Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 4, Issue of February 25, pp. 1610-1612, 1982
Printed in U.S.A.

Transforming Activity of Mercury-Substituted DNA Synthesized *in vitro* by Permeable Cells of *Bacillus subtilis**

(Received for publication, December 4, 1981)

Sudha Bhattacharya and Nilima Sarkar

From the Department of Metabolic Regulation, Boston Biomedical Research Institute, Boston, Massachusetts 02114 and the Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115

Mercurated DNA was synthesized in permeable cells of *Bacillus subtilis*, using 5-mercurideoxycytidine triphosphate as one of the substrates, and was separated from parental unsubstituted DNA by isopycnic centrifugation in CsCl gradients. The ability of mercurated DNA to transform auxotrophic strains of *B. subtilis* to prototrophy was compared with that of normal DNA and was 10-20% of the latter. Mercurated and normal DNA bound with similar affinities to identical surface receptors of the recipient cells, but the efficiency of the bound mercurated DNA in promoting transformation was one-tenth to one-fifth of that of normal DNA. The transforming activity of mercury-substituted DNA synthesized in an *in vitro* system opens the way for the use of mercury as a probe to study the mechanism of bacterial transformation and various other kinds of genetic exchange.

In 1973, Dale and Ward (1) introduced mercury-substituted nucleotides as probes for the study of nucleic acid metabolism. We have recently shown that 5-mercurideoxycytidine triphosphate was effectively utilized as a substrate for semiconservative DNA replication in permeabilized *Bacillus subtilis* cells and that the newly synthesized mercurated DNA could be separated from parental DNA by isopycnic centrifugation (2). This provided an opportunity to examine the biological activity of *in vitro* synthesized mercurated DNA by measuring its potential for genetic exchange by transformation, as a first step in exploiting the unique properties conferred by mercury substitution for the study of the mechanism of bacterial transformation.

EXPERIMENTAL PROCEDURES

Materials—Deoxyribonucleotides were purchased from P-L Biochemicals, [*methyl*-^{3}H]dTTP (22 Ci/mmol) from New England Nuclear, Hg-dCTP,[1] lysozyme, and Proteinase K from Boehringer-Mannheim Biochemicals, heparin from Organon, Bio-Gel P4 (200-400 mesh) from Bio-Rad, CsCl from Pierce, and 3,5-diaminobenzoic acid dihydrochloride from Aldrich.

Synthesis and Isolation of Mercurated and Normal DNA—*B. subtilis* ATCC 23857 was grown and treated with toluene as described before (2). Mercurated DNA was synthesized in 5×10^8 toluene-treated cells of *B. subtilis*; the incubation mixture (1.0 ml) contained 70 mM potassium phosphate, pH 7.5, 1.5 mM ATP, 13 mM $MgSO_4$, 2 mM dithiothreitol, 5 mM 2-mercaptoethanol, 40 μM each of dATP, dGTP, Hg-dCTP, and 40 μM of [^{3}H]dTTP (100 μCi/μmol) (2). After 30 min at 37 °C, cells were lysed by adding 0.6 ml of 0.3 M NaCl, 0.2 M EDTA (pH 8.0) and 0.15 ml of 6 mg/ml of lysozyme. Incubation was continued for 30 min, followed by a further 40 min after adding sarkosyl (10 mg/ml), heparin (0.1 mg/ml), and proteinase K (0.5 mg/ml). The lysate was desalted by passage through a column of Bio-Gel P-4 equilibrated with 10 mM Tris-HCl, pH 7.5, and 0.1 M NaCl. The excluded fractions were pooled, adjusted to a final volume of 3.69 ml in 1 mM EDTA (pH 8.0), mixed with 4.6 g of CsCl in a siliconized nitrocellulose tube, and centrifuged at 25 °C in a Beckman 50 Ti rotor at 33,000 rpm for 62 h. Fractions (0.12 ml) were collected from the bottom of the tube. Mercurated DNA was defined by the distribution of radioactivity in the fractions, whereas the position of normal unmercurated DNA was defined by comparison with a parallel CsCl gradient that contained normal *B. subtilis* DNA (2.4 absorbance units at 260 nm). The fractions (from the same gradient) containing mercurated and normal DNA were pooled and dialyzed against 10 mM, Tris-HCl, pH 7.5, 10 mM KCl, and 1 mM EDTA. DNA concentration was determined fluorimetrically with a Perkin-Elmer fluorometer; excitation was 408 nm and emission was 500 nm (3).

