Detection of RNA–protein complex in vaccinia virus core in vitro transcription system

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The incubation of vaccinia virus cores in appropriate conditions promotes the release of core proteins into a supernatant fraction. Under transcription assay conditions core mRNAs are extruded in association with viral core proteins, however the presence of these proteins within the core particle is not essential for RNA synthesis and extrusion. The RNA–protein complex is resistant to micrococcal nuclease. Five proteins of 60K, 43K, 28K, 18K and 14.5K with RNA-binding abilities have been identified by [32p]RNA overlay protein blot assays. These proteins are likely to be a component of the viral ribonucleoprotein complex since core basic proteins with similar Mr,s have been identified and at least one RNA-binding protein is predicted in the vaccinia virus genome.

Introduction

The poxviruses are a large family that infect both vertebrate and invertebrate hosts (for review see Moss, 1990). They are distinguished by their large size, complex morphology and cytoplasmic site of replication. Studies with vaccinia virus, the prototype of the family, have contributed to our understanding of the strategies used by these viruses to replicate and express their genome. The infection of tissue culture cells with vaccinia virus results in profound cytopathic effects, such as changes in membrane permeability and inhibition of host protein, RNA and DNA synthesis (Bablanian, 1984).

Studies on vaccinia virus transcription have been facilitated by purification of the virus particle, and the ability of the cores to synthesize RNA in vitro (Kates & McAuslan, 1967; Munyon et al., 1967). These transcripts have been characterized by sedimentation (Paoletti, 1977). RNA–DNA hybridization (Boone & Moss, 1978; Cabrera et al., 1978) and translation in cell-free systems (Cooper & Moss, 1978; Pelham et al., 1978).

The core transcription system was re-evaluated in terms of RNA synthesis in the presence of Mg2+ or Mn2+ and the effect of polyamines on the assay system (Moussatché, 1985). In addition, it was also verified that some core proteins which might contribute to the regulation of early transcription are phosphorylated during the initiation of RNA synthesis (Moussatché & Keller, 1991). Recently, we have shown that when vaccinia virus cores are incubated in the presence of nucleotides, several core-associated proteins are released into the supernatant fraction (Dâmasso & Moussatché, 1992). SDS-PAGE analysis of the supernatant revealed the presence of 17 polypeptides, four of which are phosphorylated.

In this report we show that vaccinia virus core mRNAs extruded during in vitro transcription are associated with core proteins. In the presence of viral proteins the mRNA can be protected from micrococcal nuclease digestion and two peptides of 18K and 14.5K are u.v. crosslinked to [32p]RNA. The identification of the RNA-binding proteins was also performed by RNA overlay protein blotting (Northwestern blot). This procedure revealed the presence of five proteins with apparent Mr,s of 60K, 43K, 28K, 18K and 14-5K with affinity for viral core mRNA. A ribonucleoprotein (RNP) with a typical RNA-binding motif and an Mr of 14-2K was predicted within the genome of vaccinia virus Copenhagen strain (A31R) (Goebel et al., 1990) and an equivalent protein was found in the WR strain (SalL1R) with an apparent Mr of 14-9K (Smith et al., 1991). The possibility of this protein being one of the proteins identified in this work, and the formation of RNA–protein complexes during in vitro mRNA synthesis, are discussed.

Methods

Virus. Vaccinia virus (WR strain) was purified from infected HeLa cells after a 48 h infection as described previously (Moussatché & Keller, 1991). The virus was diluted to 2 x 1011 particles/ml, assuming that 1.0 A260 unit corresponds to 1.2 × 1011 particles/ml. The 35S-
labelled vaccinia virus was produced by a 48 h incubation of BHK-21 cells at an m.o.i. of 0.1 p.f.u./cell in the presence of 10 μCi/ml [35S]methionine during the final 24 h of infection.

Preparation of vaccinia virus cores for in vitro assays. The vaccinia virus cores used in the in vitro transcription system were prepared by incubating purified virus (2 x 10^11 particles/ml) in 50 mM-Tris-HCl pH 7.5, 10 mM-DTT, 0.05% NP40 for 10 min at 37°C and processing as described by Moussatché (1985).

