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Expression of Interferon-Inducible Genes in RD-114 Cells

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RD-114 is a cell line which is partially responsive to interferon (IFN). Although both IFN-α and IFN γ inhibit production of the resident retrovirus, they do not inhibit replication of other viruses, such as vesicular stomatitis virus and encephalomyocarditis virus, in these cells. In the studies reported here, we studied the characteristics of induction of seven IFN-inducible mRNAs in RD-114 cells. We observed that mRNAs 561, 6-16, 1-8, 2A, and 6-26 have similar induction characteristics in RD-114 cells and in HeLa cells, a fully responsive line. mRNA 2′-5′-oligo-adenylate synthetase (2-5(A) synthetase), however, was induced more efficiently by IFN-α in HeLa cells than in RD-114 cells. The same was true for the induction of metallothionein II mRNA by IFN-γ. However, the latter mRNA was induced equally strongly in both lines when $ZnCl_2$ was used as the inducer, suggesting that the gene is not defective in RD-114 cells. Although IFN-α induced 2-5(A) synthetase mRNA poorly and IFN-γ did not induce it at all in these cells, a mixture of IFN-α and IFN-γ induced this mRNA quite effectively, to a level of induction comparable to that in HeLa cells. Only 1 U of IFN-γ per ml was sufficient to elicit this synergism, and the data suggested that an IFN-γ-inducible protein was needed for this process. Induction of mRNA 561 by IFN-α in RD-114 cells, unlike that in HeLa cells, did not need ongoing protein synthesis. Once induced, this mRNA turned over rapidly in both cell lines, and this turnover could be slowed down by inhibiting protein synthesis in either cell line. IFN-induced mRNAs, such as 561 and 1-8, were polysome associated in IFN-treated RD-114 cells, suggesting that they were actively translated. Therefore, it is unlikely that the products of these IFN-inducible genes, by themselves, mediate the inhibition of replication of those viruses which are insensitive to IFN action in RD-114 cells.

Interferons (IFNs) have a variety of biological activities, including actions against viruses which use different replication strategies (12, 18). All actions of IFN are thought to be mediated by products of a specific set of genes whose expression is induced or at least augmented in IFN-treated cells. Much of the current research on the mechanism of action of IFN is focused on identifying these genes, understanding how IFN enhances gene expression, and evaluating the specific contributions of the products of these genes toward various actions of IFN.

We have been using partially IFN-responsive cell lines to study the action of IFN (1, 8, 20–22). In these cells, IFN would inhibit replication of some viruses but not of others. The human rhabdomyosarcoma line RD-114 is one such line. These cells are responsive to both IFN-α and IFN-γ, which are known to act through different receptors. Production of the resident retrovirus RD-114 by this cell line is inhibited by either IFN-α or IFN-γ as efficiently as in any other cell line (8). However, replication of vesicular stomatitis virus (VSV) and of encephalomyocarditis virus (EMCV) in this cell line is not sensitive to IFN treatment (8). Similarly, IFN-α and IFN-γ had little anticellular effect on these cells, as measured by the rate of cell multiplication and [^{3}H]thymidine incorporation into DNA (22). Although these cells contained the usual quantity of RNase L, the amount of 2′-5′-oligo-adenylate synthetase (2-5(A) synthetase) activity induced by either IFN was quite low, and the level of double-stranded-RNA-dependent protein kinase was barely detectable even in IFN-treated RD-114 cells. These cells are therefore only partially responsive to IFN-α and IFN-γ.

Recently, cDNA clones corresponding to several IFN-inducible mRNAs have been isolated (2, 6, 11, 13, 15–17, 19, 25). These cDNA clones can now be used as hybridization probes to assess the induced synthesis of the corresponding mRNAs in response to IFN treatment of any cell line. In the work presented in this paper, we have investigated whether seven mRNAs which are usually inducible in fully responsive cell lines are indeed induced in RD-114 cells in response to treatment with IFN-α or IFN-γ. We also examined whether the induced mRNAs are translated in these cells, thereby producing functional proteins. Results from these studies suggest that certain IFN-inducible gene products are not sufficient to inhibit replication of VSV or EMCV.

Synergism between IFN-α and IFN-γ has been reported both for antiviral and anticellular actions (3, 5). Here we present results demonstrating such synergism at the molecular level. 2-5(A) synthetase mRNA induction by IFN-α was strongly boosted by a very low dose of IFN-γ in RD-114 cells. Our results indicate that an IFN-γ-inducible protein is needed for this process.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., and International Biotechnologies, Inc. Radioactive chemicals were obtained from New England Nuclear Corp. For radiolabeling cDNA probes, a nick translation kit from Bethesda Research Laboratories was used.

IFNs. Pure recombinant IFN-αA was a gift from Sidney Pestka, Rutgers Medical School, Piscataway, N.J. and pure recombinant IFN-γ was a gift from Genentech, Inc.

Cell culture. The two cell lines used here, HeLa and RD-114 (10, 22), were both grown in monolayer cultures in 100- or 150-mm-diameter plates with minimum essential medium containing 10% fetal bovine serum. When the cells were subjected to a series of treatments with different inhibitors or IFNs, cells were washed three times with warm culture medium between treatments.

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cDNA probes. Isolation of cDNA clone P561 has been described previously (10). The cDNA clone corresponding to 2-5(A) synthetase mRNA (cDNA 6-2) was a gift from Bryan Williams, The Hospital for Sick Children, Toronto, Ontario, Canada (17). cDNA clones 1-8, 6-16, 6-26, 2A, and metallothionein II (MTII) were gifts from George Stark and Ian Kerr, Imperial Cancer Research Funds, London, England (6). For probes for Northern and dot blot analyses, the cDNA inserts from these clones were isolatd by digestion with the appropriate restriction enzymes followed by electrophoresis on an agarose gel before the cDNA inserts were nick translated.

