Indiscriminate Degradation of RNAs in Interferon-Treated, Vaccinia Virus-Infected Mouse L Cells

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In this report we used Northern blot hybridization analysis to characterize the fate of several species of viral RNA transcribed from internal and terminal regions of vaccinia DNA in interferon-treated, infected mouse L cells grown in suspension. All species of viral RNAs were expressed but were reduced in amount. Larger-sized RNAs were reduced more than smaller-sized RNAs. This reduction appears to be related to the activation of the interferon-mediated double-stranded RNA-dependent 2-5A synthetase-endoribonuclease system, as the rRNA cleavage pattern characteristic of this system was observed early in infection and in cell extracts in response to exogenous 2-5A. Thus, in interferon-treated, vaccinia-infected mouse L cells in suspension, there is indiscriminate degradation of viral and cellular RNAs, and this RNA breakdown might play a role in the interferon-mediated inhibition of protein synthesis.

It has been known for a long time that interferon (IFN) drastically inhibits vaccinia virus protein synthesis in some mouse L cells (16, 23). This block on translation is not selective, since viral and cellular protein synthesis are equally inhibited. The nature of this inhibition is not yet known.

Two double-stranded RNA (dsRNA)-dependent enzymes have been related to inhibition of viral protein synthesis in IFN-treated, infected cells (reviewed in references 2 and 21). The first is a 2-5A synthetase, which synthesizes from ATP a series of oligonucleotides (18, 19) that activate a latent endoribonuclease. This endoribonuclease in turn leads to the degradation of mRNA and rRNA, thus inhibiting protein synthesis (3, 28, 43). The second enzyme is a protein kinase which phosphorylates the small subunit α of the initiation factor eIF-2 (13) and inhibits protein synthesis (20, 31, 44).

The aim of this investigation was to determine whether the 2-5A synthetase-endoribonuclease pathway is activated in IFN-treated, vaccinia virus-infected mouse L cells grown in suspension, and if so, if there is a difference in degradation of viral and cellular RNAs. This has been carried out by examining the fate of various species of viral RNAs and of ribosomal RNAs. The mouse L cell line used for this investigation is the same as that previously described (23). There are several important features of this virus-cell system. Vaccinia virus RNA synthesis reaches a peak at ca. 30 min postinfection (p.i.), and host shut-off is complete by 40 min p.i. (12, 23). If these cells are pretreated with IFN and subsequently infected with vaccinia virus, viral RNA synthesis continues unabated, whereas overall protein synthesis is blocked by 20 min p.i. (23). This inhibition has been related to the failure of the small ribosomal subunit to bind with viral mRNA (24).

To measure the effect of IFN on the fate of vaccinia virus RNAs in the course of infection, mouse L cells were treated with IFN (50 to 500 U/ml) in suspension, at a concentration of 1.5×10^6 cells per ml, in Eagle medium plus 10% newborn calf serum for 4 h at 37°C. The cultures were then diluted with an equal volume of medium, and incubation was

continued for a further 16 h. Control cells were similarly treated but with the omission of IFN. Mouse IFN (6×10^7 U/mg; containing 15% α and 85% β species) was prepared and partially purified on an antibody affinity column (generously provided by the late K. Pauker). Titrations were carried out in mouse L cells with vesicular stomatitis virus as a challenge virus against a mouse IFN standard (G 002-90511) from the Antiviral Substances Program, National Institutes of Health, and they are given in reference units. The viability of the cell populations (IFN treated and mock-treated) at the end of the 20-h treatment was >99%, as determined by trypan blue staining. The vaccinia yield in the presence of 50 U of IFN per ml was reduced by >90% when titrated in monkey BSC-40 cells. At the end of IFN treatment, cells were harvested by centrifugation (1,000 rpm, 5 min), resuspended at 10^7 cells per ml in Puck medium containing 1% fetal calf serum and 20 mM $MgCl_2$, and infected with 500 particles per cell of purified vaccinia virus (particle-to-PFU ratio of 35:1). After 15 min of virus adsorption with continuous stirring (designated zero time), cells were diluted to 10^6 /ml in Eagle medium containing 2% newborn calf serum. At various times p.i. total RNA was isolated by the guanidinium-cesium chloride method (22), which removes viral DNA. These RNAs (20 μ g) were then electrophoresed through 1% agarose gels containing formaldehyde (34), transferred to nitrocellulose paper, and hybridized to several cloned restriction fragments of vaccinia DNA that had been labeled with ^{32}P by nick-translation (30). The cloned fragments of vaccinia DNA chosen were an *EcoRI* 10-kilobase (kb) fragment containing the inverted terminal repetition (kindly provided by B. Moss) and the *HindIII* fragments F, G, I, J, and L. Vaccinia mRNAs mapping within and proximal to the *EcoRI* 10-kb terminal repeat, as well as the 5-kb *HindIII*-J region of the vaccinia genome, have been characterized (10, 15, 36, 37, 41, 42). Information regarding the function of the mapped viral mRNAs is only available for the 590-base pair virus thymidine kinase gene in *HindIII*-J (14, 15, 36, 37).

