The c-*MYC* NHE III_1: Function and Regulation

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Key Words

G-quadruplex, nucleolin, CNBP, hnRNP K, superhelicity, NM23-H2

Abstract

c-MYC is an important regulator of a wide array of cellular processes necessary for normal cell growth and differentiation, and its dysregulation is one of the hallmarks of many cancers. Consequently, understanding c-*MYC* transcriptional activation is critical for understanding developmental and cancer biology, as well as for the development of new anticancer drugs. The nuclease hypersensitive element (NHE) III_1 region of the c-*MYC* promoter has been shown to be particularly important in regulating c-*MYC* expression. Specifically, the formation of a G-quadruplex structure appears to promote repression of c-*MYC* transcription. This review focuses on what is known about the formation of a G-quadruplex in the NHE III_1 region of the c-*MYC* promoter, as well as on those factors that are known to modulate its formation. Last, we discuss the development of small molecules that stabilize or induce the formation of G-quadruplex structures and could potentially be used as anticancer agents.

INTRODUCTION

Proto-oncogene: a normal gene that can acquire mutations or dysregulated expression to become an oncogene, which can then contribute to the development of cancer

B-form DNA: a right-handed double-helical conformation of DNA

Understanding the role of c-MYC has been a critical issue in cancer biology since the discovery of the human homolog of *v-gag-myc* in 1982 (1). Aberrant c-*MYC* expression is a common feature in a number of human malignancies, including breast, colon, cervix, small-cell lung cancers, osteosarcomas, glioblastomas, and myeloid leukemias (2–4). It has been estimated that as many as one-seventh of all cancer deaths (70,000 deaths annually in the United States) are associated with alterations in the c-*MYC* gene or its expression (5).

Over 19,000 papers have been published about MYC (6). In the pursuit to understand this complex proto-oncogene, we now appreciate that it plays critical roles in diverse cellular processes. Describing the varied pathways resulting in c-*MYC* expression has also become an important topic in cancer biology because it appears that changes in c-*MYC* expression underlie its propensity to promote tumorigenesis. This is in contrast to other commonly characterized oncogenes, such as the human RAS oncogene, where the primary mechanism of tumor promotion is through the acquisition of activating mutations. In this review, we examine the complex regulation of c-*MYC* expression through the formation of non–B-form DNA structures in the c-*MYC* promoter and how the formation of such structures presents an opportunity for the potential therapeutic modulation of c-*MYC* expression.

FUNCTIONS OF C-MYC

A number of comprehensive reviews of the overall properties of c-MYC are available (7–12); therefore, we give only a brief outline of c-MYC functions here. The c-*MYC* proto-oncogene encodes a multifunctional transcription factor that plays a critical role in a broad range of cellular processes, including the regulation of cell cycle progression, cell growth, differentiation, transformation, angiogenesis, and apoptosis (12, 13). c-MYC is able to activate a number of genes by forming heterodimeric complexes with other transcription factors such as MAX or MAD that interact with specific DNA sequences, such as the E-box sequence (14). For example, the c-MYC–MAX heterodimeric complex has been shown to promote cell proliferation by activating cyclins (cyclin D1, cyclin D2, cyclin E1, cyclin A2) and cyclin-dependent kinases (CDK4) that are required for cell-cycle progression (10), while repressing the transcription of cell-cycle checkpoint genes (GADD45 and GADD153) and inhibiting the function of cyclin-dependent kinase inhibitors (p15 and p21) (14–16).

However, just as c-MYC can induce proliferation, it can also stimulate cellular differentiation, depending on the level and duration of c-*MYC* expression and activation (6, 11, 17). Recently, it has been shown that even transient deactivation of c-*MYC* is sufficient to allow cells to escape the cell cycle and undergo differentiation (17), which illustrates the potential utility of even short-term downregulation of c-*MYC* in cancer therapy. Furthermore, it was recently shown that overexpression of c-*MYC*, along with three other transcription factors, is sufficient to de-differentiate mature fibroblasts into a more primitive state (18).

Similarly, changes in the quantity of c-*MYC* have been shown to play a critical role in apoptosis (19). For example, just as maintaining c-*MYC* expression results in resistance of the cell to undergo apoptosis, the reduction of c-*MYC* expression has been associated with the induction of apoptosis, as well as cell sensitization to a variety of apoptotic agents (20–22). In addition, in some systems, inappropriate expression or a change in the rate of c-*MYC* expression also appears to lead to programmed cell death (23, 24).

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DYSREGULATION OF C-*MYC* EXPRESSION

As noted above, the c-MYC protein plays a central role in a multitude of diverse biological processes including cell proliferation, differentiation, and apoptosis. Consequently, it is usually subject to tight transcriptional regulation. Dysregulation of c-*MYC* can arise through a variety of mechanisms, including chromosomal translocation (25), gene amplification (26), and increased transcription (27–30), as well as a higher rate of translation and enhanced protein stability (31–33). However, c-*MYC* is usually dysregulated indirectly through alterations in upstream cell signaling pathways that lead to an increase in its transcription (16).

Negative superhelicity: the force that results when right-handed DNA is underwound upstream of a transcribing RNA polymerase, facilitating nucleosome rearrangement

REGULATION OF C-*MYC* PROMOTER ACTIVITY

The mechanisms that govern c-*MYC* transcription are complex and involve multiple promoters (P_0, P_1, P_2, P_3) and start sites (**Figure 1**). In addition, the promoter region of c-*MYC* contains a number of *cis*-elements that have been shown to assume either a single-stranded or a non–B-DNA conformation under negative superhelicity, which is naturally generated behind RNA polymerase complexes during transcription (34–38). For example, the far upstream element (FUSE) that is located 1.7 Kb upstream of the c-*MYC* P_2 promoter has been reported to become single-stranded owing to negative superhelical forces that are generated during c-*MYC* transcription, but to remain in a double-stranded conformation if c-*MYC* is not being expressed (37–39). In other words, the FUSE functions as a physical sensor of ongoing transcriptional activity. In addition, this element is regulated by the FUSE-binding protein (FBP), which binds to the single-stranded FUSE to further activate and maintain c-*MYC* transcription, whereas the FBP-interacting repressor (FIR) binds to FBP and returns c-*MYC* transcription to basal levels (38, 39). Together, the FUSE-FBP-FIR system functions as a mechanosensor mechanism in which undulating superhelical stress controls the firing rate of the c-*MYC* promoter.

