Samuels, M., Fire, A., & Sharp, P. A. (1982) *J. Biol. Chem. 257*, 14419-14427.
Sawadogo, M., Lescure, B., Sentenac, A., & Fromageot, P. (1981) *Biochemistry 20*, 3542-3547.
Schaller, H., Nusslein, C., Bonhoeffer, F. J., Kurz, C., & Nietzschmann, I. (1972) *Eur. J. Biochem. 26*, 474-481.
Seifart, K. H., Kuhasz, P. P., & Benecke, B. J. (1973) *Eur. J. Biochem. 33*, 181-191.
Sekeris, C. E., Schmid, W., & Roewekamp, W. (1972) *FEBS Lett. 24*, 27-31.
Sekimizu, K., Yokoi, H., & Natori, S. (1982) *J. Biol. Chem. 257*, 2719-2721.
Shibata, T., Das Gupta, C., Cunningham, R. P., & Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1638-1642.
Spindler, S. R. (1979) *Biochemistry 18*, 4042-4048.
Symons, R. H. (1977) *Nucleic Acids Res. 4*, 4347-4355.
Ueno, K., Sekimizu, K., Mizuno, D., & Natori, S. (1979) *Nature (London) 277*, 145-146.
Vannice, J. L., Taylor, J. M., & Ringold, G. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4241-4245.
von Hippel, P. H., & McGhee, J. D. (1972) *Annu. Rev. Biochem. 41*, 231-300.
Ward, D. F., & Gottesman, M. E. (1981) *Nature (London) 292*, 212-215.
Weiner, J. H., Bertsch, L. L., & Kornberg, A. (1975) *J. Biol. Chem. 250*, 1972-1980.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981) *J. Biol. Chem. 256*, 8829-8834.
Weisbrod, S., & Weintraub, H. (1981) *Cell (Cambridge, Mass.) 23*, 391-400.
Williams, R. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2311-2315.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197-203.
Wyers, F., Sentenac, A., & Fromageot, P. (1976) *Eur. J. Biochem. 69*, 377-383.

Base-Pairing Properties of N^4-Methoxydeoxycytidine 5'-Triphosphate during DNA Synthesis on Natural Templates, Catalyzed by DNA Polymerase I of *Escherichia coli*†

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ABSTRACT: N^4-Methoxydeoxycytidine 5'-triphosphate (mo^{4}dCTP) was synthesized by reaction of dCTP with methoxyamine and then purified by high-performance liquid chromatography (HPLC) and used to analyze the specificity of mo^{4}dCMP incorporation during polymerization on natural templates, catalyzed by DNA polymerase I of *Escherichia coli*. Elongation of synthetic 5'-^{32}P-labeled primers, annealed to single-stranded DNA of bacteriophage M13, was carried out in the presence of only three of the four normal dNTPs; then, reaction products were displayed by high-resolution gel electrophoresis and visualized by autoradiography. By measuring primer elongation in each of the four "minus" reactions with and without added mo^{4}dCTP, we examined the specificity of mo^{4}dCMP incorporation at different positions along the M13 template. The results of this experimental approach indicated that (i) mo^{4}dCTP is utilized most readily (although at low efficiency) in place of dTTP during DNA synthesis, (ii) the analogue can also replace dCTP during primer elongation, although at barely detectable efficiency, and (iii) the ease at which both mo^{4}C·A and mo^{4}C·G pairs are formed during DNA synthesis on natural templates is markedly influenced by the nucleotide sequence of the template.

Many mutagenic agents are believed to act by producing chemical modifications of purine and pyrimidine residues on the DNA template or dNTP[1] precursors. Altered or ambiguous base-pairing specificities of these residues during DNA replication can lead to heritable changes in nucleotide sequence (Drake, 1970; Drake & Baltz, 1976, Singer & Kusmierek, 1982; Singer & Grunberger, 1983). We recently developed an electrophoretic assay of misincorporation that can be used to directly examine the base-pairing specificity of chemically modified dNTPs during DNA synthesis catalyzed by purified DNA polymerases on natural DNA templates (Hillebrand et al., 1984; Revich et al., 1984). The primer-template employed in this approach consists of a discrete restriction fragment or synthetic oligonucleotide (labeled with ^{32}P at the 5'-terminus), annealed to a circular template strand (extracted from a single-stranded DNA bacteriophage). The rate of elongation of 5'-^{32}P-labeled primer in the presence of only three of the four normal dNTPs (monitored by electrophoresis/autoradiography) reflects the rate of misincorporation in place of the missing dNTP. To examine the specificity of incorporation of a chemically modified nucleotide during DNA synthesis, each of the four "minus" reactions is carried out in the absence

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[1] Abbreviations: dNTP, 2'-deoxynucleoside 5'-triphosphate; HPLC, high-performance liquid chromatography; mo^{4}C, N^4-methoxycytosine; ho^{4}C, N^4-hydroxycytosine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; ddNTP, 2',3'-dideoxynucleoside 5'-triphosphate.

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and presence of the modified dNTP. The extent to which primer elongation in a given "minus" reaction is stimulated by addition of the analogue is a measure of the potential for incorporation of the chemically modified nucleotide in place of the missing dNTP.