Assay of Transformation by DNA—Competent recipient cells were prepared and transformation assays were done as described by Sonenshein *et al.* (4) with minor modifications as suggested by Dr. A. L. Sonenshein. Transformation basal medium (T base) contained 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 6 g of KH_2PO_4, and 1 g of trisodium citrate dihydrate/liter. For SpC medium, T base was supplemented with 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, 0.025% Casamino acids, 0.005% required amino acids, and 0.2% nutrient broth. SpT medium was the same as SpC except that the concentration of $MgSO_4 \cdot 7H_2O$ was 0.082%, Casamino acids 0.01%, nutrient broth 0.1%, and it contained in addition 0.5 mM $CaCl_2$. Competent cells were grown in SpC medium until the end of log phase. A 5-fold dilution of the culture into SpT medium was incubated with shaking at 37 °C for about one doubling of cell mass. These competent cells were centrifuged and suspended in 0.1 volume of the same culture medium to which 10% glycerol (v/v) had been added and aliquots were stored frozen at −80 °C and used as detailed by Dubnau *et al.* (5).

For transformation, thawed competent cells (about 10^8 cells/ml) were incubated in SpT medium containing 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid with 10–20 ng of DNA with shaking at 37 °C for 30 min. The transformed cells were then centrifuged, resuspended in the same volume of T base and plated on selective agar.

RESULTS AND DISCUSSION

Mercurated DNA was synthesized in toluene-treated cells of *B. subtilis* 168 (indole$^-$) with Hg-dCTP replacing dCTP as one of the substrates (2). As shown in Fig. 1, the newly synthesized mercurated DNA could be resolved from parental light DNA by equilibrium sedimentation in a CsCl gradient. The fractions of mercurated DNA that were free of parental DNA, as well as those containing parental DNA, were separately pooled and their ability to transform auxotrophic strains of *B. subtilis* was compared. The transformation of three separate auxotrophic markers was studied (lys-1, aroD120, metB10), and the efficiency of transformation by mercurated DNA was found to be 10–20% of that of normal DNA (Table I). The dependence on DNA concentration of transformation of the metB10 marker is illustrated in Fig. 2*a*. Normal and mercurated DNA produced half-maximal transformation at similar concentrations (60 and 110 ng/ml, re-

* This investigation was supported by Grant GM 25022 from the National Institute of General Medical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviation used is: Hg-dCTP, 5-mercurideoxycytidine 5'-triphosphate.

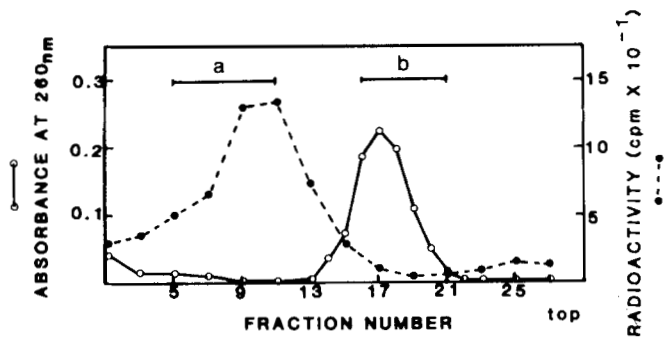

FIG. 1. **Separation of normal and mercurated DNA by isopycnic centrifugation.** Mercurated DNA was synthesized as described under "Experimental Procedures." The Hg-DNA in excluded fractions of Bio-Gel P4 column were pooled, adjusted to a final volume of 3.69 ml in 1 mM EDTA (pH 8.0) and mixed with 4.6 g of CsCl in a siliconized nitrocellulose tube and centrifuged at 25 °C in a Beckman 50 Ti rotor at 33,000 rpm for 62 h. Fractions (0.12 ml) were collected from the bottom of the tube and radioactivity in 20 μl was determined with a scintillation counter (●---●). The radioactive fractions corresponding to mercurated DNA were pooled as indicated by (*bar a*). The position of parental nonmercurated DNA in the same gradient was defined by centrifuging normal *B. subtilis* DNA (2.4 absorbance units at 260 nm) in a parallel CsCl gradient and measuring the distribution of absorbance at 260 nm (○——○). The fractions from the original gradient indicated by (*bar b*) were then pooled for subsequent transformation experiments.

