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Enhanced stabilization of the triplexes $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ and poly r(U:A•U) by water structure-making solutes[1☆]

Laurence Lavelle*,[2], Jacques R. Fresco*

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

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Abstract

A variety of organic cations, cationic lipids, low molecular weight alcohols, sodium dodecylsulfate, trehalose, glycerol, low molecular weight polyethylene glycols, and DMSO were tested for their ability to modulate the stability of the triplexes $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$, poly r(U:A•U) and their respective core duplexes, $d(A\text{-}G)_6$•$d(C\text{-}T)_6$, $d(A)_{21}$•$d(T)_{21}$, poly r(A•U). Very substantial enhancement of triplex stability over that in a physiological salt buffer at pH 7 is obtained with different combinations of triplex and high concentrations of these additives, e.g. trimethylammonium chloride and $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$; 2-propanol and $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$; ethanol and poly r(U:A•U). Triplex formation is even observed with a 1:1 strand mixture of $d(A\text{-}G)_6$ and $d(C\text{-}T)_6$ in the presence of dimethylammonium, tetramethylammonium, and tetraethylammonium-chloride, as well as methanol, ethanol, and 2-propanol. Triplex stability follows the water structure-making ability (and in some cases the duplex unwinding ability) of the organic cations, the low molecular weight alcohols and other neutral organic compounds,

Abbreviations: MA–Cl, Methylammonium Chloride; DMA–Cl, Dimethylammonium Chloride; TriMA–Cl, Trimethylammonium Chloride; TMA–Cl, Tetramethylammonium Chloride; $(TMA)_2$–SO_4, Tetramethylammonium Sulfate; TEA–Cl, Tetraethylammonium Chloride; TPA–Cl, Tetrapropylammonium Chloride; CTriMA–Cl, Cetyltrimethylammonium Chloride; DidecylA–Cl, Didecylammonium Chloride; TridodecylMA–Cl, Tridodecylmethylammonium Chloride; DOSPA, 2,3-dioleyloxy-*N*-[2(sperminecarboxamido) ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate; SDS, Sodium Dodecylsulfate; MeOH, Methanol; EtOH, Ethanol; 2-PrOH, 2-Propanol; 1-BuOH, 1-Butanol; Trehalose, α-D-glucopyranose α-D-glucopyranoside; DMSO, Dimethyl Sulfoxide; PEG 200, poly(ethylene glycol), ave. MW = 200; MB, mixing buffer (contains 0.15 M NaCl + 0.005 M $MgCl_2$ + 0.01 M cacodylate, pH 7.0). Triplexes are symbolized by their base triplets, such that the third strand residue is located to the left of the purine residue of the Watson-Crick base pair, separated by a colon, i.e. U:A•U, C^+:G•C, T:A•T, etc.

☆ This paper is dedicated to Walter Kauzmann on the occasion of his 85th birthday.

*Co-corresponding author. Tel.: +1-310-825-2083; fax: +1-301-206-4038 (L. Lavelle). Tel.: +1-609-258-3927; fax: +1-609-258-2759 (J.R. Fresco).

E-mail address: lavelle@mbi.ucla.edu (L. Lavelle).

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[2] Present address: Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, CA 90095, USA.

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whereas water structure-breaking additives decrease triplex stability. These findings are consistent with those reported in the accompanying paper that triplex formation occurs with a net uptake of water. Since the findings suggest that third strand-binding is facilitated by unwinding of the target duplex, it is inferred that triplex formation may be enhanced by nucleic acid binding proteins operating similarly.

Keywords: Nucleic acid triplexes; Nucleic acid duplexes; Triplex stabilization; Triplex-stabilizing additives

1. Introduction

By virtue of its remarkable specificity [1–4], nucleic acid third strand-binding to target Watson–Crick duplex sequences offers opportunities for modulating replication [5]; for silencing genes by blocking their transcription [6–9]; for accurate delivery of genome-cleaving reagents [10,11]; for delivery of photochemical and other reactive reagents that can damage specific gene sites for the purpose of inducing point mutations via the intercession of genome-repair enzymes [12,13]; for specific gene knockout [14]; and for creating a novel class of convenient cytogenetic probes [15]. In addition, knowledge of the spectrum of substances that can enhance third-strand-binding affinity in vitro and in vivo can provide a better understanding of the physical-chemistry of the binding process itself.

In this report, we describe the results of an extensive survey of ionic and non-ionic solute additives investigated for their potential to enhance the third strand-binding affinity of a particular class of third strands that give rise to triplexes in the pyrimidine/parallel motif, along with an interpretation of aspects of the enhancing effects.

The stabilizing effect of the chloride salts of the organic cations MA^+, DMA^+, $TriMA^+$, TMA^+, TEA^+ and TPA^+ on triplex and duplex stability was first determined. As all but TPA^+ enhance triplex stability, the chloride salts of the cationic lipids (used as transfection agents) $CTriMA^+$ ($TriMA^+$ with one C_{16} tail), $TridodecylMA^+$ (MA^+ with three C_{12} tails), and $DOSPA^{4+}$ (essentially a spermine head with two C_{18} tails) were studied in the hope of achieving even greater triplex stability.

Whereas the accompanying paper [16] reports the stabilizing effect of salts with systematically varied anions that do not interact with the negatively charged triplex backbone, here the varied organic cations do interact with that backbone. The stabilizing effect of the neutral molecules trehalose, glycerol, PEG and DMSO was also examined. The disaccharide trehalose stabilizes proteins at high temperatures and is used for long term storage of dried formulations [17,18] because of its chemical stability (non-reducing disaccharide) and glass forming (T_g 110 °C) and water replacing ability [19]. Glycerol was explored because of its strong dehydrating capacity. PEG is well known to facilitate crystallization of nucleic acids, while DMSO is a broad spectrum solvent of organic and inorganic compounds with a strong hydrogen (H–) bonding ability; it is metabolized [20] and promotes microtubule assembly in plant protoplasts [21].

Three types of triplexes were studied at pH 7. Maximum enhanced stability for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ was observed with 3.0 M TMA−Cl, $T_m = 50$ °C vs. 5 °C in 3.0 M NaCl; for $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ in 50 vol.% 2-propanol + 0.15 M NaCl + 0.005 M $MgCl_2$, $T_m = 65$ °C vs. 23 °C in 0.15 M NaCl + 0.005 M $MgCl_2$; for poly r(U:A•U) in 50 vol.% ethanol + 0.016 M NaCl, $T_m = 53$ °C vs. 26 °C in 0.016 M NaCl. Since the T:A•T and U:A•U base triplets do not require third strand base protonation, whereas the C^+:G•C base triplet does, the stability of $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ increases further with decreasing pH, with a maximum T_m of 75 °C in 3.0 M TMA-Cl at pH 3.7.

An important conceptual finding of this investigation is that the enhancement of triplex stability

by the various compounds correlates with their water structure-making tendencies. Preliminary reports of this work have appeared [22,23].

2. Materials and methods

2.1. Sample preparation, UV spectroscopy, melting profiles and CD spectroscopy

Materials and methods were as described in the accompanying paper [16] and [24]. The UV melting profiles, wavelength scans and difference spectra were not smoothed. Since triplex stability could not be studied below 0 °C, 'no triplex formation' implies 'above 0 °C'. The highest concentration of an additive for which a T_m value is reported is the maximum concentration at which it remains soluble over the entire temperature range. Since triplex stability is dependent on third strand concentration as well as total oligonucleotide concentration, all triplex mixtures contained equimolar proportions of the three strands, and at the same total concentration. Because of their UV absorbance, bromide, iodide and thiocyanide salts could not be investigated.

3. Results

3.1. Identification of transitions

3.1.1. $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$

Tables 1–4 list the T_m values for this triplex and Tables 5 and 6 the values for the comparable duplex mixtures. All these T_m values were derived from UV melting profiles whose transitions were identified [16,24] from the unique spectral characteristics of third strand dissociation from the triplex and of dissociation of the target duplex itself. All transitions were so analyzed. Some among the examples in Figs. 1–4 were the most difficult to analyze as they involve a $3 \rightarrow 1+1+1$ transition and because some of the additives (e.g. CTriMA) also undergo their own temperature-dependent UV absorbance changes. Many triplex transitions were confirmed by lowering the solution pH and observing an increase in triplex stability [24] without an increase in duplex stability.

