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Beta and Gamma Interferons Act Synergistically To Produce an Antiviral State in Cells Resistant to Both Interferons Individually

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We showed previously that the mouse fibroblastoid cell line Ltk-aprt- is resistant to the antiviral effects of beta interferon. This lack of response reflects a partial sensitivity to the interferon that is accompanied by a failure to activate expression of several interferon-regulated genes, although certain other genes respond in a normal manner. We show here that Ltk-aprt- cells were also unable to establish an antiviral state and to activate expression of 2,5-oligo(A) synthetase when treated with gamma interferon. Strikingly, however, treatment with a combination of beta interferon and gamma interferon provided complete protection against viral replication. Although the cells were completely insensitive to up to 250 U of the interferons per ml added singly, essentially complete protection from viral cytopathic effects was achieved when as little as 10 U of each of the interferons per ml were combined. Expression of 2,5-oligo(A) synthetase was also sensitive to this synergistic effect. Activation of an antiviral state could also be achieved by sequential treatment, first with gamma interferon and then with beta interferon. Partial protection against viral replication could be achieved by pretreatment with gamma interferon for as little as 1 h before incubation with beta interferon and could be blocked by the addition of specific antibodies or by cycloheximide, indicating that gamma interferon induces the synthesis of a protein which can act synergistically with a signal produced by the beta-interferon receptor. We suggest that Ltk-aprt- cells suffer from defects in one or more components of the gene activation pathways for both type I and type II interferons. Nonetheless, gamma interferon is able to activate the expression of a gene encoding a protein required for signal transduction. This protein acts synergistically with a transient signal produced in response to beta interferon, thereby activating the expression of a further group of genes.

Interferons (IFNs) induce the production of an antiviral state by binding to high-affinity cell surface receptors and thereby activate the expression of several genes encoding enzymes with antiviral capacities (25). Although several IFN-responsive genes have been cloned and their upstream regulatory elements have been defined, little is known about the process of signal transduction which couples the IFN receptors with transcription activation factors. Characterization of these signals and the transcription factors with which they interact is a major goal. Achieving this aim would be facilitated by the availability of cell variants which are defective in their responses to IFNs but, preferably, can be manipulated to respond under appropriate stimuli. We have been studying the effects of IFNs on a variant cell line which fails to produce an antiviral state when treated with either beta or gamma IFN (IFN-β or IFN-γ) (30, 31). As we show here, however, a strong antiviral effect was produced when the cells were exposed to a combination of these two IFNs. Our results suggest that IFN-γ induces the synthesis of a protein which acts synergistically with a signal induced by IFN-β to activate gene expression. This cell line may be ideally suited for dissecting the pathways by which IFNs modulate gene expression, in order to identify the signals and transcription factors involved.

To understand these pathways completely, it will be necessary to account for the effects of the different classes of IFNs on the various genes which they regulate. Three types of IFNs are recognized, according to the nature of the producing cells and the stimulus for production. Thus, IFN-α is produced by virus-infected leukocytes while IFN-β is synthesized by fibroblastoid cells exposed either to viruses or to double-stranded RNA. These IFNs are very similar chemically and genetically and indeed bind to the same cell surface receptors in both human and mouse cells (5, 20). IFN-γ is quite distinct from these type I IFNs since it is produced by a subpopulation of lymphocytes stimulated with mitogens or specific antigens and appears to be involved in immune and inflammatory responses. IFN-γ not only differs from IFN-α and IFN-β in its chemical and genetic properties but also binds to a separate cell surface receptor (1, 3, 5, 20, 37, 41). Although all three species of IFNs induce the expression of a similar set of proteins, there are several differences in the nature of the responses observed (48). Some proteins are induced preferentially by IFN-γ (7, 38), while other proteins are induced by IFN-α and IFN-β but not by IFN-γ (8, 21, 44). Moreover, the relative potency of different IFNs in activating particular responses (e.g., activation of major histocompatibility antigen expression) varies (47). For genes regulated by all three classes of IFNs, the mechanisms by which type I and II species bring about induction may be somewhat different, since protein synthesis inhibitors block induction of some genes by IFN-γ while the response to IFN-α is not affected (11, 21–23). It has also been shown that the simultaneous addition of different IFNs can lead to synergistic effects (10, 13–15, 23, 52), suggesting that different mechanisms of action may be involved.

We have previously characterized a mutant mouse fibroblastoid cell line, Ltk-aprt-, which is refractory to the antiviral effects of IFN-β (30, 31) while still exhibiting other responses, including cell growth inhibition and activation of at least one gene, I-8, to the same level as that seen in sensitive cells (42). By transfecting specific DNA sequences into these cells we have been able to restore the capacity of

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IFN-β to activate antiviral responses (26, 30, 31). The lack of antiviral effects in Ltk-aprt- cells correlates with a failure of IFN-β to induce at least three enzymes with established antiviral properties (2, 8, 31). Several other genes which are usually regulated by IFN are also refractory to induction in this cell line, including both positively (42) and negatively (B. Shan and J. A. Lewis, manuscript in preparation) modulated species. Since several genes are affected it is likely that some step in the signaling pathway between the cell surface receptor and the genome is involved. The fact that the cells are at least partially responsive to IFN-β (42) indicates that functional cell surface receptors are present. As we show here, Ltk-aprt- cells are also resistant to the antiviral effects of murine IFN-γ. Remarkably, however, treatment of these cells with a combination of type I and type II IFNS provides complete protection against viral infection and induces mRNAs in a manner similar to that seen in cells which respond normally to IFNs.

MATERIALS AND METHODS

Cells, cell growth, and IFNs. The origin of the Ltk-aprt- cells and conditions of growth have been described previously (30). Murine IFN-β (5.6×10^7 U/mg of protein) was purchased from Lee Biomolecular Research, San Diego, Calif., and the titer was determined against the National Institutes of Health murine IFN-β standard preparation (Gb02-902-511) on L-929 cells with vesicular stomatitis virus (VSV) as described previously (27). Murine IFN-γ was prepared from supernatants of a Chinese hamster ovary cell line expressing a recombinant murine IFN-γ cDNA under control of the simian virus 40 late promoter (35). This cell line was a generous gift from Alan Morris (University of Warwick, Coventry, England). Partial purification was achieved by chromatography on Cibacron Blue-Agarose and elution with 50% ethylene glycol in 2.0 M NaCl to give a preparation with a specific activity of 5×10^6 U/mg of protein. Titers are reported with respect to the mouse IFN-β standard and were determined by using the same assay described for IFN-β, so the antiviral potencies of our IFN-β and IFN-γ preparations were equivalent. A polyclonal anti-IFN-β serum was purchased from Lee Biomolecular Research. A monoclonal antibody, HB107, specific for murine IFN-γ was prepared from culture supernatants of a rat-mouse hybridoma, HB107 (43).

