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A Triplex DNA Structure of the Polypyrimidine: Polypurine Stretch in the 5' Flanking Region of the Sea Urchin Arylsulfatase Gene

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ABSTRACT—Previously we reported that a long (522 bp) polypyrimidine: polypurine stretch in the 5' flanking region of the arylsulfatase gene of the sea urchin, *Hemicentrotus pulcherrimus*, took an unusual, perhaps triplex, DNA structure, when subjected to an acidic pH (pH 5) (Yamamoto *et al.*, 1994). In the present study we have isolated a polypyrimidine: polypurine containing fragment from the arylsulfatase gene and surveyed the sensitivities of the polypyrimidine: polypurine stretch to base modification by diethylpyrocarbonate and osmium tetroxide under various levels of negative supercoiling. Based on the sensitivity of highly negatively supercoiled DNA to these base-modifying reagents, we conclude that, when highly negatively supercoiled, the polypyrimidine: polypurine stretch can take a triplex DNA structure even at a neutral pH and under physiological ionic strength in the presence of Mg^{2+}.

INTRODUCTION

Eukaryotic genomes often contain polypyrimidine: polypurine sequences that, in many cases, are located in or around transcription-regulating regions of genes (Mirkin *et al.*, 1987; Sinden, 1994; Wells *et al.*, 1988) and are thus believed to be related to regulation on transcription. Though little evidence is available on the role of these polypyrimidine stretches in gene transcription, many investigators report that a polypyrimidine sequence inserted into a plasmid can take an unusual DNA structure distinct from a usual B-structure (Hentschel, 1982; Htun *et al.*, 1984; Kato *et al.*, 1990; Kohwi-Shigematsu and Kohwi, 1985; Mace *et al.*, 1983: Nickol and Felsenfeld, 1983; Pulleyblank *et al.*, 1985; Schon *et al.*, 1983). Mirkin *et al.* (1987) and Wells *et al.* (1988) showed that a region with a mirror-repeat sequence within a polypyrimidine stretch forms a triplex DNA structure through Hoogsteen base pairings, and stability of triplex DNA structure is dependent on the degree of negative supercoiling of the plasmid as well as pH and ionic strength of the DNA solution (Hanvey *et al.*, 1988; Htun and Dahlberg, 1988; Mirkin *et al.*, 1987; Pulleyblank *et al.*, 1985; Wells *et al.*, 1988).

The arylsulfatase (Ars) gene of the sea urchin, *Hemicentrotus pulcherrimus*, which is expressed in a developmental stage-specific (Sasaki *et al.*, 1988) as well as tissue-specific manner (Akasaka *et al.*, 1990), contains a long polypyrimidine:polypurine (Pyr-Pur) stretch in its 5' flanking region (Akasaka *et al.*, 1994) and in its 1st intron (unpublished data). Because the DNA region with an unusual structure may have a specific function in regulation of transcription, we have undertaken a series of studies to explore the possible structure of the polypyrimidine stretch in the sea urchin Ars gene. Through an S1 nuclease assay at pH 5, we previously reported that a part of the polypyrimidine stretch of the sea urchin Ars gene can take an unusual DNA structure different from B-DNA, when the stretch is in a negatively supercoiled plasmid (Yamamoto *et al.*, 1994). However, the reaction conditions such as pH 5 and a low ionic strength in an S1 nuclease assay obviously do not reflect *in vivo* conditions.

To approach to the question of the biological function of Pyr-Pur region in the 5' flanking region of the Ars gene, DNA structure of the Pyr-Pur region has been re-examined by measuring sensitivities of the Pyr-Pur region to base modification by osmium tetroxide and diethylpyrocarbonate under physiological ionic strength at neutral pH and in the presence of 5 mM Mg^{2+}. The results suggest that, if the stretch is highly negatively supercoiled, a part of the polypyrimidine stretch takes triplex DNA structure even under physiological conditions.

