T4 RNA ligase catalyzed synthesis of base analogue-containing oligodeoxyribonucleotides and a characterization of their thermal stabilities

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ABSTRACT

Self-complementary oligodeoxyribonucleotides containing the base analogues 2-aminopurine, 2,6-diaminopurine, N⁶-methyladenine, uracil, and 5-bromouracil were synthesized by a general method that allows incorporation of the analogues at specific positions. The method uses chemically synthesized partial sequences but circumvents the need for protected base analogues by incorporating their unprotected 3',5'-bisphosphate derivatives enzymatically. T4 RNA ligase was used to add the analogues to the oligodeoxyribonucleotides with yields from 54 to greater than 95 percent. Oligodeoxyribonucleotides were joined to the oligodeoxyribonucleotides containing the analogues at their 3'-termini in yields from 22 to 81 percent. The high yields obtained in these joinings suggest that RNA ligase should be of general use for the specific incorporation of other deoxyribonucleoside analogues into oligodeoxyribonucleotides. The oligodeoxyribonucleotides containing the base analogues were characterized by their mobilities during HPLC, nucleoside compositions, sequences, and thermal stabilities.

INTRODUCTION

Oligodeoxyribonucleotides and DNA containing base analogues are useful for a variety of biochemical studies (1). Presently such oligonucleotides and DNA may be synthesized by either chemical or enzymatic techniques (2-8). For chemical syntheses, the appropriately protected nucleoside derivative is required. These protected analogues are neither readily available nor are they trivial to prepare (8). For enzymatic syntheses, a deoxyribonucleoside triphosphate derivative of the analogue is required for use with a DNA polymerase (2,3,7) or a diphosphate derivative for use with polynucleotide phosphorylase (4). The 5'-phosphate derivatives are sometimes difficult to prepare and, more importantly, are not always efficient substrates for the enzymes (3,4). The enzymatic techniques are also limited in their ability to incorporate base analogues into specified sites in the DNA sequence.

We describe here a general method for the synthesis of oligodeoxyribonucleotides that contain base analogues at specified positions. The method employs unprotected base analogues and combines a chemical and enzymatic

Figure 1. General synthetic scheme for the incorporation of a base analogue into oligodeoxyribonucleotides. RNA L, T4 RNA ligase; APase, alkaline phosphatase; N, base analogue.

approach. T4 RNA ligase catalyzes the formation of a 3' + 5' phosphodiester bond between an oligoribonucleotide acceptor (three or more nucleotides long) containing a 3'-hydroxyl group and another, the donor, bearing a 5'-phosphate (9). (See reviews 10,11.) RNA ligase can synthesize oligodeoxyribonucleotides of defined sequences (12-15) and will also join a variety of base analogues and compounds to RNA molecules (11). In the method described here, RNA ligase is used to add the 3',5'-bisphosphate derivative of the deoxyribonucleoside analogue to the 3'-terminus of an unprotected oligodeoxyribonucleotide and to join this fragment, after removal of its 3'-phosphate, to another unprotected fragment to create the final oligonucleotide. The bisphosphate derivative of the analogue and the appropriate fragments of oligonucleotides are prepared chemically. Figure 1 outlines the general synthetic scheme. By varying the starting substrates, the analogues can be incorporated at defined positions. The method is general, the yields of the enzymatic steps are reasonably high, and modified oligonucleotides can be made at the nmol scale with readily attainable amounts of RNA ligase (16).

We have used this methodology to synthesize octanucleotides containing the *EcoRI* restriction endonuclease and modification methylase recognition sequence with base analogue substitutions at specific sites. We present here the details of the syntheses, purification, and characterization of these oligonucleotides as well as the effects of the analogues upon the thermal stabilities of the oligonucleotides.

MATERIALS AND METHODS

Materials: The oligodeoxyribonucleotides, d(G-G-A) [d(GGA)]³ and d(GGAA) were gifts from B. Haas, M. Schnolzer, and H. Seliger (Ulm, FRG). The base,

2-aminopurine (2AP) was purchased from Vega Biochemicals and converted to the 2'-deoxyribonucleoside by R. Blakley (Memphis, TN) using trans-N-deoxyribosylase. The nucleoside, 2'-deoxy-N⁶-methyladenosine (m⁶A) was purchased from Vega Biochemicals. All other nucleosides were purchased from Pharmacia P-L Biochemical. Polystyrene with the protected 3'-deoxynucleoside attached and 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole were purchased from Bachem Inc. The protected mononucleotides, dinucleotides, and 1-(p-toluenesulfonyl)-4-nitroimidazole were purchased from Pharmacia P-L Biochemical. The protected nucleoside of m⁶A was a gift from R. Naylor of Pharmacia P-L Biochemical. Pyrophosphoryl chloride was a gift from W. Wittenberg and is available from Sigma. The method of Johnson and Walseth (17) was used to prepare [γ -³²P]ATP. DEAE-Elutip columns were provided by D. Bacon, Schleicher and Schuel, Inc. Homomixes were prepared as described (18). Tetrabutylammonium hydroxide was purchased from Fluka. T4 RNA ligase was purified as described (16). T4 polynucleotide kinase and T4 *Pse*T1 polynucleotide kinase were gifts from O. Uhlenbeck and D. Soltis. All other RNA ligase reaction components and the enzymes used for analyses were as in (12-16).

