ON THE MECHANISM OF SINGLE-STRANDED RNA SYNTHESIS BY ENCEPHALOMYOCARDITIS VIRUS REPLICATION COMPLEXES

Preferential inhibition by adenylyl (β, γ-methylene)-diphosphonate

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1. Introduction

RNA-synthesizing preparations containing endogenous viral RNA templates, the so called replication complexes (RC), can be extracted from picornavirus-infected cells (reviewed [1,2]). The membrane-free RCs can in turn be separated, by ultracentrifugation, into fractions synthesizing preferentially either single-stranded (ss) or double-stranded (ds) viral RNA species [3–6]. What are the differences in the mechanisms responsible for the formation of these two classes of viral RNA. One such difference, which seemed to us worth investigating, is the additional energy demand for the synthesis of ssRNA molecules as compared to the synthesis of dsRNA species.

Here we present data indicating that adenylyl (β, γ-methylene)-diphosphonate, an ATP analog possessing a non-hydrolysable linkage between the β and γ phosphorus atoms, is an acceptable substrate for the synthesis of encephalomyocarditis (EMC) virus-specific dsRNA species but cannot substitute for ATP in the ssRNA-synthesizing reaction. This result suggests that the synthesis of picornaviral ssRNA species requires, in addition to RNA-polymerase, an ATP-dependent function (cf. [7]).

2. Methods

Crude membrane-free preparations of RCs were isolated from EMC virus-infected Krebs-2 cells as in [6,8] which included the treatment of the cell homogenate pellet (40 000 × g, 15 min) with deoxycholate and dextran sulfate and ethanol precipitation. To fractionate these complexes further, they were subjected to centrifugation in a 10–50% sucrose concentration gradient prepared in 0.004 M $MgCl_2$, 0.01 M Tris–HCl (pH 7.5) (Spinco rotor SW 27, 15 000 rev./min, 17 h, 2°C). RNA polymerase activity was assayed as in [6,8]; in most experiments, however, ATP was 125 μM in the incubation mixture instead of 250 μM. The RNA products were deproteinized by phenol extraction and centrifuged in a 5–20% sucrose concentration gradient prepared in 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris–HCl (pH 7.4) (Spinco rotor SW 50.1, 45 000 rev./min, 150 min, 4°C). Radioactivity in the acid-insoluble material was determined before and after treatment with pancreatic RNase (10 μg/ml, 30 min, 37°C).

3. Results

A preparation of membrane-free EMC virus RC was subjected to sucrose density gradient centrifugation and the RNA-synthesizing activity was assayed in each fraction of the gradient using either ATP or $AppCH_2p$ as 1 of 4 ribonucleoside triphosphate substrates. Fig.1a shows that the RNA polymerase activity was distributed over the entire gradient, the maximal activity being sedimented at ~200 S. Substitution of $AppCH_2p$ for ATP resulted in a severe depression of the RNA polymerase activity which was especially apparent in the relatively fast-sedimenting RC, whereas the polymerase activity of the slowly sedimenting RC was changed only slightly, if at all. A dif-

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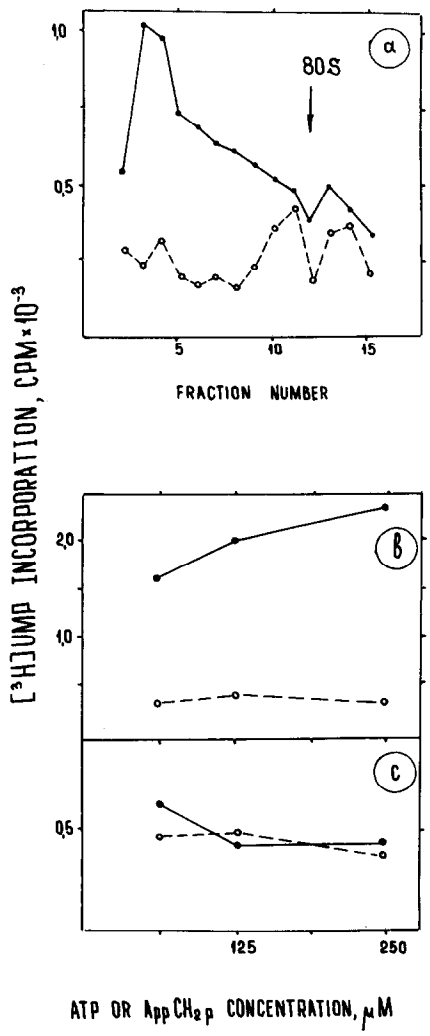

