Deoxyribonucleic Acid Modification Methylase from *Bacillus stearothermophilus*†

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ABSTRACT: A modification methylase was isolated from *Bacillus stearothermophilus* 1503-4R (*Bst*1503I) and purified to homogeneity. The enzyme is an acidic protein and composed of a subunit with a molecular weight of 105 000, and only the tetrameric form was detected in solution. The methylase exhibited maximal activity between 54 and 61 °C and between pH 8.1 and 9.3. In contrast to *Bst*1503I endonuclease [Catterall, J. F., & Welker, N. E. (1977) *J. Bacteriol. 129*, 1110–1120], the methylase is completely inactivated when exposed to temperatures near the optimal growth temperature (63–67 °C). The methylase was also inactivated when exposed to temperatures below the minimal growth temperature (48–53 °C). The thermostability of the methylase is significantly enhanced by Na^+, K^+, or NH_4^+. Membrane-bound methylase is resistant to heat inactivation at temperatures near the maximum growth temperature (73–75 °C). The methylase functions as a tetramer. The initial rates of methyl transfer are first order in methylase concentration, and the enzyme obeys Michaelis–Menten kinetics with respect to DNA but not to *S*-adenosyl-L-methionine.

The *Bst*1503I[1] methylase is part of the restriction and modification system in *Bacillus stearothermophilus* 1503-4R (Catterall et al., 1976).

The *Bst*1503I restriction enzyme appears to recognize the hexanucleotide sequence d(pG-G-A-T-C-C), with cleavage at the G–G bond leaving single-stranded 5′-phosphoryl termini (Catterall & Welker, 1977, 1980).

Although a relatively large number of type II endonucleases have been reported, there is little or no direct genetic or biochemical evidence for the presence of a restriction and modification system. The *Eco*RI restriction and modification system has been the subject of extensive study (Modrich & Zabel, 1976; Greene et al., 1975; Rubin & Modrich, 1977), and, other than this one system, there have been no reports on the physical and catalytic properties of type II modification methylases.

In this paper, we describe the purification to homogeneity of the modification methylase *Bst*1503I from the obligate thermophile *B. stearothermophilus* 1503-4R, as well as some of its properties. A preliminary report of this work has been published (Levy & Welker, 1978).

Experimental Procedures

Bacterial Strains, Phage, and Growth Medium. Bacillus stearothermophilus strains 1503-4R R^+M^+ (Catterall & Welker, 1977) and $4SR^-_{22}M^-$ (Catterall et al., 1976) were grown in 2% trypticase peptone (BBL), 0.5% yeast extract, and 0.5% glucose containing $FeCl_3 \cdot 6H_2O$ (7 mg/L), $MnCl_2 \cdot 4H_2O$ (1 mg/L), and $MgSO_4 \cdot 7H_2O$ (15 mg/L). *Bacillus amyloliquefaciens* H (RUB 500) was obtained from Dr. G. Wilson.

The growth and plaque assays of thermophilic bacteriophage TP-1C were described by Welker & Campbell (1965). Phages propagated on strain $4SR^-_{22}M^-$ contained unmethylated, linear duplex DNA having a molecular weight of 12×10^6 (Catterall et al., 1976; J. F. Catterall, unpublished experiments; Welker & Campbell, 1965).

Isolation of Phage Tp-1C DNA. Phages were collected by a modification of the method of Yamamoto et al. (1970). Lysates were incubated at 37 °C with 1 μg mL ribonuclease and 1 μg/mL deoxyribonuclease for 1 h. Solid NaCl was added to 0.8 M, and the cell debris was removed by centrifugation. Poly(ethylene glycol) (40% w/v; average molecular weight 6000–7500; Matheson, Coleman and Bell) was added to a final concentration of 15%, and the mixture was incubated at 4 °C for 1 h. The precipitate was collected by centrifugation at 8000*g* for 15 min, and the pellet was suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl and 1 mM $CaCl_2$ (TNC buffer). The recovery of bacteriophage was between 90 and 95%.

DNA was isolated and purified following the procedure of Streips & Welker (1969) as modified by Catterall & Welker (1977). Purified DNA was stored in 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0).

Enzymes and Proteins. Restriction endonuclease *Bst*1503I was provided by J. F. Catterall (Catterall & Welker, 1977). DNA methylase activity was prepared from cells of RUB 500 grown in antibiotic medium 3 (Difco) at 37 °C by using the procedure described for *Bst*1503I modification methylase. The DNA methylase preparations (pooled fractions from the first Bio-Gel fractionation) were judged to be essentially free of *Bam*HI endonuclease activity and nonspecific nucleases.

Human cold-insoluble globulin was obtained from Dr. L. Lorand. Beef liver catalase, rabbit muscle lactic dehydrogenase, and ovalbumin were purchased from Sigma Chemical Co., bovine serum albumin and hog stomach pepsin were from Mann Research Laboratories, and β-galactosidase was from Worthington Biochemical Corp. Restriction endonuclease *Bam*HI was a generous gift from Bethesda Research Laboratories.

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[1] This nomenclature follows the suggestions of Smith & Nathans (1973). Roberts (1978) has designated *Bst*1503 restriction endonuclease (Catterall & Welker, 1977) as *Bst*I. In the interest of maintaining a uniform nomenclature, we use *Bst*1503I to describe the restriction endonuclease or modification methylase present in *B. stearothermophilus* 1503-4R.

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Chemicals. AdoMet[2] stocks were stored in 0.2 N H_2SO_4 at −15 °C. $NaDodSO_4$ (99%) was purchased from Gallard-Schlesinger.

Modification Methylase Assay. Modification methylase activity was determined by measuring the extent of protection of DNA from cleavage by *Bst*1503I restriction endonuclease. Methylase reaction mixtures contained 35 μL of 83 mM Tris base (pH 8.7), 17 mM EDTA, 33 mM 2-mercaptoethanol, 5 μL of 60 mM AdoMet, 10 μL of TP-1C DNA (3.7 μg), and 10 μL of enzyme (final pH 8.7) in a capped, 0.5-mL microcentrifuge tube. The reaction mixture was incubated at 55 °C.

Parks & Schlenk (1958) reported that relatively little destruction of AdoMet occurs in 50 mM phosphate buffer (pH 8.8) at 30 °C (less than 1% in 1 h). We examined the inactivation of AdoMet (unable to serve as a methyl donor in the methylase reaction) in methylase assay buffer (pH 8.7) over a 30-min period, and we found no inactivation at 55 and 60 °C, 5% inactiation at 65 °C, and 15% inactivation at 70 °C.

