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# Temperature and salt dependence of higher order structure formation by antisense c-*myc* and c-*myb* phosphorothioate oligodeoxyribonucleotides containing tetraguanylate tracts

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## ABSTRACT

**The use of complementary RNA or DNA sequences to selectively interfere with the utilization of mRNA of a target gene is an attractive therapeutic strategy. Two well-studied targets for oligonucleotide therapy are the c-*myc* and c-*myb* proto-oncogenes. It has been reported that sequences which contain four contiguous Gs can elicit a non-antisense response, due to the formation of a homotetrameric G quartet structure. Therefore, it was of interest to determine whether anti-c-*myc* and anti-c-*myb* phosphorothioate DNAs including tetraguanylate form higher order structures under physiologically relevant salt conditions and temperature. First, the identity of the higher order structure was established and was found to be a tetraplex. Employing intracellular (high $K^+$), extracellular (low $K^+$) and normal saline (no $K^+$) salt mixtures, native gel electrophoresis revealed no tetraplex formation at 37°C, the physiologically relevant temperature. On the other hand, tetraplex structure formation was observed at 4 and 23°C. Hence, the potential for these sequences to form tetraplex structures at lower temperatures may not be relevant for their activity in cells and animals at physiological temperature.**

## INTRODUCTION

The potential of oligonucleotides to achieve inhibition of gene expression selectively is being studied intensively (1,2). To be active as an antisense agent, an oligonucleotide has to enter and travel within a cell to reach RNA in the cytoplasm or nucleus and form a specific duplex with the target mRNA. In early experiments, this laboratory reported that targeting the human c-*myc* proto-oncogene with complementary DNA phosphodiesters inhibited proliferation of PMA-stimulated human peripheral blood lymphocytes (3) and human promyelocytic leukemia cells (4). The same sequence, as a DNA methylphosphonate, inhibited MYC protein levels in peripheral and splenic lymphocytes of an Eμ-*myc* transgenic mouse model for Burkitt's lymphoma (5). Similarly, the anti-c-*myc* sequence, as a DNA phosphorothioate, inhibited arterial restenosis by smooth muscle cells in a porcine model (6). In parallel studies, tumorigenesis by human leukemia cells (7) and human melanoma cells in SCID mice (8) was inhibited by targeting the c-*myb* proto-oncogene with complementary DNA phosphorothioates. Recently, this laboratory found that targeting murine c-*myc* with complementary DNA phosphorothioates in the Eμ-*myc* mouse lymphoma model led to a prophylactic effect against lymphoma development (9).

The antisense oligonucleotide sequences used in the experiments above (Table 1) contained contiguous tetraguanosine tracts, which have been found capable of sequence-independent antiproliferative effects on cultured human epithelial cells (10). Similarly, it was observed that high concentrations (30–50 μM) of antisense phosphorothioate DNAs directed against c-*myc* and c-*myb* showed non-antisense activity in primary cultures of smooth muscle cells *in vitro* and in arteries *ex vivo* (11). The antiproliferative effects were ascribed to the contiguous tetraguanylates present in those antisense sequences. Rows of guanosines are necessary for telomere function and are known to form a G quadruplex or tetraplex structure (12). Formation and dissociation rates of G tetraplex structures are deceptively slow and vary widely depending upon the 5′ and 3′ flanking sequences (13–15). For example, full reassociation of dTGGGT phosphodiester single strands in 1 M KCl, 0.1 mM EDTA, 10 mM $K_2HPO_4$, pH 7, at 0°C, required 20 h (13). For dTTGGGGTT phosphorothioate single strands in phosphate-buffered saline (PBS) at 37°C, the fourth order association rate constant was $6\times 10^4/M^3$ s (15).

Thus it was suggested that since the c-*myc* and c-*myb* antisense sequences contain G4 tracts, they could form tetraplex structures, interfering with base pairing with the target mRNA, exclusively imparting their therapeutic effect through a non-antisense mechanism (11). On the other hand, it has not been demonstrated that these sequences act via a non-antisense, G quartet mechanism at the low concentrations (0.1–10 μM) used in many other systems where these sequences were found to be effective (3–9). Furthermore, it has been found that tetraguanylates do not always give rise to a non-antisense effect (16). Therefore, it was of interest to explore the formation of higher order structures by the c-*myc* and c-*myb* phosphorothioate DNA antisense sequences *in*

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*vitro* under intracellular and extracellular salt conditions at a variety of temperatures, especially at the physiologically relevant 37°C, by native gel electrophoresis. At the onset the identity of the tetraplex band was unambiguously established. Although these tetraguanylate-containing sequences form tetraplexes and another structure with the mobility of a duplex in certain salts at 4 and 23°C, the tetraplex structures were not observed at 37°C under physiologically relevant conditions.

