Abortive Transcription Products of Vaccinia Virus Are Guanylylated, Methylated, and Polyadenylylated

ALAN GERSHOWITZ AND BERNARD MOSS*

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Received for publication 22 February 1979

Abortive transcription products were synthesized in vitro by UV-irradiated vaccinia virus particles that were incubated with all four ribonucleoside triphosphates or by unirradiated particles that were incubated in reaction mixtures deficient in CTP or UTP. The RNA sedimented at 4 to 6S in sucrose gradients, suggesting that premature termination had occurred, presumably in one case because the DNA contained UV-induced pyrimidine dimers and in the other case because a ribonucleoside triphosphate was present at limiting concentration for transcription. Nevertheless, the short transcripts were capped, methylated, and polyadenylylated, indicating that neither completion of an RNA chain nor processing from a polycistronic precursor was required for modification of either end of the RNA. In addition, the finding of mG(5')pppA" and mG(5')pppGm at the 5' ends of the short RNA molecules implied that transcription was initiated with both ATP and GTP. The presence of the polyadenylic acid tract suggested that a slow-down or cessation of transcription, rather than a specific 3'-terminal sequence, served as a signal for polyadenylation.

Vaccinia virus provides a unique in vitro system for studying transcription of a DNA genome (9, 15). Mature mRNA, about 1,000 nucleotides long, containing a methylated cap structure at the 5' end (25) and 100 or more adenylate residues at the 3' end (8), is formed by virus core-associated enzymes. The detection of high-molecular-weight RNA intermediates when suboptimal levels of ATP are used for transcription (18), as well as evidence that ATP with a hydrolyzable γ-phosphate is specifically required for RNA synthesis (5), for termination of transcription or processing of high-molecular-weight RNA precursors (5, 18) or both, and for extrusion of RNA from the virus core (24), indicates the complexity of the transcription system.

We considered that information regarding the signals involved in initiation of RNA synthesis, capping, and polyadenylylation could be obtained by analyzing the formation of prematurely terminated transcripts. Such transcripts, if less than unit mRNA length, would presumably lack the normal 3'-terminal sequence and could not be formed by processing of polycistronic precursors. In bacterial and eucaryotic systems, premature termination has been achieved by UV irradiation (6, 7, 21). At the sites of UV-induced DNA lesions, believed to be pyrimidine dimers, both RNA polymerase and prematurely terminated transcripts are released.

That a similar situation might occur when vaccinia virus particles are UV irradiated was suggested by analyzing the proteins formed in a coupled transcription-translation system (3, 19). In the present study, the nature of the RNA formed after UV irradiation has been determined.

Vaccinia virus particles were irradiated with UV light for lengths of time varying from 10 s to 5 min. Even the shortest time, however, was sufficient to reduce infectivity by more than 99%. Incorporation of [3H]UTP by irradiated particles was drastically depressed (Fig. 1). Analysis by sucrose gradient centrifugation of RNA synthesized by UV-irradiated virus indicated that the inhibition was accompanied by a reduction in size of the transcripts consistent with premature termination. After irradiation for 0.5 min, the sedimentation rate of the RNA that formed dropped sharply from 13S to 8S (Fig. 2). A shift to 7S occurred after irradiation for 1 min (Fig. 2) and to 4 to 6S after 5 min (not shown).

By contrast to the severe effect on total RNA synthesis, incorporation of S-adenosyl[methyl-3H]methionine ([3H]AdoMet) was only slightly affected by UV irradiation (Fig. 1). Nevertheless, the methyl-labeled RNA also sedimented at 4 to 6S (not shown). After digestion of [3H]AdoMet-labeled RNA with nuclease P1 and alkaline phosphatase, all of the radioactivity was recovered in
5'-terminal cap structures. 7-Methylguanosine (m7G), 2'-O-methylguanosine (Gm), and 2'-O-methyladenosine (A') derived from m7G(5')pppGm and m7G(5')pppA' were identified by paper chromatography after further digestion with snake venom phosphodiesterase and alkaline phosphatase. The data in Table 1 show that the ratio of Gm to A' was not significantly different in RNA formed by unirradiated or UV-irradiated virus particles. Similar results were obtained with particles irradiated for as long as 5 min, suggesting that initiation occurs with ATP and GTP and that abortive transcripts can be capped and methylated.

Sheldon and Kates (22) reported that polyadenylic acid [poly(A)] synthesis by vaccinia virus particles incubated with ATP alone was very resistant to UV irradiation. Similarly, we noted that incorporation of [3H]ATP in the presence of the other three ribonucleoside triphosphates was affected to a far lesser extent than was [3H]UTP incorporation (Fig. 1). To determine the relative amounts of poly(A) and RNA formed, the [3H]AMP-labeled material was digested with RNase A, and T1 in the presence of 0.3 M KCl. The results (Table 2) indicate that 28.2% of the labeled material made by non-irradiated virus was RNase-resistant poly(A). This corresponds to 255 adenylate residues (or a total of approximately 1,018 nucleotides) in RNA for every 100 adenylate residues in poly(A). By contrast, 92.1% of the labeled material made by virus that was irradiated for 2 min consisted of RNase-resistant poly(A). This corresponds to only 8 adenylate residues (or approximately 32 nucleotides) in RNA for every 100 adenylate residues in poly(A).

