Nienhaus, G. U. (1987) Ph.D. Dissertation, Universität Münster.
Ormos, P., Braunstein, D., Hong, M. K., Lin, S. L., & Vittitow, J. (1986) in *Biophysical Studies of Retinal Proteins* (Ebrey, T. G., Frauenfelder, H., Honig, B., & Nakanishi, K., Eds.) pp 238–247, University of Illinois Press, Urbana, IL.
Ormos, P., Braunstein, D., Frauenfelder, H., Hong, M. K., Lin, S. L., Sauke, T. B., & Young, R. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8492–8496.
Ormos, P., Ansari, A., Braunstein, D., Cowen, B. R., Frauenfelder, H., Hong, M. K., Iben, I. E. T., Sauke, T. B., Steinbach, P. J., & Young, R. D. (1990) *Biophys. J. 57*, 191–199.
Parkhurst, L. (1979) *Annu. Rev. Phys. Chem. 30*, 503–546.
Petrich, J. W., Poyart, C., & Martin, J. L. (1988) *Biochemistry 27*, 4049–4060.
Petrich, J. W., Lambry, J.-C., Kuczera, K., Karplus, M., Poyart, C., & Martin, J.-L. (1991) *Biochemistry* (preceding paper in this issue).
Phillips, S. E. V. (1980) *J. Mol. Biol. 142*, 531–554.
Phillips, S. E. V. (1981) *The X-ray Structure of Deoxy-Mb (pH 8.5) at 1.4 Å Resolution*, Brookhaven Protein Data Bank.
Potter, W. T., Tucker, M. P., Houtchens, R. A., & Caughey, W. S. (1987) *Biochemistry 29*, 4699–4707.
Redi, M. H., Gerstman, B. S., & Hopfield, J. J. (1981) *Biophys. J. 35*, 471–484.
Richert, F., & Bässler, H. (1990) *J. Phys.: Condens. Matter 2*, 2273–2288.
Rousseau, D., & Argade, P. V. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1310–1314.
Sassaroli, M., & Rousseau, D. (1987) *Biochemistry 26*, 3092–3098.
Sassaroli, M., Dasgupta, S., & Rousseau, D. L. (1986) *J. Biol. Chem. 261*, 13704–13713.
Sato, F., Shiro, Y., Sakaguchi, Y., Suzuki, T., Iizuka, T., & Hayashi, H. (1990) *J. Biol. Chem. 265*, 2004–2010.
Srajer, V., Schomaker, K. T., & Champion, P. M. (1986) *Phys. Rev. Lett. 57*, 1267–1270.
Srajer, V., Reinisch, L., & Champion, P. M. (1988) *J. Am. Chem. Soc. 110*, 6656–6669.
Stein, D. L., Palmer, R. G., van Hemmen, J. L., & Doering, C. R. (1989) *Phys. Lett. A136*, 353–357.
Steinbach, P. J. (1990) Ph.D. Dissertation, University of Illinois at Urbana–Champaign.
Stetzkowski, F., Banerjee, R., Marden, M. C., Beece, D. K., Bowne, S. F., Doster, W., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., & Jung, C. (1985) *J. Biol. Chem. 260*, 8803–8809.
Young, R. D., & Bowne, S. F. (1984) *J. Chem. Phys. 81*, 3730–3737.
Zallen, R. (1983) *The Physics of Amorphous Solids*, John Wiley & Sons, New York.
Zwanzig, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2029–2030.

Properties of 2′-Fluorothymidine-Containing Oligonucleotides: Interaction with Restriction Endonuclease *Eco*RV

David M. Williams, Fritz Benseler, and Fritz Eckstein*

Abteilung Chemie, Max-Planck-Institut für Experimentelle Medizin, Hermann-Rein-Strasse 3, D-3400 Göttingen, FRG

Received October 18, 1990; Revised Manuscript Received December 19, 1990

ABSTRACT: 2′-Fluorothymidine (T_f) was synthesized via an improved procedure with (diethylamino)sulfur trifluoride. The compatibility of the analogue with DNA synthesis via the phosphoramidite method was demonstrated after complete enzymatic digestion of the oligonucleotides $d(T_{f_{11}}T)$ and $d(T_{f_3}T)$, the sole products of which were 2′-fluorothymidine and thymidine in the expected ratio. The 2′-fluorothymidine was also incorporated into the *Eco*RV recognition sequence (underlined), within the complementary oligonucleotides d(CAAACCGATATCGTTGTG) and d(CACAACGATATCGGTTTG). Thermal melting characteristics of these duplexes showed a significant decrease in stability only when both of the thymidine residues in one of the strands were replaced. In contrast, when all of one strand of a duplex contained 2′-fluorothymidine, as in $d(T_{f_{11}}T)\cdot d(A_{12})$, a substantially higher T_m and cooperativity of melting was observed relative to the unmodified structure. *Eco*RV cleaved a duplex that contained a 2′-fluorothymidine at the scissile linkage in each strand at two-thirds of the rate obtained for the unmodified structure. A duplex containing two 2′-fluorothymidine residues in one strand and none in the other was cleaved at one-third of the rate in its unsubstituted strand, whereas the cleavage rate was reduced to 22% in its modified strand.

The differences in both the structure and the properties of ribo- and deoxyribonucleosides and their polymers may be attributed to the nature of the 2′-substituent, –H or –OH. Thus, RNA exists almost exclusively in the A-form in which the ribose moiety exhibits a 3′-endo (*N*) conformation (Saenger, 1984). In contrast, DNA most commonly displays a 2′-endo (*S*) sugar conformation although under certain conditions (in A- and Z-DNA) the 3′-endo conformation is adopted (Saenger, 1984). These differing structural preferences result in the ability of several drugs such as netropsin (Kopka et al., 1985) to recognize and bind exclusively within the minor groove of the B-DNA double helix and also the highly specific cleavage of double-stranded DNA by type II restriction endonucleases (Maass, 1987).

0006-2960/91/0430-4001$02.50/0

In order to understand this structural and functional diversity between DNA and RNA, there has been a growing interest in the synthesis and properties of several 2′-modified nucleoside analogues. Of these, the 2′-deoxy-2′-fluoronucleosides have received the most attention, since fluorine has a van der Waals radius similar to that of hydrogen (1.35 Å compared to 1.20 Å) but an electronegativity more akin to that of a hydroxyl group.

The 2′-deoxy-2′-fluoro analogues of uridine (Codington et al., 1964), cytidine (Doerr & Fox, 1967), thymidine (Codington et al., 1964), adenosine (Ikehara & Miki, 1978), and guanosine (Ikehara & Imura, 1981) have all been prepared, and the crystal structures reveal that a 3′-endo conformation is adopted by these analogues (Hakoshima et al., 1981; Marck et al., 1982b; Suck et al., 1974). NMR[1] data (Catlin & Guschlbauer, 1975; Cushley et al., 1968; Guschlbauer & Janikowski, 1980) indicate that this same conformational preference exists in solution, often resulting in more than 90% of the *N*-conformer being present. Such findings are also consistent with reported theoretical predictions of the preferred conformations adopted by substituted furanoses (Olson, 1982).

The polymers of the nucleotides of 2′-deoxy-2′-fluorocytidine (Guschlbauer et al., 1977; Kakiuchi et al., 1982), 2′-deoxy-2′-fluorouridine (Janik et al., 1971), 2′-deoxy-2′-fluoroinosine (Kakiuchi et al., 1982), and 2′-deoxy-2′-fluoroadenosine (Ikehara et al., 1978) have all been prepared from their respective diphosphate analogues by the use of polynucleotide phosphorylase. The CD spectra of such polymers show more resemblance to RNA than to DNA, and the NMR data for the hexamer $d(CG_f)_3$ (Fazakerley et al., 1985) indicate a 3′-endo conformation and an A-form structure. In general, these polymers possess thermal stabilities similar to or higher than those of the corresponding RNA duplexes and are also more stable to nucleases and alkali than the corresponding DNA duplexes (Guschlbauer et al., 1977; Ikehara et al., 1978; Janik et al., 1971). Antibodies raised against poly(rI)·poly(rC) also recognized the corresponding 2′-deoxy-2′-fluoronucleoside-containing polymers but not poly(dI)·poly(dC) (Kakiuchi et al., 1982), further supporting the view that such polymers are more comparable to RNA than to DNA. However, the A → Z transition in $d(CG_f)_3$ (Fazakerley et al., 1985) and the ability of 2′-deoxy-2′-fluoronucleoside-substituted polymers of poly(dA)·poly(dT) or poly(dI)·poly(dC) to interact with drugs such as netropsin (Marck et al., 1982a; Guschlbauer, 1982) are characteristics normally associated with DNA rather than RNA and indicate that these polymers may display characteristics of both types of nucleic acid.

