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DNA methylases separated through the HeLa cell cycle methodology show allosteric properties

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Two DNA methylases (DNAmets) can be separated through the cell cycle. The first appears as a minor peak in G_1, the second as a major peak in S. Both enzymes protect from *Hpa*II a plasmid (H_{31}), constructed with the pBR322 vector (4.3 kbp) and the inverted A_γ fragment of the human globin gene (3.5 kbp), inserted at its *Hin*dIII site (the vector carries several *Hpa*II sites, the insert only one *Hpa*II site). DNAmets G_1 and S show distinct K_m values and different kinetics vs the ionic strength of the medium, while their Michaelis-Menten and Lineweaver-Burk plots are sigmoidal and hyperbolical curves, respectively. This is the first suggestion about the allosteric nature of the eukaryotic DNAmet system.

DNA methylase; Cell cycle; Allostery

1. INTRODUCTION

Following the demonstration that newly replicating DNA is semi-conservatively modified during the S-phase [1], it was suggested that the DNA methylase (DNAmet) system should involve more than one enzyme in eukaryotes. In fact, while one DNAmet functioned with a higher efficiency on GC-rich sequences replicating in early S-phase, another one functioned with a higher efficiency on AT-rich sequences replicating in late S-phase [2]. This was supported by the observation that the DNAmet kinetics may depend on the base composition of given co-polymers [3–5]. On this basis, we believed that the enzyme methylating the newly replicating DNA [1,6] might differ from that methylating the repair patches [7,8]. The present contribution shows that two DNAmets can be separated during the HeLa cell cycle: one in G_1 and the other in S [9]. They both appear to be allosteric enzymes.

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2. MATERIALS AND METHODS

DNAmet *Hpa*II from *Haemophilus parainfluenzae* [10], *Hpa*II, *Hin*dIII and calf thymus DNA were purchased from Boehringer, Mannheim. H_{31} was a gift from Professor S. Ottolenghi, University of Milan [11]. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]SAM, 77 Ci/mmol, 0.5 mCi/ml) was obtained from Amersham, England.

Cell growth and synchronization were performed in suspension as in [1]. Protein content was determined as in [12].

To obtain a DNAmet sample, 8×10^7 cells were washed with 0.9% NaCl, harvested for 10 min at $1000 \times g$, resuspended in 10 ml hypotonic solution (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), disrupted in a Dounce homogenizer, and sonicated for 30 s with a Labsonic 1510 Braun instrument (250 W).

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To assay DNAmet activity, 50 μl of this crude extract was added (in triplicate) to 50 μl incubation mixture (1 ml of 0.1 M Tris-HCl, pH 8.0, contained 200 μg calf thymus DNA and 40 μl [^{3}H]SAM) for 1 h at 37°C. The reaction was stopped by the addition of 200 μl cold 10% trichloroacetic acid-0.01 M sodium pyrophosphate. The precipitated material was filtered on 0.45 nm Millipore disks, dissolved in Bray's solution, and tested for radioactivity in a Packard 460 CD spectrometer.

For methylation of H_{31}, the crude DNAmet samples were further purified on DE-52 [13]. Then, equal volumes of H_{31} and purified DNAmet were maintained overnight at 37°C in 50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 5 mM mercaptoethanol, and 1 μCi [^{3}H]SAM/μg H_{31}. The reaction was stopped by increasing the temperature to 65°C for 20 min. The incubated sample was cooled in ice, treated with *Hpa*II [10], dialyzed for 10 h vs 10 mM Tris-1 mM EDTA, digested with *Hin*dIII [11,14] and, finally, run in 0.8% agarose gel for 2 h using as buffer a solution of 0.089 M Tris-borate, pH 8.2, containing 0.01 M EDTA. The M_r of the purified enzymes was checked as described in [15], while the cytosol and nuclear fractions were obtained as in [1].

3. RESULTS

Fig.1 shows that, as expected from the pattern of DNA methylation [1], DNAmet activity increases strongly as the S-phase progresses, reaching a maximum at the end of this phase and dropping thereafter. In the G_2, M and early G_1 stages, by contrast, there is no significant DNAmet activity. A lower peak of DNAmet activity appears in late G_1.

