Esnault, C., Roques, B. P., Jacquemin-Sablon, A., & Le Pecq, J. B. (1984) *Cancer Res. 44*, 4355.
Gaugain, B., Barbet, J., Capelle, N., Roques, B. P., Le Pecq, J. B., & Le Bret, M. (1978) *Biochemistry 17*, 5079.
Gaugain, B., Markovits, J., Le Pecq, J. B., & Roques, B. P. (1981) *Biochemistry 20*, 3035.
Gaugain, B., Markovits, J., Le Pecq, J. B., & Roques, B. P. (1984) *FEBS Lett. 169*, 123.
Guillerez, J. (1984) Doctorat d'Etat ès Sciences, Université Paris VII.
Hansen, J. B., & Buchardt, O. (1983) *J. Chem. Soc., Chem. Commun.*, 162.
Hansen, J. B., Thomsen, T., & Buchardt, O. (1983) *J. Chem. Soc., Chem. Commun.*, 1015.
Hélène, C. (1977) *FEBS Lett. 74*, 10.
Hinkle, D. C., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157.
Irvin, J. L., & Irvin, E. M. (1950) *J. Am. Chem. Soc. 72*, 2743.
Jain, S. C., & Sobell, H. M. (1972) *J. Mol. Biol. 68*, 1.
Le Pecq, J. B., Le Bret, M., Barbet, J., & Roques, B. P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2915.
Lin, S. R., & Riggs, A. D. (1972) *J. Mol. Biol. 72*, 671.
Markovits, J., Gaugain, B., Barbet, J., Roques, B. P., & Le Pecq, J. B. (1981a) *Biochemistry 20*, 3042.
Markovits, J., Gaugain, B., Roques, B. P., & Le Pecq, J. B. (1981b) *Jerusalem Symp. Quantum Chem. Biochem. 14*, 285–298.
Markovits, J., Ramstein, J., Roques, B. P., & Le Pecq, J. B. (1983) *Biochemistry 22*, 3231.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431.
McGhee, J. D., & Von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469.
Mirau, P. A., & Kearns, D. R. (1983) *Nucleic Acids Res. 11*, 1931.
Müller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251.
Müller, W., & Crothers, D. M. (1975) *Eur. J. Biochem. 54*, 267.
Ohlendorf, D. H., Anderson, W. F., Fisher, R. G., Takeda, Y., & Matthews, B. W. (1982) *Nature (London) 298*, 718.
Pelaprat, D., Delbarre, A., Le Guen, I., Roques, B. P., & Le Pecq, J. B. (1980) *J. Med. Chem. 23*, 1336.
Record, M. T., Jr., Lohman, T. M., & De Haseth, P. (1976) *J. Mol. Biol. 107*, 145.
Revet, B., Schmir, M., & Vinograd, J. (1971) *Nature (London), New Biol. 229*, 10.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67.
Roques, B. P., Pelaprat, D., Le Guen, I., Porcher, G., Gosse, C., & Le Pecq, J. B. (1979) *Biochem. Pharmacol. 28*, 1811.
Rosowsky, A., Chen, K. K. N., & Papathanasopoulos, P. (1976) *J. Heteterocycl. Chem. 13*, 727.
Saucier, J. M., Festy, B., & Le Pecq, J. B. (1971) *Biochimie 53*, 973.
Schwartz, G. (1968) *Rev. Mod. Phys. 40*, 206.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465.

DNA Methylation in Mammalian Nuclei†

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ABSTRACT: A novel system to study the methylation of newly synthesized DNA in isolated nuclei was developed. Approximately 2.5% of cytosine residues incorporated into nascent DNA became methylated by endogenous methylase(s), and the level of DNA modification was reduced by methylation inhibitors. DNA synthesis and methylation were dependent on separate cytosol factors. The cytosol factor or factors required for DNA methylation were sensitive to trypsin digestion and were precipitable by $(NH_4)_2SO_4$, suggesting that they were proteinaceous. Time-course experiments revealed a short lag of approximately 20 s between synthesis and methylation in nuclei. The DNAs produced in these nuclei were a mixed population of low molecular weight fragments and higher molecular weight fragments shown to be short extensions of existing replicons. The methylation level found in low molecular weight DNA was lower than that found in bulk L1210 DNA, indicating that further methylation events might take place after ligation of small fragments. These data suggest that newly synthesized DNA is a good substrate for methylase enzymes and that nuclear cytoplasmic interactions may be important in controlling inheritance of methylation patterns.

Alteration of cytosine methylation patterns present in vertebrate DNA has been suggested to be a necessary but not sufficient requirement for altering gene expression (Razin & Riggs, 1980; Doerfler, 1983). The majority of the methylcytosine residues are found in CG sequences (Doskocil & Sorm, 1962), which are underrepresented in eukaryotic DNA (Josse et al., 1961). Hemimethylated sites can be converted into symmetrically methylated sites by "maintenance methylases", thus maintaining the fidelity of the methylated state (Pfeifer et al., 1983).

Studies in many laboratories using inhibitors of 5-methylcytosine formation have shown an inverse correlation between methylation and gene expression (Jones, 1985). This inverse

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correlation between DNA methylation and gene activation cannot, however, be generalized to every case. Studies of the vitellogenin gene in chicken liver showed that changes in methylation pattern occurred considerably later than when transcriptional activation of the gene was induced by estrogen (Wilks et al., 1984).

