*CONCERNING THE MECHANISM OF ACTION OF INTERFERON**

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Communicated by Harry Eagle, June 10, 1966

Interferons are simple proteins (mol wt about 35,000, isoelectric point about pH 6.5)[1-3] formed in response to infection with both RNA and DNA viruses. There is no doubt that interferons are coded by the host cell genome since they display absolute cell species specificity[1] and since their formation, even in cells infected with RNA-containing viruses, is inhibited by actinomycin D.[4-6] Interferons fail to affect virus multiplication if messenger RNA[7] and protein[8, 9] synthesis are inhibited: it is therefore postulated that interferons induce the synthesis of protein(s) which is the actual inhibitor of virus multiplication.

The mechanism by which the inhibitory protein acts is unknown. Neither infectious progeny RNA nor replicative intermediate are synthesized in interferon-treated cells;[10-12] and there is some autoradiographic evidence that viral DNA replication is also inhibited.[13] Further, it has been shown that an early function of RNA genomes, namely the shutting off of host cell RNA synthesis, is prevented in interferon-treated cells.[14, 15] These observations are in line with an earlier suggestion[16] that since interferons prevent the multiplication of both RNA and DNA viruses, the most likely locus of action would be the earliest common event, namely, the exercise of messenger RNA function: in the case of RNA viruses, that of the parental viral RNA itself; in the case of DNA viruses, that of the messenger RNA transcribed from parental genomes.

In L cells infected with vaccinia virus, the transcription and translation of genetic message from both parental and progeny genomes is readily studied. Such cells therefore represent a promising system for investigating the mechanism of action of the protein induced by exposure to interferon. This paper describes experiments suggesting that a primary defect is failure of early viral messenger RNA and ribosomes to combine to form polyribosomes.

Materials and Methods.—Cells and virus: Mouse L cells were propagated in Spinner culture in Eagle's medium[17] containing 10% fetal calf serum. The WR strain of vaccinia virus was grown in HeLa cells and purified according to the method of Joklik.[18] One highly purified preparation containing 3×10^{11} virus particles[19] per ml was used. The virus particle/PFU ratio was 40:1 when titrated on chick embryo fibroblasts. Cells were infected by procedure A as described by Becker and Joklik.[20] Multiplicities are hereafter expressed in terms of the number of *virus particles adsorbed per cell.* The techniques used for pulse-labeling, breaking of cells, and the preparation of cytoplasmic fractions, as well as the techniques for sucrose and sucrose-SDS density gradient centrifugation, have been fully described,[21, 22] but there were the following modifications: L cells were always broken in RSB containing NaCl rather than KCl, and were allowed to swell at 0° for 5 min rather than 10 min. Cytoplasmic fractions were the supernatants after centrifuging at 600 *g* for 2 min.

Interferon: Mouse interferon was prepared by Newcastle disease virus infection of L cell monolayers. After 24 hr at 37° the tissue culture supernatants were pooled and acidified to pH 2 with 0.1 *N* HCl, and after 5 days at 4° were neutralized with 0.1 *N* NaOH. Some of this material was applied to a column of the Amberlite ion exchange resin, XE-64, and eluted with a succinate gradient as described elsewhere.[1] Fractions containing the maximum amounts of interferon were pooled and dialyzed against Eagle's medium (MEM) prior to use. The activity of these prepara-

tions ranged between 1/3,000 and 1/10,000 when measured in an assay employing bovine vesicular stomatitis virus and an endpoint of 50% plaque reduction on L cell monolayers.

In order to determine the effect of interferon under the experimental conditions used here, interferon was assayed as follows: Replicate 50-ml aliquots of an L cell spinner culture containing 5×10^5 cells/ml were exposed to various dilutions of interferon for 24 hr. The cells were then centrifuged, resuspended at 10^7 cells/ml, and infected with vaccinia virus at a multiplicity of 80. After a 15-min adsorption period, during which 50% of the virus adsorbed, the cells were diluted to 5×10^5/ml and incubated for 24 hr. The cells were then centrifuged, resuspended in 5 ml of medium, and intracellular virus was assayed after sonication. At a dilution of 1 in 500, the interferon preparation which was used for most of this work reduced the yield of vaccinia virus by 90%, at a dilution of 1 in 100 by 98%.