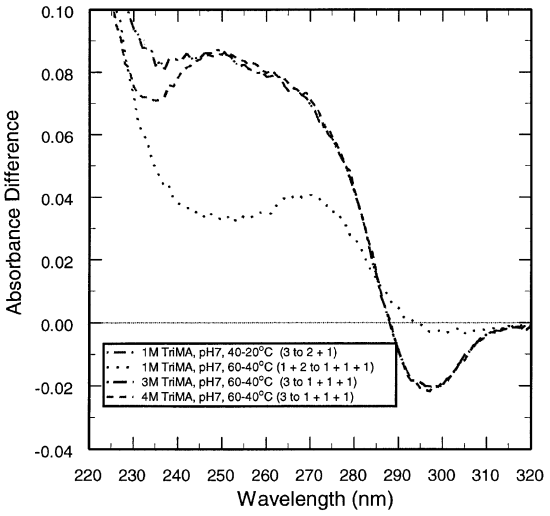

Fig. 1. Temperature difference spectra for the triplex $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ and duplex $d(A\text{-}G)_6$•$d(C\text{-}T)_6$ in TriMA–Cl.

The difference spectra for the triplex mixture in 1 M TriMA–Cl at pH 7.0. (Fig. 1) confirms that

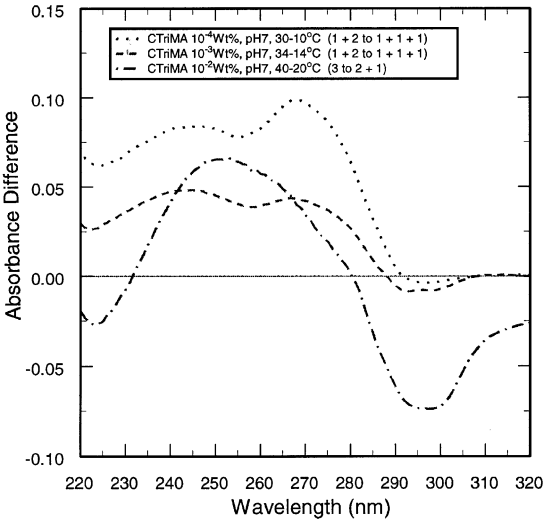

Fig. 2. Temperature difference spectra for the triplex $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ and duplex $d(A\text{-}G)_6$•$d(C\text{-}T)_6$ in CTriMA–Cl.

Table 1
T_m values for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$

Conditions[b]	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
1.0 M NaCl	**9**	**17**	**54**	**12**
1.0 M MA–Cl	18	13	53	8
2.0 M	19	12	53	8
3.0 M	20	13	52	6
4.0 M	19	11	51	8
1.0 M DMA–Cl	20	11	53	8
2.0 M	23	10	52	8
3.0 M	26	9	51	6
4.0 M	27	10	49	8
1.0 M TriMA–Cl	28	10	52	6
2.0 M	36	9	52	7
3.0 M (pH 3.7)	72[a]	19	–	–
3.0 M (pH 4.9)	67[a]	15	–	–
3.0 M (pH 5.8)	58[a]	15	–	–
3.0 M	50[a]	17	–	–
3.0 M (pH 7.4)	36	8	51	7
3.0 M (pH 7.8)	–	–	52	8
4.0 M	53[a]	16	–	–
4.0 M (pH 7.4)	36	8	50	6
1.0 M TMA–Cl	31	10	54	9
2.0 M	29	9	55	7
3.0 M (pH 3.7)	75[a]	18	–	–
3.0 M (pH 4.9)	72[a]	16	–	–
3.0 M (pH 5.8)	61[a]	14	–	–
3.0 M	30	10	56	9
4.0 M	43	9	59	7
6.0 M	50[a]	23	–	–
6.0 M (pH 6.0)	67[a]	23	–	–
0.5 M TEA–Cl	16	10	43	6
1.0 M	22	13	43	10
1.6 M	26	9	37	7
2.0 M	Insoluble			
0.1 M TPA–Cl	–	–	29	8
0.5 M	–	–	31	8
0.9 M	–	–	28	8
0.9 M (pH 8.5)	–	–	27	8
1.0 M	Insoluble			

[a] $3 \rightarrow 1$ transition.
[b] 0.01 M cacodylate, pH 7.0 or as otherwise stated.

the first transition ($T_m = 28$ °C), characterized by a peak at 245–250 nm and a trough at 295–300 nm, is due to dissociation of the third strand, while the second ($T_m = 52$ °C), characterized by a peak at 270 nm and no trough, is due to dissociation of the core duplex. Similarly, the difference spectra for the triplex mixture in 3.0 and 4.0 M TriMA–Cl confirm that their monophasic melting profiles, with T_m values of 50 and 53 °C, respectively, are due to direct triplex melting to single strands.

Table 2
T_m values for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$

Conditions[f]	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
MB	**11**	**12**	**50**	**10**
10^{-4} wt.% CTriMA–Cl (3×10^{-6} M)	–	–	20	10
10^{-3} wt.% (3×10^{-5} M)	–	–	23[a]	5
10^{-2} wt.% (3×10^{-4} M)	28[b]	5	–	–
10^{-1} wt.% (3×10^{-3} M)	Insoluble, micelle formation			
10^{-4} wt.% CTriMA–Cl + MB	10	11	50	13
10^{-3} wt.% + MB	22[c]	8	52	14
10^{-2} wt.% + MB	Insoluble, micelle formation			
10^{-3} wt.% CTriMA–Cl + 0.02 M TMA-Cl + MB	12	8	51	10
10^{-3} wt.% + 0.1 M + MB	14	11	51	9
10^{-3} wt.% + 0.2 M + MB	15	10	52	8
10^{-3} wt.% + 0.4 M + MB	16	11	52	8
10^{-2} wt.% + 0.1 M + MB	45	11	64	11
10^{-2} wt.% + 0.2 M + MB	Insoluble, micelle formation			
10^{-4} wt.% TridodecylMA-Cl (2×10^{-6} M)	–	–	20	10
10^{-3} wt.% (2×10^{-5} M)	–	–	20	10
10^{-3} wt.% (pH 6.0)	41[d]	14	–	–
10^{-2} wt.% (2×10^{-4} M)	Insoluble, micelle formation			
10^{-4} wt.% TridofecylMA-Cl + MB	10	10	51	11
10^{-3} wt.% + MB	11	9	51	10
10^{-2} wt.% + MB	Insoluble, micelle formation			
10^{-4} wt.% DOSPA (7×10^{-7} M)	–	–	21	11
10^{-4} wt.% (pH 6.0)	40[d]	10	–	–
10^{-3} wt.% (7×10^{-6} M)	22[d]	7	–	–
10^{-3} wt.% (pH 6.0)	40	6	79	7
10^{-2} wt % (7×10^{-5} M)	27	6	77	25[e]
10^{-1} wt.% (7×10^{-4} M)	Insoluble, micelle formation			
10^{-4} wt.% DOSPA + MB	10	11	50	16
10^{-3} wt.% + MB	12	7	50	15
10^{-2} wt.% + MB	Insoluble, micelle formation			

[a] See Fig. 2, duplex melting but possibly a very small amount of triplex melting.
[b] $3\rightarrow2+1$ transition (see Fig. 2), phase transition of CTriMA masks duplex transition.
[c] Very broad transition (3–40 °C).
[d] $3\rightarrow1$ transition.
[e] Significant overlap with phase transition of DOSPA.
[f] 0.01 M cacodylate pH 7.0 when other pH or MB not stated.

