Methylation of the Deoxyribonucleic Acid of *Physarum polycephalum* at Various Periods during the Mitotic Cycle

HELEN H. EVANS AND THOMAS E. EVANS

From the Departments of Radiology, Biochemistry, and Microbiology, Case Western Reserve University, Cleveland, Ohio 44106

SUMMARY

The methylation of major nuclear DNA-cytosine was found to occur throughout the mitotic cycle of *Physarum polycephalum*, during the period of DNA synthesis (S), as well as during the remainder of interphase (G2) when essentially no synthesis de novo of the major nuclear DNA takes place. After incubation of the mold with methionine methyl-3H, 5-methylcytosine was the only radioactive methylated minor base detected in major nuclear DNA, although 3H was also found to be incorporated during the S period into DNA-adenine, -guanine, and -thymine, presumably by conversion of the methionine methyl group to 1-carbon intermediates incorporated into DNA nucleotides during synthesis de novo. During the G2 period, methylation of major nuclear DNA occurred at a relatively constant rate which was approximately 50% of that observed during the S period. Incorporation of 3H from methionine methyl-3H into 5-methylcytosine of both mitochondrial DNA and the nuclear heavy satellite DNA was also observed. It was estimated that the three DNA fractions of *Physarum* contain 1 methyl-cytosine residue for every 12 to 24 cytosine residues.

The enzymatic methylation of pre-formed DNA was first described by Gold, Hurwitz, and Anders (2). Subsequently it has been reported that DNA methylation appears to be associated with DNA synthesis in both prokaryotic and eukaryotic cells: Lark (3) and Billen (4) have demonstrated that DNA is normally methylated as it is replicated in *Escherichia coli*, while inhibitors of DNA synthesis added to cultures of mammalian cells have been found to reduce the level of DNA methylation by 60 to 85% (5, 6). In the present work, we have studied the methylation of DNA at various periods during the mitotic cycle of *Physarum polycephalum*, a myxomycete whose nuclei undergo naturally synchronous division and in which a clearly defined DNA synthetic period (S) occurs immediately following mitosis. There is no G1 period in *Physarum*. We have found that methylation of the major nuclear DNA occurs not only during the S period but also during the G2 period, a time when essentially no synthesis de novo of the major nuclear DNA takes place. The rate of DNA methylation during the G2 period, which was found to be relatively constant, was about 50% of the rate observed during the S period.

MATERIALS AND METHODS

Growth of Organism—Stock shake cultures were maintained axenically as microplasmodia grown at 23° in semidefined medium in the dark as described by Daniel and Baldwin (7). Synchronously dividing microplasmodia were prepared by fusion of microplasmodia on filter paper surfaces (Carl Schleicher and Schuell Company, Keene, New Hampshire, type 576) supported on a layer of glass beads, as described by Nygaard, Güttes, and Rusch (8). The time of mitosis was ascertained by microscopic examination of alcohol-fixed smears. Interdivision time under our conditions was approximately 7 hours.

Incorporation of Radioactive Precursors—Labeled precursors, generally obtained from New England Nuclear or Schwarz BioResearch, were added to the medium at the desired time at the following levels: thymidine methyl-3H, 5 μCi per ml; methionine methyl-3H, 5 to 30 μCi per ml; sodium formate-14C, 2 μCi per ml; phosphate-32P, 10 μCi per ml. The specific activity of the labeled precursors varied but was found not to affect the level of incorporation, probably because of the content of these precursors in the semidefined medium. At the end of the incubation period, shake cultures were harvested by a brief centrifugation, after which the pellets were frozen. Macroplasmodia were scraped from the filter paper into a cold solution composed of 0.15 m NaCl and 0.015 m sodium citrate, which was then frozen.

Hot Acid Extraction of Nucleic Acids—In experiments involving the incorporation of thymidine-3H, molds were washed with cold 4% trichloracetic acid in acetone and then with cold 0.6 N HClO4. Total nucleic acids were extracted from the acid-insoluble pellet by twice heating at 85° for 15 min in the presence of 0.6 N HClO4. The hot acid extract was assayed for radioactivity and for DNA by the Burton modification of the diphenylamine reaction (9). In experiments involving the incorporation of formate-14C, molds were washed with cold acid as described.