We have recently used this experimental approach to examine the mispairing potential of br^{5}dUTP, io^{5}dUTP, and 1,N^6-etheno-dATP during DNA synthesis catalyzed by DNA polymerase I of *Escherichia coli* (Hillebrand et al., 1984; Revich et al., 1984). In this paper we report the use of the electrophoretic assay of misincorporation to examine the base-pairing properties of N^4-methoxydeoxycytidine 5′-triphosphate (mo^{4}dCTP) during DNA synthesis. This nucleotide derivative was prepared by reaction of dCTP with the mutagen *O*-methylhydroxylamine (methoxyamine) and extensively purified by HPLC prior to use in the electrophoretic assay of misincorporation. The mutagenic effect of hydroxylamine and methoxyamine (which produce almost exclusively C → T transitions when bacteriophage or nucleic acids are directly treated) has been attributed to pairing of N^4-hydroxy(methoxy)cytidine [which exists predominately as the imino tautomer (Brown et al., 1968)] with A during DNA synthesis (Singer & Frankel-Conrat, 1969; Kochetkov & Budowsky, 1969; Frenkel-Conrat & Singer, 1972). Previous work has shown that mo^{4}C·A (ho^{4}C·A) pairing occurs during RNA synthesis (Phillips et al., 1965; Budowsky et al., 1971b; Singer & Spengler, 1981). Most recently, Singer et al. (1984) reported that mo^{4}dCTP is utilized (specifically in place of dTTP) during synthesis of deoxy copolymers, catalyzed by DNA polymerase I of *E. coli*. The results reported here confirm that the mo^{4}C·A base pair can form during DNA synthesis (as well as the mo^{4}C·G base pair at much lower frequency). Furthermore, the propensity (and specificity) of mo^{4}dCMP incorporation during DNA synthesis varies greatly at different positions along natural DNA templates.

Materials and Methods

The unmodified dNTPs used in this work consisted of commercially HPLC-purified preparations (obtained from P-L Biochemicals), which we further purified by HPLC, as described previously (Hillebrand et al., 1984; Revich et al., 1984). Methoxyamine hydrochloride was purchased from Chemical Dynamics Corp. Sephadex G-10 and DEAE-Sephacel were obtained from Pharmacia. The Whatman Partisil SAX column was obtained from Custom LC, Houston, TX.

Routine measurements of UV absorption were made with a Zeiss Model PM6 spectrophotometer, whereas UV absorption spectra of purified mo^{4}dCTP (at pH 1, 6, and 12) were taken with a Varian Series 634 scanning spectrophotometer. Conductivity was measured with a Yellow Springs Instruments Model 31 conductivity bridge. HPLC was performed as described previously (Hillebrand et al., 1984; Revich et al., 1984) with a Perkin-Elmer Series 4 liquid chromatograph and a Hewlett-Packard Model HP1040 spectrophotometric detection system.

DNA polymerase I "large fragment" from *E. coli* was obtained from P-L Biochemicals and Bethesda Research Laboratories. Bacteriophage M13 DNA and primer–templates were prepared and used in the electrophoretic assay of misincorporation as described previously (Hillebrand et al., 1984). In the nomenclature used to described primer–templates (e.g., S17–M13mp9), the first part refers to the primer (S17 stands for synthetic 17-mer), and the second part refers to the template (e.g., DNA of bacteriophage M13mp9). Synthetic primer S17 (obtained from P-L Biochemicals) anneals to the M13mp9 template such that the first incorporation during DNA synthesis occurs at position 6254 in the nucleotide sequence of M13mp9 (van Wezenbeek et al., 1980; Messing, 1983). Synthetic primer S16 (the "hybridization probe" primer obtained from New England Biolabs) anneals to M13mp7 DNA such that the first residue incorporated during chain elongation is at position 6204 in the M13mp7 genome.

Results

Preparation and Purification of mo^{4}dCTP. A total of 28 mg (50 μmol) of dCTP was dissolved in 1.0 mL of aqueous 2.7 M methoxyamine hydrochloride (pH 5.0). The mixture was incubated at 30 °C. At various times, 1-μL samples were withdrawn and added to 99 μL of H_2O (on ice). To monitor the progress of the reaction, 5 μL of the diluted mixture was injected onto a 4.6 × 250 mm Whatman Partisil SAX column and eluted isocratically at 1.5 mL/min with 750 mM KH_2PO_4, pH 3.7. Panel A of Figure 1 shows the UV absorption profile obtained during chromatography of sample taken at 24 h. At this time no detectable dCTP remained in the reaction mixture. UV absorption spectra (at pH 3.7) obtained during passage of each component through the flow cell of the diode array detector are displayed at the top of panel A (solid line), in comparison with the absorption spectrum (pH 3.7) of the final purified product (dotted line). Peak 4 (after further purification) was identified as mo^{4}dCTP from its UV absorption spectra taken at pH 1, 6, and 12, compared with published spectra (Budowsky et al., 1971a) of N^4-methoxycytidine 5′-phosphate. The spectrum exhibited by this major product in the HPLC mobile phase (pH 3.7) was similar to that obtained at pH 6. At pH 1 the material purified from peak 4 exhibited a major absorption maximum near 280 nm and a minor maximum near 220 nm, as reported by Singer et al. (1984) for mo^{4}dCTP. Peak 1 was probably the diphosphate of mo^{4}dC, formed by reaction of methoxyamine with dCDP present in the initial preparation of dCTP (and perhaps with some contribution via hydrolysis of the modified triphosphate). Peaks 2 and 3, which were not studied further, exhibited spectral properties characteristic of the 5,6-saturated bis product known to be formed by reaction of methoxyamine with the cytosine ring, N^4-methoxy-6-(methoxyamino)-5,6-dihydrocytosine (Budowsky et al., 1971a; Singer et al., 1984).

