Interferon Induction by Viruses. II. Sindbis Virus: Interferon Induction Requires One-Quarter of the Genome—
Genes G and A

By PHILIP I. MARCUS AND FREDERICK J. FULLER

Microbiology Section, U-44, Biological Sciences Group,
The University of Connecticut, Storrs, Connecticut 06268, U.S.A.

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SUMMARY

We have measured the amounts of interferon formed by chick cells ‘aged’ in vitro in response to different amounts of infectious wild-type Sindbis virus. Our results suggest that one plaque-forming unit is enough to induce maximum interferon formation. With higher m.o.i. the yield of interferon is less.

To inactivate the interferon-inducing activity of Sindbis virus, four times more u.v.-radiation was needed than to inactivate the infectivity of the virus. This suggests that only 25% of the virus genome need be intact in order to induce interferon. Temperature-sensitive Sindbis virus mutants from the three RNA+ complementation groups, C, D and E, gave rise to interferon in chick cells incubated at a non-permissive temperature. Similarly, mutants from two of the RNA- groups, B and F, gave rise to interferon, but not mutants from groups G and A.

We conclude that no pre-formed inducer of interferon is present in Sindbis virus. It appears, however, that genes G and A represent a special one-quarter of the genome which must be functional in order to synthesize an interferon-inducing moiety. We suggest that this moiety is a double-stranded RNA molecule formed after synthesis of a segment of RNA complementary to the genome.

INTRODUCTION

There have been many attempts to define what molecules and synthetic events are involved in the production of interferon in response to viruses (see reviews by Burke, 1973; Johnston & Burke, 1973). Most investigators have concluded that some early virus functions are required in order to synthesize a threshold amount of virus double-stranded (ds) RNA—presumed to be the inducer. We have recently provided compelling evidence that dsRNA is indeed the inducer and that a single molecule per cell is the threshold amount required to induce synthesis of interferon (Marcus & Sekellick, 1977; Marcus et al. 1978). We demonstrated that [+]-RNA defective-interfering (DI) particles of vesicular stomatitis virus (VSV), strain DI-oii, which contain covalently-linked self-complementary [+]-message and [-] anti-message (genome) RNA in a single-stranded ribonucleoprotein complex within the subvirion (Lazzarini et al. 1975; Perrault & Leavitt, 1978) are extraordinarily efficient inducers of interferon. A single non-replicating physical particle of DI-oii induced a quantum response of interferon, and thus represents an interferon-inducing particle (i.f.p.). Conventional [-]-RNA DI particles with the same virus polypeptide composition as [+]-RNA DI particles did not induce interferon. We postulated that a single molecule of dsRNA, which is presumed to form when an interferon-inducing particle enters the cell, constitutes the
actual (or proximate) moiety inducing interferon synthesis (Marcus & Sekellick, 1977; Marcus et al. 1978). This conclusion was based primarily on the unusual nature of the dose (multiplicity)-response (interferon yield) curve and its good fit to the Poisson distribution of cells infected with only one DI particle (Marcus & Sekellick, 1977).

We have now sought to determine whether this unusual dose-response curve is unique to non-replicating [±]RNA DI-011 particles and for comparison we have used infectious Sindbis virus, an excellent inducer of interferon.

We show that (i) a single infectious particle of Sindbis virus is sufficient to induce a quantum response of interferon, (ii) only a quarter of the virus genome need be functional to produce the interferon-inducing moiety and (iii) the virus genes defined by complementation groups G and A appear to represent this quarter of the genome. We also provide further evidence suggesting that a single molecule of dsRNA is the actual interferon-inducing moiety formed in response to a virus.