The restriction step of the assay was initiated by the addition of 60 μL of 10 mM Tris-HCl (pH 7.8), 13 mM $MgCl_2$, and 6.6 mM 2-mercaptoethanol and 10 μL of *Bst*1503I restriction endonuclease (1 nM in 20 mM potassium phosphate, pH 6.8, containing 0.2 mM EDTA, 0.5 mM dithiothreitol, 300 mM KCl, and 10% glycerol) to the methylation reaction mixture (final pH 8.3). The mixture was incubated at 55 °C. The reaction was stopped by the addition of 10 μL of 0.1 M EDTA containing 37% sucrose and 0.125% bromophenol blue.

When *Bam*HI restriction endonuclease or DNA methylase preparations were used, identical conditions were employed, except incubations were run at 37 °C.

The time of incubation was varied to ensure that all unmodified DNA was restricted, and appropriate controls were run to verify that the various experimental conditions did not inhibit restriction endonuclease activity.

Aliquots of 100 μL of each sample (2.6 μg of DNA) were analyzed by agarose gel electrophoresis (Sharp et al., 1973). Agarose gels (1%) were prepared in 40 mM Tris base, 5 mM sodium acetate (pH 7.9), 1 mM EDTA, and 1 μg/mL ethidium bromide. Samples were electrophoresed at 6 mA/gel (16 °C) until the marking dye reached the bottom of the gel (6.5 cm). Gels were photographed under ultraviolet illumination (Ultra Violet Products Model C-61 transilluminator) with Polaroid type 47 film with a Tiffen series 6, number 15G orange filter or were examined for the intensity of fluorescence.

A Gilford Model 2000 recording spectrophotometer equipped with a model 2410 linear transport was used to scan agarose gels.

The linear transport was adapted to scan gels for fluorescence by using a convenient modification of the apparatus described by Oliver & McLaughlin (1977). A black plastic tube was placed into the opening of the side plate of the transport chamber adjacent to the photodetector. An aperture plate (0.2 mm wide and 2.3 mm high; Gilford Instrument) was cut to fit inside the plastic tube. A Geletran Du Pont Mylar filter (medium Lemon no. 40; Berkey Photo Inc., Burbank, CA) was placed immediately behind the aperture plate (facing the photodetector) and secured with a thin section of black rubber tubing. The aperture plate was positioned in the plastic tube so that the slit was perpendicular to the plane of movement of the sample transport and flush with the inside wall of the chamber. A wooden cuvette holder was mounted on the sample transport by means of the indexing pins. The cuvette was held in position at least 2 mm in front of the aperture plate. A wooden holder for a portable short-wave ultraviolet lamp (Mineralight UVS-12, Ultra Violet Products, Inc., San Gabriel, CA) was made to fit into the top of the scanning chamber. Strips of black felt cloth were added to the lamp holder to eliminate extraneous light. The cuvette holder and the lamp holder were stained black.

Fluorescent scanning of the gels was accomplished by using the procedure of Oliver & McLaughlin (1977). Gels were scanned at a rate of 2 cm/min with a chart speed of 2.5 cm/min.

The area under the tracing fragments 5, 6, and 7 (see Figure 1) of TP-1C DNA digested with the corresponding restriction endonuclease allows a quantitative estimate of modification methylase activity. The area under the peaks was determined by cutting them out and weighing the paper. Alternatively, multiple copies of the tracings were made on a commercial copying machine to reduce weighing errors.

The intensity of fluorescence (area) is proportional to the amount of undigested phage TP-1C in the agarose gel between 0.05 and 1.1 μg.

A unit of *Bst*1503I modification methylase activity is defined as that amount of enzyme required to produce a 1% decrease in the amount of fragments 5, 6, and 7 after digestion with *Bst*1503I restriction endonuclease for 1 min at 55 °C. Units of enzyme activity were determined by measuring the initial velocity of the protective methylation reaction. One unit of methylase activity is equivalent to 0.12 pmol of methyl groups added to TP-1C DNA under the standard conditions.

Polyacrylamide Gel Electrophoresis. The homogeneity of the methylase was established by nondenaturing polyacrylamide gel electrophoresis by using the method of Davis (1962) as described by Maurer (1971; gel system 1) except that a spacer gel was not used. The separation gel contained buffer of pH 8.9 and 7.5% acrylamide monomer (medium pore). Samples (50–150 μL) in 20% glycerol were layered on top of each gel, and electrophoresis was performed at 4 mA/gel in 5 mM Tris-HCl and 38 mM glycine, pH 8.3 (4 °C). The upper buffer (cathode) contained 0.02 mg/L bromophenol blue. Electrophoresis was terminated after the marking dye migrated approximately three-fourths of the length of the gel (50 mm). Gels were stained in 1.6 M acetic acid in 50% methanol containing 0.25 g/L Coomassie brilliant blue R-250 for 3 h at room temperature.

Gels were destained by immersion in acetic acid–methanol–water (1.5:1.0:17.5) to which was added a swatch of nylon "panty hose". Nylon binds Coomassie blue more efficiently than most commercially available destaining materials and reduces the destaining time by 50% (W. P. Levy, unpublished experiments).

The aggregate molecular weight of the modificiation methylase was determined by using a variation of the procedure of Ferguson (1964). Polyacrylamide gel electrophoresis of protein standards and the methylase was performed as described above with the following exceptions. Aliquots of 2–7 μg of protein in 40 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 5 mM 2-mercaptoethanol (TEM buffer) containing 10% sucrose were electrophoresed through individual gels containing 5, 7.5, or 10% acrylamide monomer. Gels were cut at the position of the marking dye, stained, and destained. The mobility of each protein was determined, and data were analyzed according to the method of Hendrick & Smith (1968).

[2] Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; Ado-[*methyl*-^{3}H]Met, *S*-adenosyl-L-methionine with the methyl group of methionine labeled with ^{3}H; $NaDodSO_4$, sodium dodecyl sulfate.

$NaDodSO_4$–polyacrylamide gel electrophoresis was used to determine the subunit composition of the modification methylase (Weber & Osborn, 1969). Samples (3–10 μg of protein in 10 mM sodium phosphate, pH 7.0, 0.1% $NaDodSO_4$, and 0.1% 2-mercaptoethanol) were incubated at 37 °C for 4 h. Glycerol and bromophenol blue were added to a final concentration of 20% and 0.02%, respectively. Electrophoresis was performed according to Weber & Osborn (1969), except that polymerized gels contained 6% acrylamide monomer.