The reaction mixture at 24 h was desalted by passage over a 0.7 × 30 cm column of Sephadex G-10, eluted with 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. The main peak of conductivity eluted shortly after the peak of A_{240}. The resulting solution (10.5 mL) was loaded onto the Whatman Partisil SAX column (equilibrated with 25 mM KH_2PO_4, pH 3.7) by repeated injection of 1.75-mL aliquots. After the final injection, the column was washed for an additional 5 min with low-strength mobile phase; then, the material was isocratically eluted with 750 mM KH_2PO_4, pH 3.7. Panel B shows the plot of absorption (260 ± 40 nm) vs. time obtained during the first preparative HPLC run. UV absorption spectra (upper part of panel B) identified the major peaks as those seen in the 24-h reaction mixture (panel A). The main peak fractions were reinjected onto the column, resulting in the profile seen in panel C. Comparison of panels B and C (signals attenuated to give comparable magnitude of the main peak) reveals that the contaminating material was nearly completely removed during the first preparative HPLC run [spectra (not shown) of peaks marked 1–4 in panel C were identical with those of the corresponding peaks in panels A and B]. Peak fractions collected during the second HPLC run were pooled, desalted by DEAE-Sephacel chromatography (Hillebrand et al., 1984; Revich et al., 1984), dissolved in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and stored at −20 °C. This material was used

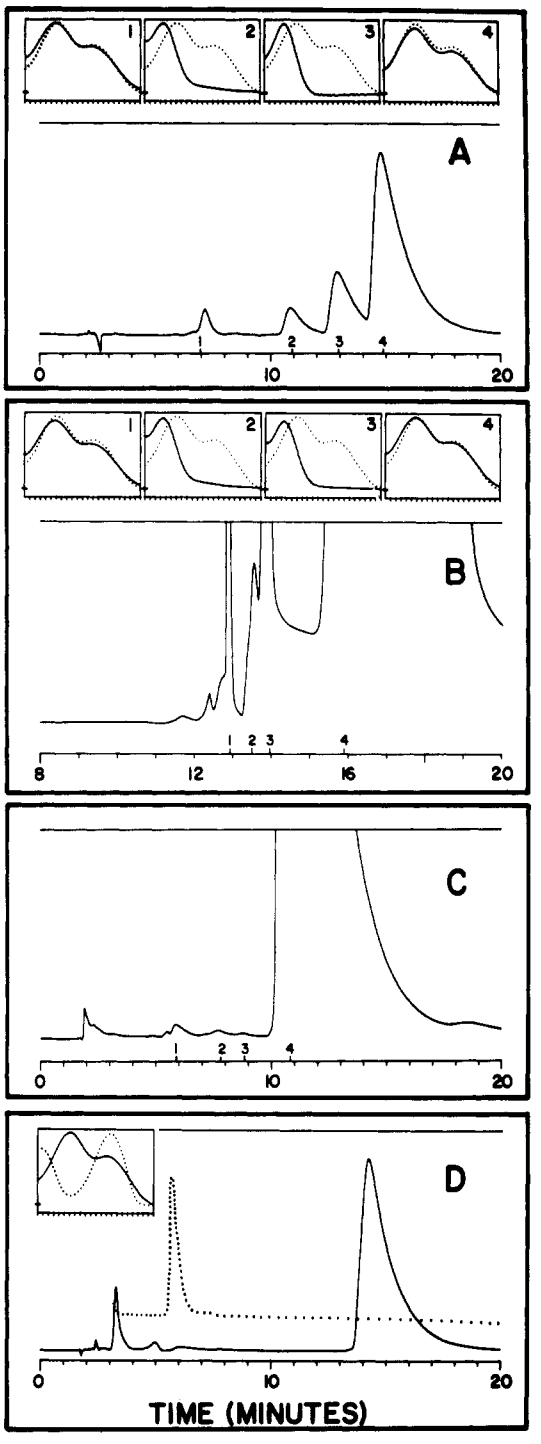

FIGURE 1: HPLC purification of mo⁴dCTP. Separations were achieved on a 4.6 × 250 mm column of Whatman Partisil SAX, eluted at room temperature with 750 mM KH_2PO_4, pH 3.7. After reaction of dCTP with methoxyamine for 24 h, a small quantity of reaction mixture was diluted and injected onto the column, producing the UV absorption vs. time profile shown in panel A. At the top are UV absorption spectra (210–320 nm) acquired by the diode array detector during the separation (solid lines), plotted along with the spectrum (pH 3.7) of purified mo⁴dCTP (dotted lines). Peaks and corresponding spectra are numbered. After removal of salt by Sephadex G-10 chromatography, the reaction mixture was loaded onto the column and then eluted with the above mobile phase, producing the UV absorption vs. time profile and spectral data shown in panel B. Peak 4 material from the first preparative separation was rechromatographed to produce the absorption profile shown in panel C. Peak fractions from the second preparative run were desalted by DEAE-Sephacel chromatography and used in the electrophoretic assay of incorporation. Panel D shows absorption vs. time profiles and UV spectra obtained during analytical HPLC of dCTP (dotted lines) and purified mo⁴dCTP (solid lines).

for subsequent electrophoretic assays of the base-pairing specificity of mo⁴dCTP during DNA synthesis.