METHODS

**Cells and medium.** Primary chick embryo cells from 9- to 10-day-old embryos were plated in 50 mm plastic dishes containing 5 ml of NCI medium (Marcus & Carver, 1965) with 6% calf serum, incubated at 37.5 °C in an incubator flushed with a humidified mixture of 5% CO₂-95% air and ‘aged’ *in vitro* for 6 to 9 days (Carver & Marcus, 1967; Marcus & Sekellick, 1977). A dish inoculated with about 1 × 10⁷ cells produced a confluent monolayer within 1 or 2 days and contained a relatively constant number of cells thereafter, about 1 × 10⁷ per plate, with few, if any, mitotic cells. Cells cultured and ‘aged’ under our conditions produce relatively large amounts of interferon upon appropriate treatment with inducers and are more responsive to the action of interferon (Carver & Marcus, 1967; Marcus & Sekellick, 1977).

**Viruses.** Wild type Sindbis virus was obtained originally from John F. Enders. The ‘heat-resistant’ (HR) wild type Sindbis virus and its temperature-sensitive (ts) mutants were kindly provided by Elmer R. Pfleiferkorn (Pfeiferkorn & Burge, 1967) and by Ellen and James Strauss (Strauss et al. 1976). Stock virus was grown in baby hamster kidney (BHK-21) cells inoculated at a m.o.i. for chick cells (m.p.f.u.) of ≈ 0.01 and incubated at 30 °C for 36 to 40 h, and contained 0.5 to 1 × 10⁹ p.f.u./ml, with very few (if any) defective particles (Schlesinger et al. 1972). Some virus stocks of ts mutants were initiated from individual plaques to minimize the number of wild type revertants. Some ts mutants inherently produced stocks with a relatively high frequency of wild type revertants, which, however, usually contributed little or nothing to the interferon yield because interferon was induced at a m.p.f.u. of approx. 1. One p.f.u. equals 1 plaque-forming particle.

**Inactivation of virus by u.v. radiation.** Wild type Sindbis virus was inactivated with u.v. radiation, as previously described (Marcus & Sekellick, 1975). The germicidal lamp produced a flux of 83 ergs/mm²/10 s.

**Interferon induction.** Monolayers of aged chick cells were drained and inoculated with 0.3 ml of attachment solution (NCI medium + 6% calf serum) containing the desired amount of Sindbis virus to allow attachment. After 30 min at 4 °C, unadsorbed virus was aspirated, 3.0 ml of NCI medium warmed to 40.5 °C were added and the plates were incubated for 24 h, a time which we have determined is sufficient to obtain peak yields of interferon (data not shown). Serum was omitted from the medium because some batches had a deleterious effect on the yield of interferon (P. I. Marcus, M. J. Sekellick and F. J. Fuller, unpublished observations). Unless otherwise noted interferon was induced at 40.5 °C—a temperature which produced maximal yields of interferon by wild type virus (cf. Fig. 3).

**Interferon assays.** Interferon samples were prepared for assay by treatment with perchloric acid at 0.15 M final concentration for 15 to 20 h at 4 °C, followed by neutralization.
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Interferon was assayed on monolayers of ‘aged’ chick cells as follows: 0.1 ml of an appropriate dilution of interferon (in NCI medium) was added directly to the 5 ml of spent growth medium in the assay plate. Following incubation for 24 h at 37.5 °C, the plates were drained and challenged with about 100 p.f.u. of wild type vesicular stomatitis virus (VSV), Indiana strain, prepared as described by Marcus & Sekellick (1977). The stock VSV did not itself induce interferon (Sekellick & Marcus, 1979). All assays were carried out in duplicate. Plots of the reciprocal of the interferon dilution versus the per cent reduction in plaque number were used to determine the 50% plaque reduction titre (PR$_{50}$). Yields of interferon were recorded in terms of the $1 \times 10^7$ cells which constituted the induction monolayer. However, under most induction conditions not all cells were infected and hence potentially capable of contributing to the final yield of interferon – only the fraction representing the $r \geq 1$ class in the Poisson distribution (Marcus, 1959; Marcus & Sekellick, 1977). Medium from mock-induced cell monolayers never contained any interferon.