The recovery of modification activity from polyacrylamide gels was used to identify the protein-staining band as the methylase. Polyacrylamide gel electrophoresis was performed by using the procedure of Williams & Resifield (1964) as described by Maurer (1971; gel system 6) except that the spacer gel was omitted. The separation gel contained buffer of pH 7.5 and 7.5% acrylamide monomer (medium pore). The electrophoresis buffer was 8.3 mM Tris-HCl and 30 mM diethylbarbituric acid (pH 7.0). Gels were cleared of contaminating material by electrophoresis at 4 mA/gel for 2 h at 4 °C.

Duplicate gels were prepared as follows. Samples of methylase (0.4 μg) containing 6% sucrose and 0.005% bromophenol blue were layered on the gel and subjected to electrophoresis at 4 mA/gel at 4 °C until the marking dye reached the bottom of the gel (60 mm).

One gel was cut at the position of the marker dye, stained, and destained. A duplicate gel was sliced into 2-mm segments. Each segment was placed in a test tube containing 75 μL of TEM buffer, macerated, and held at 4 °C for 22 h, and assayed for modification methylase activity.

Absorption profiles of stained polyacrylamide gels at 550 nm were obtained by using a Gilford Model 2000 spectrophotometer equipped with a Gilford Model 2410 linear transport.

Isoelectric Focusing in Polyacrylamide Gels. A modification of the procedure described by Wrigley (1969) was used to determine the isoelectric point of the enzyme. Gels contained 7.5 g of acrylamide monomer and 5 mL of Ampholine (40%; pH 3.5–10; LKB Produkter) per 100 mL of solution. Samples of the enzyme (7 μg) in 25% glycerol were layered under a protective solution of 2.5% Ampholine in 10% glycerol. The upper chamber contained 1 mM HCl (pH 3; anode), and the lower chamber contained 1 mM NaOH (pH 10; cathode). Electrophoresis was carried out for 1 h at 16 °C at a constant voltage of 100 V followed by 3 h at 200 V.

Duplicate gels were treated as follows. One gel was sliced into 2-mm segments, and each segment was placed into a test tube containing 1 mL of distilled water. The segments were macerated and held at 25 °C for 15 h. Samples from each tube were assayed for modification activity, and the pH of the remaining solution was measured. The other gel was soaked in 20% trichloroacetic acid (Cl_3CCOOH) for 12 h to remove the ampholytes and to fix the proteins, stained in 20% Cl_3CCOOH containing 0.05% Coomassie brilliant blue for 3 h, and destained in 20% Cl_3CCOOH.

Isolation of Membranes. Membranes were prepared from cells obtained from 3 L of culture by using the procedure described by Bodman & Welker (1969). After the membranes were washed with 40 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ (TM buffer), they were suspended in 30 mL of the TM buffer and divided into three equal fractions. Membrane suspensions were stored at 5 °C.

Purification of Bst1503I Modification Methylase. Cultures of *B. stearothermophilus* strain 1503-4R were grown as described by Catterall & Welker (1977). Cells were harvested by centrifugation when the culture was in the late exponential phase of growth [(2–3) × 10^8 viable cells/mL of culture].[3] Approximately 25 g (wet weight) of cells was obtained from 12 L of culture.

(*1*) *Crude Extract.* Cells were washed in TNC buffer at 4 °C and suspended in 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.5 mM EDTA, 50 mM NaCl, and 10% glycerol (sonication buffer; 2 mL/g wet weight of cells). A 1-mg sample of lysozyme was added per gram of cells, and the mixture was incubated at 37 °C until the cells were converted to protoplasts. The sample was equilibrated at 4 °C, and the protoplasts were disrupted by ultrasound (Sonifer, Branson Ultrasonic Corp.). Protplast rupture was monitored with a phase-contrast microscope. All subsequent steps were performed at 4 °C.

Cell debris was removed by centrifugation at 105000*g* for 1 h. Nucleic acids were precipitated from the supernatant fluids by the addition of freshly prepared 10% streptomycin sulfate (1 mL for each 1500 absorbance units at 260 nm) with stirring for 1 h followed by centrifugation at 35000*g* for 20 min.

(*2*) *Ammonium Sulfate Fractionation.* Solid ammonium sulfate (144 g/L; enzyme grade) was added over a period of 20 min with constant stirring. The mixture was stirred for an additional 30 min, and the precipitate was removed by centrifugation at 35000*g* for 20 min. Solid ammonium sulfate (267 g/L) was added to the supernatant fluids, and the precipitate was collected by centrifugation and stored at −20 °C.

The remainder of the purification procedure was developed for one-fourth of the precipitate.

The ammonium sulfate precipitate was suspended in 1.5 mL of 10 mM potassium phosphate (pH 7.1), 0.5 mM EDTA, 5 mM 2-mercaptoethanol, and 5% glycerol (PEM buffer). The sample was dialyzed twice (4 and 14 h) against 100 volumes each of PEM buffer.

(*3*) *Gel Filtration.* The sample was applied to a column (37 × 2.5 cm) of Bio-Gel A-5M, previously equilibrated with PEM buffer, and eluted with PEM buffer. The activity was eluted at a flow rate of 25 mL/h. Active fractions were pooled and concentrated by ammonium sulfate precipitation (430 g/L). The precipitate was collected by centrifugation, suspended in 2 mL of PEM buffer, applied without dialysis to a Bio-Gel A-5M column, and eluted as before. Active fractions were pooled, concentrated by ammonium sulfate precipitation, and subjected to a third cycle of Bio-Gel A-5M filtration. The methylase was collected by ammonium sulfate precipitation, suspended in 0.7 mL of PEM buffer, and dialyzed twice (4 and 14 h) against 500 volumes each of PEM buffer.

(*4*) *Phosphocellulose Chromatography.* The sample was applied to a column (6 × 0.5 cm) of phosphocellulose (Whatman P11) which was previously equilibrated with PEM buffer. Modification methylase activity passed through the column, and active fractions were pooled.

(*5*) *DEAE-cellulose Chromatography.* A column (6 × 0.5 cm) of DEAE-cellulose was equilibrated with PEM buffer, and the enzyme was applied at 50 mL/h. The column was washed extensively with PEM buffer, and the activity was eluted with PEM buffer containing 0.05 M KCl. Active fractions were pooled, adjusted to 50% glycerol, and stored at −20 °C.

Protein was determined spectrophotometrically by the method of Kalb & Bernlohr (1977). This spectrophotometric

[3] Subsequent experiments have demonstrated that higher cell densities [(6–8) × 10^9 viable cells/mL] can be obtained if fructose is used in place of glucose in the growth medium.

