Substitution of Adenine by Purine-2,6-diamine Improves the Nonenzymatic Oligomerization of Ribonucleotides on Templates Containing Thymidine

Preliminary Communication

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Dedicated to Professor *Albert Eschenmoser* on the occasion of his 75th birthday

A standard DNA sequencer was used as a novel and highly efficient tool to study the template-controlled polymerization of RNA. When labeled with appropriate fluorescent dyes, primers and their extension products could be separated and quantified with excellent sensitivity, reproducibility, and speed. The new technique was applied to compare the template-controlled incorporation of adenosine mononucleotide **2** and its purine-2,6-diamine analogue **3**, the latter being capable of forming three H-bonds with thymidine or uridine residues. The rates and yields of incorporation are similar when only one thymidine unit is available for pairing in the template (see template **6** and *Table 2*). However, on template **7** with two consecutive thymidine residues, purine-2,6-diamine is clearly ahead of adenine (see *Table 3*). This advantage is most pronounced when the template contains stretches of three and four thymidine moieties (see templates **8** and **9** and *Tables 4* and *5*, resp.).

1. Introduction. – Nonenzymatic oligomerization [1][2] and ligation reactions [3][4] of nucleic acids have been extensively studied in several laboratories. A prominent example is the efficient chain formation from 2-methyl-1*H*-imidazole-activated guanosine-5′-monophosphate ((2-MeIm)pG; **1**) in the presence of oligo(cytidylic acid) [5][6]. However, all attempts to generate self-replicating systems based on this chemistry have proved elusive so far. Important obstacles are the self-complementarity of oligo(GC) sequences and the tendency of oligo(guanylic acids) to form G quartets, thus lowering their utility as templates. The latter problem can be in part resolved by reducing the concentration of K^+ and Na^+ ions [6]. Nevertheless, to enlarge the scope of the template-controlled RNA oligomerization, the availability of additional base-pair types would be of great importance. Unfortunately, the adenosine · thymidine pair does not fit the given requirements. *Orgel* and co-workers have found that the assembly of (2-MeIm)pA (**2**) monomers opposite to thymidine residues in the template proceeds in a rather sluggish way [7]. The incorporation of (2-MeIm)pU opposite to adenosine is even more difficult to achieve [7].

Compared to G · C, the stability of A · T and A · U base pairs is inferior due to their reduced number of H-bonds. An obvious way to increase the strength of association is to install a second amino group in the framework of adenosine (see *Fig. 1*). The base pairs of the resulting purine-2,6-diamine nucleoside (abbreviated as D) with T and U are expected to be stronger than A · T but less stable than G · C because of negative secondary interactions [8]. In a recent report, *Kozlov* and *Orgel* have shown that templates containing D in place of A indeed facilitate the incorporation of (2-MeIm)pU [9]. Here we present the complementary experiment: oligomerization