The triplex mixture in 3×10^{-6} M (10^{-4} wt.%) CTriMA–Cl, pH 7.0, displays a transition with T_m of 20 °C, which is shown in Fig. 2 to be due to dissociation of the duplex, i.e. $1+2\rightarrow1+1+1$. However, on increasing CTriMA–Cl concentration 100 fold, triplex is formed, which melts with a T_m of 28 °C, and a difference spectrum that is clearly due to a $3\rightarrow2+1$ transition. However, T_m for the subsequent duplex transition is masked by a phase transition of CTriMA. In fact, CTriMA, TridodecylMA, and DOSPA, each with a hydrophilic positively charged nitrogen head and a hydrophobic tail self-aggregate to micelles. The transition for CTriMA is readily distinguished from

Table 3
T_m values for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$

Conditions[c]	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
MB	**11**	**12**	**50**	**10**
0.1 wt.% SDS (4×10^{-3} M)+MB	–[a]	–	51	12
1 wt.% (4×10^{-2} M)+MB	–[a]	–	51	11
10 wt.% (4×10^{-1} M)+MB	–[a]	–	54	12
0.1 M $(TMA)_2$-SO_4	16	7	48	21
0.5 M	20	17	56	11
1.0 M	25	14	57	11
1.5 M	$(TMA)_2$-SO_4 precipitates			
0.75 M Trehalose +MB	12	7	47	10
1.5 M +MB	12	4	44	10
2.0 M +MB	13	4	41	9
10 vol.% Glycerol+MB	11	9	49	10
20 vol.% +MB	12	9	45	10
30 vol.% +MB	12	6	42	12
30 vol.% +MB+1.0 M TriMA–Cl	19	8	45	9
20 vol.% PEG200 +MB	18	11	44	10
40 vol.% +MB	33[b]	23	–	–
20 vol.% PEG400 +MB	22	22	48	17
20 vol.% +MB	24	15	49	14
10 vol.% DMSO +MB	15	12	48	12
20 vol.% +MB	17	11	45	12
40 vol.% +MB	20	12	41	12
50 vol.% +MB	27[b]	17	–	–
60 vol.% +MB	15[b]	12	–	–
60 vol.% +MB (pH 6.0)	36[b]	24	–	–

[a] SDS appears to inhibit triplex formation; however, transitions below 20 °C may occur that are not observed as the solution solidifies below ~20 °C.
[b] $3\rightarrow1$ transition.
[c] 0.01 M cacodylate pH 7.0 when MB not stated.

those for nucleic acids, as it shows a very broad difference spectrum between 220 and 320 nm, whereas nucleic acid transitions show a maximum hyperchromic change near 260 nm and almost none at 320 nm.

The difference spectra for the triplex mixture in 10^{-3} and 10^{-4} wt.% TridodecylMA-Cl (Fig. 3) confirm an absence of triplex at pH 7.0, the observed transition being due to duplex dissociation ($1+2\rightarrow1+1+1$). However, at pH 6.0 in the 10^{-3} wt.% solution, triplex is formed, as shown by a difference spectrum characteristic of a $3\rightarrow1+1+1$ transition (Fig. 3).

In 40% PEG 200+MB at pH 7.0, the triplex mixture shows only one transition, $T_m=33$ °C, with a characteristically large hypochromicity (23%) and a $3\rightarrow1+1+1$ difference spectrum (Fig. 4).

3.1.2. $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$

Table 7 lists T_m values for this triplex and its core duplex. As discussed in the accompanying paper [16], their CD-spectra are readily distinguished.

Fig. 5 shows the CD-spectra of the duplex and triplex mixtures in MB and in 2 M TriMA–Cl+

Table 4
T_m values for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$

Conditions[d] $V_{alcohol}/V_{total}$	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
MB	**11**	**12**	**50**	**10**
10% MeOH + MB	15	13	51	11
20% + MB	15	14	48	11
30% + MB	15	12	44	12
60% + MB	16	7	37	7
70% + MB	16	7	35	7
80% + MB	Soluble, no transitions			
10% EtOH + MB	12	12	48	10
20% + MB	13	12	44	11
30% + MB	15	6	41	11
40% + MB	15	7	36	10
50% + MB	21	23	38	8
60% + MB	40[a,c]	31	–	–
70% + MB	Soluble, no transitions			
5% 2-PrOH + MB	9	7	49	7
10% + MB	11	14	47	12
20% + MB	17	9	43	13
30% + MB	20	11	40	12
40% + MB	27[b]	31	39[b]	15
50% + MB	40[c]	38	–	–
60% + MB	Insoluble			
0.1% 1-BuOH + MB	8	10	51	9
1% + MB	7	9	50	9
5% + MB	7	10	47	9
10% + MB	Phase separation			
30% 2-PrOH + 20% EtOH + 3 M TMA-Cl	32[c]	10	–	–
40% + 3 M TMA-Cl	Phase separation			
50% + 3 M TMA-Cl	Insoluble			
50% EtOH + 1.5 M TriMA–Cl	30[c]	16	–	–

[a] Broad transition (20–60 °C).
[b] Overlapping transitions.
[c] 3→1 transition.
[d] 0.01 M cacodylate, pH 7.0 when MB not stated.

MB. In both solvents, the CD spectrum of the duplex has a positive band at 217 nm and a negative band at 247 nm; those for the triplex are quite similar, but for an additional negative band at 209 nm. These CD spectra for $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$, and $d(A)_{21}$•$d(T)_{21}$, are similar to those for poly d(T):d(A)•d(T) and poly d(A)•d(T) [25]. The CD spectra in Fig. 5 confirm that the single transition with T_m of 66 °C in the UV melting profile of the triplex mixture in 2 M TriMA–Cl + MB is 3→1+1+1. As is the case for all 3→1 transitions for this triplex, a significantly greater percent hypochromicity is observed (Table 7).

Similarly, for the triplex mixture in 50% methanol + MB, which displays a single transition with T_m = 38 °C and 36% hypochromicity, the CD spectrum confirms the presence of $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ (Fig. 5).

The UV spectra of triplex mixtures were also analyzed to determine the number of temperature-dependent conformational equilibria. Since each of

Table 5
T_m values for d(A-G)$_6$•d(C-T)$_6$

Conditions[a]	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
MB (pH 7.0)	–	–	**48**	**15**
MB (pH 5.0)	–	–	48	18
MB (pH 7.5)	–	–	48	15
0.4 M NaCl	–	–	51	15
0.5 M	–	–	52	15
0.8 M	–	–	54	15
0.9 M	–	–	54	16
1.0 M	–	–	53	16
2.0 M	–	–	52	16
3.0 M	–	–	52	15
1.0 M MA–Cl	–	–	52	16
2.0 M	–	–	52	16
3.0 M	–	–	52	16
4.0 M	–	–	50	15
1.0 M DMA–Cl	–	–	51	16
2.0 M	–	–	51	16
3.0 M	23	2	50	15
4.0 M	–	–	48	16
1.0 M TriMA–Cl	–	–	50	14
2.0 M	–	–	52	16
3.0 M	–	–	51	17
4.0 M	–	–	52	19
1.0 M TMA–Cl	33	2	54	14
2.0 M	33	2	57	14
3.0 M	33	2	55	17
4.0 M	43	3	58	15
0.5 M TEA–Cl	–	–	41	13
1.0 M	23	3	40	13
1.6 M	26	4	36	10
0.1 M TPA–Cl	–	–	29	13
0.5 M	–	–	30	15
0.9 M	–	–	27	16
0.9 M (pH 8.5)	–	–	26	16

[a] MB at indicated pH or 0.01 M cacodylate pH 7.0 or as stated.

the two helical species in equilibrium has a characteristic spectrum and their proportion changes with increasing temperature, sets of spectra arise that intersect at a common isosbestic wavelength. Thus, for each equilibrium there will be at least one isosbestic wavelength. For example, Fig. 6 contains the UV melting profile at 260 nm for the triplex d(T)$_{21}$:d(A)$_{21}$•d(T)$_{21}$ in 1.0 M TMA–Cl, while Fig. 7 shows spectra with an isosbestic point at 284 nm that results from the equilibrium between the duplex and single strands.

