Simultaneous Measurements of DNA Synthesis and DNA Methylation in Higher Organisms

Dieter Lutz *, Klaus Engel, and Hans Kröger

Robert Koch-Institut, Berlin-West

(Z. Naturforsch. **28 c**, 747–749 [1973]; received September 5, 1973)

Dedicated to Prof. Dr. Georg Henneberg on the occation of his 65th birthday

DNA synthesis, DNA methylation, higher organisms

Different ways for studying DNA synthesis and DNA methylation were investigated. On the one hand the reactions were measured by incorporation of several specific precursors, taking into account that pool alterations might influence the results. The best way to avoid this is the direct conversion of DNA cytosine to DNA 5-methyl-cytosine by using [^{14}C]-cytidine. According to our results we propose to calculate the correct DNA methylation rate in term of the specific radioactivity of the separated DNA bases.

Introduction

DNA methylation is a specific enzymatic reaction at the polymeric level. After the synthesis of the polynucleotide few of the newly incorporated cytosine bases are modified to 5MC by the transfer of the methyl group of S-adenosylmethionine to DNA cytosine [1–9].

In this paper we want to show different ways of simultaneous measurements of DNA synthesis and DNA methylation *in vivo*. Therefore different radioactive labelled precursors were used to compare DNA synthesis and DNA methylation in control organs and in organs with an enhanced nucleic acid metabolism *i. e.* in the antigen stimulated mouse spleen and in the regenerating liver. Furthermore, we are dealing with the question how the methylation rate of DNA should be expressed in a way avoiding additionally methodical errors.

Methods

For hepatectomy experiments two lobes of the liver of female Wistar rats (80–100 g) were removed by the method of Higgins and Anderson [10]. 24 hours thereafter the animals were killed by decapitation and the residual lobes of the liver were used for DNA isolation.

For immunization each mouse (NMRI-breed, approx. 20 g) was injected i.p. by a single dose of 300 μg bovine serum albumin 24 hours before being killed. The spleens of 4 mice were pooled and used as one sample for DNA isolation.

DNA isolation from rat liver was performed by the Colter procedure [11] and the DNA purified by incubation at 37 °C in SSC for 1 hour with ribonuclease (50 μg/ml) and for additional 16 hours with pronase (1 mg/ml). This incubation mixture was deproteinized by an equal volume of chloroform – isoamylalcohol (24 : 1), the DNA was precipitated by a double volume of ethanol. Spleen DNA was isolated by the method of Marmur [12] with an additionally pronase incubation for 16 hours at 37 °C following the ribonuclease treatment (1 hour).

Base separation was performed after formic acid hydrolysis of the DNA by thinlayer chromatography as reported elsewhere [13]. 5MC was the only methylated base detected in DNA of normal and regenerating liver in agreement with Craddock [14].

Materials

L-methionine (methyl-T) with a specific radioactivity of 2.9 Ci/mmole (Batch 23) and [2-^{14}C]-thymidine with a specific radioactivity of 60 mCi/mmole were obtained from Radiochemical Center, Amersham, England. Endoxan (Cyclophosphamide) is a gift from ASTA-Werke, Brackwede, Germany. Bovine serum albumin was purchased from Boehringer, Mannheim, Germany. [U-^{14}C]-deoxycytidine with a specific radioactivity of 512 mCi/mmole (Batch 8) was obtained from the Radiochemical Center, Amersham, England.

* Dr. Dieter Lutz, Ludwig Boltzmann-Institut für Leukämieforschung u. Hämatologie im Hanusch-Krankenhaus, *A-1140 Wien*, Heinrich Collinstraße 30.

Abbreviations: A, adenine; DNA, deoxyribonucleic acid; 5MC, 5-methylcytosine; BSA, bovine serum albumin; SSC, standard saline citrate solution; T, thymine.

Requests for reprints should be sent to the Robert Koch-Institut, *1 Berlin 65*, Nordufer 20.

Results

a. Double label pulse experiments

In these experiments [^{14}C]-thymidine incorporation was used as measure for DNA synthesis and the labelling of 5MC derived from [^{3}H]methylmethionine served as measure for DNA methylation. 24 hours after hepatectomy DNA synthesis is much more elevated than DNA methylation in comparison with the control values (Table 1). Additionally, applying Endoxan, DNA methylation is reduced to a higher extent than DNA synthesis in the regenerating liver.