In panel D are shown UV absorption vs. time profiles and spectral data (pH 3.7) for the dCTP reactant (dotted lines) and for the final purified product (solid lines). The small peak eluting at 3 min during HPLC of dCTP corresponds to dCDP. These data show that under the chromatographic conditions used for purification of the product, mo⁴dCTP was very well separated from any possible contamination by unreacted dCTP. Furthermore, the product was also well resolved from any possible contamination by dTTP, which would have eluted immediately after dCTP with the mobile phase used (data not shown). The latter point is important in light of the results of the electrophoretic assay of misincorporation (described below) that suggest base pairing of mo⁴dCTP with adenine residues in the template during DNA synthesis. (Contamination of mo⁴dCTP with dTTP would have produced artifactual chain elongation upon addition of the analogue to a "–T" polymerase reaction.) The peaks eluting at less than 5 min during HPLC of the final product (panel D, solid line) were identified as buffer components [the major absorbing species being EDTA (data not shown)]. The trace of material in the final preparation of mo⁴dCTP that had a retention time similar to that of dCTP exhibited a UV spectrum close to that of the 5,6-saturated bis derivative, entirely distinct from that of dCTP or dTTP (data not shown).

Electrophoretic Analysis of Base-Pairing Properties of mo⁴dCTP during DNA Synthesis. Figure 2 shows the effect of mo⁴dCTP on elongation of primer S17 on the M13mp9 template by *E. coli* DNA polymerase I, as measured by gel electrophoresis. Control reactions (compare lanes 13 and 14) demonstrated that the analogue (at 10 μM) did not inhibit polymerization in the presence of all four normal dNTPs. To examine the potential of the analogue to be incorporated in place of each of the four normal dNTPs, we conducted a series of "minus" reactions in which only three of the four dNTPs were present (at 10 μM) and tested the effect of added mo⁴dCTP (at 10 μM) on the extent of primer elongation. The "dideoxy" sequencing reactions (Sanger et al., 1977) were included to aid in the interpretation of the data. Each ddNTP reaction generates a series of primers, terminated by elongation up to successive template positions complementary to the 2′,3′-dideoxy analogue. Thus, the dideoxy lanes mark the positions at which the "missing" nucleotide would normally be incorporated during primer elongation (lines connect the T residues in the template sequence with corresponding "ddA" bands in lane 1 of Figure 2). As evidenced by the nearly identical autoradiographic banding patterns in lanes 2 and 3, mo⁴dCTP did not stimulate elongation in the "–A" reaction, above that due to misincorporation of the three unmodified dNTPs in place of dATP. Similarly, mo⁴dCTP did not significantly replace dGTP during primer elongation (lanes 5 and 6). The slightly greater overall chain elongation seen when mo⁴dCTP was added to the "–C" reaction (compare lanes 8 and 9) suggests that the analogue can be incorporated at detectable (although extremely low) efficiency opposite G residues in the template.

The only "minus" reaction in which elongation of the S17 primer was markedly stimulated by addition of analogue was the –T reaction (compare lanes 11 and 12). Thus, the results obtained with primer–template S17–M13mp9 suggest that incorporation of mo⁴dCMP residues occurs most readily opposite adenine in the template during DNA synthesis, in agreement with earlier findings for RNA synthesis and with the recent experiments of Singer et al. (1984), which measured

