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Effect of Human Alpha A Interferon on Influenza Virus Replication in MDBK Cells

RICHARD M. RANSOHOFF,[1,2] PATRICIA A. MARONEY,[1] DEBI P. NAYAK,[3] THOMAS M. CHAMBERS,[3] AND TIMOTHY W. NILSEN[1]*

Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine,[1] and Department of Neurology, Cleveland Clinic Foundation,[2] Cleveland, Ohio 44106, and Department of Microbiology and Immunology, University of California at Los Angeles School of Medicine, Los Angeles, California 90024[3]

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To determine the molecular mechanism whereby interferon induces resistance to influenza virus, we began an investigation of influenza virus replication in MDBK cells treated with recombinant human alpha A interferon. Negative- and positive-strand virus-specific RNA accumulation was monitored by blot hybridization with cloned probes. Primary transcription (transcription of infecting viral negative strands by the virion-associated polymerase) was inhibited by interferon treatment of MDBK cells. At moderate levels of interferon treatment (10 U/ml), this inhibition was restricted to transcripts of polymerase genes, whereas at higher levels of interferon treatment (50 U/ml), accumulation of all primary transcripts was markedly inhibited. Secondary transcripts and viral negative strands did not accumulate to any significant extent in interferon-treated MDBK cells. These results suggest that interferon-induced mechanisms which inhibit influenza virus replication in MDBK cells act at the level of primary transcription.

Although the antiviral action of interferon was initially detected in experiments with UV-inactivated influenza virus (10), the mechanism by which interferon inhibits influenza virus is not well understood. The best-characterized interferon-induced mechanism, the 2′,5′-oligo(A) synthetase-RNase L pathway (1, 15), is not likely to be effective as a general mechanism of influenza virus inhibition, since interferon treatment of embryonal carcinoma cells induces 2′,5′-oligo(A) synthetase (28) and confers a limited antiviral state in which cells are protected against reovirus and encephalomyocarditis virus but remain susceptible to influenza virus (19).

The specific mechanism by which interferon treatment inhibits influenza virus replication is of particular interest because influenza virus is the sole nononcogenic RNA virus which carries out important steps in replication within infected cell nuclei (7). To date, there is no consensus about the stage in influenza virus replication at which inhibition occurs in interferon-treated cells. Bean and Simpson (2) observed inhibition of primary transcription in interferon-treated, influenza-infected chick embryo cells. However, Repik and co-workers (22) found that inhibition of influenza replication probably occurred at a stage after primary transcription in interferon-treated, influenza-infected chicken embryo fibroblasts and L cells.

Conflicting results have also been obtained in studies of the mechanism whereby interferon inhibits influenza replication in murine cells bearing the dominant allele *Mx*, which confers interferon-dependent resistance to orthomyxoviruses (5, 6). Experiments by Meyer and Horisberger (17) in influenza-infected *Mx*-bearing murine macrophages indicate that primary transcription is largely unaffected by interferon treatment, whereas viral protein synthesis is virtually abolished. In contrast, Krug and co-workers (14) have recently found that the rate of synthesis of influenza primary transcripts in influenza-infected *Mx*-bearing mouse embryo cells treated with interferon is dramatically reduced.

* Corresponding author.

To determine what mechanism of interferon action operates to bring about inhibition of influenza replication in other animal systems, we studied the effect of recombinant human alpha A interferon on influenza virus replication in MDBK cells. MDBK cells were selected for study because they are permissive for influenza virus strain WSN (12) and are highly responsive to human alpha A interferon (27). Our initial approach was to determine what stage in the influenza replicative scheme is specifically inhibited in interferon-treated cells. To do so, we first evaluated accumulation of viral negative strands in infected cell nuclei at a time (2 h) before significant amplification of infecting viral genomic RNA (Fig. 1). Hybridization probes specific for a polymerase segment (PA [2.2 kilobases]) and the major nonstructural protein (NS_1 [0.9 kilobases]) were used to determine the effect of interferon treatment on accumulation of segments of different sizes. We also did assays at 5 h postinfection to assess the effect of interferon on negative-strand amplification. There was no effect of interferon treatment on nuclear accumulation of negative strands at the 2-h time point, whereas there was a markedly reduced amount of viral negative strands in samples from interferon-treated cells at 5 h postinfection (Fig. 1). Since there was essentially no amplification of negative strands between 2 and 5 h postinfection in samples from interferon-treated cells (Fig. 1), it seems likely that the negative strands detected in samples from interferon-treated cells and in control cells at 2 h postinfection represent the infecting viral genome. If this is the case, this experiment provides an indirect assessment of adsorption, penetration, and uncoating, since these processes are required precedent events for nuclear accumulation of influenza virus genomic RNA (4). Results of this assay accordingly indicate that these early processes are unaffected by interferon treatment of MDBK cells, whereas subsequent amplification of negative strands is dramatically inhibited. We also assayed the level of influenza virus-specific mRNAs in interferon-treated and untreated cells by blot hybridization analysis of cytoplasmic poly(A)$^+$ RNA prepared at the point of maximal mRNA accumulation (7 h).

