Molecular Aspects of Interferon
Induction by Viruses

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ABSTRACT Virus-induced interferon formation depends on the presence within the cell of a viral ribonucleic acid. This RNA may either be double stranded or, in certain cases, single stranded. The double-stranded RNA can be derived from a virus, such as reovirus, which contains this type of RNA, or it may be synthesized within the cell using viral single-stranded RNA as a template. Single-stranded RNA must possess a stable configuration in solution to be active, and certain viral RNA molecules appear to be active for this reason. The presence of this RNA triggers a derepression event, which is probably nuclear, by an unknown mechanism, and this is followed by the production of an interferon messenger RNA and its translation. Little is known of the derepression event or the events that follow it.

Interferon was discovered during a study of virus-induced interference, and despite the recent spate of work using polynucleotides as inducers, they are still probably the most widely studied and best understood class of interferon inducers. They have a number of advantages as inducers; there are very many viruses which differ not only as to the type of nucleic acid they contain, but as to whether the nucleic acid is single or double stranded or whether it is a single polynucleotide chain or in pieces. Since temperature-sensititive mutants of a number of animal viruses have now been isolated and characterized, these can be used too; and finally it must be remembered that all the available evidence suggests that it is the viruses that are responsible for interferon formation in vivo, and in studying virus-induced interferon formation we are looking at a process which occurs under normal physiological conditions.

The induction of interferon by viruses may be represented diagrammatically as shown in the Fig. 1. The virus, which may be either infective or, in certain cases, inactivated, enters the cell and there uncoats. This nucleic acid then interacts either directly or indirectly with the host genome to induce the formation of a new messenger RNA, which then codes for the protein interferon. The process can thus be divided into the following three stages: the events prior to the derepression step, the derepression step itself, and the...
subsequent translation of the interferon messenger RNA. It is convenient to consider these stages in turn.

EARLY EVENTS IN INTERFERON FORMATION

When a virus particle invades a cell, it may initiate one or both of the following two chains of events: those leading to virus multiplication and those leading to interferon formation. The general approach has been to discover what stages in virus multiplication are essential for the formation of interferon, i.e., at what stage do the two processes leading to interferon formation and virus multiplication part company?

A number of lines of evidence pointed to the importance of the viral nucleic acid as the inducer (reviewed in reference 1), and the interferon inducing capacity of the synthetic polynucleotides strongly support this idea. But there is more direct evidence, for the double-stranded nucleic acid from reovirus and the double-stranded replicative forms synthesized during replication of MS2 bacteriophage, of encephalomyocarditis virus, or of vaccinia virus, all induce interferon formation in vivo (2–5). The viral coat proteins are, therefore, not essential for the induction of interferon formation. In all these cases the inducer was a double-stranded RNA, and since single-stranded viral nucleic acids failed to produce interferon formation (5, 6), it was suggested that the formation of double-stranded ribonucleic acid is essential for inter-
feron formation. If this is so, then it is easy to see how double-stranded RNA viruses can induce interferon formation, but how do the single-stranded RNA viruses and the double-stranded DNA viruses induce the formation of interferon?

As mentioned above, the double-stranded replicative forms which can be isolated from cells infected with single-stranded RNA viruses were potent interferon inducers, and since such species may be formed during the replication of all single-stranded RNA viruses, it is possible that these viruses have to form double-stranded RNA before they can induce interferon formation. Experimental evidence to support this idea came from the work of Skehel and Burke (7, 8). They found that when Semliki Forest virus, which had been inactivated by hydroxylamine, was used for infection of cells in tissue culture that the capacity of the virus to produce infectious virus, virus hemagglutinin, virus RNA synthesis, virus RNA polymerase, and interferon were all inactivated with first order kinetics. This suggested that functional viral RNA was required for interferon induction as well as for the other viral coded properties measured. But what function did the viral RNA have to carry out? Interferon formation in this system was induced in a temperature-shift system, in which the cells were infected at 37°C and then incubated at 42°C. Under these conditions, interferon, but not virus, was produced. Further examination of the system showed that there was a requirement for incubation at 37°C (Table I); if the cells were infected at 42°C and then incubated at 37°C for a period as short as 15 min, then interferon was formed. It was also shown that virus RNA synthesis, which cannot proceed at 42°C, occurred during a short incubation at 37°C, and it appeared that synthesis of viral RNA in the infected cells was essential for interferon formation. It is known that three species of viral RNA are made in the infected cells: two single-stranded species and one double-stranded species (9). It seemed likely that it was the formation of the double-stranded RNA that was the essential step in interferon formation.

