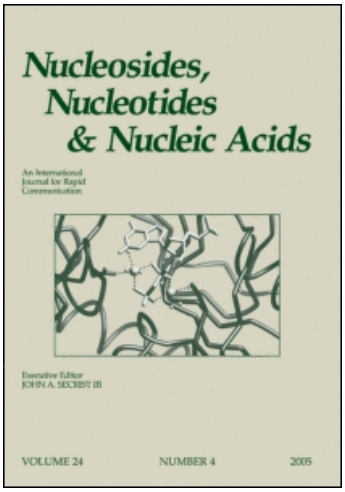

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Systematic Mutation in the Third Strand of a Purine Motif DNA Triple Helix: a Story of a Molecule Which Hides Its Tail

Martin. Mills[a]; Horst H. Klump[a]
[a] Department of Biochemistry, University of Cape Town, Rondebosch, South Africa

To cite this Article Mills, Martin. and Klump, Horst H.(1998) 'Systematic Mutation in the Third Strand of a Purine Motif DNA Triple Helix: a Story of a Molecule Which Hides Its Tail', Nucleosides, Nucleotides and Nucleic Acids, 17: 9, 1919 — 1936
To link to this Article: DOI: 10.1080/07328319808004731
URL: http://dx.doi.org/10.1080/07328319808004731

NUCLEOSIDES & NUCLEOTIDES, 17(9-11), 1919-1936 (1998)

SYSTEMATIC MUTATION IN THE THIRD STRAND OF A PURINE MOTIF DNA TRIPLE HELIX:A STORY OF A MOLECULE WHICH HIDES ITS TAIL[1]

Martin Mills & Horst H. Klump[2]

Department of Biochemistry

University of Cape Town

Private Bag 7700

Rondebosch

South Africa

ABSTRACT

A DNA triple helix formed according to the Purine-motif can accommodate both purines and pyrimidines in the third strand in a pH independent manner. This motif is thus a more versatile means of targeting double stranded DNA than the pH dependent Pyrimidine motif. In this paper we assess the impact of systematically replacing thymine with adenine, inosine or cytosine in the third strand. To this aim we have designed a double length, 22-

[1] *Supported by the South African Foundation for Research Development (FRD)*

[2] *Corresponding author*

mer "purine" strand to target a 9-mer pyrimidine strand such that the extending tail acts as the third strand (reversed-Hoogsteen strand) which is antiparallel to the purine strand of the underlying WC duplex. By systematically replacing thymines with adenines in the reversed-Hoogsteen strand there is an increase in the stability (T_m) of the triplex, particularly when the sequence closest to the loop consists of a stack of purines. Further substitution towards the 3' end of the third strand reverses the stability. Systematic mutations in the third strand next to the loop reveal that the stability of the triads can be ranked according to their effect on T_m in the following order: A-AT > T-AT = I-AT . > C-AT where C is considered a mismatch.

INTRODUCTION

Synthetic DNA oligonucleotides (ODNs) are being developed as drugs called antisense and triplex agents. An antisense oligonucleotide is designed to bind specifically by Watson-Crick base pairing (Watson & Crick, 1953) to a target messenger RNA (sense strand) preventing the mRNA from being translated into the diseased protein. Triplex therapy aims to inhibit unwanted protein by stalling transcription of the gene (review Chan & Glazer, 1997). Triplex strategy has attractive application in directing desirable chemical reactions to DNA target sites (Moser & Dervan, 1987) for chromosome analysis and gene mapping (Hélène *et al.*, 1989; Vasquez *et al.* 1995).