RNA synthesis by core-associated RNA polymerase. Vaccinia virus cores were assayed for in vitro RNA synthesis as described (Moussatché, 1985) with some modifications. The transcription reaction mixtures contained 50 mM-Tris–HCl pH 7.5, 10 mM-DTT, 5 mM-MgCl2, 2 mM-azididine, 20 mM-KCl, 2 mM-ATP, 2 mM-GTP, 2 mM-CTP, 0.1 mM-[3H]UTP (35 c.p.m./pmol) and 4 x 10^10 vaccinia virus core particles/ml. In some experiments the [3H]UTP was replaced by [α-32P]UTP (250 µCi/ml). The 100 µl reaction assays were incubated at 37°C and the reaction was stopped by a 2 min centrifugation in an Eppendorf microfuge. Each of the supernatant fractions was removed, precipitated with 1 ml of cold 5% TCA and collected in a nitrocellulose filter (Schleicher & Schuell, BA-85). The acid-precipitated samples were then treated as described (Moussatché, 1985) and the vaccinia virus core mRNA isolated as described (Moussatché & Keller, 1991).

Nitrocellulose filter-binding assay. To measure RNA-protein interaction in solution, a nitrocellulose filter-binding assay was used. Samples (100 µl) of the supernatant fraction, radioactively labelled in vitro, were removed and directly applied to nitrocellulose filters, which had been pre-moistened in binding buffer containing 50 mM-Tris–HCl pH 7.5, 5 mM-MgCl2, 20 mM-KCl. After filtering, the nitrocellulose filters were washed with 2 ml of binding buffer, dried and the radioactivity incorporated was measured in an LS-7000 Beckman liquid scintillation counter.

Micrococcal nuclease treatment of the supernatant fraction. The RNA-protein complex was analysed for resistance to micrococcal nuclease treatment. The vaccinia virus [32P]RNA-protein complex was separated from the cores by a 2 min centrifugation. The sample was incubated with 0.75 mM-CaCl2 and Staphylococcus aureus nuclease (1250 units/ml, Boehringer Mannheim). At a given time, the reaction was stopped by the addition of 2.5 mM-EGTA and the samples were prepared for electrophoresis in 5% non-denaturing polyacrylamide gel (Konarska, 1989). In another experiment, the sample was placed under a u.v. lamp (1.2 x 10^7 J/m2; UVIS-11 mineralight) for 20 min on ice before micrococcal nuclease digestion. After the treatment, the samples were prepared and analysed in a 15% polyacrylamide gel (Laemmli, 1970).

PAGE. The supernatant fraction from the core system was treated with 5 volumes of acetone for 18 h at -40°C. After centrifugation, the precipitate was resuspended in loading buffer containing 10% glycerol, 100 mM-DTT, 2% SDS, 0.02% bromophenol blue and heated at 100°C for 5 min. The samples destined for electrophoresis were not heated prior to loading on the gel. The polypeptides were separated using a 12.5% or a 15% SDS-polyacrylamide gel as described by Laemmli (1970). Following electrophoresis, the gel was fixed with 20% TCA, dried and exposed to an Omat-K Kodak X-ray film or treated for electroblotting. The laser densitometer scan was performed using an UltraScan apparatus (model 2202, LKB).

Protein blotting. Vaccinia virus core-released proteins (20 µg) were prepared for electrophoresis as described before. After electrophoresis, the polypeptides were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell) (4 h, 225 mA, 4°C) in a buffer containing 192 mM-glycine, 25 mM-Tris and 20% methanol (Towbin et al., 1979). The membrane was incubated in a blotting solution containing 10 mM-Tris–HCl pH 7.5, 50 mM-NaCl, 1 mM-EDTA, 0.02% BSA, 0.02% Ficol, 0.02% polyvinylpyrrolidone (Bowen et al., 1980) in a sealed plastic bag for 2 h at room temperature, with gentle shaking. The solution was replaced with the blotting solution containing vaccinia virus core [32P]RNA (15 x 10^6 c.p.m.) and 15 µg/ml of tRNA as carrier. The membrane was incubated for 90 min at room temperature in a sealed plastic bag with gentle shaking. After this period the membrane was washed four times, for 30 min each, with 10 mM-Tris–HCl pH 7.5, 50 mM-NaCl, 1 mM-EDTA, then dried at room temperature and exposed to an X-ray film as described before.

