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c-myc Suppression in Burkitt's Lymphoma Cells

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The purpose of the study was to elucidate how DNA tetraplex (also referred to as G-quadruplex)-forming oligonucleotides mediate suppression of the human c-myc gene at the level of transcription initiation. A 22-base-long oligonucleotide, which is rich in guanines and folds into an intrastrand DNA tetraplex under physiological conditions, was administered to a Burkitt's lymphoma cell line overexpressing a (8:14) translocated c-myc allele. Administration of the oligonucleotide at nanomolar concentrations to the surrounding medium resulted in efficient cellular uptake, and was accompanied by a substantial concentration- and conformation-dependent decrease in growth rate. We discuss how c-myc transcription is initiated at the molecular level and speculate that the oligonucleotide exerts a dual effect on c-myc expression *in vivo*. © 2002 Elsevier Science

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Illegitimate activation of the c-myc oncogene is a hallmark of tumor development and progression (1), and has prompted attempts to specifically suppress c-myc expression. In general, the activation of genes is greatly alleviated by chromatin unfolding, and for the transcription machinery to assemble at a gene promoter chromatin needs to unfold locally. This may cause nuclease hypersensitive sites to form, and the formation of one in the promoter of the human c-myc gene consistently precedes c-myc expression (2). The c-myc nuclease hypersensitive site comprises a DNA sequence of uneven base composition; the strand that serves as template for c-myc transcription is rich in guanines and folds into a DNA tetraplex (Fig. 1), also known as G-quadruplex, under physiological conditions (3, 4), whereas the other strand is cytosine-rich and has the ability to form several distinct i-motif structures (5). These non-B-DNA structures cannot be packaged into nucleosomes, and their formation may lead to chromatin unfolding and account for hypersensitivity toward nucleases. They are also substrates for transcription factors that act together to serve as a docking platform for the assembly of transcriptionally engaged RNA polymerase II (6). Since this site appears pivotal to c-myc expression, it has been targeted by synthetic oligonucleotides with the purpose to abolish the production of c-myc mRNA. Despite some success on tumor cell lines over-expressing c-myc (7), the precise modes of function have proven elusive. Here we examine oligonucleotide mediated c-myc suppression in more detail, by administering a 22-base-long synthetic DNA molecule to the Burkitt's lymphoma cell line Ramos, which carries a constitutively expressed (8:14) translocated c-myc allele (8) still retaining the nuclease hypersensitive site.

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MATERIALS AND METHODS

The 22-base-long oligonucleotide is identical to the guanine-rich strand of the c-myc nuclease hypersensitive site and has the sequence 5′-GGG GAG GGT GGG GAG GGT GGG G-3′. It was synthesized at 1 μmol scale by Eurogentec (Eurogenetec, Belgium), as were 5′-fluorescein conjugated oligonucleotides of the same sequence and 22-base-long oligonucleotides of random sequence. All variants were purified by standard denaturing gel electrophoresis, recovered from gels by electroelution, ethanol precipitated, and dissolved in 18.2 MΩ water prior to use. The DNA tetraplex conformation was induced as previously described (3).

The Burkitt's lymphoma cell line Ramos was cultivated at +37°C in RPMI 1640 medium (Life Technologies, Scotland) with 10% fetal calf serum (HyClone, U.S.A.) supplemented with L-glutamine (Sigma, U.S.A.) and 1% penicillin/streptomycin solution (Life Technologies, Scotland). To minimize initial effects of high local concentrations, the oligonucleotides were diluted in medium just prior to administration. Duplicate samples were taken from the cultures at 0, 12, 24, 36, 48, and 72 h after oligonucleotide administration. Cell growth was measured by cell count, and cell viability was immediately checked by the trypan blue exclusion test.

Fluorescence microscopy was performed using a Leica LEITZ-DMRB microscope (Leica, Germany) equipped with a Hamamatsu 4800 cooled CCD camera (Hamamatsu, Japan).