Triple helical complexes were first observed by Felsenfeld *et al.* in 1957 formed by two strands of Uracil (poly U) and on of Adenine (poly A). A homopolypyrimidine third-strand binds via Hoogsteen hydrogen bonding (Hoogsteen, 1959) to the major groove H-bonding sites of a homopurine-homopyrimidine Watson-Crick (WC) double helix now

called the Pyrimidine motif. In 1988 Kohwi and Kowi-Shigematsu described a triple helical complex called H-DNA formed *in vitro* within supercoiled plasmids. It is alleged to form by rearranging part of the duplex sequence, which disproportionates in such a way that one strand dissociates to form a triplex with an intact part of the double helix while the other strand remains single stranded. This triplex conformation has a homopurine third-strand binding via Reverse-Hoogsteen hydrogen bonding (RHG) now called the Purine motif (Frank-Kamenetskii & Mirkin, 1995). The purine motif is of considerable interest as it forms at neutral pH opposed to the pyrimidine motif which consists of C^+-GC and T-AT triads where cytosine must be protonated to form the triplex (Letai *et al.*, 1988).

The purine motif consists of G-GC, T-AT (Beal & Dervan, 1992) and A-AT triads respectively (Dayn *et al.*, 1992). G-GC triads have been characterised by vacuum CD spectroscopy (Johnson *et al.*, 1992), by high-resolution Nuclear magnetic resonance (van Meervelt *et al.*, 1995) and by FTIR spectroscopy (White & Powell, 1995). NMR studies show that the T-G.C triad can also be accommodated (Radhakrishnan & Patel, 1994). Eventually a protonated purine triplex can also form with A^+-G.C triads but only at very low pH (Malkov *et al.*, 1993). Interestingly RNA sequences cannot form the purine triplex motif (Semerad & Maher, 1994).

Formation of purine triplexes is dependent on the presence or absence of certain metal ions. Monovalent cations can inhibit purine triplex formation with K^+ and Rb^+ being most effective followed by NH^{4+}, while Na^+ and Li^+ have little or no adverse effect (Cheng & van Dyke, 1993). Competing equilbria are possible in the presence of Na^+ as guanine rich

strands favour quadruplex formation via G-quartets over triplex formation (Scaria *et al.*, 1992). Stretches of GA repeats within a sequence are susceptible to self-structure formation (Noonberg *et al.*, 1995) and clusters of cytosines in the pyrimidine strand can lead to parallel duplexes with topologically linked $C.C^+$ base pairs (Olivas & Maher, 1995). H-DNA formation was shown to be Mg^{2+} dependent besides requiring supercoiling and switching from Mg^{2+} to Zn^{2+} affected the equilibrium between isoforms. (Kohwi and Kohwi-Shigematsu, 1993).

The effect of sequence composition on purine triplex stability has become a topic of recent interest (Cheng & Van Dyke, 1994; de Bizemont & Hélène, 1996 & Faucon & Hélène, 1996).

In this paper we examine the influence of systematically exchanging thymines for either adenines, inosines or cytosine on the third strand binding energy of a purine motif triple helix. We use a model oligonucleotide system in which a 22-mer oligopurine strand targets a partly complementary single pyrimidine 9-mer strand to form an antiparallel purine triplex (Vo *et al.*, 1995; Mills & Klump, 1997). The pyrimidine sequence is asymmetrical to allow a core WC duplex to form with the 5' side of the 22-mer without interference from the extending RHG strand. Systematic substitutions are made only in the RHG strand. The WC duplex sequence and the number of guanines in the system are invariant.

The technique of UV-melting was used to determine the melting temperature (T_m) of the triplex-coil and duplex-coil transitions. The impact of replacing thymine in the Reverse-

Hoogsteen orientation with adenine, inosine or cytosine is reflected in the T_m and van't Hoff enthalpy (Marky & Breslauer, 1987). It is assumed that the ordered state is the triplex and the unfolded states are two unequally long coiled single strands, and no intermediate state is populated during the transition.

MATERIALS AND METHODS

(A) DNA preparation. Oligonucleotides listed in Table 1 were synthesised by conventional phosphoramidate chemistry on a Beckman 1000M DNA synthesiser and purified by anion exchange HPLC or ordered pure from Eurogentec (Belgium).