The experiments in which we used H_{31} as substrate, programmed with unique specificity

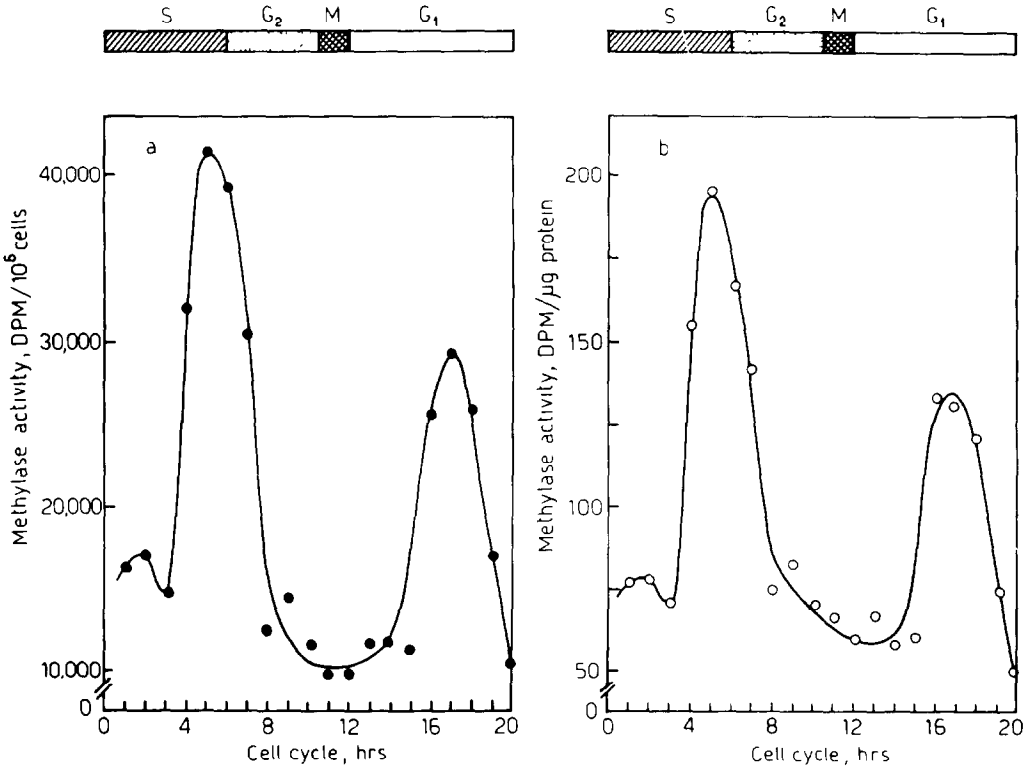

Fig.1. Development of the DNAmet system as a function of cell cycle. Using calf thymus DNA as a substrate, DNAmet activity was measured in crude extracts at 1 h intervals from the time of entering S-phase and expressed in dpm incorporated $\cdot h^{-1} \cdot (10^6 \text{ cells})^{-1}$ (a) and $\cdot h^{-1} \cdot \mu g^{-1}$ protein (b).

against *Hpa*II, suggested some similarity between the catalytic centers carried by the S and G_1 DNAmets. Unmethylated H_{31}, as digested by *Hpa*II and *Hind*III, is cleaved in fragments of 1.9, 1.3, 0.3 kbp etc. (fig.2A). Alternatively, when H_{31} is previously modified with the S (fig.2B) or G_1 (fig.2C) DNAmets, treatment with the two restrictases yields only fragments of 4.3 and 3.5 kbp. This is due to the cuts by *Hind*III, which releases A_γ from pBR322. *Hpa*II, which recognizes only one site on A_γ and several sites on pBR322 [14], is unable to digest these two methylated moieties further. The H_{31} protection by the two HeLa DNAmets is similar to that which we know is provided by the bacterial DNAmet [10], used here also for checking the M_r of the cleaved fragments (fig.2D).