Figure 1 shows a representative experiment with Northern blot hybridization analysis with RNAs prepared at 3 h p.i. and hybridized to vaccinia *HindIII*-J (5 kb), *HindIII*-I (7 kb), the *EcoRI* terminal repeat (10 kb), and *HindIII*-F (13 kb).

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FIG. 1. Identification of vaccinia virus RNAs synthesized in untreated and IFN-treated, infected cells by Northern blot analysis. RNAs were prepared at 3 h p.i. by lysis of 3×10^8 mouse L cells in 4 M guanidinium thiocyanate. RNA was separated from the cell lysates by pelleting through a cesium chloride cushion (5.7 M) at $150,000 \times g$ for 18 h (22). Northern blots and hybridization conditions followed standard protocols (22, 34). The concentrations of IFN used to treat the cells (0, 50, and 500 U/ml) and the hybridization probes are indicated on top of each autoradiogram. The molecular lengths in nucleotides are given for the five species of vaccinia RNAs corresponding to the 5-kb *Hind*III fragment J (4) and for two RNAs corresponding to the 10-kb *Eco*RI terminal fragment (41). All gels were run separately. Two exposures, 16 and 48 h ($\times 3$), are shown for RNAs corresponding to vaccinia *Hind*III-F and *Hind*III-I to facilitate visualization of less abundant RNA species. With IFN doses of 50 to 500 U/ml, protein synthesis, as measured by pulse-labeling infected cells with [35 S]methionine, was inhibited by $>90\%$ at 1 h p.i. Densitometric analysis of the autoradiograms showed that in IFN-treated (50 to 500 U/ml), infected cells, RNAs (3,840 to 1,790 base pairs) were reduced in amount by 40 to 80%, and RNAs (1,790 to 590 base pairs) were reduced by 30 to 60% when compared with RNAs from untreated, infected cells.

Five species of viral RNAs are found within vaccinia *Hind*III fragment J (Fig. 1A). IFN reduced the amount of all five RNAs, even at low concentrations. When RNAs corresponding to vaccinia *Hind*III fragment I were analyzed,

three species of RNAs were observed (Fig. 1B). IFN decreases the amount of these RNAs. Similar reductions were observed with other regions of the viral genome, including the *Eco*RI 10-kb inverted terminal repetition (Fig. 1C) specific for three RNA species and the large vaccinia *Hind*III fragment F specific for at least eight RNAs. With RNAs prepared from untreated and IFN-treated, infected cells at 2 h p.i. or from the same cells treated for 4 h with an inhibitor of protein synthesis such as cycloheximide, we also observed similar reduction of viral RNAs in IFN-treated, infected cells (data not shown).

The relative abundance of hybridized vaccinia RNAs corresponding to the viral *Hind*III fragments F, G, I, J, and L and the *Eco*RI terminal repeat was determined after densitometric analysis of the autoradiograms. All classes of viral RNAs were reduced in amount; larger-sized RNAs were reduced more than smaller-sized RNAs. The extent of the reduction was not changed by RNA concentrations of 5, 10, or 20 μ g per gel. This reduction was IFN dependent and could be neutralized by antiserum to mouse IFN but not to human IFN- α .