Furthermore, there are seven nuclease hypersensitive elements (NHEs) in the c-*MYC* promoter (**Figure 1**, top), and one of these, the NHE III_1 region, has been shown to have the ability to form non–B-DNA structures, whose formation is known to be facilitated by negative superhelical stress (40–45). This region is located –142 to –115 base pairs upstream of the P_1 promoter and has been shown to control up to 90% of the total c-*MYC* transcription (46, 47). NHE III_1 consists of a cytosine-rich (C-rich) coding strand and a guanine-rich (G-rich) noncoding strand

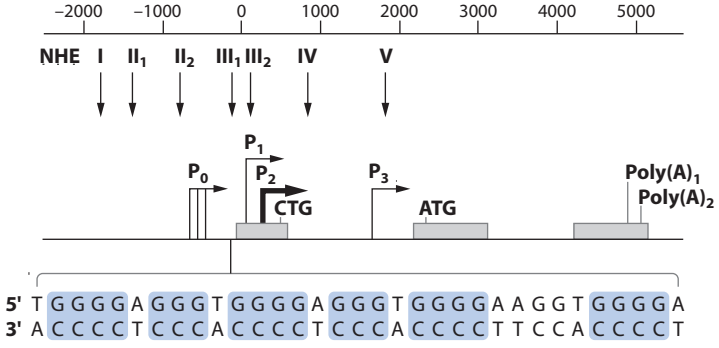

Figure 1

Promoter structure of the c-*MYC* gene and location of the NHE III_1 region. The inset shows the sequence of the NHE III_1. G-rich and C-rich tracts are shown in blue boxes. Arrows indicate the location of the different nuclease hypersensitive elements within the c-*MYC* promoter.

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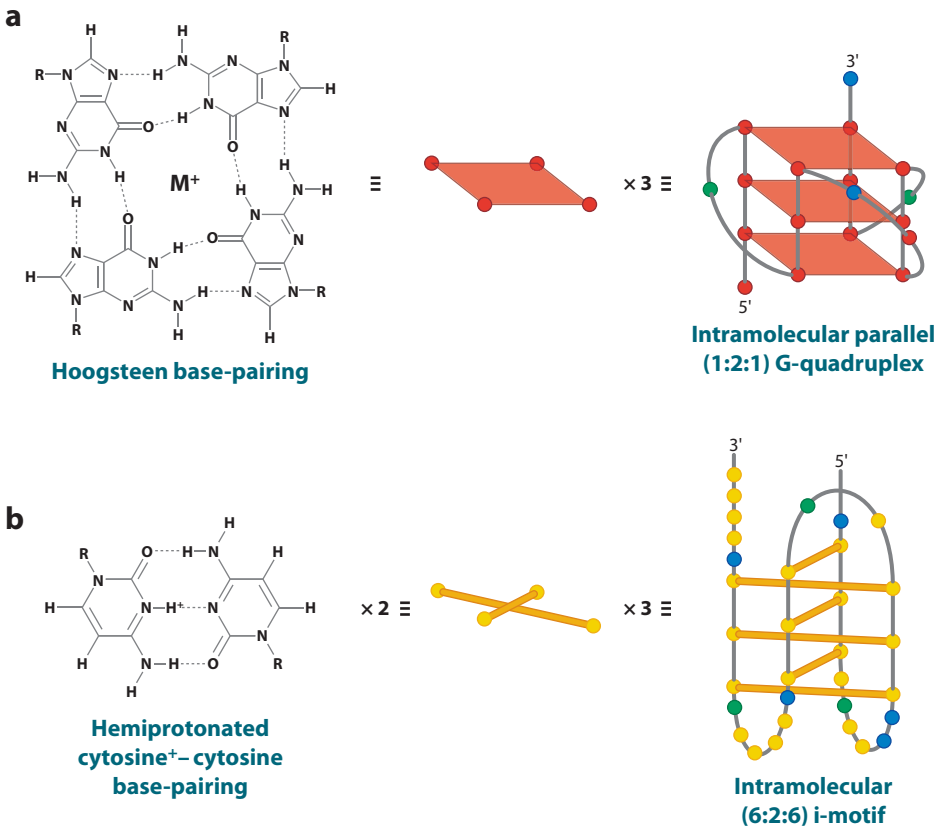

Figure 2

Structural representations of the non–B-DNA secondary structures formed in the c-*MYC* NHE III_1 region. (*a*) G-tetrad, the building block of the G-quadruplex structure, showing the guanine-guanine Hoogsteen base-pairings. (*b*) The hemiprotonated cytosine$^+$-cytosine base-pairing that leads to the formation of the i-motif structure.

G-quadruplex: four-stranded structure formed in guanine-rich DNA or RNA consisting of two or more stacked G-tetrads

i-motif: four-stranded DNA structure consisting of intercalated hemiprotonated cytosine$^+$-cytosine base pairs that are zipped together in an antiparallel orientation

that are capable of engaging in a slow equilibrium between B-form duplex DNA, single-stranded DNA, and tetra-stranded DNA. Specifically, the guanine tracts of the G-rich strand can form a G-quadruplex structure, whereas the cytosine tracts in the complementary C-rich strand can form an i-motif or i-tetraplex (**Figure 2**). An excellent review describing the organization and regulation of the c-*MYC* promoter has been published recently (48). In our review, therefore, we focus on describing the regulation of the c-*MYC* promoter by the NHE III_1 and provide new results that have appeared recently in the literature.

FORMATION OF A G-QUADRUPLEX WITHIN THE C-*MYC* NHE III_1

G-quadruplexes are four-stranded DNA structures consisting of two or more G-tetrads. A G-tetrad is made up of four hydrogen-bonded guanines in a planar arrangement (**Figure 2*a***). The formation of such structures is facilitated by the negative superhelical stress produced during transcription and stabilized by monovalent cations, such as K^+ and Na^+, that intercalate between the G-tetrads and coordinate bonds with the guanine carbonyl groups (49–52).