(B) UV-melting. Thermal denaturation studies were carried out on a Uvikon 940 spectrophotometer interfaced to an IBM-AT personal computer. The temperature of the cell holder was maintained by a Haake P2 waterbath and controlled by a Haake PG 20 thermoprogrammer to give a cooling and heating cycle at a rate of 0.2 °C /min. Samples contained 2.5μM oligonucleotide strands in 100mM LiAc, 20mM $MgCl_2$, 20mM NaCacodylate at pH 6.0. Absorbencies were recorded at 260nm and subtracted from a baseline recorded at 500nm.

(C) Gel shift experiments (PAGE). Electrophoresis was performed with a 0.5cm thick, 15% polyacrylamide/bisacrylamide (19:1) non-denaturing gel in a 50mM 2-(N-morpholino)ethane-sulfonic acid (MES) buffer (pH 6) containing 10mM $MgCl_2$. Oligonucleotides were ^{32}P labelled with T_4 kinase. Samples were denatured and allowed to cool slowly to anneal with incubation overnight at 4°C in 50mM MES (pH 6)

Table 1. Oligodeoxynucleotide sequences and computed extinction coefficients[1].

Name	Sequence 5'-3'	ε ($\times 10^5$ M^{-1} cm^{-1})
9Y	CCTCTCTTC	0.72
9R	GAAGAGAGG	1.19
22RT	GAAGAGAGG CCTT GGTGTGTTG	2.44
$22RA_5$	GAAGAGAGG CCTT GGTG**A**GTTG	2.50
$22RA_{3,5}$	GAAGAGAGG CCTT GG**A**G**A**GTTG	2.57
$22RA_{7,8}$	GAAGAGAGG CCTT GGTGTG**AA**G	2.57
$22RA_{3,5,7}$	GAAGAGAGG CCTT GG**A**G**A**G**A**TG	2.64
22R	GAAGAGAGG CCTT GG**A**G**A**G**AA**G	2.70
$22RI_5$	GAAGAGAGG CCTT GGTG**I**GTTG	2.47
$22RI_{3,5}$	GAAGAGAGG CCTT GG**I**G**I**GTTG	2.51
$22RC_3$	GAAGAGAGG CCTT GGTG**C**GTTG	2.42

[1] Extinction computed using C, 7400; T, 8700; G, 11500; A 15400; I, 12200 $M^{-1}cm^{-1}$ (Cantor & Schimmel, 1980)

10mM $MgCl_2$, 10% sucrose, 1μM carrier oligo dT_{26}, 1μM cold oligo, then loaded and run at 120 V for 6 hours.

RESULTS AND DISCUSSION

(A) Characterisation of the reference purine triplex (9Y/22RT) with respect to the underlying duplex (9Y/9R).

Figure 1 depicts the proposed folding pathways for a triple helix based on the purine motif

