# THE SPECIFICITY OF INTERFERON INDUCTION IN CHICK EMBRYO CELLS BY HELICAL RNA*

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*Abstract.*—Treatment of chick embryo cells growing in culture with rI:rC and many other RNA-like polymers results in the induction of interferon. DEAE dextran is required to facilitate the uptake of the RNA into the cells. Interferon-inducing activity is found with a variety of double-stranded helical polynucleotides, provided that all the sugar residues are ribose. However, the effectiveness of different active polynucleotides at a given concentration varies considerably. The differences in activity among the various polynucleotides do not appear to reflect differences in the rate or amount of uptake into the cells or in the rate of intracellular breakdown. The high degree of specificity of the induction process is consistent with the existence of a specific intracellular receptor site, which may be a protein.

*Introduction.*—An early response to the infection of an animal by some viruses is the appearance of a nonviral protein (interferon) which conveys protection from infection to uninfected cells.[1] Interferon is species-specific[2] and its induction requires the synthesis of RNA and protein.[3, 4] Interferon may be induced by many active and inactive animal viruses, many bacteria, rickettsiae, and fungal viruses.[5–7] Bacterial endotoxin, phytohemagglutinin, and polycarboxylate copolymers have also been shown to induce interferon.[5–7] Hilleman and his coworkers have recently demonstrated the induction of interferon by double-stranded RNA from a variety of sources[8–10] and by double-stranded synthetic polyribonucleotides.[11, 12] The interferon-inducing activity of the double-stranded homopolymer pair, rI:rC, has been confirmed in several other laboratories.[13–16]

The common characteristic of the nonreplicating interferon inducers is that they are all polyanionic macromolecules. Two general models for the induction of interferon suggest themselves: (1) The induction may occur by a nonspecific process which requires only that a large polyanion enter the cell. (2) There might be a specific receptor site inside the cell which recognizes the inducer and thereby allows the synthesis of interferon. We have studied the interferon-inducing activity of a number of synthetic single- and double-stranded polynucleotides which differ from each other by one or a few atoms in their mononucleotide units in the polymer. Our results are consistent with the second model and suggest that the receptor site inside the cell is a protein molecule.

*Materials and Methods.*—*Induction of interference by synthetic polynucleotides:* Primary cultures of chicken embryo fibroblasts were prepared according to the method of Rein and Rubin.[17] Cells were plated in 50-mm Petri dishes at $4 \times 10^6$ cells per dish in NCI medium (Schwarz BioResearch), containing 2% tryptose phosphate broth (Difco), and 1% of calf and chicken serum (Grand Island Biological Co.). After 5 days the medium was replaced with 3 ml NCI medium containing 6% calf serum (growth medium) and appro-

priate amounts of DEAE dextran (mol wt 2 × $10^6$, Pharmacia) and the specific polynucleotides to be tested. After a 24-hr induction period the cultures were washed twice with growth medium and assayed for resistance to viral infection.

*Plaque reduction assay:* Washed cultures were challenged with 50–100 plaque-forming units (PFU) of Sindbis virus in 0.3 ml of growth medium for 30 min. The cultures were then washed with this medium and overlaid with growth medium containing 0.7% agar. After 36 to 48 hr the cultures were stained with 0.02% neutral red and the plaques were counted.

*Titer reduction assay:* Washed cultures were treated with $10^7$ PFU Sindbis virus in 0.3 ml of growth medium. After 30 min the medium was removed and the cultures were washed twice with growth medium. Three ml of growth medium was added and the cultures were incubated at 37°C. After 22 hr the medium was collected and the titer of Sindbis virus was determined by infecting confluent secondary cultures of chick fibroblasts with appropriate dilutions of medium. Infection and overlay conditions were as described in the previous section.

*Determination of uptake and breakdown of $P^{32}$-labeled polymers:* Cell cultures were treated with $P^{32}$-labeled polymers as described above. At appropriate times, the excess labeled polymer was removed and the labeled cultures were washed three times with ice-cold physiological saline buffered at pH 7.4 with 0.1 *M* Tris-HCl. One ml of cold 5% trichloroacetic acid was added and the dishes were placed in the cold for 30 min. The supernatant solution (cold acid-soluble fraction) was removed, combined with 12 ml scintillation fluid,[18, 19] and counted in a liquid scintillation spectrometer. The dishes were then washed three times with ice-cold 5% trichloroacetic acid and 2 ml of 10% trichloroacetic acid was added. The dishes were incubated at 65°C for 1 hr; this led to the solubilization of all residual labeled material. An aliquot of this solution (cold acid-insoluble fraction) was counted as above.

