions and spermidine on the stability of the PyPuPy-triplexes. In our first photofootprinting experiments, we demonstrated the striking effect of the protection of DNA duplexes against the formation of photoproducts (such as (6–4) photoproducts) within the triplexes (13). Because the stability of PyPuPy triplexes, which contain protonated CGC⁺-triads, strongly depends on pH (2), we were not able to observe at that time the triplex formation at a pH above 5.5 with the photofootprinting method. This was confirmed by the experiments presented in Fig. 1. The data show a clear-cut protection effect, due to triplex formation at pH 5.0 and 5.5 (lanes 3 and 4), and no such effect at pH 6.0 (lane 5). Fig. 1 also clearly shows that the formation of the UV-photoproducts in regions outside the insert did not depend on pH. The presence of the

Fig. 1. Photofootprinting of the intermolecular triplex formed between pTC45 linearized plasmid and the d(TC)₅ oligonucleotide. The strand carrying the pyrimidine part of the d(CT)₂₂·d(GA)₂₂ insert in the duplex DNA was studied. Irradiation was performed in 50 mM of sodium acetate, 1 mM EDTA, the pH values are indicated. Lanes 6–8 are the standard Maxam-Gilbert ladders. The 5'-end is at the top.

Fig. 2. The formation of the intermolecular PyPuPy triplex between two d(TC)₁₆·d(AG)₁₆ inserts and a d(TC)₅ oligonucleotide, in the presence of either 10 mM MgCl₂ (a), or 1 mM spermidine (b). Irradiation was performed in 10 mM NaAc buffer for pH 5.0; 10 mM Na-MOPS for pH 6.0 and 7.0; 10 mM Tris·HCl for pH 8.0. The 5'-end is at the top.

$d(CT)_5$ oligonucleotide did not influence the photodamage of DNA in regions flanking the $d(CT)_{22} \cdot d(AG)_{22}$ insert.

To form triplexes at pH values closer to neutrality, we stabilized the triplexes with magnesium ions or spermidine. We used a plasmid carrying two $d(CT)_{16} \cdot d(AG)_{16}$ inserts plus $d(CT)_5$ and/or $d(TC)_5$ oligonucleotides. Fig. 2 shows that the photofootprinting assay revealed triplex formation up to pH 6.0 in the presence of 10 mM Mg^{++} (Fig. 2, a) or 1 mM spermidine (Fig. 2, b). Similar results were obtained when 10 mM Mg^{++} plus 1 mM spermidine were added (data not shown). The same triplex was stable only at pHs below 5.5 in 0.05 to 0.2M Na^+ (see (13) and Fig 1). Thus, the substitution of 10 mM Mg^{++} or 1 mM spermidine for 0.05 to 0.2 M Na^+ made it possible to extend the pH range of triplex stability by at least 0.5 pH units.

It was observed in our previous work, that low ionic strength decreases triplex stability (2). The stability of the triplex that forms between the $d(CT)_{16} \cdot d(GA)_{16}$ insert and the $d(CT)_5$ oligonucleotide was studied at 10 mM Mg^{++} , 10 mM Na-MOPS (pH 6.0) and increasing concentrations of NaCl, in order to determine whether the effect of magnesium and spermidine is specific, or just reflects the increase of ionic strength (Fig. 3 a). In the absence of NaCl, the complex was formed (Fig. 3

a, lanes 2). However, with increasing NaCl concentrations, the effectiveness of photoprotection in the $d(CT)_{16}$ regions decreased (or the yield of photoproducts increased) (Fig. 3 a, compare lanes 3, 4, and 5). This result was obtained under conditions in which the medium contained both Na^+ and Mg^{++} ions. Therefore, when considering the result, one must keep in mind the possible competition between mono- and divalent ions for this range of salt concentrations at this temperature.

We also studied the influence of Mg^{++} concentration on the stability of the triplex. Fig. 3 b shows that at pH 6.0 and low ionic strength (10 mM NaCl), the $d(TC)_{16} \cdot d(GA)_{16} \cdot d(CT)_5$ triplex was formed at Mg^{++} concentrations higher than 4 mM. One can conclude, therefore, that the mechanisms of stabilization of $d(TC)_x \cdot d(GA)_x \cdot d(CT)_x$ -triplexes by Mg^{++} and Na^+ -ions differ.

