

# Purification and Characterization of a Protein Synthesis Inhibitor Associated with Vaccinia Virus\*

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A protein synthesis inhibitor, solubilized from vaccinia virus (Ben-Hamida, F., Person, A., and Beaud, G. (1983) *J. Virol.* 45, 452–455), has been purified to homogeneity, yielding a basic protein with molecular mass of 11 kDa. This purified protein migrates as a single spot in two-dimensional gel analysis (isoelectric point above 8.6). It is phosphorylated by the vaccinia-associated protein kinase, and it aggregates in the absence of reducing agents. This 11-kDa protein inhibits protein synthesis when added to a reticulocyte lysate at a stoichiometric ratio of approximately one protein molecule/ribosome, and it associates with the ribosome fraction after incubation in reticulocyte lysates or in Ehrlich ascites tumor cell lysates. As previously described for the inhibitor associated with vaccinia cores, the purified inhibitor inhibits the formation of the 40 S ribosomal subunit-Met-tRNA<sub>i</sub> ribosomal initiation complex. It has no detectable effect on the formation of the ternary complex (Met-tRNA<sub>i</sub>-GTP-eucaryotic initiation factor 2). This inhibitor associated with vaccinia virus particles may be involved in the shutoff of host protein synthesis and may also be responsible for the absence of virus replication in some cell-virus systems.

Productive infection of cells by vaccinia virus generally results in viral mRNA translation with a concomitant shutoff of host protein synthesis (1). This shutoff was first proposed to result from an accelerated degradation of cellular mRNA, although the mechanism which might bring about this reduction in half-life of the mRNAs remains unknown (2, 3). Supporting this hypothesis is the fact that selective translation of vaccinia early mRNA was not observed in extracts prepared from vaccinia virus-infected cells (4).

When viral gene expression is blocked in infected cells by treatment with actinomycin D (5–7) or cycloheximide (8), an efficient shutoff of host cell protein synthesis still occurs. It was thus proposed that translation inhibition might be mediated by viral RNAs (9) or by the abortive transcription products which are synthesized both *in vivo* (7) and *in vitro* (10) in the presence of actinomycin D. Since these short transcripts are capped, methylated, and polyadenylated (10, 11), they might compete with cellular mRNA in binding to ribosomes. Supporting this hypothesis, it has also been reported that some vaccinia virus transcripts from productive

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infections inhibit selectively mRNA translation *in vitro* (12).

However, an inhibition of host protein synthesis was also observed in infected cells exposed to cordycepin (13, 14), i.e. in the absence of a detectable synthesis of viral mRNA and poly(A), suggesting that a factor responsible for this inhibition of translation was associated with the virions themselves. Since cellular mRNA remains stable under these conditions (2, 7, 14), it could be concluded that such an inhibition factor was not an RNase. A corresponding inhibition of initiation of protein synthesis was also observed in cell-free extracts prepared from vaccinia virus-infected cells exposed to cordycepin (15). Furthermore, purified vaccinia cores inhibited translation in rabbit reticulocyte lysates (16), and a similar inhibition was also suggested to occur in a coupled transcription-translation system directed by vaccinia virus cores (17). We reasoned that the purification of this component would answer the question concerning the molecular nature of the vaccinia-associated inhibitor and would also provide further data on its mechanism of action. To this end, a protein synthesis inhibitor associated with vaccinia virus particles was solubilized from purified virions and from viral cores in the course of an endogenous protein kinase reaction (19–21). As had been previously observed during translation inhibition in vaccinia virus-infected cells exposed to cordycepin and in reticulocyte lysates incubated with purified vaccinia subviral particles (18), the soluble inhibitor blocked the formation of the ribosomal 40 S-Met-tRNA<sub>i</sub><sup>1</sup> initiation complex (21). In these experiments, the vaccinia soluble inhibitor was only partially purified and contained several virion structural proteins, three of which were phosphorylated *in vitro* (21). Here we report the purification to homogeneity of the virion-associated inhibitor, SVI, and discuss its possible physiological role during infection.

## EXPERIMENTAL PROCEDURES

**Preparation of Purified Cores**—Vaccinia cores were prepared from purified virus by treatment with Triton X-100 and β-mercaptoethanol as described (16).

**Cell-free Systems**—Rabbit reticulocytes lysates were prepared according to Housman *et al.* (22). Ehrlich ascites tumor (EAT) cell lysates were prepared as previously described (15). Protein synthesis in reticulocyte lysates was assayed by the incorporation of L-[<sup>35</sup>S]methionine in the presence of: 20 mM Hepes, pH 7.5, 5 mM dithiothreitol, 5 mM magnesium acetate, 100 mM potassium acetate, 1 mM ATP, 0.5 mM GTP, 8 mM creatine phosphate, 50 μg/ml creatine phosphokinase, 19 unlabeled amino acids at 40 μM, and 200 μCi/ml L-[<sup>35</sup>S]methionine (1000 Ci/mmol, Amersham Corp.). The effect of

<sup>1</sup> The abbreviations used are: 40 S-Met-tRNA<sub>i</sub>, methionyl-initiator tRNA bound to native 40 S ribosomal subunits; eIF2, eucaryotic initiation factor 2; SVI, soluble vaccinia inhibitor of protein synthesis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EAT, Ehrlich ascites tumor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

SVI on the endogenous translation in reticulocyte lysates was measured after a 10-min preincubation step, as previously described (16).

