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# INHIBITORY EFFECT OF INTERFERON-γ ON ADENOVIRUS REPLICATION AND LATE TRANSCRIPTION

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**Abstract**—We have previously shown that human interferon-γ inhibited adenovirus multiplication *in vitro* in a dose-dependent fashion. This action was previous to capsid proteins synthesis and did not involve virus adsortion nor penetration. In this report we have analysed viral mRNA levels at early (7 hr post infection (p.i.)) or late (20 hr p.i.) times, as well as DNA replication in Wish cells pretreated with interferon-γ and infected with adenovirus 5. Controls included untreated cells as well as cells treated with interferon-α, to which adenovirus are reported to be resistant. Transcription of adenovirus regions E1, E4, L1 and L2 has been analysed by Northern blot. Adenovirus DNA replication was determined by DNA–DNA hybridization with total adenovirus 2 DNA. We have also searched for adenovirus E1A proteins by immunoblot with a specific monoclonal antibody.

Although pretreatment of cells with either interferon-α or interferon-γ resulted in reduced amounts of E1 and E4 mRNA in the early phase of infection (7 hr p.i.), the near complete inhibition of viral DNA and late transcription was only achieved by interferon-γ. Immunoblot has shown the absence of the 48-kD E1A protein in cells pretreated with interferon-γ. The lack of this regulatory adenovirus protein may be involved in the inhibitory mechanism of interferon-γ on adenovirus.

Interferon-γ (IFN-γ) is a lymphokine with pleiotropic immunoregulating actions [1], initially defined as a member of the IFN‡ family by its antiviral activity. IFNs can prevent virus replication by interfering with various steps in viral life cycle. Which, if any, of these impairments is the major one depends primarily on the virus in question [2]. Despite many similarities in the antiviral actions of IFN-γ and IFN-α, there are a number of differences great enough to imply that each of them may trigger specific mechanisms [3]. These differences include their molecular structure (both have been cloned and are currently produced by recombinant DNA technology), the specific receptors for each of them in the surface of target cells, the spectrum of susceptible viruses, and probably the transduction and intracellular amplification steps [4].

We have previously shown that human recombinant IFN-γ (rIFN-γ) inhibits adenovirus multiplication *in vitro* [5], the $ID_{50}$ being 25–30 IU/ml, with a plateau over 700 IU/ml. On the contrary, pretreatment with IFN-α does not modify adenovirus yield in infected cells [5, 6].

The lytic infection by adenovirus might be schematically divided in three phases. The early phase includes the events from penetration up to the transcription of viral E1 to E4 genes. The intermediate phase begins with the onset of viral DNA replication [7], which is associated with the switch from host and viral gene expression to that of late viral genes [8]. The late phase is characterized by the maximal synthesis of the products of late viral genes [7]. The expression of the immediate early gene E1A is responsible for the transcriptional activation of all other early viral regions [9]. E1A products regulate also the expression of some cellular genes [10] and are involved in cellular transformation by adenovirus [11].

In this report we show that pretreatment with either rIFN-α or rIFN-γ decreased equally E1A mRNA. Both IFNs did also reduce E4 mRNA. On the other hand, only rIFN-γ inhibited adenovirus DNA replication. Late transcription was completely blocked by rIFN-γ, while rIFN-α resulted only in a partial decrease. IFN-γ abolished the expression of the 48 kD E1A protein, suggesting that an effect on this key viral protein might be involved in the other effects reported herein.

## MATERIALS AND METHODS

*Cells and virus.* Human amniotic Wish cells (Flow) and FS-4 human fibroblasts (Flow) were grown as monolayers in Earle's Minimal Essential Medium (MEM, Boehringer) supplemented with 10% foetal calf serum (Flow), 2 mM glutamine (Gibco) and 1 mg/l non-essential aminoacids (Seromed). Monolayers were treated overnight with either IFN-α or IFN-γ and, after removal of IFN, the cells were infected with human adenovirus 5 at different multiplicities, as indicated in the text, or mock infected. Untreated infected cells were used as positive control.