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Simonsson and colleagues (53) proposed the first intramolecular G-quadruplex structure within the c-*MYC* NHE III_1 region, which was described as an antiparallel-stranded structure involving three G-tetrads formed from four G-tracts linked by two lateral loops and a central diagonal loop. However, further examination by chemical footprinting, circular dichroic (CD), and nuclear magnetic resonance studies revealed that there is a single G-quadruplex isomer of parallel topology containing three lateral loops (a 1:2:1 loop isomer) in the c-*MYC* NHE III_1 region (reviewed in 54).

Although much of the structural work on G-quadruplexes has been done in vitro, evidence suggests that these structures exist in vivo (55–57). For example, the identification of antibodies and proteins that preferentially bind to, stabilize, unwind, or cleave G-quadruplexes provides evidence for their existence in vivo (for review, see 58). In addition, recent reports have demonstrated that putative G-quadruplex motifs are highly prevalent in human promoter regions, with as many as 40% of human gene promoters containing at least one of these elements (59–61). Potential G-quadruplex–containing promoters have been found to associate with nuclease hypersensitive sites, suggesting that the formation of these structures may be favored in sequences dynamically equilibrating between duplex and G-quadruplex chromatin conformations in vivo (60). In addition, the presence of G-quadruplex motifs has been shown to be correlated with gene function, because oncogenes have a disproportionately high incidence of G-quadruplex motifs in their promoters, whereas the promoters of tumor suppressors exhibit an extremely low potential for G-quadruplex formation (62). Most importantly, the topological diversity of these structures that arises from variations in strand directionality, loop length, and sequence, as well as the number of tetrad stacks, provides an opportunity for the rational development of molecules that can modulate the formation or stability of these structures to regulate gene expression.

G-tetrad (guanine tetrad)/G-quartet (guanine quartet): consists of four guanine bases in a coplanar arrangement in which each guanine shares four hydrogen bonds with two other guanines. A G-quartet is the building block of all G-quadruplex structures

FORMATION OF AN I-MOTIF WITHIN THE C-*MYC* NHE III_1

The C-rich strand of the c-*MYC* NHE III_1 can form another non–B-DNA structure: the cytosine-intercalated tetraplex, also known as the i-motif (63). C-rich DNA strands can associate both inter- and intramolecularly to form i-motifs, whose building block is the hemiprotonated cytosine$^+$-cytosine base pair (**Figure 2*b***) (64). However, C-rich single-stranded DNA can form i-motif structures only under acidic conditions because the protonation of N3 is essential for the stability of the structure and enables the formation of three hydrogen bonds between the two cytosines (65).

Recently, our group performed plasmid Br_2 footprinting experiments on the c-*MYC* NHE III_1 C-rich region to elucidate the c-*MYC* i-motif folding pattern under negative superhelicity. From these experiments, we determined that in the presence of continuous negative superhelical forces, i-motif formation is possible under neutral pH conditions (52). Our results demonstrate that under negative superhelicity, one major form of the i-motif utilizes four tracts of three cytosines, thereby increasing the loop sizes (6:2:6 loop isomer) relative to those found under acidic conditions (**Figure 2*b***). This important result demonstrates that i-motifs, as well as G-quadruplexes, can form in promoter regions under conditions of transcriptionally induced negative superhelicity and may therefore displace transcriptional factors such as the cellular nucleic-acid-binding protein (CNBP) or heterogeneous ribonucleoprotein K (hnRNP K), which are known to bind to the single-stranded NHE III_1 to activate c-*MYC* transcription (66, 67).

REGULATION OF C-*MYC* EXPRESSION THROUGH THE NHE III_1

The discovery of the G-quadruplex and i-motif within the NHE III_1 region of the c-*MYC* promoter has led us and others to hypothesize about the role these structures play in the regulation of c-*MYC*.

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One model that has been proposed suggests the presence of three DNA structural populations within the NHE III_1: two that can cause activation and one that results in repression of c-*MYC* (67) (**Figure 3*a–c***). **Figure 3*a*** depicts the relative location of the NHE III_1 with respect to the FUSE and the P_1 and P_2 promoters in the duplex form of the c-*MYC* promoter in the absence of transcription factors. Activation of c-*MYC* expression from duplex B-form DNA can be induced through the interaction of the Sp1 transcription factor with the double-stranded NHE III_1, which contains several Sp1 binding sites (**Figure 3*b***) (68). The NHE III_1 is also capable of forming a denatured, or open, form that is involved in the activation of c-*MYC* transcription owing to the recognition and coregulation by two single-stranded binding proteins (**Figure 3*c***). Specifically, CNBP binds to the G-rich strand of the NHE III_1, whereas hnRNP K binds to the complementary C-rich strand (66, 67). In addition, it has been hypothesized that the induction of the G-quadruplex and complementary i-motif leads to the silencing of c-*MYC* expression (**Figure 3*d***). Consistent with this hypothesis, destabilization of the G-quadruplex by point mutations results in increased transcriptional activity of a luciferase reporter gene carrying the c-*MYC* promoter (69). Conversely, stabilization by G-quadruplex-interactive compounds reduces transcriptional activity (69).

TRANSCRIPTION FACTORS THAT MODULATE THE C-*MYC* NHE III_1

Previous studies showed that Sp1, Sp3, CNBP, NM23-H2, and hnRNP K bind to the NHE III_1 region of the c-*MYC* promoter (see references in each subsection below). Further characterization of these proteins and the identification of other proteins that bind to this region are likely to better define the role that various DNA structures have in c-*MYC* expression.

Sp1 and Sp3

Sp1 and Sp3 are transcription factors that are ubiquitously expressed in mammalian cells. They are involved in the activation or repression of a number of genes that are key to the regulation of cell growth and proliferation and are essential during embryogenesis (70). These two proteins are structurally similar. Their DNA-binding domain contains a combination of three conserved Cys2His2 zinc fingers, and they share more than 90% sequence homology. Accordingly, Sp1 and Sp3 bind with similar affinities to GC-rich promoter elements to regulate the expression of a number of target genes, including c-*MYC*. Although Sp1 and Sp3 share similar structures and binding sites, their regulatory functions are different and are dependent on the particular promoter and the cellular context (71, 72). In mammalian cells, Sp1 usually functions as a transactivator, whereas Sp3 behaves as a repressor or weak activator.