*Polymers:* rI, rC, rA, and rU polymers, purchased from Miles Chemical Co., were prepared for use as previously described.[20] Homopolymer pairs[21] and alternating polymers[22–24] were also prepared as described. Polymer concentrations are given in equivalents of nucleotide phosphorus.

*Results.—Induction by rI:rC of resistance to virus in chick embryo cells:* After chick embryo cells are exposed to rI:rC, they become resistant to infection with Sindbis virus (Table 1). Thus, 10 μg/ml of rI:rC eliminates plaque formation by the low dose of virus used in the plaque reduction assay, while the titer of virus produced after heavy infection of cultures treated at this polynucleotide concentration is reduced 20,000-fold (titer reduction assay). Treatment of the cultures with 1 μg/ml of rI:rC does not reduce the number of plaques produced by the virus significantly, although there is a fourfold reduction of virus titer.

*Enhancement of rI:rC directed interference by DEAE dextran:* Reports of enhanced activity with polynucleotides in the presence of DEAE dextran[13, 16] led

TABLE 1. *Induction by rI:rC of resistance to Sindbis virus in chick embryo cells.*

| rI:rC (μg/ml) | DEAE dextran (μg/ml) | Plaques | Sindbis titer (PFU/ml) |
|---|---|---|---|
| None | None | 52 | $3.5 \times 10^8$ |
| 10 | None | 0 | $1.2 \times 10^4$ |
| 1 | None | 51 | $6.3 \times 10^7$ |
| None | 10 | 48 | $2.4 \times 10^8$ |
| 10 | 10 | 0 | <10 |
| 1 | 10 | 0 | <10 |
| 0.1 | 10 | 0 | $2 \times 10^2$ |
| 0.01 | 10 | 0 | $1.2 \times 10^4$ |
| 0.001 | 10 | 36 | $1.4 \times 10^7$ |

us to examine its effect in the chick embryo cell system. When 10 μg/ml of DEAE dextran were included in the induction medium, there was no effect on the ability of these cells to support the growth of Sindbis virus (Table 1) and there were no visible cytopathic effects. The inclusion of DEAE dextran in the induction medium led to a striking increase in the resistance induced by rI:rC. Even 0.001 μg/ml of rI:rC confers detectable resistance to Sindbis infection, as monitored by both plaque reduction and yield reduction assays. No Sindbis plaques can be found on cells treated with 10 μg/ml of DEAE dextran and rI:rC at any concentration between 0.01 and 10 μg/ml. However, the increasing protection conferred by increasing the rI:rC concentration is signaled by the steady drop of virus yield over the same range of polymer concentrations. Thus, the titer reduction assay allows a much more quantitative estimate of the potency of the polynucleotide in producing resistance to virus infection than the plaque reduction assay allows.

We studied the effect of DEAE dextran on the rate of uptake of $P^{32}$-labeled rI:rC into chick embryo cells. The inclusion of 10 μg/ml of DEAE dextran in the medium led to a 20-fold increase in the rate of uptake of polynucleotide into these cells. A similar enhancement of the total amount of polymer taken up by the cells after 24 hours was also found. We conclude that a primary role of DEAE dextran in enhancing the effectiveness of polynucleotides is to increase both the rate and the extent of uptake of polynucleotide into the cell.

*Is interference by rI:rC due to interferon?* Medium removed from chick embryo cell cultures treated with rI:rC and with the other helical RNA inducers described below contains a nondialyzable, RNase-resistant, trypsin-sensitive, species-specific material that confers protection against virus infection to cells treated with it. The synthesis of this material is abolished by pretreatment of chick embryo cells with 1 μg/ml actinomycin D. The properties of this material are those of interferon. However, the amount of interferon released into the medium of polynucleotide-treated chick embryo cells is quite small. Hence, the titer reduction assay for intracellular interferon was used for screening the effectiveness of the polynucleotides described below.

