Formation of a Combined H-DNA/Open TATA Box Structure in the Promoter Sequence of the Human Na,K-ATPase a2 Gene*

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Abstract

Structural variation of DNA within the promoter of the human Na,K-ATPase a2 gene, which contains a 35-base pair (bp) homopyrimidine-homopurine (Py-Pu) tract adjacent to a TATA box has been studied. The Py-Pu tract contains a 26-bp quasi-mirror repeat sequence with a potential for intramolecular triplex formation. As analyzed by two-dimensional agarose gel electrophoresis, a plasmid containing 151 bp of the promoter sequence including the 35-bp Py-Pu tract undergoes structural transitions under moderately acidic pH. Chemical probing with chloroacetaldehyde, dimethyl sulfate, and potassium permanganate is consistent with the formation of triplex DNA within the Py-Pu tract at native superhelical density as isolated from Escherichia coli. Chemical probing was used to determine a supercoil dependence for the formation of this combined unwound structure. At the superhelical density sufficient to locally unwind DNA, an H-y5 isomer of intermolecular triplex likely forms. However, at higher superhelical tension an H-y5 structure forms in the Py-Pu tract, and with increasing supercoiling the local DNA unwinding extends into the abutting TATA box. The H-y5/open TATA box combination structure might be favorable at higher superhelical densities since it relaxes more supercoils. The possible involvement of the H-y5/open TATA box structure in transcription is discussed.

Homopyrimidine-homopurine (Py-Pu)1 tracts capable of forming triple-stranded DNA structures exist in the genomes of various organisms. In eukaryotes, the Py-Pu tracts of at least a few dozen base pairs (bp) long constitute up to 1% of the entire genome (1–6). Py-Pu tracts with mirror repeat symmetry may form H-DNA structures composed of intramolecular triple-helical and single-stranded regions (7). A computer analysis of the human genome suggests the presence of one H-DNA forming sequence in every 50,000 bp (8). H-DNA may be involved in key biological processes such as transcription, replication, and recombination (for reviews, see Refs. 9–12).

The Py-Pu tracts often occur in 5' flanking regions of eukaryotic genes, and a number of these tracts have been shown to be sensitive to the single-strand-specific nuclease, suggesting that alternative non-B-DNA structures can form in these sequences (13–20). More extensive studies showed that many of the naturally occurring Py-Pu tracts can adopt a supercoil-dependent H-DNA structure (21–26). Detailed structure-function analyses for many eukaryotic genes have demonstrated the importance of the Py-Pu sequences for promoter function. A partial loss of transcription efficiency was observed when the Py-Pu tracts were deleted from promoter regions of human epidermal growth factor receptor; (27), c-myc (18, 28), et-s2 (29), and decorin genes (30); rat neuronal cell adhesion molecule gene (31); mouse c-Ki-ras (20) and transforming growth factor-p3 genes (32); and Drosophila hsp26 (19, 23) and actin genes (33). Contradictory results on the importance of H-DNA in the regulation of transcription have been reported (23, 26, 34, 35). In Drosophila, a (GA)n, tract is likely involved in gene expression in its B-conformation, representing a protein binding site, rather as an H-DNA structure (23). A similar conclusion has been drawn for the Py-Pu tracts in the neuronal cell adhesion molecule (31) and c-Ki-ras promoters (26). On the other hand, mutations in a promoter of the γ-globin gene, which would reduce the probability of forming H-DNA, led to the expression of the gene when it should be turned off (34). For the c-myc promoter, H-DNA formation in vitro correlated positively with the potential of the Py-Pu tract to promote the transcription in vivo (35). Thus, the mechanisms of an influence of H-DNA forming sequences on gene expression are not yet well understood (11). Therefore, new information on mechanisms of H-DNA formation, its stabilization, and influence on conformations of adjacent regulatory sequences can advance our understanding of the potential involvement of H-DNA in biological processes.

