Mechanism of Synthesis of Vaccinia Virus Double-Stranded Ribonucleic Acid In Vivo and In Vitro

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The synthesis of vaccinia virus double-stranded ribonucleic acid (RNA) in infected HeLa cells was sensitive to actinomycin D, suggesting that a deoxyribonucleic acid-dependent reaction is involved. Some double-stranded RNA was made in the presence of cytosine arabinoside in infected cells. Double-stranded and complementary RNA were synthesized in vitro by using vaccinia cores. These two observations indicate that some of the double-stranded RNA is read from “early” genes. The double-stranded RNA synthesized in vitro had the same properties as that made in vivo. At least 70% of the double-stranded RNA made in vivo was in ribonuclease-resistant form prior to sodium dodecyl sulfate-phenol extraction. In addition, there was a complementary RNA in infected cells which could be converted to double-stranded RNA by annealing.

Vaccinia is a large deoxyribonucleic acid (DNA)-containing virus which replicates in the cytoplasm of infected cells and which stimulates those cells to synthesize the antiviral protein, interferon. Field et al. (5) postulated that a DNA ribonucleic acid (RNA) hybrid might be the inducer of interferon in cells infected with a DNA virus; however, DNA-RNA-like synthetic polynucleotides were found not to induce interferon in chick embryo fibroblasts.

Recently, Colby and Duesberg (3) reported finding ribonuclease-resistant RNA in vaccinia virus-infected chick cells. The RNA was characterized as double-stranded RNA and it was shown to be virus specific; i.e., it was hybridized with vaccinia virus DNA. This RNA is an active inducer of interferon.

The presence of double-stranded RNA in cells infected with a DNA virus is not restricted to eucaryotic cells. Bøvre and Szybalski (1) found complementary RNA synthesized from the b2 region of coliphage λ, and Jurale et al. (9) found virus-specific double-stranded RNA in T4-infected E. coli. Thus, the mechanism of synthesis of the vaccinia virus double-stranded RNA may reflect a generally interesting biological phenomenon.

MATERIALS AND METHODS

HeLa cells were grown in monolayers in 32-oz (ca. 900 ml) prescription bottles in 40 ml of Eagle's medium containing 5% calf serum treated for 1 hr at 56 C. Vaccinia virus (WR strain) was grown in HeLa cells and purified as described by Joklik (7). Confluent monolayers were infected with 100 virus particles per cell for 1 hr in 1.0 ml of medium.

Radioactive RNA was prepared by decanting the medium and incubating the monolayers for 15 min with 100 mCi of 3H-uridine (New England Nuclear Corp.) in 5 ml of medium. The radioactive medium was removed, and the cells were washed with 25 ml of ice-cold 0.15 M NaCl solution. The monolayers were then treated with 1.5 ml of ice-cold 0.01 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), 0.001 M MgCl2 and allowed to stand on ice for 3 min. The cells were removed from the surface of the glass by scraping and disrupted by treatment with a rotating Dounce homogenizer. The nuclei were removed by centrifugation, and sodium dodecyl sulfate (SDS) and Pronase were added to the cytoplasmic extract to final concentrations of 0.5% (w/v) and 0.5 mg/ml, respectively. This extract was incubated for 30 min at 37 C. The cytoplasmic nucleic acids were purified by three extractions with phenol followed by ethanol precipitation.

Double-stranded RNA was prepared exactly as described previously (3). DNA and single-stranded RNA were digested with deoxyribonuclease and ribonuclease, respectively, and, after removing the nucleases by three phenol extractions, the remaining nucleic acids and oligonucleotides were applied to a 6% agarose column (3).

The RNA was characterized as an RNA duplex by the following criteria: (i) nuclease sensitivity, (ii) thermal denaturation, (iii) Cs2SO4 equilibrium density gradient centrifugation, (iv) induction of interferon in chick embryo cells, (v) self-annealing after removal of possible contaminating DNA strands (3, 4). It is important to note that only the material which is
phenol-SDS-purified, resistant to digestion with deoxyribonuclease and ribonuclease, and excluded from a 6% agarose column will be referred to below as vaccinia virus double-stranded RNA.

