Inhibition of Early Vaccinia Virus Ribonucleic Acid Synthesis in Interferon-Treated Chicken Embryo Fibroblasts

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Pretreatment of chicken embryo cells with homologous but not heterologous interferon inhibits the synthesis of vaccinia virus early messenger ribonucleic acid (mRNA). This inhibition is seen in the presence of cycloheximide, i.e., in the absence of protein synthesis, suggesting that the virion-bound transcriptase may be the target of the antiviral activity associated with interferon treatment. The inhibition of viral mRNA synthesis is dependent on the amount of chicken interferon used. The nonviral interferon inducer, polyriboinosine·polyribocytidine, similarly inhibits viral early mRNA synthesis in a dose-specific manner. The helical polynucleotide polydeoxyinosine·polyribocytidine, which is not an effective interferon inducer in chicken embryo cells, has no effect on viral ribonucleic acid synthesis.

Recent experiments by Marcus et al. (P. I. Marcus et al., *Science*, *in press*) demonstrate that the virion-bound transcriptase of vesicular stomatitis virus (VSV) is inhibited *in vivo* by pretreatment of the cells with interferon. Since vaccinia virus also contains a virion-bound transcriptase (13) and since the replication of vaccinia virus in the cytoplasm of infected cells is sensitive to the antiviral action of interferon (10), we have tested the sensitivity of the vaccinia transcriptase *in vivo* to pretreatment of the cells with two interferon preparations and with helical polynucleotides.

MATERIALS AND METHODS

Chicken interferon was prepared from the medium of Sindbis virus-infected aged chicken embryo fibroblasts (1) by the method of Lampson et al. (14). Mouse interferon was prepared by the same method from the medium of mouse L cells infected with ultraviolet (UV)-irradiated Newcastle disease virus. The plaque reduction assay (1 PR₅₀ unit = the concentration of interferon that reduces the plaque titer of a challenge virus by 50%) was used with both interferons; vaccinia was the challenge virus in the case of chicken interferon, and VSV was the challenge virus for mouse interferon.

Vaccinia virus (strain WR) was grown in suspension cultures of HeLa cells and purified by the method of Joklik (8). The virus was plaque-titered on chicken embryo cells. Vaccinia deoxyribonucleic acid (DNA) was prepared from purified virions by the method of Kates and Beeson (11). The methods for preparing pulse-labeled vaccinia virus ribonucleic acid (RNA) from the cytoplasm of infected cells and for

hybridizing this RNA to vaccinia DNA have been described (3, 5).

Polyriboinosine[poly(rI)]·polyribocytidine[poly(rC)] was purchased from P-L Biochemicals. Polydeoxyinosine[poly(dI)]·polyribocytidine[poly(rC)] was obtained from M. J. Chamberlin. Procedures for inducing the interferon response in chicken embryo cells with helical polyribonucleotides and diethylaminoethyl-dextran have been described (2).

RESULTS

Secondary cultures of chicken embryo cells were exposed to interferon for 20 hr. Interferon-treated and untreated cultures were then infected with vaccinia virus, (35 plaque-forming units/cell), in the presence of 50 µg of cycloheximide per ml. The use of this antimetabolite to eliminate protein synthesis insures that the synthesis of vaccinia messenger RNA (mRNA) is catalyzed only by the virion-bound polymerase (12). We allowed 1 hr for virus penetration and cell-mediated removal of the viral envelope, and followed viral RNA synthesis by pulsing the cells for 12 min with ³H-uridine. Vaccinia RNA and cellular transfer RNA are the only RNA species in the cytoplasm that are labeled under these conditions (9).

The RNA in the cytoplasm was extracted and purified by sodium dodecyl sulfate-Pronase-phenol treatment and precipitated with ethanol. Table 1 presents the results from such an experiment. It can be seen that pretreatment of the cells with 400 units of chicken interferon apparently

TABLE 1. *Effect of interferon pretreatment on vaccinia virus early messenger ribonucleic acid^a*

Vaccinia (PFU/cell) ^b	Chicken interferon (PR ₅₀ vaccinia) ^c	Mouse interferon (PR ₅₀ VSV) ^d	Acid-insoluble cytoplasmic ³ H (counts per min)
	400		2,018
		1,000	3,580
35			3,860
35		1,000	27,440
35	400		28,220
			12,800