Although DNA methylation has been intensely studied for the past decade, many questions still remain unresolved, and the mechanisms involved in the formation of methylation patterns of DNA are poorly defined. Several reports propose a close coupling between synthesis and methylation. Observations made in CHEF/18 cells suggest that the processes of DNA synthesis and methylation are both carried out by an enzyme complex termed a "replitase" (Noguchi et al., 1983). Other studies suggest that methylation can be divided into two stages, one which lags shortly behind DNA synthesis (Gruenbaum et al., 1983) and a later stage in which the remaining sites are finally methylated (Woodcock et al., 1982). In addition, little is known about the role of the cytoplasm in the development of methylation patterns.

Further understanding of the mechanisms responsible for the inheritance of methylation patterns in replicating chromatin would be facilitated by the availability of subcellular systems in which these complex-coordinated reactions can be dissected. We have therefore developed a DNA replication system using isolated nuclei that is capable of methylating newly synthesized DNA in the presence of cytoplasmic factors. Methylation of replicating DNA was found to lag 20 s or less behind DNA synthesis, such that DNA modification occurred primarily on DNA undergoing continuous synthesis on preexisting replicons. DNA synthesized noncontinuously was not as good a substrate for methylation. On the basis of our observations we propose a model to explain the temporal order of events taking place on newly synthesized DNA as they relate to DNA methylation.

Materials and Methods

Preparation and Treatment of L1210 Cells. Male CD2F1 mice (Charles River Labs, Wilmington, MA) were injected intraperitoneally with 5×10^5 L1210 cells in 0.2 mL of PBS,[1] and mice were kept for 7 days before cells were harvested. Intraperitoneal injections with 0.2 mL of either 5-aza-CR (1 mg/kg) in 0.45% NaCl, or salt solution alone, were administered 18 h before cells were harvested (10^8 cells/mouse).

DNA Replication Assay. All chemicals and supplies were purchased from Sigma (St. Louis, MO), unless otherwise specified. Nuclei and cytosol fractions were prepared from approximately (1–4) $\times 10^9$ L1210 cells by the method of Hershey et al. (1973). Cells were lysed in hypotonic buffer B [10 mM Tris-HCl, 4 mM $MgCl_2$, and 1 mM EDTA (pH 7.8)] by using 26 strokes of a glass Dounce homogenizer. Nuclei were pelleted by centrifugation for 10 min at 800*g* at 4 °C. The supernatant from the above procedure was centrifuged for 1 h in an SW 50.1 rotor (Beckman, Irvine, CA) at 200000*g* at 4 °C, and the resulting supernatant was termed the cytosol fraction.

The concentration of dCTP present in the cytosol was determined by the method of Lowe & Grindey (1976) to be 15 ± 6 pmol/10^6 cells. This value allowed us to determine the specific activity of [^{32}P]dCTP in the assay and to calculate the in vitro rate of synthesis.

Isolated nuclei (10^6) were incubated in a reaction mixture containing 33 mM HEPES (pH 7.8), 50 mM NaCl, 6.7 mM $MgCl_2$, 5 mM ATP, 100 μM dGTP, 100 μM dTTP, 100 μM dATP, 50 μM SAM, 0.28 μM [^{32}P]dCTP (800–2000 Ci/mmol; ICN, Irvine, CA), and 40 μL of cytosol fraction in a final volume of 90 μL. In one set of experiments 5 μg of activated DNA (calf thymus DNA nicked with DNase I from Sigma, St. Louis, MO) was substituted for nuclei. The reaction mixture was incubated for 1 h at 37 °C unless otherwise specified.

Reactions were terminated by the addition of 7 μL of 5 M NaOH and 11 μL of 0.2 M EDTA (pH 8.0) and incubated for 1 h at 65 °C. The mixture was neutralized by the addition of 7 μL of 5 M HCl and 20 μL of 0.1 M Tris-HCl (pH 7.6). Proteinase K (20 μL, 7.5 mg/mL) was added and the mixture incubated 1 h at 65 °C. DNA was precipitated in 10% TCA and washed 3 times in 5% TCA followed by washing in 95% ethanol. The amount of ^{32}P incorporation was determined by hydrolysis of the samples in 5% TCA for 30 min at 100 °C. The radioactivity present in the acid-soluble material was determined by liquid scintillation counting in 5 mL of Safety Solve (Research Products International Corp., Mount Prospect, IL).

Sedimentation Analysis of Newly Synthesized DNA. L1210 cells were harvested from mice and preincubated with BUdR (10 μg/mL) for 2 h at 37 °C in RPMI media containing 10% heat-inactivated fetal calf serum. Nuclei were isolated and incubated in the DNA replication assay for 30 min at 37 °C. Reactions were stopped by addition of 20 μL of 0.2 M EDTA and chilled on ice. One of the samples was irradiated with thymidine-filtered 313-nm light for 1 h as previously described (Plank & Mueller, 1977), while the other sample was left to stand in the dark.

Assays were layered on top of 5–20% alkaline sucrose gradients of 30 mL, containing 0.3 M NaOH, 0.5 M NaCl, and 1 mM EDTA, over a 4-mL cushion of 2 M sucrose. After being allowed to stand for 6 h at 40 °C, samples were centrifuged for 8 h in an SW 27 rotor (Beckman) at 90000*g* at 20 °C. Gradients were fractionated by piercing the tube bottoms with an 18-gauge needle and collecting 16-drop fractions.