Assays of antiviral activities. VSV was grown in L-929 cells, and the titer was determined by conventional plaque assay in the same cells (27). Sensitivity to virus infection was assayed by measuring cytopathic effects, using methyl violet staining (27). Briefly, cells were grown to confluency in 24- or 96-well dishes, treated with combinations of IFNs for the times indicated, and then infected with VSV at 10 PFU per cell. After adsorption for 1 h, the virus inoculum was removed and virus growth was allowed to proceed for 24 to 48 h before staining with 0.25% methyl violet in 50% ethanol–0.9% NaCl–2% formaldehyde. The dishes were thoroughly washed in H_2O, and the dye was eluted in 50% ethanol–0.5 M NaCl and quantitated by A_{570}. Assays of virus protein synthesis were performed in 24-well cultures. Cells were treated with IFNs, infected with 10 PFU of VSV per cell, and radiolabeled from 3.5 to 6 h postinfection with 10 μCi of [^{35}S]methionine and [^{35}S]cysteine (^{35}S-Translabel; ICN) per ml in medium lacking methionine. The cells were lysed in 1% Nonidet P-40–0.25% sodium deoxycholate–10 mM Tris hydrochloride (pH 7.5)–15 mM NaCl–1.5 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride–15 U of Trasylol per ml, and after removal of nuclei by centrifugation the extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (27).

Assay of 2,5-oligo(A) synthetase. 2,5-Oligo(A) synthetase assays were performed by a modification of a procedure described previously (31). Extracts were prepared exactly as described (31), but synthesis of ^{3}H-labeled 2,5-oligo(A) was performed in a solution assay. The extracts (25 μl) were incubated in a final volume of 50 μl containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH (pH 7.5)–90 mM KCl–10 mM magnesium acetate–7 mM 2-mercaptoethanol–1 mM [^{3}H]ATP (40 Ci/mol) with or without poly(I · C) (10 μg/ml). Incubation was at 30°C for 60 min, and reactions were terminated by heating to 90°C. Denatured protein was removed by centrifugation, and ^{3}H-labeled 2,5-oligo(A) was determined by binding to DEAE-cellulose and elution with 0.34 M KCl as described previously (31).

Measurement of mRNA levels. Monolayers of Ltk-aprt- cells (10-cm culture dishes) were treated with IFNs and washed with cold phosphate-buffered saline, and the cells were lysed in 4 M guanidinium isothiocyanate–100 mM 2-mercaptoethanol. The extracts were layered over a cushion of 5.7 M CsCl–25 mM sodium acetate and centrifuged at 35,000 rpm in a Beckman SW50.1 rotor for 18 h at 20°C. The pelleted RNA was dissolved in 0.3 M sodium acetate, ethanol precipitated, and analyzed by electrophoresis on 1.3% agarose gels after denaturation in formamide-formaldehyde. The RNA was transferred to Nytran paper (Schleicher & Schuell, Inc., Keene, N.H.) and hybridized with a nick-translated probe as described previously (28).

RESULTS

Ltk-aprt- cells are resistant to the antiviral effects of IFN-γ. We have demonstrated previously that Ltk-aprt- cells fail to establish an antiviral state when treated with up to 2,000 U of IFN-β per ml (30). The parental L-929 cell line is strongly

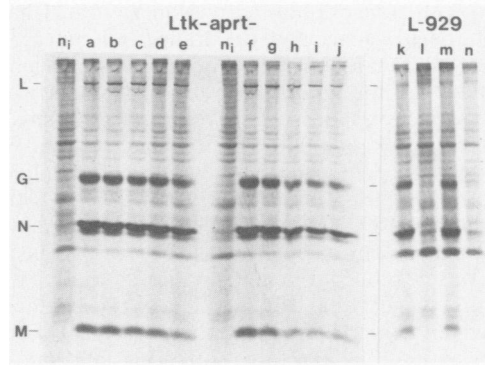

FIG. 1. Ltk-aprt- cells are resistant to the antiviral effects of IFN-γ. Ltk-aprt- cells were treated for 18 h with IFN-β (lanes a to e) or IFN-γ (lanes f to j) at 0 (lanes a and f), 5 (lanes b and g), 25 (lanes c and h), 100 (lanes d and i), and 250 (lanes e and j) U/ml and then infected with VSV (10 PFU per cell). The cells were radiolabeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine between 3.5 and 6 h after infection, and extracts were prepared for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The positions of viral proteins are indicated at the left. Extracts of uninfected cells are shown (n_i). To demonstrate the efficacy of the IFNs, their effects on VSV protein synthesis in L-929 cells are shown: no addition (lane k), 100 U of IFN-β per ml (lane l), 100 U of IFN-β per ml with polyclonal antibody to IFN-β (lane m), and 100 U of IFN-γ per ml (lane n).

TABLE 1. Synergistic effects of IFN-β and IFN-γ in preventing VSV replication[a]

Treatment	VSV yield (PFU/ml)	Fold reduction
None	5.8×10^8	
100 U of IFN-β/ml	5.6×10^8	1
100 U of IFN-γ/ml	1.4×10^8	4
100 U of IFN-β/ml + 100 U of IFN-γ/ml	1.6×10^6	363

[a] Ltk-aprt- cells were treated with IFNs for 18 h and then infected with VSV (10 PFU per cell). After 24 h the monolayers were frozen and thawed in their medium and the yield of VSV was determined by plaque assay on L-929 cells.

protected against VSV and mengo virus infection by 5 to 10 U of IFN-β per ml under the same conditions, with our laboratory endpoint being typically 3 reference units per ml. When Ltk-aprt- cells were treated with up to 250 U of IFN-γ per ml there was only a very slight reduction in the synthesis of VSV proteins (Fig. 1), indicating that these cells are also essentially unable to respond to IFN-γ by producing an antiviral state. In contrast, 100 U of IFN-γ per ml completely abolished synthesis of VSV proteins in the sensitive L-929 cell line. Measurements of virus yield also showed that neither IFN-β nor IFN-γ was able to inhibit virus replication significantly in Ltk-aprt- cells (Table 1). The very slight inhibition (two- to fourfold) of VSV production by IFN-γ (Table 1 and Fig. 1) is discussed below. This failure to activate an appreciable antiviral state in Ltk-aprt- cells was also seen when the cells were challenged with a different virus such as Mengo virus (results not shown). As we have shown previously, IFN-β also fails to activate the expression of several genes in Ltk-aprt- cells, although at least one gene, I-8, is sensitive to induction (42).

