Bolotina, I. A., Chekov, V. O., Lugauskas, V., Finkel'shtein, A. V., & Ptitsyn, O. B. (1980) *Mol. Biol. (Moscow) 14*, 891-901.
Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J. 133*, 529-539.
Bulleid, N. J., & Freedman, R. B. (1988) *Nature 335*, 649-651.
Burns, L. D., & Schachman, H. K. (1982) *J. Biol. Chem. 257*, 8638-8647.
Chen-Kiang, S., Cardinale, G. J., & Udenfriend, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4420-4424.
Correa-Freire, M. C., Barenholz, Y., & Thompson, T. E. (1982) *Biochemistry 21*, 1244-1248.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404-427.
Freedman, R. B. (1989) *Cell 57*, 1069-1072.
Goldberger, R. F., Epstein, C. J., & Anfinsen, C. B. (1963) *J. Biol. Chem. 238*, 628-635.
Goldberger, R. F., Epstein, C. J., & Anfinsen, C. B. (1964) *J. Biol. Chem. 239*, 1406-1410.
Helmkamp, G. M., Jr. (1986) *J. Bioenerg. Biomembr. 18*, 71-91.
Hillson, D. A., Lambert, N., & Freedman, R. B. (1984) *Methods Enzymol. 107*, 281-294.
Kaska, D. D., Myllyla, R., Gunzler, V., Gibor, A., & Kivirikko, K. I. (1988) *Biochem. J. 256*, 257-263.
Kivirikko, K. I., Myllyla, R., & Pihlajaniemi, T. (1989) *FASEB J. 3*, 1609-1617.
Koivu, J., Myllyla, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K., & Kivirikko, K. I. (1987) *J. Biol. Chem. 262*, 6447-6449.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
Livingston, D. M. (1974) *Methods Enzymol. 34*, 723-731.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
McLean, L. R., & Hagaman, K. A. (1989) *Biochemistry 28*, 321-327.
Morishima, N., Nakagawa, K., Yamamoto, E., & Shibata, T. (1990) *J. Biol. Chem. 265*, 15189-15197.
Pownall, H. J., Hickson, D., Gotto, A. M., Jr., & Massey, J. B. (1982) *Biochim. Biophys. Acta 712*, 169-176.
Roseman, M. A., & Thompson, T. E. (1980) *Biochemistry 19*, 439-444.
Rothman, J. E. (1989) *Cell 59*, 591-601.
Steiner, R. F, De Lorenzo, F., & Anfinsen, C. B. (1965) *J. Biol. Chem. 240*, 4648-4651.
Tuderman, L., Oikarinen, A., & Kivirikko, K. I. (1977) *Eur. J. Biochem. 78*, 547-556.
Wallace, B. A., & Teeters, C. L. (1987) *Biochemistry 26*, 65-70.
Wetterau, J. R., & Zilversmit, D. B. (1984) *J. Biol. Chem. 259*, 10863-10866.
Wetterau, J. R., & Zilversmit, D. B. (1985) *Chem. Phys. Lipids 38*, 205-222.
Wetterau, J. R., & Zilversmit, D. B. (1986) *Biochim. Biophys. Acta 875*, 610-617.
Wetterau, J. R., Combs, K. A., Spinner, S. N., & Joiner, B. J. (1990) *J. Biol. Chem. 265*, 9800-9807.
Wetterau, J. R., Aggerbeck, L. P., Laplaud, P. M., & McLean, L. R. (1991) *Biochemistry 30*, 4406-4412.
Wirtz, K. W. A. (1982) in *Lipid-Protein Interactions* (Griffith, O. H., & Jost, P. C., Eds.) Vol. 1, pp 151-231, John Wiley & Sons, New York.
Yang, J. T., Wu, C.-S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208-269.

Study of a Hammerhead Ribozyme Containing 2'-Modified Adenosine Residues†

David B. Olsen,‡ Fritz Benseler, Helle Aurup, Wolfgang A. Pieken, and Fritz Eckstein*
Max-Planck Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein Str. 3, W-3400 Göttingen, FRG
Received May 20, 1991; Revised Manuscript Received July 12, 1991

ABSTRACT: The improved synthesis of 2'-fluoro-2'-deoxyadenosine (2'-FA) starting from adenosine is described. This compound was converted to the phosphoramidite and incorporated into a hammerhead ribozyme RNA with the use of automated RNA synthesis techniques. Ribozymes containing 2'-deoxyadenosine (2'-dA) were prepared in a similar manner. A kinetic rate comparison of the unmodified ribozyme with two ribozymes that had every adenosine replaced with 2'-FA or 2'-dA revealed a large decrease in catalytic efficiency (k_{cat}/K_m) for the modified ribozymes resulting from a drop in k_{cat}. The kinetic analysis of a number of partially substituted 2'-FA or 2'-dA containing hammerheads revealed that the decrease in activity was not associated with any particular residue but was the result of the accumulation of modified nucleosides within the structure.

Several different satellite RNAs, associated with a number of plant viruses, can form a hammerhead ribozyme structure that undergoes self-catalyzed cleavage yielding an RNA with a 5'-OH and an RNA with a 2',3'-cyclic phosphate [for reviews see Bruening (1989) and Sheldon et al. (1990)]. The study of this cleavage reaction is of great interest for two reasons. First, the catalytic mechanism of this novel intramolecular RNA self-cleavage reaction is poorly understood. Second, these compounds are considered to be potential therapeutic agents targeted against viral diseases such as hepatitis B and AIDS (Cotten, 1990; Rossi & Sarver, 1990; Sarver et al., 1990).

Recent structure–function studies of hammerhead ribozymes have explored the effect of incorporation of phosphorothioate groups into the catalytic RNA (Buzayan et al., 1988, 1990; Ruffner & Uhlenbeck, 1990). This research has revealed that

† This work was supported by the Deutsche Forschungsgemeinschaft.
‡ Present address: Merck Sharp & Dohme Research Laboratories, Department of Virus and Cell Biology, West Point, PA 19486.

