CORRECTIONS

NEUROSCIENCE. For the article “AIPL1, the protein that is defective in Leber congenital amaurosis, is essential for the biosynthesis of retinal rod cGMP phosphodiesterase,” by Xiaoqing Liu, Oleg V. Bulgakov, Xiao-Hong Wen, Michael L. Woodruff, Basil Pawlyk, Jun Yang, Gordon L. Fain, Michael A. Sandberg, Clint L. Makino, and Tiansen Li, which appeared in issue 38, September 21, 2004, of Proc. Natl. Acad. Sci. USA (101, 13903–13908; first published September 13, 2004; 10.1073/pnas.0405160101), the authors note that “r/\(r_{\text{peak}}\)” incorrectly appeared as “pA” for the ordinate label in Fig. 5B. The corrected figure and legend appear below. This correction does not affect the conclusions of the article.

Fig. 5. Photoresponses of single rods. (A) Averaged, normalized flash responses of a mutant rod (thick traces) and a WT rod (thin traces). The maximum response amplitude was 11.4 pA for the mutant rod and 15.9 pA for the WT rod. (B) Averaged single-photon responses of mutant (thick trace) and WT (thin trace) rods. Averaged dim flash responses from mutant and WT rods were scaled to the average ratio of the ensemble variance to mean amplitude for rods of each type and then normalized by 0.65 pA, the mean value obtained for the mutant rods. Flashes generating responses with mean amplitudes <0.2 \(r_{\text{max}}\) were considered to be dim. The times to peak were 215 and 130 msec, and the integration times were 545 and 259 msec for the mutant and WT rods, respectively. (C) Stimulus-response relations of cells in A. Results were fit with: \(r/\(r_{\text{max}}\) = 1 - \exp(-k/i)\), where \(i\) is the flash strength, \(k\) is ln(2)/\(i_{1/2}\), and \(i_{1/2}\) is the flash strength producing a half-maximal response. \(i_{1/2}\) values were 28 and 50 photons \(\mu\)m\(^{-2}\) for the mutant and WT rods, respectively.

BIOPHYSICS. For the article “An acoustic microscopy technique reveals hidden morphological defenses in Daphnia,” by Christian Laforsch, Wilfred Ngwa, Wolfgang Grill, and Ralph Tollrian, which appeared in issue 45, November 9, 2004, of Proc. Natl. Acad. Sci. USA (101, 15911–15914; first published November 1, 2004; 10.1073/pnas.0404860101), the authors note that the following statement should be added to the acknowledgements: “We thank the Deutsche Forschungsgemeinschaft for funding the project (Grant TO 171 4-1). The address where the induction experiments were performed is as follows: Ludwig Maximilians University Munich, Department of Biology II, Grosshadernerstrasse 2, 82152 Planegg-Martinsried, Germany.”

PHYSIOLOGY. For the article “Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice,” by R. C. Marians, L. Ng, H. C. Blair, P. Unger, P. N. Graves, and T. F. Davies, which appeared in issue 24, November 26, 2002, of Proc. Natl. Acad. Sci. USA (99, 15776–15781; first published November 13, 2002; 10.1073/pnas.242322099), the authors note that due to an inadvertent duplication made during the assembly of Fig. 7A, lanes 4–6 (wtR, hR, and koR) are identical to lanes 1–3 (wt, h, and ko). The corrected figure and its legend appear below. This correction does not affect the conclusions of this article.
RETRACEMENTS

MEDICAL SCIENCES. For the article “Mutations in the G-quadruplex silencer element and their relationship to c-MYC overexpression, NM23 repression, and therapeutic rescue,” by Cory L. Grand, Tiffanie J. Powell, Raymond B. Nagle, David J. Bearss, Denise Tye, Mary Gleason-Guzman, and Laurence H. Hurley, which appeared in issue 16, April 20, 2004, of Proc. Natl. Acad. Sci. USA (101, 6140–6145; first published April 12, 2004; 10.1073/pnas.0400460101), the authors wish to note the following: “After an unsuccessful effort to expand the observations that were reported in our article, we have determined that certain data contained in the manuscript are incorrect. The error is a result of contamination of genomic DNA with plasmid DNA, which affects the results represented in Fig. 2 and Table 1 of this article. Our conclusion that the erroneous results were due to a plasmid contamination has been confirmed by an independent laboratory, at our request. We have been unable to reproduce the experiments indicating the presence of tumor-derived mutations in the G-quadruplex silencer element of the nuclease hypersensitivity element region of the c-MYC gene in the tumor samples reported in the article or in additional tumor samples that we have analyzed. We therefore retract the article. We deeply regret this error and apologize for any inconvenience publication of this study may have caused.”