Fig.1. Effect of substitution of $AppCH_2p$ for ATP on the RNA polymerase activity of the heavy and light RC of EMC virus. A crude preparation of RC was subjected to centrifugation in a sucrose concentration gradient as in section 2. RNA polymerase activity was determined in each fraction of gradient by measuring the incorporation of the label from [^{3}H]UTP into acid-insoluble products during a 30 min incubation at 37°C under standard conditions [6] (——) and after substitution of $AppCH_2p$ for ATP (– – –). ATP or $AppCH_2p$ in the incubation mixture was 125 μM. The position of 80 S ribosomes, run in a parallel gradient, is indicated by the arrow. (b) Aliquots of the heavy RC (material from fraction 4 of the gradient is shown in fig.1a) were incubated in the standard RNA polymerase assay mixture containing different concentrations of either ATP (——) or $AppCH_2p$ (– – –). (c) Aliquots of the light RC (material from fraction 11 of the gradient shown in fig.1a) were incubated in the standard RNA polymerase assay mixture containing different concentrations of either ATP (——) or $AppCH_2p$ (– – –).

ferential sensitivity of the RNA polymerase activities of the fast-sedimenting (heavy) and slowly-sedimenting (light) RC to the substitution of $AppCH_2p$ for ATP was also shown in an experiment presented in fig.1b,c, where the effect of varying concentrations of ATP or $AppCH_2p$ in the incubation mixture on the RNA-synthesizing capacity of separated heavy and light RC was investigated. Since the heavy and light picornaviral RC were known to synthesize preferentially either ssRNA or dsRNA species, respectively [3–6], the results presented in fig.1 suggested that $AppCH_2p$ cannot be used as a substrate for the formation of viral ssRNA species, being an acceptable substrate for dsRNA synthesis.

To test this suggestion, the effect of substitution of $AppCH_2p$ for ATP on the nature of the products formed by the heavy and light RC was studied. Fig.2a demonstrates that the heavy RC, in the pres-

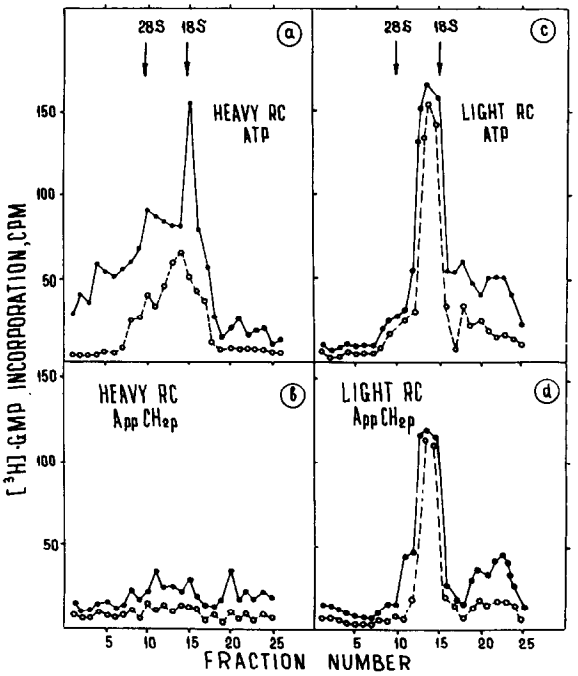

Fig.2. Effect of substitution of $AppCH_2p$ for ATP on the nature of products synthesized by the heavy and light RC of EMC virus. Preparations of the heavy (a,b) or light (c,d) RC (materials from fractions 2 + 3 and 11, respectively, of the gradient shown in fig.1a) were incubated in the standard RNA polymerase assay mixture containing 125 μM of either ATP (a,c) or $AppCH_2p$ (b,d). [^{3}H]GTP was used as labeled substrate. RNA products were analysed as in section 2. Radioactivity in the acid-soluble material was determined before (——) and after (– – –) treatment with pancreatic RNase.