Synergistic effects of IFN-β and IFN-γ. Although Ltk-aprt- cells were resistant to the effects of IFN-β and IFN-γ when each was added singly, treatment with a combination of these agents brought about complete protection against the cytopathic effects of VSV (Fig. 2). If cells were treated with 100 U of IFN-β per ml together with as little as 5 U of IFN-γ per ml, partial protection against virus-induced cytopathic effects was observed, while treatment with 100 U of both IFNs per ml afforded complete protection. IFNs added singly to the cells at a concentration of 100 U/ml were totally ineffectual (Fig. 2). A more detailed analysis of the dose requirements for the two IFNs is shown in Fig. 3. When equal concentrations of the two IFNs were serially diluted, 50% of the cells were protected from viral cytopathic effects by approximately 6 U of each per ml (Fig. 3a). Dilution of one of the IFNs in the presence of a constant amount of the other indicated that a combination of 10 U of IFN-β per ml and approximately 5 U of IFN-γ per ml was sufficient to protect 50% of the cells against the cytopathic effects of VSV (Fig. 3b). In the presence of 10 U of IFN-γ per ml significant protection of cells could be achieved with less than 1.0 U of IFN-β per ml (Fig. 3c) even though no protection was afforded by 100 U of either of the IFNs per ml added singly (Fig. 3d). Thus, Ltk-aprt- cells can be protected against viral infection by relatively low concentrations of each of the two

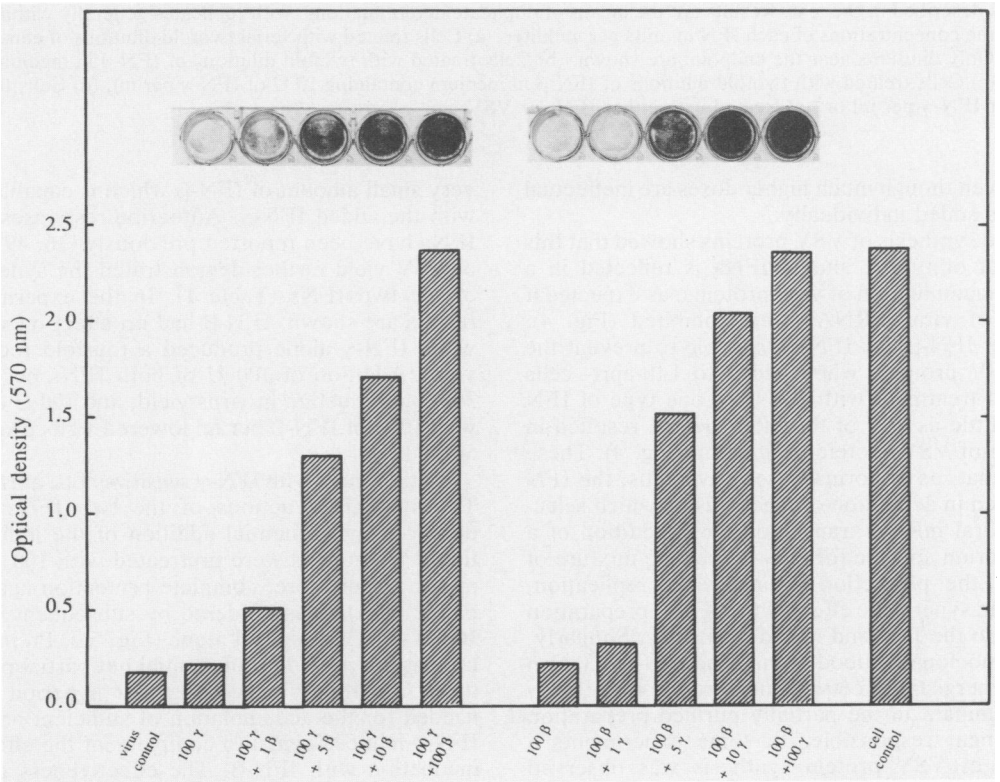

FIG. 2. Synergistic effects of IFN-β and IFN-γ on Ltk-aprt- cells. Ltk-aprt- cells were treated with mixtures of IFN-β and IFN-γ for 18 h as indicated below each bar. Numbers refer to the concentration of each IFN in units per milliliter. Cells were then infected with VSV (10 PFU per cell) and stained 48 h later, and the dye was eluted and quantitated as described in the text. A photograph of the wells is shown at the top of the figure, with each well corresponding to the bar beneath it. The data are from a typical experiment.