TABLE I

Transforming activity of normal and mercurated DNA

Normal and mercurated DNA was isolated from toluene-treated cells of *B. subtilis* ATCC 23856 (*aro*$^+$ *lys*$^+$ *met*$^+$) as described in the legend to Fig. 1. The pooled samples from the CsCl gradient were dialysed for 24 h against 0.01 M Tris-HCl (pH 7.5), 0.01 M KCl, and 1 mM EDTA and DNA concentration was determined fluorimetrically (3). Transforming activity was measured using *B. subtilis* strains TAL (*trpC2 aroD120 lys-1*) and BR151 (*trpC2 metB10 lys-3*) as recipients for the *aro* and *lys* markers and for the *met* marker, respectively, as described under "Experimental Procedures." Competent cells (2×10^8 in 1 ml) were incubated with 10–20 ng of DNA. After 30 min, the cells were collected by centrifugation, resuspended in 1 ml of T base, and samples (0.1 ml) were plated on selective agar plates. The addition of mercurated DNA had no effect on the total number of colonies (transformed and untransformed) observed in control experiments.

DNA	*aro*$^+$	*lys*$^+$	*met*$^+$
	transformants/ng DNA		
Normal	512	410	353
Mercurated	69	86	29

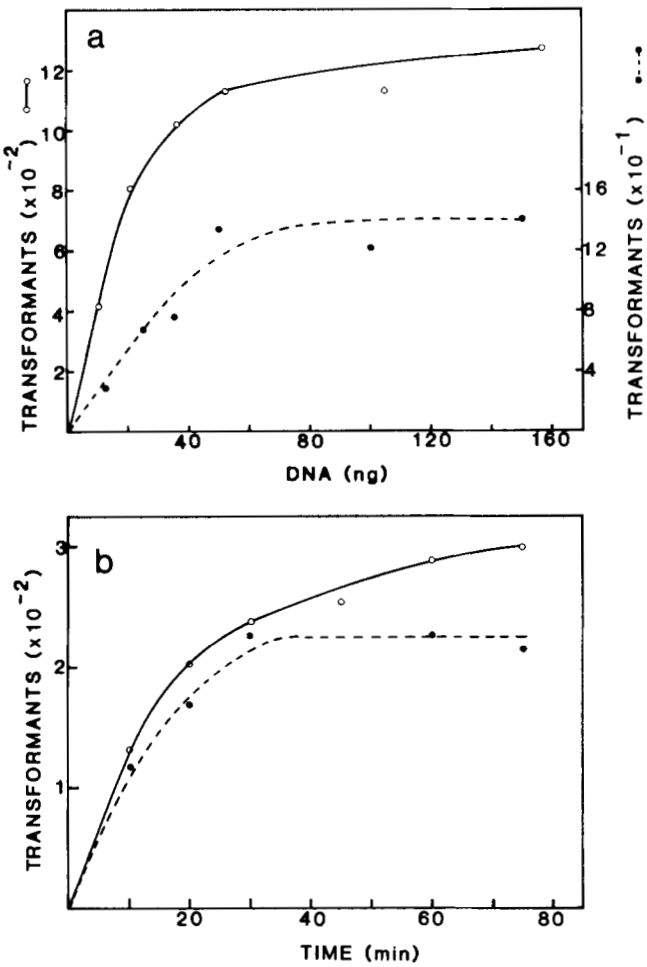

FIG. 2. *a*, effect of DNA concentration on transforming activity. Normal and mercurated DNA and competent cells of *B. subtilis* BR151 were prepared as described in the legends to Fig. 1 and Table I, except that the competent cells were diluted 10-fold in SpT medium. The indicated amounts of DNA were added to 0.25 ml of cells and, after 30 min at 37 °C, the mixtures were diluted with 2.3 ml of T base. Samples (0.2 ml) were plated and scored for *met*$^+$ transformants. *b*, time course of interaction of DNA with competent cells. Normal DNA (1.3 ng) and mercurated DNA (8.4 ng) were added to 0.5 ml of competent *B. subtilis* BR151 cells. After incubation for the indicated time periods, the cells were collected by centrifugation, resuspended in 0.5 ml of T base, and samples (0.1 ml) were plated and scored for *met*$^+$ transformants. (○——○), normal DNA; (●---●), mercurated DNA.

