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5-METHYLCYTOSINE FORMATION IN WHEAT EMBRYO DNA

Gudrun Theiss and Hartmut Follmann

Fachbereich Chemie, Arbeitsgruppe Biochemie, Philipps-Universität
D-3550 Marburg, Germany

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SUMMARY. The methylation of cytosine residues in wheat DNA was studied in isolated embryos during the first 30 hrs of germination. L-Methionine, but also L-serine serve as methyl group donors in vivo. DNA methylation reaches high values after the maximum of DNA replication at 18 hrs. Isolated nuclei contain S-adenosyl methionine-dependent methyltransferase activity at all stages of germination, a significant increase is observed after the 22nd hour. The plant enzyme can methylate thymus DNA in vitro, it is inhibited by S-adenosyl methionine analogs.

INTRODUCTION

Plant deoxyribonucleic acids contain a much higher proportion of 5-methylcytosine (m^5Cyt)* than bacterial or animal DNA. Base analyses have shown the presence of 5 - 7 mol% m^5Cyt besides 13 - 17 mol% of the parent nucleotide, deoxycytidylate (1,2). Despite this fact, which should favor experimental study of the methylation mechanism, the reaction has found more attention in animal tissues (3-7) which contain only up to 0.9 mol % m^5Cyt. Surprisingly little is known about formation, by DNA methyltransferases, or function of the modified nucleotide in plant cells (8-10).

We have initiated an analysis of cytosine methylation in germinating wheat. This system was chosen because the enzymes responsible for deoxyribonucleotide (11), DNA (12,13), and RNA synthesis (14) show pronounced activity changes during the

* Abbreviations: Cyt, cytosine; m^5Cyt, 5-methylcytosine; SAM, S-adenosyl methionine; TCA, trichloroacetic acid.

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early phases of seed germination. Slight differences in the m^5Cyt content of nucleotide sequences in seeds and in 3 day-old seedlings have been noted (10) but properties and time course of DNA methyltransferase in wheat are entirely unknown.

MATERIALS AND METHODS

Winter wheat (Triticum aestivum, "Kormoran" variety) was kindly provided by v.Lochow-Petkus, Northeim, Germany. Viable embryos were mechanically produced (15) and germinated at 25° on agar plates. Radioactive amino acids, nucleosides, and nucleotides were obtained from Amersham-Buchler, Braunschweig. [Methyl-^{3}H]SAM (spec.activity, 5-15 Ci/mmole) was rechromatographed if less than 90% pure. Percoll is a product of Pharmacia, Sweden.

Extraction and purification of wheat DNA have been described (11). DNA was estimated fluorimetrically after reaction with 3,5-diaminobenzoic acid·2 HCl (16). Formic acid hydrolysates (88% HCOOH, 45 min at 175°) were first fractionated on Dowex 1x2(Cl^-) anion exchanger in 0.2 M NH_4Cl-NH_3 buffer (pH 10.2), and m^5Cyt was then separated from cytosine by HPLC on an Aminex A7 column (60°, flow rate 0.35 ml/min) in 0.1 M K-borate buffer (pH 7.55); retention times: Cyt, 45 min; m^5Cyt, 62 min. Radioactivity of DNA precipitated on glass fiber filters was counted in toluene-based scintillator, and of the fractionated bases in Unisolve 1 (Zinsser, Frankfurt).

500 mg-batches (dry weight) of germinating wheat embryo were incubated in 2 ml of a 2% glucose solution with 250 µCi of tritiated or 100 µCi of C-14-labeled precursor, respectively. Incorporation of radioactivity was approximately linear between 30 min and 3 hrs. After 1 hr at 25°C the embryos were rinsed, frozen in liquid nitrogen, and homogenized (11).

For preparation of nuclei, 4 g of germinated embryos were suspended in 15 ml of grinding medium (50 mM Tris-HCl, 2 mM $CaCl_2$ 0.8 M sucrose, pH 7.0), homogenized at high speed for 30 sec, and filtered through two layers of miracloth. The filtrate was centrifuged through 1.2 M sucrose/2 mM $CaCl_2$, the pellet resuspended in grinding medium and the procedure repeated twice. The pellet was then suspended in 1 ml Honda medium (25 mM Tris-HCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.25 M sucrose, 5 mM dithiothreitol, pH 8.0) and layered upon a step gradient (80%/50%) of Percoll (17). After centrifugation for 45 min at 8000 x g the nuclei are concentrated in a narrow band at the 80% percoll (1.13 gm/cm^3 density) border and are recovered with a pipet. They are washed twice in Honda medium and finally suspended in 1-2 ml of 0.30 mM Tris-HCl/0.25 M sucrose (pH 8.0) solution. Protein and DNA are determined and serve to adjust, by appropriate dilution, the concentration of nuclei when different organelle preparations have to be compared.

