Polyamine effects on purine–purine–pyrimidine triple helix formation by phosphodiester and phosphorothioate oligodeoxyribonucleotides

Marco Musso and Michael W. Van Dyke*

Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Received December 20, 1994; Revised and Accepted May 5, 1995

ABSTRACT

Utilization of oligodeoxyribonucleotides to inhibit specific gene transcription *in vivo* (antigene strategy) requires the efficient formation of triple helices under physiological conditions. However, pyrimidine-motif triplexes are not favored at physiological pH, and physiological concentrations of potassium cations hamper purine-motif triplex formation. Here we investigated the effects of polyamines on promoting triplex formation by G/T-rich oligodeoxyribonucleotides containing either phosphodiester or a diastereomeric mixture of phosphorothioate linkages. Compared with Mg^{2+} , equimolar concentrations of polyamines greatly facilitated purine-motif triplex formation with the following order of effectiveness: spermine > spermidine > putrescine. At low polyamine concentrations, phosphorothioate oligonucleotides were better at triplex formation than the corresponding phosphodiester oligonucleotides. Kinetic studies indicated that polyamines facilitated triplex formation by increasing the rate of oligonucleotide–duplex DNA association. However, triplex accumulation with either oligonucleotide was still low under physiological conditions (140 mM K^+ , 10 mM Mg^{2+} , 1 mM spermine). The inhibitory effects of K^+ could be partially overcome with high concentrations of Mg^{2+} or spermine, with phosphodiester oligonucleotides being better able to form triplexes than phosphorothioates under these conditions.

INTRODUCTION

One approach to the therapeutic regulation of specific gene expression, the antigene strategy, involves oligonucleotide-mediated triple helix formation with targeted genomic DNA sequences and a subsequent local inhibition of the transcriptional process (1,2). Two motifs of DNA triple helices, or triplexes, have been described. In the pyrimidine motif, thymidine- and cytosine-rich oligonucleotides bind with a parallel orientation to runs of purine acceptors in the major groove of duplex DNA through Hoogsteen base pairing (3,4). Typically this involves thymidine

binding to adenosine in an A•T Watson–Crick base pair (T:A•T) and either protonated cytidine or 5-methyl-cytidine binding to guanosine ($C^+ : G \cdot C$) in the DNA duplex. Unfortunately, this requirement for cytidine protonation can limit the utility of pyrimidine-motif triplexes under physiological conditions. In the purine motif, guanosine-rich oligonucleotides bind with an antiparallel orientation to runs of purine acceptors in the major groove of duplex DNA through reverse Hoogsteen base pairing (5,6). Base pairing in this motif involves guanosine binding to guanosine ($G : G \cdot C$) and either adenosine or thymidine binding to adenosine ($A : A \cdot T$ or $T : A \cdot T$). These purine–purine–pyrimidine (Pu–Pu–Py) triplexes have been shown to exist at physiological pH, Mg^{2+} and polyvalent cation concentrations, though not at physiological monovalent cation concentrations (7,8).

Several laboratories have reported the use of purine motif triplex-forming oligonucleotides to inhibit transcription from specific genes *in vivo*. Examples include inhibition of human *c-myc* (9), *IL2R α* (10), HIV (11), progesterone receptor-dependent (12), 6-16 (13,14) and SV40 early promoter (15) transcription. While triplex-dependent displacement of transcription factors has been suggested as the cause for the observed inhibition of transcription, how this process occurs in spite of the presence of inhibitory concentrations of monovalent cations *in vivo* remains to be determined. One possible explanation is that certain cellular components facilitate Pu–Pu–Py triplex formation. Polyamines such as spermidine and spermine would be likely candidates, given their high ambient concentrations in eukaryotic nuclei (16,17) and their proven ability to stabilize both duplex (18,19) and pyrimidine-motif triplex DNAs (20,21).

To further our understanding of triplex formation under physiological conditions, we investigated the effects of various polyamines on Pu–Pu–Py triplex formation. G/T-rich triplex-forming oligonucleotides containing either phosphodiester or phosphorothioate linkages were used, the latter being potentially more relevant to *in vivo* applications given their intrinsic resistance to many deoxyribonucleases (22,23). We found that polyamines facilitated Pu–Pu–Py triplex formation to different extents, dependent on the oligonucleotide and polyamine present. We also found that, in the presence of physiological K^+ concentrations, phosphodiester oligonucleotides were consistently

* To whom correspondence should be addressed

better at triplex formation than phosphorothioate oligonucleotides.