*Interferons.* Human rIFN-γ (Roussel-Uclaf, Romainville, France) was used at 700 units/ml; its specific activity was $4 \times 10^7$ units per mg protein. Human rIFN-α2a (Hoffmann-La Roche, Basel, Switzerland) was used at 1000 units/ml; its specific

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‡ Abbreviations used: IFN, interferon; m.o.i., multiplicity of infection; p.i., post infection; SDS, sodium dodecyl sulphate.



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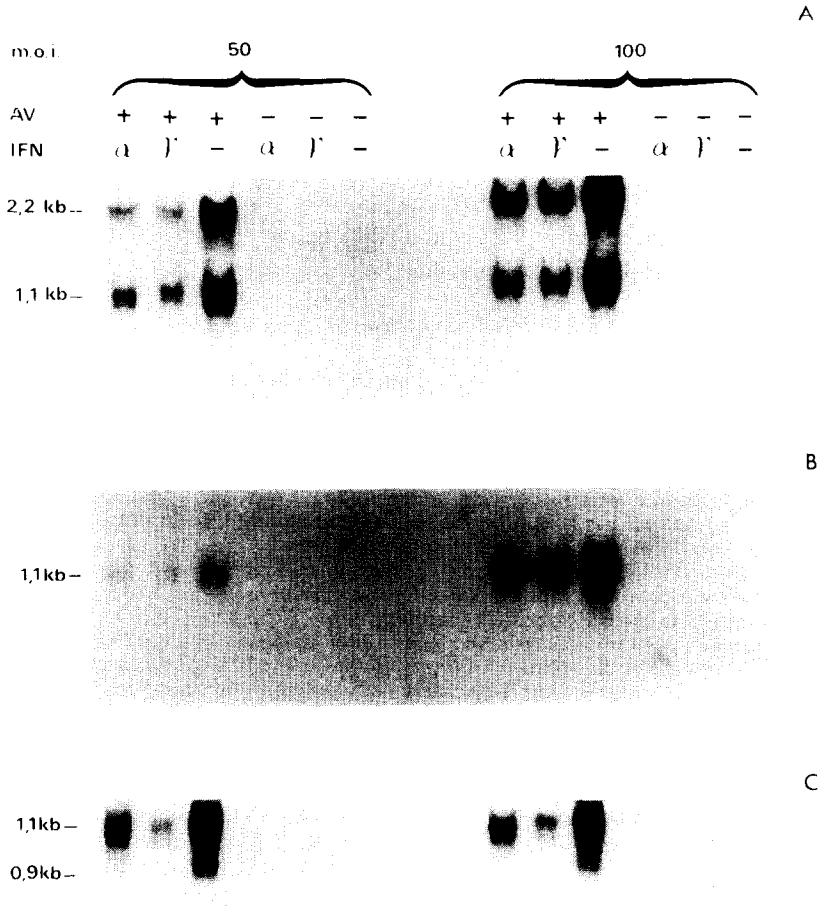


Fig. 1. Effect of rIFN-$\alpha$ (1000 IU/ml) and rIFN-$\gamma$ (700 IU/ml) on E1A and E1B mRNA expression in Wish cells infected with adenovirus 5, by Northern blot analysis. (A) The probe is the Bcl I-C restriction fragment of adenovirus 2, at 7 hr p.i. (B, C) The probe is E1A cDNA and the times are 7 hr p.i. or 20 hr p.i., respectively. Abbreviations: m.o.i., multiplicity of infection; AV, adenovirus; IFN, interferon.

activity was $2 \times 10^8$ units per mg protein. Both IFNs are produced in *Escherichia coli*.