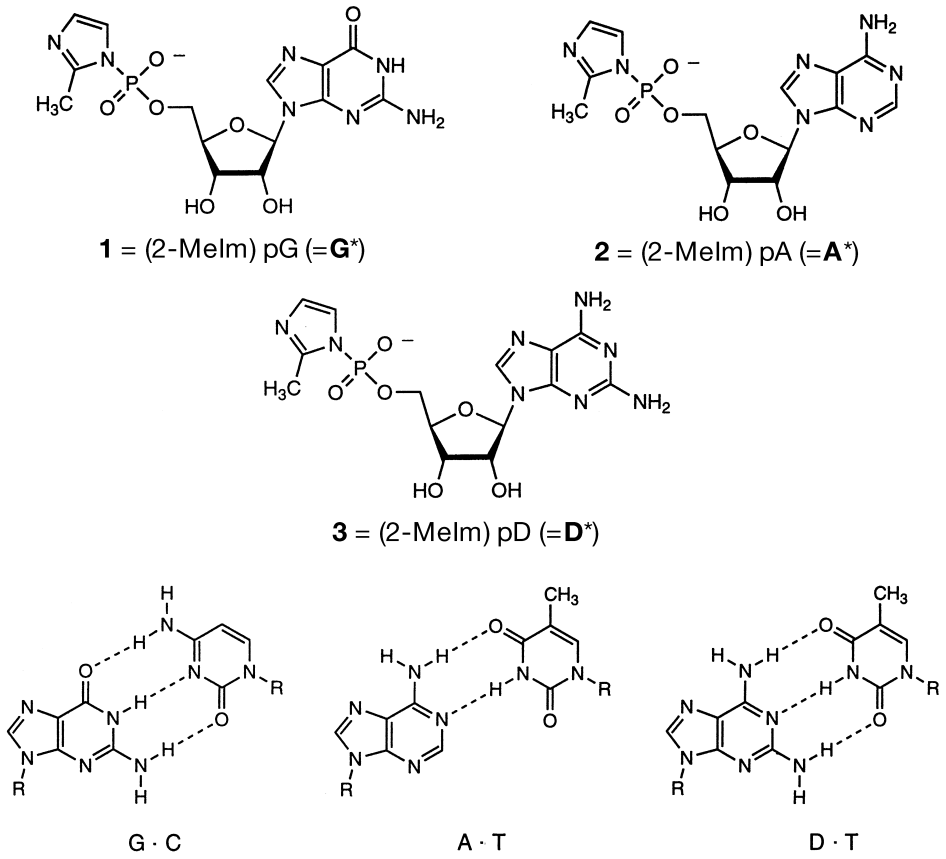

Fig. 1. *Hydrogen bonds in G · C, A · T, and D · T base pairs*

reactions of (2-MeIm)pD (**3**) controlled by thymidine-containing templates[1]). In addition, we wish to demonstrate that automated DNA sequencers may be used as a novel and highly efficient tool for the analysis of RNA-chain extension.

2. Oligomerization Experiments. – To determine the rates of RNA-chain extension, we first applied our existing methods [6]: a short acridine-labeled primer was incubated with the activated mononucleotides (2-MeIm)pG (= **G***; **1**), (2-MeIm)pA (= **A***; **2**), or (2-MeIm)pD (= **D***; **3**) in the presence of different template strands. Product mixtures were then analyzed by reversed-phase HPLC. Unfortunately, the peak of the fully extended primer chain often became broad and poorly resolved. While the general results of these experiments could be confirmed by the findings presented below, the

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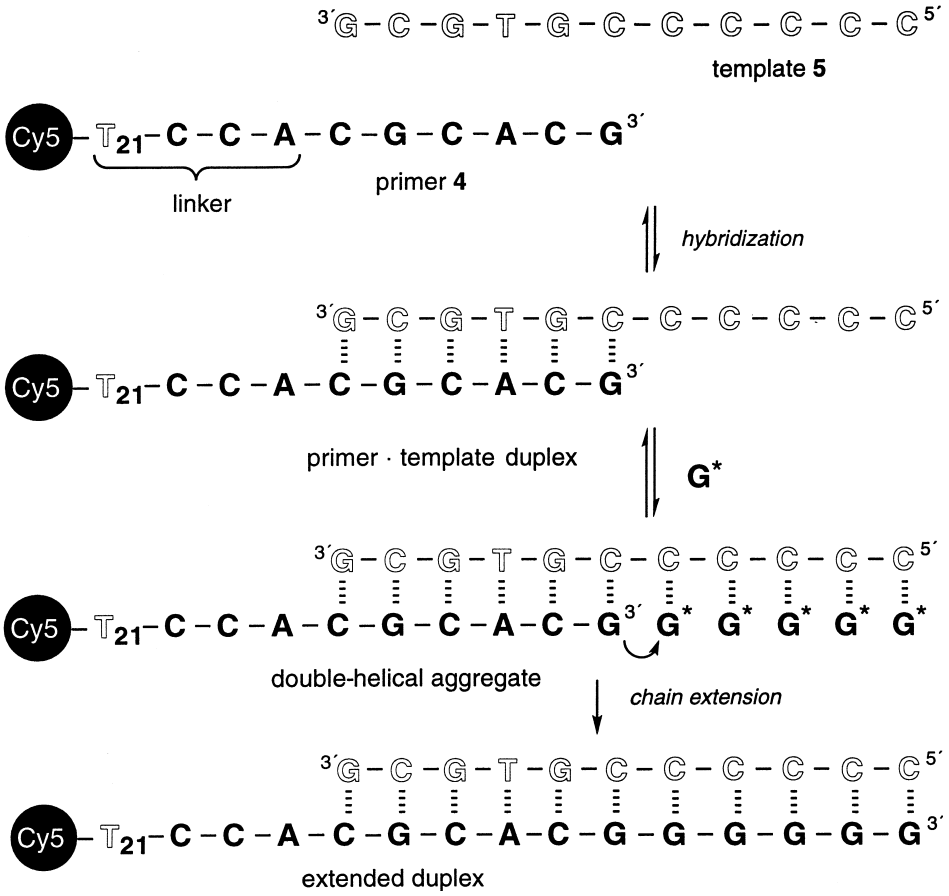
[1]) Purine-2,6-diamine has been incorporated into different types of natural [10] and unnatural [11][12] nucleic-acid structures. First attempts to oligomerize (Im)pD and (2-MeIm)pD (**3**) on a poly(U) template were done in *Orgel*'s laboratory almost two decades ago [13]. However, only moderate yields of dimers and trimers could be obtained in these experiments.

