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MECHANISM OF INTERFERON ACTION
Inhibition of Vesicular Stomatitis Virus in Human Amnion U Cells by Cloned Human Leukocyte Interferon

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SUMMARY. The effects of a subsaturating, long treatment (24 h) dose of a highly purified cloned subspecies of human leukocyte interferon (IFN-αA) on vesicular stomatitis virus (VSV) primary macromolecular synthesis in tsG41-infected human amnion U cells were examined. IFN-αA, under these conditions, was found to inhibit primary VSV protein synthesis ten-fold while producing no detectable effect on the amount or integrity of primary viral message transcripts. There was no selective reduction by IFN-αA of the VSV G or M proteins.

Interferons (IFN) are a set of extracellular regulatory proteins that act upon a variety of target cells and profoundly affect a number of biological functions (1). Most notably, in many animal cells IFNs inhibit the replication of a wide range of RNA and DNA viruses. Studies of IFN production and action have frequently made use of the rhabdovirus VSV, a negative-stranded RNA virus. The suitability of VSV as a challenge virus is due to its acute sensitivity to the antiviral effect of IFN, its broad host range and its rapid multiplication. Earlier reports on the mechanism of the antiviral action of IFN variously attributed IFN-induced inhibition of VSV replication to an effect on VSV transcription (2,3), mRNA cap methylation (4), mRNA translation (5-7), or glycosylation and incorporation of the envelope glycoprotein G into assembled virions (8,9).

Using a molecularly cloned subspecies of human leukocyte IFN, IFN-αA, we have recently shown that in human amnion U cells the kinetics of induction of antiviral activity against VSV are biphasic over a period of 24 h of IFN treatment (10). Similar biphasic kinetics also have been observed with other cloned

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human leukocyte IFN subspecies, IFN-αB, αC, αF, αI and αJ (11). We have examined in detail the first phase of IFN-αA-induced inhibition of VSV replication in U cells (0-6 h IFN treatment) and found that this inhibition occurs principally, and probably solely, at the level of viral protein synthesis (10,12). No significant first phase effect was observed on adsorption, penetration and uncoating of infecting virions, nor was there an inhibition of transcription *in vivo* or a reduction of the *in vitro* translational activity of viral mRNA synthesized *in vivo*. As well, progeny VSV released from IFN-treated cells, although greatly reduced in number, were found to be equally as infectious as VSV released by untreated cells.

Although the first phase of the antiviral action against VSV accounts for the major extent of inhibition of replication of this virus, the biphasic kinetics of induction of IFN action raised the possibility that, in different time frames, two or more IFN-induced activities might play a role in the inhibition of VSV multiplication. In this communication we report that treatment of U cells with a subsaturating dose of highly purified, cloned IFN-αA for a long time period (24 h) did not unmask any effects not detected following only 6 h of treatment with a saturating dose of IFN-αA. We have also extended our analysis of VSV transcription to include the NS and G RNAs in addition to the N and M RNAs analyzed earlier (12).

MATERIALS AND METHODS

Materials. Plasmids containing cloned cDNAs to the N, NS, M and G mRNAs of VSV (13-15) were generously provided by Dr. J.K. Rose, Salk Institute, San Diego, CA. L-[^{35}S]methionine (1060 Ci/mmol) was from New England Nuclear; [α-^{32}P]dTTP (3000 Ci/mmol) was from Amersham. The sources of all other materials and reagents have been described previously (10,12).

Cells, Viruses, and IFN. The growth of human amnion U cells in static monolayer culture and the growth and preparation of wild type VSV and the VSV mutant tsG41(IV) (both Indiana serotype) were as described previously (10,12) except that U cells were maintained in Eagle's MEM supplemented with 5% fetal calf serum instead of 7% newborn calf serum. Highly purified (~90% pure) cloned human leukocyte IFN-αA (equivalent to IFN-α2) was generously provided by Dr. D.V. Goeddel, Genentech, S. San Francisco, CA (16).

Assay of Viral Protein Synthesis In Vivo. Primary viral protein synthesis in U cells infected with VSV tsG41 was measured as described in detail elsewhere (12). Briefly, duplicate U cell monolayers (21 cm^2 LUX dishes), which had been treated for various times with IFN-αA at 37°C, were infected at a multiplicity of 30 pfu/cell for 1 h at 37°C and then were shifted to 39.5-40°C.

