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AVIAN ONCORNAVIRUS ASSOCIATED N^2-METHYLGUANINE TRANSFERASE, LOCATION AND ORIGIN*

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SUMMARY. The location of the N^2-methylguanine transferase associated with Avian Myeloblastosis virus was determined by comparison with that of viral reverse transcriptase. The kinetic constants of the enzyme from Avian Myeloblastosis virus and from Rous Sarcoma Virus were determined and compared to those of N^2-methylguanine transferase from chick embryo fibroblast membrane fractions. Our results suggest that viral methylases unlike reverse transcriptase have a cellular origin.

INTRODUCTION

Various tRNA methylase activities associated with avian oncornaviruses have been reported, and the existence of an N^2-methylguanine transferase associated with AMV and with RSV has been demonstrated (1-3). - As no specific function for this enzyme in the virus replication cycle could be assessed, it may be asked, whether it plays a role in this cycle, and whether its association with the virus is not simply a result of the budding process by what viral maturation occurs ? Indeed, the only enzyme known to be coded for

*Dedicated to Professor Edgar Lederer on the occasion of his 70th birthday.

Avian Myeloblastosis Virus = AMV ; Rous Sarcoma Virus = RSV ; Chick embryo fibroblast = CEF ; RNA dependent DNA polymerase or reverse transcriptase = RT ; S-adenosyl-L-homocysteine = SAH ; 5'-deoxy-5'-S-isobutyl-adenosine = SIBA ; Ethylenediamine tetraacetic acid = EDTA ; S-adenosyl methionine = SAM ; Nonidet P 40 = NP 40 ; Sodium docecyl sulfate = SDS ; N^2methylguanine = N^2mG ; N^2,N^2-dimethylguanine = N^2,N^2dim ; Guanine = G ; 7-methylguanine = 7mG ; 1-methyladenine = 1mA ; 5-methylcytosine = 5mC ; 3-methylcytosine = 3mC ; 2-methyladenine = 2mA ; Uracil = U ; 5-methyluracil = 5mU ; N^6-methyladenine = N^6mA ; N^6,N^6-dimethyladenine = N^6N^6 dimA.

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by the viral genome in oncornaviruses is reverse transcriptase (RT) associated with nuclease H (4). Two other viral genes code for the viral internal proteins (gag) and envelope glycoproteins (env), and another (src) for the transforming capacity of avian sarcoma viruses (5). As N^2-methylguanine transferase activty was found to be also associated with non transforming oncornaviruses, it is probably not coded for by the src gene. On the other hand, oncornavirus particles are formed by budding from the cell surface, and components of the outer cell membrane become part of the viral envelope. So an enzyme close to the cell membrane may be trapped non specifically by the virus. - In this paper, we describe the presence of an N^2 methylguanine transferase in membrane fractions of CEF used for producing RSV. The comparison of the characteristics of this enzyme with those of the enzymes associated with AMV or RSV, strongly suggests the origin of the viral methylases is the host.

MATERIALS AND METHODS

Chemicals : S-adenosyl-L-homocysteine and 5'-deoxy-5'-S-isobutyl-adenosine were from Sefochem Fine Chemicals (Israel). E.coli K_{12}tRNA was generously supplied by Dr. Escaut (ICSN, CNRS, Gif sur Yvette) tRNA and RNA from Virginia Creeper and ethiolated wheat seedlings were provided by Dr. F. Vedel (University of Paris-Sud, Orsay)
Cells and viruses : Growth of CEF, preparation of cell-free extracts, and production of RSV (clonal line from SR-RSV-D)SR4 were as described earlier (6) Plasma from chickens infected with AMV was generously provided by Dr. J.W. Beard (Duke University, USA). Virus purification was performed as described by Bolognesi (7). The final pellet was resuspended in NTE buffer (150 mM NaCl, 10 mM Tris/HCl pH : 7.4 1 mM EDTA) to obtain a final concentration of 3-5 mg/ml of virus protein, as estimated by the Lowry procedure (8). The virus suspension was stored at - 40°C.
Membrane fractions from normal and RSV-transformed CEF were prepeared as described by Atkinson and Summers (9).

tRNAs from normal and RSV transformed CEF were extracted by a modified phenol procedure (10).
RNA-dependent DNA polymerase, or reverse transcriptase (RT), activity was assayed using the procedure of Temin and Mizutani (11). Under these assay conditions incorporation of 1 pmole of (^{3}H) thymidine 5' triphosphate (specific activity 10 Ci/mM, Amersham, G.B.) per 50 µl reaction mixture corresponded to 6700 cpm.

tRNA methylase activity was measured as described by Gantt (2). Under the assay conditions incorporation of 1 pmole of (^{3}H)-S-adenosyl methionine (5 Ci/mM) per 50 µl of reaction mixture corresponded to 3500 cpm. Specific activity was expressed in pmoles of (^{3}H) methyl groups incorporated into 50 µg of tRNA in one hour at 37° per mg of protein. Controls without tRNA were deduced from each value.