In all experiments described below, two samples of cells were removed from the stock culture. One was exposed to interferon at a dilution of 1 in 100, while the other served as the control. After 16–20 hr, the cells were centrifuged, resuspended in adsorption medium at 10^7 cells/ml, and infected. All subsequent operations were carried out as described above.

Mengovirus: Mengovirus was kindly supplied by Dr. D. Summers, Department of Microbiology, Albert Einstein College of Medicine. Its titer was about 10^9 PFU/ml.

DNA polymerase assay: DNA polymerase was assayed as described by Jungwirth and Joklik,[23] with the following modifications: 40 μmoles each dATP, dGTP, and dCTP and 10 μmoles of H^3-dTTP (3.5×10^7 cpm/μmole); and 15 μg heated calf thymus DNA.

Results.—Induction of the synthesis of DNA polymerase: The activity of DNA polymerase increases at about 1 hr after the infection of HeLa cells with vaccinia virus.[23] For this and other reasons,[24] DNA polymerase is regarded as an "early" enzyme coded for by vaccinia virus DNA. A similar increase is found after infection of L cells with vaccinia virus, but not in interferon-treated cells (Fig. 1). Under the conditions used here, the synthesis of virus-coded DNA polymerase is reduced by interferon pretreatment to at most $^1/_{20}$ of that found in control cells.

Vaccinia virus DNA replication: Replication of vaccinia virus DNA can be followed by pulse-labeling cells with C^{14}-dT for 10 min, breaking open the cells, and measuring the amount of radioactive label incorporated into the cytoplasmic fraction.[25] In uninfected cells, less than 3 per cent of the incorporated radioactivity appears in the cytoplasmic fraction. In infected cells, the increased amount of radioactivity incorporated into this fraction gives a measure of the rate of viral DNA replication. Under the conditions used here, the maximum rate occurred between 1 $^1/_2$ and 2 $^1/_2$ hr after infection. In interferon-treated cells, the rate of viral DNA replication was at most 5 per cent of that in control cells and generally no more than 2 per cent. Inhibition of the synthesis of virus-coded DNA polymerase thus entails absence of viral DNA replication.

Vaccinia messenger RNA formation: Vaccinia messenger RNA synthesis can be followed by pulse-labeling infected cells with C^{14}-uridine for 15 min or less.[20] The only labeled RNA species found in the cytoplasm under such conditions are vaccinia messenger RNA and a small amount of 4*S* transfer RNA (Fig. 2*A*); host cell messenger RNA and ribosomal RNA, which are synthesized in the nucleus, do not migrate to the cytoplasm in detectable amounts within 15 min.[21, 22] Evidence that the labeled RNA species found in the cytoplasm under these conditions is indeed vaccinia messenger RNA has been presented elsewhere.[20]

The pattern of vaccinia messenger RNA formation in L cells differs somewhat from that previously described in HeLa cells.[20] At a multiplicity of 150–200, relatively little messenger RNA is formed, and its rate of formation remains fairly constant for the first 4 hr after infection. The characteristic shift in size which is

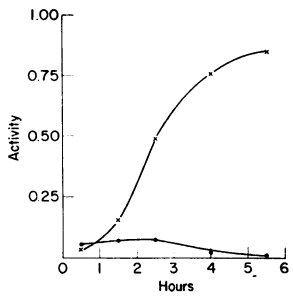

FIG. 1.—Synthesis of DNA polymerase in normal and interferon-treated L cells infected with vaccinia virus. Multiplicity: 500. Activity is expressed as mμmoles H^3-dTTP rendered acid-insoluble/mg protein/30 min/37°. The amount of protein in the $5^1/_2$ hr interferon-treated cell sample was only 13% of that in the $^1/_2$ hr sample owing to cell degradation (see below). *Crosses*, no interferon; *circles*, interferon-treated. The source of enzyme was the cytoplasmic fraction of cells disrupted by Dounce homogenization as described in *Materials and Methods*.

noted in infected HeLa cells is also observed here: most of the messenger RNA molecules formed by 1 hr after infection have an *S* value of about 10, whereas at 4 hr after infection, the median *S* value is about 18 (Fig. 2*B*). The amount of messenger RNA formed under these conditions is in general too small for adequate investigation. When larger multiplicities of infection are used (750–1000), there occurs a very large burst in vaccinia messenger RNA synthesis within the first hour of infection, all of which is of the small, 10–12*S* type (Fig. 2*A*). However, this large multiplicity of infection drastically limits the formation of late messenger RNA. Most of the experiments described below were carried out at a multiplicity of infection of 500, which was intended to serve as a compromise between the two extreme situations just described.