* This work was supported by Contract W-31-109-Eng-78 with the United States Atomic Energy Commission, Report COO-78-230. A preliminary report of this work has been presented (1).
above, and RNA and DNA were then separated by the Schmidt-
Thannhauser procedure (10). Both RNA and DNA were then
concentrated under reduced pressure and subjected to hydrolysis
and base separation as described subsequently.

Extraction of Native DNA—In experiments involving the
incorporation of methionine methyl-3H or phosphate-3P, a more
rigorous purification of DNA was employed. Total DNA was
extracted according to the method described by Evans (11).
The heavy satellite DNA and mitochondrial DNA were se-
lectively extracted in some experiments by the method of
Braun and Evans (12). In experiments involving the incorpora-
tion of 3P, the extracted DNA was subjected to chromatog-
raphy on a column, 2 X 15 cm, of Sepharose 6-B (Pharmacia)
at 40° to separate the DNA from radioactive low molecular
weight contaminants. The DNA samples were added to
solutions of Radio-Tracer Grade CsCl (Harshaw Chemical
Company, Cleveland, Ohio) which were subsequently adjusted
to the appropriate density (1.701 to 1.712 g per ml) and con-
centrated under reduced pressure and subjected to hydrolysis
and chromatographic identification of these compounds. The
DNA, or RNA, was hydrolyzed to free bases by heating for 1 hour
in the presence of 0.04 ml of 70% HClO4 at 100°. The hydrolysate
was centrifuged and the supernatant solution was subjected to
two-dimensional chromatography on Whatman No. 4 chroma-
tographic paper, with the use of isopropyl alcohol-HCl-H2O
(66:1:33) (15) as the solvent. The separated deoxyribonucleotides
were eluted from the paper with H2O, concentrated under reduced
pressure, and chromatographed on a strip of Whatman No. 4 paper with
isobutyric acid-concentrated NH4OH-H2O (66:1:33) (15) as the
solvent. The separated deoxyribonucleotides were eluted from
the paper with H2O, concentrated under reduced pressure, and

Hydrolysis and Chromatographic Separation of Bases and
Nucleotides—Before hydrolysis of the isolated DNA samples,
nonradioactive bulk Physarum DNA was added to bring the
total amount of DNA to 0.5 to 1.0 mg. Carrier 5-methylcyto-
sine (0.42 pmole) or 5-methylcytosine deoxyribonucleotide
(0.15 pmole) was also added to facilitate subsequent chromatographic
identification of these compounds. The DNA, or RNA, was hydrolyzed
to yield free bases by heating for 1 hour in the
presence of 0.04 ml of 70% HClO4 at 100°. The hydrolysate
was centrifuged and the supernatant solution was subjected to
two-dimensional chromatography on Whatman No. 4 chroma-
tography paper, with the use of isopropyl alcohol-HCl-H2O
(170:41:39) (13) in the first direction and 86% butanol-H2O
(140:27:11) (16) in the second direction. Adenine,
thymine, cytosine, and 5-methylcytosine were eluted with 0.1
M HCl, and guanine was eluted with 2 M HCl. When complete
separation of the bases was not obtained, chromatography was
repeated with the isopropyl alcohol-HCl-H2O solvent system.
The absorbance at 260 nm and the radioactivity of the eluted
bases were then determined.

DNA was hydrolyzed to deoxyribonucleotides by enzymatic
digestion with DNase I (EC 3.1.4.5, Worthington) and snake
venom phosphodiesterase (EC 3.1.4.1, Worthington). DNase
I (100 µg) and 1 mg of DNA were dissolved in a 1-ml solution
composed of ammonium acetate (0.01 M, pH 7.0), MgCl2 (0.025
M), and NaF (0.025 M). The solution, which was incubated for
2 hours at 37° was then heated rapidly to 70° and maintained at
this temperature for 5 min. After cooling, the pH was adjusted
to 9.0 with NH4OH, phosphodiesterase (0.123 mg) was added,
and the solution was incubated first at 37° for 90 min and then
at 70° for 5 min. After clarification by centrifugation, the
supernatant solution was concentrated under reduced pressure
and chromatographed on a strip of Whatman No. 4 paper with
isobutyric acid-concentrated NH4OH-H2O (96:1:33) (15) as the
solvent. The separated deoxyribonucleotides were eluted from
the paper with H2O, concentrated under reduced pressure, and