*Northern blot analysis.* At early or late times (7 or 20 hr p.i., respectively), total cell RNA has been extracted by guanidinium isothiocyanate lysis and cesium chloride fractionated [12]. RNAs were denaturated by heating at 65° per 10 min in glyoxal-DMSO. Samples (20 $\mu$g per slot) have been loaded into a 1% agarose gel, run, and then transferred by the Northern blot method [13] to Gene Screen™ membranes (Dupont). After transfer, membranes were baked for 2 hr at 80°, prehybridized for 6 hr at 42° in a buffer containing 50% formamide, 20 mM Tris–HCl pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 0.2% Denhardt's solution (1× is 0.02% polyvinylpyrrolidone–0.02% bovine serum albumin–0.02% Ficoll), 200 $\mu$g/ml of denatured salmon sperm DNA and 5% dextran sulfate. Hybridization was performed for 18 hr at 42° in the same buffer containing between 0.5 and $1 \times 10^6$ cpm of the following [$^{32}$P] DNA probes: E1A cDNA, E4 cDNA, $\beta$-actin cDNA, and restriction fragments Bcl I-C (from 0 to 11.6 m.u., corresponding to E1 region), Pvu I-C (21.4 to 34.2 m.u., corresponding to L1 region), and Pvu I-D (40.4 to 51.2 m.u., corresponding to L2 region). Restriction fragments were from adenovirus 2 DNA (BRL). Probes were labelled by nick-translation to specific activity between $1 \times 10^8$ and $1 \times 10^9$ cpm per $\mu$g with a commercial kit (Amersham). The membranes were then washed for 30 min at room temperature in 2× standard saline citrate (SSC, 1× is 0.15 M NaCl, 0.015 M sodium citrate) and twice for 30 min in 0.1× SSC at 65°. The dry membranes have been autoradiographed on Hyperfilm™ (Amersham) with intensifying screen at −70°. Quantitative measures have been made by densitometric scanning.

*Dot-blot hybridization.* Low molecular weight DNA was extracted at 20 hr p.i. by the method of Hirt [14]. In brief, after lysis in 10 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.06% SDS, nucleic acids were precipitated in 1 M NaCl. High molecular weight DNA was eliminated by centrifugation and the supernatant was digested with proteinase K

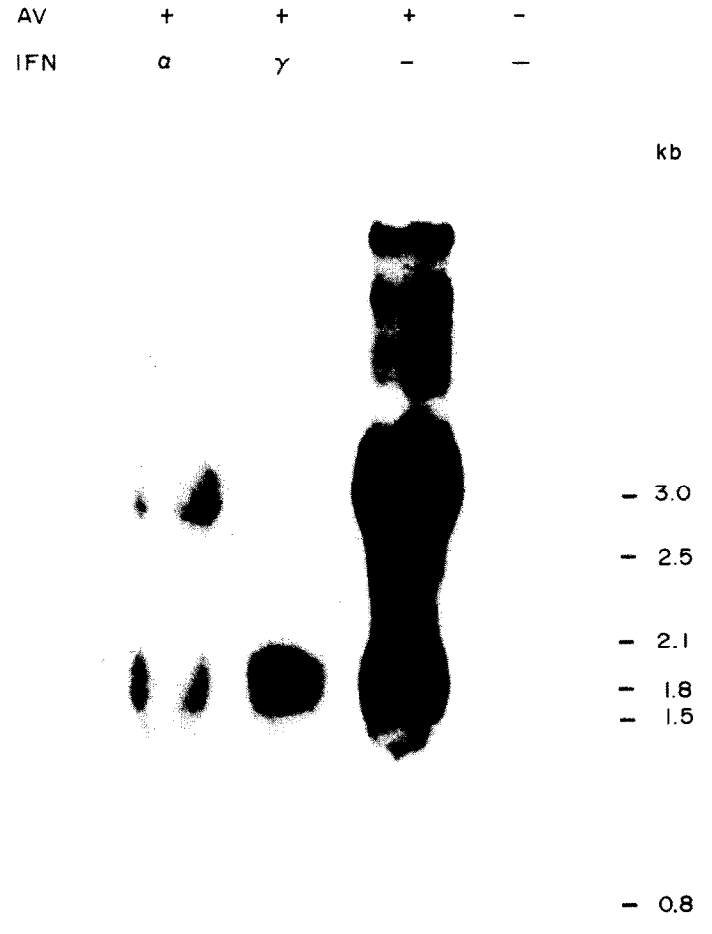


Fig. 2. Effect of rIFN-α and rIFN-γ on E4 mRNA expression in Wish cells infected with adenovirus 5 (m.o.i. = 50 virus per cell). Total cytoplasmic RNA was extracted at 7 hr p.i. and analysed by Northern blot with E4 cDNA. Abbreviations and IFN dose as in Fig. 1.

