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S-Adenosyl-L-Homocysteine as a Stimulator of Viral RNA Synthesis by Two Distinct Cytoplasmic Polyhedrosis Viruses

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An in vitro RNA-synthesizing system was used to study the effects of S-adenosyl-L-homocysteine, S-adenosyl-L-methionine, and adenosine on the methylation and synthesis of single-stranded RNA by two different cytoplasmic polyhedrosis viruses.

A great deal of interest has been shown in the 5'-terminal structure of many RNA species (21), particularly with regard to the relevance which modifications such as methylation may have on transcription (3) and translation (3, 4, 7, 10, 13, 21-23). Early studies with the cytoplasmic polyhedrosis virus (CPV) of the silkworm *Bombyx mori* (3, 12, 24) showed that, unlike reovirus (10, 20) or wound tumor virus (18), synthesis of mRNA by CPVs is greatly stimulated (about 60-fold) by the addition of the methyl donor S-adenosyl-L-methionine (SAM) to an in vitro RNA-synthesizing system. As RNA synthesis by the CPV was stimulated to such a great extent by SAM and the methyl group from SAM was incorporated at an early stage into the synthesized RNA (3, 4), it was suggested that transcription of the RNA genome is dependent on the methylation of the 5' terminus of the mRNA (3). It was also found that S-adenosyl-L-homocysteine (SAH), a competitive inhibitor of methylation (25), did not reduce the stimulation of transcription caused by SAM. Therefore, it was concluded that methylation in this system was somehow resistant to inhibition by SAH (3) and therefore was different from any methylation previously studied (1, 2, 11, 25).

We report on recent investigations in our laboratory which have shown that the addition of SAH effectively inhibits the incorporation of methyl groups, but at the same time stimulates RNA synthesis by CPVs. Two virus types, which appear similar on morphological grounds but which are biochemically distinct (17), are shown to differ in their response to SAH and structurally related chemicals.

CPV types 1 and 2 (17) were grown in larvae of *Bombyx mori* and *Mamestra brassicae*, respectively. Polyhedra were purified from the infected insects as described previously (14). Virus particles were liberated from polyhedra by treatment with 0.2 M sodium carbonate buffer, pH 10.8, followed by purification on sucrose gradients (14).

When virus particles from CPV type 1 were incorporated into a standard polymerase assay system (Fig. 1), the level of RNA synthesis, measured by UMP incorporation, was approximately 60 times greater when SAM was included at 0.5 mM than when it was omitted (Fig. 1B), and this high level of RNA synthesis was maintained over a wide SAM concentration range (Fig. 1B). These results confirm those obtained by Furuichi (3). In contrast, RNA synthesis by CPV type 2 showed two distinct features: measurable RNA synthesis occurred in the absence of SAM, and when SAM was included at 0.5 mM, only threefold stimulation was observed (Fig. 1A).

When the competitive inhibitor of methylation, SAH, was added in place of SAM, it also produced a large stimulation of RNA synthesis. With type 1 CPV, SAH appeared to be a slightly more effective stimulator than SAM both at low concentrations and in the maximum level of RNA synthesis produced (Fig. 1B). With type 2 CPV, SAH and SAM appeared to produce very similar effects, with only minor differences in their maximum stimulations of RNA synthesis (Fig. 1A). It seems that whatever the overall mechanics of the stimulation process, type 1 CPV shows a higher response to SAH than to SAM, whereas type 2 CPV shows a similar response to both molecules. It is possible that these effects are caused by differences in the relative affinities of the two viruses for these compounds.

The effect of SAH on methylation of mRNA was examined in a series of dual-label experiments using [α-^{32}P]UTP to measure RNA synthesis and [*methyl*-^{3}H]SAM to measure methylation (Fig. 2). As with reovirus (20), the addition of SAH effectively inhibited incorporation

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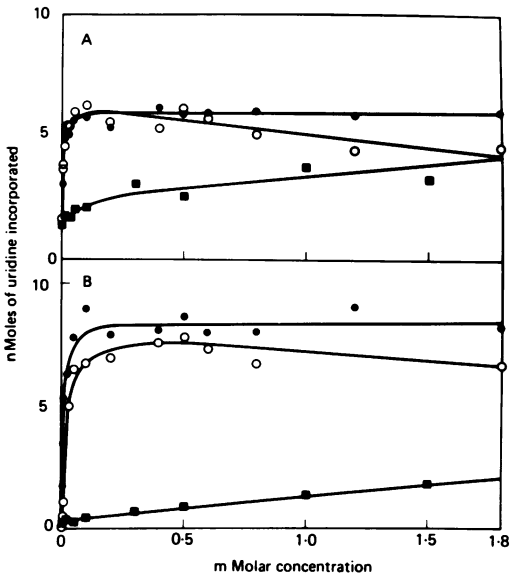

