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VACCINIA VIRAL CORE INHIBITS Met-tRNA$_f$·40S INITIATION COMPLEX FORMATION WITH PHYSIOLOGICAL mRNAs

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Vaccinia viral core inhibits protein synthesis in reticulocyte lysates. In partial reactions using micrococcal nuclease treated reticulocyte lysates, the viral core inhibits Met-tRNA$_f$ binding to 40S ribosomes in response to physiological mRNAs such as globin mRNA, cowpea mosaic viral RNA, and brome mosaic viral RNA but not in response to a trinucleotide codon, AUG. The core has also no effect on Met-tRNA$_f$ binding to 40S ribosomes in a partial reaction using partially purified peptide chain initiation factors and AUG codon.

The present observation of preferential inhibition by vaccinia viral core of Met-tRNA$_f$·40S initiation complex formation with physiological mRNAs and not with an artificial mRNA such as AUG codon, suggests that the viral core inhibits some step(s) in peptide chain initiation involved in the recognition of structural feature(s) unique to physiological mRNAs.

The infection of animal cells with viruses is often accompanied by shut-off of host protein synthesis, (for a review see Ref. 1). There are indications that some step(s) in peptide chain initiation is involved in this inhibition (1). In the case of vaccinia virus, this inhibition occurs in the absence of viral RNA or protein synthesis (2-7) and the time required for the establishment of shut-off corresponds to the first stage of uncoating, i.e., the release of cores into the cytoplasm (8,9). Using an in vitro protein synthesizing system, Ben Hamida and Beaud observed that the vaccinia viral core was strongly inhibitory to protein synthesis (10). Recently, these authors have reported that the vaccinia viral core inhibits Met-tRNA$_f$·40S initiation complex formation in reticulocyte ly-

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sates (11). Pelham, Sykes and Hunt (12) have reported that the vaccinia viral core inhibits protein synthesis in reticuloyte lysates and several factors including double-stranded RNA activated protein synthesis inhibitor (dsI) and endonuclease are possibly involved in this inhibition.

In this communication, we report that vaccinia viral core inhibits Met-$tRNA_f$·40S complex formation in reticulocyte lysates in response to physiological mRNAs and not in response to AUG codon. Also, the viral core does not inhibit Met-$tRNA_f$ binding to 40S ribosomes in the presence of AUG codon and partially purified peptide chain initiation factors. The trinucleotide AUG codon is known to act as initiation codon and can substitute physiological mRNAs in all the partial peptide chain initiation reactions except in those cases which involve specific recognition of structural features unique to physiological mRNAs. The present observation of preferential inhibition by vaccinia viral core of Met-$tRNA_f$·40S initiation complex formation with physiological mRNAs and not with an artificial mRNA such as AUG codon strongly suggests that the component(s) in viral core which inhibits protein synthesis in reticulocyte lysates is not a general protein synthesis inhibitor such as dsI (double-stranded RNA activated protein synthesis inhibitor) but inhibits some step in the initiation process involved in the recognition of physiological mRNAs.

MATERIALS AND METHODS

[^{35}S] Methionine and [^{14}C] leucine were purchased from Amersham/Searle and New England Nuclear respectively. Brome mosaic virus and cowpea mosaic virus were gnerously provided by Dr. Leslie Lane, University of Nebraska (Lincoln) and Dr. G. Bruening, University of California, Davis. Viral RNAs were isolated by the phenol extraction procedure (13).

Preparations of Rabbit Reticulocyte Lysates and Assay of Protein Synthesis

The procedures for the preparation of reticulocytes and reticulocyte lysates and the incubation mixtures for protein synthesis have been described (14-16). Protein synthesis was assayed by the incorporation of [^{14}C] leucine into hot trichloroacetic acid-insoluble protein as described previously (15). In some experiments, the reticulocyte lysates were treated with micrococcal nuclease to degrade endogenous globin mRNA following the procedure of Pelham and Jackson (17) and the nuclease treated lysates were then used for studies of protein synthesis with exogenously added mRNAs.