Table I. Measurements of DNA synthesis and DNA methylation by double pulse labelling with [^{14}C]-thymidine and [^{3}H]-methionine in control and hepatectomized rats 24 hours after operation. Endoxan (100 μg/g body weight), [^{3}H]-methylmethionine (5 μCi/g body weight) and [^{14}C]-thymidine (0.1 μCi/g body weight) were injected 2 hours, 60 and 20 min, respectively, before decapitation. Mean values of 4–6 rats.

	DNA Methylation	DNA-Synthesis		
	[^{3}H] 5MC	[^{14}C] T	[^{3}H] T	[^{3}H] A
	[dpm/μmole base]			
Control	4495	8367	292	23
24 h	21225	49533	2225	118
24 h + Endoxan	6750	19600	805	51

Beside 5MC, thymine and adenine were labelled by [^{3}H]methionine, too. This is achieved by the conversion of the methyl group of methionine over the C_1-pool to the adenine and thymine precursors for DNA synthesis. The specific radioactivity of these both bases can be used as an intrinsic measure for DNA synthesis. As shown in Table I the [^{3}H]-labelling of thymine and adenine corresponds to the values of DNA synthesis obtained by the [^{14}C]-thymidine incorporation.

b. Experiments with [^{14}C]-deoxycydidine

Applying [^{3}H]methionine, respectively [^{14}C]-thymidine, an altered behaviour of the single precursor pool can influence the correlation between DNA methylation and DNA synthesis. Such pool alteration can be avoided by labelling with [^{14}C]-deoxycytidine. In this case the direct conversion of DNA-cytosine to DNA-5MC at the polymer level can be measured. 24 hours after BSA immunization this conversion is elevated compared to the control animals. This is indicated by measuring the DNA methylation rate ($[MC \cdot 100]/[MC + C]$) as dpm per μmole base (Table II). If this DNA methylation rate is calculated only by the counts found in cytosine and 5MC (dpm) deviated results in the single experiments are achieved. Such expressions would lead to a similar DNA methylation rate in control and immunized mice. This effect depends on the different base contents of each isolated DNA as shown in the base analysis data of Table II.

Discussion

The methylation of DNA in comparison to DNA synthesis in higher organisms can be measured in different ways. If one uses double label pulse ex-

Table II. Analysis of cytosine and 5-methoxycytosine of the spleen DNA in single experiments and different calculation of their conversion rate. [^{14}C]-deoxycytidine (0.1 μCi/g body weight) was injected i.p. 60 min before killing.

Experiment No.		DNA-Analysis [μmole · 10^{-4}]	DNA-Analysis [% of total base content]	DNA-Analysis [dpm]	DNA-Methylation DNA-Synthesis $\frac{[MC \cdot 100]}{[MC + C]}$ %	$\frac{[MC \cdot 100]}{[MC + C]}$ dpm in 5MC %	dpm/μmole in 5MC
				Control mice			
1	Cytosine	1244.0	20.50	1174	4.39		80.7
	5MC	71.7	1.18	54			
2	Cytosine	1280.0	20.50	1040	3.97		79.4
	5MC	67.3	1.08	43			
				BSA-immunized mice			
3	Cytosine	1384.0	10.8	896	4.27		94.8
	5MC	65.3	0.98	40			
4	Cytosine	1344.0	20.30	577	5.09		103.8
	5MC	69.4	1.05	31			

periments pool alteration should be considered. For this we determined the specific radioactivity of each base after application of [^{14}C]thymidine and [^{3}H]-methionine. Additionally to 5MC, DNA thymine and DNA adenine were labelled by [^{3}H]methionine. This results from the transfer of the methyl group of methionine via the C_1-pool to thymine and adenine precursors for DNA synthesis [15]. Consequently their [^{3}H]labelling rates are conform to the rate of DNA synthesis indicated as [^{14}C]thymidine incorporation. If there were any pool alterations as shown under the influence of diverse drugs by Puschendorf *et al.* [16], any differences should be observed in the three determined DNA synthesis rates. In our example (Table I) the DNA synthesis rates are well corresponding in their stimulation after hepatectomy and in their inhibition by Endoxan [17]. This indicates that there are no different alterations of these precursor pools involved. In contrast to the stimulation of DNA synthesis the labelling of DNA-5MC is less stimulated in the regenerating liver and is decreased to a higher extent by the action of Endoxan. This result let us propose that 24 hours after hepatectomy DNA methylation is reduced or incompletely terminated.

