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Selective inhibition of cytosine-DNA methylases by polyamines

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Abstract We have advanced the hypothesis that polyamines affect DNA methylation and thus promote the expression of developmentally controlled genes. We demonstrate that the activity of cytosine-DNA methyltransferases *Hpa*II, *Hha*I, *Hae*III and *Sss*I is inhibited by physiological concentrations of polyamines. On the other hand, activity of the adenine-DNA methyltransferase *Eco*RI, and restriction enzymes *Hpa*II, *Hha*I, *Hae*III and *Eco*RI, is insensitive to polyamine concentrations up to 40 mM. Our results indicate that the effect of polyamines on cytosine-DNA methyltranferases is rather selective and suggest a possible mode of action in vivo.

Key words: Putrescine; Spermidine; DNA-methylation; Restriction enzyme

1. Introduction

Polyamines are essential metabolites which play important roles in the growth of all prokaryotic and eukaryotic cells (see reviews in [1–3]). It has been considered that, because of their basic net charge, polyamines exert their multiple functions by binding to negatively charged macromolecules and cellular structures. Most of the polyamine activities are related to their ability to bind to nucleic acids. In *Escherichia coli*, 90% of the total spermidine is bound to RNA, 5% to DNA and only 0.8 to membrane lipids [4]. Polyamines protect DNA from enzymatic degradation, X-ray irradiation and mechanical shearing. They stabilize RNA and prevent ribosomal dissociation. A role for polyamines in DNA replication has also been established in vivo by use of inhibitors of their biosynthetic enzymes. In vitro they stimulate DNA and RNA synthesis, and increase translation fidelity. More recently it has been suggested that they protect replicating DNA from oxidative damage [5,6].

Polyamines are also involved in development and cell differentiation [1,2]. Their precise mode of action in these functions remains uncertain, although a considerable body of information suggests that specific inhibition of their biosynthesis interferes with the development of almost all the eukaryotic systems analyzed thus far. We have reported that, prior to each differentiation step in the development of a significant number of fungal systems, increases in the pools of polyamines and in the activity of ornithine decarboxylase (ODC) [7,8] take place. Addition of ODC inhibitors blocks differentiation but affects growth to a much lesser extent [9–11]. Studying the effect of ODC inhibitors on the levels of methylation of total DNA [12] and selectively expressed genes [13], and its reversal by the DNA-methylation inhibitor 5-methyl cytosine [9,11,12], we have suggested that polyamines affect the methylation state of DNA [12–14]. In many biological systems it has been demonstrated that the state of DNA methylation may regulate gene expression at different developmental stages [15,16].

Accordingly, a generalized hypothesis would be that the role of increased levels of polyamines in development is to inhibit DNA methylation, thus permitting the expression of specific genes. Among the possible mechanisms, the simplest one is a direct effect of polyamines on DNA methylases. As a model system to test this hypothesis, we have made use of the cytosine-DNA methylases *Hpa*II, *Hha*I, *Hae*III and *Sss*I, which recognize the sequences CpCpGpG, GpCpGpC, GpGpCpC and CpG, respectively. This selection was based on the fact that most methylcytosine residues in animal [15] and fungal [17] systems occur in the CpG sequence. The rationale for using bacterial cytosine-DNA methylases as the test system lies in the observation that cytosine-DNA methylases from bacterial, animal and plant origin are structurally similar, containing ten conserved motifs [18,19]. Accordingly, the results could be extrapolated to other systems. Furthermore, it has been described that *Hpa*II methylase is able to methylate DNA from human chromosomes in situ [20]. As controls we included *Eco*RI methylase which modifies the internal adenine residue of the sequence GpApApTpTpC, and the restriction enzymes specific for the sequences modified by the corresponding methylases. We tested only the effect of putrescine and spermidine, since these are universally present in all organisms. Most fungi do not contain spermine [21].

2. Materials and methods

2.1. Restriction analyses

Incubation mixtures in a final volume of 10 μl contained the buffer solutions provided by the restriction enzyme supplier (Boehringer), 0.5 μg of λ DNA (Boehringer), 3 U of restriction enzyme and polyamines where indicated. Polyamines (hydrochlorides; Sigma) did not produce any change in pH of the reaction mixtures. After 3 h at 37°C, samples were subjected to agarose gel (0.8%) electrophoresis in the presence of ethidium bromide, and photographed under UV light.

2.2. Determination of DNA methylation by its resistance to restriction enzymes

Reaction mixtures in a final volume of 10 μl contained the corresponding buffer solutions provided by the methylase supplier (Bio-Labs), 0.5 μg λ DNA, 2 U of methylase, 80 μM SAM, and polyamines where indicated. After 4 h at 37°C, samples were heated at 65°C for 15 min. Nuclease buffer (40 μl) containing 5 U of the corresponding restriction endonuclease was added, samples were incubated for 2 h, and analyzed by agarose gel-electrophoresis.

