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Intracellular Production of Virus Particles and Viral Components in NIH/3T3 Cells Chronically Infected with Moloney Murine Leukemia Virus: Effect of Interferon

MORDECHAI ABOUD,[1] RUTH KIMCHI,[2] MARY BAKHANASHVILI,[2] AND SAMUEL SALZBERG[2]*

Microbiology and Immunology Unit, Faculty of Health Sciences, Ben-Gurion, University of the Negev, Beer-Sheva,[1] and Life Sciences Department, Bar-Ilan University, Ramat-Gan 52100,[2] Israel

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The effect of interferon on the biochemical properties and the maturation process of intracellular viral particles isolated from the cytoplasmic fraction of NIH/3T3 cells chronically infected with Moloney murine leukemia virus was investigated. By labeling these virions with either [^{35}S]methionine or [^{3}H]glucosamine, we demonstrated that they contain the same viral proteins and glycoproteins found in extracellular virions. Interferon treatment was found to reduce the rate of intracellular virus assembly. This effect was not a consequence of an interferon inhibition of viral RNA synthesis or its translation or a consequence of an interference with the posttranslational cleavage processing of viral precursor proteins, since all of these steps were not affected by interferon. However, the reduced rate of virus assembly could be attributed to the inhibition of viral protein glycosylation observed in interferon-treated cells. Nevertheless, despite this reduced rate, virus particles accumulated in interferon-treated cells. This accumulation was probably due to the strong inhibition of their final release from such cells.

Interferon (IFN) has been shown by several laboratories to interfere with the replication of retroviruses (8, 17, 18). However, the mechanism of its action against these viruses is not completely understood. For exogenous infection, it has been demonstrated that IFN interferes with the early stage of the virus replication cycle, probably before the integration of provirus DNA into the host genome (1-3, 5, 25, 26). Some investigators regard this early interference as a minor effect of IFN, whereas the major target of its action is claimed to reside at the late stage of virus replication (29, 30, 38). Likewise, different inhibitory effects of IFN have been described in chronically infected cells. Some studies strongly suggest that IFN inhibits the final release of virus particles from the cells (9-11, 19, 20), resulting in an accumulation of cell-associated virions (9-11, 15, 33, 38). Others indicate that the primary result of IFN action is the production of defective noninfectious virus particles (23, 27-29, 31, 38), apparently due to interference with the processing of viral proteins (13) and their assembly into complete virions (7, 14, 27, 28, 37). It is not clear whether these effects of IFN are a consequence of its action on a single target or whether they indicate that IFN acts on various targets during the virus replication cycle. Furthermore, it is possible that these effects are a result of a cellular interaction with IFN rather than a result of the antiviral activity of IFN, as recently suggested by Czarniecki et al. (16).

We recently reported data demonstrating the existence of intracellular reverse transcriptase containing virus particles in NIH/3T3 cells chronically infected with Moloney murine leukemia virus (NIH/3T3[MLV] cells) (33). We showed that IFN treatment of the cells results in an accumulation of these intracellular virions (33).

In this study, we further characterized the biochemical properties of these viral particles and attempted to elucidate the mode of IFN action against their formation.

MATERIALS AND METHODS

Cells. NIH/3T3 mouse fibroblasts chronically infected with NIH/3T3(MLV) cells were used throughout this study. The cells were maintained in Dulbecco modified Eagle medium containing 10% newborn calf serum. In experiments with IFN, the serum concentration was reduced to 2.5%.

Interferon. Mouse IFN was prepared by infecting CCL-1 cells with Newcastle disease virus and was partially purified as described elsewhere (4). The IFN preparation used in this study contained 10^6 IU per mg of protein and was applied at 80 U/ml for 24 h. Such IFN treatment of NIH/3T3(MLV) cells resulted

in 90 to 95% inhibition of virus release estimated by viral reverse transcriptase activity in the culture medium, as previously described (4, 32).

Antisera. Goat anti-p30 and anti-gp69/71 monospecific antisera and a polyvalent anti-MLV antiserum were obtained from the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute, Bethesda, Md.

