MESSENGER ACTIVITY IN MAMMALIAN CELL-FREE EXTRACTS OF REOVIRUS SINGLE-STRANDED RNA PREPARED IN VITRO

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SUMMARY

Reovirus ssRNA synthesized in vitro by a viral-specific RNA transcriptase displays messenger activity when incubated with supplemented S150 extracts and purified ribosomes prepared from L-cells. Pre-treatment of the ssRNA with HCHO enhances amino acid incorporation several-fold. 80S ribosomes and 60S and 40S ribosomal subunits are equally effective in supporting polypeptide synthesis.

INTRODUCTION

Reovirus RNA transcriptase is found within the virus and becomes active when the outer shell of capsomeres is removed exposing a relatively stable SVP* containing the enzyme and all of the dsRNA genome (1-3). Purification of the SVP from extracts of reovirus-infected L-cells yields a particulate enzyme which efficiently transcribes in vitro multiple ssRNA copies of 1 strand of each of the 10 segments of the dsRNA genome (4). By several criteria, the ssRNA species produced in vitro were found to be identical to reovirus-specific ssRNA isolated from polyribosomes of infected cells (4-6). These findings suggested that the in vitro transcriptase products might serve as messengers

*Abbreviations: SVP, subviral particles; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; mRNA, messenger RNA; DTT, dithiothreitol; TCA, trichloroacetic acid; DOC, sodium desoxycholate; rRNA, ribosomal RNA; L-cells, L-929 mouse fibroblasts.

when added to supplemented mammalian cell-free extracts. The data presented here demonstrate the ability of the in vitro ssRNAs to stimulate polypeptide synthesis in the presence of S150 supernatant enzymes and either 80S ribosomes or 60S and 40S ribosomal subunits derived from L-cells. The extent of amino acid incorporation was considerably enhanced by pre-treatment of the presumptive mRNA with HCHO (7).

METHODS AND MATERIALS

Reovirus production in L-cells and the isolation from infected cultures of SVP containing RNA transcriptase have been described (4). The in vitro synthesis and purification of reovirus RNA transcriptase ssRNA products were carried out as previously reported (4). For the preparation of S150 extracts and ribosomes, washed L-cells were disrupted in a Dounce homogenizer and the nuclei and cellular debris removed from the crude extract by low-speed centrifugation (4). After further centrifugation at 150,000 g for 2 hours, the top half of the resultant supernate (S150) was dialyzed vs 25 mM Tris (pH 7.5)-50 mM KCl-1 mM $MgCl_2$-3 mM DTT-10 % glycerol. For stripped 80S ribosomes, an S20 extract prepared from the crude extract by centrifugation at 20,000 g for 10 min was supplemented with the components for in vitro polypeptide synthesis (see Table I) and 25 μg/ml of puromycin (8), and preincubated at 37°. After 30 min, DOC was added to a final concentration of 0.8 % and the ribosomes sedimented through 25 % sucrose containing 25 mM Tris (pH 7.5)-5 mM $MgCl_2$-100 mM KCl-2 mM DTT (RB) at 150,000 g for 2 hours; the ribosomes were resuspended in RB and their homogeneity confirmed by sucrose gradient analysis. In the preparation of preincubated 80S ribosomes for the experiment described in Table I, the puromycin and DOC steps were omitted resulting in ribosomes in which some endogenous mRNA was retained. The 60S and 40S ribosomal subunits were prepared from stripped 80S ribosomes by

TABLE I

Effect of HCHO-treatment of reovirus ssRNA on polypeptide synthesis *in vitro*

RNA added	HCHO treatment	pMoles ^{3}H-leucine incorporated
- - -	- - -	1.35
ssRNA	-	3.05
	+	12.90
18S rRNA	-	1.53
	+	1.47
28S rRNA	-	1.27
	+	0.74

The incubation mixtures contained the following in a final vol of 50 µl: 20 mM HEPES buffer (pH 7.7), 1 mM ATP, 0.2 mM GTP, 11 mM $MgCl_2$, 120 mM KCl, 5 mM DTT, 6 mM creatine phosphate, 4 nmoles each of 19 amino acids, 0.8 nmoles of ^{3}H-leucine (1510 cpm/pmole), 15 µg of L-cell tRNA, 0.8 A_{260} units of preincubated 80S ribosomes, 5 µg creatine phosphokinase, 0.4 A_{260} units of ssRNA or rRNA, and 40 µg of L-cell S150 extract protein. Incubation was at 37° for 1 hour. Reaction terminated by addition of TCA to a final concentration of 10 % followed by heating at 90° for 15 min. The residue was collected on Millipore membranes, washed with 5 % TCA, and counted in a liquid scintillation spectrometer (4). The extent of polypeptide synthesis is defined as the amount of radioactivity incorporated into hot TCA-precipitable material. All values are corrected for zero-time controls.

the method of Martin and Wool (8). ^{3}H-leucine was obtained from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Effect of HCHO-treatment of reovirus ssRNA on its messenger efficiency in vitro: The addition of reovirus ssRNA prepared *in vitro* to supplemented S150 extracts and partially stripped 80S ribosomes derived from L-cells stimulated polypeptide synthesis approximately 2-fold (Table I). After treatment of the purified ssRNA with HCHO (7), the incorporation of ^{3}H-leucine

into polypeptide increased to 10-fold over the control. By contrast, 18S and 28S rRNA extracted from L-cell ribosomes did not stimulate polypeptide synthesis in this system. In all subsequent experiments described here, HCHO-treated ssRNA was utilized exclusively.