The c-*MYC* promoter contains five Sp1 binding sites, three of them upstream of the P_1 promoter and two others upstream of P_2. The binding sites at the NHE III_1 and the one located at –44 base-pairs from the P_1 promoter are high-affinity Sp1 binding sites, whereas the others display only low Sp1 affinity (73, 74). The c-*MYC* promoter is not occupied by Sp1 in quiescent cells that express low levels of c-*MYC*; however, induction of c-*MYC* transcription by serum stimulation results in binding of Sp1 to the c-*MYC* NHE III_1 region, suggesting that Sp1 is involved in the serum-induced activation of c-*MYC* transcription (75). Cotransfection experiments in mammalian and insect cells indicate that Sp1 transactivates the c-*MYC* promoter (**Figure 3*a***) (76), whereas Sp3 does not. In addition, enforced expression of Sp3 repressed Sp1-mediated activation of c-*MYC* (76). Interestingly, it has been reported that for promoters containing multiple Sp1 binding sites, such as the c-*MYC* promoter, Sp1 exerts its transcriptional synergism through direct protein-protein interaction, in which Sp1 forms higher-order complexes able to bind to multiple sites (77, 78).

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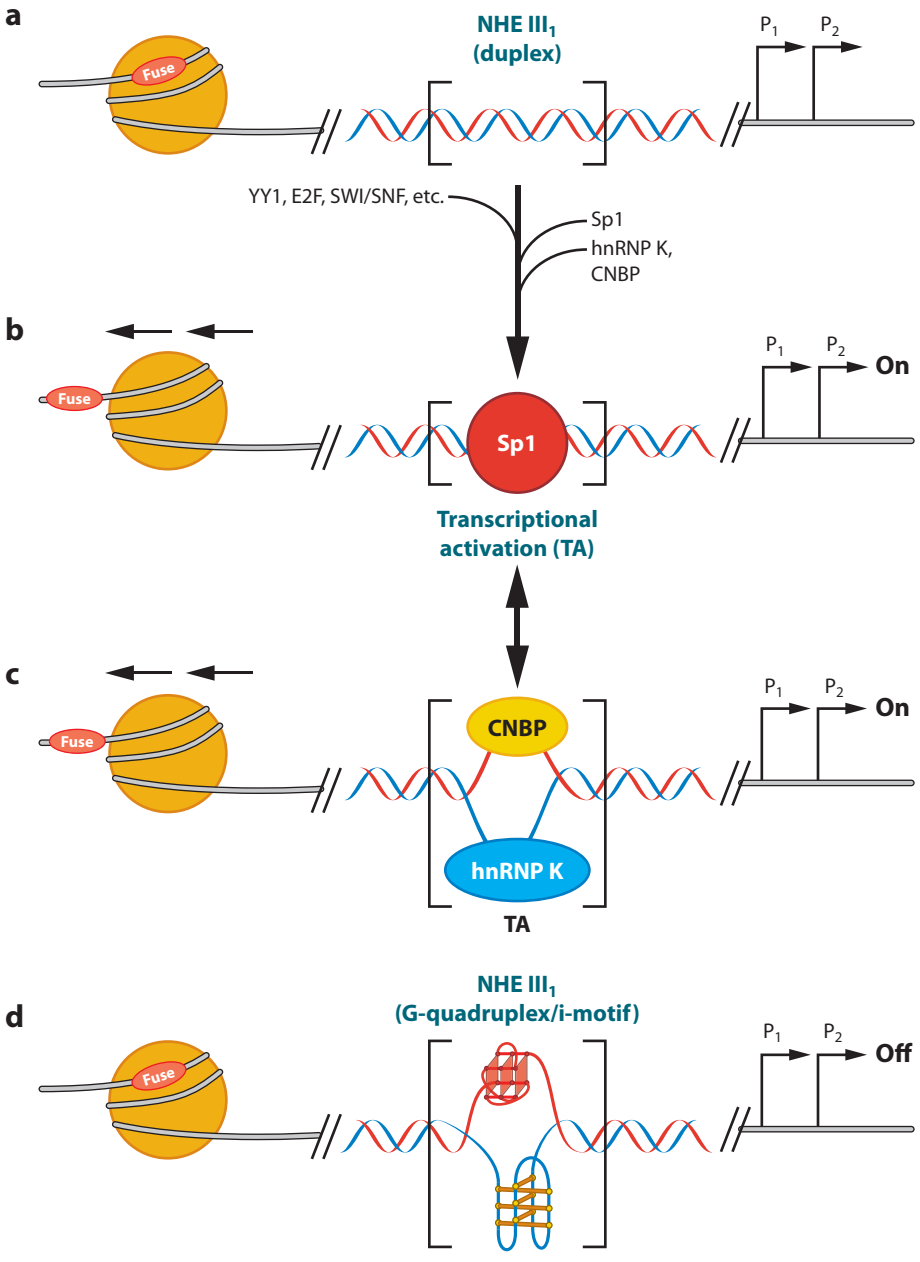

Figure 3

Models of the different promoter forms within the c-*MYC* NHE III_1. (*a*) Duplex representation of the promoter without any proteins bound. (*b*) Binding of Sp1 to the duplex structure, leading to activation of c-*MYC* expression. (*c*) Binding of hnRNP K and CNBP to the single-stranded C- and G-rich regions, respectively, leading to activation of c-*MYC* transcription. (*d*) Repression of c-*MYC* transcription when Sp1, hnRNP K, and CNBP are not bound, leading to the formation of the G-quadruplex and i-motif.

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In addition, there is evidence that Sp1 first forms tetramers and then assembles multiple tetramers at the DNA-binding site (77, 78). Sp3, on the other hand, is unable to form multimers or synergistically activate transcription of promoters containing multiple Sp1 binding sites (72). Sp3 has been shown to bind to the Sp1 binding sites as a monomer and repress Sp1-dependent transcription by competing with Sp1 for the binding sites (72, 79). However, Sp3 does not always act as a repressor. For example, in the case of the p21 promoter, Sp3 activates, rather than represses, transcription (80). In other words, the functions of Sp1 and Sp3 cannot be simplified by classifying these proteins as either an activator or a repressor, respectively. Instead, it appears that their actions are dependent on the promoter and cellular context.