2.3. Determination of DNA methylation by a radioactive assay

Incubation mixtures (20 μl) containing the corresponding buffers

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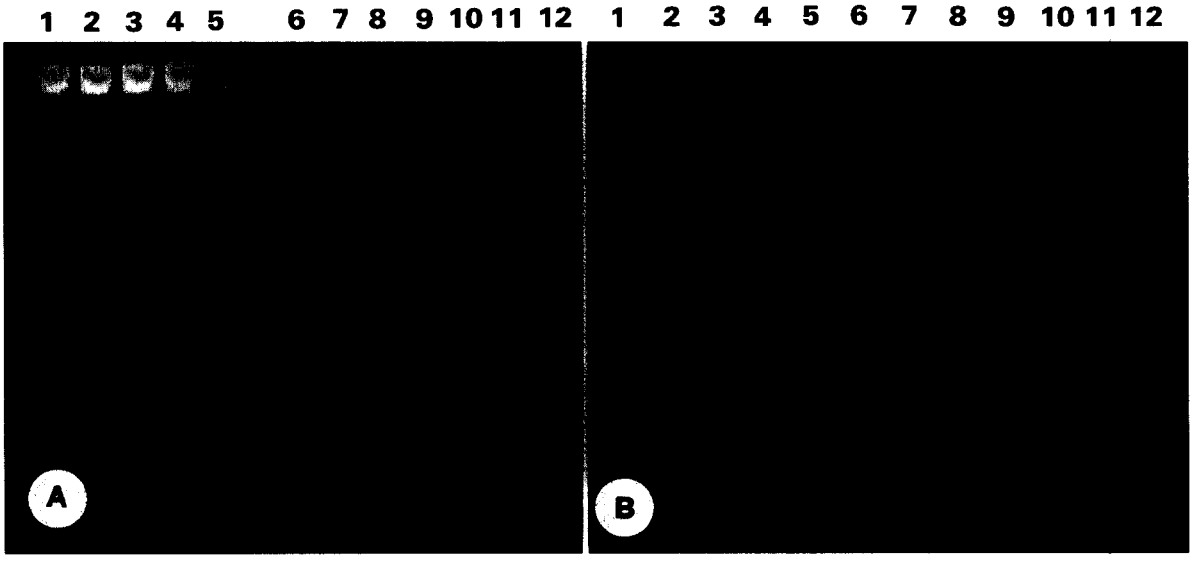

Fig. 1. Effect of putrescine and spermidine on the activity of restriction enzymes. Conditions as described in section 2. (A) Lanes 1–5, *Eco*RI. Lanes 7–11 *Hpa*II. Lanes 6 and 12, λ DNA digested with *Hin*dIII as M_r markers. Lanes 1 and 7, no polyamine; lanes 2 and 8, 20 mM putrescine; lanes 3 and 9, 40 mM putrescine; lanes 4 and 10, 10 mM spermidine; lanes 5 and 10, 30 mM spermidine. Note that spermidine induces unspecific activity of *Hpa*II. (B) Lanes 1–5, *Hha*III. Lanes 7–11, *Hae*I. Lanes 6 and 12, M_r standards. Polyamine concentrations as in A.

provided by the methylase supplier, 20 μg *E. coli* DNA (Sigma), 80 μM SAM, and 2 U of each methylase, were incubated at 37°C for 15 min. At this time, *S*-adenosyl L-[methyl-^{3}H]methionine (1 μCi, 3.03 TBq/mmol; Amersham) and where indicated spermidine trihydrochloride, were added (addition of spermidine did not bring about any change in pH). Samples were incubated at 37°C for 2 h more, and DNA was precipitated with 120 μl of 0.5 M sodium acetate and 1 ml of isopropanol. DNA was sedimented by centrifugation, washed 3 times with cold 70% ethanol, resuspended in 100 μl of water, and mixed with Readysafe (Beckman) scintillation fluid. Radioactivity was measured in a liquid scintillation spectrometer.

3. Results and discussion

The activity of restriction enzymes *Hpa*II, *Hha*I, *Hae*III and *Eco*RI was not inhibited by concentrations of spermidine and putrescine up to 30 and 40 mM, respectively (Fig. 1). In the presence of spermidine *Hpa*II digested the DNA abnormally. Whether this effect is due to a change in specificity of the enzyme or the activation of a contaminating nuclease remains unknown.