MATERIALS AND METHODS

Plasmid DNA

A region containg 522 bp Pyr-Pur stretch (−2, 201 to −1, 680) of the Ars gene of the sea urchin (*Hemicentrotus pulcherrimus*) was excised and inserted into pBluescript SK− (pBSK) vector (Stratagene Cloning Systems, USA) to construct the plasmid pBSK-AvNs (Fig. 1). Various degrees of negative supercoiling were introduced into pBSK-AvNs by treating 20 μg/ml of pBSK-AvNs with topoisomerase I at 37°C for 1 hr in the presence of various concentrations of ethidium bromide. After extracting the supercoiled

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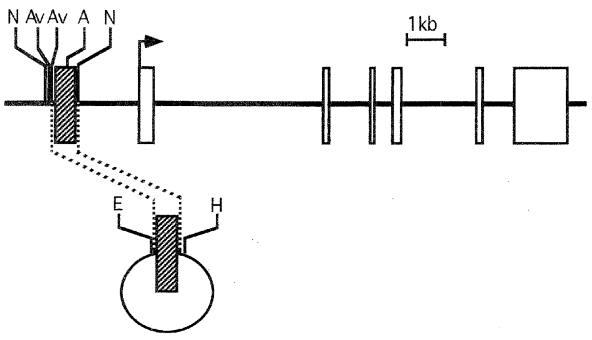

Fig. 1. Restriction sites around the Pyr-Pur region in the Ars gene and in the pBSK-AvNs. Slashed box; the Pyr-Pur region, open boxes; exons, a thick line; 5' flanking region and introns, a thin line; pBSK vector. A; *Apa*I, H; *Hin*dIII, N; *Nsp*7524I, Av; *Ava*I, E; *Eco*RI.

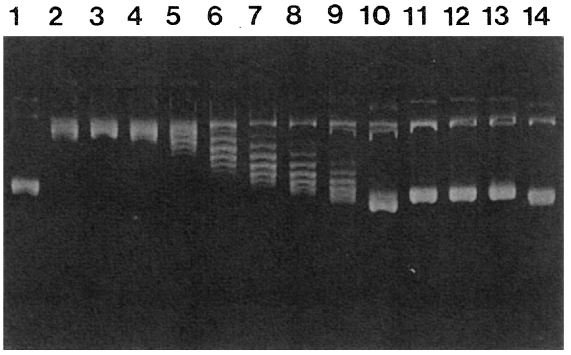

Fig. 2. Electrophoretic mobility of pBSK-AvNs changes depending on degree of negative supercoiling. Various degrees of negative supercoiling were produced in pBSK-AvNs by treating the plasmid with topoisomerase I in the presence of various concentrations of ethidium bromide. Electrophoresis was carried out in 1.5% agarose gel. Concentration of ethidium bromide, lane 2; without ethidium bromide, lane 3; 0.2 μg/mg, lane 4; 0.4 μg/ml, lane 5; 0.6 μg/ml, lane 6; 0.8 μg/ml, lane 7; 1.0 μg/ml, lane 8; 1.2 μg/ml, lane 9; 1.4 μg/ml, lane 10; 2 μg/ml, lane 11; 5 μg/ml, lane 12; 8 μg/ml, lane 13; 10 μg/ml. Lanes 1 and 14; pBSK-AvNs before treatment with topoisomerase I.

DNA by phenol/chloroform, ethidium bromide was removed by water-saturated n-butanol, and DNA was precipitated and washed with ethanol, and dried *in vacuo*.

The density of negative supercoiling was determined by two dimensional electrophoresis (Pulleyblank *et al.*, 1985). A mixture of variously supercoiled plasmids was first electrophoresed on 1.5% agarose in 0.5×TBE, then gels were equilibrated with 1 μg/ml chloroquine in 0.5×TBE. The second electrophoresis was performed in the presence of 1 μg/ml chloroquine in 0.5×TBE. The DNA bands were detected by southern blotting with digoxygenin-labeled pBSK-AvNs as the probe.

Diethylpyrocarbonate (DEPC) and osmium tetroxide reactions

Modification of purine bases (especially adenine) by DEPC and pyrimidine bases (especially thymine) by osmium tetroxide in the negatively supercoiled pBSK-AvNs were carried out as described by Hanvey *et al.* (1988) with some modifications.