FIG. 1. *Stimulation in UMP incorporation produced in the polymerase assay system by the addition of SAM (○), SAH (●), or adenosine (■). (A) Type 2 CPV; (B) type 1 CPV. The standard assay (3) was carried out at 31°C for 5 h in a total volume of 250 μl. The assay contained 2 mM ATP, 2 mM CTP, 2 mM GTP, 0.4 mM UTP, 0.5 μCi of [^{3}H]UTP or [α-^{32}P]UTP (final activity, 5 mCi/mmol), 12 mM $MgCl_2$, 60 mM Tris-hydrochloride buffer (pH 8.1), 10 μg of actinomycin D, and 10 μg of virus particles. The incorporation of label into RNA was measured by trichloroacetic acid precipitation in 10% trichloroacetic acid at 4°C for 1 h, followed by filtration onto glass-fiber disks, which were washed with 40 ml of 10% trichloroacetic acid and 10 ml of ethanol. The disks were dried and counted in a liquid scintillation spectrometer. The rate of incorporation was found to be constant for up to 10 h and linear with respect to virus addition up to 20 μg. SAM, SAH, and adenosine were added to the standard assay system in varying concentrations when required.*

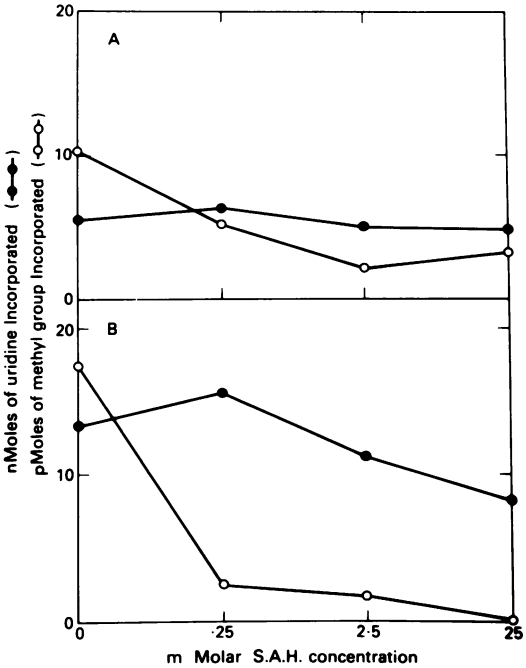

FIG. 2. *Effect of SAH addition on methyl group incorporation (○) and UMP incorporation (●) into RNA by type 2 CPV (A) and type 1 CPV (B). A modified version of the standard assay system was used to analyze the effects of SAH addition on methyl group incorporation into the synthesized RNA. These assays were run for 8 h to ensure a high incorporation of label. A 0.5-μCi amount of [α-^{32}P]UTP was used to measure RNA synthesis, and [methyl-^{3}H]SAM was added at a specific activity of 5 μCi/0.011 μmol and at a final concentration of 0.044 mM. This concentration of SAM was used as it is suboptimal for RNA synthesis (Fig. 1) and therefore permits any additional stimulation of RNA synthesis by SAH to be observed. Other conditions were the same as for the standard assay system. Unless RNA was specifically required for reannealing purposes, bentonite was omitted from the assays, as it appears to adsorb labeled SAM and therefore produced artifically high background levels of incorporation of this molecule.*

of the methyl group into the newly synthesized RNA. At the same time, RNA synthesis was stimulated to the maximum level by the addition of 0.25 mM SAH and then decreased again at the higher concentration used in this experiment. Thus, although RNA synthesis by CPVs (particularly type 1 CPV) shows some dependence on the presence of SAH or SAM, methylation is not a prerequisite for efficient transcription of the CPV genome. In this experiment (Fig. 2) it appeared that SAH was a more effective inhibitor of methylation in type 1 than in type 2 CPV. Once again, this supports the idea (Fig. 1) that in type 1 CPV some part of the virus directly involved in the stimulation of RNA synthesis and in methylation (possibly the methylase) has a higher affinity for SAH than for SAM, whereas type 2 CPV appears to have a similar affinity for both molecules.

Several other molecules were tested for their ability to increase RNA synthesis. However, when AMP, GMP, UMP, CMP, uridine, cytosine, adenosine, guanosine, homocysteine, and methionine were added to the standard assay system at 0.5 mM, the only compound that caused any detectable increase in RNA synthesis was adenosine. However, when adenosine was added to the assay system in place of SAH or SAM, the level of RNA synthesis was much reduced (Fig. 1).

Under conditions in which SAM or SAH was

TABLE 1. *Comparison of the stimulation of RNA transcription produced by SAH, SAM, and adenosine added separately or together*

Addition to standard assay system	nmol of UMP incorporated	
	Type 1 CPV	Type 2 CPV
Zero	0.38	0.78
SAH, 0.5 mM	5.84	2.44
SAM, 0.5 mM	4.54	1.60
Adenosine, 1.5 mM	1.98	2.07
SAH, 0.5 mM, + adenosine, 1.5 mM	4.78	2.11
SAM, 0.5 mM, + adenosine, 1.5 mM	5.22	1.75
SAM, 0.5 mM, + SAH, 0.5 mM	5.27	2.13

included at optimal concentration, no additive effects were observed (Table 1). Thus, SAH or SAM appears to stimulate the system to a maximum level, above which no further increase in RNA synthesis can be caused by the addition of the remaining compound or adenosine. This suggests that the three compounds may well affect RNA synthesis by a similar process.