100 µl of suspended nuclei (0.8-1 mg protein) were incubated, in a total volume of 0.40 ml 30 mM Tris-HCl/0.25 M sucrose (pH 8.0) containing 40 µM SAM plus 2.5 µCi [methyl-^{3}H] SAM. The reaction was stopped in 0.3 ml ice-cold 2 M NaCl containing 30 µl of a 10% SDS solution, the samples were mixed with an equal volume of chloroform/isoamyl alcohol (24:1, v/v)

and shaken for 15 min. After brief centrifugation DNA was precipitated with an equal volume of 2-propanol, collected after 30 min standing at 0°, and then redissolved in 0.40 ml of 0.15 M NaCl/0.015 M Na-citrate. DNA was purified by treatment with 100 µg ribonuclease and 200 µg pronase (1 hr at 37°) (11). It was applied to Whatman GF/C filters which were washed in 10% TCA, 5 % TCA, ethanol/ether (3:1), and ether, and then subjected to DNA and radioactivity determination.

RESULTS AND DISCUSSION

The synthesis of macromolecules is initiated in a stepwise fashion (RNA; protein; DNA) after imbibition of wheat seeds (18).To establish the time of maximum DNA methylation in germinating embryos we have first compared the incorporation of radioactive thymidine into DNA,and of methyl groups derived from methionine into 5-methylcytosine (Fig.1A). The prominent

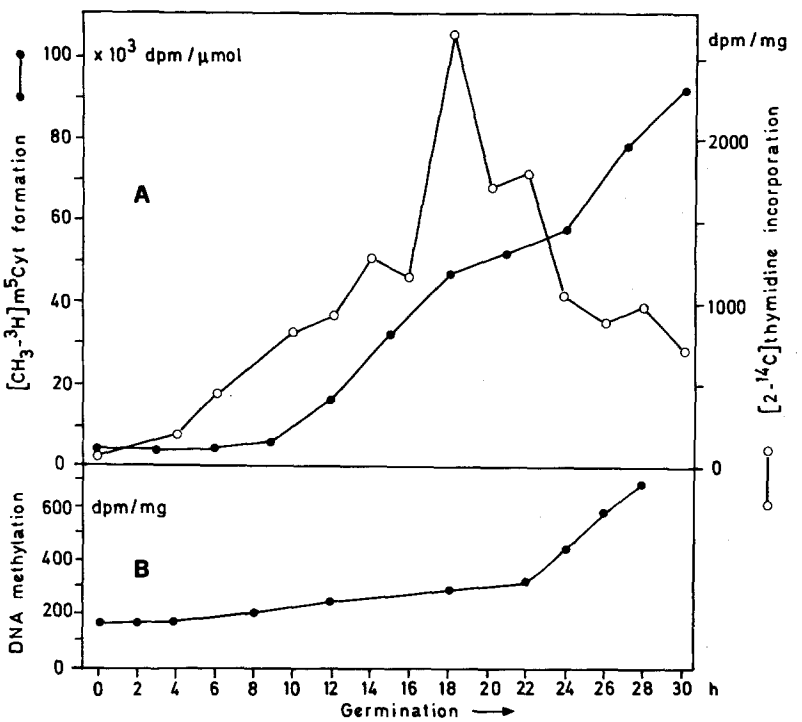

Figure 1. A: Incorporation of radioactive thymidine into DNA of germinating wheat embryo (right scale), and specific radioactivity of 5-methylcytosine isolated after 1 hr administration of radioactive methionine (left scale). B: DNA labeling by [methyl-^{3}H]S-adenosyl methionine in wheat nuclei prepared at different germination times (expressed in dpm/mg nuclear protein). The DNA was purified by ribonuclease and pronase treatment.

peak of thymidine incorporation at 18 hrs, indicating the first wave of nuclear DNA synthesis, is in agreement with previous observations (11-13). In contrast, the specific radioactivity of m^5Cyt, isolated after acid hydrolysis of DNA, shows no such maximum but increases continually up to the 30th hour, where thymidine incorporation is back to a lower level. This time course is compatible with DNA methylation as a post-replicational event. Whereas DNA labeling is low during the first 6 hrs of germination, thymidine uptake and m^5Cyt formation are not negligible between 8 and 16 hrs. A plausible explanation for early incorporation of methyl groups is modification of the cytoplasmic DNA fraction which appears in wheat embryo at 6-12 hrs, before nuclear DNA is synthesized (18). The time lag between thymidine and methyl group incorporation supports this view. In contrast, there is no evidence for originally undermethylated DNA in wheat seeds (10) which could require early methylation.