Labeling and isolation of intracellular and extracellular virions. Cells were labeled with 50 μCi of [^{35}S]methionine (690 Ci/mmol, New England Nuclear, Boston, Mass.) or [^{3}H]glucosamine (59 Ci/mmol, New England Nuclear) per ml for the indicated time. In chase experiments, the labeled cells were washed four times with phosphate-buffered saline and further incubated with unlabeled medium for the required time. This chase procedure immediately stops further incorporation of the radioactive labels into total trichloroacetic acid (TCA)-precipitable material. At the indicated times, the culture medium was collected and cleared of floating cell debris by centrifugation. The cells were washed four times with phosphate-buffered saline to remove residual free extracellular virions and were dispersed by 0.4% trypsin. This trypsinization procedure removes most of the external cell-associated viral buds. The cells were washed once more with phosphate-buffered saline and then suspended in a hypotonic buffer (10 mM Tris-hydrochloride [pH 7.5], 10 mM NaCl, and 15 mM $MgCl_2$). After the cells were swelled for 10 min at 4°C they were homogenized by 20 strokes in a Dounce glass homogenizer with a tightly fitted pestle (Wheaton Scientific, Millville, N.J.). The cell homogenate was strongly blended in a Vortex mixer for 5 min. The cell membranes were removed by centrifugation at 4,000 × *g* for 10 min, and mitochondria were removed from the supernatant by a subsequent centrifugation for 20 min at 20,000 × *g*. This postmitochondrial cytoplasmic fraction was treated with 5 mM EDTA for 15 min to dissociate nascent-labeled proteins from polyribosomes. The cytoplasmic fraction and the cleared culture medium were centrifuged for 60 min at 105,000 × *g* in a Beckman SW41 rotor through a 20% (wt/vol) sucrose cushion prepared in NTE (100 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], and 1 mM EDTA) to sediment virus particles. The viral pellets were suspended in a small volume of NTE and centrifuged through 15 to 60% sucrose gradients prepared in NTE at 105,000 × *g* for 2 h in an SW41 rotor. The labeled virions were detected by TCA precipitation of samples from each fraction collected from the bottom of the gradients.

Immunoprecipitation of viral proteins and glycoproteins. Samples containing 5 × 10^5 cpm in 20 μl of a postmitochondrial cytoplasmic fraction prepared from [^{35}S]methionine- or [^{3}H]glucosamine-labeled cells were reacted with 10 μl of anti-p30 or anti-gp69/71 antisera in 300 μl of phosphate-buffered saline containing 1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Trasylol (aprotinin, Sigma Chemical Co., St. Louis, Mo.). Similar reactions were carried out with normal goat serum for control. After 1 h of incubation at 37°C, the immunocomplexes were precipitated by adding 1.5 ml of the same buffer and 50 μl of a 10% (wt/vol) suspension of a protein A-rich Cowan I strain of *Staphylococcus aureus* prepared according to the procedure of Kessler (21). After 1 h of incubation at 4°C, the immunoprecipitates were washed twice with phosphate-buffered saline containing 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, and 1 M NaCl and were washed two more times with a similar buffer but without 1 M NaCl. The precipitates were suspended in phosphate-buffered saline, and samples were counted in Instagel scintillation cocktail (Packard Instrument Co.).

SDS-PAGE. Labeled immunoprecipitates were boiled for 3 min in a small volume of a buffer containing 1 M Tris-hydrochloride (pH 6.8), 10% glycerol, 0.1% SDS, 0.3% β-mercaptoethanol, and 0.1% bromphenol blue and were subjected to SDS–10% polyacrylamide slab gel electrophoresis (PAGE) as described by Laemmli (22). For molecular weight estimation, we used bovine serum albumin (molecular weight, 68,000), ovalbumin (molecular weight, 45,000), and hemoglobin (molecular weight, 15,000) as markers. [^{35}S]methionine-labeled virions purified from the culture medium of NIH/3T3(MLV) cells were used for viral protein markers. The lanes of the gel were sectioned into 2-mm slices which were extracted for 16 h in 1 ml of Soluene (Packard Instrument Co., Rockville, Md.) and counted in a toluene-based scintillation liquid.

