High Molecular Weight Virion-associated RNA of Vaccinia

A POSSIBLE PRECURSOR TO 8 TO 12 S mRNA

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The high molecular weight virion-associated RNA synthesized by vaccinia in vitro can be cleaved into smaller components, some of which are extruded from the virus as 8 to 12 S RNA. The high molecular weight virion-associated RNA fails to bind appreciably (5%) to poly(U) filters indicating that it is not polyadenylated. Its cleavage products will, however, bind to poly(U) (40 to 50%) after processing in the presence of ATP. The high molecular weight virion-associated RNA is methylated by the virus, and purified unmethylated RNA can be methylated by detergent-solubilized extracts of vaccinia virus cores. In the presence of GTP, methylation is stimulated 3-fold. The level of methylation of purified unmethylated high molecular weight RNA achieved by soluble core extracts is approximately 80% of the level of methylation achieved with purified unmethylated 8 to 12 S viral RNA, suggesting that more residues than the primary 5' termini became methylated. Approximately 85% of the unmethylated RNA binds to poly(U) when purified high molecular weight RNA is processed with soluble core extracts in the presence of S-adenosyl[methyl-3H]methionine, GTP, and ATP, conditions which also cleave the RNA. Nucleic acid hybridization-competition studies indicate that virion-extruded 8 to 12 S mRNA contains sequences found in the high molecular weight virion-associated RNA.

Evidence for the origin of mRNA from large precursor RNA molecules has been reported for eukaryotic as well as viral systems (for a recent review, see Refs. 1 and 2). In the accompanying communication (3), data were presented on the synthesis and characterization of a high molecular weight virion-associated RNA synthesized by vaccinia in vitro. Pulse-chase analysis indicated that some of this RNA was cleaved into smaller 8 to 12 S RNA by an endogenous viral system. This suggested to us that the high molecular weight virion-associated RNA might function as a precursor to the virion-extruded 8 to 12 S mRNA. If this hypothesis were correct, the precursor or the cleavage products would have to fulfill some of the characteristics currently ascribed to functional mRNA. The RNA cleavage products would have to be released from the virus, and should become polyadenylated at the 3' terminus and blocked and methylated at the 5' terminus. Further, nucleic acid hybridization studies should reveal an effective competition between the extruded 8 to 12 S viral mRNA and the high molecular weight virion-associated RNA.

This communication presents some experiments directed toward preliminary answers to the above questions.

EXPERIMENTAL PROCEDURES

Cells and Virus—Vaccinia virus was grown and purified as described in the accompanying communication (3).

Isolation of Virus from RNA Polymerase Reaction—Virus was sedimented from an RNA polymerase reaction mixture at 38,000 x g for 30 min and resuspended in 50 mM Tris/HCl (pH 8.4) and 5 mM EDTA by sonication. It was then resedimented, washed in 50 mM Tris/HCl (pH 8.4) and 1 mM EDTA, and finally resuspended in Tris buffer.

Purification of RNA—RNA was synthesized in vitro as described (3). Extruded 8 to 12 S viral RNA was purified from the virus-free supernatant of an RNA polymerase reaction mixture after 15 min of synthesis. High molecular weight virion-associated RNA was derived from virus repurified from an RNA polymerase reaction mixture after 40 min of synthesis. The viral pellet was resuspended with sonication and solubilized with sodium deoxycholate. The RNA preparations were extracted three times using the hot phenol-SDS method as described by Girard (4). RNA was precipitated from the aqueous layer with ethanol followed by two cycles of precipitation from 2 M LiCl (5). The RNA was subsequently purified through one SDS-sucrose gradient and two Me2SO-sucrose gradients (3). All RNA preparations used for the hybridization studies were digested with DNase, re-extracted with phenol, and desalted on Sephadex G-50. Concentrations were determined using 1 A260 unit as equivalent to 38.4 μg of RNA/ml. A260/A230 ratios were approximately 0.5 for all RNA preparations used.