Studying DNA methylation in the spleen of immunized mice, we observed a reduced [^{3}H]methionine labelling of DNA-5MC, thymine and adenine compared to the control animals [18]. On the other hand we could demonstrate an increased thymidine incorporation into spleen DNA after immunization. This indicates a variation or a different behaviour of the single precursor pools. To avoid such pool alterations we measured the direct conversion of DNA cytosine to DNA-5MC applying [^{14}C]d-cytidine (Table II). The correlation of the radioactivity quantitatively analysed (Goldamnn-Ménagé [11], p. 258, found in DNA-5MC to DNA cytosine corresponds to the DNA methylation rate at the newly synthesized polynucleotide. Indications are given by the base analysis that small deviations either in the base composition of each isolated DNA batch or in the base separation should be considered. It is well known that 5MC is not uniformly distributed over the whole DNA, on the contrary, it is clustered in some DNA segments, as for example in the mouse satellite DNA which contains relatively about 3 times more 5MC than the rest of the DNA [19]. Therefore it seems to be obvious that the isolated DNA may differ in its content of 5MC from one experiment to the other. So, it does not suffice to correlate only the radioactivity found in the DNA bases cytosine and 5MC of a given DNA amount. The radioactivity must be refered to the content of the separated bases. We want to propose the expression of the correct DNA methylation rate in the correlation of $[5MC \cdot 100]/[MC + C]$ as dpm/μmole base.

Using this way of calculation similar values are obtained for the enhanced spleen DNA methylation in BSA immunized mice in the single experiments. The differences in the values calculated either from the radioactivity found in the bases of a defined amount of DNA and from the specific radioactivity of each base are demonstrated in the two last columns of the Table II.

Supported also by grants from Deutsche Forschungsgemeinschaft and Bundesministerium für Forschung und Technologie.

[1] M. Gold, M. Gefter, R. Housmann, and J. Hurwitz, J. gen. Physiol. **49**, 5 [1966].
[2] E. Borek and P. R. Srinivasan, Annu. Rev. Biochem. **35**, 275 [1966].
[3] R. H. Burdon and R. L. P. Adams, Biochim. biophysica Acta [Amsterdam] **174**, 322 [1969].
[4] C. Lark, J. molecular Biol. **31**, 389 [1968].
[5] D. Billen, J. molecular Biol. **31**, 477 [1968].
[6] T. W. Sneider and R. van Potter, J. molecular Biol. **42**, 271 [1969].
[7] F. Kalousek and N. R. Morris, J. biol. Chemistry **243**, 2440 [1968].
[8] F. Kalousek and N. R. Morris, Science [Washington] **164**, 721 [1969].
[9] D. Drahovsky and N. R. Morris, J. molecular Biol. **57**, 475 [1971].
[10] G. M. Higgins and R. M. Anderson, Arch. Pathology **12**, 186 [1931].
[11] J. S. Colter, R. A. Brown, and K. A. O. Ellem, Biochim. biophysica Acta [Amsterdam] **55**, 31 [1962].
[12] J. Marmur, J. molecular Biol. **3**, 208 [1961].
[13] D. Lutz, M. Löwel, H. Kröger, D. Kubsch, and W. Uecker, Z. Naturforsch. **27 b**, 992 [1972].
[14] V. M. Craddock, Biochim. biophysica Acta [Amsterdam] **240**, 376 [1970].
[15] S. Kit, Ch. Beck, O. L. Graham, and A. Gross, J. biol. Chemistry **233**, 944 [1958].
[16] B. Puschendorf and H. Grunicke, Biochim. biophysica Acta [Amsterdam] **272**, 16 [1972].
[17] D. Lutz, H. Grahn, and H. Kröger, Z. Naturforsch. **28 c**, 460 [1973].
[18] D. Lutz, H. Kröger, W. Uecker, D. Kubsch, and E. Mertens, in preparation.
[19] R. Salomon, A. M. Kaye, and M. Herzberg, J. molecular Biol. **43**, 581 [1969].