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Intracellular human γ-interferon triggers an antiviral state in transformed murine L cells

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ABSTRACT **Interaction of human γ-interferon (IFN-γ) with a cell-surface receptor is known to be essential for the cell to become resistant to viral infection. Here we demonstrate that IFN-γ, when present inside the cell, is also capable of inducing a permanent antiviral state. Mouse cells transformed with a truncated human cDNA encoding a mature IFN-γ protein lacking the signal peptide accumulate high levels of intracellular human IFN-γ. Not only do these cells acquire a permanent resistance to viral infection, they also exhibit all the biochemical characteristics normally observed after exposure to exogenous IFN. The observed loss of species specificity normally associated with IFN-γ suggests that this restriction is strictly dependent on the interaction of the molecule with the cell-surface receptor.**

It is generally accepted that the first and obligatory step for interferon (IFN) to exert its antiviral effect resides in its binding to specific receptors located on the cell membrane (1). Some evidence demonstrates that IFN acts from outside the cell in the same manner as many polypeptide hormones (2). In this model after viral infection, those cells that produce IFN would not become resistant to the virus but secrete the molecule, thus protecting other uninfected cells exhibiting the IFN receptor.

The availability of important amounts of homogeneous IFN has made it feasible to study the nature of these receptor complexes. As a result, two different receptors have been reported (3, 4): one specific for the two antigenically distinct IFN-α and IFN-β, and a second one that is recognized specifically by IFN-γ.

Two models for the mechanism of action of IFN are postulated: interaction with the IFN receptor is sufficient to generate the primary signal(s), or internalization of IFN is required to trigger the cellular response.

Recently, it has been shown that the initial receptor–ligand complex was rapidly internalized under physiological conditions by virtue of a well-documented phenomenon known as receptor-mediated endocytosis (5).

The internalization of IFN-α (6) is followed by its subsequent degradation, presumably after transportation into lysosomes. Similar results have been observed for human IFN-γ (HuIFN-γ) (7). However, murine IFN-γ (MuIFN-γ) appears to be stable in L1210 cells for as long as 12 hr after uptake of the molecule (8).

It has recently been demonstrated that specific receptors for both IFN-β and IFN-γ could be detected on the nuclear membrane of L cells (9, 10). This would suggest that the internalized MuIFN is able to modulate regulatory functions of the nucleus as well as the expression of specific genes.

Independent of the nature of these signals, the three types of IFN are capable of activating the expression of a common set of genes, among which the most studied have been the (2′-5′)oligoadenylate synthetase and a 67-kDa protein kinase (11). Recently, IFNs were also shown to activate expression of major histocompatibility complex (MHC) genes (12, 13) and to reduce expression of various genes such as c-*myc* (14–17), although the response depended on the type of IFN and the cell line used.

We have previously reported our observations using murine cell lines after transformation with the HuIFN-γ cDNA gene (18). Some of these clones appeared to be permanently protected against viral infections. This phenomenon was strictly dependent on the presence of intracellular HuIFN-γ and could be correlated with increased levels of (2′-5′)oligoadenylate synthetase and 67-kDa protein kinase activity. These results would favor a model in which IFN-γ can act intracellularly after internalization by receptor-mediated endocytosis. The previously reported stability of MuIFN-γ inside the cell may well be in agreement with these observations (8).

To study the proposed model, we established murine cell lines transformed with an expression vector containing a truncated HuIFN-γ cDNA encoding a mature protein that was expected to be accumulated inside the cell. A number of biochemical and biological parameters, characteristic for the antiviral state of these cells, were studied in comparison with transformed cells that secrete IFN-γ via the classical pathway.

MATERIALS AND METHODS

Cell Cultures and Transformation. LTK^- mouse cells, obtained from J. A. Lewis (State University of New York), were cultured in minimal essential medium containing Earle's salts (Boehringer Mannheim) and supplemented with 10% newborn calf serum (Biopro, Strasbourg, France), penicillin (100 units/ml) and streptomycin (50 μg/ml). LTK^- cells (1×10^6) were transformed according to the calcium phosphate precipitation method as described (19) using 9 μg of high molecular weight LTK^- carrier DNA, 50 ng of linearized pTG301, and 1 μg of the IFN-γ constructions pTG11-Sau800 and pTG11-Sau720. LTK^+ clones were selected in the presence of hypoxanthine (15 μg/ml), aminopterin (1 μg/ml), and thymidine (5 μg/ml), repicked after 15–20 days, and expanded individually in culture flasks (Nunc).