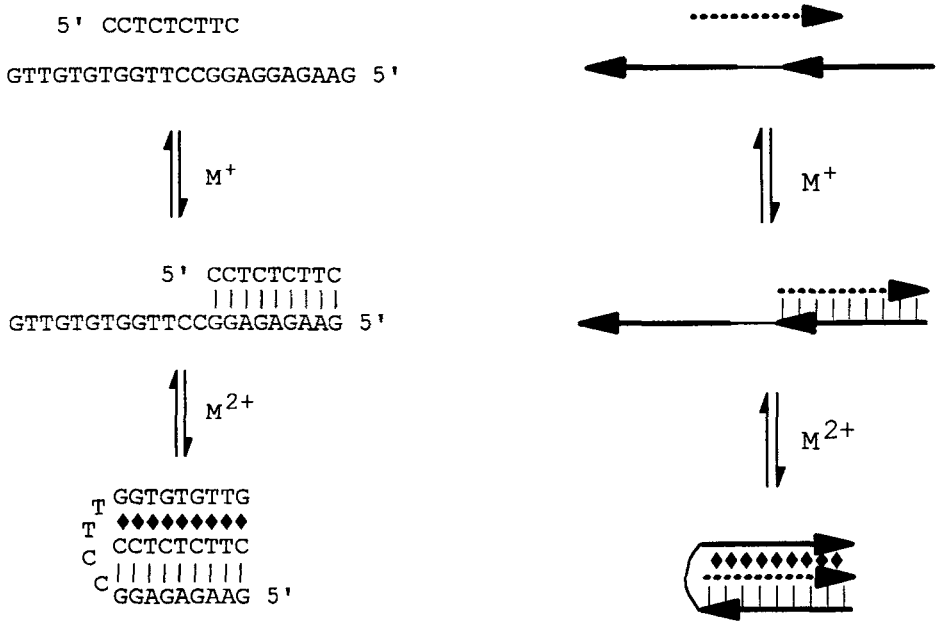

Figure 1. Proposed folding pathway for the formation of a triple helix based on the Purine Motif.

constructed by linking the Crick and Hoogsteen strands via a loop to target the Watson, pyrimidine strand. In the presence of monovalent cations the complementary Watson and Crick sequences bind to form a 9-mer duplex with a 3' single strand extension of the Crick strand. On addition of divalent cations the tail folds on to the duplex to form a stable Reverse-Hoogsteen (RHG) triple helix with G-GC and T-AT triads.

The melting behaviour of the triplex is shown in comparison to the duplex and the purine-rich single strand (22RT) in Figure 2. The insert shows normalised-absorbance at 260nm *vs.* temperature profiles for the 22mer strand alone (22RT, filled triangles) which shows some gradual increase in absorbance but no co-operative melting curve, opposed to the

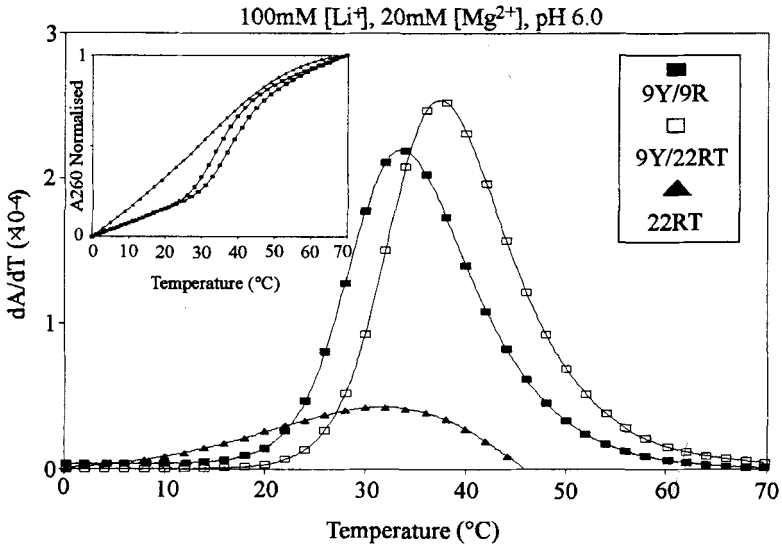

Figure 2. First derivative melting profiles with an insert of normalised melting curves of duplex (9Y/9R, filled squares), purine triplex (9Y/22RT, empty squares) and single strand (22RT, filled triangles).

duplex (filled squares) and purine triplex (empty squares). Both order/disorder transitions are monophasic and this is consistent with the related system of *Vo et al.* (1995) and the intermolecular and intramolecular systems of Svinarchuck *et al.* (1995) and Chen (1991) respectively. The profiles produced on cooling are identical to those produced on heating (0.2 °C min^{-1}) for the short duplex and all triplexes indicating rapid and reversible complex formation. The derivative melting profiles are very similar in shape. This can be seen as an indication that the triplex melts directly to the coil state and that there is no duplex-with-a-tail intermediate at pH 6.0.

