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Quantitative measurements on the duplex stability of 2,6-diaminopurine and 5-chloro-uracil nucleotides using enzymatically synthesized oligomers

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2,6-Diaminopurine and 5-chloro-uracil 2'-deoxynucleoside 5'-triphosphates were synthesized from their 2'-deoxynucleosides. Using a method of creating oligonucleotides by enzymatic primer extension, dodecanucleotides representing an *XbaI/SalI* site and the complementary *SalI/XbaI* site were generated containing these base modifications. Their duplex stability was quantitatively compared by thin-layer chromatography to oligomers containing 2'-deoxyadenosine and 2'-deoxythymidine. The two unmodified oligomers already showed significant differences in dissociation temperature and binding equilibrium. Substitution with 5-chloro-2'-deoxyuridine did not affect the dissociation temperature of either oligomer, the 2,6-diaminopurine, however, led to an increase of 1.8°C or 1.5°C per modified base, respectively. While in the *XbaI/SalI* oligomer both base modifications changed the binding equilibrium, the 2,6-diaminopurine by a factor of 1.32, the 5-chloro-uracil by 0.65, no such effect was found with the complementary oligomer.

Thin-layer chromatography; 2,6-Diaminopurine; 5-Chloro-uracil; Thermodynamics

1. INTRODUCTION

Hybridization of short oligonucleotide probes has become a major tool in molecular biology, particularly more recently progressing towards the use of very short oligomers. Studier [1], for instance, proposed an improved sequencing strategy, based on enzymatic primer extension with dideoxynucleotides but using octanucleotide primers. Another new application is the physical mapping of large genomes by 'oligomer fingerprinting' whose practical feasibility has been demonstrated [2]. Also, large scale sequencing using hybridization of genomic DNA fragments to an array of oligomers (e.g. all 65 536 octamers) has been proposed [3,4]. All these methods, but also standard procedures like the polymerase chain reaction, for instance, would gain from an improvement in duplex stability of oligonucleotides. A way to achieve this is the substitution of natural nucleotides by base modifications, like pyrimidines with a halogen at the C^5-position, which reportedly influences the base stacking [5], and 2,6-diaminopurine with a third hydrogen bond in its base pairing with thymine [6,7] which is known to thermally stabilize DNA-duplexes [8,9]. Since both 2,6-diaminopurine and 5-chloro-uracil 2'-deoxynucleosides are not available as phosphoramidites for a standard chemical synthesis of oligonucleotides, their 5'-triphosphates were synthesized, being an ample source of nucleotide for the generation of different oligomers bearing modified (or unmodified) bases by means of a conventional fill-in reaction. Dodecamers representing the respective strand of an *XbaI/SalI* site were used to quantitatively determine how the base substitutions affect the dissociation temperature (T_d) and the binding equilibrium.

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2. MATERIALS AND METHODS

2.1. *Synthesis of nucleoside triphosphates*

5-Chloro-2'-deoxyuridine (5-Cl-dUrd) and 2,6-diaminopurine 2'-deoxynucleoside (2-NH_2-dAdo) were from Sigma. Phosphorylation was carried out following a protocol modified from Goody and Isakov [10] and Ludwig [11]. 50 mg dry 2-NH_2-dAdo was taken up in 500 μl dry triethyl phosphate stirring under argon. 25 μl $POCl_3$ were added and the mixture was incubated at −20°C. In the meantime, 1 mmol pyrophosphoric acid was dissolved in 0.95 ml tri-*n*-butylamine and 2 ml methanol and dried in a rotary evaporator. Subsequently it was dried by evaporation twice from 5 ml pyridine, with 70 μl tri-*n*-butylamine also added before the second time. Finally, it was dissolved in 2 ml dry dimethyl formamide.