(Boehringer). Low molecular weight DNA was concentrated by ethanol precipitation, denaturated, neutralized, spotted onto Gene Screen™ membranes (Dupont) and hybridized as described [15]. As probe we have used total adenovirus 2 DNA cut with Sma I and nick-translated. Autoradiographies were exposed for 6 h with intensifying screen at −70°.

*Preparation of cell lysates and immunoblotting of proteins.* At different times after infection (3, 6, 24, or 30 hr p.i.), cells were lysed in 20 mM Tris–HCl pH 7.6:50 mM KCl:2 mM $MgCl_2$:1% Nonidet-40 (Sigma):0.2 mM phenoxyphenylsulphonylfluoride. Proteins were quantified and 120 μg of protein from each sample were seeded in 7.5–15% polyacrylamide-SDS gels. Electrophoresis was performed for 20 hr at 2.5 V/cm. Proteins were then electrophoretically transferred to nitrocellulose paper [16] by using a Hoefer Scientific Instruments transfer apparatus. After transfer, the nitrocellulose paper was soaked in a 5% nonfat dry milk solution in PBS pH 7.4 for 18 hr with gentle shaking. Nitrocellulose filters were then incubated with the monoclonal antibody clone M-73 (Oncogene Sciences Inc., New York, U.S.A.), specific for adenovirus 2 E1A proteins [17], at 2 μg/ml in PBS, for 60 min at 37°. Filters were then washed twice and incubated with 2 μCi of [$^{125}$I]-protein A (Amersham) for 60 min at 37°. After two washes, filters were dried and exposed in Hyperfilm™ (Amersham) in the presence of an intensifying screen.

## RESULTS

We began the study by the analysis of IFN effect in the early phase of adenovirus cycle. The effect of IFN-α and -γ on early transcription is shown in Fig. 1. When analysed at 7 hr p.i. for the E1 region (A), pretreatment with both IFNs resulted in a similar decrease in its mRNA level; either at m.o.i. of 50 or 100. This effect was equally evident on E1B 2.2 kb mRNA (22 S) and on 1.1 kb mRNA (13 S). Since 1.1 kb mRNA could correspond to either E1A or E1B mRNA, we have studied the former with E1A cDNA. Figure 1 (B) again shows a similar decrease in E1A mRNA for both IFNs. E1A gene continues to be transcribed and translated in the late phase [18]. Once the gene is actively transcribing, the E1A gene products return to the nucleus to enable autoamplification of E1A gene expression and to induce

