Induction of Duplex to G-quadruplex Transition in the c-myc Promoter Region by a Small Molecule*

Received for publication, July 7, 2000, and in revised form, October 11, 2000
Published, JBC Papers in Press, October 16, 2000, DOI 10.1074/jbc.M005962200

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A major control element of the human c-myc oncogene is the nuclease-hypersensitive purine/pyrimidine-rich sequence. This double-stranded DNA fragment, corresponding to the 27-base pair segment in the nuclease-hypersensitive element of the c-myc promoter region, forms a stable Watson-Crick double helix under physiological conditions. However, this duplex DNA can be effectively converted to G-quadruplex DNA by a small molecular weight ligand. Both intermolecular and intramolecular G-quadruplex forms can be induced by this ligand. Similar transitional changes are also observed with the duplex telomeric sequence from the Oxytricha species. These results provide additional support to the idea that G-quadruplex structures may play structural roles in vivo and also provide insight into novel methodologies for rational drug design. These structurally altered DNA elements might serve as regulatory signals in gene expression or in telomere dynamics and hence are promising targets for drug action.

The protein product of the c-myc protooncogene plays a vital role in the process of cellular growth and differentiation (1). Deregulation of c-myc expression has been detected in many cancers and is believed to be an important step in tumorigenesis (2). The control of c-myc gene expression is a complex process and occurs at various steps of transcription, such as initiation, elongation, and attenuation, as well as during the post-transcriptional stages. Although the mechanisms involved in this regulation are not yet completely understood, a major control element of the human c-myc oncogene has been localized. This is a purine/pyrimidine-rich region located 115 bases upstream from the P1 promoter, which controls up to 95% of the total c-myc transcription (3, 4). This DNA segment is highly sensitive to DNase I and S1 nuclease (5, 6) and is termed the nuclease-hypersensitive element (NHE).1 The appearance of this hypersensitive site is coupled with transcription activation of the c-myc oncogene. Structural variations in the NHE can influence the binding of transcription factors. Transcription factors such as heterogenous nuclear ribonucleoprotein K and nucleoside diphosphate kinase B (7) bind sequence specifically to the pyrimidine-rich strand of the NHE and activate c-myc transcription (8). The transacting factors heterogenous nuclear ribonucleoprotein A/B (9) and cellular nucleic acid-binding protein (10) bind to the NHE and are shown to augment c-myc expression in vitro. Apart from protein factors, antisense oligonucleotides bind to the NHE and repress c-myc transcription in vitro (11, 12). Formation of a colinear triplex between the synthetic oligonucleotide and the NHE was proposed to cause this observed repression in transcription.

The NHE has a high potential to form atypical DNA structures under superhelical stress. It was proposed to be in a slow equilibrium between a Watson-Crick base-paired double helix and an atypical DNA structure (6). Many models have been suggested to explain the conformational changes observed in the NHE. The marked disparity in the nucleotide composition of the two strands of the NHE prompted suggestion of an H-DNA structure as a model for the noncanonical NHE structure (13). This H-DNA structure is an intramolecular pyrimidine-purine-pyrimidine triplex. Alternatively, a purine-pyrimidine-purine triplex has also been proposed as an explanation for the observed nuclease hypersensitivity (11). Both of these structures require nonphysiological conditions to be stable, either low pH for the pyrimidine triplex or very high magnesium concentration for the purine triplex, and are therefore highly unlikely to form in vivo.

Recently the NHE fragment of DNA has been shown to adopt an intrastraand fold-back DNA tetraplex under physiological conditions (14). According to the proposed model, an interconversion of the NHE between a normal B-DNA conformation and a very stable atypical G-quadruplex DNA conformation can recruit transcription factors and activate c-myc transcription. Involvement of G-quadruplex structures in the regulation of c-myc transcription opens up an interesting area for the design of small molecules that can selectively interact with the G-quadruplex structure.