Three μg of negatively supercoiled pBSK-AvNs was dissolved in 100 μl of 0.3 M Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 0.25 M KCl, and incubated for 1 hr at 13°C. The DEPC reaction was started by adding 10 μl of DEPC to the mixture, followed by 1-min of vortexing and a 30-min incubation at 22°C. The osmium tetroxide reaction was started by addition of 2 μl of pyridine and 10 μl of 20 mM OsO_4 to the mixture, followed by 1-min vortexing and 30-min incubation at 22°C. The plasmid DNA was then cleaved at modified nucleotides by piperidine, and sites of modification were determined on a denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Electrophoretic mobility of negatively supercoiled pBSK-AvNs

pBSK-AvNs was negatively supercoiled by DNA topoisomerase I treatment in the presence of ethidium bromide and electrophoresed on 1.5% agarose gel containing TBE buffer at pH 8.2. Increasing the concentration of ethidium bromide, increased the electrophoretic mobility of pBSK-AvNs (Fig. 2), indicating that the level of negative supercoiling of pBSK-AvNs is positively dependent on the concentration of ethidium bromide in the topoisomerase reaction. Electrophoretic mobility of the plasmid decreased by increasing ethidium bromide from 2 μg/ml to 10 μg/ml, suggesting that the high negative supercoiling (σ<–0.0385, as determined in the presence of chloroquine by two dimensional electrophoresis, see Materials and Methods) causes a severe conformational change of the plasmid that leads to reduction of the electrophoretic mobility. A plasmid with this level of negative supercoiling is hereafter referred to as highly negatively supercoiled, with the supercoiling density of σ=–0.0357 as a native level of supercoiling.

Chemical modification of bases in negatively supercoiled pBSK-AvNs

It is known that diethylpyrocarbonate (DEPC) carboxyethylates adenines at their N7 and cleaves an imidazole ring, while OsO_4 reacts with thymines at their C5 and C6 to form osmate esters, when DNA is in a non-B structure (Johnston, 1988; Vojtiskova and Palecek, 1987; Wells *et al.*, 1988). Thus the sensitivity of DNA to these reagents is a good measure to estimate its structure.

Modification by DEPC and OsO_4 of nucleotide bases in a linearized pBSK-AvNs, a native pBSK-AvNs (with a native level of supercoiling) and a highly negatively supercoiled pBSK-AvNs was surveyed under physiological ionic strength and Mg^{2+} concentration similar to *in vivo* levels. The experimental results are shown in Figure 3, and the positions of modified bases are summarized schematically in Figure 4.

Clustered adenines in the purine strand of a linear DNA were chemically modified while any bases in the pyrimidine strand were not modified (Fig. 4a). At present we do not have any evidence to explain increased accessibility of DEPC to adenines in linear DNA fragments, but it is conceivable that consecutive adenines tend to cause DNA bending (Sinden, 1994) that would make adenine bases accessible to DEPC.

In the case of a native pBSK-AvNs (Fig. 4b), though the purine strand was modified by DEPC in a manner similar to