absolute precision of the data was not always satisfying. We, therefore, developed an improved analytical technique based on gel electrophoresis on a commercial DNA sequencer. To operate the instrument under (almost) standard conditions, a minimal primer length is required.

In addition, depending on the laser system in use, each type of sequencer calls for a specific dye. Good results were obtained with primer **4**, consisting of an RNA part and a DNA linker connecting it to a 5′-terminal dye label (Cy5; excitation wavelength 633 nm). Primer **4** was incubated with one of the template strands **5**–**9** and (2-MeIm)pG (**1**), (2-MeIm)pA (**2**), or (2-MeIm)pD (**3**). The mechanism of chain extension is outlined in *Scheme 1:* at the same time as primer **4** and template **5** hybridize, mononucleotides **1** (= **G***) associate with the single-stranded region to form a double-helical aggregate. The proximity of functional groups in these complexes facilitates the nucleophilic attack of 3′ alcohol functions at the activated phosphate esters of **1**, **2**, or **3**, while the sequential arrangement of the monomers is given by the

Scheme 1. *Template* **5** *Controlling the Chain Extension of Primer* **4** (see *Table 1*). Ribonucleotides are symbolized by bold, deoxyribonucleotides by outlined letters.



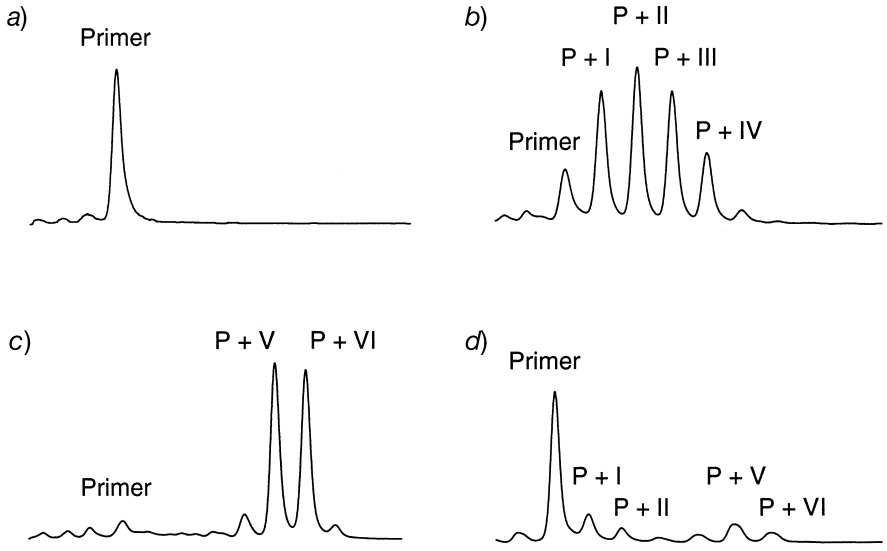


Fig. 2. *Typical curves obtained from the DNA sequencer:* a) *primer* **4** *without templates and activated nucleotides* (control), b) *template* **9** *controlling the chain extension of primer* **4** *with (2-MeIm)pD* (**3**) (after 7 d; see *Scheme 2*,*d*, and *Table 5*), c) *template* **7** *controlling the chain extension of primer* **4** *with (2-MeIm)pG* (**1**) *and (2-MeIm)pD* (**3**) (after 2 d; see *Scheme 2*, *b*, and *Table 3*), d) *template* **7** *controlling the chain extension of primer* **4** *with (2-MeIm)pG* (**1**) *and (2-MeIm)pA* **2** (after 2 d; see *Scheme 2*, *b*, and *Table 3*)

base-pairing rules. The chemical consequence thus is template-controlled RNA oligomerization. In principle, any type of labeling and electrophoretic separation could be used to analyze the resulting mixtures. The excellent sensitivity, speed, and sample throughput, however, are important features of a sequencer, making it the instrument of choice. Some typical results are shown in *Fig. 2*.