Determination of DNA Methylation Levels. DNA precipitated in 5% TCA from the above assay was digested with DNase I and snake venom phosphodiesterase as previously described (Christman, 1982). Samples were applied to cellulose plates (E. Merck, Darmstadt, Germany) along with dCMP and dmCMP standards (10 μg each for UV detection) and separated by two-dimensional thin-layer chromatography as previously described (Gruenbaum et al., 1981). Plates were developed twice in isobutyric acid–water-concentrated ammonium hydroxide (66:20:1) followed by saturated ammonium sulfate–1 M sodium acetate–2-propanol (80:18:2) in the second dimension. Autoradiographs of each plate were made by using XRP-5 film (Kodak, Rochester, NY), and the exposed areas corresponding to dCMP and dmCMP were scraped and extracted in 0.1 M HCl (1.0 mL) and dissolved in 15 mL of Saftey Solve. The radioactivity extracted from a randomly

[1] Abbreviations: PBS, 0.227 M KCl, 0.015 M KH_2PO_4, 0.080 M Na_2HPO_4, and 1.37 M NaCl; 5-aza-CR, 5-azacytidine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; dCTP, 2′-deoxycytidine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ATP, adenosine 5′-triphosphate; dGTP, 2′- deoxyguanosine 5′-triphosphate; dTTP, thymidine 5′-triphosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; SAM, *S*-adenosyl-L-methionine; TCA, trichloroacetic acid; dCMP, 2′-deoxycytidine 5′-monophosphate; BUdR, bromodeoxyuridine; dmCMP, 2′-deoxymethylcytidine 5′-monophosphate; SSC, 0.15 M NaCl and 0.015 M sodium citrate; *ara*-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; HPLC, high-performance liquid chromatography; SAE, *S*-adenosyl-L-ethionine; SAH, *S*-adenosyl-L-homocysteine.

selected area of similar size was subtracted from the values of radioactivity found for dmCMP and dCMP on the plate. To determine the percentage of DNA methylation, the normalized dmCMP radioactivity was divided by the sum of the normalized dmCMP and dCMP radioactivities.

Trypsinization of Cytosol. Cytosol isolated from L1210 cells was incubated for 1 h at 37 °C with either trypsin (200 μg/mL) or soybean trypsin inhibitor (2 mg/mL). After 1 h, soybean trypsin inhibitor (2 mg/mL) was added to the trypsinized cytosol, and both samples were added to complete replication assays. Each assay was carried out at 37 °C for 1 h and the percent methylcytosines incorporated into newly synthesized DNA determined.

Ammonium Sulfate Precipitation of Cytosol. Cytosol was precipitated with sequentially increasing percentages (40, 60, and 100% saturation) of $(NH_4)_2SO_4$ and pelleted by centrifugation for 10 min at 1200*g* at 4 °C. These pellets were resuspended in buffer B and chromatographed on a Sephadex G-50 column to remove salt. Fractions were collected (0.5 mL), and those fractions that contained protein ($OD_{280} > 2.5$) were pooled. Approximately 100 μg of protein, as determined by the method of Hartree (1972), from each fraction was added to replication assays and incubated 1 h at 37 °C. The methylation activity for each sample was determined.

Analysis of Methylation Levels of Different DNA Size Classes. Six gradients derived from the above procedure were pooled into two fractions, those consisting of low molecular weight DNA (fractions 24–29) and those of higher molecular weight DNA (fractions 12–22). These samples were dialyzed extensively against 0.1 × SSC by using dialysis tubing with a M_r 3500 cutoff (Spectrapore, Los Angeles, CA). Samples were made >70% with respect to ethanol and centrifuged 1 h in a Ti60 rotor (Beckman) at 300000*g* at 4 °C. The precipitated material was resuspended in 300 μL of 0.1 × SSC and again precipitated by addition of ethanol. The precipitate was resuspended in 100 μL of digestion buffer (50 mM Tris-HCl, pH 8.0, 5 mM potassium phosphate, pH 8.0, and 5 mM $MgCl_2$), digested with nucleases, and separated by two-dimensional thin-layer chromatography as described above. The levels of DNA methylation were then determined.

RESULTS

A replication system using nuclei isolated from growing L1210 cells was developed to study the methylation of newly synthesized DNA. The maximum rate of incorporation of deoxynucleoside triphosphates into acid-insoluble material required the presence of cytosol, and a lower rate of incorporation was seen in the absence of this fraction (Figure 1). The synthesis rate operated at approximately 5–7% of the in vivo rate and declined after 20 min.

The system was dependent upon the presence of all four nucleoside triphosphates, ATP, and divalent cations (Table I). These results resembled those reported for HeLa cell nuclei (Mueller et al., 1981), and depletion of a single nucleoside triphosphate (e.g., dCTP) did not inhibit DNA synthesis as severely as when several nucleoside triphosphates were omitted. *ara*-CTP strongly inhibited DNA synthesis in the absence of dCTP, and the inhibition was partially reversed by the addition of dCTP as has been found in HeLa nuclei (Wist et al., 1976). The variability seen among these assays probably reflected the different nucleotide concentrations present in individual cytosol preparations.

The size distribution of DNA synthesized in the assay was characterized by using previously described techniques (Plank & Mueller, 1977). These experiments (not shown) demonstrated the synthesis observed represented the extension of

