Nodule DNA in the (GA)$_{37}$*(CT)$_{37}$ Insert in Superhelical Plasmids*

(Received for publication, August 19, 1991)

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Di- or trivalent metal ions stabilize a supercoil-dependent transition in pGA37, which contains the (GA)$_{37}$*(CT)$_{37}$ insert, at neutral and basic pH. The structure formed is different from the well known protonated triplexes (H-DNA) adopted at low pH by polypurine-polypyrimidine (Pyr-Pyr) inserts in plasmids. DNA samples must be preincubated in the presence of multivalent ions at 50 °C for the new transition to occur. At neutral pH in the presence of Co hexamine, both strands of the insert have modification maxima situated at one-third of the distance from both ends. We propose the formation of a new structure called nodule DNA which consists of both Pyr-Pur-Pyr and Pur-Pyr-Pyr triplexes and does not contain continuous single-stranded regions. At basic pH (>8.5) in the presence of magnesium ions, the modification pattern corresponds to Pur-Pur-Pyr triplex formation in the whole insert. At neutral pH in the presence of magnesium, both nodule DNA and the Pur-Pur-Pyr triplex can be formed in the insert. We also observed a magnesium-dependent transition at neutral pH in the other Pur-Pyr insert containing plasmids. These data demonstrate that Pur-Pyr sequences can adopt several non-B conformations at close to \textit{in vivo} conditions.

\textbf{RESULTS}

\textbf{Insert after Preincubation}

\textbf{Plasmids—}pGA37 (16) and pGA20 (13) containing (GA)$_{37}$*(CT)$_{37}$ and (GA)$_{19}$*(CT)$_{19}$ inserts in the EcoRI site of pUC9 were described.

\textbf{Chemical Modifications—}For the standard experiments, a sample contained 1 µg of the plasmid in 40 mM Tris.HCl buffer (pH 7.5 or 9.0), 20 mM NaCl plus one of the following: 10 mM MgCl$_2$, 10 mM CaCl$_2$, 1 mM Co hexamine (Sigma), or 1 mM spermine (Sigma). Modifications by OsO$_4$, DEPC, and KMnO$_4$ were performed as described (20). Briefly, samples were modified in the total volume of 50 µl with 2.5 mM OsO$_4$, plus 2.5 mM 22'-dipryridyl for 5 min, 2 mM KMnO$_4$ for 2 min, or 1 µl of DEPC for 5 min, all at room temperature. OsO$_4$ reactions were stopped by ether extraction. DEPC and KMnO$_4$ reactions were stopped by addition of β-mercaptoethanol to 1 M and ethanol precipitation. All samples were purified by gel filtration through Sephadex G-50 microcolumns equilibrated with H$_2$O (21). Digestion by P$_1$ nuclease (BRL) was performed in a buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM MgCl$_2$ with 15 units of P$_1$ nuclease in a total volume of 50 µl. Reactions were stopped by extraction with phenol chloroform and DNA was purified by gel filtration through G-50 microcolumns.

In the majority of the experiments for the analysis of the modification in the Pur-Pyr inserts, modified plasmids were cut by Sau3A, labeled at the 3'-end (for Pur-strand analysis) by the Klenow fragment of DNA polymerase I (U. S. Biochemicals) or at the 5'-end (for Pur-strand analysis) by T4 polynucleotide kinase (U. S. Biochemicals), and cut by HindIII. After purification on G-50 microcolumns, heating in piperidine and lyophilizations, samples were analyzed on sequencing gels without fragment purification, allowing the shorter (15 base pairs) fragment to run off the gel. In other cases, the shorter HindIII-EcoI fragment of the modified pGA37 was purified by polyacrylamide with subsequent analyses on sequencing gels. The autoradiograms of the gels were quantitated as described (22).

\textbf{RESULTS}

\textbf{Transition in (GA)$_{37}$*(CT)$_{37}$ Insert after Preincubation in Magnesium—}OsO$_4$, KMnO$_4$, DEPC, and P$_1$ nuclease were used as probes for the structures of the Pur-Pyr inserts since they are sensitive reagents for bases in unusual conformations (20, 24, 26). OsO$_4$ and KMnO$_4$ were used as probes for the pyrimidine strand of the inserts since they react predomi-

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* This work was supported by Grant GM30822 from the National Institutes of Health, National Science Foundation Grant 86-07785, and the Robert A. Welch Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Pur-Pyr, polypurine-polypyrimidine; DEPC, diethyl pyrocarbonate.