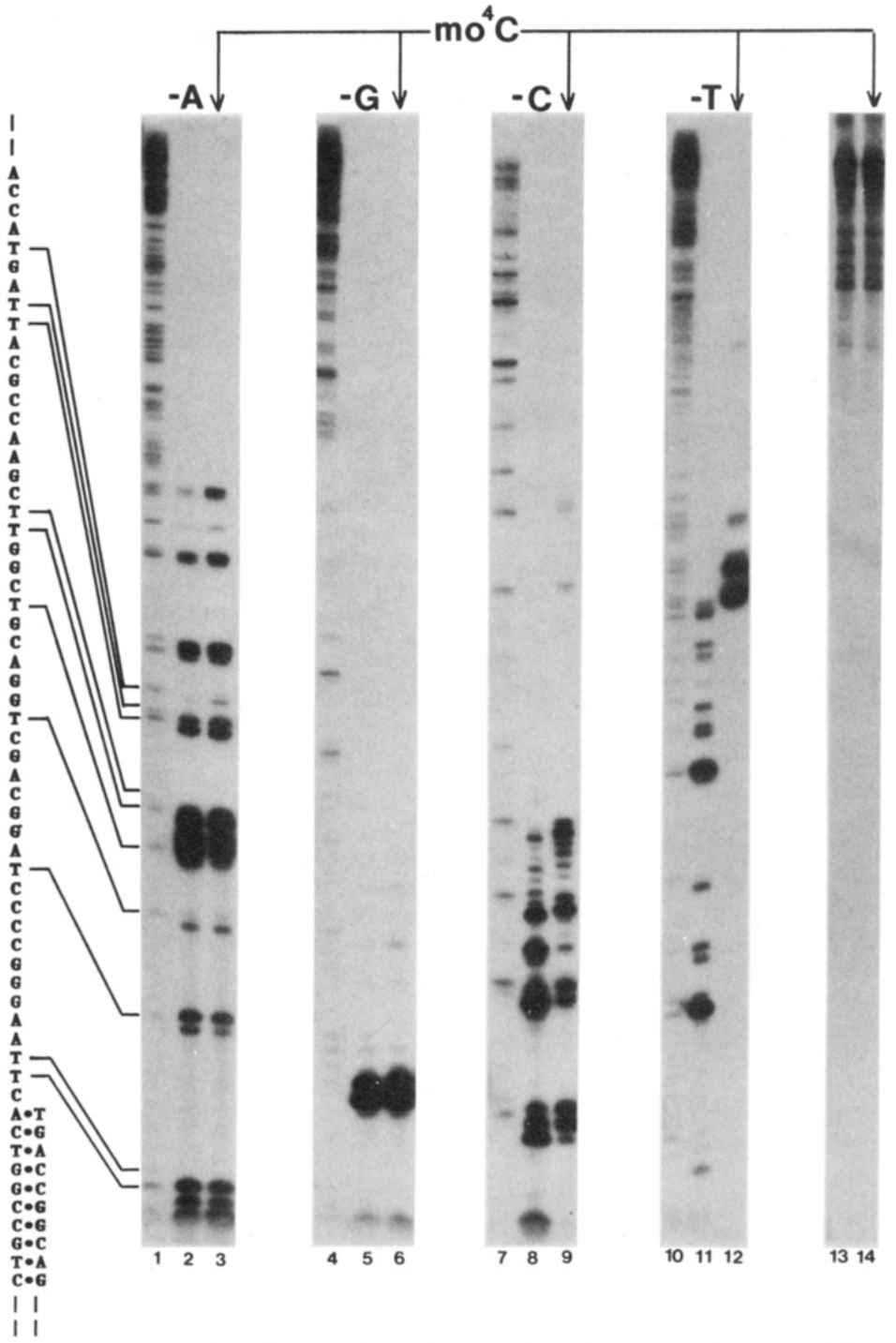

FIGURE 2: Electrophoretic analysis of mo^{4}dCMP incorporation during elongation of S17–M13mp9 by *E. coli* DNA polymerase I. Lanes 1, 4, 7, and 10 represent ddATP, ddGTP, ddCTP, and ddTTP sequencing reactions, respectively. The sequence of the M13mp9 template near the 3′-OH end of the S17 primer is displayed along the left side, with lines connecting T residues in the template with corresponding "ddA" bands in lane 1. Lanes 2 and 3 represent –A reactions conducted in the absence and presence of mo^{4}dCTP, respectively. Lanes 5 and 6 represent –G reactions conducted in the absence and presence of mo^{4}dCTP, respectively. Lanes 8 and 9 represent –C reactions conducted in the absence and presence of mo^{4}dCTP, respectively. Lanes 11 and 12 represent –T reactions conducted in the absence and presence of mo^{4}dCTP, respectively. Lanes 13 and 14 represent reactions conducted for 5 min in the presence of all four normal dNTPs, in the absence and presence of mo^{4}dCTP, respectively. (All other reactions were carried out for 30 min.) See Hillebrand et al. (1984) for details of the reaction conditions.

incorporation of the analogue into synthetic deoxy copolymers, catalyzed by DNA polymerase I of *E. coli*.

A notable feature of the utilization of mo^{4}dCTP in place of dTTP during synthesis on the M13mp9 template is the strong influence of nearest-neighbor sequences on the propensity for analogue incorporation. Accumulation of the two dark bands in lane 12 suggests the existence of two positions on the M13mp9 template at which formation of mo^{4}dCTP·A base pairs during chain elongation was strikingly disfavored, compared with this type of pairing at preceding A residues in the template.

The results obtained with another primer–template (S16–M13mp7) are shown in Figure 3. As with the other primer–template, mo^{4}dCTP was utilized for chain elongation predominantly in place of dTTP (compare lanes 11 and 12). Again, formation of mo^{4}dCTP·A base pairs during DNA synthesis exhibited a great deal of sequence dependence, as reflected by the strikingly nonuniform autoradiographic band