RGG domain: RNA-binding domain rich in arginine, glycine, and aromatic amino acids

Heregulin: a 45-kDa secreted protein similar to the members of EGF ligand family

CNBP

CNBP, also known as ZNF9, is a multifunctional protein composed of seven cysteine-cysteine-histidine-cysteine zinc knuckles and an arginine-glycine-glycine (RGG) domain. This protein has been shown to play an essential role in embryonic development, especially in forebrain and craniofacial development, by controlling cell proliferation and survival (81, 82). These functions have been suggested to be mediated by c-*MYC* because CNBP binds to the purine-rich single strand of the c-*MYC* NHE III_1 region to induce c-*MYC* expression (**Figure 3*c***) (67, 83). In addition, $CNBP^{-/-}$ mouse embryos have a substantial reduction in cell proliferation, which was found to correlate with the absence of c-*MYC* expression (81). Functional mutation analysis of CNBP has revealed that whereas the zinc knuckles of the protein contribute partially to its nucleic acid binding activity and induction of c-*MYC* expression, the RGG domain is essential for these activities (84). Truncated forms of CNBP lacking the RGG domain have been shown to occur naturally, and it is speculated that the biochemical activity of CNBP may be regulated through proteolytic mechanisms (85).

hnRNP K

hnRNP K has been implicated in the regulation of transcription and translation and is a participant in a variety of signaling systems (86, 87). This 463-residue modular protein is characterized by the presence of several K homology domains that mediate its interactions with single-stranded DNA elements (88). The stereotypical folding of the KH domains forms an elongated groove on the surface of the protein, where it interacts with single-stranded nucleic acids via hydrogen bonds and van der Waals contacts (88). hnRNP K binds preferentially to single-stranded nucleic acids; therefore, DNA binding by hnRNP K must be coupled with nuclear stress that results in negative superhelicity to melt the duplex DNA (89).

hnRNP K has been shown to bind specifically to the pyrimidine-rich single strand of the c-*MYC* NHE III_1 and to activate the c-*MYC* promoter both in vivo and in vitro (89–91) (**Figure 3*c***). Accordingly, hnRNP K increases the endogenous c-*MYC* mRNA and protein expression. For example, serum stimulation of rat hepatoma HTC-IR cells induces binding of hnRNP K to the c-*MYC* promoter, strongly suggesting that it may be involved in the serum activation of c-*MYC* transcription (92, 93). In addition, the anti-EGFR antibody C225 and the anti-HER2 antibody Herceptin/Trastuzumab inhibit hnRNP K mRNA and protein expression, as well as c-*MYC* mRNA expression, strongly suggesting that EGF and heregulin induce c-*MYC* transcription via the EGFR pathway (94).

NM23-H2

The ubiquitous human nonmetastatic 23 isoform 2 protein (NM23-H2) is a hexamer composed of identically folded 17-kDa subunits. This protein is also known as nucleoside diphosphate (NDP)

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kinase, PuF (purine-binding factor), and nucleoside diphosphate kinase B (NDPK-B). It is a multifunctional protein that has been shown to play a role in nucleotide metabolism, cell development, proliferation, metastasis, and apoptosis (for review, see Reference 95). Some of these functions may be mediated by c-*MYC* because NM23-H2 can activate the transcription of c-*MYC* via the NHE III_1 (**Figure 4*a***) (47, 96, 97). However, there is little consensus about how NM23-H2 regulates c-*MYC* transcription. For example, Postel and coworkers suggested that NM23-H2 may activate c-*MYC* by removing nontypical secondary DNA structures on the c-*MYC* NHE III_1 region by cleaving and rejoining the DNA strands (98). In addition, they reported that the binding of NM23-H2 to the C-rich strand, the G-rich strand, and the duplex NHE III_1 was rather similar (98). In contrast, Raveh and colleagues observed that NM23-H2 had a low affinity for double-stranded DNA compared with other transcription factors and that it bound preferentially to single-stranded DNA with no apparent sequence specificity (99). Finally, another report concluded that NM23-H2 does not directly stimulate c-*MYC* transcription through the NHE III_1 (100).

In an attempt to address some of the controversy behind the functions of NM23-H2 on c-*MYC* regulation, our laboratory expanded on the studies of the effect of NM23-H2 on c-*MYC* transcription. Our results confirmed that NM23-H2 binds to the single-stranded G- and C-rich strands of the c-*MYC* NHE III_1 (**Figure 4*a***, center, and **4*b***), but not to the duplex NHE III_1 (101). In addition, we found that potassium ions and the G-quadruplex–stabilizing agent TMPyP4 reduce binding of NM23-H2 to the G- and C-rich strands of the NHE III_1, suggesting that stabilization of the G-quadruplex and i-motif structures within the NHE III_1 region hinders the recognition and remodeling functions of NM23-H2 in relation to the G- and C-rich strands (101). Furthermore, we discovered that the previously detected DNA cleavage activity associated with NM23-H2 was due to a minor contaminant associated with the recombinant protein or to an accessory protein that is lost on more extensive purification or on mutation of NM23-H2 (101, 102). Specifically, the results of this investigation demonstrated that the peaks of DNA binding activity and DNA cleavage activity were not coincident during heparin affinity chromatography, which provides evidence against the former proposition that NM23-H2 possesses an inherent nuclease activity. On the basis of these results and molecular modeling studies, we can hypothesize that NM23-H2 induces c-*MYC* transcription by trapping the NHE III_1 region in a single-stranded conformation and allowing single-stranded transcription factors such as CNBP or hnRNP K to bind to and activate c-*MYC* transcription (**Figure 4*b***). In addition, we provide our working model for how stabilization of the G-quadruplex or i-motif structures formed within the c-*MYC* gene promoter region can inhibit NM23-H2 from activating c-*MYC* gene expression (**Figure 4*a***).