To check the equivalence of our old and new experimental setup, primer **4** was hybridized with template **5** and incubated with (2-MeIm)pG (**1**), Mg^{2+}, and buffer. Template **5** gave rise to a primer extension by five guanosine units (see *Scheme 1*). The duplex-forming part of primer **4** was constructed entirely from ribonucleotides to ensure an A-type conformation, a crucial condition for efficient chain extension [6][14]. The results, summarized in *Table 1*, are in good agreement with our previous data obtained from acridine-labeled primers [6]. All elongation steps except the last were very fast. The combined yield of (**4** + IV) and (**4** + V), therefore, is a good criterion for the success of the template-controlled reaction. It surpassed 95%. After long incubation times, minor amounts of the 'overextended' product (**4** + VI) became visible, an effect well-documented in the literature [5][6].

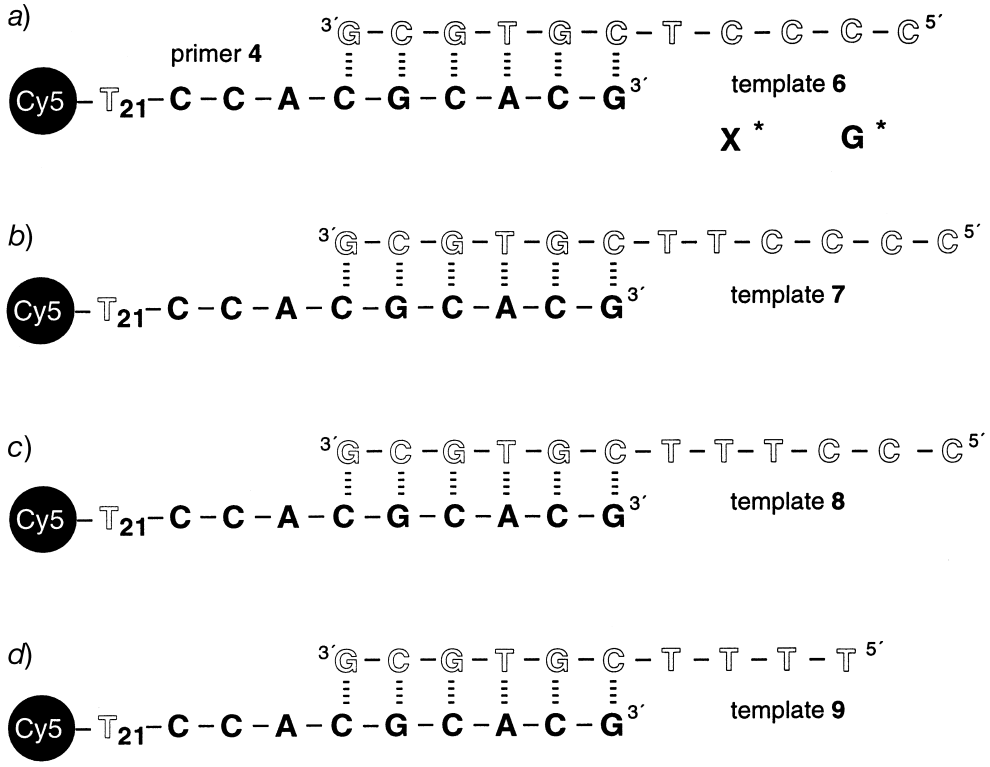
Having established the analytical tools, primer **4** was reacted next with a mixture of mononucleotides **1** and **2** (=**G*** and **A***) or **1** and **3** (=**G*** and **D***) in the presence of template **6** (*Scheme 2*, *a*). Template **6** should lead to extension by one adenosine (or D, resp.) and four guanosine units (*Table 2*). (2-MeIm)pD (**3**) reacted slightly faster than the adenosine derivative **2**, but the difference was not pronounced. After several days,

Table 1. *Extension of the Duplex* **4·5**: *Product Distribution* [%] *after Constant Time* [a])

Time	**X***	Primer **4**	**4** + I	**4** + II	**4** + III	**4** + IV	**4** + V
1 d	**G***	2.5	0.5	0.5	1.0	21.5	74.0
2 d	**G***	2.0	0.5	0.5	1.0	15.0	81.0
7 d	**G***	1.0	0.5	0.5	1.0	7.0	90.0
14 d	**G***	0.5	0.5	0.5	1.0	6.0	91.5

[a]) Conditions: 30 μM primer **4**, 60 μM template **5**, 50 mM **1**, 0.25M *Tris*·HCl (pH 7.7), 0.2M Mg^{2+}, 10°.