Nucleic Acid Hybridization—The basic filter technique (6, 7) using DNA immobilized on filters was employed for the hybridization studies. DNA-containing filters or blank filters were exposed for 20 to 24 h at 57° to varying RNA concentrations in 0.25 ml of 1.0 M NaCl, 4 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.5), 0.1% SDS, and 50% formamide. The unlabeled RNA solutions were removed and filters challenged with saturating levels of labeled RNA for another 20 to 24 h. Finally the filters were placed in 4 ml of 2 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 10 μg/ml of heat-inactivated RNAse (8), digested for 1 h at room temperature, and washed extensively with 2 x SSC.

Poly(U) Filter Binding—Poly(U) was bound to GF/C filters as described by Sheldon et al. (9). RNA samples were tested for their ability to be selected on poly(U) filters using the modified conditions of Sheldon et al. (9) as described by Wei and Moss (10).

Methylation by Soluble Detergent-disrupted Core Extracts—Soluble preparations of detergent-disrupted vaccinia cores were prepared as described (11). The methylation assay consisted of purified RNA (4.8 to 24 μg/ml), 50 mM Tris/HCl (pH 8.4), 10 mM Mg2+, 10 mM dithiothreitol, 0.05% Nonidet-P40, 4 mM ATP, 2 mM GTP, 0.6 μM [3H]AdoMet (specific activity 8.8 Ci/mmol), and soluble enzyme preparation at an equivalent virus concentration of 10 A260 units/ml.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; Me2SO, dimethyl sulfoxide; [3H]AdoMet, S-adenosyl[methyl-3H]methionine.
RESULTS

Cleavage and Extrusion of High Molecular Weight Virion-associated RNA - Pulse-chase analysis indicated that the high molecular weight virion-associated RNA was cleaved to smaller components in the presence of ribonucleoside triphosphates (3). It was of interest to determine whether the 8 to 12 S cleavage products would be extruded from the virus or remain virion-associated. Steady state labeled high molecular weight virion-associated RNA was obtained by repurifying the virus from an RNA polymerase reaction. The purified virus was reincubated in an RNA polymerase reaction mixture either in the presence or the absence of the four ribonucleoside triphosphates and the fate of the labeled RNA was followed. As is demonstrated in Fig. 1, Panel A, the bulk of total RNA remains large after reincubation in the absence of ribonucleoside triphosphates, whereas in the presence of ribonucleoside triphosphates some of the RNA is cleaved and sediments at about 8 to 12 S. Aliquots from the above reaction mixtures were taken, the virus removed by centrifugation, and a portion of the virus-free supernatants were analyzed on SDS-sucrose gradients. As is demonstrated in Fig. 1, Panel B, when the virus was reincubated in the absence of ribonucleoside triphosphates, essentially all of the RNA remained high molecular weight and virion-associated. On the other hand, when the virus was reincubated in the presence of all four ribonucleoside triphosphates, the RNA was cleaved and approximately 50% of the RNA was extruded from the virus. The bulk of this extruded RNA sedimented at about 8 S. As indicated previously (3), the cleavage of the virion-associated high molecular weight RNA requires ribonucleoside triphosphates. The requirement for ribonucleoside triphosphates for extrusion of the RNA confirms the findings of Kates and co-workers (12, 13). In a number of similar experiments we have found the amount of RNA cleaved and extruded to vary from 25 to 85%. The reason for this variation is not clear but may reflect damage to the virus in repurifying it from an RNA polymerase reaction.

Polyadenylation of Cleavage Products from High Molecular Weight Virion-associated RNA - With few exceptions, most eukaryotic and viral mRNAs contain a polyadenylate chain covalently linked at the 3' terminus (14, 15). The poly(A) is believed to be added post-transcriptionally (16). In order to determine whether the high molecular weight virion-associated RNA or its cleavage products were polyadenylated, we took advantage of the ability of poly(A)-containing RNA to bind to poly(U) filters. As is shown in Table I, approximately 5% of the virion-associated RNA bound to poly(U) filters. When this RNA is processed by the virus in the presence of GTP, CTP, UTP, and ATP, the RNA is cleaved and a considerable portion (44%) of this RNA became polyadenylated, as judged by its ability to bind to poly(U)-containing filters. The requirement for ATP suggested that the poly(A) moiety was newly synthesized. In a similar experiment with extruded RNA, the presence of ATP affected poly(U) binding but not cleavage and extrusion (Table I).