The detailed descriptions of the protein synthesis assay procedures are also given in the legends of the tables.

Peptide Chain Initiation Assays

Met-tRNA$_f$·40S initiation complex formation in the presence of different physiological mRNAs and AUG codon was studied using micrococcal nuclease treated reticulocyte lysates. The lysates (nuclease treated, Pelham and Jackson (17)) were preincubated for 10 min at room temperature with or without vaccinia cores in the presence of indicated mRNA and 80 mM KCl. At the end of the incubation, Met-tRNA$_f$ and a master mix containing KCl, GTP, 19 amino acids (-methionine), creatine phosphate, Tris-HCl, pH 7.8, and magnesium acetate were added. The final reaction mixture (60 µl) containing 33% by volume of reticulocyte lysate, 10 mM Tris-HCl, pH 7.8, 1 mM magnesium acetate, 100 mM potassium chloride, 0.2 mM GTP, 30 µM concentrations each of 19 amino acids (-methionine), 4 pmol of Met-tRNA$_f$ (25,000 cpm/pmol), 5 mM creatine phosphate, 10 µg/ml of creatine phosphokinase and 20 µM hemin and, where indicated, 0.39 A_{260} unit of vaccinia cores, and mRNA. The reaction mixtures were incubated at 30 C for 5 minutes.

The reaction was stopped by the addition of 100 µl of 20 mM potassium phosphate buffer, pH 6.6 and 150 µl of the reaction mixure was layered on 5 ml of sucrose density gradient (4 → 27%) (containing 20 mM potassium phosphate pH 6.6, 100 mM KCl, 1 mM dithiothreitol, 5 mM Mg^{2+}) and centrifuged at 45,000 rpm for 90 min in a SW 50.1 rotor. The gradients were fractionated by using an ISCO density gradient fractionator, and 0.3 ml fractions were collected in 0.5 ml of 0.5 M sodium acetate, pH 5.0 containing 0.5 µg/ml of carrier yeast RNA. The fractions were precipitated by addition of 0.5 ml aliquots of 2% CTAB and processed as described by Legon et al. (18).

Met-tRNA$_f$ binding to 40S ribosomes in the presence of partially purified peptide chain initiation factors and AUG codon was assayed by the standard two stage Millipore filtration assay method described by our laboratory (19). The components in stage 1 incubation contained (in a total volume of 0.07 ml): 20 mM Tris-HCl, pH 7.8, 80 mM KCl, 2 mM Mg^{2+}, 2 mM dithiothreitol, 0.2 mM GTP, 0.02 A_{260} unit of AUG, 0.08 A_{260} unit of 40S ribosomes, 12 µg of crude initiation factors (Fraction III (19)), 10 µg of bovine serum albumin, 8 pmol [^{35}S] Met-tRNA$_f$ (20-25,000 cpm/mol) and, where indicated, 0.39 A_{260} unit of vaccinia cores. All tubes were preincubated at room temperature for 10 min in the presence of buffer, KCl, bovine serum albumin, dithiothreitol, crude initiation factors and, where indicated, 0.39 A_{260} unit of vaccinia cores. Stage 1 incubation was carried out at 37°C for 5 min. At the end of the incubation, 5 µl of $MgCl_2$ solution (45 mM) was added and the incubation was continued in an ice bath for 10 min. The reaction was terminated with addition of 3 ml of buffer (20 mM Tris-HCl, pH 7.8, 100 mM KCl, 1 mM Mg^{2+}) and processed using a standard Millipore filtration technique (19).

Preparation of Viral Cores

Vaccinia virus was isolated and purified according to the method of Joklik (20,21). To isolate viral cores, purified virus was incubated with periodic shaking at 37°C for 30 min in 50 mM Tris-HCl, pH 8.4, 50 mM dithiothreitol and 0.5% nonidet P-40. Cores were purified by sedimentation through a sucrose cushion (36%, w/v in 10 mM Tris-HCl, pH 8.4 and 1 mM dithiothreitol) at 15,000 rpm in a SW 25 rotor for 30 min. Purified cores were resuspended by sonication in 10 mM Tris-HCl, pH 8.4 and 1 mM dithiothreitol.