It has been demonstrated that several type II restriction endonucleases, including *Eco*RI, are able to cleave the DNA strand of DNA·RNA hybrids when the enzyme concentration is about 20–50 times higher than that normally required to cleave the natural DNA duplex (Molloy & Symons, 1980). However, under such circumstances, it was not known if the RNA strand was also cleaved. Although Ohtsuka and coworkers (1984) have previously examined the rates of cleavage by *Eco*RI of the two fluoronucleoside-containing self-complementary oligonucleotide duplexes $d(GGA_fATTCC)$ and $d(GG_fAATTCC)$, no definite conclusions were drawn from their results. The first was hydrolyzed slightly slower by *Eco*RI than the unmodified substrate while in the latter case the cleavage rate was slightly enhanced.

We have recently become interested in the properties of 2′-deoxy-2′-fluoronucleosides, in particular, whether or not they can inhibit restriction endonuclease catalyzed cleavage of DNA. Here we describe an improved synthesis of 2′-fluorothymidine and its subsequent incorporation into oligonucleotides via standard, automated phosphoramidite chemistry. The thermal stabilities of several such duplexes have been examined. In addition, the *Eco*RV cleavage of suitable oligonucleotide duplexes containing this analogue has been studied.

Materials and Methods

Acetonitrile (0.01% H_2O) from Baker (Gross-Gerau, FRG) was stored over activated molecular sieves (3 Å). Dichloromethane (Merck, Darmstadt, FRG) was passed down a column of basic alumina immediately prior to use. Toluene and THF (all less than 0.01% H_2O) (Merck) were stored over activated 4-Å molecular sieves (Merck). Analytical grade DMF (Merck) was distilled under reduced pressure with a dry argon bleed and a fractionating column filled with glass helices. Pyridine was obtained from Merck. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane, methanesulfonyl chloride, and DAST were purchased from Merck. 9-Chloro-9-phenylxanthene (pixyl chloride) was purchased from Fluka AG (Neu-Ulm, FRG). TBAF (1 M in THF) and β-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite were obtained from Aldrich Chemie (Steinheim, FRG). Sep-PAK C-18 cartridges were from Millipore-Waters (Boenningstedt, FRG). ODS-Hypersil (5 μm) was obtained from Shandon (Runcorn, England). LiChrospher 100 RP-18, Kieselgel 60 (<63 μm), and precoated silica gel F_{254} plates for thin-layer chromatography (TLC) were obtained from Merck. Szintillator 303 scintillation fluid was obtained from Riedel-de-Haen (Seelze, FRG). Alkaline phosphatase (molecular biology grade from calf intestine, 22 units/μL) and snake venom phosphodiesterase (*Crotalus durissus*, 2 mg/mL) were obtained from Boehringer (Mannheim, FRG). T4 polynucleotide kinase (30 units/μL) was obtained from U.S. Biochemicals (Bad Homburg, FRG). *Eco*RV endonuclease (260 μM dimer in 50% glycerol/water containing 200 mM NaCl, 20 mM KH_2PO_4, pH 7.4, 2 mM DTE, and 1 mM EDTA) was kindly provided by B. A. Connolly, Department of Biochemistry, Southampton University, England. The enzyme was diluted to 50 μM in the same buffer, which contained DTT in place of DTE. Immediately prior to reaction, this stock solution was diluted to give 2.5 μM enzyme in 50 mM HEPES/KOH, pH 7.5, containing 10 mM $MgCl_2$ and 100 mm NaCl.

Melting points were determined on a Reichert melting point apparatus and are uncorrected. ^1H NMR spectra were recorded at 360.13 MHz on a Bruker WH 360 spectrometer with tetramethylsilane as the internal standard. CD_3OD was added to ^1H NMR samples for the identification of exchangeable protons. ^{19}F and ^{31}P NMR spectra were recorded on the same instrument at 338.87 and 145.79 MHz, respectively, with ^1H decoupling and C_6F_6 as the internal standard

[1] AraT, 1-β-D-arabinofuranosylthymine; dA, 2′-deoxyadenosine; dC, 2′-deoxycytidine; dG, 2′-deoxyguanosine; dT, thymidine; dAf, 2′-deoxy-2′-fluoroadenosine; dGf, 2′-deoxy-2′-fluoroguanosine; T_f, 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)thymine (referred to throughout text as the trivial name 2′-fluorothymidine); CD, circular dichroism; DAST, (diethylamino)sulfur trifluoride; DMF, *N*,*N*-dimethylformamide; DMT, 4,4′-dimethoxytrityl group; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, disodium ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate; EtOAc, ethyl acetate; Et_3N, triethylamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; MeOH, methanol; NMR, nuclear magnetic resonance; mp, melting point; PxCl, pixyl chloride (9-chloro-9-phenylxanthene); TBAF, tetrabutylammonium fluoride; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; THF, tetrahydrofuran; TLC, thin-layer chromatography; T_m, melting temperature midpoint; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; Tris-borate, Tris(hydroxymethyl)aminomethane borate.

or 85% H_3PO_4 as the external standard. ^{19}F and ^{31}P chemical shift values that are upfield of the respective standards possess a negative value.

Silica TLC plates were developed by use of one of the following solvent systems: S1, CH_2Cl_2/MeOH 9/1 (v/v); S2, CH_2Cl_2/MeOH 4/1 (v/v); S3, CH_2Cl_2/MeOH 19/1 (v/v); and S4, CH_2Cl_2/MeOH/Et_3N 19/19/2 (v/v/v). Compounds were visualized under UV light or by being sprayed with 5% aqueous sulfuric acid followed by heat. Flash column chromatography was performed on Kieselgel 60. Preparative HPLC for 2′-fluorothymidine was performed on a column (21 mm × 305 mm) containing LiChrospher 100 RP-18 material by use of a Du Pont 8800 instrument equipped with preparative pump heads (40 mL) and an 8-mL injection loop and coupled to a Du Pont 8800 UV detector. HPLC for oligonucleotides was carried out with two Waters Associates Model 6000A pumps, controlled by a Model 680 automated gradient programmer and coupled to a Waters 484 UV detector. Analytical and preparative separations were performed on 5-μm ODS-Hypersil, in a column of dimensions 250 × 4 mm or 250 × 10 mm, respectively. The following HPLC gradients were employed: gradient I, an isocratic gradient of water for 5 min, followed by a linear gradient of acetonitrile (0–15% in 30 min) in water, with a flow rate of 15 mL/min (it is preferable to use water as solvent A and an aqueous acetonitrile solution as solvent B); gradient II, a linear gradient of acetonitrile (3.5–56% in 30 min) in 50 mM TEAA, pH 7.0, with a flow rate of 2 mL/min; gradient III, a linear gradient of acetonitrile (3.5–56% in 30 min) in 100 mM TEAB, pH 7.5, with a flow rate of 5 mL/min; gradient IV, a linear gradient of acetonitrile (0–3.25% in 15 min, then 3.25–48.75% in a further 5 min) in 50 mM TEAA, pH 7.0, with a flow rate of 2 mL/min; gradient V, a linear gradient of acetonitrile (3.5–56% in 30 min) in 100 mM TEAA, pH 7.0, with a flow rate of 2 mL/min; and gradient VI, a linear gradient of acetonitrile (0–16% in 10 min) in 100 mM TEAB, pH 7.5, with a flow rate of 2 mL/min.

Radioactive gel pieces were counted in 5 mL of scintillation fluid for 5 min, with a Beckman LS 6000LL counter.

An LKB Ultroscan XL laser densitometer was used for densitometric scanning of autoradiographs.

T_m determinations were obtained as previously described (Pörschke, 1982) with a Perkin-Elmer λ9 UV spectrophotometer.

Oligonucleotide synthesis was performed by use of an Applied Biosystems 380B DNA synthesizer; standard phosphoramidite chemistry was employed.