Scheme 2. *Templates* **6–9** *Controlling the Chain Extension of Primer* **4** (see *Tables 2–5*). For symbols, *see Scheme 1*.



the results became almost identical. In both cases, excellent yields of the fully elongated strands were obtained.

In the following experiment with template **7**, the incorporation of two consecutive A or D residues and four G units was studied (*Scheme 2, b*; *Table 3*). In agreement with earlier results [7], the primer extension by (2-MeIm)pA (**2**) and (2-MeIm)pG (**1**) proceeded slowly. Only traces of the final product could be detected after one day. The yield improved to moderate values upon standing for two weeks. Replacing **2** by (2-MeIm)pD (**3**) caused a dramatic change. More than 97% of primer **4** reacted forming up to 94% combined yield of the extension products (**4** + V) and (**4** + VI) (*cf. Fig. 2, c* and *d*).

Table 2. *Extension of the Duplex* **4**·**6**: *Product Distribution* [%] *after Constant Time* [a])

Time	X*	Primer **4**	**4** + I	**4** + II	**4** + III	**4** + IV	**4** + V
1 d	**A*/G***	8.0	4.5	2.5	8.0	47.5	30.0
	D*/G*	5.0	2.0	1.0	5.0	46.0	41.0
2 d	**A*/G***	5.0	3.0	1.0	4.0	34.0	53.0
	D*/G*	3.0	1.5	1.0	3.5	33.0	58.0
7 d	**A*/G***	2.5	1.5	0.5	1.5	12.5	81.5
	D*/G*	2.0	1.0	1.0	1.5	13.5	81.0
14 d	**A*/G***	2.0	1.5	0.5	1.5	7.5	87.0
	D*/G*	1.5	1.0	1.0	1.5	9.0	86.0

[a]) Conditions: 30 μM primer **4**, 60 μM template **6**, 25 mM **1**, 25 mM **2** or **3**, 0.25M *Tris*·HCl (pH 7.7), 0.2M Mg^{2+}, 10°.

Table 3. *Extension of the Duplex* **4**·**7**: *Product Distribution* [%] *after Constant Time* [a])

Time	X*	Primer **4**	**4** + I	**4** + II	**4** + III	**4** + IV	**4** + V	**4** + VI
1 d	**A*/G***	72.5	15.5	5.0	1.5	1.5	3.0	1.0
	D*/G*	10.5	2.0	2.0	3.0	10.0	47.0	25.5
2 d	**A*/G***	59.0	15.0	6.5	2.0	4.0	9.0	4.5
	D*/G*	5.5	1.0	0.5	1.0	6.0	41.0	45.0
7 d	**A*/G***	42.5	11.5	5.5	2.5	6.5	13.0	18.5
	D*/G*	3.0	1.5	0.5	0.5	2.0	16.5	76.0
14 d	**A*/G***	31.5	11.0	5.5	2.0	4.0	13.5	32.5
	D*/G*	2.5	1.0	0.5	0.5	1.5	10.5	83.5

[a]) Conditions: 30 μM primer **4**, 60 μM template **7**, 25 mM **1**, 25 mM **2** or **3**, 0.25M *Tris*·HCl (pH 7.7), 0.2M Mg^{2+}, 10°.

The difference in reactivity between (2-MeIm)pA (**2**) and (2-MeIm)pD (**3**) became most prominent with template **8** (*Scheme 2*, *c*). The sequence TTT completely blocked the primer extension by nucleotides **1** and **2** (*Table 4*). When (2-MeIm)pD (**3**) substituted nucleotide **2**, a slow but efficient process started producing a final combined yield of (**4** + V) and (**4** + VI) of more than 90%!

Encouraged by this result, primer **4** was hybridized with template **9** containing four consecutive T residues (*Scheme 2*, *d*, and *Table 5*). Even in this desperate case,

Table 4. *Extension of the Duplex* **4**·**8**: *Product Distribution* [%] *after Constant Time* [a])

Time	X*	Primer **4**	**4** + I	**4** + II	**4** + III	**4** + IV	**4** + V	**4** + VI
1 d	**A*/G***	83.5	16.0	0.5	0	0	0	0
	D*/G*	23.0	12.0	3.0	6.0	12.5	31.0	12.5
2 d	**A*/G***	77.5	22.0	0.5	0	0	0	0
	D*/G*	12.0	6.0	1.0	3.0	10.0	39.0	29.0
7 d	**A*/G***	61.5	31.5	5.0	1.0	0.5	0.5	0
	D*/G*	4.0	2.0	1.0	1.0	3.5	21.0	67.5
14 d	**A*/G***	53.5	35.5	7.5	1.0	1.0	1.0	0.5
	D*/G*	3.5	1.0	0.5	0.5	2.0	13.0	79.5

[a]) Conditions: 30 μM primer **4**, 60 μM template **8**, 25 mM **1**, 25 mM **2** or **3**, 0.25M *Tris*·HCl (pH 7.7), 0.2M Mg^{2+}, 10°.