0006-2960/91/0430-9735$02.50/0

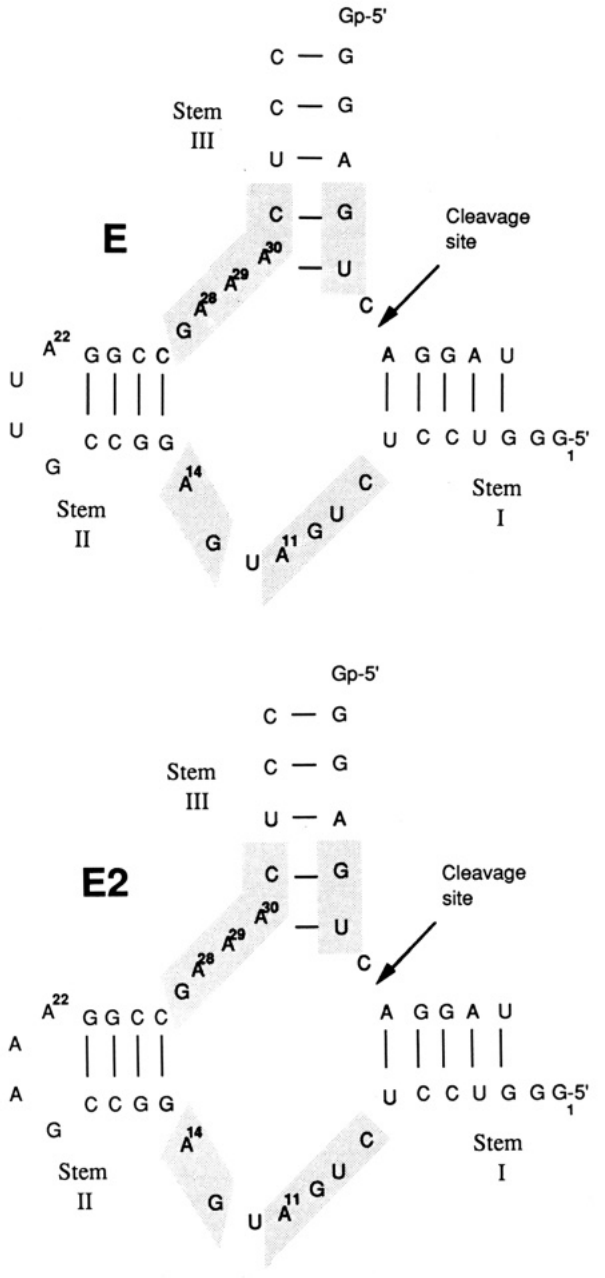

FIGURE 1: Secondary structure of the two hammerhead ribozymes considered in this paper. The conserved central core region is shaded. Ribozyme-E2 differs from ribozyme-E only in the sequence of the loop of stem II.

specific phosphate positions 5′ to three adenosine residues, A_{14} (Buzayan et al., 1990; Ruffner et al., 1990) and A_{28} and A_{29} (Ruffner et al., 1990), located within the conserved central core sequence (Figure 1, shaded regions) of the hammerhead, may be required for the proper formation of the tertiary structure of the RNA (Ruffner & Uhlenbeck, 1990). In addition, it has been determined that the presence of a phosphorothioate at the position of cleavage causes a reduced rate of hammerhead induced self-cleavage (Buzayan et al., 1988; Slim & Gait, 1991).

In another study, it has been shown that the substitution with 2′-deoxynucleosides at multiple positions within the single-stranded core region of the ribozyme can cause a several orders of magnitude decrease in catalytic efficiency (Perreault et al., 1990). These experiments, however, did not investigate the effect of deoxynucleoside residues incorporated at single specific positions.

We recently determined that the substitution of all the pyrimidine nucleoside positions within a hammerhead sequence with 2′-deoxy-2′-fluoro analogues decreases catalytic efficiency, relative to an unsubstituted counterpart ($E_{unmodified}$), by an order of magnitude (Pieken et al., 1991). This confirmed the idea that the replacement of the 2′-OH group with a fluorine atom is a fairly conservative change in terms of the structure of the RNA (Saenger, 1984). It was also reported that when 2′-fluoro-2′-deoxyadenosine was substituted for all the adenosines in the hammerhead ribozyme sequence (E-FA_{total}), the catalytic activity of the RNA was almost abolished. In order to study the influence of specific 2′-OH groups, we report here the kinetic characterization of hammerhead ribozymes substituted at specific positions with 2′-deoxy- or 2′-fluoro-2′-deoxyadenosine.

Experimental Procedures

Materials and Methods. Precoated Merck silica gel F_{254} plates were used for thin-layer chromatography (TLC). The compounds were visualized by the use of UV light or by spraying with 5% aqueous sulfuric acid followed by heating. The following solvents were used to develop the TLC plates: solvent 2, CH_2Cl_2/MeOH, 8:2; solvent 3, CH_2Cl_2/MeOH, 95:5; solvent 4, CH_2Cl_2/ethyl acetate, 9:1; solvent 5, CH_2Cl_2/ethyl acetate, 95:5. Flash column chromatography was performed on Kieselgel 60 (<63 μm; Merck, Darmstadt, FRG). 5′,2′-Bis(*tert*-butyldimethylsilyl)adenosine was synthesized according to Hakimelahi et al. (1982). 9-Chloro-9-phenylxanthene (pixyl chloride) was obtained from Fluka, and (diethylamino)sulfur trifluoride (DAST) was supplied by Merck. Tetra-*n*-butylammonium fluoride (TBAF, 1.1 M solution in THF) was from Aldrich. Ribonuclease *Phy* M (sequencing grade) was from Pharmacia.

Synthesis of Nucleoside Analogues. The synthesis of the nucleoside analogue 2′-fluoro-2′-deoxyadenosine is given below. This method is an improvement of previously published procedures (Auer et al., 1989; Ikehara et al., 1980; Ikehara & Miki, 1978). The 2′-fluoro-2′-deoxyadenosine 3′-phosphoramidite was prepared by the method of Sinha et al. (1984).