Construction of the IFN-γ Expression Plasmids. A HuIFN-γ cDNA clone was previously isolated from a cDNA library prepared with mRNA from phytohemagglutinin-induced human peripheral blood lymphocytes (20). The sequence of the 1100-base-pair (bp) insert corresponded to the published

Abbreviations: IFN, interferon; HuIFN, human IFN; MuIFN, murine IFN; VSV, vesicular stomatitis virus; TK, thymidine kinase; MHC, major histocompatibility complex.

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sequence (21) starting at nucleotide position 80, except for an adenine to guanine transition at position 588 changing a glutamic acid residue to arginine. A *Bgl* II restriction site was created 16 nucleotides upstream of the ATG codon by site-directed mutagenesis on a single-stranded M13 template. Digestion of the resulting plasmid construction with *Sau*3A generates an 800-bp fragment containing the complete coding sequence for HuIFN-γ.

To delete the signal peptide, a *Bgl* II site followed by an ATG codon was created in the last three codons (Cys-Tyr-Cys) of the signal sequence. Digestion with *Sau*3A gave a 720-bp fragment encoding a mature HuIFN-γ protein preceded by an ATG-initiator resulting in the NH_2-terminal amino acid sequence Met-Gln-Asp-Pro-. . . .

Both *Sau*3A fragments of 800 and 720 bp were inserted in the *Bgl* II site of pKSV-10 (Pharmacia) and selected for the correct orientation by restriction with *Bgl* I (Fig. 1).

Plasmid pTG301, containing the thymidine kinase (TK) gene and used in cotransfection for the selection of LTK$^+$ clones, was constructed by insertion of a 3.16-kilobase (kb) *Bam*HI fragment of the herpes simplex virus genome (22) into the *Bam*HI site of pBR328.

IFN Assays. IFN-γ activity was titrated by cytopathic effect inhibition on human WISH cells using vesicular stomatitis virus (VSV) as challenge (20). Antiviral activity produced by transformed LTK$^+$ cells was determined in culture supernatants of confluent monolayers 24 hr after the last medium change. Intracellular IFN was assayed by lysing 1×10^7 cells in 200 μl of hypotonic buffer (10 mM Hepes, pH 7.4/10 mM NaCl/2 mM $MgCl_2$) in the presence of phenylmethylsulfonyl fluoride (50 μg/ml) (Sigma). Ionic concentrations were adjusted to 20 mM Hepes, pH 7.4/140 mM NaCl, 20% (vol/vol) glycerol was added, and antiviral activity was titrated immediately (final vol, 250 μl).

Natural MuIFN-α/β, used to analyze the induction of the antiviral state in transformed LTK$^+$ cells, was prepared from supernatants of CCL1 cells (Flow Laboratories) infected with Newcastle disease virus as described (23). Natural MuIFN-γ was produced in suspension cultures of spleen cells treated with phytohemagglutinin (3 μg/ml) (Wellcome) and purified on blue Sepharose CL-6B (24).

(2'-5')Oligoadenylate Synthetase. Activity of this enzyme was assayed in extracts of monolayer cultures. Cells (1×10^7) were lysed in 1 ml of 10 mM Hepes, pH 7.5/100 mM KCl/7 mM 2-mercaptoethanol/2 mM $MgCl_2$/0.5% Nonidet P-40. Extracts were centrifuged at 8000 $\times$ *g*. The (2'-5')oligoadenylate synthetase was determined in 10 μl of cell extracts as described by Merlin *et al.* (25). The oligoisoadenylate phosphatase-resistant cores were recovered from alumina columns with 1 M glycine hydrochloride (pH 2) and counted in a scintillation counter.