Here we have studied structural variation of DNA within the promoter of the human Na,K-ATPase a2 gene (see Fig. 1) which contains a 35-bp Py-Pu tract adjacent to a TATA box (36). The Py-Pu tract contains a 26-bp quasi-mirror repeat sequence with a potential for H-DNA formation. As analyzed by two-dimensional agarose gel electrophoresis, a plasmid containing a 151-bp fragment of promoter sequence undergoes a supercoil-dependent structural transition under moderately acidic pH. Chemical probe analyses with chloroacetaldehyde, dimethyl sulfate, and potassium permanganate (KMnO4) are consistent with the formation of H-DNA at the Py-Pu tract. Yet, the local DNA unwinding is not limited to the Py-Pu tract; the sequence of the neighboring TATA box becomes unwound as well. Chemical modification experiments with plasmid DNA of different superhelical densities show that at moderate superhelical tension an H-y5 isomer of intramolecular triplex forms. At higher superhelical tension, the H-y5 isomer is predominant, and the TATA box adjoining a single strand in this H-DNA isomer becomes unwound leading to the further relaxation of supercoils.

EXPERIMENTAL PROCEDURES

Materials—Plasmid pBB151-8, in which a 151-bp fragment of the promoter region of the human Na,K-ATPase a2 gene (36) was cloned

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§ The abbreviations used are: Py-Pu, homopyrimidine-homopurine; bp, base pair(s).
the BamHI site of plasmid pUC8, was a generous gift from Dr. J. B. Lingrel (University of Cincinnati). PvuII (New England Biolabs), Taq DNA polymerase (Life Technologies, Inc.), and Stoffel fragment of AmpliTaq DNA polymerase (Perkin-Elmer Corp.) were used according to the manufacturer's specifications. Double-distilled chloroacetaldehyde was a generous gift from Dr. A. Bacolla (Institute of Biosciences and Technology, Texas A&M University).

Two-dimensional Agarose Gel Electrophoresis—Topoisomers of particular superhelical densities were generated by incubating supercoiled plasmid with a topoisomerase extract from HeLa cells in the presence of varying concentrations of ethidium bromide as described (37). Plasmid DNA (4 μg) containing a mixture of topoisomers with a broad distribution of superhelical densities was incubated at 37 °C in 10 μl of the first-dimension electrophoresis buffer for 1 h to form H-DNA. After cooling to room temperature and addition of 5 μl of dye mixture, the sample was loaded onto a 1.75% agarose gel in TBE buffer (90 mM Tris-borate [pH 8.3], 2.5 mM EDTA) with sequencemarkers obtained by chemical probing. The gel was washed extensively to remove the chloroquine, stained with ethidium bromide, and photographed.

Chemical Probing—In a standard experiment, each sample contained 2.5 μg of plasmid in 40 μl of 13.5 mM Tris acetate (pH 4.2 or 5.0), 1 mM magnesium acetate. To probe DNA in the duplex conformation, plasmid DNA was dissolved in 20 μl of HEPES, 50 mM NaCl (pH 7). After a 1-h incubation at 37 °C, samples were treated with chemicals at room temperature by adding either 2 μl of 2% dimethyl sulfate, 1.6 μl of 50% chloroacetaldehyde, or 2 μl of 20 mM KMnO4. After 10 min of dimethyl sulfate or 5 min of KMnO4 modification, reactions were stopped by adding 8 μl of 2.5 M β-mercaptoethanol. After 2.5 h chloroacetaldehyde modification, the reaction was stopped by extraction with diethyl ether. Following two ethanol precipitations, modified DNAs were digested overnight with PvuII and the reaction mixtures then extracted with phenol-chloroform. After two additional ethanol precipitations, samples were resuspended in 40 μl of TE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA), and 10 μl was used for primer extension analysis of modification products as described (38, 39). Two 23-nucleotide-long primers that hybridized approximately 150 nucleotides away from the Py tract of plasmid in 40 μl of 13.5 mM Tris acetate (pH 4.2) were used for primer extension analysis of modification products. Gels were dried and exposed to x-ray film or a PhosphorImager plate for analysis of the radioactivity pattern using ImageQuant software (Molecular Dynamics).