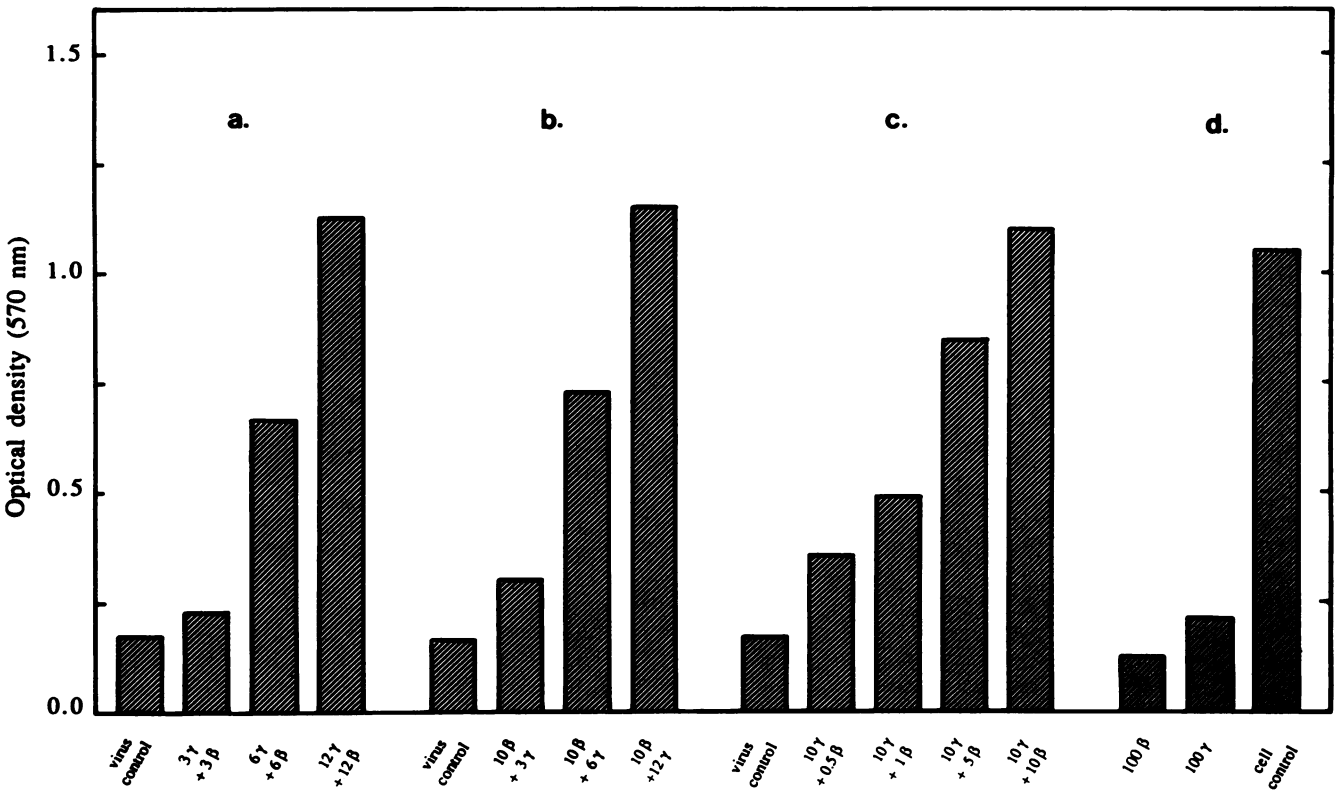

FIG. 3. Synergistic effects of different combinations of IFN-β and IFN-γ. Ltk-aprt- cells cultured in a 96-well dish were treated with mixtures of IFN-β and IFN-γ for 18 h and then infected with VSV (1 PFU per cell). After 48 h the cells were stained, and the dye was eluted and quantitated as described in the text. Results are the means of duplicate determinations, with replicates generally within 7% of the mean. Numbers refer to the concentrations of each IFN in units per milliliter. (a) Cells treated with serial twofold dilutions of equal titers (100 U/ml) of the two IFNs. Only dilutions near the endpoint are shown. (b) Cells treated with twofold dilutions of IFN-γ in medium containing 10 U of IFN-β per ml. (c) Cells treated with twofold dilutions of IFN-β in medium containing 10 U of IFN-γ per ml. (d) Cells treated with 100 U of either IFN-β or IFN-γ per ml or not treated with either IFNs or VSV (cell control).

IFNs together even though much higher doses are ineffectual when the IFN is added individually.

Analysis of the synthesis of VSV proteins showed that this synergistic effect of type I and II IFNs is reflected in a decrease in the accumulation of viral proteins, as expected if the translation of viral mRNAs were inhibited (Fig. 4). Although neither IFN-β nor IFN-γ was able to prevent the synthesis of VSV proteins when added to Ltk-aprt- cells alone, combined treatment with 100 U of one type of IFN per ml with as little as 5 U of the other per ml resulted in strong inhibition of VSV protein production (Fig. 4). These results suggest that, as in normally sensitive cells, the IFN treatment resulted in activation of mechanisms which selectively prevent viral mRNA translation (26). Addition of a polyclonal antiserum specific for IFN-β with the mixture of IFNs abolished the protection against virus replication, indicating that the synergistic effect of the IFN-β preparation was indeed due to the IFN and not to impurities. Similarly, addition of a monoclonal antibody to murine IFN-γ (43) also abolished the synergistic effects, demonstrating that IFN-γ and not a contaminant in the partially purified preparation was the component responsible. In some experiments a slight reduction of VSV protein synthesis was observed when cells were treated with IFN-γ alone (Fig. 1 and Table 1), but this effect could be eliminated if polyclonal antibodies specific for IFN-β were included in the medium (Fig. 4). This suggests that the Ltk-aprt- cells constitutively produce a very small amount of IFN-β which is capable of synergizing with the added IFN-γ. Autocrine responses to endogenous IFNs have been reported previously (16, 49). Measurement of VSV yield further demonstrated the synergistic capacity of the two IFNs (Table 1). In the experiment for which results are shown, IFN-β had no effect on virus replication while IFN-γ alone produced a fourfold reduction in VSV yield. Addition of 100 U of both IFNs per ml produced a 360-fold reduction in virus yield, and 100 U of IFN-γ per ml with 10 U of IFN-β per ml lowered virus production at least 50-fold.

Pretreatment with IFN-γ sensitizes Ltk-aprt- cells to IFN-β. The synergistic actions of the two IFNs could also be observed by sequential addition of the individual preparations. When cells were pretreated with 100 U of IFN-γ per ml for 2 h or more, complete protection against viral cytopathic effects was rendered by subsequent incubation with 100 U of IFN-β per ml alone (Fig. 5a). Preincubation for as little as 1 h provided substantial but partial protection under these conditions, suggesting that a period of 1 to 2 h is needed for the accumulation of sufficient amounts of some IFN-γ-induced signal to complement the effects of separate incubation with IFN-β. The effectiveness of the pretreatment varied somewhat from one experiment to another, and in some cases only partial protection was achieved by preincubating with 100 U of IFN-γ per ml for 4 h (see below). With 10 U of IFN-γ per ml longer periods of preincubation

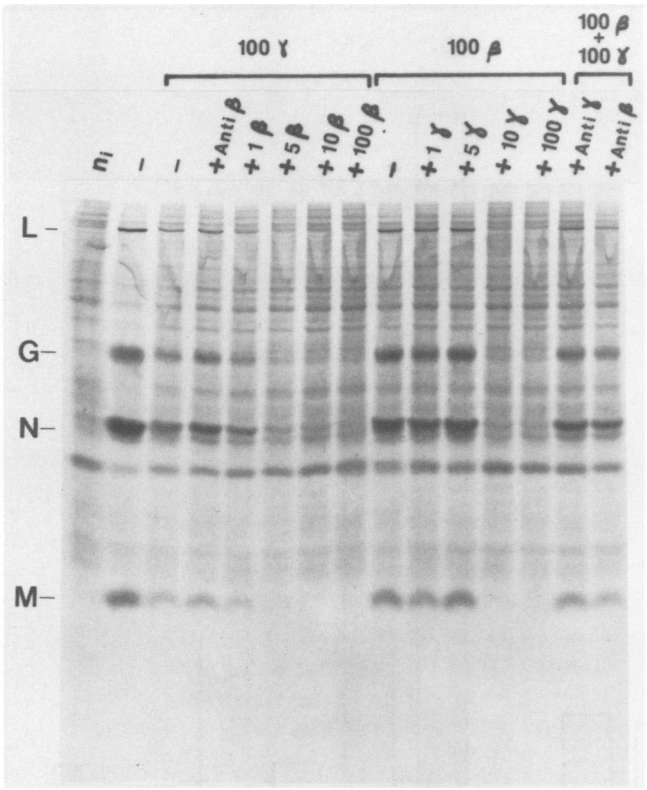

FIG. 4. Synergistic effect of IFN-β and IFN-γ on synthesis of VSV proteins in Ltk-aprt- cells. Cells were treated with mixtures of IFNs and antibodies as indicated for 18 h. After infection with VSV (10 PFU per cell) the cells were radiolabeled and analyzed as described in the text. The profile of proteins synthesized by uninfected cells (n_i) is shown for comparison, and the positions of VSV proteins are indicated at the left. Cells were either untreated (−) or treated as indicated with 100 U of IFN-γ per ml and 0, 1, 5, 10, or 100 U of IFN-β per ml or with 100 U of IFN-β per ml and 0, 1, 5, 10, or 100 U of IFN-γ per ml. Cells were also treated with 100 U of both IFN-β and IFN-γ per ml together with monoclonal antibodies to IFN-γ (Anti-γ) or polyclonal antibody to IFN-β (Anti-β).

were necessary to achieve the same level of protection (Fig. 5a) and complete inhibition of viral cytopathic effects was only seen after a 24-h exposure. After pretreatment with IFN-γ, inclusion of monoclonal antibodies specific for IFN-γ in the subsequent incubation with IFN-β did not significantly affect the development of an antiviral state (Fig. 5b), indicating that the effects were due to sequential activities and not to carry-over of the IFN-γ into the second incubation. As expected, the addition of monoclonal antibodies to IFN-γ during the pretreatment did block development of an antiviral state (Fig. 5b).