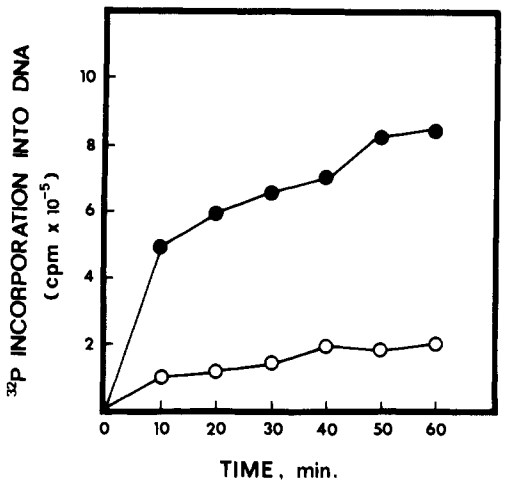

FIGURE 1: Effects of added cytoplasmic fraction on the kinetics of [^{32}P]dCTP incorporation into L1210 nuclei. Nuclei isolated from growing cells, were assayed for replication activity in the presence (●) or absence (○) of cytoplasmic fraction. Each reaction contained 10^6 nuclei, 20 μCi of [^{32}P]dCTP (0.07 μM) and other components of the reaction mixture as indicated in Table I. Reactions contained 40 μL of cytoplasmic fraction or buffer B in a final volume of 90 μL and were incubated at 37 °C at the times indicated. Results are expressed as the amount of [^{32}P]dCTP incorporated into the acid-precipitable material. The data are averages of two experiments run in triplicate.

Table I: Optimal Conditions for DNA Synthesis[a]

assay conditions	percentage of ^{32}P incorporated into DNA ± SD
complete system	100
-dATP, dTTP, dCTP	40 ± 20
-ATP	13 ± 7
+EDTA	2 ± 3
-dCTP	70 ± 10
+*ara*-CTP (100 μM) - dCTP	10 ± 1
+*ara*-CTP (100 μM) + dCTP	50 ± 5

[a] Nuclei prepared from L1210 cells were assayed for DNA synthetic activity in the presence or absence of added cofactors. The complete system contained 10^6 nuclei, 20 μCi of [^{32}P]dGTP (0.28 μM), 33 mM HEPES (pH 7.8), 50 mM NaCl, 6.7 mM $MgCl_2$, 5 mM ATP, 100 μM each of dATP, dCTP, and dTTP, and 40 μL of the cytoplasmic fraction in a final volume of 90 μL. Data are expressed as a percentage of the complete system which incorporated 218 000 cpm/40-min reaction at 37 °C. Results are expressed as averages of two experiments run in triplicate.

preexisting replicons (450–10 000 nucleotides) with some formation of short Okazaki fragments (100–450 nucleotides). These observations were consistent with and supported the contention that DNA synthesis took place within L1210 nuclei in this test tube reaction.

A two-dimensional thin-layer chromatography system to detect the methylation of incorporated deoxycytidine was also developed so that the relationship between DNA synthesis and methylation could be investigated. DNA isolated from mouse cells was nick translated in the presence of [α-^{32}P]dCTP (Maniatis et al., 1982) and was incubated for 1 h at 37 °C in reaction mixtures containing a crude maintenance methylase and SAM (Taylor & Jones, 1982). The reaction product was then digested sequentially with DNase I and snake venom phosphodiesterase, and the resulting nucleoside 5′-monophosphates were separated by two-dimensional thin-layer chromatography. This method separated dmCMP from dCMP (Figure 2), and the identity and homogeneity of the separated compounds were determined by using HPLC (data not shown). Other radioactive spots that were sometimes present on the plates and contained <5% of the total radio-

Table II: Factors Inhibiting DNA Methylation in Nuclei[a]

assay conditions	% [dmCMP/(dmCMP + dCMP)] ± SD	n	DNA polymerization ± SD (% of control)
complete system + SAM (50 μM)	2.5 ± 0.3	16	100
complete system (without added SAM)	2.5 ± 0.2	8	90 ± 2
complete system + SAM (50 μM) + DNA methyltransferase	1.0 ± 0.7	3	77 ± 22
complete system + SAM (50 μM) + nuclei from cells pretreated with 5-aza-CR	1.0 ± 0.4	4	80 ± 12
complete system (without added SAM) + SAE (100 μM)	1.7 ± 0.5	4	89 ± 14
complete system (without added SAM) + SAH (100 μM)	0.9 ± 0.3	4	93 ± 19
complete system − cytosol	0.3 ± 0.3	4	29 ± 13
complete system − cytosol + SAM (50 μM) + DNA methyltransferase	0.6 ± 0.2	4	52 ± 4
complete system − cytosol + albumin (1 mg/assay)	0.10 ± 0.10	3	47 ± 14
complete system (cytosol with soybean trypsin inhibitor and trypsin)	2.2 ± 0.2	3	90 ± 10
complete system (cytosol with trypsin)	0.9 ± 0.1	3	87 ± 27
complete system (without nuclei) + SAM (50 μM) + cytosol + activated DNA	0.13 ± 0.13	3	

[a] Nuclei prepared from L1210 cells were assayed for their abilities to methylate cytosine residues in vitro under various conditions. DNA polymerization was expressed as the percentage of [^{32}P]dCTP incorporated for each assay, relative to the complete system with added SAM. In one set of experiments activated DNA (5 μg/assay) was substituted for nuclei. The assay conditions were identical with those outlined for the complete system in Table I, except 20 μCi of [^{32}P]dCTP (2000 Ci/mmol) was substituted for labeled dGTP. Results are expressed as the percentage of dmCMP divided by the sum of both dmCMP and dCMP. Results are averages of n experiments.

activity were assumed to be deamination products resulting from impurities in the venom phosphodiesterase (Laskowski, 1966).

We next investigated whether cytosine residues incorporated into DNA in the nuclear system could be methylated by endogenous enzymes (Table II). The complete system, with or without added SAM, was capable of methylating a maximum level of 2.5% of incorporated cytosine residues. The levels of SAM in the cytosol fraction was apparently high enough so that additional SAM was not necessary. Addition of exogenous maintenance methylase did not increase the methylation up to the 3.62% level normally seen in L1210 cells (Wilson et al., 1983), rather it inhibited the methylation process. This inhibition may represent a competition between the added methylases and the endogenous methylases for methylation factors residing in the cytosol.