All moisture-sensitive reactions were performed under an atmosphere of dry argon, with oven-dried glassware. All nucleosides were dried in a high-vacuum drying oven over sodium hydroxide at 40 °C for 20 h.

Ribofuranosylthymine (**1**, Figure 1) was prepared according to the procedure of Vorbrüggen and Bennua (1978).

2,2′-Anhydro-1-β-D-arabinofuranosylthymine (**2**). To a solution of ribofuranosylthymine (8 g, 31 mmol) in pyridine (140 mL) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (9.37 mL, 31.15 mmol) and the mixture stirred at room temperature for 5 h. The reaction was monitored by TLC (S1). The solution was then evaporated and the residue taken up in EtOAc (130 mL). This was successively washed with cold, aqueous 1 N HCl (110 mL), saturated, aqueous $NaHCO_3$ (110 mL), and saturated, aqueous NaCl (110 mL), then dried over Na_2SO_4, and evaporated to dryness. The resulting foam was dissolved in pyridine (130 mL) and the solution stirred with methanesulfonyl chloride (3.1 mL, 40.3 mmol) for 16 h at room temperature. The reaction was followed by TLC (S3). Water (10 mL) was added, the solvents were removed by evaporation, and the residue was dissolved in toluene (130 mL). The organic phase was extracted twice with 5% aqueous $NaHCO_3$, dried over Na_2SO_4, and evaporated to dryness. Residual pyridine was coevaporated with toluene (50 mL) and the residue subsequently dissolved in THF (130 mL). To this solution was added a 1 M solution of tetra-*n*-butylammonium fluoride in THF (46.5 mL, 46.5 mmol). The reaction was followed by TLC (S2). After 60 min, the solvent was removed by evaporation and the residue was dissolved in water (150 mL) and extracted with EtOAc (2 × 150 mL). The aqueous phase was evaporated and residual water removed by coevaporation three times with ethanol. The product was purified by flash chromatography with the use of a stepwise gradient of 4%, 8%, 12%, and 16% MeOH in CH_2Cl_2 and eluted between 12% and 16% MeOH. Product-containing fractions were pooled and evaporated to dryness, and the residue was recrystallized from ethanol (40 mL): yield 5.35 g (72%); mp 215–218 °C lit. mp 227–228 °C (Codington et al., 1964)]; ^1H NMR δ (DMSO-d_6) 7.74 (d, 1 H, H6), 6.29 (d, 1 H, J = 5.7 Hz, H1′), 5.18 (d, 1 H, H2′), 4.38 (d, 1 H, H3′), 4.06 (pt, 1 H, H4′), 3.27 (dd, 1 H, H5′), 3.17 (dd, 1 H, H5′), 1.79 (d, 3 H, CH_3).

3′,5′-Di-O-pixyl-1-β-D-arabinofuranosylthymine (**3**). Pixyl chloride (8 g, 27.5 mmol) was added to a solution of **2** (3 g, 12.5 mmol) in pyridine (90 mL) and the solution stirred at room temperature for 3 h. During this time, a white, voluminous precipitate was obtained. Water (10 mL) was then added, and the solvents were removed by evaporation. The residue was taken up in toluene (150 mL), the solution was extracted twice with water (150 mL), and the organic phase was evaporated to dryness. The white solid obtained was suspended in MeOH (150 mL) and refluxed for 2 h with 1 N aqueous NaOH (61 mL, 61 mmol). The resulting white precipitate was filtered off and washed with a mixture of MeOH/water (3/1 v/v) at 4 °C: yield 7.5 g (78%); R_f values (S1) of 0.00 for **2** and 0.40 for **3**; ^1H NMR δ (DMSO-d_6) 7.40–6.87 (m, 27 H, pixyl, H6), 5.97 (d, 1 H, J = 3.2 Hz, H1′), 3.56 (dd, 1 H, H2′), 3.33 (d, 1 H, H3′), 3.95 (m, 1 H, H4′), 2.63 (dd, 1 H, H5′), 2.50 (dd, 1 H, H5′), 1.60 (s, 3 H, CH_3).

2′-Fluorothymidine (**4**). DAST (428 μL, 3.22 mmol) was added to an ice-cooled suspension of **3** (500 mg, 0.64 mmol) in CH_3CN (10 mL) and DMF (746 μL, 9.67 mmol). The clear solution obtained was stirred for 10 min at 0 °C and then for 18 h at room temperature. The reaction was monitored by silica TLC; with S3, R_f values of 0.40 for **3** and 0.73 for the 3′,5′-dipixyl derivative of **4**, or with S2 (after treatment of the components on the TLC plate with 10% dichloroacetic acid/CH_2Cl_2), R_f values of 0.39 for **3** and 0.61 for **4**. (The reaction proceeds via two intermediates as observed by TLC; with S3, R_f values of 0.51 and 0.62.) The solution was then cooled to 0 °C; triethylamine (200 μL) and methanol (2 mL) were added, and solvents were removed by evaporation. The residue was dissolved in methanol (5 mL) and diethyl ether (50 mL) and the solution extracted with water (50 mL). The organic phase was evaporated, and the residue was redissolved in methanol (30 mL) and put on ice for 10 min before 1 N HCl (3 mL) was added. After reaction for 30 min at room temperature, the solution was neutralized with 0.1 N NaOH and then evaporated. The residue was suspended in water (50 mL) and the suspension extracted with CH_2Cl_2 (50 mL). The aqueous layer was evaporated, and water (25 mL) was added to the residue. The white precipitate formed was then filtered

off and washed with water. Residual pixyl material was removed by Sep-Pak C-18 filtration. The product was eluted with 10% methanol in water and then purified by preparative HPLC using gradient I (retention time, 30 min). The product-containing peak was collected and then evaporated: yield 72 mg (57%); the compound crystallized from water as white needles; mp 185–188 °C [lit. mp 185–188 °C (Codington et al., 1964)]; ^{1}H NMR δ (DMSO-d_6) 7.80 (d, 1 H, H6), 5.93 (dd, 1 H, J_{HH} = 2.2 Hz, J_{HF} = 17.6 Hz, H1′), 5.02 (dd, 1 H, J_{HF} = 53.2 Hz, H2′), 4.20 (ddd, 1 H, J_{HF} = 20.1 Hz, H3′), 3.88 (broad pseudodoublet, 1 H, H4′), 3.79 (dd, 1 H, H5′), 3.61 (dd, 1 H, H5′), 1.77 (d, 3 H, CH_3); ^{19}F NMR δ (DMSO-d_6) −201.95 (s).

5′-O-(4,4′-Dimethoxytrityl)-2′-fluorothymidine (**5**). 2′-Fluorothymidine (**4**) was protected as its 5′-*O*-(4,4′-dimethoxytrityl) ether by use of standard conditions (Jones, 1984), and the crude product was purified by flash chromatography: yield quantitative; ^{1}H NMR δ (DMSO-d_6) 11.50 (s, 1 H, NH), 7.53 (d, 1 H, H6), 7.41–6.88 (m, 13 H, DMT), 5.92 (dd, 1 H, J_{HH} = 1.1 Hz, J_{HF} = 20.0 Hz, H1′), 5.67 (d, 1 H, 3′-OH), 5.12 (dd, 1 H, J_{HH} = 4.6 Hz, J_{HF} = 53.3 Hz, H2′), 4.30 (m, 1 H, H3′), 4.02 (m, 1 H, H4′), 3.74 (s, 6 H, 2 DMT/MeO), 3.28 (m, 2 H, H5′, H5″), 1.46 (d, 3 H, CH_3).