Reannealing studies confirmed that the radioactive counts incorporated into trichloroacetic acid-precipitable material by the two viruses in the presence of SAH and SAM were found in virus-specific single-stranded RNA. Single-stranded RNA was synthesized by using a standard polymerase assay system scaled up by a factor of 10. Virus particles were removed by centrifugation at 80,000 × *g* for 1 h, and RNA was extracted by a hot phenol-sodium dodecyl sulfate method (16). Reannealing to heat-denatured viral RNA was carried out as described elsewhere (16). With CPV type 1 under constant reannealing conditions, single-stranded RNA synthesized in the presence of SAH or SAM reannealed to viral RNA to the same extent. When ^{3}H-methylated RNA was used, the calculated average number of methyl groups to each RNA chain was 1.26 before reannealing and 0.84 after annealing, confirming that the labeled methyl group from SAM was also incorporated into virus-specific RNA.

From the experiments described above, a number of conclusions can be drawn. (i) SAH and SAM are both highly effective stimulators of in vitro RNA synthesis by the virion-associated RNA polymerase of both type 1 and type 2 CPVs. (ii) Type 2 CPV has a higher level of RNA synthesis in the absence of SAH or SAM than does type 1 CPV. (iii) Type 2 CPV shows a maximum stimulation of RNA synthesis by SAH or SAM of only 3-fold, whereas type 1 CPV is stimulated up to 60-fold. (iv) With type 1 CPV, SAH is a more effective stimulator of RNA synthesis than is SAM, whereas with type 2 CPV both compounds appear to be equally effective. (v) SAH is an effective inhibitor of methylation of CPV RNA, although apparently more efficient in type 1 than in type 2 CPV. (vi) Adenosine has a low level of stimulating activity, less than 1% of that of SAH or SAM.

As the 5′-terminal nucleotide in the uncapped RNA or type 1 CPV is adenosine (4), it was possible that the three compounds stimulated transcription by acting as initiators for RNA synthesis (9). However, preliminary experiments using *S*-adenosyl-L-[2(*n*)-^{3}H]methionine or [2-^{3}H]adenosine failed to show any significant incorporation of tritium into the synthesized RNA. It therefore appears that the stimulation does not entail a physical incorporation of the molecules into RNA. Instead, we suggest that the presence of SAM or a similar molecule at the active site of the methylase induces a conformational change in the enzyme. This change allows RNA synthesis to proceed more rapidly, possibly as a result of a physical interaction between the proteins involved in the methylase and polymerase functions of the CPV virion. This is in direct contrast to reovirus, in which the two enzyme functions appear to be distinct with little or no interregulation (5, 6). In type 2 CPV the requirement of RNA synthesis of induction of the conformational change may be less rigid. This would explain a higher background level of polymerase activity in the absence of SAH or SAM and a low overall factor of stimulation when either of these compounds is added.

In type 1 as compared with type 2 CPV, the methylase may have a higher affinity for SAH than for SAM. This results in a more effective inhibition of methylation and a greater stimulation of RNA synthesis by SAH than by SAM.

As adenosine lacks either the homocysteine or the methionine side chains, it may have a low affinity for the active site of the enzyme, thus making it a less effective stimulator of RNA synthesis. To test this idea, *S*-adenosyl-L-ethionine or *S*-adenosyl-D-homocysteine was added to the standard assay system. It was found that both compounds were more effective as stimulators of RNA synthesis than was adenosine, producing a level of synthesis directly comparable to that in the presence of SAH or SAM.

From the dual-label experiments (Fig. 2) it is possible to calculate the number of methyl groups incorporated per RNA molecule. With the uridine content of type 2 CPV RNA taken as 31.5% (15) and that of type 1 CPV taken as 28.5% (11) and the molecular weights of type 1 and 2 CPV RNA taken as 14.6 × 10^6 and 14.36

$\times 10^6$, respectively (17), it was calculated that 0.84 methyl group to each RNA chain was incorporated by type 1 CPV and 1.3 methyl groups were incorporated by type 2 CPV. These estimates agree well with those made by Furuichi (3) of an average of one methyl group to each RNA chain synthesized. However, two per molecule would be expected with the 5'-terminal structure ($m^7G^5ppp^5Am$) found in CPV RNA (4). The incomplete methylation observed may well be due to the presence of SAH in the assay, either as an end product of transmethylation or as a contaminant of SAM (19). This would inhibit methylation but still permit a high level of RNA synthesis.

It has been found in other studies of transmethylation (2) that a naturally occurring enzyme can remove SAH by hydrolysis and will produce an enhanced rate of methylation. Thus, by control of the relative and overall levels of SAH and SAM at the site of synthesis, we can envisage a possible mechanism by which the levels of synthesis and methylation of CPV RNA could be regulated independently. This could, in turn, affect the relative amounts of RNAs with complete and incomplete cap structures and have considerable relevance in the control of transcription and translation of the CPV genome.

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