RESULTS

In agreement with the previous workers, we have noted that addition of vaccinia viral core inhibited protein synthesis in reticulocyte lysates and such

TABLE I

EFFECTS OF ADDITION OF VACCINIA VIRAL CORES ON ENDOGENOUS GLOBIN mRNA TRANSLATION IN RETICULOCYTE LYSATES

Experiment No.	Additions	[^{14}C] leucine incorporated/ 5 µl incubation mixture (pmol)	Percent Inhibition
1	none	108	20
	cores	87	
2	none	100	48
	cores	52	

[^{14}C] Leucine incorporation into proteins was determined using reticulocyte lysates as described under Materials and Methods. Reaction mixtures (25µ l) contained 50% by volume of reticulocyte lysate, 10 mM Tris-HCl, pH 7.8, 1 mM magnesium acetate, 100 mM potassium chloride, 0.2 mM GTP, 30 µM concentrations each of 19 amino acids (-leucine), 57 µM [^{14}C] leucine (250 cpm/pmol), 5 mM creatine phosphate, 10 µg/ml creatine phosphokinase, 20 µM hemin and, where indicated, 0.13 A_{260} unit of vaccinia cores. The reaction mixture in experiment 2 was preincubated at room temperature for 10 min in the presence of KCl, hemin, reticulocyte lysate and, where indicated, vaccinia cores. The reaction mixtures were then mixed with other components of protein synthesis and were incubated at 30°C for 40 min. [^{14}C] Leucine incorporation in protein was determined in a 5 µl aliquot following the method of Ernst et al. (15).

inhibition was more pronounced when the viral core was preincubated in the reticulocyte protein synthesizing system (Table I). Protein synthesis in this experiment was measured in response to endogenous globin mRNA. We also performed similar experiments using micrococcal nuclease treated reticulocyte lysate and studied the effects of vaccinia viral core on protein synthesis directed by exogenously added mRNAs such as globin mRNA, brome mosaic viral (BMV) RNA and cowpea mosaic viral (C_pMV) RNA. Addition of viral core inhibited protein synthesis in each case and the extent of inhibition varied between 40% for C_pMV RNA to 60% for globin mRNA and BMV RNA. Also, in each case, the inhibition was more pronounced when the viral core was preincubated in the amino acid incorporating system. All these experiments were repeated at least three times. The extent of inhibition observed in each case was essentially the same.

In agreement with Ben Hamida and Beaud (10), we also concluded that protein synthesis inhibition by the viral core is not due to nucleolytic degradation of mRNAs by nuclease present in the core, but involves inhibition of some specific

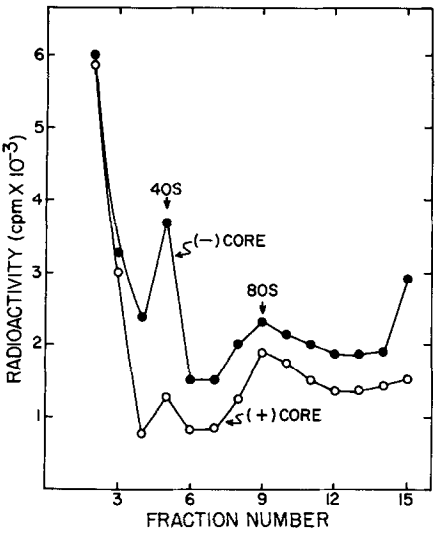

Figure 1: Effects of addition of vaccinia viral core on Met-tRNA$_f$·40S initiation complex formation in the presence of globin mRNA.

The experimental procedures were the same as described under Materials and Methods. Micrococcal nuclease treated reticulocyte lysates were used in the presence of 1 μg of globin mRNA.

step in peptide chain initiation. Several lines of investigations were performed (data not shown): (i) The characteristics of protein synthesis inhibition by the viral core were the same whether the mRNA was present or absent during preincubation of the viral core in the micrococcal nuclease treated reticulocyte lysates. (ii) No significant degradation of [^{125}I] labelled globin mRNA was observed when the radioactively labelled mRNA was incubated with the viral core under the reaction conditions. (iii) Viral core did not inhibit poly r (U) directed polyphenylalanine synthesis in reticulocyte lysates.