The experiments in which the enzyme activity was tested against the ionic strength of the medium revealed, however, some dissimilarity between the S and G_1 DNAmets. In Tris-HCl, pH 7.9, DNAmet G_1 shows two optima of activity (near 0.01 and 0.1 M), whilst DNAmet S shows one optimum between these molarities (fig.3). Data from the literature show that the M_r of the eukaryotic DNAmet oscillates between 90000 and 180000 [16]. In conformity with this, we found that G_1 protein is well purified from the cytosol and shows an M_r close to 92000, while the S protein is well purified from nuclei and shows an M_r 2-fold greater. All this might thus suggest a quaternary structure of the S-phase methylase, assembled in the nuclei using subunits coming from the G_1 cytosol.

This idea fitted fairly well with the discovery that DNAmets S and G_1 reveal an allosteric behaviour. Actually, for both of them, while the Michaelis-Menten plots are sigmoidal curves, the Lineweaver-Burk plots are unequivocal hyperbolic curves (fig.4). K_m is about 1.7 mM for DNAmet S and about 1.4 mM for DNAmet G_1.

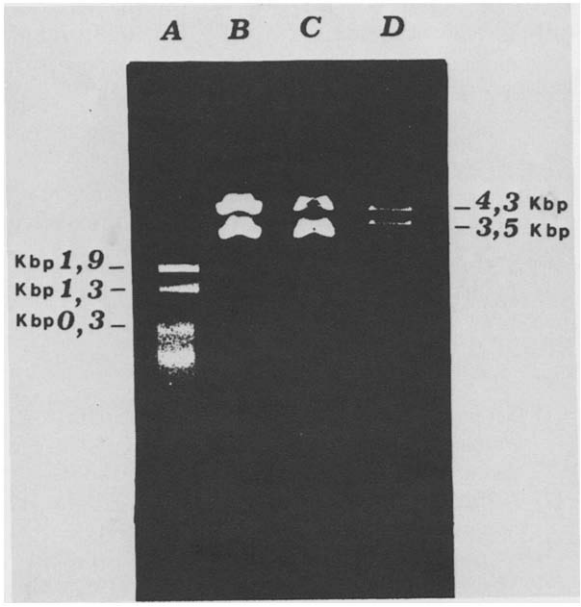

Fig.2. Protection of H_{31} by DNAmets S and G_1 against *Hpa*II. (A) Unmethylated H_{31}; (B,C) H_{31} treated with purified DNAmets S and G_1, respectively; (D) H_{31} treated with *H. parainfluenzae* DNAmet *Hpa*II. Amounts of DNA per lane: 0.8 μg for A–C and 0.2 μg for D.

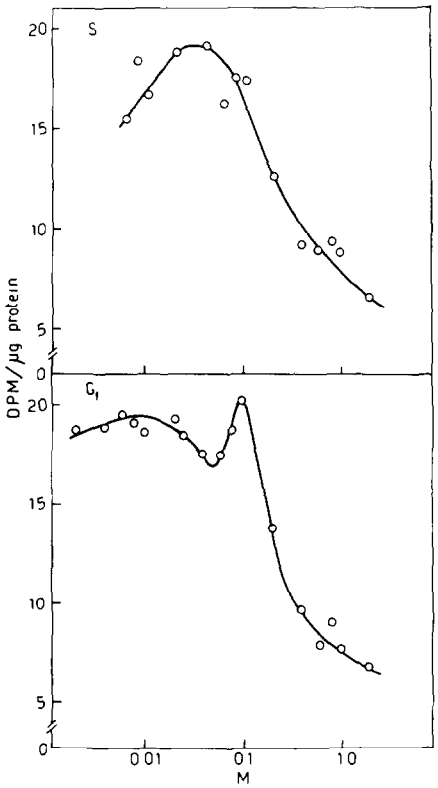

Fig.3. Specific activities of DNAmets S and G_1 as a function of the ionic strength of the medium. The enzyme activity was measured against increasing concentrations (M) of Tris-HCl, pH 7.9, and expressed in dpm incorporated $\cdot h^{-1} \cdot \mu g^{-1}$ protein.