*Induction of interference by other polynucleotides:* Using conditions derived from the above studies, we have measured the effectiveness of a series of synthetic polynucleotides in the induction of resistance to Sindbis virus in chick embryo cells. Whenever a polynucleotide was found to induce interferon, the relationship between polymer concentration and titer reduction was measured (Table 2). Several points are immediately clear: (1) Only double-stranded helical ribonucleotides are active under the conditions used. (2) The specific activity of different polynucleotides varies considerably. (3) No activity is found with a variety of helical polynucleotides whose physical and chemical structures are very similar to those of the active polynucleotides. In particular, all the sugar residues in the polynucleotide must be ribose residues.

*Kinetics of uptake of inducing and noninducing polynucleotides:* A trivial explanation for the differences between various polynucleotides with respect to their ability to induce interferon is that inducing polymers are taken up by the cells, while noninducing or less active polymers are excluded or are taken up

TABLE 2. *Activity of different helical polynucleotides in inducing resistance to Sindbis virus.*

| Inducing Polynucleotides | | | Noninducing Polynucleotides | |
|---|---|---|---|---|
| Polynucleotide | ($\mu$g/ml) | Sindbis titer (PFU/ml) | Polynucleotide (10 $\mu$g/ml) | Sindbis titer (PFU/ml) |
| rIC | 1.0 | <10 | rI | $3 \times 10^8$ |
| | 0.1 | $5.2 \times 10^2$ | rC | $1.8 \times 10^8$ |
| | 0.01 | $8 \times 10^5$ | rA | $4 \times 10^8$ |
| | 0.001 | $4 \times 10^8$ | rU | $5.7 \times 10^8$ |
| $rI\overline{BC}$ | 1.0 | <10 | rG | $2.4 \times 10^8$ |
| | 0.1 | $5.6 \times 10^3$ | rI:dC | $3.3 \times 10^8$ |
| | 0.01 | $2.5 \times 10^8$ | dI:rC | $2.1 \times 10^8$ |
| | 0.001 | $3.7 \times 10^8$ | $dI_2$:rC | $3.0 \times 10^8$ |
| rG:rC | 1.0 | <10 | dI:dC | $1.5 \times 10^8$ |
| | 0.1 | $3.1 \times 10^3$ | dG:dC | $2.8 \times 10^8$ |
| | 0.01 | $1.3 \times 10^5$ | rA(dU) | $4.7 \times 10^8$ |
| | 0.001 | $4 \times 10^7$ | rA:$rU_2$ | $1.4 \times 10^8$ |
| rAU | 10 | $1.3 \times 10^2$ | dAT | $4.1 \times 10^8$ |
| | 1.0 | $1.7 \times 10^3$ | $\lambda$DNA | $2.7 \times 10^8$ |
| | 0.1 | $1.2 \times 10^6$ | None | $4 \times 10^8$ |
| | 0.01 | $4.1 \times 10^7$ | ... | ... |
| | 0.001 | $2.5 \times 10^8$ | ... | ... |
| $rA\overline{BU}$ | 10 | $<10^2$ | ... | ... |
| | 1.0 | $1.3 \times 10^2$ | ... | ... |
| | 0.1 | $1.7 \times 10^6$ | ... | ... |
| | 0.01 | $1.1 \times 10^7$ | ... | ... |
| | 0.001 | $3.5 \times 10^8$ | ... | ... |
| rA:rU | 10 | $10^5$ | ... | ... |
| | 1.0 | $2.8 \times 10^8$ | ... | ... |
| | 0.1 | $4 \times 10^8$ | ... | ... |

much more slowly. To test this possibility we followed the kinetics of uptake into chick embryo cells of $P^{32}$-labeled rIC, an active inducer, and $P^{32}$-labeled rI:dC, an inert homopolymer pair. Figure 1*A* shows that rI:dC at a concentration of 1 $\mu$g/ml is taken up by chick embryo cells at essentially the same rate as rIC. Furthermore, the amount of polymer which accumulates inside the cells is the same in each case. We conclude that the difference in activity between these two polymers cannot be explained on the basis of differences in either the rate or the extent of uptake.