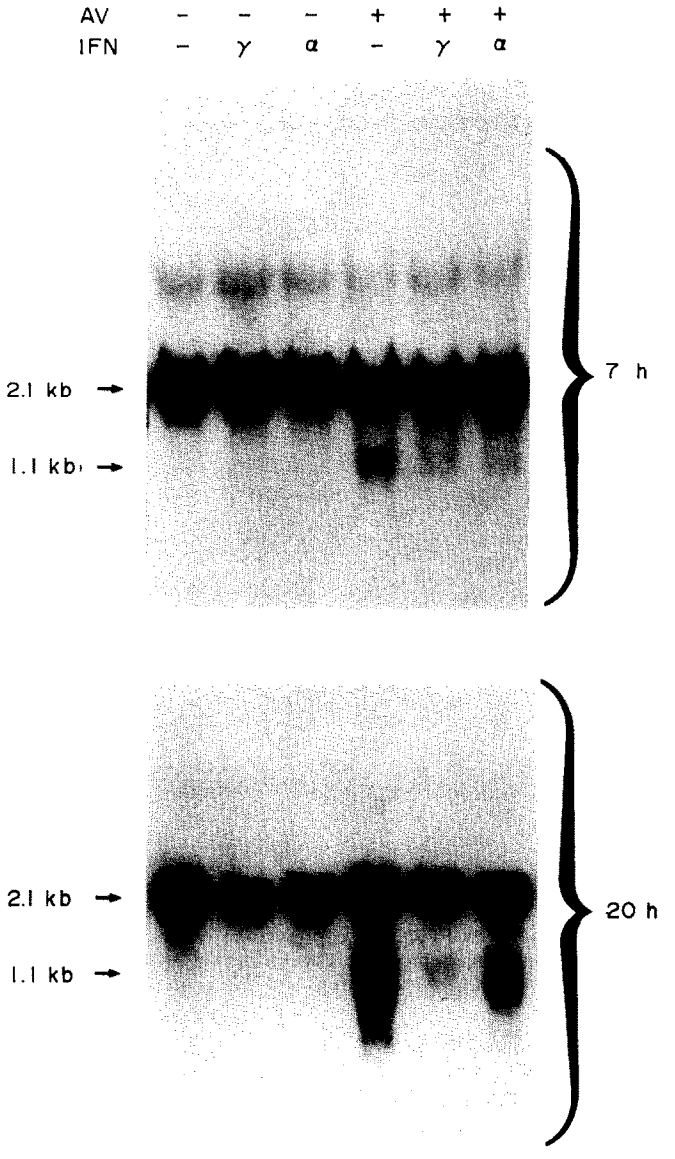


Fig. 3. Analysis of β-actin and E1A mRNA by Northern blot in Wish cells infected with adenovirus 5 (m.o.i. = 50 virus per cell). The time of exposition is not optimal for E1A mRNA (1.1 transcript). Abbreviations and IFN dose as in Fig. 1.

transcription of other early E1A-dependent adenovirus genes [19]. The late transcription of E1A gene was analysed at 20 hr p.i. As presented in Fig. 1 (C), E1A transcription is strongly inhibited in cells pretreated with rIFN-γ, even at high multiplicities. The quantification of this decrease by densitometric scanning showed that while rIFN-α resulted in only a three-fold decrease in E1A mRNA level, rIFN-γ induced a greater effect of 11-fold decrease.

In the absence of E1A, the expression of all the other early adenovirus genes is very inefficient and the virus fails to propagate [9]. To assess whether the effect of rIFN-γ was restricted to the amount of E1A mRNA, we have looked for other adenoviral early transcripts. The analysis of the complex E4 unit at 7 hr p.i. (time of its maximal expression, reference 20) is shown in Fig. 2. In untreated cells, adenovirus infection produces several mRNA species, at least three easily shown (i.e. 3 kb, 2.5 kb, and the superimposition of 1.8 and 1.5 kb). IFN-α and IFN-γ decrease the level of E4 transcripts, but with a different pattern. IFN-α reduces substantially all E4 species, while IFN-γ reduces partially the 1.8–1.5 kb complex and abrogates the more heavy transcripts.

To study the virus specificity of this finding, we have simultaneously hybridized the extracted RNAs with E1A and β-actin cDNAs. As shown in Fig. 3, at 7 hr p.i., cytoplasmic actin mRNA level is the same in rIFNs-pretreated cells (whether -α or -γ) and in untreated cells. In the late phase (at 20 hr p.i.),

