The Effect of Interferon on the Synthesis and Activity of an RNA Polymerase Isolated from Chick Cells Infected with Semliki Forest Virus

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SUMMARY

The effect of interferon on the activity and synthesis of a specific Semliki forest virus RNA polymerase has been studied. Highly purified and semi-purified interferons were without effect on the action of the viral RNA polymerase in the in vitro assay, while crude interferon preparations were inhibitory. Polymerase activity was low in interferon-treated infected cells, but this did not result from the presence of enzyme inhibitors in the preparations. Attempts to demonstrate an inhibitor of the polymerase in interferon-treated uninfected cells were unsuccessful. The inhibition of viral RNA synthesis observed in interferon-treated cells may result from an inhibition of the synthesis of the viral RNA polymerase rather than from an inhibition of the action of this enzyme.

INTRODUCTION

In the previous paper (Mécs et al. 1967) we showed that the synthesis of Semliki forest viral RNA was inhibited in chick embryo fibroblasts treated with interferon. This inhibition could result equally well from an impairment of the function or of the synthesis of the enzymes responsible for viral RNA replication, and it was proposed that the latter possibility was more likely. However, this suggestion can only be directly tested by examining the effect of interferon on the synthesis of a particular virus specific protein when viral RNA and protein synthesis can be dissociated. Alternatively the effect of interferon pretreatment on the levels of the polymerase responsible for the synthesis of viral RNA could be studied, since it should be possible to determine whether the action of this enzyme is sensitive to interferon, or to an inhibitor possibly produced in the cell in response to interferon.

Martin & Sonnabend (1967) describe the isolation and properties of a virus specific RNA polymerase from chick embryo fibroblasts infected with Semliki forest virus. The activity of this enzyme depends on the addition of all four ribonucleoside triphosphates and requires Mg++, an ATP generating system and 2-mercaptoethanol for maximum activity. It is not affected by actinomycin D or deoxyribonuclease, and

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only partially inhibited by ribonuclease. It catalyses the synthesis of a single species of RNA resistant to ribonuclease and identical in properties to the double-stranded form of viral RNA found in cells infected with Semliki forest virus.

The replication of viral RNA probably involves the participation of two enzymes (Lodish & Zinder, 1966; Martin, 1966). One may be a polymerase which uses single-stranded viral RNA as a template for the synthesis of a double-stranded intermediate RNA; this is the enzyme we believe we have isolated from cells infected with Semliki forest virus. The second may be a polymerase which uses this intermediate RNA as a template for the synthesis of single-stranded progeny RNA. Attempts to demonstrate the activity of this second enzyme in Semliki forest virus infected cells have not so far been successful.

In this communication we report the results of studies on the effect of interferon on the synthesis and activity of the first type of viral RNA polymerase. Treatment with interferon caused a marked decrease in polymerase activity; evidence is presented which shows that this effect is more probably a result of an inhibition of polymerase synthesis than of polymerase activity.

**METHODS**

*Virus and cells.* The preparation of Semliki forest virus and chick embryo fibroblasts, and the conditions for the growth and assay of virus, were described in the previous paper (Mécs *et al.* 1967).

*Interferon.* Partially and highly purified interferon were prepared from influenza virus (B/England/939/59) infected allantoic fluid by the method of Fantes (1965). The semi-purified interferon (specific activity \(2.9 \times 10^4\) units/mg. protein) was prepared without DEAE chromatography, and consisted of bulked off-peak fractions from a CM-Sephadex column. The highly purified sample (specific activity \(1.2 \times 10^6\) units/mg. protein) was obtained by subjecting crude interferon to the whole purification process. Crude interferon (2000 units/ml.) was prepared by infecting monolayers of chick embryo fibroblast with Chikungunya virus at a multiplicity of 0.1 p.f.u./cell, and harvesting the culture fluid 48 hr later. This was dialysed at pH 2 for 24 hr against HCl (0.01N) and then against Gey's solution buffered with tris + HCl (pH 7-6), then concentrated by pressure dialysis. Interferon was assayed as described previously (Mécs *et al.* 1967).