Anion exchange high performance liquid chromatography (HPLC) was carried out on aminopropylsilica (APS) columns purchased from either Dupont Co. (Zorbax NH₂) or Shandon Southern Instruments, Inc. (APS Hypersil). Reverse phase HPLC was performed using Ultrasphere octylsilica (OS) and Ultrasphere octadecylsilica (ODS) from Beckman Instruments, Inc. and ODS Hypersil from Shandon Southern Instruments, Inc. The following solvent systems were used for HPLC. I: Buffer A: 0.05 M potassium phosphate, pH 4.5, 10% methanol. Buffer B: 0.9 M potassium phosphate, pH 4.5, 10% methanol. II: Buffer A: 50 mM sodium phosphate, pH 5.9. Buffer B: 50 mM sodium phosphate, pH 5.9, 25% methanol. III: Buffer A: 10 mM ammonium phosphate, pH 7.0. Buffer B: 10 mM ammonium phosphate, pH 7.0, 20% acetonitrile. IV: Buffer A: 10 mM ammonium acetate, pH 5.9. Buffer B: 10 mM ammonium acetate, pH 5.9, 50% acetonitrile. V: Buffer A: 5 mM tetrabutylamine (TBA), 5 mM sodium phosphate adjusted to pH 6.5 with H₃PO₄. Buffer B: 5 mM TBA, 5 mM sodium phosphate, 70% acetonitrile, similarly adjusted to pH 6.5.

Synthesis of Substrates: The 2'-deoxyribonucleosides were converted to 2'-deoxyribonucleoside 3',5'-bisphosphates (pdNp) with pyrophosphoryl chloride by the procedure of Barrio et al. (19) and were purified as described (12). The nucleoside 3',5'-bisphosphate of 5-bromodeoxyuridine (brU) was prepared from pdUp and bromine by the method of Riley et al. (20) and purified as in (12).

The oligonucleotides, d(TTCC)rC, d(TCC)rC, and d(CC)rC were synthesized in solution by phosphate-phosphotriester chemistry (21,22). The fully deprotected oligonucleotides were purified by Dowex AG1-2X column chromatography with a linear gradient of 50 mM ammonium chloride, pH 8.0, 40% ethanol to 500 mM ammonium chloride, pH 8.0, 40% ethanol and desalted by Sephadex G10 chromatography in 30 mM triethylammonium bicarbonate (TEABC), pH 8.0, 20% ethanol. The purity was checked by reverse phase HPLC on an ODS column in solvent system III using a gradient of 5 to 62.5% buffer B over 45 minutes at a flow rate of 1 ml/minute. Nucleoside compositions were determined as described below and for d(TTCC)rC the ratio of dT:dC:rC was 1.94:2.03:1.00; for d(TCC)rC, 1.05:2.00:1.00, and for d(CC)rC, dC:rC, 2.03:1.00. The 3'-terminal ribonucleosides were removed from these oligonucleotides by periodate oxidation and β -elimination as described by Winter and Brownlee (23). The products after oxidation and elimination were purified on DEAE Sephadex A-25 columns using a linear gradient of 0.1 to 1.0 M TEABC, pH 7.5. To convert d(TTCCp), d(TCCp), and d(CCp) to donor molecules for RNA ligase reactions, they were phosphorylated by incubation with PseTl polynucleotide kinase [a mutant kinase that lacks 3'-phosphatase activity (24)] and a 10-fold excess of ATP as described (15). The phosphorylated products were separated from ATP on DEAE Sephadex A-25 columns as described above. Because, d(pCCp) and ATP coeluted from the DEAE A-25 column, d(pCCp) was purified by APS HPLC using solvent system I with a gradient of 0 to 100% buffer B over 30 minutes at a flow-rate of 3 ml/minute (25) and desalted by descending paper chromatography on Whatman 3 MM paper in 1-propanol:ammonia:water:: 55:35:10. Radiolabelled [5'-³²P]d(pTTCCp), [5'-³²P]d(pTCCp), and [5'-³²P]d(pCCp) were prepared using PseTl polynucleotide kinase and [γ -³²P]ATP and purified by paper chromatography in the system described above.