A number of G-quadruplex-interactive agents, exemplified by porphyrins (15), anthraquinones (16), perylenes (17), and carbocyanines (18), have been developed and are shown to promote and/or stabilize these secondary DNA structures. N,N'-Bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER) is a small molecule from the class of perylene compounds that is well characterized for G-quadruplex interactions (17). For example, it is shown to act as a “driver” in accelerating the assembly of G-quadruplex structures from single-stranded DNA (19). In this respect, the role of PIPER is very analogous to the β-subunit of the Oxytricha telomere-binding protein, altering the dimerization kinetics from second to first order. This implies that PIPER may elicit biological effects by promoting G-quadruplex structures analogous to the chaperone proteins. In addition, once the G-quadruplex structure is formed, PIPER prevents G-quadruplex unwinding by Sgs1, a G-quadruplex-specific helicase (20).

* This work was supported by National Institutes of Health Grant CA49751. 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: NHE, nuclease-hypersensitive element; PIPER, N,N'-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide; DMS, dimethyl sulfate.
In this study we demonstrate that a small molecule such as PIPER can facilitate the formation of G-quadruplex structures not only from G-rich single-stranded DNA but also from the Watson-Crick base-paired G-rich duplex DNA. This is the first evidence that a small ligand can cause the transition from Watson-Crick base-paired duplex DNA to G-quadruplex DNA under physiological conditions.

**EXPERIMENTAL PROCEDURES**

Poly nucleotide and PIPER Preparation—The 27- and 24-base pair duplex DNA oligomers (for gel electrophoresis experiments) NHE-27, mutated NHE-27, OT-24 (OT = *Oxytricha* telomeric), mutated OT-24, and HT-24 (HT = human telomeric), shown in Scheme I, were synthesized on a DNA synthesizer (PerSeptive Biosystems Expedite 8909) and purified using 20% denaturing polyacrylamide gel electrophoresis. About 400 ng of DNA was 5'-end-labeled with 32P using T4 polynucleotide kinase (New England Biolabs) and subsequently annealed with the complementary strand. The duplex was purified using 20% native polyacrylamide gel electrophoresis, diluted to 40 μM, and dispensed into small aliquots. The duplex DNAs for NMR experiments, d(TAGGGTT-A-d(TAACCCTA) and d(TTGGGTT-T-GGTTGGG), were synthesized on a DNA synthesizer, purified by reverse phase high performance liquid chromatography on a C18 column (Dynamax 300A), and dialyzed extensively. Solid supports and phosphoramidites were purchased from Glen Research and PerSeptive Biosystems. PIPER (Scheme ID) was synthesized and purified as described previously (17). A 1 mM stock solution of PIPER prepared in distilled water was stored at −20 °C and diluted to required concentrations immediately before use.

**Electrophoretic Mobility Shift Assays—**32P-end-labeled duplex DNA at a concentration of 4 μM was heated to 95 °C for 10 min in a 1 × KCl/TE buffer (10 mM Tris HCl, 1 mM EDTA, 100 mM KCl, pH 8.0). After the DNA was cooled to room temperature, 2 μl of stock PIPER was dispensed to each sample to obtain the concentrations specified in the figures at a total volume of 20 μl. For the concentration-dependent experiments, the incubation time was 10 h at either 28 °C or 55 °C. For the time-course experiments, the samples were taken at the times specified in the figures and the incubation was carried out at either 28 °C or 55 °C. The incubation was terminated by the addition of 5 μl of gel loading buffer (30% glycerol, 0.1% bromphenol blue, and 0.1% xylenol cyanol). Eight microliters of each sample was then loaded onto a 16% native polyacrylamide gel that was prerun for 30 min. The samples were electrophoresed for 15 h at 4 °C in a 1 × TBE/KCl running buffer (90 mM Tris borate, 90 mM boric acid, 2 mM EDTA, pH 8.3, and 100 mM KCl). Gels were dried, exposed to a phosphor screen, and visualized on a PhosphorImager (Molecular Dynamics model 445SI).

**NMR Spectroscopy—**NMR experiments were performed on a Varian UNITY Plus 500-MHz spectrometer. All titration experiments were carried out in a 90% H2O, 10% D2O solution containing 100 mM KCl, 25 mM KH2PO4, and 1 mM EDTA (pH 7.0). A standard 1–1 echo pulse sequence with a maximum excitation centered at 12.0 ppm was used for water suppression.