To determine whether poly(A) was actually attached to abortive transcription products, methyl-labeled RNA was chromatographed on polyuridylic acid-Sepharose (Table 3). We found that at least 78 to 87% of the methyl-labeled RNA formed by virus particles irradiated for 2
TABLE 2. RNase resistance of [3H]ATP-labeled RNA

<table>
<thead>
<tr>
<th>UV (min)</th>
<th>Total RNA (cpm)</th>
<th>RNase resistance (cpm)</th>
<th>% RNase resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21,873</td>
<td>6,176</td>
<td>28.2</td>
</tr>
<tr>
<td>2</td>
<td>9,121</td>
<td>7,219</td>
<td>92.1</td>
</tr>
</tbody>
</table>

* RNA was synthesized in standard reaction mixtures containing 1 mM ATP (50 Ci/mol), 1 mM GTP, 1 mM CTP, 1 mM UTP, and non-irradiated virus or virus irradiated for 2 min. After 30 min, the reaction mixture was treated with EDTA and sodium dodecyl sulfate and was extracted with phenol-chloroform (1:1). After ethanol precipitation, the RNA was dissolved in 1 ml of water, and duplicate 0.1-ml samples were added to separate tubes containing 0.1 M Tris-hydrochloride (pH 7), 0.3 M KCl, 50 µg of carrier poly(A), 10 µg of RNase A, and 1 µg of RNase T1, in a total volume of 1 ml. Duplicate samples were also added to tubes lacking RNase. After 30 min at 37°C, the material was precipitated with trichloroacetic acid and counted. The average values are shown.

TABLE 3. Polyuridylic acid-Sepharose chromatography of methyl-labeled RNA

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>UV (min)</th>
<th>Poly(A)− (cpm)</th>
<th>Poly(A)+ (cpm)</th>
<th>% Poly(A)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>295</td>
<td>1,165</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>130</td>
<td>894</td>
<td>87</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>13,575</td>
<td>93,510</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7,964</td>
<td>27,980</td>
<td>78</td>
</tr>
</tbody>
</table>

<sup>a</sup> A column of polyuridylic acid-Sepharose (0.7 by 1.5 cm) was equilibrated with NETS buffer containing 10 mM Tris-hydrochloride (pH 7.5), 0.12 M NaCl, 10 mM EDTA, and 0.1% sodium dodecyl sulfate. Methyl-labeled RNA, synthesized by non-irradiated or UV-irradiated virus and purified by phenol-chloroform-sodium dodecyl sulfate extraction and ethanol precipitation, was applied in NETS buffer. The unbound RNA is listed as poly(A)−. After washing with NETS buffer, poly(A)+ RNA was eluted with 90% formamide containing 10 mM Tris-hydrochloride (pH 7.5) and 10 mM EDTA. Trichloroacetic acid-precipitable counts per minute are shown.

<sup>b</sup> In experiment 2, which was an entirely separate experiment, poly(A)+ RNA was eluted with 75% formamide in buffer containing 10 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)] and 10 mM EDTA. Subsequently, a portion of the RNA was heated for 2 min at 60°C in 75% formamide, then diluted with 4 volumes of NETS containing 0.15 M NaCl, and reapplied to a polyuridylic acid-Sepharose column. A total of 86% of the poly(A)+ RNA synthesized by non-irradiated virus and 92% of the poly(A)+ RNA synthesized by irradiated virus rebound.

To determine the nature of the RNA formed to 6S size of the RNA formed after prolonged UV irradiation. Although these experiments indicate that the abortive transcripts are polyadenylated, they do not exclude the possibility that "free poly(A)" is also made by UV-irradiated particles. In this respect, omission of GTP, CTP, and UTP reduced the incorporation of [3H]ATP by less than 50%.

Attempts were made to produce premature chain termination by additional methods. Mixtures of cordycepin triphosphate and ATP in various ratios were ineffective because RNA synthesis was severely inhibited at cordycepin triphosphate concentrations that did not affect the sedimentation rate of the RNA formed. Initial results obtained by omitting one or more of the four ribonucleoside triphosphates, however, appeared encouraging. Without either CTP or UTP, methyl incorporation was only reduced 40% and 81%, respectively (Table 4). Deletion of ATP or GTP or of both CTP and UTP, however, reduced methyl incorporation by more than 99% (Table 4). The reason for complete inhibition in the absence of GTP or ATP is evident since without GTP the cap structure cannot form, and omission of ATP completely prevents RNA synthesis (5). The absence of methyl incorporation when CTP and UTP were omitted indicates that if a significant amount of free poly(A) was formed it was not capped. Further analysis indicated that the methyl label was incorporated into cap structures when either CTP or UTP was omitted and that the ratio of G<sup>M</sup> to A<sup>M</sup> was not significantly changed (Table 4). In addition, the methyl-labeled RNA that formed in the absence of either CTP or UTP sedimented at about 6S and 54 to 85% bound to oligodeoxythymidylic acid-cellulose in 0.5 M NaCl, indicating that most of it was polyadenylated (not shown). Short polyadenylated transcripts have also been obtained under similar conditions by Paoletti (personal communication).