Methylation of Vaccinia High Molecular Weight Virion-associated RNA - The 5' terminus of vaccinia mRNA (17) as well as other viral and eukaryotic mRNAs (18-25) has recently been shown to be blocked and methylated. To determine whether the high molecular weight virion-associated RNA is methylated, vaccinia RNA was continuously labeled with [a-32P]UTP and [3H]AdoMet, then purified and analyzed on 5 to 20% sucrose gradients in 99% dimethyl sulfoxide. As is demonstrated in Fig. 2, the ratio of 3H to 32P increases with decreasing sedimentation rate, and precipitable 3H counts were found.
Possible Precursor to Vaccinia 8 to 12 S mRNA

**Experiment I**—Total steady state labeled RNA was derived from a 40-min synthesis period. Virion-associated high molecular weight RNA was pulse-labeled for 10 min after 30 min of synthesis with cold ribonucleoside triphosphates. The virus containing the pulse-labeled high molecular weight RNA was repurified from the polymerase reaction as described under “Experimental Procedures,” and aliquots were reincubated at 37° either in the absence or presence of ribonucleoside triphosphates as indicated.

**Experiment II**—In a separate experiment, virion-associated high molecular weight RNA was pulse-labeled for 10 min after 30 min of unlabeled synthesis. The virus containing this pulse-labeled RNA was repurified as described under "Experimental Procedures" and aliquots reincubated at 37° either in the absence or presence of ribonucleoside triphosphates as indicated. The virus was removed by centrifugation and the extruded RNA recovered and analyzed.

wherever precipitable 32P counts were found suggesting that all of the RNAs are methylated.

RNA pulse with [3H]AdoMet and then reincubated by repurifying the virus from the reaction mixture was cleaved, and the 8 to 12 S cleavage products were found to be methylated when reincubated in the presence of [3H]AdoMet and the four ribonucleoside triphosphates. It is not clear from such studies, whether it is the high molecular weight virion-associated RNA cleavage products, or 8 to 12 S RNA that is still virion-associated that is actually methylated. In an attempt to resolve this question, the following experiment was done. Pulse-labeled high molecular weight virion-associated RNA was purified and tested for its ability to be methylated by detergent-solubilized extracts of virion cores, which are known to contain the pertinent enzymes (26). As is shown in Table II, this purified unmethylated RNA can be methylated by solubilized core extracts of vaccinia. Incorporation of methyl groups is stimulated 3-fold when GTP is present. Addition of ribonucleoside triphosphates also results in the cleavage of the high molecular weight RNA by solubilized core extracts.

Wei and Moss (17) have shown that the viral RNA is blocked with [3H]UTP and then reincubated by repurifying the virus from the reaction mixture was cleaved, and the 8 to 12 S S RNA was pulse-labeled for 10 min after 30 min of unlabeled synthesis. The virus containing this pulse-labeled RNA was repurified as described under "Experimental Procedures" and aliquots reincubated at 37° either in the absence or presence of ribonucleoside triphosphates as indicated. The virus was removed by centrifugation and the extruded RNA recovered and analyzed.

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>RNA fraction</th>
<th>Acid-precipitable</th>
<th>Bound to poly(U)</th>
<th>Per cent RNA bound to poly(U)</th>
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<tbody>
<tr>
<td>Total steady state labeled RNA</td>
<td>20,751</td>
<td>17,537</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Virion-associated high molecular weight RNA</td>
<td>2,681</td>
<td>124</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Processed in absence of triphosphates</td>
<td>2,728</td>
<td>277</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Processed with GTP, CTP, UTP</td>
<td>9,665</td>
<td>1,177</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Processed with GTP, CTP, UTP, ATP</td>
<td>2,681</td>
<td>124</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Acid-precipitable radioactivity</th>
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</thead>
<tbody>
<tr>
<td>[3H]AdoMet</td>
<td>480</td>
</tr>
<tr>
<td>[3H]AdoMet plus CTP, UTP, ATP</td>
<td>671</td>
</tr>
<tr>
<td>[3H]AdoMet plus CTP, UTP, ATP, GTP</td>
<td>1,796</td>
</tr>
</tbody>
</table>