RNA analysis. Total cytoplasmic RNA was isolated as described previously (23). Northern analyses were done with RNAs which had been denatured in the presence of formaldehyde. Procedures for dot blot analysis and precautions taken to ensure a linear response were as described previously (10).

Analysis of polyribosomes. HeLa and RD-114 cells (1.6×10^8 for polysome fractionation and 0.8×10^8 for total polysome isolation) treated with IFN-α for 6 h were used for this experiment. Just before harvest the cells were treated for 15 min with 100 μg of cycloheximide per ml to prevent ribosome runoff during fractionation. The cells were cooled quickly on ice, collected by centrifugation, and suspended (1.6×10^8 cells per ml) in 10 mM Tris chloride (pH 7.5)–10 mM NaCl–1.5 mM $MgCl_2$–5 mM vanadyl ribonucleoside complex–100 μg of cycloheximide per ml. Triton X-100 was added to the suspension to a final concentration of 0.5%, and the mixture was kept on ice for 3 min. Concentrations of Tween 40 and sodium deoxycholate were then adjusted to 1 and 0.5%, respectively, and cells were lysed by 10 strokes with a Dounce homogenizer. The nuceli and mitochondria were removed by centrifugation at $10,000 \times g$ for 10 min in a Sorvall SS34 rotor, and the supernatant fluid was used for either total polysome isolation or polysome fractionation. All the operations were performed at 4°C.

Total polysomal RNA was prepared by sedimenting cytoplasmic extract through a 4-ml cushion of 30% (wt/wt) sucrose in 10 mM NaCl–1.5 mM $MgCl_2$–50 μg of dextran sulfate per ml for 2 h at 49,000 rpm in a Beckman SW50.1 rotor. Polysomal pellets were suspended in 10 mM Tris chloride (pH 7.5)–150 mM NaCl–1 mM $MgCl_2$–0.05% Triton X-100. Sodium dodecyl sulfate and EDTA concentrations were adjusted to 0.5% and 10 mM, respectively. RNA was then extracted by the method described above.

For polysome fractionation, the supernatant fluid was layered on top of a 38-ml 10 to 50% linear sucrose density gradient containing 10 mM Tris chloride (pH 7.5), 500 mM KCl, and 5 mM $MgCl_2$. The gradients were centrifuged at 26,000 rpm in the Beckman SW28 rotor at 4°C for 270 min. The gradient was drained at a constant rate by a pump, and fractions of 1 ml were collected. A_{260} of each fraction was measured, making any necessary dilutions. The fractions were pooled into groups of five, and 5 ml of 2× PK buffer (200 mM Tris chloride [pH 7.5], 25 mM EDTA, 300 mM NaCl, and 2% sodium dodecyl sulfate) was added to each pool, and RNA was extracted by the method discussed above. Equal portions of RNA from each pooled fraction were used for dot blot analysis.

RESULTS

Induction of different mRNAs by IFN-α and IFN-γ in RD-114 cells. RD-114 cells in monolayer cultures were treated with 500 U of IFN-αA or IFN-γ per ml for various times before harvesting. Cytoplasmic RNA was isolated, and identical amounts of different RNA samples were electrophoresed for Northern analysis. cDNA clones corresponding to various inducible mRNAs were radiolabeled by nick translation and used as probes. For comparison, similar experiments were done with HeLa cells, which are fully responsive to IFN. The results are discussed below.

mRNA 561, an IFN-inducible mRNA of about 1.8-kilobases (kb) length, encodes a protein of 56 kilodaltons. It was induced strongly by IFN-α in both HeLa and RD-114 cells. The maximum level was reached by 6 h of IFN-α treatment. The level then decreased rapidly, almost reaching the uninduced level by 24 h of IFN treatment. IFN-γ did not induce detectable amounts of mRNA 561 in either HeLa or RD-114 cells (Fig. 1A).

Fig. 1B shows induction profiles of mRNA 2A, a member of the HLA class I family of about 1.7-kb length, and mRNA 1-8 of about 0.8-kb length, which encodes an unidentified protein. mRNA 2A was induced to a much greater extent by IFN-γ than by IFN-α in both cell lines. The kinetics of induction of mRNA 2A by IFN-γ was slower than that observed for mRNA 561 induction by IFN-α; the maximum level was reached by about 24 h. mRNA 1-8 was induced by both IFN-α and IFN-γ in both the cell lines. The uninduced level of mRNA 1-8 was considerably higher in HeLa cells. The induced level reached the maximum by about 12 h and did not decrease by 24 h. The extent of induction by IFN-α and IFN-γ was comparable.

Induction of mRNA 6-16, an mRNA of 1.0 kb encoding an unidentified protein, is shown in Fig. 1C. The uninduced level of this mRNA was almost undetectable in both HeLa and RD-114 cells, and it was strongly induced by IFN-α_A in both the lines. The level peaked by 6 h and remained the same for 24 h. IFN-γ also induced this mRNA in both lines but to a much lower extent and with slower kinetics, the maximum level being reached by 12 h. mRNA 6-26, which encodes thymosin, is induced by IFN in certain cell lines. However, the uninduced level of this mRNA was high in both HeLa and RD-114 cells, and this level was not increased further in either cell line by treatment with IFN-α or IFN-γ (Fig. 1D; data not shown). It should be noted that in Fig. 1B and C, lanes 1 to 11 are from one experiment and lanes 12 to 15 are from another. The exposure times for the autoradiographs from the two experiments were different, making direct comparisons of the intensities of the bands between the two experiments unreliable. This is demonstrated by examining the intensities of the same band in lanes 8 and 12 of Fig. 1B, in which the same sample was analyzed in the two experiments.