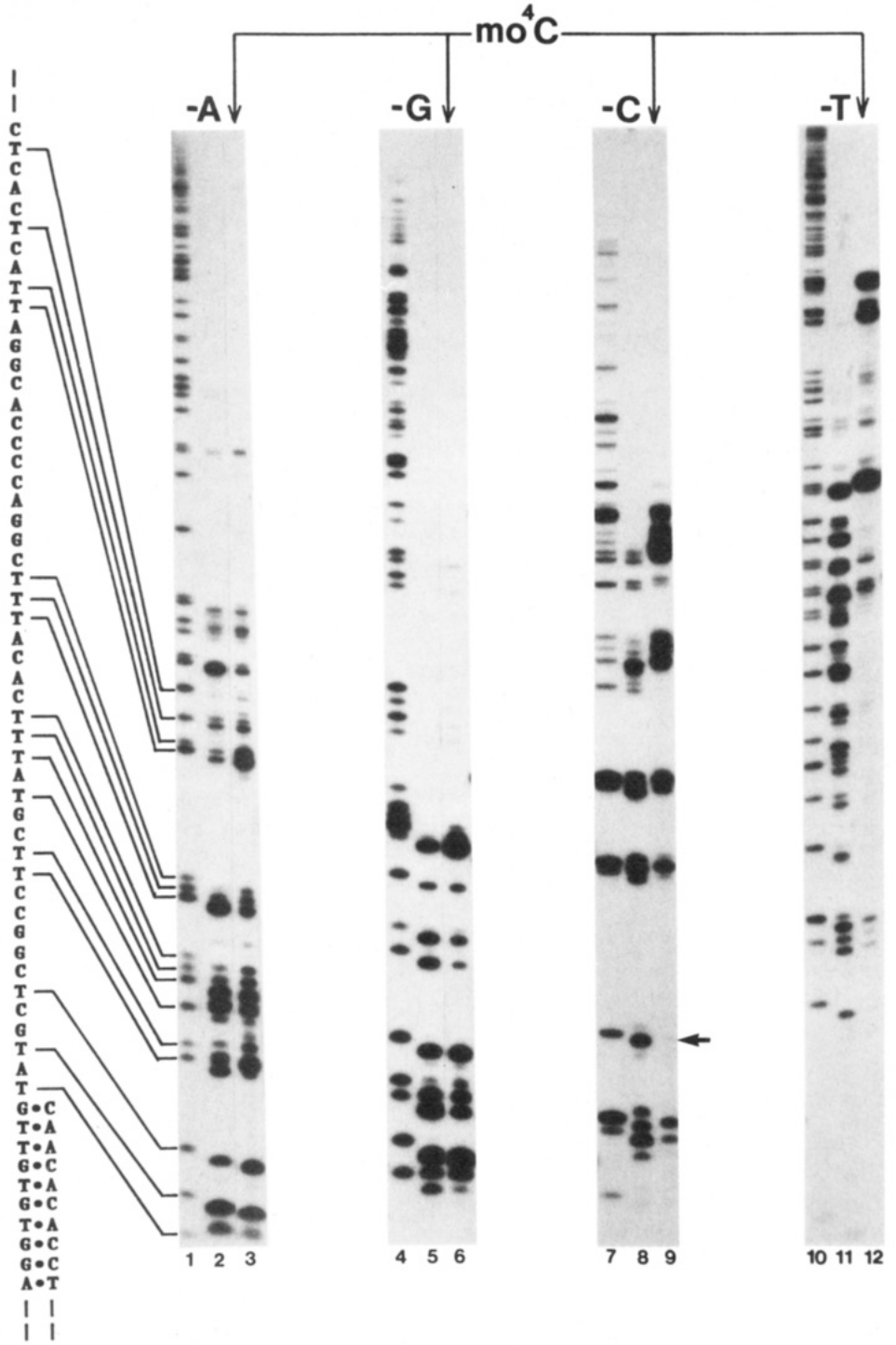

FIGURE 3: Electrophoretic analysis of mo^{4}dCMP incorporation during elongation of S16–M13mp7 by DNA polymerase I of *E. coli*. Lanes 1, 4, 7, and 10 represent ddATP, ddGTP, ddCTP, and ddTTP sequencing reactions, respectively. Lanes 2 and 3 represent –A reactions without and with addition of mo^{4}dCTP, respectively. Lanes 5 and 6 represent –G reactions without and with addition of mo^{4}dCTP, respectively. Lanes 8 and 9 represent –C reactions without and with addition of mo^{4}dCTP, respectively. Lanes 11 and 12 represent –T reactions without and with addition of mo^{4}dCTP, respectively.

intensities in lane 12. In the region of the M13mp7 template examined (which consists of the carboxy terminal region of the lacI gene), some replacement of dCTP by mo^{4}dCTP clearly occurred (compare lanes 8 and 9), although at low efficiency. The arrow to the right of lane 9 marks an apparent "hot spot" for incorporation of the analogue in place of dCTP (the prominent –C band in lane 8 was not produced in the presence of mo^{4}dCTP).

Discussion

We have utilized a sensitive electrophoretic assay of chain elongation to investigate the base-pairing potential of a mutagen-induced dNTP product, mo^{4}dCTP, during DNA synthesis catalyzed by purified DNA polymerase I of *E. coli*. A major benefit of this approach in studies of mutagenic mechanisms is the ability to test specific hypotheses regarding the base-pairing (or mispairing) properties of a given modified nucleotide during DNA synthesis. In this regard, our results bear on the notion that mutagenesis induced by hydroxylamine (and its *O*-methyl derivative, methoxyamine) arises largely via pairing of mutagen-induced ho^{4}C (or mo^{4}C) residues in the template with dATP during DNA synthesis. This prediction

arose from a variety of genetic and biochemical studies:

Genetic analysis of mutations arising from direct treatment of bacteriophage genomes or virions with hydroxylamine revealed that the predominant base change was G·C → A·T transition (Singer & Fraenkel-Conrat, 1969; Kochetkov & Budowsky, 1969). Treatment of conidia of *Neurospora crassa* with hydroxylamine or methoxyamine also caused predominantly G·C → A·T transitions (Malling, 1967, 1971). Studies of the products formed by reaction of hydroxylamines with nucleic acids and their constituents suggested a mechanism for mutagenesis (Budowsky et al., 1971a; Fraenkel-Conrat & Singer, 1972). The major product was N^4-hydroxycytosine, which was shown to exist predominantly as the imino tautomer (Brown et al., 1968), which should base pair like thymine (or uracil) during DNA (or RNA) synthesis. The tautomeric equilibrium of N^4-methoxycytosine is shifted even more strongly toward the imino form. That the base-pairing property of cytosine does indeed change upon formation of the N^4-hydroxy (or N^4-methoxy) derivative was first demonstrated in studies of RNA synthesis. Singer & Spengler (1981) showed that during transcription of ribo copolymers composed of C and ho^{4}C by DNA-dependent RNA polymerase in the presence of Mn^{2+}, AMP (and to a lesser extent GMP) was incorporated opposite the ho^{4}C residues in the template. When copolymers containing mo^{4}C residues were transcribed, the only misincorporation detected was that of AMP. The same authors subsequently showed (Spengler & Singer, 1981) that ribo copolymers of U and mo^{4}C annealed with poly(A) and were transcribed with ATP and RNA polymerase to form duplex RNA in which mo^{4}C was retained within the helix.