Since the restriction enzymes were resistant to polyamines, we measured the effect of putrescine and spermidine on methylation by an indirect method. We incubated the DNA substrate with each of the methylases in the presence or absence of polyamines. Methylases were heat-inactivated, DNA was digested with the corresponding restriction enzymes, and the products were analyzed by agarose gel-electrophoresis. The activity of the the three cytosine-DNA methylases was extremely sensitive to polyamines, mainly spermidine (Fig. 2). On the other hand, *Eco*RI methylase was insensitive to these concentrations of polyamines (Fig. 2). No differences were observed when DNA from λ bacteriophage (Fig. 2) or a pBR322-derived plasmid, grown in *Escherichia coli* 607119 deficient in methylases (not shown), were used as methylation substrates.

Quantitative data on the effect of polyamines on DNA methylases were obtained by measuring the incorporation of radioactive methyl groups from *S*-adenosyl L-[methyl ^{3}H]methionine (SAM-^{3}H) into *E. coli* DNA. The results confirmed that spermidine inhibited DNA methylation by cytosine-DNA methylases (Table 1). By contrast, no inhibition of *Eco*RI methylase by spermidine concentrations up to 40 mM was observed when incubated under the same conditions (Table 1). Two- to three-times higher levels of inhibition were observed when spermidine was mixed with DNA before the addition of methylase, as compared to reaction mixtures to which the polyamine was added once the methylation reaction was in progress (Table 2). Accordingly it appears that polyamines inhibit both the binding and activity of the methylases.

The kinetics of inhibition of *Hpa*II and *Hha*I methylases by spermidine as a function of DNA concentration were complex but clearly non-competitive (Fig. 3). A significant difference in

Table 1
Inhibition of cytosine-DNA methylases by spermidine using a radiolabel method

Spermidine (mM)	Methyl groups incorporated (pmol)					Inhibition (%)				
	*Hae*III	*Hha*I	*Hpa*II	*Sss*I	*Eco*RI	*Hae*III	*Hha*I	*Hpa*II	*Sss*I	*Eco*RI
None	37.6	30.3	11.38	426.0	25.4	–	–	–	–	–
5	20.6	25.3	86.6	316.2	26.7	45	17	24	26	0
10	8.5	22.4	40.8	92.5	25.3	51	26	64	55	0.4
20	9.4	17.6	24.9	50.8	29.9	74	42	78	88	0
30	3.1	7.0	10.8	31.5	25.2	92	77	91	93	0.8

Methylation was measured by the transfer of methyl groups from SAM-^{3}H as described in section 2.

