Magnesium-dependent supercoiling-induced transition in $(dG)_n \cdot (dC)_n$ stretches and formation of a new G-structure by $(dG)_n$ strand

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ABSTRACT

Plasmids containing $(dG)_{27} \cdot (dC)_{27}$ inserts (pPG27), $(dG)_{37} \cdot (dC)_{37}$ inserts (pPG37), and $(dG)_{24} \cdot (dC)_{21} \cdot (dC)_{24} G(dC)_{21}$ inserts (pPG460) were constructed for the study of structural transitions within $(dG)_n \cdot (dC)_n$ stretches. Two-dimensional gel electrophoresis has shown that a Mg^{2+} -dependent supercoiling-induced structural transition takes place at pH 8 in plasmid pPG460. The transition occurs at $\sigma = 0.06$ and involves a supercoiling release corresponding to 5 superhelical turns.

After denaturation of the restriction fragments containing $(dG)_n \cdot (dC)_n$ inserts, the strands do not renature completely and the $(dG)_n$ -containing strand migrates in PAGE much faster than the $(dC)_n$ -containing one. Chemical modification experiments with the $(dG)_n$ -strand have revealed the periodic nature of the protection of n guanines against dimethyl sulfate methylation. The $(dG)_n$ strand in the presence of Mg^{2+} forms complexes with the complementary $(dC)_n$ strand, which differ from the native duplex in mobility. We believe these effects to be due to the formation of an intrastrand structure within the $(dG)_n$ strand stabilized by G·G interactions (we called it G-structure), which in the presence of Mg^{2+} forms an interstrand complex with the $(dC)_n$ strand.

INTRODUCTION

Regular repetitive sequences are common with eukaryotic genomes. One of the motives is the repetition of purines along one strand and, accordingly, pyrimidines along the other [1]. In some cases rather long fragments have been revealed which contain almost exclusively G·C pairs ($(dG)_n \cdot (dC)_n$ stretches). For instance, the sea urchin histone gene spacer carries a $(dG)_{11} \cdot (dC)_{11}$ sequence [2], the crab satellite DNA, a $(dG)_{23} \cdot (dC)_{23}$ sequence [3], and the chicken β^A -globin gene, a $(dG)_{26} \cdot (dC)_{26}$ sequence [4]. Interest in such sequences dramatically increased

after it was discovered that, incorporated into plasmid DNAs, they may adopt conformations other than the B form under superhelical stress. The most deeply studied structural transition is that taking place with reducing pH, when the $(dC)_n$ strand becomes partially protonated and forms a $dC \cdot dG \cdot dC^+$ triple helix with the 3' half of the $(dG)_n$ strand [5]. The superhelical density at which such a transition takes place strongly depends on pH, and at physiological pH values the transition is practically undetectable.

It was recently found that a pH-independent structural transition takes place in $(dG)_n \cdot (dC)_n$ in the presence of millimolar concentrations of Mg^{2+} [6]. It was suggested on the basis of experiments on chloroacetaldehyde modification of supercoiled DNA that in the presence of Mg^{2+} the $(dG)_n$ strand forms a $dG \cdot dG$ -stabilized hairpin while the 3' halfstrand of the $(dC)_n$ strand joins the hairpin to form a $dG \cdot dG \cdot dC$ triple helix. However, the question of whether this transition is an equilibrium process occurring under supercoiling or it is induced by chloroacetaldehyde, as well as the question concerning the energy parameters of the transition, has been remaining open.

Another example of a structure stabilized with participation of $dG \cdot dG$ pairs is given by telomere sequences. Most of telomeres studied so far are made up of variable number of tandemly repeated, simple (G+C)-rich sequences [7]. Such sequences, added to the ends of chromosomes by a specific enzyme [8], are supposed to be capable of forming hairpins due to the formation of $dG \cdot dG$ pairs. Hairpins were revealed in synthetic telomere sequences by a change in the electrophoretic mobility in PAG [7].

It has been found [9] that synthetic oligonucleotides with the sequences of the switch regions of Ig genes can form complexes of four molecules stabilized by four-stranded helices produced by the interaction of the $(dG)_n$ stretches contained therein. A hypothesis has been put forward that such structures take part in the coupling of homologous chromosomes during meiosis.

The above facts suggest that $(dG)_n$ stretches may play an important role in various processes of genome functioning, by

virtue of the possibility of formation of unusual dG·dG-stabilized structures.