**Preparation of Crude SVI**—The purified cores (40–60 A<sub>260</sub> units/ml) were suspended in 50 mM Tris-HCl, pH 7.5, 100 mM potassium acetate, 3.5 mM magnesium acetate, 5 mM dithiothreitol, 15 mM creatine phosphate, 50 µg/ml creatine phosphokinase, 0.2 mM GTP, and either 0.5 mM unlabeled ATP or 0.2 mM ATP and 300–700 µCi/ml [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol, Amersham Corp.). After 30 min at 37 °C, the mixture was centrifuged at 12,000 × g for 15 min to remove the vaccinia cores. The supernatant was designated crude SVI.

**DEAE-Sephadex Chromatography**—The crude SVI containing about 300 µg of protein/ml was loaded on a 2-ml DEAE-Sephadex (Pharmacia) column preequilibrated with 0.15 M NaCl, 0.15 M Tris-HCl, pH 8.6, 6% glycerol, 0.1% Triton X-100, 5 mM dithiothreitol, and 1 mM EDTA. The flow rate was 6 ml/h, and the initial effluent was collected.

**Carboxymethyl-Sephadex Chromatography**—The fractions from the effluent of the DEAE-Sephadex column containing proteins were pooled, adjusted by dilution to the concentration of the C-25 equilibrium buffer 0.05 M NaCl, 0.05 M Tris-HCl, pH 8.6, 2% glycerol, 0.05% Triton X-100, 5 mM dithiothreitol, 1 mM EDTA, and then loaded on a 1.5-ml column, with a flow rate of 4 ml/h. After collecting the effluent and washing the column with 3 volumes of C-25 equilibrium buffer, the inhibitory activity was step-eluted by equilibrium buffer adjusted to 0.3 M NaCl.

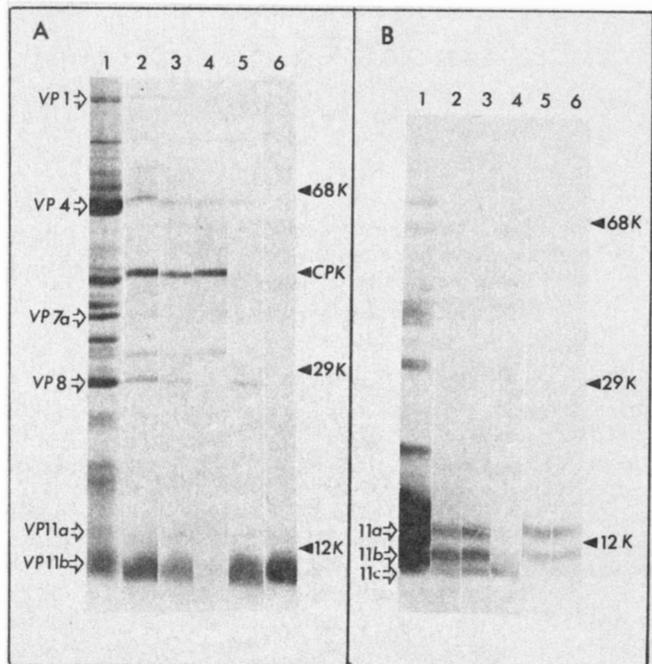
**Sephacryl S200 Gel Filtration**—The inhibitory peak eluted from the C-25 column was dialyzed against the buffer used for Sephadryl S200 chromatography (150 mM potassium acetate, 20 mM Tris-HCl, pH 7.5, 9 mM dithiothreitol, and 0.01% Triton X-100). The dimensions of the column were 0.7 × 7 cm, the flow rate was 3 ml/h, and 0.15-ml fractions were collected.

**Ternary Complex Formation**—Ternary complex formation was assayed as described (23). The incubation mixture (20 µl), containing 4 pmol of purified eIF-2 (23), 5 pmol of [<sup>35</sup>S]Met-tRNA<sub>i</sub> ( $1.5 \times 10^4$  cpm/pmol), and 15 µM GTP, was incubated in the presence of SVI for 10 min at 30 °C. It was then diluted 20-fold and filtered through a Millipore membrane (0.45-µ pore size).

**GTPase Activity Assay**—1 µl of each fraction was incubated in 25 mM Tris-HCl, pH 7.5, 2 mM magnesium acetate, 2.5 mM dithiothreitol, and 100 mM potassium acetate in a final volume of 3 µl with 100 pmol of [<sup>3</sup>H]GTP (5000 cpm/pmol) for 15 min at 35 °C. At the end of the incubation, 20 nmol of unlabeled GTP and GDP were added, and the mixture was chromatographed on polyethyleneimine-cellulose (Merck) in 1 M LiCl. The spots were visualized under UV light, before excision and counting.