Estimation of the functional half-life time of viral RNA. Total RNA synthesis was stopped by 2.5 μg of actinomycin D per ml. This dose of the drug immediately arrests over 97% of [^{3}H]uridine incorporation into TCA-precipitable material. At various times after actinomycin D addition, the cells were pulse-labeled for 10 min with 50 μCi of [^{35}S]methionine per ml. Total radioactivity incorporation was determined by TCA precipitation of a sample of the labeled cells. Cytoplasmic fractions prepared from these cells were assayed for *gag* and *env* gene products by immunoprecipitation with anti-p30 and anti-gp69/71 antisera, respectively. Nonspecific precipitable counts per minute obtained with normal goat serum were subtracted from each value of the specific immunoprecipitates to estimate the amount of viral proteins synthesized at various times after viral RNA synthesis was blocked. SDS-PAGE analysis of each of the immunoprecipitates confirmed that 80 to 85% of the immunoprecipitable labeled material represented viral proteins.

Estimation of the rate of viral RNA synthesis. Cells were labeled for 30 min with 20 μCi of [^{3}H]uridine (50 Ci/mmol, New England Nuclear Corp.) per ml. Total RNA was extracted as described by Salzberg et al. (32, 33). Various amounts of the labeled RNA were hybridized with 0.2 μg of unlabeled viral cDNA synthesized in vitro by detergent-disrupted purified Moloney MLV, as described by Salzberg et al. (34) except that no labeled nucleotide was included in the reaction mixture. The amount of unlabeled cDNA was estimated from a parallel small reaction by using [^{3}H]-TTP as previously described (33). The hybridization procedure was as described by Salzberg et al. (34), except that the extent of hybridization was estimated by the fraction of labeled RNA rendered resistant to RNase A (Sigma Chemical Co.). Background values of self-annealed RNA obtained under similar conditions in the absence of cDNA were subtracted.

RESULTS

Radiolabeling of intracellular and extracellular virions. We recently demonstrated the existence of intracellular reverse transcriptase containing virions in NIH/3T3(MLV) cells (33). To further characterize these virions, we labeled NIH/3T3(MLV) cells for 4 h with either-[^{35}S]methionine (Fig. 1A) or [^{3}H]glucosamine (Fig. 1B). Labeled virions were isolated from the culture medium and the cytoplasmic fraction of these cells and were analyzed in 15 to 60% sucrose gradients. Intracellular virions cosedimented with extracellular virions with both labels, banding at a typical density of 1.12 to 1.14 g/cm^3 (Fig. 1).

SDS-PAGE analysis of the labeled virions revealed that intracellular virions resembled extracellular particles in their proteins (Fig. 1C) and in their glycoprotein composition (Fig. 1D). In some experiments, the labeled virions were lysed with SDS and their proteins were immunoprecipitated with a polyvalent goat anti-Moloney MLV antiserum before being subjected to SDS-PAGE, and the same results were obtained (data not shown). These sedimentation and structural properties, together with our previous findings that viral RNA and reverse transcriptase packaged in intracellular virus particles, suggest that these are actually completed virions.