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FIG. 1. The guanine tetrad base-pairing scheme (top) makes the guanine-rich strand of the c-myc nuclease-hypersensitive site fold into a DNA tetraplex containing three stacked guanine tetrads, which coordinate two potassium ions (bottom).

The Basic Local Alignment Search Tool program suite (9; http://www.ncbi.nlm.nih.gov/BLAST/) version 2.2.1 was used to search for the occurrence of the oligonucleotide sequence in the current draft of the human genome, as well as genomes of other organisms.

RESULTS

The synthetic guanine-rich 22-base-long oligonucleotide was administered to cultures of the Burkitt's lymphoma cell line Ramos either in an unstructured single stranded conformation or as a preformed DNA tetraplex, and random sequence oligonucleotides were administered to cell cultures as controls. Effects on growth rate were then monitored continuously for 72 h, and duplicate samples were taken at regular intervals. Administration of control 22-base-long random sequence oligonucleotides did not significantly affect growth rates at concentrations up to 100 nM (Fig. 2; open and filled triangles), and cells treated with them exhibited population doubling times comparable to those of untreated cells (~24 h) (Fig. 2; filled diamonds). In stark contrast the guanine-rich c-myc specific oligonucleotide had a prominent effect on cell growth rates, and already at 10 nM concentrations treated cells displayed an almost twofold increase in

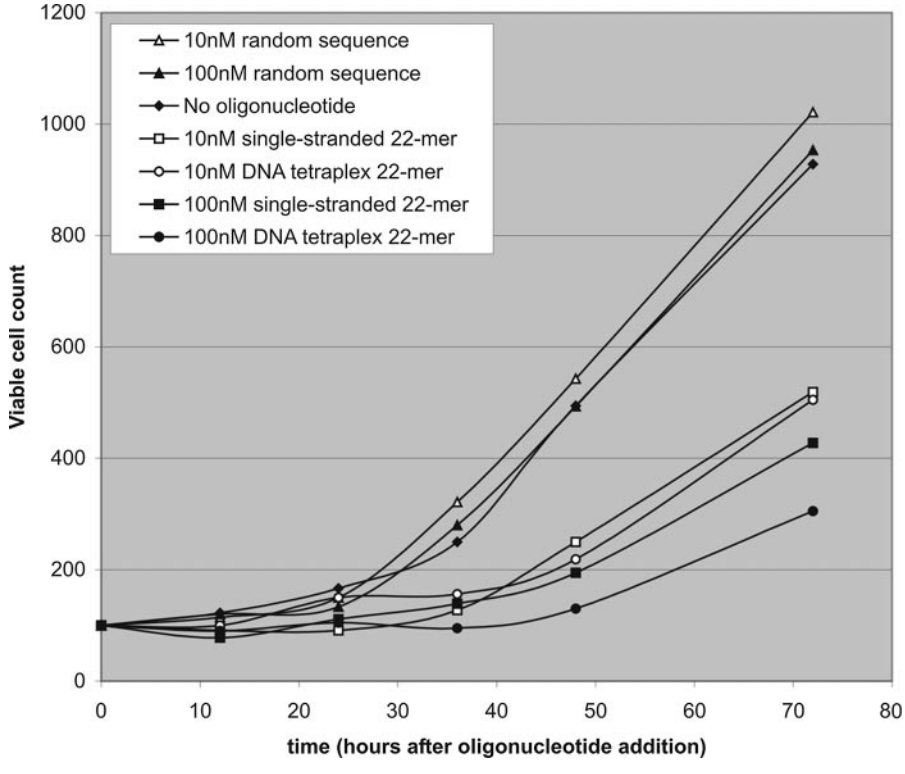

FIG. 2. Administrations of synthetic oligonucleotides to Burkitt's lymphoma Ramos cells affect their growth rate in an oligonucleotide concentration- and conformation-dependent manner. In comparison to the growth rate in absence of oligonucleotides (filled diamonds), random sequence oligonucleotides have virtually no effect at concentrations up to 100 nM (open and filled triangles). At 10 nM concentrations, the effect of the unstructured oligonucleotide (open square) is similar to the DNA tetraplex conformation (open circle), whereas at 100 nM the effect of the DNA tetraplex conformation (filled circle) becomes much more pronounced than does the unstructured oligonucleotide (filled square).