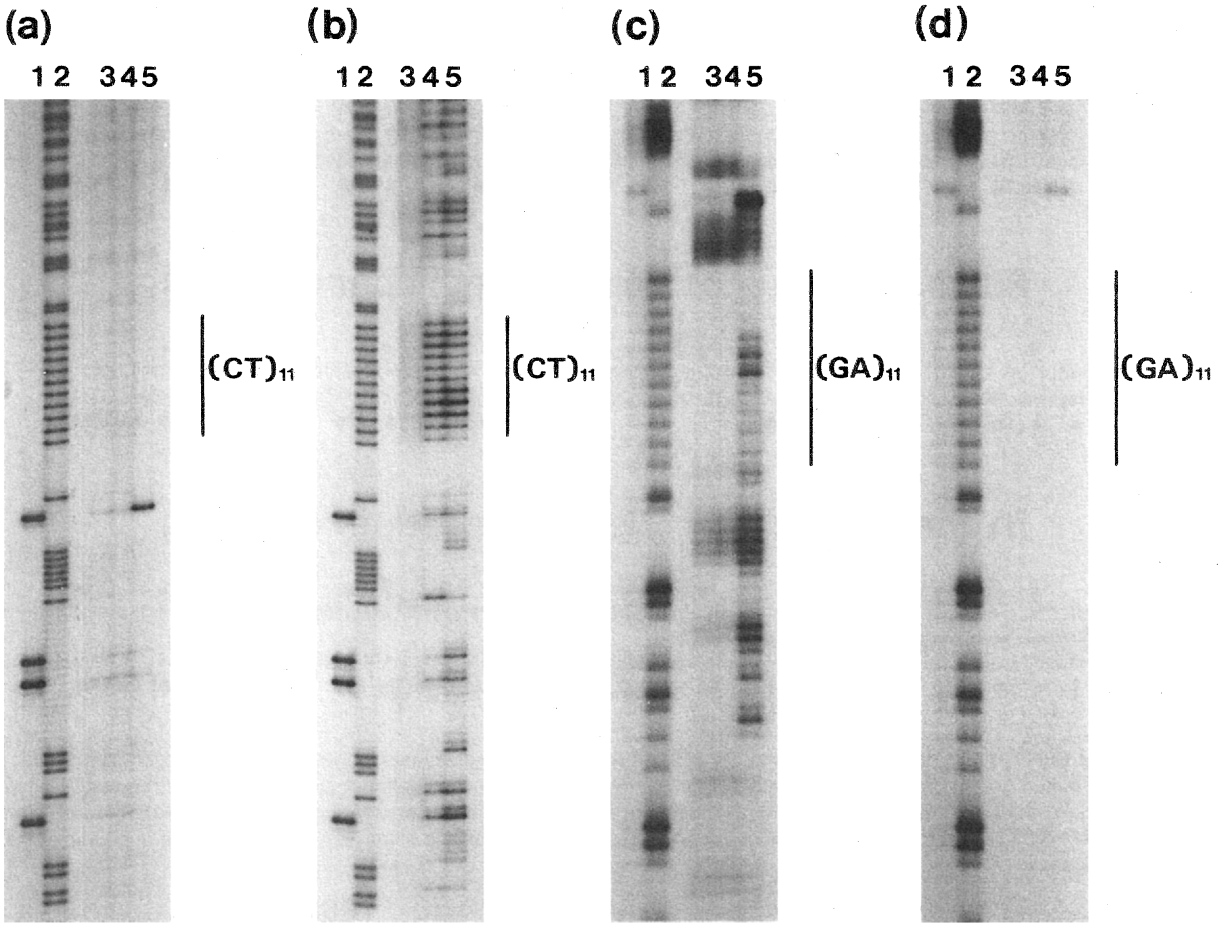

Fig. 3. Base modificaiton of Pyr-Pur region in negatively supercoiled plasmid by DEPC or by OsO_4. pBSK-AvNs in linear, native and highly negatively supercoiled forms were treated with DEPC or with OsO_4, cleaved by *Hin*dIII, labeled with ^{32}P at the 3' end of the pyrimidine strand, and the labeled *Hin*dIII-*Apa*I fragments were isolated (a and b). In the case of preparation of the ^{32}P-labeled *Hin*dIII-*Apa*I fragments from the purine strand (c and d), the plasmid was first cut by *Apa*I, and the labeled fragment was prepared in a similar way. The labeled fragments were cleaved by piperidine at modified bases and electrophoresed on 4% denaturing polyacrylamide gel (a and b) and on 6% denaturing polyacrylamide gel (c and d). Sites of $(CT)_{11}$ or $(GA)_{11}$ were indicated by vertical lines on the right side of each column. Lanes 1 and 2; Maxam-Gilbert ladders or A+G and C in (a) and (b) and C+T and G in (c) and (d), respectively. Lane 3; linearized pBSK-AvNs, lane 4; native pBSK-AvNs, lane 5; highly negatively supercoiled pBSK-AvNs. (a) DEPC-sensitive sites in the pyrimidine strand. (b) OsO_4-sensitive sites in the pyrimidine strand. (c) DEPC-sensitive sites in the purine strand. (d) OsO_4-sensitive sites in the purine strand.

a linear plasmid, thymines in the pyrimidine strand that were not accessible to OsO_4 in a linear plasmid became accessible to the reagent. It is of interest that, while the $(CT)_{11}$ tract was extensively modified by OsO_4, the complementary $(GA)_{11}$ tract was not modified by DEPC, and thymine stretches flanking both ends of the $(CT)_{11}$ tract were protected from modification. This result suggests that even a native level of negative supercoiling can produce a conformational change in the Pyr-Pur region which results in a DNA structure slightly deviated from B-form.

By imposing higher negative supercoiling than native level, the accessibility of base-modifying reagents to the plasmid increased in the purine strand. As depicted in Figure 4c, while little change was observed in the thymine modification of the pyrimidine strand as compared with a plasmid with a native level of supercoiling, a marked change in adenine modification was observed in a restricted region of the purine strand. Adenines in the 3' half of $(GA)_{11}$ in the purine strand were modified and the adenines upstream of $(GA)_{11}$ (from –1,827 to –1,807) became more accessible to DEPC. That is, adenines between –1,827 and –1,793 were all modified, indicating the single-stranded nature of the purine strand of this region under high negative supercoiling.