RNA was synthesized in vitro from vaccinia virus cores as described previously (10, 11). The RNA was synthesized in reaction mixtures which routinely contained the following components, in 0.4 ml: 2 × 10⁶ cores, 0.01 M 2-mercaptoethanol, 0.005 M MgCl₂, 0.05 M Tris-hydrochloride (pH 8.4), 1 μmole of adenosine triphosphate, 0.5 μmole each of cytosine triphosphate (CTP) and guanosine triphosphate (GTP), 0.02 μmole of uridine triphosphate (UTP), and 2 μCi of ³H-UTP. When ³H-CTP or ³H-GTP was used as the labeled substrate, UTP was at 0.5 μmole per 0.4 ml and the labeled nucleotide was at 0.02 μmole per 0.4 ml and 2 μCi per 0.4 ml. Incubation was for 25 min at 37 C. Purification of the RNA after synthesis was by phenol extraction, and ethanol precipitation was as previously described (2).

RESULTS

Effect of inhibition of RNA synthesis on the synthesis of double-stranded RNA. Since vaccinia virus replicates in the cytoplasm of infected cells, one can follow viral RNA synthesis with 15-min pulses of ³H-uridine. Vaccinia messenger RNA (mRNA) and a small amount of transfer RNA are the only RNA species in the cytoplasm that are labeled under these conditions (8).

The double-stranded vaccinia virus RNA could arise by two different mechanisms. One strand could be transcribed from vaccinia DNA, and an RNA-dependent RNA polymerase could catalyze the synthesis of the complementary strand of RNA. Actinomycin D would not be expected to inhibit the synthesis of the double-stranded RNA for this mechanism. Alternatively, both strands might be copied from complementary regions of the vaccinia DNA, in which case actinomycin D should inhibit the synthesis of the double-stranded RNA.

Actinomycin D (10 μg/ml) was added to the culture medium of vaccinia virus-infected HeLa cells 4 hr after infection. At 5 hr after infection, ³H-uridine-labeled cytoplasmic RNA (15-min pulse) was prepared as described above. Controls included ³H-RNA prepared from infected and uninfected HeLa cells not treated with actinomycin D.

The results presented in Table 1 indicate that there was a very small amount of ³H-ribonuclease resistant RNA in 60-min pulse-labeled uninfected HeLa cells, confirming the results previously reported by Montagnier (12). Infection with vaccinia caused a 35-fold increase in ³H-ribonuclease-resistant RNA. Treatment of vaccinia virus-infected HeLa cells with actinomycin D resulted in a 95% inhibition of RNA synthesis as measured by the incorporation of ³H-uridine. The synthesis of vaccinia virus-directed double-stranded RNA was also inhibited more than 90%.

In other experiments when vaccinia mRNA synthesis was inhibited 98% by actinomycin D, the synthesis of double-stranded RNA was inhibited 95%. These results are similar to those previously obtained with vaccinia-infected chick embryo cells (4) and suggest that the DNA-dependent mechanism is the one of choice.

Effect of inhibition of DNA synthesis on the synthesis of double-stranded RNA. At a concentration of 10 μg/ml, cytosine arabinoside inhibits host cell and vaccinia virus DNA synthesis by 99% (13). Only early vaccinia virus mRNA is transcribed in the presence of cytosine arabinoside (13). It was previously suggested (4) that the double-stranded RNA is a “late” product of viral intracellular biosynthesis. If this is true, one would expect cytosine arabinoside to abolish the synthesis of vaccinia double-stranded RNA.

Cytosine arabinoside (10 μg/ml) was added to the culture medium of HeLa cells immediately after infection with vaccinia virus. At 3 hr after infection, the cultures were pulse-labeled with ³H-uridine, and the total cytoplasmic RNA and double-stranded RNA were prepared. The amount of ³H-labeled vaccinia mRNA in the cytosine arabinoside-treated cells was reduced to one-third of that in the untreated infected cells (Table 1). However, of the RNA which was labeled, a significant proportion was double-stranded. Thus, it appears that some of the

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>³H counts/min in phenol extract</th>
<th>³H counts/min after nucleases and agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa, 60 min, labeling total nucleic acids</td>
<td>4.6 × 10⁵</td>
<td>195</td>
</tr>
<tr>
<td>HeLa, 15 min, labeling cytoplasmic extract</td>
<td>2.8 × 10⁴</td>
<td>35</td>
</tr>
<tr>
<td>HeLa + vaccinia</td>
<td>4.5 × 10⁵</td>
<td>5,300</td>
</tr>
<tr>
<td>HeLa + vaccinia + actinomycin (10 μg/ml), 4 to 5 hr postinfection</td>
<td>2 × 10⁴</td>
<td>520</td>
</tr>
<tr>
<td>HeLa + vaccinia + cytosine arabinoside (10 μg/ml), 0 to 5 postinfection</td>
<td>1.5 × 10⁵</td>
<td>820</td>
</tr>
</tbody>
</table>

a For the first treatment, uninfected HeLa cells were labeled for 1 hr with 25 μCi of ³H-uridine. For the remaining treatments, cultures were labeled for 15 min with 100 μCi of ³H-uridine and cytoplasmic RNA was prepared. All RNA preparations were purified, subjected to nuclease digestion, and eluted from a 6% agarose column as previously described (3).
vaccinia double-stranded RNA is made from early genes.