population doubling times. Albeit at this lower concentration, there was no discernible difference between the two possible conformations of the oligonucleotide (Fig. 2; open squares and circles). However, at a 10-fold higher concentration the preformed DNA tetraplex variant of the oligonucleotide exhibited a conspicuously superior antiproliferative effect (Fig. 2; filled circles) in comparison to that of the unstructured single-stranded conformer (Fig. 2; filled squares). This was manifested in a further increase in population doubling time to almost three times that of untreated cells or cells treated with random sequence oligonucleotides.

To monitor their sub-cellular location we also administered fluorescein labeled oligonucleotides to the cell cultures, and following fluorescence microscopy revealed that they accumulated preferentially to the nucleus within 1 h, confirming previous reports of efficient cellular uptake (10; data not shown).

Standard nucleotide-nucleotide BLAST searches (nBLAST) failed to detect the occurrence of the oligonucleotide sequence elsewhere in the human genome, suggesting this control element is exclusive to the c-myc promoter. However, when the search was extended to also include other available genomes, we detected its presence in the upstream control region of c-myc genes from other primates and found variants of it in c-myc control regions of other eukaryotes. This could indicate that the sequence is an evolutionary conserved upstream control element that is highly specific for the control of c-myc expression.

DISCUSSION

The current model for c-myc activation from the nuclease hypersensitive site of interest can be depicted as follows (Fig. 3): When organized into a regular chromatin fiber, c-myc is silent. To activate the gene it is necessary that chromatin unfolds in the c-myc promoter region. What triggers this event is presently unclear, but the first phase likely entails chromatin-remodeling complexes, and could possibly involve changes in intracellular potassium levels since potassium ions help induce the DNA tetraplex conformation in the c-myc promoter (3). As chromatin unfolding proceeds, the nucleosome that covers the nuclease hypersensitive site is dislodged (2, 11), releasing DNA from constraints that keep it in the transcriptionally silent B-form duplex. At this stage the multifunctional protein nucleoside diphosphate kinase isoform B (NDPK-B) comes into play (12, 13). Among its functions is a helicase activity (14, 15) that stimulates c-myc expression indirectly (16). The NDPK-B hexamer separates the two DNA strands and binds preferentially to the cytosine-rich strand. The displaced guanine-rich strand is now free to adopt the DNA tetraplex conformation. It may do so spontaneously (3), or this event may be catalyzed by the heterogeneous ribonucleoprotein particle A/B (hnRNP A/B). The hnRNP A/B is a positive regulator of c-myc transcription (17) that belongs to a family of proteins that bind sequence specifically to guanine-rich strands and promotes the formation of DNA tetraplex structures (18–20). The factor that appears most central to initiation of c-myc transcription from the nuclease hypersensitive site is the heterogeneous nuclear ribonucleoprotein K (hnRNP K). It is a ubiquitous enzyme, which is oblivious to the nuclease hypersensitive site when residing in a double-stranded B-DNA conformation, but binds sequence specifically to the cytosine-rich strand with high affinity (21). Upon doing so, the hnRNP K recruits the RNA polymerase II machinery to the promoter (22) and transcription starts. The formation of a nuclease hypersensitive site has now activated the c-myc gene and it may undergo multiple rounds of transcription until the nuclease hypersensitive site reverts to a silent B-DNA conformer, which can again be accommodated in a regular chromatin fiber.