Fig. 1. Separation of the DNA fractions of Physarum by CsCl
density gradient centrifugation. A, first density gradient cen-
trifugation of bulk DNA extracted from a shake flask incubated
for 24 hours with 10 µCi per ml of methionine methyl-3H. Fra-
cions 10 to 12 were combined for the heavy satellite DNA, Fra-
cions 14 to 18 for major nuclear DNA, and Fractions 24 to 28 for
mitochondrial DNA. The increase in absorbance in Fractions 34
to 40 is due to a polysaccharide contaminant of the DNA prepara-
tion. The initial density of the CsCl solution was 1.707 g per ml.
B, third and final density gradient centrifugation in the purifica-
tion of mitochondrial DNA. Fractions 20 to 25 were combined
for base analysis. Nonradioactive bulk Physarum DNA, 100 µg,
was added as an absorbance marker. The initial density of the
CsCl solution was 1.707 g per ml. C, third and final density
gradient centrifugation in the purification of the heavy nuclear
satellite DNA. Fractions 21 to 24 were combined for base analy-
sis. Nonradioactive bulk Physarum DNA, 100 µg, was added as
an absorbance marker. The initial density of the CsCl solution
was 1.712 g per ml.
DNA Methylation during Mitotic Cycle of Physarum

DNA was isolated from a shake culture incubated for 24 hours in the presence of 5 μCi per ml of methionine methyl-\(^3\)H.

### TABLE I

| Base                  | % total DNA radioactivity |
|-----------------------|----------------------------|
| Guanine               | 1.4                         |
| Adenine               | 26.8                        |
| Thymine               | 24.0                        |
| Cytosine              | 0.2                         |
| 5-Methylcytosine\(^c\) | 47.6                        |
| 6-Methylaminopurine\(^e\) | 0                       |

\(^a\) Total \(^3\)H in the bases was 1248 cpm.

\(^b\) Since no correction for recovery of bases was made in this experiment, the low amount of \(^3\)H found in guanine might be accounted for by low recovery of this base.

\(^c\) Added to the hydrolysate for visualization upon chromatography.

### TABLE II

Incorporation of methionine methyl-\(^3\)H into amino acids of total protein of Physarum

Total protein was isolated from a shake culture incubated for 24 hours in the presence of 10 μCi per ml of methionine methyl-\(^3\)H. Protein, 2 mg, was subjected to amino acid analysis.

| Amino acid              | Amount | Radioactivity | Specific activity |
|-------------------------|--------|---------------|-------------------|
|                         | μmoles | cpm           | cpm/μmole         |
| **Short column**        |        |               |                   |
| Unknown 1               | 4.88   | 799           |                   |
| Lysine                  | 0.81   | 837           | 1,030             |
| Histidine               | 0.26   | 221           | 850               |
| NH\(_3\)                 | 1.24   | 0             | 0                 |
| Unknown 2               | 0.54   | 182           |                   |
| Arginine                | 0.54   | 0             | 0                 |
| **Long column**         |        |               |                   |
| Unknown 3               | 63     |               |                   |
| Methionine sulfoxides   | 0.02   | 227           | 11,350            |
| Aspartic acid           | 1.21   | 85            | 70                |
| Threonine               | 0.63   | 0             | 0                 |
| Serine                  | 0.50   | 502           | 626               |
| Glutamic acid           | 0.56   | 0             | 0                 |
| Proline                 | 0.57   | 0             | 0                 |
| Glycine                 | 1.02   | 0             | 0                 |
| Alanine                 | 1.12   | 0             | 0                 |
| Valine                  | 0.45   | 0             | 0                 |
| Methionine              | 0.22   | 7,163         | 39,500            |
| Isoleucine              | 0.34   | 0             | 0                 |
| Leucine                 | 1.01   | 0             | 0                 |
| Unknown 4               | 0.67   | 697           |                   |
| Tyrosine                | 0.37   | 0             | 0                 |
| Phenylalanine           | 0.48   | 0             | 0                 |

HCl and H\(_2\)O. Material adsorbed on the charcoal was eluted with 50% ethanol-0.15 M NH\(_4\)OH, the eluate was concentrated under reduced pressure, and the residue was hydrolyzed by heating with 70% HClO\(_4\) at 100° for 1 hour. After two-dimensional chromatography as described in the preceding section, adenine was eluted and assayed for ultraviolet absorbance and radioactivity.