5′-O-(4,4′-Dimethoxytrityl-2′-fluorothymidine 3′-O-(β-Cyanoethyl N,N-diisopropylphosphoramidite) (**6**). Compound **6** was prepared with some modifications of the literature procedure (Sinha et al., 1984) as follows: 5′-*O*-(4,4′-dimethoxytrityl)-2′-fluorothymidine (**5**) (470 mg, 0.835 mmol) in dry CH_2Cl_2 (8 mL) was treated with *N,N*-diisopropylamine (428 μL, 2.5 mmol) and β-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (279 μL, 1.25 mmol) and stirred at room temperature for 1 h. MeOH (2 mL) was then added, and after a further 3 min, the solution was evaporated. The crude product was purified by flash chromatography (Kieselgel 60, 15 g) and the product eluted with CH_2Cl_2/hexane (1/1 v/v) containing 2% Et_3N. The purified product was then dissolved in 5 mL of toluene and evaporated. The residue was redissolved in toluene (5 mL) and precipitated by dropwise addition into hexane (100 mL at 0 °C). The white precipitate was filtered and dried: yield 453 mg (72%); with S4, R_f values of 0.36 and 0.32 for **6** (two diastereomers), a trace of phosphonate impurity was seen at R_f 0.22; ^{31}P NMR δ (DMSO-d_6) 151.00 (d, J_{PF} = 9.8 Hz), 150.54 (d, J_{PF} = 8.3 Hz), 15.32 (d, J_{PF} = 10.1 Hz, 5% phosphonate impurity); ^{31}P NMR δ ($CDCl_3$) 157.53 (d, J_{PF} = 9.2 Hz), 157.26 (d, J_{PF} = 12.4 Hz), 20.47 (phosphonate); ^{19}F NMR δ (DMSO-d_6) −196.44 (d, J_{PF} = 9.7 Hz), −197.48 (d, J_{PF} = 8.2 Hz).

Synthesis of Oligonucleotides. The following oligonucleotides were synthesized as their 5′-dimethoxytrityl derivatives on a 0.2-μmol scale: oligo A, d(CAAACCGATATCGTTGTG); oligo B, d(CACAACGATATCGGTTTG); oligo Af, d(CAAACCGAT$_f$ATCGTTGTG); oligo Bf, d(CACAACGAT$_f$ATCGGTTTG); oligo Bff, d(CACAACGAT$_f$AT$_f$CGGTTTG); oligo C, d(T$_{f_{11}}$T); oligo D, d(T$_{f_3}$T); d(A$_{12}$); and d(T$_{12}$). For the oligonucleotides d(T$_{f_3}$T) and d(T$_{f_{11}}$T), the coupling times were extended from 15 s to 5 min.

Purification of Oligonucleotides. All oligonucleotides were treated overnight at 55 °C with 32% ammonia solution except d(T$_{12}$), d(T$_{f_{11}}$T), and d(T$_{f_3}$T), which were treated for only 3 h. Each crude dimethoxytritylated oligonucleotide was examined by analytical HPLC with gradient II and was then subsequently purified by preparative reversed-phase HPLC with gradient III. The appropriate peak was collected and the solution evaporated. Residual buffer was removed by coevaporation with methanol and the dimethoxytrityl group removed by treatment with 80% acetic acid for 30 min at room temperature. Residual acetic acid was removed by coevaporation with water, and the residue was finally redissolved in water (1 mL) and extracted with ether (2 × 5 mL). The purified deprotected oligonucleotides were then further examined by analytical HPLC with gradient II. From 0.2 μmol of support, typically 16 A_{260} units for the 18-mers and 13 A_{260} units for the 12-mer (oligonucleotide C) were obtained. Typical retention times for the DMT-oligos and the deprotected oligos were 18 and 8.5 min, respectively.

Determination of Hypochromicities of Oligonucleotides. The appropriate oligonucleotide (0.5 A_{260} unit) in 1 mL of water was digested at room temperature with snake venom phosphodiesterase (4 μg) until a constant A_{260} value was obtained. The hypochromicity values were determined by the ratio $A_{initial}/A_{final}$ and the oligonucleotide extinction coefficients calculated by multiplying the respective hypochromicity values by the sum of the constituent nucleoside micromolar extinction coefficients at 260 nm [dA, 15.3; dC, 7.7; dG, 11.9; dT, 9.3; T$_f$, 8.57 (Codington et al., 1964)].

Characterization of Oligonucleotides. The appropriate oligonucleotide (0.5 A_{260} unit) in 50 mM Tris-HCl, pH 7.5 (10 μL), was digested by adding snake venom phosphodiesterase (3 μg, 1.5 μL) for 3 h at 37 °C. Alkaline phosphatase (11 units, 0.5 μL) was then added and the solution volume made up to 15 μl containing 10 mM $MgCl_2$. After a further 0.5 h, the sample was examined by analytical HPLC with gradient IV (oligos A, B, Af, Bf, and Bff) or gradient V (oligos C and D). The nucleoside compositions were calculated from the peak area integrals from the nucleoside extinction coefficients (listed above). They were consistent with the proposed sequences. Typical retention times with gradient IV were 4.3 min (dC), 10.0 min (dG), 11.2 min (dT), 14.3 min (T$_f$), and 16.0 min (dA) and with gradient V were 2.9 min (standard araT), 3.3 min (dT), and 3.9 min (T$_f$).

5′ Labeling of Oligonucleotides. Each oligonucleotide was 5′-^{32}P-labeled by treating the oligonucleotide (200 pmol) in 50 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$/10 mM β-mercaptoethanol with [γ-^{32}P]ATP (10 pmol, 50 μCi) in the presence of T4 polynucleotide kinase (5 units) to give a total volume of 12 μL, for 1.5 h at 37 °C. After this time, 1 μL of 10 mM unlabeled ATP was added and the mixture left for a further 0.5 h. After this time, the solution was heated at 75 °C for 15 min and then placed in ice. The phosphorylated oligonucleotide was purified from unreacted ATP by applying the whole reaction mixture to a Sep-Pak C-18 column that had been prewashed with 2% acetonitrile in 100 mM TEAB, pH 7.5. The column was then washed with 30 mL of the same solution and the oligonucleotide eluted with 40% acetonitrile in 100 mM TEAB, pH 7.5. Effluent was collected dropwise in Eppendorf tubes, those containing the desired material being identified with a Geiger counter. Pooled fractions were evaporated and redissolved in water, the radioactivity was determined, and the amount of material was quantified at 260 nm. The solutions of 5 pmol/μL typically had about 2800 counts s^{-1} μL^{-1}. The oligonucleotides were also 5′-phosphorylated with unlabeled ATP by treating the oligonucleotide (4 pmol) in 50 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$/12 mM β-mercaptoethanol with ATP (20 μmol) in the presence of T4 polynucleotide kinase (100 units) to give a total volume of 260 μL, for 2 h at 37 °C. After this time, the solution was heated at 75 °C for 15 min and then placed in ice. The phosphorylated oligonucleotide was purified from unreacted ATP on an analytical HPLC column with gradient

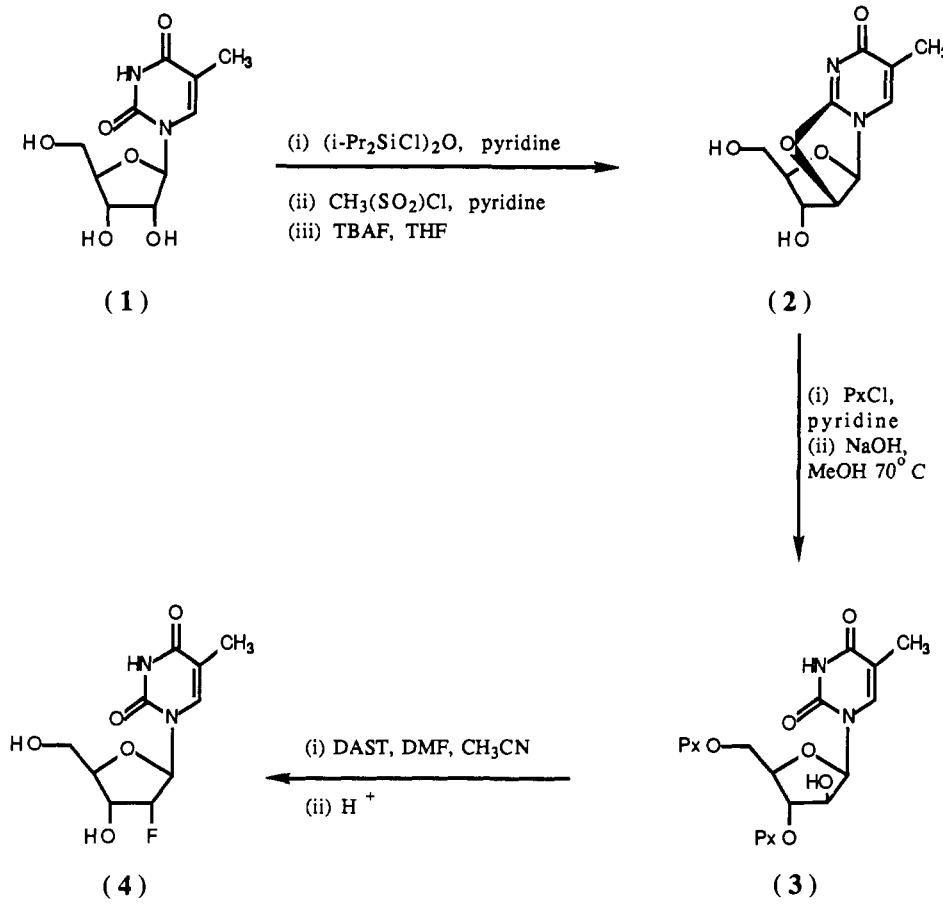

FIGURE 1: Synthesis of 2'-fluorothymidine.