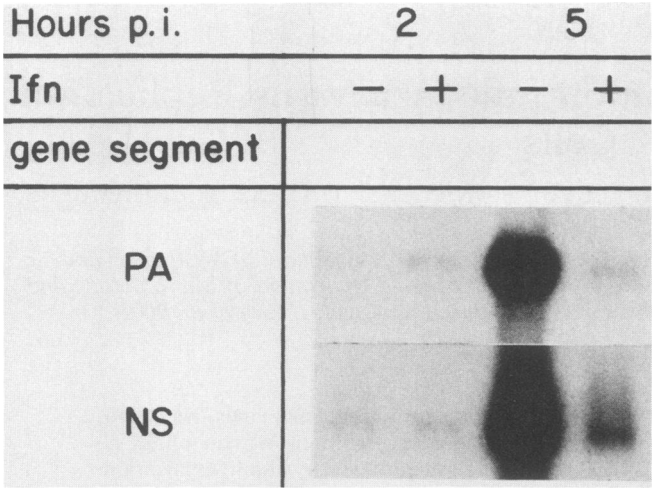

FIG. 1. Inhibition of influenza virus negative-strand accumulation in interferon-treated MDBK cells. Influenza A virus strain WSN and MDBK cells were both obtained from Robert Lamb (Northwestern University, Evanston, Ill.). WSN virus was amplified on MDBK cells as previously described (12). MDBK cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Recombinant human alpha A interferon was provided by Sidney Pestka (Hoffmann-La Roche Inc., Nutley, N.J.). The concentration of interferon required to protect MDBK cells from the cytopathic effect of influenza virus was assayed by endpoint dilution as previously described (19). Confluent cell monolayers were incubated with 50 U of interferon per ml for 16 h and infected as previously described (12). At the indicated time points, detergent-washed nuclei were prepared (20) and nuclear RNA was extracted with guanadinium isothiocyanate-hot phenol, as previously described (16). RNA (2.5 μg) was fractionated by electrophoresis on 1.3% agarose-6% formaldehyde gels and transferred to Genescreen (New England Nuclear Corp., Boston, Mass.) as previously described (16). Filters were prehybridized for 4 to 6 h and then hybridized for 14 to 16 h with 4×10^6 cpm of plus-strand DNA prepared by primer extension with genome-sense M13 clones (18, 24) as template. Primer extension was performed as previously described (J. B. Virgin, B. J. Silver, A. R. Thomason, and J. H. Nilson, J. Biol. Chem., in press), except that 2.5 μg of template DNA and 1.0 ng of single-stranded primer were used in a final reaction volume of 20 μl. The double-stranded product was denatured by suspension in 60 mM NaOH-, 12 mM EDTA-, 12% glycerol-0.1% bromocresol green and incubation at 95°C for 5 min. The strands were separated by electrophoresis on a 1% agarose gel in 1× TBE (100 mM Tris, 100 mM borate, 2 mM EDTA [pH 8.3]) and the single-stranded probe was isolated from the appropriate gel slice and dissolved in hybridization buffer in a boiling water bath. Hybridization probes used in this assay detect gene segments coding for a polymerase (PA) and nonstructural (NS) proteins. Filters were washed and exposed at −80°C to photographic film (Kodak XAR-5) with two Cronex intensifier screens. No material hybridizing with influenza virus-specific cDNAs was present in mock-infected controls (data not shown).