Several compounds known to inhibit DNA methylation in vivo also inhibited this process in isolated nuclei although they did not change the rate of DNA synthesis (Table II). If L1210 cells were preincubated with 5-aza-CR before the nuclei were isolated and added to the replication assay containing cytosol from untreated cells, the degree of methylation of newly synthesized DNA was decreased to 0.97%. Since previous studies have shown that incorporation of 5-aza-CR into DNA results in a loss of active methylase enzyme (Taylor & Jones, 1982; Creusot et al., 1982; Tanaka et al., 1980), this result suggested that the presence of the fraudulent base in DNA led to a loss of available enzyme in the nuclei. SAE and SAH also inhibited the methylation reaction at high concentrations, which was similar to the results seen in rat liver nuclei (Cox et al., 1977; Cox & Irving, 1977).

Deletion of cytosol not only drastically reduced DNA synthesis but also reduced DNA methylation (Table II). This decrease was not reversed significantly by addition of SAM and a crude preparation of DNA methylase, nor was it simply due to the loss of nonspecific proteins, since it was not overcome by the addition of albumin. Preincubation of cytosol with trypsin also reduced DNA methylation but did not significantly affect DNA synthesis. Therefore, the cytosolic factors necessary for DNA methylation were proteins and were more sensitive to trypsin digestion than the factors required for DNA synthesis.

Incubation of purified activated DNA with cytosol showed that cytosol contained high levels of polymerase activity (result not shown) but did not contain methylase activity, since no methylation of newly synthesized DNA occurred (Table II).

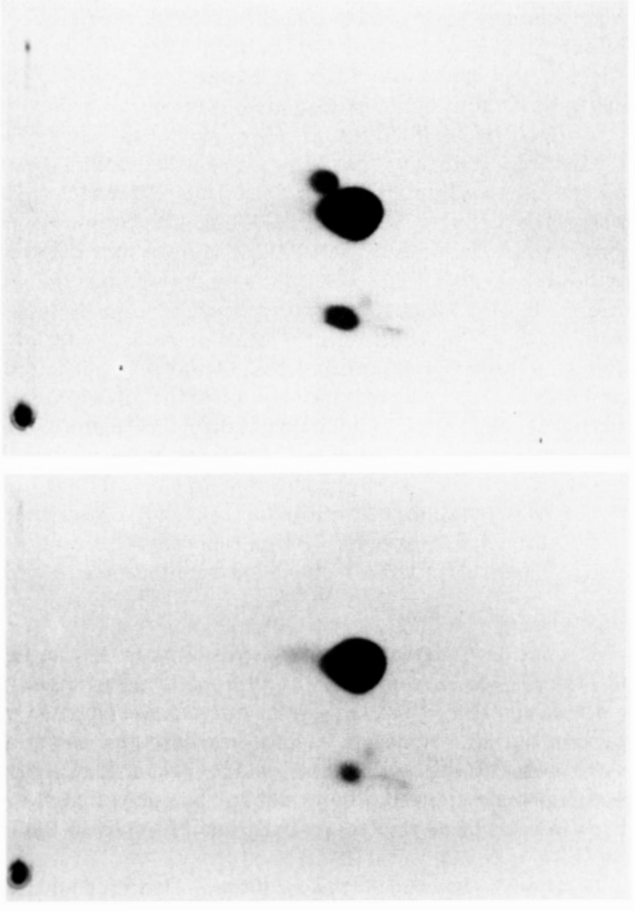

FIGURE 2: Separation of dCMP and dmCMP using two-dimensional thin-layer chromatography. DNA nick translated with [^{32}P]dCTP was incubated with SAM in the presence (top) or absence (bottom) of a crude maintenance methylase for 1 h. The DNA was digested with nucleases and deoxynucleoside monophosphates separated on cellulose plates using a two-dimensional buffer system. The autoradiographs shown were exposed for 6 h at room temperature.

Thus, DNA synthesized in nuclei became methylated by an endogenous methylase in the presence of cytoplasmic proteins. However, the cytoplasmic proteins themselves were not capable of methylating DNA.

Cytosolic proteins from L1210 cells were partially purified by precipitating with $(NH_4)_2SO_4$, and the resulting fractions were desalted and added to replication assays (Table III). The proteins required for methylation of newly replicated DNA

Table III: Partial Purification of Cytosolic Factor(s) Required for Methylation[a]

sample	total protein (mg)	total activity [nmol of CH_3 (mg of DNA)$^{-1}$ h^{-1}]	sp act. of [nmol of CH_3 (mg of DNA)$^{-1}$ h^{-1} (mg of protein)$^{-1}$]	yield (%)
cytosol	40	2000	50	
0–40%	18.7	48	2.5	3
40–60%	13.6	1300	94	65
60–100%	3.3	5	1.5	0.3

[a] The cytosolic factor(s) necessary for methylation of newly replicating DNA in L1210 nuclei were partially purified by precipitation with $(NH_4)_2SO_4$. These pellets were desalted by elution through a Sephadex G-50 column and added to the replication assay (100 μg of protein/assay). The methylation activity in each fraction was reported as nanomoles of CH_3 incorporated per milligram of DNA synthesized per hour, and the specific activity was that value divided by the milligrams of protein. These data represent the average of three separate experiments.

were precipitated only after the salt concentration reached 40% saturation and precipitation past 60% saturation did not increase the yield of proteins which aided the methylation process. Thus, $(NH_4)_2SO_4$ precipitation was a good first method for the purification of the proteins.

