DNA METHYLASE ACTIVITY ASSOCIATED WITH ROUS SARCOMA VIRUS

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1. Introduction

More than 15 enzyme activities associated with purified preparations of C-type RNA tumor viruses have been reported [1]. Some of these enzymes function in synthesis or in modification of proteins, while others are involved in nucleic acid synthesis. However, the only one definitely known to be coded for by the viral genome is RNA-directed DNA polymerase (reverse transcriptase) associated with nuclease H [2]. In contrast, most of the other virus-associated enzymes are presumably coded for by the host cell. We have shown recently that the N^2-methylguanine transferase associated with avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV) which methylates tRNA of *Escherichia coli*, presumably originates from the host cell [3]. An RSV-associated tRNA methylase which incorporates a small amount of radioactivity from *S*-adenosyl-methionine (SAM) into *E. coli* DNA was described [4].

S-Adenosyl-homocysteine (SAH) the demethylated derivative of SAM is the natural inhibitor of nearly all transmethylases in vitro [5,6]. Some synthetic analogs of SAH inhibit nearly completely RSV-induced cell transformation and concomitant virus replication [7,8], although SAH has no such activity, due to its rapid intracellular degradation [9]. The ability of synthetic methylase inhibitors to block both the production of RSV and cell transformation by this virus, has prompted us to further study the RSV-associated DNA methylase. The function of this enzyme might be to methylate viral cDNA. This methylation might be required for subsequent steps of viral replication and cell transformation and might be inhibited by the analogs. Our results show that the activity of the RSV-associated DNA methylase is dependent on the presence of detergent, and is distinct from the virus-associated tRNA methylase.

The enzyme, which appears to be located in the outer envelope or in the surface projection of the virus does not methylate cDNA, and the synthetic SAH analogs which inhibit virus replication and cell transformation have only a weak affinity for this methylase.

2. Materials and methods

Different DNAs were obtained from the following commercial sources: *Micrococcus lysodeikticus*, Miles Laboratories Inc., USA; calf thymus, Choay, France; *E. coli B* and salmon sperm, Sigma, USA. DNA from normal and RSV-transformed chick embryo fibroblasts (CEF) was extracted as in [10]. cDNA from the same cells was prepared with reverse transcriptase as in [11] in final vol. 500 μl (extraction as above).

2.1. *Chemicals*

Compounds 2,3,4 listed in table 3 were synthesised in our laboratory [8]. SAH and SIBA came from

Sefochem Fine Chemicals, Israel. Unlabelled deoxyribonucleotides came from Sigma, USA. [^{3}H]-Thymidine-5′-triphosphate (spec. act. 10 or 30 Ci/mM) and *S*-adenosyl [*methyl*-^{3}H]methionine (spec. act. 10 Ci/mM) came from Amersham, England. The non-ionic detergent Nonidet P 40 (NP 40) was from Shell, France.

2.2. *Cell cultures and virus production*

Infection of CEF by RSV strain SRD and virus purification were performed as in [7].

2.3. *Separation of viral components*

Virus protein concentration was adjusted to 3 mg/ml, estimated as in [12]. The purified virus preparation was then incubated with various concentrations of NP 40 at 37°C for 30 min. 500 μg were put on top of a 30–66% linear sucrose density gradient. Sedimentation was run for 16 h in a Spinco ultracentrifuge, in a SW 41 rotor, at 30 000 rev/min. Fractions of 300 μl were then collected from the top to the bottom of the gradient and the A_{280} monitored.

2.4. *DNA methylase assay*

The incubation mixture in 50 μl had the following composition: 50 mM Tris–HCl, pH 8.3, 60 mM NaCl, 16 mM $MgCl_2$, 3 mM dithiothreitol, 50 μg heat denatured calf thymus DNA, 0.2% NP 40, 1.6 μCi methyl-labelled SAM. The mixture was incubated at 37°C for 1 h. Blanks were run under the same conditions, without DNA. The reaction was stopped by adding cold 5% trichloroacetic acid (TCA), then the precipitate was filtered on GF/C Whatman filters, thoroughly washed by cold 5% TCA and counted. Blank values were deducted in order to determine the incorporation of [^{3}H]methyl from SAM into DNA; specific activity is expressed in pmol [^{3}H]methyl groups incorporated into 50 μg DNA in 1 h at 37°C.