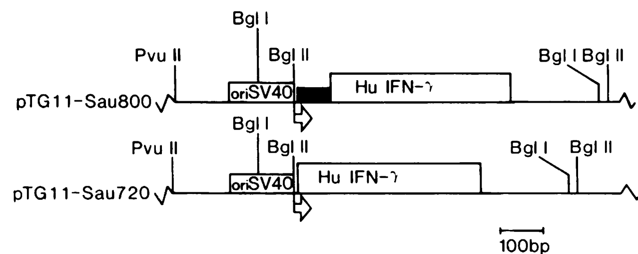

FIG. 1. Partial map of pKSV-10 derivatives directing the synthesis of extra- versus intracellular HuIFN-γ after transformation in mouse LTK$^-$ cells. *Sau*3A fragments of 800 and 720 bp were inserted downstream of the simian virus 40 (SV40) early promoter (arrow) into the unique *Bgl* II site of pKSV-10. The shaded area in the pTG11-Sau800 represents the signal peptide originally present in the HuIFN-γ cDNA.

RNA Analysis. Total RNA was extracted by the guanidinium thiocyanate procedure (26) and enriched for polyadenylylated RNA on oligo(dT)-cellulose columns (27). RNA preparations for RNA blot analysis were glyoxylated, fractionated on 1.1% agarose gels, transferred to nylon membranes (Genescreen; New England Nuclear), and hybridized with a nick-translated DNA fragment (28).

Nuclease S1 mapping on HuIFN-γ transcripts produced by various LTK$^+$ clones was performed (29) by hybridizing 20 μg of poly(A)$^+$ RNA with 5 ng of the end-labeled probe (specific activity, 2×10^6 cpm/μg) in 50% formamide/400 mM NaCl/40 mM Pipes, pH 6.8/1 mM EDTA, at 42°C for 18 hr. Nuclease S1 digestion was done in 200 μl with 5 units of enzyme for 30 min at 37°C.

RESULTS

Intracellular IFN-γ in Murine Cells Transformed with a Truncated HuIFN-γ cDNA Sequence. LTK$^+$ clones obtained after transformation with either pTG11-Sau800 or pTG11-Sau720 (see *Materials and Methods* and Fig. 1) were analyzed for integration of the HuIFN-γ sequence by dot blot hybridization on total DNA (data not shown). Those that gave a positive signal were analyzed for the presence of IFN-γ activity both in culture supernatants and in cellular extracts (Fig. 2). The clones that had integrated the complete HuIFN-γ structural gene including the sequence for the signal peptide (clones 6.58 to 6.68) secreted HuIFN-γ up to 250 units/ml in the culture medium with a very low antiviral activity detectable in the corresponding cell extracts. In contrast, all clones transformed with the HuIFN-γ cDNA lacking the leader sequence (6.7 to 6.12) contained HuIFN-γ in the cell extracts, while almost no antiviral activity could be detected in the culture supernatants. The control clones (6.35 and 6.39), which have integrated only the selection plasmid, did not show any significant activity either in the supernatant or in the cell extract. In all cases, the antiviral activity could only be titrated on human WISH cells by the cytopathic effect inhibition assay using VSV as a challenge and corresponded to HuIFN-γ based on its species specificity and antigenic properties (data not shown).

Transcription of the HuIFN-γ cDNA in Transformed Cells. Analysis of the genomic DNA of the clones described above by Southern blot hybridization confirmed the presence of an intact copy of both IFN cDNAs used in transformation of these cells (data not shown).

The transcription of these genes was analyzed both by RNA blotting and nuclease S1 mapping. Hybridization of a ^{32}P-labeled HuIFN-γ cDNA probe with RNA blots gave the expected band of 3500 bp, corresponding to a transcript initiated from the simian virus 40 promotor and extending to

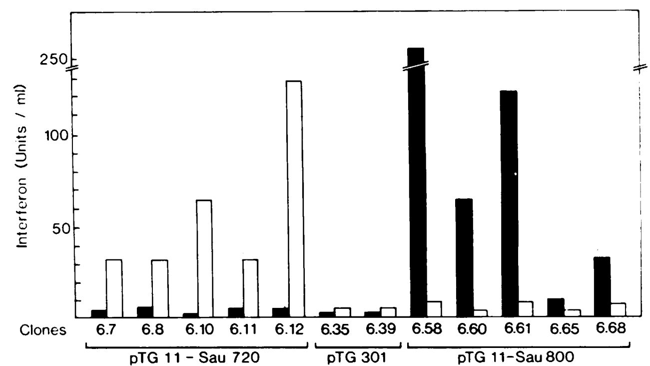

FIG. 2. Extra- and intracellular HuIFN-γ activity in LTK$^+$ clones transformed with pTG11-Sau800 or pTG11-Sau720. ■, Antiviral activity titrated in the culture supernatants 24 hr after the last medium change; □, antiviral activity in cellular extracts.