- High molecular weight virion-associated RNA labeled with [3H]UTP was purified and methylated using soluble vaccinia core extracts. Ribonucleoside triphosphates were present at 2 mM and S-adenosyl[methyl-3H]methionine at 6 μM.
- S-Adenosyl[methyl-3H]methionine.
Each purified RNA was included in the reaction at 4.8 µg/ml. The soluble fraction of detergent-disrupted vaccinia cores was used as enzyme source for the 30 min incubation at 37°C. S-Adenosyl[3H]methionine (specific activity 8.8 Ci/mmol) was present at 6 µM, ATP at 5 mM, and GTP at 2 mM.

Soluble fraction of detergent-disrupted vaccinia cores was used as enzyme source for the 30-min incubation at 37°C. S-Adenosyl[3H]methionine (specific activity 8.8 Ci/mmol) was present at 6 µM, ATP at 5 mM, and GTP at 2 mM.

Extruded (8 to 12 S) RNA can be both methylated and polyadenylated. Further data will be presented in a future communication.

Nucleic Acid Hybridization-Competition — Much of the data presented in this and the accompanying communication are compatible with the hypothesis that the large molecular weight virion-associated RNA is a precursor of at least some of the extruded 8 to 12 S mRNA species. In order to test whether the two RNA classes contained identical sequences, nucleic acid hybridization-competition studies were performed. Extruded 8 to 12 S RNA was labeled for 10 min after 5 min of synthesis with cold substrates. The virus was removed by centrifugation and the RNA purified as described. High molecular weight virion-associated RNA was pulsed for 10 min after 30 min of cold synthesis; the virus was removed from the polymerase reaction mixture by centrifugation and the RNA was extracted. The RNAs were then individually purified through one SDS- and two 99% Me2SO-sucrose gradients. The sedimentation profiles of the purified RNA species used for the hybridization studies are shown in Fig. 3. Unlabeled total RNA, unlabeled 8 to 12 S RNA, and unlabeled high molecular weight RNA compete effectively with labeled 8 to 12 S RNA (Fig. 4A). Unlabeled purified high molecular weight virion-associated RNA competed effectively with labeled 8 to 12 S extruded RNA, and unlabeled 8 to 12 S extruded RNA competed effectively with labeled virion-associated high molecular weight RNA (Fig. 4B). The presence of identical RNA sequences in both size classes of RNA is clearly established by these hybridization-competition studies. This finding suggests that at least some of the 8 to 12 S extruded RNA sequences can be found in the high molecular weight virion-associated RNA. Although an effective competition is observed, it is not clear whether there are also unique species of RNA represented in the various size classes of RNAs tested, since the competition of hybridization in the homologous systems is 70 to 90% in our hands. The reason for this somewhat low level of competition by the homologous systems is not clear. Further work determining the complexity of these RNA classes is currently in progress.

**DISCUSSION**

This communication extends the studies on the high molecular weight virion-associated RNA synthesized by vaccinia *in vitro* presented in the accompanying paper (3). This RNA was shown to be cleaved by an endogenous viral system and the cleavage products were released from the virus as 8 to 12 S RNA. Both cleavage and extrusion required nucleoside triphosphates.

The high molecular weight virion-associated RNA was found to be methylated, and purified unmethylated RNA could be methylated by detergent-solubilized extracts of vaccinia cores. Although the nature of the methylated sites have not yet been determined, they presumably occur at 5' termini. The levels of methylation observed using soluble core extracts and purified unmethylated high molecular weight virion-associated RNA or purified unmethylated released 8 to 12 S RNA are consistent with methylation of post-transcriptionally formed 5' termini derived from cleavage of the high molecular weight RNA. If indeed post-transcriptionally formed 5' termini are blocked and methylated, then one must propose a mechanism for modifying the phosphate content of the newly formed 5'
FIG. 4 Nucleic acid hybridization-competition between purified viral RNA-size classes. Purified unlabeled vaccinia RNA was annealed to filters containing approximately 0.3 μg of vaccinia DNA for 90 to 94 h, as described under "Experimental Procedures," and subjected to a second round of hybridization with saturating quantities of purified labeled RNA. A, saturation curve of purified 8 to 12 S RNA, ○—○. Hybridization of labeled 8 to 12 S RNA competed with unlabeled 8 to 12 S RNA, O—O; unlabeled total RNA, ▲—▲; and unlabeled purified high molecular weight virion-associated RNA, △—△. Filters were challenged with 10 μg of labeled 8 to 12 S RNA at a specific activity of 3.2 × 10⁴ cpm/μg. B, competition between unlabeled purified high molecular weight virion-associated RNA and labeled purified 8 to 12 S RNA, □—□; competition between unlabeled 8 to 12 S RNA, and labeled purified high molecular weight RNA, ●—●.