Table I: Purification of *Bst*1503I Modification Methylase

purification step	volume (mL)	protein (mg)	units	sp act. (units/mg)	% yield
ammonium sulfate	4.2	140	15 500	110	100
Bio-Gel I	64	96	14 700	150	95
Bio-Gel II	34	15	13 600	910	88
Bio-Gel III	15	7	13 000	1 900	84
phosphocellulose	2	6.3	12 000	1 900	77
DEAE-cellulose	8	0.07	1 400	20 000	9

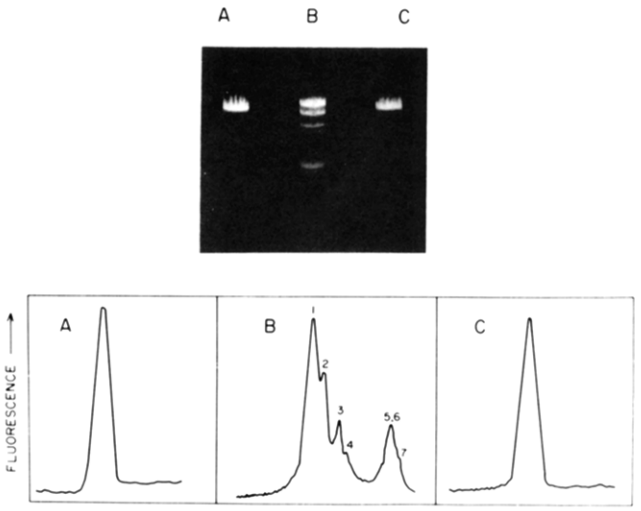

FIGURE 1: Restriction and modification of phage TP-1C DNA. Samples of TP-1C (2.7 μg of DNA) were analyzed by agarose gel electrophoresis. Agarose gels were photographed, and a fluorescence profile was measured. (A) TP-1C DNA incubated at 55 °C for 110 min; (B) TP-1C DNA incubated at 55 °C for 90 min and with *Bst*1503I endonuclease for 20 min; (C) TP-1C DNA incubated with *Bst*1503I methylase for 90 min and with *Bst*1503I endonuclease for 20 min. Fragment 1 (numbering from the top of the gel) migrates with undigested DNA, and fragments 5 and 6 comigrate in a single band. It is possible to resolve fragments 5 and 6 by electrophoresis in agarose gels 15–20 cm in length.

assay significantly decreases interference by nucleic acids.

Results

Purification of Bst15031. Table I summarizes the results of a purification procedure in which *Bst*1503I methylase was purified 180-fold with a 9% recovery. Modification methylase activity in crude extracts cannot be accurately determined because of the presence of nonspecific nucleases.

The methylase activity does not bind to DEAE-cellulose unless the preparation is first passed through phosphocellulose. It is possible that phosphocellulose removes residual DNA present in the Bio-Gel fractions which interferes with the binding of the methylene to DEAE-cellulose. Contaminating proteins were detected in some methylase preparations when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. These proteins, however, can be removed by a second DEAE-cellulose chromatography step. The recovery of methylase activity can be significantly improved by increasing the concentration of KCl in the eluting buffer. Since contaminating proteins also elute with the higher salt concentration, we elected to recovery less enzyme having a higher specific activity.

Approximately 0.3 mg of methylase was obtained from 24 g of cells in about 6 days. This purification procedure can be easily modified for use with a larger amount of cells. The enzyme can be stored in DEAE-cellulose elution buffer (PEM buffer plus 50 mM KCl) containing 50% glycerol at −20 °C for 3 weeks (50% inactivation) or containing bovine serum

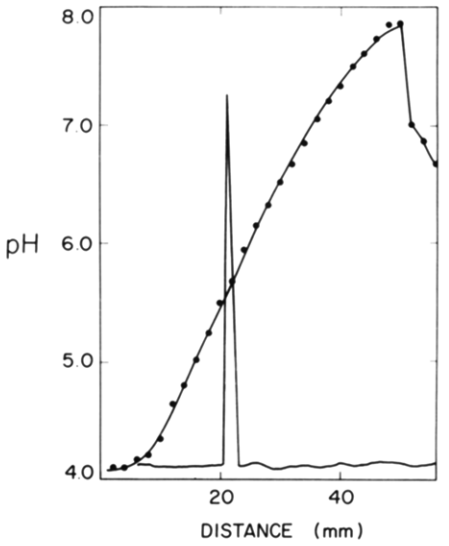

FIGURE 2: Isoelectric focusing of *Bst*1503I modification methylase. *Bst*1503I methylase (7 μg) was subjected to isoelectric focusing on polyacrylamide gels (pH range from 3.5 to 10). One gel was stained and the absorption profile at 550 nm measured (stippled area), and a duplicate gel was sliced into 2-mm segments. Each segment was extracted with distilled water and the pH gradient (●) estimated as described under Experimental Procedures.

albumin (1 mg/mL) at 4 °C for at least 2 years without significant inactivation.

*Bst*1503I endonuclease limit digests of phage TP-1C DNA contain seven fragments (Figure 1). *Bst*1503I endonuclease did not digest TP-1C DNA modified by the *Bst*1503I methylase. *Bam*HI restriction endonuclease did not digest *Bst*1503I-modified DNA. A crude preparation of DNA methylase from RUB 500 was found to protect phage TP-1C DNA from cleavage by *Bst*1503I restriction endonuclease. These results were not unexpected since the *Bst*1503I and *Bam*HI restriction endonucleases are isoschizomers (Catterall & Welker, 1977).

Homogeneity of Bst105I. Purity of methylase preparations (1–17 μg) was determined by polyacrylamide gel electrophoresis. A single protein-staining band was observed, indicating a homogeneous preparation. Duplicate gels cut into 2-mm segments showed that methylase activity coincided with this band.

The methylase was also analyzed by isoelectric focusing on polyacrylamide gels. A single protein-staining band was observed with an isoelectric point (p*I*) of about 5.5–5.7 (Figure 2). A duplicate gel cut into 2-mm segments showed that the methylase activity coincided with the band.

The *Bst*1503I methylase did not contain any detectable *Bst*1503I endonuclease activity or nonspecific nuclease activity as judged by prolonged incubation with linear φX174 DNA with an excess of methylase. The *Bst*1503I methylase was also judged free of nonspecific methylase activity since no methylation (incorporation of Ado[*methyl*-^{3}H]Met) was observed wih *Bst*1503I endonuclease digested TP-1C DNA.

Any impurity present must have the same mobility as the methylase or be present at a concentration of less than 0.35 μg (limit of resolution of the scanning procedure). Any protein contaminat would, therefore, account for less than 2% of the total protein.