The effect of pretreatment with interferon on the rate of vaccinia messenger RNA synthesis is shown in Figure 3. At a high multiplicity of infection (750), there is no detectable difference between treated and untreated cells early during the infection cycle. In both cases the rate of formation of messenger RNA decreases from about 1 hr after infection on, the rate of decrease being more rapid in interferon-treated cells. As the multiplicity of infection is decreased, the rate of formation of vaccinia messenger RNA in untreated cells decreases at a more rapid rate than in interferon-treated cells, until at a multiplicity of infection of 200 or less, vaccinia messenger RNA formation is very low in normal L cells, but still rapid in interferon-treated cells. The reason for this accelerated rate of viral messenger RNA formation in interferon-treated cells is not clear at the moment (see *Discussion*).

Rate of protein synthesis: Although pretreatment with interferon does not decrease the rate of formation of vaccinia messenger RNA, there is a dramatic decrease in the rate of protein synthesis in infected pretreated cells (Fig. 4). The decrease is evident when the multiplicity of infection is as low as 50, a multiplicity at which it is almost impossible to detect any synthesis of vaccinia messenger RNA. The higher the multiplicity of infection, the more rapid the decrease in the rate of protein synthesis.

Occurrence of polyribosomes: Polyribosomes are readily detectable in normal L cells. On infection with vaccinia virus there is a small decrease in the number of polyribosomes, but the profile remains essentially unchanged for the first 4 hr of the infection cycle. Infection of interferon-treated L cells with vaccinia virus, however, leads to a rapid disaggregation of polyribosomes (Fig. 5). Even at 1 hr after infection, no polyribosomes are usually detectable in L cells pretreated with interferon. This disaggregation of polyribosomes occurs even at multiplicities of infection as low as 50. Experiments were carried out to establish the identity of physical and functional polyribosomes. In these experiments cells were pulsed for 3

min with a uniformly C^{14}-labeled mixture of amino acids, and the optical density and radioactivity profiles of the polyribosomes were compared. The two profiles were identical, indicating the absence of polyribosomes not functioning in polypeptide chain synthesis. The polyribosome profiles of uninfected normal and interferon-treated L cells are indistinguishable, showing that interferon itself has no effect on host cell polyribosomes.

Destruction of cells: Interferon-treated cells rapidly disintegrate beginning at about 3 hr after infection. Figure 6 illustrates this phenomenon. The number of cells that can be classified visually as "intact" decreases somewhat when normal L cells are infected with high multiplicities of vaccinia virus; however, this effect is greatly magnified for interferon-treated cells. Even at multiplicities of infection as low as 50, practically all cells have been converted to "ghosts" by 6 hr after infection.

Effect of mengovirus infection on polyribosomes in normal and interferon-treated cells: The drastic disaggregation of polyribosomes in interferon-treated cells infected with vaccinia virus suggested the possibility that interferon pretreatment initiated a mechanism causing disaggregation of host cell polyribosomes when followed by virus infection. In order to test this concept, the effect of infection with mengovirus on the polyribosome pattern was determined in normal cells and in cells treated with interferon. Table 1 shows the heights of the 74*S* ribosomal monomer peaks in sucrose density gradients as recorded by a Gilford-Beckman recording spectrophotometer. This technique provides a sensitive measure of the movement of ribosomes out of and into polyribosomes: disaggregation of polyribosomes results in an increase in the number of 74*S* ribosomal monomers and thus an increase in the height of the corresponding peak, and vice versa. During the first 2 hr of infection with mengovirus, the number of 74*S* ribosomes increases equally in normal and interferon-treated L cells. The higher the multiplicity, the more rapid is the disaggregation of host cell polyribosomes. This is followed by the appearance of the characteristic mengovirus RNA polyribosomes in normally infected cells, but not in cells pretreated with interferon. This experiment demonstrates that pretreatment with interferon does not *per se* lead to an accelerated breakdown of host cell polyribosomes when followed by infection; rather, it suggests that infection with vaccinia virus causes a rapid disaggregation of host cell polyribosomes, and that the liberated ribosomes rapidly reform polyribosomes with the large amounts of vaccinia messenger RNA which is being transcribed. This reformation does not occur in interferon-treated cells, so that the disaggregation of host cell polyribosomes is clearly unmasked.