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nantly with thymines, whereas DEPC was used as a probe for the purine strand since it reacts with adenines and guanines in non-B conformations.

Fig. 1 shows modification of pGA37 at native superhelical density (~0.055) by different probes. For the samples marked −, MgCl₂ was added at room temperature just prior to modification. Samples marked + were preincubated in the presence of MgCl₂ at 50 °C, cooled to room temperature, and then modified. Only background levels of modification were found for the samples that were not preincubated (lanes 1, 4, 6, and 8). However, after preincubation in the presence of magnesium, a substantial increase in reactivity was observed with all probes in the central part of the insert. OsO₄ modification (lane 2) reveals three maxima; one is situated at the center of the insert and two others are at one-third of the way from the ends. However, after additional preincubation in the presence of EDTA, the modification returns to the background level (lane 3).

KMnO₄ and DEPC modification patterns (lanes 5 and 9) are similar to those found with OsO₄, except that the one-third maximum closer to the 3'-end (for GA-strand) is weaker than the one closer to the 5'-end. In the case of P₁ nuclease, the central one-third of the insert is hypersensitive to the enzyme with the clear maxima at the center (lane 7).

Thus, preincubation of pGA37 in the presence of magnesium results in a dramatic increase in the reactivity of certain bases, reflecting a change in the structure of the (GA)₃₇(CT)₃₇ insert.

Kinetics of the Structural Changes—Fig. 2 presents the time course of the OsO₄ modification of the plasmid preincubated in a buffer containing 10 mM MgCl₂ at 50 °C. Only a background level of reactivity of the CT-strand of the insert was found without preincubation. However, after as little as 2 min of preincubation, an increase in the reactivity of thymines inside the central one-third of the insert is observed but there is no distinct maxima or minima. After 5 min of preincubation, three maxima of modification inside the insert are found. These maxima get sharper with an increase in the preincubation time. No differences were observed between the modification patterns after 30 and 80 min of preincubation. After 80 min preincubation at 37 °C, the modification pattern resembles that found after 2 min preincubation at 50 °C with no distinct maxima (data not shown).

Effect of Different Ions—Fig. 3 (left panel) shows the OsO₄ and DEPC modification patterns of pGA37 at native superhelical density in the presence of 1 mM Co hexamine. In both cases, the EaeI-HindIII fragment containing the insert was labeled on the purine (3'-EaeI) or pyrimidine (3'-HindIII) strand, purified, and analyzed on a sequencing gel. Only background levels of modification were observed after the reactions without preincubation (lanes 2 and 4). After preincubation in the presence of Co hexamine, both strands have

**Fig. 1.** Fine mapping of chemically modified sites in supercoiled pGA37 at neutral pH. pGA37 was modified by OsO₄ (lanes 1–3), KMnO₄ (lanes 4 and 5), P₁ nuclease (lanes 6 and 7), and DEPC (lanes 8 and 9). All reactions were performed at room temperature in buffers containing 10 mM MgCl₂ (see “Materials and Methods”). The samples in lanes 2, 5, 7, and 9 were preincubated at 50 °C for 30 min before modification. The sample in lane 3 was preincubated in the presence of MgCl₂ followed by incubation at 50 °C for 30 min in the presence of 20 mM EDTA. Samples were analyzed on sequencing gels without fragment purification (see “Materials and Methods”). The brackets indicate the (GA)₃₇(CT)₃₇ insert. The arrows show the positions of the maximum amount of modification.

**Fig. 2.** Time course of OsO₄ modification. pGA37 was modified by OsO₄ after different times of preincubation in MgCl₂ containing Tris buffer at 50 °C. The brackets indicate the (GA)₃₇(CT)₃₇ insert. The arrows show the positions of the maximum amount of modification.
maxima of modification situated one-third of the way from the ends.

