Viral Events Necessary for the Induction of Interferon in Chick Embryo Cells

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Temperature-sensitive mutants of Sindbis virus were employed to investigate the nature of the viral event(s) which induces chick-embryo cells to produce interferon. Chick embryo cells induced by the parental heat-resistant strain of Sindbis virus produced essentially equal amounts of interferon at 29 and 42 C. An RNA- and three RNA+ strains [temperature-sensitive mutants unable (RNA-) and able (RNA+) to make ribonucleic acid] produced interferon at 29 C but not at 42 C. It is concluded that viral RNA per se and the replication of viral RNA do not induce interferon production by chick embryo cells.

Burke et al. (2) showed that chick-embryo cells which were infected with Semliki Forest virus at 37 C for 1 hr and then transferred to 42 C produced interferon but no infectious virus. Skehel and Burke (8) established the need for viral events which could occur at 36 C but not at 42 C. Only 2 hr at 36 C were necessary for maximal interferon to be made during additional incubation at 42 C. A mixture of single- and double-stranded viral ribonucleic acid (RNA) was synthesized during the 2-hr period at 36 C. In light of the recent reports (5, 6, 9) which suggested that double-stranded polynucleotides induce interferon formation, Skehel and Burke (8) suggested that the formation of double-stranded viral RNA might be the first stage in interferon production.

Although the formation of double-stranded viral RNA may be required for interferon formation, it does not completely explain the induction process. By studying the ability of several temperature-sensitive mutants of Sindbis virus to induce interferon, we obtained results which indicate that viral processes other than viral RNA synthesis are required for cells to make their full yields of interferon.

MATERIALS AND METHODS

Cells. Primary cultures of chick embryo fibroblasts were prepared from 10-day-old decapitated chick embryos according to the method of Dulbecco and Vogt (3).

Viruses. Temperature-sensitive mutants and a heat-resistant wild type (HR) of Sindbis virus were kindly given to us by E. R. Pfefferkorn, Hanover, N.H.

Stock virus preparations were produced in chick embryo cells.

Medium and solutions. Eagle's medium (4) containing 3% calf serum (EC medium) was used throughout this study. Virus dilutions were carried out in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin fraction V (BSA). This solution was also used to wash monolayers. For plaque titrations, cultures were overlaid with 1% agar in Eagle's medium. Cycloheximide was purchased from the Sigma Chemical Co., St. Louis, Mo.

Interferon. Interferon was prepared by infecting CE cultures with virus and incubating them with 5 ml of EC medium for 24 to 48 hr, depending on the incubation temperature. The fluid was then collected. To inactivate the virus in the fluid, 5-ml samples in a 100-mm petri plate were exposed to an 8-w General Electric germicidal lamp for 5 min at a distance of 15 cm. To determine the interferon titer, the irradiated fluids were diluted in twofold steps in EC medium, and 2 ml was added to chick embryo cultures. After overnight incubation, the cultures were challenged with >10 plaque-forming units (PFU) per cell of Sindbis HR virus and incubated an additional 24 hr. The monolayers were then examined for cytopathic effects, and the greatest dilution capable of protecting the cells was considered to contain one protective unit (PU) of interferon per ml.

Temperature shifts. To shift cultures from 29 to 42 C, the plates were first moved to a warm plate (43 C). The cells were washed once with PBS + BSA at 45 C. EC medium (5 ml) warmed to 45 C was then added to the cultures and they were quickly transferred to an incubator at 42 C.

RESULTS

Ability of chick embryo cells to make interferon at 29 and 42 C. The Sindbis HR virus, which multiplies extensively both at 42 C and at lower temperatures, was used. Chick embryo cells were

1 Contribution no. 1467.
infected with multiplicities of virus in excess of 10 PFU per cell and incubated at either 29 C or 42 C. Previous experiments indicated that 48 hr of incubation was required at 29 C to be certain that maximal interferon titters were reached. At 37, 40, and 42 C, maximal titters were assured after 24 hr of incubation. At these times, fluids were harvested for the collection of data. The data in Table 1, which are an average of 7 and 10 experiments at 29 C and 42 C, respectively, indicate that infected chick embryo cells make almost equal amounts of interferon at the two temperatures.

Interferon production by an RNA\(^{-}\) temperature-sensitive mutant. Pfefferkorn et al. (7) showed that one of the mutants unable to make RNA at 40 C (RNA\(^{-}\)) was blocked in its ability to form double-stranded RNA at 40 C.

We used an RNA\(^{-}\) temperature-sensitive mutant to see whether interferon could be induced under nonpermissive conditions. Mutant ts 15, an RNA\(^{-}\) mutant, was chosen. It was verified as an RNA\(^{-}\) mutant by showing that it was unable to make viral RNA at 42 C (Fig. 1). It was found further that 37 C was a permissive temperature for both virus and interferon production, whereas 42 C was not. Data from a number of experiments (Table 2) indicate that, under permissive conditions, cells infected with mutant ts 15 produced an average of 32 units per ml of interferon. At nonpermissive conditions, an average of 1.5 units per ml of interferon was produced. However, cells which were infected and incubated 2 hr at permissive conditions and then shifted to 42 C produced an average of 13.3 units per ml of interferon. Clearly, some early viral events necessary for the stimulation of interferon production had occurred during the 2-hr period at 29 C. We have been unable to find the production of any recognizable large molecular weight viral RNA during that time.