### TABLE III

Measurement of Radioactivity—Radioisotopes were assayed by liquid scintillation counting in a solution containing 125 g of naphthalene, 67.6 g of Omnifluor (New England Nuclear), 100 ml of absolute methanol, and 40 g of Cab-O-Sil (Cabot Corporation, Boston, Massachusetts) per liter of p-dioxane. Counts were corrected for quenching by means of an external standard.

### RESULTS

DNA Bases Labeled after Incubation of Physarum with Methionine Methyl-\(^3\)H—In an initial experiment, the occurrence of the methylation of DNA in Physarum was determined. A shake culture was incubated with methionine methyl-\(^3\)H for 24 hours, and the distribution of \(^3\)H in the individual bases of major nuclear DNA was determined. The results are shown in Table I. Most of the radioactivity was located in adenine, thymine, and 5-methylcytosine. In this experiment, 6-methylaminopurine, in addition to 5-methylcytosine, was added to the hydrolysate before chromatography. No \(^3\)H was found in the re-isolated 6-methylaminopurine. In order to detect contamination of adenine with other methylated minor bases, the adenine fraction was further chromatographed with 86% butanol-H\(_2\)O (14), 1-propanol-HCl-H\(_2\)O (32:5:18) (18), and isopropanol alcohol-NH\(_4\)OH-0.1 M H\(_2\)BO\(_3\) (30:5:18) (18). The \(^3\)H remained with the adenine in all of the chromatograms. Thus, no radioactive methylated minor base other than 5-methylcytosine was detected in major nuclear DNA after incubation of Physarum with methionine methyl-\(^3\)H.

Conversion of Methionine Methyl Group to 1-Carbon Intermediates—It is probable that the radioactivity incorporated into DNA adenine, -guanine, and -thymine arises through conversion of the methyl group of methionine to 1-carbon intermediates utilized subsequently in the formation of purine and thymine nucleotides which would be incorporated into DNA during its synthesis de novo. The occurrence of these reactions in Physarum was indicated indirectly by the finding that \(^3\)H from methionine methyl-\(^3\)H was incorporated into serine of total protein and into acid-soluble adenine compounds. Amino acid analysis of the total protein and the distribution of \(^3\)H in the amino acids are shown in Table II. The labeled fractions were methionine, methionine sulfoxides, serine, aspartic acid, lysine, histidine, two unknown basic amino acids, and two unknown ninhydrin-negative fractions. The \(^3\)H in the lysine and histidine is probably due to the presence in these fractions of methylated lysine and methylated histidine, which have been found to occur in the proteins of a variety of tissues and which would not be separated from lysine and histidine under the...
conditions of our chromatography (19). Aspartic acid might be labeled by conversion of serine to pyruvate, oxalacetate, and aspartate. Unknowns 1 and 2, which were ninhydrin-positive, were eluted with the basic amino acids from the short column in the order listed. Unknowns 3 and 4, which were ninhydrin-negative, were eluted from the long column in the order listed.

To investigate the incorporation of \(^{3}H\) from methionine methyl-\(^{3}H\) into acid-soluble adenine compounds, synchronously dividing plasmodia were incubated with \(38 \, \text{aCi per ml of methionine methyl-}^{3}H\) during the \(S\) period. Total acid-soluble adenine was found to have a specific activity of \(30,000 \, \text{cpm per \(\mu\)mole}, while the specific activity of DNA-adenine was \(2,400 \, \text{cpm per \(\mu\)mole}.

**DNA Methylation during Mitotic Cycle**—The occurrence of DNA methylation at various periods throughout the mitotic cycle was determined by incubating macroplasmodia with methionine methyl-\(^{3}H\) for successive periods of 14 hours each following mitosis. Two small portions of each plasmodium were also incubated in media containing thymidine methyl-\(^{3}H\) for successive periods of 3, 7, 12, and 24 hours following mitosis. Two small portions of each plasmodium were also incubated in media containing thymidine methyl-\(^{3}H\) for successive periods of 14 hours each following mitosis. Two small portions of each plasmodium were also incubated in media containing thymidine methyl-\(^{3}H\) for successive periods of 3, 7, 12, and 24 hours following mitosis. Two small portions of each plasmodium were also incubated in media containing thymidine methyl-\(^{3}H\) for successive periods of 14 hours each following mitosis.