Overall the results showed that DNA was methylated during DNA synthesis in nuclei and that agents known to inhibit DNA methylation in intact cells inhibited methylation in isolated nuclei. In addition, cytosol was found to contain factors necessary for methylation of newly synthesized DNA.

Nearest-neighbor analysis in which radioactive dGTP was used as a precursor in place of dCTP (Gruenbaum et al., 1983) revealed that 35% of the CpG sequences were methylated in our system (results not shown). This is lower than the 66% value of CpG methylation reported for permeabilized cells (Gruenbaum et al., 1983). We calculated that 80% of the sites methylated in our assay were at CpG sites by comparing the ratios of determined and maximum values for CpG methylation with that of cytosine methylation.

The kinetics of DNA methylation were studied to determine whether a lag time existed between DNA synthesis and methylation (Figure 3, top). Deoxycytidine incorporated into DNA at short time periods was methylated less efficiently than at longer times (>5 min). Methylation levels increased with time to a maximum of 2.5% under the standard assay conditions. This result suggested that DNA methylation lagged behind DNA synthesis at the replication fork.

Assays were carried out for short time periods (<5 min) and the levels of DNA methylation determined at each time point. The results were plotted as the maximum percent methylation divided by the difference between the maximum value and the value at each time point as a function of time (Figure 3, bottom). An apparent lag time of approximately 20 s was determined from this plot. Since the times required to initiate and terminate DNA synthesis and methylation were short, the apparent lag time determined should be close to the actual value. Thus, DNA methylation lagged behind DNA synthesis in isolated nuclei in a similar manner to that previously described in living cells (Gruenbaum et al., 1983).

The levels of cytosine methylation in different size classes of DNA synthesized by isolated nuclei were also determined. DNAs isolated from either the middle (fractions 12–22) or top of gradients (fractions 24–29) were each precipitated in 70% ethanol and concentrated by high-speed centrifugation. DNA isolated from the middle of the gradients had methylation levels of 2.24%, similar to the levels seen overall in newly synthesized DNA (Table II). On the other hand, DNA isolated from the tops of the gradient had a methylation level of 1.26%, which was lower that seen in overall newly synthesized DNA. These results suggested that DNA methylation occurred primarily on DNA strands produced through continuous synthesis on replicons which had been active before the start of the nuclear isolation.

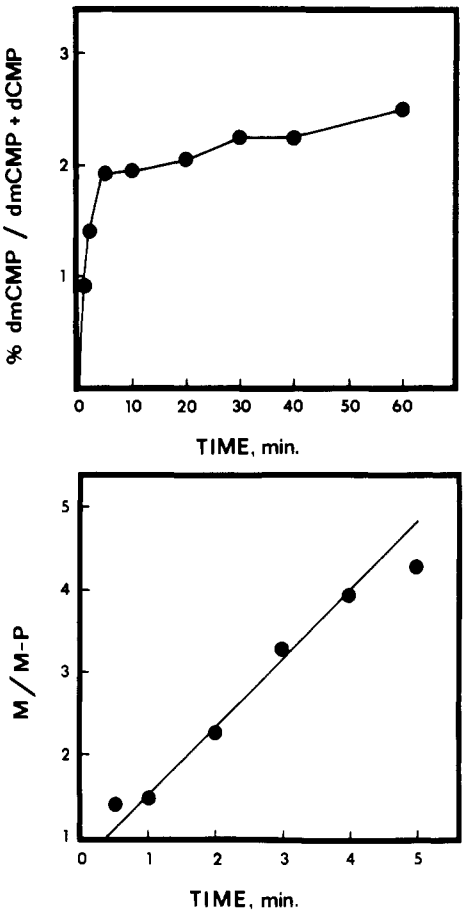

FIGURE 3: Time dependency of methylation of newly synthesized DNA in L1210 nuclei. Assays were incubated at 37 °C for the times indicated (top) and terminated by the addition of NaOH and EDTA. Methylation levels were determined for each time point as described under Materials and Methods. Results are averages of at least two experiments run in triplicate. Similar experiments were carried out at shorter time periods, and the DNA methylation levels for each sample were determined. On the basis of these results the apparent lag time between DNA synthesis and methylation were obtained (bottom) by plotting $M/(M - P)$ vs. time, where P is the percent methylation at each time point and M is the maximum level of DNA methylation (2.50%). Each point represents the average values of three or more experiments.

Discussion

Our previous work with a static system in which DNA was not being synthesized showed that chromatin was a poor substrate for methylating enzymes (Kautiainen & Jones, 1985). We have therefore developed a dynamic system using L1210 cells which established basic parameters of DNA synthesis and methylation in nuclei from replicating cells. This system in which newly incorporated cytosine residues became enzymatically modified may be used to explore further the factors responsible for the methylation of newly synthesized

DNA. The experiments showed that replication in isolated nuclei extended from sites which were actively replicating DNA in living cells. In addition to requiring the usual low molecular weight precursors and cofactors necessary for DNA synthesis, synthesis and methylation were dependent on the availability of soluble proteins present in the cytosol. Although others have observed DNA methylation in isolated nuclei (Tosi et al., 1972; Burdon & Douglas, 1974; Adams & Hogarth, 1973), our study is the first to investigate methylation of newly synthesized DNA in a dynamic system. The system was capable of methylating 2.5% of the total cytosine residues present in newly replicated DNA under optimal conditions. Furthermore, the majority of methylation took place at CpG sites.

The finding that methylation of newly synthesized DNA required cytoplasmic factors is novel and has important implications in gene control. These factors did not, in themselves, contain methylase activity, and although it is possible, they may have leaked out of the nucleus during fractionation, we consider this unlikely because only 5% contamination of nuclear proteins into the cytoplasm was found by Hudack et al. (1963) using similar fractionation techniques with Ehrlich ascites cells.