In order to investigate the structural transitions in $(dG)_n \cdot (dC)_n$ stretches and the conditions of formation and stability of the dG·dG pairs, we constructed plasmids carrying $(dG)_n \cdot (dC)_n$ stretches of different lengths. In this paper, structural transitions in the insert-carrying plasmids have been studied through two-dimensional electrophoresis of topoisomers. In addition, we have observed formation of intrastrand complexes within single-stranded fragments containing the $(dG)_n$ inserts, and binding in the presence of Mg^{2+} of the complementary $(dC)_n$ strand to these complexes.

MATERIALS AND METHODS

Plasmids

To obtain plasmids with $(dG)_n \cdot (dC)_n$ inserts, we cleaved pUC18 with PstI (Pharmacia), divided the sample into two parts, and attached $(dC)_n$ to one of them at the 3' end and $(dG)_n$ to the other, using terminal deoxynucleotidyl transferase (Olaive, USSR) as described in Ref.[10]. 0.1- μ g aliquots of the samples were mixed together and incubated at 100°C for 1 min in 0.1M NaCl, whereupon they were slowly cooled down to room temperature. The preparation obtained was used to transform JM83 cells [10]. Insert-containing clones were selected on an L agar with IPTG and X-gal.

Plasmid DNA was isolated from the 24 selected clones, cleaved with EcoRI and HindIII (Pharmacia), and labeled with the Klenow fragment of DNA polymerase I (Pharmacia) and $\alpha^{32}[P]dATP$ (Izotop, USSR). A 6%-PAG electrophoretic analysis of the lengths of the fragments cleaved out revealed 11 clones containing plasmids carrying 16 to 47-bp inserts. Sequencing was according to Maxam and Gilbert [11]. Part of the polylinker sequence and the sequences of the inserts are shown in Fig.1.

Plasmids were isolated in preparative quantities by double precipitation in PEG 6000 [12]. Topoisomers with various superhelical density were obtained as described in Ref.[13]. In some cases positive topoisomers were obtained using reverse

Figure 1. The pUC18 polylinker sequence and the sequences of inserts into the *PstI* site for plasmids pPG46C, pPG37, and pPG27. The sequence of the *PstI* site partially duplicated in the course of cloning (shown in *italio*).

gyrase (the preparation kindly provided by Dr A.Slesarev, IMG, USSR Ac.Sci.).

DNA Fragments

To obtain $(dG)_n \cdot (dC)_n$ -containing DNA fragments, we cleaved appropriate plasmids at the polylinker with a restriction enzyme (see Fig.1). The fragments were 5'-end labeled with polynucleotide kinase or 3'-end labeled with the Klenow fragment of *E.coli* DNA polymerase I. After that, the fragments were cleaved by another restriction enzyme and the digest was electrophoresed through 6% PAG. The shorter fragment was eluted from the gel [11], precipitated in ethanol, and transferred into a proper buffer by gel filtration on Sephadex G50 microcolumns [10]. The $(dG)_n \cdot (dC)_n$ -containing fragment at concentration 0.1-0.2 $\mu\text{g/ml}$ was heated at 100°C for 1 min in a buffer containing 40 mM Tris, 5 mM NaAc, 0.1 mM EDTA, then rapidly chilled and loaded on 6% PAG. Then the band corresponding to the $(dG)_n$ -containing strand was eluted from the gel.

Electrophoresis

Two-dimensional separation of topoisomers was performed as described in Ref.[14]. When electrophoresis was carried out in the presence of MgAc, a precipitate appeared on the negative electrode. However, the concentration of Mg^{2+} in the buffer, measured by titration with eriochrome black, did not decrease to within 10%. Gels were stained with ethidium bromide and photographed on Mikrat 300 film through a red filter.

DNA fragments were electrophoresed at room temperature on a nondenaturing PAG in a TE buffer containing 40 mM Tris, 5 mM

Figure 2. Two-dimensional electrophoretic separation of pUC18 (A) and pPG460 (B) topoisomers on a 0.5%-agarose/2%-acrylamide gel in a buffer containing 36 mM Tris, 30 mM NaH_2PO_4 , 0.2 mM EDTA, pH 7.7. Separation in the first dimension was top to bottom, in the second, left to right. Topoisomer -10 is marked. OC, open circular molecules.

NaAc, 0.1 mM EDTA, pH 8. Denaturing gels were prepared with 8.3 M urea and thermostatted with a water envelope at 60°C.