## MATERIALS AND METHODS

### Oligodeoxynucleotide synthesis and purification

Sequences synthesized (Table 1) included human (HMYC) (3) and mouse (MMYC) (9) c-*myc* antisense oligonucleotides against codons 1–5, reversed (MREV) and scrambled (MSCR) (9) mouse antisense c-*myc* sequences, a human c-*myb* antisense oligonucleotide (8) against codons 3–7 (HMYB) and a sequence (QG14) with two tetraguanylate tracts previously shown to form a tetraplex structure (17), used as a positive control sequence. A sequence complementary to the MSCR sequence was synthesized to allow formation of a duplex control. Also, MYC10, a 3′ decamer fragment of MMYC, was synthesized for heterogeneous tetraplex formation with MMYC.

**Table 1.** Oligodeoxynucleotide sequences

| Description | Label | Sequence |
|---|---|---|
| Mouse c-*myc* (antisense) | MMYC | 5′-dCACGTTGA**GGGG**CAT |
| Mouse c-*myc* (reverse) | MREV | 5′-dTAC**GGGG**AGTTGCAC |
| Mouse c-*myc* (scrambled) | MSCR | 5′-dCTGCTGAGAGTCGAG |
| Human c-*myc* (antisense) | HMYC | 5′-dAACGTTGA**GGGG**CAT |
| Human c-*myb* (antisense) | HMYB | 5′-dGTGCC**GGGG**TCTTCG |
| Decamer c-*myc* (antisense) | MYC10 | 5′-dTGA**GGGG**CAT |
| Decamer c-*myc* (scrambled) | SCR10 | 5′-dCTGCTGAGAG |
| Tetraplex control[a] | QG14 | 5′-dT**GGGG**AGCT**GGGG**T |

[a]Phosphodiester sequence, from Sen and Gilbert (17). All other sequences are phosphorothioates. The tetraguanylate portions in the sequences are underlined.

Oligodeoxynucleotides were synthesized by standard phosphoramidite coupling to produce normal phosphodiesters (18) or phosphorothioates (19). When desired, oligonucleotides were 5′-radiolabeled with [$\gamma$-$^{33}$P]ATP (2000 Ci/mmol; no. NEG-602H; DuPont/New England Nuclear, Boston, MA) using T4 polynucleotide kinase (no. M410A; Promega, Madison, WI) (4). Full-length oligonucleotides were purified from failure sequences by electrophoresis on denaturing 20% polyacrylamide gels in 89 mM Tris–borate, pH 8.3, 1 mM EDTA (TBE) plus 7.0 M urea, then extracted by crushing and soaking. The purified DNAs were desalted using $C_{18}$ Sep-Pak cartridges (no. 51910; Waters, Milford, MA). Oligonucleotides were quantitated by absorbance at 260 nm as described (20). Oligonucleotide purities were re-analyzed, following purification and desalting, by 20% polyacrylamide–7 M urea denaturing gel electrophoresis. Oligonucleotide bands were visualized with Stains-All (no. 19171; BioRad, Richmond, CA) following the standard protocol supplied by the manufacturer. A calibration experiment for linearity of tetraplex staining with 0.1, 0.3, 0.5, 0.7 and 0.9 nmol MMYC pre-incubated in solution A and analyzed at 4°C displayed linear densitometry results with a correlation coefficient of 0.964. Gels with [5′-$^{33}$P]oligonucleotides were quantitated with a PhosphorImager 445 SI (Molecular Dynamics, Palo Alto, CA).