VI. The appropriate peak was collected and evaporated and residual buffer removed by coevaporation with water.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE was performed on 20% gels containing 8 M urea (denaturing) or no urea (nondenaturing) in plates of dimension 20 cm (width) × 40 cm. Gels were run for 1 h at 50 W with 0.89 M Tris–borate, pH 8.3, running buffer. After electrophoresis, the gels were autoradiographed for 12 h with Kodak X-OMAT film.

Oligonucleotide Duplex Stability. (i) Nondenaturing Gel of Oligonucleotide Duplexes. One ^{32}P-labeled oligonucleotide (215 pmol) and its phorphorylated, complementary oligonucleotide (215 pmol) were annealed in 100 mM HEPES/KOH, pH 7.5, containing 20 mM $MgCl_2$ and 200 mM NaCl in a total volume of 19.5 μL by cooling from 70 °C to room temperature over 1 h. A 3-μL aliquot of this solution was then mixed with 3 μL of water and 4 μL of glycerol/water (1/1 v/v) containing 0.025% (w/v) bromophenol blue and xylene cyanol (loading mix). For single-stranded marker oligonucleotide, 33 pmol of ^{32}P-labeled oligonucleotide in 6 μL of water was mixed with 4 μL of loading mix. The samples were then subjected to 20% PAGE.

(ii) T_m Determinations. T_m determinations were performed by measuring the absorbance change every 20 s at 260 nm and by using temperature increments of 0.1 °C/min. Solutions contained 1.95 μM oligonucleotide duplex in 50 mM HEPES/KOH, pH 7.5, containing 10 mM $MgCl_2$ and 100 mM NaCl. Solutions were annealed in this buffer in a volume of 36 μL as described above and then diluted with this buffer to 1.2 mL.

EcoRV Cleavage Reactions. In each experiment a ^{32}P-labeled oligonucleotide (10 pmol) was mixed with identical unlabeled, phosphorylated oligonucleotide (490 pmol) and annealed (as described above) to its phosphorylated complement (500 pmol) in 100 mM HEPES/KOH, pH 7.5, containing 20 mM $MgCl_2$ and 200 mM NaCl (2× buffer) in a volume of 20 μL. After annealing was complete, 20 μL of water was added and the mixture preincubated at 37 °C for 3 min before the addition of *Eco*RV endonuclease (10 μL, 2.5 μM dimer) in 1× buffer to give a total volume of 50 μL, an oligonucleotide concentration of 10 μM (duplex), and an enzyme concentration (dimer) of 500 nM in 1× buffer. Aliquots of 5 μL were removed at various time points and heated for 5 min at 100 °C with 10 μL of 95% formamide/10 mM EDTA. After denaturing 20% PAGE, bands corresponding to both product and starting material were located by autoradiography and excised and the radioactivity was determined as described above.

Results

The first reported preparation of 2'-fluorothymidine involved ring opening of 2,2'-anhydro-1-β-D-arabinofuranosyluracil with liquid HF (Codington et al., 1964). More recently (Herdewijn et al., 1989), a number of sugar-fluorinated nucleosides have been synthesized by employing the fluorinating agent DAST (Card, 1985). We have used this reagent for an improved and convenient synthesis of 2'-fluorothymidine from ribofuranosylthymine as shown in Figure 1. After purification by reversed-phase HPLC, the 2'-fluorothymidine (**4**) was obtained in 35% overall yield from ribofuranosylthymine (**1**). When **3** was protected with groups other than pixyl, the yield obtained in the fluorination reaction was decreased. The 2'-fluorothymidine possessed a melting point identical with that reported previously (Codington et al., 1964) and the expected $J_{H,F}$ coupling constant values in its proton NMR spectrum (Cushley et al., 1968). The 5'-dimethoxytrityl β-cyanoethyl *N,N*-diisopropylphosphoramidite of **4** was obtained by a pro-

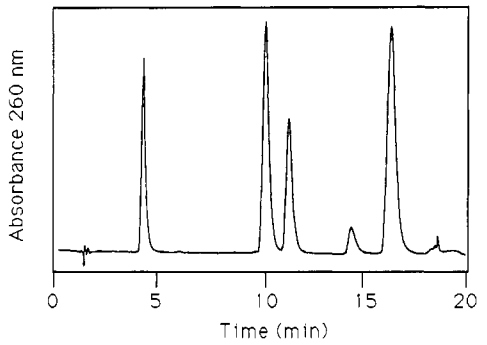

FIGURE 2: HPLC profile of oligonucleotide Af after digestion to constituent nucleosides (gradient IV): dC (4.3 min), dG (10.0 min), dT (11.2 min), T_f (14.3 min), and dA (16.0 min).

cedure analogous to that described in the literature (Sinha et al., 1984). In both the ^{31}P and the ^{19}F NMR spectra of this compound, each phosphoramidite diastereomer displayed a doublet with phosphorus–fluorine coupling constants of about 10 Hz.

In order to investigate the *Eco*RV cleavage reaction, the complementary, unmodified oligonucleotides A and B were synthesized in addition to oligomers Af and Bf, in which the underlined thymidine residues in the recognition sequence d(GATATC) were replaced by 2′-fluorothymidine. Oligonucleotide Bff, in which both of the thymidine residues within the recognition sequence were substituted by 2′-fluorothymidine, was also synthesized (see Materials and Methods for oligonucleotide sequences).

Krug et al. (1989) have recently reported that when 2′-deoxy-2′-fluorouridine was incorporated as the 3′-terminal residue of oligonucleotides, it was converted to 1-β-D-arabinofuranosyluracil during the ammonia deprotection step, the resulting oligonucleotide displaying a different mobility with reversed-phase HPLC. Thus, it was expected that oligonucleotides $d(T_{f_{11}}T)$ and $d(T_{f_3}T)$, which were also synthesized, would be suitable for assessing the compatibility of this analogue with automated DNA synthesis. The coupling time was increased from 15 s to 5 min for these oligomers in order to ensure efficient, successive coupling reactions, thereby affording a good yield of oligonucleotide product. Removal of the base- and phosphate-protecting groups and cleavage from the support were accomplished by treatment with aqueous ammonia solution overnight at 55 °C, or for 3 h at 55 °C for oligonucleotides C and D. The resulting DMT-protected oligonucleotides were then purified by reversed-phase HPLC and the DMT groups removed with 80% acetic acid. The 2′-fluorothymidine-containing oligonucleotides were obtained in yields comparable to those of the unmodified oligomers. All oligonucleotides appeared pure, as judged from analytical HPLC.

The HPLC profiles for the crude 5′-dimethoxytritylated $d(T_{12})$ and $d(T_{f_{11}}T)$, respectively, were essentially indistinguishable. In order to further demonstrate that the analogues had not been chemically modified during the synthesis, the compositions of all of the oligonucleotides were determined by HPLC analysis of the products obtained after complete digestion with snake venom phosphodiesterase, followed by alkaline phosphatase. The nucleoside compositions were calculated from the peak area integrals, with use of the nucleoside extinction coefficients, and were consistent with the proposed sequences. The HPLC trace of oligonucleotide Af after digestion is shown in Figure 2. For oligomers C and D, the expected 2′-fluorothymidine/thymidine ratios of 11/1 and 3/1 were obtained, with no detectable 1-β-D-arabinofuranosylthymine. Additionally, after treatment of the tetramer with ammonia at 55 °C for 16 h, followed by complete enzymatic digestion, the same result was obtained.