MATERIALS AND METHODS

Oligodeoxyribonucleotides and DNA probes

Both G/T-rich, triplex-forming oligodeoxyribonucleotides (TFO) used in this study contained the sequence 5'-TG₃TG₄TG₄TG₃T-3' and either phosphodiester (OPO) or a diastereomeric mixture of phosphorothioate (OPS) linkages. OPO and OPS oligonucleotides were synthesized by phosphoramidite chemistry on an Applied Biosystems DNA synthesizer using 0.017 M I₂ in tetrahydrofuran/pyridine/H₂O (88:10:2) or 0.05 M 3*H*-1,2-benzodithiole-3-one, 1,1-dioxide (Glen Research) in anhydrous acetonitrile, respectively, as oxidizing solutions. Further purification was by *n*-butanol precipitation (24). Concentrations were determined spectrophotometrically, using an average nucleotide molar extinction coefficient at 260 nm of $3.3 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. The duplex DNA probe (T1) used in DNase I cleavage protection assays consisted of the 201 bp *EcoRI*-*PvuII* fragment from pBluescript II SK, containing a 19 bp polypurine-polypyrimidine cassette, 5'-AG₃AG₄AG₄AG₃A-3' and its complement, cloned into the *SacI* site as previously described (8). This probe was singly 3' end-labeled at the *EcoRI* site by Klenow end-filling and purified by NA-45 membrane (Schleicher and Schuell) following the manufacturer's instructions. For electrophoretic mobility shift assays (EMSA), a duplex DNA probe (T4) consisting of the oligonucleotides 5'-TCGAAG₃AG₄AG₄AG₃A-3' and 5'-TCGA-TC₃TC₄TC₄TC₃T-3' was 3' end-labeled by Klenow end-filling and purified by ethanol precipitation.

DNase I cleavage protection (footprinting) assays

Triplexes were formed by incubating 50 nM probe T1 and 1 μM TFO for 60 min at 30°C in a 20 μl volume containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 10 mM MgCl₂, 5 mM dithiothreitol and other components as described in the figure legends. DNase I (2 ng) was then added to each sample, and cleavage was allowed to proceed for 30 s at room temperature and then terminated by the addition of 3 μl stop buffer (3.0 M ammonium acetate, 0.5 M EDTA, 1.0 mg/ml tRNA). Adenine-specific chemical cleavage reactions were used as markers (25). Samples were purified by phenol-chloroform extraction and ethanol precipitation, dried briefly, resuspended in 95% formamide, and heated at 95°C for 4 min before being loaded onto a denaturing 8% polyacrylamide gel. Electrophoretically separated DNA cleavage products were visualized by autoradiography and quantitated by densitometric scanning. Determination of the extent of triplex formation followed normalization of DNase I cleavage efficiency using sites on either side of the footprints.

Electrophoretic mobility shift assays (EMSA)

For our standard EMSA reaction conditions, 1 nM probe T4 and 1 μM TFO were incubated for 60 min at 30°C in a 10 μl volume containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 10 mM MgCl₂ and 0.01% NP-40 to effect triplex formation. These samples were then loaded directly onto a 30%:1% acrylamide-bis-acrylamide gel containing 50 mM Tris-borate, 2 mM MgCl₂ and 5% glycerol, which was electrophoresed at 10 V/cm for 3 h at room

temperature. The dried gels were visualized by autoradiography and quantitated by densitometry.