RESULTS

A Supercoil-dependent Transition within the 35-Bp Region of the Human Na,K-ATPase a2 Gene—The promoter region of the human Na,K-ATPase a2 gene contains a 35-bp PyPu tract upstream of the TATA box (Fig. 1). There is an imperfect mirror repeat element within this sequence which could participate in the formation of various intramolecular triplex (H-DNA) structures. Two-dimensional gel electrophoresis is a convenient method of measuring the relaxation of superhelical tension associated with local unwinding of the DNA double helix (40). Intramolecular triplex formation in a 35-bp PyPu tract and partial supercoil relaxation should result in an abrupt change in the mobility of topoisomers containing the triplex. Fig. 2 shows that at pH 4.2 a structural transition began with topoisomer −8 (superhelical density, ρs = −0.030). The observed relaxation was about two and a half supercoils, suggesting an unwound region 26–27 bp long, which may correspond to the length of a quasi-mirror repeat (26 bp). An unusual feature of this transition is that topoisomers −9 through −11 do not show the increase in mobility in the first dimension expected for molecules that relaxed a fixed amount of supercoils. Apparently, additional supercoil relaxation occurred after the initial transition, which suggests an additional structural transformation. At pH 5.0 a structural transition occurred at ρs = −0.049 (data not shown), as expected from the dependence of ρs on pH (21, 41).

Chemical Probing at pH 4.2 Shows the Presence of H-DNA—An Open TATA Box in the Topoisomers of Native Superhelical Density—At pH 4.2 the chemical modification patterns (Fig. 3, A and B) for DNA of native superhelical density, as isolated from Escherichia coli (ρs = −0.055), were consistent with the local unwinding of the double helix and the subsequent pairing of the 5’ part of the Py strand to the 5’ part of the Pu strand to form an H-y5 isomer of intramolecular triplex. Chloroacetaldehyde, which recognizes unpaired adenines and cytosines, and, to a lesser extent, guanines (42), and KMnO4, which recognizes unpaired thymines (43), were used to reveal single-stranded regions. Protection from dimethyl sulfate modification indicated specific guanines involved in hydrogen bonding with the incoming third strand upon triplex formation (39). Adenines and guanines in the 3’ part of the Pu-rich strand (sequence –21 to –50) were reactive to chloroacetaldehyde at pH 4.2, indicating that these bases were unpaired (Fig. 3A, lane 3). Guanines in this region of the Pu strand were partially reactive with dimethyl sulfate at pH 4.2 (Fig. 3A, lane 5), consistent with a single-strand character of this sequence. At the same time, guanines were protected from reaction with dimethyl sulfate in the 5’ part of the Pu strand (sequence –52 to
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**Fig. 3.** Mapping of chemical modification sites in pBB151-8 plasmid. Modification at pH 4.2 and primer extension reactions for a plasmid DNA of native superhelical density were performed as described under “Experimental Procedures.” Panel A, chemical reactivity of bases in the purine-rich strand. Lanes 1 and 2, T and C sequencing reactions for a complementary strand; lanes 3 and 4, chloroa acetatedehyde (CAA) modification at pH 4.2 and 7.0, respectively; lanes 5 and 6, dimethyl sulfate (DMS) modification at pH 4.2 and 7.0, respectively. Panel B, chemical reactivity of bases in the pyrimidine-rich strand. Lanes 1 and 2, G and A sequencing reactions for a complementary strand; lanes 3 and 4, chloroa acetatedehyde modification at pH 4.2 and 7.0, respectively; lanes 5 and 6, dimethyl sulfate modification at pH 4.2 and 7.0, respectively. Panel C, suggested intramolecular triplex (H-DNA) structure with the key triplex-specific reactivities. Chloroa acetatedehyde and KMnO₄ reactivities are shown with open and filled arrowheads, respectively; guanines protected from dimethyl sulfate modification are shown in italics. Watson-Crick hydrogen bonding is shown with lines; Hoogsteen hydrogen bonding is shown with (·) for T+G, (·) for CG+CT, and (·) for possible CG+CT triad with one Hoogsteen hydrogen bond.

