Temperature-sensitive Mutants of Newcastle Disease Virus Affecting Interferon Induction

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SUMMARY

Temperature-sensitive (ts) mutants of Newcastle disease virus (NDV) were isolated and studied for interferon (IFN) induction in primary chick embryo (CE) cells. At the non-permissive temperature (41 °C), there was no viral RNA synthesis or IFN induction by u.v.-treated virions except for ts-3 (RNA+), which did synthesize RNA at 41 °C, and whose u.v.-treated virions did induce IFN at this temperature. Another mutant (ts-4) induced IFN without irradiation, at the permissive temperature (37 °C). The minimum u.v. target size for IFN inducibility was unaffected by the mutation and corresponded to about 5% of the genome required for the expression of infectivity. These results support the hypothesis that the appearance of NDV RNA immediately after infection (primary transcription) plays a key role in IFN induction.

INTRODUCTION

Newcastle disease virus (NDV), a paramyxovirus, has been used extensively to study the induction of interferon (IFN) in various animal cells. Several reports have suggested that the action of the virion-associated RNA polymerase, and hence the early appearance of the complementary RNA strand of plus polarity, is involved in the events leading to cellular IFN synthesis (Clavell & Bratt, 1971; Meager & Burke, 1972; Sheaff et al., 1972).

One of the many ways of investigating the suggested dependence of IFN induction on viral RNA synthesis is to use virus mutants whose functions, including the synthesis of RNA, are restricted under definable conditions. With mutants of NDV that were defective in replication and RNA synthesis at the non-permissive temperature (42 °C), Kowal & Youngner (1978) showed that IFN induction by NDV in chick embryo (CE) cells occurred only when synthesis of viral RNA occurred. However, their temperature-sensitive (ts) mutants of RNA− phenotype induced IFN when the CE cells had been primed with IFN prior to inoculation. This observation appeared to add another complexity to the cellular events related to IFN induction. They interpreted the results by postulating that pretreatment with IFN resulted in an increased reactivity of CE cells so that a very small amount of the inducing substance(s) registered as an IFN-inducing stimulus.

The purpose of the present studies was to investigate early viral functions needed for cells to commence IFN synthesis.

METHODS

Cells. CE cells were prepared as described previously (Kohno et al., 1968) and were grown in Eagle's minimum essential medium (MEM) supplemented with 2% heated calf serum (CS).

Virus. The Miyadera strain of NDV, which had been plaque-puriﬁed three times, was used as wild-type (ts+) virus. Mutants were selected from progeny of the wild-type virus which had been grown in CE cells in the presence of 200 μg/ml 5-fluorouracil (5-FU) and 5% CS at 37 °C. Possible ts mutants were selected from plaques that showed no enlargement after transfer from 37 °C to 41 °C. They were further characterized as temperature-sensitive by defective growth at 41 °C (less than 1/100), and then plaque-puriﬁed three times.
**Seed stocks.** These were prepared by inoculating the mutants into the chorioallantoic sacs of 11-day-old fertile hens' eggs. The viruses were purified from the allantoic fluids of surviving eggs at 2 days post-infection as described previously (Kohase & Vilček, 1979). The revertant level of each seed stock virus was determined to be lower than 1/1000.

**Ultraviolet irradiation.** The virus suspension (2 ml) was appropriately diluted with phosphate-buffered saline (PBS), and was placed in a plastic dish (38 mm diam.) and mounted on a constantly shaking table (about 1 cycle/s), and irradiated by a 15 W u.v. lamp at a distance of 15 cm (about 6-5 J/m²/s). In experiments to determine u.v. target size, 1 ml of diluted virus suspension was irradiated in a glass dish with a flat bottom (10 mm diam.).

**Test for IFN production.** CE cells grown in plastic plates were used on the 3rd to 10th day of culture. Duplicate cell cultures were used in each sample. Cells were inoculated with u.v.-irradiated NDV (or live NDV, when appropriate) and incubated for 1 h at 4 °C. Subsequently, the cells were fed with MEM containing 5% CS and incubated for 20 h at 37 °C or 41 °C, by immersing the cell culture in water baths accurate to ±0.2 °C. The culture fluids were then harvested, heated at 60 °C for 1 h to eliminate any residual infectivity and stored at −20 °C until assayed.