Methionine has served as methyl donor in these experiments, indicating that wheat DNA methyltransferase uses the same substrate as mammalian enzymes (3-5). However, in the whole embryo serine is also a good precursor of m^5Cyt (Table 1). Although the base is preferentially labeled by methionine, its activity exceeds that of thymine even in the presence of the C_1 donor

Table 1. Specific activity of methylpyrimidines in wheat DNA after labeling of embryos (at 20 hrs germination) with radioactive C_1 precursors for 1 hour at 25°C.

Base	L-[methyl-^{14}C]methionine dpm/µmol	% of total	L-[3-^{14}C]serine dpm/µmol	% of total
m^5Cyt	52 000	65	20 000	43
thymine	12 300	16	16 000	34

of thymidylate synthesis, serine. DNA precursor utilization in Chlorella pyrenoidosa also suggests a distribution of radioactive methyl groups over the entire C_1 pool (9).

Surprisingly, formation of labeled thymine was not suppressed in the presence of up to 10 mM thymidine. This raised the question whether the base could be formed by deamination of labeled m^5Cyt in a post-replicational process (7), or as an artefact. We have indeed found that,contrary to general belief, HCOOH treatment at 175° converts up to 10 % of m^5Cyt into thymine. Therefore, and because the radioactivities of purified wheat DNA and the isolated m^5Cyt fractions always correlated we found it justified to perform routine assays of DNA methylation by omitting the acid hydrolysis step.

Cell nuclei from gram amounts of wheat embryo were prepared by centrifugation of crude homogenate fractions in Percoll (colloidal silica) gradients. The nuclear fraction was homogenous as judged by electron microscopy and by the absence of mitochondrial enzymes. It catalyzed efficient [^{3}H]UTP incorporation into acid-precipatable material, in accord with the high RNA polymerase activity of wheat seeds (14).

Nuclei prepared from embryos at the 22nd hr of germination were used to characterize m^5Cyt formation in vitro.S-Adenosyl methionine concentrations of 40 μM proved saturating for the methyltransferase reaction which proceeded linearly for at least 3 hrs at 25°C. It was not affected by the presence or absence of K^+ (0-0.1 M) and Mg^{++} ions (0-10 mM) or Triton X-100 (0.1 %). Ca^{++} ions were found inhibitory at 1 mM and higher concentration. Spermine and spermidine caused 50 % inhibition at 0.5 and 6 mM concentration,respectively.Even stronger inhibition was produced by the two SAM analogs,S-adenosyl

ethionine and S-adenosyl homocysteine; at 40 μM substrate and 20 μM inhibitor concentration, the former caused 50 % and the latter (which is a possible contaminant of SAM solutions) caused 65 % inhibition. These data are comparable to the behaviour of liver DNA methylases (3,19). The enzyme can be extracted from wheat nuclei by treatment with 1 M NaCl/ 5 % Triton X-100; the obtained fraction is capable of methylating calf thymus DNA besides its homologous substrate DNA.

We have finally measured DNA methylation in wheat nuclei prepared at different germination stages (Fig.1B). In this case m^5Cyt formation increases little up to the 22nd hour, reflecting the fact that cytoplasmic or mitochondrial DNA modification cannot be observed here. The more than twofold increase between 22 and 28 hrs should then represent methylation of the newly replicated DNA, but one cannot yet differentiate whether the figures indicate an increase in enzyme activity, the doubled amount of available DNA, or both. It is important to note that m^5Cyt formation is already measurable in nuclei of dry wheat seeds. DNA methyltransferase thus resembles wheat DNA polymerases (13) and RNA polymerase II (14) whereas ribonucleotide reductase appears much later (11).

Taken together, our results indicate that the larger quantity of m^5Cyt in plant DNA is not associated with qualitative differences of the enzyme apparatus. The terminal methylation step is unlikely to exert any material control over DNA replication in wheat like DNA precursor biosynthesis. Further purification of the methyltransferase, however, should lead to an evaluation of its methylation site specificities.

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