RESULTS

K⁺ inhibits Pu-Pu-Py triplex formation by phosphodiester- and phosphorothioate-containing oligonucleotides

It has been found that moderate concentrations of certain monovalent cations (e.g., K⁺) inhibit Pu-Pu-Py triplex formation with phosphodiester oligonucleotides possessing either natural or modified nucleic acid bases (7,8). We chose to investigate whether changes in the TFO backbone, specifically substitution of phosphodiester linkages for phosphorothioates, relieved this inhibition. The G/T-rich oligodeoxyribonucleotide 5'-TG₃TG₄TG₄TG₃T-3' was chosen for these studies because of its strong, sequence-specific binding to a complementary homopurine-homopyrimidine duplex DNA target, both as a phosphodiester- and as a phosphorothioate-containing oligonucleotide (5,26,27). TFOs were incubated with labeled duplex DNA under conditions favoring triplex formation (10 mM Mg²⁺ buffered to pH 7.3). The extent of triplex formation was determined by DNase I cleavage protection and quantitated by densitometry. As shown in Figure 1A and B, samples containing either phosphodiester or phosphorothioate TFOs exhibited complete DNase I cleavage protection specifically over the GA strand of the homopurine target on the duplex DNA probe. No qualitative differences were observed in the DNase I cleavage patterns for either TFO. For both OPO and OPS TFOs, increasing concentrations of K⁺ similarly diminished this cleavage protection.

DNase I cleavage protection, while allowing the visualization of triplex formation at specific locations along a duplex DNA probe, is not readily amenable to quantitation, especially under conditions of incomplete triplex formation. For the purposes of this study, we used EMSA to quantitate triplex formation. EMSA allows accurate quantitation of multiple nucleic acid species, both bound and unbound, and may be performed in the presence of many solution components (e.g., polyamines) that would otherwise interfere with the endonuclease cleavage reactions required by DNase I and restriction endonucleases. As shown in Figure 1C and D, nearly quantitative formation of triplexes was observed with either OPO or OPS oligonucleotides under our standard reaction conditions. This data also demonstrates that purine-motif triplexes are essentially stable throughout the course of electrophoresis, thus validating the use of EMSA in analyzing these nucleic acid complexes. Increasing concentrations of K⁺ inhibited triplex formation for both OPO and OPS TFOs, with half-maximal inhibition occurring at 7.6 and 6.6 mM K⁺, respectively. Note that these data differ slightly from those obtained from DNase I footprinting, especially at the lower K⁺ concentrations (1–10 mM). This difference may result from the existence of a weakly bound DNA species that, while capable of preventing DNase I cleavage during the course of a 30 s reaction, is not sufficiently stable to survive gel electrophoresis for several hours at room temperature.

Differential facilitation of Pu-Pu-Py triplex formation by polyamines

Polyamines are well recognized for stabilizing both double-helical (18,19) and pyrimidine-motif triplex DNAs (20,21); however their effects on purine-motif triplex formation are not as

Figure 1. Inhibition of Pu-Pu-Py triplex formation by potassium ions. (OPO), triplex-forming oligonucleotide containing phosphodiester linkages; (OPS), TFO containing phosphorothioate linkages. (A) DNase I cleavage protection analysis. Triplex formation was performed in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 10 mM MgCl₂, 5 mM dithiothreitol, 1 nM probe T1, 1 μM TFO and K⁺ at the concentration indicated above each lane. Lane A, products of an adenine-specific cleavage reaction; lane D, control DNase I cleavage pattern. The solid bar at right indicates the region protected against DNase I cleavage by oligonucleotide binding. (B) Quantitation of data from DNase I cleavage protection assays. (C) EMSA. Buffer conditions were identical to those used in DNase I footprinting, except for the use of duplex oligonucleotide probe T4. Lane C, probe alone control. T, triplex species; D, duplex species. (D) Quantitation of data from EMSA.

well established. We used EMSA to investigate the effects of various polyamines on triplex formation by both phosphodiester and phosphorothioate G/T-rich oligodeoxyribonucleotides. Reactions were performed under standard conditions [40 mM Tris-Cl⁻ (pH 7.3, 30°C), 1–10 mM polycation, 0.01% NP-40, 1 nM probe and 0–1 μM TFO], with different combinations of polycation and TFO. Reaction mixtures containing the polyamine spermine were supplemented with 1 μM of the oligonucleotide PA1 (5'-GATC-CTCTAGAGTC-3'), which served as a carrier to prevent probe precipitation when very low concentrations (<10 nM) of total nucleic acids were present. Triplex formation was visualized by autoradiography and quantitated by densitometry. The results were plotted as a function of the final TFO concentration and subjected to non-linear regression analysis with curve fitting to a standard saturation binding curve.