This analysis revealed that influenza virus mRNA synthesis was essentially abolished in interferon-treated cells (Fig. 2).

Failure to accumulate viral RNA in interferon-treated cells could result from inhibition of any step in viral replication. However, it was of particular interest to assay primary transcription in view of previous findings by Bean and Simpson (2) and Krug and co-workers (14) in other cell systems (see above). Because influenza replication past the stage of primary transcription requires protein synthesis, infection in the presence of cycloheximide arrests influenza virus replication at that point (23), and primary transcripts accumulate in linear fashion for at least 7 h (25). Accordingly, primary transcription was assayed at 7 h postinfection by blot hybridization in cells infected in the presence of cycloheximide. Cells were pretreated with either 10 or 50 U of interferon per ml; controls infected in the presence of cycloheximide were not pretreated.

At the lower level of interferon treatment, primary transcripts from polymerase gene segments (PB_2, PA) were significantly inhibited, whereas transcripts from three other gene segments (NS, [nonstructural] M [matrix], NA [neuraminidase]) were either unaffected or increased (Fig. 3). The higher level of interferon treatment led to dramatic inhibition of accumulation of transcripts from all influenza gene segments (Fig. 3). Results observed with a hybridization probe for mRNA for PB_1, the third polymerase protein, were identical with those seen with probes for PB_2 and PA mRNAs (data not shown).

Together with providing information relevant to the stage

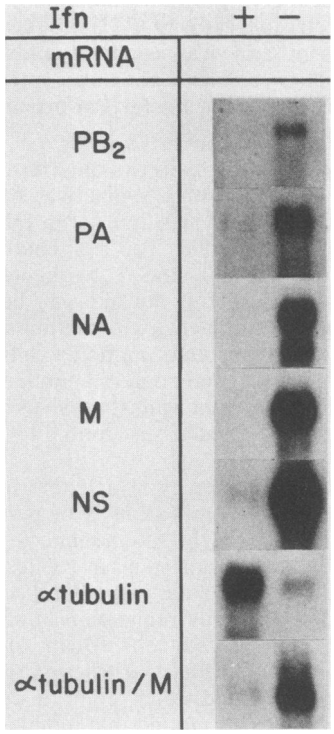

FIG. 2. Inhibition of influenza virus mRNA accumulation in interferon-treated MDBK cells. MDBK cells were treated with interferon, infected, and harvested at 7 h postinfection. The postnuclear supernatant was extracted twice with phenol-chloroform, ethanol precipitated, and dissolved in water. PolyA^+ RNA was isolated by oligo(dT)-cellulose affinity chromatography, ethanol precipitated, and dissolved in water. Poly$(A)^+$ RNA (0.2 μg) was subjected to blot hybridization analysis as described in the legend to Fig. 1, except that mRNA-sense M13 clones were used as templates for primer extension to generate the hybridization probe. Blots analyzed for alpha tubulin mRNA contained 0.75 μg of RNA per lane. Hybridization probes used in this experiment are designated by standard abbreviations for the corresponding influenza proteins: PA, polymerase protein PA; PB_2, polymerase protein PB_2; NA, neuraminidase; M, matrix protein; NS, nonstructural protein 1. The filter previously hybridized with the alpha tubulin probe was rehybridized with a probe for M mRNA, demonstrating directly the presence of intact influenza mRNA in cells in which degradation of cellular mRNA had occurred.