TABLE I
EFFECT OF INCUBATION AT DIFFERENT TEMPERATURES ON INTERFERON FORMATION

| Temperature of infection | Time incubated at 37°C | Incubation temperature | Interferon yield (PDD10) |
|--------------------------|------------------------|------------------------|-------------------------|
| 42°C                     | 15 min                 | 42°C                   | <5                      |
| 42°C                     | 30 min                 | 42°C                   | 66                      |
| 42°C                     | 60 min                 | 42°C                   | 127                     |
| 42°C                     | 90 min                 | 42°C                   | 187                     |

Cells were infected at 42°C and incubated at 37°C for different times before reincubation at 42°C and interferon assay.
Interferon production induced by the related Sindbis virus was also shown by Lockart, Bayliss, Toy, and Yin (10) to be dependent on a short period of incubation at a temperature permissive for virus multiplication. These authors showed too that viral protein synthesis was essential for interferon formation, and this would be expected since viral RNA polymerase must be formed before viral RNA synthesis is possible. They explored the situation further by making use of a series of temperature-sensitive mutants of Sindbis virus. The wild type multiplies and produces interferon at 42°C, but an RNA+ mutant, which did not multiply at 42°C but made viral RNA, did not make interferon at 42°C, and the authors concluded that viral processes, other than RNA synthesis, were required for cells to make their full yields of interferon. This was a very puzzling finding, and the situation was further examined by Lomniczi and Burke using a series of temperature-sensitive mutants of Semliki Forest virus. They found that the wild type and all the RNA- mutants could make interferon if viral RNA synthesis was allowed to occur (Table II), and that the RNA+ mutants could also make interferon if viral RNA synthesis was allowed to occur and if the multiplicity of infection was sufficiently high (Table III). At lower multiplicities of infection with the RNA+ mutants, virus RNA but not interferon was formed. Thus in cells infected with either Sindbis or Semliki Forest viruses, the RNA+ mutants were poorer interferon inducers than the wild type. The reason for this difference is not known; it could be that the infection with the RNA+ mutants led to the formation of smaller amounts of double-stranded viral RNA than the wild type, although Tan, Sambrook, and Bellett (11) did not detect any large difference in the proportions of the various species or viral RNA synthesized in infected cells. However, it does appear that the failure of the RNA+ mutants to make interferon is a qualitative rather than quantitative defect. Lomniczi and Burke also found that the wild type and the mutants induced interferon formation without any detectable viral RNA synthesis if the multiplicity was sufficiently high, and they concluded that in these cases the RNA of the input virus was the inducer. Thus it appears that a low multiplicities (less than 10 p.f.u. per cell), the RNA of the infecting virus does not provide enough inducer for interferon formation to be detectable, and virus RNA synthesis is required in order to amplify the effect, but at higher multiplicities the RNA of the infecting virus is an adequate stimulus.