N^6,3′-Dipixyl-9-β-D-ribofuranosyladenine. 5′,2′-Bis(*tert*-butyldimethylsilyl)adenosine (4 g, 8.1 mmol) was dissolved in pyridine (150 mL) and treated with pixyl chloride (5 g, 17 mmol; Fluka) overnight. The reaction was followed by TLC (solvent 3, R_f of starting material 0.31 and R_f of product 0.86). Methanol (20 mL) was added, and the reaction mixture was evaporated to dryness. The residue was taken up in toluene (150 mL) and extracted with saturated aqueous $NaHCO_3$ (150 mL) followed by saturated aqueous NaCl (150 mL), the organic layer was evaporated to dryness, and the residue was coevaporated twice with toluene. After the oily residue was dissolved in THF (150 mL), a 1.1 M solution of TBAF/THF (12 mL, 12 mmol) was added. The reaction was followed by TLC (solvent 3, R_f of product 0.54). After 1 h, methanol (20 mL) was added and the solution was evaporated to dryness, the residue was redissolved in ethyl acetate containing 1 mL of triethylamine and extracted with saturated aqueous $NaHCO_3$ (150 mL) and saturated aqueous NaCl (150 mL), and the organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in CH_2Cl_2 (10 mL), methanol (50 mL) was added, and the product was crystallized at 5 °C overnight. Yield: 5.9 g (93%) mp: 177–178 °C. The ^1H NMR spectrum is in agreement with the structure.

N^6,5′,3′-Tripixyl-9-β-D-ribofuranosyladenine. To N^6,3′-dipixyl-9-β-D-ribofuranosyladenine (5.67 g, 7.3 mmol) dissolved in pyridine (80 mL) was added pixyl chloride (2.24 g, 7.66 mmol) portionwise over 60 min, and the reaction was followed by TLC (solvent 3, R_f of product 0.85). After 6 h,

methanol (20 mL) was added and the solution was evaporated to dryness. The white solid residue was suspended in ethyl acetate (80 mL) and methanol (30 mL) and refluxed for 5 min. After 2 h at 5 °C, the white precipitate was filtered off and washed with a cooled mixture of ethyl acetate and methanol (8:2 v/v). Yield: 6.5 g (86%). The ^{1}H NMR spectrum is in agreement with the structure.

N^6,5′,3′-Tripixyl-9-β-D-arabinofuranosyladenine was prepared according to a modified procedure originally described by Hansske et al. (1984). CrO_3 (1.5 g, 15 mmol) was suspended in CH_2Cl_2 (40 mL) and cooled in an ice bath, and acetic anhydride (1.43 mL, 15 mmol) was added dropwise to the solution. After 5 min, pyridine (2.42 mL, 30 mmol) was added and the dark brown solution was treated for 3 min in an ultrasonic bath and stirred vigorously for 5 min in an ice bath before N^6,5′,3′-tripixyl-9-β-D-ribofuranosyladenine (5.15 g, 5 mmol) was added. The reaction was followed by TLC (solvent 4, R_f of starting material 0.35 and R_f of the keto intermediate 0.70). After 45 min, ethanol (120 mL), precooled in an ice/NaCl bath, was added and the solution was put into an ice/NaCl bath before $NaBH_4$ (375 mg, 10 mmol) dissolved in ethanol (20 mL) was added dropwise over a period of 15 min. The reaction was followed by TLC (solvent 4, R_f of product 0.84). After 30 min, the solution was mixed with ethyl acetate (550 mL) containing 1% triethylamine and the dark brown precipitate was removed by filtration over Kieselgel 60 (60 g) packed in ethyl acetate. The filtrate was evaporated and the residue was purified by flash column chromatography on Kieselgel 60 (45 g). The product eluted with CH_2Cl_2/*n*-hexane (40:60 v/v) and was obtained as a white foam after evaporation. Yield: 3.2 g (62%). The ^{1}H NMR spectrum is in agreement with the structure.

2′-Fluoro-9-β-D-ribofuranosyladenine. To a solution of N^6,5′,3′-tripixyl-9-β-D-arabinofuranosyladenine (2.76 g, 2.7 mmol) in CH_3CN (45 mL), CH_2Cl_2 (6 mL), and DMF (3.2 mL) was added DAST (1.78 mL, 13.4 mmol), and the solution was stirred at 35 °C for 10 h, after which time TLC indicated completion of the reaction (solvent 5, R_f of starting material 0.50 and R_f of major compound 0.68; solvent 2, R_f of starting material 0.22 and R_f of major compound 0.44, after removal of the pixyl groups by treatment of the applied material with a solution of 10% dichloroacetic acid in CH_2Cl_2 before development of the plate). TLC analysis indicates that the reaction proceeds through at least one intermediate. After the solution was placed on ice for 15 min, methanol (8 mL) and triethylamine (0.8 mL) were added. The solution was evaporated almost to dryness, transferred to an extraction funnel with ethyl acetate (10 mL), and extracted with diethyl ether (80 mL) and water (80 mL). The organic phase was evaporated to dryness, coevaporated with ethyl acetate/ethanol (1:1 v/v) to remove triethylamine, and then dissolved in CH_2Cl_2 (3 mL) and methanol (36 mL). To the oily residue was added concentrated HCl (0.330 mL, resulting in an 0.1 M solution of HCl), and the solution was stirred in an ice bath. After 1 h, the resulting white suspension was neutralized with 1 N NaOH, evaporated almost to dryness, taken up in water (20 mL), and placed on ice for 1 h. The precipitate was filtered off and washed with cold water. The filtrate was washed once with ethyl acetate (20 mL), evaporated to about 3 mL, and filtered through LiChrospher 100 RP-18 (3 g; 5 μm; Merck, Darmstadt), eluted with water containing 5% methanol, and evaporated to about 3 mL. The product was purified by preparative HPLC on a Waters Delta Prep 3000 instrument connected to a Waters 484 tunable absorbance detector using a Delta Pak C-18 cartridge (WATO 15401, 57 mm × 30 cm, 100 Å, 15 μm) in a Waters 1000 Prep Pak Module. For elution a linear gradient of 0–30% CH_3CN in water over 200 min at a flow rate of 45 mL/min was applied. It is advisable to use an aqueous solution of 70% CH_3CN to form the gradient. The product eluted between 60 and 70 min. After evaporation of the product-containing fractions, the white solid residue was suspended in ethanol (5 mL), refluxed for 2 min, and recrystallized at 4 °C overnight. Yield: 310 mg (43%). Mp: 232–233 °C [lit. (Ikehara & Miki, 1978) mp 231–233 °C]. ^{1}H NMR (DMSO-d_6)δ 8.36 (s, 1 H, H8); 8.15 (s, 1 H, H2); 6.23 (dd, 1 H, $J_{HH2'}$ = 3.03 Hz, J_{HF} = 16.79 Hz, H1′); 5.43 (ddd, 1 H, $J_{HH1'}$ = 3.22 Hz, $J_{HH3'}$ = 4.35 Hz, J_{HF} = 52.95 Hz, H2′); 4.48 (ddd, 1 H, $J_{HH2'}$ = 4.58 Hz, $J_{HH4'}$ = 6.23 Hz, J_{HF} = 17.30 Hz, H3′); 3.99 (br pd, 1 H, H4′); 3.75 (dd, 1 H, $J_{HH5'A}$ = 12.38 Hz, $J_{HH4'}$ = 2.68 Hz, H5′B); 3.58 (dd, 1 H, $J_{HH5'B}$ = 12.37 Hz, $J_{HH4'}$ = 3.87 Hz, H5′A). ^{19}F NMR (DMSO-d_6 standard C_6F_6) δ −204.1 (s).