To determine the nature of the RNA formed

TABLE 4. Effect of nucleoside triphosphate deletion on incorporation of [3H]AdoMet

<table>
<thead>
<tr>
<th>NTP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>cpm</th>
<th>% mG&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% G&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% A&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A,G,U,C</td>
<td>8,040</td>
<td>54.4</td>
<td>30.1</td>
<td>15.5</td>
</tr>
<tr>
<td>A,G</td>
<td>4,831</td>
<td>50.8</td>
<td>29.3</td>
<td>19.9</td>
</tr>
<tr>
<td>A,G,C</td>
<td>1,498</td>
<td>51.2</td>
<td>29.9</td>
<td>19.0</td>
</tr>
<tr>
<td>A,G</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G,C,U</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A,C,U</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Ribonucleoside triphosphates (NTP) purified by high-pressure liquid chromatography (ICN) were used at 2 mM where indicated. RNA labeled with [3H]-AdoMet was analyzed as in Table 1.
when CTP or UTP was omitted, [\(\alpha\)\textsuperscript{32}P]GTP was used for labeling. As expected, incorporation was severely reduced relative to a control containing all four ribonucleoside triphosphates (Table 5). Nearest-neighbor analysis was performed by digestion of the RNA with RNase T\(_2\), followed by high-voltage paper electrophoresis. To our surprise, virtually the same ratios were obtained whether all four ribonucleoside triphosphates were used for RNA synthesis or whether CTP or UTP was omitted (Table 5). Evidently, despite our use of ribonucleoside triphosphates obtained from ICN that were specially purified by high-pressure liquid chromatography to eliminate cross-contamination, the RNA polymerase managed to scavenge trace CTP and UTP from the virus particle itself if not from one or more of the added ribonucleoside triphosphates. Thus, in our experiments, transcription was affected by a deficiency rather than a complete absence of CTP or UTP.

The RNA labeled with [\(\alpha\)\textsuperscript{32}P]GTP was also used to determine the chain length, exclusive of the poly(A) segment. The RNA was digested with nuclease P\(_1\), and labeled m'G(5')pppG\(_m\), m'G(5')pppAm, and pG were resolved by electrophoresis on DEAE-paper in 7% formic acid. The number of 5' ends was determined by adding together the counts associated with m'G(5')pppAm and half of the counts associated with m'G(5')pppG\(_m\). The counts associated with pG were multiplied by 3.3, based on the nearest-neighbor ratios in Table 5, to provide a measure of the total RNA formed. From the ratios of total RNA to 5' ends, we determined that the average product made without added CTP was about 300 nucleotides long, whereas that made without added UTP was 170 nucleotides long. In contrast, RNA made with all four ribonucleoside triphosphates was calculated to be 1,360 nucleotides long.

The simplest interpretation of our results is that short transcripts are formed abortively by premature termination, in one case because of pyrimidine dimers produced by UV irradiation.

### Table 5. [\(\alpha\)\textsuperscript{32}P]GTP incorporation and nearest-neighbor analysis

<table>
<thead>
<tr>
<th>NTP(^a)</th>
<th>Incorporation (cpm (\times 10^6))</th>
<th>% (\textsuperscript{32}P) associated with</th>
</tr>
</thead>
</table>

\(^a\) Ribonucleoside triphosphates (NTPs) were present at 2 mM except for [\(\alpha\)\textsuperscript{32}P]GTP, which was present at 0.5 mM (160 Ci/mol). Nearest-neighbor analysis was carried out as previously described (5).
leading to a block in transcription similar to that which occurs because of UV-induced pyrimidine dimers. Although, at that time, RNA was not known to be capped and methylated, we suggested that the small RNA molecules might compete with cellular mRNA for ribosome binding and contribute to the profound inhibition of host protein synthesis that occurs upon high-multiplicity infection in the presence of actinomycin D (12, 20). This hypothesis was supported by the finding of the short RNA molecules associated with ribosomes (20). Similarly, the inhibition of host protein synthesis that occurs after infection with UV-irradiated vaccinia virus (12) could at least partly result from abortive transcription. Inhibition of host protein synthesis also occurs during normal infection, however, and a variety of factors may be involved in this poorly understood process.

In conclusion, we wish to point out that the abortive transcription products synthesized in vitro or in vivo may be useful for mapping promoter sites on the vaccinia virus genome.

LITERATURE CITED