**RESULTS**

The role of PIPER in inducing the formation of G-quadruplex structures from Watson-Crick base-paired duplex DNA was examined using electrophoretic mobility shift assays and NMR studies. The double-stranded DNA fragment corresponding to the 27-base pair segment in the NHE of the c-myc promoter region was used in the first part of this study (see Scheme I). The purine-rich strand of this double-stranded fragment has the ability to form at least two types of G-quadruplex DNA, namely, the tetramolecular G-quadruplex and the intramolecular foldover G-quadruplex (Scheme II). To provide additional insights into dynamic requirements for PIPER-driven conversion of Watson-Crick base-paired duplex to G-quadruplex, two more duplexes, namely, the ciliate telomeric sequence from the *Oxytricha* species and the human telomeric sequence, were compared for their propensity to form G-quadruplex DNA in the presence of PIPER.

All the experiments were carried out at 100 mM concentrations of potassium. Experiments carried out in the absence of potassium showed patterns similar to those seen in the presence of potassium; however, the intensity of the G-quadruplex bands was weaker in the absence of potassium.

**Incubation of the 27-mer NHE Duplex with PIPER Results in Conversion to G-quadruplex Structures—**Watson-Crick base-paired duplex DNA was incubated with increasing concentrations of PIPER at 55 °C. In this experiment the 5'-end of the G-rich strand of the duplex DNA was radiolabeled. As the concentration of PIPER was increased to 32 μM, there was increased formation of a slow migrating species, indicative of a higher order structure (corresponding to T in Fig. 1A). It is significant that the intensity of only a single band is increased, indicating that PIPER may be promoting a specific type of structure, which may be a G-quadruplex. The diffuse bands which occur in the free DNA labeled *isomeric forms* in Fig. 1A are most likely to be tetramolecular G-quadruplex structures in which there is misalignment of one or more of the G-rich strands. Parallel experiments in which the 5'-end of the C-rich strand of the Watson-Crick base-paired duplex DNA was radiolabeled were performed under identical conditions. The intensity of the band corresponding to single-stranded C-rich DNA (SS in Fig. 1B) increased at elevated concentrations of PIPER. Therefore, a higher order structure is formed by the...
G-rich strand, leaving an excess of single-stranded C-rich DNA. Previous studies with G-rich single-stranded DNA indicated that PIPER binds tightly and specifically to G-quadruplexes and acts as a driver in the assembly of G-quadruplex structures (19). Hence, the structure formed by the G-rich strand in our experiments is most likely to be a G-quadruplex structure, which is consistent with the results from the DMS chemical footprinting experiments.2

Besides the tetramolecular G-quadruplex, PIPER induces the formation of an intramolecular foldover G-quadruplex structure. This was deduced from the experiments carried out at 28 °C using the Watson-Crick base-paired duplex. The G-rich strand of NHE-27 was radiolabeled (Fig. 1, C and D). These experiments exhibited the formation of an intramolecular G-quadruplex (T in Fig. 1C) and another species with a higher mobility (M in Fig. 1C). In accordance with the literature (21), the compact intramolecular G-quadruplex has faster mobility than either the double-stranded DNA or the nonstructured single-stranded DNA. Results from the DMS footprinting experiments were consistent with the idea that the M band is a G-quadruplex structure.2

That the intramolecular G-quadruplex structure was not observed in the experiments carried out at 55 °C suggests that the intramolecular G-quadruplex is less stable than the tetramolecular G-quadruplex.

A Mutant 27-mer NHE Duplex That Is Incapable of Adopting G-quadruplex Structures Does Not Form Higher Order Structures in the Presence of PIPER—The specificity of PIPER to unwind G-rich Watson-Crick base-paired duplexes was determined by modifying the NHE-27 duplex used in the previous experiments. It was modified such that neither strand had runs of two or more consecutive guanines and was thus incapable of forming G-quadruplex structures. Incubation of this mutated NHE-27 Watson-Crick base-paired duplex (Scheme IA) under conditions similar to those of the nonmutant NHE-27 neither affected the duplex bands nor indicated the appearance of new faster or slower migrating species (Fig. 2, A and B). This demonstrates that PIPER may be specific in promoting the formation of a G-quadruplex DNA structure from the Watson-Crick base-paired G-rich duplexes.