The oligodeoxyribonucleotides d(GGAATTCC) and d(GGm⁶AATCC) were synthesized by solid phase phosphate-phosphotriester synthesis (26). The oligodeoxyribonucleotides were deprotected except for the 5'-trityl group, and passed over a Sephadex G50 column in 10 mM TEABC, pH 8.0. They were concentrated by evaporation under vacuum and the TEABC removed by coevaporation with methanol. The oligonucleotides were further purified by ion-paired reverse phase HPLC on an ODS column in solvent system V using a gradient of 50 to 60% buffer B over 15 minutes then to 80% buffer B over 20 minutes at a flow rate of 1.5 ml/minute. The UV absorbance profile at 254 nm showed one major peak eluting late in the gradient for each

oligonucleotide. This material was collected, concentrated by evaporation under vacuum, and then treated with 80% acetic acid to remove the trityl group (26). The products were again desalted by Sephadex G50 chromatography. Final purification was by reverse phase HPLC on an ODS column in solvent system IV using a gradient from 0 to 25% buffer B over 25 minutes then to 100% buffer B over 5 minutes at flow rate of 1.5 ml/minute. The samples were heated at 65°C just prior to injection onto the column and the column was heated to 45°C to prevent the self-complementary oligonucleotides from forming duplexes. The major peak was again collected and concentrated by evaporation. The purity was checked by reverse phase HPLC on ODS and OS columns and found to be greater than 95%. The compositions of the oligonucleotides were verified by nucleoside analyses and the sequences determined by the wandering spot method (see below).

Addition of Nucleotide Analogues with RNA Ligase: The addition of 2'-deoxyribonucleoside 3',5'-bisphosphates to oligodeoxyribonucleotides using RNA ligase was performed as described in Brennan et al. (16). Product formation was monitored by HPLC on APS columns using solvent system I and a gradient of 0 to 45% buffer B over 45 minutes at a flow rate of 2 ml/minute at 35°C. The products were purified by chromatography on DEAE Sephadex A-25 columns using a linear gradient of either 0.05 to 0.8 M TEABC, pH 7.5 or 0.1 to 1.0 M TEABC, pH 7.5. The 3'-phosphates were removed from the products by treatment with alkaline phosphatase (AP) as described (15) and the oligonucleotides purified by descending paper chromatography on Whatman 3 MM paper in 1-propanol:ammonia:water:: 55:10:35. For reactions in which the unreacted donor was not recovered, the RNA ligase mixture was treated with AP prior to purification of the product. The products were characterized by their mobility on HPLC, UV spectral ratios, and in some initial cases the nucleoside compositions were analyzed and the sequences determined.

Coupling Oligodeoxyribonucleotides with RNA Ligase: The oligodeoxyribonucleotides to oligodeoxyribonucleotides joining reactions were performed as described (16) except that the concentration ratios of acceptor:donor:ATP were 1:1:0.04. In reactions using d(pCCp) as the donor, single nucleotide addition reaction conditions were used and the ratios of acceptor:donor:ATP were 1:4:0.2. Product formation was monitored using either anion exchange HPLC on APS columns using solvent system I and a gradient of 0 to 100% buffer B over 30 minutes at a flow rate of 3 ml/minute at 35°C or by gel electrophoresis on 20% polyacrylamide, 7 M urea gels (27). For the oligodeoxyribonucleotides joining reactions, 5'-³²P labelled donors were used and product formation was

quantified using the radioactivity of the HPLC fractions or in the bands cut from a gel. These products were purified as described above. When unreacted acceptor and donor were recovered, the reaction mixture was applied to an APS HPLC column and the appropriate fractions collected. The collected sample was diluted to 0.2 M potassium phosphate, applied to a DEAE Elutip, washed with 0.2 M TEABC, pH 8.0 and the oligonucleotide eluted with 1.0 M TEABC. The TEABC was removed by coevaporation with methanol. The product was treated with AP and purified by polyacrylamide gel electrophoresis. When the acceptor and donor were not recovered, the entire RNA ligase reaction mixture was treated with AP. Proteins and salts were removed by applying the sample and eluting it from a DEAE Elutip as above. The mixture was then fractionated by electrophoresis on a polyacrylamide gel and the product located by autoradiography. The product was eluted from the gel by soaking the gel piece in 0.5 M ammonium acetate, 1.0 mM EDTA, pH 8.0 at 37°C overnight and was then desalted on DEAE Elutips.