### Native gel electrophoresis

Samples were incubated in three different salt mixtures: intracellular salt, 150 mM KCl, 10 mM $Na_2HPO_4$, 1 mM EDTA, pH 7.0 (solution A); extracellular salt, 150 mM NaCl, 10 mM $K_2HPO_4$, 1 mM EDTA, pH 7.0 (solution B); control lithium, 150 mM LiCl, 10 mM $Li_2HPO_4$, 1 mM EDTA, pH 7.0 (solution L); normal saline, 137 mM NaCl, 1.5 mM $K_2HPO_4$ and 8 mM $Na_2HPO_4$, pH 7.0 (solution S). In each case, the 1 nmol samples (2.0 µl at 0.5 mM, tightly sealed to prevent evaporation) were denatured at 90°C for 4 min, then incubated in the given salt solution for 24 h, unless otherwise specified, at 4, 23 or 37°C. Formation of higher order structures was studied by non-denaturing electrophoresis on 20% polyacrylamide gels, $10 \times 8 \times 0.1$ cm, in TBE in a Protean II electrophoresis chamber (BioRad, Hercules, CA). In this apparatus, the glass plates containing the gel are immersed on both sides in running buffer, which serves as a heat sink to dissipate the heat generated by electrophoresis and equilibrate the temperature of the gel with that of the buffer. Electrophoretic analyses were carried out in a cold room at 4°C for 12 h or on a laboratory bench at 23°C for 8 h or in a warm room at 37°C for 5 h, because oligonucleotide mobility increases with temperature. Native gels were also electrophoresed at 50°C in a water bath for 3 h after incubating the samples at 4°C for 48 h. The gels and the running buffers were pre-run and pre-equilibrated at the corresponding temperature for at least 4 h, until current, voltage and temperature were equilibrated, before the samples were loaded onto the gels. All gels were run with samples at a constant electric field of 6 V/cm, at a current of 20 mA, dissipating power of 1.0 W (0.24 cal/s) for an 8 cm gel. At this heating rate, the 800 ml of upper and lower reservoir buffer would be predicted to increase in temperature by 0.0003°C/s, or 1.1°C/h, unless cooled by the surrounding air. The temperatures of the electrophoresis buffers, which are presumed to be in equilibrium with the immersed gels, were monitored during electrophoretic analyses and were never found to vary by >1°C from the 4, 23 or 37°C temperatures of their environments.

### Tetraplex formation by the anti-c-*myc* sequence

To demonstrate tetraplex formation by the anti-c-*myc* phosphorothioate pentadecamer, MMYC was mixed in 1:1 molar ratio with a decamer, MYC10, lacking the five 5′ residues, then heat denatured at 90°C for 4 min and annealed at 4°C for 48 h in the presence of 150 mM KCl, 10 mM $Na_2HPO_4$, 1 mM EDTA, pH 7.0 (solution A). The mixture was analyzed by electrophoresis on a 20% polyacrylamide native gel at 4°C.

### Tetraplex formation by anti-c-*myc* phosphorothioate DNA collected from micro-osmotic pumps

Alzet micro-osmotic pumps (no. 2002; Alza, Palo Alto, CA) are utilized in this laboratory as a controlled slow release device for the delivery of oligonucleotides in transgenic mice (9). The pumps were removed from subjects under anesthesia 2 weeks after s.c. implantation. Immediately after removal, the pumps were placed in a 37°C room. Residual oligonucleotides were

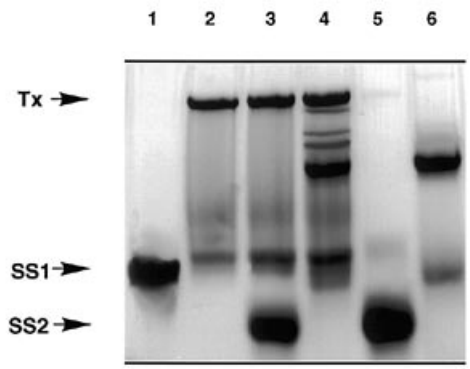


**Figure 1.** Molecularity of higher order structure formation by anti-c-*myc* sequence MMYC, tested by formation of hybrid complexes with MYC10. Samples (0.5 mM) were incubated for 24 h at 4°C in intracellular salt, 150 mM KCl, 10 mM $Na_2HPO_4$, 1 mM EDTA, pH 7.0 (solution A), then analyzed by non-denaturing gel electrophoresis at 4°C and visualized with Stains-All. Lane 1, MSCR (SS1); lane 2, MMYC, (Tx and SS1); lane 3, MMYC (Tx and SS1) and SCR10 (SS2); lane 4, MMYC and MYC10; lane 5, SCR10 (SS); lane 6, MYC10 (Tx and SS). Lane 4 shows six bands, $(MMYC)_4$, $(MMYC)_3(MYC10)_1$, $(MMYC)_2(MYC10)_2$, $(MMYC)_1(MYC10)_3$ and $(MYC10)_4$ and SS1, implying tetraplex formation.