ence of normal substrates, formed a heterogeneous set of RNA molecules displaying a considerable, although not complete, sensitivity to the hydrolytic action of pancreatic RNase. The relative proportion of the full-length ssRNA molecules was in this experiment somewhat lower than in [6,8], which might, perhaps, be due to a lower ATP concentration in the incubation mixture used for the RNA synthesis here. In the presence of $AppCH_2p$ the RNA polymerase of the heavy RC was nearly inactive (fig.2b). The light RC synthesized, under control conditions, almost exclusively a 20 S dsRNA species (fig.2c) and substitution of $AppCH_2p$ for ATP exerted no appreciable effect on this RNA accumulation (fig.2d).

4. Discussion

These experiments were undertaken to test the hypothesis according to which an energy-dependent reaction is specifically involved in the synthesis of picornaviral ssRNA species. It was thought that this reaction might require splitting the β–γ phosphodiester bond in ATP molecules. Therefore, $AppCH_2p$ was expected:

(i) To fail to substitute for ATP in the ssRNA-synthesizing reaction, but

(ii) To serve as an acceptable substrate for dsRNA synthesis.

These results are in full accord with these expectations. The nature of the ATP-requiring reaction is not clarified here, however. Different hypotheses may be put forward.

It may, for example, be suggested that the synthesis of viral ssRNA and dsRNA species is carried out by two distinct RNA polymerases and that $AppCH_2p$ is an acceptable substrate only for the dsRNA-synthesizing enzyme. However, we are aware of no sound evidence for the existence of two distinct picornaviral RNA polymerases, except, perhaps, some indirect genetic data [9]. Moreover, $AppCH_2p$ differs from ATP in possessing a non-hydrolysable linkage between the β and γ phosphorus atoms and, therefore, is expected to inhibit or to fail to serve as a substrate for an enzyme which is normally involved in the splitting β–γ phosphodiester bond in ATP molecules. In fact, an ATPase activity, as revealed by ATP→ADP conversion, appears to be present in the gradient fractions containing EMC virus RC, and $AppCH_2p$ inhibits this activity (not shown). It should be noted, however, that the distribution of ATPase activity over the gradient upon centrifugation of crude membrane-free preparations of EMC virus RC was different compared to the distribution of RNA polymerase activity. Moreover, an ATPase activity was also present in preparations obtained from uninfected cells using the protocol for isolation of viral RC (data not shown). Nevertheless, it is tempting to draw a functional analogy between the ATP-requiring protein suggested to participate in picornaviral ssRNA synthesis and the *rep* protein of *Escherichia coli*, which performs an ATP-dependent unwinding of DNA duplex during genome replication of some bacteriophages [10]. These results, however, are also compatible with the assumption that the ATP-requiring function in question is a protein kinase or some other enzyme which splits the γ phosphate residue from ATP molecule.

Another question concerns the origin, viral or host cell, of the ATP-dependent protein participating in the synthesis of viral ssRNA. The possibility that a viral function may be specifically involved in the ssRNA synthesis is indicated by the existence of poliovirus mutants which display an impaired ssRNA, but not dsRNA synthesis under restrictive conditions [9]. On the other hand, antibodies raised against cellular proteins preferentially inhibit the synthesis of ssRNA species in preparations of EMC virus RC [8], implying that a host cell protein may have a special role in the ssRNA formation. If the protein under discussion is really of host cell origin, an attractive function for it in uninfected cells may be unwinding of regions with secondary structure in mRNA molecules; in other words, it may be regarded as an auxiliary elongation factor of translation (discussed [7]).

Although the present experiments are unable to solve these important problems, they clearly point to a difference in the mechanisms of picornaviral ssRNA and dsRNA synthesis and suggest that the synthesis of ssRNA requires, in addition to RNA polymerase, an ATP-dependent function.

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