^a Interferons in the amounts indicated in the table were added to 100-mm dishes of confluent chicken embryo cultures in 8 ml of NCI medium (Schwartz BioResearch) containing 2% heated calf serum. After incubation for 24 hr at 39 C to allow the establishment of the interferon block, the medium was removed and 8 ml of NCI $\pm$ 2% heated calf serum containing 50 μ g of cycloheximide per ml was added to the cultures. The dishes were incubated for 10 min at 37 C, after which the medium was removed and 0.8 ml of NCI plus 2% heated calf serum plus cycloheximide (50 μ g/ml) plus vaccinia virus strain WR was added. The virus was allowed to adsorb to the cells for 30 min at 37 C. The medium was replaced and incubation continued for an additional 60 min, after which the medium was removed from all plates and 2 ml of a labeling solution, containing NCI plus 2% dialyzed calf serum, 50 μ g of cycloheximide per ml, and 100 μ Ci of ³H-uridine per ml was added. The dishes were incubated at 37 C for 12 min with agitation every 5 min and then transferred to an ice-slurry. The labeling solution was removed and the plates were washed with 5 ml of ice cold phosphate-buffered saline (PBS). An additional 5 ml of cold PBS was then added and the cells were removed from the plate with a rubber policeman. The plates were washed with cold PBS and the combined suspension of cells was centrifuged for 3 min at 2,000 rev/min. The cellular pellet was resuspended in 2 ml of cold hypotonic buffer of the following composition: 0.025 M KCl, 0.002 M MgCl₂, 0.005 M CaCl₂, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), 0.1% Triton X-100, and allowed to swell on ice for 5 to 10 min before being gently dounced 12 to 15 times. Nuclei and cellular debris were removed by centrifugation and to the supernatants were added sodium dodecyl sulfate, (0.5% final concentration) and Pronase (0.5 mg/ml final concentration). After incubation at 37 C for 30 min, the solutions were extracted three times with phenol, and the ribonucleic acid (RNA) was precipitated by the addition of 2 volumes of cold absolute ethanol. The RNA was collected by centrifugation and redissolved in 0.01 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 0.001 M ethylenediaminetetraacetic acid.

^b Plaque-forming units per cell.

TABLE 2. *Dose-response inhibition of vaccinia virus early messenger ribonucleic acid synthesis by interferon^a*

Vaccinia (PFU/cell)	Chicken interferon (PR ₅₀ vaccinia/10 ⁷ cells)	Acid-insoluble ³ H counts per min (post-agarose)
		8,200
35		30,700
35	80	24,300
35	160	13,500
35	320	5,300

^a The cultures were treated and the ribonucleic acid (RNA) was prepared as described in footnote a, Table 1, except that the final RNA solution was further purified by gel filtration on a 6% agarose column (3).

^b Plaque-forming units per cell.

^c See Table 1, footnote c.

inhibits the viral enzyme by 50%, whereas pretreatment of the cells with 1,000 units of heterologous mouse interferon has no effect on early vaccinia virus RNA synthesis.

We then treated chicken cells with various concentrations of interferon, infected then with vaccinia virus in the presence of cycloheximide, and extracted the pulse-labeled cytoplasmic RNA as before. This RNA was then subjected to gel filtration on a 6% agarose column. The low-molecular-weight RNA is retarded on such a column, whereas vaccinia mRNA appears in the void volume (3). The results presented in Table 2 show the dose-dependent inhibition of vaccinia virus early mRNA synthesis by pretreatment with interferon. We routinely find 80 to 85% inhibition of early mRNA synthesis by pretreatment with 320 units of chicken interferon when the RNA is purified in this manner.

A trivial explanation of these results is that interferon pretreatment and vaccinia virus infection markedly alters the intracellular pools of uridine, rendering the incorporation of ³H-uridine into acid-insoluble material an inaccurate measure of RNA synthesis. We therefore measured the soluble intracellular pools of uridine under the experimental conditions described above and found fluctuations of less than 10%. These data are presented in Table 3. We therefore conclude that our measurements reflect the rate of RNA synthesis in these cells.

^c One plaque-reduction unit (1 PR₅₀) equals the concentration of interferon that reduces the plaque titer of vaccinia virus by 50%.

^d One PR₅₀ VSV unit equals the concentration of interferon that reduces the plaque titer of vesicular stomatitis virus by 50%.

TABLE 3. *Effect of interferon pretreatment and vaccinia virus infection on the intracellular pools of uridine^a*

Vaccinia (PFU/cell) ^b	Chicken interferon (PR ₈₀ vaccinia) ^c	Intracellular uridine (³ H counts per min × 10 ⁵)
		1.66
	400	1.55
35		1.66
35	400	1.52

^a The conditions of interferon treatment, infection with vaccinia virus, and a period of labeling with ³H-uridine were as described for Table 1. The intracellular soluble pools of H³-uridine were determined as previously described (4). The values in the table represent an average of duplicate experiments. The efficiency of uptake of ³H-uridine by the cells during the 12-min pulse was 6%.

^b Plaque-forming units per cell.

^c See Table 1, footnote c.

The RNA from these experiments was tested for its sequence homology with vaccinia virus DNA by DNA-RNA hybridization. The results of these experiments (Table 4) show that approximately 50% of the pulse-labeled ³H-RNA extracted from cells which had been infected with vaccinia was able to hybridize with vaccinia DNA, whereas none of the RNA extracted from the uninfected cells was capable of forming such a hybrid. The fact that a constant fraction of the input RNA was hybridizable indicates that only a small amount of chicken RNA is present in these samples.

