The influence of subcellular fractions on the enzymic methylation of
DNA in ascites cell nuclei

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ABSTRACT

Soluble factors appear to be present in both nuclei and cytoplasm of Krebs 2 ascites tumour cells capable of stimulating the enzyme catalysed methylation of DNA in isolated nuclei from these cells.

INTRODUCTION

5-methyl cytosine in mammalian DNA is formed by the selective methylation by specific DNA methylases of cytosine residues in certain nucleotide sequences of DNA.¹⁻⁴ As yet the function of this genomic modification is not understood although suggestions have been put forward concerning a possible role in cellular differentiation and gene activity.^{5,6} Indeed recent data indicate the 5-methyl cytosine content of mammalian DNA to be both species and tissue specific.^{7,8}

Previous studies have shown that the DNA methylase activity of mammalian cells is located in the nuclear chromatin fraction.¹⁻⁴ More recent studies with rat liver however have shown that the DNA methylase can be solubilised from the chromatin but only under stringent extraction conditions. This achievement has led to its partial purification and to detailed studies on the nature of its interaction with its substrate DNA.⁹ On the other hand little is yet known regarding possible regulatory factors that might control DNA methylation in the intact chromatin. This report points to the existence of factors in soluble preparations from the nucleus and cytoplasm of mouse ascites tumour cells which stimulate the methylation of endogenous DNA nuclei isolated from these cells.

METHODS

Nuclei were prepared from Krebs 2 ascites tumour cells using Tween 80 by the method described previously.³ Either these were suspended in 10 vol TME buffer (0.02M tris-HCl buffer pH 7.8 - 0.01M 2-mercaptoethanol) for direct use or were subsequently 'extracted' two times by homogenisation in 10 vol TME buffer in a glass-teflon homogeniser followed by centrifugation at 600g for 10 min. After the second 'extraction' the sedimented extracted nuclei were resuspended in 10 vol TME buffer. The supernatant fluids from each centrifugation were combined to yield a preparation henceforth termed the nuclear extract.

Crude cytoplasm was prepared from Krebs 2 cells by first homogenising the cells in ice-cold water to cause their disruption¹⁰ and then after making the homogenate 0.02M with respect to tris buffer pH 7.8 centrifuging at 600 g for 10 min to yield a supernatant fluid. This is referred to henceforth as the crude cytoplasm and in certain cases was centrifuged at 140,000 g for 3 hr to yield a cytosol fraction.¹¹

The methylation of endogenous DNA of chromatin was determined using S-adenosyl-L-[Me-³H] methionine (Radiochemical Centre, Amersham; sp.act. 530mC/mole) and the SDS/phenol extraction method already described.³

The level of S-adenosyl-L-methionine clearing activity in the various subcellular fractions was determined by the method described by Geftter *et al.*¹²

The estimation of DNA and protein in various subcellular fractions was carried out by the methods of Burton¹³ and Lowry *et al.*¹⁴ respectively.

RESULTS AND DISCUSSION

Figure 1 shows the time course of transfer of methyl groups from S-adenosyl-L-[Me-³H]methionine to endogenous DNA as catalysed by nuclei of Krebs 2 ascites tumour cells. Whilst there is extensive transfer up until around 4 hr at 37°, this is not the case if the nuclei are initially extensively extracted with buffer of low ionic strength.

Figure 1. Time course of incorporation of [^3H]methyl groups into DNA of Krebs 2 cell nuclei. Each reaction mixture (0.5ml) was 0.02M with respect to tris-HCl pH7.8, 0.01M with respect to 2-mercaptoethanol and contained 5 μC S-adenosyl-L-methionine and 0.2 ml of the appropriate suspension of nuclei (equivalent to 70 μg DNA). In the case of the intact Krebs 2 cell nuclei the protein to DNA ratio (by weight) was 14.6 whereas in the case of the extracted nuclei it was 10.3. Where indicated 0.25 ml of nuclear extract (0.4 mg protein) was added to the extracted nuclei. Incubation in all cases was at 37° and each reaction was subsequently analysed using the phenol extraction procedure already described. The results are expressed as pmoles CH_3 incorporated into DNA per mg DNA, by isolated Krebs 2 nuclei ●—●, by extracted Krebs 2 nuclei ○—○, and by extracted nuclei plus nuclear extract, △—△

This yields a crude chromatin like preparation (see ref.3) in which transfer of methyl groups to endogenous DNA is somewhat reduced (see Fig.1) and in any case ceases after only 1 hr. Addition of the nuclear extract to these extracted nuclei has a restorative effect on DNA methylation. Prior dialysis (Table 1) against TME buffer of the nuclear extract, (which itself has no detectable ability, under these conditions, to catalyse the methylation of added Krebs 2 DNA),

Table 1

The effect of various subcellular fractions and cyclic nucleotides on the methylation of DNA by nuclei of Krebs 2 cells.