Graphs of the data are shown in Figure 2. For both OPO and OPS TFOs, polyamines facilitated complete triplex formation with an observed order of effectiveness: spermine > spermidine > putrescine, with the divalent cation Mg²⁺ having an effectiveness intermediate between the last two. For example, 50% triplex formation occurred at 90 nM OPO TFO in the presence of 10 mM

Mg²⁺ and at only 12 nM OPO TFO with 1 mM spermine present. Similar results were also found with these two cations and the OPS TFO. However, significant differences in triplex formation potentials for the OPO and OPS TFOs were observed when the polyamines putrescine and spermidine were present. For example, in the presence of 1 mM spermidine, 50% triplex formation occurred at 95 nM OPO and 10 nM OPS TFO. Similarly, with 10 mM putrescine, 20% triplex formation occurred with 120 nM OPS and 830 nM OPO TFO.

This apparent difference in triplex facilitation actually reflected the different optimal polyamine concentrations required by phosphodiester and phosphorothioate oligonucleotides. As shown in Figure 3, maximal triplex formation was observed in the presence of 1 mM spermidine and 0.1 mM spermine for phosphorothioate oligonucleotides, while 3 mM of either spermidine or spermine was optimal for phosphodiester oligonucleotides. Increasing triplex formation was observed with both types of oligonucleotides with increasing Mg²⁺ or putrescine concentrations to 100 mM, although putrescine preferentially facilitated triplex formation by phosphorothioates. The decrease in triplex formation observed at the higher polyamine concentra-

Figure 2. Multivalent cation effects on Pu-Pu-Py triplex formation. Shown are plots of data obtained by EMSA performed with either OPO (○) or OPS (●) triplex-forming oligonucleotides. Triplex formation was performed in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 0.01% NP-40 and 1 nM probe T4, OPO or OPS at the concentrations indicated below and the following multivalent cations: (A) 10 mM Mg^{2+} , (B) 10 mM putrescine, (C) 1 mM spermidine, (D) 1 mM spermine. Incubations with spermine also contained 1 μM carrier oligonucleotide PA1 to prevent probe precipitation.

tions reflected a preferential loss of the triplex species under these conditions, apparently through selective precipitation of the triplex species. This effect is shown quite dramatically in Figure 4, where preformed triplexes become increasingly less capable of entering the gel during electrophoresis following their incubation with higher spermine concentrations. Note that this effect was considerably more pronounced for phosphorothioate-containing triplexes with significant precipitation occurring even at 1 mM spermine.

Previously we had found that the extent of purine-motif triplex formation was primarily determined by the rate of association between TFO and duplex DNA (8). Under our standard reaction conditions, 50% maximal triplex formation was observed after 12 min whereas <10% dissociation of triplexes occurred after 24 h. This difference in kinetics is indicative of considerable hysteresis with these triplexes and that we are not observing their formation under equilibrium conditions. To determine whether an increased association rate was also responsible for the increase in triplex formation observed in the presence of polyamines, we investigated the kinetics of triplex formation in their presence. Reaction mixtures containing either 0.1 μM OPO or OPS TFO and either 10 mM Mg^{2+} or 1 mM spermidine were incubated for various times (0–60 min) at 30°C before being stopped by the addition of

K^+ (100 mM final concentration). Moderate concentrations of K^+ have been shown to rapidly and completely prevent further purine-motif triplex formation without causing significant dissociation of existing triplexes (8). Reaction products were analyzed by EMSA, as described above, with the exception that the percentage of total probe present in a triplex species was plotted as a function of incubation time and subjected to non-linear regression analysis with curve fitting to an exponential association curve. The results are shown in Figure 5. Note that a best fit was obtained when a brief (5–10 min) lag period was introduced for the data from OPO TFO in either Mg^{2+} or spermidine or the OPS TFO in Mg^{2+} . As seen in these examples, spermidine increased triplex accumulation to a greater extent than Mg^{2+} for both OPO and OPS TFOs. However, this increase was more pronounced for the OPS TFO, in good agreement with the increased extent of triplex formation found for OPS TFOs with low concentrations of spermidine (Figs 2 and 3). Note that under these reaction conditions complete triplex formation could be achieved with either oligonucleotide or cation after a sufficient period of time (e.g. 600 min). Thus the differences we observed most likely reflect differences in the initial rates of triplex formation for different combinations of cations and oligonucleotides.