The oligodeoxyribonucleotides products were phosphorylated using polynucleotide kinase and a 10-fold excess of ATP over oligonucleotide. The product was purified from ATP and unphosphorylated oligonucleotide by ion-paired reverse phase HPLC on an ODS column using solvent system V and a gradient of 50 to 65% buffer B over 15 minutes at a flow-rate of 2 ml/minute and desalted by Sephadex G25 spin-columns and/or DEAE Sephadex A-25 spin-columns. Sephadex was packed into 1 ml syringes as described (27) and equilibrated with 10 mM TEABC, pH 8.0. The oligonucleotides were applied in a volume of 100-200 μ l and the column centrifuged at 1500 rpm for 3 minutes in a Damon-IEC HN-S centrifuge. The column was similarly washed several times with 100 μ l aliquots of 10 mM TEABC and fractions collected. Oligonucleotides eight nucleotides or longer eluted in the first fraction and the salt began eluting in the third fraction. DEAE Sephadex A-25 spin-columns were used to purify samples that contained a high concentration of salt. The resin (0.1-0.2 ml) was packed into 1.0 ml syringes, washed with 1.2 M TEABC, pH 8.0, and equilibrated in 0.2 M TEABC. The samples were diluted to bring the salt concentration to 0.2 M and applied to the columns which were centrifuged at 1000 rpm for 1 minute. The columns were then washed several times with 100 μ l 0.2 M TEABC, followed by 0.5 M TEABC. The oligonucleotides were eluted with 1.2 M TEABC and the TEABC removed by coevaporation with methanol. Products labelled with ^{32}P were prepared in the same way except that equimolar oligonucleotide and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2-5 Ci/mmol) were used.

Product Characterization: The oligodeoxyribonucleotides were quantified by measuring the absorbance at 260 nm and using reported extinction coefficients when available (28). The extinction coefficient of oligonucleotides for which no values were reported were estimated by using published extinction coefficients to determine an average extinction coefficient at 260 nm at pH 7-9 for each base in oligonucleotides of various lengths (29). The extinction coefficient for an oligonucleotide was calculated by summing the average value for each base in an oligonucleotide of that length. The error in the extinction coefficient calculated in this way was no more than 10% and usually less than 5% (29). Predicted spectral ratios were calculated by taking the weighted average of the spectral ratios for each base in the oligonucleotide (30).

The nucleoside composition of an oligodeoxyribonucleotide was determined by incubating the oligonucleotide (0.1-0.5 A_{260} units) with 0.5 mg/ml venom phosphodiesterase (VPD) and 0.1 mg/ml AP in 10 mM sodium glycinate, pH 9.2, 5 mM $MgCl_2$ for two hours at 37°C. The resultant nucleosides were separated by chromatography by OS reverse phase HPLC in solvent system II using a gradient of 0 to 60% buffer B over 20 minutes, then to 100% buffer B over 10 minutes at a flow-rate of 1 ml/minute (H. Coyer, Pharmacia P-L Biochemical, personal communication). The identity of the nucleosides was determined by comparison of the peak retention times with those of standards. The molar ratios were calculated by dividing the integrated peak area by the extinction coefficient at 254 nm for each nucleoside and normalizing the numbers. The extinction coefficient for each nucleoside was determined at 254 nm in 50 mM sodium phosphate, pH 5.9. The sequences of the synthesized oligodeoxyribonucleotides were verified by the wandering spot method as described by Frank and Blocker (31).

Thermal Stability of Octanucleotides: Melting curves for the 5'-phosphorylated octanucleotides were obtained by measuring the absorbance at 260 nm as a function of temperature from 5 to 65°C. The measurements were taken in a Beckman DU-8 spectrophotometer. The melting curves were determined over a concentration range of 3 to 30 μM of strands of octanucleotide in 250 to 300 μl of 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA. A plot of f , the fraction of duplex, versus temperature was calculated from the absorbance versus temperature profiles as described by Uhlenbeck et al. (32). The melting temperature, T_m , is the temperature at which $f = 0.5$.

Table 1. Single Addition Reactions^a

No. ^b	Acceptor	Donor	Product or Derivative	Yield ^c (%)	Time of Reaction (Days)
1.	dGGA	pdAp	dGGAAp	86	7
2.	dGGA	pd2APp ^d	dGGA2AP	66	6
3.	dGGA	pd2APp	dGGA2APp	54	6
4.	dGGA	pd2,6APp	dGGA2,6AP	76	3
5.	dGGA	pd2,6APp	dGGA2,6APp	66	6
6.	dGGA	pdm ⁶ Ap	dGGAm ⁶ A	59	6
7.	dGGA	pdm ⁶ Ap	dGGAm ⁶ Ap	65	6
8.	dGGAA	pdTp	dGGAATp	89	3
9.	dGGAA	pdUp	dGGAAUp	90	3
10.	dGGAA	pdbrUp	dGGAAbrUp	80	5
11.	dGGAA	pdbrUp	dGGAAbrUp	78	3
12.	dGGAAT	pdUp	dGGAATUp	>95	1
13.	dGGAAT	pdbrUp	dGGAATbrUp	90	3
14.	dGGA2AP	pdTp	dGGA2APTp	41	7
15.	dGGA2,6AP	pdTp	dGGA2,6APTp	60	5
16.	dGGAm ⁶ A	pdTp	dGGAm ⁶ ATp	71	7

^aReactions were performed as described in Materials and Methods.

