A Temperature-sensitive Event in Interferon Production

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SUMMARY

The production of interferon by chick embryo cells infected with Semliki Forest virus and incubated at 42° is dependent on a period of incubation at 36°. During incubation at 36° virus-directed RNA synthesis occurs and it is suggested that the temperature-sensitive event in interferon production is an early stage in virus multiplication rather than a process controlled by the cell genome.

INTRODUCTION

Both virus multiplication and interferon production are initiated by virus infection, although it is possible to inhibit one or the other selectively. In chick cells infected with Semliki Forest virus, both processes depend on infection by virus with functional RNA (Skehel & Burke, 1968), which suggests that a virus-directed process may be essential for interferon formation. This paper reports an examination of the temperature sensitivity of Semliki Forest virus multiplication, and the demonstration of a temperature sensitive stage in interferon formation.

METHODS

Materials. Actinomycin D was given by Merck, Sharp and Dohme Ltd. [5-3H] uridine (17·3 c/m-mole) and [2-14C]uridine (56·4 Mc/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks. [3H]UTP (2·24 c/m-mole) was bought from Schwarz Bioresearch Ltd, New Jersey, U.S.A.; and the nucleoside triphosphates from Sigma, London.

Media, cells and viruses. These were previously described by Walters, Burke & Skehel (1967).

Interferon production and assay were previously described by Skehel & Burke (1968).

Estimation of viral RNA synthesis. (1) Pulse-labelling method. Chick cell monolayer cultures (approximately 8 × 10⁶ cells/culture) were treated for 4 hr before infection with 0·5 µg./ml. actinomycin D at 36°. Following infection with Semliki Forest virus (5 p.f.u./cell) for 1 hr at 36° the cultures were washed twice and overlaid with 1 ml. of maintenance medium. They were then incubated at either 36° or 42° and at various times [3H]uridine (1·0 µC/culture) was added to triplicate cultures for 20 min. periods. The cultures were then washed, extracted as described previously (Skehel et al. 1967) and the radioactivity of the acid insoluble, defatted residue was determined. (2) Cumulative count method. Chick cell monolayers were treated for 2 hr with 0·5 µg./ml.
actinomycin D at 36°. They were then either incubated at 36° or at 42° for 1 hr before infection with Semliki Forest virus. Following adsorption for 1 hr at the required temperature the virus was removed, the cultures were washed twice, and then overlaid with 1 ml. of maintenance medium. [3H]uridine (0.6 μCi) was then added to each culture to give a final uridine concentration of 10^{-4} M (in order to maintain a linear rate of incorporation over the period of the experiment), and at various times afterwards triplicate cultures were removed for radioactivity determination. Incubation and wash media, virus innocula, and isotope solutions were heated to the required temperature before use. Further incubation details are given in the text.

Extraction of RNA and sucrose gradient analysis. Following the required incubations, monolayer cultures (120 × 10^6 cells/culture) were washed twice with ice-cold saline and then with buffer (0.1 M-KCl; 0.001 M-EDTA; 0.01 M-tris+HCl pH 7.5). The cells were scraped from the glass and bentonite and sodium dodecyl sulphate were added to the suspension to final concentrations of 0.05 and 0.5% respectively. The cell suspension was then extracted with water-saturated phenol for 10 min., centrifuged, and the resulting aqueous phase again extracted with phenol. Residual phenol was removed from the final aqueous phase by ether extraction. Samples (0.2 ml.) of RNA solution thus prepared were layered on to linear sucrose gradients (5 to 20% sucrose, 0.1 M-KCl; 0.001 M-EDTA; 0.01 M-tris+HCl pH 7.5) and the gradients were centrifuged at 4° for 3 hr at 38,500 rev./min. in the SW39 rotor of the Spinco L2 preparative ultracentrifuge.

After centrifuging 0.25 ml. fractions were collected into 5% trichloracetic acid by puncturing the tubes. Bovine plasma albumin (1.0 mg./ml.) was added and the resulting precipitates were collected on glass fibre discs (Whatman GF/C). The discs were washed with 5% trichloracetic acid, ethanol and ether, dried, and toluene scintillation mixture added. Radioactivity was determined using a Packard spectrometer Series 3000. For estimation of the RNase sensitivity of the RNA fractions, samples were incubated with 1 μg./ml. RNase at 30° for 20 min. before trichloracetic acid precipitation.

Preparation and assay of viral RNA polymerase activity were described by Martin & Sonnabend (1967) and Skehel & Burke (1968).