Mock-infected controls were treated identically. Proteins were labeled from 4-4.5 h post-infection at 39.5-40°C with methionine-free MEM containing 0.5 μg/ml actinomycin D and 46.6 μCi/ml [^{35}S]methionine, after which the label was chased for 1 h by incubation with maintenance medium. NP40 extracts were then prepared. ^{35}S-labeled polypeptides were analyzed directly by NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography, or were first immunoprecipitated with anti-VSV antiserum and formalin-fixed Staphylococcus aureus before gel electrophoresis (10,12).

VSV-specific Antiserum. VSV virions were purified (18) and UV-inactivated, and 1 mg of this preparation, in Freund's complete adjuvant, was used to subcutaneously immunize a 2 kg New Zealand albino rabbit. Booster immunizations (1 mg) were given at 2 and 6 week intervals; blood was collected 7, 10 and 14 days after the last injection.

RNA Blots and Hybridizations. Analysis of primary viral RNA synthesized in U cells infected with VSV tsG41 was as described previously (12). U cell monolayers (147 cm^2 LUX dishes) were treated with IFN-αA and infected with VSV tsG41 exactly as for protein labeling. At 4 h post-infection (at 39.5-40°C), cells were harvested and whole cytoplasmic RNA was isolated. RNA samples were reacted with glyoxal and fractionated on 1.2% agarose gels prior to transfer to nitrocellulose. Alternatively, for quantitative estimates, glyoxylated RNA samples were serially 2-fold diluted with 20X SSC and transferred directly to nitrocellulose using a BRL Hybri-Dot Manifold. Probes for hybridization were prepared from cDNAs to the N, NS, M and G mRNAs of VSV, which had been excised with PstI from their parent plasmids pN4, pNS319 and pM309 (13,14), or with EcoRI from pGR125 (15). The cDNA inserts were nick translated (19) in the presence of [α-^{32}P]dTTP to yield probes of 3-5 x 10^8 cpm/μg DNA.

RESULTS AND DISCUSSION

The induction of antiviral activity against VSV in human amnion U cells treated with varying doses of highly purified, cloned human IFN-αA was determined by the single-cycle yield reduction assay. As shown in Table 1, ~90% pure IFN-αA, used in 24 h treatments, inhibited wild type VSV replication in a

Table 1

Antiviral Activity of Highly Purified IFN-αA Against Wild Type VSV in Human Amnion U Cells[a]

IFN-αA dose (ng/ml)	viral yield (pfu/ml)	$\log_{10}$ reduction
0	2.0 x 10^9, 2.0 x 10^9	--
0.3	5.4 x 10^8	0.57
1.0	3.1 x 10^8	0.81
3.0	1.9 x 10^8	1.02
10.0	4.4 x 10^7	1.66

[a]Single-cycle (12 h) viral yields were measured as previously described (10,17), except that IFN treatments were for 24 h and cultures were infected with VSV at a multiplicity of 30 pfu/cell.

concentration-dependent manner. IFN concentrations are expressed in nanograms of protein per ml because activity unitage varies widely among human cell lines and with different challenge viruses (11,20-22). In all following experiments, a subsaturating dose, 3.0 ng/ml, was used.

In order to examine directly the relative effects of IFN-αA-treatment on VSV transcription and protein synthesis, we used the VSV mutant tsG41, which at the nonpermissive temperature (39.5-40°C) carries out transcription but does not detectably synthesize genome-length 42S (+) or (-) strand species of viral RNA (23,24). This restricted our analysis to primary viral transcription and translation, thereby allowing us to distinguish between a true effect of IFN on VSV transcription and an indirect inhibition of secondary viral transcription brought about by IFN-induced inhibition of viral protein synthesis (3,12).