^bEach is a separate reaction.

^cYields are based on amount of acceptor converted to product as determined from ion exchange HPLC profiles.

^dAbbreviations are shown in footnote 3.

RESULTS AND DISCUSSION

Single Addition Reactions: The addition of 2'-deoxyribonucleoside 3',5'-bisphosphates (donor molecules) to oligodeoxyribonucleotides (acceptor molecules) was performed using T4 RNA ligase. Previous work showed that RNA ligase adds any common pdNp to oligodeoxyribonucleotides in good yields (12,13,15). Here we have used the enzyme to add a variety of base analogues to oligodeoxyribonucleotides and obtained yields from 54 to greater than 95% (Table 1). A concentration excess of nucleoside bisphosphate donor over acceptor and the presence of the acceptor at mmolar concentrations are required for high yields. The requirement for a high concentration of acceptor precludes the use of the enzyme to add nucleotide analogues to macromolecular DNA.

Figure 2. Anion exchange HPLC of RNA ligase single nucleotide addition reactions. Reactions and chromatography were performed as described in Materials and Methods. A: Reaction mixture for the addition of pd2APp to dGGA before incubation with RNA ligase. B: As in A after incubation with RNA ligase for 146 hours. C: Addition of pd2,6APp to dGGA before incubation with enzyme. D: As in C after incubation with enzyme for 146 hours. E: Addition of pdm⁶Ap to dGGA before incubation with enzyme. F: As in E after incubation with enzyme for 146 hours.

The progress of the reactions was monitored by analyzing aliquots of the reaction mixtures using anion exchange HPLC. Figure 2 shows HPLC analyses of the reactions of the acceptor d(GGA) with the pdNp derivatives of 2-aminopurine (pd2APp), 2,6-diaminopurine (pd2,6APp), and N⁶-methyladenosine (pdm⁶Ap). The top panels (A, C, and E) show the reaction components before RNA ligase was added; the peaks, in order of times of elution, correspond to the acceptor d(GGA), the donor pdNp, and ATP. After incubation of the reaction mixtures with RNA ligase (Panels B, D, and F), there was a decrease in the acceptor and the appearance of a new peak that eluted later and corresponded to the product d(GGANp). For these reactions, the product yields

Figure 3. Reverse phase HPLC of the nucleoside analysis of dGGA2,6AP. Analysis was performed as described in Materials and Methods. A: dGGA2,6AP with no treatment. B: dGGA2,6AP after treatment with VPD and AP. C: Standards of the nucleosides dG, d2,6AP, and dA in the order of their elution.

after incubation with RNA ligase for 146 hours were from 54 to 66%.

The products were characterized by their mobilities on anion exchange HPLC, UV spectral ratios, and nucleoside compositions. Figure 3 shows the nucleoside composition analysis of d(GGA2,6AP). Panel A gives the elution profile of d(GGA2,6AP) before treatment with VPD and AP and shows the product to be greater than 95% pure. Panel B shows the elution profile of the

Table 2. Oligonucleotide to Oligonucleotide Joining Reactions^a

No.	Acceptor	Donor	Product	Yield ^b (%)	Time of Reaction (Days)
1.	dGGA2AP	pdTTCCp	dGGA2APTTCp	60	7
				35	5
				68	5
2.	dGGA2,6AP	pdTTCCp	dGGA2,6APTTCp	75	7
				35	5
				81	5
3.	dGGAm ⁶ A	pdTTCCp	dGGAm ⁶ ATTCCp	78	7
				22	5
				42	5
4.	dGGAAU	pdTCCp	dGGAAUTCCp	54	7
				58	6
5.	dGGAAbR	pdTCCp	dGGAAbRUTCCp	54	7
				65	6
6.	dGGAATU	pdCCp	dGGAATUCCp	41 ^c	3-7
				45	6
7.	dGGAATbrU	pdCCp	dGGAATbrUCCp	60 ^c	3-7
				66	6

^aReactions were performed as described in Materials and Methods.

^bYields were based on the labelled donor converted to product. Separate entries indicate different reactions.

^cAverage yield of several time points.

nucleosides resulting from the treatment of the oligonucleotide with VPD and AP. Panel C is the elution profile of standards of 2'-deoxyguanosine, 2,6-diaminopurine-2'-deoxyriboside and 2'-deoxyadenosine (in order of elution). Based on the elution times, the nucleosides in panel B were identified. The molar ratio of dG:d2,6AP:dA was 2.21:0.91:1.00.