Cory L. Grand
Tiffanie J. Powell
Raymond B. Nagle
David J. Bearss
Denise Tye
Mary Gleason-Guzman
Laurence H. Hurley

GENETICS. For the article “Detecting patterns of protein distribution and gene expression in silico,” by Michael T. Geraghty, Doug Bassett, James C. Morrell, Gregory J. Gatto, Jr., Jianwu Bai, Brian V. Geisbrecht, Phil Hieter, and Stephen J. Gould, which appeared in issue 6, March 16, 1999, of Proc. Natl. Acad. Sci. USA (96, 2937–2942), the undersigned authors wish to note the following: “Fig. 1 was reported to show the subcellular distribution of peroxisomal proteins fused to green fluorescent protein in wild-type yeast cells and yeast cells mutant for the PEX3 gene. Fig. 1 A, D, and F were labeled as showing localization of proteins LYS1, LYS4, and YGL184C, respectively. Identical images showing the localization of different proteins in different cell strains were published in two other papers [Geisbrecht, B. V., Schulz, K., Nau, K., Geraghty, M. T., Schulz, H., Erdmann, R. & Gould, S. J. (1999) Biochem. Biophys. Res. Commun. 260, 28–34; Geisbrecht, B. V., Zhu, D., Schulz, K., Nau, K., Morrell, J. C., Geraghty, M., Schulz, H., Erdmann, R. & Gould, S. J. (1998) J. Biol. Chem. 273, 33184–33191]. The images in Fig. 1 were the data presented supporting the identification of peroxisomal proteins found by using a computer algorithm. Therefore, we are retracting the paper. We apologize for this error.”

Doug Bassett
James C. Morrell
Gregory J. Gatto, Jr.
Jianwu Bai
Brian V. Geisbrecht
Phil Hieter
Stephen J. Gould

www.pnas.org/cgi/doi/10.1073/pnas.0407487101
Mutations in the G-quadruplex silencer element and their relationship to c-MYC overexpression, NM23 repression, and therapeutic rescue

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We have demonstrated that a parallel G-quadruplex structure in the c-MYC promoter functions as a transcriptional repressor element. Furthermore, a specific G-to-A mutation in this element results in destabilization of the G-quadruplex repressor element and an increase in basal transcriptional activity. To validate this model in an in vivo context, we have examined the sequence of this region in human colorectal tumors and the surrounding normal tissue. We have found that ~30% of tumors contain one of two specific G-to-A mutations, not present in the surrounding normal tissue, that destabilize the parallel G-quadruplex, which would be expected to give rise to abnormally high expression of c-MYC in these cells. In contrast, G-quadruplex-disruptive mutations were absent in 20 colon adenomas, suggesting that these mutations occur late in tumorigenesis. We have also demonstrated that these same mutations are found in established colorectal cell lines. NM23-H2 levels are lower in cancer tissues and cell lines that harbor these mutations. In cells with repressed levels of NM23-H2, the mutated and destabilized G-quadruplex silencer element can be reinstated by the addition of G-quadruplex-stabilizing compounds, providing an opportunity for therapeutic intervention for patients carrying these mutations.

It is well established that overexpression of the c-MYC proto-oncogene is associated with a significant number of human malignancies, including colon and cervical cancers, myeloid leukemias, B and T cell lymphomas, and glioblastomas (1, 2). The underlying cause of this overexpression is known in many cases. For example, c-MYC expression can be increased through physical changes in chromosomal DNA, such as amplification (3–6) or translocation (7, 8), or through simple up-regulation of transcription (1, 9). However, in the latter case, how this increase in c-MYC expression is produced is often uncertain, and elucidation of its mechanism would be important in designing strategies for the treatment of patients with specific genetic alterations.