Incorporation of thymidine methyl-\(^{3}H\) into bulk Physarum DNA during various periods of mitotic cycle

| Time in cycle | Specific activity of DNA base |
|---------------|-------------------------------|
| After Mitosis II | Phase | 5-Methylcytosine | Cytosine | Thymine | Guanine |
| brs           | 0-11/2 | S       | 17.5 | 29.2 | 1.4 | 11.5 | 37.5 |
|               | 11/2-3 | S-G2    | 9.9 | 4.0 | 1.3 | 2.7 | 4.7 |
|               | 3-41/2 | G2      | 7.6 | 2.7 | 2.1 | 6.0 | 2.1 |
|               | 41/2-6 | G2      | 8.0 | 1.6 | 1.8 | 6.0 | 2.1 |
|               | 6-71/2 | G2-S    | 9.1 | 22.1 | 7.3 | 29.1 |

** TABLE V **

Incorporation of formate-\(^{14}C\) into Physarum nucleic acids during various periods of mitotic cycle

| Time in cycle | Specific activity $^a$ |
|---------------|------------------------|
| After Mitosis II | Phase | RNA-adenine | DNA-adenine | DNA-thymine |
| brs           | 0-11/2 | S       | 53.8 | 81.0 | 47.0 |
|               | 11/2-3 | S-G2    | 50.9 | 10.4 | 12.8 |
|               | 3-41/2 | G2      | 71.9 | 7.4  | 4.1  |
|               | 41/2-6 | G2      | 47.2 | 12.7 | 10.1 |
|               | 6-71/2 | G2-S    | 33.6 | 94.0 | 31.0 |

$^a$ No \(^{14}C\) was found in DNA-5-methylcytosine
TABLE VI

Incorporation of $^3$H of methionine methyl-$^3$H into three DNA components of Physoarum during G2 period

Synchronously dividing plasmodia were incubated in medium containing 30 μCi per ml of methionine methyl-$^3$H during the G2 period (3 to 6 hours following Mitosis II).

| DNA fraction          | Specific activity | 5-Methylcytosine | Adenine | Thymine | Guanine |
|-----------------------|-------------------|------------------|---------|---------|---------|
|                       | cpm/μg DNA        | cpm/munole base  |         |         |         |
| Major nuclear         | 9.0               | 2.5              | 1.4     | 0.9     |         |
| Mitochondrial$^a$     | 2.3               | 5.2              | 12.4    |         |         |
| Nuclear satellite$^a$ | 2.9               | 10.7             | 24.0    | 2.2     |         |

$^a$ Specific activities may be inaccurate as a result of the large dilution factor caused by adding a large amount of carrier DNA to a very small amount of labeled material.

TABLE VII

5-Methylcytosine content of three DNA components of Physoarum estimated from $^{32}$P content after uniform labeling

| DNA Fraction         | Experiment number | Total radioactivity | dCMP | dMCMP$^a$ | dMCMP$^b$ |
|----------------------|-------------------|---------------------|------|-----------|-----------|
|                      |                   | cpm                | cpm  | cpm       | cpm       |
| Major nuclear        | 1                 | 652                | 18   | 36        |           |
|                      | 2                 | 3,541              | 310  | 13        |           |
|                      | 3                 | 2,586              | 73   | 35        |           |
|                      | 4                 | 463                | 22   | 21        |           |
|                      | 5                 | 41,248             | 3,250| 15        |           |
|                      | 6                 | 15,740             | 706  | 22        |           |
|                      |                   | Mean = 24 ± 8     |      |           |           |
| Mitochondrial        | 1                 | 805                | 71   | 12        |           |
| Nuclear satellite$^a$| 1                 | 440                | 4.2  | 33        |           |
|                      | 3                 | 540                | 40.6 | 13        |           |
|                      |                   | Mean = 23 ± 10     |      |           |           |

$^a$ Counts in dMCMP were corrected for contamination with dCMP as described in the text.