IFN-γ induces the synthesis of a protein that acts synergistically with IFN-β. If the pretreatment with IFN-γ was carried out in the presence of cycloheximide for 4 h, the synergistic effect was blocked (Fig. 5b), suggesting that IFN-γ induces the synthesis of a protein (or several proteins) which is required for the synergistic interaction with responses produced by IFN-β in the second incubation. In this particular experiment the 4-h pretreatment provided only partial protection against VSV, and this was completely abolished by cycloheximide. In other instances, more complete protection was afforded by the short pretreatment (Fig. 5a), but cycloheximide only partially inhibited production of the ensuing antiviral state (results not shown). This finding is consistent with the induced synthesis and accumulation of a specific mRNA during the incubation with IFN-γ and cycloheximide and subsequent translation of the mRNA into the active protein during the second incubation with IFN-β after removal of the cycloheximide. The ability of IFN-γ to elicit synthesis of a protein when added to Ltk-aprt- cells alone indicates the presence of functional receptors for IFN-γ on the surface of these cells, as shown previously for IFN-β (42). Although IFN-γ is unable to activate an antiviral state, the Ltk-aprt- cells express a partial response to it involving the production of some factor or signal, either a protein or a product of a newly synthesized enzyme, which permits synergistic interaction with signals produced in response to IFN-β.

IFN-γ elicits the production of a stable factor which sensitizes Ltk-aprt- cells to IFN-β. To obtain further information on the nature of the factor produced by pretreatment with IFN-γ, we performed chase experiments (Fig. 6a). A strong antiviral effect could be achieved by treatment with IFN-γ for 20 h followed by withdrawal of the stimulus for 8 h before the addition of IFN-β. The level of protection achieved against virus infection was only slightly lower than in cells treated with IFN-γ and then treated immediately with IFN-β. Withdrawal for shorter times, such as 6, 4, and 2 h, had no effect on the antiviral state (results not shown). Remarkably, pretreatment with IFN-γ for 4 h followed by withdrawal for 24 h and then incubation with IFN-β gave the same level of antiviral activity as did a 4-h pretreatment followed immediately by the addition of IFN-β (Fig. 6a). This indicates that the factor produced in response to IFN-γ is relatively stable. Most likely this reflects the synthesis of a long-lived protein, although we cannot exclude the synthesis of a stable mRNA.

IFN-β acts synergistically with IFN-γ by producing a transient signal. In contrast to the results discussed above, pretreatment with IFN-β for 24 h or for shorter times did not permit the establishment of an antiviral state when cells were subsequently incubated with IFN-γ (Fig. 6b). The distinct behaviour of IFN-γ and IFN-β in this type of assay suggests differences in the nature of the signals induced by these two agents and indicates that IFN-β produces a transient signal which can interact with a stable protein induced by IFN-γ. Since double-stranded RNA has been reported to act as a signal for induction of genes regulated by IFNs (46, 54), we tested its ability to act synergistically with IFN-β and IFN-γ. Partial protection against viral cytopathic effects could also be obtained by treating Ltk-aprt- cells with poly(I · C) in the presence of DEAE-dextran for 1 h followed by incubation with IFN-γ but not IFN-β (Fig. 6b). Since this effect of poly(I · C) could be prevented by including polyclonal antibodies against IFN-β (Fig. 6b), the antiviral response was almost certainly due to induction of IFN-β production and not to a direct effect of double-stranded RNA. We have previously determined that the Ltk-aprt- cells used in our laboratory (30) secrete IFN in response to poly(I · C) treatment (J. A. Lewis, unpublished observations).

Induction of gene expression by combined treatment with IFN-β and IFN-γ The level of 2,5-oligo(A) synthetase activity in extracts of Ltk-aprt- cells treated with the two types of IFNs is shown in Table 2. Neither IFN-β nor IFN-γ was capable of eliciting an increase in expression of this enzyme when added to the cells individually. The addition of 100 U of both types of IFN per ml together, however, led to a significant increase in activity. A combination of 100 U of one IFN per ml with 10 U of the other per ml was sufficient to produce a lower level of induction. The stimulation of