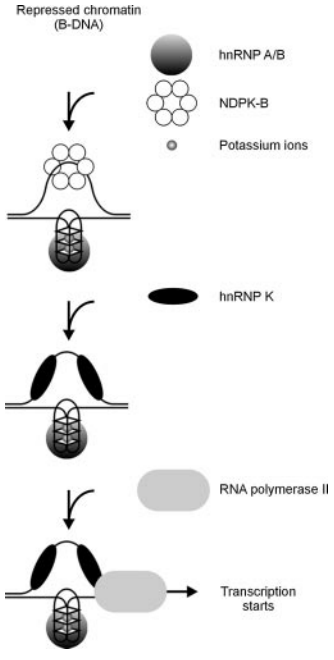

FIG. 3. A model for c-myc activation (see Discussion for details).

Even though the oligonucleotide was either a largely unstructured single-strand, or had adopted the DNA tetraplex conformation prior to administration, it does not remain trapped in whichever state inside the cell nucleus. As the two possible conformations approach a physiological equilibrium in cell nuclei, they will coexist at a certain ratio irrespective of conformational origin. The molecular framework for c-myc activation then allows identification of two possible points of attack of the 22-base-long oligonucleotide, one for each conformation. If the oligonucleotide is in the unstructured single-stranded conformation, it can hybridize to