In our assay, 1 PR$_{50}$ unit was equivalent to 10 to 25 units, in terms of the research reference chick interferon standard 63/A, kindly supplied by the National Institutes of Health Resources Center. As an internal control to monitor the sensitivity of the assay cells, we routinely included our own standard of chick interferon in all assays.

RESULTS

Interferon induction with Sindbis virus: dose (multiplicity)-response (interferon yield) curve

The results illustrated in Fig. 1 are typical of eight independent experiments. They show the characteristic dose-response curve obtained with ‘aged’ chick cells infected at various multiplicities with a high titred (low DI particle) stock of wild type Sindbis virus. The amount of interferon in the medium 24 h p.i. was critically dependent upon the multiplicity of infection. Initially, an increase in the multiplicity (measured as p.f.u.) led to a precipitous increase in the yield of interferon, with the peak at a multiplicity of approx. 1 (the values ranged from $m \simeq 0.5$ to 2 in the eight experiments). With further increases in multiplicity, the yield of interferon fell rapidly, and at $m \simeq 5$, the amount accumulated was often 100-fold less than the peak yield. In some experiments this decline phase was followed by a plateau, indicating that cells infected with $m \geq 5$ continued to produce 20 to 30% of the peak yield of interferon.

This dose-response curve is similar to that described earlier for a non-replicating inducer of interferon, the $[\pm ]$RNA defective-interfering particles of VSV strain DI-011, where yields of interferon peaked at about 1 physical particle per cell and the shape of the curve allowed definition of the population of virions in terms of i.f.p. (Marcus & Sekellick, 1977). In five experiments, the Sindbis virus dose-response curves best fitted a similar model. However, with some lots of cells, although the general shape of the dose-response curve remained characteristically the same, the best fit was to a model in which it is assumed that the virus population contained two to three times more i.f.p. than p.f.u. and that peak yields of interferon were obtained from those cells infected with 2 or 3 i.f.p. (representing, respectively, the $r = 2$ and $r = 3$ class, of infected cells as calculated from the Poisson distribution, $P_r = e^{-m} m^r/r$, where $m =$ multiplicity of i.f.p.; Marcus, 1959; Marcus & Sekellick, 1977).

Ultraviolet light survival curves for plaque-forming particle and interferon-inducing particle activity of Sindbis virus

The survival curves for Sindbis virus p.f.u. and i.f.p. activity were determined by exposing stock wild type virus for various intervals to an u.v. lamp. In Fig. 2, the survival curve for p.f.u. activity represents the average of eight different experiments, with individual points varying less than $\pm 15\%$ from the mean value. The linear curve indicates that a single u.v.
Fig. 1. Dose-response curve relating multiplicity of infection of 'aged' chick embryo cells with wild type Sindbis virus to the interferon yield.

![Dose-response curve](image)

Fig. 2. U.v. survival curves for wild-type Sindbis virus p.f.u. activity (●—●) and i.f.p. activity (○—○). Surviving p.f.u. activity was determined by plaque assay on chick embryo cells with or without 0.01 µg/ml actinomycin D included in the agar overlay (Carver & Marcus, 1967). When i.f.p. activity was measured after different doses of radiation, cell monolayers were infected (induced) with the same constant dilution of virus, which with unirradiated virus induced maximal levels of interferon (mp.f.u.m.i.p. = 1).

The curve for residual i.f.p. activity was obtained by infecting cells with a constant amount of virus, equivalent to m.p.f.u.m.i.p. = 1 for the unirradiated sample. The 24 h yields of interferon induced by irradiated virus are plotted in Fig. 2 as the fraction of surviving activity. The experimental points on the i.f.p. curve represent the average of seven different experiments in which individual determinations varied by ±50% from the mean value. The 'hit' to the virus genome inactivates infectivity; the u.v. dose for the 37% (1/e) survival was 101 ergs/mm².
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Table 1. Interferon-inducing particle phenotypes of Sindbis virus ts-mutants