Synthesis of double-stranded and complementary RNA in vitro. Vaccinia virions contain an RNA polymerase activity which directs the synthesis of early viral mRNA (10, 11). Table 2 shows that the RNA made in vitro contains approximately equal amounts of the three nucleotides uridine monophosphate, cytidine monophosphate, and guanosine monophosphate. $^3$H-ATP was not used because of the formation of poly A (10). Some of the RNA made in vitro was resistant to a mixture of pancreatic and T1 ribonuclease at a high salt concentration (Table 2). However, when we subjected the RNA to annealing conditions, there was a sixfold increase in the level of ribonuclease-resistant RNA. Finally, thermal denaturation rendered both annealed and nonannealed RNA susceptible to ribonuclease degradation.

The level of ribonuclease-resistant $^3$H-RNA after annealing varied from 3 to 10% of the total synthesis in different experiments. The annealing reaction is dependent on the concentration of the input RNA. Figure 1A shows that the level of annealed ribonuclease-resistant $^3$H-RNA decreased with second-order kinetics as the $^3$H-RNA was diluted. Figure 1B shows that the annealing reaction was also time-dependent.

Table 2. Effect of annealing on the amount of ribonuclease-resistant RNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^3$H-UTP (counts/min)</th>
<th>$^3$H-CTP (counts/min)</th>
<th>$^3$H-GTP (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Trichloroacetic acid-pretreated RNA purified by phenol extraction and ethanol precipitation</td>
<td>151,589</td>
<td>113,984</td>
<td>109,644</td>
</tr>
<tr>
<td>B. Ribonuclease-treated RNA</td>
<td>2,432</td>
<td>1,931</td>
<td>1,539</td>
</tr>
<tr>
<td>C. Anneal ribonuclease-treated RNA</td>
<td>14,271</td>
<td>11,825</td>
<td>8,674</td>
</tr>
<tr>
<td>D. Melt ribonuclease-treated RNA</td>
<td>206</td>
<td>217</td>
<td>208</td>
</tr>
</tbody>
</table>

*Purification of the RNA after synthesis was by phenol extraction and ethanol precipitation, the RNA was resuspended in 2X SSC (SSC = 0.15 M NaCl plus 0.15 M sodium citrate), and the samples were treated as follows. (A) The 5% trichloroacetic acid-insoluble $^3$H-RNA was determined. (B) Ribonuclease was treated with 10 µg of pancreatic ribonuclease plus 3 µg of T1 ribonuclease per ml at 37°C for 15 min in 2X SSC, and the 5% trichloroacetic acid precipitable counts per minute were determined. (C) The RNA was annealed for 6 hr at 65°C in 2X SSC and treated as in B. (D) Samples diluted to 0.1X SSC before or after annealing (shown here for after annealing) were heated to 100°C, cooled, and treated as in B.

$^3$H-RNA as previously described (3). A portion of the $^3$H-RNA was excluded from the agarose column in both cases, indicating the presence of high-molecular-weight ribonuclease-resistant RNA with and without annealing. Only 0.074% of the $^3$H-RNA which had no prior annealing was excluded from the column, whereas the amount excluded increased to 0.34% for $^3$H-RNA which was annealed before ribonuclease treatment.

Table 3 indicates that the excluded RNA was resistant to both ribonuclease and ribonuclease plus deoxyribonuclease, but it became completely sensitive to ribonuclease after thermal denaturation. The melting temperature of the ribonuclease-resistant RNA excluded by the agarose column...
is determined from resistant with the was sucrose transition with (Fig. 3).

\[
\text{A. Trichloroacetic acid ppt.} \\
\text{B. Ribonuclease} \quad 1,542 \\
\text{C. Ribonuclease + deoxy-ribonuclease} \quad 1,437 \\
\text{D. Melt and ribonuclease} \quad 74
\]

- Annealed RNA was prepared and subjected to ribonuclease digestion and agarose chromatography. The excluded RNA was dialyzed versus 0.01 M Tris (pH 7.4), M NaCl. Samples were treated as follows. (A) Five per cent trichloroacetic acid precipitable counts per minute were determined. (B) Ribonuclease was treated with 20 \(\mu\)g of pancreatic ribonuclease plus 2 \(\mu\)g of T RNase per ml in 0.1 M Tris (pH 7.4), 0.2 M NaCl for 20 min at 37 C. Then treatment proceeded as in A. (C) As in B, but the solution was made 0.004 M MgCl\(_2\) and 50 \(\mu\)g of DNase per ml (ribonuclease-free, Worthington) was added for 20 min at 37 C. (D) Sample was heated to 100 C for 15 min, cooled, and treated as in B.

was determined as previously described (3). A sharp transition with a \(T_m\) of 76 C was observed (Fig. 2).