A triplex DNA structure of the Pyr-Pur region

A triplex DNA structure through intramolecular formation of Hoogsteen base-pairings (Hoogsteen, 1963) seems to be a most plausible DNA model that accomodates most of the above experimental results on the accessibility of the Pyr-Pur region to base-modifying reagents. A possible model of a triplex DNA structure taken by the Pyr-Pur region of the sea urchin Ars gene is depicted in Figure 5. In this model, the purine strand between –1,829 and –1,787 is single-stranded, while the pyrimidine counterpart winds up around

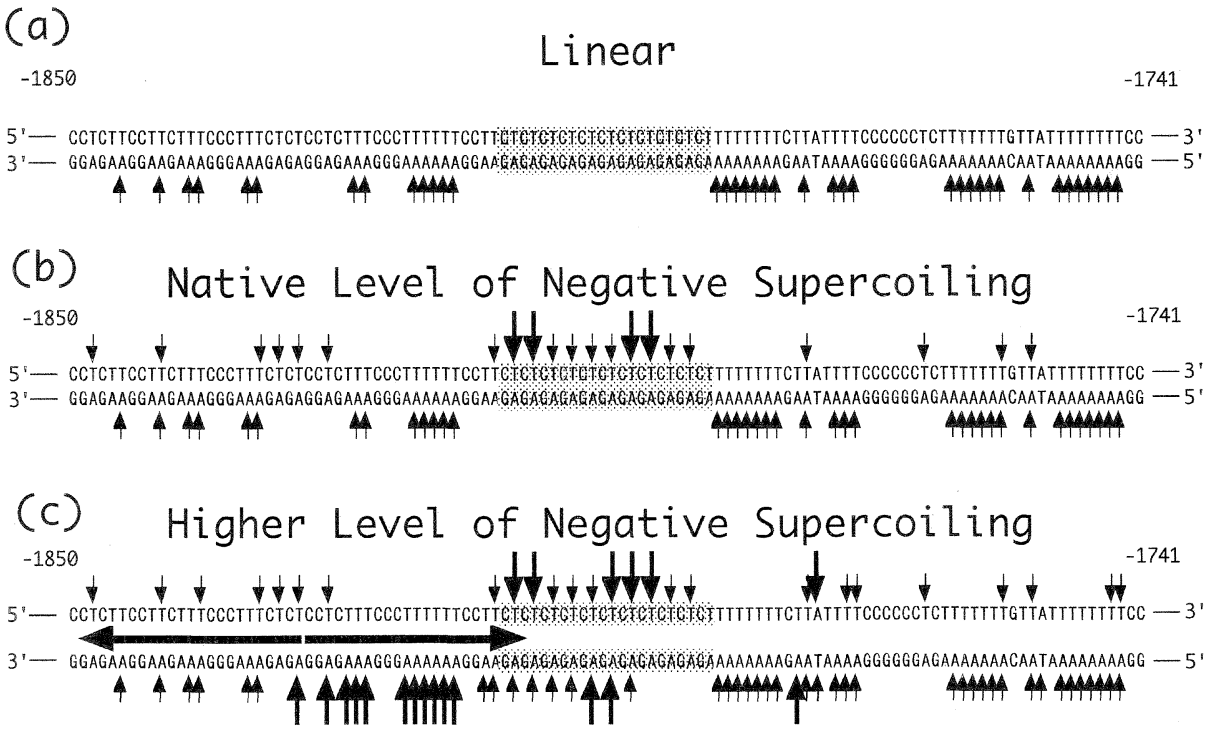

Fig. 4. Mapping of sites of base modification in *Hin*dIII-*Apa*I fragments in linear, native and highly negatively supercoiled plasmids. The sites of base modification determined in experiments in Fig. 3 are mapped in the nucleotide sequence of AvNs fragments. Sites of base modification in (a) a linearized plasmid, (b) a native plasmid, and (c) a highly negatively supercoiled plasmid. Thin and short arrows indicate weakly sensitive sites, and thick and long arrows indicate highly sensitive sites. Very thick horizontal arrows indicate a mirror repeat sequence.