Fig. 3 (right panel) shows a summary of the OsO₄ modification patterns of the CT-strand after preincubation in the presence of different ions. The distributions of reactivity are the same after preincubation in the presence of CaCl₂ and MgCl₂; both contain three distinct maxima inside the insert. Preincubation in the presence of 1 mM Co hexamine results in a modification pattern which is different from the one obtained after preincubation in the presence of MgCl₂. In this case, only two maxima are situated at one-third distance from the ends of the insert, whereas the central maxima almost disappeared. A similar distribution of reactivity is observed after preincubation in the presence of spermidine but the intensity of modification of thymines was significantly lower.

Influence of Negative Supercoiling—Fig. 4 shows the amount of OsO₄ modification of plasmid preparations with different average superhelical densities. All samples were preincubated at 50 °C for 30 min in a buffer containing 10 mM MgCl₂. The midpoint of the transition in the insert is estimated as −0.055.

The structural changes in pGA37 also were analyzed by...
two-dimensional gel electrophoresis of topoisomers in the presence of magnesium (data not shown). No transition was observed in topoisomers up to number −15 which corresponds to a superhelical density of −0.056. However, more information could not be derived from the two-dimensional gels because of the low resolution of the highly negative supercoiled topoisomers. Thus, we took advantage of the relatively long kinetics of pGA37 in order to determine the amount of relaxation caused by the transition.

Fig. 5 shows the separation of the products of relaxation of pUC9 and pGA37 relaxation by topoisomerase I in an agarose gel. A Gaussian distribution of pUC9 topoisomers corresponding to the completely relaxed state was observed (lane 1). This family was shifted to the positive values because of the presence of chloroquine in the gel. However, pGA37 that was relaxed in the presence of magnesium (lane 3) or Co hexamine (lane 5) was composed of two Gaussian distributions of topoisomers. The stronger one corresponded to the relaxed state at the conditions of relaxation. The weaker one was shifted to negative supercoil values. This second distribution almost disappeared when the topoisomerase reaction was performed in the absence of multivalent ions (lane 2) and was completely absent in the case of previously relaxed DNA (lane 4). We believe that the second maxima in topoisomer distribution corresponds to the molecules that had an altered conformation of the insert even after relaxation by topoisomerase, which was feasible because of the slowness of the transition. After applying the DNA onto the gel in the presence of chloroquine, the insert returns to the unperturbed conformation which causes the shift of topoisomers to the negative supercoiled values. The distance between the two Gaussian distributions and therefore the amount of relaxation introduced by the transition corresponds to 6.5 topoisomers for both magnesium and Co hexamine containing samples.

Transition at Basic pH—Fig. 6 shows the modification of pGA37 by OsO4 and DEPC at pH 9.0. For the analysis of the purine strand, the Eae1-HindIII fragment labeled at the 3′-Eae1 end was purified. The pyrimidine strand was analyzed without fragment purification (see “Materials and Methods”). If the samples were not preincubated, then only a background level of modification was observed. Reaction of preincubated DNA with DEPC revealed only one maximum of modification which was at the center of the purine strand, whereas the rest of the insert was protected from modification even in comparison with the unpreincubated sample. OsO4 modification revealed the maximum at the center of the pyrimidine strand and stronger reactivity of thymines with the 5′-half compared with 3′-half of the insert.

Experiments with Other Plasmids—OsO4 modification experiments were also carried out with pGA20, pRW1724, and pRW1716. pGA20 is the same as pGA37, except that it contains (GA)20 insert instead of (GA)37. pRW1724 and pRW1716 were obtained by introducing (AGG)6TCC(AGG)8 and (GAA)8TTCGC(GAA)8 inserts into the single BamHI site of the pRW791 vector (20). Thus, inserts were studied with approximately 33, 50, and 66% G + C. Fig. 7 presents OsO4 modification patterns of pGA20, pRW1724, and pRW1716. Preincubation of pGA20 at 50 °C in the presence of 10 mM MgCl2 results in maximum modification at the center of the (GA)20–(CT)20 insert and some protection of the thymines in the rest of the insert. The intensity of the modification maximum in the case of pGA20 was considerably less than found with pGA37.

In the case of pRW1724 and pRW1716, the modification of the (AGG)6TCC(AGG)8 and (GAA)8TTCGC(GAA)8 inserts, respectively, was analyzed. Preincubation of pRW1724 in the presence of 10 mM MgCl2 resulted in hypermodification of thymine in the TCC sequence situated between the two runs of thymines. No increase in reactivity of thymines between the two runs of purines was observed for pRW1716. Also, the background modification of purines decreased after preincubation in the presence of magnesium.