PIPER Facilitates the Formation of G-quadruplex Structures and Interconversion between Different G-quadruplex Structures from the 27-mer NHE Duplex DNA—PIPER has been shown to play a driver role in the formation of G-quadruplex structures from single-stranded DNA (19). It promotes the formation of both dimeric and tetrameric G-quadruplexes from DNA oligomers containing two repeats of telomeric DNA from the Oxytricha species, while converting the reaction kinetics of oligomer dimerization from second to first order (19). To investigate the ability of PIPER to induce interconversion between different types of G-quadruplex structures in the presence of a complementary DNA strand, the 27-mer NHE Watson-Crick base-paired duplex DNA was incubated with 8 μM PIPER at 28 °C for 32 h. At incubation times of up to 12 h, the tetramolecular G-quadruplex is the predominant structure; however, at times greater than 24 h, the intramolecular foldover G-quadruplex becomes the predominant structure (Fig. 3B).

Piper-mediated Interconversion of Tetramolecular G-quadruplex to the Intramolecular G-quadruplex Structure Does Not Occur in the Absence of the Complementary C-rich Strand—The involvement of a G-rich single strand as an intermediate in the PIPER-mediated interconversion was evaluated. The design of this experiment was analogous to that for Fig. 3 (A and B), except that single-stranded G-rich DNA was substituted for Watson-Crick base-paired duplex DNA. The G-rich single strand of NHE-27 was incubated in the absence (Fig. 3C) and in the presence (Fig. 3D) of PIPER for 32 h at 28 °C. As expected, the results show that the presence of 8 μM PIPER does facilitate the formation of G-quadruplex structures. However, somewhat surprisingly, the tetramolecular G-quadruplex was the only structure formed over the 32-h period, suggesting that the presence of a complementary strand affects the overall transi-
Duplex DNA (Watson-Crick duplex; M) 100 mM KCl/TE buffer for the specified times.

A single-stranded DNA (G-rich 27-mer NHE over extended periods of time is shown in Fig. 3). The G-quadruplex formed from the single-stranded G-rich genomic regions apart from the 27-mer NHE duplex was also investigated. The intrinsic ability of PIPER to drive the conversion of human and Oxytricha Watson-Crick base-paired duplexes to G-quadruplex was evaluated.

A graphical representation comparing the formation of different G-quadruplex structures from the Watson-Crick base-paired imino protons involved in the G-quadruplex predominate at the 1:1 DNA:PIPER concentrations. It is important to note that the Hoogsteen base-paired imino protons are shifted upfield, indicating the probable interaction of PIPER with the G-quadruplex.

Ciliate Telomeric Duplex Sequences Can Also Be Effectively Converted to G-quadruplex Structures in the Presence of PIPER—The Watson-Crick base-paired duplex DNA OT-24 (Scheme IB), containing one repeat of the Oxytricha telomeric sequence, was incubated with increasing concentrations of PIPER at 55 °C. Experiments were carried out in which either the 5'-end of the G-rich strand (Fig. 4A) or the 5'-end of the C-rich strand (Fig. 4B) was radiolabeled. As the concentration of PIPER increased, the formation of a slow mobility species corresponding to the tetramolecular G-quadruplex structure (T in Fig. 4A) was observed. However, there was no effect on a mutated OT-24 (Fig. 4C), which did not contain two or more runs of guanines.

Parallel 1H NMR titration experiments were carried out using the Watson-Crick base-paired duplex sequence d(TTGGGGTT)-d(AACCCCAA) (containing one repeat of the Oxytricha telomeric sequence) by titrating the duplex against PIPER. The one-dimensional spectrum of the Watson-Crick base-paired duplex is shown in Fig. 5A. Inspection of the imino proton region (10–14 ppm) indicates the presence of equilibrium between the Watson-Crick base-paired duplex and G-quadruplex structures. The Watson-Crick base-paired imino protons are observed between 12.5 and 13.2 ppm, and the imino protons involved in the G-quadruplex Hoogsteen base pairing are observed in the region of 10.6 to 11.6 ppm. Upon titration with increasing concentrations of PIPER (Fig. 5B), the signals from the Watson-Crick base-paired imino protons are reduced and imino protons involved in the G-quadruplex predominate at the 1:1 DNA:PIPER concentrations. It is important to note that the Hoogsteen base-paired imino protons are shifted upfield, indicating the probable interaction of PIPER with the G-quadruplex.