In contrast to the examples given so far, induction patterns of mRNAs 2-5(A) synthetase and MTII in RD-114 cells and in HeLa cells were quite different (Fig. 2). Three distinct 2-5(A) synthetase-related mRNAs of approximately 3.6-, 2.5-, and 1.6-kb sizes were induced strongly in HeLa cells in response to IFN-α, the maximum level being reached between 6 to 12 h (lanes 12 to 15). The same mRNAs were also induced by IFN-γ in HeLa cells, but the magnitude of induction was considerably lower and the kinetics were somewhat slower (lanes 8 to 11). Induction of these mRNAs was much poorer in RD-114 cells. IFN-γ did not induce them at all (lanes 5 to 7), and although IFN-α induced them (lanes 1 to 4), the level of induction was low. To obtain a better quantitative measure of the extent of induction of 2-5(A) synthetase mRNAs in the two cell lines in response to IFN-α, twofold serial dilutions of the respective RNA samples were dot blotted and hybridized to the labeled cDNA

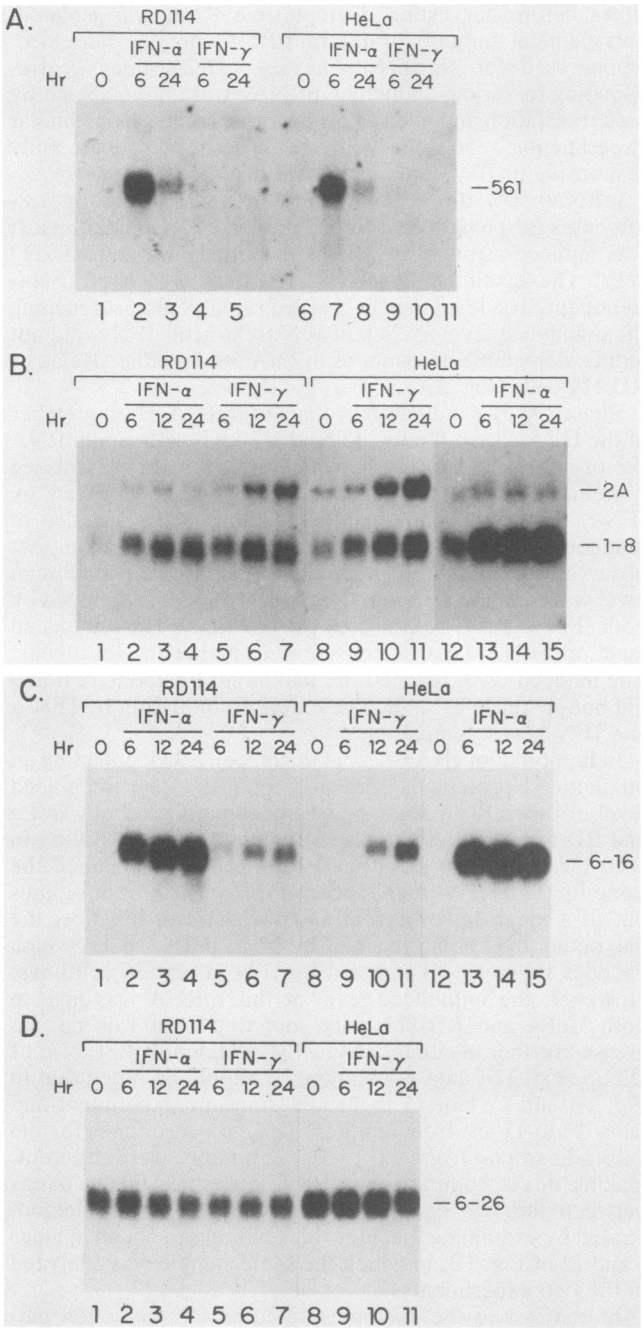

FIG. 1. Kinetics of synthesis of five IFN-inducible mRNAs in IFN-α- or IFN-γ-treated RD-114 and HeLa cells. Cells were treated with 500 U of IFN-αA or IFN-γ per ml for the indicated times. Total cytoplasmic RNA was isolated. Of total RNA, 20 μg was denatured in the presence of formaldehyde and electrophoresed for Northern analysis. The blots were probed with cDNA 561 (A), cDNAs 2A and 1-8 (B), cDNA 6-16 (C), and cDNA 6-26 (D).

probe. Densitometric scanning of the autoradiogram revealed that the levels of 2-5(A) synthetase mRNAs in untreated HeLa and RD-114 cells were similarly low, and this level was increased 16-fold at 6 h of IFN-α treatment in HeLa cells and about threefold in RD-114 cells (data not shown).

mRNA MTII which encodes metallothionein was strongly induced by IFN-γ in HeLa cells (Fig. 2; lanes 8 to 11). The

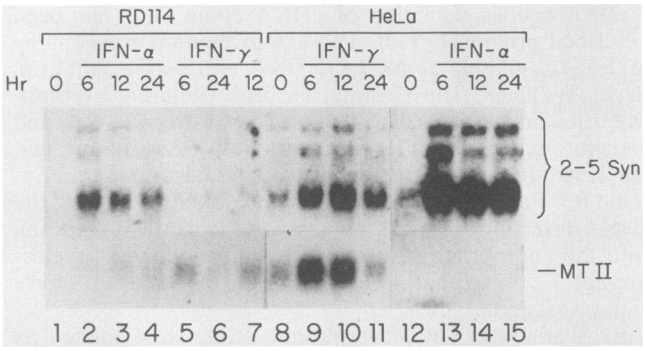

FIG. 2. Kinetics of induction of mRNAs 2-5(A) synthetase and MTII in RD-114 and HeLa cells. Conditions were similar to those described in the legend to Fig. 1. Two separate blots were probed with cDNA 2-5(A) synthetase (2-5 Syn) and cDNA MTII. The relevant portions of the autoradiogram are shown here.

level peaked at around 6 to 12 h and then decreased to the uninduced level by about 24 h. In RD-114 cells, this mRNA was very poorly induced, if at all, by IFN-γ. IFN-α did not induce this mRNA appreciably in either cell line.