U cell monolayers were treated for 4, 8 or 24 h with 3.0 ng/ml of IFN-αA at 37°C and then were infected with VSV tsG41 and shifted to 39.5-40°C. Cells were labeled with [^{35}S]methionine from 4-4.5 h post-infection, and viral proteins were immunoprecipitated and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1A). Primary synthesis of VSV polypeptides decreased progressively over the course of 24 h of IFN treatment. The extent of protein synthesis inhibition at 24 h was seen to be approximately ten-fold by comparison with viral polypeptides immunoprecipitated from a ten-fold dilution of extract from untreated, infected cells diluted with extract from untreated, uninfected cells (Fig. 1A, lanes g-l). A longer autoradiographic exposure of these samples (Fig. 1A, lanes g'-l') showed that inhibition of viral protein synthesis was uniform; no individual viral polypeptide (in particular, G or M protein) was selectively reduced by IFN treatment. Analysis of cell extracts prior to immune precipitation (Fig. 1B) also revealed that 24 h of IFN-αA treatment of U cells had no effect on cellular protein synthesis. Clearly, the reduction of VSV protein synthesis was not due to a general anticellular effect of the IFN.

Whole cytoplasmic RNA was isolated at 4 h post-infection from IFN-αA-treated, VSV tsG41-infected U cell monolayers parallel to those in which