Nuclei	Addition	p moles CH ₃ incorporated/mg DNA
<u>extracted nuclei</u>	none	9.2
"	<u>nuclear extract</u>	16.3
"	" (dialysed)	17.2
"	" (heat treated)	9.8
<u>intact nuclei</u>	none	34.7
"	<u>crude cytoplasm</u>	69.0
"	<u>crude cytoplasm</u> (heat treated)	65.5
"	<u>cytosol</u>	57.3
	<u>cytosol</u> (dialysed)	59.5
	<u>cytosol</u> (heat treated)	55.4
	cAMP(10^{-6} M)	32.0
	cGMP(10^{-6} M)	31.9

Each reaction mixture (0.5ml) was 0.02M with respect to tris-HCl pH 7.8, 0.01M with respect to 2-mercaptoethanol and contained 5 μ C S-adenosyl-L-[Me-³H]methionine and 0.2ml of the appropriate nuclear suspension (equivalent to 70 μ g DNA). 0.2ml portions of nuclear extract, crude cytoplasm, and cytosol (equivalent to 0.4mg protein) were added where indicated. Dialysis was for 24 h against TME buffer and heat treatment for 3 min at 100°. Incubation in all cases was at 37° for 4 hr and subsequent analysis as described for Figure 1.

has no effect on its ability to stimulate the methylase activity of the extracted nuclei. Heat treatment however at 100° for 3 min abolished its stimulatory effect. Addition of low levels of pancreatic DNase to the extracted nuclei is without effect on their ability to methylate endogenous DNA (Fig.2) suggesting that the stimulatory effect of the nuclear extract was probably not trivial and simply explained by the presence of some nuclease in the nuclear extract. Pancreatic RNase (Fig.2) was also without effect.

Figure 2. The effect of DNase and RNase on the ability of extracted Krebs 2 nuclei to incorporate [3 H]methyl groups into endogenous DNA. The assays were carried out for 4 hours using the extracted nuclei under the conditions described for Figure 1. The results are expressed as pmoles CH_3 incorporated into DNA per mg DNA by extracted Krebs 2 nuclei in the presence of pancreatic DNase o—o; or in the presence of pancreatic RNase Δ — Δ .

Additionally it could be demonstrated that the extracted nuclei contained negligible levels of S-adenosyl-L-methionine cleaving activity (only 2% of the substrate was degraded per hr at 37°) so the stimulatory effects of the nuclear extract could not be readily explained by the presence of inhibitors of S-adenosyl-L-methionine cleaving enzymes.

Since neither the crude cytoplasm or the cytosol fraction from Krebs 2 cells contain readily detectable levels of DNA methylase activity it was also of interest to determine whether these preparations contained any stimulatory activity for the nuclear DNA methylation. From Table 1 it can be seen that both the crude cytoplasm and cytosol fractions are stimulatory. Thus the cytoplasm also appears to contain soluble stimulatory factors, but unlike the nuclear factors they are heat stable. On the other hand neither cyclic AMP or cyclic GMP could mimic the stimulatory effect of the cytosol and subsequent dialysis studies suggest the stimulatory factor to have a higher molecular weight than such nucleotides.

Thus there appear to be distinctive factors from both the nucleus and cytoplasm of Krebs 2 cells capable of stimulating the methylation of DNA in nuclei of these cells. It cannot yet be decided whether these effects are mediated by a direct interaction with the chromatin DNA methylase or merely result from an alteration brought about in the tertiary structure of chromatin which simply allows more DNA methylation to occur. Also it remains to be seen whether cytosine residues on different types of DNA sequence are involved. In addition in view of the suggested role of 5-methyl cytosine in differentiation it will be of interest to examine more closely the effect of cytoplasmic preparations from cells other than the Krebs 2 strain. For instance preliminary studies with mouse liver cytosol (heat treated at 100° for 3 min to destroy S-adenosyl-L-methionine cleaving activity) show it also capable of stimulating the DNA methylase activity of isolated Krebs 2 cell nuclei.

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