The transcriptional regulation of c-MYC expression is complex and involves multiple promoters and transcriptional start sites. P1 and P2 seem to be the predominant promoters (for reviews, see refs. 1, 9, and 10). The nucleosome hypersensitivity element (NHE) III1 of the c-MYC promoter controls 85–90% of c-MYC transcription and has been the subject of considerable research over the past two decades (11–21). Originally identified as a major site of DNase I hypersensitivity (13), the NHE III1 is a 27-bp sequence located −142 to −115 bp upstream from the P1 promoter (Fig. 1A). Recently, we uncovered a means of regulation of the c-MYC proto-oncogene: a facile transformation of a purine-rich sequence of the NHE III1 to a kinetically favored (NHE) III1 of the c-MYC promoter functions as a transcriptional repressor element. Furthermore, a specific G-to-A mutation in this element results in destabilization of the G-quadruplex repressor element and an increase in basal transcriptional activity. To validate this model in an in vivo context, we have examined the sequence of this region in human colorectal tumors and the surrounding normal tissue. We have found that ~30% of tumors contain one of two specific G-to-A mutations, not present in the surrounding normal tissue, that destabilize the parallel G-quadruplex, which would be expected to give rise to abnormally high expression of c-MYC in these cells. In contrast, G-quadruplex-disruptive mutations were absent in 20 colon adenomas, suggesting that these mutations occur late in tumorigenesis. We have also demonstrated that these same mutations are found in established colorectal cell lines. NM23-H2 levels are lower in cancer tissues and cell lines that harbor these mutations. In cells with repressed levels of NM23-H2, the mutated and destabilized G-quadruplex silencer element can be reinstated by the addition of G-quadruplex-stabilizing compounds, providing an opportunity for therapeutic intervention for patients carrying these mutations.

Methods

Analysis of Colorectal Tumor Specimens. Tissue acquisition. Appropriate tumor and polyp specimens were selected by R.B.N. of the University of Arizona Tissue Acquisition and Cellular/Molecular Analysis Shared Service. Paraaffin blocks were cut into 3-μm-thick slides, stained with hematoxylin and eosin for visualization, and microdissected as described below.

Laser capture microdissection. Approximately 1,500 normal and tumor cells were microdissected from different areas of the same slide for later sequence analysis, using an Arcturus PixCell II Laser Capture Microdissection instrument. Polyp cells were later collected in the same fashion. DNA extraction was performed by using the PicoPure DNA extraction kit (Arcturus, Mountain View, CA).

PCR sequencing. Primers used for 45-cycle PCR amplification of the NHE III1 region were 5′-GACAGGAGGCGGTGTTCGTCA-3′ (NHEseqfwd) and 5′-GAGATAGCGAGAGAGAGAGAC-3′ (NHEseqrev). The PCR products were sent to the University of Arizona Sequencing Facility for sequence determination.

Immunohistochemistry. Immunohistochemistry was performed by the University of Arizona Tissue Acquisition and Cellular/Molecular Analysis Shared Service. Briefly, staining was performed on 3-μm sections of formalin-fixed, paraffin-embedded colon tissue after antigen retrieval with microwaving in 0.01 M sodium citrate buffer, pH 6.0. Staining of c-MYC and β-catenin was accomplished by using antibodies 9E10 (Dako) and sc-1496 (Santa Cruz Biotechnology), respectively. Subsequent detection used the LSAB II kit and DAB (Dako), followed by counterstaining with hematoxylin.

RT-PCR for c-MYC, NM23-H1, and NM23-H2 in Patient Tissues and Established Cell Lines. Total RNA was isolated from patient samples or established cell lines. The RNA was used as a template for reverse transcription. PCR was performed by using...
Destabilization of the G-quadruplex structure.