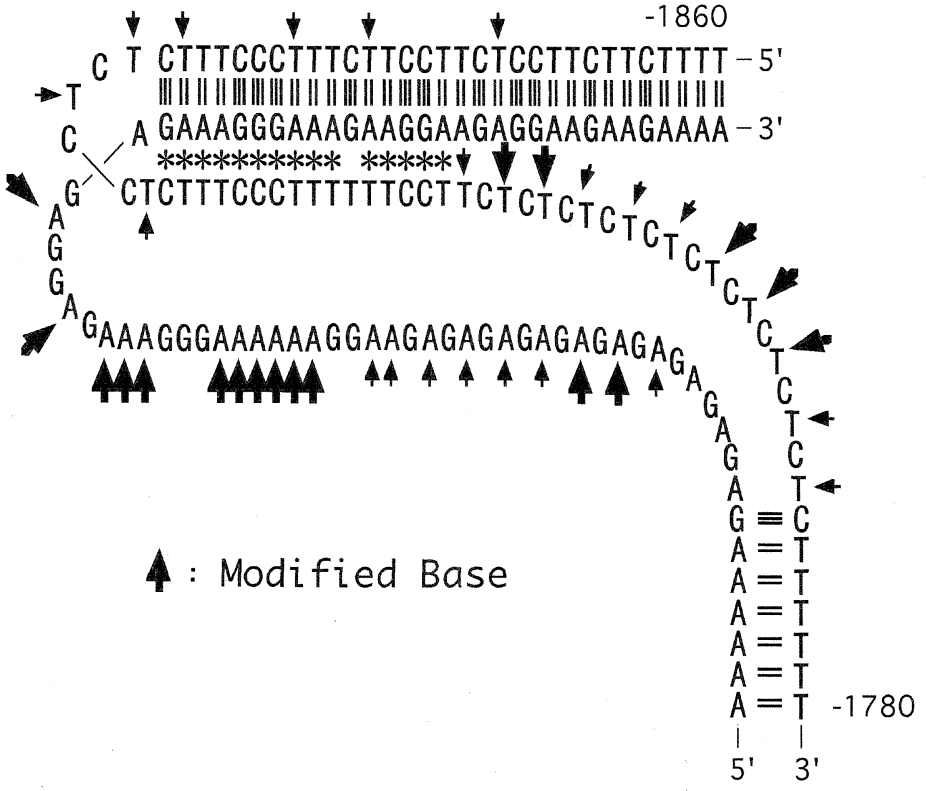

Fig. 5. A possible H-DNA model formed in polypyrimidine region of the sea urchin Ars gene. Small arrows indicate bases weakly accessible to base-modifying reagents when the polypyrimidine region is highly negatively supercoiled, and large arrows indicate bases highly accessible to the reagents.

the upstream double helical DNA along its major groove to form a triple-stranded DNA structure. Thymines in the third pyrimidine strand of this structure would be eventually protected from attack by OsO_4.

The existene of a mirror repeat sequence (from –1,849 to –1,804; Fig. 4c) in the Pyr-Pur region supports this model. If strand separation occurs in the right half of the mirror repeat accompanied by folding back of its single-stranded pyrimidine counterpart along a major groove of the left half of the mirror repeat, 21 Hoogsteen base-pairings containing C-G-C and T-A-T would be formed between the purine strand of the double helix and the pyrimidine strand that folded back over it, as suggested by Wells *et al.* (1988). In spite of the presence of Mg^{2+}, H-DNA structure appears to be formed through C-G-C and T-A-T in the Pyr-Pur region of the sea urchin Ars gene, while Kohwi and Kohwi-Shigematsu (1988) showed that in the presence of Mg^{2+} an isomer of H-DNA is formed that contains C-G-G in place of C-G-C. Alternately-arranged T-A-T's may stabilize the C-G-C structure.

Biological significance of a triplex DNA structure is not clear though its possible functions in transcription, replication and recombination have been discussed (Sinden, 1994). Michel *et al.* (1993) suggested that a triplex DNA region may act as a transcriptional insulator by insulating some genetic loci from influences of other genes through its unusual steric conformation, while Baran *et al.* (1991) showed that DNA synthesis is arrested at sites of DNA triplex formation. It is also possible that sharp bends of DNA in these structures may help interaction of protein factors that bind to distantly separated *cis*-elements.

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