Effect of IFN on intracellular virus for-

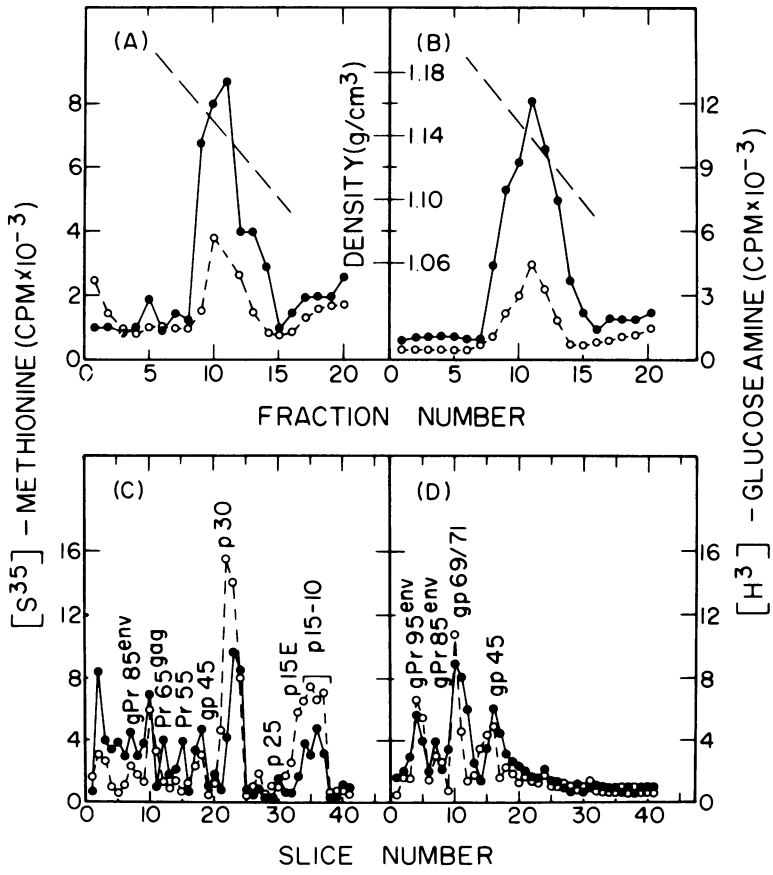

FIG. 1. *Isolation and SDS-PAGE analysis of labeled virions. NIH/3T3(MLV) cells were labeled for 4 h with either [^{35}S]methionine or [^{3}H]glucosamine. Labeled virions were pelleted from the cytoplasmic fractions (●) or the culture medium (○) through a 20% (wt/vol) sucrose cushion as detailed in the text. [^{35}S]methionine-labeled (A) and [^{3}H]glucosamine-labeled (B) viral pellets were analyzed on 15 to 60% sucrose gradients. Peak fractions of each gradient were pooled and diluted, and virus particles were collected by centrifugation. [^{35}S]methionine-labeled viral proteins (C) and [^{3}H]glucosamine-labeled viral glycoproteins (D) were analyzed by SDS-10% PAGE. Each lane of the gel with radioactive proteins was cut into 2-mm slices which were extracted for 16 h in Soluene and counted in a toluene-based scintillation liquid.*

mation. We previously showed that intracellular virus particles accumulate in IFN-treated NIH/3T3(MLV) cells (33). However, since this accumulation could result from the strong inhibition of virus release from such cells, it is unclear whether IFN affects the formation rate of these virions. Therefore, it was necessary to determine the initial rate of intracellular virus formation because otherwise the subsequent intracellular virus accumulation in IFN-treated cells would complicate the measurement of the virus formation rate in these cells. Thus, IFN-treated and untreated cells were pulse-labeled for 10 min with [^{35}S]methionine, washed, and further incubated with unlabeled medium. The incorporation of labeled proteins into intracellular virions was determined at various times thereafter. The rate of virus assembly during the initial 80 min of chase was remarkably reduced in IFN-treated cells (Fig. 2). This reduction was not a consequence of a lower [^{35}S]methionine uptake by IFN-treated cells, since its incorporation into total proteins was identical in both IFN-treated and untreated cells (data not shown).

Rate of viral RNA synthesis in IFN-treated and untreated cells. To determine whether IFN-mediated inhibition of intracellular viral formation resulted from an inhibition of viral RNA synthesis, IFN-treated and untreated cells were labeled for 30 min with [^{3}H]uridine. Total RNA was extracted and various amounts of the labeled RNA were hybridized with 0.2 μg of virus-specific unlabeled cDNA. The fraction of hybridized RNA was practically the same (3%) throughout the RNA concentration range used, indicating that cDNA was present in sufficient excess to anneal all viral RNA sequences (Fig. 3). Moreover, this experiment shows that IFN has no effect on viral RNA synthesis.

Functional half-life time of viral RNA. It was essential to determine whether IFN affects the protein synthesis capacity of viral RNA or its stability. To investigate this possibility, we compared the functional half-life time of viral RNA in IFN-treated and untreated cells. Since the synthesis of *gag* and *env* gene products is directed separately by two different viral RNA species (12, 24), it was important to determine the stability of both of these. Thus, RNA synthesis was blocked by adding actinomycin D, and at various times thereafter, the cells were labeled for 10 min with [^{35}S]methionine. Total incorporation was determined by TCA precipitation of samples of the labeled cells to estimate the average functional half-life time of cellular mRNA molecules. Postmitochondrial cytoplasmic fractions prepared from the cells were assayed for *gag* and *env* gene products by immunoprecipitation with anti-p30 and anti-gp69/71