*Preparation and assay of RNA polymerase.* About \(2 \times 10^6\) cells were harvested 6 hr after infection with Semliki forest virus and washed twice with 0.15 M-NaCl and once, rapidly, with 0.001 M-tris + HCl buffer (pH 8.3) containing 0.01 M-2-mercaptoethanol and 0.001 M-MgCl\(_2\). They were suspended in the tris-mercaptoethanol MgCl\(_2\) medium and disrupted with a Dounce homogenizer. Concentrated solutions of tris + HCl (pH 8.3), 2-mercaptoethanol and MgCl\(_2\) were added to give final concentrations of 0.04 M, 0.01 M and 0.002 M respectively. Cell debris and nuclei were spun off (600g for 10 min.) and the supernatant fluid centrifuged at 10,000g for 20 min. The pellet, suspended in 0.04 M-tris + HCl, 0.01 M-2-mercaptoethanol and 0.002 M-MgCl\(_2\), was used as the source of RNA polymerase; it was stored at \(-70^\circ\). Protein concentration was estimated from the total nitrogen content of the suspension, determined by the method of Lang (1958). Details of the polymerase assay method, briefly summarized here, are reported elsewhere (Martin & Sonnabend, 1967). Polymerase (0.3 to 1.0 mg.
Effect of interferon on RNA polymerase

Protein) was incubated for 15 min. at 37 °C with [\(^{3}H\)]GTP (50 \(\mu\)moles, specific activity 25 C./mole), ATP, CTP and UTP (50 \(\mu\)moles each), \(\text{MgCl}_2\) (0.8 \(\mu\)moles), phosphoenol pyruvate (5 \(\mu\)moles), pyruvate kinase (10 \(\mu\)g.), actinomycin (1 \(\mu\)g.), 2-mercaptoethanol (7 \(\mu\)moles) and tris+ HC1 (pH 8.6, 35 \(\mu\)moles), in a final volume of 0.35 ml. The reaction was stopped by the addition of 0.5\(\text{H}_2\text{O}\) containing 0.125 M-\(\text{Na}_4\text{P}_2\text{O}_7\), and RNA extracted with 5% trichloracetic acid; its radioactivity was measured in a Packard ‘Tricarb’ scintillation counter. In experiments on the direct effect of interferon on polymerase activity, mercaptoethanol was omitted from all reagents used in the preparation and assay of the enzyme.

For the examination of enzyme reaction products by sucrose gradient analysis [\(^{3}H\)]GTP of specific activity 100 C./mole was used, and the quantities of reactants increased threefold. After incubation the reaction mixture was extracted with sodium dodecyl sulphate by the method of Dalgarno et al. (1966), and analysed on sucrose gradients as described previously (Meces et al. 1967).

Materials. The materials used were those described by Martin & Sonnabend (1967).

RESULTS

Direct effect of interferon on polymerase activity

The effect of adding interferon to the reaction mixture during the assay of polymerase activity was examined. In these experiments mercaptoethanol was omitted during the preparation and assay of the enzyme, as it is known to inactivate interferon (Fantes & O'Neill, 1964; Merigan, Winget & Dixon, 1965). Six units of highly purified interferon (specific activity 1.2 \(\times\) 10\(^6\) units/mg, protein) had no effect on the activity of the polymerase in the in vitro assay, while 128 units of the semi-purified material (specific activity 2.9 \(\times\) 10\(^4\) units/mg, protein) caused only an 8% inhibition (Table 1). The amount of polymerase in each incubation represented material from about 10\(^7\) cells: one unit of interferon caused an 80% decrease in virus titre in this number of cells. Sucrose gradient analysis of the products formed when purified interferon was

<table>
<thead>
<tr>
<th>Interferon added</th>
<th>Polymerase activity* ((\mu)moles GTP incorporated/mg. protein/15 min.)</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly purified†</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>Highly purified</td>
<td>1.5</td>
<td>69</td>
</tr>
<tr>
<td>Highly purified</td>
<td>3.8</td>
<td>62</td>
</tr>
<tr>
<td>Highly purified</td>
<td>7.5</td>
<td>62</td>
</tr>
<tr>
<td>Expt. 2</td>
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<td></td>
</tr>
<tr>
<td>Highly purified</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>Highly purified</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Highly purified</td>
<td>6</td>
<td>51</td>
</tr>
<tr>
<td>Semi-purified‡</td>
<td>128</td>
<td>42</td>
</tr>
<tr>
<td>Crude</td>
<td>50</td>
<td>17</td>
</tr>
</tbody>
</table>

* Each value is the mean of duplicate assays.
† Specific activity of highly purified interferon was 1.2 \(\times\) 10\(^6\) units/mg, protein.
‡ Specific activity of semi-purified interferon was 2.9 \(\times\) 10\(^4\) units/mg, protein.