DNA methylation in the absence of cytosol was not restored by addition of a crude maintenance methylase and SAM, indicating that the methylation of newly synthesized DNA in isolated nuclei is more complex than the methylation that occurs in simpler systems (Taylor & Jones, 1982). Cytoplasmic factors may therefore modulate the process of DNA methylation in nuclei, and the specific cytoplasmic proteins responsible for this phenomenon are presently being purified.

Studies have indicated that DNA synthesis occurs at fixed sites within a nuclear matrix (Vogelstein et al., 1980; Pardoll et al., 1980). This matrix may also sequester methylase enzymes at fixed sites downstream from the polymerase, thus accounting for the 20-s lag between synthesis and methylation, which was slightly shorter than the values previously reported in permeabilized L cells (Gruenbaum et al., 1983). Given this lag time coupled with our rate of synthesis which was similar to that reported in chick red blood cells (Weintraub & Holtzer, 1972), we predict that newly synthesized DNA must travel a distance of eight nucleosome units before associating with the methylase enzyme, prior to its release from the matrix. The enzyme may therefore be fixed on the nuclear matrix rather than "walking" along the DNA as previously proposed (Drahovsky & Morris, 1972).

Our model argues against the existence of an enzyme complex that contains both polymerase and methylase activity (Noguchi et al., 1983), since DNA polymerase and methylase can act independently in our system. Pretreatment of cells with 5-aza-CdR showed that DNA synthesis can be relatively uneffected while DNA methylation is significantly inhibited.

Methylation levels varied among different size classes of DNA. DNA that extended continuously from replicons existing prior to their utilization in the assay had high levels of methylation relative to small DNAs produced by continuous or noncontinuous means. The question of whether Okazaki fragments become methylated during DNA synthesis is still a controversial issue. A report that labeled cells in vivo with [^{3}H]methionine concluded that Okazaki fragments were methylated during synthesis (Kiryanov et al., 1980), while other investigators reported considerably lower levels of methylation among Okazaki fragments (Marinus, 1976; Adams, 1974; Drahovsky & Wacker, 1975). Our observations suggest that these fragments were hypomethylated with respect to other newly synthesized DNA.

Reports from several laboratories have shown that DNA becomes associated with histones within minutes of its replication (Ryoji & Worcel, 1984; Annunziato et al., 1981). We have previously reported that in a static system a competition may exist for potentially methylatable sites between methylating enzymes and histones, since removal of histones from hemimethylated DNA stimulated methylase activity in a concentration-dependent manner (Kautiainen & Jones, 1985). Furthermore, linker histones were more potent inhibitors of DNA methylation than core histones. Histones have been shown to be deposited sequentially on newly synthesized DNA (Worcel et al., 1978; Jackson & Chalkley, 1981). Core histones are deposited within the first 10 min of DNA replication, while linker histones are deposited 10–20 min after replication. Thus, the association of histones, particularly linker histones, may signal the end of the initial phase of DNA methylation.

The observation that a portion of DNA methylation takes place after synthesis, but before the next replication cycle (Woodcock et al., 1982), may reflect the need for cells to compensate for this lack of complete methylation during the initial synthesis phase. Therefore, levels of initial DNA methylation may be determined by the relative concentrations of methylase and histones present in the nucleus and the temporal order of histone association to DNA during replication. Studies are now in progress to test this possibility.

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Registry No. dATP, 1927-31-7; dTTP, 365-08-2; dCTP, 2056-98-6; dGTP, 2564-35-4; 5′-ATP, 56-65-5; cytosine, 71-30-7.

REFERENCES

Adams, R. L. P. (1974) *Biochim. Biophys. Acta 335*, 365–373.
Adams, R. L. P., & Hogarth, C. (1973) *Biochim. Biophys. Acta 331*, 214–220.
Annunziato, A. T., Schindler, R. K., Thomas, C. A., Jr., & Seale, R. L. (1981) *J. Biol. Chem. 256*, 11880–11886.
Burdon, R. H., & Douglas, J. T. (1974) *Nucleic Acids Res. 1*, 97–103.
Christman, J. K. (1982) *Anal. Biochem. 119*, 38–48.
Cox, R., & Irving, C. C. (1977) *Cancer Res. 37*, 222–225.
Cox, R., Prescott, C., & Irving, C. C. (1977) *Biochim. Biophys. Acta 474*, 493–499.
Creusot, F., Acs, G., & Christman, J. K. (1982) *J. Biol. Chem. 257*, 2041–2048.
Doerfler, W. (1983) *Annu. Rev. Biochem. 52*, 93–124.
Doskocil, J., & Sorm, F. (1962) *Biochim. Biophys. Acta 55*, 953–959.
Drahovsky, D., & Morris, N. R. (1972) *Biochim. Biophys. Acta 277*, 245–250.
Drahovsky, D., & Wacker, A. (1975) *Naurwissenschaften 62*, 189–190.
Gruenbaum, Y., Stein, R., Cedar, H., & Razin, A. (1981) *FEBS Lett. 124*, 67–71.
Gruenbaum, Y., Szyf, M., Cedar, H., & Razin, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4919–4921.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
Hershey, H. V., Stieber, J. F., & Mueller, G. C. (1973) *Eur. J. Biochem. 34*, 383–394.
Hudack, E. D., Eros, D. E., & Brummond, D. O. (1963) *Exp. Cell. Res. 29*, 343–348.
Jackson, V., & Chalkley, R. (1981) *Cell (Cambridge, Mass.) 23*, 121–134.