Therefore, preincubation in the presence of magnesium caused structural changes not only in (GA)37 but also in the other Pur–Pyr inserts. For these changes to occur, a Pur–Pyr insert must be GC-rich. Also the distribution of OsO4-hypersensitive sites is different for (GA)37 and the shorter inserts, which reflects the difference in the nature of structural changes (see “Discussion”).

**DISCUSSION**

These data demonstrate that supercoil-dependent structural transitions occur in a (GA)37–(CT)37 insert at neutral and basic pH in the presence of di- or trivalent ions. These transitions are quite slow, requiring preincubation of the plasmids at 50 °C for at least 20 min. Preincubation of pGA37 in the presence of MgCl2 at pH 9.0 results in a DEPC modification maximum at the center of the purine strand and an increase of reactivity with OsO4 at the 5′-half of the pyrimidine strand with the maximum reactivity at the center of the insert. This modification pattern corresponds to the formation of an intramolecular Pur–Pur–Pyr triplex between the 3′-half of the insert and the 5′-half of the pyrimidine strand with the maximum reactivity at the center of the insert. The modification pattern corresponds to the formation of an intramolecular Pur–Pur–Pyr triplex between the 3′-half of the insert and the 5′-half of the purine strand with the 5′-half of the pyrimidine strand remaining single-stranded. The 5′-half of the purine strand (the third strand of the triplex) binds to the 3′-half of the insert via Hoogsteen-like hydrogen bonds, forming G·G·C and A·A·T base triads.
(27). The formation of co-linear intermolecular Pur-Pur-Pyr triplexes stabilized by these triads was described (28, 29). In the case of an intramolecular triplex, the two purine strands are antiparallel and therefore part of the purines in the third strand must be in the syn conformation (30). This type of triplex was observed previously for a G₃-C₉ insert in the presence of magnesium at neutral pH (8, 12).

Preincubation of pGA37 at pH 7.5 in the presence of Co hexamine results in two maxima of reactivity with both OsO₄ and DEPC (for the pyrimidine and the purine strands, respectively) situated one-third of the way from both ends of the insert. To explain these results, we propose a novel bitriplex structure formed by strand exchange, called nodule DNA (Fig. 8). This structure is composed of both a protonated Pyr-Pur-Pyr triplex and a Pur-Pur-Pyr triplex. Nodule DNA does not contain regions of single-stranded DNA except in the loop regions located at a distance of one-third from the ends of the insert. These loops are hypersensitive to the chemical and enzymatic probes. The formation of nodule DNA reduces the distance between the 5'- and 3'-ends, therefore causing a shortening of DNA molecule. Previous studies (17) have characterized more simple types of bitriplexes which do not require strand exchange.

The formation of nodule DNA and the Pur-Pur-Pyr triplex is in agreement with the amount of relaxation caused by the transition of the insert as determined by agarose gel relaxation studies. Both nodule DNA and a Pur-Pur-Pyr triplex are topologically equivalent to the melted state of the insert. Melting of the 74-base pairs insert corresponds to the relaxation of 7.0 turns, assuming 10.5 base pairs per turn in the duplex state. This value is close to the 6.5 turns of relaxation which were experimentally observed (Fig. 5). The discrepancy of 0.5 turns may be caused by distortions at the ends and/or by the difference from 10.5 for the number of base pairs per turn for the duplex state of the insert. In any case, the amount of relaxation observed reveals that the transition involves the entire insert. Therefore, possible models that consider triplex formation in two-thirds of the (GA)₃₇ insert can be ruled out.

The modification pattern observed at neutral pH in the presence of MgCl₂ corresponds to a mixture of the Pur-Pur-Pyr triplex and nodule DNA. Fig. 9 illustrates the different structures than can be formed in the (GA)₃₇- (CT)₃₇ insert. At a pH lower than 6.5 (16), the Pyr-Pur-Pyr triplex which requires protonation of cytosines was formed. At basic pH, the Pur-Pur-Pyr triplex was formed which required magnesium or other multivalent ions for stability. At neutral pH, nodule DNA, containing both types of triplexes, can be formed. All these structures are separated by high energetic
Fig. 7. Fine mapping of OsO₄ modification sites in the supercoiled pGA20, pRW1724, and pRW1716. Reactions were performed at room temperature in the presence of 10 mM MgCl₂ (see "Materials and Methods"). Samples in lanes 1, 3, and 5 were preincubated at 50 °C for 30 min before modification. The brackets indicate the (CT)₂₀, (AGG)₅TCC(AGG)₅, and (GAA)₅TTCGC(GAA)₅ inserts, respectively.