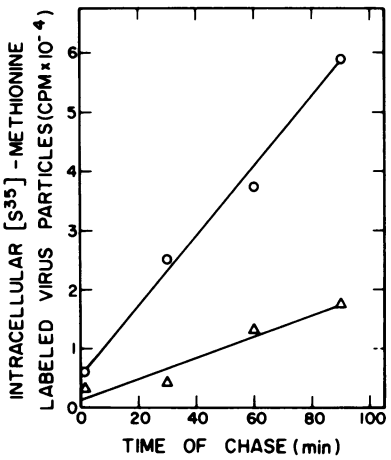

FIG. 2. *Effect of IFN on the initial rate of intracellular virus assembly. IFN-treated* (△) *and untreated* (○) *cells were pulse-labeled for 10 min with [^{35}S]methionine and subsequently chased with a nonradioactive medium. At the indicated times of chase, sampled cells were collected, and total [^{35}S]methionine incorporation was determined by TCA precipitation. Intracellular labeled virions were isolated from the cytoplasmic fraction through a 20% sucrose cushion and banded in 15 to 60% sucrose gradients. Peak fractions from density 1.12 to 1.14 g/cm^3 were pooled, and virus was quantitated by TCA precipitation and counted in a toluene-based scintillation fluid.*

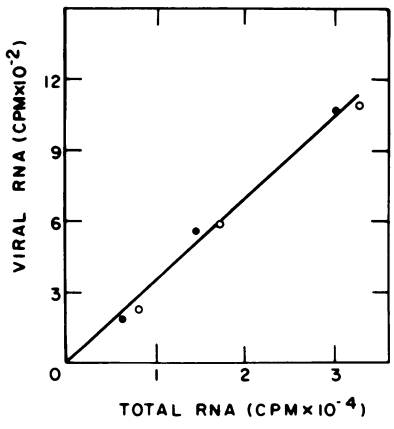

FIG. 3. *Rate of viral RNA synthesis in IFN-treated and untreated cells. IFN-treated* (○) *and untreated* (●) *cells were labeled for 30 min with [^{3}H]uridine. Total RNA was extracted, and increasing amounts were analyzed for virus specificity by hybridization with 0.2 μg of unlabeled Moloney MLV-specific cDNA.*

antisera, respectively. Nonspecific precipitates obtained with normal serum were subtracted. The range of these background values was 10 to 20%. SDS-PAGE analysis of each immunoprecipitate confirmed that 80 to 85% of the precipitated label represented virus-specific proteins (data not shown). The functional half-life time of both viral RNA species is quite similar to that of the average cellular mRNA (Fig. 4). Moreover, IFN appears to have no effect on the stability of any of the viral RNA species or on their ability to direct the synthesis of virus-specific proteins.

Posttranslational cleavage of viral proteins in IFN-treated and untreated cells. Next, we investigated the possibility that IFN interfered with the posttranslational cleavage process of the *gag* and *env* precursor proteins. For this purpose, cells were pulse-labeled for 15 min with [^{35}S]methionine and chased by washing and incubation with unlabeled medium. At various times thereafter, postmitochondrial cytoplasmic fractions were prepared from sampled cells and analyzed for *gag*- and *env*-specific proteins by immunoprecipitation with anti-p30 and anti-gp69/71 antibodies and SDS-PAGE of the immunoprecipitates. Among the viral proteins precipitated with anti-p30 antiserum, there was a protein with a molecular weight higher than 130,000, which appeared in the first one to two slices of the gel. This could be either the *gag-pol* precursor protein reported by Wood and Arlington (39) or the p30-reverse transcriptase complex reported by Bandyopadhyay et al. (6, 7). Nevertheless, IFN appears to have no effect on the cleavage processing of either the *gag* or *env* gene products (Fig. 5).