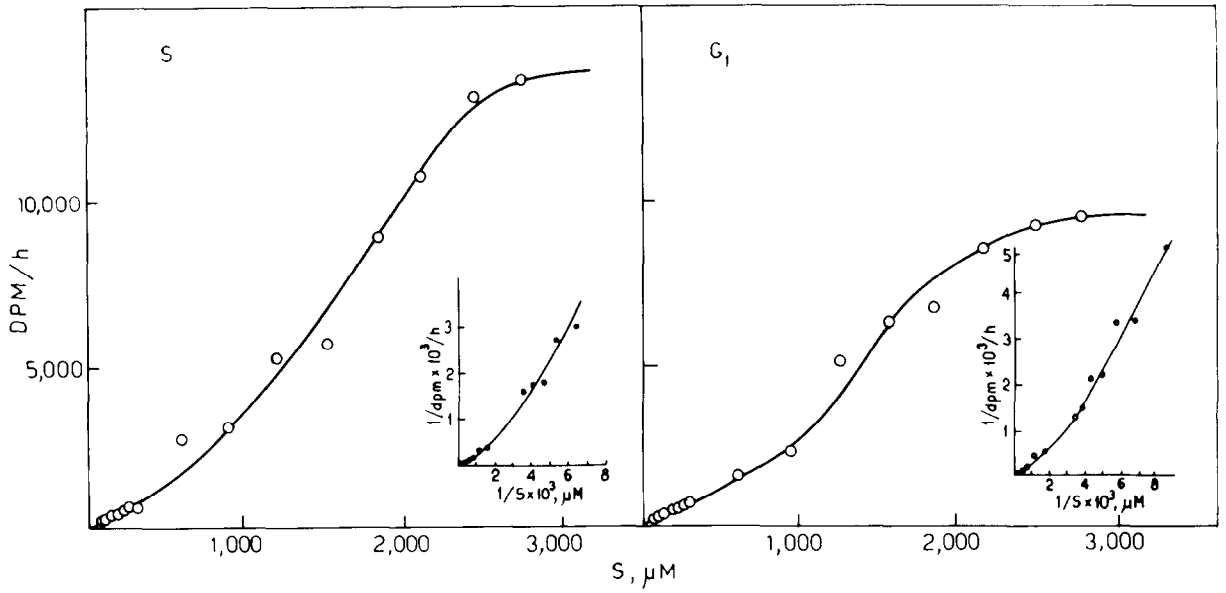

Fig.4. Allosteric properties of S and G_1 DNAmets. Enzyme activity was measured against the calf thymus DNA concentrations (S) and expressed in dpm incorporated $\cdot h^{-1} \cdot \mu g^{-1}$ protein. (Insets) Lineweaver-Burk plots.

4. DISCUSSION

Previous work from our laboratory showed that DNA polymerase α is well expressed during the S-phase, while DNA polymerase β – although with a relatively higher expression at the threshold of S – is produced at a rather constant rate throughout the whole interphase [17]. Therefore, fig.1 signifies first of all that a full coupling occurs, in S, between the synthesis of DNA polymerase α (needed for DNA replication) and that of DNAmet S (needed for modification of newly replicating DNA). The DNAmet activity detectable in late G_1 (fig.1) apparently couples with that of DNA polymerase β at least when it is increased at the threshold of S [17]. However, the fact that the DNA repair synthesis is less than 10% of the whole DNA synthesis [7], without excluding such direct coupling, might rather suggest the assembly of G_1 subunits into S DNAmet quaternary structures. Alternatively, the G_1 DNAmet activity should be correlated with the extra-S-phase DNA methylation [1], the nature of which has remained unknown so far.

The other interest of this paper concerns the hitherto undescribed allosteric properties of DNAmet. As far as we know, this is a rare example of allostery dealing with a polymeric substrate like DNA (although it is obvious that along the chain only one monomer, i.e. cytosine, is transformable into 5-methylcytosine). Work is in progress to identify the presumed allosteric effector.

ACKNOWLEDGEMENT

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