## RESULTS

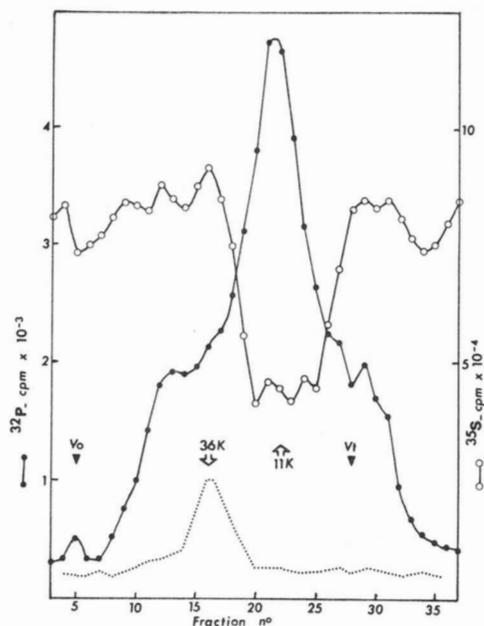
**Purification of the Soluble Vaccinia Inhibitor**—SVI was obtained after incubating vaccinia cores in the presence of ATP, as described under "Experimental Procedures." During this solubilization incubation, only 8–10% of core proteins were solubilized, as compared to 30–40% solubilized core proteins when the incubation was done in the presence of Nonidet P-40 (21). However, the amount of inhibitory activity in the soluble fraction was the same in both cases, indicating that incubation of the cores without detergent resulted in a more specific solubilization of the protein synthesis inhibitory activity. Fig. 1A, lane 2, shows the pattern of solubilized proteins analyzed by SDS-PAGE and Coomassie Blue staining. It can be seen that the major viral protein which is released from the cores in the soluble fraction is a protein of low molecular mass (11 kDa), together with other minor proteins. Most of the other viral proteins were found in the pellet fraction (Fig. 1A, lane 1). After DEAE-Sephadex chromatography of crude SVI to remove any contaminating nucleic acids, essentially the same proteins were recovered in the effluent (Fig. 1A, lane 3), which also contained the inhibitory activity (see Table I). Upon CM-Sephadex chromatography of the DEAE-Sephadex effluent, approximately 60% of the proteins were recovered in the flow-through fraction (Fig. 1A, lane 4), with a relatively low amount of SVI, whereas the proteins eluting at 0.3 M NaCl contained most of the inhibitory activity (Table I). This fraction was composed of the



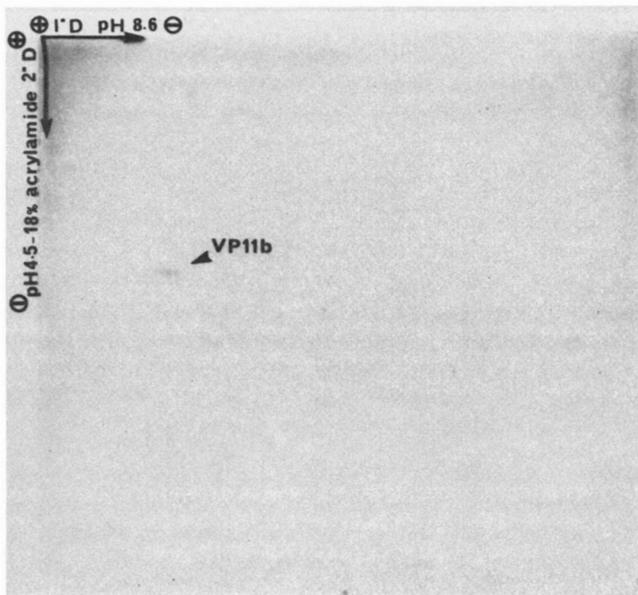
**FIG. 1. SDS-PAGE analysis of SVI at successive stages of purification.** The purification steps are described under "Experimental Procedures." A, Coomassie Blue staining of the proteins; B, autoradiogram of the phosphoproteins labeled with [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol). Lanes 1, pellet containing the proteins which remained associated with the viral cores after the solubilization reaction; lanes 2, supernatant from the solubilization reaction containing the crude SVI (CPK is the added creatine phosphokinase); lanes 3, flow-through fraction from DEAE-Sephadex; lanes 4, flow-through fraction from Sephadex C-25; lanes 5, 0.3 M NaCl step-eluted fraction from the Sephadex C-25; lanes 6, gel filtration inhibitory peak fractions from Sephadryl S200. A and B show two different purification experiments. VP1, VP4, VP7a, VP8, VP11a, and VP11b denote vaccinia virion proteins according to the nomenclature of Sarov and Joklik (26). SDS-PAGE electrophoresis (15% polyacrylamide gel) was carried out as previously described (15).

major 11-kDa protein together with three other minor proteins (Fig. 1A, lane 5) and was further purified by filtration through a column of Sephadryl S200. The peak of inhibitory activity thus obtained (Fig. 2), corresponded to a molecular mass in the range of 10–13 kDa, as calculated by calibration of the column with markers of known molecular mass. The SDS-PAGE analysis of the fractions from Sephadryl-S200 containing the highest inhibitory activity revealed a single protein band with molecular mass of 11 kDa (Fig. 1A, lane 6), indicating that SVI activity was associated with a monomeric protein. Moreover, as shown in Fig. 3, a single spot corresponding to a isoelectric point higher than 8.6 was observed when purified SVI was analyzed by a two-dimensional electrophoresis procedure combining nonequilibrium pH gradient electrophoresis and electrophoresis at pH 4.5. When purified SVI was analyzed by a two-dimensional system including isoelectric focusing in the first dimension, no detectable spot was observed after Coomassie Blue staining (data not shown). These two-dimensional analyses, carried out by B. Buendia and J. J. Madjar (Faculté de Médecine, Université de Lyon I, Lyon, France), strongly suggested that the 11-kDa protein associated with purified SVI was homogenous.