The products of the single addition reactions were treated with AP to remove the terminal 3'-phosphate in order to convert them to acceptors for use in subsequent RNA ligase reactions. Oligodeoxyribonucleotides with base analogues at their 3'-termini were reactive as acceptors in subsequent RNA ligase reactions using single deoxynucleoside donors (Table 1, Nos. 14 and 15). RNA ligase allows the stepwise synthesis of oligonucleotides of defined sequence. For example, the oligonucleotide d(GGAATbrU) was synthesized by first adding pdTp to d(GGAA) to form the oligonucleotide d(GGAATp). After treatment of this oligonucleotide with AP to give d(GGAAT),

Figure 4. Anion exchange HPLC of the RNA ligase oligonucleotide joining reaction. Reaction and chromatography were performed as described in Materials and Methods. A: Reaction mixture for the joining of pTTCCp to dGGA2,6AP before incubation with RNA ligase. B: As in A after incubation with RNA ligase for 5 days.

pdbrUp was added with RNA ligase and the product treated with phosphatase to give d(GGAATbrU).

Oligodeoxyribonucleotide Joining Reactions: Previous work showed that T4 RNA ligase catalyzes the intermolecular joining of oligodeoxyribonucleotides (14,15). The oligodeoxyribonucleotide joining reaction is less efficient than the single addition reaction and the limiting factor is the poor reactivity of DNA acceptor molecules. We have found that acceptors with base analogues at their 3'-termini are also reactive in RNA ligase reactions (Table 2). Oligodeoxyribonucleotide donors were joined to oligodeoxyribonucleotides with 3'-terminal analogues in yields of 22 to 81%.

The oligodeoxyribonucleotide joining reactions were analyzed either by anion exchange HPLC or polyacrylamide gel electrophoresis. Figure 4 shows an analysis by APS HPLC of the joining of [5'-³²P]d(pTTCCp) to d(GGA2,6AP). Panel A shows the elution profile of the reaction components before enzyme was added; the peaks in order of increasing elution times are ATP, d(GGA2,6AP), and d(pTTCCp). Panel B shows the reaction components after incubation with RNA ligase for 5 days. A new peak eluting later in the gradient appeared and was the product d(GGA2,6APTCCp). Quantitation of the radioactivity in the column fractions showed that before enzyme was added, 98% of the radioactivity coeluted with the d(pTTCCp) peak. After incubation with RNA ligase, 54% of the radioactivity coeluted with d(pTTCCp) and 33% coeluted with the product

Figure 5. Wandering-spot sequence analysis of $[5'-^{32}\text{P}]\text{pdGGA2,6APTCC}$. **A:** Autoradiograph of a 2-dimensional separation of a VPD partial digest of $[5'-^{32}\text{P}]\text{pdGGA2,6APTCC}$. 1 represents the direction of electrophoresis and 2 that of the homochromatography. **B:** Pattern predicted for a VPD partial digest of pdGGAATTCC (31).

peak. The remaining 13% was not retained on the column.

The octanucleotide products were treated with AP to remove the terminal 3'-phosphates, treated with ATP and polynucleotide kinase to phosphorylate the 5'-termini, and characterized by their mobilities on HPLC, UV spectral ratios, and sequences. After purification, the phosphorylated oligonucleotides were greater than 95% pure as determined by ion-paired HPLC. Figure 5A shows an autoradiogram of the 2-dimensional sequence analysis of $[5'-^{32}\text{P}]\text{d(pGGA2,6APTCC)}$. The pattern of spots agrees with that predicted for the sequence d(pGGAATTCC) (Figure 5B)(31). None of the incorporated base analogues significantly modified the wandering spot pattern from that predicted for d(pGGAATTCC) .

Chemical Synthesis of d(GGAATTCC) and $\text{d(GGm}^6\text{AATTCC)}$: The oligodeoxyribonucleotides d(GGAATTCC) and $\text{d(GGm}^6\text{AATTCC)}$ were synthesized chemically (26). Although RNA ligase is a useful tool for the incorporation of base analogues into defined sequences, the amounts synthesized enzymatically are limited (6-22 nmol). Chemical synthesis allows the preparation of μmol amounts of oligodeoxyribonucleotides and is thus the preferred method when analogues are not to be incorporated or when the appropriately blocked based analogues are available. These oligonucleotides after deprotection and purification

Figure 6. Plots of T_m^{-1} versus the log concentration of strands. T_m determined for pdGGAATTCC, ●; pdGGm⁶AATTCC, ▲; pdGGAm⁶AATTCC, ◇; pdGGA2APTCC, △; pdGGA2,6APTCC, ◆; pdGGAAUTCC, ■; pdGGAATbrUCC, □.