Figure 3 shows the magnesium concentration dependency of the stability of purine triplex 9Y/22RT compared to the duplex (9Y/9R). In the absence of Mg^{2+} the melting

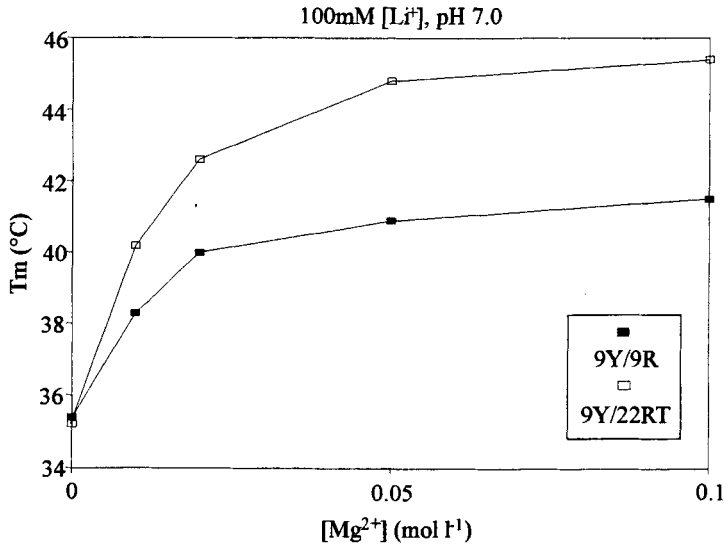

Figure 3. Melting temperatures (T_m) of duplex-coil and purine triplex-coil transitions *vs.* magnesium concentration in 100mM LiAc, 20mM Tris-Ac (pH 7.0).

temperatures are identical indicating that the triplex does not form but the double helix with the 3' extension is present. Both duplex and triplex are not significantly stabilised with addition of more than 20mM Mg^{2+}. The dT_m/dlog [Mg^{2+}] between 10mM and 50mM Mg^{2+} is 2.2 (±0.1) °C and 6.5 (±0.7) °C for duplex and triplex respectively in 100mM [Li^+] at pH 7.0.

Figure 4 shows a scan of a non-denaturing gel carried out at 4°C in which the concentration of labelled target strand (9Y*) is kept constant and either the same strand or complementary strand (9R) or the 22mer ligand (22RT) are added in increasing concentration. Three distinct bands are observed and labelled as single strand (ss), duplex (ds) and triplex (ts) exhibiting increasing retardation related to the size of the complex.

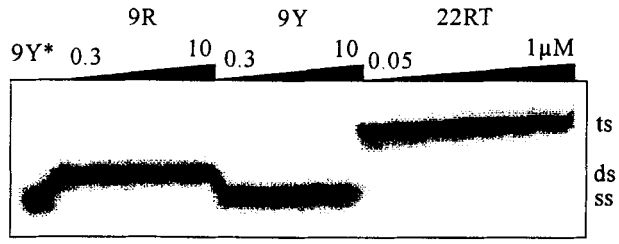

Figure 4. 15% native PAGE of ^{32}P labelled 9mer strand alone (9Y*) with increasing concentration (0.3-10 μM) of complementary strand (9R) and 22mer ligand (22RT, 0.05-1μM) to form the duplex and purine triplex respectively. Addition of more of 9Y has no complex forming influence.

(B) Influence of adenine for thymine exchange on stability of the Purine triplex motif.

Table 2 lists the thermodynamic data ordered according to decreasing T_m due to systematic replacement of the "third-strand" thymines with adenine or inosine. One would think that introducing a large purine base into a purine strand in place of the smaller pyrimidine base should increase the stability of the triplex by gain in stacking interaction. The hypothesis holds true for a first and second replacement of the thymines closest to the loop. A stability maximum is reached at this point. A decrease in T_m follows, however, with further substitution until the all-purine triplex (9Y/22RA) is similar in stability to the G, T triplex (9Y/22RT). A similar trend was observed at pH 7.0 (Mills & Klump, 1997). It appears that an optimum stacking unit is formed when 2 T's nearest the loop are replaced by A's and further substitution may distort the underlying Watson-Crick duplex. In line with this explanation is the observation that the T_m is 4°C lower for 2 T's replaced furthest from the loop (9Y/ $22RA_{7,8}$). To further explore the special role of the 2 T's closest to the loop they were replaced either by inosine or a cytosine mismatch.