taken out of the pumps and the concentrations measured by UV absorption. The concentration of oligonucleotides remaining inside the pumps after 2 weeks varied from 4.5 to 5.0 mM. Samples were analyzed on native polyacrylamide gels at 37°C. Aliquots of the collected samples were also incubated at 4°C for 24 h and analyzed on native polyacrylamide gels at 4°C.

### Tetraplex formation in serum

Aliquots of oligonucleotides collected from micro-osmotic pumps were incubated in 50% heat-inactivated fetal bovine serum, 50% PBS for 1, 4 or 24 h at 37°C. The samples were then analyzed by electrophoresis under native conditions at 37°C. These samples were also incubated at 4°C for 24 h, then incubated for 1, 4 and 24 h at 37°C and subjected to electrophoresis at 37°C.

### Temperature-dependent transitions of higher order structures

Oligonucleotides were incubated in the three salt mixtures (A, B and S) at 4°C for 7 days to maximize higher order structure formation and then analyzed by electrophoresis at 4, 23, 37 and 50°C, to observe any transition of structure from the complexes formed after 7 days incubation.

## RESULTS

### Tetraplex formation by anti-c-*myc* sequence

To confirm that the anti-c-*myc* sequence MMYC forms a tetraplex structure, MMYC was mixed with MYC10, which lacks the first five 5′ nucleotides of MMYC but includes the tetraguanylate tract, and incubated for 48 h at 4°C in solution A, mimicking intracellular salt. The interstrand tetraplex structures if formed would yield five different combinations, $(MMYC)_4$, $(MMYC)_3(MYC10)_1$, $(MMYC)_2(MYC10)_2$, $(MMYC)_1(MYC10)_3$ and $(MYC10)_4$, resulting in five distinct bands. Similar patterns have been observed before with other tetraguanylate sequences, such as QG14 (17,21). The five bands predicted for a tetraplex were observed upon native gel electrophoresis at 4°C (Fig. 1). This result is consistent with the model that the slowest moving band formed by the anti-c-*myc* pentadecamer MMYC is indeed due to a tetraplex structure.

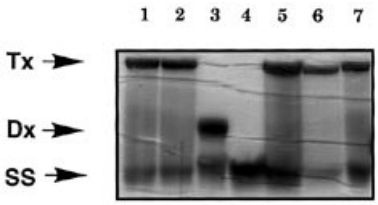


**Figure 2.** Tetraplex formation by tetraguanylate sequences, incubated for 24 h at 4°C in solution A, then analyzed by non-denaturing gel electrophoresis at 4°C, as in Figure 1. Lane 1, MMYC (Tx and SS); lane 2, HMYC (Tx and SS); lane 3, MSCR duplex (Dx); lane 4, MSCR (SS); lane 5, HMYB (Tx and SS); lane 6, QG14 (Tx); lane 7, MREV (Tx and SS).

### Effects of salt on higher order structure formation

Formation of higher order structures, such as tetraplexes, is often studied using high salt conditions (12), which may not be relevant to salt conditions *in vivo*. One tetraplex hypothesis holds that ion fluctuations (especially $K^+$) play a role in the control of nucleic acid-dependent physiological functions via their effect on the structure of G-rich sequences (22). Therefore, physiologically relevant salt conditions were employed in order to determine whether these particular DNA sequences form higher order structures under salt conditions encountered *in vivo*. The sequences may exist in three forms: tetraplex structure (Tx), which migrates on a native gel similarly to a tetraplex formed by QG14 (17); an intermediate structure (Dx), which migrates similarly to a control 15 bp duplex of the MSCR scrambled control and a complement of that sequence; a single strand (SS), which migrates similarly to MSCR, which has not displayed any higher order structures under any of the temperature and salt conditions used. We found that the tetraguanylate sequences QG14, MMYC, MREV, HMYC and HMYB all formed higher order structures (Tx) after 24 h incubation at 4°C under solution conditions mimicking intracellular salt composition (solution A) and native gel electrophoresis at 4°C (Fig. 2). The same tetraplexes were also observed in extracellular salt (solution B) (not shown) and normal saline (solution S) (not shown) after 24 h incubation at 4°C. These results establish that these sequences have the ability to form tetraplex structures, as would be expected from their sequences. Next, the incubation and electrophoretic temperatures were varied to examine the role of temperature in higher order structure formation.