Aggregate and Subunit Molecular Weight. The aggregate molecular weight of the methylase was determined by polyacrylamide gel electrophoresis. The molecular weight was found to be 425 000 by comparison of its mobility to those of proteins of known molecular weights. No other form of the

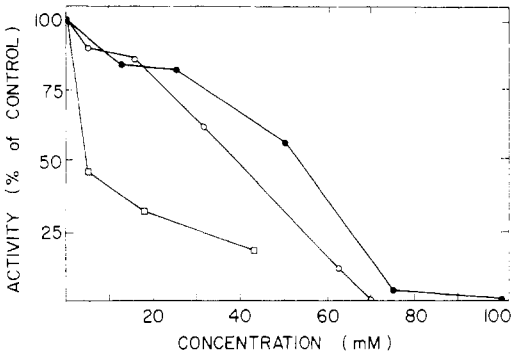

FIGURE 3: Effect of Na^+, K^+, and Mg^{2+} concentration on *Bst*1503I activity. *Bst*1503I methylase (1.2 μg) activity was measured in modification methylase assay buffer, pH 8.7, containing Mg^{2+} (□), K^+ (●), or Na^+ (○) at 55 °C. The methylase preparation contributed 8.3 mM and 0.083 mM K^+ and EDTA, respectively, to the methylase reaction mixture.

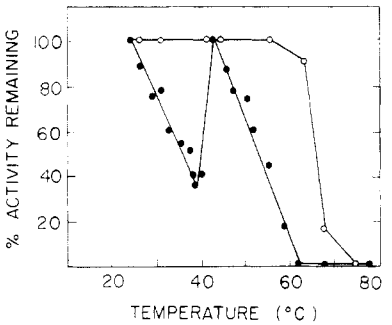

FIGURE 4: Salt-induced thermostability of *Bst*1503I. *Bst*1503I methylase (8.5 μg) in PEM buffer (25 mM KCl and 50% glycerol) containing 100 mM NaCl (○) or no added NaCl (●) was incubated at temperatures between 24 and 78 °C for 3 h. The enzyme was diluted in PEM buffer and the activity measured in methylase assay buffer at 55 °C. The 100 mM samples contributed 2.9 mM Na^+ to the methylase reaction mixture.

enzyme was observed at concentrations between 0.7 and 4.2 nM. During the purification of *Bst*1503I, the activity appeared just after the void volume on Bio-Gel A-5M. This indicates an apparent molecular weight in excess of 400 000.

A single protein-staining band was observed when the methylase was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The molecular weight of the subunit was found to be 105 000 by comparison of its mobility to those of proteins of known molecular weight.

General Properties of Bst1503I. *Bst*1503I methylase was active over a pH range of 7.3–9.9 with maximum activity between pHs 8.1 and 9.3. The methylase was sensitive to Na^+ and K^+ concentrations with a 50% inactivation observed around 40 mM Na^+ and 53 mM K^+ (Figure 3).

*Bst*1503I methylase was sensitive to Mg^{2+} concentration with a 50% inactivation observed at 5 mM.

*Bst*1503I methylase was active over a temperature range of 40–80 °C, with maximal activity between 54 and 61 °C. No activity was detected at 35 °C or below. The thermostability of *Bst*1503I methylase (8.3 μg) in PEM buffer containing 25 mM KCl and 50% glycerol was determined by incubating samples of enzyme at various temperatures. Samples were removed at intervals up to 4 h, diluted, and assayed at 55 °C (concentration of KCl in the reaction mixture was 21 mM). Enzyme incubated at 40 and 55 °C for 30 min was not inactivated. Incubation of methylase for 30 min at 61, 68, and 78 °C resulted in a 20, 58, and 65% inactivation, respectively. Greater than 95% inactivation of the methylase occurred after 3 h of incubation at 61 °C, 1.5 h at 58 °C, and 1 h at 78 °C.

Table II: Thermostability of Membrane-Bound *Bst*1503I

membrane[a] treatment	*Bst*1503I methylase activity (total units)[b]
incubation at 20 °C for 3 h followed by treatment with ultrasound	5230
treatment with ultrasound followed by incubation at 60 °C for 3 h	0
incubation at 60 °C for 3 h followed by treatment with ultrasound	5010

[a] Membranes were prepared as described under Experimental Procedures. Membrane suspensions (10 mL) were treated as shown in the first column. Prior to treatment with ultrasound, the concentration of dithiothreitol, phenylmethanesulfonyl fluoride, NaCl, and glycerol was adjusted to that found in the sonication buffer. EDTA was added to a final concentration of 50 mM. [b] *Bst*1503I methylase released by treatment with ultrasound was purified through step 2 of the purification procedure and dialyzed against PEM buffer. Methylase activity was measured as described under Experimental Procedures.

Methylase was also inactivated when incubated for 30 min at temperatures between 24 and 38 °C. An examination of a plot showing the inactivation of methylase after incubation at temperatures between 24 and 78 °C for 3 h reveals that the enzyme undergoes significant inactivation below as well as above 40 °C (Figure 4).

No inactivation of the enzyme occurs at 24 °C or below. A dramatic change in the apparent thermostability of the methylase occurs between 38 and 42 °C. Polyacrylamide gel electrophoresis of methylase incubated at 38 °C for 3 h (65% inactivation) did not reveal any protein-staining bands having a mobility different from that of native enzyme. enzyme incubated at 38 °C for 3 h could not be reactivated by incubation at 24 or 42 °C. These preliminary results indicate that the inactivation of methylase as 38 °C is irreversible. The thermostability of the methylase below and above 40 °C is dramatically enahnced by the addition of 100 mM NaCl. Identical results were obtained with KCl and NH_4Cl (data not shown).

Although purified *Bst*1503I methylase undergoes significant heat inactivation at temperatures around the optimal temperature of growth of this strain (63–65 °C), it does appear to function in cells of cultures growing at 65 or 68 °C (N. E. Welker, unpublished experiments). In vivo, the methylase must be protected from heat inactivation by cellular components. The most likely candidate for this role is the membrane. In our initial attempts to isolate *Bst*1503 methylase, we observed that the enzyme was tightly bound to the membrane. This observation is in contrast to that observed for *Bst*1503I endonuclease (N. E. Welker, unpublished experiments) where the enzyme is released upon rupture of the protoplast.

Membranes of *B. stearothermophilus* 1503-4R R^+M^+ were prepared as described under Experimental Procedures and treated as described in Table II. *Bst*1503I methylase was released by treatment with ultrasound and purified through step 2 of the purification procedure. Methylase activity was measured in each sample, and the amount of enzyme activity recovered was calculated. In the membrane, *Bst*1503I methylase was protected from heat inactivation at 60 °C whereas methylase activity in crude preparations was inactivated. Similar results were observed when membranes were held at 65 or 70 °C.