Table 2: Thermodynamic data for purine triplex motifs with mutations in the Reverse Hoogsteen Strand ranked according to melting temperature (T_m) in the presence of 100mM LiAc, 20mM $MgAc_2$, 20mM NaCacodylate with 1.25μM strands at pH 6.0.

Ligand Name	Reverse Hoogsteen Strand 5'-3'	T_m[a] (°C)	δT[b] (°C)	$-\Delta H_{vH}$[c] (Kcal mol^{-1})
$22RA_{3,5}$	...GG**A**G**A**GTTG	45.7	20.0	61
$22RA_5$	...GGTG**A**GTTG	43.5	18.5	65
$22RA_{3,5,7}$	...GG**A**G**A**G**A**TG	42.0	20.0	60
$22RA_{5,7}$	...GGTGTG**AA**G	41.4	23.0	52
22RA	...GG**A**G**A**G**AA**G	40.0	21.0	56
$22RI_5$	...GGTG**I**GTTG	39.8	19.5	60
$22RI_{3,5}$	...GG**I**G**I**GTTG	39.1	19.5	60
22RT	...GGTGTGTTG	38.3	19.0	61
$22RC_5$	...GGTG**C**GTTG	35.8	23.5	49
9R	GAAGAGAGG	35.2	18.5	62

[a] Uncertainties in T_m and ΔH values are estimated at 0.5°C and 10% respectively.

[b] δT is the melting range

[c] $\Delta H_{vH} = 2(n+1)\bullet R\bullet(T_m^2/\delta T)$ where n is the number of oligonucleotides binding (n=2), R is the gas constant (2 Kcal mol^{-1} K^{-1}) and T_m is in Kelvin.

(C) Influence of inosine for thymine exchange and inosine for adenine exchange on the stability of a purine triplex.

According to Figure 5 and Table 2 replacing one or two thymines with inosine has hardly any effect on the T_m or van't Hoff enthalpy of the triplex motif. This is a surprising result as one would expect to lose a hydrogen bond when presenting inosine in the Reverse-Hoogsteen orientation. Nevertheless inosine can be accommodated. It's influence can only be seen as negative when replacing adenine. There is a drop in T_m of 3.7°C on replacing an A-AT triad for a I-AT triad. Accordingly there is a drop of 6.6°C for replacing 2A's with 2I's.

(D) Effect of exchanging thymine and adenine for cytosine on triplex stability.

Cytosine has to be considered a mismatch as it does not hydrogen-bond within the purine motif at all and this is evident with a drop in T_m of 2.5°C and a reduction in ΔH_{vH} of 12 Kcal for replacing a single T. Replacing an A with C drops the T_m by 7.7°C and decreases ΔH_{vH} by 16 Kcal. A C-AT mismatch is even 4°C less stable than an I-AT triad.

CONCLUSION

A triple helix constructed from a purine-rich 22mer ligand targeting an all-pyrimidine 9mer strand (Purine Motif) is a sensitive model system to investigate the role of mutations in the Reverse-Hoogsteen (third) strand. Purine motif DNA triplexes can accommodate A-AT, T-AT, I-AT and G-GC triads. By systematically replacing thymines with adenines in the reversed-Hoogsteen strand there is an increase in the stability of the triplex until half of the third strand, actually the sequence closest to the loop, consists of purines. Further substitution in the other section of the third strand lowers the stability of the triplex. This