Sucrose gradient sedimentation in 15 to 30% sucrose was carried out on the ribonuclease-resistant RNA excluded by agarose, indicating a range from 7.5 to 10.5S for core double-stranded RNA (Fig. 3). This value is in good agreement with the sedimentation coefficient of the vaccinia double-stranded RNA synthesized in vivo (3).

**Effect of annealing on the amount of vaccinia virus \(^3\)H-double-stranded RNA made in vivo.** Our results with the vaccinia RNA synthesized in vitro prompted us to reinvestigate the kinetics of appearance of the vaccinia virus double-stranded RNA in the infected cell. Confluent monolayers of HeLa cells were infected with vaccinia virus. At 1, 3, and 5 hr after infection, cultures were pulse-labeled with 100 \(\mu\)Ci of \(^3\)H-uridine, and the total cytoplasmic nucleic acids were prepared. After removing the contaminating DNA by digestion with deoxyribonuclease (100 \(\mu\)g/ml, 60 min, 37 C), each \(^3\)H-cytoplasmic RNA preparation was purified by three phenol extractions and three ethanol precipitations. The purified RNA was divided into two equal samples. From one, double-stranded RNA was prepared as above. The other was allowed to self-anneal for 6 hr and then double-stranded RNA was prepared. The results are presented in Table 4. It is clear that complementary RNA is made throughout the infection cycle.

**Is there double-stranded RNA inside the cell?** It may be argued that only complementary RNA exists inside the cells and that the SDS-phenol extraction catalyzes the annealing of the RNA to give helical structures. This argument was tested by the following experiment. \(^3\)H-cytoplasmic RNA was prepared as above. Before extraction with SDS and phenol, NaCl and ribonuclease were added to final concentrations of 0.25 M and 100 \(\mu\)g/ml, respectively. After incubating the mixture at 37 C for 60 min, double-stranded RNA was prepared by the standard SDS-phenol, nuclease digestion, agarose chromatography technique. As a control, duplicate cultures were infected at the same time and double-stranded RNA was prepared as usual. At least 70% of the double-stranded RNA purified by this technique was in a form that is resistant to ribonuclease digestion and agarose chromatography.

### Table 3. Nuclease sensitivity of purified ribonuclease-resistant RNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^3)H-counts/ min</th>
<th>Per cent of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Trichloroacetic acid ppt.</td>
<td>1,604</td>
<td></td>
</tr>
<tr>
<td>B. Ribonuclease</td>
<td>1,542</td>
<td>93.7</td>
</tr>
<tr>
<td>C. Ribonuclease + deoxy-ribonuclease</td>
<td>1,437</td>
<td>82.5</td>
</tr>
<tr>
<td>D. Melt and ribonuclease</td>
<td>74</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of temperature on ribonuclease-resistant RNA. RNA excluded by agarose as in Table 2 was dialyzed versus 0.02 M Tris (pH 7.4), 0.01 M NaCl. Samples were made 0.001 M ethylenediaminetetraacetic acid and heated to the indicated temperature for 10 min and rapidly cooled on ice. Each sample was made 0.2 M NaCl and treated with ribonuclease as in Table 2. The trichloroacetic acid-insoluble RNA was determined.
VOL. 0.01 M
27,000 rev/min was Bacillus subtilis RNA containing RNA resistant helical RNA inside the it is considering extraction before isolation. Therefore, DNA-dependent a 15
ethylenediaminetetraacetic acid for 16 hr at 27,000 rev/min at 25 C in a Spinco SW27 rotor. Bacillus subtilis RNA was used as a marker.

TABLE 4. Effect of annealing on the amount of \(^3\)H-double-stranded RNA made in vivo

<table>
<thead>
<tr>
<th>Time after infection (hr)</th>
<th>(^3)H counts/min after phenol and deoxyribonuclease</th>
<th>(^3)H counts/min after ribonuclease and agarose</th>
<th>(^3)H counts/min after annealing, ribonuclease, and agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 \times 10^4</td>
<td>560</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>2.0 \times 10^4</td>
<td>2,400</td>
<td>3,600</td>
</tr>
<tr>
<td>5</td>
<td>1.8 \times 10^4</td>
<td>2,440</td>
<td>4,600</td>
</tr>
</tbody>
</table>

before extraction with phenol (Table 5). Considering the severity of the ribonuclease digestion, it is likely that most, if not all, of the double-stranded RNA isolated by this technique is helical RNA inside the cell.