Need for protein synthesis during the permissive period. Cycloheximide was added at a concentr-

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**TABLE 1. Interferon and virus titers at 29 C and 42 C from chick embryo cells infected with Sindbis virus\(^{a}\)**

<table>
<thead>
<tr>
<th>Temp</th>
<th>No. of experiments</th>
<th>Avg titters</th>
<th>Virus (PFU/ml) (\times 10^6)</th>
<th>Interferon (PU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7</td>
<td>9.7 ± 5.1</td>
<td>123 ± 91</td>
<td></td>
</tr>
<tr>
<td>29 C</td>
<td>10</td>
<td>0.9 ± 0.42</td>
<td>112 ± 63</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) The HR strain which multiplies at 29 C and 42 C was used.

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**FIG. 1. Cultures of chick embryo cells were infected with 20 PFU per cell of Sindbis virus strain HR which multiplies at 42 C, or of ts 15 which does not. The cultures were kept at 42 C at all times after infection. At 2 hr after inoculation, 25 \(\mu\)c of \(^{3}H\)uridine was added to each culture. After 3 hr, the cultures were removed and the RNA was extracted by phenol and sodium dodecyl sulfate and analyzed on 15 to 30% sucrose gradients containing 0.1 m NaCl, 0.01 m tris(hydroxymethyl)aminomethane, 5 \(\times\) \(10^{-3}\) m ethylenediaminetetraacetate, and 0.5% sodium dodecyl sulfate, at pH 7.4. The gradients were centrifuged for 16 hr at 22,000 rev/min in an SW25.3 rotor at 25 C. The RNA was collected and precipitated by cold trichloroacetic acid onto membrane filters (Millipore Corp., Bedford, Mass.), and the radioactivity was counted in a liquid scintillation counter.**

**TABLE 2. Interferon and virus produced at permissive and nonpermissive conditions by chick embryo cells infected with RNA\(^{-}\) mutant ts 15**

<table>
<thead>
<tr>
<th>Temp</th>
<th>No. of experiments</th>
<th>Avg titters</th>
<th>Virus (PFU/ml)</th>
<th>Interferon (PU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3</td>
<td>2.8 ± 0.2 (\times 10^6)</td>
<td>32.0 ± 0</td>
<td></td>
</tr>
<tr>
<td>37 C</td>
<td>8</td>
<td>9.1 ± 8.8 (\times 10^4)</td>
<td>1.5 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>29 (2 hr)</td>
<td>3</td>
<td>2.2 ± 3.9 (\times 10^4)</td>
<td>13.3 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>42 (22 hr)</td>
<td>3</td>
<td>9.2 ± 3.9 (\times 10^4)</td>
<td>13.3 ± 4.6</td>
<td></td>
</tr>
</tbody>
</table>

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The induction of 100 \(\mu\)g per ml and was present between 1 and 2 hr after the addition of RNA\(^{-}\) virus mutant ts 15 while the cultures were incubated at the permissive temperature. The cycloheximide was removed and half of the cultures were in-
cubated for 22 hr at 42 C (nonpermissive), while the others were incubated for a similar period at 37 C (permissive). No interferon was produced at the nonpermissive temperature, whereas 32 units per ml was produced at the permissive temperature (Table 3). Protein synthesis was required for the events which occurred at the permissive temperature and which resulted in 16 and 4 units per ml of interferon, since the presence of cycloheximide (during the 1 hr) inhibited subsequent interferon formation at 42 C. The effect of cycloheximide was reversible because subsequent incubation at 37 C resulted in 32 units per ml of interferon. The inhibition of subsequent interferon production by cycloheximide during that period of infection clearly shows the need for early viral protein synthesis for interferon induction.

**Ability of RNA+ mutants to induce interferon at several temperatures.** The preceding data do not make clear whether the protein synthesized or the subsequent effects of the protein are responsible for the actual induction activity, or whether viral RNA synthesis is required. To see whether viral RNA synthesis was somehow responsible for interferon induction, we turned to another group of temperature-sensitive mutants. If viral RNA, per se, were the inducer, temperature-sensitive mutants able to make viral RNA (RNA+ mutants) should be able to induce interferon production under nonpermissive conditions. This prediction was not borne out. Three different strains of RNA+ mutants were used. Each is defective because of change in a different cistron (1). Analyses of the RNA production and the nature of the defects have been made (10; B. W. Burge and E. R. Pfefferkorn, submitted for publication). Data showing the ability of these three mutant strains to induce chick embryo cells to make interferon at 29 and 42 C are shown in Table 4. Clearly, the three mutant strains are almost completely deficient in their ability to produce infectious virus and to induce interferon formation at 42 C. However, at 42 C, mutant strains ts 2, ts 20, and ts 23 produced 82% (average of four experiments), 17% (average of three experiments), and 25% (average of three experiments), respectively, as much viral RNA as that produced by the wild-type virus strain. Thus, no interferon was produced, even though the replication of viral RNA occurred.

**Discussion**

Our data confirm those of Skehel and Burke (8) which showed that early viral events are needed to induce interferon production in chick cells. Also, viral RNA replication, or its accumulation in the cell (10), is insufficient to account for interferon production; if it is assumed that double-stranded RNA is a necessary intermediate for replication, these molecules are probably not the actual inducers of interferon production.

The complete explanation of the nature of the induction process for interferon formation is still in doubt. It seems evident that at least some step in virus multiplication which requires protein synthesis must occur to initiate the induction process. However, since the defects in the temperature-sensitive mutants result from the pro-
duction of proteins which are nonfunctional at the higher temperatures, some viral proteins, or the processes for which they are necessary, must be required for interferon induction.

**Literature Cited**