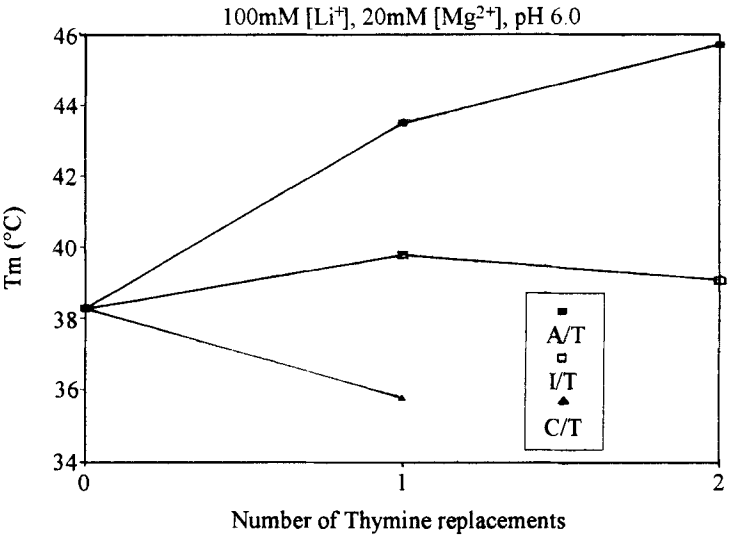

Figure 5. Comparing stability's of the purine triplex with G, T third strand (9Y/22RT) with mutations of one or two Thymines exchanged for Adenine, Inosine or Cytosine.

may be due to distortion of the underlying Watson-Crick double helix. Systematic mutations in the third strand closest to the loop reveal that the triads can be ranked according to the stability of the resulting triplex indicated by T_m in the following order A-AT > T-AT = I-AT . > C-AT where C is considered a mismatch.

ACKNOWLEDGEMENTS

M.M. acknowledges the assistance of Dr. J-L. Mergny of the Laboratoire de Biophysique, Muséum d'Histoire Naturelle (Directors T. Garéstier & C. Hélène), the University Research Council (URC) of UCT and the Foundation for Research Development (FRD) Open Programme for financial support. These results were displayed at the conference entitled "Nucleic Acids and Related Macromolecules: Synthesis, Structure, Function and Applications" in Ulm, Germany in September 1997.

REFERENCES

Beal, P.A. & Dervan, P.B. (1992) The influence of single base triplet changes on the stability of a Pur.Pur.Pyr triple helix determined by affinity cleaving.
Nucleic Acids Res. 20, 2773-2776

Cantor, C.R., & Schimmel, P.R. (1980)
Biophysical Chemistry Vol.1, San Fransisco: Freeman

Chan, P. P. & Glazer, P. M. (1997) Triplex DNA: fundamentals, advances, and potential applications for gene therapy.
J Mol Med 75, 267-282

Chen, F-M (1991) Intramolecular Triplex Formation of the Purine-Purine-Pyrimidine Type
Biochemistry 30, 4472-4479

Cheng, A.J. & Van Dyke, M.W. (1993) Monovalent cation effects on intermolecular purine-purine-pyrimidine triple-helix formation
Nucleic Acids Research 21, 5630-5635

Cheng, A.J. & Van Dyke, M.W. (1994) Oligodeoxyribonucleotide length and sequence effects on intermolecular purine-purine-pyrimidine triple-helix formation
Nucleic Acids Research 22, 4742-4747

Dayn, A., Malkhosyan, S., Mirkin, S.M. (1992) Transcriptionally driven cruciform formation in vivo.
Nucleic Acids Research 20, 5991-5997

de Bizemont, T., Duval-Valentin, G.. Sun, J. S., Bisagni, E., Garéstier T. & Hélène C. (1996) Alternate strand recognition of double-helical DNA by (T,G)-containing oligonucleotides in the presence of a triple helix-specific ligand.
Nucleic Acids Res, 24, 1136-1143

Faucon, B., Mergny J. L. & Hélène C. (1996) Effect of third strand composition on the triple helix formation: purine versus pyrimidine oligodeoxynucleotides.
Nucleic Acids Res 24, 3181-3188

Felsenfeld, G., Davies, D.R., & Rich, A. (1957) Formation of a three stranded polynucleotide molecule
J. Am. Chem. Soc. 79, 2023-2024

Frank-Kamenetskii, M.D. & Mirkin, S.M. (1995) Triplex DNA Structures
Annu. Rev. Biochem. 64, 65-95

Hélène, C., Thuong, N.T., Saison-Behmoaras, T., & Francois, J.C. (1989) Sequence specific artificial endonucleases
Trends Biotechnol. 7, 310-315

Hoogsteen, K. (1959) The structure of crystals containing a hydrogen-bonded complex of 1-methylthymine and 9-methyladenine
Acta Crystallogr. 12, 822-823