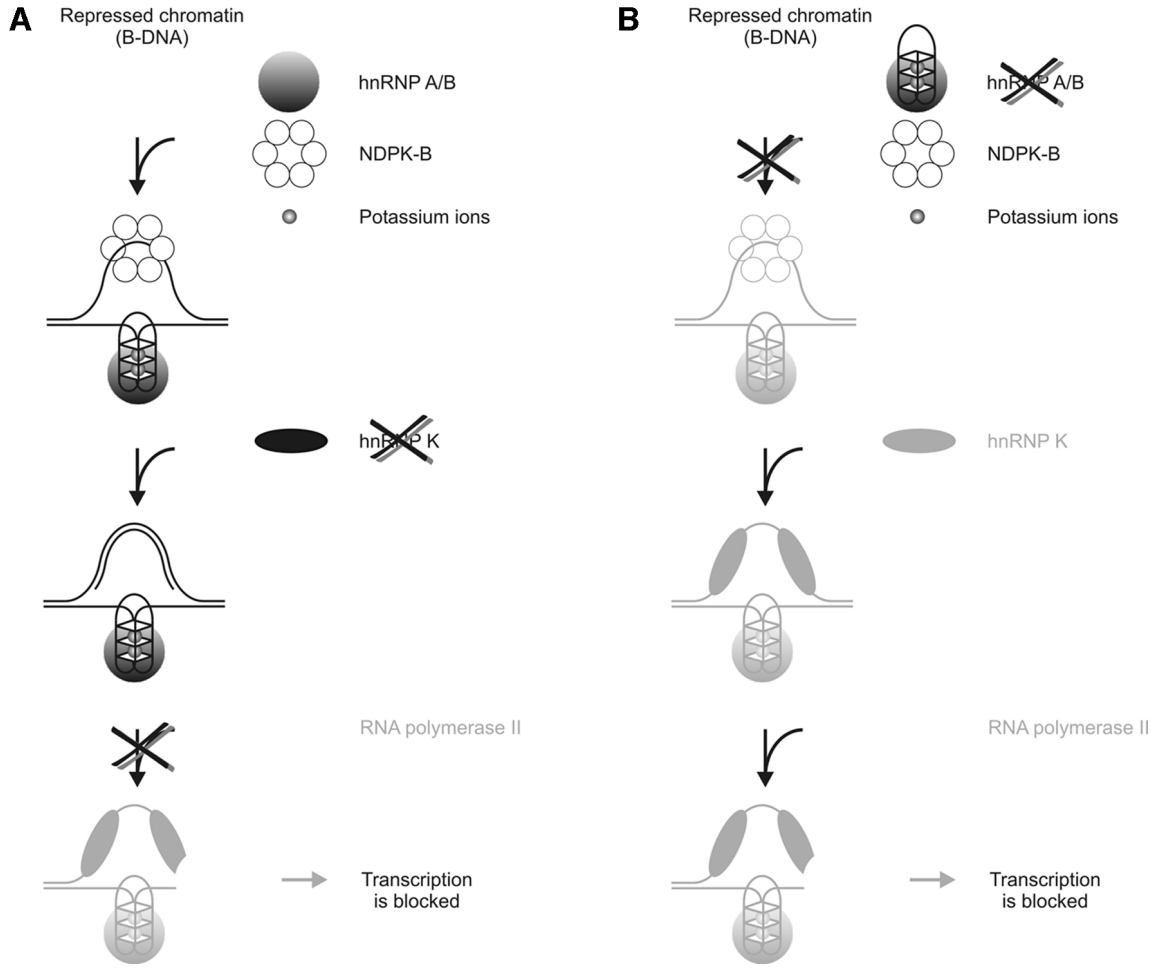

FIG. 4. The synthetic 22-base-long oligonucleotide exerts a dual inhibitory effect on the c-myc-overexpressing Burkitt's lymphoma cell line Ramos. (A) If the oligonucleotide is in an unstructured single-stranded conformation, it can hybridize to the cytosine-rich strand of the nuclease hypersensitive site. The hnRNP K then remains oblivious to the site, preventing assembly of the RNA polymerase II transcription machinery at the promoter. (B) When in a DNA tetraplex conformation, the oligonucleotide sequesters transcription factors specific for the guanine-rich strand of the nuclease-hypersensitive site (hnRNP A/B), which similarly blocks c-myc activation.

the complementary cytosine-rich strand of the nuclease hypersensitive site (Fig. 4A). In this case, the hnRNP K remains oblivious to its native target DNA and is unable to recruit the RNA polymerase II transcription machinery to the promoter. If on the other hand the administered oligonucleotide is folded into the DNA tetraplex structure (Fig. 4B), it serves as a transcription factor decoy, competing effectively with the native target DNA sequence for available hnRNP A/B. In accord with our observations, this effect should unveil only when the oligonucleotide concentration is sufficiently high to sequester most of the hnRNP A/B in the cell nucleus.

To the best of our knowledge the 22-base-long oligonucleotide sequence is unique to the c-myc promoter within the human genome, and inhibitory effects due to hybridization should therefore be c-myc specific. However, the number of genes that are controlled by the hnRNP A/B are unknown, and at increasing concentrations the DNA tetraplex conformation of the oligonucleotide may sequester enough hnRNP A/B to influence the expression of other genes controlled by it. Consequently, at this point we cannot state with absolute certainty that the collective *in vivo* antiproliferative effect of the oligonucleotide is c-myc specific.

Aside from hybridizing to complementary cytosine-rich strands of nuclease hypersensitive sites and sequestering transcription factors that are specific for guanine-rich strands, synthetic oligonucleotides comprising DNA tetraplex forming sequence motifs have two general advantages in relation to oligonucleotides without such motifs. First, they confer resistance against nucleases and are remarkably stable in a cellular environment (23). Second, they penetrate into the cell nucleus with greater efficacy, probably by active transport mechanisms (24), which should potentially allow lower dosage and ultimately make treatment less toxic.

Finally, in addition to the advantages above DNA tetraplex forming oligonucleotides have the capacity to inhibit telomerase (25), which is essential for transformation and immortalization of most neoplastic cell types (26). In the context of the recent important link that the c-MYC protein regulates expression of the telomerase catalytic subunit (27), we conjecture that the DNA tetraplex forming oligonucleotides we use to suppress c-myc, directly or indirectly may also influence telomerase activity. This potential synergistic effect warrants further investigation, and in conclusion the possible future clinical utility of DNA tetraplex forming oligonucleotides for cancer therapy looks promising.

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