G-to-A mutations found in the colorectal cancers that are associated with relevant parallel G-quadruplex structure, showing the sites (12 and 17) of two posed looped out G-quadruplex structure, which stabilizes the silencer element for transcriptional activation (22, 35). Interaction of the parallel G-quadruplex (gene off) to unstructured purine and pyrimidine single-stranded DNA forms role of NM23-H2 in conversion of the paranemic secondary DNA structures for the activation and repression of gene transcription involving the accessory sequence of the purine-rich strand upstream of the P1 promoter (9). (Fig. 1)

NM23-H2 Protein Levels in SW480 and SW620. Protein expression was determined by using Western blot analysis. Nuclear and whole cell proteins were isolated from SW480 and SW620 established cell lines by using Nonidet P-40 lysis buffer. A total of 50 μg per lane was separated on a 4–12% Bis-Tris Gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% (wt) milk in 1× TBST overnight at 4°C and probed for 2 h with an NM23-H2 antibody (Seikagaku no. H2–206) at a 1:1,000 dilution. Actin was used as a control (Abcam no. 6276). Abcam (no. 6728) rabbit polyclonal antihouse-IgG was used as a secondary antibody at a 1:4,000 dilution in blocking buffer, and was added to the membrane for an additional hour. Proteins were detected by using the photo-ter-horseradish peroxidase Western blot detection system (Cell Signaling Technology, Beverly, MA) followed by exposure to radiographic film.

Luciferase Reporter Experiments. Site-directed mutagenesis. The Del-4 plasmid was a gift from Bert Vogelstein (Johns Hopkins University, Baltimore). Single guanine mutations were made to the NHE III1 of this plasmid by using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s protocol. Transfection and luciferase assays. Protocol from Siddiqui-Jain et al. (22) was followed. HeLa S3 cells were transfected by using the Effectene lipid-based system (Qiagen) according to the manufacturer’s protocol. A total of 0.1 μg of pRL-TK (Renilla luciferase reporter plasmid) and 0.9 μg of the mutated plasmids was used.

Statistical Treatment of Data. This manuscript was submitted to the University of Arizona Biometry Shared Service for review, and the results from a Fisher’s exact test (P = 0.02) were deemed statistically significant.

Results and Discussion
Two Specific G-to-A Mutations Found in Colorectal Cancer Specimens Destabilize the Silencer Element of c-MYC and Result in Overexpression of c-MYC. Having established that a parallel G-quadruplex exists naturally in the c-MYC promoter and that disruption of this structure leads to increased gene expression, we speculated that such mutations in vivo would have similar consequences, i.e., up-regulation of c-MYC expression. This would provide a specific mutational means by which cells can overexpress c-MYC and thus be selected for during tumorigenesis. To explore this possibility, we have examined the NHE III1 sequence in 21 colorectal cancer specimens taken from patients, as well as in the corresponding surrounding normal tissue. Colon adenomas (polyps) were also examined to determine whether mutations to the quadruplex-forming region of the c-MYC promoter might be early events in the genesis of colorectal cancer. We chose colorectal cancer for our initial study for several reasons. First, c-MYC expression is known to be elevated up to 40-fold in primary colorectal cancers, but significantly, this elevation is not caused by amplification or translocation of the genomic c-MYC sequence (24). Therefore, we suspected that if G-quadruplex mutation is indeed a means by which tumor cells up-regulate this

5'-AGAGAAGCTGCGCTCTTACC-3' (c-myc-1), 5'-AGCCTTTGTGCCTTGCTG-3' (c-myc-2), 5'-CTGCAAGCGAGTTCATACC-3' (NM23H1–1), 5'-GTCTGCGCTCTTACCTACGC-3' (NM23H2–2), 5'-TCCTCCTGCTCTCAGCGGCG-C3' (NM23H2–1), and 5'-CCGTGCCTGAAAGGACTGCTT-3' (NM23H2–2). PCR amplification was performed by using 25 cycles with a 59°C (c-MYC) or 62°C (NM23-H1 and -H2) annealing temperature and a 72°C extension temperature, using an MJ Research Thermodcycler PTC-200. PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and products were run on a 1% agarose gel. β-actin primers (Ambion, Austin, TX) were included as a control.