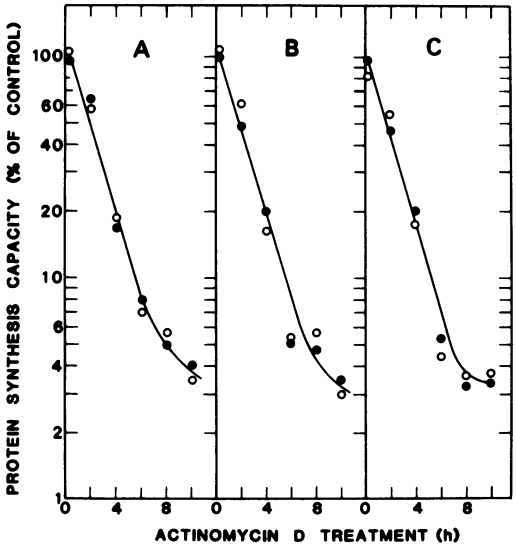

FIG. 4. *Functional half-life time of viral mRNA. RNA synthesis was arrested in IFN-treated* (○) *and untreated* (●) *cells by 2.5 μg of actinomycin D per ml. At various times thereafter the cells were pulse-labeled with [^{35}S]methionine. Total incorporation was determined by TCA precipitation of a sample of the labeled cells (A). Postmitochondrial cytoplasmic fractions were prepared, and samples containing 3 × 10^5 cpm were immunoprecipitated with either anti-p30 (B) or anti-gp69/71 (C) goat antiserum.*

Effect of IFN on the glycosylation of viral proteins. Another posttranslational processing of viral proteins involves glycosylation to produce glycoproteins of the *env* gene products. To investigate the effect of IFN on this process, IFN-treated and untreated cells were labeled with [^{3}H]glucosamine. At various times of labeling, samples of cytoplasmic fractions prepared from sampled cells were immunoprecipitated with anti-gp69/71 antiserum, and the immunoprecipitates were analyzed by SDS-PAGE. IFN significantly inhibits viral protein glycosylation during the initial stage of the labeling (Fig. 6). This inhibition was not a consequence of a reduced uptake of [^{3}H]glucosamine by the cells, since no difference in its incorporation into total cellular glycoproteins could be detected during this time between IFN-treated and untreated cells (data not shown).

Effect of IFN on the accumulation of intracellular labeled virions and their release. Finally, to follow the intracellular virions in IFN-treated and untreated cells, the cells were pulse-labeled for 10 min with [^{35}S]methionine. After the cells were washed, they were chased with unlabeled medium. At various times thereafter, intracellular labeled virions were isolated from the cytoplasmic fraction of sampled cells and extracellular virions were isolated from their culture medium. The appearance of intracellular labeled virions was detected in untreated cells long before the appearance of labeled virions in the culture medium of these cells (Fig. 7). After reaching a maximal level at 3 h of chase, the intracellular virions gradually disappeared, whereas a parallel increase in the amount of labeled virions was concomitantly observed in the culture medium. In IFN-treated cells, the intracellular labeled virions remained at their maximal level within the cells, whereas virus release into the culture medium was strongly inhibited.

DISCUSSION

In this study, we extended our investigations on the nature of the intracellular virus particles isolated from the cytoplasmic fraction of NIH/