Table 1 summarizes the data presented in Fig. 1 and 2. Of the seven mRNAs tested, five were induced similarly in both cell lines. mRNA 561 was induced only by IFN-α. mRNA 6-16 was induced much more efficiently by IFN-α, compared with IFN-γ, whereas the opposite was true for mRNA 2A. mRNA 1-8 was induced well by either IFN, and mRNA 6-26 was not induced above the high constitutive level in these cells. Induction of 2-5(A) synthetase mRNA was greater by IFN-α than by IFN-γ, and it was greater in HeLa cells than in RD-114 cells. On the other hand, mRNA MTII was induced to a great extent only in IFN-γ-treated HeLa cells. Our results are in general agreement with those of Kelly et al. (9), who studied the induction patterns of several IFN-inducible mRNAs in HeLa cells. However, in contrast to our results, they did not observe induction of MTII mRNA by IFN-γ to a great extent.

Induction of MTII mRNA by other agents in RD-114 cells. The inability of IFN-γ to induce MTII mRNA synthesis in RD-114 cells could be due to a defect in the MTII gene itself or to a defect in the requisite signals generated by IFN-γ in these cells. To distinguish betwen the possibilities, we tested whether the same mRNA can be induced by other agents known to do so in other cell lines. mRNA MTII levels were estimated in HeLa and RD-114 cells which had been treated with IFN-α, IFN-γ, dexamethasone, or $ZnCl_2$ (Fig. 3). In HeLa cells, IFN-γ and Zn^{2+} were very good inducers of mRNA MTII, whereas dexamethasone was a moderate

TABLE 1. Induction of different mRNAs by IFN-α and IFN-γ in HeLa and RD-114 cells

mRNA	Induction[a]			
	IFN-α		IFN-γ	
	HeLa	RD-114	HeLa	RD-114
561	++++	++++	−	−
6-16	++++	+++	+	+
2A	+	+	+++	+++
1-8	+++	++++	+++	++++
6-26	−	−	−	−
2-5(A) synthetase	++++	+	+	−
MTII	+	+	++++	+

[a] Stimulation over uninduced level indicated semiquantitatively.

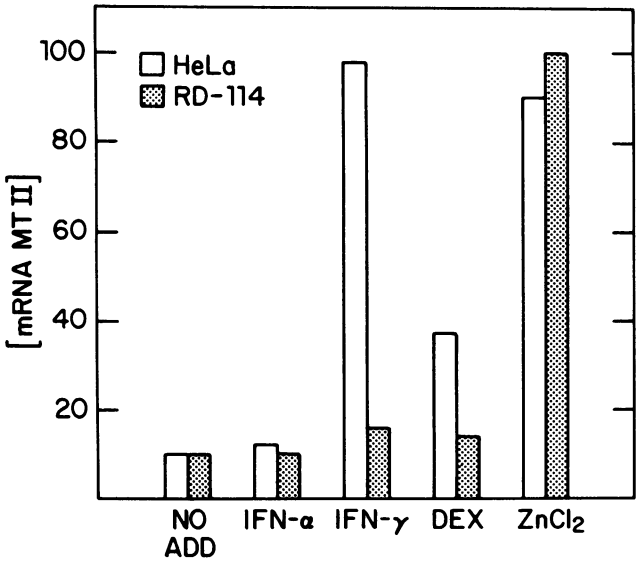

FIG. 3. Induction of mRNA MTII in HeLa and RD-114 cells in response to various agents. Cells were treated with 500 U of IFN-αA per ml, 500 U of IFN-γ per ml, 1 μM dexamethasone (DEX) or 100 μM $ZnCl_2$ for 3 h before harvesting. Total cytoplasmic RNA was used for dot blot analysis with MTII cDNA probe. Results are presented in arbitrary units.

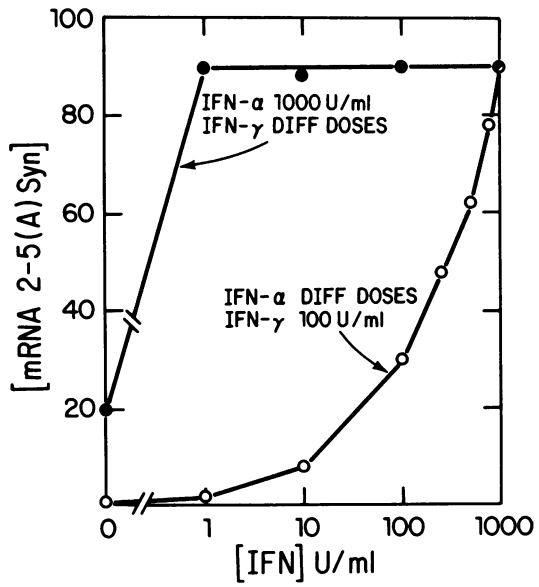

FIG. 4. IFN dose response for synergistic induction of 2-5 (A) synthetase mRNA in RD-114 cells. RD-114 cells were treated with indicated doses of IFN-α and IFN-γ for 6 h, and the cytoplasmic levels of 2-5(A) synthetase mRNA were determined by dot blot analyses. These levels are presented in arbitrary units, and the IFN doses are presented on a logarithmic scale. The level of this mRNA in untreated cells was 1 unit.

inducer. Although IFN-γ could not induce this mRNA effectively in RD-114 cells, it was induced by $ZnCl_2$ to the same extent as in HeLa cells, suggesting that the MTII gene in RD-114 cells does not have any gross defects in the regulatory region.

Synergism between IFN-α and IFN-γ for induction of 2-5(A) synthetase mRNA in RD-114 cells. We were interested to determine whether the level of induction of 2-5(A) synthetase mRNA in RD-114 cells could be augmented by any means. During these studies, we observed that although IFN-γ by itself could not induce this mRNA in RD-114 cells, it could elevate the level of induction by IFN-α (Table 2). Thus, about fourfold more 2-5(A) synthetase mRNA accumulated in RD-114 cells treated with both IFN-α and IFN-γ, compared with cells treated with IFN-α alone (plate 6). In HeLa cells, no such synergistic induction was observed.