A summary of the purification steps of SVI is shown in Table I. Assuming a molecular mass of 11 kDa for SVI and a concentration of ribosomes of 1.5 mg/ml in the reticulocyte lysate, it can be calculated from the specific activity of Sephadryl S200-purified SVI (Table I) that the amount of SVI



**FIG. 2.** Sephadryl S200 gel filtration of the solubilized inhibitor (labeled with  $^{32}\text{P}$ ). The gel filtration on a Sephadryl S200 column is described under "Experimental Procedures." Each fraction (150  $\mu\text{l}$ ) was assayed for: 1)  $^{32}\text{P}$  radioactivity by Cerenkov counting (●—●), 2) inhibitory activity on the endogenous protein synthesis of the reticulocyte lysate (○—○), and 3) GTPase activity, as described under "Experimental Procedures" (····). The peak value corresponds to 50% GTP hydrolyzed in the assay.



**FIG. 3.** Two-dimensional analysis of SVI-associated protein. Approximately 4  $\mu\text{g}$  of Sephadryl S200 purified protein were applied on the gel. Electrophoresis in the first dimension (horizontal axis) was by nonequilibrium pH gradient gel electrophoresis (27). Electrophoresis in the second dimension was at pH 4.5 through an 18% acrylamide gel (28). VP11b designates the 11-kDa SVI protein. The Coomassie Blue-stained gel is shown.

protein required to produce a 50% inhibition of protein synthesis corresponds to a stoichiometric ratio of approximately one 11-kDa molecule/ribosome. It can also be calculated from Table I that the amount of purified 11-kDa protein recovered from 10 mg of purified vaccinia virus corresponds to 170,000 molecules of SVI/viral plaque-forming unit. Since infections are usually carried out at 3 plaque-forming units/cell, it can

**TABLE I**  
Purification of the inhibitor of protein synthesis associated with vaccinia cores

The initial amount of vaccinia cores was 7 mg, corresponding to approximately 10 mg of purified vaccinia virus.

Fraction	Total protein $\mu\text{g}$	Total SVI activity <sup>a</sup> units	Specific activity units/ $\mu\text{g}$	Yield of SVI %
Crude soluble inhibitor	650	3260	5.4	100
DEAE effluent	560	3160	5.6	87
0.3 M C-25 elution	116	1720	14.8	47
S200 gel filtration peak	34	1298	38.2	36

<sup>a</sup> 1 unit represents the amount of inhibitor required for a 50% inhibition of protein synthesis in 5  $\mu\text{l}$  of reticulocyte lysate.

be concluded that physiologically significant amounts of SVI protein may be present in vaccinia virus-infected cells, considering the number of ribosomes in these cells.

In order to analyze the phosphorylated proteins present in the different SVI fractions, the solubilization reaction was also carried out in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP, and the phosphoproteins were then detected by autoradiography after separation by SDS-PAGE. We have previously shown that crude SVI contained three phosphorylated viral proteins of low molecular mass (10–13 kDa), which we designated as VP11a, VP11b, and VP11c based on their electrophoretic mobilities (21, 24). These proteins were phosphorylated *in vitro* by the core-associated protein kinase and released from the cores. Fig. 1B, lane 2, shows that, in the absence of detergent during the solubilization, there are also three phosphoproteins detected in the soluble fraction from the vaccinia cores. VP11c does not copurify with SVI after CM-Sephadex chromatography (Fig. 1B, lane 4), whereas phosphorylated VP11a and VP11b remain unseparated from SVI even after the last purification step (lane 6). However, the  $^{32}\text{P}$ -labeled VP11a band does not correspond to a protein band detectable by Coomassie staining (Fig. 1A). Therefore, this band represents a highly phosphorylated protein, which has properties similar to those described for the phosphate acceptor II protein in the vaccinia protein kinase *in vitro* reaction system (25). Moreover, as shown below, we could eliminate phosphorylated VP11a from SVI. When a two-dimensional electrophoretic analysis similar to that presented in Fig. 3 was carried out on crude SVI prepared in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP, it was observed that the  $^{32}\text{P}$ -labeled spot corresponding to a 11-kDa protein phosphorylated *in vitro* migrated as a slightly less basic protein after nonequilibrium pH gradient electrophoresis than the corresponding 11-kDa protein stained with Coomassie Blue but did not correspond to a detectable stained spot.<sup>2</sup> This experiment strongly suggested that the *in vitro* phosphorylated 11-kDa protein present in purified SVI results from the phosphorylation of minor amounts of SVI-associated protein by the protein kinase associated with the vaccinia cores.