The viral genes expressed during the replication cycle of vaccinia virus have been classified into three categories (immediate early, delayed early, and late) on the basis of the pattern of their sensitivity to inhibitors of macromolecular synthesis (25). As observed (Fig. 1), all RNAs corresponding to vaccinia DNA *Hind*III restriction fragments F, I, and J and to the *Eco*RI termini were expressed in IFN-treated, infected cells, in spite of severe inhibition ($\sim 90\%$) of viral and cellular protein synthesis (see the legend to Fig. 1). By definition, these RNAs are classified as immediate-early genes (25). The immediate-early genes constitute about half of the genetic capacity of the virus (5), and they are transcribed by the virion DNA-dependent RNA polymerase and are distributed over almost all regions of the viral genome (7). Other workers have mapped the five immediate-early RNAs within *Hind*III-J and the three immediate-early RNAs within the *Eco*RI terminal fragment of vaccinia DNA (4, 37, 41). Since inhibitors of protein synthesis are known to stimulate transcription of vaccinia virus during infection (17, 39), the reduced level of viral RNAs seen in IFN-treated, infected cells might be explained by decreased virus adsorption to cells, inhibition of viral transcription, RNA breakdown, or all three.

The possibility that reduction of vaccinia RNAs could be caused by IFN-mediated changes in virus adsorption to mouse L cells, as has been observed for vesicular stomatitis virus (40), was examined. Cells were treated with cycloheximide to prevent release of viral DNA, and the extent of vaccinia virus penetration into cells was determined by sucrose gradient analysis (1). Figure 2 shows that the amount of vaccinia virus that adsorbs, penetrates, and forms cores in IFN-treated, infected cells was similar to that in untreated, infected cells. It was concluded that IFN does not alter vaccinia virus penetration into mouse L cells.

Data which show that IFN has no effect on transcription of vaccinia virus in this particular virus-cell system, but rather that viral transcription is enhanced, has been documented in earlier publications based on kinetic studies (23, 24). Recently (11), we have shown that RNAs from IFN-treated, infected mouse L cells grown in suspension, where there is a 90% inhibition of protein synthesis *in vivo*, can be translated in a rabbit reticulocyte cell-free system into a large number of viral polypeptides. Saturating concentrations of RNAs from IFN-treated, infected cells translate as efficiently as those RNAs from untreated, infected cells. Only with nonsat-

FIG. 2. The effect of IFN on vaccinia virus penetration into infected cells. Mouse L cells growing in suspension were pretreated with 500 U of interferon per ml, and 18 h later cells were infected with 20 PFU per cell of [^3H]thymidine-labeled vaccinia virus (specific activity, 3×10^5 cpm/ μg of DNA) in the presence of cycloheximide (100 $\mu\text{g}/\text{ml}$). At 4 h p.i. cytoplasmic cell extracts were prepared, and cell-associated virus was characterized by centrifuga-

urating concentrations of IFN RNAs was there a major decline in the amount of polypeptides in the high-molecular-weight range. The polypeptide products directed *in vitro* by saturating concentrations of IFN RNAs from cycloheximide-treated infected cells (to block translation but not viral transcription) were identical in size and amount to those of control RNAs. These results showed the biological competency of the IFN RNAs as mRNAs and indicate that the inhibition of protein synthesis that occurred *in vivo* is not due to an effect of IFN on viral transcription (11).