term, since a diphosphate-terminated polynucleotide has been shown to be the substrate required for the mRNA guanylyltransferase and mRNA (guanine-7-methyltransferase purified from vaccinia (28). Similarly, studies using specifically labeled compounds showed that only the ß-phosphate from the blocking GTP is incorporated into the triphosphate bridge of m⁷G(5')pppAmp (26). One could postulate the modification of the phosphate content of post-transcriptionally derived 5' termini in several ways, but it is not readily clear how this modification would be compatible with the data of Moss and co-workers (26, 28). Hypermethylated fragments or internal methylations, however, must also be considered.

The results on polyadenylation suggest that it is a post-transcriptional event. Polyadenylation probably proceeds by the terminal addition of adenosine residues to the 3' terminus of the RNA. A poly(A) polymerase has been purified from vaccinia cores (27) and has been shown to be stimulated by primer polynucleotides (29). Although this is probably the method of polyadenylation of the cleavage products from the high molecular weight RNA by both intact virus and soluble extracts of vaccinia cores, other mechanisms of poly(A) addition, such as ligation of a preformed poly(A) to the 3' terminus of an RNA fragment, cannot yet be excluded.

Although hybridization-competition studies indicate competitive competition between the 8 to 12 S extruded RNA and the virion-associated high molecular weight RNA, the extent of homology is uncertain, since in the homologous systems only 70 to 90% competition was observed. It is not clear whether there are two modes of transcription in this system, one mode resulting in the synthesis of monocistronic messages, the other in polycistronic messages followed by cleavage. It is interesting to note that Oda and Joklik (30) have reported that in vivo, early mRNA is 8 to 12 S but that later in infection these early sequences are found in much larger RNA. The significance of this transition in vivo is not clear. It will be of interest to look for high molecular weight RNA in vivo at early times of infection.

Recently Jaureguiberry et al. (31) reported the presence of high molecular weight RNA synthesized by vaccinia in vitro, and their studies on in vitro translation showed that similar polypeptides were obtained from the various size classes of RNA. This is not surprising, since the high molecular weight RNA contains sequences present in the 8 to 12 S RNA. It is not apparent why these investigators found high molecular weight RNA in the extruded RNA fraction, since in our studies the high molecular weight RNA remains virion-associated. It should be noted that their incubation periods were relatively long and some of the viral cores may have become fragile with time, releasing large uncleaved RNA.

If processing of RNA is a requisite in the biogenesis of mRNA, it is not surprising that vaccinia should contain the appropriate enzymatic machinery. Vaccinia is generally considered to be a cytoplasmic virus and the nature of this site of replication in the cell would impose certain requirements upon the virus. As an infectious unit in the cytoplasm, the uncoated viral core can be considered as an alien nucleus and would be
expected to contain the enzymatic machinery required for the biogenesis of RNA.

Although the biological significance of the high molecular weight virion-associated RNA synthesized by the virus in vitro has not been firmly established, much of the data presented are compatible with the working hypothesis that it is a precursor RNA and that it is cleaved to 8 to 12 S mRNA fragments which are modified at both the 5' and 3' termini and extruded from the virion. Many critical experiments remain to be done, but if their results confirm this hypothesis, vaccinia is a unique model for studying the biogenesis of mRNA in vitro.

REFERENCES
2. Darnell, J. E., Jr. (1973) Harvey Lect. 69, 1-47
High molecular weight virion-associated RNA of vaccinia. A possible precursor to 8 to 12 S mRNA.

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