Fig. 8. Model for nodule DNA. The Watson-Crick paired duplexes of the vector are shown at the top left and bottom right. To form the nodule conformation, the central one-third of the insert must pass through an unpaired intermediate and the pyrimidine strand (shaded) forms the Pur-Pur-Pyr triplex with the 5'-third of the insert stabilized by T-A-T and C-G-C* base triads (top) while the purine strand (unshaded) forms the Pur-Pur-Pyr triplex with the 3'-third of the insert stabilized by A-A-T and G-G-C triads (bottom).

Nodule DNA does not contain continuous single-stranded regions and, in this sense, must be more energetically favorable than the Pur-Pur-Pyr triplex at neutral pH. On the other hand, it is composed of two triplexes which are in very close proximity. The addition of trivalent ions, which are stronger than divalent ions in neutralizing phosphate charges, reduces the repulsion between these two triplexes. Hence, Co hexamine changes the ratio between nodule DNA and the Pur-Pur-Pyr triplex in favor of the former. At basic pH, nodule DNA is not stable because of the high energetic cost of cytosine protonation.

We believe that the reason why the kinetics of formation of nodule DNA and the Pur-Pur-Pyr triplex are considerably slower than for H-DNA (23) is that a significant number of purines must change their conformation from anti (in B-DNA) to syn (the third strand of Pur-Pur-Pyr triplex) during the transition. This phenomenon was observed for G-G-C triplex formation in a G₅-C₅ insert in superhelical DNA (12) and for the transition in oligo(dG) from the hairpin stabilized by G-G base pairs to a tetraplex structure (33). On the other hand, formation of nodule DNA may occur through melting of the central one-third of the insert. This melting would also contribute to the high activation energy and, consequently, to the substantial time required for nodule DNA formation. The long kinetics of nodule DNA formation may be overcome in vivo by enzymes (conformases) that could accelerate the transition.

Our results show that a magnesium-induced transition occurs in other inserts as well. The (GA)₂₀ insert is probably too short to be able to fold into nodule DNA, whereas the insert in pRW1724 has an interruption in the center that makes the nodule conformation not favorable. Instead, these inserts appear to form another structure after preincubation in magnesium, most likely Pur-Pur-Pyr triplexes. At the same time, no transition was detected in the insert of pRW1716, which is more AT-rich than the insert in pRW1724. Therefore, the G-G-C triads may provide the major contribution to the stabilization of Pur-Pur-Pyr triplexes.

Long (GA)ₙ tracts are not the only sequences that are consistent with the requirements for nodule DNA formation. A Pur-Pyr sequence composed of three consecutive mirror repeats (1, 2, 14) can potentially adopt nodule DNA; a number of barriers. Therefore, once one of these conformations is adopted by a given sequence, it is kinetically trapped, and therefore two or even three structures may coexist in the same plasmid population.

Numerous examples of the occurrence of long (GA)ₙ sequences in regulatory regions of eukaryotic genomes are known (1, 31, 32). The structural polymorphism (four conformations, Fig. 9) of these sequences may be involved in genetic regulation. Different proteins may selectively bind to the different conformations of (GA)ₙ. Alternatively, the transition to the non-B conformations may cause a displacement of proteins (i.e. histones) that normally bind to DNA.
of these types of simple repetitive sequences are known which 
are functionally important (1). For example, purine-rich re-
petitive sequences from recombination regions of herpes sim-
plex virus 1 (34, 35) and the related viruses pseudorabies (36) 
and varicella-zoster (37) also contain at least three Pur·Pyr 
mirror repeats separated by several nucleotides of random 
DNA and, therefore, can potentially adopt the nodule struc-
ture. These correlations and the strand exchange feature 
suggest a possible role of nodule DNA in recombination. 

Addendum—A model for a double triplex structure which is similar 
to nodule DNA was described recently for the G6·G6 insert (38).

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