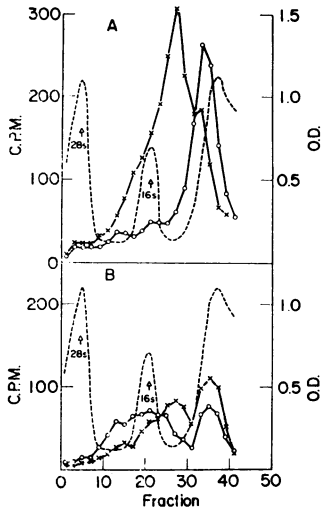

FIG. 2.—Vaccinia messenger RNA in L cells. The cytoplasmic fraction of 2×10^7 cells (1.5 ml, rendered 1% with respect to SDS) was layered onto each gradient; each cell sample had been pulsed for 10 min with 5 μc UR-C^{14}. *A*, Uninfected cells (*circles*) and cells infected for $1^1/_2$ hr at a multiplicity of 750 (*crosses*); *B*, cells infected at a multiplicity of 150 for 1 (*crosses*) and 4 hr (*circles*). *Broken curves*, optical density. Gradients: 15–30% sucrose–0.5% SDS, centrifuged at 25,000 rpm for $18^1/_2$ hr at 27°.

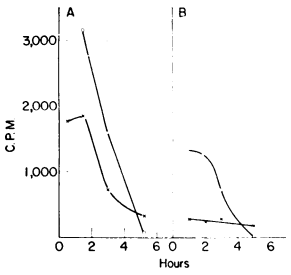

FIG. 3.—Rate of formation of vaccinia messenger RNA in normal and interferon-treated cells. Each point was derived from summation of the appropriate area of an SDS-sucrose density gradient profile obtained as described in Fig. 2. *A*, Multiplicity of infection, 750; *B*, multiplicity of infection, 175. *Crosses*, no interferon; *circles*, interferon-treated.

Attachment of messenger RNA to polyribosomes: From the work presented thus far it is clear that in interferon-treated L cells there are synthesized large amounts of vaccinia messenger RNA, but that no polyribosomes can be found as early as 1 hr after infection. This suggests that in interferon-treated cells the vaccinia messenger RNA is incapable of attaching to ribosomes to form polyribosomes. This was tested directly in the following manner. Normal and interferon-treated cells were infected with vaccinia virus at a multiplicity of 500 and pulse-labeled with C^{14}- uridine for 10 min at 1 1/2 hr after infection. The cytoplasmic extracts were then analyzed on sucrose density gradients centrifuged under conditions such that 74*S* ribosomal monomers migrated to the bottom fifth of the centrifuge tube. In such gradients free vaccinia messenger RNA remains in the top portion of the tube, whereas vaccinia messenger RNA in polyribosomes is deposited with the pellet.[21, 22] Table 2 shows the percentage of vaccinia messenger RNA in polyribosomes in both normal and interferon-treated cells. It is clear that this percentage is much greater in infected untreated L cells than in interferon-treated L cells, irrespective of the duration of the pulse. Any transient association of vaccinia messenger RNA with ribosomes is thus ruled out. The situation here is therefore different from that in HeLa cells treated with isatin-β-thiosemicarbazone, where vaccinia messenger RNA is initially able to form polyribosomes, but which then rapidly decay.[26]

Half life of vaccinia messenger RNA in normal and interferon-treated L cells: An experiment was carried out to determine whether in interferon-treated cells vaccinia messenger RNA, which does not attach to ribosomes to form polyribosomes, has a shorter half life than vaccinia messenger RNA in infected untreated L cells, which does form polyribosomes. Vaccinia messenger RNA was labeled for 10 min with C^{14}-uridine at 1 1/2 hr after infection; actinomycin D was then added to a final concentration of 5 μg/ml, and samples of the cultures were analyzed at various times thereafter in SDS-sucrose density gradients. The rate of disappearance of label from the vaccinia messenger RNA fraction was the same in untreated and interferon-treated L cells (half life 60–90 min).

TABLE 1

RELATIVE AMOUNTS OF FREE 74*S* RIBOSOMES IN NORMAL AND INTERFERON-TREATED L CELLS INFECTED WITH MENGOVIRUS

Interferon	Time after infection (hr)	OD of 74*S* peak
+ or −	0	2.08
−	1	2.74
+	1	2.82
−	2	3.96
+	2	4.04
−	4	2.18
+	4	4.14

Cytoplasmic fractions of 5 × 10^7 cells were treated with sodium deoxycholate (final concentration 0.1%) and centrifuged on 15–30% sucrose density gradients as described for Fig. 5. The maximum height of the 74*S* peak is recorded in col. 3.