Comparison of 80S ribosomes with 60S and 40S subunits: The efficiency of stripped 80S ribosomes and ribosomal subunits in polypeptide synthesis with HCHO-treated ssRNA as messenger was compared (Table II). Ribosomal subunits were slightly more effective than 80S ribosomes in promoting ^{3}H-leucine incorporation into peptides. When assayed separately, the 60S and 40S subunits were ineffective. The slight stimulation observed with 60S particles was due to a small contamination by 40S subunits.

TABLE II

Comparison of 80S ribosomes and 60S and 40S subunits during polypeptide synthesis

Ribosomes	pMoles ^{3}H-leucine incorporated	
	-ssRNA	+ssRNA
40S	0.07	0.11
60S	0.19	0.59
40S + 60S	0.20	4.81
80S	0.18	4.11

The incubation is described in Table I. Stripped 80S ribosomes, 0.8 A_{260} units; for subunits, 0.5 A_{260} units of 60S and 0.25 A_{260} units of 40S.

Requirements for protein synthesis: As shown in Table III, polypeptide synthesis is completely dependent upon the presence of mRNA, ribosomes, $MgCl_2$, and S150 extract enzymes. For maximal activity, the addition of tRNA

TABLE III

Requirements for polypeptide synthesis

Components	pMoles ^{3}H-leucine incorporated
Total system	11.33
-mRNA	0.23
-ribosomes	0.11
-tRNA	2.89
-$MgCl_2$	0.15
-S150 extract	0.14
-regenerating system	1.69

The incubation mixture and assay are described in Table I.

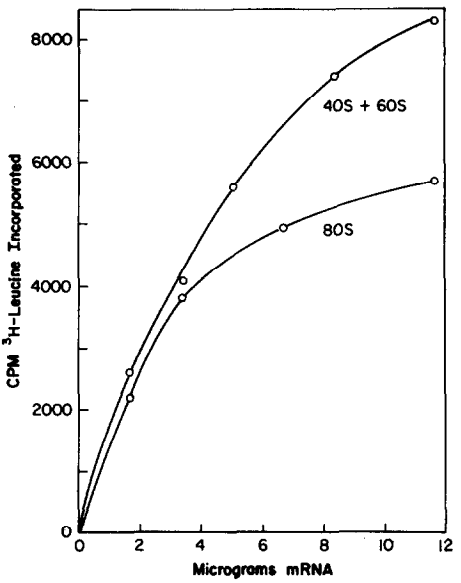

Fig. 1. Dependence of polypeptide synthesis on reovirus ssRNA concentration Assays are described in Table I; ribosome concentrations in Table II.

and an energy regenerating system was required. The extent of ^{3}H-leucine incorporation was dependent upon the concentration of HCHO-treated ssRNA

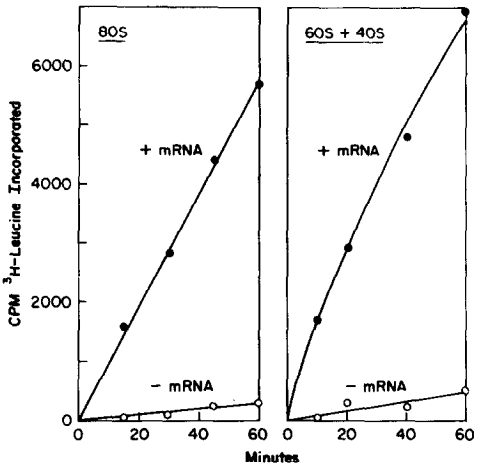

Fig. 2. Kinetics of polypeptide synthesis. Assays are described in Table I, ribosome concentrations in Table II. At indicated time points, aliquots were removed for analysis.

(Fig. 1). In the presence of excess ssRNA, polypeptide synthesis continued for more than 60 min with either 80S ribosomes or ribosomal subunits (Fig. 2).

DISCUSSION

The data reported here indicate that the ssRNA products synthesized in vitro by a particulate reovirus RNA transcriptase can function as messengers when incubated with L-cell extracts supplemented with all of the components required for polypeptide synthesis. In the presence of stripped 80S ribosomes, the addition of reovirus ssRNA stimulated ^{3}H-leucine incorporation as much as 50-fold over a control from which ssRNA was omitted. A similar stimulation was obtained when 80S ribosomes were replaced by 60S and 40S ribosomal subunits. With partially stripped ribosomes (Table I), a 10-fold stimulation over background was obtained. Analysis of the products by acrylamide gel electrophoresis revealed a large proportion of the radioactive peptides in a broad peak with an average molecular weight of 10-15,000; traces of radioactive peptides were found at the 60,000 molecular weight range. In comparison,

the molecular weight range of reovirus proteins is 40,000-150,000.

Lodish has reported that mild HCHO treatment of bacteriophage f2-RNA considerably enhanced specific initiation and translation of f2-RNA cistrons in E. coli extracts owing to a partial disruption of the secondary structure (7). In the experiments described here, a similar stimulation of polypeptide synthesis was observed after treatment of the reovirus ssRNA with HCHO. This suggests that secondary structure in the reovirus ssRNA may exert a regulatory function in translation.

In the experiments described here the optimal Mg^{++} concentration was 11 mM with either 80S or 60S and 40S ribosomal subunits. Other investigators have demonstrated a requirement for ribosomal factors in the initiation of hemoglobin chains in rabbit reticulocyte cell-free systems at lower Mg^{++} concentrations (9, 10). Preliminary experiments in our own laboratory suggest that at 5 mM Mg^{++}, reovirus ssRNA-directed polypeptide synthesis is stimulated by the addition of a 0.5 M KCl wash of crude ribosomes (9, 10). Further work is underway and will be reported elsewhere.

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