<table>
<thead>
<tr>
<th>Phenotype*</th>
<th>Complementation group†</th>
<th>ts-mutant designation</th>
<th>Interferon yield at 40.5 °C (units/10⁷ cells)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>tsRNA−,t.l.p.−</td>
<td>G</td>
<td>ts7</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ts18</td>
<td>660</td>
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<tr>
<td></td>
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<td>ts15</td>
<td>1000</td>
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<td></td>
<td></td>
<td>ts24</td>
<td>&lt;150</td>
</tr>
<tr>
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<td>A</td>
<td>ts11</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ts6</td>
<td>26100</td>
</tr>
<tr>
<td>tsRNA+,t.l.p.+</td>
<td>B</td>
<td>ts5</td>
<td>28808</td>
</tr>
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<tr>
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<td></td>
<td>57000</td>
</tr>
<tr>
<td>Wild type</td>
<td>W+(Enders)</td>
<td></td>
<td>48000</td>
</tr>
</tbody>
</table>

* RNA− mutants (tsRNA−) at non-permissive temperatures generally produce ≤1% of the level of RNA synthesized by wild type virus, whereas RNA+ mutants (tsRNA+) synthesized 50 to 100% of wild type levels (Pfefferkorn & Burge, 1967; Atkins et al. 1974; Strauss et al. 1976).
† Complementation groups and mutant designations are according to Pfefferkorn & Burge (1967) and Strauss et al. (1976). Mutants ts11(B) and ts6(F) were once considered as members of complementation groups designated A′ and B, respectively (Zuckerbraun & Marcus, 1971).
‡ Monolayers of aged chick embryo cells were infected at mₚₜₖₕₐₜ= 1 with wild type or ts mutant Sindbis viruses and incubated for 24 h.
§ Atkins et al. (1974) reported that mutant ts5(C) was as good an interferon inducer as wild type virus at 39°C, but induced little, if any, interferon at 42°C. Our relatively low yield of interferon at 40.5°C suggests that ts5 induces interferon at elevated temperatures, albeit much less efficiently.

amount of interferon induced in the different experiments by the non-irradiated virus varied between 15 x 10³ and 15 x 10⁴ PR₅₀ units/10⁷ cells. The survival curve again is one-hit in nature, indicating that a single hit suffices to inactivate the capacity of the virion to induce interferon. However, the 37% (1/e) survival dose of 399 ergs/mm² shows that four times more u.v. radiation is required to inactivate i.f.p. activity than to inactivate infectivity (p.f.u.). Thus, only a quarter of the virus genome need be intact to induce interferon (see Discussion). We next sought to determine whether this quarter of the genome represents a particular region of the virus RNA.

Interferon induction by ts mutants of Sindbis virus

Ts mutants representative of the seven known complementation groups of Sindbis virus (Burge & Pfefferkorn, 1968; Strauss et al. 1976) were tested for their capacity to induce interferon at a permissive (30°C) and a non-permissive (40.5°C) temperature. Tests were conducted at mₚₜₖₕₐₜ= 1 in order to eliminate or minimize the contribution of revertants to wild type virus and genetic-leak to the induction process. Under these conditions all the ts mutants and wild type virus gave rise at 30°C to comparable large amounts (several thousand units/10⁷ cells) of interferon (data not shown). When tested at 40.5°C, only ts mutants in RNA− complementation groups G (ts7, ts18) and A (ts15, ts24) failed to induce significant levels of interferon (Table 1). Our results with mutants ts7 (group G) and ts15 (group A) confirm those of Atkins et al. (1974) carried out at m = 5 (and in the case of ts7, also at mₚₜₖₕₐₜ = 50). In addition, they reported ts24 (group A) as a non-inducer. We can therefore designate these mutants in groups G (ts7, ts18) and A (ts15, ts24) as having a tsRNA− phenotype. We infer that the induction of interferon by Sindbis virus requires functional proteins encoded by genes G and A and that these two genes represent the one-fourth of the virus genome which must survive u.v. radiation to express the i.f.p. phenotype (see Discussion).