Specificity of interferon: The interferon preparations used here do not inhibit vaccinia virus multiplication in HeLa cells, do not adversely affect the polyribosome population in HeLa cells, and do not inhibit the induction of the synthesis of virus-coded DNA polymerase or vaccinia virus DNA replica-

TABLE 2

PERCENTAGE OF VACCINIA MESSENGER RNA ASSOCIATED WITH POLYRIBOSOMES IN NORMAL AND INTERFERON-TREATED CELLS

Duration of pulse (min)	No interferon	Interferon-treated
4	41	16
9	57	17
15	66	18

tion. Bearing in mind the demonstrated absolute species specificity of interferon, this provided conclusive evidence that the effects described above are due to interferon and not to some nonspecific inhibitor.

Discussion.—The experiments here reported demonstrate that a primary defect in vaccinia virus development caused by exposure of cells to interferon is the abolition of the ability of viral messenger RNA and ribosomes to combine to form polyribosomes. As a result, virus-coded DNA polymerase is not synthesized, and consequently there is no replication of vaccinia virus DNA.

Certain findings warrant specific comment:

1. Host cell polyribosomes disaggregate rapidly following infection of L cells treated with interferon. This disaggregation probably also occurs in normally infected cells, but is masked there by the rapid reformation of polyribosomes on vaccinia messenger RNA which is synthesized in large amounts very soon after infection. This is strongly suggested by experiments with mengovirus, in which the disaggregation of host cell polyribosomes is clearly apparent in cells untreated with interferon: interferon does not potentiate this process. Infection of interferon-treated cells is therefore not inevitably followed by abnormally rapid disaggregation of host cell polyribosomes. The fact that disaggregation of host cell polyribosomes

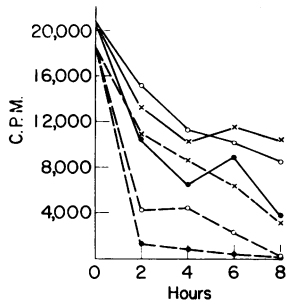

FIG. 4.—Rate of protein synthesis in normal and interferon-treated L cells infected with vaccinia virus. Each point represents the amount of radioactivity incorporated into the cytoplasmic fraction of 1.35×10^7 cells in Eagle's medium containing one tenth of the usual concentration of amino acids, pulsed for 4 min with 15 μc of a uniformly C^{14}-labeled amino acid mixture (1.5 mc/mg). *Continuous curves*, no interferon; *broken curves*, interferon-treated. *Crosses*, multiplicity of infection 50; *open circles*, 150; *closed circles*, 500.

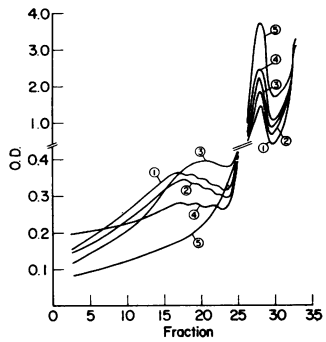

FIG. 5.—Polyribosomes in normal and interferon-treated L cells infected with vaccinia virus. Each profile was derived from a 15–30% sucrose density gradient charged with the cytoplasmic fraction of 5×10^7 L cells; 2 hr centrifugation at 25,000 rpm at 2°. Multiplicity of infection, 50. *Curve 1*, uninfected cells, normal or interferon-treated; *curves 2, 3*, and *4*, normal cells infected for 1, 2, and 4 hr, respectively; *curve 5*, interferon-treated cells infected for 1, 2, or 4 hr.