RESULTS

Virus multiplication at 42°

We reported earlier that when chick cells were infected with Semliki Forest virus at 36°, and then incubated at 42°, interferon but not virus was produced (Burke, Skehel & Low, 1967). We also reported that no virus-directed RNA synthesis could be detected by a pulse-labelling method in actinomycin-treated cells infected at 36° and incubated at 42°, and have confirmed this. When infected cells were moved to 42° at various times during virus multiplication, the rate of virus-directed RNA synthesis was at first increased, and then, after about 1 hr at the higher temperature, rapidly diminished (Fig. 1). A similar result was obtained when isotope was added to every culture immediately after infection and samples taken at different times thereafter (cumulative count procedure): virus-directed synthesis ceased about 1 hr after increasing the incubation temperature (Fig. 2a). However, a small amount of RNA synthesis was detectable in cells moved to 42° at the end of the 1 hr period of infection
Fig. 1. Effect of different incubation temperatures on the rate of [³H]uridine incorporation into RNA of chick cells. The isotope was added to the cells for 20 min. at the times shown. Cells infected at 36 ° and incubated at 42 ° (O—O); or moved back to 36 ° after 100 min. at 42 ° (□—□). Cells infected at 36 ° and incubated at 36 ° (●—●); or moved to 42 ° after 100 min. at 36 ° (■—■). The arrow indicates the time of the temperature change.

Fig. 2. Effect of different incubation temperatures on the rate of [³H]uridine incorporation into RNA of chick cells. (a) Actinomycin-treated cells infected at 36 ° and incubated at 36 ° (O—O); or moved to 42 ° 80 min. after infection (□—□). (b) Actinomycin-treated cells infected at 36 ° and then incubated at 42 ° immediately after infection (●—●); or moved to 36 ° 80 min. (□—□), or 155 min. (■—■) after infection. Cells infected and incubated at 42 ° (O—O). All additions of isotope were made immediately after infection. The arrows indicate the times of temperature changes.
which was not detectable in cells infected and incubated at 42° (Fig. 2b). This will be referred to again below. The rapid decrease in the rate of virus-directed RNA synthesis when the cells were moved to 42° could be due to an effect on either the formation or the action of the virus-induced RNA polymerase or on both formation and action. The enzymic activity of a cell-free preparation of the virus-induced polymerase at 42° was about 35% of the maximum enzymic activity at 34° and about 40% of the activity of the enzyme at 36° (Fig. 3). Since the action of RNA polymerase in vivo probably exerts a cascade effect this reduction in activity would be sufficient to affect

![Fig. 3. Effect of different incubation temperatures on the activity of RNA polymerase induced by Semliki Forest virus. Two different polymerase preparations were obtained from infected cells 5 hr after infection.](image)

RNA synthesis substantially within 1 hr. However, the possibility that synthesis of RNA polymerase is also inhibited at 42° cannot be excluded. Thus we conclude that virus RNA synthesis, and hence virus multiplication, is inhibited at 42° because of an effect on the action, and also possibly on the synthesis, of RNA polymerase.

**Interferon production at 42°**

The data of Fig. 2b indicated that infection at 36°, followed by incubation at 42°, was accompanied by a small amount of virus-directed RNA synthesis. This did not occur when the cells were infected and incubated at 42°, conditions which also prevented interferon formation (Table I). This failure to induce RNA synthesis or interferon production was not due to failure of the virus to adsorb or penetrate at 42°, since cells infected at 42° and then incubated at 36° produced as much virus and interferon as cultures maintained throughout at 36° (Table I). These results suggested that interferon synthesis was preceded by an event which could not occur at the
higher temperature, and other results shown in Table 1 confirmed this. Cells were incubated at 42°C for 1 hr and then infected with prewarmed virus for 1 hr. They were then either held at 42°C for 8 hr or moved to 36°C immediately following infection for varying periods of time and then reincubated at 42°C until 8 hr after infection. The amount of interferon produced increased with the time of incubation at 36°C, maximum yields being produced when the cells were incubated for 2 hr at 36°C before re-incubation at 42°C. The rate and time-course of interferon production were largely independent of the conditions before the final incubation at 42°C (Table 2).