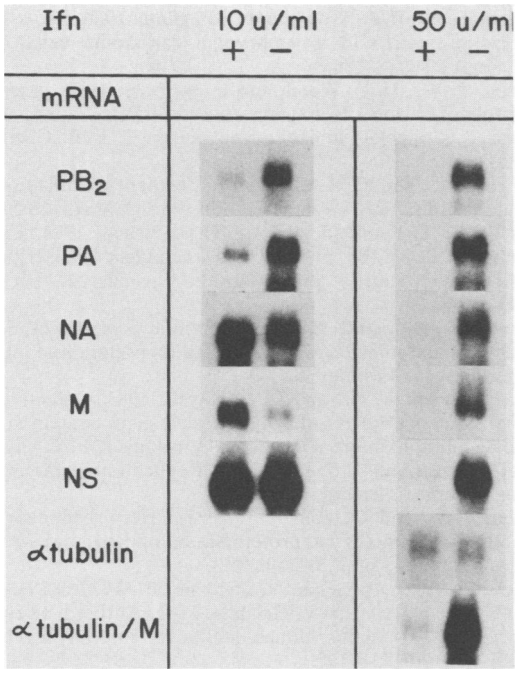

FIG. 3. Inhibition of influenza virus primary transcript accumulation in interferon-treated MDBK cells. MDBK cells were treated with 10 or 50 U of interferon per ml as described in the legend to Fig. 1. Ten minutes before infection, cells were treated with 100 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml. Cells were washed and infected, as described in the legend to Fig. 1, with 100 μg of cycloheximide per ml in the infecting inoculum, and cells were maintained thereafter at 100 μg of cycloheximide per ml until harvesting at 7 h postinfection. Treatment of MDBK cells with 100 μg of cycloheximide per ml inhibited cellular protein synthesis by 97% as measured by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable counts (data not shown). Extraction and blot hybridization analysis of poly(A)$^+$ RNA was done exactly as described in the legend to Fig. 2, except that lanes in Fig. 3 contained 0.5 μg of RNA. In MDBK cells infected in the presence of cycloheximide, production of viral mRNAs was inhibited drastically. Autoradiograms shown in Fig. 3 were generated by exposing filters to X-ray film 10 to 50 times longer than those shown in Fig. 2. Blots analyzed for alpha tubulin mRNA contained 0.75 μg per lane. The filter previously hybridized with the alpha tubulin probe was rehybridized with a probe for matrix protein (M) mRNA. PB_2, Polymerase protein PB_2; PA, polymerase protein PA; NA, neuraminidase; NS, nonstructural protein 1.

in influenza replication inhibited by interferon, the experiments described above offered an opportunity to evaluate the effect of abortive influenza infection on the metabolism of cellular mRNA. Both Inglis (9) and Katze and Krug (11) have demonstrated enhanced turnover of host mRNA in influenza-infected cells, although the precise processes during the infectious cycle which mediate this turnover are unclear. We evaluated the effect of prior interferon treatment on the level of alpha tubulin mRNA in infected cells (Fig. 2 and 3). Significantly greater amounts of alpha tubulin mRNA were present in interferon-treated infected cells than in untreated infected controls (Fig. 2). Amounts of alpha tubulin mRNA were also assayed at 7 h postinfection in cells infected in the presence of cycloheximide with or without interferon treatment. Similar levels of alpha tubulin were detected in both samples (Fig. 3). Identical results are obtained in both experiments when levels of beta actin were measured (data not shown). These experiments indicate that enhanced turnover of cellular mRNA in influenza-infected cells requires steps in viral replication subsequent to primary transcription and does not occur in interferon-treated cells.

Our results indicate that primary transcripts do not accumulate in interferon-treated MDBK cells. These observations are consistent with the findings of Bean and Simpson (2) in chicken embryo fibroblasts and of Krug and co-workers in *Mx*-bearing mouse embryo cells (14). In the *Mx* system, Haller and co-workers (8) have demonstrated that the inhibition of influenza replication in interferon-treated *Mx*-bearing cells can be directly correlated with the induction of a 72-kilodalton protein. Furthermore, they have recently described an interferon-induced homolog to this murine protein in human cells (26). It seems possible that a similar protein may also be induced by interferon in bovine cells and may contribute to inhibition of influenza replication in MDBK cells.

The observed failure to accumulate primary transcripts in interferon-treated cells could be explained either by direct inhibition of transcription or increased lability of transcripts that are synthesized. Our experiments cannot distinguish between these alternatives. Furthermore, there is no obvious explanation for the intriguing observation that accumulation of transcripts of the polymerase genes is preferentially inhibited at moderate doses of interferon. Primary transcription of influenza virus has been well characterized in vitro (3, 13, 21), and its seems likely that these questions could be resolved if the phenomenon observed in vivo can be reproduced by addition of extracts from interferon-treated cells to an in vitro transcription system.

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