RESULTS

Supercoiling-induced Structural Transition in Inserts

The most informative technique for investigating the thermodynamics and kinetics of structural transitions in supercoiled DNA is two-dimensional electrophoresis of DNA topoisomers [16,17]. A set of topoisomers with widely varying ΔLk ($\Delta\text{Lk} = \text{Lk} - \text{Lk}_0$, where Lk is the linking number of the topoisomer, Lk_0 , the linking number of the relaxed molecules under specified conditions) can be separated on a gel, the mobility rising as $|\Delta\text{Lk}|$ rises. But the mobility of topoisomers that have undergone a structural transition may reduce as part of the superhelical tension is released. Such topoisomers migrate along with the topoisomers whose $|\Delta\text{Lk}|$ is smaller by the value of this release. For comigrating topoisomers to be separated, perpendicular electrophoresis is performed in the presence of an intercalator which reduces the superhelical tension so as to block any structural transitions.

The first dimension mobility of the topoisomers of both pUC18 (Fig.2,B) and pPG46C (Fig.2,A) gradually increased up to the topoisomer with $\Delta Lk=-16$, when electrophoresis was carried out without Mg^{2+} at room temperature. The mobility jump observed further is identical for both plasmids and seems to be associated with the formation of a cruciform in the palindrome of pUC18 (1385-1409), the same as that of pBR322 [18,19].

If the first-dimension electrophoresis is performed with 5 mM MgAc at room temperature, the separation patterns of pPG46C and pUC18 topoisomers significantly differ (Fig.3). While the mobility jump in pUC18 is the same as that observed without Mg^{2+} (Fig.3 A), in pPG46C it occurs at topoisomer -15 and, most dramatically, the mobility drop corresponds to a supercoiling release of 5 superehelical turns (Fig.3 B). Thus, in the presence of Mg^{2+} , plasmid pPG46C undergoes a structural transition not observed with the original pUC18 vector.

When the first-dimension electrophoresis is performed at 6°C, both in the presence (data not shown) and even in the absence of Mg^{2+} (Fig.4), the structural transition in pPG46C is observed only if the sample had been preincubated at 37°C for 30 min with 10 mM $MgCl_2$ before loaded on the gel. Hence the characteristic time of the Mg^{2+} -induced transition at 6°C exceeds the duration of electrophoresis (i.e. 20 hours).

Figure 3. Two-dimensional electrophoretic separation of pUC18 (A) and pPG46C (B) topoisomers. Separation in the first dimension was with 5 mM MgAc. Samples were loaded in TE buffer. Other conditions are the same as in Fig.1.

When studying pPG37 and pPG27 plasmid topoisomers by two-dimensional electrophoresis, we failed, within the sensitivity of the technique, to unambiguously detect a Mg-induced transition other than the structural transition in pUC18.

The Properties of the $(dG)_n \cdot (dC)_n$ -containing Linear Fragments

Since a new structural transition was only observed for pPG460 plasmid DNA, differing from the original pUC18 by the presence of a long $(dG)_n \cdot (dC)_n$ insert, we have investigated some properties of the fragments containing such inserts (see Fig.1).

a.Denaturation and renaturation. During the sequencing of the $(dG)_n$ strand according to conventional procedure by Maxam and Gilbert [11], distinct bands were only observed for the terminal ca. 30 deoxyguanosine residues at the labeled end, the rest producing a diffuse background (Fig.5,). A similar phenomenon has been observed by other authors (S.M.Mirkin, personal communication). Distinct bands can be observed for all inserted deoxyguanosines by elevating the temperature of the gel up to

Figure 4. Two-dimensional electrophoretic separation of pPG460 topoisomers. Sample A was preheated at 37°C for 30 min in a TE buffer with 5 mM $MgCl_2$, sample B was done the same but without $MgCl_2$. Separation in the first dimension was at 6°C with 5 mM $MgAc_2$. The arrow shows the topoisomers that have undergone a structural transition.

Figure 5. 6% denaturing polyacrylamide gel electrophoresis of the EcoRI-PstI fragment of plasmid pPG37 labeled at the 3'-EcoRI end and cleaved with piperidine after treatment with dimethyl sulfate according to Maxam & Gilbert [11].

60°C. Since the bands corresponding to cytosines under the sequencing of $(dC)_n$ -containing strand are all resolved including the topmost ones without thermostating gel, the possibility of imperfect denaturation or partial renaturation of the $(dG)_n$ - and $(dC)_n$ -containing strands during electrophoresis is ruled out.