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<th>Temperature of incubation (°C)</th>
<th>Incubation time after infection (min.)</th>
<th>Incubation temperature (°C)</th>
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<th>Interferon yield (PDD 50)</th>
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The amount of virus-directed RNA synthesis occurring under these different conditions was measured by pretreating the cells with actinomycin D and using the cumulative count procedure described in Methods section. No interferon was produced in these experiments because of the pretreatment with actinomycin D, but it is assumed that any viral functions required for interferon induction would have occurred before the actinomycin sensitive stage of interferon production. The results showed that RNA synthesis started during the period of incubation at 36°C, and increased with the time of incubation at the lower temperature (Fig. 4). Examination of this RNA by sucrose gradient centrifugation showed the presence of RNA species...
sedimenting at about 20 to 26S, at all times after 40 min. incubation at 36° (Fig. 5) and approximately 20% resistant to the action of ribonuclease. These species of RNA therefore contained ribonuclease-resistant double-stranded 20S RNA as well as 26S single-stranded RNA (Sonnabend, Martin & Mecs, 1967). Virus-induced RNA polymerase must have been formed during the short incubations at 36° but it could not be detected, presumably because of the small amount of enzyme and/or RNA primer present. No virus-induced proteins could be detected by the double-labelling method of Hay, Skehel & Burke (1968) unless the cells were incubated at 36° for 90 min. when virus structural proteins were detectable (A. J. Hay, unpublished). Thus the formation of increasing amounts of interferon was accompanied by the formation of increasing amounts of viral RNA; interferon production, therefore, may have depended on a small amount of viral RNA synthesis.

**Host cell RNA synthesis at 42°**

The temperature sensitive stage in interferon production may have been due to inhibition of the synthesis of one or more species of host cell RNA or protein at 42°. However, it was found that the rate of [3H]uridine incorporation increased markedly when either infected or uninfected cells were moved from 36° to 42° (Fig. 6). This increased incorporation was not a consequence of an increase in the rate of uridine
Temperature and interferon production

Fig. 5. Sucrose gradient centrifugation of RNA extracted from actinomycin-treated chick cells infected for 1 hr with Semliki Forest virus. [3H]uridine was added at the end of the infection and the cells harvested after 2 hr incubation. (a) Cells infected at 36°C and incubated at 36°C. (b) Cells infected at 36°C and incubated at 42°C. (c) Cells infected at 42°C, incubated for 1 hr at 36°C and then at 42°C. O—O, 3H-virus-directed RNA; •—•, uninfected chick cell ribosomal RNA labelled with [14C]uridine.
incorporation into the trichloracetic-acid-soluble, nucleotide-precursor fraction. Similar results were obtained when the rate of protein synthesis was measured. Examination by sucrose gradient centrifugation of the RNA species produced in uninfected cells during incubation at 42° showed that the rate of synthesis of both ribosomal and soluble RNA was stimulated to the same extent. Moreover, no new species of RNA was detected at 42° and the stimulations in the rate of ribosomal and soluble RNA synthesis accounted for the increase in RNA synthesis.

**Fig. 6.** Effect of temperature on the rate of incorporation of [H]uridine into the RNA of uninfected chick cells. Cells incubated at 36° (O—O), or moved to 42° at zero time and then pulsed with isotope at the times shown (□—□). At the time indicated cultures were moved from 42° to 36° for 1 hr and then back to 42° (△—△).

**DISCUSSION**

Use of a cumulative count method has demonstrated that, when chick cells were infected with Semliki Forest virus at 36°, and then incubated at 42°, virus-directed RNA synthesis could be detected. This RNA synthesis could not be detected by use of the less sensitive pulse-labelling method (Burke et al. 1967) and did not occur when the cells were infected at 42°. Under these latter conditions, no interferon was formed, and these findings suggest that an early stage in virus replication is required before interferon induction. Since the virus-directed RNA was shown to contain ribonuclease-resistant RNA, and since the product of the polymerase enzyme is largely ribonuclease-resistant RNA, in the cell-free system at any rate (Martin & Sonnabend, 1967), such an early stage could be the formation of a double-stranded RNA species. Indeed, the recent reports that double-stranded polynucleotides, rather than single-stranded molecules induce interferon formation support this suggestion (Field et al. 1967). If this were so, then the first stage in interferon induction by viruses containing single-stranded RNA would be the formation of a double-stranded RNA.
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However, it has been reported (Gharpure, 1965) that cellular functions required for virus multiplication are adversely affected by short periods of incubation at 45°, and also that nucleolar RNA synthesis is inhibited at elevated temperature (Simard & Bernhard, 1967). When chick cells were pre-incubated and infected at 42° and then incubated at 36°, the yields of both virus and interferon were the same as those from cultures incubated throughout at 36°, showing that the effect on virus growth and interferon production were reversible. Moreover, the rates of cellular RNA and protein synthesis were stimulated twofold at 42°, compared with the rates at 36°, the rate of synthesis of all detectable RNA species being increased to the same extent. It is therefore unlikely that the temperature-sensitive event in interferon production is cell-directed, and the increased rate of interferon production by cells incubated at 42° may be a consequence of the general increase in macromolecular metabolism.

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REFERENCES


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