to −65), indicating that a 5′ part of the Py strand folds back to form Hoogsteen hydrogen bonds with guanines of the 5′ part of the Pu strand. Probing of the complementary Py strand revealed a single guanine (−51), which interrupts a homopyrimidine sequence, and a cytosine (−44), which is 6 bases away, which were strongly reactive to chloroa acetatedehyde (Fig. 3B, lane 3). In addition, cytosines in the central part of this strand (sequence −46 to −50) were weakly reactive toward chloroa acetatedehyde. The dimethyl sulfate reactivity for this strand (Fig. 3B, lanes 5 and 6) was identical for the triplex-favoring (pH 4.2) and duplex-favoring conditions (pH 7). Note that in addition to the bases that belong to the Py-Pu tract, chloroa acetatedehyde reactivity was observed for adenines contained within the TATA box sequence (Fig. 3, A and B, lanes 3). The H-y5 isomer of intramolecular triplex DNA shown in Fig. 3C can explain the key base modifications and protections. In this structure the entire 35-bp Py-Pu tract participates in a triplex formation rather than only the bases of the quasi-mirror repeat sequence. In addition, the local unwinding extends into the A + T sequence of the TATA box, where unpaired adenines are susceptible to modification with chloroa acetatedehyde. Probing of unpaired thymines with KMnO₄ (see Figs. 4B and 5C) is consistent with this model. Note that cytosines in the single-stranded loop at the tip of triplex are only partially modified with chloroa acetatedehyde, which suggests that they may participate in a structure different from that shown in Fig. 3C (e.g. hydrogen-bonded protonated C+C⁻ hairpin; Refs. 44 and 45). In three mismatched base triads, interactions between the duplex guanines and third-strand thymines might be partially stabilizing, and a formation of a partially stable C+T triad with one Hoogsteen hydrogen bond is indicated by a colon in Fig. 3C, showing a suggested intramolecular triplex structure (see also “Discussion”).
3' end of Py strand (sequence -67 to -69) was still observed. Thus, at intermediate superhelical densities, an H-y3 isomer gives rise to chloroacetaldehyde reactivity at these adenes. However, at higher supercoiling this chloroacetaldehyde reactivity becomes relatively weak, and the H-y5 isomer/open TATA box structure with chloroacetaldehyde reactive bases at 5' part of the Py strand and in the TATA box sequence becomes predominant. This correlates with observed reactivity of thymines (-22 to -23, -27) which comprise the TATA box (Fig. 4B, lanes 5 and 6). Note also that the reactivity of cytosines (-47 to -50) which supposedly occupy positions in the single-stranded tip of triplex are weak, suggesting that this single strand does not have a truly open conformation.

Chemical modification patterns for the bases in the Pu-rich strand are shown in Fig. 5. A high background of chloroacetaldehyde reactivity was observed in the Pu strand in linear plasmid and lower density topoisomers. This could be due to a low ionic strength buffer employed in our studies and may be similar to the significant susceptibility of a linear Py-Pu tract to S1 nuclease at low salt (46). This background reactivity was still observed at a higher ionic strength (120 mM NaCl); however, the formation of an unwound structure was significantly shifted to higher superhelical densities (data not shown). Another contribution to a high background may come from the guanine-rich sequence itself as the primer extension through this sequence usually results in a higher background compared with other sequences. As shown in Fig. 5A (lane 4), at average superhelical density, \( \sigma = -0.030 \), where the transition occurred in a two-dimensional gel, the 3' part of the Pu sequence
(--30 to --50) was modified with chloroacetaldehyde, suggesting its unpaired character. This is expected for an H-y5 triplex isomer. The 5' part of this strand (--50 to --65) was also susceptible to chloroacetaldehyde as expected for an H-y3 isomer, although to a lesser extent. Thus, in agreement with the chemical probing data for the Py strand, the chloroacetaldehyde reactivity of bases in the Pu strand shows that the H-y5 and H-y3 isomers of intramolecular triplex coexist at intermediate superhelical densities. At higher superhelical densities (Fig. 5A, lanes 5 and 6), the H-y5 isomer predominates since the pattern of chloroacetaldehyde reactivity shows that the 5' part of the Pu strand is hydrogen bonded, and its 3' part is unpaired. The involvement of the 5' part of the Pu strand in the triple helix is consistent with the observed protection of guanines (--52 to --65) in this strand from dimethyl sulfate modification (Fig. 5B, lanes 5 and 6). The KMnO4 modification pattern shows that, at the abrupt transition point (Fig. 5C, lane 4), thymines (--67 to --68) adjoining the Pu sequence at its 5' end and a cytosine interrupting the Pu strand are reactive. At higher superhelical densities (Fig. 5C, lanes 5 and 6), this reactivity is relatively weak; however, a prominent reactivity of thymines (--24 to --26, --28) in the TATA box sequence appears. Thus, the reactivity pattern for the Pu strand is consistent with the presence of a combined H-y5 triplex/open TATA box structure at high superhelical density.