Fig. 1. (A) Promoter structure of the c-MYC gene. (Inset) The 27-mer sequence of the purine-rich strand upstream of the P1 promoter (9). (B) Model for the activation and repression of gene transcription involving the accessory role of NM23-H2 in conversion of the paranemic secondary DNA structures (gene off) to unstructured purine and pyrimidine single-stranded DNA forms for transcriptional activation (22, 35). Interaction of the parallel G-quadruplex structure with TMPyP4 stabilizes the gene-off form by conversion to a proposed looped out G-quadruplex structure, which stabilizes the silencer element and results in transcriptional inhibition. (C) Cartoon of the biologically relevant parallel G-quadruplex structure, showing the sites (12 and 17) of two G-to-A mutations found in the colorectal cancers that are associated with destabilization of the G-quadruplex structure.
gene, colorectal cancer specimens would be the most likely tissue type in which to find evidence of this. Second, the progression of human colorectal cancer has been characterized extensively by the Vogelstein group (25) and is one of the best-characterized human tumor systems. Thus, early precancer forms of this tumor, adenomatous polyps, are known and can be included in our study (25).

Laser-capture microdissection was performed to specifically dissect out either tumor or nontransformed cells. The genomic DNA was harvested from these cells, and PCR was performed to amplify the NHE III region of the c-MYC promoter. DNA sequencing analysis was then carried out on the PCR products. A flowchart of this procedure is shown in Fig. 2A. Of the 21 tumor samples, six were found to bear mutations, which do not appear in the normal tissue, that are known to disrupt the parallel G-quadruplex structure (see Fig. 1C). In previous studies, we have demonstrated that these same mutations lead to a 3-fold increase in gene expression (22). These sequences and the two specific G-to-A mutations are shown in Table 1. Although only 23 bases are shown in Table 1, the mutations found were the only ones in >200 bp of the c-MYC promoter sequence. In none of the 21 tumor samples did we find a mutation in nontransformed cells that would disrupt the parallel G-quadruplex, indicating that these mutations are selected for in tumor cells. For each of the six pairs of tissue samples from each patient, c-MYC expression levels were determined (Fig. 2B). In each case, the cancer tissue showed a higher level of expression.

Tumor stage at resection was also examined to determine whether these mutations correlate with the degree of invasiveness or aggression of the tumors. Although the tumors with parallel G-quadruplex mutations did show appreciable involvement of the colon tissue (T2 and T3 in Table 1) and local lymph nodes (N2 in two cases), the same was found for tumors without parallel G-quadruplex mutations, indicating that there is no correlation between the mutations and degree of tumor severity in this limited group of tumor samples.

**Subcellular Localization of β-Catenin Correlates with the Mutational Status of the c-MYC Silencer Element.** A major alternate pathway through which c-MYC may also be up-regulated in colorectal tumors involves abrogation of the adenomatous polyposis coli (APC) pathway (25). To investigate whether disruption of normal APC function correlates with the G-quadruplex mutations found in this study, immunohistochemistry for the APC-regulated protein responsible for c-MYC expression, β-catenin, was performed (26). The results are shown in Table 1. In 5 of the 21 tumors, β-catenin was not found in the nucleus, where it is expected to localize if it is playing a role in regulation of the c-MYC promoter. Of these five tumors, three exhibited G-quadruplex destabilizing mutations. This suggests that disruption of the repressive parallel G-quadruplex structure in the NHE III can act as a surrogate or complement for APC mutations in up-regulating c-MYC expression. c-MYC up-regulation may occur through a number of means, including retroviral insertion, amplification, and dysregulation of up-stream pathways (1, 9). It now appears that G-quadruplex disruption can be added to this list.

**G-to-A Mutations in the Colorectal Tumors Studied Exhibit Heterozygosity.** As c-MYC is known as an oncogene, we might expect those mutations that are suspected to up-regulate c-MYC to be found in only one of the two alleles. To determine whether the mutations are heterozygous, the PCR products used for sequencing were cloned into a shuttle vector, pCR2.1 TOPO, so that individual sequences could be examined. Bacteria were

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**Table 1. c-MYC NHE III1 mutations in human colorectal tumors**