Results

Vaccinia core RNA–protein complex formation

Purified vaccinia virus core particles when incubated in the presence of the four ribonucleotides synthesize mRNA (Kates & Beeson, 1970). The RNA extruded from the core can be separated from core particles by centrifugation. Recently, we reported that the incubation of vaccinia virus cores in the presence of nucleotides also promotes the release of viral proteins from the core (Dámaso & Moussatché, 1992). Analysis by SDS–PAGE indicates the presence of approximately 17 peptides in the supernatant fraction. The release of core proteins during viral transcription suggests the occurrence of RNA–protein complexes as shown in other systems (reviewed in Dreyfuss, 1986). To investigate this hypothesis, we utilized the nitrocellulose filter-binding assay, in which the protein-associated RNA is retained on the filters. Protein-free RNA does not bind to the filters under this assay condition (Richter & Smith, 1983). Phenol-extracted vaccinia virus core RNA also does not bind to nitrocellulose filters (data not shown). Purified cores were incubated in a transcription assay system as described in Methods. At a given time, fractions were removed, the cores were pelleted by centrifugation, and the amount of RNA present in the supernatant fraction was measured by acid precipitation or by nitrocellulose filter-binding assay. No core contamination was found in the supernatant fraction after centrifugation (data not shown). During the 15 min incubation most of the synthesized RNA extruded was associated with proteins since no difference in total counts was observed using two different procedures (Fig. 1). After this period, most of the newly synthesized RNA was extruded free of proteins. Only half of the [3H]RNA counts were retained on the nitrocellulose filters when compared to the total amount of acid-precipitated [3H]RNA.

Incubation of vaccinia virus cores with ATP promotes the release of proteins into the supernatant fraction without RNA synthesis (Dámaso & Moussatché, 1992). These proteins seem to be similar to those released during in vitro transcription, prompting us to verify whether cores recovered from ATP pre-incubation were
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Fig. 1. Retention on nitrocellulose filters of the RNP complex released from vaccinia virus cores. Vaccinia virus cores were incubated in the standard reaction mixture for transcription as described in Methods. At the indicated times samples were removed, centrifuged and the supernatants were precipitated with 5% TCA (O) or used in the nitrocellulose filter-binding assay (●) as described in Methods.

able to synthesize and extrude mRNA. In this experiment, cores were pre-incubated in the presence of ATP for 20 min, recovered by centrifugation and resuspended in the reaction mixture for transcription, as described in Methods. As shown in Fig. 2, pre-incubated cores were in fact capable of synthesizing and extruding RNA from the particle when transcription was measured by acid precipitation. Most of the transcripts were extruded free of protein since a small amount of RNA was able to bind to the nitrocellulose filter. We then determined whether this RNA could reassociate with the proteins previously released during pre-incubation, by adding the protein supernatant fraction (10 μg/ml) to the RNA synthesized from pre-incubated cores. The RNA–protein complex was reconstituted, since the retaining capacity of the nitrocellulose filters was restored (Fig. 2).

RNA–protein complex analysis

To study the formation of the RNA–protein complex, we tested the effect of different treatments in reconstituting the RNP complex. For this assay we utilized 2.8 μg of [3H]RNA/ml corresponding to the total amount synthesized during a 20 min incubation. RNA was combined with the total proteins released during pre-incubation (10 μg/ml). Table 1 shows that 20% of the RNA

Table 1. Effect of different treatments on the reconstitution of the RNA–protein complex

<table>
<thead>
<tr>
<th>Complex fraction</th>
<th>Treatment*</th>
<th>Radioactivity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>50 mM-EDTA</td>
<td>45</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>500 mM-NaCl</td>
<td>42</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>30 mg/ml Heparin</td>
<td>46</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>120 mg/ml Heparin</td>
<td>38</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>500 mg/ml Heparin</td>
<td>22</td>
</tr>
<tr>
<td>RNA</td>
<td>10 mg/ml BSA</td>
<td>28</td>
</tr>
<tr>
<td>RNA</td>
<td>500 mg/ml BSA</td>
<td>29</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>30 mg/ml tRNA</td>
<td>40</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>30 mg/ml Globin mRNA</td>
<td>50</td>
</tr>
<tr>
<td>RNA + supernatant</td>
<td>2.8 mg/ml Core RNA</td>
<td>50</td>
</tr>
</tbody>
</table>

* Vaccinia virus cores were pre-incubated in the presence of ATP for 20 min. The supernatant fraction was removed by centrifugation and the core fraction was resuspended in the transcription reaction mixture in the presence of [3H]UTP for 20 min. After this time RNA extruded from the cores was removed by centrifugation and assayed for binding to nitrocellulose filters in the absence or in the presence of the core supernatant fraction obtained during the pre-incubation period as described in Methods. The treatment was performed by mixing the agent with the supernatant fraction before [3H]RNA addition.
synthesized from pre-incubated cores was still retained on the nitrocellulose filters. The addition of 50 mM-EDTA or 500 mM-NaCl during reconstitution of the complex reduced the binding capacity to 45 and 42% respectively.