Human Telomeric Duplex DNA Cannot Be Converted to G-quadruplex, Even in the Presence of PIPER—The Watson-Crick base-paired human telomeric duplex DNA d(TAGGGTTA)-d(AACCCCAA) (containing one human telomeric repeat) was titrated against PIPER under conditions similar to those of the previous NMR experiment. The one-dimensional NMR spectra are shown in Fig. 6A. Unlike the Oxytricha telomeric sequence, the human telomeric duplex was predominantly in Watson-Crick base-paired duplex form. These Watson-Crick base-paired duplex DNA resonances were unaffected upon titration.
Duplex to G-quadruplex Transition

Fig. 5. A, one-dimensional NMR spectrum of the free Watson-Crick duplex containing the *Oxytricha* telomeric sequence showing the imino protons for Watson-Crick base pairing (12–14 ppm) and for Hoogsteen base pairing (10–12 ppm). B, titration of the *Oxytricha* telomeric sequence d(TTGGGTT)-d(AACCCCAA) with PIPER.

Fig. 6. A, one-dimensional NMR spectra showing the imino proton region of the free DNA and the 1:1 DNA:PIPER sample of the human telomeric duplex d(TAGGGTTA)-d(TAACCCTA). B, effect of increasing PIPER concentration on HT-24 duplex DNA. The G-rich strand of HT-24 was end-labeled and incubated at 55°C with increasing amounts of PIPER. One band (DS) was observed (* indicates the radiolabeled end).

with PIPER, even at a 1:1 DNA:PIPER concentration. This suggests that the human telomeric G-quadruplex may be less stable than the Watson-Crick base-paired duplex and hence is not observed. Additionally, since the 1H NMR resonance signals of the Watson-Crick base-paired imino protons are unchanged, this indicates the high selectivity of PIPER for its interaction with G-quadruplex versus Watson-Crick base-paired duplex DNA. Electrophoretic mobility shift assays carried out with the four-repeat sequence of the human telomere HT-24 (see Scheme IC) also indicated that there were no transitional changes with increasing concentrations of PIPER (Fig. 6B), a result that is consistent with the 1H NMR data in Fig. 6A.

**DISCUSSION**

Structural heterogeneity in DNA is evidenced by its capability to adopt several types of conformation (22). These include cruciform DNA, Z-DNA, triplex DNA, and G-quadruplex DNA, apart from the usual B-DNA. Repetitive sequences in the telomeric and promoter regions, as well as in the coding regions of the eukaryotic genome, have a high potential to form unusual structures (23). Although the biological significance of these unorthodox structures is not yet completely understood, they are often associated with functional roles in transcription, gene regulation (24), and tumor-associated genetic changes (25). These alternative structures may be stabilized in vivo by the presence of a specific protein or a small peptide. The promoter regions are often guanine-rich on one strand and exhibit nuclease hypersensitivity, emphasizing the dynamic nature of the DNA structure (26).

For this study, we chose the NHE fragment of the c-myc promoter on the basis of the observation that NHE can be in a conformational equilibrium between two states, a nuclease-sensitive state and a nuclease-insensitive state. Moreover, it was proposed that in vivo endogenous RNA molecules could bind to the regulatory region of c-myc and affect this conformational equilibrium (6). This prompted the idea that factors stabilizing or destabilizing the SI nuclease-sensitive conformer of NHE might have an effect on the expression of the c-myc oncogene. Indeed, switching of DNA secondary structures is shown to affect the transcription response of many genes (24, 27, 28).