See Methods for preparation of crude interferon.
present in the reaction mixture showed that the product was identical to that formed in the absence of interferon (see below). Hence, we conclude that interferon has no direct effect on the activity of the RNA polymerase preparations. Fifty units of the crude interferon caused a marked inhibition of polymerase activity. Glasky, Simon &

Fig. 1. Polymerase activities in infected control and interferon-treated cells during growth of virus. Control and interferon-treated (5 units/ml.) cells were harvested at various times after infection with Semliki forest virus (40 p.f.u./cell). RNA polymerase was prepared and assayed as described in the Methods section. RNA polymerase activity of infected untreated (●—●) and interferon-treated (○—○) cultures. Results of assays performed without addition of three nucleoside triphosphates are shown for untreated (▲) and interferon-treated (△) cells. Virus titres in control (+—+) and interferon-treated (■—■) cultures.

Holper (1964) reported that crude chick and calf interferons inhibited the incorporation of labelled nucleoside triphosphates into RNA by RNA polymerases prepared from homologous cells infected with Newcastle disease or influenza viruses. This observation was surprising as studies with metabolic inhibitors had shown that a period of host-cell RNA and protein synthesis was necessary before interferon could exert its antiviral action (Taylor, 1964; Levine, 1964; Lockart, 1964; Friedman & Sonnabend, 1965). Our observations with crude interferon suggest that the results obtained by Glasky et al. (1964) could have been due to impurities in their interferon preparations.
Effect of interferon on RNA polymerase activity during infection

When the polymerase was prepared from cells infected for various times with Semliki forest virus, its activity rose sharply after 1½ hr to a maximum at 6 hr, reaching a value 10 times that found in the uninfected cells (Fig. 1). Incorporation by pre-

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Fig. 2. Sucrose gradient analysis of reaction products formed by polymerases from control and interferon-treated infected cells. Polymerase preparations from infected control and interferon-treated (10 units/ml) cells were incubated with [3H]GTP and the products extracted with sodium dodecyl sulphate as described in the Methods section. The extracts were mixed with similar extracts from actinomycin-treated infected cells which had been labelled with [14C]uridine from 4 to 6 hr after infection. The mixed extracts were sedimented through 5 to 20 % sucrose gradients (see Methods) and the distribution of [3H] and [14C]-radioactivity determined. 30 S and 18 S refer to the peaks of optical density (260 m\(\mu\)) of chick ribosomal RNA added as a marker. Fraction number 1 is from the bottom of the gradient. A: Polymerase from untreated infected cells; insert—distribution of radioactivity after incubation of mixed extracts with ribonuclease before centrifugation. B: Polymerase from interferon-treated infected cells. [3H]-radioactivity (polymerase reaction product) (●—●). [14C]-radioactivity (virus-specific RNA labelled in vivo) (○—○). [14C]-radioactivity after incubation of fractions with 2 \(\mu\)g./ml. of ribonuclease (△—△).
parations from uninfected cells was not caused by a heteropolymerase, as it was not affected by the omission of the other three nucleoside triphosphates (Martin & Sonnabend, 1967). When the cells were incubated with 20 units of interferon before infection, polymerase activity was almost abolished (Fig. 1). Much of the incorporation obtained with preparations from the interferon-treated cells was found to be non-specific, i.e. it was not affected by omission of the three unlabelled nucleoside triphosphates. The reaction products formed by polymerases from infected control and interferon-treated cells were analysed by sedimentation on sucrose gradients (Fig. 2). The control preparation showed a single peak of radioactivity with an S value of about 20 S; it was resistant to incubation with 2 μg./ml. of ribonuclease at 30 °, and sedimented in an identical position to the ribonuclease-resistant RNA component found in Semliki forest virus-infected cells pulse-labelled with [3H]adenosine (Fig. 2a). Martin & Sonnabend (1967) found this product to be virus specific double-stranded RNA. On the other hand the product catalysed by a polymerase preparation from interferon-treated cells exhibited only small amounts of 20 S RNA (Fig. 2b); the major RNA species formed was a low molecular weight component of about 2 to 6 S. Much of the polymerase activity seen in infected interferon-treated cells (Fig. 1) was therefore probably due to incorporation of precursor into non-viral RNA.