After 90 min at −20°C the phosphorylation mixture was evaporated to remove excess $POCl_3$ and the tri-*n*-butylammonium pyrophosphate in dimethyl formamide was added. Incubation was for 1.5 min at room temperature. The reaction was stopped by addition of 5 ml 0.2 M triethylammonium bicarbonate (pH 7.6) and kept on ice for 4 h. For 5-Cl-dUrd the conditions were identical, but 50 μl $POCl_3$ were added and the phosphorylation was carried out at room temperature for 4 h.

After the hydrolysis, the mixture was evaporated, the pH adjusted to 7.5, and extracted with 1 volume diethyl ether. Separation of the

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I. 5'-TCTAGA GTCGAC-3'

II. 3'-AGATCT CAGCTG-5'

(XbaI) (SalI)

Fig. 1. Sequences of the two complementary dodecanucleotides used as probes. The exact positions of the incisions by *Xba*I and *Sal*I in double stranded DNA are indicated.

products was on a (2.5 × 20 cm) Q-Sepharose column using a linear gradient of 0.15 M to 0.8 M triethylammonium bicarbonate.

Yields of 8.7 μmol 2-NH_2-dATP (5%) and 27 μmol 5-Cl-dUTP (14.2%) were achieved. Though less than reported for deoxyadenosine (dAdo) and derivatives of deoxyuridine [11], even the former yield provides enough material for more than 4300 reactions of 50 μl containing 40 μM nucleotide. Stored frozen, the nucleotides were found to be stable over long periods of time.

2.2. *Oligonucleotide probes*

Oligonucleotides were generated and labeled to high specific activity as described [12]. Using a primer/template system, primer extension was carried out in the presence of modified or unmodified nucleotides and α-^{32}P label. The newly synthesized DNA was subsequently released by cleavage with a mixture of uracil-DNA glycosylase and endonuclease IV, and isolated from 20% acrylamide gels. As is the case with deoxythymidine (dThd = 5-methyl-dUrd), 5-Cl-dUrd was found to be no substrate for the uracil-DNA glycosylase.

2.3. *Thin-layer chromatography*

Thin-layer chromatography (TLC) was performed as described in detail elsewhere [13]. Single stranded target-DNA was created by either *Hin*dIII or *Eco*RI cleavage of plasmid pJDH118 [14] followed by exonuclease III digestion as described [15]. Samples of 3.1 fmol probe annealed to 0.94 pmol plasmid-DNA were spotted on TLC-plates (Polygram CEL300, DEAE/HR-1/15). The plates were developed in 8 × SSC (1.2 M NaCl, 0.12 M Na-citrate) perpendicular to a temperature gradient. All free oligomer was removed by the buffer, while only oligomer bound to the plasmid-DNA remained at the origin. The plates were autoradiographed together with different dilutions of labeled probe of known concentration and the autoradiograms were scanned for quantification.

3. RESULTS

Fig. 1 shows the two complementary dodecanucleotides used as probes. They were chosen for the reasons that (i) all four bases occur three times in either sequence, and (ii) once each in a terminal position in one of the oligomers; (iii) their target sequence is the *Xba*I/*Sal*I site of the pJDH118 (and pUC18) polylinker, which allows easy access to template-DNA; (iv) after cleavage of pJDH118 with either *Xba*I or *Sal*I and subsequent single strand creation by exonuclease III, the hybridization of undecanucleotides could be checked in the same probe/template system.

To check the efficiency of incorporation of 2-NH_2-dATP and 5-Cl-dUTP by Klenow enzyme, which was