A similar result was obtained with $P^{32}$-labeled rAU. The kinetics of uptake of

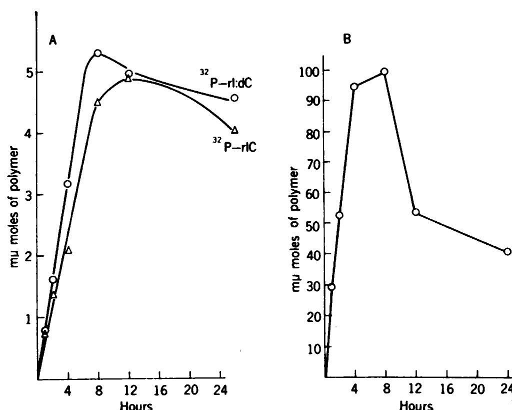


FIG. 1.—Uptake of $P^{32}$ polynucleotides into chick embryo cells.

(*A*) Cells were treated with 10 $\mu$moles of $P^{32}$-rIC or $P^{32}$-rI:dC plus 10 $\mu$g/ml DEAE dextran. At the times indicated, the amount of trichloroacetic acid-insoluble radioactivity inside the cells was determined as described in *Materials and Methods.*

(*B*) Cells were treated with 100 $\mu$moles $P^{32}$-rAU plus 10 $\mu$g/ml DEAE dextran as in (*A*).

$P^{32}$ rAU at 1 μg/ml are similar to those of rIC and rI:dC. At 10 μg/ml, where rAU shows a viral yield reduction equivalent to 0.1 μg/ml rIC, the rate and extent of uptake of $P^{32}$ polymer are enhanced about tenfold (Fig. 1*B*). Thus the rate and extent of polymer uptake at these concentrations appears to be directly proportional to the concentration in the medium. We conclude that a much higher intracellular concentration of rAU relative to rIC is required to induce a given level of resistance.

*Kinetics of breakdown of inducing and noninducing polynucleotides:* A second explanation for the observed differences in activity of different polymers is that, owing to some feature of their structure, the noninducing polymers are more rapidly degraded inside the cell than are the inducing polymers. An alternative form of this hypothesis is that polymer cleavage would be required for activity and that inert polymers are resistant to the putative nuclease involved.

We have followed the rate of breakdown of representative active and inactive polymers *in situ* in chick embryo cells. No qualitative differences were detected between the breakdown of rIC and rI:dC after uptake by these cells (Fig. 2).

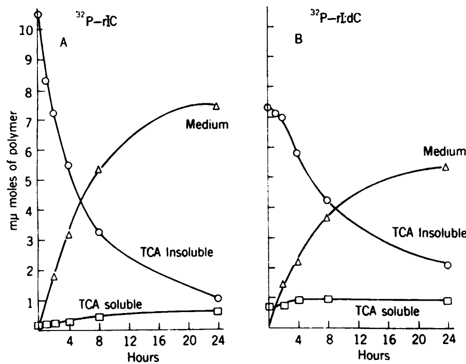


FIG. 2.—Intracellular breakdown of $P^{32}$-rIC and $P^{32}$-rI:dC. Cells were treated with 2.5 μmoles $P^{32}$-polynucleotide plus 10 μg/ml DEAE dextran in 3 ml growth medium for 4 hr. The cells were washed extensively and fresh growth medium was added. At the times indicated, the amounts of trichloroacetic acid-soluble and -insoluble radioactivity inside the cells and the radioactivity in the medium were determined as described in *Materials and Methods.*

Quantitatively, rIC appears to be broken down slightly faster than rI:dC, although the differences are not great. In both cases, as the polynucleotides are broken down the soluble pools become saturated and the amount of radioactivity which appears in the medium accounts quantitatively for the amount of internal acid-insoluble material that is lost. We conclude that no large differences exist in the rate of breakdown of these two polymers and that, at the gross level of sensitivity, breakdown is neither required nor does it restrict the activity of the helical polynucleotides tested.

*Discussion.*—Interferon induction by helical RNA in the chick embryo cell system is similar in many ways to that found *in vivo* and in culture with other cells. In particular, the amounts of helical RNA required to confer resistance are exceedingly low. While many polyanions, including synthetic polycarboxylates, can give rise to interferon production, they are active only at concentrations $10^5$ times higher than those required for helical RNA. This suggests immediately that the helical RNA is acting with much greater specificity, and it is tempting to assume that it acts by mimicking in some way the natural induction process triggered by a replicating virus.