Johnson, K.H., Durland, H.R., Hogan, M.E. (1992) The vacuum UV CD spectra of G.G.C triplexes
Nucleic Acids Research 20, 3859-3864

Kohwi, Y. & Kohwi-Shigematsu, T. (1988) Magnesium ion-dependent triple-helix

structure formed by homopurine-homopyrimidine sequences in supercoiled plasmid DNA
PNAS. USA. 85, 3781-3785

Kohwi, Y. & Kohwi-Shigematsu, T. (1993) Structural Polymorphism of homopurine-hompyrimidine sequences at neutral pH. *J. Mol. Biol. 231*, 1090-1101

Letai, A.G., Palladino, M.A., Fromm, E., Rizzo, V. & Fresco, J. R. (1988) Specificity in formation of triple stranded nucleic acid helical complexes: studies with agarose-linked polyribonucleotide affinity columns
Biochemistry 27, 9108-9112

Malkov, V.A. *et al.* (1993) Protonated pyrimidine-purine-purine triplex
Nucleic Acids Research 21, 105-111

Marky, L.A. & Breslauer, K.J. (1987) Calculating thermodynamic data for transitions of any molecularity from equilibrium melting curves.
Biopolymers 26, 1601-1620

Mills, M. & Klump H.H. (1997) A new twist to an old tale: The influence of the exchange of thymine for adenine on the stability of a purine motif DNA triple helix.
South African Journal of Chemistry (in press)

Moser, H.E., & Dervan, P.B. (1987) Sequence-specific cleavage of double-helical DNA by triple helix formation.
Science 238, 645-650

Noonberg, S.B., Francois, J-C., Garéstier, T. & Hélène, C. (1995) Effect of competing self-structure on triplex formation with purine rich oligodeoxynucleotides containing GA repeats
Nucleic Acids Research 23, 1956-1963

Olivas, W.M., & Maher III, J.L. (1995) Competitive Triplex/Quadruplex Equilibria involving guanine-rich oligonucleotides
Biochemistry 34, 278-284

Radhakrishnan I., Patel, D.J. (1994) Solution Structure and Hydration Patterns of a Pyrimidine.purine.pyrimidine DNA Triplex containing a novel T.GC Base-triple
J.Mol.Biol. 241, 600-619

Scaria, P.V., Shire, S.F. & Schafer, R.H. (1992) Quadruplex structure of (G_2T_4G) stabilised by K^+ or Na^+ is an asymmetric hairpin dimer
PNAS. USA. 89, 10336-10340

Semerad, C. L. & Maher, L. J. (1994) Exclusion of RNA strands from a purine motif triple helix.
Nucleic Acids Res 22, 5321-5325

Svinarchuck, F., Paoletti, J. & Malvy, C. (1995) An unusually stable purine(purine-pyrimidine) short triplex
J. Biol. Chem. 270, 14068-14071

Van Meervelt, L., Vlieghe, D., Dautant, A., Galloia, B., Precigoux, G. & Kennard, O. (1995) High-resolution structure of a DNA helix forming (C.G)*G base triplets
Nature 374, 742-744

Vasquez, K.M., Wensel, T.G., Hogan, M.E., Wilson. J.H. (1995) High-Affinity Triple Helix Formation by Synthetic Oligonucleotides at a site within a selectable mammalian gene
Biochemistry 34, 7243-7251

Vo, T., Wang, S. & Kool, E.T. (1995) Targeting pyrimidine single strands by triplex formation: structural optimisation of binding
Nucleic Acids Research 23, 2937-2944

Watson, J.D. & Crick, F.H.C. (1953) Genetic implications of the structure of deoxyribonucleic acids.
Nature (London) 171, 964-967

White, A.P. & Powell, J.W. (1995) Observation of the hydration-dependent conformation of the $(dG)_{20}(dG)_{20}(dC)_{20}$ oligonucleotide triplex using FTIR Spectroscopy
Biochemistry 34, 1137-1142