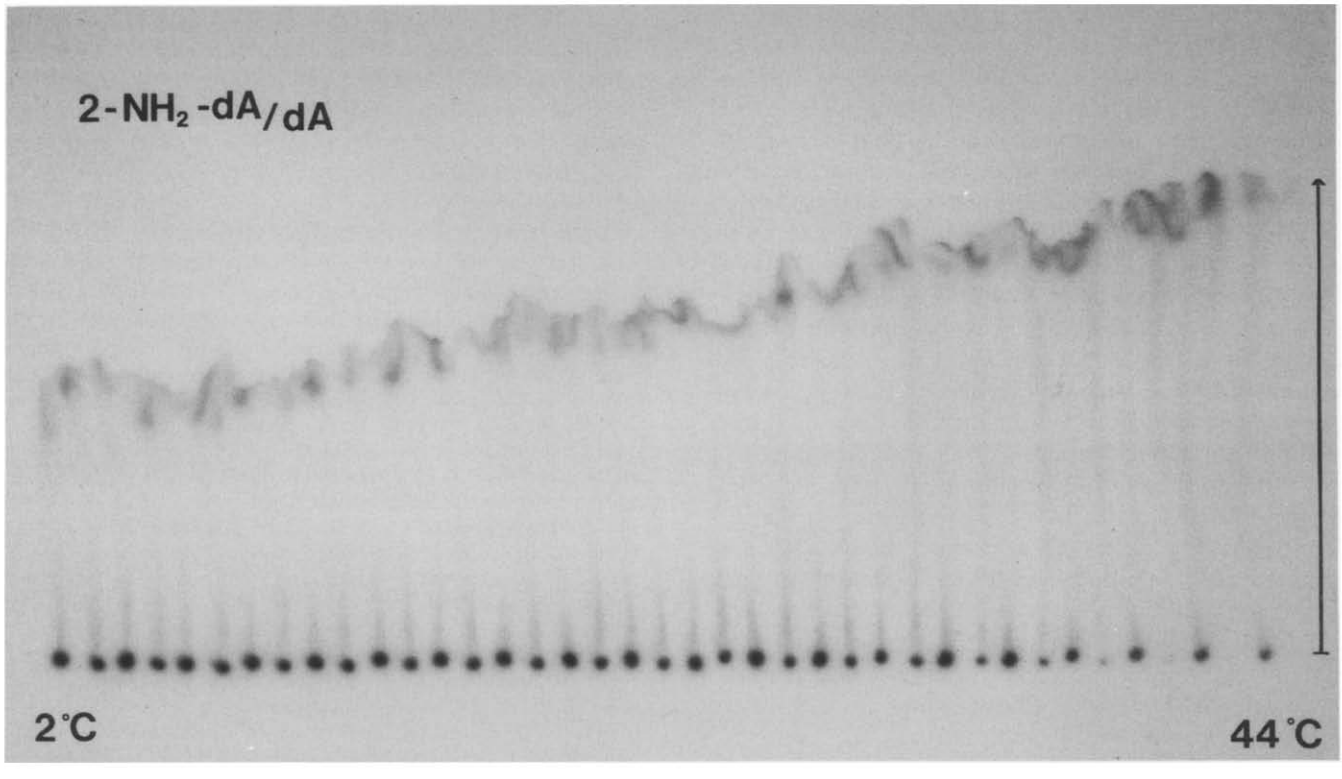

Fig. 2. TLC of oligomer I containing 2-NH_2-dAdo (slightly upper row) or dAdo (lower row), respectively. The direction of TLC was bottom to top, the temperature gradient being 2°C (left) to 44°C (right). The base of the arrow on the right marks the position of the spotted samples, its top the buffer front.

used to generate the oligonucleotides, reverse sequencing primer (New England Biolabs) was annealed to single stranded pJDH118, the primer's 3′ end 85 bp distant from the DNA-terminus created by the *Hin*dIII-cut before the exonuclease III reaction (see section 2). Extension in the presence of 40 μM and 180 μM of the respective modified nucleotide resulted in blunt-ended molecules and showed no difference in comparison to dATP or dTTP (results not presented).

Measuring the melting behaviour of oligonucleotides by TLC perpendicular to a continuous temperature gradient proved to be very useful, because a whole temperature range is examined in a single experiment and different probes can be used simultaneously (e.g. Fig. 2). Probe concentrations similar to those in actual filter hybridizations can be used, enabling a direct application of the results to real hybridization experiments [13].