In studies of RNA synthesis using the modified CTP derivatives, Budowsky et al. (1971b) showed that both ho^{4}CTP and mo^{4}CTP substituted for either CTP or UTP during transcription of bacteriophage T2 DNA, although the methoxy derivative was incorporated predominantly in place of UTP (as expected from its existence as the imino tautomer). The deoxynucleotide derivative (ho^{4}dCTP) was employed in DNA synthesis to achieve site-directed mutagenesis in the β-globin gene (Muller et al., 1978; Weissman et al., 1979; Weber et al., 1981). In that work, ho^{4}dCMP residues were found to be incorporated opposite either G or A residues in the β-globin template strand. Replacement of dCTP by ho^{4}dCTP during "nick translation" synthesis resulted in A·T → G·C transition.

The electrophoretic assay of chain elongation provided a sensitive means to characterize the base-pairing potential of mo^{4}dCTP during DNA synthesis. The results indicate that mo^{4}dCMP residues are incorporated most readily in place of dTTP during chain elongation catalyzed by *E. coli* DNA polymerase I, in agreement with the recent report of Singer et al. (1984) that mo^{4}dCTP substituted for dTTP during synthesis on poly[d(A-T)], catalyzed by *E. coli* DNA polymerase I. The fact that some incorporation of the mo^{4}C analogue in place of dCTP was detected during primer elongation on natural DNA templates suggests that the tautomeric equilibrium of the mo^{4}C derivative is not shifted so far toward the imino form that ambiguous base pairing is totally precluded during DNA synthesis. The reported lack of incorporation of mo^{4}dCMP during synthesis on poly[d(G-C)] by *E. coli* pol I (Singer et al., 1984) does not conflict with our findings, in light of the small amount of mo^{4}C·G pairing detected by the electrophoretic assay and the large influence of DNA sequence on analogue incorporation revealed by our studies.

mo^{4}dCTP was utilized at very low efficiency during DNA synthesis, even in place of dTTP. Overall, the extent of chain elongation observed in −T reactions containing 10 μM mo^{4}dCTP would be produced by addition of dTTP at 2–3 orders of magnitude lower concentration (data not shown). The poor efficiency of incorporation of the mo^{4}C analogue probably stems largely from steric effects of the methoxy group, which exhibits strong preference for orientation syn to N3 of the cytosine ring (Birnbaum et al., 1979; Kierdaszuk et al., 1983). The strong tendency of mo^{4}C to exist as the imino tautomer would further act to "freeze" the syn rotomeric form, thus diminishing the probability of mo^{4}C·A base pairing during DNA synthesis. If this type of the base pairing is involved in methoxyamine-induced mutagenesis, it most likely occurs during replication past mutagen-induced mo^{4}C residues in the template, in which case chain elongation past the modified template residue would result in a heritable change in nucleotide sequence (C → T) at high probability.

An interesting phenomenon revealed by use of the electrophoretic assay of chain elongation was that the ease of formation of mo^{4}C·A (and mo^{4}C·G) base pairs during polymerization on natural DNA templates exhibited pronounced sequence dependence. These results add to the emerging view that the type of mispairing that occurs during DNA synthesis is strongly influenced by the nucleotide sequence of the template.

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REFERENCES

Birnbaum, G. I., Kulikowski, T., & Shugar, D. (1979) *Can. J. Biochem. 57*, 308–313.

Birnbaum, G. I., Kierdaszuk, B., & Shugar, D. (1984) *Nucleic Acids Res. 12*, 2447–2460.

Brown, D. M., Hewlins, M. J. E., & Schell, P. (1968) *J. Chem. Soc. C*, 1925–1929.

Budowsky, E. I., Sverdlov, E. D., Shibaeva, R. P., Monastyrskaya, G. S., & Kochetkov, N. K. (1971a) *Biochim. Biophys. Acta 246*, 300–319.

Budowsky, E. I., Sverdlov, E. D., & Spasokukotskaya, T. N. (1971b) *FEBS Lett. 17*, 336–338.

Drake, J. (1970) *The Molecular Basis of Mutation*, Holden-Day, San Francisco.

Drake, J., & Baltz, R. H. (1976) *Annu. Rev. Biochem. 56*, 11–37.

Fraenkel-Conrat, H., & Singer, B. (1972) *Biochim. Biophys. Acta 262*, 264–268.

Hillebrand, G. G., McCluskey, A. H., Abbott, K. A., Revich, G. G., & Beattie, K. L. (1984) *Nucleic Acids Res. 12*, 3155–3171.

Kierdaszuk, B., Stolarski, R., & Shugar, D. (1983) *Eur. J. Biochem. 130*, 559–564.

Kochetkov, N. K., & Budowsky, E. I. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 403–438.

Malling, H. V. (1967) *Mutat. Res. 4*, 559–565.

Malling, H. V. (1971) *Hereditas (Lund, Swed.) 68*, 219–234.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Muller, W., Weber, H., Meyer, F., & Weissman, C. (1978) *J. Mol. Biol. 124*, 343–358.

Phillips, J. H., Brown, D. M., Adman, R., & Grossman, L. (1965) *J. Mol. Biol. 12*, 816–828.