3.1.3. Poly r(U:A•U)

T_m values for poly r(U:A•U) and poly r(A•U) are listed in Table 8. For triplex mixtures showing two transitions at 260 nm, the second was confirmed to be due to dissociation of the duplex by comparison with the melting profile of the com-

Table 6
T_m values for d(A-G)$_6$•d(C-T)$_6$

Conditions[d] $V_{alcohol}/V_{total}$	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
MB	**11**	**12**	**50**	**10**
10% MeOH + MB (pH 6.7)	20	8	48	15
10% + MB	13	5	47	17
20% + MB	15	5	45	18
30% + MB	15	5	42	18
60% + MB	17	7	38	13
70% + MB	17	6	35	12
80% + MB	Soluble, no transitions			
10% EtOH + MB	–	–	46	20
20% + MB	–	–	41	20
30% + MB	–	–	38	21
40% + MB	20[a]	7	38[a]	7
50% + MB	19	11	39	12
60% + MB	42[b]	30	–	–
70% + MB	Soluble, no transitions			
5% 2-PrOH + MB	–	–	48	19
10% + MB	–	–	46	19
20% + MB	–	–	42	20
30% + MB	–	–	39	20
40% + MB	21[c]	4	41[c]	15
50% + MB	32[c]	16	44[c]	10
60% + MB	Insoluble			
0.1% 1-BuOH + MB	–	–	50	17
1% + MB	–	–	49	17
5% + MB	–	–	44	17
10% + MB	Phase separation			

[a] Broad and overlapping transitions.
[b] 3 → 1 transition.
[c] Significant overlap of transitions resulting in possible overestimation of T_m of second transition.
[d] MB pH 7.0 or as stated.

parable duplex mixture. For triplex mixtures showing just one melting transition, the greater percent hypochromicity (double or more) identified them as 3 → 1 + 1 + 1 transitions (Table 8).

3.2. Effects of additives on triplex stability

3.2.1. $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$

The organic salts, MA–Cl, DMA–Cl, TriMA–Cl, TMA–Cl and TEA–Cl all enhance triplex stability (Table 1, Fig. 8) relative to the same molar concentrations of NaCl (cf. Table 2 in [16]). Consistent with the enhancing effect, DMA–Cl, TMA–Cl and TEA–Cl drive the duplex mixture to partially disproportionate to triplex and single strand, as indicated by a smaller hypochromicity for the first transition (Table 5).

The organic salt TPA–Cl inhibits triplex formation at ≥0.1 M. Whereas TEA–Cl and TPA–Cl decrease duplex stability with increasing concentration, the other organic salts have little effect (Table 5). While the organic cation TMA^+ (relative to Na^+) and the inorganic anion SO_4^{2-} (relative to Cl^-) each enhance triplex stability, an additive effect was not obtained with $(TMA)_2$–SO_4, e.g. T_m of 25 °C in 1.0 M $(TMA)_2$-SO_4 vs 29 °C in 2.0 M TMA–Cl (Fig. 8).

At pH 7.0 the methyl substituted nitrogen com-

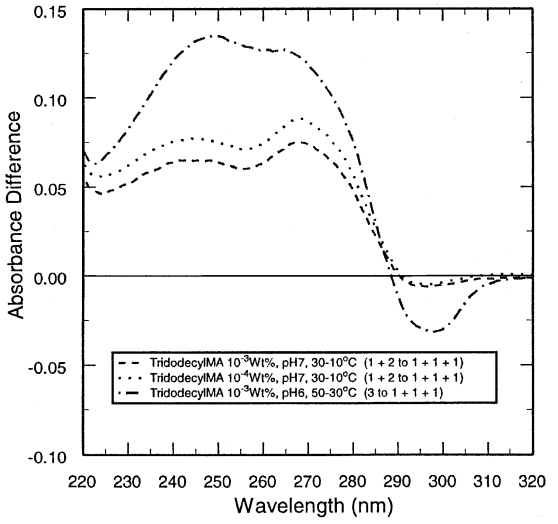

Fig. 3. Temperature difference spectra for the triplex $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ at pH 6.0 and the duplex $d(A\text{-}G)_6$•$d(C\text{-}T)_6$ in TridodecylMA-Cl at pH 7.0.

pounds MA (pK_a 10.7), DMA (pK_a 10.7), and TriMA (pK_a 9.8) are cations, as are the quaternary nitrogen compounds TMA, TEA and TPA. Since triplex stability is significantly enhanced by $TriMA^+$ and TMA^+, it was thought that chemical species with head groups of similar positive charge and molecular volume might be made even more effective by a hydrophobic hydrocarbon tail that could facilitate displacement of the water H-bonded to the bases in the major groove of the target duplex, as required for third strand-binding. As the nucleic acid bases are H-bonded to water in a single nucleic acid strand, interstrand H-bonding can be viewed as the consequence of an analogous displacement, with base pairing between the strands being driven by the strong preference of the bases for the hydrophobic environment in the center of the helix. In view of these considerations, the triplex stabilizing ability of several cationic lipids was examined: $CtriMA^+$ (TriMA with one C_{16} tail), $TridodecylMA^+$ (MA with three C_{12} tails), $DOSPA^{4+}$ (essentially a spermine head with five positive charges at pH 7 and two C_{18} tails). Didecylamine, with a positively charged NH_2 head and two C_{10} tails proved too insoluble in water to be useful.

Despite their very low solubilities, these cationic lipids exert a definite triplex stabilizing effect (Table 2). At pH 7.0 triplex $T_m = 28$ °C with 10^{-2} wt.% CTriMA–Cl (3×10^{-4} M), 22 °C with 10^{-3} wt.% DOSPA (7×10^{-6} M), 27 °C with 10^{-2} wt.% DOSPA. Lowering the pH to 6.0 further enhances triplex stability; $T_m = 41$ °C with 10^{-3} wt.% TridodecylMA-Cl (2×10^{-5} M), 40 °C 10^{-4} wt.% and 10^{-3} wt.% DOSPA. The only additive effect was obtained with 10^{-2} wt.% CTriMA–Cl + 0.1 M TMA-Cl + MB, in which $T_m = 45$ °C.

These cationic lipids were difficult to study. Even at very low concentrations they show UV transitions in the melting profiles which superimpose the triplex and duplex transitions. Also, their limited solubility (10^{-5} or 10^{-4} M) is often insufficient to significantly stabilize the three negatively charged phosphodiester backbones; and addition of MB to provide sufficient cations in some cases further reduces their solubility. These factors prevented systematic investigation of the effect of changing size and shape of the organic cation head group and the length, number and orientation of the lipid tails.

An interesting aside is that the fusion lipid, DOSPA, which is a very good transfection agent

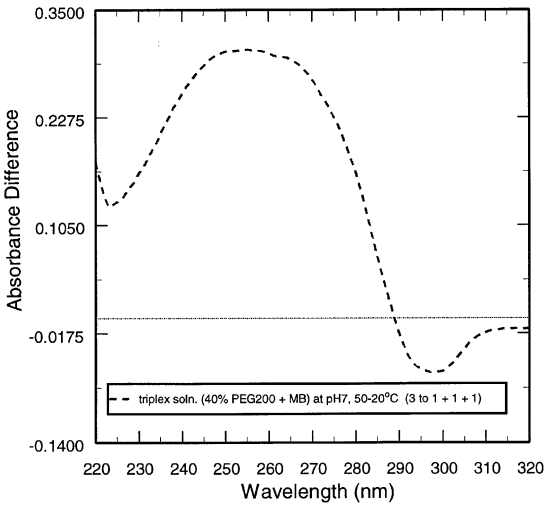

Fig. 4. Temperature difference spectrum for the triplex $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ in 40% PEG 200 + MB.