A rather similar conclusion was reached as a result of a study of myxovirus-induced interferon formation. Neither influenza nor parainfluenza virus induce interferon formation until after they have been irradiated with ultraviolet light, and both lose their inducing ability after longer periods of irradiation.
**TABLE II**

INTERFERON PRODUCTION AND RNA SYNTHESIS BY RNA− MUTANTS

| Mutant | Multiplicity of infection | Multiplicity of infection | Transferred to 30°C | Transferred to 39°C | Transferred to 42°C |
|--------|--------------------------|--------------------------|---------------------|---------------------|---------------------|
|        |                          | Interferon titre*        | RNA synthesis†      | Interferon titre*   | RNA synthesis†      |
|        |                          |                          |                     |                     |                     |
| Wild   | 30                       | 1280                     | <100                | 640                 | 100                 |
|        | 6                        | 1280                     | <100                | 640                 | 100                 |
|        | 1                        | 640                      | <100                | 320                 | 40                  |
| ts 5   | 30                       | 680                      | <100                | 7                   | <1                  |
|        | 5                        | 400                      | —                   | 0                   | 0                   |
| ts 10  | 40                       | <1280                    | —                   | 0                   | 0                   |
|        | 5                        | 640                      | 80                  | 5                   | <5                  |

Cells were preincubated and infected at 42°C and then shifted to 30°C (the permissive temperature) or to 39°C (the nonpermissive temperature) for 2 hr before reincubation at 42°C or incubated at 42°C throughout. Interferon was measured after 12 hr. RNA synthesis was measured by means of addition of uridine-5H to actinomycin-treated cells.

* PDD50.

† As per cent of wild type using multiplicity of 6 p.f.u. per cell and shifted to 39°C.

**TABLE III**

INTERFERON PRODUCTION AND RNA SYNTHESIS BY RNA+ MUTANTS

| Mutant | Multiplicity of infection | Multiplicity of infection | Transferred to 30°C | Transferred to 39°C | Transferred to 42°C |
|--------|--------------------------|--------------------------|---------------------|---------------------|---------------------|
|        |                          | Interferon titre*        | RNA synthesis†      | Interferon titre*   | RNA synthesis†      |
|        |                          |                          |                     |                     |                     |
| ts 4   | 25                       | <1280                    | —                   | 280                 | —                   |
|        | 5                        | 280                      | >100                | 50                  | 100                 |
|        | 1                        | 70                       | >100                | 5                   | 60                  |
| ts 13  | 90                       | 800                      | >100                | 340                 | 92                  |
|        | 5                        | 260                      | >100                | 34                  | 62                  |
|        | 1                        | 140                      | 50                  | 5                   | 18                  |

For details see Table II.

* PDD50.

† As per cent of wild type using multiplicity of 6 p.f.u. per cell and shifted to 39°C.

The failure of unirradiated virus was shown by Gandhi and Burke (15) to be due to the depression in the rate of host cell RNA and protein synthesis that follows virus infection, and when this inhibitory effect was destroyed by ultraviolet irradiation of the virus, interferon was produced. The irradiated virus is completely noninfective, and no viral RNA synthesis could be detected in interferon-producing cells, suggesting that, in this system
too, interferon formation was induced by the RNA of the infecting virus. However, Huppert, Hillova, and Gresland (16) reported that ultraviolet irradiated Newcastle disease virus could induce the formation of small amounts of viral RNA in infected cells, and although they made no measurements of interferon formation in this system, they suggested that interferon formation was dependent on the formation of double-stranded RNA, made either by trace amounts of viral polymerase or else by a host enzyme (17). It is not possible to prove that no viral RNA synthesis occurs in cells infected with Semliki Forest virus at the nonpermissive temperatures or with irradiated myxoviruses, for in theory it is possible that the formation of a single complementary strand of RNA is sufficient to induce interferon formation; however, it is unlikely that viral RNA synthesis should cease after this amount of virus RNA synthesis if polymerase is present, and if it continued it would be expected to have been detected by the methods which had been used. In addition, Dianzani, Gagnoni, Buckler, and Baron (18) have shown, by use of metabolic inhibitors, that Newcastle disease virus can initiate the derepression step without a requirement for protein synthesis, and this makes it unlikely that induction depends on the formation of polymerase and hence on viral RNA synthesis in this system also.