the first polyadenylylation site present in pKSV-10 (data not shown). The correct structure of the IFN-γ transcripts was confirmed in more detail by nuclease S1 mapping using a single-stranded ^{32}P-labeled probe complementary to the NH_2-terminal part of the IFN-γ mRNA and derived from an *Hin*fI restriction fragment as shown in Fig. 3. Correctly initiated RNA is expected to give a protected fragment of 490 bp in the case of mRNA from pTG11-Sau800-transformed cells (6.58, 6.68) and also from phytohemagglutinin-stimulated lymphocytes, while a fragment of 400 bp can be expected in the case of mRNA from pTG11-Sau720-transformed cells. The sizes of the bands (Fig. 3) are in agreement with the values calculated, indicating the correct structure of the sequence at the 5′ end of the HuIFN-γ transcripts in secreting (clones 6.58 and 6.68) and nonsecreting cells (clones 6.10 and 6.12).

Antiviral State of Cells That Express IFN-γ Activity Intracellularly. In a first set of experiments, the sensitivity of various TK$^+$ clones to two types of viruses, VSV and Mengo virus, was studied. The clones secreting IFN-γ (6.58, 6.61, 6.68) generated similar or higher yields of viruses than the control clones (6.35, 6.39). In contrast, the clones 6.8, 6.10, and 6.12 produced significantly lower amounts of both VSV and Mengo virus, as shown in Table 1. Pretreatment with MuIFN-γ further reduced the viral replication in cell lines 6.10 and 6.12 to levels identical to those observed for secreting clones after preincubation with MuIFN-γ. Control LTK$^+$ clones, which had not integrated any of the IFN-γ cDNAs, showed consistently 1/10th the virus yield after exposure of the cells to MuIFN-γ. On the other hand, preincubation of murine cells with up to 20,000 units of HuIFN-γ per ml did not modify the virus yield.

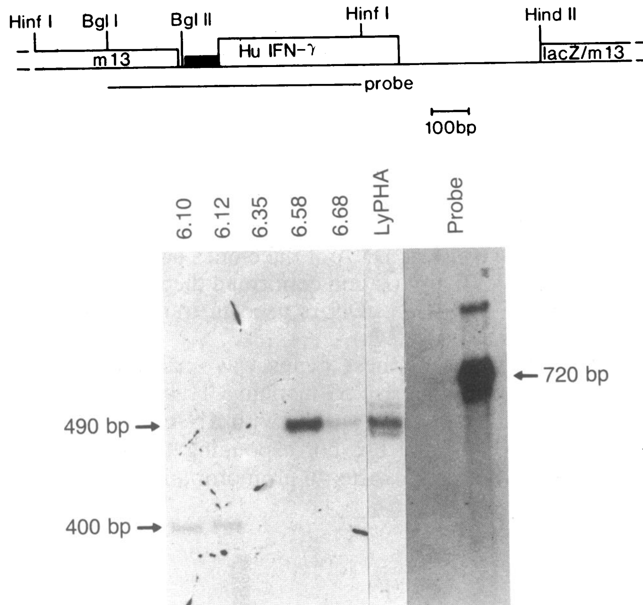

FIG. 3. Nuclease S1 mapping on the IFN-γ transcripts in LTK$^+$ clones transformed with pTG11-Sau800 and pTG11-Sau720, respectively. (*Upper*) Construction of a single-stranded probe containing 200 bases of the M13mp8 vector upstream of the 5′ region of the HuIFN-γ cDNA as present in pTG11. (*Lower*) Experimental results with RNA from LTK$^+$ clones and from stimulated human blood lymphocytes (LyPHA). Clones 6.10 and 6.12 were transformed with pTG11-Sau720, resulting in a protected transcript of 400 bp. Clones 6.58 and 6.68 contain the complete cDNA giving a transcript of 490 bp. Clone 6.35 corresponds to LTK$^+$ cells, which had not integrated the HuIFN-γ sequence. RNA preparation from 6.12 was slightly contaminated with poly(A)$^+$ RNA from clone 6.58 due to passage over the same oligo(dt) column. This contamination explains the presence of a faint band at 490 bp in the transcript mapping of clone 6.12.