### Higher order structure formation is very dependent on temperature

After incubation of the candidate sequences in solution A at 37°C for 24 h, native gel electrophoresis at 37°C revealed only the intermediate structure (Dx) and single strand (SS) (Fig. 3). The same pattern was observed after incubation at 37°C in extracellular salt (solution B) (not shown) and normal saline (solution S) (not shown). To extend the dynamic range of detection below that possible with Stains-All detection, the 37°C incubation in solution A was repeated with [5′-$^{33}$P]MMYC and [5′-$^{33}$P]QG14. In each case, labeled oligonucleotide was added to unlabeled

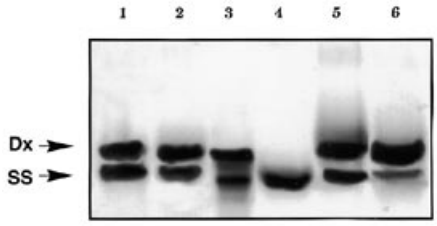


**Figure 3.** Lack of stable tetraplex structures in tetraguanylate sequences, incubated for 24 h at 37°C in solution A, then analyzed by non-denaturing gel electrophoresis at 37°C, as in Figure 1. Lane 1, MMYC (Dx and SS); lane 2, HMYC (Dx and SS); lane 3, MREV (Dx and SS); lane 4, MSCR (SS); lane 5, HMYB (Dx and SS); lane 6, MSCR duplex (Dx and minor SS).

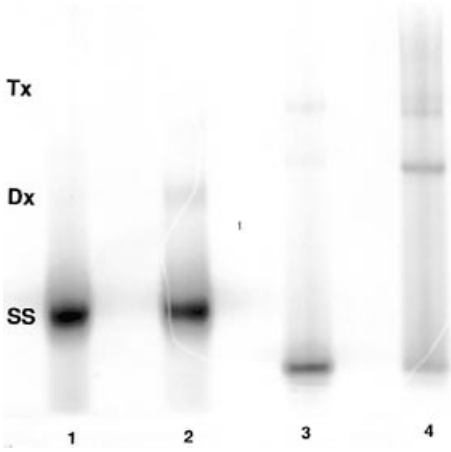


**Figure 4.** Tetraplex formation by [5′-$^{33}$P]tetraguanylate sequences, incubated for 24 h at 37°C in solution A (intracellular salt) or solution L (physiological LiCl), then analyzed by non-denaturing gel electrophoresis at 37°C, as in Figure 1, but visualized by PhosphorImager analysis. Lane 1, MMYC (solution L, SS); lane 2, MMYC (solution A, Dx and SS); lane 3, QG14 (solution L, SS); lane 4, QG14 (solution A, Tx and SS).

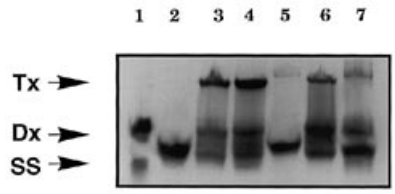


**Figure 5.** Tetraplex formation by tetraguanylate sequences, incubated for 24 h at 23°C in solution A, then analyzed by non-denaturing gel electrophoresis at 23°C, as in Figure 1. Lane 1, MSCR duplex (Dx and SS); lane 2, MSCR (SS); lane 3, HMYC (Tx, Dx and SS); lane 4, MMYC (Tx, Dx and SS); lane 5, MMYC, denatured at 90°C just before loading (SS); lane 6, MREV (Tx, Dx and SS); lane 7, HMYB (Tx, Dx and SS).