2.5. *Reverse transcriptase assay*

This was performed as in [11]. RNA methylase was assayed as in [5].

2.6. *Thin-layer chromatography*

The hydrolysate of DNA was chromatographed with a mixture of known bases in the following solvents: first dimension, ethyl acetate, methanol, H_2O, formic acid (100 : 25 : 20 : 1); second dimension, acetonitrile, ethyl acetate, 2-propanol, 1-butanol, 34% ammonium hydroxide (40 : 30 : 20 : 10 : 37.5).

The spots were detected in ultraviolet, scraped and counted.

3. Results and discussion

We reported recently that an N^2-methylguanine transferase associated with AMV and with RSV is located inside the viral envelope but outside the viral core, contrary to reverse transcriptase which is in the core [3]. In order to know whether the DNA methylase activity associated with RSV is distinct from the RNA methylase, we have studied its location by sucrose density gradient centrifugation of purified viruses in the presence of detergent (NP 40) under conditions allowing the separation of virions, viral cores and intermediate particles. Before performing this study, we checked that no methylase activity was associated with purified concentrated viruses. As seen in fig.1a when 1% NP 40 was used, reverse transcriptase and RNA methylase activities remained together, at a density of 1.15 g/cm^3, while the DNA methylase activity sedimented at 1.16 g/cm^3. With 2% NP 40, DNA methylase activity remained at a density of 1.16–1.17 g/cm^3, while RNA methylase activity sedimented at a density of 1.23 g/cm^3 and reverse transcriptase activity at 1.26 g/cm^3, corresponding to that expected for viral cores. (fig.1b). These results suggest that the two methylases are distinct proteins, which are not adsorbed onto the virions since their activity is detected only following treatment with NP 40. Furthermore, neither activity sediments with the viral core but at lower densities suggesting that the tRNA methylase may be located inside the outer viral envelope, whereas the DNA methylase may be associated with this envelope, or with its surface projections. Indeed, treatment of AMV with NP 40 releases the surface projections in a distinct aggregate which can be separated completely from other virus material by density gradient centrifugation [13].

We also looked for the presence of a DNA methylase activity in other RNA viruses such as Rous-associated leukemia virus (RAV_1) avian myeloblastosis virus (AMV) murine sarcoma virus (MSV) and vesicular stomatitis virus (VSV). Only RSV and RAV_1