Heparin is a polyanion that has been used to block the formation of nucleic acid–protein complex (Dynan & Burgess, 1979). Our results show that increasing concentrations of heparin gradually prevented reconstitution of the RNA–protein complex. When a very high concentration of heparin (500 µg/ml) was used, reconstitution of the complex was prevented. The core protein fraction could not be replaced by high concentrations of BSA. In competition experiments, addition of the same amount of unlabelled vaccinia virus RNA (2.8 µg/ml) reduced the binding capacity to 50%. However, tRNA and globin mRNA must be present in at least a 10-fold excess over 32P-labelled vaccinia virus RNA to lower the amount of radioactivity retained on the filter by 40 and 50% respectively.

**Micrococcal nuclease digestion of the RNA–protein complex**

To examine the RNA–protein complex formed in vitro we used the u.v. crosslinking approach described by Dreyfuss (1986). The supernatant fraction from the vaccinia virus core transcription system was isolated as described in Methods. The RNA–protein complex and purified vaccinia virus core RNA were digested with micrococcal nuclease and analysed by PAGE as described. Fig. 3(a) shows that purified vaccinia virus RNA was completely digested by the micrococcal nuclease after a 5 min treatment. However, the RNA–protein complex was partially resistant to 20 min of micrococcal nuclease treatment. Alternatively, the protein was u.v. crosslinked to the newly synthesized core 32P-RNA and was also analysed by SDS–PAGE after nuclease digestion as described in Methods. As shown in Fig. 3(b) viral peptides crosslinked to 32P-RNA were radioactively labelled after micrococcal nuclease treatment. Fig. 3(c) shows the laser densitometer scanning of the autoradiogram shown in Fig. 3(b, lane 5). Two major complexes of 32P-RNA–protein with M₈s of 14.5K and 18K and a minor peak of 28K were observed after 20 mins of nuclease digestion.

**Detection of vaccinia virus core RNA-binding protein**

As previously described, 17 polypeptides were released when the cores were pre-incubated in the presence or absence of factors enabling viral transcription. To analyse which of these proteins could associate with the viral RNAs, the proteins were separated by SDS–PAGE and electobotted onto nitrocellulose filters as described in Methods. The resulting blots were assayed for the binding of 32P-RNA free of proteins prepared from pre-incubated cores. Fig. 4 shows the identification of five
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Fig. 4. Detection of vaccinia virus core nucleic acid-binding proteins by the RNA overlay protein blot assay. Vaccinia virus core proteins released after pre-incubation with ATP were separated by 12.5% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were incubated with [32P]RNA synthesized by pre-incubated cores in vitro as described in Methods. (a) Lane 1, [35S]methionine-labelled vaccinia virus; lane 2, vaccinia virus cores; lane 3, supernatant fraction. (b) Autoradiogram of the membrane after exposure for 40 h with an intensifying screen as described in Methods. (c) Autoradiogram of the membrane after exposure for 24 h as described in Methods. The arrows indicate the 18K and 14.5K bands.

Table 2. Hybridization of the vaccinia virus RNP complex

<table>
<thead>
<tr>
<th>Mr</th>
<th>Arbitrary units*</th>
<th>Percentage of total</th>
</tr>
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<tbody>
<tr>
<td>60K</td>
<td>3.1</td>
<td>0.23</td>
</tr>
<tr>
<td>43K</td>
<td>1.0</td>
<td>0.07</td>
</tr>
<tr>
<td>28K</td>
<td>9.5</td>
<td>0.71</td>
</tr>
<tr>
<td>18K</td>
<td>567.8</td>
<td>42.18</td>
</tr>
<tr>
<td>14.5K</td>
<td>764.6</td>
<td>56.81</td>
</tr>
</tbody>
</table>

* The autoradiogram shown in Fig. 4 was subjected to densitometry in an UltraScan laser densitometer (LKB). The values presented as units were obtained by cutting and weighing the absorbance peaks recorded during the scanning of each band of the autoradiograph.

proteins with apparent Mr of 60K, 43K, 28K, 18K and 14.5K with viral RNA binding capability (Fig. 4a, lane 2). In a different gel exposure the two bands of 18K and 14.5K were more distinguishable. The autoradiogram was scanned in a laser densitometer and the peak areas corresponding to the RNA–protein complexes were determined in arbitrary units. Table 2 shows that 99% of the radioactivity was linked to proteins with Mr of 18K and 14.5K whereas the other three protein bands represented 1% of the total radioactivity.