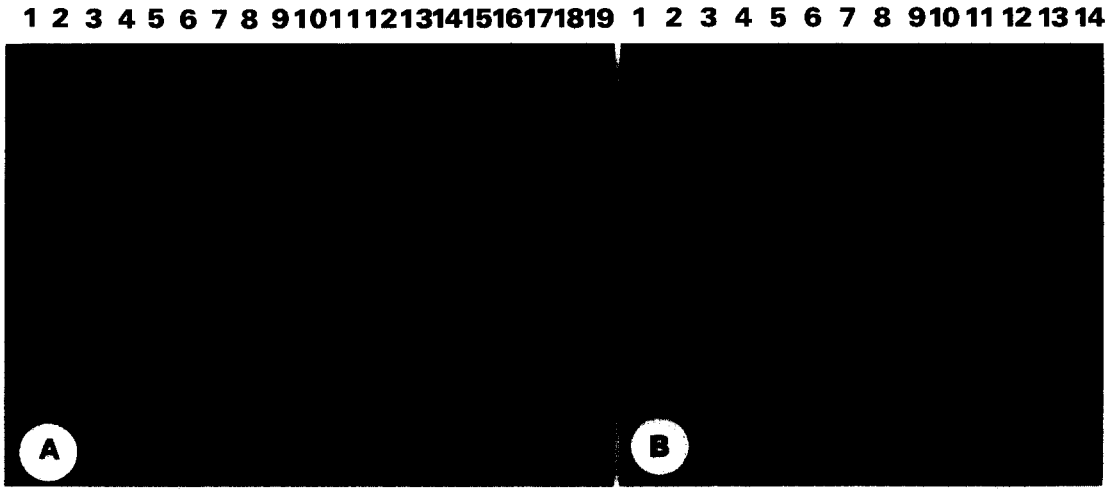

Fig. 2. Effect of polyamines on DNA methylases. Conditions as described in section 2. (A) Lanes 1–7, DNA methylated with *Eco*RI methylase, and digested with *Eco*RI. Lanes 12–18, DNA methylated with *Hpa*II methylase and digested with *Hpa*II. Lane 8, undigested λ DNA. Lane 9, unmethylated DNA digested with *Eco*RI. Lane 10, unmethylated DNA digested with *Hpa*II. Lanes 11 and 19, M_r markers. Lanes 1 and 12, no polyamine; lanes 2 and 13, 10 mM putrescine; lanes 3 and 14, 20 mM putrescine; lanes 4 and 15, 40 mM putrescine; lanes 5 and 16, 2 mM spermidine; lanes 6 and 17, 4 mM spermidine; lanes 7 and 18, 8 mM spermidine. (B) Lanes 2–7, DNA methylated with *Hha*I methylase and digested with *Hha*I. Lanes 9–14, DNA methylated with *Hae*III methylase and digested with *Hae*III. Lanes 1 and 8, M_r standards. Lanes 2 and 9, no polyamine; lanes 3 and 10, 10 mM putrescine; lanes 4 and 11, 20 mM putrescine; lanes 5 and 12, 10 mM spermidine; lanes 6 and 13, 20 mM spermidine; lanes 7 and 14 unmethylated DNA digested with *Hha*III or *Hae*I, respectively.

the K_i value of spermidine was observed between both enzymes (Fig. 3). The kinetics of inhibition of *Hha*I as a function of SAM concentration was of the mixed type; and the one from *Hpa*II was non-competitive (Fig. 4).

Owing to their polycationic nature, polyamines bind to negatively charged molecules. It has been suggested that polyamines bind to the minor groove of B-DNA, moving to the major groove in the A-form [22]. This binding appears to be responsible for their multiple effects. However, clear-cut examples that polyamines recognize selective sites in the DNA are rare. The most striking specific interactions of polyamines with nucleic acids occur during transcriptional and translational regulation of their own synthesis [23,24]. Additional examples of a certain degree of specific interaction between polyamines and DNA is the observation that some tricationic polyamines bind selectively to GC sequences [25], and a report describing the

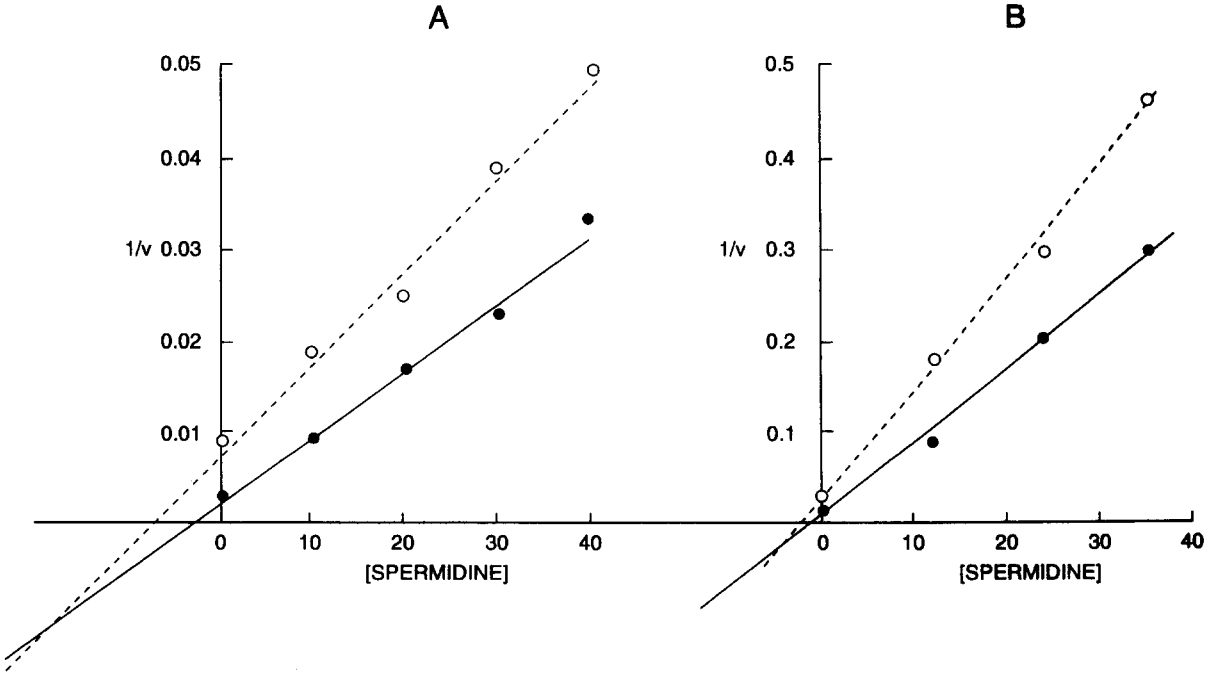

Fig. 3. Kinetics of inhibition of *Hha*I and *Hpa*II methylases by spermidine as a function of SAM concentration. Conditions as described in section 2. (A) *Hha*I methylase. ○, 30 μM SAM; ●, 100 μM SAM. (B) *Hpa*II methylase. ○, 20 μM SAM; ●, 50 μM SAM. Data are plotted according to Dixon [28]. Spermidine is expressed in mmolar concentrations.