It is noteworthy that other cellular proteins present in these crude preparations did not enhance the thermostability of the

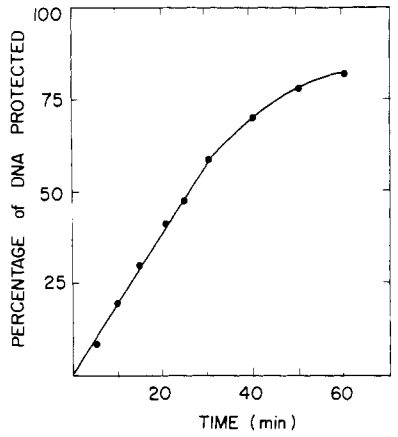

FIGURE 5: Methylation of phage TP-1C DNA by *Bst*1503 modification methylase. Phage TP-1C DNA (3.7 μg) was incubated with *Bst*1503I methylase (2 nM) in modification methylase buffer at 55 °C for various times. Each sample was analyzed by agarose gel electrophoresis, and a fluorescent profile was measured. The relative decrease in the proportion of DNA fragments 5, 6, and 7 with time of incubation with the methylase was calculated, and the data are presented as the percentage of DNA protected.

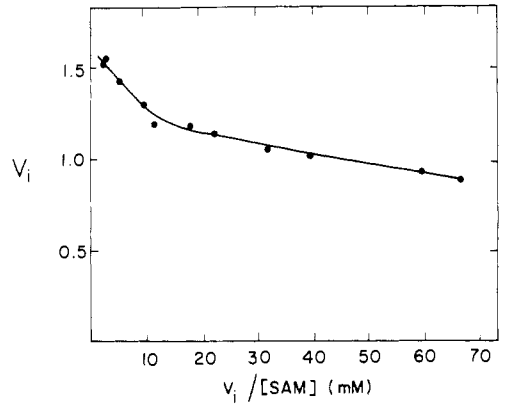

FIGURE 6: Effect of *S*-adenosyl-L-methionine of *Bst*1503 methylase activity. *Bst*1503I methylase (2 nM) was assayed in modification methylase buffer containing various concentrations of AdoMet at 55 °C. Initial velocities (V_i) are in enzyme units.

methylase over that of a homogeneous preparation.

Catalytic Properties of Bst1503I. The time course of methylation of phage TP-1C DNA by *Bst*1503I methylase is shown in Figure 5. Methylation proceeds at a linear rate for about 30 min. In this experiment, complete protection was observed after 90–100 min of incubation.

The rate of methylation of phage TP-1C DNA was measured at enzyme concentrations up to 4.2 nM. The initial reaction rate was first order up to a concentration of 3.5 nM. Since the methylase is tetrameric at concentrations between 0.7 and 4.2 nM, we conclude that the active form of the methylase is the tetramer.

The initial velocity of methylation at various concentrations of AdoMet and phage TP-1C DNA was determined, and the data are represented by Eadie–Hofstee plots. The enzyme obeyed Michaelis–Menten kinetics with respect to DNA (data not shown) but not with respect to AdoMet (Figure 6). These results indicate that the methylase has a single binding site for DNA and more than one binding site for AdoMet.

Discussion

A relatively simple procedure was developed for obtaining homogeneous *Bst*1503I methylase of high specific activity in good yield. The yield of enzyme is comparable to that reported by Rubin & Modrich (1977) for *Eco*RI methylase. Recovery of *Bst*1503I endonuclease can be accomplished by a modification of the procedure described for the preparation of a crude extract. As described previously, the methylase is bound to the membrane, and the cognate endonuclease is released upon rupture of the protoplast. The two enzymes can be resolved by separating protoplast membranes (methylase) from the supernatant fluids (endonuclease) before exposure to ultrasound.

Preliminary experiments revealed that crude extracts of strain 1503-4R R^+M^+ contain considerable DNA methylase activity. For this reason, the protective methylation assay was utilized instead of the standard assay for methylase activity which measures the transfer of [^{3}H]methyl groups to DNA. Protective methylation was determined by measuring the decrease in the amounts of fragments 5, 6, and 7 with time of incubation with methylase. The amounts of fragments 3 and 4 were also observed to decrease to a similar extent. These results indicate that at least five of the six recognition sites in TP-1C DNA are methylated by the methylase with an equal probability.

Despite their ability to recognize a common hexanucleotide sequence, the *Bst*1503I methylase and endonuclease are quite distinct in their physical properties. The endonuclease is composed of a subunit with a molecular weight of 46 000 and exists in solution as dimers or tetramers (Catterall & Welker, 1977). The methylase is composed of a subunit with a molecular weight of 105 000, and only the tetrameric form has been detected in solution. The endonuclease exhibited maximal activity between 60 and 65 °C and was not inactivated after exposure at 55 or 65 °C up to 10 h (Catterall & Welker, 1977). The methylase, on the other hand, exhibited maximal activity between 54 and 61 °C and was completely inactivated after exposure at 55 °C for 6–7 h and 61 °C for 1.5 h. In addition, the methylase exhibited an unexpected thermolability below 40 °C.

The thermostability of the methylase is significantly enhanced by Na^+, K^+, or NH_4^+. The role of monovalent cations in enhancing thermostability has been demonstrated for K^+ and NH_4^+ but not for Na^+ in the case of formyltetrahydrofolate synthetase from the thermophile *Clostridium thermoaceticum* (Shoaf et al., 1974) and for Na^+ and NH_4^+ in the case of glyceraldehyde-3-phosphate dehydrogenase from a facultatively thermophilic strain of *Bacillus coagulans* (Crabb et al., 1975, 1977). The role of monovalent cations in enhancing thermostability is not restricted to proteins isolated from thermophilic sources. Very similar results have been reported for *Bam*HI endonuclease from the mesophile *B. amyloliquefaciens* (Smith & Chiritkjian, 1979) and formyltetrahydrofolate synthetase from the mesophile *Clostridium formicoaceticum* (O'Brien et al., 1976).

Monovalent cations not only enhance the thermostability of *Bst*1503I methylase but also inhibit methylase activity. These results are consistent with the proposal that neutral salts bring about a perturbation in the macromolecular structure of the enzyme.