To investigate this phenomenon, we denatured the EcoRI-HindIII fragment of pPG46C, which carries the $(dG)_n \cdot (dC)_n$ insert, sharply chilled it and loaded it on a gel (Fig.6). After denaturation of the fragment labeled on the $(dG)_n$ -containing strand, an additional band arises whose mobility is appreciably higher than that of the native fragment (Fig.6 D,E). When the fragment whose $(dC)_n$ -containing strand has been labeled is denatured, again, a band arises that migrates faster than the native fragment but slower than the band observed in the case of the $(dG)_n$ -labeled fragment (Fig.6 B,C). Since the newly arising bands have different mobility, they correspond to nonrenatured $(dG)_n$ and $(dC)_n$ strands of the fragment. At the same time, a

Figure 6. 6% polyacrylamide gel electrophoresis of the ECORI-HindIII fragment of pPG46C (B,C,D,E): B and C, labeled at the 5'-ECORI end ((dC)_n-labeled fragment); D and E, labeled at the 3'-ECORI end ((dG)_n-labeled fragment). A, MspI fragments of pUC18 as length markers; F and G, the 110-bp MspI fragment of pUC18 isolated individually. The samples in lanes C,E,G were preheated at 100°C for 1 min and sharply chilled in liquid nitrogen.

fragment as long (110 bp) which carries no (dG)_n·(dC)_n insert produces no additional bands, i.e., completely renatures before electrophoresis (Fig.6 F,G). Furthermore, there is no complete renaturation of (dG)_n·(dC)_n-containing fragments even after incubation at 40 and 70°C for 10 min (Fig.7 B,A), though the radioactivity in the band of the native fragment slightly increases with raising temperature.

Similar results have been obtained with other (dG)_n-containing restriction fragments - ECORI-PstI, Sali-PstI (Fig.1) - and with the same fragments containing 27- and 37-bp-long (dG)_n·(dC)_n inserts. In all cases there is no complete renaturation of (dG)_n and (dC)_n-containing strands.

b. Influence of magnesium ions. If denaturation is followed by incubation of the ECORI-PstI fragment of pPG46C in the

Figure 7. 6% polyacrylamide gel electrophoresis of the SalI-HindIII fragment of pPG460 labeled at the 3'-SalI end ((dG)_n-labeled fragment) (A,B,C) and of the G-hairpin of this fragment, extracted from gel (D,E). All samples were preheated at 100°C for 1 min and sharply chilled by immersing into liquid nitrogen. After that, the samples were incubated for 10 min at 70°C (A,D) and 40°C (B,E). NF marks the native fragment; GS, the (dG)_n strand.

presence of 10 mM Mg^{2+} , then the band corresponding to the (dG)_n strand in the pattern of electrophoresis with Mg^{2+} is accompanied by additional bands. The mobility of these bands is the same for the (dC)_n-labeled fragment (Fig.8 A,B) and (dG)_n-labeled fragment (Fig.8 C,D). This suggests that these additional bands correspond to complexes that include both (dG)_n- and (dC)_n-containing strands. These complexes are only stable in the presence of Mg^{2+} , as they are not observed if electrophoresis is carried out without magnesium ions (data not shown). It should be noted that no additional bands are observed with the (dG)_n·(dC)_n-containing EcoRI-HindIII fragment of the same plasmid after renaturation with magnesium.

c. The distribution of the modification of guanine bases.

The G-specific chain splitting was performed by treatment of the (dG)_n·(dC)_n-containing fragment and of the free (dG)_n-strand with dimethyl sulfate followed by heating in piperidine according to Maxam and Gilbert [11]. After modification of the native EcoRI-HindIII fragment the splitting efficiency monotonically decreases as one moves away from the labeled end (Fig.9 B,D). This is due to the fact that there is more than one cleavage per fragment on an average.

Figure 8. 6% polyacrylamide gel electrophoresis with 10 mM MgAc of the EcoRI-PstI fragment of plasmid pPG46C labeled at the 5'-EcoRI end (A,B,C) and at the 3'-EcoRI end (D,E,F). C and F, native fragments; B and E, heated at 100°C for 1 min and sharply chilled; A and D, heated at 100°C for 1 min, sharply chilled, and then incubated at 50°C for 10 min with 10 mM MgCl₂. G, MspI fragments of pUC18 as length markers. Complexes containing both strands are marked (K1,K2).

In the case of the isolated (dG)_n-strand there is periodicity in the cleavage intensity: 2 maxima are situated at the ends and 3 in the middle of the insert in an approximately equidistant manner (Fig.9 A,C). It should be emphasized that the number of maxima is the same for (dG)_n strands of different lengths, though the distance between them is shorter for pPG27 than for pPG37. In addition, a much greater portion of the (dC)_n strand - the topmost band, which corresponds to the uncleaved (dG)_n strand, - remains unmodified in the case of an isolated (dG)_n-strand as compared to the native duplex. This points to the protection of deoxyguanosines within an isolated (dG)_n-strand against the attack of dimethyl sulfate, i.e. to the existence in this strand of some specific regular secondary (tertiary?) structure.