In conclusion, SVI has been extensively purified to yield a basic protein of 11 kDa which appears homogeneous on SDS-PAGE and two-dimensional gel analysis. It contains also a 11-kDa phosphoprotein phosphorylated *in vitro*.

*Properties of Vaccinia Soluble Inhibitor*—When SDS-PAGE of the soluble inhibitor was carried out in the absence of a reducing agent in the sample buffer, a polymerization of the 11-kDa protein was observed. Fig. 4A, lane S+, shows the migration of  $^{32}\text{P}$ -labeled SVI as a 11-kDa protein after its reduction with  $\beta$ -mercaptoethanol, whereas it migrates as a

<sup>2</sup> B. Buendia, A. Person-Fernandez, G. Beaud, and J. J. Madjar, manuscript in preparation.

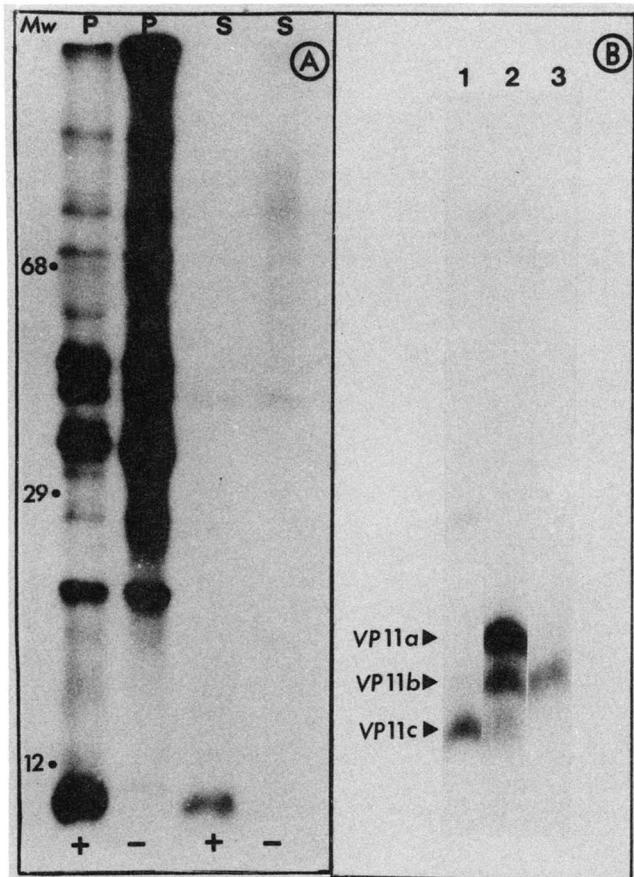


FIG. 4. A, autoradiogram from an SDS-PAGE analysis comparing the migration of the proteins from the core pellet fraction and from the soluble supernatant fraction, reduced or not reduced before electrophoresis. The solubilization reaction was carried out as described under "Experimental Procedures" in the presence of [ $\gamma$ -<sup>32</sup>P]ATP (250 Ci/mmol). The fractions containing the pellet (*P*) or the supernatant (*S*) from the solubilization were then heated 2 min at 100 °C in 0.1% SDS with (+) or without (-) 50 mM  $\beta$ -mercaptoethanol, before being analyzed on SDS-PAGE. In this experiment, phosphorylated VP11a and VP11b were not separated. B, specific precipitation of VP11b protein under nonreducing buffered conditions. The purified SVI fraction eluted from Sephadex C-25 chromatography showed visible aggregates after several freeze-thaw cycles. These aggregates were pelleted by 10-min centrifugation at 12,000  $\times g$ , and the supernatant fraction (*lane 2*) and pellet fraction (*lane 3*) were analyzed by SDS-PAGE. *Lane 1* corresponds to the flow-through fraction from the Sephadex C-25 chromatography. SVI was labeled with <sup>32</sup>P during the solubilization step, and an autoradiogram of the gel is presented.

polymer of high molecular mass (90–120 kDa) when the reduction step by  $\beta$ -mercaptoethanol is omitted (Fig. 4A, *lane S-*). This observation was confirmed by Coomassie Blue staining after SDS-PAGE of SVI (data not shown). Moreover, a similar effect was obtained when these experiments were carried out on the <sup>32</sup>P-labeled proteins remaining associated with viral cores (Fig. 4A, *lanes P+* and *P-*) and was also confirmed after Coomassie Blue staining (data not shown). This specific aggregation of the 11-kDa protein suggested a possible means for eliminating phosphorylated VP11a from SVI. In addition, we also observed the formation of a precipitate in the solution of crude or purified SVI after several freeze-thaw cycles. This precipitate could be collected by centrifugation and dissolved in a buffer containing  $\beta$ -mercaptoethanol or dithiothreitol. As expected, it contained only phosphoprotein VP11b, as shown in Fig. 4B (*lane 3*), and corresponded to a single band stained by Coomassie Blue (not shown). The supernatant remaining after centrifugation con-

tained the unprecipitated phosphoproteins VP11a and VP11b (Fig. 4B, *lane 2*), and also the Coomassie Blue-stained 11-kDa band (not shown). Furthermore, the protein synthesis inhibitory activities present in the fractions corresponding to the supernatant and solubilized pellet were measured. As expected, approximately half of the total inhibitory activity was recovered in the pellet fraction, demonstrating that phosphorylated VP11a was unrelated to SVI.