Table 1. Virus yield after infection of HuIFN-γ-secreting (6.58, 6.61, 6.68) and nonsecreting (6.8, 6.10, 6.12) LTK$^+$ clones

	Virus yield relative to the control TK$^+$ clones*		Mengo virus yield after 24-hr incubation with MuIFN-γ†	
TK$^+$ clone	Infection with VSV	Infection with Mengo	No treatment	Incubation with MuIFN-γ at 100 units/ml
6.58	88 ± 6	140 ± 8	100	0.1
6.61	124 ± 11	117 ± 11	120	0.4
6.68	107 ± 7	158 ± 7	120	1
6.8	57 ± 7	29 ± 2	—	—
6.10	18 ± 9	20 ± 7	6	1
6.12	15 ± 9	8 ± 6	3	0.3
6.35‡	100 ± 7	100 ± 3	100	0.04
6.39‡	100 ± 7	100 ± 3	100	0.04

Cells were infected with VSV or Mengo virus at a multiplicity of infection of 1 for 1 hr, washed, and supplied with fresh medium for 18 hr. Virus yield in the supernatants was determined on mouse L929 fibroblasts.

*Medium contained a cocktail of anti-MuIFN-α/β/γ and anti-HuIFN-γ antibodies to exclude interference with endogenously produced interferon. Results are expressed as percentage of the titre obtained after infecting control LTK$^+$ clones with VSV or Mengo virus (5×10^6 and 7.2×10^7 plaque-forming units/ml, respectively). Mean ± SD of six experiments.

†Results are expressed as percentage relative to the nontreated control clones infected with Mengo virus. Mean of duplicate experiments.

‡Control LTK$^+$ clones that had not integrated the human IFN-γ cDNA sequence.

The inhibition of viral replication observed in clones 6.10 and 6.12 suggested the possibility that these cells were in a permanent antiviral state. This suggestion could be confirmed by analyzing various clones with respect to their (2′-5′)oligoadenylate synthetase expression, normally stimulated by exogenous IFN (11). Lysates of 6.10 and 6.12 cells showed an enhanced level of the (2′-5′)oligoadenylate synthetase (6- and 3-fold, respectively) compared to the controls or IFN-secreting clones (Table 2). Treatment with MuIFN-γ resulted in increased levels of (2′-5′)oligoadenylate synthetase (from 3- to 14-fold) only in the controls and IFN-secreting clones. On the other hand, treatment with MuIFN-α/β resulted in an important increase in the enzyme activity for all cell lines studied.

Finally, it was interesting to determine whether intracellular HuIFN-γ was capable of modulating the expression of murine genes encoding MHC class I and class II antigens. We observed increased levels of MHC class I mRNA in the nonsecreting cells compared to the control and IFN-secreting cells (data not shown). In addition, whereas the messenger for class II antigens was hardly detectable in the control and in IFN-secreting cells, we found high levels expressed in the nonsecreting clones, suggesting that intracellular HuIFN-γ induces a permanent transcript of H2 class II genes (Fig. 4). It has been reported that IFN-γ specifically activates the expression of MHC class II antigens, resulting in a concomitant increase in the amount of cytoplasmic mRNA encoding these proteins (12).

DISCUSSION

The results presented here strongly confirm our previous observations, suggesting that the constitutive expression of intracellular HuIFN-γ activity can be correlated to a permanent partial resistance to viral infection. In the clones described here, the permanent antiviral state was shown to be associated to the continuous activation of various murine genes due to the presence of intracellular HuIFN-γ.