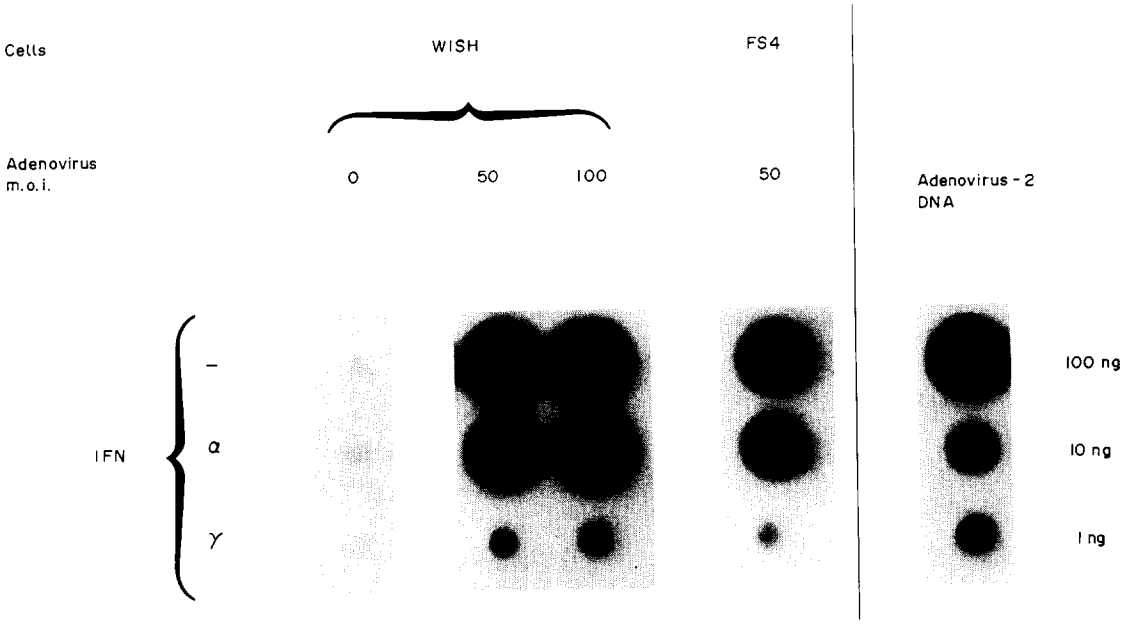


Fig. 4. Inhibition by rIFN-γ of adenovirus DNA replication. Low molecular weight DNA extracted from either Wish cells at 20 hr p.i. or FS-4 fibroblasts at 72 hr p.i., pretreated or not with the indicated IFNs and infected with adenovirus, has been dotted and hybridized with adenovirus 2 genome. A simultaneous control with unlabelled adenovirus 2 genome (1 to 100 ng per spot) has been performed. Abbreviations and IFN dose as in Fig. 1.

while the decrease of E1A mRNA with rIFN-γ is very impressive, the level of actin mRNA is slightly lower than in control cells. rIFN-α did also reduce partially actin mRNA, and to greater extent, E1A mRNA. The decrease in E1A mRNA seems thus unlikely to be related to an overall perturbation of cellular transcription.

Since the effect of IFN-γ on early genes transcription was different from that of IFN-α only in the late phase, we have studied the intermediate phase, which is characterized by viral DNA replication. Adenovirus DNA replication was analysed at 20 hr p.i. by dot blots. The search for viral DNA at this time in Wish cells resulted in its easy detection, which is consistent with the known time-course of the adenovirus lytic cycle [21]. As shown in a typical experiment presented in Fig. 4, viral DNA is not greatly reduced by IFN-α, while IFN-γ markedly inhibited its replication, even at 100 pfu per cell. This action of rIFN-γ was evident in Wish cells and in fibroblasts, and related inversely with adenovirus multiplicity.

Since DNA replication was blocked by IFN-γ, could it also inhibit late gene transcription? The presence of adenoviral late mRNAs was assayed at 20 hr p.i. by Northern blot. As expected, late mRNAs L1 and L2 were undetectable in cells pretreated with IFN-γ, and this is illustrated in Fig. 5 (A and B, respectively). This was a consistent finding, even at the highest multiplicity tested (100 pfu per cell). On the contrary, IFN-α did only induce a partial decrease.

The regulation of adenovirus gene expression has become one of the most important systems for the study of the control of eukaryotic transcription [22]. This control is performed by E1A polypeptides [23], the first viral proteins to be synthesized in the course of the infection. To determine whether rIFN-γ was able to affect these proteins, E1A polypeptides were analysed by Western blot with a specific monoclonal antibody [17]. As shown in Fig. 6, the major E1A polypeptide (the 48 kD E1A protein) is absent in rIFN-γ pretreated cells at 24 hr p.i. In rIFN-α pretreated cells there is also a decrease in 48 kD protein level, but only partial. Similar results have been obtained at 30 hr p.i.; at earlier times, the amount of E1A proteins were below the detection level by immunoblot. In addition to E1A polypeptides, a doublet is detected at about 70 kD, probably reflecting the cellular 70 kD heat shock proteins, which are non-specifically precipitated by the monoclonal antibody [17].