In the experiment shown in Fig. 4, we measured the dose response of this synergism with respect to IFN-α and IFN-γ. A dose of 100 U of IFN-γ per ml induced little 2-5(A) synthetase mRNA, but the level of induction increased with increasing doses of IFN-α added along with IFN-γ. At the highest dose of IFN-α tested (1,000 U/ml), the induced level was still increasing. In contrast, 1,000 U of IFN-α per ml by itself induced about 4.5-fold less 2-5(A) synthetase mRNA, compared with that at 100 U of IFN-γ per ml and 1,000 U of IFN-α per ml. However, the addition of only 1 U of IFN-γ per ml (the lowest dose tested) along with IFN-α maximally enhanced the induction, which was not increased any further by increasing the IFN-γ dose. These results demonstrated that minute amounts of IFN-γ could dramatically increase the potency of IFN-α under these conditions. Thus 20 U of IFN-α per ml could induce, in the presence of IFN-γ, as much 2-5(A) synthetase mRNA (20 arbitrary units) as that induced by 1,000 U of IFN-α per ml alone.

Several characteristics of 2-5(A) synthetase mRNA induc-

TABLE 2. Synergism between IFN-α and IFN-γ for induction of 2-5(A) synthetase mRNA in RD-114 cells

Plate no.	Treatment time[a] (h)		Time of harvest (h)	mRNA 2-5(A) synthetase level[b]	
	IFN-α	IFN-γ		HeLa	RD-114
1			0	5	4
2	0–6		6	100	16
3		0–6	6	20	4
4	0–12		12	108	14
5		0–12	12	36	5
6	0–12	0–12	12	138	62

[a] 500 U of IFN-α or IFN-γ per ml was used where indicated.
[b] mRNA levels are in arbitrary units, and the values are directly comparable only within this experiment.

TABLE 3. Characteristics of synergistic induction of 2-5(A) synthetase in RD-114 cells

Plate no.	Treatment time[a] (h)			Time of harvest (h)	mRNA 2-5(A) synthetase level[b]
	IFN-α	IFN-γ	Cyclo-heximide		
1				0	8
2	0–6			6	30
3	0–6		0–6	6	26
4		0–6		6	10
5	0–6	0–6		6	100
6	0–12	0–12		12	95
7	0–24	0–24		24	91
8	6–12	0–6		12	114
9	6–12	0–6	6–12	12	128
10	6–12	0–6	0–12	12	25
11	8–14	0–6	0–6 8–14	14	120

[a] 500 U of IFN-α per ml, 100 U of IFN-γ per ml, and 50 μg of cycloheximide per ml were used where indicated.
[b] mRNA levels are in arbitrary units, and the values are directly comparable only within this experiment.

tion in RD-114 are shown in Table 3. The steady-state cytoplasmic levels of this mRNA in cells treated with both IFN-α and IFN-γ reached the maximum by about 6 h (plate 5) and remained relatively unchanged over a period of 24 h (plates 6 and 7). The treatments with IFN-α and IFN-γ did not have to be simultaneous, because treatment with IFN-γ followed by IFN-α was equally effective (plate 8). Induction of a low level of 2-5(A) synthetase mRNA in response to IFN-α alone did not need ongoing protein synthesis (plate 3). Similarly, for synergistic induction with a sequential IFN treatment protocol, protein synthesis was not needed during IFN-α treatment (plate 9). When protein synthesis was blocked during the entire period of treatment with IFN-γ and IFN-α, no synergistic induction was observed, although the basal level of induction by IFN-α was still there (plate 10). This suggested that IFN-γ-induced synthesis of a putative mRNA whose translation product was needed for synergistic interaction with IFN-α Consistent with this model, synergistic induction of 2-5(A) synthetase mRNA was observed in plate 11, in which IFN-γ treatment was done in the presence of cycloheximide, allowing synthesis of the putative mRNA but not its translation product; IFN-γ and cycloheximide were removed, allowing translation of the preformed putative mRNA already induced by IFN-γ, and finally IFN-α was added along with cycloheximide.

Turnover of mRNA 561. Some of the IFN-inducible mRNAs are quite stable, whereas others turn over rapidly (9). mRNA 561 has a short half-life in HeLa cells, but it can be stabilized by inhibiting protein synthesis once the mRNA has been induced (10). In the experiments shown in Fig. 5, we investigated whether the characteristics of turnover of mRNA 561 in RD-114 cells were similar to those in HeLa cells. Induced synthesis of mRNA 561 in response to IFN-α in RD-114 cells, unlike that in HeLa cells, did not need ongoing protein synthesis. We have reported previously that the need for ongoing protein synthesis in HeLa cells could be obviated by pretreating the cells with IFN-γ, which by itself did not induce mRNA 561 (10). For these reasons, for the turnover experiments shown in Fig. 5, untreated RD-114 cells and IFN-γ-pretreated HeLa cells were used. In HeLa cells, accumulation of mRNA 561 continued up to 6 h after treatment with IFN-α and cycloheximide began, when a stable plateau was reached. When synthesis of the mRNA was stopped at 3 h by withdrawing IFN-α and adding actinomycin D, the accumulated mRNA 561 was reasonably stable if cycloheximide was retained. However, if cycloheximide was removed at 3 h along with IFN-α, the accumulated mRNA 561 turned over rapidly. Similar characteristics of mRNA 561 turnover were also observed in RD-114 cells; accumulated mRNA 561 was stable in the presence of cycloheximide but turned over rapidly in its absence. These results suggest that the synthesis of a functionally labile protein, perhaps an IFN-inducible one, is needed for the rapid turnover of mRNA 561 in both cell lines.