Table I: Hypochromicity and Molar Extinction Coefficients of Oligonucleotides

oligo	sequence	extinction coeff (M^{-1} cm^{-1} × 10^3)	hypochromicity[a] (%)
A	d(CAAACCGATATCGTTGTG)	154.2	23.4
B	d(CACAACGATATCGGTTTG)	153.9	23.6
Af	d(CAAACCGAT$_f$ATCGTTGTG)	154.8	23.1
Bf	d(CACAACGAT$_f$ATCGGTTTG)	154.1	23.5
Bff	d(CACAACGAT$_f$AT$_f$CGGTTTG)	152.6	24.2
C	$d(T_{f_{11}}T)$	96.1	7.5
	$d(T_{12})$	104.5	6.4

[a] Determined after complete digestion with snake venom phosphodiesterase followed by alkaline phosphatase (see also Materials and Methods).

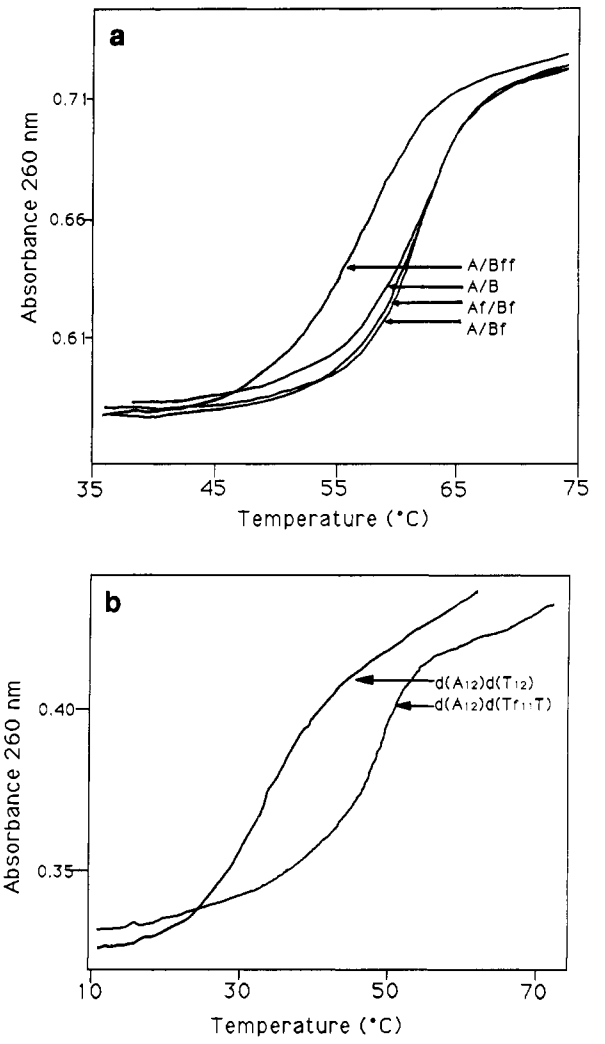

FIGURE 3: Melting profiles of (a) oligonucleotide duplexes A/B, A/Bf, Af/Bf, and A/Bff and (b) oligonucleotide duplexes $d(A_{12})$·$d(T_{f_{11}}T)$ and $d(A_{12})$·$d(T_{12})$.

The hypochromicity values and molar extinction coefficients were calculated for all five oligonucleotides from the increase in UV absorbance obtained after enzymatic digestion to the constituent nucleosides (Table I). The larger hypochromicity of 2′-fluorothymidine-containing oligonucleotide C (7.5%), compared to that of $d(T_{12})$ (6.4%), indicates a greater degree of base stacking for this oligomer.

The melting profiles for oligonucleotides A/B, A/Bf, Af/Bf, and A/Bff are shown in Figure 3a and those of $d(A_{12})$·$d(T_{f_{11}}T)$

Table II: Melting Characteristics of the Oligonucleotide Duplexes[a]

duplex	sequence	T_m (°C)	ΔH (kJ/mol)
A/B	5′-d(CAA ACC GAT ATC GTT GTG) 3′-d(GTT TGG CTA TAG CAA CAC)	62.9	435
A/Bf	5′-d(CAC AAC GAT ATC GGT TTG) 3′-d(GTG TTG CTA T_fAG CCA AAC)	63.5	460
Af/Bf	5′-d(CAA ACC GAT$_f$ ATC GTT GTG) 3′-d(GTT TGG CTA T_fAG CAA CAC)	63.5	438
A/Bff	5′-d(CAA ACC GAT ATC GTT GTG) 3′-d(GTT TGG CT$_f$A T_fAG CAA CAC)	58.7	274
	$d(A_{12})$·$d(T_{12})$	32.6	50.5
	$d(A_{12})$·$d(T_{f_{11}}T)$	48.3	63.2

[a] Solutions contained 1.95 μM oligonucleotide duplex concentration in 50 mM HEPES/KOH, pH 7.5, containing 10 mM $MgCl_2$ and 100 mM NaCl (see also Materials and Methods).

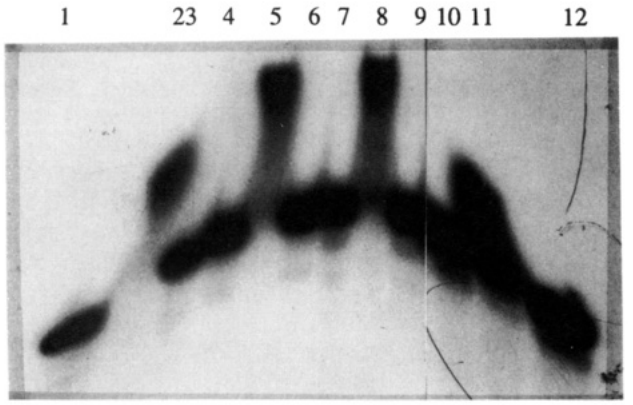

FIGURE 4: Autoradiograph of a nondenaturing polyacrylamide gel of the oligonucleotide duplexes: lanes 1 and 3, oligo B; lane 2, oligos B and A; lanes 4 and 6, oligo Bf; lane 5, oligos Bf and A; lanes 7 and 9, oligo Af; lane 8, oligos Bf and Af; lanes 10 and 12, oligo Bff; lane 11, oligos Bff and A. (Oligonucleotide duplexes B and A in lane 2 have shifted to the right during the running of the gel. The faster running band in lanes 10 and 12 represents 4% of the material in these lanes, as determined by scanning densitometry.)

and $d(A_{12})$·$d(T_{12})$ are shown in Figure 3b. The melting characteristics of the duplexes are summarized in Table II. The ΔH values were calculated assuming a two-state model as described previously (Pörschke, 1982), and they reflect the cooperativity of the melting process. The single substitution of a 2′-fluorothymidine in one or both strands does not significantly affect the T_m values (oligo sets A/Bf and Af/Bf, respectively). However, a significant decrease in thermal stability is observed for the duplex formed from the doubly substituted 2′-fluorothymidine-containing oligonucleotide with its complementary unmodified strand (A/Bff). The relative thermal stabilities of the various duplexes were also investigated by annealing one 5′-^{32}P-labeled strand to its unlabeled, phosphorylated complement and then examinign the mixture on a nondenaturing polyacrylamide gel (Figure 4). In each case, the mobility of the annealed oligonucleotide set was retarded relative to the respective single-stranded species, indicating the two strands to be hybridized. The duplex A/Bff was seen to be less stable than duplexes A/B, A/Bf, and Af/Bf, which ran as distinct, retarded bands. This result was consistent with the T_m data.