Automated Synthesis of Oligoribonucleotides. Automated oligoribonucleotide synthesis was carried out with an Applied Biosystems 380B DNA synthesizer on a 1-μmol scale with use of the monomeric ribonucleotide phosphoramidites and control pore glass columns with the ribonucleoside coupled to it as supplied by Milligen/Biosearch.

Deprotection and Purification of the Oligoribonucleotides. The oligoribonucleotides were base deprotected by incubation in 3 mL of aqueous concentrated ammonia/ethanol (3:1) overnight at 55 °C in a screw-cap glass vial. After removal of the solvent by Speed-Vac evaporation (rotor RH 24-18), the residue was taken up in 1 mL of 1.1 M TBAF in THF and left at 25 °C for 48 h. The RNA was then purified by Qiagen ion-exchange chromatography followed by UV shadowing as previously described (Scaringe et al., 1990). After the final purification step, a small portion of the oligonucleotide was ^{32}P-end-labeled and subjected to 20% denaturing PAGE to verify the homogeneity of the product.

Determination of Ribozyme Steady-State Parameters. Kinetic reactions were performed essentially as described by Fedor and Uhlenbeck (1990). Stock solutions of 75 nM ribozyme and 1 μM substrate were prepared in 50 mM Tris-HCl (pH 7.5), preheated separately at 95 °C for 1 min, and cooled to 25 °C for 15 min. The ribozyme stock solution was adjusted to 10 mM $MnCl_2$ or $MgCl_2$ and incubated at 25 °C for 15 min. The substrate stock solution was made 10 mM in $MnCl_2$ or $MgCl_2$ immediately prior to use. The cleavage reactions were performed by use of five different concentrations of 5′-^{32}P-labeled substrate between 50 and 750 nM. Each reaction mixture contained between 1 and 10 nM ribozyme, 50 mM Tris-HCl (pH 7.5), and 10 mM metal ion. The cleavage reactions were performed at 25 °C in a volume of 20 or 30 μL and were initiated by the addition of substrate to the solutions containing ribozyme. Aliquots were removed at appropriate time intervals between 0.5 and 13 min and quenched with an equal amount of stop mix. The cleavage reactions were followed by denaturing PAGE analysis and quantitated by use of laser densitometry techniques as described previously (Pieken et al., 1991). All kinetic parameters were determined from Eadie–Hofstee plots, and for any given ribozyme k_{cat}, K_m, and k_{cat}/K_m were found to vary approximately by a factor of 2.

Ribonuclease Reaction with the Modified and Unmodified Oligoribonucleotides. The ^{32}P-end-labeled $E_{unmodified}$ and the E-FA_{total} ribozymes were purified by 20% denaturing PAGE as previously described (Pieken et al., 1991). The end-labeled oligoribonucleotides were mixed with 50 μL of stop mix buffer containing 3.5 M urea, 0.5 mg/mL carrier tRNA, 0.02%

xylene cyanol FF, 0.04% bromophenol blue, 16 mM sodium citrate (pH 5), and 0.8 mM EDTA and heated at 55 °C for 5 min before being placed on ice. Aliquots (10 μL) of the RNA were placed into four microcentrifuge tubes and ribonuclease *Phy* M was added to the first tube to a concentration of 2000 units/mL. The enzyme was serially diluted to the fourth tube to a concentration of 10 units/mL. The tubes were incubated at 55 °C for 30 min and loaded immediately onto a 20% denaturing polyacrylamide gel and analyzed by standard autoradiography techniques. Mn^{2+}-catalyzed cleavage for sequencing was carried out by heating a solution containing 10 μM RNA and 10 mM $MnCl_2$ for 3 min at 95 °C (Pieken et al., 1991).

Results

The introduction of a fluorine atom to the 2′-position of adenosine has so far relied on fluorination using tetrabutylammonium fluoride (Ikehara et al., 1980; Ikehara & Miki, 1978; Ranganathan, 1977). The development of (diethylamino)sulfur trifluoride (DAST) as a fluorinating reagent has offered an alternative route for the synthesis of fluorinated nucleosides [Middleton, 1975; for a review see Tsuchiya (1990)]. The use of this methodology for the synthesis of 2′-deoxy-2′-fluorothymidine has already been described (Williams et al., 1991).

Auer et al. (1989) have reported in an abstract that the reaction of 9-[3′,5′-bis-*O*-(monomethoxytrityl)-β-D-arabinofuranosyl]adenine with DAST yielded 2′-fluoro-2′-deoxyadenosine in only 16% yield. The authors suggest that obtaining higher yields is improbable due to unfavorable dipolar and steric interactions. We have found that the nature of the protecting group was critical for obtaining high yields. Thus, our best results involved the reaction of N^6-5′,3′-tripixyl-9-β-D-arabinofuranosyladenine with DAST, which resulted in a 43% yield of the desired compound. The pixyl protecting group proved superior to alternative groups such as the benzoyl, acetyl, and tetraisobutyldisilyl group. As discussed by Tsuchiya et al. (1990), the reaction proceeds through at least one intermediate and close monitoring of the progress of the reaction by TLC was necessary to ascertain that it had gone to completion. After conversion of 2′-fluoro-2′-deoxyadenosine to the phosphoramidite using established procedures (Sinha et al., 1984), the 2′-fluoro-2′-deoxyadenosine analogues were incorporated into RNA oligoribonucleotides according to standard automated synthesis techniques.