Although the methylase is inactivated at temperatures below the optimal growth temperature (63–67 °C), membrane-bound *Bst*1503I methylase is resistant to thermal inactivation at temperatures very near the maximum growth temperature (73 °C). The alkaline phosphatase and the NADH oxidase of this strain are also protected from heat inactivation because of their association with the cytoplasmic membrane (Wisdom & Welker, 1974).

The subunit molecular weights of *Bst*1503I endonuclease (46 000) and the methylase (105 000) are considerably larger

than those reported for the corresponding type II enzymes isolated from other sources. The subunit molecular weights of other endonucleases are 22 000 for *Bam*HI (Smith & Chirikjian, 1979), 25 000 for *Bcl*I (Bingham et al., 1978), 28 500 for *Eco*RI (Modrich & Zabel, 1976), 42 500 for *Eco*RII (Bingham & Atkinson, 1978), and 60 000 for *Bgl*I (Johannssen et al., 1979).

The modification methylases *Eco*RI and *Hpa*II have a subunit molecular weight of 39 000 (Rubin & Modrich, 1977) and 38 800 (Yoo & Agarwal, 1980), respectively. In contrast to *Bst*1503I methylase, the active species of the *Eco*RI and *Hpa*II methylases is the monomer.

Clarke & Hartley (1979) reported that the physical and catalytic properties of *Bst*I isolated from *B. stearothermophilus* N.C.I.B. 8924[4] (NCA 1503) were very different from those reported by Catterall & Welker (1977). The subunit molecular weight was found to be 26 000, and it differed substantially from *Bst*1503I in optimum temperature and pH for activity and isoelectric point. In addition, in the presence of high concentrations of enzyme or glycerol, DNA cleavage occurred at secondary sites.

*Bst*1503I endonuclease was isolated from 100 g of *B. stearothermophilus* 1503–4R, and a number of physical and catalytic properties were determined (N. E. Welker, unpublished experiments). The properties were identical with those reported by Catterall & Welker (1977). It is likely that the strain used by Clarke & Hartley (1979) was different than the one used by Catterall & Welker (1977), and their enzyme should be designated *Bst*8924I.

For investigation of the identity of the base methylated by *Bst*1503I methylase, phage TP-1C DNA was incubated with methylase and Ado[*methyl*-^{3}H]Met and analyzed (Randerath & Randerath, 1957) for ^{3}H-containing bases after acid hydrolysis (Wyatt, 1951). The only ^{3}H-methylated base detected (recovery 93% of the counts applied to the chromatographic sheet) was N^6-methyladenine. These results are in conflict with the action of the modification methylase *Bam*HI, reported by others.

Hattman et al. (1978) have proposed that a DNA–cytosine methylase is the modification enzyme (*Bam*HI methylase) in *B. amyloliquefaciens* H, and the modified sequence would be d(pG-G-A-T-m^{5}C-C). Evidence cited was that *B. amyloliquefaciens* H DNA contains only 5-methylcytosine (0.75% of the cytosine residues are methylated) and no DNA adenine methylase activity was detected, *Bam*HI endonuclease will cleave DNA in which the external cytosines of the recognition site are methylated (Mann & Smith, 1977), and methylation of some of the adenine residues within this sequence does not protect DNA against *Bgl*II endonuclease [recognition sequence contains the central tetranucleotide (GATC) of the *Bam*HI and *Bst*1503I recognition sequence; Pirrotta, 1976].

In addition, Dreiseikelman et al. (1979) have also shown that *Bam*HI endonuclease will cleave DNA in which the adenine residue within the recognition site is methylated.

We consider it unlikely that a contaminating methylase is responsible for the methylation of adenine. It is possible that *Bst*1503I methylase is methylating adenine residues outside of the recognition sequence. Studies are now in progress to determine the nature and location of the bases methylated by *Bst*1503 methylase.

Although detailed kinetic studies have not been done, evidence was presented that the methylase contains more than one binding site for AdoMet and that it functions as a tetramer. Studies to further investigate the catalytic properties of the methylase must be done by using as a substrate a circular DNA genome having a single *Bst*1503I site.

[4] N.C.I.B., National Collection of Industrial Bacteria (Aberdeen, Scotland).

References

Bingham, A. H. A., & Atkinson, T. (1978) *Biochem. Soc. Trans. 6*, 316.

Bingham, A. H. A., Atkinson, T., Sciaky, D., & Roberts, R. J. (1978) *Nucleic Acids Res. 5*, 3457.

Bodman, H., & Welker, N. E. (1969) *J. Bacteriol. 97*, 924.

Catterall, J. F., & Welker, N. E. (1977) *J. Bacteriol. 129*, 1110.

Catterall, J. F., & Welker, N. E. (1980) *Methods Enzymol. 65*, 167.

Catterall, J. F., Lees, N. D., & Welker, N. E. (1976) in *Microbiology* (Schlessinger, D., Ed.) p 358, American Society for Microbiology, Washington, DC.

Clarke, C. M., & Hartley, B. S. (1979) *Biochem. J. 177*, 49.

Crabb, J. W., Murdock, A. L., & Amelunxen, R. E. (1975) *Biochem. Biophys. Res. Commun. 62*, 627.

Crabb, J. W., Murdock, A. L., & Amelunxen, R. E. (1977) *Biochemistry 16*, 4840.

Davis, B. J. (1962) *Disc Electrophoresis*, Distillation Products Division, Eastman Kodak Co., Rochester, NY.

Dreiseikelmann, B., Eichenlaub, R., & Wackernagel, W. (1979) *Biochim. Biophys. Acta 562*, 418.

Ferguson, K. A. (1964) *Metab., Clin. Exp. 13*, 985.

Greene, P. J., Poonian, M. S., Nussbaum, A. L., Tobias, L., Garfin, D. E., Boyer, H. W., & Goodman, H. M. (1975) *J. Mol. Biol. 99*, 237.

Hattman, S., Keister, T., & Gottehrer, A. (1978) *J. Mol. Biol. 124*, 701.

Hendrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155.

Johannssen, W., Schutte, H., Mayer, F., & Mayer, H. (1979) *J. Mol. Biol. 134*, 707.

Kalb, V. F., Jr., & Bernlohr, R. W. (1977) *Anal. Biochem. 82*, 362.

Levy, W. P., & Welker, N. E. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1414.

Mann, M. B., & Smith, H. O. (1977) *Nucleic Acids Res. 4*, 4211.

Maurer, H. R. (1971) *Disc Electrophoresis*, p 44, de Gruyter, New York.

Modrich, P., & Zabel, D. (1976) *J. Biol. Chem. 251*, 5866.

O'Brien, W. E., Brewer, J. M., & Ljungdahl, L. G. (1976) *Experientia, Suppl. 26*, 249.