Table 2. Effect of increasing amounts of interferon on polymerase activity and on virus growth

<table>
<thead>
<tr>
<th>Interferon* added (units/ml.)</th>
<th>Polymerase activity†</th>
<th>Virus yields‡</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>μmoles GTP/ mg. protein/ 15 min.</td>
<td>Percentage of control</td>
</tr>
<tr>
<td>0</td>
<td>135·9</td>
<td>100</td>
</tr>
<tr>
<td>2·5</td>
<td>49·7</td>
<td>36·8</td>
</tr>
<tr>
<td>5</td>
<td>24·0</td>
<td>17·7</td>
</tr>
<tr>
<td>20</td>
<td>11·6</td>
<td>8·5</td>
</tr>
</tbody>
</table>

Cells were infected at a multiplicity of 80 p.f.u./cell.
* Semi-purified interferon. Specific activity 2·9 × 10⁴ units/mg. protein.
† Polymerase extracted 6 hr after infection.
‡ Virus yields assayed 8 hr after infection.

The degree of inhibition produced by interferon on the activity of RNA polymerase in infected cells depended on the amount of interferon used, and at all doses was inhibited less than the production of infectious virus (Table 2). This is consistent with the results in the preceding communication (Mécs et al. 1967), which showed that the inhibition of virus growth by interferon was much greater than the inhibition of 20 S ribonuclease-resistant RNA synthesis.

Absence of an inhibitor of RNA polymerase in preparations from interferon-treated cells

The low levels of incorporation of [3H]GTP by enzyme prepared from interferon-treated cells could have resulted from the action of an inhibitor in the preparations. This possibility was investigated by assaying an active enzyme in the presence of
Effect of interferon on RNA polymerase

preparations derived from interferon-treated uninfected cells, and from normal cells. Material from untreated cells and cells treated with 20 units of interferon isolated in the same way as the enzyme had no inhibitory effect on the active polymerase. Furthermore, low activity enzyme prepared from infected interferon-treated cells did not inhibit incorporation by active enzyme preparations (Fig. 3).

![Graph](image)

Fig. 3. Effect of extracts from interferon-treated cells in the *in vitro* polymerase assay. The large-particle cytoplasmic fractions were prepared from infected cells (1), uninfected cells (2) and uninfected cells which had been incubated for 4 hr with 20 units/ml. of interferon (3). The time-course of incorporation of [\(^{3}H\)]GTP into acid-insoluble products was measured in the assay system described in Methods. Incorporation of GTP by polymerase from infected cells alone (●—●), or when approximately equal amounts of similar preparations from uninfected cells (○—○) or interferon-treated cells (△—△) had been added to the assay. Polymerase activity of preparations from uninfected cells (●—●) and interferon-treated cells (▲—▲) when assayed individually. The polymerase activities of the mixed preparations are given as \(\mu\)moles GTP incorporated per mg. of protein in the infected cell preparation.

**DISCUSSION**

The observations that interferon caused a decrease in the level of the virus-specific RNA polymerase and that it had no inhibitory action on the activity of this enzyme, either directly or by inducing the synthesis of an inhibitor within the cell, give strong support to the suggestion that it is the *synthesis* rather than the activity of the RNA
polymerase that is blocked in interferon-treated cells. They do not prove this—an inhibitor of polymerase action may be unstable or be located in a part of the cell other than the fraction tested, and, as pointed out previously (Mécs et al. 1967), an inhibition of polymerase action would also result in a diminished synthesis of the polymerase. Nevertheless, in the absence of studies on suitable systems where parental messenger RNA function can be directly observed, we feel that our hypothesis is a reasonable one. It implies that interferon inhibits the synthesis of all proteins specified by the viral RNA. In particular, production of the polymerase which uses double-stranded RNA as a template for the synthesis of single-stranded progeny viral RNA should also be inhibited to the same extent as the polymerase examined in the present study; this possibility is being investigated. This hypothesis also provides an explanation for the general antiviral action of interferon against a wide variety of both RNA and DNA viruses. Inhibition of a virus specific RNA polymerase could not account for its action against DNA viruses, whereas the synthesis of specific proteins specified by a viral RNA, derived either directly from the invading virus or synthesized on a viral DNA template, is a step common to both types of virus, and its inhibition would have similar consequences.

Note added in proof: Essentially similar results to those described here were reported by N. Miner, W. J. Ray & E. H. Simon (1966. Biochem. biophys. Res. Commun. 24, 264), who found that the synthesis of RNA polymerase in Mengo virus infected L cells was inhibited by interferon.

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REFERENCES


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