Intermolecular PyPuPu Triplexes

The $d(C)_n \cdot d(G)_n \cdot d(G)_n$ -triplexes. Kohwi and Kohwi-Shigematsu (17) observed the formation of intramolecular PyPuPu triplexes in the presence of Mg^{++} in a supercoiled plasmid, which carried a $d(G)_n \cdot d(C)_n$ insert. In contrast to the usual H form, which includes the protonated CGC⁺ base-triads, the PyPuPu triplex does not depend on pH. These findings indicated the possibility that intermolecular CGG-triplexes can be formed in the presence of Mg^{++} at neutral pH. However, except for a solitary report (3), all attempts to observe intermolecular CGG-triplexes previously, have failed. Fig. 4 shows that, at a high enough concentration of $d(G)_{10}$, this kind of intermolecular triplex actually forms in the $d(C)_{18} \cdot d(G)_{18}$ insert, and a significant protection effect is observed. Fig. 4 also shows that in the presence of 10 mM $MgCl_2$, the yield of photoproducts in the $(C)_{18}$ region decreased with increasing concentrations of the $d(G)_{10}$ oligonucleotide. Without Mg^{++} ions, the effect of protection was not observed, even at very high concentrations of $d(G)_{10}$ (data not shown). Thus, we conclude that the

Fig 3. The influence of concentrations of NaCl (in the presence of 10 mM $MgCl_2$) (a) and $MgCl_2$ (without NaCl) (b) on the formation of intermolecular triplexes between two $d(TC)_{16} \cdot d(AG)_{16}$ inserts and a $d(CT)_5$ oligonucleotide at pH 6.0 (10 mM Na-MOPS).

Fig. 4. The formation of intermolecular PyPuPu triplex at pH 8.0 between the DNA carrying the $d(C)_{18} \cdot d(G)_{18}$ insert and the $d(G)_{10}$ oligonucleotide, in the presence of 10 mM $MgCl_2$, 10 mM Tris·HCl. The pG18 plasmid (0.05 pmole) was incubated with increasing concentrations of $d(G)_{10}$.

intermolecular PyPuPu triplexes do form, and can be detected with the photofootprinting assay, just as the PyPuPy triplexes.

The $d(TC)_n \cdot d(GA)_n \cdot d(GA)_n$ Triplex. PyPuPu triplexes probably are not limited to the $d(G)_n \cdot d(C)_n$ sequence, because the formation of UAA triplexes has been convincingly demonstrated at the polynucleotide level (18). However, our assay apparently cannot be used to study triplex formation in $d(A)_n \cdot d(T)_n$ tracts because (6-4)-photoproducts are not formed in such a stretch (13, 14).

Bernues et. al. (19) claimed to observe the formation of a $(TC)_n \cdot (GA)_n \cdot (GA)_n$ intramolecular triplex in $d(TC)_n \cdot d(GA)_n$ inserts in the presence of Zn^{++} ions. Thus, we studied the formation of intermolecular triplexes between a piece of plasmid DNA carrying two $d(TC)_{16} \cdot d(GA)_{16}$ inserts and the $d(AG)_5$ oligonucleotide in the presence of 10 mM Mg^{++} (Fig. 5, lane 3) or 10 mM Zn^{++} (Fig. 5 lane 5). In contrast to the CGG-triplex, the presence of 10 mM Mg^{++} did not stimulate the formation of $(CT)_n \cdot (GA)_n \cdot (GA)_n$ triplexes with $d(AG)_5$. Nevertheless, the substitution of Zn^{++} for Mg^{++} ions was sufficient to facilitate triplex formation (lane 5). The $d(TC)_n \cdot d(GA)_n \cdot d(GA)_n$ triplex is readily formed after the addition of 4 mM $ZnCl_2$ (data not shown). We conclude that the PyPuPu triplex formation depends in a very peculiar way on the nature of divalent ions added, as well as on DNA sequence.