**DISCUSSION**

Non-B-DNA Structures Form in the Promoter Sequence of the Human Na,K-ATPase α2 Gene—As indicated from two-dimensional gel analysis (Fig. 2), alternative DNA structures form, with increasing superhelical density, within a 35-bp PyPu region from the human Na,K-ATPase α2 gene promoter. Pu-Py sequences with mirror repeat symmetry can form a number of intramolecular structures (10–12). The imperfect nature of a 26-bp mirror repeat in the Na,K-ATPase α2 promoter and the presence of the larger PyPu tract provide the opportunity for the formation of many different intramolecular triplex structures. Analysis of this region reveals about 20 PyPu*Pu and about 10 PuPy*Py triplexes that may form, many of which may have only slight energetic differences. We show that this 35-bp PuPy tract, which is 2 bp upstream of the TATA box in this human Na,K-ATPase α2 gene, forms at least two very different intramolecular triplex structures as a function of negative DNA supercoiling. At intermediate levels of supercoiling (x = --0.02 to --0.03) a H-y3 triplex is formed which may be stabilized, in part, by unusual C-G*T triads. At higher, native superhelical densities (x = --0.041) a H-y5 intramolecular triplex is formed, and as the negative superhelical density increases the TATA box becomes unwound. Such structural transitions in vivo could have profound implications for the regulation of gene expression from this promoter.

At lower superhelical densities, several possible H-y3 intramolecular triplex isomers could form. Since the 26-bp PyPu quasi-mirror repeat has an imperfect mirror symmetry, a usually predominant H-y3 isomer (47) of the intramolecular triplex at this sequence would have nine perfect T-A*T and C-G*C' triads and two mismatched triads (Fig. 6A). The Py strand would presumably fold into a structure with a 4-nucleotide single-stranded loop. Another possible folding scheme in the 35-bp PyPu tract shows that the Py strand might be aligned such that the H-y3 intramolecular triplex would contain 10 perfect triads and three mismatched triads with a 3-nucleotide loop (Fig. 6B). This folding scheme includes a part of pyrimidine sequence beyond the mirror repeat. The chloroacetaldehyde reactivity of adenosines at the 3' side of the Pu sequence (Fig. 4A, lanes 3 and 4) and KMnO4 reactivity at the 5' side of the Pu sequence (Fig. 5C, lane 4) indicate the presence of the latter triplex structure at intermediate superhelical densities. However, having three mismatched triads such a structure would not be very stable unless there were an additional stabilizing contribution. It is possible that some of the mismatched triads are less triplex-stabilizing than others. For example, the C-G*T triad was the least destabilizing of the mismatches in some intermolecular triplexes (48–50). It was also less destabilizing than other triads in H-DNA (51). The reason for an intermediate stability of this triad is that G and T may form a single hydrogen-bonded Hoogsteen pair (Fig. 6C). Possible partial hydrogen bonding in the C-G*T triad is indicated with a colon in Fig. 6B.

In DNA of native superhelical density (x = --0.055), an H-y5 intramolecular triplex isomer (Fig. 3C) forms within the entire 35-bp PyPu sequence, not just the 26-bp region containing mirror symmetry. In addition, the local DNA unwinding present in the intramolecular triplex expands into the abutting TATA box. Analysis of folding schemes for the H-y5 triplex isomer shows a possibility of forming at least five structures with comparable numbers of perfect base triads, mismatched base triads, and unpaired bases at the tip of triple helix. Our
experimental data are best explained by the model shown in Fig. 3C. The absence of strong chloroacetalddehyde reactivity of cytosines in a suggested Py loop is tentatively explained by their participation in a protonated C+C+ hairpin (44, 45) at pH 4.2, which is close to the pK_a value for protonation of the cytosines. The possibility of forming C-G+T triads is supported by the absence of KMnO_4 reactivity of thymines in that part of Py sequence which serves as a third strand in the suggested triple helix.