Table 7
T_m values for $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ and $d(A)_{21}$•$d(T)_{21}$

Conditions[d]	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
d(T)$_{21}$•d(A)$_{21}$•d(T)$_{21}$				
MB	**23**	**18**	**53**	**15**
1.0 M TriMA–Cl + MB	45	16	61	11
2.0 M + MB	66[a]	27	–	–
3.0 M + MB	70[a]	26	–	–
1.0 M	39	17	63	18
1.0 M TMA–Cl	28	17	65	19
6.0 M	95[a]	33[b]	–	–
1.0 M $(TMA)_2$-SO_4	54	10	74	13
1.5 M	$(TMA)_2$-SO_4 precipitates			
50 vol.% MeOH + MB	38[a]	38	–	–
50 vol.% EtOH + MB	53[a]	74	–	–
50 vol.% 2-PrOH + MB	65[a]	74	–	–
20 vol.% PEG200 + MB	39[a]	25	–	–
40 vol.% + MB	41[a]	45	–	–
20 vol.% PEG600 + MB	52[a]	54	–	–
30 vol.% DMSO + MB	34[c]	18	44[c]	16
40 vol.% + MB	38[a]	35	–	–
50 vol.% + MB	15	9	34	28
d(A)$_{21}$•d(T)$_{21}$				
MB	–	–	**53**	**28**
1.0 M TriMA–Cl + MB	–	–	60	23
2.0 M + MB	–	–	65	25
3.0 M + MB	–	–	70	23

[a] 3→1 transition.
[b] Obtained by extrapolation.
[c] Overlapping transitions.
[d] 0.01 M cacodylate, pH 7.0 when MB not stated.

[26], significantly enhances duplex stability (Table 2).

The anionic lipid SDS appears to inhibit triplex formation (Table 3). However, as the solution solidifies below ~20 °C, transitions below that temperature could not be detected.

The effects of varying concentrations of trehalose, glycerol, DMSO and PEG were measured in the presence of MB, as none of these compounds ionize. While trehalose is a protein stabilizer, this sugar has no effect on triplex stability, though it does destabilize the duplex (Fig. 9, Table 3). While glycerol absorbs water up to 50% of its weight, it behaves as does trehalose towards this triplex and duplex (Fig. 9, Table 3). These observations show that agents that stabilize proteins, presumably by virtue of their dehydrating ability, do not necessarily stabilize nucleic acid triplexes or duplexes.

Given that 30 vol.% glycerol + MB does not affect triplex stability, it is surprising that addition of 1.0 M TriMA–Cl to that solvent reduces the T_m enhancement due to 1.0 M TriMA–Cl alone by 9 °C (Table 3). This is a striking example of a compound that has no effect on triplex stability by itself, yet decreases stability in the presence of another compound.

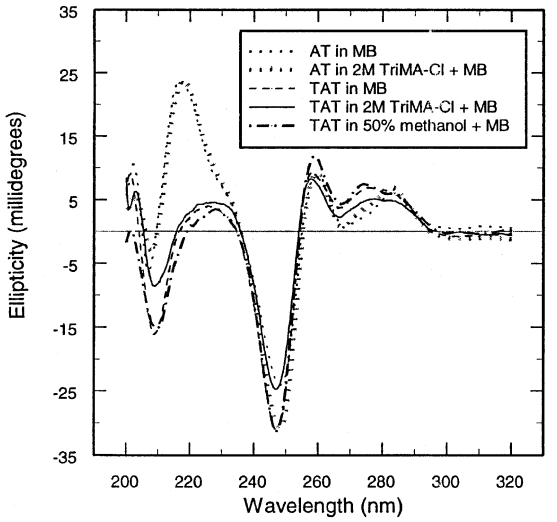

Fig. 5. CD spectra at 1 °C of the duplex $d(A)_{21}$•$d(T)_{21}$ and the triplex $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$.

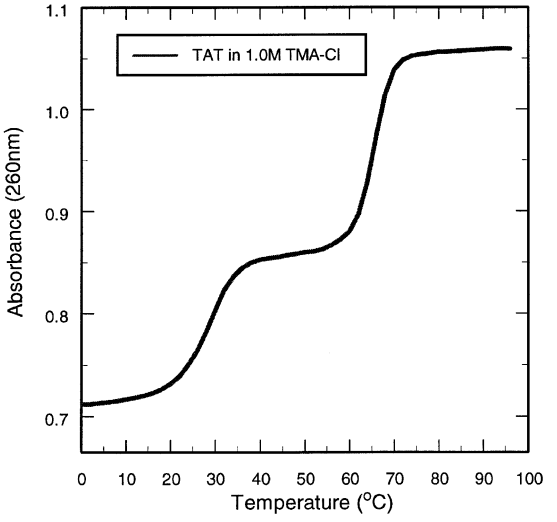

Fig. 6. UV melting profile of the triplex $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ in 1.0 M TMA–Cl.

Both PEG and DMSO enhance triplex stability (Fig. 9, Table 3). A similar effect has been observed with PEG 3400 [27]. With increasing PEG molecular weight (20 vol.% PEG 200, 400, 600), stability of both triplex (T_m = 18, 22, 24 °C, respectively) and duplex (T_m = 44, 48, 49 °C, respectively) increase. For DMSO, triplex stability rises to a maximum at 50 vol.% DMSO + MB (T_m = 27 °C), and then decreases. In addition, to the difference spectral analysis to identify this triplex (see above), the single transition in 60 vol.% DMSO was shown to be acid stabilized, as is characteristic for triplexes with third strand C residues (60 vol.% DMSO, pH 7.0, T_m = 15 °C; pH 6.0, T_m = 36 °C). While increasing triplex stability, DMSO decreases duplex stability (10, 20, 40 vol.%, T_m = 48, 45, 41 °C, respectively).

Of the alcohols MeOH, EtOH, 2-PrOH and 1-BuOH (Table 4, Fig. 10), only 1-BuOH significantly destabilizes the triplex. Studies with 1-pentanol were not possible due to its phase separation from water. Maximum triplex stability was attained in 70 vol.% MeOH + MB (T_m = 16 °C), 60 vol.% EtOH + MB (T_m = 40 °C), and 50 vol.% 2-PrOH + MB (T_m = 40 °C). Various combinations of EtOH and 2-PrOH with TMA-Cl and TriMA–Cl produced no additive effect (Table 4).

In addition to their triplex stabilizing ability, MeOH, EtOH and 2-PrOH drive the equilibrium in a duplex mixture towards triplex. Table 6 includes data indicating the ability of these alcohols to promote triplex formation in the duplex

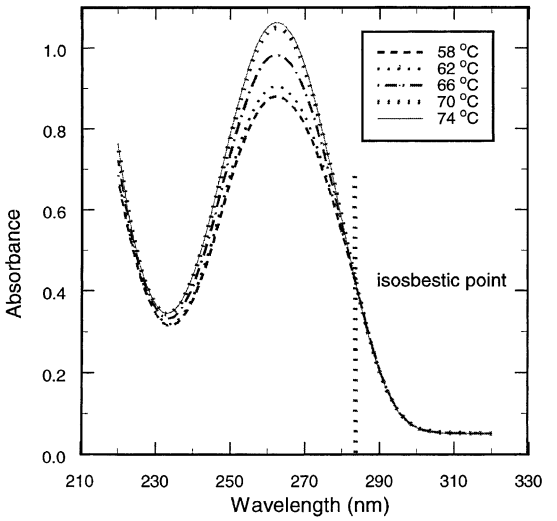

Fig. 7. UV spectra between 58 and 74 °C of the triplex mixture $d(T)_{21}$ + $d(A)_{21}$•$d(T)_{21}$ in 1.0 M TMA-Cl. Note the isosbestic point at 284 nm.