**IFN assay.** IFN was titrated by the dye-uptake inhibition method (Finter, 1969), with some modifications. We used 96-well microplates to grow CE cells and infected them with Sindbis virus, as described in detail elsewhere (Kohase et al., 1982). The titres were converted to international reference units (MRC Research Standard A, 62/4).

**Early viral RNA synthesis (primary transcription).** NDV (ts+ or ts−) was added to CE cells at 4 °C using about 200 to 500 p.f.u./cell. After adsorption for 1 h, the inocula were removed and the cells were immediately fed with MEM containing 5 μg/ml actinomycin D and 50 μg/ml cycloheximide. Incubation was continued at 37 °C or 41 °C for 30 min prior to the addition of 40 μCi/bottle of 3H-labelled uridine. After labelling at 37 °C or 41 °C for 3-5 h, the acid-insoluble fraction of the cells was assayed for the radioactivity. The difference in ct/min between infected and control (uninfected) cells was regarded as virus-specific. In some experiments, late viral RNA synthesis was determined for confirmatory purposes.

**Chemicals.** [5-3H]Uridine was the product of Amersham International, cycloheximide and 5-FU were obtained from Sigma and actinomycin D was purchased from MAKOR Chemicals, Israel.

**RESULTS**

**Initial characterization of ts mutants**

Synthesis of the late viral RNA (5 to 9 h post-infection) was temperature-sensitive (RNA−) with most of the mutants. However, in CE cells infected with wild-type (ts+) strain or the ts-3 mutant, viral RNA was synthesized at an enhanced rate at 41 °C, indicating the RNA+ phenotype of ts-3 (data not shown).

Rates of viral primary transcription occurring at very early times after infection were estimated by the procedures given in Methods. Table 1 summarizes the results obtained with some representative mutants. The table shows that the early viral RNA was synthesized at both 37 °C and 41 °C in cells infected with either ts+ or ts-3 strains. Although leakiness was seen to various degrees with some mutants, primary transcription by all ts mutants was found to be inhibited by more than 80% of that at the non-permissive temperature. The results in the table are summarized from separate experiments, so that the exact counts incorporated can not be directly compared.

Complementation analysis of the ts mutants (data not shown) suggested that all RNA− mutants were in the same complementation group, while the ts-3 (RNA+) mutant showed a very high efficiency of complementation with the rest of the mutants, indicating that ts-3 is in a separate group. Since the growth of this mutant can be arrested by raising the incubation temperature to 41 °C at any time after infection, its temperature-sensitive lesion appears to be expressed at a later step in replication.

**IFN induction**

As has been reported with various strains of NDV (Youngner et al., 1966; Lomniczi, 1973), u.v. irradiation converted our mutants (other than ts-4) into inducers of IFN in CE cells (Table 1). The exceptional mutant, ts-4, like the heat- or low pH-treated wild-type Miyadera strain of NDV (Kohno et al., 1969), did not require u.v. treatment to induce IFN, indicating that a viral
### Table 1. Phenotype of RNA synthesis and IFN inducibility of ts mutants

<table>
<thead>
<tr>
<th>Virus clone</th>
<th>Virus-specific RNA synthesis (0.5 to 4 h; ct/min)</th>
<th>Ratio 41°C/37°C</th>
<th>RNA phenotype of primary transcription</th>
<th>IFN yield (units)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3,849/4455</td>
<td>1.15 +</td>
<td>Unirradiated 37°C 41°C Irradiated 37°C 41°C</td>
<td></td>
</tr>
<tr>
<td>ts-1</td>
<td>7,019/363</td>
<td>0.05 -</td>
<td>&lt;10 10 &lt;10 10 &lt;10 10 &lt;10 10</td>
<td></td>
</tr>
<tr>
<td>ts-2</td>
<td>2,617/126</td>
<td>0.05 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-3</td>
<td>8,673/8051</td>
<td>0.93 +</td>
<td>&lt;10 10 &lt;10 10 10 200 &lt;10 10</td>
<td></td>
</tr>
<tr>
<td>ts-4</td>
<td>2,486/0</td>
<td>0.01 -</td>
<td>96 &lt;10 10 &lt;10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-8</td>
<td>1,057/128</td>
<td>0.12 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-10</td>
<td>1,5428/2,346</td>
<td>0.15 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-117</td>
<td>1,050/1449</td>
<td>0.14 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-119</td>
<td>1,983/336</td>
<td>0.17 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-126</td>
<td>2,387/445</td>
<td>0.19 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
<tr>
<td>ts-128</td>
<td>2,238/283</td>
<td>0.13 -</td>
<td>&lt;10 10 &lt;10 10 10 &lt;10 10 &lt;10</td>
<td></td>
</tr>
</tbody>
</table>