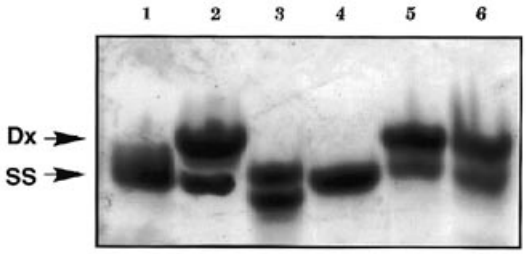


**Figure 6.** Lack of stable tetraplex structures in tetraguanylate sequences, incubated for 24 h at 4°C in solution A, then analyzed by non-denaturing gel electrophoresis at 37°C, as in Figure 1. Lane 1, MMYC, denatured at 90°C just before loading (SS); lane 2, MMYC (Dx and SS); lane 3, HMYC (Dx and SS); lane 4, MSCR (SS); lane 5, HMYB (Dx and SS); lane 6, MREV (Dx and SS).

oligonucleotide in order to maintain an incubation concentration of 0.5 mM. Parallel incubations were carried out in 0.15 M LiCl (solution L), because $Li^+$ disfavors tetraplex formation (12).

Native gel electrophoresis at 37°C, followed by PhosphorImager analysis (Fig. 4) revealed 56% of total label as tetraplex and higher order structures for the tetraplex-forming sequence QG14 (17) in solution A (0.15 M KCl) at 37°C, compared with 2.5% of total labeled QG14 in solution L (0.15 M LiCl). For labeled MMYC, no higher order structures were detectable by eye in solution A or solution L. By PhosphorImager analysis, the ratio of background density in the tetraplex mobility region to the single-stranded MMYC band in solution L (lane 1) was 0.00084. In solution A (lane 2), the ratio of background density in the tetraplex mobility region to the single-stranded MMYC band was 0.00175. Subtraction of the solution L ratio from the solution A ratio yielded an upper limit of 0.00091 possible tetraplex fraction in 0.5 mM MMYC in solution A after 24 h at 37°C.

Thus, although the anti-c-*myc* and anti-c-*myb* sequences have the potential to form tetraplexes at lower temperatures, they were not detected at 37°C, the physiologically relevant temperature, in physiologically relevant salt concentrations. However, after incubation of the candidate sequences in solution A at 23°C for 24 h, native gel electrophoresis at 23°C displayed all three structural forms (Fig. 5). This pattern was repeated after 24 h incubation at 23°C in extracellular salt (solution B) (not shown) and normal saline (solution S) (not shown).

When tetraplex formation was favored as much as possible by incubating at 4°C for 48 h in solution A, but the samples were then analyzed by electrophoresis at 37°C, the non-permissive temperature, only the Dx and SS forms were detected (Fig. 6). This result suggests that the tetraplex structure is not stable at physiological temperature and quickly redistributes to the intermediate structure Dx and single strand. The presence of two distinct bands and the absence of a visible smear implies rapid dissociation of Tx in the sample well before the bands entered the gel. However, these experiments were done after only 48 h incubation at the relevant temperature, which may not necessarily be long enough to achieve equilibrium for a tetraplex structure. This result, following a relatively short period of incubation on the tetraplex time scale, raises the question of what may happen after prolonged incubation under physiological temperature and salt conditions in a therapeutic situation.

### Tetraplex formation by oligonucleotides after 14 days inside Alzet pumps

Alzet micro-osmotic pumps are utilized in this laboratory for long-term delivery of oligonucleotides to Eμ-*myc* transgenic mice (9). These micropumps are implanted s.c. and are designed to deliver oligonucleotides at a steady rate for 14 days. After the end of one such experimental period the micropumps were removed from anesthetized mice, immediately put into a 37°C

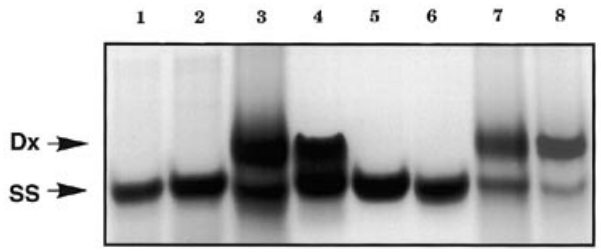


**Figure 7.** Lack of stable tetraplex structures in tetraguanylate sequences recovered from micro-osmotic pumps after 14 days implantation in mice, then analyzed by non-denaturing gel electrophoresis at 37°C, as in Figure 1. Lanes 1, 2 and 5, MSCR recovered from three different mice (SS); lane 6, pure MSCR standard (SS); lanes 3, 4 and 7, MMYC recovered from three different mice (Dx and SS); lane 8, MSCR duplex (Dx and SS).