Table 2. Level of (2′-5′)oligoadenylate synthetase in transformed LTK+ clones expressing intra- or extracellular HuIFN-γ activity

Preincubation	Intracellular HuIFN-γ activity 6.10	6.12	Extracellular HuIFN-γ activity 6.58	6.61	6.68	Control TK+ clone 6.39	Extract† of L929 cells
No IFN	15,235	8,220	2,175	3,740	2,150	3,300	2,435
MuIFN-γ*	20,995	7,260	10,610	23,830	9,890	10,365	34,425
MuIFN-α/β*	79,075	115,650	49,915	41,705	41,935	85,070	81,880

Results are expressed in cpm and represent the mean of triplicate determinations.
*Preincubations were done for 24 hr with IFN at 100 units/ml.
†Reference for (2′-5′)oligoadenylate synthetase activity.

The protein appears to be similar to native IFN-γ with respect to its receptor binding and triggering the antiviral state when added to human cells. We also demonstrate an increased level of (2′-5′)oligoadenylate synthetase activity, a phenomenon implicated in the establishment of the antiviral state (31–33). In clones 6.10 and 6.12, intracellular HuIFN-γ induces the antiviral state and increases the level of (2′-5′)oligoadenylate synthetase activity. Exogenous MuIFN-γ does not change the levels; however, treatment of these cells with MuIFN-α/β results in an important additional increase of the (2′-5′)oligoadenylate synthetase activity. These results suggest that IFN-γ may induce the antiviral state by a different pathway to that of IFN-α and -β. We also studied in these cells the level of response of both MHC class I and class II genes. While in control and secreting clones MHC class I antigens were expressed at a low level and class II antigens could hardly be detected, the constitutive presence of intracellular HuIFN-γ activated the expression of both genes. The intracellular HuIFN-γ appears to induce the transcription of class II genes and thus conforms with the pattern of MHC gene expression observed when type I or type II IFN is added to the cells (12, 13).

These results demonstrate that the activation of the target cell, normally mediated by endocytosis of the IFN–receptor complex, can be alternatively triggered by intracellular HuIFN-γ. This contrasts with results showing that type I IFN could not induce an antiviral state after microinjection of this molecule in murine or human cells (34, 35). Some data suggest that processing of MuIFN-γ may not be similar to that of IFN-α and IFN-β and that the former is stably maintained for a long time after internalization (8), suggesting that HuIFN-γ constitutively expressed in murine cells could have an important role with regard to specific changes in gene expression.

A last interesting observation is that HuIFN-γ when present inside the cell has lost its species specificity and activates the murine genes involved in antiviral as well as cellular responses. It would seem, therefore, that the species specificity exhibited by IFN-γ is determined by its interaction with the cell-surface receptor. A recent report presents evidence that activation of human and murine macrophages by intracellularly delivered recombinant IFN-γ encapsulated in liposomes occurred with apparently no species specificity (36).

In summary, we conclude from our data that HuIFN-γ, expressed intracellularly in mouse cells, can induce those genes that are specifically involved in the establishment of the antiviral state.

Whether the molecule affects gene regulation via interaction with a potential nuclear receptor (9, 10) or whether alternative modes of signal transfer exist inside the cytoplasm remains an open question.

It will indeed be necessary to elucidate these basic mechanisms to understand how hormones, in this case IFN-γ, can regulate gene expression on the molecular level.

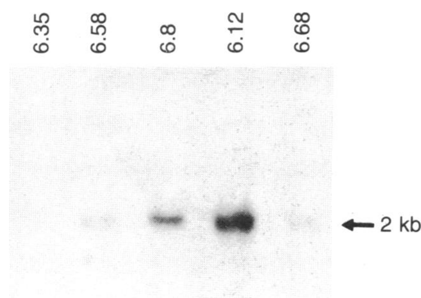

FIG. 4. Identification of MHC class II transcripts in cells accumulating high levels of intracellular HuIFN-γ activity. Clones 6.8 and 6.12 were transformed with a truncated cDNA lacking the signal peptide sequence. Clones 6.58 and 6.68 secreted HuIFN-γ into the medium. Control cells (6.35) did not contain an integrated copy of the HuIFN-γ cDNA sequence. Blot was probed with a nick-translated 775-bp *Pst* I fragment from the MHC Aαd cDNA (30).

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