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>Sequence*</th>
<th>Tumor grade</th>
<th>Heterozygosity</th>
<th>Nuclear β-catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TGGGGAGGGTGGAGGGTGG</td>
<td>T2N0</td>
<td>8/10 mutant</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>TGGGGAGGGTGGAGGGTGG</td>
<td>T3N0</td>
<td>4/10 mutant</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>TGGGGAGGGTGGAGGGTGG</td>
<td>T3N0</td>
<td>5/10 mutant</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>TGGGGAGGGTGGAGGGTGG</td>
<td>T3N0</td>
<td>9/10 mutant</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>TGGGGAGGGTGGAGGGTGG</td>
<td>T3N2</td>
<td>7/10 mutant</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>TGGGGAGGGTGGAGGGTGG</td>
<td>T3N2</td>
<td>6/10 mutant</td>
<td>–</td>
</tr>
</tbody>
</table>

*G-to-A mutations are shown in bold.
transformed with the plasmid plus insert constructs, and 10 individual colonies, each representing a single PCR product, were selected. These colonies were grown overnight, and the plasmid DNA was extracted and again subjected to sequencing. The expected heterozygosity was found, such that between 40% and 90% of the individual colonies were found to have the identical mutation found previously (Table 1). Thus, the mutated allele appears to be overrepresented in these human samples, suggesting that it may be present in more than a single copy, perhaps present in five times as many copies as the wild type.

**Human Colon Adenoma Samples Do Not Carry the Mutations Found in the Adenocarcinoma Tissue Samples.** To determine the point in a tumor’s development at which these mutations occur, 20 human colon adenoma samples were procured and tested in the same manner as the colorectal tumors described before. In contrast to the colorectal cancer specimens, mutations to the NHE III1 were not found in any of the 20 colon adenomas. This indicates that G-quadruplex-disruptive mutations in the c-MYC promoter are a later event in tumorigenesis. The absence of mutations in these polyp samples was not unexpected. It is widely accepted that c-MYC has effects beyond induction of proliferation; it also activates apoptotic pathways, possibly to safeguard against up-regulation of c-MYC in the absence of appropriate growth signals, perhaps through interactions with Bin-1, pRb, p53, and p73, and through activation of p53 expression (9, 27-29). In colorectal cancer, p53 mutation or inactivation is a late event in tumorigenesis (25); therefore, overexpression of c-MYC may be expected to occur later in a tumor’s development, after such apoptotic checkpoints have been compromised. Also, other means of c-MYC up-regulation exist, as discussed earlier. These mechanisms may be in effect in earlier precancerous lesions, including adenomatous polyps, such that c-MYC is up-regulated to some degree without the presence of G-quadruplex-specific mutations. The specific G-to-A mutations uncovered here that disrupt the parallel G-quadruplex structure increase basal transcriptional activity only by ~3-fold. These mutations may act as a multiplier by being a late-stage event, where amplification or dysregulation occurs as an upstream event.

**NM23-H2, But Not NM23-H1, Expression Levels Are Reduced in Mutant Colorectal Tumor Cells Relative to Normal Cells.** The NM23 gene was originally identified as a potential metastasis suppressor gene because it has reduced expression in metastatic melanoma and breast carcinoma cells (30–32). Within the family of NM23 proteins, NM23-H1 and -H2 are involved in cancer and have been associated with transcriptional regulation of PDGF-A and c-MYC. The Postel laboratory (33) has demonstrated that NM23-H2 recognizes the NHE III1 of c-MYC and activates transcription. However, NM23-H2 is not a conventional transcriptional factor, but appears to be involved in remodeling the silencer element to convert it to the transcriptionally active forms (21). We evaluated whether transcriptional levels of NM23-H2 are reduced in cells that carry a G-to-A mutation, because c-MYC expression levels are higher in these cells relative to their normal counterparts. NM23-H1 levels were also measured in the same patient samples as an internal control. The results (Fig. 3) show a preferential reduction of up to 30% of NM23-H2 expression levels in the mutation-bearing cancer cells relative to normal cells. In contrast, NM23-H1 expression levels were not significantly affected (i.e., ~2% to +6% change in expression levels) in the same patient samples (Fig. 3). In the remaining patient samples, which had wild-type sequences in the silencer element, NM23-H2 levels did not significantly differ between cancer and adjacent normal tissues (unpublished results). Thus, there appears to be an inverse relationship between NM23-H2 expression and c-MYC levels, where G-to-A mutations exist in the silencer element. The underlying mechanism for this provocative relationship is not clear at this time.