Surprisingly, the two unmodified oligomers (Fig. 1) were found significantly different in both T_d and binding equilibrium (Fig. 3). Although identical in base composition and very similar in sequence, with only the two blocks of hexanucleotides having swapped position, the difference in T_d was determined being 7.9°C [34.0°C (±0.35°C) for oligomer I to 41.9°C (±1.2°C) for oligomer II], and 65% (±0.6%) of oligomer II were bound below 10°C, that is by a factor of 1.2 more than the 54% (±1.5%) of oligomer I.

An autoradiogram is shown in Fig. 2 of an actual TLC experiment comparing the stability of oligomer I containing either dAdo or 2-NH_2-dAdo. Directly from the intensities of the radioactivity remaining at the origins, it is obvious that the substitution of 2-NH_2-dAdo for dAdo affected the oligomer's stability. The T_d increased by 5.5°C from 34.0°C (±0.35°C) of the unmodified oligomer to 39.5°C (±0.7°C) (Fig. 3A).

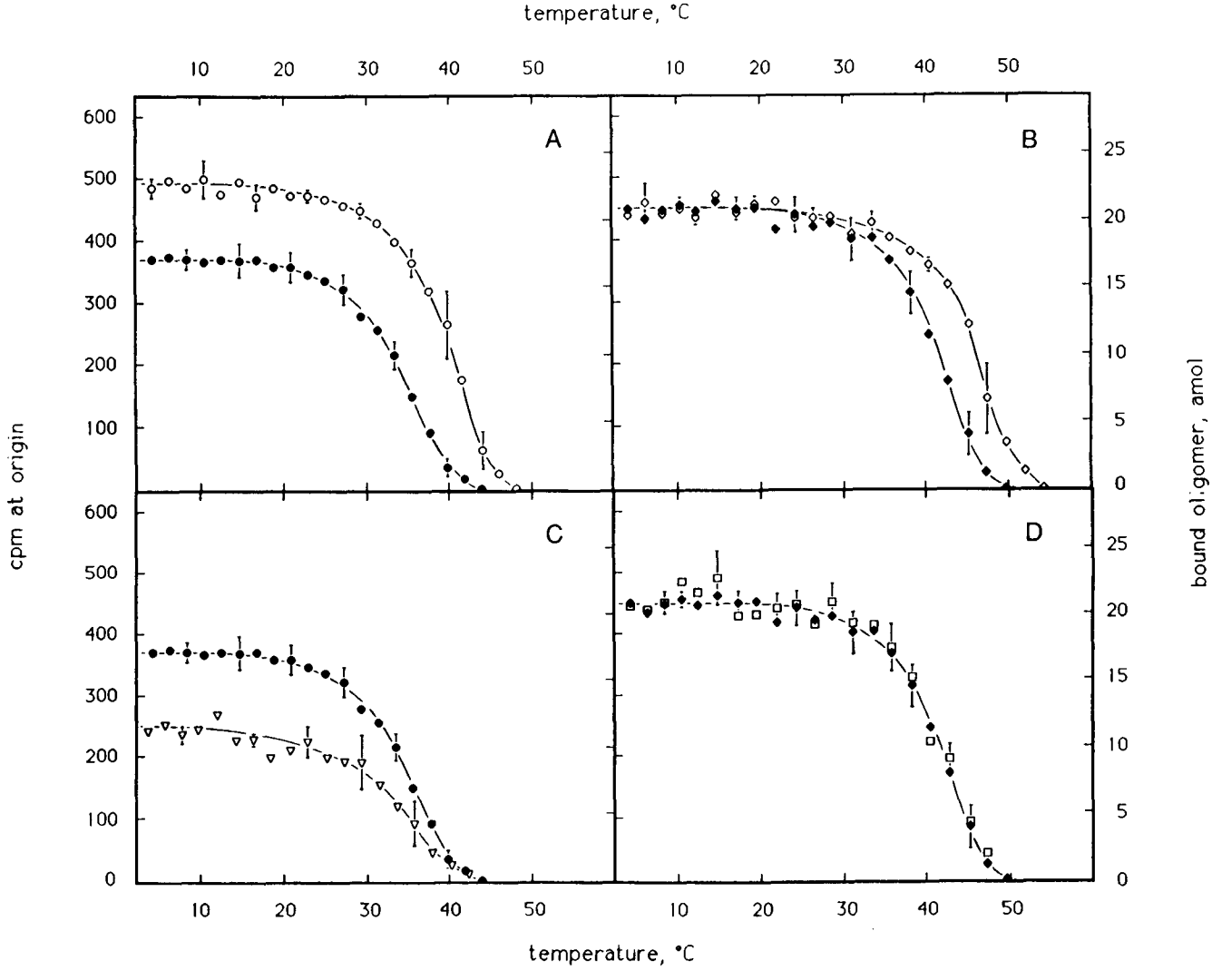

Fig. 3. Melting curves of oligomer I (A,C) and oligomer II (B,D). The two upper panels show the effect of substitution of dAdo (●, ♦) by 2-NH_2-dAdo (○, ◇), the lower panels the melting curves obtained with 5-Cl-dUrd (▽, □) instead of dThd (●, ♦). Each curve is the result of at least three entirely independent experiments, each point representing the average of 9 to 15 measurements; for each third point, the standard deviation is given.

Additionally, the binding equilibrium was improved over the whole temperature range. Below 10°C, for instance, 71.3% (±0.6%) of the modified oligomer was bound in comparison to only 54% (±1.5%) of the unmodified one. Using the oligomer II, the absolute difference in T_d was smaller with 4.6°C (41.9°C (±1.2°C) to 46.5°C (±0.6°C)). The amount of oligonucleotide bound below 10°C, however, did not change by substitution with 2-NH_2-dAdo (Fig. 3B).