Confirmation of the presence of the 2′-modified adenosine residues in the synthetic RNA products was facilitated by digestion of the ^{32}P-end-labeled RNA by ribonuclease digestion. Initially, we tried to partially digest the ribozymes using the enzyme RNase U_2, which is reported to be a purine-specific endoribonuclease (Uchida & Egami, 1971). In our hands, this enzyme cleaved primarily at Gp▼Np sites while Ap▼Np sites remained resistant to hydrolysis (data not shown). We therefore employed ribonuclease *Phy* M for the digestion reactions. This enzyme, when incubated in the presence of urea, cleaves RNA preferentially at Ap▼Np and Up▼Np sites (Kuchino & Nishimura, 1989). Figure 2 shows the result of a RNase *Phy* M digest of $E_{unmodified}$ and E-FA_{total}. A serial dilution of the RNase was used, similar to the RNase A digestion conditions originally described by Donis-Keller et al. (1977). All cleavage products were not observed with the use of only one concentration of enzyme since there are preferential cleavage sites for the enzyme. For example, the unmodified ribozyme shows greater cleavage at A_{22} than at the neighboring positions U_{20-21}. However, for $E_{unmodified}$ there was a cleavage product observed for each expected position,

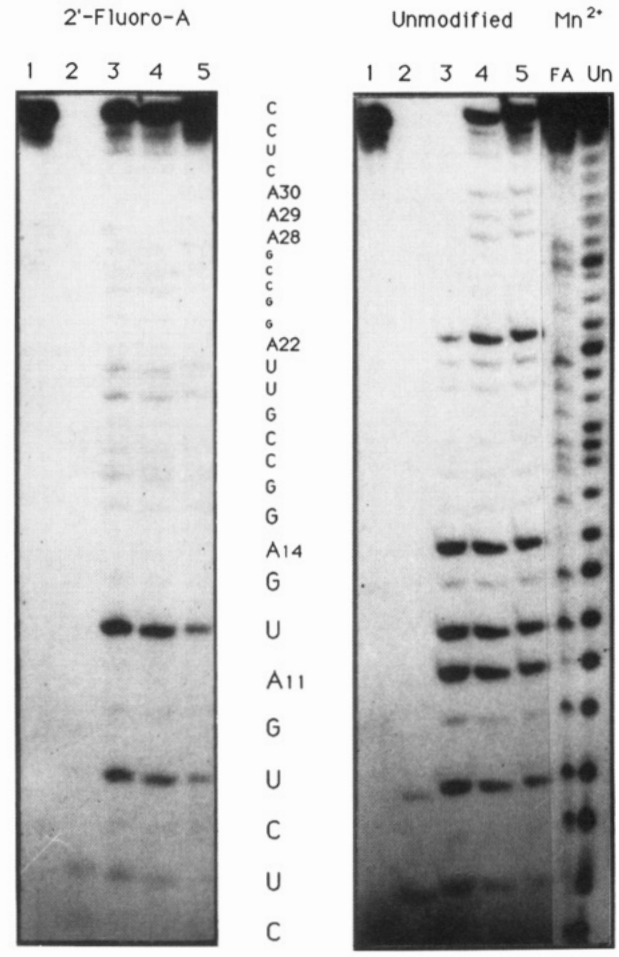

FIGURE 2: Polyacrylamide gel electrophoretic analysis of ribonuclease *Phy* M digested $E_{unmodified}$ and E-FA_{total}. Experimental details are given under Materials and Methods. In lane 1, no enzyme was added; lanes 2–5 contained 20, 3, 0.6, and 0.1 units of enzyme, respectively. Numbering of the bands was facilitated by Mn^{2+}-catalyzed hydrolysis of the modified (FA) and unmodified (Un) ribozymes. Cleavage bands are not visible for positions corresponding to 2′-fluoro-2′-deoxyadenosine because the 2′-OH group required for hydrolysis is not present.

Table I: Kinetic Parameters for Unmodified and 2′-Fluoro-2′-deoxyadenosine-Containing Hammerhead Ribozymes[a]

	ribozyme	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot min^{-1}$)
1	$E_{unmodified}$	220	41	186
		(217)	(2.8)	(13)[b]
2	E-FA_{total}	276	0.2	1[c]
		(nd)	(nd)	(nd)[b,d]
3	E-$FA_{22,28-30}$	107	0.7	7[c]
4	E-$FA_{11,14}$	187	10	53
5	E-FA_{14}	269	38	140[c]
		(321)	(4)	(12)[b]
6	E-FA_{28}	243	22	90
7	E-FA_{29}	187	16	86
8	E-FA_{30}	91	4	44

[a] The reaction conditions are as given under Materials and Methods, with Mn^{2+} as cofactor except where indicated. [b] The data given in parentheses are the kinetic parameters using Mg^{2+} as the metal cofactor. [c] Taken from Pieken et al. (1991). [d] nd indicates that no ribozyme-catalyzed cleavage product was detected.

whereas for E-FA_{total} there were no observable bands corresponding to cleavage 3′ to the positions of 2′-fluoro-2′-deoxyadenosine substitution. The Mn^{2+}-catalyzed hydrolysis of the modified and unmodified RNA also yielded a footprinting pattern consistent with the correct incorporation of the 2′-fluoro-2′-deoxyadenosine residues (Figure 2).

Table II: Kinetic Parameters for Unmodified and 2′-Deoxyadenosine-Containing Hammerhead Ribozymes[a]

	ribozyme	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})
1	E-dA$_{total}$	157	0.6	4
2	E-dA$_{11,14}$	133	8	57
3	E-dA$_{14}$	264	21	79
4	E-dA$_{28}$	168	14	86
5	E-dA$_{29}$	179	20	114
6	E-dA$_{30}$	58	5	89

[a] The reaction conditions are as given under Materials and Methods, with Mn^{2+} as cofactor.

Table III: Comparison of the Kinetic Parameters for the Hammerhead Ribozyme-E2 with an Unmodified or a 2′-Fluoro-2′-deoxyadenosine-Containing Stem II Loop[a]

	ribozyme	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})
1	E2$_{unmodified}$	178	22	120
2	E2-FA$_{20-22}$	184	25	136

[a] The reaction conditions are as given under Materials and Methods, with Mn^{2+} as cofactor.