Nucleolin

Nucleolin is a 110-kDa multifunctional nucleolar phosphoprotein that plays a role in chromatin decondensation, ribosome biogenesis, transcriptional regulation, cell proliferation, differentiation and maintenance of neural tissue, and apoptosis (103–107). It is a modular protein that can be structurally divided into three domains: the N-terminal, which is made up of highly acidic regions interspersed with basic sequences and contains multiple phosphorylation sites; the central domain, which contains four RNA-binding motifs; and the C-terminal, defined by spaced RGG repeats interspersed with amino acids that are often aromatic (107). Interestingly, CD spectropolarimetry and homology studies of the C-terminal of nucleolin suggest that this domain adopts a helical conformation made of repeated beta-turns. It has also been suggested that the regularity of arginine and phenylalanine side chains projecting outside the central core of the spiral structure creates electrostatic and hydrophobic ridges that are prone to interact nonspecifically with

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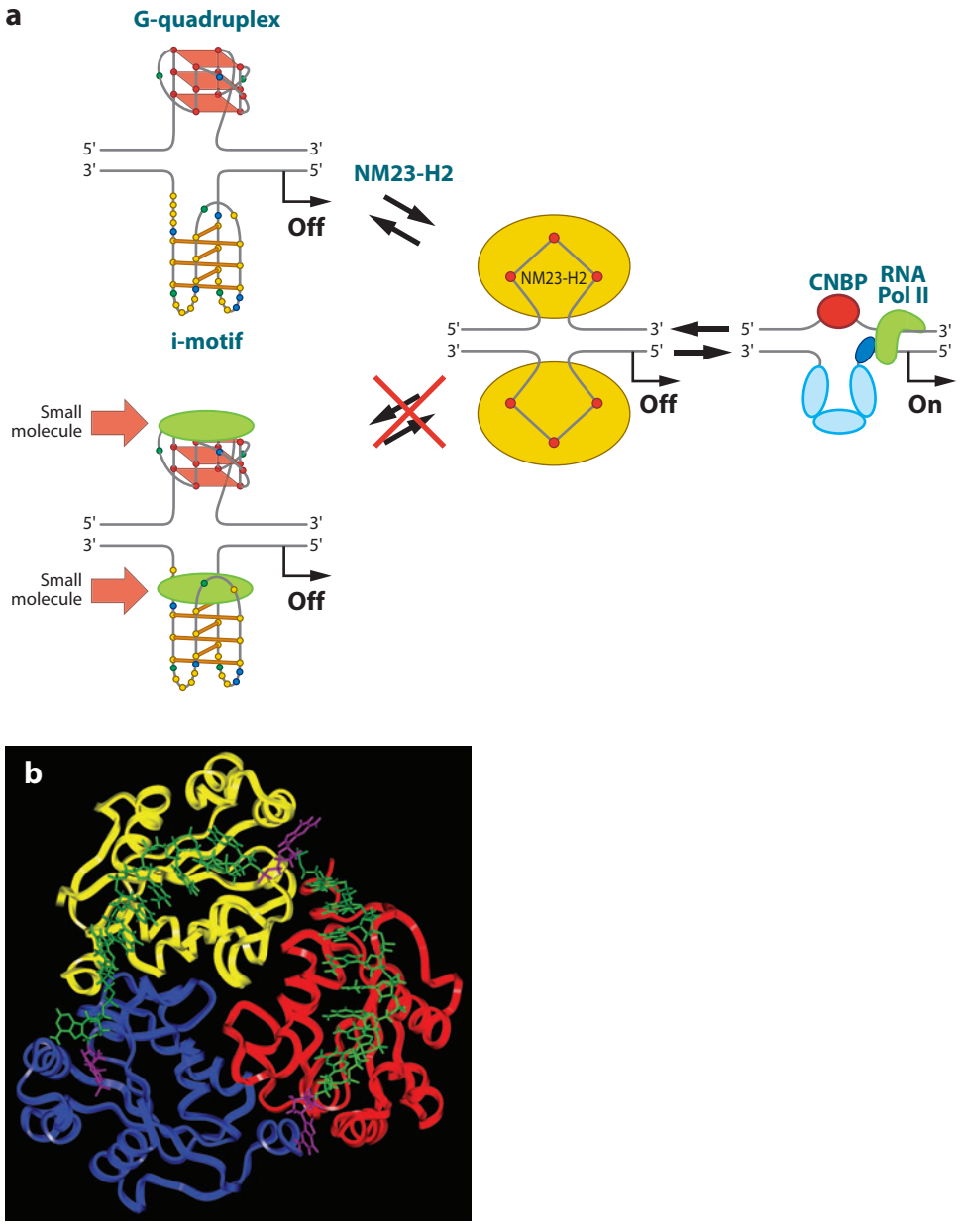

Figure 4

Proposed mechanism for the transcriptional activation of c-*MYC* via the NM23-H2–DNA complex (*a*) and molecular modeling of NM23-H2 and single-stranded DNA. (*b*) Model of c-*MYC* G-rich sequence with NM23-H2. The DNA strand is shown as capped sticks in green, with flipped guanines shown in magenta. A trimer of NM23-H2 is shown as a backbone ribbon (side chains not shown). Also shown in (*a*) is the mechanism for inhibition of the activity of NM23-H2 by stabilization of the G-quadruplex or i-motif through binding of small molecules.

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RNA and DNA (108, 109). However, this domain has been proven essential for the interaction of several G-quadruplex–binding proteins with their respective targets, and it appears to be responsible for the binding specificity to certain nucleic acid sequences. We have recently shown that nucleolin binds preferentially to the G-quadruplex conformation of the c-*MYC* NHE III_1 (**Figure 5*a***) (110). By performing CD studies, we have shown than the interaction of nucleolin with the c-*MYC* G-quadruplex is unable to unwind the G-quadruplex; instead, nucleolin induces the formation of a G-quadruplex from single-stranded NHE III_1 DNA (**Figure 5*b*,*c***) (110). Furthermore, we demonstrated that nucleolin does not bind to all G-quadruplexes to the same extent, but discriminates between different G-quadruplexes, and it appears to preferentially bind to parallel G-quadruplexes with short loops such as the c-*MYC* G-quadruplex (**Figure 5*d***) (110). In summary, the high affinity and selectivity of nucleolin for the c-*MYC* G-quadruplex structure strongly suggest that this protein may regulate c-*MYC* transcription by modulating the structure of the NHE III_1.