The reduced amount of vaccinia RNAs in IFN-treated, infected cells could be due to RNA degradation through activation of the 2-5A synthetase-endoribonuclease system. Activation of the endoribonuclease by 2-5A leads to generalized degradation of both viral and cellular RNAs, as determined in cell extracts (21). Only in IFN-treated, simian virus 40-infected monkey cells, encephalomyocarditis virus-infected mouse or human cells, and reovirus-infected HeLa cells has degradation of RNAs been observed in intact cells (26, 27, 29, 32, 33, 38). Previous studies have shown that 2-5A causes a unique rRNA cleavage pattern (43). Since these cleavage patterns of rRNA correlate with intracellular levels of 2-5A (32), we examined the cleavage pattern of rRNA in the intact vaccinia virus-infected mouse L cells. In IFN-treated, vaccinia virus-infected cells, extensive cleavage of 28S and 18S rRNA was observed early in infection (Fig. 3). These cleavage patterns could be demonstrated by autoradiography after prelabeling IFN-treated cells with $^{32}\text{P}_i$ (Fig. 3) and by ethidium bromide staining of cytoplasmic RNA (11). Other evidence for enhanced degradation of RNAs is provided by a chase experiment with an inhibitor of virus transcription. Actinomycin D (20 $\mu\text{g}/\text{ml}$) was added at 30 min p.i. (at the time of maximal viral RNA synthesis; see reference 23) to block further viral transcription in untreated and IFN-treated, infected cells, and cytoplasmic RNAs were isolated at 3 h p.i. The corresponding controls were RNAs isolated at 30 min p.i. RNAs were run in agarose gels, and the degree of RNA breakdown was monitored by ethidium bromide staining (as an index of rRNA cleavage) and by Northern blot (as an index of the abundance of viral RNAs), with *Hind*III-J vaccinia DNA as a probe. These RNAs were also tested in a rabbit reticulocyte cell-free system. The results showed that IFN had no effect on viral transcription at 30 min p.i., whereas there was enhanced degradation of RNAs from IFN-treated, infected cells after the chase with actinomycin D. There was no degradation with RNAs from untreated, infected cells (data not shown).

To demonstrate that cleavage of rRNA was mediated by the 2-5A-dependent endoribonuclease, we measured the rRNA cleavage products after addition of 2-5A to cell-free systems. Cell extracts prepared from uninfected and virus-infected cells with or without IFN treatment were mixed with ^{32}P -labeled ribosomes and purified 2-5A. After incubation, total RNA was extracted and analyzed on agarose gels, and cleavage patterns were examined by autoradiography. The same unique rRNA cleavage patterns were seen in RNAs incubated with cell extracts from IFN-treated, uninfected and IFN-treated, virus-infected cells (Fig. 4). Similar patterns of rRNA cleavage have been observed in RNA

tion analysis on 20 to 45% (wt/vol) sucrose gradients (1). The position of virus particles (I) and viral cores (II) is indicated. Their identity was established by electron microscopy. The amount of radioactivity found across the gradients was (in cpm): 366,228 (virus), 60,852 (IFN 0), 58,853 (IFN 50), 51,110 (IFN 500), and 67,772 (IFN 2000).

FIG. 3. Cleavage patterns of rRNA from untreated and IFN-treated, vaccinia virus-infected cells. Mouse L cells in phosphate-free medium were prelabeled with 20 μ Ci of 32 Pi per ml in the absence or presence of IFN for 18 h. Conditions for virus infection were the same as those described in the legend to Fig. 1. The isolation, denaturation, and electrophoresis of RNA in agarose gels and autoradiography was carried out essentially as described previously (11, 32). Samples (5,000 cpm) of RNA in 6 μ l of water were denatured by incubation for 1 h at 50°C with 5 μ l of 10 M urea, 15 μ l of dimethylsulphoxide, and 5 μ l of deionized glyoxal containing 65 mM sodium phosphate (pH 7.0). Marker (1 μ l of 0.1% bromophenol blue) was added to each sample. Electrophoresis was in 1.8% agarose gel for 18 h at 10 mA in 10 mM sodium phosphate (pH 7.0). The buffer was recirculated to prevent a pH gradient across the gel. The gels were dried and exposed to X-ray film. The numbers on the top denote the times p.i. Zero time is designated after 15 min of virus adsorption. Symbols: -, untreated; + IFN treated. Lane 4+ was loaded with half the amount of labeled RNA.

isolated from IFN-treated, encephalomyocarditis virus-infected cells (32, 33, 43). In the encephalomyocarditis virus-cell system it has been proposed that IFN treatment results in the prevention of virus-mediated inhibition of the 2-5A-dependent endoribonuclease, rather than the induction of an alteration in the level of the 2-5A synthetase (32). However, in the vaccinia virus-cell system described here, the 2-5A-dependent endoribonuclease activity was the same in untreated, virus-infected cells as in IFN-treated, virus-infected cells (Fig. 4, lanes 5 and 6); unlike encephalomyocarditis, vaccinia virus did not inhibit the level of the 2-5A-dependent endoribonuclease *in vitro* (Fig. 4; data not shown).