Intramolecular triplexes

The H Form. As was expected, the yield of pyrimidine photoproducts in the H form, detected by piperidine treatment,

Fig. 5. The formation of PyPuPu intermolecular triplexes between two $d(TC)_{16} \cdot d(GA)_{16}$ inserts and a $d(AG)_5$ oligonucleotide, in the presence of 10 mM $MgCl_2$ (lanes 2 and 3) and 10 mM $ZnCl_2$ (lanes 4 and 5). Irradiation was performed at pH 5.5 in a 10 mM NaAc buffer.

was dramatically decreased when compared to duplex DNA because nearly the entire pyrimidine strand participates in triplex formation. Our data show that this is actually the case for the $d(CT)_{22} \cdot d(GA)_{22}$ insert (the same insert which was studied in Fig. 1). The H form is definitely present under these conditions (pH 4.5), as indicated by our data on the chemical modification by diethylpyrocarbonate (data not shown). Fig. 6 (lanes 1-6) demonstrates the clear-cut protection effect for the whole insert, with decreasing pH in supercoiled plasmid. This protection encompasses the entire pyrimidine strand, including the half that enters the Watson-Crick part of the triplex, and the half that lies in the major groove forming Hoogsteen pairs with the duplex. However, we found that at this pH, the UV-protection of the H form was not as complete as it was for the intermolecular triplexes (compare with Fig. 1). We believe that this is due to the presence of a small proportion of nicked molecules in plasmid preparation. These molecules do not form H-DNA at pH 4.5 and, as a result, their insert remains in the B form. This assumption is supported by the data in lane 10 of Fig. 6, which shows that in linear DNA at pH 4.0, where the insert is in the H form (see (20)), we observe virtually complete protection, with the exception of several bands in the middle. These bands might be due to looped out pyrimidine nucleotides in H-DNA.

Note that in contrast to all our results with intermolecular triplexes, where no protection was observed outside the insert,

Fig. 6. Photofootprinting of intramolecular triplex (H-DNA) in superhelical (lanes 1-6) and linear (lanes 10-14) pTC45 DNA. Below pH 6.0 the same buffer as in Fig. 1 was used. Above pH 6.0 the buffer was used 20 mM MOPS, 50 mM NaCl, and 1 mM EDTA. The pH values are indicated. The 5'-end is at the top.

we observed a clear-cut protection effect in the 5'-flanking region of the insert in linear DNA, but not in supercoiled DNA. (compare the yield of photoproducts marked with an arrow in lanes 1 to 6 and 10 in Fig. 6). This effect can be explained as a result of the formation of the alternative form of H-DNA (see discussion).

Similar data on UV-protection of the pyrimidine chain in the H form was observed in the case of $d(C)_{31} \cdot d(G)_{31}$ (data not shown).

The PyPuPu intramolecular triplexes. We have also used the photofootprinting assay to study the formation of the PyPuPu intramolecular triplexes (e.g., so-called *H structure) in homopurine-homopyrimidine sequences in the presence of Mg^{++} cations at pH 8.0 (17). Because only half of the pyrimidine strand enters the triplex in these structures, we expected and found protection of this half of the strand (Fig. 7, lane 3). One can see a clear-cut asymmetry of the UV-damage of the $d(C)_{31}$ insert in superhelical DNA, in the presence of Mg^{++} ions. The 3'-half of the $d(C)$ -strand is protected, whereas, the 5'-half is heavily damaged by UV-irradiation, in full agreement with the data on chemical modifications of triplexes (17). Single-stranded DNA is known to be a very good target for UV-damage (25). We were not able to detect the *H form in the $d(CT)_{16} \cdot d(GA)_{16}$ insert at pH 5.5, in the presence of 10 mM Mg^{++} or Zn^{++} .

Fig. 7. The detection of intramolecular triplexes in superhelical DNA carrying the $d(G)_{31} \cdot d(C)_{31}$ insert. Irradiation was performed in 0.2M NaCl and 10 mM Tris·HCl at pH 8.0 (lane 1); in 0.2 M NaAc at pH 4.2 (lane 2); and in 10 mM $MgCl_2$, 10 mM Tris·HCl at pH 8.0 (lane 3).

DISCUSSION

Using the photofootprinting assay for detection of DNA triplexes originates from our recent observation that the triplex formation protects the duplex against UV-damage (13). In our experiments, we detect photoproducts by piperidine treatment. Such treatment is known to reveal basically one type of DNA photoproducts, (6-4) pyrimidine photoproducts (7).

The photofootprinting assay has advantages over other methods that are used to detect DNA triplexes: (i) rapid measurement, about 1 min in our experiments, which may be further reduced dramatically by using laser sources; (ii) the method may be used under arbitrary ambient conditions in the presence of any ions or proteins and even within the cell; (iii) there is a possibility of studying the fraction of complexed molecules in quantitative terms.