The question of whether single-stranded RNA can induce interferon formation is of some importance, for it shows whether interferon formation is a response to the presence of double-stranded ribonucleic acids or to RNA molecules with conformational stability. The studies with synthetic polynucleotides would suggest that single-stranded, conformationally-stable polyribonucleotides can induce interferon formation (19), and the results obtained with the viral inducers suggest a similar conclusion for the RNA of both the myxoviruses and arboviruses are known to have stable conformations (20, 21).

How do DNA viruses induce interferon formation? The results obtained with the synthetic polynucleotides suggest that since polydeoxyribonucleotides are such poor inducers, it is unlikely that the inducer is the double-stranded viral DNA, and it is more likely to be due to a newly synthesized RNA molecule. Colby and Duesberg (5) have reported the formation of double-stranded RNA in vaccinia virus-infected cells, and they suggested that this was the inducer, although they never actually showed that their virus-infected cells produced interferon. This may well be so, although it is not clear what role this double-stranded RNA plays in vaccinia virus multiplication. Pusztai, Beladi, Bakay, and Mucsi (22) showed that adenovirus induced interferon formation in chick cells, but that the inducing ability was lost on trypsin treatment of the virus without any effect on virus infectivity, and that ultraviolet irradiation destroyed infectivity faster than interferon
inducing ability. The authors suggested that the penton antigen of the virus was responsible for interferon induction, and further investigation of this interesting system would be of interest.

In summary, it is certain that double-stranded RNA molecules can induce interferon formation, and it is likely that single-stranded RNA molecules with a stable secondary structure can also do so. But is viral protein synthesis necessary for interferon formation? When RNA synthesis is required for interferon formation, then formation of viral polymerase is obviously also necessary. Hay and Burke (unpublished data) looked for viral protein synthesis in the system induced by Semliki Forest virus, but they were unable to detect viral protein synthesis under conditions where interferon was formed. It was concluded that no substantial amount of viral protein synthesis was essential for interferon formation, and it appears likely that it is the nucleic acid that triggers the derepression event rather than a protein coded by the nucleic acid. This is, of course, consistent with the results obtained with the synthetic polyribonucleotides which would hardly be expected to code for a biologically functional protein.

**DEREPRESSION EVENT**

How does the viral nucleic interact with the host genome with the formation of the interferon messenger RNA? Does the RNA pass into the nucleus and interact directly with the genome, or does it interact with some cytoplasmic material which is responsible for the repression of the nuclear gene? Or is the event nuclear at all? These and many other questions have hardly begun to be answered.

The basis for the formulation of interferon formation as a derepression event is the inhibition by actinomycin and the species specificity of interferon. Actinomycin is well known as an inhibitor of DNA-directed RNA synthesis, and although it does exert some secondary effects, it does seem very likely that its inhibitory effect on interferon formation is due to its primary effect. But is this DNA in the nucleus, or is it possibly mitochondrial? There is, of course, very little genetic information within mitochondrial DNA, and it seems unlikely that one of the proteins coded for is interferon, especially when interferon is not found in the microbial world from which source the mitochondria are probably derived. It has been reported too that chick erythrocytes produce chick interferon after fusion with human cells (23), and since chick erythrocytes do not contain mitochondria, a mitochondrial interferon gene appears unlikely. It has also been reported (24) that production of interferon in the mouse shows simple Mendelian genetics, suggesting that an interferon gene does exist, but little more than that is known. There has been one report (23) of an attempt to investigate the control of this gene by the
widely used cell fusion technique, and it was claimed that cells which had previously been incapable of producing interferon produced interferon after fusion with an interferon producing cell, suggesting that the control was cytoplasmic; nonproducing cells either failed to produce a substance that carried the inducer into the nucleus or produced a cytoplasmic repressor of interferon formation which was neutralized or diluted out after fusion. Clearly, much more information about this part of the induction process is needed.