Virus core preparation. The method described by Coffin and Temin (12) was used. Fractions of 0.4 ml were collected from the bottom of the

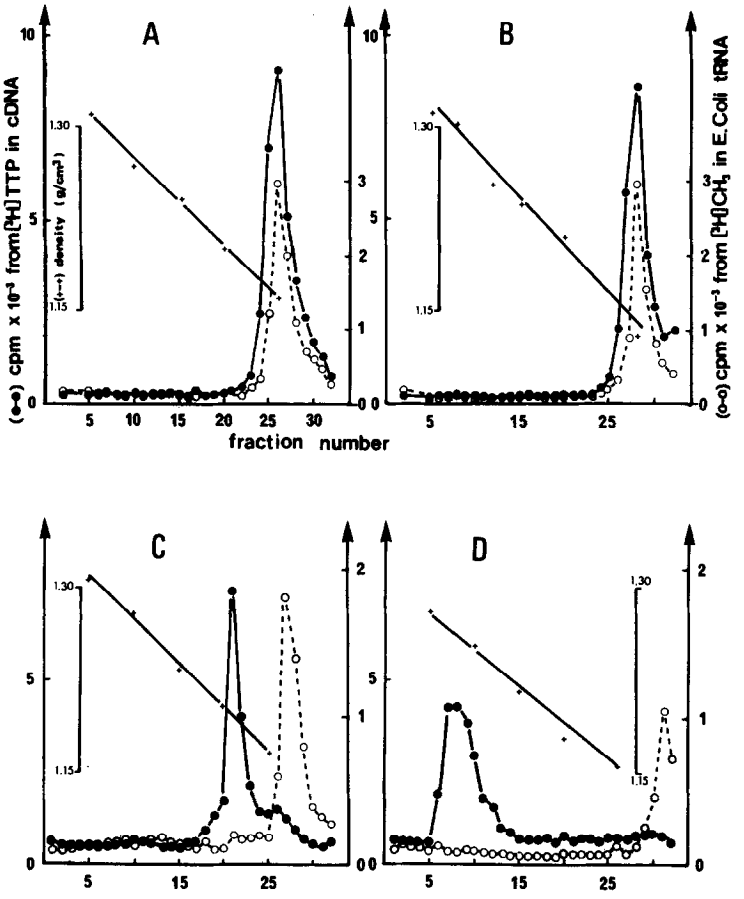

Fig. 1 : Sucrose density gradients of NP 40 disrupted virus.

Mixture of purified virus were layered on sucrose gradients and centrifuged after treatment with A) buffer, B) NP 40 0,1 %, C) NP 40 5,0 %, and D) NP 40 10 %. Reverse transcriptase (O-O) and tRNA methylase activities (O-O) were determined for all fractions by incorporation of (^{3}H) TTP into cDNA and of (^{3}H) CH_3 from SAM into E.coli K_{12} tRNA. Refractive indexes of some fractions were determined and the density in grams/cm^3 was plotted (+-+) AMV and RSV gave similar results.

sucrose gradient. Refractive index of some selected fractions was measured and the density curve plotted. Two 25 µl aliquots of each fraction were assayed for RT and tRNA methylase activities in the presence of 0.1 % NP 40.

Identification of methylated bases : A 500 µl standard reaction mixture with (^{3}H) SAM (10 Ci/mM) was incubated for 3 or 4 hours at 37°. 500 µl of buffer containing 0.4 % Sodium dodecyl sulfate was then added and tRNAs extracted. The final precipitate was washed with 70 % ethanol, dried and resuspended in 1 ml of 88 % formic acid. Hydrolysis, two dimmensional chromatography and autoradiography were performed as described by Munns et al. (13) and by Randerath (14).

Table I. Comparison of kinetic constants of N^2 methylguanine transferase from AMV, RSV and CEF membrane

	AMV	RSV	CEF membrane
Vmax pmole	10	12	22
Km SAM µM	1.5	1.3	1.0
KiSAH µM	3.5	2.8	2.5
Ki SIBA* µM	4000	5200	3800

*SIBA an analogue of SAH inhibits cell tranformation by RSV (6)