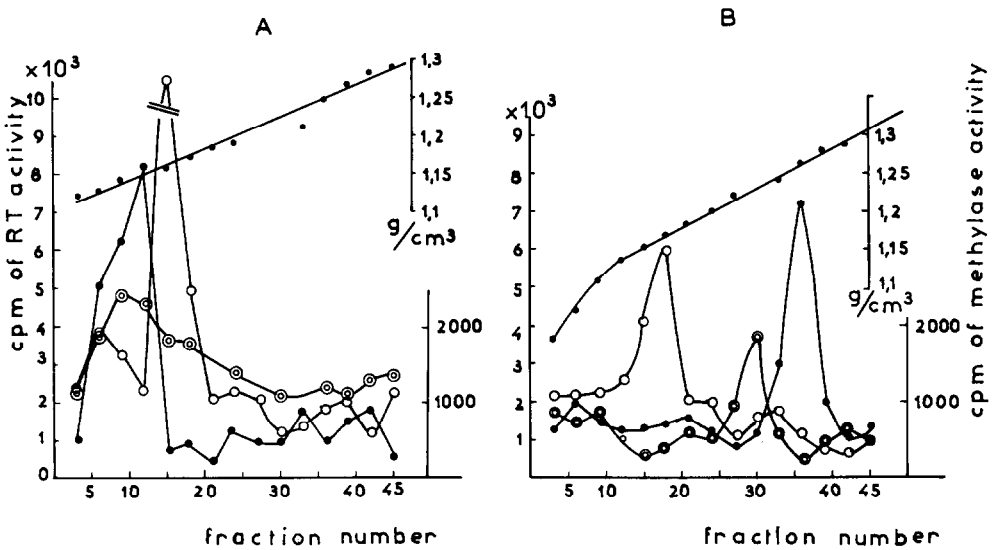


Fig.1. Localization of enzymatic activities in disrupted viral particles. A: treatment with 1% NP 40. B: treatment with 2% NP 40. Reverse transcriptase (•——•). RNA methylase activity (◎——◎). DNA methylase activity (○——○).

showed this activity. The fact that the virus associated DNA methylase appears to be located on the outer membrane of the virus or its projections led us to consider, whether this enzyme might have a cellular origin. However, no DNA methylase activity was found in purified CEF cell membranes prepared as in [14].

The main characteristics of this DNA methylase were subsequently determined.

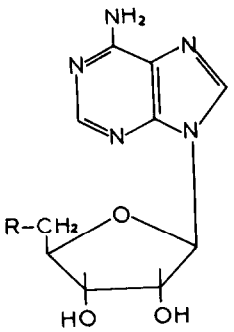
The following specific activities were obtained with DNAs from different sources: heat-denatured calf thymus, 340; RSV-transformed CEF, 175; salmon sperm, 165; *E. coli B*, 95; normal CEF, 68; *M. lysodeikticus*, 50; RSV cDNA, 3.6.

The best substrate is thus heat-denatured calf thymus DNA, whereas cDNA from RSV is not a good substrate. A linear relationship was obtained between the enzyme activity and the protein concentration up to 200 μg/ml and between enzyme activity and incubation time. The optimal pH range of the DNA methylase with heat-denatured calf thymus DNA was between 8.0 and 8.4. No enzyme activity could be detected without NP 40 and the optimal activity was obtained when the concentration of the non-ionic detergent was between 0.1% and 0.2% (results not shown).

The products of this DNA methylase have been determined. After extraction [15] the [^{3}H]methyl-labelled calf thymus DNA was treated with 88% formic acid for 2 h at 174°C. A two-dimensional thin-layer chromatography of the hydrolysate revealed that 40% of the radioactivity is incorporated into 5-methyl-cytosine and 38% into 1-methyl-adenine. 22% of the radioactivity was distributed to about the same proportion between *N*-6 methyl-adenine, 2-methyl-adenine, *N*-6,*N*-6-dimethyl-adenine, *N*-2-methyl-guanine and 5-methyl uracil. It is rather unprobable that the C-5 methylation of cytosine and the *N*-1 methylation of adenine are catalyzed by the same enzyme. The methylation of adenine by a DNA methylase is rather unexpected; however, 1-methyl-adenine was a minor product of in vivo methylation of hamster liver DNA by the carcinogen dimethyl-nitrosamine [16].

Since structural analogs of SAH are good inhibitors of cell transformation, we have tried to correlate this inhibition with their effect on the DNA methylase. Table 1 shows that all the analogs tested have low affinities for this enzyme in vitro. Hence, these results suggest that this methylase contrary to cellular tRNA and protein methylases is not the main target of the inhibitors.

Table 1
Effect of SAH and some of its analogs on RSV associated DNA methylases



No.		R	K_i (μM)
1	SIBA	H_3C \ HC–CH_2S– / H_3C	4310
2	ISOSIBA	CH_3–CH_2–CH(–CH_3)–S–	6000
3		CH_2=CH–CH_2S–	380
4		$HOH_2C(HO)HC$–CH_2S–	500
5	SAH	HOOC CH(NH_2)–$(CH_2)_2S$–	1.5

SAH and the analogs behave like competitive inhibitors with respect to SAM. The K_m value for SAM is 0.72 μM. The substrate used was heat-denatured calf thymus DNA

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