Tables I and II summarize the results of the kinetic characterization of the ribozymes seen in Figure 1. The unmodified ribozyme and the 2′-deoxy- and the 2′-fluoro-2′-deoxyadenosine-containing ribozymes exhibited insignificant variations in K_m values. In contrast, k_{cat} was found to differ considerably depending upon the nubmer and location of 2′-substituted analogues incorporated into the RNA.

From our previous study (Pieken et al., 1991), we knew that the fully 2′-fluoro-2′-deoxyadenosine-substituted hammerhead ribozyme (E-FA$_{total}$) exhibited a drastic decrease in catalytic efficiency relative to its unmodified counterpart (Table I). The ribozyme was therefore divided into two regions in order to localize the site(s) of perturbation caused by the incorporation of the 2′-modified residues. 2′-Fluoro analogues were either substituted into the single-stranded region between stems I and II (E-FA$_{11,14}$) or between stems II and III (E-FA$_{22,28-30}$) of the RNA. The catalytic parameters determined for these two ribozymes revealed that substitution of the A$_{22,28-30}$ adenosine residues resulted in the most significant reduction in catalytic efficiency (nearly 25-fold). The presence of 2′-fluoro-modified groups at positions A$_{11,14}$ caused a 3.5 reduction of catalytic efficiency. This effect stemmed from a decrease in k_{cat} while K_m remained unaltered (Table I).

Site-specific incorporation of single 2′-fluoro-2′-deoxyadenosine groups into positions A$_{28-30}$ revealed that the incorporation of a modified residue at position A$_{28}$ or A$_{29}$ decreased catalytic efficiency by a factor of 2, which was not more significant than our experimental error. Substitution at A$_{30}$ decreased catalytic efficiency by approximately a factor of 4.

Ribozyme-E2 (Figure 1), which had the two uracil nucleosides within the loop of stem II replaced by adenosine residues, was found to have kinetic parameters very similar to those of E$_{unmodified}$ (Tables I and III). Ribozyme E2-FA$_{20-22}$, with the three adenosine residues in this loop substituted with 2′-fluoro-2′-deoxyadenosine groups, did not exhibit any decrease in catalytic efficiency relative to its unmodified counterpart (Table III).

The kinetic characterization of single 2′-deoxyadenosine-substituted hammerhead ribozymes (Table II) revealed an interesting parallel to those for the 2′-fluoro-2′-deoxyadenosine substituted counterparts. The fully substituted, E-dA$_{total}$, and partially substituted ribozymes E-dA$_{11,14}$, E-dA$_{14}$, E-dA$_{28}$, E-dA$_{29}$, and E-dA$_{30}$ do not show significant variation in catalytic efficiency relative to the values obtained for the 2′-fluoro-2′-deoxyadenosine-containing ribozymes given in Table I.

Discussion

It has been shown previously that the hammerhead ribozyme can be divided into a 34-nucleotide catalytic domain and a 12-nucleotide RNA substrate domain (Fedor & Uhlenbeck, 1990). Extensive research has revealed that alteration of any of the conserved core nucleotides of the hammerhead, prepared by runoff transcription, can cause a drastic decrease in self-splicing activity (Koizumi et al., 1989; Sheldon & Symons, 1989; Ruffner et al., 1990). This indicates the essential role of the bases of these nucleosides for the proper formation of the tertiary structure. An additional study of the nonconserved nucleotides of the hammerhead (Fedor & Uhlenbeck, 1990) revealed that changes in the sequence yielded only moderate changes in catalytic activity. For these RNAs, K_m values were found to fluctuate approximately 60-fold while k_{cat} remained largely unchanged.

With respect to sugar ring conformation, the substitution of 2′-deoxy-2′-fluoronucleosides for unmodified ribonucleosides is a very conservative exchange because these compounds have similar sugar ring conformations (Tsuchiya, 1990). Mei et al. (1989) have indicated that at least one or more 2′-OH groups might be involved in the formation of the correct tertiary structure of the hammerhead. Since the ribozyme is very sensitive to minor changes in its primary structure, as mentioned above, it seemed reasonable that the replacement of adenosine residues with 2′-deoxy-2′-fluoroadenosine or the less conservative 2′-deoxyadenosine residues might lead to the identification of single sites involved in essential tertiary structure interactions within the RNA. We have prepared the hammerhead ribozyme catalytic domain (E$_{unmodified}$) with the highest catalytic efficiency (k_{cat}/K_m) characterized by Fedor and Uhlenbeck (1990) by automated RNA synthesis techniques. This ribozyme sequence was used to study the effect of the incorporation of 2′-modified nucleotides on catalysis.

Automated synthesis of RNAs yielded large amounts of material that were easily purified from byproducts by use of standard UV shadowing techniques. Automated RNA synthesis also allowed the facile preparation of the fully and partially substituted 2′-deoxy- or 2′-fluoro-2′-deoxyadenosine-containing ribozymes in this study. The results from partial digestion of 5′-^{32}P-labeled synthetic RNA (Figure 2) revealed that the ribozymes had the expected length and sequence and that the 2′-modified adenosine residues had been incorporated at the expected position.

The kinetic characterization of the ribozymes presented in this paper was carried out with use of Mn^{2+} as the required metal ion cofactor. To date, the role of the metal ion for ribozyme cleavage remains uncertain (Heus & Pardi, 1991). Several research groups have indicated that one possible function is the stabilization of the pentacoordinated phosphate in the transition state by coordination with the pro-R oxygen atom of the phosphorus that undergoes hydrolysis (Slim & Gait, 1991; Mei et al., 1989). It remains unclear why Uhlenbeck (1987) observed a 10-fold decrease in $t_{1/2}$ for hammerhead-assisted RNA cleavage when Mg^{2+} was replaced with Mn^{2+}. We have also observed an increase in the rate of cleavage by an order of magnitude after substituting Mn^{2+} for Mg^{2+} in the cleavage reaction of several hammerheads (Pieken et al., 1991) (Table I). This increase in rate was found to be due to an increase in k_{cat} while K_m remained largely unaffected. Since some of the 2′-substituted hammerhead

ribozymes exhibited very slow turnovers, we decided to characterize each catalytic RNA using Mn^{2+} as the metal cofactor.