Activation of the 2-5A synthetase requires dsRNA (2, 21). It is known that small amounts of dsRNA can be found in vaccinia RNAs extracted from virus-infected cells (6, 8, 9, 35), but it remains to be established whether these viral dsRNA forms exist *in vivo*. Our findings indicate the occurrence of viral dsRNA in the intact cells. In IFN-treated, infected mouse L cells in suspension, protein synthesis is inhibited (11; see legend to Fig. 1), viral RNA synthesis is prolonged (23, 24), and extensive rRNA cleavage occurs (Fig. 3).

Inhibition of viral and cellular protein synthesis occurs within 20 min p.i. in this IFN-treated virus-cell system (11, 23). This rapid block in translation cannot be caused by inhibition of viral transcription or by structural alterations of

viral mRNAs (i.e., capping or methylation), since IFN enhances viral transcription (11, 23, 24), and these viral RNAs are biologically active when translated in cell-free systems (11). Degradation of RNAs or activation of the protein kinase system, or both, might be responsible for inhibiting translation. Our studies show that degradation of RNAs (viral and ribosomal) occurs in IFN-treated, infected cells, supporting the hypothesis that breakdown of RNAs is a mechanism for limiting the occurrence of viral RNAs during infection and impairing the function of ribosomes. The contribution of the protein kinase to inhibition of vaccinia protein synthesis has not been established. Previous observations have shown that polyribosomes from IFN-treated, infected mouse L cells were disaggregated (16, 24) and that binding of the 40S subunit to vaccinia mRNA was blocked (24). One might postulate that the protein kinase is involved in this event.

The results presented here showed that in IFN-treated, infected cells all species of viral RNA examined were expressed but were reduced in amount, that large-sized

FIG. 4. 2-5A-dependent endoribonuclease activity in cell extracts from IFN-treated, vaccinia virus-infected cells as measured by the rRNA cleavage assay. Preparation of 10,000 $\times g$ post-mitochondrial (S_{10}) supernatants from uninfected and virus-infected cells was done as described (33). 32 P-labeled ribosomes were pelleted by centrifugation at 100,000 $\times g$ for 2 h at 4°C and resuspended in 3 mM magnesium chloride–20 mM Tris-hydrochloride (pH 7.6)–100 mM potassium chloride–0.2 mM EDTA. Post-mitochondrial supernatants (12.5 μ l, each adjusted to the same protein concentration) were incubated for 2 h at 30°C in a final volume of 25 μ l with 0.2 μ g of rRNA (109,000 cpm) and 1 μ M 2-5A (trimer and higher oligomers) by using protein synthesis conditions but without creatine phosphokinase. The RNA was extracted with phenol and electrophoresed under denaturing conditions in agarose gels. Lane 1. 32 P-labeled ribosomes isolated from IFN-treated, infected cells at 2 h p.i.; lanes 2 through 6, 32 P-labeled ribosomes from uninfected cells, which were incubated with cell extracts without (lane 2) or with (lanes 3 through 6) 2-5A. The origin of the cell extract used was uninfected cells without (lane 3) and with (lane 4) IFN treatment and vaccinia virus-infected cells without (lane 5) and with (lane 6) IFN treatment. Letters indicate the positions of rRNA cleavage products observed *in vivo*. The cell extracts shown were prepared at 4 h p.i., but similar results were observed at earlier times p.i.

RNAs were reduced more than smaller-sized RNAs, that there was extensive breakdown of rRNA, and that the 2-5A-dependent endoribonuclease was activated, all strongly suggesting that there is indiscriminate degradation of RNAs and that this effect might play a role in inhibiting protein synthesis in this virus-cell system.

We thank B. Moss for his gift of cloned *EcoRI* termini of vaccinia DNA and Victoria Jimenez for skilled technical assistance.

This research was supported by Public Health Service grant AI-16780 from the National Institutes of Health. J.B. was supported by a fellowship from Fondo para la Formacion del Personal Investigador, Spain. E.P. was supported by a fellowship from the Consejo Superior de Investigaciones Cientificas, Spain.

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