warm room and residual oligonucleotides were collected by syringe. In this case, the micropumps mimicked the situation of incubating the oligonucleotides at 37°C for 14 days in PBS at 5 mM concentration. Aliquots of the oligonucleotides were analyzed on a 20% polyacrylamide–7 M urea denaturing gel and found to be essentially intact. These samples, when analyzed on a native gel at 37°C, did not show any evidence of tetraplex formation (Fig. 7). The results were identical to the situation where the samples were incubated and analyzed at 37°C. However, when samples of these collected oligonucleotides were incubated at 4°C for 24 h and the gel run at 4°C, there was evidence of substantial tetraplex formation (data not shown). This result demonstrates that although the MMYC anti-c-*myc* sequence has a propensity for tetraplex formation at lower temperatures, tetraplexes were not observed at 37°C, even after 2 weeks in micropumps in mice. This result is significant because the anti-c-*myc* sequence may be free to act as an antisense agent if it is not involved in tetraplex structure, but is primarily single stranded. These *in vitro* situations may not always be comparable with *in vivo* conditions, because proteins and other factors present in serum may facilitate higher order structure formation, as it is known that proteins often mediate the formation of structures by nucleic acids.

### Serum incubation does not facilitate tetraplex formation

In mammalian cell culture or *in vivo* the temperature is usually near 37°C and the oligonucleotides are exposed to a mixture of proteins that might mediate or stabilize a preformed higher order structure. It would be difficult to test this hypothesis *in vivo*, as extraction of the structured oligonucleotides from whole cells or tissues without denaturation, i.e. disruption of the higher order structure(s), may not be possible. Hence, to mimic a similar condition, we incubated the oligonucleotides in the presence of serum. The oligonucleotides collected from the micropumps were incubated in 50% fetal bovine serum, 50% solution S at 37°C for different time periods and then analyzed on native polyacrylamide gels at 37°C. Again, no tetraplex formation was detected (data not shown). Identical bands were observed with or without incubation in serum. Therefore, the serum did not facilitate tetraplex formation. However, in this case the oligonucleotides were not initially in tetraplex form; it was also necessary to examine the case where the tetraplex structure was preformed and then incubated with serum at physiological temperature. Oligonucleotides were first incubated in high $K^+$ intracellular salt (solution A) at 4°C for >48 h to allow substantial tetraplex formation. This incubation was followed by a second incubation at 4°C in 10% fetal bovine serum in RPMI medium. The samples were then analyzed by electrophoresis at either 4 or 37°C. Again, the results were similar to those in the absence of serum (Figs 2 and 6). The samples analyzed at 4°C showed almost complete tetraplex formation, but those analyzed at 37°C did not show any tetraplex, implying that serum proteins under the conditions employed did not stabilize tetraplex formation at 37°C.

### The higher order structure with faster mobility (Dx) is stable even at 50°C

In order to determine the stability of the intermediate structure with the mobility of a pentadecamer duplex formed by all the tetraguanylate-containing sequences, the native gels were also analyzed at 50°C, after preforming the tetraplex structure by incubating in high $K^+$ salt (solution A) for 48 h at 4°C. We found that even at 50°C the tetraplex structure redistributed to the intermediate structure, so that both the single-stranded and the intermediate structure co-existed at 50°C, with a predominance of the latter (data not shown).

## DISCUSSION

Short complementary oligonucleotide sequences targeting c-*myc* and c-*myb* mRNA have been employed as gene-specific inhibitory agents both in cell culture and in animal experiments (3–9). The sequences used for this purpose contain four guanylates in a row, which have the potential to form tetraplex or higher order structures (12). In this work we investigated tetraplex formation by these sequences and control sequences containing tetraguanylates at physiologically relevant temperatures and salt concentrations. Most of the previous studies of tetraplex formation were done with telomeric repeat sequences from *Oxytricha* to human (23,24). Evidence exists that tetraplex formation by a tetraguanylate-containing phosphorothioate sequence, selected by a combinatorial approach, is a potent inhibitor of HIV envelope-mediated cell fusion (25).