(Materials and Methods) were greater than 96% pure as determined by OS reverse phase HPLC. The nucleoside composition analysis of d(GGAATTCC) showed that all the phosphodiester bonds could be cleaved by VPD indicating that all the phosphate protecting groups had been removed. Nucleoside ratios of dG:dA:dT:dC of 1.09:1.00:1.00:0.87 were obtained. Similarly d(GGm⁶AATTCC) was completely degraded by VPD and gave nucleoside ratios of dG:dm⁶A:dA:dT:dC of 2.19:1.00:1.14:1.95:2.00. Both sequences were confirmed by 2-dimensional sequencing (data not shown).

Thermal Stability of the Octanucleotides: The synthesized octameric oligonucleotides will be used as substrates for the *EcoRI* restriction endonuclease and modification methylase. Since the substrate for these enzymes is a duplex (33-35), we need to ascertain the fraction of oligonucleotide strands that will be present as duplexes in the enzyme assays. For this reason, melting curves for the oligonucleotides were determined under conditions similar to those in the enzyme assays. For the oligonucleotides d(pGGAATTCC) and d(pGGm⁶AATTCC), T_m values were determined at

Figure 7. Fraction of strands in duplex versus temperature. Melting curves were determined as in Materials and Methods at strand concentrations from 3.5 to 4.6 μM (Table 3). Curves were calculated from absorbance versus temperature profiles as described (32). pdGGAATTCC, ●; pdGGm⁶AATTCC, △; pdGGAm⁶ATTCC, □; pdGGA2,6APTCC, ■; pdGGAAUTCC, ▲; pdGGAATbrUCC, ○.

several concentrations of oligonucleotide and Figure 6 shows a reciprocal T_m versus log concentration plot of these data. The enthalpies, ΔH , for these oligonucleotides were determined from the slopes of the lines. A value for ΔH of -59.2 kcal/mole was calculated for d(pGGAATTCC). This agrees well with values of -51.8 kcal/mole (36) and -58.8 kcal/mole (37) obtained for the unphosphorylated oligonucleotide d(GGAATTCC). For d(pGGm⁶AATTCC), the value for ΔH was -46.3 kcal/mole. At any desired oligomer concentration one can interpolate a T_m value from the curves in Figure 6. This T_m value can be used to generate an f versus temperature profile (32, Figure 7).

The other oligonucleotides were synthesized in limited amounts and the melting curves determined at single concentrations. The reciprocal values of the measurements are shown in Figure 6. Several studies have demonstrated that oligodeoxyribonucleotide duplexes containing mismatched bases (37,38), an extra nucleotide in one strand (37), the base analogue O⁶-methylguanine opposite any of the four regular bases (39), or 2,6AP paired with T or U (40) all have enthalpies for the helix-to-coil transition that do not differ greatly from those of the controls containing the normal base pairs. The major contribution to the enthalpy of the transition derives from the nearest neighbor stacking interactions (41). All the analogue substitutions we have

Table 3. Melting Temperatures and Enthalpies for Octanucleotides

Octanucleotide	Concentration ^a (uM)	T _m ^b (°C)	Slope at T _m (df/dT)	ΔH ^c (kcal/mole)
dpGGAATTCC	4.1	31	-0.046	-51.1
dpGGm ⁶ AATTCC	4.3	22	-0.044	-46.8
dpGGAm ⁶ ATTCC	3.6	24	-0.038	-40.0
dpGGA2,6APTCC	4.1	35	-0.044	-49.8
dpGGA2APTCC ^d	2.4-5.1	30-33	-	-
dpGGAATbrUCC	4.0	33	-0.042	-45.7
dpGGAAUTCC	4.6	29	-0.047	-51.5

^aConcentration of strands of oligonucleotide.

^bDetermined as described in Materials and Methods.

^cCalculated as described in (42).

^dPoor quality melting curves, T_m values are approximations and ΔH was not determined.

made form stable base pairs with their complements and stacking interactions should not be greatly affected. Furthermore, the shapes of the melting curves (Figure 7) are similar to that of the control octamer indicating little change in the enthalpies. The enthalpy can be calculated from the slope of the melting curve at the T_m from the relationship $\Delta H = 6RT_m^2(df/dT)_{T_m}$ (42). Although this is not the most accurate way to calculate enthalpies (42), the values determined in this manner are all similar to that of d(pGGAATTCC) (Table 3). These data support the proposition that, with the exception of N⁶-methyladenine, ΔH is not significantly affected by the analogue substitutions. In any event, small changes in ΔH will not drastically affect the T_m estimations. For example, if we extrapolate from the melting temperature measured at 3.6 uM for d(pGGAm⁶ATTCC) to its T_m at 0.25 uM using the ΔH for d(pGGAATTCC) (-59.2 kcal/mol), we obtain a value of 16°C. If we do the extrapolation using the ΔH for d(pGGm⁶AATTCC) (-46.3 kcal/mol), the T_m is 14°C. The difference of 2°C is within experimental error. Therefore, in order to derive the melting temperatures of the oligonucleotides at concentrations other than the one measured, we have used the enthalpy value for the control d(pGGAATTCC).