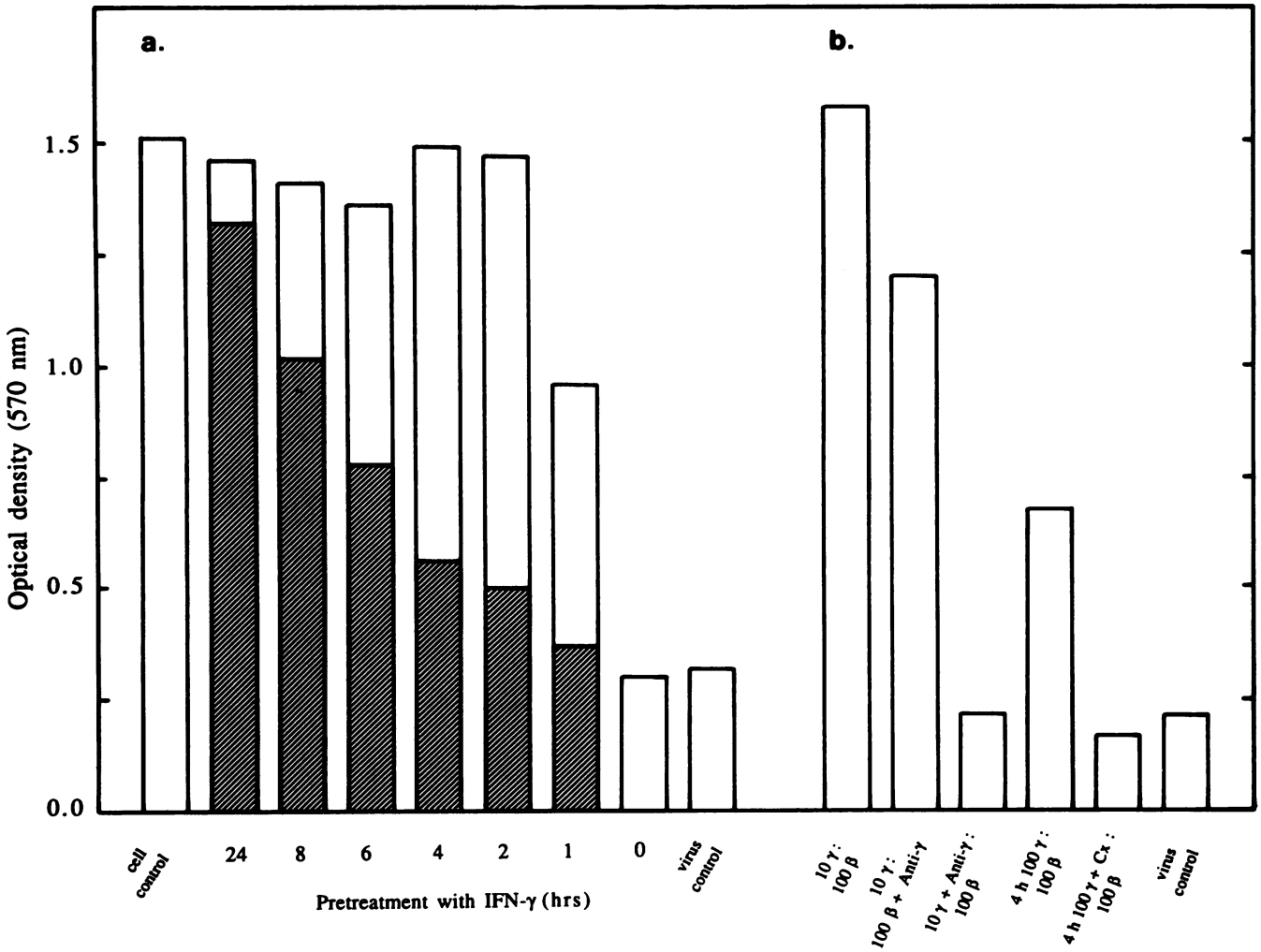

FIG. 5. Pretreatment of Ltk-aprt- cells with IFN-γ permits establishment of an antiviral state on subsequent exposure to IFN-β. (a) Ltk-aprt- cells were preincubated with 100 U (open bars) or 10 U (hatched bars) of IFN-γ per ml for the times indicated. The IFN was removed and replaced by medium containing 100 U of IFN-β per ml. After 18 h the cells were infected with VSV (10 PFU per cell), and 48 h later they were stained and the dye was quantitated. Each bar represents the mean of duplicate determinations. (b) Treatment of Ltk-aprt- cells was as follows: pretreatment for 24 h with 10 U of IFN-γ per ml followed by 100 U of IFN-β per ml without (10 γ:100 β) or with (10 γ:100 β + Anti γ) monoclonal antibodies to IFN-γ; pretreatment for 24 h with 10 U of IFN-γ per ml with monoclonal antibodies to IFN-γ followed by 100 U of IFN-β per ml (10 γ + Anti-γ:100 β); pretreatment for 4 h with 100 U of IFN-γ per ml in the absence (4 h 100 γ:100 β) or presence (4 h 100 γ + Cx:100 β) of 35 μg of cycloheximide per ml followed by 100 U of IFN-β per ml; or no pretreatment (virus control). Incubations with IFN-β were for 18 h, and cells were then infected with VSV and cytopathic effects were determined as described in the text.

2,5-oligo(A) synthetase expression, however, was weak when compared with the levels seen in L-929 cells treated with either IFN alone. Combined treatment of L-929 cells also gave a synergistic effect on the level of enzyme activity (Table 2).

Northern (RNA) blot analysis of the effects of IFN on gene expression is shown in Fig. 7. The mRNA for 2,5-oligo(A) synthetase was undetectable in untreated Ltk-aprt- and L-929 cells. Only an extremely weak induction of the 1.8-kilobase mRNA could be detected in Ltk-aprt- cells treated with either IFN-β, as reported previously (28, 42), or IFN-γ. However, a significant level of induction was seen in Ltk-aprt- cells treated simultaneously with both IFNs, although the level of accumulation of 2,5-oligo(A) synthetase mRNA was lower than that observed in L-929 cells treated with IFN-β alone. This is in accord with the observations presented above for the level of enzyme activity. Blots probed for β-actin showed no difference in signal intensity, indicating that the same amount of RNA was loaded in different lanes.

DISCUSSION

The mechanisms by which interaction of IFNs with cell surface receptors leads to modulation of gene expression are poorly understood. After binding to the high-affinity receptors, IFNs are internalized (50, 51, 53), but definitive proof as to whether uptake of IFN into the cell is necessary for further events has been elusive (2, 6, 17, 51, 53). Microinjection of IFN-α and IFN-β into cells does not produce antiviral effects (18, 19), but several reports have suggested that intracellular IFN-γ may be capable of activating gene expression and an antiviral state (12, 40). Several lines of