Substitution of several 2′-fluoro-2′-deoxyadenosine residues into distinct regions of the RNA revealed that the most critical region, in terms of disruption of catalytic efficiency, was the conserved single-stranded region between stem II and stem III (Table I). The decrease in k_{cat} observed fro E-$FA_{22,28-30}$ appeared to be associated with one or more of the adenosine nucleosides at positions A_{28-30}. The kinetic characterization of three ribozymes containing single 2′-fluoro-2′-deoxyadenosine-modified substitutions (E-FA_{28}, E-FA_{29}, E-FA_{30}) revealed that modified groups at positions A_{28} or A_{29} do not perturb catalysis within experimental error. This result is in contrast to the large reduction in self-cleavage observed for a hammerhead ribozyme containing an Rp-phosphorothioate group substituted at these same positions (Ruffner & Uhlenbeck, 1990). The cause for the small decrease in activity associated with substitution at A_{30} is unknown.

It is known that 2′-fluoro-substituted nucleosides have the ability to form hydrogen-bound water bridges (Kakiuchi et al., 1982; Janik et al., 1972) similar to those found in unmodified tRNAs or polyribonucleotides (Bolton & Kearns, 1978). On the other hand, Perreault et al. (1990) have shown that a partially 2′-deoxynucleotide-containing hammerhead ribozyme shows reduced catalytic function compared to its all-ribo counterpart. To test the possibility of H bonding to the 2′-position, we also prepared and characterized hammerhead ribozymes containing 2′-deoxyadenosine residues at specific positions. The results (Table II) for these modified ribozymes have similar catalytic efficiencies compared to those of their 2′-fluoro-2′-deoxyadenosine-containing counterparts. This suggests that no single 2′-OH group of the adenosine residues tested is essential for activity.

It is interesting that the incorporation of a 2′-deoxy- or a 2′-fluoro-2′-deoxyadenosine residue at positions $A_{11,14}$ (Tables I and III) did only cause a nearly 3-fold reduction in catalytic efficiency. Nucleoside A_{11} has been implicated in the binding of the metal ion required for catalysis, albeit via an N^3 base interaction (Mei et al., 1989). In addition, introduction of a phosphorothioate at position A_{14} has also resulted in a large decrease in catalytic activity (Ruffner & Uhlenbeck, 1990; Buzayan, 1990). During the course of this investigation, Perreault et al. (1991) reported that a related hammerhead ribozyme carrying a 2′-deoxyadenosine at position A_{14} showed 20-fold-reduced catalytic efficiency compared to that of the unmodified counterpart in the presence of Mg^{2+}. In our system, neither a ribozyme modified with 2′-fluoroadenosine at A_{14} in the presence of Mg^{2+} or Mn^{2+} nor a species carrying a 2′-deoxy modification at A_{14} in the presence of Mn^{2+} shows reduced catalytic efficiency compared to that of $E_{unmodified}$. Our results indicate that the 2′-OH groups at positions 11 and 14 do not appear to be directly involved in either catalysis or the formation of essential hydrogen bonds involved in folding.

The influence on catalysis of modified groups present in the loop of stem II was tested by comparison of the kinetic parameters of E2 (Figure 1) and E2-FA_{20-22} (Table III), which carry adenosines at positions 20–22. Interestingly, ribozyme E2-FA_{20-22} is as active as E2 even though it has been reported that nucleotides in small RNA loops prefer an C-2′-endo sugar conformation (Tinoco et al., 1990). However, NMR spectroscopy, circular dichroism, and X-ray crystallographic data indicate that a C-3′-endo puckering for the 2′-fluoro-2′-deoxyadenosine is preferred due to the presence of the highly electronegative fluorine atom (Kakiuchi et al., 1982; Morishita et al., 1984, 1990; Uesugi et al., 1979). It is unknown what conformation the 2′-modified sugars have acquired in the loop; however, it is obvious that the presence of three 2′-fluoro-2′-deoxyadenosine residues does not effect the catalytic activity of the hammerhead ribozyme.

The results reported here indicate that the extremely low catalytic activity of E-FA_{total} and E-dA_{total} must be the result of an accumulative effect of the 2′-modification, an interpretation that is supported by the decreasing activity of the ribozymes with increasing numbers of modified positions. A comparison with the results obtained with the 2′-fluoropyrimidine nucleotide containing ribozymes (Pieken et al., 1991) shows that these modifications, even at total substitution, interfere much less with ribozyme activity than does the same modification at the adenosines reported here. This might be a consequence of the larger number of adenosines present at the invariant positions and of the fact that, in the sequence of the hammerhead investigated by us, the pyrimidines are largely concentrated in the two stem regions that form upon annealing with the substrate.

In summary, we have prepared the phosphoramidite of 2′-fluoro-2′-deoxyadenosine. A catalytically active hammerhead ribozyme of 34 nucleotides in length containing 2′-deoxy- or 2′-fluoro-containing modified adenosines was accomplished by use of automated synthesis techniques. Kinetic analysis of the ribozymes having every adenosine replaced by either of these two modified groups resulted in a dramatic decrease in catalysis. Further analysis of partially substituted hammerheads indicated that there is no single position responsible for the decrease in activity. Unlike the bases of the conserved central core region, the 2′-OH group of the adenosines is not essential for catalysis or required for the proper formation of the hammerhead tertiary structure.

ACKNOWLEDGMENTS

We express our gratitude to Annette Fahrenholz and Ursula Kutzke for the expert technical assistance and thank the Deutsche Forschungsgemeinschaft for support (Ec28-14/1).

REFERENCES

Auer, M., Rosch, P., Sczakiel, G., & Goody, R. S. (1989) *Nucleosides & Nucleotides 8*, 1041–1042.

Bolton, P. H., & Kearns, D. R. (1978) *Biochim. Biophys. Acta 517*, 329–337.

Bruening, G. (1989) in *RNA Processing* (Dahlberg, J. E., & Abelson, J. N., Eds.) pp 546–558, Academic Press, Inc., San Diego.

Buzayan, J. M., Feldstein, P. A., Segrelles, C., & Bruening, G. (1988) *Nucleic Acids Res. 16*, 4009–4023.

Buzayan, J. M., Tol, H. v., Feldstein, P. A., & Bruening, G. (1990) *Nucleic Acids Res. 18*, 4447–4451.

Cotten, M. (1990) *Trends Biotechnol. 8*, 174–178.

Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.

Fedor, M. J., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1668–1672.