Table 8
T_m values for Poly r(U:A•U) and Poly r(A•U)

Conditions[c]	1st Transition		2nd Transition	
	T_m °C	Hypochromicity %	T_m °C	Hypochromicity %
Poly r(U:A•U)				
0.016 M NaCl	**26**	**17**	**40**	**23**
0.020 M TriMA-Cl + 0.016 M NaCl	34	21	42	18
0.053 M + 0.016 M	44[a]	37	–	–
0.600 M + 0.016 M	69[a]	41	–	–
0.020 M TMA + 0.016 M NaCl	31	18	40	17
10^{-4} Wt% CtriMA-Cl (3×10^{-6} M) + 0.016 M NaCl	27	16	40	20
10^{-3} Wt% (3×10^{-5} M) + 0.016 M	38[b]	12	63[b]	18
10^{-2} Wt% (3×10^{-4} M) + 0.016 M	Insoluble, micelle formation			
10 vol.% EtOH + 0.016 M NaCl	39[a]	37	–	–
20 vol.% + 0.016 M	42[a]	39	–	–
30 vol.% + 0.016 M	45[a]	40	–	–
50 vol.% + 0.016 M	53[a]	54	–	–
60 vol.% + 0.016 M	Insoluble			
Poly r(A•U)				
0.016 M NaCl	–	–	**40**	**25**
0.020 M TriMA-Cl + 0.016 M NaCl	–	–	41	25
0.053 M + 0.016 M	–	–	45	24
0.600 M + 0.016 M	–	–	69	28
10 vol.% EtOH + 0.016 M NaCl	–	–	39	26
20 vol.% + 0.016 M	–	–	42	26
30 vol.% + 0.016 M	–	–	46	26
50 vol.% + 0.016 M	–	–	58	26
60 vol.% + 0.016 M	Insoluble			

[a] $3\rightarrow1$ transition.
[b] Overlapping transitions.
[c] 0.01 M cacodylate pH 7.0.

mixture (by disproportionation), and also shows that decreasing duplex stability parallels increasing triplex stability. In the equimolar strand mixture with 60 vol.% EtOH + MB, the extreme case of only triplex formation is observed, i.e. a $3(+1)\rightarrow 1+1+1(+1)$ transition at 42 °C.

Of the dehydrating compounds trehalose, glycerol, PEG, DMSO, MeOH, EtOH, 2-PrOH, 1-BuOH, only PEG, DMSO, MeOH, EtOH, and 2-PrOH enhance the stability of $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$.

3.2.2. $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$

The organic cations $TriMA^+$ and TMA^+ stabilize the triplex (Fig. 11, Table 7), but to a lesser extent than equivalent concentrations of NaCl, i.e. at 1.0 M, TMA–Cl (T_m = 28 °C), TriMA–Cl (T_m = 39 °C), NaCl (T_m = 49 °C). However, stabilization by NaCl begins to level off above 2.0 M (Fig. 5 in [16]), reaching a maximum T_m of 72 °C in 5.0 M NaCl, whereas in 6.0 M TMA–Cl, T_m increases to 95 °C. This difference between NaCl and TMA–Cl stabilization indicates that the charge density of organic cations such as $TriMA^+$ and TMA^+ is less than that of Na^+, but that charge screening is not the only way that cations stabilize triplexes.

MeOH, EtOH and 1-PrOH also stabilize this triplex (in 50 vol.% + MB: MeOH (T_m = 38 °C), EtOH (T_m = 53 °C), 2-PrOH (T_m = 65 °C) (Fig. 12), and to a greater extent than they do $d(C^+\text{-}$

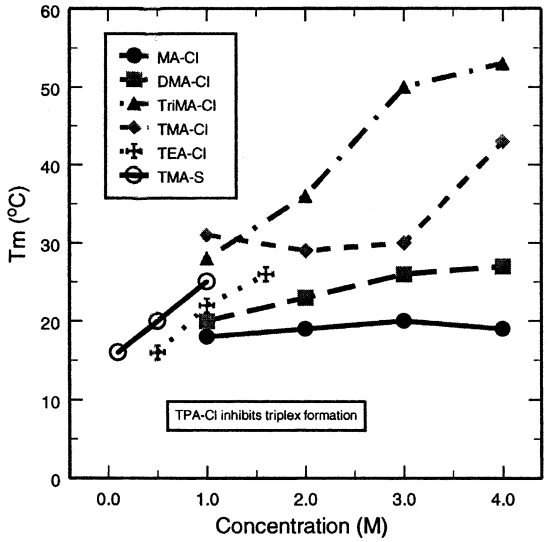

Fig. 8. Plots of T_m vs. concentration of various organic salts for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$.

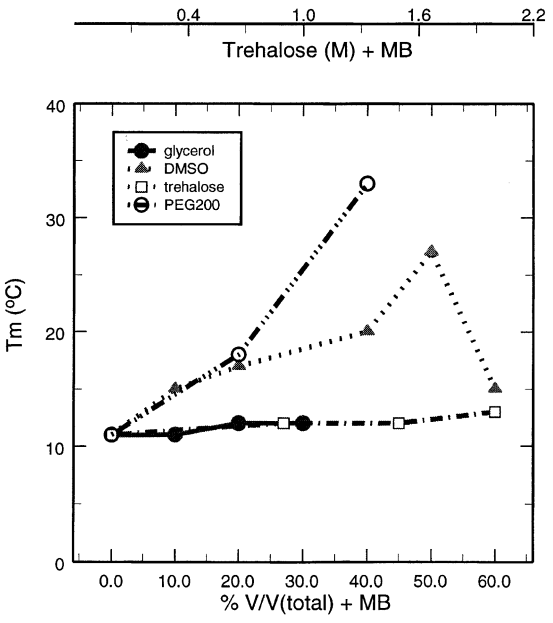

Fig. 9. Plots of T_m vs. vol.%(neutral organic molecule)/Vol(total) + MB and vs trehalose concentration + MB for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$.

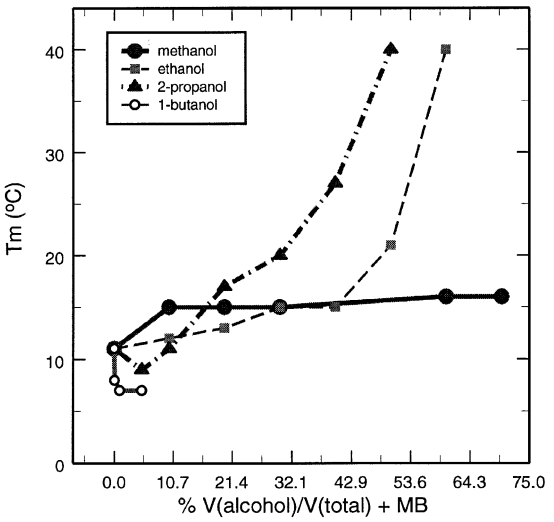

Fig. 10. Plots of T_m vs. vol.%(EtOH)/Vol(total) + MB for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$.

$T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, which is shorter and requires third strand C residue protonation for third strand-binding.

Increasing triplex stability is observed with increasing % PEG and PEG molecular weight (Fig. 12, Table 7). DMSO also increases the

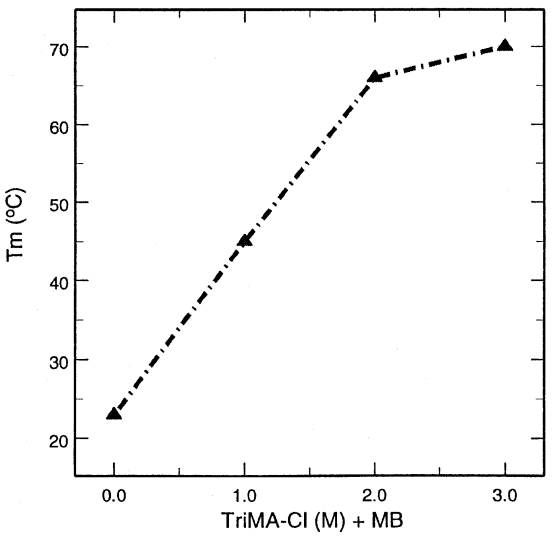

Fig. 11. Plot of T_m vs. TriMA–Cl concentration + MB for $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$.

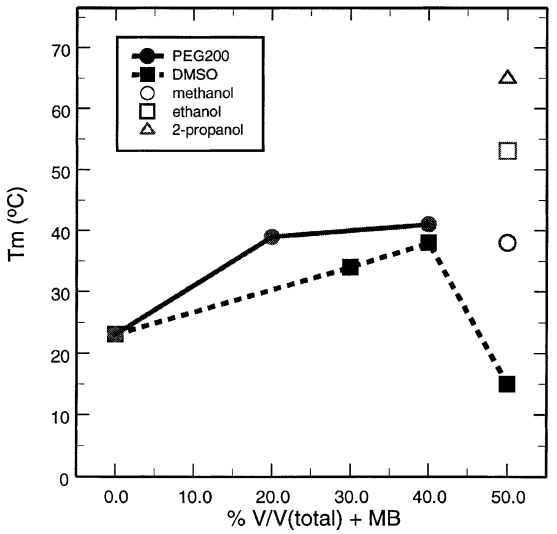

Fig. 12. Plots of T_m vs. vol.%(neutral organic molecule)/Vol(total)+MB for $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$.