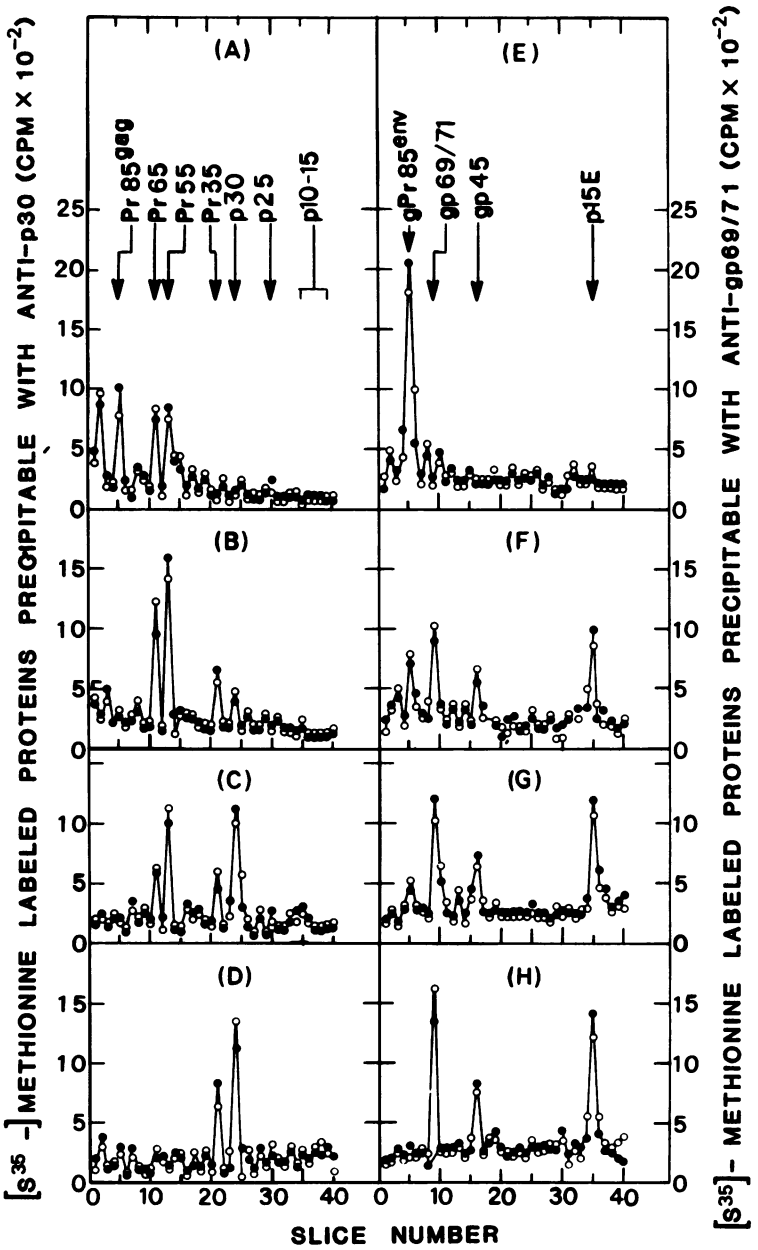

FIG. 5. *Posttranscriptional cleavage of viral precursor proteins. IFN-treated* (○) *and untreated* (●) *cells were pulse-labeled for 15 min with [^{35}S]methionine. The labeled cells were washed and chased with unlabeled medium. At 0 (A and E), 15 (B and F), 30 (C and G), and 60 (D and H) min of chase, postmitochondrial cytoplasmic fractions were prepared from sampled cells and immunoprecipitated with either anti-p30 (A, B, C, and D) or anti-gp69/71 (E, F, G, and H) goat antiserum. The immunoprecipitates were extracted, and samples containing 10^4 cpm of each were analyzed by SDS-PAGE.*

3T3(MLV) cells (33). These particles were labeled with either [^{35}S]methionine or [^{3}H]glucosamine and found to resemble extracellular virions in their sedimentation properties in sucrose gradients and in their protein and glycoprotein composition.

Intracellular labeled virions could be detected long before the appearance of labeled virions in