The characterization of NHE under physiological conditions showed that it could adopt a G-quadruplex structure (14). It was observed that potassium ions stabilized the G-quadruplex structure. In isolation, the formation of G-quadruplex structures is a slow process. In fact, the inability of plasmid NHE, but not the single-stranded NHE (G-rich), to form G-quadruplex structures was attributed to the slow kinetics of G-quadruplex formation as well as to the competitive binding of the complementary strand (14). In this study we have demonstrated that PIPER is able to facilitate the formation of two secondary structures from Watson-Crick base-paired duplex DNA (Scheme III). On the basis of the electrophoretic mobility, sequence variation, and DMS footprinting results, the lower mobility species is assigned as a tetramolecular G-quadruplex, and the structure with higher mobility is more likely to be a foldover G-quadruplex. Although the tetramolecular G-quadruplex is the kinetically favored species (Fig. 3B), the foldover G-quadruplex structure is thermodynamically favored. As a possible explanation for this observation, we propose that early on in the incubation there is sufficient PIPER to bind to and stabilize the tetramolecular structure (III in Scheme III), but as the concentration of this species increases, there is insufficient PIPER to bind to and stabilize this species. The tetramolecular species then dissociates, releasing ligand-free single-stranded DNA, which can fold into the thermodynamically stable foldover structure (upper pathway in Scheme III). Significantly, only the tetramolecular G-quadruplex structure is formed from the single-stranded DNA in the presence of excess PIPER. This is because less PIPER is bound up in the disordered single-stranded DNA-PIPER conjugates (lower pathway in Scheme III) and is therefore available to stabilize the tetramolecular structure, which favors an equilibrium in which little of the free tetramolecular G-quadruplex is found. Consequently, little, if any, of the foldover structure is formed. Alternatively, it can be speculated, based on this model, that at low concentrations of DNA the parallel species will be the predominant structure, while at higher DNA concentrations at longer periods of time, the intramolecular foldover structure will predominate. This model also identifies a mechanism for the facilitation of G-quadruplex formation. Based on this model, ordered PIPER molecules stack in between parallel duplex molecules to facilitate formation of G-G parallel duplex molecules, which dimerize to form the G-quadruplex structures with dissociation of the ordered stacked PIPER molecules (I → II → III in Scheme III).

The induction of duplex to G-quadruplex by PIPER cannot be generalized to all G-rich-containing duplex sequences capable of forming G-quadruplexes, as indicated by the experiments...
using telomeric DNA. The ciliate telomeric duplex DNA is in a state of equilibrium between the duplex and G-quadruplex structure (Figs. 4 and 5), and PIPER can cause a transition from the duplex to G-quadruplex. However, the human telomeric duplex DNA (Fig. 6) is present predominantly in the Watson-Crick base-paired duplex form. Thus, it appears that the human telomeric G-quadruplex may be less stable than the duplex and PIPER cannot effectively induce the transition of the duplex to a G-quadruplex structure. This indicates that the inherent ability of certain duplexes to be in equilibrium with a G-quadruplex may be a requirement for PIPER to cause a transition from a duplex to a G-quadruplex structure.

The role of PIPER in promoting and stabilizing G-quadruplex structures is analogous to the chaperone action observed with the β-subunit of the Oxytricha telomere binding protein (29). Hence it is conceivable that PIPER can mimic the activity of a G-quadruplex binding protein in vivo. PIPER, therefore, can be used to probe for regions in the genome that have a potential to be in equilibrium between normal B-DNA and G-quadruplex DNA. Such regions include the immunoglobulin switch region, triplet repeats of the fragile X syndrome, and the G-quadruplex DNA. Such regions may include the telomeric G-quadruplex at the end of G-rich DNA in equilibrium between normal B-DNA and G-quadruplex DNA. Hence it is significant promise for clinical success, but the conversion of this technology to therapeutic agents still remains elusive. In this study we achieved the specific recognition and folding of a 27-base pair sequence by a small molecule. In contrast, if we relied on the direct duplex sequence recognition approach, a molecule the size of PIPER could possibly recognize and interact with a trinucleotide sequence at most.

Studies are in progress to evaluate the effect of G-quadruplex driver molecules on in vitro transcription of the c-myc oncogene. These studies will provide insight into the possible roles of G-quadruplex structures in c-myc transcription regulation.

**ACKNOWLEDGMENTS**—We thank Dr. Brent Iversen (University of Texas, Austin, TX) for important insights into the effects of limiting concentrations of PIPER on G-quadruplex transitions and for the development of the model for conversion of Watson-Crick base-paired duplex to G-quadruplex by PIPER. We are grateful to Dr. David Bishop for proofreading, editing, and preparing the final version of the manuscript and figures.

**Note Added in Proof**—Recently, Simonson et al. (33) have shown that the C-rich strand of the c-myc NHE can form an intramolecular i-motif structure. We have also recently demonstrated that G-quadruplex-interactive compounds such as TMPyP4 can also facilitate the formation of i-motif DNA and bind to the resulting structure by a nonintercalative mechanism (34). This provides an additional mechanism for intervention with small molecules in the conversion of c-myc NHE between duplex and alternative secondary structures.

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