The major shortcoming of the method is that it, like all the other footprinting assays, is a negative one. It detects the lack of photoreactivity of the DNA stretch covered with the oligonucleotide and, to obtain convincing results, a significant portion of molecules in the sample must form triplexes. This negative character of the assay is the major obstacle to its wide use *in vivo*. However, the results presented above demonstrate that the method is extremely effective in detecting triplexes of different kinds *in vitro*.

We have extended our previous study of PyPuPy triplex formation. These triplexes are extremely stable at acid pH, but their stability dramatically decreases with increasing pH (2). Our goal was to discover ambient conditions, making it possible to observe triplexes of this type at pH values closer to neutrality. Dervan, et. al., (1, 6) demonstrated that polyvalent cations, such as spermidine, greatly stabilize these complexes. In agreement with this observation, we have shown that Mg^{++} , as well as spermidine, separately and together increase the triplex stability range by 0.5 pH units for the case of $d(CT)_{16} \cdot d(AG)_{16} \cdot d(CT)_5$, as compared with the buffer containing 0.2 M Na^+ (see Fig. 2). This effect cannot simply be attributed to an increase of ionic strength because the addition of Na^+ in the presence of Mg^{++} destabilizes the triplex, most probably due to the displacement of magnesium (see Fig. 3, a and b).

Although the possibility of the formation of PyPuPu triplexes has been anticipated for some time (3, 17-19), the lack of a powerful stabilizing factor, such as pH, which was very important for the stability of PyPuPy triplexes, hampered the systematic studies of the PyPuPu triplexes. We have demonstrated that the formation of PyPuPu triplexes dramatically decreases the yield of photodimers, just as in the case of PyPuPy triplexes (see Fig. 4). We believe, therefore, that a similar mechanism of protection works in both cases. That is, the triplex formation significantly reduces the duplex flexibility, thus preventing bases from adopting conformations favorable for photodimerization (13).

The data on intermolecular triplexes indicated that divalent cations stabilize the PyPuPu triplexes (17, 19). Our results confirm this. Specifically, Mg^{++} ions stabilize the $d(C)_{18} \cdot d(G)_{18} \cdot d(G)_{10}$ triplexes (Fig. 4), whereas, they do not favor the formation of $d(CT)_{16} \cdot d(AG)_{16} \cdot d(AG)_5$ triplexes. However, substitution of Zn^{++} for Mg^{++} leads to very efficient formation of the $d(CT)_{16} \cdot d(AG)_{16} \cdot d(AG)_5$ triplex (Fig. 5). This peculiar effect of different cations agrees with available data on intramolecular PyPuPu triplexes (11, 13). Our data provide the first unambiguous indication of the formation of intermolecular PyPuPu triplexes for sequences which have both A and G

residues. Such a possibility has long been anticipated because nearly isomorphous CGG and TAA base triads could be formed (26).

Our data cannot answer the intriguing question of whether purine strands are parallel or antiparallel in the PyPuPu triplexes which we observed, because we used only regular sequences in this study. We plan to answer this question using irregular ones.

Our data show that the photofootprinting assay is applicable to the study of intra- as well as intermolecular triplexes. We have been able to demonstrate this for all known types of intramolecular triplexes, including the H form in $d(CT)_x \cdot d(GA)_x$ and $d(C)_n \cdot d(G)_n$ sequences at low pH, and the CGG triplex in the presence of Mg^{++} . In the case of H-DNA (PyPuPy intramolecular triplex), virtually the entire pyrimidine chain is protected against photodamage. As one would expect from the data of Kohwi and Kohwi-Shigematsu (16), in the case of the CGG intramolecular triplex, the 5'-half of the pyrimidine chain is significantly damaged by UV-irradiation because it is in single-stranded form.

We also observed the H-DNA formation in linear DNA (Fig. 6, lane 10) and photoprotection of the short region of DNA outside of the H-form. We suggest that it is a consequence of the formation of isomeric H form in this region. The influence of the H form extrusion on the 3'-flanking region of the pyrimidine chain (or 5'-flanking region of the purine chain) is well known from data on chemical modification (17, 20–24). It was interpreted as the result of the transient or stable opening of base pairs adjacent to the duplex end, when the isomer carrying the triplex on the 3'-end of the insert is formed. Since we observe the effect on the opposite end of the insert, it is tempting to assume that, in our case, the other isomer is formed. This assumption is in agreement with the data of Htun and Dahlberg (24), who observed the formation of the other isomer (with the triplex at the 5'-end of the insert) by chemical modification when the H form was extruded in the linear DNA at pH 4.0.

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