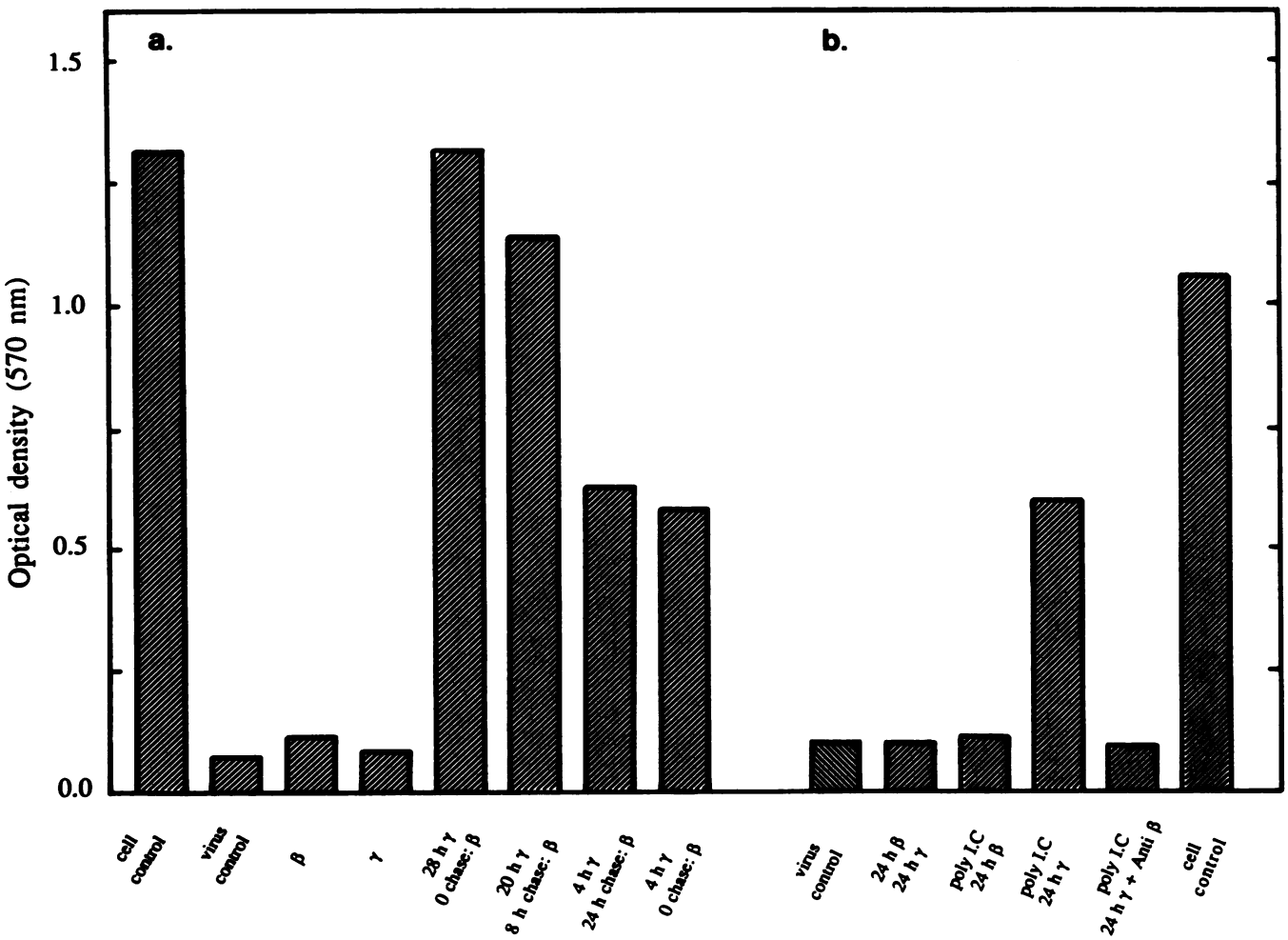

FIG. 6. Stability of an IFN-γ-induced factor which acts synergistically with IFN-β and effects of pretreatment with IFN-β or poly(I · C). (a) Ltk-aprt- cells were treated as follows: pretreatment for 28 h with IFN-γ followed by IFN-β (28 h γ:0 chase:β); pretreatment for 20 h with IFN-γ followed by an 8-h chase in growth medium and then an 18-h incubation with IFN-β (20 h γ, 8 h chase:β); pretreatment for 4 h with IFN-γ followed by a 24-h chase with growth medium and then an 18-h incubation with IFN-β (4 h γ, 24 h chase:β); pretreatment for 4 h with IFN-γ followed immediately by incubation for 18 h with IFN-β (4 h γ, 0 chase:β). Cells were infected with VSV, and cytopathic effects were assayed as described in the text. The cell control and virus control and treatment with either IFN-β or IFN-γ are shown. In all cases the IFNs were used at a concentration of 100 U/ml. (b) Ltk-aprt- cells were treated with 100 U of IFN-β per ml for 24 h followed by 100 U of IFN-γ per ml (24 h β, 24 h γ) or with poly(I · C) (50 μg/ml in 50 μg of DEAE-dextran per ml) for 1 h followed by a 24-h incubation with 100 U of IFN-β (poly I · C, 24 h β) or IFN-γ (poly I · C, 24 h γ) per ml or 100 U of IFN-γ per ml with polyclonal antibodies to IFN-β (poly I · C, 24 h γ + Anti β). Cells were then infected with VSV, and cytopathic effects were assayed as described in the text. Virus and cell controls are also shown.

evidence suggest that IFN-α and IFN-β operate through somewhat different pathways than IFN-γ: the receptors of type I and type II IFNs are separate entities (1, 3, 5, 20, 37, 41), the kinetics of intracellular degradation of IFN-γ in mouse cells are much slower than those for type I IFNs (50), and internalized IFN-γ has been reported to be transported to the nucleus (32). Differences in the sensitivity of various genes to induction by type I and II IFNs and in the ability of cycloheximide to block gene expression suggest that distinct mechanisms are involved in gene activation (11, 21, 22).

It is generally supposed that binding to the receptors is followed by transmission of a signal or signals which in some way modulate gene expression, perhaps by causing alterations in transcriptional activation factors. Recently, protein factors which bind to upstream regulatory elements have been detected in extracts of cells treated with IFNs (9, 24, 36, 39). The IFN-induced appearance of such transcription factors must result from activation of preexisting proteins by posttranslational modification (45) possibly accompanied by de novo synthesis of additional factors (11, 21–23). The differential effects of cycloheximide on activation of some genes suggest that the signals generated by occupancy of type I and type II IFN receptors may not be identical, consistent with reports of synergistic effects of the different types of IFNs on cell growth inhibition and antiviral activity (10, 13–15, 52) and on the induction of 2,5-oligo(A) synthetase (13, 23). Also, more than one pathway is likely to be involved in the activation of different genes by type I IFNs (21, 22, 33, 34, 42), and therefore different sets of signals may exist, with a degree of functional overlap between those generated by type I and type II IFNs. Analysis of variant cell lines and the effects of cycloheximide have shown that some genes can be activated without a need for protein synthesis,

TABLE 2. Synergistic effects of IFN-β and IFN-γ on 2,5-oligo(A) synthetase levels[a]

Cells and treatment	2,5-Oligo(A) synthetase activity (cpm/mg of protein per 60 min)	
	−Poly(I · C)	+Poly(I · C)
Ltk-aprt-		
None	224	322
100 U of IFN-β/ml	164	337
100 U of IFN-γ/ml	212	439
100 U of IFN-β/ml + 100 U of IFN-γ/ml	369	3,590
100 U of IFN-β/ml + 10 U of IFN-γ/ml	398	1,760
100 U of IFN-γ/ml + 10 U of IFN-β/ml	466	1,088
L-929		
None	665	385
100 U of IFN-β/ml	450	6,796
100 U of IFN-γ/ml	433	1,781
100 U of IFN-β/ml + 100 U of IFN-γ/ml	554	15,985

[a] Ltk-aprt- and L-929 cells were treated with IFNs at the concentrations indicated for 18 h and harvested for enzyme assay as described in the text. Each extract was incubated in the absence or presence of 10 μg of poly(I · C) per ml. Levels of radioactivity determined in control reaction mixtures lacking extract were approximately 400 cpm with or without poly(I · C).

i.e., a primary response (11, 21, 22). For other genes, activation may require the production of a protein(s) induced by IFNs, i.e., a secondary response. Sen and colleagues (22, 23, 46) have shown that in some cells the activation of gene 561 expression by IFN-α depends on a combination of multiple signals, including a protein that is synthesized in response to IFN-α. This protein can also be induced by IFN-γ, which alone fails to activate expression of gene 516. Subsequent treatment with IFN-α (22) or other agents such as double-stranded RNA and growth factors (46) causes the activation of gene expression even in the presence of cycloheximide. Thus, a protein induced by IFN-α and IFN-γ interacts with various signals to promote expression of gene 561.

Our results with Ltk-aprt- cells have established that this cell line is partially sensitive to IFNs. Although it lacks the capacity to establish an antiviral state and to induce expression of a particular set of genes (e.g., for 2,5-oligo(A) synthetase, eucaryotic initiation factor 2 kinase, and major histocompatibility complex antigens) when treated with IFN-β and IFN-γ, it still responds to IFN-β by a reduction in the rate of cell growth and by normal induction of gene I-8 (42). These results cannot be explained on the basis of defective cell surface receptors, and the inability of Ltk-aprt- cells to respond to added cadmium by increased expression of metallothionein (29), the gene for which is also regulated by IFN, lends further support to the idea that the defect is not at the level of receptor functioning. The results presented here support this hypothesis. The dramatic effect of combined treatment with IFN-β and IFN-γ shows that both sets of receptors are indeed functional. In an earlier report (42), we showed that IFN-β failed to activate transcription of several genes in Ltk-aprt- cells, and therefore the defective responses of this line are due either to alterations in upstream regulatory elements or to a failure to activate particular transcriptional activation factors. Since many genes (at least eight) are affected, the latter explanation seems most likely. The observations presented here suggest that IFN-γ can overcome this failure to activate transcription factor(s) in Ltk-aprt- cells treated with IFN-β. These cells therefore provide an excellent system for studying the mechanism by which signal transduction is coupled to gene activation.