## DISCUSSION

In the analysis of rIFN-γ mechanism against adenovirus, several points have been shown to be modulated by this IFN. Early adenovirus transcription is equally inhibited by rIFN-γ and rIFN-α. Since then, divergence in their action becomes evident. The difference is amplified during adenovirus DNA replication, which is substantially inhibited by rIFN-γ but not by rIFN-α, and is even more evident in late transcription. Moreover, at late time there is an absence of the regulatory 48 kD E1A protein.

As previously reported, the viral yield of cells

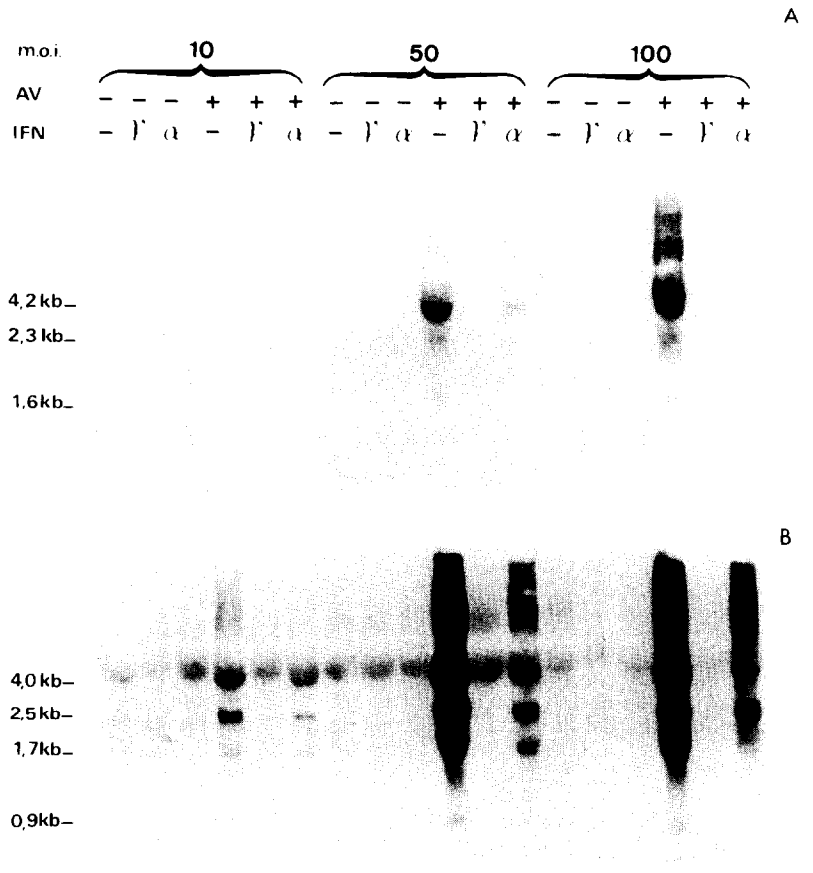


Fig. 5. Decrease by rIFN-$\gamma$ of late transcript level in adenovirus-infected Wish cells. Northern blot analysis of cytoplasmic RNA in the late phase of infection (20 hr p.i.). (A) Effect of rIFN-$\alpha$ and -$\gamma$ on the late region L1 mRNA; (B) effect on L2 mRNA. Probes: restriction fragments Pvu I-C (for L1) and Pvu I-D (for L2). Abbreviations and IFN dose as in Fig. 1.

infected with adenovirus is not modified by pretreatment with rIFN-$\alpha$ [5, 6], Surprisingly, rIFN-$\alpha$ does decrease the amount of early and late viral mRNA, at least in Wish cells. These apparently contradictory results can be explained by the fact that, in untreated cells, viral proteins are synthesized in large excess and only a small percentage (1 to 5%) is actually assembled into infectious particles [24]. The complete lack of late mRNA from cells pretreated with rIFN-$\gamma$ is consistent with the absence of capsid polypeptides under the same conditions [25].