RESULTS AND DISCUSSION

Until now the exact position of the N^2methylguanine transferase in the virus particles was not known. We have now localized this enzyme in the purified AMV particles relatively to RT which is a known core enzyme. - Wu and Gallo (15) have described three rules for localizing viral enzymes. If the activity is detected in the absence of nonionic detergent, the enzyme is probably on the outer envelope. If the activity requires nonionic detergent but is not in the core fraction, the enzyme is probably located inside the outer envelope, otherwise, it is in the viral core. - Virus particles were treated at 0°C for 5 min with NP40 and the treated particles were banded in a sucrose density gradient. In the absence of NP40, the proteins corresponding to the two activities cosedimented at a density of 1.16 g/cm^3, which is the density of intact virus particles (Fig 1/A). With 0.1 % NP40 the two activities still banded with the same density of 1.14 g/cm^3 (Fig 1/B). However in the presence of 5.0 % NP40, the two activities were separated : tRNA methylase remained at 1.14 g/cm^3, while the polymerase (RT) sedimented at 1.2 g/cm^3 (Fig 1/C). At higher concentration of NP40 (10 %), the methylase activity decreased about two fold. Whereas RT banded at 1.26 g/cm^3, which is the density of virus core and the residual methylase activity was found at the top of the gradient

Table II. Dependence of N^2 methylguanine transferase activity on the presence of detergent

NP 40 %	AMV	RSV	CEF membrane*
	(% of total activity)		
0	4	0	21
0.025	41	3	58
0.100	96	89	77
0.200	100	100	100

*Cellular cytoplasmic methylases are not stimulated by NP40. The methylase in the membrane fraction represents 5 % of the total cellular methylase activity

(Fig 1/D). At this concentration of detergent, RT activity decreased less than the methylase activity. This is probably due to the proximity of the NP40 layer to the top of the gradient. Anyhow these results indicate that the virus associated N^2 methylguanine transferase of AMV must be located inside the viral envelope, contrary to RT which is in the viral core. We further compared some kinetic constants of the N^2methylguanine transferases from AMV, RSV, and the CEF cell membrane. Table I shows that the Km for SAM and the Ki for SAH are identical for the three enzymes. The V_{max} was, however, two fold higher in the membrane preparation than in virus particles. Table II shows that the three methylase activities are stimulated to a comparable degree in the presence of 0.1-0.2 % of NP40. However the enzyme from the CEF membrane is less dependent on detergent than the viral enzyme. This may be due to the fact that the membrane fraction could not entirely be freed from cytoplasmic methylases. - Studies on substrate specificity with the three enzymes further revealed, that the best substrates for all three are tRNA from E.coli K_{12} or E.coli B. Only a weak activity was observed with yeast tRNA and no activity with tRNAs of the following origin : wheat seedlings, calf thymus, CEF, RSV transformed CEF, Virginia Creeper, or with ribosomal RNAs from wheat seedlings and Virginia Creeper.

Table III : Analysis of the different bases of E.coli K_{12} tRNA methylated in vitro by the different methylases.

Base	AMV	RSV	CEF membrane normal cells	CEF cytoplasm normal cells	CEF cytoplasm transformed cells
N^2mG	86.0	75.0	87.0	38.0	58.0
N^2N^2 dimG	5.0	5.0	1.9	41.0	16.0
G	0.4	1.8	0	0	0.4
7mG	0.5	5.0	2.2	0.7	2.0
1mA	0.8	1.1	2.1	4.0	7.0
5mC	1.4	0.4	0.8	11.0	9.0
3mC	0.5	0.2	0.3	0	0
2mA	0.7	0.5	0.5	0	0
U	0.4	0	0	0	0
5mU	1.4	1.0	2.0	0.8	0.5
N^6mA	1.3	1.1	0.4	3.0	5.0
N^6N^6dimA	0.2	0.4	0.1	0	0
origin	0.4	6.0	0.3	0.6	0.9

Results are given as percentage of total counts.

The analysis of the different bases of E.coli K_{12} tRNA methylated in vitro by the three enzymes and by cell free extracts of normal and transformed-cells is shown in table III. 75 to 87 % of the total radioactivity were found in N^2 mG with the two viral enzymes and with the CEF membrane methylase, and no significant methylation was observed on the other bases. In contrast, cytoplasmic enzymes from normal or transformed CEF methylated three other bases significantly.

All these results strongly suggest that the virus associated methylase has host origin. The question is, whether this activity is due to a membrane contaminant which is purified together with the virus, as the densities of oncornavirus particles and membrane

preparations are very close. The different response to the action of NP40 suggests it is not the case. The exact location of N^2 methyl guanine transferase inside the cells is unknown, although over 90 % of the total methylase activity is assumed to be cytoplasmic. Cell membranes purified by the method used by us are contaminated by endosplasmic reticulum (16) but our results suggest that a part of N^2 methylguanine transferase may be located close to the membrane structure and trapped during the maturation of virus particles. Whether the enzyme thus trapped can methylate viral RNA remains a matter of conjecture.

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