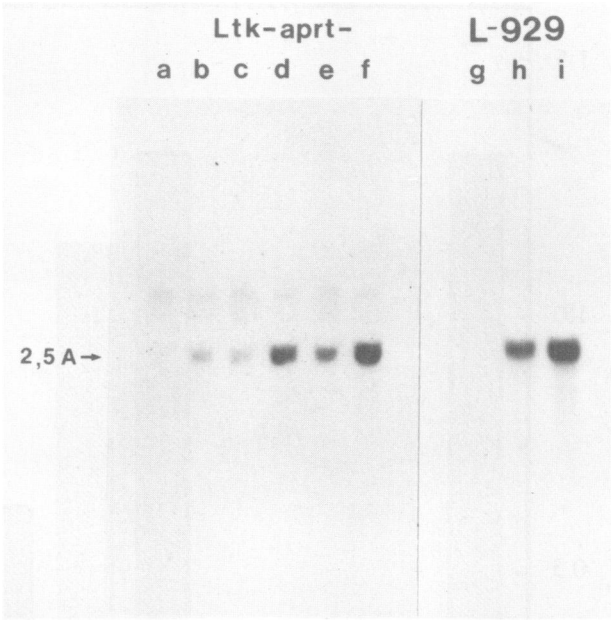

FIG. 7. IFN-β and IFN-γ act synergistically to induce expression of the 2,5-oligo(A) synthetase gene. RNA was extracted from cells treated with combinations of IFNs as described in the text. Northern blots were hybridized with a probe specific for 2,5-oligo(A) synthetase (2,5 A). Ltk-aprt- cells were untreated (lane a) or treated for 16 h with 100 U of IFN-β per ml (lane b), 100 U of IFN-γ per ml (lane c), 100 U of IFN-β per ml plus 10 U of IFN-γ per ml (lane d), 100 U of IFN-γ per ml plus 10 U of IFN-β per ml (lane e), or 100 U of both IFN-β and IFN-γ per ml (lane f). As a control L-929 cells were treated with IFN-β for 0 (lane g), 8 (lane h), or 16 (lane i) h.

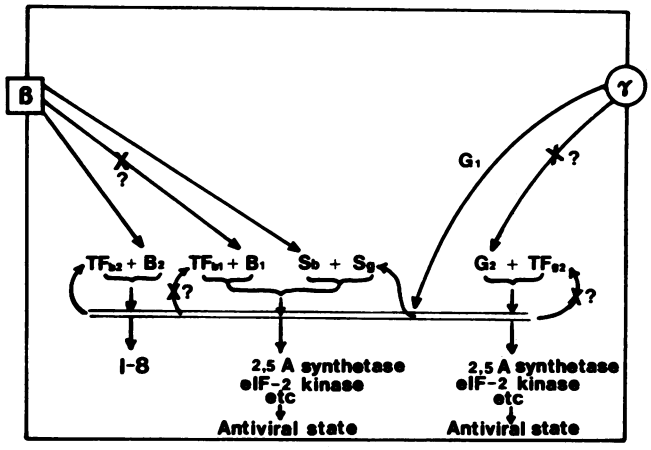

FIG. 8. Schematic model for activation of gene expression of IFN-β and IFN-γ. Receptors for IFN-β (square) and IFN-γ (circle) in the cell membrane are shown; upon activation by binding of the IFNs, they give rise to signals (denoted by the arrows) which interact with transcription activation factors (TFs) causing alterations in gene expression. Potential blocks in these pathways are shown by X's. Although signals are shown as single lines, multiple steps may be involved in fulfilling any path. eIF-2, Eucaryotic initiation factor 2.

To account for the insensitivity of Ltk-aprt- cells to IFNs, we propose that a defect in signal processing exists for both type I and II IFNs, resulting in an inability to effect certain of the normal responses, although some signaling pathways are apparently intact and thus able to activate expression of cytostatic responses and induction of gene I-8 (42). A schematic model is shown in Fig. 8 that has some similarities to one proposed by Kusari and Sen (22) based on their studies of gene 561 induction in HeLa cells. Both models propose that IFNs activate multiple signals and that IFN-γ exerts its effect in part by inducing the synthesis of a protein. We suggest that type I and II IFNs activate separate but functionally overlapping sets of signals and the absence of a required component in one set may be complemented by the other when both types of IFNs are present. Activation of a particular gene or group of genes by a single type of IFN requires the interaction of one or more signals with a specific transcription factor. In Ltk-aprt- cells at least one of these components, a signal or a transcription factor, is absent and hence expression of the dependent genes is not possible. The complementary IFN has a similarly defective but distinct set of signals, one of which can replace the defective component.

Our results indicate that IFN-γ produces at least one signal (G1 in Fig. 8) which induces the synthesis of a protein (Sg, for IFN-γ-induced synergistic factor). This protein is relatively stable, as seen in the pretreatment and withdrawal experiments, and its production is blocked by cycloheximide. We propose that protein Sg is able to interact with signal Sb produced by the IFN-β receptor and thus activate expression of the 2,5-oligo(A) synthetase gene and other genes, with the production of an antiviral state. Activation of an antiviral state by IFN-γ normally depends on the production of signal G2, which activates transcription factor TFg2, but one of these is defective in Ltk-aprt- cells. Induction of 2,5-oligo(A) synthetase and major histocompatibility complex gene expression by IFN-γ has been shown to be sensitive to cycloheximide in other cells (4, 11), in contrast to induction by IFN-α. This could reflect a need to synthesize TFg2, which is activated by a second signal (G2) from the IFN-γ receptor. If Ltk-aprt- cells fail to generate G2, it is possible that Sg and TFg2 are identical and thus Sg may be a transcription factor analogous to TFb1.

The IFN-β receptor in Ltk-aprt- cells activates transcription of gene I-8 through signal B2, which does not need complementation. Induction of an antiviral state by IFN-β requires activation of a constitutively expressed transcription factor, TFb1, by signal B1. Signals B1, B2, and Sb may be identical if Ltk-aprt- cells fail to synthesize TFb1. In this case a transient signal (B1 = B2 = Sb) would be generated but could only activate those genes regulated by TFb2 and thus an antiviral state would not be produced. In the presence of IFN-γ, however, Sg is produced and this can be activated by Sb, leading to expression of gene products with antiviral capacities. If Sg is the same protein as TFg2, Sb would effectively replace signal G2.

Whether the signals which are defective in Ltk-aprt- cells are intermediates in the pathway or are factors which interact directly with gene regulatory elements remains to be established, and the Ltk-aprt- cells provide an excellent system for studying the nature of these factors. We are currently attempting to identify Sg and determine whether factors capable of binding to upstream regulatory elements are induced in Ltk-aprt- cells after treatment with various combinations of IFNs.

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