Oliver, S. G., & McLaughlin, C. S. (1977) *Anal. Biochem. 82*, 271.

Parks, L. W., & Schlenk, F. (1958) *J. Biol. Chem. 230*, 295.

Pirrotta, V. (1976) *Nucleic Acids Res. 3*, 1747.

Randerath, K., & Randerath, E. (1967) *Methods Enzymol. 12*, 323.

Roberts, R. J. (1978) *Gene 4*, 183.

Rubin, R. A., & Modrich, P. (1977) *J. Biol. Chem. 252*, 7265.

Sharp, P. A., Sugden, B., & Sambrook, J. (1973) *Biochemistry 12*, 3055.

Shoaf, W. T., Neece, S. H., & Ljungdahl, L. G. (1974) *Biochim. Biophys. Acta 334*, 448.

Smith, H. O., & Nathans, D. (1973) *J. Mol. Biol. 81*, 419.

Smith, L. A., & Chirikjian, J. G. (1979) *J. Biol. Chem. 254*, 1003.

Streips, U. N., & Welker, N. E. (1969) *J. Bacteriol. 99*, 344.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Welker, N. E., & Campbell, L. L. (1965) *J. Bacteriol. 89*, 175.

Williams, D. E., & Reisfeld, R. A. (1964) *Ann. N.Y. Acad. Sci. 121*, 373.
Wisdom, C., & Welker, N. E. (1974) *J. Bacteriol. 120*, 748.
Wrigley, C. W. (1969) *Sci. Tools 15*, 17.
Wyatt, G. R. (1951) *Biochem. J. 48*, 584.
Yamamoto, K., Alberts, B., Benzinger, R., Hawthorne, L., & Treiber, G. (1970) *Virology 40*, 734.
Yoo, O. J., & Agarwal, K. I. (1980) *J. Biol. Chem. 255*, 6445.

Ambiguity and Transcriptional Errors as a Result of Modification of Exocyclic Amino Groups of Cytidine, Guanosine, and Adenosine†

B. Singer* and S. Spengler

ABSTRACT: Ribopolynucleotides containing nucleosides with modified exocyclic amino groups were transcribed by using DNA-dependent RNA polymerase in the presence of Mn^{2+} and all four ribonucleoside triphosphates. Nearest-neighbor analysis of the products revealed a variety of effects. N^4-Modified cytidines can act preferentially like uridine (U) as a result of the known tautomerism, but ambiguity is also observed in the case of N^4-hydroxycytidine and N^4-methylcytidine. No ambiguity results from N^4-methoxycytidine which acts only like U. N^4-Acetylcytidine, known to be in the anti conformation, base pairs only with guanosine (G), as expected. N^2-Methylguanosine acts ambiguously in directing all four nucleosides into a transcript. However, it shows preference for incorporation of adenosine (A). Three different size substituents on the N^6 of A did not affect A·U pairing, indicating a conformation in which these modifications must lie anti to the ring N-1. Our results on fidelity may be explained in terms of the tautomerism of cytidine and orientation of the substituent. N^4-Methoxycytidine, predicted to prefer the imino form, appears to have the substituent anti to the ring N-3, resulting in strict U-like behavior. For the amino forms, modifications lying anti to the Watson–Crick side permit normal base pairing. However, substituents in the syn position may block normal pairing; this leads to the ambiguity observed for N^4-hydroxycytidine, N^4-methylcytidine, and N^2-methylguanosine. If the group is both large and syn, e.g., N^6-isopentenyladenosine, the attempt to transcribe the base may result in an inactivating event, such as a frame shift or termination. For this large substituent, the isopentenyl group must be anti when unambiguous base pairing occurs. As a general hypothesis, ambiguity, which may lead to point mutations, will result when hydrogen bonds of the appropriate number or length cannot be formed. This may arise from either steric hindrance and electronic shielding of the sites or loss of the appropriate donor or receptor. The continuation of transcription when noncomplementary nucleotide incorporation occurs may not require that any hydrogen bonds be formed, but that only stacking and other energy considerations are involved.

The misincorporation of noncomplementary bases during in vitro transcription of polydeoxynucleotides has been found to occur (Paetkau et al., 1972; Sirover et al., 1979; Seal et al., 1979), and it is postulated that the polymerases used have an error rate which is influenced by the transcription conditions. Such errors are usually corrected during in vivo transcription. However, when they are not repaired, mutation may result. When the template contains modified bases, the error rate or infidelity of transcription can be greatly increased (Sirover & Loeb, 1974).

Polyribonucleotides containing specifically modified bases can also be transcribed by using DNA-dependent RNA polymerase in the presence of Mn^{2+}, and the transcript can be analyzed for noncomplementary bases. Such a technique has proven useful in studying the effect of many different modified bases in transcription [reviewed by Singer & Kröger (1979)].

In the previous paper from this laboratory, Kröger & Singer (1979b) used as templates polynucleotides containing about 10% of 3-methylcytidine, 3-methyluridine, or 1-methyladenine. These derivatives were chosen because the methyl group blocks one of the essential hydrogen-bonding sites, and it had been considered that all were lethal modifications. However, it was found that all three methylated derivatives act with complete ambiguity and can direct any nucleotide into the transcript. None appeared to stop transcription.

The present series of experiments was designed to investigate whether modification of any exocyclic amino group causes ambiguity even though a single substitution of the N^2 of G, N^6 of A, or N^4 of C should not affect normal base pairing unless the substituent is oriented syn to the base-pairing side. For the purpose of comparison, we also studied several derivatives modified on sites which do not block Watson–Crick base pairing. These included 5-substituted pyrimidines, isoadenosine, 4-thiouridine, and 2′-*O*-methylcytidine. Nearest-neighbor analysis of transcription products was used as a sensitive test for misincorporation.

Materials and Methods

Chemicals and Polyribonucleotides. [α-^{32}P]GTP (20–30 Ci/mmol) was purchased from New England Nuclear. N^4-Hydroxy-CDP was prepared according to Janion & Shugar (1968). N^4-Methoxy-CDP was prepared in an analogous way by using methoxyamine (*O*-methylhydroxylamine). N^2-Methyl-GDP, 5-hydroxy-UDP, N^6-(hydroxyethyl)-ADP, and iso-ADP were generous gifts from Dr. A. M. Michelson, Institut de Biologie, Paris. 4-Thio-UDP, N^6-isopentenyl-ADP, and unmodified nucleoside diphosphates were purchased from P-L Biochemicals. We are also indebted to Dr. A. M. Michelson for poly(C,N^4-acC) and poly(A,N^4-acC). Poly(2′-*O*-

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