THE C-*MYC* G-QUADRUPLEX AS A DRUG TARGET

The G-quadruplex that forms within the NHE III_1 region of the c-*MYC* promoter has been demonstrated to function as a silencer element (69). Consequently, it appears logical that compounds that can stabilize this structure could potentially be used to specifically repress c-*MYC* expression, which may be an effective approach to targeting human malignancies that overexpress c-*MYC*. In fact, using TMPyP4, our group was able to decrease c-*MYC* expression at both the mRNA and protein levels, as well as lower the level of several c-*MYC*–regulated genes (111). By contrast, TMPyP2, a structural isomer of TMPyP4 that lacks the ability to interact with the G-quadruplex, had a much reduced effect on c-*MYC* transcription (112). Furthermore, our laboratory has determined the importance of the NHE III_1 region of c-*MYC* for gene silencing by using TMPyP4 in combination with two Burkitt's lymphoma cell lines, Ramos and CA46, which have retained or lost, respectively, the NHE III_1 region in one of the alleles because of different translocation break points (**Figure 6*a***) (69). As predicted, when the NHE III_1 was deleted, as in the CA46 cell line, TMPyP4 had little effect on c-*MYC* expression; whereas in the Ramos cell line, in which the NHE III_1 was present, TMPyP4 lowered c-*MYC* transcriptional activation much more significantly (**Figure 6*b***). Taken together, these results provide convincing evidence that specific G-quadruplex structures within the NHE III_1 of the c-*MYC* promoter represent the silenced state of the gene that can be stabilized with small molecules. However, the high incidence of G-quadruplex–forming motifs in eukaryotic promoters (60, 61) suggests that drug selectivity can be difficult, but the diversity of folding patterns, number of G-tetrads, loop length, and loop sequence offer opportunities to distinguish between these structures.

CONCLUDING REMARKS

Enormous progress has been made in our understanding of the role that c-MYC plays in normal cellular processes such as proliferation, differentiation, and apoptosis, as well as in tumorigenesis. Attempts to understand how the c-*MYC* proto-oncogene is regulated have led us and others to identify the NHE III_1 region as a critical regulator of expression. It is now becoming clear that this regulation can be influenced by secondary DNA structures such as the G-quadruplex and i-motif, which are in turn influenced by the proteins that bind to the region. However, whereas the proposed model shown in **Figure 3** provides a greater understanding of how c-*MYC* is regulated, numerous questions remain. Are there other proteins that bind to the i-motif and G-quadruplex? What is the composite structure of the G-quadruplex–i-motif complex? Does the magnitude of

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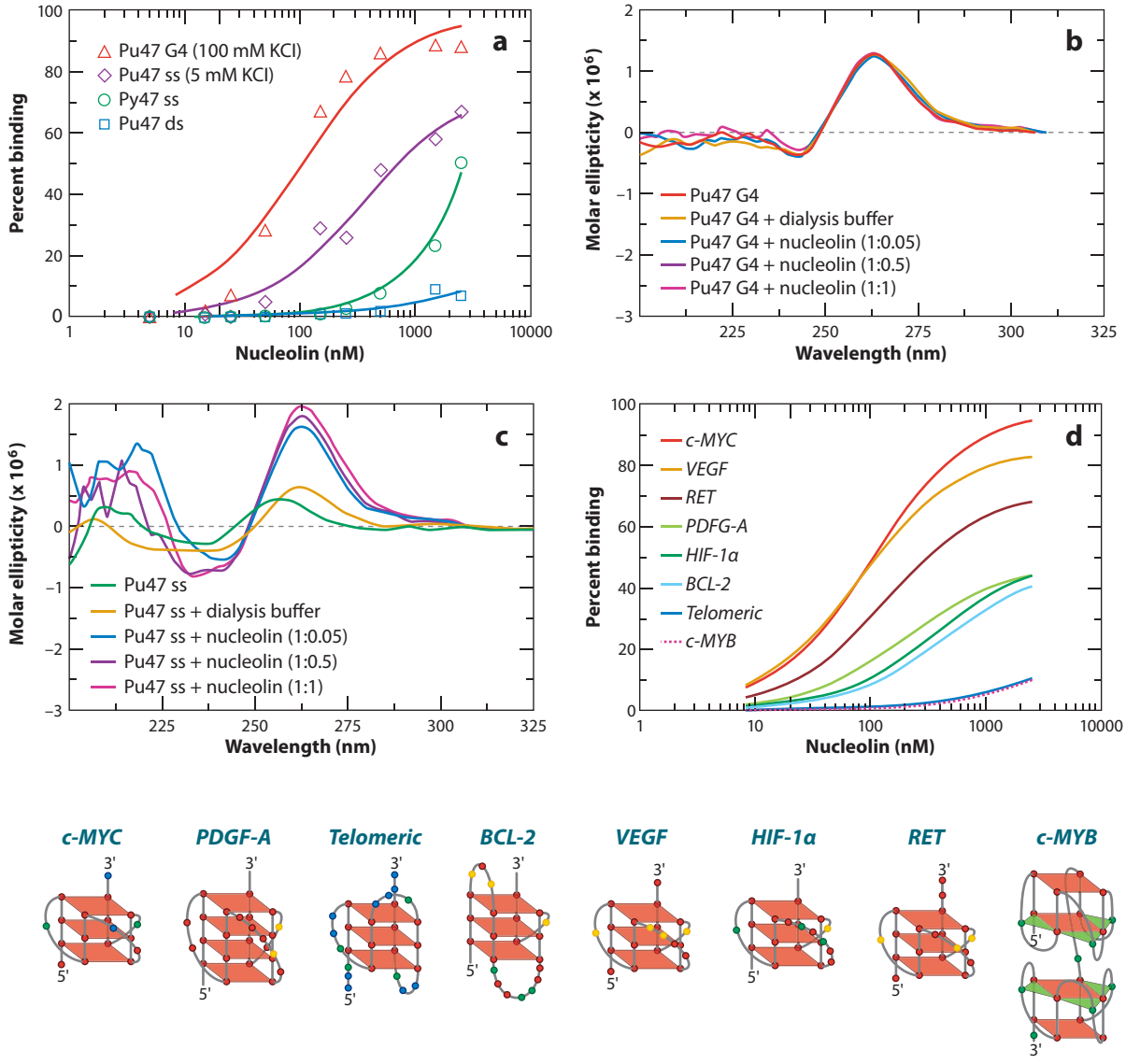