Hakimelahi, G. H., Proba, Z. A., & Ogilvie, K. K. (1982) *Can. J. Chem. 60*, 1106–1113.

Hansske, F., Madej, D., & Robins, M. J. (1984) *Tetrahedron 40*, 125–135.

Haseloff, J., & Gerlach, W. L. (1988) *Nature 334*, 585–591.

Heus, H. A., & Pardi, A. (1991) *J. Mol. Biol. 217*, 113–124.

Ikehara, M., & Miki, H. (1978) *Chem. Pharm. Bull. 26*, 2449–2453.

Ikehara, M., Hasegawa, A., & Imura, J. (1980) *J. Carbohydr., Nucleosides, Nucleotides 7*, 131–140.

Janik, B., Kotik, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., & Wilson, D. P. (1972) *Biochem. Biophys. Res. Commun. 46*, 1153-1160.
Jeffries, A. C., & Symons, R. H. (1989) *Nucleic Acids Res. 17*, 1371-1377.
Kakiuchi, N., Marek, C., Rousseau, N., Leng, M., De Clercq, E., & Guschlbauer, W. (1982) *J. Biol. Chem. 257*, 1924-1928.
Koizumi, M., Iwai, S., & Ohtsuka, E. (1988) *FEBS Lett. 239*, 285-288.
Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H., & Ohtsuka, E. (1989) *Nucleic Acids Res. 17*, 7059-7071.
Kuchino, Y., & Nishimura, S. (1989) *Methods Enzymol. 180*, 154-163.
Mei, H.-Y., Kaaret, T. W., & Bruice, T. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9727-9731.
Middleton, W. J. (1975) *J. Org. Chem. 40*, 574-578.
Morishita, K., Hakoshima, T., Fujiwara, T., Tomita, K., Kaneyasu, T., Uesugi, S., & Ikehara, M. (1984) *Acta Crystallogr., Sect. C 40*, 434-436.
Perreault, J.-P., Wu, T., Cousineau, B., Ogilvie, K. K., & Cedergren, R. (1990) *Nature 344*, 565-567.
Perreault, J.-P., Labuda, D., Usman, N., Yang, J.-H., & Cedergren, R. (1991) *Biochemistry 30*, 4020-4025.
Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H., & Eckstein, F. (1991) *Science 253*, 314-317.
Ranganathan, R. (1977) *Tetrahedron Lett. 15*, 1291-1294.
Rossi, J. J. & Sarver, N. (1990) *Trends Biotechnol. 8*, 179-183.
Ruffner, D. E., & Uhlenbeck, O. C. (1990) *Nucleic Acids Res. 18*, 6025.
Ruffner, D. E., Stromo, G. D., & Uhlenbeck, O. C. (1990) *Biochemistry 29*, 10695-10702.
Saenger, W., Ed. (1984) in *Principles of Nucleic Acid Structure*, Springer Verlag, New York, Berlin, Heidelberg, and Tokyo.
Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, R. A., Stephens, D. A., & Rossi, J. J. (1990) *Science 247*, 1222-1224.
Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res. 18*, 5433-5441.
Sheldon, C. C., & Symons, R. H. (1989) *Nucleic Acids Res. 17*, 5679-5685.
Sheldon, C. C., Jeffries, A. C., Davies, C., & Symons, R. H. (1990) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) pp 227-242, Springer-Verlag, Berlin, Heidelberg.
Sinha, N. D., Biernat, J., & Köster, H. (1984) *Nucleic Acids Res. 12*, 4539-4557.
Slim, G., & Gait, M. J. (1991) *Nucleic Acids Res. 19*, 1183-1188.
Tinoco, I., Puglisi, J. D., & Wyatt, J. R. (1990) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) pp 204-226, Springer-Verlag, Berlin, Heidelberg.
Tsuchiya, T. (1990) in *Advances in Carbohydrate Chemistry and Biochemistry* (Tipson, R. S., & Horton, D., Eds.) pp 91-277, Academic Press, San Diego.
Uchida, T., & Egami, F. (1971) in *Hydrolysis* (Boyer, P. D., Ed.) pp 205-250, Academic Press, New York and London.
Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., & Kyogoku, Y. (1979) *Tetrahedron Lett. 42*, 4073-4076.
Uhlenbeck, O. C. (1987) *Nature 328*, 596-600.
Williams, D. M., Benseler, F., & Eckstein, F. (1991) *Biochemistry 30*, 4001-4009.

DNA Topoisomerase I Cleavage Sites in DNA and in Nucleoprotein Complexes†

Arndt Richter* and Jürgen Ruff
Division of Biology, University of Konstanz, D-7550 Konstanz, FRG
Received April 22, 1991; Revised Manuscript Received July 15, 1991

ABSTRACT: The intracellular substrate for eukaryotic DNA topoisomerases is chromatin rather than protein-free DNA. Yet, little is known about the action of topoisomerases on chromatin-associated DNA. We have analyzed to what extent the organization of DNA in chromatin influences the accessibility of DNA molecules for topoisomerase I cleavage in vitro. Using potassium dodecyl sulfate precipitation (Trask et al., 1984), we found that DNA in chromatin is cleaved by the enzyme with somewhat reduced efficiency compared to protein-free DNA. Furthermore, using native SV40 chromatin and mononucleosomes assembled in vitro, we show that DNA bound to histone octamer complexes is cleaved by topoisomerase I and that the cleavage sites as well as their overall distribution are identical in histone-bound and in protein-free DNA molecules.

Transcription and replication of cellular DNA require the separation of complementary strands of the DNA double helix. This unwinding in topologically fixed molecules causes a coiling of the helix axis behind and/or in front of the advancing transcription complex or replication fork. These topological constraints may inhibit both processes and are relaxed by topoisomerases. It is widely accepted that topoisomerase I is one of the major cellular enzymes performing this function in transcription and replication [summarized in Wang (1987), Liu (1989), Richter and Knippers (1989), Hsieh (1990), and Austin and Fisher (1990)].

The catalytic activity of eukaryotic topoisomerase I has been extensively characterized (Vosberg, 1985; Wang, 1985). The enzyme initiates the reaction cycle by binding to the substrate DNA followed by the introduction of a nick into one strand

† This work was supported by the Deutsche Forschungsgemeinschaft through Grant SFB 156.

0006-2960/91/0430-9741$02.50/0