Table 5. *Extension of the Duplex* **4**·**9**: *Product Distribution* [%] *after Constant Time* [a])

Time	X*	Primer **4**	**4** + I	**4** + II	**4** + III	**4** + IV
1 d	**A***	84.5	15.0	0.5	0	0
	D*	33.0	40.5	19.0	6.5	1.0
2 d	**A***	78.0	21.0	1.0	0	0
	D*	23.0	38.0	24.0	12.0	3.0
7 d	**A***	64.0	32.0	4.0	0	0
	D*	10.5	24.0	27.5	23.5	14.5
14 d	**A***	58.0	35.0	6.5	0.5	0
	D*	8.5	20.0	26.5	24.5	20.5

[a]) Conditions: 30 μM primer **4**, 60 μM template **9**, 50 mM **2** or **3**, 0.25M *Tris*·HCl (pH 7.7), 0.2M Mg^{2+}, 10°.

considerable amounts of the fully extended product were formed by (2-MeIm)pD (**3**) (see *Fig. 2, b*).

Discussion. – When dipolar functional groups form H-bonds, the most simple interpretation is coulombic attraction between the complementary partial charges of the directly interacting atoms. At first glance, the base pairs G·C and D·T seem to be equivalent due to their identical number of H-bonds. However, even the simplified partial-charge model immediately shows that an important difference arises from secondary (crosswise) interactions. While, in the case of G·C, two repulsive and two attractive interactions compensate each other, four repulsive secondary interactions clearly destabilize the D·T base pair (*Fig. 3*). Detailed Monte Carlo simulations of base-pair stabilities in $CHCl_3$ suggest that the energy gain of D·U over A·U due to the third H-bond is completely lost as a result of this effect [8]. In the light of such predictions and of experimental host-guest stability constants (summarized in [8]), the substitution of A by D should not have a major impact. On the other hand, there is good evidence from nucleic-acid chemistry for increased base-pair stability of D·U compared to A·U [10][11]. The distinct advantage of (2-MeIm)pD (**3**) over (2-MeIm)pA (**2**), nevertheless, is a positive surprise. Thus, (2-MeIm)pD opens interesting possibilities to expand the field of template-controlled RNA oligomerization. The potential and limitations of this approach are currently under investigation.

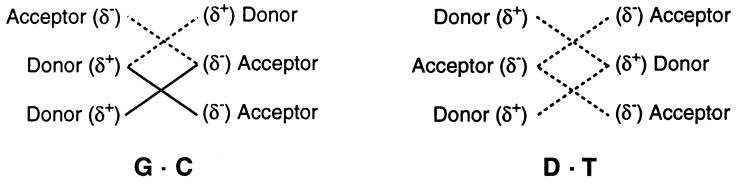


Fig. 3. *Secondary electrostatic interactions in triply hydrogen-bonded complexes* (– attractive; ····· repulsive).

Financial support by the *Fonds der Chemischen Industrie*, Frankfurt, Germany, is gratefully acknowledged.

Experimental Part

General. Water bath: *Lauda RMT 6*; precision ±0.2°. TLC: *Merck* aluminium sheets coated with silica gel *F 254/366*. 1×TBE Buffer: 100 mM *Tris*·HCl, 80 mM boric acid, 1 mM EDTA. Sequencer: *ALFExpress* from *AP Biotech* with *ALFWin* Instrument Control, Version 2.00.15a; Prep. reversed-phase HPLC: *Waters* pump *P-590*, RI-detector *Waters* differential refractometer *R-401*; column: *Dr. Maisch Reprosil C18-AQ* (10 μm), 250 × 20 mm. FT-IR: *Perkin-Elmer 1600*; in $\tilde{\nu}$ [cm^{-1}]. ^{1}H-NMR: *Bruker-AM-250* or *Bruker-AMX-400* spectrometer; chemical shifts δ in ppm rel. to (D_6) DMSO (2.50 ppm) or HDO (4.52 ppm) as internal standards, *J* in Hz. ^{31}P-NMR: *Bruker AMX 400* (161.98 MHz); δ rel. to phosphoric acid as external standard (0.00 ppm). ESI-MS: *Fisons VG* Platform II.