5-Cl-dUrd (Fig. 3C) had no significant effect on the T_d of either oligomer. However, in oligomer I the substitution of 5-Cl-dUrd for dThd at positions 3 and 8 of the dodecamer reduced the amount of oligomer bound below 10°C by a factor of 0.65 to 35.1% (±0.8%) (the first nucleotide of the dodecamer could not be substituted, because it originated from the primer molecule used for its synthesis [12]). Again, there was no such effect in the complementary oligomer II, with the binding equilibrium being unchanged by the base modification (Fig. 3D).

4. DISCUSSION

The combination of the relatively simple phosphorylation of nucleosides to generate nucleotide triphosphates and the creation of oligomers by a conventional fill-in reaction provides a system to easily synthesize oligomers containing base modifications. Its use is even more versatile, because all components necessary - nucleotide triphosphates, template/primer oligonucleotides, uracil-DNA glycosylase - are stable if stored appropriately (uracil-DNA glycosylase is meanwhile commercially available from Boehringer-Mannheim). Here, the system was used to measure the effect of 2-NH_2-dAdo and 5-Cl-dUrd on the duplex stability of dodecamers.

Using 2-NH_2-dAdo, a considerable improvement in the duplex stability of short oligomers is achieved. Its incorporation should allow either more stringent conditions for primer annealing, thereby improving the specificity of the duplex formation and suppressing background problems, or the use of shorter oligomers. The lack of any significant difference in their effect on T_d between the halogen substitution of 5-Cl-dUrd and the 5-methyl group of dThd is in agreement with similar data obtained for the 5-bromo and 5-methyl derivatives of deoxycytidine [13].

The occurrence of changes in the binding equilibrium appears to be dependent on the sequence. This, and the considerable difference in duplex stability of the two unmodified dodecamers is the more surprising, since according to data from nearest neighbour analysis [16] the only difference in the sequence of the two oligomers (the central dinucleotide 'AG' or 'CT', respectively) should not matter. Further investigations into this direction are necessary to make the duplex formation of very short oligomers understood.

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