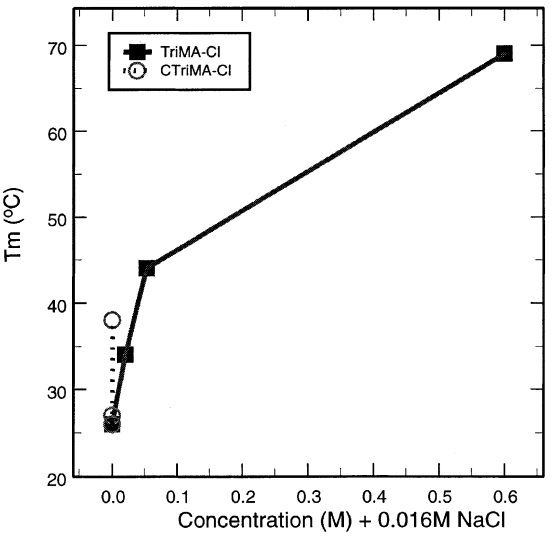

Fig. 13. Plots of T_m vs. concentration of organic salt+0.016 M NaCl for poly r(U:A•U).

stability of this triplex up to a maximum in 40 vol.% DMSO+MB and then decreases it (Fig. 12).

3.2.3. Poly r(U:A•U)

As expected for a polymeric triplex with no C^+:G•C triplets, the stability of poly r(U:A•U) is very sensitive to ionic strength. Thus, poly r(U:A•U) shows T_m dependence on the concentration of TriMA–Cl and TMA-Cl (Fig. 13) similar to that on NaCl concentration. However, in 0.053 M TriMA+0.016 M NaCl, the triplex melts $3\rightarrow 1+1+1$, whereas in 0.069 M NaCl [28] it melts $3\rightarrow 2+1$ and only at higher NaCl concentration does it melt $3\rightarrow 1+1+1$. This must be because TriMA–Cl stabilizes triplexes but destabilizes duplexes, which NaCl does not do.

Enhanced stability ($T_m=38$ °C) in 10^{-3} wt.% CTriMA–Cl (3×10^{-5} M)+0.016 M NaCl is observed in comparison to $T_m=26$ °C for the same concentration of NaCl. Because CTriMA–Cl forms micelles, studies at higher concentrations were not possible.

Just as for the other triplexes, the stability of poly r(U:A•U) is enhanced in the presence of EtOH (Fig. 14).

4. Discussion

4.1. General findings

Tested on three triplexes with different types of homopyrimidine third strands, the responses to

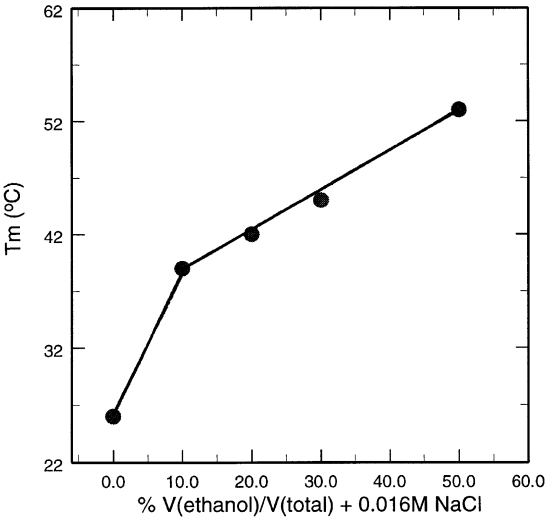

Fig. 14. Plot of T_m vs. concentration of EtOH+0.016 M NaCl for poly r(U:A•U).

most of the additives were quite similar. However, a few differences are worthy of note. In the accompanying paper [16], it was shown that while NaCl and $(NH_4)Cl$ stabilize $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$, they destabilize $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, which is reasonable given its protonated third strand dC residues [24]. In this investigation, we have found that, while both DMSO and ethanol stabilize all the triplexes, the effects increase with triplex length. For example, a maximum effect is observed with 50 vol.% DMSO for $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ and 40 vol.% with $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$; also, whereas it takes 50 vol.% or more ethanol to enhance the stability of $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, it only requires 10 vol.% for poly r(U:A•U).

4.2. pK_a and triplex stability

Although the organic salts MA–Cl, DMA–Cl, TriMA–Cl, TMA–Cl and TEA–Cl enhance the stability of $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, they do not do so by raising the pK_a of the dC residues, as these salts also enhance the stability of $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ which contains no protonated third strand residues (maximum T_m of 72 °C in 5.0 M NaCl, Fig. 11 vs. maximum T_m of 95 °C in 6.0 M TMA-Cl, Table 7). Moreover, $d(C^+\text{-}T)_6$:d(A-G)6•$d(C\text{-}T)_6$ is still responsive to lower pH, e.g. $T_m = 72$ °C at pH 3.7 in 3.0 M TriMA–Cl (vs. 50 °C at pH 7.0) and 75 °C at pH 3.7 in 3.0 M TMA–Cl (vs. 30 °C at pH 7.0). This would not be the case if these salts raised the pK_a of the third strand dC residues.

4.3. Water structure-making organic cations

Whereas the stability of duplex DNA is not greatly affected by the type of cation at very high concentration (see Table 5 and [29]), *it has been shown here that the organic cations have a particularly strong effect on triplex stability.* This stabilizing ability can be interpreted in terms of the ion-water model described in the accompanying paper [16]. Thus, for these organic cations V_{caged} is dominant, and water 'structure-making' occurs as a result of their hydrophobicity. Their relative effect on the stability of $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$ at pH 7.0 is: TPA–Cl < NaCl < MA–Cl < DMA–Cl < TEA–Cl < TMA–Cl ≤ TriMA–Cl.

Duplexes, and triplexes even more so, have high negative charge densities, so that cations of sufficient positive charge density are required to stabilize them. Therefore, while the partial molal volume ($cm^3.mole^{-1}$) is negative [30b] (see footnote 6 in [16]) for the organic cations because of their water structure-making tendency, e.g. TMA^+ −21.1, TEA^+ −17.6, and TPA^+ −23.7, TPA^+ must not have a sufficient positive charge density to stabilize the triplex. At pH 7.0, these cations all have one positive charge, so their charge density scales inversely with their surface area (calculated using ChemPlus 1.6): MA^+ (178 Å²), DMA^+ (208 Å²), $TriMA^+$ (232 Å²) TMA^+ (252 Å²), TEA^+ (325 Å²), and TPA^+ (383 Å²). This implies that $TriMA^+$ and TMA^+ have the optimum combination of size and charge to stabilize the triplex backbone phosphates.

The observed enhanced triplex stability in the presence of water structure-making solutes (organic cations and neutral molecules) is consistent with the observed enhanced triplex stability in the presence of water structure-making anions [16]. However, unlike those anions, which do not interact with the highly negatively charged triplex, so that $\Delta\Delta S°$ values can be calculated for them, such values cannot be calculated for the organic cations and neutral molecules because they do interact with the triplex.

4.4. Water structure-making neutral organic additives

Neutral polar organic additives are also classified as water structure-breaking (chaotropes) or water structure-making (kosmotropes) [31]. The low molecular weight alcohols are water structure-making, as is the neutral hydrophilic polymer PEG, and the strong H-bond acceptor DMSO. The rank order of triplex stabilization at pH 7.0 for these compounds follows.

$d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$	MB < MeOH < EtOH < 2-PrOH > 1-BuOH[3]
Low mol wt. alcohols + MB:	MB < MeOH < EtOH < 2-PrOH > 1-BuOH[3]
20 vol.% PEG + MB:	MB < PEG200 < PEG400 < PEG600
vol.% DMSO + MB:	0% < 10% < 20% < 40% < 50% > 60%
$d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$	
50 vol.% alcohol + MB:	MB < MeOH < EtOH < 2-PrOH
vol.% DMSO + MB:	0% < 30% < 40% > 50%
Poly r(U:A•U)	
Vol.% EtOH + 0.016 M NaCl:	0% < 10% < 20% < 30% < 50%

Based upon the foregoing observations, we conclude that water structure-making additives enhance triplex stability.