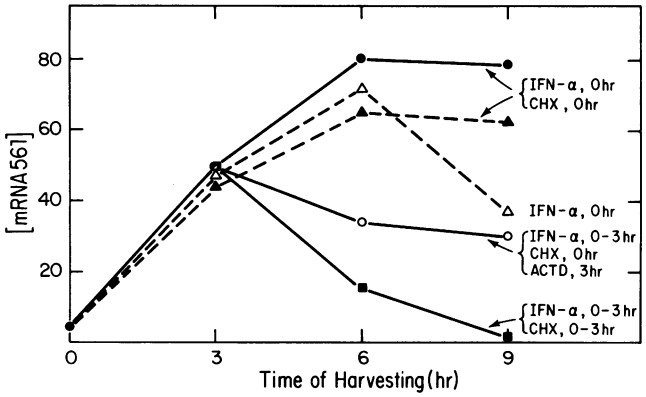

FIG. 5. Turnover of mRNA 561 and effects of cycloheximide. Untreated RD-114 cells (-----) or HeLa cells that had been pretreated with 500 U of IFN-γ per ml for 6 h (——) were treated as indicated. 500 U of IFN-α per ml, 50 μg of cycloheximide (CHX) per ml, and 1 μg of actinomycin D (ACTD) per ml were used where indicated. Symbols: ●, ○, and ■, HeLa cells; ▲ and △, RD-114 cells.

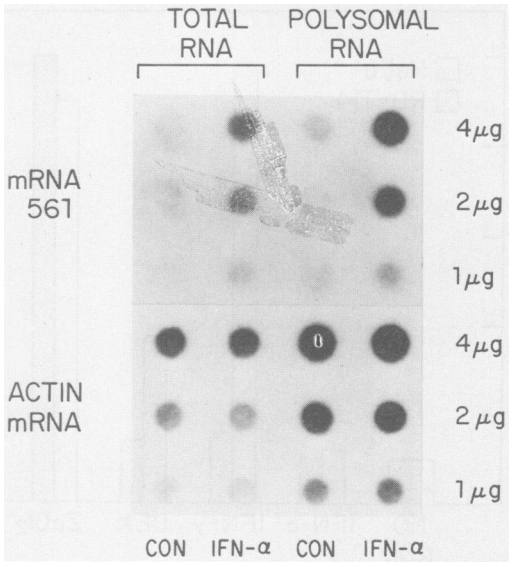

FIG. 6. Association of mRNA 561 and mRNA actin with polysomes in RD-114 cells. Total cytoplasmic RNA and polysomal RNA were isolated from IFN-αA-treated and control RD-114 cells. Serial dilutions of these RNAs were blotted and probed with cDNA 561 or cDNA actin. CON, Control.

Translation of induced mRNAs in RD-114 cells. One reason for determining the pattern of induction of different mRNAs in RD-114 cells was to assess the roles of their products in the activiral actions, since the replication of viruses such as VSV, EMCV, or influenza virus is not inhibited in RD-114 cells by IFN treatment. It was important for this purpose, however, to determine whether these mRNAs were translated in these cells. But without the availability of any specific reagents, i.e., antibodies, there was no direct way to achieve this goal. Association of mRNAs with polysomes is a good index for their active translation in the cells. We therefore examined the polysome association of mRNA 561 in IFN-treated RD-114 cells. As positive controls for this experiment, the association of mRNA 561 with polysomes in HeLa cells and of actin mRNAs with polysomes in both cell lines was examined. Fig. 6 shows the association of mRNA 561 and actin mRNA with total polysomes in IFN-treated and untreated RD-114 cells. mRNA 561 was partitioned into the polysome fraction as much as actin mRNA was, suggesting that they were being translated equally well. The same distribution of mRNA 561 and actin mRNA was observed in IFN-treated HeLa cells (data not shown).

To assess the degree of association of these two mRNAs with polysomes of different sizes, cell extracts were analyzed by centrifugation through linear sucrose gradients. To prepare these cell extracts, conditions were chosen which