The rates of cleavage of oligonucleotide duplexes A/B, Af/Bf, and A/Bff by the *Eco*RV endonuclease were determined by use of one radioactively labeled oligonucleotide annealed to its phosphorylated complement. The cleavage reactions were performed in the same buffer that was used for the T_m determinations and at a duplex concentration of 10 μM and 500 nM enzyme (dimer concentration). At various time points, aliquots were removed, heated with stop mix, and subjected to PAGE. The bands corresponding to product and starting material were excised, and the amount of radioactivity was determined. From this the amount of duplex cleaved at each time point was calculated. The rates of cleavage of both the 2′-fluorothymidine-containing strand and the unmodified strand were determined for duplex A/Bff. The relative cleavage rates of the oligonucleotides in comparison to the unmodified duplex are summarized in Table III. The Bf/Af duplex, in which the thymidine residues at the *Eco*RV cleavage site within each strand were replaced by 2′-fluorothymidine, was cleaved at approximately two-thirds of the rate of the unmodified duplex. However, when two such substitutions were made in one strand, as in duplex A/Bff, a 3-fold reduction in the cleavage rate was obtained for the unmodified strand, while the modified strand was cleaved at 22% of the rate obtained for the A/B duplex.

Table III: Relative Rates of Cleavage of Duplexes by *Eco*RV

duplex	recognition sequence	cleavage rate[a] (pmol/min)	relative cleavage rate (%)
*A/B[b]	*5′-d(GATATC) 3′-d(CTATAG)	71.6 (±3.2)	100
Af/*Bf	*5′-d(GAT$_f$ATC) 3′-d(CTAT$_f$AG)	42.1 (±6.2)	59
*A/Bff	*5′-d(GATATC) 3′-d(CT$_f$AT$_f$AG)	25.9 (±1.3)	36
A/*Bff	5′-d(GATATC) *3′-d(CT$_f$AT$_f$AG)	15.9 (±1.8)	22

[a] Determined from gradients of the plots of cleaved substrate against time. The standard errors obtained are indicated in parentheses. [b] The 5′ radioactively labeled strand is designated by the asterisk.

Discussion

The restriction endonuclease *Eco*RV recognizes the sequence d(GATATC) and catalyzes the hydrolysis of the central phosphodiester bond of each strand of a double-stranded substrate (D'Arcy et al., 1985; Taylor & Halford, 1989). The importance of the interactions of *Eco*RV with either of the dA-dT base pairs within both the major (Fliess et al., 1986; Mazzarelli et al., 1989; Nwosu et al., 1988) and the minor groove (Cosstick et al., 1990) of the DNA has been previously demonstrated with several nucleoside analogue containing oligonucleotide duplexes. In addition, it has recently been shown that a single phosphorothioate substitution between the central thymidine and 2′-deoxyadenosine residues within one strand of a DNA duplex dramatically reduces the rate of *Eco*RV-catalyzed hydrolysis (Olsen et al., 1990). We became interested in the possibility of inhibiting the *Eco*RV-catalyzed cleavage of DNA by the incorporation of 2′-fluorothymidine residues. For this study we synthesized 18-mer oligonucleotides containing 2′-fluorothymidine substitutions within the *Eco*RV recognition sequence. In these oligonucleotides, one or both of the thymidine residues (oligonucleotides Af and its complement Bf and oligonucleotide Bff, respectively) were replaced by 2′-fluorothymidine. The unmodified oligonucleotides A and B were also synthesized, in addition to $d(T_{f_{11}}T)$ and $d(T_{f_3}T)$. The 2′-fluorothymidine was obtained from ribofuranosylthymine in good yield via the route illustrated in Figure 1.

The hypochromicity values of the oligonucleotides A, B, Af, and Bf, obtained after complete enzymatic digestion to the constituent nucleosides, are all very similar. The hypochromicity of Bff was slightly larger (Table I). These values indicate that the degrees of base stacking in these oligonucleotides are all very similar. Comparison of the hypochromicity values of oligonucleotides $d(T_{f_{11}}T)$ and $d(T_{12})$ indicates that a higher degree of base stacking for that containing

2′-fluorothymidine occurs. This result is consistent with the proposals of Melcher (1970) that the 2′-substituent of the sugar interacts with the π-electron system of the base attached to the residue on its 3′-side and that this interaction increases with increasing substituent electronegativity.

On the basis of earlier work by Janik et al. (1971), who reported that poly(rA)·poly(Uf) has a T_m 17 °C higher than that of poly(rA)·poly(rU) and 19 °C higher than that of poly(rA)·poly(dU), it was expected that the analogue-containing duplexes would display melting temperatures similar to or higher than that of the unmodified duplex, A/B. As indicated in Table II, the presence of a single 2′-fluorothymidine residue in either one or both strands (A/Bf and Af/Bf, respectively) does not significantly alter the T_m or the melting enthalpy (ΔH) values from those of the A/B duplex. however, the T_m of the A/Bff duplex is over 4 °C lower than that of the A/B duplex, but more importantly its melting enthalpy is considerably reduced, suggesting a large decrease in the cooperativity of the melting process. It is likely that the 2′-fluorothymidine residues within the A/Bf and Af/Bf duplexes, like the unmodified 2′-deoxyribonucleosides in these duplexes, may adopt a 2′-endo conformation and thereby allow a normal B-DNA duplex to be formed. The reduced cooperativity of melting shown by the A/Bff duplex results in a value that is more comparable to that expected for two 9-mers (Pörschke, 1977). It thus appears that a local destabilization of the duplex occurs when more than one analogue is incorporated into a strand. This may be as a result of the preference of 2′-fluorothymidine for the 3′-endo conformation (Guschlbauer, 1982). However, in contrast, a considerable increase in both the stability and the cooperativity of melting of oligo duplex $d(A_{12})\cdot d(T_{f_{11}}T)$ compared to $d(A_{12})\cdot d(T_{12})$ was observed. The former duplex displayed an increased T_m of 16 °C and an increased ΔH value of 13 kJ/mol. Presumably in this situation the whole of the 2′-fluorothymidine-containing strand may adopt the ribo-like 3′-endo conformation and the resulting duplex would therefore be expected to display the higher thermal stability that distinguishes RNA·DNA hybrids from DNA duplexes.

The relative cleavage rates of the duplexes obtained with *Eco*RV are summarized in Table III. The values obtained reflect the relative order of the thermal stabilities of the duplexes. The fact that the Af/Bf duplex was cleaved at two-thirds of the rate of the unmodified A/B duplex indicates that such single substitutions, although at the cleavage site, have only minor consequences. However, the A/Bff duplex, which the melting studies suggest might have an altered conformation in the region of the modified thymidine analogues, was cleaved at one-third of the rate and one-fifth of the rate of the A/B duplex in the unmodified and modified strands, respectively. It seems that a local conformational change of the oligonucleotide induced by the substitution of the two thymidines by 2′-fluorothymidine may be more important for inhibition than the presence of the analogue on the 5′-side of the scissile bond of each strand. Thus, in order to inhibit the rate of *Eco*RV-catalyzed cleavage of DNA, it appears that several such 2′-deoxy-2′-fluoronucleoside substitutions are required. The increased resistance to cleavage by restriction enzymes of RNA·DNA hybrids compared to that of DNA duplexes (Molloy & Symons, 1980) probably also results from the altered conformation of such duplexes rather than the presence of an electronegative 2′-substituent.

Although the specific base–protein interactions are important for normal *Eco*RV-catalyzed cleavage to occur (Taylor & Halford, 1989; Fliess et al., 1986; Mazzarelli et al., 1989; Nwosu et al., 1988; Cosstick et al., 1990), this study indicates that the conformation of the DNA is also an important factor for this and perhaps other restriction endonuclease catalyzed cleavage reactions.

Acknowledgments

We thank Dr. B. A. Connolly for kindly supplying the *Eco*RV endonuclease and Dr. D. Pörschke for measuring the melting characteristics of the oligonucleotide duplexes. We are additionally grateful to Drs. D. B. Olsen, J. R. Sayers, and W. A. Pieken for reading the manuscript.