4.5. Relation between triplex-stabilizing effect of additives and their solvent-compressing ability or effect on water surface tension

Molar ionic solutions have been shown to compress or distort the structure of water to an extent equivalent to that due to or exceeding a thousand atmospheres and to cause ion-specific water structure changes in accord with the Hofmeister series of anions [32,33]. However, any correlation between compression and the Hofmeister series is of limited significance because pressure increases are observed for both NaCl (approx. 1400 atm) and Na_2SO_4 (approx. 1700 atm) [32]. Consequently, in accord with Le Chatelier's principle, both these anions should favor or induce molecular structures with the smallest volume. Since NaCl inhibits formation of $d(C^+\text{-}T)_6$:$d(A\text{-}G)_6$•$d(C\text{-}T)_6$, while Na_2SO_4 promotes its formation, triplex formation in the presence of Cl^- would be expected to cause a positive molar volume change, but in the presence of SO_4^{2-} a negative molar volume change. Such a severe molar volume difference for the same triplex in the presence of different anions seems highly unlikely. In fact, since both duplex and triplex formation in the presence of NaCl result in negative molar volume changes [34,35], it follows that increasing NaCl concentration should favor formation of both. Instead, in this and the accompanying paper [16], whereas triplex stability is shown to be highly dependent on both salt and solute type, duplex stability under comparable conditions is far less dependent.[4]

[3] Clearly, solute-size, -charge density, and -solubility are contributing parameters that determine the effect of a solute on triplex stability. For example, as discussed in the previous section, the structure-making TPA^+, with a partial molal volume of -23.7 cm^3.mole^{-1}, destabilizes the triplex $d(C^+-T)_6$:$d(A-G)_6$•$d(C-T)_6$ and the duplex $d(A-G)_6$•$d(C-T)_6$. Similarly, size and solubility must play a role for the larger alcohols, 1-BuOH, etc. that also destabilize the same triplex and duplex. Our data clearly show that there are limits to the observed trends, be it lowered stability in the presence of TPA^+, or on going from 50 to 60% DMSO, from 2-PrOH to 1-BuOH, from 70 to 80% MeOH, from 60 to 70% EtOH, or from 50 to 60% 2-PrOH, etc.

[4] Partial molal volume is a calculated number for the change in volume of an ion on going from a solid to an aqueous phase; and as noted by Millero [30a,43], the magnitude of this calculated difference is dependent on the values used for V_{ion} and V_{cryst}. However, this is not *experimental* evidence that the number of water molecules per unit volume has changed. The neutron diffraction work by Leberman and Soper [32] provides direct observation of the number of water molecules per unit volume, which increases in the presence of NaCl and Na_2SO_4. They also show that this increase in water density is equivalent to pure water under high pressure. On page 366 of [32] they refer to partial molar volumes obtained from [43], when they should have stated conventional partial molal volumes from [43]. These two quantities are different; conventional partial molal volumes represent a true volume that an ion occupies in solution under dilute conditions in which ion-ion interactions are of no concern. Illustrating this difference with Na^+, Cl^-, and SO_4^{2-} as examples, their respective partial molal volumes ($V_{ion} - V_{cryst}$) at 25 °C are -8.7, 8.3 cm^3.mole^{-1}, (no SO_4^{2-} value available) from Table 15 in [30], while their respective conventional partial molal volumes at 25 °C are -1.21, 17.83, and 13.98 cm^3.mole^{-1} from Table 8 in [30] or Table 3 in [43]. Since the conventional partial molal volume of water is ~ 18 cm^3.mole^{-1}, substituting water with Cl^- has almost no effect, while the smaller values for Na^+ and SO_4^{2-} of -1.21 and 13.98 cm^3.mole^{-1} do have a significant effect. For example, $3\times 18 = 54$ cm^3.mole^{-1} vs. $(2\times -1.21) + (13.98) = 11.56$ cm^3.mole^{-1} for aqueous Na_2SO_4; and if one thinks of $Na^+(aq)$ and $Cl^-(aq)$ 'replacing' two water molecules, then their respective conventional partial molal volumes are 16.62 vs. 36 cm^3.mole^{-1}. It is these conventional partial molal volumes that give rise to the concept of certain ions having a large electrostrictive effect on water that is likened to high-pressure conditions. Numbers aside, where we disagree with Leberman and Soper [32] is that if one uses electrostrictive-induced pressure arguments, then (1) salts like NaCl and Na_2SO_4, which act equivalent to high-pressure on water, should have similar effects on proteins and nucleic acid duplexes and triplexes, which is not the case, and (2) both salts should enhance the stability of proteins and nucleic acids with a lower molar volume in the native state, and both salts should destabilize native structures that have a higher molar volume, which is also not the case. In other words, there is a tenuous link between this 'induced pressure' model and the Hofmeister series when a salt like Na_2SO_4 is shown to enhance stability regardless of the relative molar volumes for folded/unfolded protein states and associated/dissociated nucleic acid triplex and duplex states.

Another parameter to consider is the effect of a solute on the surface tension of water. In general, higher surface tension is unfavorable for non-polar molecules due to cavity formation (cf. [36] for a discussion relevant to protein stability). Since base stacking is a driving force for duplex and triplex formation, any solute that increases water surface tension should increase duplex and triplex stability. Our results in this and the accompanying paper [16] show that only *particular* solutes and salts enhance triplex stability, whereas all salts increase water surface tension [36,37]. Therefore, there is no correlation between water surface tension and triplex stability.

4.6. Partial unwinding of the target duplex

In addition to showing that triplex-stabilizing additives are water structure-makers, we have found that this property correlates with the capacity of these additives to unwind the DNA duplex. Thus, another outcome of the water-alcohol (or PEG or DMSO) interaction is that their presence makes less water available for hydration of the nucleic acid. It is well known that DNA is sufficiently dehydrated in 60 to 70% ethanol to undergo a B→A transition, A-DNA being less hydrated than B-DNA, e.g. [38,39]. Essentially, this conformational change involves unwinding the DNA duplex so that the rotation per nucleotide changes from 36–45° to 30–33°. Such unwinding is also favored in the presence of MeOH, EtOH, ethylene glycol and DMSO, but far less so for glycerol,[5] increasing continuously in response to the concentration of the organic solvent [40]. The vol.% required for unwinding increases in the order: DMSO < MeOH ≤ EtOH < ethylene glycol. Since glycerol has a minor effect on duplex unwinding and triplex stability (Table 3), it seems likely that MeOH, EtOH, 2-PrOH, PEG and DMSO all enhance triplex stability by facilitating unwinding of the duplex, a process consistent with displacement of major groove H-bonded water by the H-bonded third strand. It is just this displacement that RecA probably facilitates [41], by providing a hydrophobic environment that fosters elimination of water from the major groove.

[5] In [40], Fig. 3 shows on the vertical axis that $\Delta Lk = 0$ for the standard condition of 0.2 M NaCl, 37 °C, 6.6 mM Mg^{2+}. At 40% (v/v) glycerol, $\Delta Lk = 0.0$, whereas for the same concentrations of ethylene glycol and methanol, it is −1.8 and −7.0, respectively, and for the highest concentrations studied, 50% (v/v), $\Delta Lk = -0.4$ for glycerol vs. −2.6 for ethylene glycol.

5. Conclusion

In sum, triplex stability is enhanced by additives that are both water structure-making and facilitate unwinding of the target duplex. While it is not known if all water structure-making additives facilitate unwinding of the target duplex, such cooperation would be consistent with the facilitation of third strand-binding. Clearly, triplexes are far more sensitive to solution conditions than duplexes, and understanding the solution conditions that enhance triplex stability should prove useful in developing applications of third strand-binding particularly in vitro (e.g. [15]) and in obtaining triplex crystals in a well-ordered lattice [42].

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