Parson, Ben Hamida and Beaud (11) have recently reported that vaccinia viral core inhibits Met-tRNA$_f$ binding to 40S ribosomes in reticulocyte lysates. We have extended these studies and have examined the characteristics of viral core induced inhibition of Met-tRNA$_f$·40S complex formation. In our studies, we have used the micrococcal nuclease treated reticulocyte lysates and have studied the characteristics of Met-tRNA$_f$·40S complex formation in response to different mRNAs. Figure 1 describes the results of a typical experiment showing the inhibition of Met-tRNA$_f$·40S complex formation in response to exogenously added

TABLE II

EFFECTS OF ADDITION OF VACCINIA VIRAL CORES ON Met-tRNA$_f$·40S COMPLEX FORMATION IN MICROCOCCAL NUCLEASE TREATED RETICULOCYTE LYSATES

mRNA Added	Core Added	[^{35}S] Met-tRNA$_f$ bound to 40S ribosomes (cpm)	Percent Inhibition
Globin	-	7,840	
	+	3,250	58
BMV RNA	-	4,960	
	+	2,310	58
C_pMV RNA	-	6,830	
	+	4,120	37
AUG	-	5,700	
	+	5,460	6

Assay conditions were the same as described under Materials and Methods. Where indicated, 1 µg globin mRNA, 0.6 µg BMV RNA, 0.5 µg C_pMV RNA or 0.02 A_{260} unit AUG was added.

globin mRNA. Table II summarizes the results of similar experiments done with globin mRNA, BMV RNA, C_pMV RNA and also triplet codon AUG. Addition of vaccinia viral core inhibited Met-tRNA$_f$·40S complex formation by approximately 60% with globin mRNA and BMV RNA, 40% with C_pMV RNA but had very little effect on Met-tRNA$_f$·40S complex formation with AUG codon. This result thus strongly suggests that the vaccinia viral core inhibits some step of the peptide chain initiation process involving recognition of physiological mRNAs.

We also tested the effect of vaccinia viral core on Met-tRNA$_f$ binding to 40S ribosomes in response to AUG codon using a Millipore filtration method previously described by our laboratory (19). Partially purified peptide chain initiation factor preparation was used in this study. Addition of AUG codon stimulated Met-tRNA$_f$ binding to 40S ribosomes approximately 7-fold and addition of viral core did not have any inhibitory effect on such binding (Table III).

DISCUSSION

The results presented in this paper clearly indicate that vaccinia viral core inhibits Met-tRNA$_f$·40S complex formation with physiological mRNAs but not with an artifical mRNA such as AUG codon.

TABLE III

EFFECTS OF ADDITION OF VACCINIA VIRAL CORES ON Met-tRNA$_f$·40S·AUG COMPLEX FORMATION IN THE PRESENCE OF PARTIALLY PURIFIED PEPTIDE CHAIN INITIATION FACTORS

Addition	[^{35}S] Met-tRNA$_f$ bound to 40S ribosomes pmol	
	-AUG	+AUG
none	0.25	1.8
cores	0.22	1.7

Met-tRNA$_f$ binding to 40S ribosomes was assayed using a two stage Millipore filtration assay method as described under Materials and Methods.

This observation, therefore, suggests that vaccinia viral core inhibits some specific step in the peptide chain initiation process involving recognition of physiological mRNAs possibly binding of physiological mRNAs to 40S ribosomes. Vaccinia viral core had no significant effect on Met-tRNA$_f$ binding to 40S ribosomes in response to AUG codon. These results clearly suggest that the inhibition produced by the viral core is not due to activation of double-stranded RNA induced protein synthesis inhibitor, dsI, as has been suggested by Pelham et al. (12). Activated dsI strongly inhibits Met-tRNA$_f$·40S complex formation in response to AUG codon (22).

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