Jones, P. A. (1985) *Cell (Cambridge, Mass.) 40*, 485–486.
Josse, J., Kaiser, A. D., & Kornberg, A. (1961) *J. Biol. Chem. 236*, 864–875.
Kautiainen, T. L., & Jones, P. A. (1985) *Biochemistry 24*, 1193–1196.
Kiryanov, G. I., Kirnos, M. D., Demidkina, N. P., Alexandrushkina, N. I., & Vanyushin, B. F. (1980) *FEBS Lett. 112*, 225–228.
Laskowski, M., Sr. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) pp 154–187, Harper and Row, New York.
Lowe, J. K., & Grindey, G. B. (1976) *Mol. Pharmacol. 12*, 177–184.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning*, pp 109–112, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marinus, M. G. (1976) *J. Bacteriol. 128*, 853–854.
Muller, M. T., Kajiwara, K., & Mueller, G. C. (1981) *Biochim. Biophys. Acta 653*, 391–407.
Noguchi, H., Prem veer Reddy, G., & Pardee, A. B. (1983) *Cell (Cambridge, Mass.) 32*, 443–451.
Pardoll, D. M., Vogelstein, B., & Coffey, D. S. (1980) *Cell (Cambridge, Mass.) 19*, 527–536.
Pfeifer, G. P., Grunwald, S., Boehm, T. L. J., & Drahovsky, D. (1983) *Biochim. Biophys. Acta 740*, 323–330.
Plank, S. R., & Mueller, G. C. (1977) *Biochemistry 16*, 2778–2782.
Razin, A., & Riggs, A. D. (1980) *Science (Washington, D.C.) 210*, 604–610.
Ryoji, M., & Worcel, A. (1984) *Cell (Cambridge, Mass.) 37*, 21–32.
Tanaka, M., Hibasami, H., Nagai, J., Ikeda, T. (1980) *Aust. J. Exp. Biol. Med. Sci. 58*, 391–396.
Taylor, S. M., & Jones, P. A. (1982) *J. Mol. Biol. 162*, 679–692.
Tosi, L., Granieri, A., & Scarano, E. (1972) *Exp. Cell Res. 72*, 257–264.
Vogelstein, B., Paradoll, D. M., & Coffey, D. S. (1980) *Cell (Cambridge, Mass) 22*, 79–85.
Weintraub, H., & Holtzer, H. (1972) *J. Mol. Biol. 66*, 13–35.
Wilks, A., Seldran, M., & Jost, J. P. (1984) *Nuceic Acids Res. 12*, 1163–1177.
Wilson, V. L., Jones, P. A., & Momparler, R. L. (1983) *Cancer Res. 43*, 3493–3496.
Wist, E., Krokan, H., & Prydz, H. (1976) *Biochemistry 15*, 3647–3652.
Woodcock, D. M., Adams, J. K., & Cooper, I. A. (1982) *Biochim. Biophys. Acta 696*, 15–22.
Worcel, A., Han, S., & Wong, M. L. (1978) *Cell (Cambridge, Mass.) 15*, 969–977.

Tissue-Specific Regulation of Two Functional Malic Enzyme mRNAs by Triiodothyronine

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ABSTRACT: Rat liver malic enzyme (ME) synthesis is known to be regulated by 3,5,3′-triiodo-L-thyronine (T_3). Hybridization of ^{32}P-labeled ME cDNA with RNA extracted from normal and T_3-induced livers (15 or 50 μg/100 g body weight for 10 days) showed an increase in the ME mRNA concentration by ~11-fold in T_3-treated rats. ME activity and ME mass were stimulated to the same degree as ME mRNA. Northern blot analysis of either total or poly(A+) RNA revealed two distinct ME mRNAs (21 and 27 S) which were equally induced by T_3 treatment. Both mRNAs were shown by in vitro translation assay to program the synthesis of the same immunoprecipitable protein corresponding to full-sized ME. From all the above, we concluded that both messages code for active enzyme. ME activity and ME mRNA were also detected in nonhepatic tissues for which different responses to T_3 induction were observed without direct correlation with their respective content of T_3 nuclear receptor. Increases in ME activity and level of hybridizable ME mRNA were seen 48 h after a single administration of T_3 (200 μg/100 g body weight) in liver, kidney, and heart (10.3- and 15.5-, 1.7- and 2.6-, and 1.72- and 3.4-fold above basal values, respectively). Lower levels of induction could already be detected after 24 h, liver being the most stimulated tissue. ME was not affected in brain, lung, testis, and spleen. Northern blot analysis showed that both ME mRNAs are present in all tissues tested, although in different relative proportions. As in liver, the two mRNAs were stimulated to the same extent in T_3-induced kidney and heart. These data provide direct evidence for pretranslational regulation by T_3 of ME synthesis through two functional mRNAs and for tissue specificity of this hormonal control. Furthermore, both malic enzyme mRNAs are under T_3 control in all responsive tissues.

In rat liver, the activities of several lipogenic enzymes such as fatty acid synthetase (Volpe & Vagelos, 1976; Morris et al., 1982; Roncari & Murthy, 1975), acetyl-CoA carboxylase (Roncari & Murthy, 1975), ATP–citrate lyase (Gibson et al., 1972; Sul et al., 1984a), and glucose-6-phosphate and 6-phosphogluconate dehydrogenases from the hexose monophosphate shunt (Tepperman & Tepperman, 1964; Diamant et al., 1972; Mariash et al., 1980; Miksicek & Towle, 1982) respond to thyroid hormone administration. Cytosolic malic

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