* Primary CE cells cultured for 3 days at 37°C in 24-well Falcon plastic plates were infected with u.v.-irradiated (15 s) or unirradiated NDV (m.o.i = 40). After 1 h adsorption at 4°C, unadsorbed viruses were removed, and the cells were re-fed with 1 ml/well of production medium (see Methods).

function inhibitory to the induction of IFN is lost through mutation. Table 1 also shows that the IFN induction by either irradiated or untreated ts (RNA-) virions is temperature-sensitive, whereas that by ts-3 (RNA+) or by the wild-type strain is not.

The inability of RNA- ts mutants to induce IFN is not due to their inactivation in situ at 41°C. CE cells, which had been inoculated with the mutants and incubated for 6 h at 41°C, produced IFN when the temperature was lowered to 37°C (data not shown).

In order to find out when the NDV viral function leading to IFN induction was expressed, CE cells were infected with ts mutants and incubated at 37°C for various time periods before the incubation temperature was shifted up to 41°C. By determining yields of IFN at 20 h (data not shown), the temperature-sensitive virus function was found to be supplied within 2 to 3 h of incubation at 37°C.

### Cellular state and IFN induction

As has been reported by Kowal & Youngner (1978) with primed CE cells, our ts mutants were also capable of inducing IFN at 41°C in CE cells when the cells had been either primed with 20 units of chick IFN overnight, or aged (Carver & Marcus, 1967) in vitro for 10 days (Table 2). However, when these cells were induced for IFN production by inoculating with ts mutants at lower m.o.i. (10 or less p.f.u., before irradiation, per cell), IFN induction was shown to be temperature-sensitive (Table 2).

### Virus genome size required for the expression of IFN inducibility

The above results supported the view that induction of IFN is related to the primary transcription of virus RNA. Accordingly, we performed a series of experiments in which the relative u.v. target size of viral infectivity and that of the RNA template for the early transcription leading to IFN induction were estimated. Virus suspensions were treated with u.v. (Methods) and inoculated on 10-day-old CE cells at a multiplicity of infection of about 2 (before irradiation). Under these conditions, maximum IFN yield was induced by virus irradiated for 10 s. Because unirradiated or weakly irradiated (less than 10 s) viruses induced insufficient IFN (as has already been found by many other investigators), for the estimation of target size we omitted these results.

Fig. 1 shows the survival of infectivity and of the potency to induce IFN after such treatment. Although the slopes of the curves differ markedly for the two parameters, in both cases decay followed first-order kinetics. In addition, it must be noted that the slopes of the decay of infectivity and of IFN inducibility were unaltered by the ts mutation. In separate experiments
### Table 2. Effect of multiplicity of infection of u.v.-irradiated ts mutants on IFN induction in primed or aged cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Virus clone</th>
<th>Primary transcription</th>
<th>Temperature (°C)</th>
<th>IFN yield (units) at m.o.i. of 40</th>
<th>IFN yield (units) at m.o.i. of 10</th>
<th>IFN yield (units) at m.o.i. of 2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primed*</td>
<td>Wild-type</td>
<td>+</td>
<td>37</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td>90</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>ts-117</td>
<td>−</td>
<td>37</td>
<td>280</td>
<td>140</td>
<td>80</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td>200</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>ts-128</td>
<td>−</td>
<td>37</td>
<td>140</td>
<td>110</td>
<td>60</td>
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<td></td>
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<td></td>
<td>41</td>
<td>140</td>
<td>30</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Aged†</td>
<td>Wild-type</td>
<td>+</td>
<td>37</td>
<td>3200</td>
<td>2500</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>ts-3</td>
<td>+</td>
<td>37</td>
<td>3500</td>
<td>1000</td>
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<td></td>
<td></td>
<td></td>
<td>41</td>
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<td>2200</td>
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<tr>
<td></td>
<td>ts-117</td>
<td>−</td>
<td>37</td>
<td>2900</td>
<td>1400</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td>1200</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>ts-128</td>
<td>−</td>
<td>37</td>
<td>3000</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td>250</td>
<td>100</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Three-day-old primary CE cells were primed with IFN (20 units) for 20 h.
† Aged for 10 days in six-well Costar plastic plates.