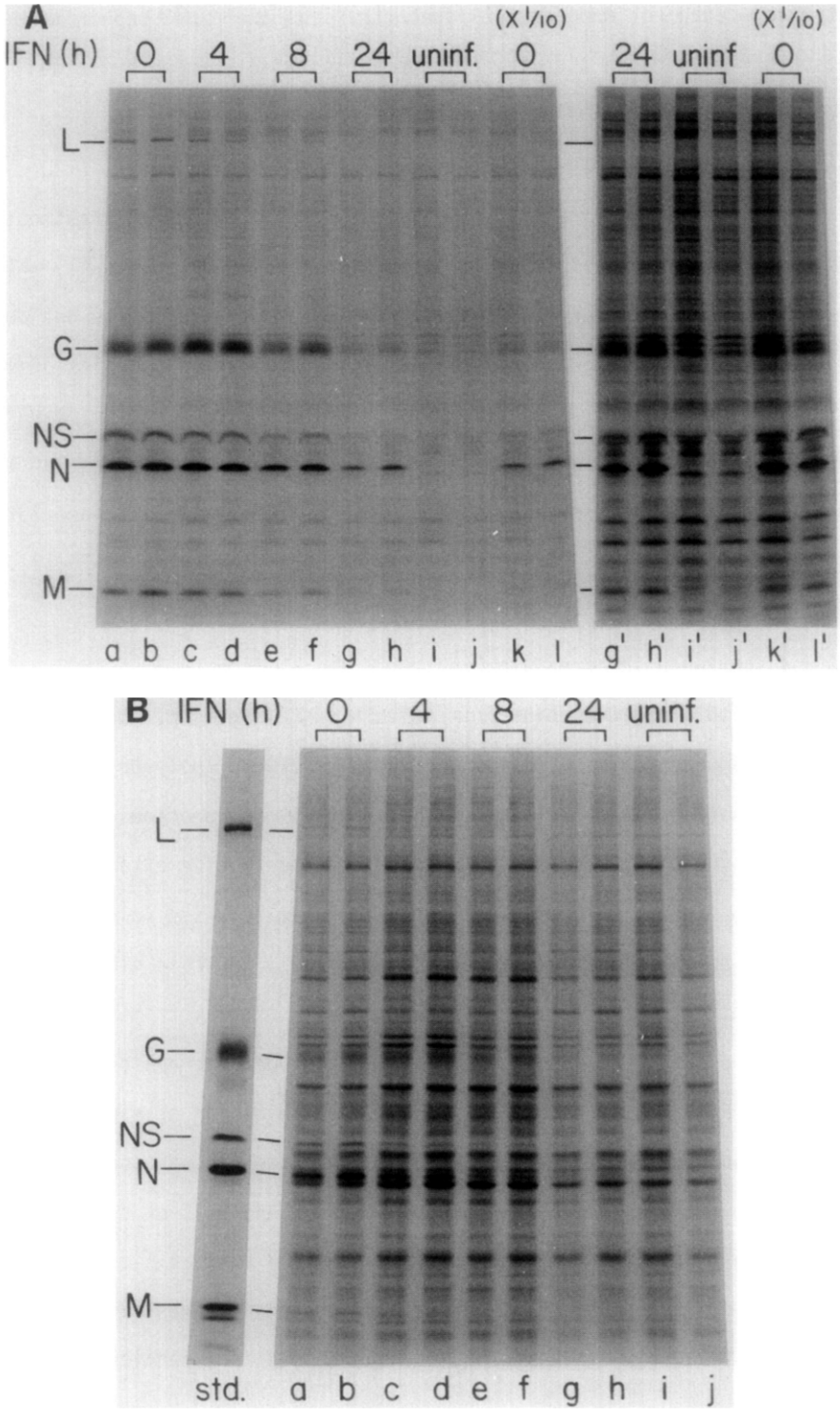

Figure 1. Primary viral protein synthesis in IFN-treated amnion U cells infected with VSV tsG41. U cell monolayers were untreated (a,b,i-l) or treated with 3.0 ng/ml of IFN-αA for 4 h (c,d), 8 h (e,f), or 24 h (g,h). Monolayers were then left uninfected (i,j) or were infected (a-h,k,l) with VSV tsG41.

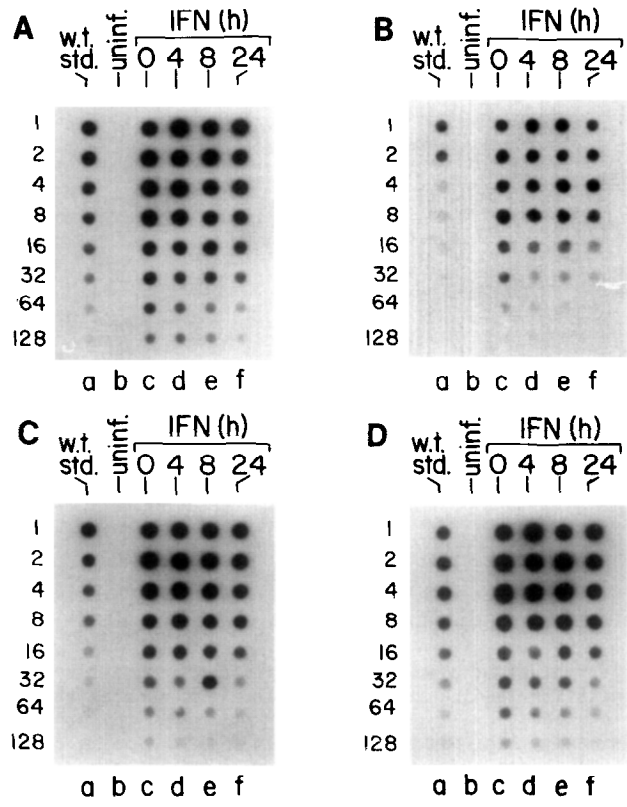

Figure 2. Dot blots of primary N, NS, M and G gene mRNA in IFN-treated amnion U cells infected with VSV tsG41. U cell monolayers (parallel cultures to those in Fig. 1) were untreated (b,c) or treated with 3.0 ng/ml of IFN-αA for 4 h (d), 8 h (e), or 24 h (f). Monolayers were then left uninfected (b) or were infected (c-f) with VSV tsG41. At 4 h post-infection (39.5-40°C), RNA was purified from cells, glyoxylated, serially diluted 2-fold, transferred to nitrocellulose, and probed with [α-^{32}P]dTTP-labeled cDNA to N (A), NS (B), M (C), or G (D) gene mRNA. Reciprocal dilutions are listed to the left of each set of dots; all undiluted samples contained 10 µg RNA, except for a standard of RNA from wild type VSV-infected U cells (a) which contained 0.24 µg RNA at the highest concentration.

primary viral protein synthesis had been labeled. Dot blots of serial two-fold dilutions of these RNA samples were hybridized to [α-^{32}P]dTTP-labeled cDNA to the VSV N, NS, M or G genes (Fig. 2). Primary viral RNA synthesis remained unaltered over the same time course of IFN-treatment (Fig. 2) which had reduced

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Cells were labeled with [^{35}S]methionine from 4-4.5 h post-infection at 39.5-40°C and harvested at 5.5 h post-infection; cell lysates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis either directly (B) or following immune precipitation (A). In (A), the material immunoprecipitated in lane k or l was a mixture of one-tenth of the amount of extract in lane a or b (untreated, infected) diluted with nine-tenths the amount of extract in lane i or j (untreated, uninfected); lanes g'-l' are a ten-fold longer autoradiographic exposure of lanes g-l. In (B), lanes a-j contain one-tenth the amount of lysate from which the material in (A) was immunoprecipitated; the standard at the left is from wild type VSV-infected mouse L_{929} cells.