To estimate the concentration dependence of the T_m values for the oligonucleotide analogues, lines were drawn that passed through the measured point

on the reciprocal T_m versus log concentration plot of each analogue (Figure 6) and were parallel to the line for d(pGGAATTCC), i.e., indicated the same ΔH . For d(pGGAm⁶ATTCC) a line parallel to that for d(pGGm⁶AATTCC) was drawn. From these lines, the melting temperature at any concentration can be estimated.

Although the enthalpy is probably not significantly affected by the analogue substitutions, the T_m values do vary. The decreased T_m values in one study have been attributed to entropic effects (37). Table 3 shows the melting temperatures for the octanucleotides determined at from 3 to 5 μ M strands. The analogues containing brU and 2,6AP both stabilize the duplex in agreement with results of Dwyer-Hallquist et al. (6) with brU in the oligonucleotide d(GGTBrUAACC) and of Gaffney et al. (40) with 2,6AP in the hexamer d(CGT2,6APCG). The homopolymers poly[d(A)]·poly[d(BrU)] and alternating copolymers of poly [d(A-BrU)] are more stable than polymers with dT in place of dBrU (20,43). The substitution of U into octanucleotides decreased the T_m values only slightly. The oligonucleotides d(GGTUAACC) (6) and d(CGU2,6APCG) (40) also have T_m values that are not significantly different than the corresponding oligonucleotides containing thymine. Similarly, homopolymer helices of dA and dU are only slightly less stable than those containing dT (43). The largest effect on the T_m values resulted from the substitutions of N⁶-methyladenine into the oligonucleotides. These oligonucleotides were much less stable than the control. The copolymers poly[d(A,m⁶A-T)] and homopolymers poly[d(A,m⁶A)]·poly[d(T)] show that the helix is destabilized in proportion to the extent of methyladenine substitution (44). Although m⁶A unwinds the DNA helix (45), the destabilization is not sufficient to disrupt Watson-Crick basepairing (44,45). The octanucleotide containing 2-aminopurine has a T_m value approximately the same as that of the control in agreement with data from the alternating copolymers of poly[r(2AP-T)] and poly[r(2AP-U)] which have T_m values only slightly lower than poly [r(A-T)] and poly[r(A-U)] (46).

CONCLUSIONS

We have presented a general method for the incorporation of deoxyribonucleoside analogues into oligodeoxyribonucleotides of defined sequences. The methodology uses chemistry to synthesize partial sequences but obviates the need for blocked base analogues by incorporating the unprotected analogues enzymatically as 3',5'-bisphosphates. The bisphosphates derivatives are readily synthesized from unprotected 2'-deoxyribonucleosides (19). T4 RNA ligase was used to add base analogues to oligodeoxyribonucleotides in yields

from 54 to greater than 95%. The products of these reactions after treatment with alkaline phosphatase, are reactive as acceptors in subsequent RNA ligase joining reactions thereby allowing sequential syntheses of oligodeoxyribonucleotides with base analogues incorporated at specific positions. Oligodeoxyribonucleotides were joined to acceptors containing analogues at their 3'-termini in yields from 22 to 81%. Since RNA ligase can join a wide variety of base analogues and compounds to the 3'-terminus of RNA molecules (11), the high yields obtained here for the joining of deoxyribonucleoside analogues to oligodeoxyribonucleotides suggests that many other base analogues could also be incorporated into oligodeoxyribonucleotides by RNA ligase.

The oligonucleotide analogues that we have synthesized are self-complementary and consequently form duplexes containing two symmetrically disposed substitutions. None of the analogue substitutions resulted in depressions of the T_m values that were large enough to prevent duplexes from existing at 20°C therefore, these oligonucleotide analogues are suitable for testing as substrates for the EcoRI restriction and modification enzymes.

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³Abbreviations: 2AP, 2-aminopurine; 2,6AP, 2,6-diaminopurine; m⁶A, N⁶-methyladenine; brU, 5-bromouracil; pNp, the 3',5'-bisphosphate derivative of the 2'-deoxyribonucleoside N; dpNNN or d(pNNN), d(pN-N-N); APS, aminopropylsilica; ODS, octadecylsilica; OS, octylsilica; HPLC, high performance liquid chromatography; TEABC, triethylammonium bicarbonate; VPD, snake venom phosphodiesterase; AP, alkaline phosphatase; T_m , melting temperature.

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