All of the other ts mutants (whether RNA− or RNA+) and wild type virus induced con-
considerable amounts of interferon under our experimental conditions and hence are classified phenotypically as \( ts^{i.f.p. +} \) (Table 1). These data essentially confirm those of Atkins et al. (1974) with respect to the Burge and Pfefferkorn (1968) \( ts \) mutant tested at 39 °C and \( m_{p.f.u.} = 5 \).

The regulation of the absolute amounts of interferon induced by different \( ts^{i.f.p. +} \) mutants appears complex and will be considered in a subsequent communication. However, we have measured the expression of the i.f.p. character from wild type and mutants of groups G, A and D as a function of temperature. Fig. 3 demonstrates that for wild type virus, increasing the induction temperature from 30 °C resulted in a steady increase in interferon yield, usually with a peak at about 40 °C, and a sharp decline thereafter – a result very similar to that reported by Isaacs (1962) for another togavirus, ‘Kumba’ (a strain of Semliki Forest virus). With the two \( ts^{i.f.p. -} \) mutants, \( ts^{15}(A) \) and \( ts^{18}(G) \), increasing the induction temperature from 30 °C resulted in peak production of interferon at 34.5 °C followed by a steady decline thereafter and no interferon at \( > 38.5 \) °C. With the one \( ts^{i.f.p. +} \) mutant tested, \( ts^{10} \) (D), the temperature optimum was 36.5 °C for peak interferon production and there was a much higher cut off temperature than with the \( ts^{i.f.p. -} \) mutants, which it otherwise resembled more closely than wild type virus.

**DISCUSSION**

The dose–response curve relating yields of interferon from ‘aged’ primary chick cells to the m.o.i. with wild type Sindbis virus has the same characteristic shape as that obtained with VSV strain DI-011 (Marcus & Sekellick, 1977). This shows that a single infectious
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particle of Sindbis virus induces a quantum response of interferon, so that virus stocks can be defined in terms of interferon-inducing particles. In cells infected with an average of two or more i.f.p. of Sindbis virus, there was a marked reduction in the yield of interferon; here again the system resembles that previously noted with VSV, strain DI-011. These results can be contrasted with the more usual dose–response curve where yields of interferon increase steadily with multiplicity until a plateau is reached at a multiplicity of five or more (Fleischmann & Simon, 1974; Marcus et al. 1978). However, we are continuing to study this ‘unusual’ dose–response curve in ‘aged’ chick cells, in the belief that it may be more representative of what is found in vivo than the more conventional response seen with cells of established lines.

We have presented evidence indicating that the DI-011 particle contains a preformed inducer of interferon (Marcus & Sekellick, 1977). However, we believe that this is not so for wild type Sindbis virus for the following reasons. First, Sindbis virus i.f.p. are sensitive to low doses of u.v. irradiation, unlike DI-011 particles. Second, pre-treatment of cells with interferon reduces the interferon-inducing capacity of Sindbis virus in proportion to the amount of interferon used (our unpublished observations).

Treatment of Sindbis virus with u.v. leads to loss of i.f.p. activity with one hit to inactivate kinetics. However, only one quarter as much irradiation is needed to render Sindbis virus incapable of replicating. Thus, a virion rendered non-infectious by u.v. radiation can still induce interferon provided that a quarter of the genome remains functional. This information does not distinguish between the requirement for survival of a particular quarter of the genome, or a quarter at random.

When we studied ts mutants of Sindbis virus at non-permissive temperatures, we found that only RNA− mutants belonging to complementation groups G and A failed to induce interferon (Table 1). These results completely confirm those of Atkins and co-workers (1974). We thus propose that polypeptides translated from genes G and A are required to produce an interferon-inducing moiety.