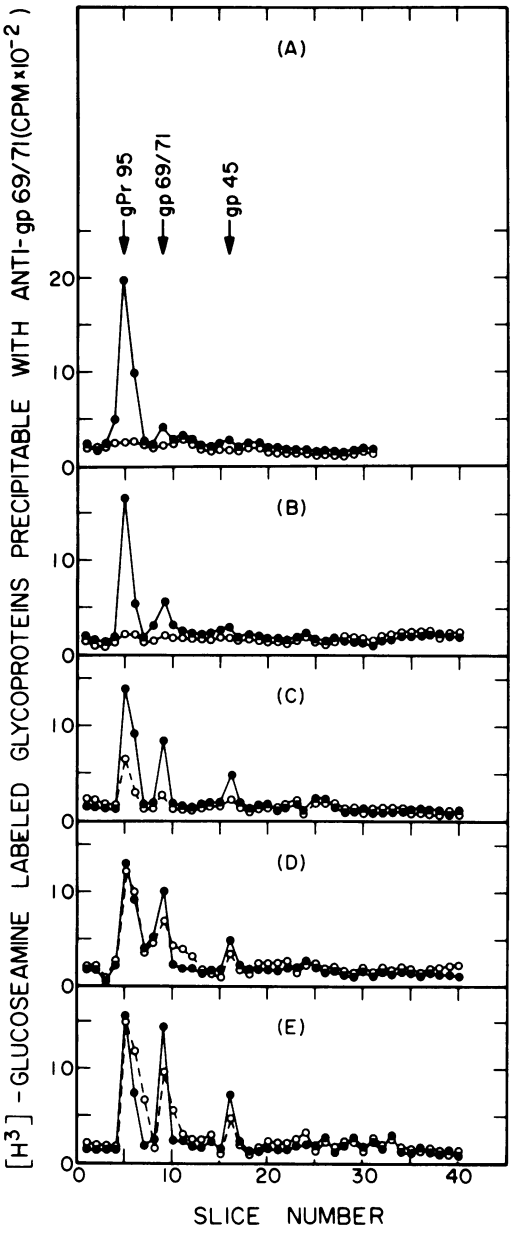

FIG. 6. *Effect of IFN on viral protein glycosylation. IFN-treated* (○) *and untreated cells* (●) *were labeled with [^{3}H]glucosamine. At 10 (A), 20 (B), 30 (C), 45 (D), and 60 (E) min of labeling, postmitochondrial cytoplasmic fractions were prepared from sampled cells and immunoprecipitated with goat anti-gp69/71. The immunoprecipitates were extracted, and samples containing 10^4 cpm were analyzed by SDS-PAGE.*

the culture medium. Moreover, in IFN-treated cells, there was a high level of intracellular labeled virions, although very few labeled virions could be detected in the culture medium of these

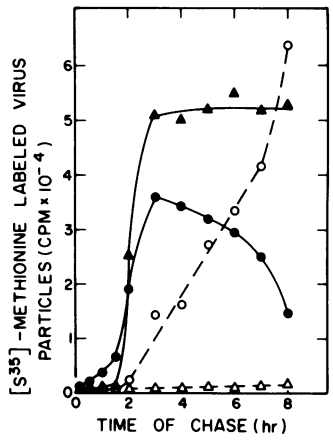

FIG. 7. *Effect of IFN on the accumulation of intracellular virions and their release. IFN-treated and untreated cells were pulse-labeled for 15 min with [^{35}S]methionine and chased with unlabeled medium. At the indicated times of chase, intracellular virions were isolated from IFN-treated* (▲) *and untreated* (●) *cells. At each of these times, the culture medium was collected from IFN-treated* (△) *and untreated* (○) *cultures, and extracellular labeled virions were isolated. Both intracellular and extracellular virions were analyzed on 15 to 60% sucrose gradients, and TCA was quantitated by precipitation of the pooled peak fractions at densities between 1.12 and 1.14 g/cm^3.*

cells. These data argue against the possibility that the cytoplasmic virions are actually extracellular virus particles that have been internalized by phagocytosis, as suggested by Sato et al. for Friend leukemia virus (35, 36). It can therefore be concluded that these virions are completed intracellularly before their release.

In our previous study (33), we demonstrated that IFN treatment of NIH/3T3(MLV) cells results in a remarkable accumulation of intracellular virions. However, this accumulation does not necessarily mean that their formation is unaffected by IFN. It might merely reflect the inhibition of their release from the cells. In fact, the present study demonstrates a considerable inhibition of the rate of virus assembly in IFN-treated cells.

IFN was found to have no effect on viral RNA synthesis or its stability or protein synthesis capacity. Nor did IFN have any effect on the posttranslational cleavage processing of viral precursor proteins. However, IFN exerted a significant inhibitory effect on the glycosylation of the *env* proteins. We could demonstrate this inhibition only during the initial stage of labeling with [^{3}H]glucosamine, since afterward the level of glycosylated viral *env* proteins in IFN-treated cells gradually approached that of control cells. However, this finding does not necessarily indi-

cate that the effect of IFN is only temporary. This effect probably continues much longer, but it is difficult to further demonstrate because of the subsequent accumulation of glycosylated *env* proteins resulting from the strong inhibition of the final virus release. This inhibition of glycosylation can explain the reduced rate of intracellular virus assembly. Such abnormal glycosylation of viral proteins may lead to the production of defective noninfectious virions, as reported by others (13, 23, 27-31, 37, 38). IFN-induced inhibition of viral protein glycosylation has also been reported to affect the assembly of vesicular stomatitis virus (23).

Finally, it is yet unclear whether the effect of IFN on virus assembly and glycosylation of viral proteins is due to a direct action of IFN on these processes or is an indirect feedback consequence of IFN-induced inhibition of the final virus release. Moreover, the effect of IFN on retroviruses might be a consequence of its interaction with the cell membrane (17, 18) or other cellular effects (16) rather than due to its antiviral activities (17).

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