This conclusion is reinforced by the high degree of specificity shown by the induction process; that is, while all double-stranded helical RNA polymers that we have tested are active, any modification of this structure results in complete loss of antiviral activity. Single-stranded RNA is inactive, as are the triple-stranded helices $rA:rU_2$ and $dI_2:rC$. Furthermore, the 2′-hydroxyl on the sugar moiety appears essential for interferon induction, since helices in which one strand is a polydeoxyribonucleotide or in which every other sugar residue is deoxyribose are inactive. While some of these alterations produce large changes in the physical and biochemical properties of the resulting helix, other changes have very little effect. For instance, X-ray diffraction studies of rI:rC and rI:dC reveal no differences in their three-dimensional helical structure; thus each has the RNA-helix configuration. Yet rI:rC is active at 0.001 μg/ml, while rI:dC is inert at 10 μg/ml. We have shown that the differences in activity between a representative inducing polynucleotide, rIC, and a noninducing homopolymer pair, rI:dC, may not be explained on the basis of differences in the rate or extent of uptake by the cells (Fig. 1*A*) or differences in the rate of intracellular breakdown (Fig. 2).

The titer reduction index of the inducing polymers varies from 6.3 for rI:rC to 0 for rA:rU; i.e., there is greater than a millionfold difference in the reduction of virus yield from cells treated with 0.1 μg/ml of the various inducing polynucleotides (Table 3). The differences in efficiency could reflect differences in size requirements for induction by the polymers or differences in the longevity of the polynucleotides inside the cell. The rigorous analysis of this question awaits the study of the size and rate of intracellular breakdown of radioactivity-labeled inducing polymers. However, we have found no correlation between the efficiency of induction of these polynucleotides and either their thermal stability or their sensitivity to pancreatic RNase (Table 3). Furthermore, when the concentration of rAU, a polynucleotide of intermediate inducing activity, was increased tenfold in the induction medium, ten times as much polynucleotide was found inside the cell after 24 hours.

A second possibility is that induction involves combination between a specific intracellular receptor site and the inducer and that the extent of induction is

TABLE 3. *Ribonuclease sensitivity and thermal stability of different helical polynucleotides.*

| Polynucleotide | Titer reduction index* | Rate of degradation (μmoles/hr/mg enzyme) | Melting temperature (°C)† |
|---|---|---|---|
| rI:rC | 6.3 | 3.8 | 60 |
| rIC | 5.9 | 17 | 60 |
| rG:rC | 5.1 | <0.02 | 136 |
| $rI\overline{BC}$ | 4.9 | 1.4 | 86 |
| rAU | 2.5 | 110 | 66 |
| $rA\overline{BU}$ | 2.4 | 41 | 79 |
| rA:rU | 0 | 13 | 57 |
| rI:dC | 0 | 0 | 52 |
| dI:rC | 0 | 11 | 35 |
| rA(dU) | 0 | 0 | 18 |

* Log (Sindbis titer from control cells)/(Sindbis titer from cells + 0.1 μg/ml polynucleotide).
† $T_m$ calculated for 0.1 $M$ $Na^+$, pH 7.8.

proportional to the amount of this complex formed. By this hypothesis the extent of binding would be sensitive to the chemical fine structure of the inducer. The high degree of specificity involved in induction by helical polynucleotides, taken with the differences in potency among active inducers, lead us to favor this hypothesis.

What is the nature of the receptor site? The specificity of the induction process suggests that the receptor is probably either a protein or a nucleic acid. The latter possibility is less likely for the following reasons. First, the specificity of nucleic acid–nucleic acid interactions lies in the specificity of base-pairing interactions, and there is no evidence that the sequence of nucleotides in the inducer is crucial to the induction process. Secondly, if base pairing were involved, the strands of the inducer molecule would have to be separated. The active helical RNA inducers differ by up to 100°C in their melting temperature and hence by up to 2 kcal/mole of base pairs in the energy required to separate their strands. Yet there is no apparent correlation between melting temperature and inducing activity (Table 3), suggesting that strand separation is not involved in inducer function. Finally, the hypothesis that the receptor is a nucleic acid does not easily account for the inactivity of the hybrid homopolymer pairs since polydeoxyribonucleotides show identical base-pairing specificity to their riboanalogues.