Figure 3. Optimal multivalent cation concentrations for Pu-Pu-Py triplex formation. Shown are plots of data obtained by EMSA performed with either OPO ($\circ$) or OPS ($\bullet$) triplex-forming oligonucleotides. Triplex formation was performed in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 0.01% NP-40, 1 nM probe T4 with the following multivalent cations and TFO concentrations: (A) Mg^{2+} and 100 nM TFO, (B) putrescine and 100 nM TFO, (C) spermidine and 10 nM TFO, (D) spermine, 10 nM TFO and 1 μ M carrier oligonucleotide PA1.

Mechanism of polyamine facilitation of Pu-Pu-Py triplex formation

Facilitation of purine-motif triplex formation by polyamines could proceed through several possible mechanisms. These include polyamine-dependent changes in the solution structure of the triplex-forming oligonucleotide that place it in a conformation amenable to triplex formation. Alternatively, polyamines may interact with the duplex DNA target, making it a better environment for associating with the G/T-rich oligonucleotide. To discriminate between these two possibilities, we performed an order-of-addition experiment. Either the TFO or the duplex DNA probe was first preincubated at 30°C with 1 mM spermidine for 5 min, the remaining nucleic acid then added, and the incubation allowed to proceed for an additional 60 min. Control reactions had both nucleic acids present in the first incubation and spermidine added during the second. These order-of-addition experiments were performed with both OPO and OPS oligonucleotides. Data from a representative experiment are shown in Figure 6A, with quantitation of triplex formation presented in Figure 6B. For both OPO and OPS TFOs, preincubation of the oligonucleotide with spermidine inhibited subsequent triplex formation, while preincubation of the duplex DNA probe with spermidine had a stimulatory effect as compared to the control. The magnitude of these effects was greatest for the OPO oligonucleotide, though in all cases the extent of triplex formation

was always greater with the OPS TFO. Note that an increase in single-stranded DNA was observed when the radiolabeled probe DNA was incubated in the absence of spermidine. We believe this results from a melting of the short (27 bp) duplex DNA under these low ionic strength conditions.

Pu-Pu-Py triplex formation under physiological conditions

Ultimately, the utility of G/T-rich oligonucleotides as antigene therapeutics requires their ability to form purine-motif triplexes *in vivo*. We found that polyamines such as spermidine and spermine facilitated triplex formation ≥ 10 -fold when present at physiological concentrations. Whether this effect was sufficient to allow purine-motif triplex formation under physiological conditions was investigated by EMSA. Various concentrations of either OPO or OPS TFO were incubated with 1 nM probe and 1 μ M carrier PA oligonucleotide in a buffer containing 140 mM K⁺, 10 mM Mg^{2+} and 1 mM spermine, ionic concentrations considered representative of intracellular conditions (28). As shown in Figure 7A, under these conditions little triplex formation was observed with even the highest concentrations of TFO, though the phosphodiester oligonucleotide appeared to perform better than the phosphorothioate oligonucleotide. We then investigated whether increasing concentrations of Mg^{2+} or spermine could overcome the inhibitory effects of physiological

Figure 4. Selective precipitation of Pu-Pu-Py triplexes by high concentrations of spermine. Triplexes were first formed following incubation for 60 min at 30°C in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 0.3 mM spermine, 0.01% NP-40, 1 nM probe T4 and 1 μM oligonucleotide OPO, OPS or PA1 (control), as indicated. Spermine (Spm), to a final concentration as indicated above each lane, was then added and the incubation allowed to proceed for an additional 60 min prior to electrophoresis. D, duplex species; T, triplex species; W, probe trapped in gel well.