spectively), but the maximum extent of transformation by mercurated DNA was 11% of that produced by normal DNA. This is not the result expected if the lower transformation seen with mercurated DNA had been due to a low level of contaminating normal DNA, for such a situation would have resulted in a 10-fold higher concentration required for half-maximal transformation but in similar transformation efficiencies at saturating DNA concentrations. Rather, it appears that mercurated DNA itself is capable of causing transformation. However, whereas the primary interaction of normal and mercurated DNA with the recipient cells is similar, their dissociation constants from the outer surface receptor sites differing by about 2-fold, the bound mercurated DNA effects transformation with an intrinsically lower efficiency. The observation that normal and mercurated DNA show nearly the same time dependence in their interaction with recipient cells (Fig. 2*b*) is consistent with this interpretation.

The question whether normal and mercurated DNA bind to the same sites on the surface of recipient cells and with similar affinities could be resolved through competition experiments. Competent cells were mixed with a nearly saturating concentration of normal transforming DNA supplemented with additional amounts of either normal or mercurated DNA and the resulting transformants were scored. As shown in Fig. 3, the addition of mercurated DNA led to a significant reduction in the number of transformants. The response to added DNA was in good agreement with that predicted if mercurated DNA bound to the same receptor site on the cell surface with one-half the affinity of normal DNA and with one-tenth the maximum capacity to produce transformation, a situation formally analogous to the competition of two substrates for an enzyme with different K_m and V_{max} values (6).

The observation that mercurated DNA binds to the cell surface receptor with almost the same affinity as normal DNA and yet promotes transformation only one-tenth as efficiently implies that mercury substitution may interfere with one of

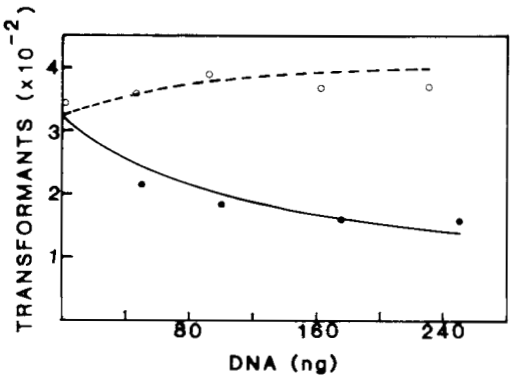

FIG. 3. **Competition of mercurated DNA with normal DNA for transformation.** Transforming DNA and competent cells of *B. subtilis* BR151 were prepared as described in the legends to Fig. 1 and Table 1. Competent cells (0.25 ml in SpT medium) were mixed with 52 ng of normal DNA plus the indicated amounts of normal (○) or mercurated (●) DNA. After 30 min at 37 °C, the mixtures were diluted with 2.3 ml of T base and samples (0.1 ml) were plated to score *met*$^+$ transformants. The curves describe the relationship between the number of transformants and DNA concentration that would result if normal and mercurated DNA bound to the same primary receptor sites so that

$$t = \frac{T_{max}^{normal}(DNA^{normal}/K_{Diss}^{normal}) + T_{max}^{Hg}(DNA^{Hg}/K_{Diss}^{Hg})}{1 + (DNA^{normal}/K_{Diss}^{normal}) + (DNA^{Hg}/K_{Diss}^{Hg})}$$

where t is the number of transformant obtained, T_{max} is the number of transformants at saturating DNA concentration, and K_{Diss} is the dissociation constant of DNA from the cell surface receptor sites. The parameters used were estimated from the results shown in Fig. 2*a* and were as follows: K_{Diss}^{normal} = 60 ng/ml; K_{Diss}^{Hg} = 112 ng/ml; $T_{max}^{Hg}/T_{max}^{normal}$ = 0.11. The *dotted curve* corresponds to the addition of further amounts of normal DNA and the *solid curve* to the addition of mercurated DNA.