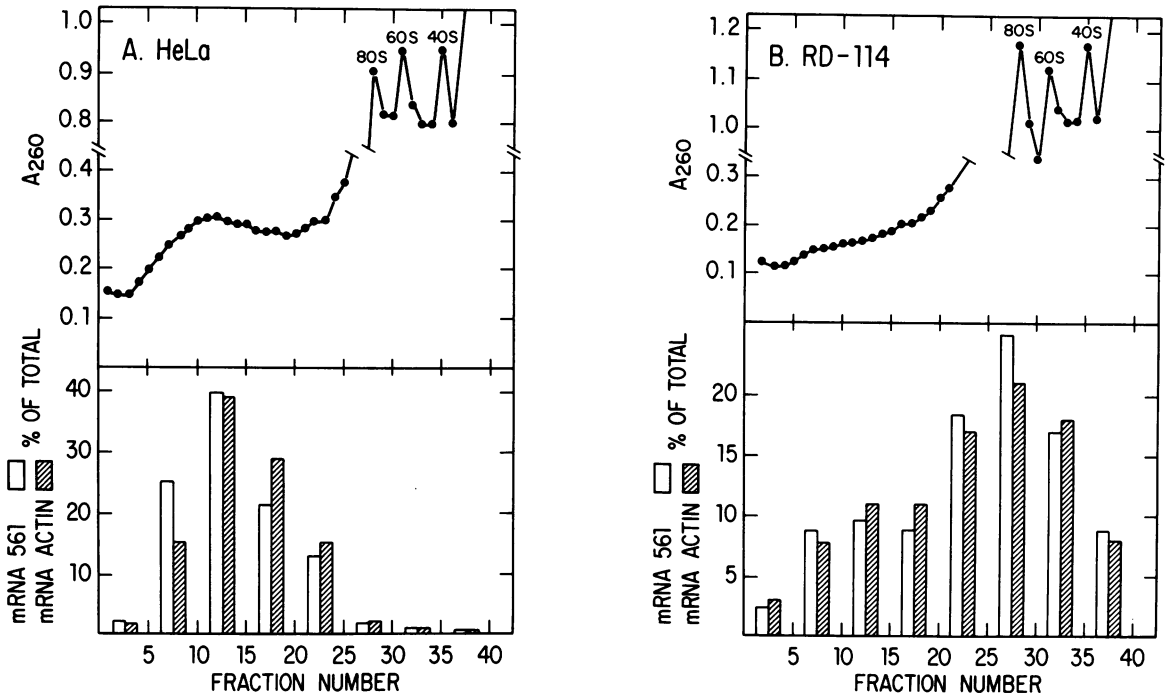

FIG. 7. Distribution of mRNA 561 and mRNA actin in polysomes of various sizes in HeLa and RD-114 cells. Methods for these analyses have been described in the Materials and Methods section. HeLa (A) and RD-114 (B) cells were both treated with IFN-αA for 6 h before making cell extracts for polysome analysis. The top of each panel shows the A_{260} profile. The bar graph shows the percentage of total mRNA 561 and mRNA actin in eight pools of five fractions each. Sedimentation was from right to left, and the positions of different ribosomal subunits are indicated.

solubilize polysomes bound to cellular membranes. After centrifugation, the gradients were separated into 40 fractions, and the A_{260} profiles were determined. The fractions were pooled into groups of five, and RNA was extracted, dot blotted, and hybridized with either actin cDNA or p561 cDNA. The concentration of each mRNA in each pooled fraction was estimated by scanning the dot blots. The data are presented in bar graphs as percentage of the total contents in Fig. 7. A well-defined polysome peak was obtained from HeLa cell extract (Fig. 7A), and almost all of the mRNA 561 and actin mRNA present in the cell extract was associated with polysomes. Moreover, the relative distributions of actin mRNA and mRNA 561 among the different fractions were very similar, suggesting that the two mRNAs were translated in vivo with equal efficiency in IFN-treated HeLa cells. The polysome profile obtained from RD-114 cell extracts was not as well-defined as that from HeLa cell extracts. We tried a variety of conditions for preparing extracts from RD-114 cells but failed to obtain a better polysome profile than the one shown in Fig. 7B. It is possible that in these cells mRNAs are not associated with as many ribosomes on the average as in HeLa cells or that our extraction procedure was degrading polysomes in RD-114 extracts but not in HeLa extracts. But no matter what the case may be, mRNA 561 and actin mRNA were similarly distributed among different fractions in RD-114 cells (Fig. 7B), indicating that mRNA 561 was translated efficiently in these cells as well. The same dot blots from HeLa and RD-114 cells were also hybridized with cDNA probe 1-8 and the distribution of mRNA 1-8 was found to be very similar to that of mRNA 561 and actin mRNA in both cell lines (data not shown).

DISCUSSION

Classifying IFN-inducible genes into sets by the property of coinduction. The nature of the signal(s) transmitted from the IFN-receptor complex on the plasma membrane to the nucleus that induces the expression of specific genes is unknown. However, some idea about the number of such signals can be obtained if we are able to show that these genes can be grouped into sets whose members are always induced simultaneously in a cell in response to IFN treatment. Following the above line of reasoning, McMahon et al. (14) classified mRNAs 2A, MTII, 2-5(A) synthetase, and 6-26 into one set and mRNAs 1-8, 9-27, and 6-16 into another. The first set was similarly induced in the wild-type and a mutant Daudi line, whereas the second set was induced only in the wild-type cells in response to IFN-α. The results presented in this paper suggest that similarly, mRNAs 561, 1-8, 6-16, 6-26, and 2A can be grouped into one set, whereas mRNAs MTII and 2-5(A) synthetase are in another set. The first set was inducible in both HeLa and RD-114 cells, whereas the second set was inducible only in HeLa cells. Further subdivisions can be done by the inducibility of these mRNAs by IFN-α and IFN-γ, which are known to act through different receptors and which induce partially overlapping sets of genes. In our studies, mRNA 1-8 was equally inducible by both IFNs, and mRNA 561 was exclusively induced by IFN-α. mRNAs 6-16 and 2-5(A) synthetase were better induced by IFN-α than by IFN-γ, and mRNAs 2A and MTII were better induced by IFN-γ. It should be kept in mind however that these patterns of induction of different mRNAs by IFN-α and IFN-γ vary from cell line to cell line. For example, IFN-γ induces

mRNA 561 and 2-5(A) synthetase mRNA well in other lines (4, 25), and IFN-α induces mRNA MTII well in some other lines (7). But the fact that some genes are induced preferentially by IFN-α in some cell lines and others are preferentially induced by IFN-γ suggests that the two types of IFN produce several second messengers, some of which could be similar but some of which are definitely not. The possibility remains open that there is more than one kind of IFN-α or IFN-γ receptor and that the interaction of IFN with one subset of receptors produces a specific signal leading to the induction of a specific set of genes. This latter scenario obviously predicts the existence of multiple second messengers which are produced in this instance by the interaction of IFN with distinct subsets of receptors. These putative second messengers may share common components, but what is suggested here is that each of these must have a distinct component not shared by others. Production of these signals in different cell lines in response to different IFNs may not always be an all-or-none response, but there could be quantitative variations leading to quantitative differences in the extent of induction of the relevant responsive gene(s). Poor induction of MTII mRNA in response to IFN-γ and of 2-5(A) synthetase mRNA in response to IFN-γ in RD-114 cells could in principle be due to defects in *cis*-acting sequences of the respective genes which make them inducible by IFN. However, this possibility seems unlikely since 2-5(A) synthetase mRNA was appreciably induced in response to a combination of IFN-α and IFN-γ, and MTII mRNA was induced very well in response to Zn^{2+}. Although we cannot rule out the possibility that the responsiveness of the MTII gene to IFN needs structural features different than those needed for response to Zn^{2+} and that these features are defective in RD-114 cells, the more likely interpretation is that the proximal signals needed to induce these genes were not produced properly by treatment of RD-114 cells with IFN.