At native superhelical density, the H-y3 and H-r5 isomers are usually preferred in perfect mirror-repeated PyPu tracts (47). However, a number of factors such as deviation from mirror symmetry and variation in the loop sequence may favor the formation of other isomers (52–55). The supercoil dependence for the formation of non-B-DNA structure shows that at the superhelical density sufficient to locally unwind DNA, an H-y3 isomer of intermolecular triplex likely forms. At higher superhelical tension the H-y5 triplex/open TATA box combination is predominant. The latter structure might be more favorable since it relaxes more supercoils. The TATA box probably opens because the double helix in the TATA box is relatively unstable, and unwinding relaxes additional DNA supercoils. In addition, the formation of H-DNA provides an unwound region adjacent to the TATA box, which further destabilizes the duplex and thus facilitates the opening of the TATA box.

Possible Biological Implications for Non-B-DNA Structures in the Promoter of the Human Na,K-ATPase α2 Gene—Na,K-ATPase is an integral membrane protein found in the cells of higher eukaryotes. It is responsible for the movement of sodium and potassium ions across the cell membrane using ATP as an energy source (56). The protein is composed of two subunits: an α subunit with a molecular mass of 113 kDa, and a glycosylated β subunit in which the protein portion accounts for 35 kDa of the total molecular mass of 55 kDa. A characteristic feature of the Na,K-ATPase is the multiplicity of α and β isoforms that are expressed in a tissue- and development-specific manner. A comparison of the 5'-flanking regions of the three isoforms of α genes of human Na,K-ATPase (36, 57, 58) shows differences in potential transcription factor binding sites which may be important in mediating the tissue- and developmental stage-specific expression of the three genes. The presence of the relatively long PyPu tract in the 5'-flanking region of the α2 gene and its ability to adopt an unusual structure comprised of the H-DNA and an unwound TATA box sequence might be also relevant to the specific expression of this gene. The details of regulation of the gene expression by either transcription factors capable of binding to multiple sites, or local unwound DNA structure are not known. Yet, some suggestions can be made about a biological significance of the above described non-B-DNA structure.

The formation of a functional preinitiation complex on eukaryotic promoters requires ordered interaction of general initiation factors in addition to RNA polymerase II (59). Recognition and binding of a TATA box-binding protein is the first step in the formation of preinitiation complex. Although TATA box-binding protein significantly distorts a bound DNA site by bending and partly unwinding it, a primary recognition process may require a regular double-helical DNA. Formation of the H-DNA in the promoter of the Na,K-ATPase gene is accompanied by unwinding of double helix in the adjacent TATA box. In such a way this site could be made incompatible with regulatory protein binding. Normal interactions between the proteins in transcription complex would be disturbed and, as a consequence, transcription would be down-regulated.

The double-stranded PyPu tract might be suitable for binding a duplex DNA-recognizing transcription factor with specificity to oligo(G) sequences (60, 61). The formation of H-DNA would make this site inappropriate for specific interaction with the protein that facilitates RNA polymerase binding. As a result, transcription would be inhibited. The promoter sequence of the human Na,K-ATPase α2 gene contains several sites that resemble those for known transcription factors (Sp1, nuclear factor-1, CACCC factors, etc.). However, none of these sites overlaps the PyPu tract, and at the present state of knowledge, a direct interference of H-DNA in the sequence studied with transcription factors is unlikely.

In another option, the strand unwinding afforded by the formation of the H-y5 structure might stimulate transcription by promoting the assembly of RNA polymerase and associated transcription factors on DNA. DNA supercoiling can alleviate the need for certain transcription factors in reconstituted transcription reactions (62). In the absence of transcription factors that may promote strand unwinding, the latter might be facilitated by using energy from DNA supercoiling. In the case of Na,K-ATPase α2 gene, increasing DNA supercoiling locally unwinds the double helix and results in the formation of the H-y5/open TATA box structure, which may serve as an RNA polymerase entry point (63). In the triplex of H-y type the Py strand comprises the long single strand. However, in the sequence studied the Py strand is the template strand, and although the long Py single strand may promote RNA polymerase binding to the region, ultimately RNA polymerase would need to bind to the Py strand for transcription to occur.

Thus, of the three options considered above, only one in which transcription might be down-regulated when normal interactions of TATA box-binding protein with the TATA box sequence are disrupted by the formation of a combined H-DNA/open TATA box structure seems possible. The described combined structure might participate in transcriptional regulation provided that there are stabilizing factors that may promote its formation in vivo (for a discussion of triplex-stabilizing factors in the cell, see Refs. 38 and 39).

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