A further indication that the inhibition of early mRNA synthesis is mediated by the interferon response comes from experiments using the non-viral interferon inducer, poly (rI)·poly (rC). The results presented in Table 5 show that pretreatment with increasing amounts of (rI)_n·(rC)_n inhibits the synthesis of early viral mRNA. An additional control was included in this experiment, namely a helical polynucleotide homopolymer pair, (dI)_n·(rC)_n, which is not an effective interferon inducer in these cells (2) and which did not reduce the level of viral mRNA.

DISCUSSION

The results of our experiments indicate that pretreatment of chicken embryo cells with interferon markedly reduces the synthesis of vaccinia virus early mRNA. This is in contrast with the results reported by Joklik and Merigan (10), who found no inhibition of pulse-labeled cytoplasmic RNA in interferon-treated mouse L cells infected with vaccinia virus. These conflicting results are

TABLE 4. *Hybridization of H³-cytoplasmic ribonucleic acid (RNA) with vaccinia deoxyribonucleic acid^a*

Source of input RNA	³ H counts per min input RNA	³ H Counts per min hybridized
Vaccinia-infected chicken cells	3,000 6,000 12,000	1,451 3,077 5,647
Vaccinia-infected chicken cells pretreated with 80 units of interferon	2,400 4,800 9,600	1,387 2,365 5,032
Vaccinia-infected chicken cells pretreated with 160 units of interferon	1,350 2,700 5,400	502 955 1,767
Vaccinia-infected chicken cells pretreated with 320 units of interferon	530 1,060 2,120	297 557 1,171
Uninfected, noninterferon-treated chicken cells	1,640 3,280	4 12

^a Vaccinia DNA (5 µg) was immobilized on each filter, and hybridization was performed as described in Materials and Methods. The time allowed for the hybridization reaction was 20 hr. The numbers in column three represent ³H counts per minute bound after subtraction of the ³H counts per minute bound to a blank filter in the same vial. The latter was routinely 25 counts per min.

most likely related to differences in the two cell systems rather than to the mechanism of action of interferon. Joklik and Merigan (10) pointed out that vaccinia virus displays severe cytotoxic effects in mouse L cells. Horak et al. (7) have recently shown that L cells pretreated with interferon and infected with vaccinia virus develop a drastic cytopathic effect, which differs morphologically from the usual cytopathic effects observed in poxvirus-infected cells. They followed the destruction of the cells by measuring the release of ⁵¹Cr from the cells into the medium and showed that the effects depended on one or more late viral functions. In an earlier report, Horak et al. (6) reported an inhibition of poxvirus RNA synthesis in mouse L cells when the cytoplasmic RNA was extracted by a gentle detergent lysis, rather than the more radical mechanical lysis used by Joklik and Merigan (10).

The hypothesis of Marcus et al. (P. I. Marcus et al., *Science*, *in press*) that transcription of early viral RNA may be a site of interferon action arose from experiments in which the virion-bound transcriptase of VSV appeared to be less active in

TABLE 5. Inhibition of vaccinia virus early messenger ribonucleic acid synthesis by poly-ribinosine [poly(rI)]·polyribocytidine [poly(rC)]^a

Vaccinia (PFU/cell) ^b	(rI) _n ·(rC) _n (μg/ml)	(dI) _n ·(rC) _n ^c (μg/ml)	Acid-insoluble cytoplasmic H ³ counts per min
			4,550
	10.0		4,106
35			6,798
35	0.1		5,721
35	1.0		3,243
35	10.0		2,990
35		10.0	6,440

^a This experiment was performed as described in Table 1, footnote *a*, except that 35-mm dishes of chicken cells were used instead of 100-mm ones. Pretreatment of the cells with the synthetic polynucleotides is described in Materials and Methods.

^b Plaque-forming units per cell.

^c Deoxyinosine (dI)·ribocytidine (rC).

interferon-treated chicken cells. This observation has been confirmed in another cell system, namely VSV-infected human cells (A. Huang and E. Manders, *personal communication*), and our experiments extend the observation to yet another virus system, namely, vaccinia-infected chicken cells. Furthermore, in the case of simian virus 40, Oxman and Levin (15) have demonstrated an inhibition of early virus-specific RNA synthesis by pretreatment by interferon. Thus, three viruses that replicate in quite dissimilar fashions, a cytoplasmic DNA virus, a cytoplasmic RNA virus and a nuclear DNA virus, show a remarkable similarity in their sensitivity to interferon.

Although these results argue for a transcriptional block as a site of action of the interferon system, they certainly do not eliminate the possibility that viral translation is also a target of interferon-induced inhibition. Finally, until one is able to demonstrate an interferon-mediated effect in vitro on either viral transcriptase systems or viral protein-synthesizing systems that initiate and terminate with fidelity, all in vivo data must be taken as indicative and not conclusive.

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