SYNTHESIS OF INTERFERON MESSENGER RNA AND INTERFERON

The induction of interferon formation is an example of the production of a specific protein by a eucaryotic cell as a response to an external stimulus. The problem is that so little interferon messenger RNA is made that it is not possible yet to detect synthesis or either the RNA or protein directly, and it is not surprising that an attempt to detect formation of a new species of RNA in interferon-producing cells failed (25). It is the production of this RNA that is inhibited by actinomycin and other inhibitors of DNA-directed RNA synthesis (26), and the production of interferon itself is inhibited by such inhibitors of protein synthesis as puromycin, p-fluorophenylalanine, and cycloheximide (27, 28).

The inhibition by cycloheximide is interesting since this inhibitor appears to inhibit cellular protein synthesis (on 80S ribosomes) more strongly than mitochondrial protein synthesis (which uses 70S ribosomes) (29), and it is, therefore, likely that interferon production uses the protein synthesizing machinery of the host rather than of the mitochondrion. However, little more is known about the details of the process, although there is some indirect evidence that a precursor to interferon is synthesized first and is later converted to interferon itself (27).

SUMMARY

The mechanism of virus-induced interferon formation may be formulated as follows. After virus infection, viral RNA, which either is derived from the infecting virus or is newly synthesized within the host cell, interacts in some way with the host genome, initiating a derepression mechanism that leads to the formation of a specific messenger RNA for interferon. Little is known of the derepression event or of the events that follow it, and future research must concentrate on this area.

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Discussion from the Floor

Dr. Paul Siminoff (Bristol Laboratories): Dr. Burke, what is your evidence that these RNA-negative mutants at high multiplicity of infection at nonpermissive temperatures are, in fact, uncoated after adsorption?

Dr. Burke: That's very hard to look at because you can't measure uncoating directly. I think what you can say is that if you infect at the higher temperature and then move to the lower temperature the virus, of course, grows, while if it were kept at a higher temperature it would not grow. So that this suggests that virus which was absorbed during the nonpermissive temperature would certainly be able to get into the cells.

Dr. Lockart: Dr. Burke, I think we obviously have the same conclusions: we don't know what's happening. I would like to comment, however, that I think you're "hung up" on the idea of the nucleic acid. I think that in your high multiplicity experiments, in addition to adding large amounts of nucleic acid, you also added large amounts of viral proteins. You decide to say that it's the nucleic acid that's active and not the other parts of the virus. I don't quite know how you can do this, other than that you choose to do so.

Dr. Burke: It's the simplest hypothesis.

Dr. Lockart: Well, I'm not sure. And I'd say secondly, for example, that if one will take the potential possibility that what causes the induction is some kind of insult to the cell, it may be this high multiplicity of other viral products that are causing this.

Dr. Burke: I think it's certainly possible—let me put it this way: we do not know what happens at the nonpermissive temperature to the invading virus particle, and this is very hard to look at. But it is very likely that if it does uncoat that the viral RNA associates with a membrane, although it may not initiate RNA viral synthesis. And this may be the specific insult that you're looking for. I'm not saying that there isn't some mechanism beyond this. What I'm trying to draw together is the way in which viruses lead in through this particular point.

Dr. Julius S. Youngner (University of Pittsburg, Pittsburg, Pa.): I'd like to follow Royce Lockart's question with one that stems from it. At the nonpermissive temperature, 42°, with the high multiplicities, have you determined that the interferon production that you get is dependent upon RNA and protein synthesis?

Dr. Burke: No we haven't done this and this could be done and it would be interesting. It would be extremely interesting if the mechanisms were different.

Dr. Samuel Baron (National Institutes of Health, Bethesda, Md.): I think it is worthwhile recalling several studies which support Dr. Burke's general interpretations. One
is the study by Kawade and associates at the University of Kyoto, where they purified MS2 phage, a single-stranded RNA phage, and demonstrated that this single-strand phage RNA was capable of inducing chick cells to produce interferon.