The 10- to 20-fold decrease in the incorporation into major nuclear DNA-purines during the G2 period as compared with the S period also suggests that this incorporation involves synthesis de novo rather than methylation of the pre-formed DNA polymer. The appearance of $^3$H in DNA-thymine might be explained either by conversion of the methionine methyl group to 1-carbon intermediates incorporated into thymidylic acid and then into DNA during synthesis de novo or by deamination of pre-formed DNA-5-methylcytosine. Sneider and Potter (5) have suggested that deamination of DNA-5-methylcytosine might occur in vivo, producing mispairing and possible initiation sites on the DNA template for replication or transcription. Burdon and Adams (6), however, found that DNA-5-methylcytosine-$^{14}$C remained stable for 7 generations following removal of the precursor. It is also possible that deamination of DNA-5-methylcytosine could occur in vitro during hydrolysis of the DNA to free bases. In Physoarum, incorporation of $^3$H from methionine methyl-$^3$H into DNA-thymine appears to be due in part to synthesis de novo of DNA from previously formed $^3$H-TMP, since inclusion of nonradioactive thymidine, fluorodeoxyuridine, and uridine in the medium reduced the incorporation into DNA-thymine by 90%, while not affecting the incorporation of $^3$H from methionine methyl-$^3$H into DNA-5-methylcytosine or the incorporation of $^{32}$P into bulk DNA. The question of whether or not $^3$H from methionine methyl-$^3$H is incorporated into DNA-thymine in Physoarum by an additional mechanism, such as deamination of pre-formed DNA-5-methylcytosine, must await further investigation.

The occurrence of methylation of the major nuclear DNA during the G2 period, in the absence of synthesis de novo of this DNA, is of interest when considering the possible function of DNA methylation. Methylation of DNA has been shown to be involved in bacterial modification-restriction processes (21), in which infecting phage DNA, methylated by permissive host enzymes, becomes resistant to host endonuclease degradation. If DNA methylation is involved with the control of nuclease action in eukaryotic organisms, the process might serve as a negative control of transcription or replication initiator endonucleases, if such exist.

DISCUSSION

The incorporation of $^3$H of methionine methyl-$^3$H into Physoarum DNA apparently involves two mechanisms: (a) conversion of the methionine methyl group to 1-carbon intermediates that are subsequently utilized for the formation of purine and thymine nucleotide precursors of DNA, and (b) methylation of cytosine residues of the pre-formed DNA polymer. Formation of 1-carbon intermediates in this organism is indicated by the experiments demonstrating the incorporation of the label into serine of the total protein and into acid-soluble adenine compounds.
12. Braun, R., and Evans, T., Biochim. Biophys. Acta, 182, 511 (1969).
13. Wyatt, G. R., Biochem. J., 48, 534 (1951).
14. Markham, R., and Smith, J. P., Biochem. J., 45, 294 (1949).
15. Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E., J. Biol. Chem., 186, 37 (1950).
16. Greenberg, D. M., and Rothstein, M., in S. P. Colowick and N. R. Kaplan (Editors), Methods in enzymology, Vol. IV, Academic Press, New York, 1957, p. 708.
17. Moore, S., and Stein, W. H., in S. P. Colowick and N. R. Kaplan (Editors), Methods in enzymology, vol. VI, Academic Press, New York, 1963, p. 819.
18. Marus, B. L., Doctoral dissertation, Western Reserve University, 1963.
19. Gershey, E. L., Haslett, G. W., Vidal, G., and Allfrey, V. G., J. Biol. Chem., 244, 4871 (1969).
20. Mittermayer, C., Braun, R., and Rusch, H. P., Biochim. Biophys. Acta, 91, 399 (1964).
21. Kühnlein, U., Linn, S., and Arber, W., Proc. Nat. Acad. Sci. U. S. A., 63, 556 (1969).
Methylation of the Deoxyribonucleic Acid of Physarum polycephalum at Various Periods during the Mitotic Cycle
Helen H. Evans and Thomas E. Evans

J. Biol. Chem. 1970, 245:6436-6441.

Access the most updated version of this article at http://www.jbc.org/content/245/23/6436

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/23/6436.full.html#ref-list-1