K⁺ concentrations. As shown in Figure 7B, increasing concentration of either Mg²⁺ or spermine alone significantly facilitated triplex formation with the OPO TFO in the presence of 140 mM K⁺, while with the OPS TFO only Mg²⁺ had an appreciable effect on this process. No significant cooperative effect was observed for triplex formation with the OPO TFO in the presence of 100 mM Mg²⁺ and increasing spermine concentration. However, addition of even low concentrations of spermine greatly reduced triplex formation with the OPS TFO, even when high concentrations of Mg²⁺ were also present. We believe this apparent lack of OPS triplex facilitation with spermine does not accurately reflect triplex formation under these conditions, but rather reflects the reduced solubility of the spermine-OPS-duplex DNA complex (see also Figs 3 and 4).

DISCUSSION

We found that millimolar concentrations of the natural polyamines spermine and spermidine promoted efficient Pu-Pu-Py triplex formation under our *in vitro* conditions. For example, comparable levels of triplex formation were observed with 1 μM TFO in the presence of 10 mM Mg²⁺ and 0.1 μM TFO in the presence of 1 mM spermine. The effectiveness of these polyamines was directly proportional to their number of potential positive charges, with the order: spermine⁴⁺ > spermidine³⁺ > putrescine²⁺ being observed. This trend is consistent with the polyamine stabilization of Watson-Crick duplex and pyrimidine-motif triplex DNAs, as determined by their thermal denaturation profiles (18–21). In our hands putrescine (1,4-diaminobutane) was actually significantly less effective than Mg²⁺ in promoting Pu-Pu-Py triplex formation. This is in contrast to the results obtained with pyrimidine oligonucleotides, where diamines were found to be more effective than inorganic cations in stabilizing triplex DNA (21). Thus not only total ionic charge but also charge density may be an important factor in determining the effectiveness of a particular polycation in facilitating either Pu-Pu-Py or Py-Pu-Py triplex formation.

Most surprisingly, with polyamines we found significant differences in the extent of triplex formation by G/T-rich

Figure 5. Kinetics of Pu-Pu-Py triplex formation in the presence of different multivalent cations. All data were obtained by EMSA. (A) Triplex-forming reactions performed in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 10 mM MgCl₂, 0.01% NP-40, 1 nM probe T4 and 0.1 μM OPO (○) or OPS (●). (B) As above, with 1 mM spermidine instead of 10 mM Mg²⁺.

oligonucleotides containing either a phosphodiester or a mixed diastereomeric phosphorothioate backbone. For example, 50% triplex formation was observed when either 10 nM OPS or 100 nM OPO was incubated with a duplex DNA target in the presence of 1 mM spermidine. This was found to result from the different optimal polyamine concentrations required for triplex formation by OPO and OPS oligonucleotides, where phosphorothioates were consistently more capable of forming purine-motif triplexes at lower polyamine concentrations than phosphodiester oligonucleotides. It is thought that the *pro-R*_P or *pro-S*_P sulfur atom in a phosphorothioate linkage possesses a greater partial negative charge and increased polarizability relative to its oxygen counterpart in a phosphodiester linkage (29). Such characteristics could affect the specificity of cations binding to these linkages and reciprocally affect the purine-motif triplex stabilization potentials of these polycations. At present the interactions between polyamines and duplex DNA are not unambiguously defined (30,31). Thus, despite the availability of high-resolution NMR structural studies for Pu-Pu-Py triplexes (32,33), beyond ascribing our observations to an increased affinity between polyamines and phosphorothioate-containing triplexes, it remains difficult to propose a mechanism for the differential effects of polyamines with OPO and OPS oligonucleotides.

To understand better how polyamines facilitate purine-motif triplex formation, we performed an order-of-addition experiment. Maximal triplex formation required pre-incubation of the duplex DNA with the polyamine; pre-incubation of either OPO or OPS oligonucleotides with spermidine resulted in significantly less triplex. These data suggest that a polyamine-duplex DNA species may favor triplex formation, whereas a potential polyamine-

Figure 6. Order-of-addition effects on Pu-Pu-Py triplex formation. (A) EMSA of triplex formation after preincubation for 5 min at 30°C in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C) and the following components as indicated: (TFO) 1 μM OPO or OPS, (Spd) 1 mM spermidine, (probe) 1 nM T4. Those components absent in the first incubation were then added prior to a second incubation at 30°C for 20 min and EMSA. Lane C, probe alone control. T, triplex species; D, duplex species; S, single-stranded species. (B) Quantitation of EMSA data.