**Colorectal Tumor Cell Lines Harbor the Same G-to-A Mutations Found in the Human Tumor Specimens.** If these data are valid, and G-quadruplex destabilization in the NHE III1 is a means by which c-MYC can be dysregulated in tumorigenesis, then we should expect to find colorectal tumor cell lines with G-quadruplex-disruptive mutations in this region as well. Six colorectal tumor cell lines, WIDR, SW480, SW620, HCT116, HT29, and Caco-2, were examined for such mutations. Two of these, SW620 and WIDR, bore the same mutation that was found in human colorectal tumor specimens 2–5 in Table 1. A third cell line, HT29, bore a heterozygous G-to-T transversion mutation in the second run of guanines, which also greatly destabilizes the parallel G-quadruplex (unpublished results). A pancreatic carcinoma cell line, AsPc-1, also bore this same G-to-T mutation (unpublished results). Therefore, 50% of the colorectal cancer cell lines examined contained G-quadruplex-disrupting point mutations, providing proof of principle that destabilization of the parallel G-quadruplex in the NHE III1 is an important event in tumorigenesis. SW480 and SW620 are cell lines established from the same patient, such that SW480 is from the primary tumor and SW620 is from a lymph node metastasis site. Only the cell line from the metastasis site carried the mutant, suggesting a late-stage event. Finally, this work suggests that it is important to experimentally determine the sequence within the NHE III1 in immortalized cell lines rather than use that found in GenBank.

**There Is an Inverse Relationship between c-MYC and NM23-H2 Levels in Cells That Carry the G-to-A Mutation.** We have shown that NM23-H2 expression levels are selectively reduced in patient cancer cells that carry G-to-A mutations in the silencer element (Fig. 3). Our observation that a cell line derived from a lymph metastasis (SW620) carried one of the two G-to-A mutations, whereas the primary tumor (SW480) from the same patient had a wild-type sequence, allowed us to compare NM23-H2 and c-MYC levels in these genetically related samples. The results in Fig. 4A show that, as expected, the mutated cell line had increased c-MYC expression levels relative to the wild-type primary tumor. There was also a significant decrease in NM23-H2 expression and protein levels in the cells from the metastasis site relative to that from the primary tumor site (Fig. 4 B and C). These results, together with those from the patient

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**Fig. 3.** NM23-H1 and -H2 expression levels relative to normal tissue in patient samples 1–6 (see Table 1). Experiments were performed in triplicate. Error bars represent 1 SD above and below the mean.
tumor samples (Fig. 3), suggest that mutations that destabilize the G-quadruplex silencer element and cause overexpression of c-MYC may be associated with the previously observed reduced expression of NM23-H2, which is normally required for remodeling of the silencer element to the transcriptionally active form of the gene (Fig. 1 A). Presumably, the requirement for this enzyme to remodel the silenced form to the transcriptionally active form would be reduced in cells where the mutant prevents formation of a stable silencer element. Because these G-to-A mutations are late events in tumorigenesis, they may also be associated with metastasis, as in the case of the SW620 cell line. It will be important to examine other pairs of primary and metastatic tissues and cell lines to establish the generality of this observation.

Stabilization of the Mutated G-Quadruplex Structure by TMPyP4 Reinstates the G-Quadruplex Structure and Silences Gene Expression.