This selective polymerization of SVI protein presumably results from intermolecular disulfide bond formation occurring after the oxydation of the  $\beta$ -mercaptoethanol initially present in the solution, suggesting that reactive SH groups may be present in SVI protein. Indeed, it has been recently shown that a 13-kDa basic phosphoprotein present in the nucleocapsid of vaccinia virus is linked to a 27-kDa protein via disulfide bridges (29). Moreover, the nucleotide sequence of the gene coding for a major late protein, a basic 11-kDa phosphoprotein, reveals the presence of 5 cysteine residues/molecule (30). These may thus be related to the 11-kDa protein associated with purified SVI.

Since the SVI protein has a high isoelectric point, it could not be completely excluded that some short viral RNA molecules, strongly associated with it, could still be responsible for the inhibitory activity of purified SVI. To be in agreement with our previous work on SVI (21), such an association with the 11-kDa protein should protect RNA molecules from RNase digestion and should be resistant to 6 M urea. However, we found that more than 60% of SVI remained active after an alkaline treatment (0.3 M KOH for 17 h at 37 °C and then neutralized with acetic acid). This result demonstrated that the inhibitory activity is not due to the presence of contaminating RNA molecules in the purified 11-kDa protein preparation.

**Site of Action of Vaccinia Inhibitor**—Our previous studies have shown that the formation of the 40 S·Met-tRNA<sub>i</sub> ribosomal preinitiation complex was inhibited by SVI (19, 21). This complex results from the binding of the ternary initiation complex (elF2·GTP·Met-tRNA<sub>i</sub>) to native 40 S ribosomal subunits. Therefore, we studied the effect of SVI on the formation of the ternary initiation complex. We first observed that crude or partially purified SVI inhibited the formation of the ternary complex, but this inhibition was released by the addition of phosphoenolpyruvate and pyruvate kinase, suggesting that a GTPase activity was involved in this inhibition. Indeed, a GTPase activity was detected in crude or partially purified SVI, and it was then important to follow the fate of GTPase activity in the last purification step (Sephacryl S200). Fig. 2 reveals a clear separation of the peak corresponding to the GTPase activity (molecular mass of approximately 36 kDa), from the peak corresponding to SVI (10–13 kDa), as assayed by protein synthesis inhibitory activity. This result showed that a contaminating GTPase activity in crude SVI was responsible for the inhibition of ternary initiation complex formation observed in the preliminary experiments. Indeed, Sephadryl S200-purified SVI had no inhibitory effect at this step (Table II, column 1). Furthermore, when the ternary initiation complex was formed in the presence of SVI (Sephacryl S200 step) and then added to a reticulocyte lysate preincubated with sparsomycin (as a source of native 40 S subunits), a 40–50% reduction in the formation of the 40 S·Met-tRNA<sub>i</sub> complex was observed (Table II, column 2). This result confirms that formation of 40 S·Met-tRNA<sub>i</sub> initiation complex is the step affected by the inhibitor of protein synthesis associated with vaccinia cores (18), whereas the preceding step, formation of the ternary complex, is not affected by SVI (Table II, column 1). On studying the

TABLE II

*Effect of crude and purified SVI on ternary complex formation and binding to 40 S ribosomal subunits*

Ternary complex formation was assayed by incubation of eIF2, as described under "Experimental Procedures." At the end of the 10-min incubation, an aliquot ( $2 \mu\text{l}$ ) was withdrawn to measure the amount of ternary complex formed. A  $25-\mu\text{l}$  aliquot of reticulocyte lysate which had been preincubated in the presence of sparsomycin (18) was added, and after a further 10-min incubation at  $30^\circ\text{C}$ , the reaction mixtures were analyzed as described previously (18).

Incubation with	$[^{35}\text{S}]$ Methionyl-tRNA present in		
	Ternary complex cpm	40 S subunits cpm	Inhibition %
Control buffer	72,000	21,500	0
Crude SVI	73,600	11,800	45
Purified SVI	79,200	12,600	42

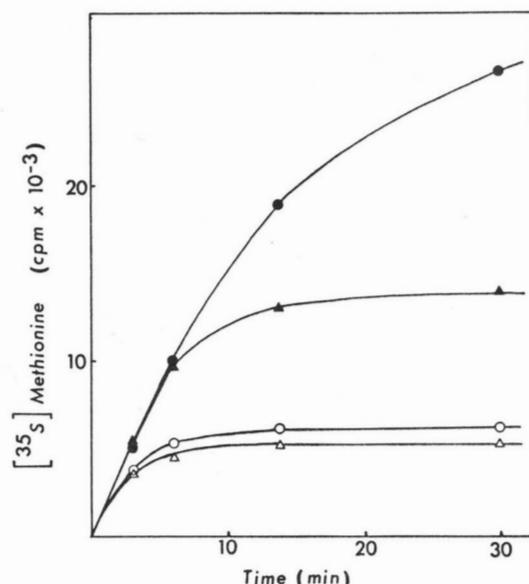


FIG. 5. Effect of purified SVI on the kinetics of protein synthesis in the presence of edeine in a reticulocyte lysate. Protein synthesis was assayed by incorporation of  $[^{35}\text{S}]$ methionine into proteins, as described under "Experimental Procedures," but omitting the preincubation step of the reticulocyte lysate with SVI. ●—●, no addition; ▲—▲,  $3 \mu\text{l}$  of purified SVI added; ○—○,  $10 \mu\text{M}$  edeine added; △—△,  $3 \mu\text{l}$  of purified SVI and  $10 \mu\text{M}$  edeine added.