E1A proteins are required for the efficient expression of early viral genes, whose products are in turn essential for viral DNA replication [9]. In absence of E1A proteins, the rate of transcription of early regions is less than 8% [9] and that from the major late promoter is greatly reduced [26]. The lack or decrease in E1A transactivation could sustain adenovirus DNA replication, but only at very low rate, depending on the virus input.

The experiments herein reported have shown the preferential inhibition by rIFN-$\gamma$ at such different levels as adenovirus DNA replication and late transcription. The common point of these different viral steps is their dependence on E1A products and suggests that E1A products could be a target of the rIFN-$\gamma$-induced protein(s) involved in its anti-adenovirus mechanism. The direct search for E1A proteins by immunoblotting did not provide support to this interpretation at early time, since E1A protein did not attain a high enough level to be detected. However, at late time (24 and 30 hr p.i.), rIFN-$\gamma$ pretreatment resulted in the lack of 48 kD E1A protein. This suggests that IFN-$\gamma$ could in addition induce post-transcriptional events that modify E1A expression. The absence of functional E1A protein at late time during adenovirus infection is further substantiated by rIFN-$\gamma$ inhibition of thymidine kinase induction (Mistchenko and Falcoff, unpublished). Thymidine kinase is a cellular enzyme induced by E1A transactivation [10].

The biological significance of E1B RNA decrease by rIFN-$\gamma$ is at present unclear. In hamster cells, adenovirus 12 produces an abortive infection which is characterized by the lack of DNA replication and undetectable expression of late genes. The blockage can be circumvented by adenovirus 2 or adenovirus 5 E1B functions [27]. Interestingly, in previous experiences we have shown that 293 line cells, which are transformed by adenovirus 5 and express E1A and E1B genes, are insensitive to the antiadenovirus action of rIFN-$\gamma$ while being able to increase HLA antigen expression upon induction with rIFN-$\gamma$ [28].

In summary, IFN-$\gamma$ seems to act on some early

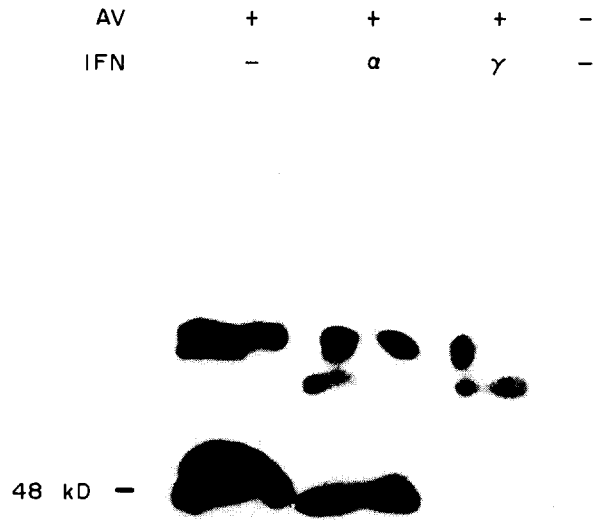


Fig. 6. Immunoblot analysis of E1A proteins in IFN-pretreated Wish cells. The monoclonal antibody M73, specific for E1A proteins, has been used to detect these proteins at 24 hr p.i.; m.o.i. = 50 virus per cell. Abbreviations and IFN dose as in Fig. 1.

step in the adenovirus cycle, probably in relation to the level and/or function of early genes E1A or E1B. Nevins and coworkers have postulated the existence of a cellular activity that inhibits adenovirus transcription, probably by regulating the availability or activity of transcriptional factors [29, 30]. One possible explanation for our findings could be for rIFN-γ to enhance this activity that interfere with E1A products. Taking into account that E1A and E1B are the oncogenes of adenovirus and that a cellular E1A-like activity has been reported in the control of cell cycle [31, 32], it would be important to explore the effect of rIFN-γ on other viral or cellular E1A activities. Further studies are currently underway to evaluate this hypothesis.

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