the later steps in transformation. Binding of transforming DNA to the cell surface (7) is known to be followed by endonucleolytic cleavage to 10–20 million dalton fragments (8), whereupon about 50% of the DNA is degraded to mononucleotides, leaving single-stranded DNA (9–11), which is complexed with intracellular components (10, 12) in order to be integrated into the bacterial chromosome. Although mercurated DNA is a substrate for many nucleases including the restriction endonucleases *Eco* RI, *Hin*dIII and *Hpa* II,[2] it is resistant to hydrolysis by the 5′ → 3′ exonucleolytic activity of *Escherichia coli* DNA polymerase (1) and it is possible that one of the nucleolytic processing steps involved in transformation is impeded by mercury substitution of transforming DNA. In this context, it is interesting to note that the mercurated DNA used in our experiments is the product of less than one cycle of semiconservative replication and thus only one of the two strands is mercury-substituted (2).

Nevertheless, our observation that mercurated DNA synthesized *in vitro* by a permeable bacterial cell system can promote bacterial transformation, albeit with a somewhat lower efficiency than normal DNA, opens the way for the use of mercurated nucleotides as a probe for the study of bacterial transformation. Despite intensive study, most molecular details of this form of genetic transfer are still vague (13). The high affinity of mercurated polynucleotides to thiol-substituted agarose permits the selective isolation of mercurated DNA (1) and offers an important tool for following the fate of mercurated transforming DNA in the recipient cells in order to analyze the complex intracellular processes that precede transformation. In addition, the experiments described here constitute the first demonstration that DNA extensively modified by an unnatural base with potential chemical reactivity (all deoxycytidylate residues being replaced by 5-mercurideoxycytidylate) has the capacity to promote bacterial transformation.

We have recently found that Hg-dCTP can successfully be used as a substrate for *E. coli* DNA polymerase I for nick translation of linear Φ29 phage DNA to produce mercurated phage DNA.[2] Thus, any sequence of DNA can be mercurated enzymatically where *in vitro* replicative synthesis is not available. This mercurated DNA can then be used as a probe to study the molecular mechanisms of various forms of genetic exchange such as transformation of bacterial cells by plasmid DNA (14) and DNA-mediated gene transfer in cultured mammalian cells (15).

[2] S. Bhattacharya and N. Sarkar, unpublished observations.

Acknowledgment—We are grateful to Dr. A. L. Sonenshein for the bacterial strains and help in transformation experiments and to Dr. H. Paulus for critical analysis of competition experiments. This investigation was supported by Grant GM 25022 from the National Institute of General Medical Sciences.

REFERENCES

1. Dale, R. M. K., and Ward, D. C. (1975) *Biochemistry* **14,** 2458–2469
2. Bhattacharya, S., and Sarkar, S. (1981) *Biochemistry* **20,** 3029–3034
3. Puzas, J. E., and Goodman, D. B. P. (1978) *Anal. Biochem.* **86,** 50–55
4. Sonenshein, A. L., Cami, B., Brevet, J., and Cote, R. (1974) *J. Bacteriol.* **120,** 253–265
5. Dubnau, D., and Davidoff-Abelson (1971) *J. Mol. Biol.* **56,** 209–221
6. Dixon, M., and Webb, E. C. (1958) *Enzymes*, p. 92, Longman Green, London
7. Garcia, E., Lopez, P., Urena, M. T. P., and Espinosa, J. (1978) *J. Bacteriol.* **135,** 731–740
8. Dubnau, D., and Cirigliano, C. (1972) *J. Mol. Biol.* **64,** 31–46
9. Joenje, H., and Venema, G. (1975) *J. Bacteriol.* **122,** 25–33
10. Piechowska, M., and Fox, M. S. (1971) *J. Bacteriol.* **108,** 680–689
11. Fornilli, S. L., and Fox, M. S. (1977) *J. Mol. Biol.* **113,** 181–191
12. Eisenstadt, E., Lange, R., and Willecke, K. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 323–327
13. Notani, N. K., and Setlow, J. K. (1974) *Prog. Nucleic Acid Res. Mol. Biol.* **14,** 39–100
14. Cohen, S. N., Chang, A. C. Y., and Hsu, L. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 2110–2114
15. Pellicer, A., Robins, D., Wold, B., Sweet, R., Jackson, J., Lowy, I., Roberts, J. M., Sim, G. K., Silverstein, S., and Axel, R. (1980) *Science* **209,** 1414–1422