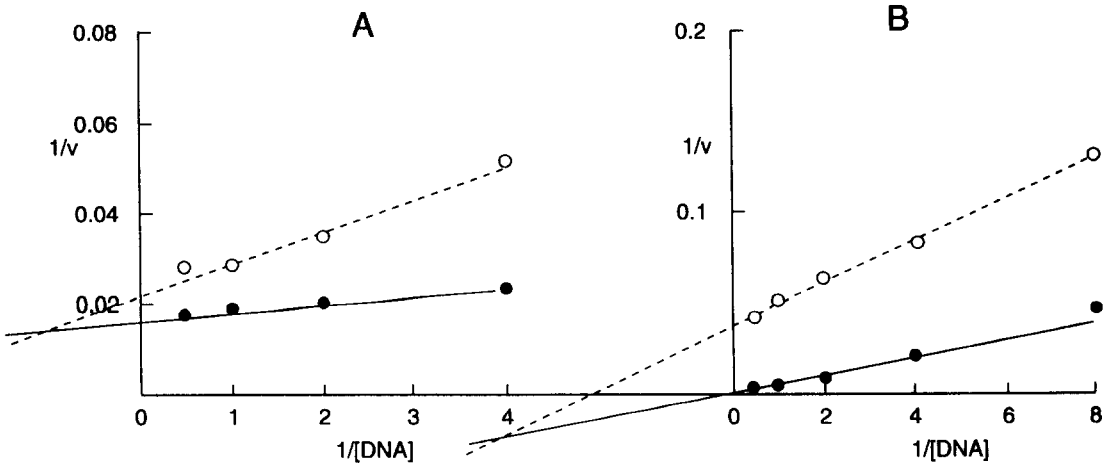

Fig. 4. Kinetics of inhibition of *Hha*I and *Hpa*II methylases by spermidine as a function of DNA concentration. (A) *Hha*I methylase. ○, 20 mM spermidine; ●, no spermidine. (B) *Hpa*II methylase. ○, 20 mM spermidine; ●, no spermidine. Data are plotted according to Lineweaver and Burk [28]. DNA concentrations are expressed as μg per μl.

stimulatory effect of spermidine on the restriction enzyme *Nae*I, mainly in the absence of activating oligonucleotides [26].

Our results demonstrate that polyamines can selectively inhibit cytosine-DNA methylases. As would be expected, inhibition is not competitive. It follows a complex kinetics, probably interfering with the interaction of both substrates at a different site other than the catalytic one. It would be tempting to suggest that polyamines selectively interfere with the recognition of DNA by one (some) of the motifs present in cytosine-DNA methylases [18,19]. In this sense it is important to recall that polyamines did not inhibit *Eco*RI, an adenine-DNA methylase. Adenine-DNA methylases do not contain those highly conserved motifs [18,19l. Also, at the concentrations used, polyamines did not affect the activity of restriction enzymes which recognize the same nucleotide sequences as the corresponding cytosine-DNA methylases.

Of particular interest is the observation that *Sss*I methylase was as sensitive to spermidine as *Hae*III and *Hpa*II. It has been reported that this enzyme methylates DNA in a processive manner, resembling mammalian methylases rather than other bacterial methylases [27].

Whether the direct mechanism of inhibition of DNA methylation by polyamines reported here is responsible for some of their effects in development remains to be determined. Nevertheless, the fact that, under the conditions tested, polyamines did not affect *Eco*RI or restriction enzymes but only cytosine-DNA methylases, suggests that their effect is selective ith respect to DNA–enzyme interaction and lends support to our working hypothesis.

Table 2
Effect of pre-mixing spermidine with DNA on the inhibition of *Hpa*II methylase activity

Spermidine (mM)	Pre-mixing DNA–polyamine	Methyl groups incorporated (pmol)	Inhibition (%)
None	No	108.2	–
2.0		96.4	11
5.0		81.3	25
2.0	Yes	70.0	35
5.0		33.0	70

Conditions are as described for Table 1. Where indicated, putrescine and DNA were pre-mixed and incubated for 15 min at 37°C, before addition of the rest of the components of the incubation mixture.

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