References

Card, P. J. (1985) *Nucleosides & Nucleotides 4*, 415–418.
Catlin, J. C., Guschlbauer, W. (1975) *Biopolymers 14*, 51–72.
Codington, J. F., Doerr, I. L., & Fox, J. J. (1964) *J. Org. Chem. 29*, 558–564.
Cosstick, R., Li, X., Tuli, D. K., Williams, D. M., Connolly, B. A., & Newman, P. C. (1990) *Nucleic Acids Res. 18*, 4771–4778.
Cushley, R. J., Codington, J. F., & Fox, J. J. (1968) *Can. J. Chem. 46*, 1131–1140.
D'Arcy, A., Brown, R. S., Zabeau, M., Resandt, R. W., & Winkler, F. W. (1985) *J. Biol. Chem. 260*, 1987–1990.
Doerr, I. L., & Fox, J. J. (1967) *J. Org. Chem. 32*, 1462–1471.
Fazakerley, G. V., Uesugi, S., Izumi, A., Ikehara, M., & Guschlbauer, W. (1985) *FEBS Lett. 182*, 365–369.
Fliess, A., Wolfes, H., Rosenthal, A., Schwellrus, K., Blöcker, H., Frank, H., & Pingoud, A. (1986) *Nucleic Acids Res. 14*, 3463–3474.
Guschlbauer, W. (1982) *Nucleic Acids Res. Symp. Ser. 11*, 113–116.
Guschlbauer, W., & Janikowski, K. (1980) *Nucleic Acids Res. 8*, 1421–1433.
Guschlbauer, W., Blandin, M., Drocourt, J. L., & Thang, M. N. (1977) *Nucleic Acids Res. 4*, 1933–1943.
Hakoshima, T., Omori, H., Tomita, K., Miki, H., & Ikehara, M. (1981) *Nucleic Acids Res. 9*, 711–729.
Herdewijn, P., VanAerschot, A., & Kernemans, L. (1989) *Nucleosides & Nucleotides 8*, 65–96.
Ikehara, M., & Imura, J. (1981) *Chem. Pharm. Bull. 29*, 1034–1038.
Ikehara, M., & Miki, H. (1978) *Chem. Pharm. Bull. 26*, 2449–2453.
Ikehara, M., Fukui, T., & Kakiuchi, N. (1978) *Nucleic Acids Res. 5*, 1877–1887.
Janik, B., Kotick, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., & Wilson, D. P. (1971) *Biochem. Biophys. Res. Commun. 46*, 1153–1160.
Jones, R. A. (1984) in *Oligonucleotide Synthesis—A Practical Approach* (Gait, M. J., Ed.) pp 27–28, IRL Press, Oxford.
Kakiuchi, N., Marck, C., Rousseau, N., Leng, M., de Clercq, E., & Guschlbauer, W. (1982) *J. Biol. Chem. 257*, 1924–1928.
Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *J. Mol. Biol. 183*, 553–563.
Krug, A., Oretskaya, T. S., Volkov, E. M., Cech, D., Shabarova, Z. A., & Rosenthal, A. (1989) *Nucleosides & Nucleotides 8*, 1473–1483.
Maass, G. (1987) in *DNA–Ligand Interactions. From Drugs to Proteins* (Guschlbauer, W., & Saenger, W., Eds.) pp 225–238, Plenum Press, New York.
Marck, C., Kakuichi, N., & Guschlbauer, W. (1982a) *Nucleic Acids Res. 10*, 6147–6161.
Marck, C., Lesyng, B., & Saenger, W. (1982b) *J. Mol. Struct. 82*, 77–94.

Mazzarelli, J., Scholtissek, S., & McLaughlin, L. W. (1989) *Biochemistry 28*, 4616–4622.
Melcher, G. (1970) *Biophysik 7*, 29–32.
Molloy, P. L., & Symons, R. H. (1980) *Nucleic Acids Res. 8*, 2939–2946.
Nwosu, V. U., Connolly, B. A., Halford, S. E., & Garnett, J. (1988) *Nucleic Acids Res. 16*, 3705–3720.
Ohtsuka, E., Ishino, Y., Ibaraki, K., & Ikehara, M. (1984) *Eur. J. Biochem. 139*, 447–450.
Olsen, D. B., Kotzorek, G., & Eckstein, F. (1990) *Biochemistry 29*, 9546–9551.
Olson, W. K. (1982) *J. Am. Chem. Soc. 104*, 278–286.
Pörschke, D. (1977) in *Molecular Biology, Biochemistry, and Biophysics* (Pecht. I., & Rigler. R., Eds.) Vol. 24, Springer-Verlag, Berlin Heidelberg.
Pörschke, D. (1982) *Nucleic Acids Res. 10*, 6163–6176.
Saenger, W. (1984) in *Principles of Nucleic Acid Structure* (Cantor, C. R., Ed.) Springer-Verlag, New York.
Sinha, N. D., Biernat, J., & Köster, H. (1984) *Nucleic Acids Res. 12*, 4539–4557.
Suck, D., Saenger, W., Main, P., Germain, G., & de Clercq, J. (1974) *Biochim. Biophys. Acta 361*, 257–265.
Taylor, J. D., & Halford, S. E. (1989) *Biochemistry 28*, 6198–6207.
Vorbrüggen, H., & Bennua, B. (1978) *Tetrahedron Lett. 15*, 1339–1342.

Two New Photoaffinity Polyamines Appear To Alter the Helical Twist of DNA in Nucleosome Core Particles[†,‡]

Elizabeth Clark,[§] Richard A. Swank,[§] James E. Morgan,[§] Hirak Basu,[∥] and Harry R. Matthews*[,§]

Department of Biological Chemistry, University of California, Davis, California 95616, and Department of Neurosurgery, University of California, San Francisco, California 94143

Received September 19, 1990; Revised Manuscript Received January 2, 1991

ABSTRACT: Two new photoaffinity derivatives of polyamines have been synthesized by the reaction of spermine or spermidine with methyl 4-azidobenzimidate. The new compounds were purified chromatographically and characterized by several methods including proton magnetic resonance spectroscopy. The spermine derivative is N^1-ABA-spermine [(azidobenzamidino)spermine], and the spermidine derivative is a mixture of N^1- and N^8-ABA-spermidine. ABA-spermine stabilizes nucleosome core particles in thermal denaturation experiments, with similar but not identical effects when compared with the parent polyamine, spermine. In circular dichroism experiments, ABA-spermine was capable of producing a B → Z transition in poly(dG-m^{5}dC) at a concentration of 30 μM, compared with 5 μM required to produce the same effect with spermine. On the other hand, ANB-spermine [(azidonitrobenzoyl)spermine; Morgan, J. E., Calkins, C. C., & Matthews, H. R. (1989) *Biochemistry 28*, 5095–5106] stabilized the B form of poly(dG-br^{5}dC). ABA-spermine is a potent inhibitor of ornithine decarboxylase from *Escherichia coli*, giving 50% inhibition at 0.12 mM, while ANB-spermine is a modest inhibitor, comparable to spermine or spermidine. Under conditions of nitrogen-limited growth, yeast take up ABA-spermine and ABA-spermidine at approximately one-third to half the rate of spermidine or spermine. In contrast, ANB-spermine was not significantly taken up. The photoaffinity polyamines were used to photoaffinity label the DNA in nucleosome core particles, and the sites of labeling were determined by exonuclease protection. All photoaffinity reagents showed both nonspecific labeling and specific sites of higher occupancy. However, the positions of the sites varied: the ANB-spermine sites confirmed those previously reported (Morgan et al., 1989); the ABA-spermine and ABA-spermidine sites were spaced at 9.8 bp intervals from the 3′ end of each DNA strand. This observation, together with the effect of spermine on the circular dichroism of DNA in nucleosome core particles, implies that polyamines alter the helical twist of DNA in nucleosome core particles. The ABA-polyamines are offered as general-purpose photoaffinity polyamine reagents.

Polyamines are small polycations found in all living cells (Tabor & Tabor, 1984). Cells may be depleted of polyamines by inhibition of their synthesis through the use of drugs or mutations (Pegg, 1988). Whenever polyamine levels are depleted, cell growth is severely inhibited or completely stopped and the cells may die (Porter & Bergeron, 1988). Ornithine decarboxylase is a very highly regulated enzyme in the polyamine synthesis pathway in mammalian cells, and its elevation is a very early response to agents that stimulate cell proliferation (Davis et al., 1988).

In spite of these indications of the essential role of polyamines in cell proliferation and other processes, very little specific information is available concerning the function(s) of polyamines in cells at the molecular level. One function emerging as essential is the modification of a protein synthesis initiation factor by the covalent addition of spermidine to a

[†] This work was supported in part by the NSF (DCB 8705378 to H.R.M.) and the NIH (CA-49409 to H.B.).
[‡] This paper is dedicated to Professor V. G. Allfrey of The Rockefeller University on the occasion of his 70th birthday.
[§] University of California, Davis.
[∥] University of California, San Francisco.

0006-2960/91/0430-4009$02.50/0