DISCUSSION

We find that the synthesis of vaccinia double-stranded RNA is inhibited by actinomycin D. Therefore, we conclude that this RNA arises via a DNA-dependent reaction mechanism.

Ribonuclease resistant RNA synthesized in vitro by vaccinia cores possesses properties similar to those of ribonuclease-resistant RNA isolated from vaccinia virus-infected cells and T4 phage-infected bacteria (3, 4, 9). It is interesting to note that the increase in ribonuclease-resistant RNA after annealing corresponds to a similar phenomenon observed for the in vivo vaccinia RNA isolated throughout infection. Since cores synthesize only "early" RNA (11), we may regard these results as proof that vaccinia virus does produce complementary RNA early in infection. Our results with cytosine arabinoside-treated cells offer further confirmation of this idea.

It is important to note that the annealing of the ribonuclease-resistant RNA made in vitro is both concentration- and time-dependent. These results support the model involving the transcription of complementary regions of vaccinia DNA and rule out the possibility that the ribonuclease resistance could be the result of a folding back of a single-stranded molecule to give a lengthy hairpin structure.

TABLE 5. Effect of pretreatment with ribonuclease before phenol extraction on double-stranded RNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^3)H counts/min in phenol extract</th>
<th>(^3)H counts/min after nucleases and agarose</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard procedure</td>
<td>3.2 \times 10^4</td>
<td>3.7 \times 10^3</td>
<td>1.16</td>
</tr>
<tr>
<td>Pretreatment with ribonuclease</td>
<td>2 \times 10^4</td>
<td>2.7 \times 10^3</td>
<td>0.84</td>
</tr>
</tbody>
</table>

FIG. 3. Sucrose gradient analysis of ribonuclease-resistant RNA excluded from 6% agarose. The RNA was run on a 15% to 30% (w/v) sucrose gradient containing 0.1% sodium dodecyl sulfate, 0.1 M NaCl, and 0.01 M ethylenediaminetetraacetic acid for 16 hr at 27,000 rev/min at 25 C in a Spinco SW27 rotor. Bacillus subtilis RNA was used as a marker.

FIG. 4. Models of convergent and divergent transcription. RNA is transcribed from both strands of the vaccinia DNA such that molecules with complementary regions are synthesized. Some of these molecules form the appropriate base pairs and are converted to intracellular double-stranded RNA with single-stranded regions (S) of 9 to 22S. During purification of the double-stranded RNA, the single-stranded portions are removed by ribonuclease digestion. In the case of convergent transcription, the terminal sequences of the complementary RNA are found in double-stranded RNA, whereas the initial sequences of complementary RNA become double-stranded RNA in the case of divergent transcription. ds-RNA, double-stranded RNA.
Duesberg and Colby (4) reported that the intracellular form of the double-stranded RNA is a heterogeneous population of molecules with sedimentation coefficients ranging from 9 to 22S. Treatment with ribonuclease converts these molecules into a homogeneous population of double-stranded molecules of 9 to 10S. Thus, the intracellular form appears to be a population of molecules sharing common size double-stranded cores and having single-stranded portions of various lengths. These data coupled with the above arguments that the double-stranded RNA is synthesized via a DNA-dependent reaction mechanism are consistent with the pattern of convergent transcription recently suggested by Bøvre and Szybalski (1) for the b2 region of coliphage λ. Alternatively, the vaccinia double-stranded RNA could arise from a pattern of divergent transcription (Fig. 4). Both models involve the transcription of complementary regions of the DNA; they differ from each other with respect to the promoter sites or regions of initiation of transcription. It should be emphasized that neither model demands the simultaneous transcription of the complementary sequences. Indeed, the finding of complementary RNA in the cytoplasm argues against simultaneous transcription.

It was previously shown that the purified vaccinia double-stranded RNA is a very potent inducer of interferon in chick cells (3). The demonstration that at least 70% of the purified double-stranded RNA is in a ribonuclease-resistant form in the cytoplasm of infected cells suggests more strongly that this population of molecules is responsible for the induction of interferon in cells infected with vaccinia virus.

ACKNOWLEDGMENTS

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LITERATURE CITED