In previous studies, we demonstrated that the cationic porphyrin TMPyP4 is able to stabilize the parallel G-quadruplex structure and repress c-MYC expression by up to 60% after 48 h (22, 23). We also demonstrated that the two G-to-A mutated parallel G-quadruplex structures reported here in the colorectal tumors could be partially reinstated by TMPyP4 (22). In these experiments, a luciferase reporter gene under the control of either the wild-type or the G-to-A mutant promoters shown in Fig. 1 C was used in plasmid transfection experiments with HeLa S3 cells. Because NM23-H2 levels were uniform under these conditions, we questioned whether the lower NM23-H2 levels in SW620 versus SW480 would affect the ability of TMPyP4 to repress c-MYC gene expression. The results shown in Fig. 4 D demonstrate that the repressed levels of NM23-H2 in the SW620 cells resulted in an enhanced effect of TMPyP4, despite the G-quadruplex-destabilizing mutation in the NHE III1. Previously, we have shown that such mutations reduce the effect of TMPyP4 on c-MYC in HeLa S3 cells in which NM23-H2 levels were constant (22). Thus, it would appear that c-MYC gene expression in cells that have lower levels of NM23-H2 may be more susceptible to G-quadruplex-stabilizing agents than cells with higher levels. Contrary to our initial expectations, these results suggest that addition of G-quadruplex-interacting compounds to cells harboring these G-quadruplex-destabilizing mutations in the NHE III1 should significantly lower c-MYC gene expression in cancer cells.

The Two Specific G-to-A Mutations Found in the Colorectal Tumors Are Those Associated with the Greatest Stimulation of Basal Gene Expression of c-MYC. It is important to address why sites 12 and 17 (Fig. 1 C) were the specific positions at which G-to-A mutations were found uniquely in cancer cells. The NHE III1 in the c-MYC promoter has three runs of four guanines and two runs of three guanines (see Table 1), providing numerous mutable sites, yet 12 and 17 are the only mutation sites found in the human colorectal tumor specimens. Significantly, these two guanines are located in equivalent, unique positions in the central tetrad crossed by the diagonal GA loop (Fig. 1 C). In contrast, although G8 and G21 are also contained in the central tetrad, they may be less critical because they are not centrally involved in the underpinning of the lattice, which folds before forming the G-quadruplex structure. To test this experimentally, each of the 14 guanines involved in the parallel G-quadruplex structure was individually mutated to an adenine and evaluated in a luciferase promoter reporter system. The G-to-A mutations at sites 12 and 17 showed the greatest stimulation of basal gene expression (Fig. 5). Thus, the selection of both of these independent mutations in different
human colorectal tumors (a 1 in 196 chance, if this were random) is done so that each maximally destabilizes the G-quadruplex structure. Furthermore, this can be predicted on the basis of our understanding of their importance to the stability of this specific G-quadruplex structure in the NHE III of the c-MYC promoter. Because all of the G-to-A mutations produce a modest degree of destabilization, this suggests that the 12 and 17 G-to-A mutations may produce additional disruption of the silencer element beyond the destabilization effect. For example, they may lead to misfolded silencer elements. Although we believe that we have provided a persuasive rationale for how these two specific G-to-A mutations can cause overexpression of c-MYC and how specific drug intervention can reinstate the silencer element, we would be remiss if we did not point to an alternative, but we believe, far less likely, explanation for the effects of these mutants on c-MYC transcriptional activation. Thus, it is conceivable that the same G-to-A mutations reported here could disrupt sequence-specific repressor binding interactions and likewise result in transcriptional activation.

Our results suggest that the parallel G-quadruplex-specific mutations found in colorectal malignancies are not simply chance single-nucleotide polymorphisms, but biologically relevant alterations that most likely have important consequences later in the development of this tumor type. To make certain of this, a number of single-nucleotide polymorphism databases were searched in regards to the NHE III region of c-MYC. In no case was any polymorphism discovered, indicating that the mutations we have found do not occur normally in healthy cells. This finding suggests that mutations in this region are not normally tolerated and, thus, are likely dealt with through apoptosis if they occur in the context of a nondysplastic cell.

**Conclusions**

We have demonstrated a unique mutational mechanism for overexpression of c-MYC in colorectal cancers. This mechanism hinges on the natural occurrence of the parallel G-quadruplex silencer element located in the NHE III of the promoter of c-MYC. Furthermore, the ability to reinstate the G-quadruplex silencer element with drugs, even in the mutated colorectal tumors, and thereby lower c-MYC expression, establishes the principle of drug intervention to reverse consequences of this late mutational event. Significantly, it has been recently demonstrated that even transient down-regulation of c-MYC results in apoptosis in a highly malignant osteogenic sarcoma (34).

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