The second is the study by Dianzani et al. (Proc. Soc. Exp. Biol. Med., 1970, in press), demonstrating that the messenger RNA for interferon was made in cells infected with Newcastle disease virus under conditions where the formation of the double-stranded replicative form of RNA was not formed. This finding also indicates that interferon was induced by the single-stranded input, viral RNA, another portion of the input NDV molecule, or a physical event associated with infection, but probably not the double-stranded viral RNA.

Finally is the finding that synthetic single-stranded RNA, under sensitive conditions of assay, can be observed to induce interferon in a variety of cells as observed in our lab and in Dr. Merigan's lab.

Dr. Burke: I would agree. I was restricting my remarks to the systems we'd worked with. But the evidence from other systems would suggest that single-stranded RNA can induce interferon, and this would be, therefore, the simplest explanation of the work in our own system, but not the only one.

Mr. Philip Roane (Microbiological Associates Inc., Bethesda, Md.): I wonder whether you would consider the possibility that some minus strands of viral RNA may have been encapsidated? And so when you use your high multiplicity approach, what you are really doing is to add plus and minus strands, which may spontaneously form double-stranded molecules.

Dr. Burke: Well, you can consider lots of possibilities. I think I would regard this as unlikely, namely because we have looked at the self hybridization of the 45S virus RNA.

And if there were a mixture of plus and minus strands in the extracted RNA, then you would expect some self-hybridization to form ribonuclease resistant material. And we did not find it.

Mrs. Marilyn Waite (Dartmouth Medical School, Hanover, N. H.): Have you looked to see whether at 42° the input RNA of your RNA-minus mutants is converted to double-stranded form?

Dr. Burke: No, we haven't. It is, in fact, very hard to do. The only way to do this is to label the viral RNA very highly with phosphorus, and then try and follow the fate of the labeled RNA. There are lots of artifacts in this system.

What we did do, as I say, was to look for viral RNA synthesis. Are you really asking us can we exclude the possibility that a single negative strand is made and the input RNA thus converted to a double-strand RNA? I would say that I feel this is unlikely on two grounds. One is that once you start a limited round of RNA synthesis I don't see why it stops at that point. And secondly, the evidence that Baron has already quoted, that suggests that certainly in the fowl plague system or the NDV system, that you don't need any viral protein synthesis, and, therefore, no polymerase.

Mrs. Waite: I'm not trying to contradict Dr. Baron's evidence; however we have found that some of our sindbis virus RNA-minus mutants are capable of continuing to synthesize single-stranded RNA if given a period at the permissive temperature to synthesize their replicative forms, but they can no longer synthesize their replicative forms. Thus RNA synthesis apparently can be interrupted in the middle of a round.
Also, another question. Is there any of your evidence that interferon induction could not be explained by failure or success of the virus in inhibiting the cells' RNA synthesis?

Dr. Burke: No, I don't think so. In fact, virus infection causes an inhibition of cell protein synthesis, and yet the cells still make interferon. I think all we can say is that the viruses are such good inducers that this inhibition doesn't matter.

Dr. Field: You just touched on the possibility of strandedness of the input viral RNA. Could you expand on this? Do you have any other related information? Have you looked for evidence of double-strandedness of that initial input RNA—melting profiles RNase resistance or something along that line?

Dr. Burke: My comment was in connection with self-annealing in hybridization. And this was looked at in quite another connection, which I won't bother to explain now. But we didn't find any self-annealing. As to the conformational stability of the viral RNA, there are two pieces of evidence that suggest that there may be some, that is the RNA of the arboviruses may have sufficient conformational stability to be active inducers. One is the observation by Sreevalsan and Lockart that the RNA of western equine encephalitis has a Tm of 57.5°C. And the second is an observation of our own which shows that between 60° and 65°C the 45S RNA splits to form half molecules. We don't know what this change involves; we think it is a hydrogen-bonded melting, and it may well be the same as the change Dr. Lockart has been observing at a similar temperature. So there are two pieces of evidence which suggest that they melt around 60°, and that the viral RNA has definite conformational stability. This might be important for the induction of interferon.