oligonucleotide species may inhibit the reaction. We have previously shown that G/T-rich oligonucleotides can themselves form an altered electrophoretic mobility species upon incubation under conditions favoring triplex formation (8). We have now identified this species as a complex of two G/T-rich oligonucleotides which, rather than being an intermediate along the reaction pathway leading to triplex formation, is actually the product of a competing side reaction (A. -J. Cheng, unpublished observations). These findings have practical implications in the design of oligonucleotide therapeutics containing a tethered polyamine moiety (34). Such molecules may prove to be less effective at triplex formation for oligonucleotides that are G/T-rich.

Polyamine facilitation of triplex formation appeared to occur at the level of oligonucleotide-duplex DNA association, a step we have shown dominates the extent to which purine-motif triplexes accumulate *in vitro* (8). Similarly, the differential effects of shorter polyamines on triplex formation observed with OPO and OPS oligonucleotides were also apparent at the level of oligonucleotide-duplex association. Data we have obtained with point-mutated G/T-rich oligonucleotides revealed that associations between the 3' end of the oligonucleotide and complementary bases on the target duplex DNA are critical for maximal purine-motif triplex formation (35). We would therefore propose

Figure 7. Pu-Pu-Py triplex formation in the presence of physiological K⁺ concentrations. OPO, triplex-forming oligonucleotide containing phosphodiester linkages; OPS, TFO containing phosphorothioate linkages. (A) Oligonucleotide titration. Triplex formation was performed in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 10 mM MgCl₂, 1 mM spermine, 0.01% NP-40, 140 mM KCl, 1 nM probe T4, 1 μM carrier oligonucleotide PA1 and a TFO concentration as indicated above each lane. T, triplex species; D, duplex species. (B) Multivalent cation titrations. Data was obtained by EMSA. Triplex formation was performed in a buffer containing 40 mM Tris-Cl⁻ (pH 7.3, 30°C), 0.01% NP-40, 140 mM KCl, 1 nM probe T4, 1 μM TFO and 1 μM carrier oligonucleotide PA1 and final Mg²⁺ and spermine (Spm) concentration as indicated.

that cation effects on purine-motif triplex formation result from their stabilizing or destabilizing effects on an intermediate involved either in initial docking between oligonucleotide and duplex DNA or early during the triplex annealing process. Polyamines could promote triplex formation by countering the high negative charge density arising from the introduction of the third strand into the major groove of the DNA duplex, whereas monovalent cations might promote oligonucleotide self-associations that are refractory to these early interactions. Ideally what may be required is a moiety tethered to the oligonucleotide 3' end that would facilitate triplex nucleation. Potential candidates include ligands such as methyl green that bind to duplex DNA through its major groove (36).

Can purine-motif triplex formation occur under physiological conditions? If the intracellular concentrations of cations are actually identical to those used in our investigations (i.e. 140 mM K⁺, 10 mM Mg²⁺, 1 mM spermine), then our data would suggest that triplex formation with G/T-rich oligonucleotides should not be very favorable. However, higher concentrations of Mg²⁺ or spermine was capable of facilitating triplex formation in the presence of physiological K⁺ concentrations. The true activity of polycations in the cell nucleus, ranging from divalent cations through basic proteins such as histones, has not been accurately

determined. Thus the formation of purine-motif triplexes *in vivo* remains an open question, though one well within the realm of possibilities.

ACKNOWLEDGEMENTS

We thank Michèle Sawadogo for critical reading of the manuscript. This work was supported by NIH grant CA16672. We also wish to acknowledge the generous support of our studies in molecular modulation of transcription by the American Cancer Society.