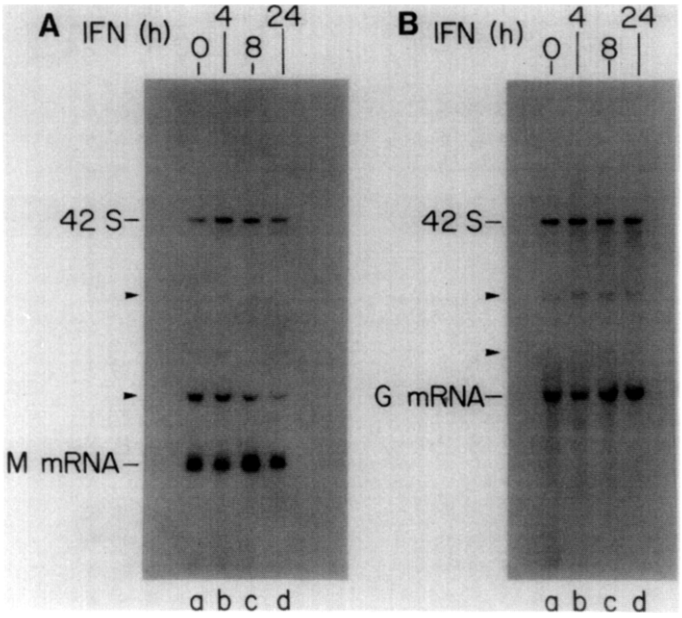

Figure 3. Northern blots of primary M and G gene mRNA in IFN-treated amnion U cells infected with VSV tsG41. RNA was from the same samples shown in Fig. 2 from VSV tsG41-infected U cells which had been untreated (a) or treated with 3.0 ng/ml of IFN-αA for 4 h (b), 8 h (c), or 24 h (d). 7.5 μg of RNA of each sample was electrophoresed through 1.2% agarose, blotted onto nitrocellulose and probed with [α-^{32}P]dTTP-labeled cDNA to VSV M mRNA (A) or VSV G mRNA (B).

primary viral protein synthesis by a factor of ten (Fig. 1). To examine the structural integrity of the viral RNA from IFN-treated cells, RNA samples were separated by agarose gel electrophoresis prior to being blotted onto nitrocellulose and probed with labeled M or G cDNA. Because M and G were the most distal of the four available probes, these would have been expected to be the most sensitive to any potential IFN-induced defect in viral mRNA synthesis due to the sequential nature of VSV transcription. As can be seen in Fig. 3, there were no significant differences in the amounts or sizes of the viral M or G message species as a function of IFN treatment, nor did IFN affect the steady-state levels of M- and G-hybridizing polycistronic VSV RNA species (ref. 12; denoted by arrows in Fig. 3). The constancy of the 42S bands observed in Fig. 3 also established that the infections with VSV tsG41 were truly only carrying out primary macromolecular synthesis, because a decreasing gradient of 42S RNA synthesis as a function of IFN treatment would have been expected if any secondary RNA synthesis had occurred.

These results demonstrate that a subsaturating IFN-αA dose administered for a long period of treatment (24 h) produced a ten-fold decrease in the amount of primary VSV protein synthesis in the absence of any detectable reduction in the amount of the four primary VSV transcripts analyzed. This argues against a role for mRNA degradation by the 2'-5' iso-oligoadenylate synthetase/nuclease system (25) in the mechanism of human leukocyte IFN inhibition of VSV in U cells. In addition, it has been suggested that subsaturating dose treatments of cells with IFN unmask an effect which selectively inhibits G and M protein incorporation into virions (8,9). Our results here and previously (10,12) rule out induction of such an activity by human leukocyte IFN-αA in human amnion U cells. The IFNα-induced inhibition of viral protein synthesis in U cells appears to be caused by an alteration of a component of the translational machinery other than the mRNA, which results in a uniform reduction in synthesis of the five VSV proteins.

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