Role of different IFN-inducible gene products in antiviral actions. Replication of VSV, EMCV, or influenza virus is insensitive to IFN-α or to IFN-γ in RD-114 cells, but retrovirus production is inhibited by both IFNs (8, 22). We measured the levels of 2-5(A) synthetase, RNase L, and double-stranded RNA-dependent protein kinase activities in IFN-treated RD-114 cells (22) and found that induction of both double-stranded RNA-dependent enzymes was poor in these cells, but RNase L level was comparable to that in L929 cells. The RNase L pathway is thought to play a crucial role in the anti-EMCV action of IFN, and therefore, resistance of EMCV replication to IFN action in these cells could be attributed to poor 2-5(A) synthetase induction. Results presented here demonstrate that a low level of this enzymic activity is a direct result of the poor induction of the corresponding mRNA in IFN-treated RD-114 cells. However, a much higher level of this mRNA was induced in RD-114 cells that had been treated with IFN-α and IFN-γ together; it would be interesting to investigate whether the replication of EMCV was impaired under these conditions.

Several mRNAs, e.g., mRNA 561, mRNA 6-16, mRNA 1-8, and mRNA 2A, were equally induced in HeLa and RD-114 cells. These results suggest that the products of these mRNAs are not by themselves sufficient to inhibit VSV, EMCV, or influenza virus replication. Similarly, it appears that induced levels of MTII mRNA are not necessary for inhibition of retrovirus production, since it is poorly induced by IFN-γ in RD-114 cells or by IFN-α in either cell line. The above conclusions assume, however, that the level of the mRNA of one of these genes is a direct measure of the level of its protein product. Some justification for such an assumption came from the experiments shown in Fig. 6 and 7, demonstrating that mRNA 561 (and mRNA 1-8) was associated with actively translating polysomes in both HeLa and RD-114 cells. McMahon et al. (14) examined the status of different IFN-inducible mRNAs in wild-type and IFN-resistant Daudi cell lines. In their system however, it would be difficult to assign roles to these mRNAs in the antiviral actions of IFN since many viruses do not multiply well in Daudi cells.

Synergism between IFN-α and IFN-γ. Synergistic antiviral and anticellular actions of IFN-α and IFN-γ have been observed before (3, 5). However, the molecular basis of such synergism was not explored. It is possible that two different biochemical pathways are induced by the two classes of IFN and that each of these pathways leads to antiviral or anticellular effects. On the other hand, it is also possible that induction of a specific IFN-inducible pathway is synergistically augmented by a combination of the two IFNs. Results presented in this paper provide strong evidence for the second possibility, demonstrating synergism at the level of induction of a specific gene.

It was remarkable that only 1 U of IFN-γ per ml was capable of potentiating induction of 2-5(A) synthetase mRNA by IFN-α to the fullest extent. Moreover, the extent of synergistic induction of this mRNA increased with increasing doses of IFN-α all the way to the highest dose tested (1,000 U/ml), whereas the low level of induction by IFN-α alone plateaued at about 200 U of IFN-α per ml and did not increase any further (data not shown). One possible interpretation of our data is that an IFN-γ-inducible protein is needed along with IFN-α for maximum induction of this mRNA in RD-114 cells. This protein is present in untreated or IFN-α-treated RD-114 cells in limited quantity, and its low concentration in IFN-α-treated cells is the reason for low induction of 2-5(A) synthetase mRNA by IFN-α alone. However, IFN-γ causes such a large increase in its concentration that enough of this protein is produced to cooperate with even 1,000 U of IFN-α per ml. This hypothesis can also explain why little synergism was observed for the induction of the same mRNA in HeLa cells. Presumably the putative IFN-γ-inducible protein is present in these cells in high quantity constitutively; therefore, IFN-α by itself can induce mRNA effectively, and IFN-γ does not boost this induction any further. Manifestation of such synergism therefore depends on the cell line in question. It is also mRNA specific; even in RD-114 cells strong synergistic induction was observed for 2-5(A) synthetase mRNA but not for mRNAs 561, 2A, MTII, 1-8, and 6-16 (data not shown).

Turnover of IFN-inducible mRNAs. Some of the IFN-inducible mRNAs, such as mRNA 561, turn over rapidly. On the other hand mRNAs such as 1-8 or 6-16 have long half-lives. We have reported that inhibitors of protein synthesis slow down the rate of turnover of mRNA 561 in HeLa cells (10). However, in these cells, protein synthesis is also needed for induction of mRNA 561, making the interpretation of results difficult. For turnover analyses, it was necessary therefore to work with IFN-γ-pretreated HeLa cells in which the need for protein synthesis is eliminated (10). In contrast, induction of mRNA 561 by IFN-α in RD-114 cells did not need ongoing protein synthesis (Fig. 5). Our experiments (Fig. 5) demonstrated that although the needs for protein synthesis for induction of mRNA 561 were different in HeLa and RD-114 cells, the turnover characteristics were similar. In both lines, mRNA 561 turned over rapidly (Fig. 1 and 5), and in both lines such rapid turnover could be

prevented by treating the cells with cycloheximide. The latter results suggest that synthesis of a functionally labile protein is needed for the turnover process, although the alternative possibility exists that the translation of mRNA 561 itself is necessary for its turnover. Are there structural features which make some of these IFN-inducible mRNAs labile? Shaw and Kamen (24) recently described a sequence motif present in the 3′ untranslated regions of many mRNAs which turn over rapidly. This motif, when artificially introduced into the 3′ untranslated region of β-globin mRNA, converted a stable mRNA into an unstable one. It is interesting that such a motif is also present in the 3′-untranslated region of mRNA 561. We are currently testing whether it contributes toward the instability of mRNA 561.

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