subsequent step, formation of the 80 S initiation complex upon addition of mRNA, we observed no inhibition in lysates from vaccinia-infected cells in the presence of cordycepin.<sup>3</sup> In addition, when the purified SVI was added to reticulocyte lysates in the presence of  $10 \mu\text{M}$  edeine (a specific inhibitor of initiation), no significant inhibition of protein synthesis was observed for the residual  $[^{35}\text{S}]$ methionine incorporation (Fig. 5). Therefore, SVI presented no detectable inhibitory activity at the step of protein chain elongation.

Since it had been shown previously that the viral protein VP11b is found in a phosphorylated form associated with ribosomes of the cells at an early time after vaccinia virus infection (24), it was of interest to confirm a possible association of the SVI-associated 11-kDa phosphoprotein with the ribosomes in EAT cell lysates. Such a study was carried out with  $^{32}\text{P}$ -labeled inhibitor incubated in EAT cell lysates (treated or not treated with micrococcal nuclease), and the results are shown in Fig. 6. The amount of added SVI in the

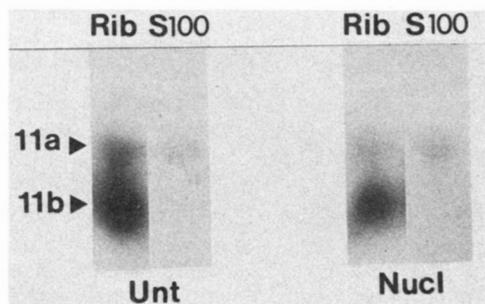


FIG. 6. Association of phosphorylated VP11b with the ribosomes in EAT cell lysates.  $30 \mu\text{l}$  of  $^{32}\text{P}$ -labeled soluble inhibitor were incubated with  $70 \mu\text{l}$  of EAT cell lysate (15), either untreated (Unt) or treated with micrococcal nuclease (Nucl), and after 15 min at  $35^\circ\text{C}$ , the lysate was diluted by the addition of 5 volumes of 20 mM Hepes, pH 7.5, 20 mM KAc, 2 mM MgAc, 2 mM dithiothreitol and centrifuged for 1 h at 50,000 rpm (SW 50.1 Beckman rotor). The ribosome pellet was rinsed and suspended in  $50 \mu\text{l}$  of the dilution buffer before being analyzed (Rib), together with  $100 \mu\text{l}$  of post-ribosomal supernatant (S100), by SDS-PAGE. The autoradiogram of the lower part of the gel is shown.

lysate correspond to an inhibition of protein synthesis of approximately 50%. Fig. 6 clearly shows that most, if not all, of the phosphorylated 11-kDa protein associates with the ribosomal fraction in the EAT cell lysates either treated or untreated with nuclease. This experiment was also carried out with unlabeled SVI, and the 11-kDa protein was then detected using an antiserum (obtained after immunization of mice with the 11-kDa protein excised from a SDS gel). The immunoblots from SDS-PAGE gels (23) confirmed that the 11-kDa protein was found only in the ribosomal fraction (data not shown). These experiments revealed that SVI-associated protein can bind to ribosomes *in vitro*.

## DISCUSSION

Purification of a protein synthesis inhibitory activity solubilized from vaccinia virus cores (SVI) yielded an apparently homogenous basic protein with a molecular mass of approximately 11,000 daltons when analyzed by SDS-PAGE and two-dimensional gel electrophoresis. SVI also contained an *in vitro* phosphorylated protein, designated in this paper as VP11b phosphoprotein (24, 26, 31), and co-migrating with the 11-kDa protein on SDS-PAGE. On nonequilibrium pH gradient gel electrophoresis, the *in vitro* phosphorylated 11-kDa protein migrated as a slightly less basic protein than SVI protein, as expected for a phosphorylated form of the latter protein, and was present in minor amount. However, the band from SDS-PAGE designated as VP11b by Sarov and Joklik (26) may contain several proteins since multiple spots corresponding to proteins of similar molecular mass have been detected on two-dimensional gel analysis of vaccinia virus structural proteins (32–35). Therefore, the relationship between the basic protein we have purified and other similar phosphorylated (or phosphorylatable) proteins previously described (31, 35–38) remains to be established. The same conclusion applies concerning a possible similarity of our protein with the 11-kDa basic protein corresponding to a gene recently sequenced (30).