Adenosin-2-amine 5′-(Dihydrogen Phosphate). Freshly distilled phosphoryl chloride (0.92 ml, 9.70 mmol) was added dropwise to a stirred suspension of adenosine-2-amine (705 mg, 2.50 mmol) in dry triethyl phosphate (10.0 ml) at 0°. After 90 min, excess phosphoryl chloride was removed *in vacuo*, and the resulting clear soln. was treated with H_2O (10 ml) for 30 min. Solvents were removed by bulb-to-bulb distillation. The white solid obtained was dissolved in 1% aq. LiOH soln. (10 ml) and poured into a stirred soln. of acetone (500 ml). The precipitate was isolated by filtration, washed with acetone, and dried *in vacuo*. The crude product was purified by prep. reversed-phase HPLC (0.05M (Et_3NH)OAc (pH 6.5) + 5% MeOH) and the colorless powder obtained dried *in vacuo:* 620 mg (66%). TLC (^{i}PrOH/NH_4OH/H_2O 55:10:35): R_f 0.55. IR (KBr): 3335*s*, 3180*s*, 2679*w*, 1708*m*, 1650*s*, 1595*s*, 1482*w*, 1406*m*, 1283*w*, 1214*m*, 1175*m*, 1040*s*, 962*m*. ^{1}H-NMR (D_2O, 250 MHz): 3.69 (*m*, 2 H–C(5′)); 4.04 (*m*, H–C(4′)); 4.19 (*dd*, *J* = 5.1, 3.4, 1 H–C(3′)); 4.47 (*t*, *J* = 5.6, H–C(2′)); 5.65 (*d*, *J* = 6.1, H–C(1′)); 7.96 (*s*, H–C(8)). ^{31}P-NMR (D_2O): 4.78 (*t*, *J* = 4.7, *s* after decoupling). ESI-MS: 361.1 (M^-; calc. 361.1).

*Adenosin-2-amine 5′-[Sodium (2-Methyl-1*H*-imidazol-1-yl)phosphonate]* (**3**·Na^+). Adenosin-2-amine (250 mg, 0.69 mmol) and 2-methyl-1*H*-imidazole (0.57 g, 6.9 mmol) were dissolved under N_2 in dry, warm DMSO (8 ml). DMF (8 ml), Et_3N (0.29 ml, 2.07 mmol), and Ph_3P (0.40 g, 1.52 mmol) were added, and the mixture was gently heated until a clear soln. was obtained. After addition of 2,2′-dithiodipyridine (0.46 g, 2.07 mmol), the yellow soln. was stirred for 2 h at r.t. and then poured into a stirred mixture of acetone (200 ml), Et_2O (125 ml), Et_3N (15 ml), and $NaClO_4$ (0.51 g, 4.14 mmol). The precipitate was isolated by filtration, washed with acetone/Et_2O 1:1 and Et_2O, and dried *in vacuo:* 288 mg (93%) of **3**·Na^+. Colorless powder. TLC (^{i}PrOH/NH_4OH/H_2O 55:10:35): R_f 0.75. IR (KBr): 3342*s*, 3204*s*, 2938*w*, 1706*w*, 1639*s*, 1479*w*, 1410*m*, 1274*m*, 1200*m*, 1103*s*, 1044*m*, 990*w*. ^{1}H-NMR ((D$_6$)DMSO, 400 MHz): 2.38 (*s*, Me); 3.64 (*m*, 1 H–C(5′)); 3.76 (*m*, 1 H–C(5′)); 3.90 (*m*, H–C(4′)); 4.03 (*dd*, *J* = 4.7, 3.0, H–C(3′)); 4.52 (*t*, *J* = 5.5, H–C(2′)); 5.28, 5.41 (2 br. *s*, exchange with D_2O, OH); 5.71 (*d*, *J* = 6.2, H–C(1′)); 5.81 (br. *s*, exchange with D_2O, NH_2); 6.60 (*t*, *J* = 1.2, 1 H, (MeI*m*)); 6.73 (br. *s*, exchange with D_2O, NH_2); 7.07 (*t*, *J* = 1.2, 1 H, (MeI*m*)), 7.94 (*s*, H–C(8)). ^{31}P-NMR ((D$_6$)DMSO): −8.81 (*t*, *J* = 6.0, *s* after decoupling). ESI-MS: 425.2 (M^-; calc. 425.1).