Factors that influence the equilibrium of formation of G tetraplex structures are different ions, DNA and ion concentrations, length of the G-rich strand, Watson–Crick complementarity and sequence differences (14,22). The findings from telomeric repeat sequences cannot necessarily be applied to the sequences we investigated, as the antisense oligomers have only one tetraguanylate in a heterogeneous sequence context and sequence plays a major role in tetraplex structure formation (14,22). In particular, tetraguanylates at the 5′-end of octadecamers were reported to favor higher order structures, while tetraguanylates at the 3′-end disfavored higher order structures (14).

The tetraplex structures in our system are presumed to be formed by four separate strands, thereby forming an interstrand G quartet structure, as all the sequences tested contain only one tetraguanylate tract. To test the molecularity of the complex, two oligonucleotide sequences of different lengths, the anti-c-*myc* pentadecamer MMYC and the homologous decamer MYC10, both containing the essential tetraguanylate tract, were incubated together at 4°C then analyzed by electrophoresis at 4°C. Five bands were observed, as predicted, due to all the combinations

possible for interstrand tetraplex formation. Such a strategy has been utilized previously to establish the identity of a tetraplex band (17,21).

In the present study, native gel electrophoresis revealed that all the tetraguanylate-containing sequences studied above are capable of forming tetraplex structures, depending on the temperature. The intracellular (high $K^+$), extracellular (low $K^+$) and normal saline (absent $K^+$) conditions used for the experiments all permitted tetraplex formation when the oligonucleotides (0.5 mM) were incubated at 4°C for 24 h and the gels were run at 4°C. When the same experiments were carried out at 23°C, three structures were observed: the tetraplex, the intermediate structure (Dx), which moves with a 15 nt control duplex, and a single strand. The intermediate structure with mobility similar to a control duplex has been observed in earlier tetraplex studies (17), but the nature of these structures has not yet been elucidated. When the incubations and electrophoresis were carried out at 37°C, only two structures were seen for the antisense sequences, the intermediate (Dx) and the single strand. Under the same conditions, the strong tetraplex control sequence QG14 displayed 56% tetraplex. Hence, higher temperature seems to disfavor the formation of tetraplex. As a negative control, pre-incubation at 37°C in the presence of $Li^+$, without $Na^+$ or $K^+$, revealed no MMYC tetraplex bands and only 2.5% QG14 tetraplex bands after electrophoresis.

Further insight into tetraplex dependence upon temperature was obtained when the samples were incubated at 4°C but analyzed by gel electrophoresis at either 23 or 37°C. In these experiments, similar band patterns were observed as were seen after incubation at 23 or 37°C respectively, indicating that the tetraplex structures rapidly redistributed to other structures when the temperature was raised, as shown by the clear presence of the relevant bands without smeared transitional forms.

It may be argued that the complex array of molecules present inside cells and in serum may stabilize a preformed higher order structure or may mediate formation of it, as evidence exists for a tetraplex binding protein observed to have a stabilizing effect on tetraplex structure (26). To test this hypothesis, the oligonucleotides would have to be retrieved from tissues without denaturation. As an alternative, we incubated the oligonucleotide samples in serum to expose them to serum proteins, to determine whether serum proteins stabilize a preformed higher order structure or mediate the formation of higher order structures. Under these conditions higher order structures were neither stabilized nor mediated by serum proteins and other factors present, though it was found previously that in telomeric repeats, tetraplex structures are clearly involved in specific interactions with protein components and proteins promoting the formation of tetraplex structures (27,28).

These findings are significant with respect to the question of the availability of tetraguanylate-containing antisense sequences in a single-stranded form. The electrophoretic analyses demonstrated that at 37°C at physiological ionic strength, under intracellular or extracellular ionic conditions, there were no detectable tetraplex structures. Even in the case where tetraplex structures already existed, due to equilibration at lower temperatures, they quickly dissociated at physiological temperature to lower order structures. One would therefore expect the same situation to exist under clinical conditions, comparable with the MMYC samples removed from a s.c. micropump after 14 days in mice, when such oligonucleotides are administered for genetic therapy or gene-specific inhibition experiments.

The structures present at physiological and higher temperatures include both the intermediate Dx and the single strand. Since the Dx structure is quite stable, even at higher temperatures, the possibility exists for non-antisense effects by the Dx fraction, in addition to antisense or non-antisense effects by the single strand fraction. The exact nature of the structure Dx needs to be elucidated in further detail, in order to help elucidate non-antisense effects observed with these sequences at high concentrations.

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