DISCUSSION

Supercoiling-induced Mg-dependent Structural Transition

The supercoiling-induced structural transition in plasmid pPG46C in the presence of Mg²⁺ takes place in topoisomers with

Figure 9. 10% polyacrylamide gel electrophoresis with 8.3 M urea. Gel was thermostated at 60°C. A, the (dG)_n strand of the ECORI-HindIII fragment of pPG37; B, the native ECORI-HindIII fragment of pPG37; C, the (dG)_n strand of the ECORI-HindIII fragment of pPG27; D, the native ECORI-HindIII fragment of pPG27. All samples were modified with dimethyl sulfate, which was followed by piperidine cleavage according to Maxam & Gilbert [11]. The boundaries of the inserts are indicated by parentheses.

$\Delta Lk \leq -15$ (under electrophoresis conditions) and results in a supercoiling release corresponding to 5 superhelical turns (Fig.3). Such a release does not contradict the formation of either dG·dG·dC [6] or dG·dC·dC⁺ (H-form [5]) triple helices within the insert. However, the fact that the transition occurs at pH 8 and is Mg-dependent, together with the data of Ref.[6] on chloroacetaldehyde modification of (dG)_n·(dC)_n inserts in superhelical DNA, argues in favor of the formation of a structure other than the H-form.

If we take topoisomer -16, for which $-\sigma = 0.06$, as the transition midpoint, then using the formula $E_s = 11RT\sigma^2$ for supercoiling energy [20] it is easy to calculate that the free energy of the structural transition under electrophoresis conditions is $E_{tr} = 36$ kcal/mol. However, this estimate should be handled with care, because, first, we are not absolutely sure that equilibrium in the structural transition has been achieved

in our experiments (this requires more detailed kinetic studies) and, second, the dependence of supercoiling energy on σ in the presence of Mg^{2+} may differ from the function presented above [21].

The Mg-dependent transition in plasmids with shorter inserts seems to be less favored than the formation of a cruciform in the 26-bp palindrome contained within the sequence of pUC18. It is possible that it does take place at high $-\sigma$ values, but it cannot be reliably detected by two-dimensional electrophoresis employed herein.

Structure of $(dG)_n$ -containing Single Strand

Incomplete renaturation of the $(dG)_n \cdot (dC)_n$ -containing fragments (Fig.6,7), together with the fact of intrastrand interaction during the sequencing of the $(dG)_n$ strand (Fig.5), made us infer that the $(dG)_n$ strand forms a stable intrastrand complex henceforth referred to as G-structure. Since G-structures appear after denaturation of the fragments within the $(dG)_n$ -strand that do not contain long complementary stretches and, moreover, in fragments containing but 6 additional nucleotides besides the $(dG)_n$ stretch (SalI-PstI fragments, Fig.1), G-structures can only be stabilized by the formation of G·G pairs. G·G-stabilized structures have been observed by electrophoresis and NMR [7] of synthetic G-rich oligonucleotides. The formation of such structures at the single-stranded G-rich ends of telomeres seems to be responsible for their inability to aggregate until heated under certain conditions [22].

The maxima observed after DMS-modification of free $(dG)_n$ strand (Fig.9 A,C) correspond to the sites worse protected against methylation. Such a distribution of the modification intensity can be attributed to the existence of a specific structure of this strand, in which the $(dG)_n$ stretch is bent thrice (the modification maxima correspond to the bends and the ends of the inserts). It cannot be excluded that this G-structure is stabilized by quadruple G·G·G·G helix [23].

It should be noted that dimethyl-sulfate modification of a single-stranded fragment of crab satellite DNA, containing a $(dG)_{23}$ stretch, has revealed a single modification-intensity maximum, found in the middle of the stretch [3]. This was interpreted as the formation of a G-hairpin in which the

G-stretch is bent once in the middle. It seems that a 23-bp G-stretch, in contrast to 37-bp and 27-bp stretches, is not sufficient for a thrice-bent structure to form.

Effect of Magnesium on Binding of G-structure to (dC)_n Strand

Complexes of the (dG)_n strand with the complementary strand, which migrate slower than the native duplex, can only be observed on the electrophoresis in the presence of magnesium (Fig.8). Such alternative complexes is rather stable since they give rise to distinct bands in the electrophoretic pattern. The magnesium stabilization of the alternative complexes between the (dG)_n and (dC)_n-strands can explain the Mg-dependent supercoiling-induced structural transition in plasmids containing (dG)_n·(dC)_n inserts. Indeed, it is the interaction between the (dG)_n and (dC)_n-strands that reduces the free energy of the transition so that it can occur at physiological values of superhelical density.

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