REFERENCES

- Hélène, C. (1991) *Anti-Cancer Drug Des.* **6**, 569–584.
- Maher, L.J., III (1992) *Bioessays* **14**, 807–815.
- Moser, H.E. and Dervan, P.B. (1987) *Science* **238**, 645–650.
- Le Doan, T., Perrouault, L., Praseuth, D., Habhoud, N., Decout, J.L., Thoung, N.T., Lhomme, J. and Hélène, C. (1987) *Nucleic Acids Res.* **15**, 7749–7761.
- Beal, P.A. and Dervan, P.B. (1991) *Science* **251**, 1360–1363.
- Durland, R.H., Kessler, D.J., Gunnell, S., Duvic, M., Pettit, B.M. and Hogan, M.E. (1991) *Biochemistry* **30**, 9246–9255.
- Milligan, J.F., Krawczyk, S.H., Wadwani, S. and Matteucci, M.D. (1993) *Nucleic Acids Res.* **21**, 327–333.
- Cheng, A.J. and Van Dyke, M.W. (1993) *Nucleic Acids Res.* **21**, 5630–5635.
- Postel, E.H., Flint, S.J., Kessler, D.J. and Hogan, M.E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8227–8231.
- Orson, F.M., Thomas, D.W., McShan, W.M., Kessler, D.J. and Hogan, M.E. (1991) *Nucleic Acids Res.* **19**, 3435–3441.
- McShan, W.M., Rossen, R.D., Laughter, A.H., Trial, J., Kessler, D.J., Zendegeui, J.G., Hogan, M.E. and Orson, F.M. (1992) *J. Biol. Chem.* **267**, 5712–5721.
- Ing, N.H., Beekman, J.M., Kessler, D.J., Murphy, M., Jayaraman, K., Zendegeui, J.G., Hogan, M.E., O'Malley, B.W. and Tsai, M.J. (1993) *Nucleic Acids Res.* **21**, 2789–2796.
- Roy, C. (1993) *Nucleic Acids Res.* **21**, 2845–2852.
- Roy, C. (1994) *Eur. J. Biochem.* **220**, 493–503.
- Hobbs, C.A. and Yoon, K. (1994) *Antisense Res. Dev.* **4**, 1–8.
- Tabor, C.W. and Tabor, C. (1984) *Annu. Rev. Biochem.* **53**, 749–791.
- Sarhan, S. and Seiler, N. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 1279–1284.
- Russell, W.C., Precious, B., Martin, S.R. and Bayley, P.M. (1983) *EMBO J.* **2**, 1647–1653.
- Thomas, T.J. and Bloomfield, V.A. (1984) *Biopolymers* **23**, 1295–1306.
- Hampel, K.J., Crosson, P. and Lee, J.S. (1991) *Biochemistry* **30**, 4455–4459.
- Thomas, T. and Thomas, T.J. (1993) *Biochemistry* **32**, 14068–14074.
- Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J.S. (1988) *Nucleic Acids Res.* **16**, 3209–3221.
- Ghosh, M.K., Ghosh, K., Dahl, O. and Cohen, J.S. (1993) *Nucleic Acids Res.* **21**, 5761–5766.
- Sawadogo, M. and Van Dyke, M.W. (1991) *Nucleic Acids Res.* **19**, 674.
- Iverson, B.L. and Dervan, P.B. (1987) *Nucleic Acids Res.* **15**, 7823–7830.
- Beal, P.A. and Dervan, P.B. (1992) *Nucleic Acids Res.* **20**, 2773–2776.
- Hacia, J.G., Wold, B.J. and Dervan, P.B. (1994) *Biochemistry* **33**, 5367–5369.
- Alberts, B. (1989) *Molecular Biology of the Cell*, Garland, NY, p 304.
- Frey, P.A. and Sammons, R.D. (1985) *Science* **228**, 541–545.
- Schmid, N. and Behr, J.P. (1991) *Biochemistry* **30**, 4357–4361.
- Egli, M., Williams, L.D., Gao, Q. and Rich, A. (1991) *Biochemistry* **30**, 11388–11402.
- Radhakrishnan, I., de los Santos, C. and Patel, D.J. (1991) *J. Mol. Biol.* **221**, 1403–1418.
- Radhakrishnan, I., de los Santos, C. and Patel, D.J. (1993) *J. Mol. Biol.* **234**, 188–197.
- Tung, C.H., Breslauer, K.J. and Stein, S. (1993) *Nucleic Acids Res.* **21**, 5489–5494.
- Cheng, A.J. and Van Dyke, M.W. (1994) *Nucleic Acids Res.* **22**, 4742–4747.
- Kim, S.K. and Nordén, B. (1993) *FEBS Lett.* **315**, 61–64.