From the various facts discussed above and findings of other workers, we present the following hypothesis to account for interferon induction by Sindbis virus and probably by other viruses as well. We have already suggested (Marcus & Sekellick, 1977) that with DI-011 particles of VSV, the actual or proximate moiety leading to induction of interferon is one molecule of dsRNA. We postulated that this is formed from one molecule of covalently linked single-stranded and self-complementary [±]RNA, when the ribonucleoprotein complex is deproteinized on entry into a cell (Lazzarini et al. 1975; Marcus & Sekellick, 1977). It is clear that when a cell is presented with more than one molecule of dsRNA made in this way, it may respond differently, and produce significantly less interferon. Because the multiplicity-interferon yield dose–response curve obtained with Sindbis virus resembles that obtained with DI-011 particles, it is tempting to suggest that the ‘aged’ chick cells similarly respond to a p.f.u. of Sindbis virus as if it was a non-replicating inducer of interferon. We suggest again that a single interferon-inducing moiety is formed de novo from each genome strand of Sindbis RNA, possibly in the manner outlined in the following paragraph.

In our studies with ts mutants of Sindbis, we found that mutants with the RNA− phenotype belonging to complementation groups B and F induced interferon in the apparent absence of RNA synthesis. We suggest that there is in fact limited synthesis of virus RNA at non-permissive temperatures by these mutants, even though the products translated from genes B and F are non-functional with respect to formation of replicating virus. The amount of RNA formed may be below the limits of biochemical detection, but the presence of a single molecule of dsRNA is detected when its action is amplified through the induction of interferon. The synthetic activity required to produce one such molecule of dsRNA would be minimal if part of the parental genome strand formed one-half of the helical structure; and indeed also if only six to twelve base pairs, or half to one turn of the helix,
are required to induce interferon, as suggested by Greene and co-workers (1978) for poly(rI). poly(rC). By drawing in part on the Simmons & Strauss (1972) scheme for the replication of Sindbis virus, we can hypothesize what initial synthetic reaction in an infected cell might produce this molecule of dsRNA. We assume that genes G and A represent the nucleotide sequences nearest to the 5' end of the genome RNA, and produce proteins G and A. These may function as polymerase (Brzeski & Kennedy, 1977) and initiate synthesis of RNA at the 3' end of the genome (where genes C, E and D are located). We postulate that this generates a single dsRNA molecule in the form of a transcriptive intermediate for synthesis of the 26S mRNA or the 42S genome strand (Simmons & Strauss, 1972), and that this intermediate is the actual interferon-inducing moiety. The relative u.v. resistance of i.f.p. activity suggests that the entire transcriptive intermediate is not required; amplified synthesis of RNA is also not involved since this would require, in addition to gene products G and A those of genes B and F, and the total target for u.v. radiation would be much larger than that observed.

The relatively small amount of u.v. required to destroy both p.f.u. and i.f.p. activity suggests that only a little damage to the Sindbis virus genome is required, most likely the formation of uracil dimers (Miller & Plagemann, 1974). Such dimers cause termination of translation and premature release of polypeptide chains (Grossman, 1963). Cancedda et al. (1975) have shown that the 42S genome mRNA of Sindbis virus contains only one initiation site at the 5' terminus. It then follows that the target for u.v. inactivation of i.f.p. activity is probably the proximal quarter of the 5' end of the Sindbis virus genome and not a quarter of the genome at random.

Comparison of our data with ts mutants and those of Atkins et al. (1974) shows significant differences in the relative amounts of interferon induced by the different mutants. No doubt this represents the particular experimental conditions used and the complex nature of the system where many factors may play a part. For example, we have found that defective particles of Sindbis virus are excellent inducers of interferon (Fuller & Marcus, 1979) and for this reason have used only high titred virus preparations derived from infection at low multiplicity in the studies reported here.

It has previously been postulated that interferon induction by viruses may require a low threshold level of RNA synthesis, occurring early in infection and that formation of virus-specified dsRNA may be the critical event (see, for example, the review by Johnston & Burke, 1973 and Atkins et al. 1974; Atkins & Lancashire, 1976). Our data confirm this view and suggest that only one molecule of dsRNA may be required to stimulate induction of interferon.

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REFERENCES


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