Figure 5

Illustration of the interaction of nucleolin with different conformations of the c-*MYC* NHE III_1 region. (*a*) Quantification of the binding of nucleolin to the different conformations of the c-*MYC* NHE III_1 region as determined by filter-binding assays, showing the preferential binding of nucleolin to the G-quadruplex (G4). Pu47 ss and Pu47 G4 correspond to the purine-rich strands in single-stranded and G-quadruplex conformations, respectively; Pu47 ss corresponds to the pyrimidine-rich single strand, and Pu47 ds corresponds to the double-stranded conformation of the NHE III_1. (*b*) CD spectra of recombinant nucleolin with G-quadruplex oligonucleotide of the c-*MYC* NHE III_1 sequence, showing that nucleolin does not unwind the G-quadruplex. (*c*) CD spectra of recombinant nucleolin with single-stranded DNA from the c-*MYC* NHE III_1 region, showing that nucleolin induces G-quadruplex formation. (*d*) Differential binding affinity of nucleolin to various G-quadruplex structures by filter-binding assay. Models of the conformations of some of these G-quadruplexes are shown for comparison.

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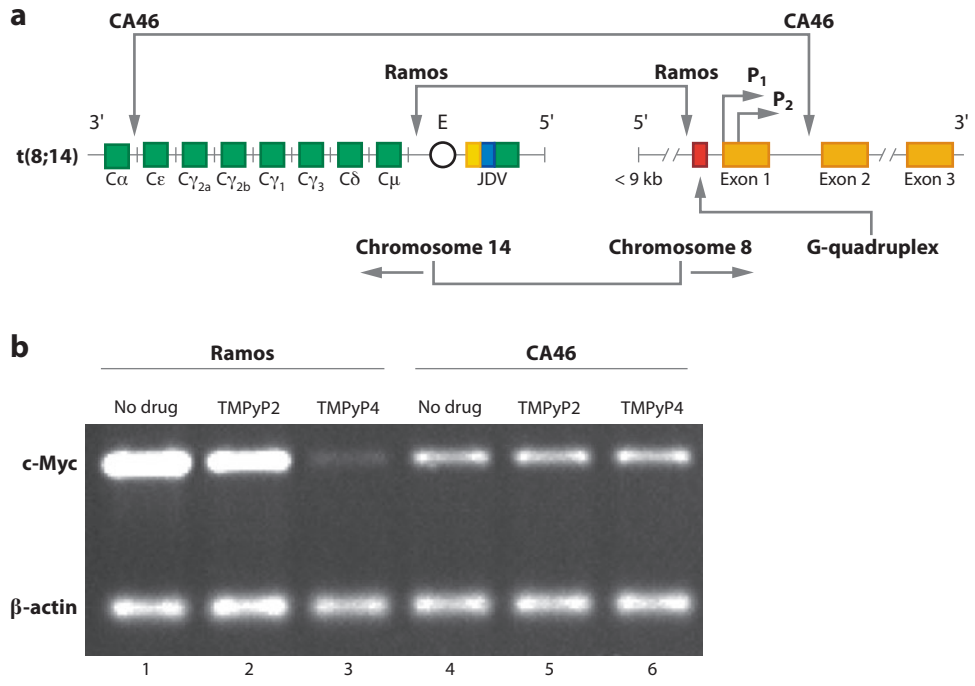

Figure 6

(*a*) Diagram of the chromosomal arrangements involved in the Ramos and CA46 Burkitt's lymphoma cell lines. Vertical arrows represent breakage and rejoining points between chromosomes 14 and 8 for each translocation. As noted, Ramos includes the NHE III_1 region in the translocation, but CA46 does not. (*b*) RT-PCR for c-*MYC* and β-actin in Ramos (lanes 1–3) and CA46 (lanes 4–6). Cell lines after no treatment (lanes 1 and 4), 48-h treatment with 100 μM TMPyP2 (lanes 2 and 5), and 48-h treatment with 100 μM TMPyP4 (lanes 3 and 6).

negative superhelicity affect the folding patterns of the G-quadruplex and i-motif? Are there naturally occurring small-molecular-weight molecules that bind to the silencer element? What other proteins are in the complex that binds to the NHE III_1 region? Does the NHE III_1 region cooperate with regions of the c-*MYC* promoter other than the FUSE to alter transcriptional regulation? It is likely that as we continue to address these questions, more complexity will arise. Yet it is our hope that as we gain a greater understanding of the regulation of c-*MYC*, practical information can be extracted to design therapeutics for use in the treatment of cancer and other diseases.

SUMMARY POINTS

1. c-*MYC* is a proto-oncogene that is dysregulated at the DNA or expression level in as many as one-seventh of all cancers in the United States; c-*MYC* is usually dysregulated indirectly through alterations in upstream signaling pathways that lead to an increase in c-*MYC* transcription.

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2. It appears that changes in c-*MYC* expression underlie its propensity to promote tumorigenesis; however, the reduction of c-*MYC* expression has been associated with the induction of differentiation and apoptosis, as well as cell sensitization to a variety of apoptotic agents.
3. A number of proteins have been shown to bind to and modulate the conformation of the c-*MYC* NHE III_1 promoter region.
4. The NHE III_1 of the c-*MYC* promoter has been shown to have the ability to form non–B-DNA structures such as the G-quadruplex and the i-motif under conditions of transcriptionally induced superhelicity and may displace transcription factors that induce c-*MYC* expression such as Sp1, CNBP, or hnRNP K, which are known to bind to and modulate the conformation of the c-*MYC* NHE III_1.
5. The c-*MYC* G-quadruplex functions as a silencer element, whose formation can be stabilized by small molecules such as TMPyP4 that could potentially be used to specifically repress c-*MYC* expression, which may be an effective approach to targeting cancers that overexpress c-MYC.

FUTURE ISSUES

1. The globular structure of the silenced form of the NHE III_1 versus the linear form of duplex DNA allows for the targeting of this element by small-molecular-weight molecules to modulate c-*MYC* gene expression.
2. Definitive evidence for how CNBP, NM23-H2, and nucleolin bind to various forms of the NHE III_1 is lacking.
3. Direct evidence for the interaction of G-quadruplex–interactive agents with the G-quadruplex element in the c-*MYC* promoter in cells is a high priority.

DISCLOSURE STATEMENT

Laurence Hurley is a shareholder in Cylene Pharmaceuticals Inc.

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