Concerning the role of the phosphorylation of the purified 11-kDa protein in its inhibitory activity, we have observed the presence in reticulocyte lysates and EAT cell lysates of protein phosphatases and kinases able to dephosphorylate and phosphorylate SVI-associated 11-kDa protein. For instance, the protein kinase present in partially purified initiation factor eIF2 preparations phosphorylates very efficiently the SVI-associated protein.<sup>3</sup> For this reason, it was not pos-

<sup>3</sup> A. Person-Fernandez and G. Beaud, unpublished results.

sible to carry out experiments which would determine whether SVI is active in its phosphorylated form, unphosphorylated form, or both. However, it should be recalled that a very early phosphorylation of VP11b does occur *in vivo*, in EAT-infected cells in the absence of viral gene expression (24).

The protein synthesis inhibitory activity of the purified SVI exhibited the same properties as those described for the shutoff of host protein synthesis in the absence of viral transcription (10, 15, 18) and for the core-mediated *in vitro* inhibition of translation (16). The additional property, that the purified inhibitor resisted alkali treatment, ruled out the possibility that viral RNA molecules were responsible for protein synthesis inhibition by SVI. These observations and the extensive purification of SVI yielding a homogenous 11-kDa protein reveal that the inhibitor of protein synthesis that we have purified from vaccinia virus is clearly different from the viral RNA (9, 12) or vaccinia surface tubules (39) which were previously suggested to be responsible for the shutoff of protein synthesis by vaccinia virus.

Our studies on the site of action of this purified inhibitor from vaccinia virus showed, as previously found in the case of infected cell extracts (18), that the formation of the 40 S-Met-tRNA<sub>i</sub> initiation complex was inhibited in reticulocyte extracts, whereas the preceding step, the formation of the ternary complex, was not affected by the purified inhibitor. Therefore, it is likely that SVI blocks initiation of protein synthesis at the stage of the attachment of ternary initiation complex to native ribosomes. Moreover, we have observed that incubation of SVI with either reticulocyte lysates or EAT cell lysate did not cause any increased phosphorylation of eIF2 (data not shown), strongly suggesting that the mechanism of action of SVI is different from that described for the hemin-regulated inhibitor (for a review, see Ref. 40). In addition, we showed that SVI-associated 11-kDa protein binds to the ribosomes after *in vitro* incubation with either reticulocyte or EAT cell lysates. It is thus possible that the binding of the 11-kDa protein to native 40 S ribosomal subunits could interfere with the attachment of the ternary complex, but additional experiments would be required to prove this hypothesis. An early transfer of viral core protein to cellular ribosomes was recently described in the case of the Sindbis virus (41) and the Semliki Forest virus (42). In the latter case, the interference of viral capsid protein with the binding of host messenger RNAs into initiation complexes was suggested to be the cause of the shutoff of host protein synthesis.

Additional observations may be made considering the mechanism of shutoff of host protein synthesis by vaccinia virus. Since SVI acts at a stage which precedes the attachment of mRNA to ribosomes, we might expect a nonselective inhibition of viral as well as host cell translation. Indeed, several experimental conditions have been reported in which infection of cells by vaccinia virus result in a total, nonselective inhibition of protein synthesis. The simplest case is the infection at a relatively high multiplicity of infection (more than 15 plaque-forming units/cell).<sup>3</sup> It was also previously shown (8, 43) that, in the absence of viral protein synthesis at the early phase of infection, the shutoff of protein synthesis is irreversible even for viral mRNAs. Moreover, the studies of restriction growth of vaccinia virus in Chinese hamster ovary cells (44), host-range rabbit poxvirus (45), and vaccinia mutants (46) led to the conclusion that the infection of nonpermissive cells was abortive because of an irreversible inhibition of both viral and cellular protein synthesis, although early viral transcription occurred efficiently. These results suggest that, in the absence of viral protein synthesis at the early stage of infection, the inhibition of translation by

vaccinia virus affects all mRNAs, including viral mRNAs. Presumably, in a productive infection the virion-mediated protein synthesis inhibition is not complete and some viral-coded early protein(s) is synthesized at a level sufficient to release the translational block (8, 43, 45). A vaccinia-dependent modification of eIF-2 occurring early in the infection has been recently observed (52), which might be directly involved in this release. The specific modifications of 40 S ribosomal proteins S2 and S13 occurring early in vaccinia infection (47)<sup>2</sup> may also have a role in this release. It is interesting to note that, if functional, these translational modifications induced early in the infection by vaccinia virus would then be directly related to the mode of action that we propose for SVI, i.e. by resulting in the restoration of the binding of initiation ternary complex to 40 S ribosomal subunits.

The presence of a virion-associated component capable of inhibiting directly cellular protein synthesis has also been reported for several other eucaryotic viruses such as herpes simplex virus type 2 (48, 49), frog *Iridovirus* type 3 (50), and Chilo iridescent virus (51). However, the viral factors responsible for protein synthesis inhibition in these cases have not yet been characterized. Our results describe for the first time the purification and characterization of the protein synthesis inhibitory activity associated with vaccinia virus, yielding a basic 11-kDa protein phosphorylatable *in vitro* by the kinase associated with viral cores.

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