(data not shown), it was found that the slopes remained unchanged when tested in the presence or absence of priming, in freshly prepared cells or at 41 °C. From the slopes, the relative target sizes for the infectivity and IFN inducibility were calculated by regression analysis as $-0.68 \pm 0.01$ and $-0.031 \pm 0.01$ respectively. The results indicate that at least 5% of the viral RNA has to be intact for the virion to act as an IFN inducer.

### DISCUSSION

Using mutagen-induced and spontaneously occurring ts mutants of NDV, Kowal & Youngner (1978) suggested an involvement of viral RNA synthesis in the events leading to an induction of IFN in CE cells. At the non-permissive temperature, where functioning of the viral RNA-dependent RNA polymerase was suppressed, their mutants (which had been irradiated with u.v.) failed to induce IFN. Similar dependence of IFN inducibility on de novo synthesis of viral RNA has been reported by others with ts mutants of Sindbis virus (Atkins & Lancashire, 1976), Semliki Forest virus (Lomniczi & Burke, 1970), and vesicular stomatitis virus (Sekellick & Marcus, 1979).

Kowal & Youngner (1978) also reported that their ts mutants induced IFN at the non-permissive temperature in CE cells which had been primed by IFN. This apparently conflicting finding led them to postulate a cellular function which in some way was involved in the process of induction and/or synthesis of IFN.

In this report, we have reproduced their finding with our ts mutants. In addition, we observed a similar rescue of IFN-inducing ability of ts virus mutants in aged CE cells. The rescue by priming or by ageing became less evident when the multiplicity of IFN-inducing virion was lowered. The RNA− mutants so far tested were as temperature-sensitive for primary transcription in primed cells (data not shown) as in unprimed young CE cells (Table 1).

These results can be interpreted as follows. Firstly, these pretreatments are known to enhance the reactivity of CE cells to IFN-inducing stimuli (Stewart et al., 1971). Secondly, a very small amount of ts virus RNA is synthesized at the non-permissive temperature. However, if this is so, the RNA must be made in such a minute quantity that it cannot activate the cells for IFN synthesis unless they have been sensitized by the above treatments. Finally, the synthesis of viral transcript(s) will increase in proportion to the multiplicity of infection up to a level recognizable
by the cells as the IFN-inducing stimulus. None of our isolates was defective for IFN induction at 37 °C. Screenings for such mutants (McKimm & Rapp, 1977; Kowal & Youngner, 1978) so far have been negative.

The emergence of IFN induction ability in u.v.-treated NDV is not a direct consequence of the loss of their infectivity. As evidenced here with ts-4 and elsewhere with some strains of NDV (Lomniczi, 1973) and with acid- or heat-treated NDV (Kohno et al., 1969), NDV virions with full infectivity occasionally have been shown to be good inducers of IFN. We have no explanation for these results at this time.

Finally, our studies revealed that IFN induction by NDV requires about 5% of the total genome, corresponding to $2.5 \times 10^5$ to $3 \times 10^6$ daltons, irrespective of the mutation and/or other experimental conditions involved. This value is smaller than that reported for vesicular stomatitis virus (10%, about $6 \times 10^5$ daltons; Marcus & Sekellick, 1980) and that determined for Sindbis virus (25%, $1 \times 10^6$ daltons; Marcus & Fuller, 1979) in aged CE cells. We also conclude that the NDV virion does not contain a preformed inducer of IFN in the CE cell system.

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REFERENCES


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