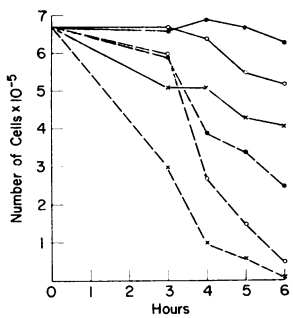

FIG. 6.—Number of "intact" cells in cultures of normal and interferon-treated L cells infected with vaccinia virus. *Continuous curves*, normal cells; *broken curves*, interferon-treated cells. *Crosses*, multiplicity of infection, 500; *open circles*, 200; *closed circles*, 100. Cell destruction was somewhat less when the initial cell concentration was 2.5×10^5 cells/ml.

is as rapid in interferon-treated cells as in untreated cells infected with mengovirus is incompatible with the hypothesis that host cell polyribosome disaggregation is an early viral function.[27] If that were so, in interferon-treated cells parental mengovirus RNA would be assuming messenger function resulting in the expression of one gene product only; for the expression of other early viral functions, such as inhibition of host cell RNA synthesis[14, 15] and formation of RNA polymerase,[11, 12] is prevented in interferon-treated cells. In view of the results obtained with vaccinia messenger RNA reported above, as well as the lack of formation of SV40 T antigen in interferon-treated cells,[28] it seems reasonable to postulate that nontranslation of viral messenger RNA's in interferon-treated cells is a widespread, if not universal, phenomenon, and that host cell polyribosome disaggregation in cells infected with mengovirus is not due to a viral function.

2. Viral messenger RNA transcription is often greater in interferon-treated cells than in untreated cells. Two possible explanations are (1) that there is a control mechanism which regulates the transcription of viral messenger RNA in untreated cells and which is absent in interferon-treated cells; or (2) that the messenger RNA transcribed in interferon-treated cells represents not only "early" viral messenger RNA, but viral messenger RNA transcribed from the whole of the parental genome. Experiments to test these two possiblities are under way.

3. Cells treated with interferon begin to disintegrate about 3–4 hr after infection, whereas untreated cells are still in reasonable shape 24 hr after infection, when the viral multiplication cycle is already long complete. Since in such cells protein synthesis is severely inhibited soon after infection, it is difficult to attribute this rapid cellular decay to a specific protein synthesized after infection, whether it is coded by the host cell or the viral genome. One explanation is that infection with vaccinia virus causes severe damage to the cell membrane of interferon-treated cells. This is being tested. Cell destruction of infected interferon-treated cells was also observed by Gauntt and Lockart.[29]

4. The work described in this paper was carried out with cells treated with large doses of interferon and infected at relatively high multiplicities. There are no data which would enable one to extend these results to cells exposed to much lower doses of interferon and infected with only one virus particle.

5. The finding that vaccinia messenger RNA does not form polyribosomes in cells pretreated with interferon can be explained by an interferon-induced alteration in either ribosomes or vaccinia messenger RNA. The experiments described here do not allow a decision as to which of these two alternatives is operative. If the alteration affects ribosomes, one would have to suppose that treatment with interferon allows ribosomes to attach to normal host cell messenger RNA but not to vaccinia messenger RNA. If the alteration concerns viral messenger RNA, the most likely change would presumably be at the initiating end of the messenger RNA molecule. Experiments are under way to decide between these alternatives. In either case, there must clearly exist a fundamental difference, probably in chemical structure, between host cell and viral messenger RNA.

The net effect of pretreatment with interferon is to convert cells into virus-inactivating structures, in which invading virus particles are uncoated, but in which no mature viral progeny formation is possible. Interferon thus does not protect cells against invasion by virus; on the contrary, cells pretreated with inter-

feron are killed and disintegrate. In this sense, cells unfortunate enough to be invaded by virus are sacrificed, but an abortive infection cycle saves the uninfected members of the population.

Summary.—Infection with vaccinia virus of L cells exposed to interferon results in a rapid and practically complete disaggregation of host cell polyribosomes and a parallel inhibition of protein synthesis. Vaccinia virus messenger RNA which is transcribed in such cells at an enhanced rate compared to that in normal cells does not reform virus-specific polyribosomes as it does in untreated L cells. Because of the lack of association between vaccinia messenger RNA and ribosomes, virus-coded DNA polymerase is not synthesized and no progeny vaccinia DNA is formed. The abortive multiplication cycle in interferon-treated cells results in complete cell destruction by 5–6 hr after infection.

Abbreviations dT, thymidine; UR, uridine; PFU, plaque-forming unit; SDS, sodium dodecyl sulfate.

* This work was supported by grants AI 04913 (to W. K. J.) and AI 05629 (to T. C. M.) from the U.S. Public Health Service. W. K. J. is the recipient of a USPHS Research Career Award (no. 1-K6-AI-22,554). The interferon used in the work reported here was prepared at Stanford; the biochemical experiments were carried out in New York.

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