Discussion

Incubation of vaccinia virus core with the four ribonucleoside triphosphates provides a useful in vitro system in which to study transcription. In this assay, since the particle is not disrupted the endogenous DNA is used and the synthesized mRNA probably resembles the transcripts made in vivo early in infection (Cooper & Moss, 1978; Kates & Beeson, 1970; Pelham et al., 1978). In this situation, core mRNA is made and extruded from the particles. Proteins from the cores can also be released when incubated in the presence of ATP (Ben-Hamida et al., 1983). Recently, we have shown that during in vitro mRNA synthesis, core proteins are also released from the particle (Damaso & Moussatché, 1992). The role of these proteins is still unknown and can probably be related to mRNA extrusion. In the present paper, we demonstrate that vaccinia virus core mRNAs are extruded in association with core proteins (Fig. 1). In addition, core RNA extruded free of proteins can reform the ribonucleoprotein complex with the core-released proteins (Fig. 2, Table 1). The RNA–protein complex is resistant to micrococcal nuclease treatment (Fig. 3) and five proteins have RNA-binding ability (Fig. 4). However, two polypeptides with apparent Mr of 18K and 14.5K have higher affinities for the viral RNA than the 60K, 43K and 28K peptides.

The data supporting the presence of non-ribosomal RNP complexes in eukaryotic cells has been reviewed by Dreyfuss (1986). In growing HeLa cells eight RNPs, ranging from 34K to 120K were identified as being associated with heterogeneous nuclear RNA (hnRNA) and also had affinity for ssDNA (Dreyfuss, 1986). Cytoplasmic non-ribosomal RNP complexes (mRNP) have also been isolated and characterized. These complexes are resistant to high concentrations of NaCl (0.5 m) and are not associated with cellular polyribosomes (Dreyfuss, 1986). Several proteins of similar Mr have been found associated with mRNA in different cell lines (Greenberg, 1977; Kumar & Pederson, 1975; Morel et al., 1971). RNP complexes were also described as being formed with viral transcripts. In cells infected with adenovirus 2 it was found that hnRNA complexes formed with both viral and cellular proteins that were probably involved in the processing and transport of the viral mRNA to the cytoplasm (Van Eekelen et al., 1981). An RNP complex
was found in vesicular stomatitis virus (VSV)-infected cells formed by the N protein, viral mRNAs and host mRNPs (Adam et al., 1986). The function of the VSV N protein–mRNA interaction is not known but it was demonstrated that this complex inhibited protein synthesis in rabbit reticulocyte lysates at the level of initiation (Rosen et al., 1984). The presence of mRNP not associated with the polyribosomes early in vaccinia virus-infected cells was demonstrated by Metz et al. (1975). These authors suggested that this structure could be a precursor of the virus mRNA found in polyribo- somes. Recently, the complete sequence of the genome of vaccinia virus was determined and an RNP with an RNA-binding signature (A31R) and an M₄ of 14·2K was predicted but not identified (Goebel et al., 1990). It is possible that the 14·5K protein identified in this communication is the same as the one identified as being encoded by the DNA of vaccinia virus.

The presence of nucleic acid-binding proteins associated with vaccinia virus has been already demonstrated. Most of these proteins were reported to bind to both ss- and dsDNA molecules (Ichihashi et al., 1984; Kao et al., 1981; Soloski et al., 1978; Yang & Bauer, 1988). Several other basic proteins associated with vaccinia virus core particles have been identified but they were not referred to as nucleic acid-binding proteins (Oie & Ichihashi, 1981). Proteins with Mₛ values ranging from 18·5K to 11K were first identified and grouped as VP9, VP10 and VP11 and correspond to 30% of total vaccinia virus proteins (Sarov & Joklik, 1972). Some of these proteins were phosphorylated and could be associated with cellular ribosomes (Sagot & Beaud, 1979). Ichihashi et al. (1984) identified four DNA-binding proteins with Mₛ values of 57K, 27K, 13·8K and 13K. All four proteins were associated with the core/lateral body fraction and were not associated with the DNA structures; this is an area which needs further investigation as the role of lateral bodies in the biology of vaccinia virus is still unknown. It is possible that some of these basic proteins could associate with viral RNA and play a role in the transport of RNA to the ribosomes or in the translation of transcripts. The latter role has already been proposed to occur in a viral system. It has been suggested that during human immunodeficiency virus infection a viral protein (tat gene) linked to the 5' end of the viral mRNAs acts as a positive translational control (Rosen et al., 1986). The role of vaccinia virus RNA-binding proteins in viral transcription and translation is currently under investigation in our laboratory.

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References


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