On the other hand, proteins are well known for their ability to recognize a particular molecular configuration, such as the double-stranded helix, as well as particular functional groups on the molecule, such as 2′-hydroxyls. The broad spectrum of activity among the inducing polynucleotides is easily visualized within the framework of the Michaelis behavior of proteins. These considerations lead us to the suggestion that the receptor is a protein. One might anticipate that at very high polynucleotide concentrations this specificity could be overridden; for example, rI:dC might trigger interferon production just as polycarboxylates are known to do. However, our present interest lies in the information about the induction process that is provided by the high specificity evident at low polymer concentrations.

Our results are consistent with the hypothesis that the agent responsible for the induction of interferon by single-stranded RNA viruses is the double-stranded replicative form. However, some DNA viruses also induce interferon. Since DNA itself does not appear to be an active inducer, one might postulate that a DNA-RNA hybrid is the inducing agent. We have tested seven different polynucleotides containing deoxyribose on one or both chains of homopolymer pairs or on alternating bases and have found them all to be noninducers (Table 3). These data suggest that DNA viruses which induce interferon do so by some agent other than the DNA-RNA hybrid.

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[1] Isaacs, A., and J. Lindenmann, *Proc. Roy. Soc.* (*London*), *Ser. B*, **147**, 258 (1957).

[2] Merigan, T. C., C. A. Winget, and C. B. Dixon, *J. Mol. Biol.*, **13**, 679 (1965).

[3] Heller, E., *Virology*, **21**, 652 (1963).

[4] Wagner, R. R., and A. S. Huang, these PROCEEDINGS, **54**, 1112 (1965).

[5] Ho, M., K. H. Fantes, D. C. Burke, and N. B. Finter, in *Interferons*, ed. N. B. Finter (Amsterdam: North-Holland Publishing Co., 1967), p. 181.

[6] Fenner, F., in *The Biology of Animal Viruses* (New York: Academic Press, 1968), vol. 1, p. 379.

[7] Hilleman, M. R., *J. Cell Physiol.*, **71**, 43 (1968).

[8] Lampson, G. P., A. A. Tytell, A. K. Field, M. N. Nemes, and M. R. Hilleman, these PROCEEDINGS, **58**, 782 (1967).

[9] Tytell, A. A., G. P. Lampson, A. K. Field, and M. R. Hilleman, these PROCEEDINGS, **58**, 1719 (1967).

[10] Field, A. K., G. P. Lampson, A. A. Tytell, M. N. Nemes, and M. R. Hilleman, these PROCEEDINGS, **58**, 2012 (1967).

[11] Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman, these PROCEEDINGS, **58**, 1004 (1967).

[12] *Ibid.*, **61**, 34 (1968).

[13] Vilcek, J., Mun H. Ng, A. E. Friedman-Kien, and T. Krawciw, *J. Virol.*, **2**, 648 (1968).

[14] Finkelstein, M. S., G. H. Bausek, and T. C. Merigan, *Science*, **161**, 465 (1968).

[15] Younger, J. S., and J. V. Hallum, *Virology*, **35**, 177 (1968).

[16] Dianzani, F., P. Cantagalli, S. Gagnoni, and G. Rita, *Proc. Soc. Exptl. Biol. Med.*, **128**, 707 (1968).

[17] Rein, A., and H. Rubin, *Exptl. Cell Res.*, **49**, 666 (1968).

[18] Patterson, M. S., and R. C. Green, *Anal. Chem.*, **37**, 854 (1965).

[19] Benson, R. H., *Anal. Chem.*, **38**, 1353 (1966).

[20] Chamberlin, M. J., and D. L. Patterson, *J. Mol. Biol.*, **12**, 410 (1965).

[21] Riley, M., B. Maling, and M. J. Chamberlin, *J. Mol. Biol.*, **20**, 359 (1966).

[22] Chamberlin, M. J., R. Baldwin, and P. Berg, *J. Mol. Biol.*, **7**, 334 (1963).

[23] Krakow, J., and M. Karstadt, these PROCEEDINGS, **58**, 2094 (1967).

[24] Chamberlin, M. J., *Federation Proc.*, **24**, 1446 (1965).