Revich, G. G., Hillebrand, G. G., & Beattie, K. L. (1984) *J. Chromatogr. 317*, 283–300.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Singer, B., & Fraenkel-Conrat, H. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 1–29.

Singer, B., & Spengler, S. (1981) *Biochemistry 20*, 1127–1132.
Singer, B., & Kusmierek, J. T. (1982) *Annu. Rev. Biochem. 52*, 655–693.
Singer, B., & Spengler, S. (1982) *FEBS Lett. 139*, 69–71.
Singer, B., & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.
Singer, B., Fraenkel-Conrat, H., Abbott, L. G., & Spengler, S. J. (1984) *Nucleic Acids Res. 12*, 4609–4619.
Spengler, S., & Singer, B. (1981) *Biochemistry 20*, 7290–7294.
van Wezenbeek, P. M. G. F., Hulsebos, T. J. M., & Schoenmakers, J. G. G. (1980) *Gene 11*, 129–148.
Weber, H., Dierks, P., Meyer, F., van Ooyen, A., Dobkin, C., Abrescia, P., Kappeler, M., Meyhack, B., Zeltner, A., Mullen, E. E., & Weissman, C. (1981) in *Developmental Biology using Purified Genes* (Brown, D. M., Ed.) pp 367–385, Academic Press, New York.
Weissman, C., Nagata, S., Taniguchi, T., Weber, H., & Meyer, F. (1979) in *Genetic Engineering, Principles and Methods* (Setlow, J. K., & Hollaender, A., Eds.) Vol. 1, pp 133–150, Plenum Press, New York.

On the Structural Specificity of Puromycin Binding to *Escherichia coli* Ribosomes†

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ABSTRACT: We have examined the structural specificity of the puromycin binding sites on the *Escherichia coli* ribosome that we have previously identified [Nicholson, A. W., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1982) *Biochemistry 19*, 3809–3817, and references cited therein] by examining the interactions of a series of adenine-containing compounds with these sites. We have used as measures of such interactions (a) the inhibition of [^{3}H]puromycin photoincorporation into ribosomal proteins from these sites, (b) the site-specific photoincorporation of the ^{3}H-labeled compounds themselves, and (c) the inhibition of peptidyl transferase activity. For the first two of these measures we have made extensive use of a recently developed high-performance liquid chromatography (HPLC) method for ribosomal protein separation [Kerlavage, A. R., Weitzmann, C., Hasan, T., & Cooperman, B. S. (1983) *J. Chromatogr. 266*, 225–237]. We find that puromycin aminonucleoside (PANS) contains all of the structural elements necessary for specific binding to the three major puromycin binding sites, those of higher affinity leading to photoincorporation into L23 and S14 and that of lower affinity leading to photoincorporation into S7. Although tight binding to the L23 and S7 sites requires both the N^6,N^6-dimethyl and 3′-amino groups within PANS, only the N^6,N^6-dimethyl group and not the 3′-amino group is required for binding to the S14 site. Our current results reinforce our previous conclusion that photoincorporation into L23 takes place from the A′ site within the peptidyl transferase center and lead us to speculate that the S14 site might be specific for the binding of modified nucleosides. They also force the conclusion that puromycin photoincorporation proceeds through its adenosyl moiety.

Photoaffinity labeling has proven itself to be an effective tool for identifying components at binding and functional sites of complex biological receptors. The antibiotic puromycin is a substrate for the peptidyl transferase center of the ribosome, and in previous work we have utilized both puromycin (Cooperman et al., 1975; Jaynes et al., 1978; Grant et al., 1979a,b) and an aryl azide derivative of puromycin, *p*-azidopuromycin[1] (Nicholson et al., 1982a,b), as photoaffinity labels for the peptidyl transferase center of the *Escherichia coli* ribosome. The major results of this work have been the following.

(1) Protein L23 is the major 50S protein labeled by both puromycin and *p*-azidopuromycin. The labeling of L23 proceeds from a site on the 50S subunit. There is a striking parallelism between the effects of added ligands on inhibiting labeling of L23 and on inhibiting peptidyl transferase, which we have taken as evidence that the labeling of L23 proceeds from the A′ site, defined as the site of binding of the 3′-end of aminoacyl-tRNA within the peptidyl transferase center.

(2) Other L proteins, particularly L18/22 and L15, also appear to be labeled by *p*-azidopuromycin from this site.

(3) Small subunit proteins are also labeled from sites on the 30S subunit, most notably S14 by puromycin and S7 and S18 by *p*-azidopuromycin.

(4) Puromycin aminonucleoside (PANS), which lacks the *O*-methyltyrosine moiety of puromycin, is almost as effective as puromycin in inhibiting L protein labeling by *p*-azidopuromycin and is a good competitive inhibitor of peptidyl transferase. It is, however, somewhat less effective in inhibiting *p*-azidopuromycin labeling of S7 and S18.

The finding that puromycin binds specifically to sites on the 30S and 50S subunits and that the full puromycin structure is not always essential for such binding raises the question of

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[1] Abbreviations: *p*-azidopuromycin, 6-(dimethylamino)-9-[3-deoxy-3-[(*p*-azido-L-phenylalanyl)amino]-D-ribofuranosyl]purine; N-AcPhe, *N*-acetylphenylalanine; PAGE, polyacrylamide gel electrophoresis; PANS, puromycin aminonucleoside; RP-HPLC, reverse-phase high-performance liquid chromatography; TP30, TP50, and TP70, total protein from 30S and 50S subunits and 70S ribosomes, respectively; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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