*Adenosine 5′-[Sodium (2-Methyl-1*H*-imidazol-1-yl)phosphonate]* (**2**·Na^+). As described above, with adenosine- 5′-(dihydrogen phosphate) (500 mg, 1.44 mmol). Precipitation yielded 570 mg (92%) of **2**·Na^+. Colorless powder. TLC: (^{i}PrOH/NH_4OH/H_2O 55:10:35). R_f 0.65. IR (KBr): 3342*s*, 3200*s*, 2936*w*, 1648*s*, 1604*m*, 1577*w*, 1479*w*, 1420*w*, 1405*w*, 1333*w*, 1261*m*, 1200*m*, 1102*s*, 1043*m*, 991*w*. ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 2.39 (*s*, Me); 3.69 (*m*, 1 H–C(5′)); 3.72 (*m*, 1 H–C(5′)); 3.94 (*m*, H–C(4′)); 4.09 (*m*, H–C(3′)); 4.59 (*t*, *J* = 5.4, H–C(2′)); 5.40 (br. *s*, exchange with D_2O, 2 OH); 5.89 (*d*, *J* = 6.0, H–C(1′)); 6.65 (*t*, *J* = 1.2, 1 H, (MeI*m*)); 7.09 (*t*, *J* = 1.2, 1 H, MeI*m*); 7.31 (br. *s*, exchange with D_2O, NH_2); 8.13 (*s*, H–C(2)); 8.40 (*s*, H–C(8)). ^{31}P-NMR ((D$_6$)DMSO): −8.70 (*t*, *J* = 6.2, *s* after decoupling). ESI-MS: 410.3 (M^-; calc. 410.3).

*Guanosine 5′-[Sodium (2-Methyl-1*H*-imidazol-1-yl)phosphonate]* (**1**·Na^+). As described previously [6].

Cy5-T_{21}r(CCA CGC ACG) (**4**). Primer **4** was assembled on a *381A DNA* synthesizer (*Applied Biosystems*) by standard phosphoramidite chemistry. The oligonucleotide was cleaved from the solid support by treatment with a mixture of aq. NH_3 soln./EtOH 3:1. This soln. was incubated at r.t. for 24 h to remove the base- and phosphate-protecting groups. Desilylation was accomplished with $Et_3N \cdot 3$ HF for 12 h, followed by desalting on a *SepPak RP-18* (*Waters*) cartridge.

DNA Oligomers d(CCCCCCGTGCG) (**5**), d(CCCCTCGTGCG) (**6**), d(CCCCTTCGTGCG) (**7**), d(CCCTTTCGTGCG) (**8**), and d(TTTTCGTGCG) (**9**) were purchased from *MWG* Biotech.

Oligomerization Experiments. Into a 1.5-ml *Eppendorf* tube were pipetted the following 3 solns.: buffer, primer **4**, one of the templates **5**–**9**. The mixture was heated to 90° for 1 min. After equilibration for 15 min at r.t., it was cooled to 10°. Finally, a freshly prepared aq. soln. of mononucleotides **1**–**3** was added, the soln. mixed ($t = 0$), and the tube sealed and maintained at 10 ± 0.2°. The final conc. were: 30 μM primer, 60 μM template, 50 mM mononucleotide, 250 mM buffer (Tris·HCl, pH = 7.7), 200 mM Mg^{2+}.

ALFExpress *Analysis of Oligomerization Experiments*. For a primary sample, an aliquot of the reaction mixture (1.0 μl) was diluted with formamide (99 μl) and stored at −30° for later use. For analysis, the primary

sample (0.25 µl) was diluted with *ALF* loading buffer (5 mg/ml dextran blue in formamide; 6.0 µl) and loaded onto a 16% denaturating polyacrylamide gel (7.0M urea, 1 × TBE buffer). *ALFExpress* run conditions: *U* 1500 V, *I* 60 mA, *P* 25 W, *T* 55°, sampling interval 2 s, 0.5 × TBE buffer. Data was collected with *ALFWin* Instrument Control, Version 2.00.15a, and the peaks obtained were integrated with *AlleleLinks*, Version 1.0 from *AP Biotech*. Product distribution was determined by dividing each integral by the sum of all areas (primer + products).

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Received July 4, 2000