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Studies on the Role of the 2′-5′-Oligoadenylate Synthetase-RNase L Pathway in Beta Interferon-Mediated Inhibition of Encephalomyocarditis Virus Replication

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Interferons inhibit the replication of vesicular stomatitis virus (VSV), but not of encephalomyocarditis virus (EMCV), in mouse JLSV-11 cells. We report the isolation of clonal derivatives from this cell line in which the replication of both viruses is impaired by interferons. These clones were selected from the parental line by virtue of their rescue by interferon treatment from the cytopathic effects of EMCV infection. In one such clone, RK8, the replication of VSV and EMCV and the production of resident murine leukemia virus were inhibited by interferon. On the other hand, in clone RK6, which was isolated without any selection, the replication of VSV, but not of EMCV, was impaired by interferons. The levels of 2′-5′-oligoadenylate synthetase mRNA and enzyme activity were similarly elevated upon interferon treatment in the two clones. However, the level of RNase L, as determined by binding and cross-linking of a radiolabeled 2′-5′-oligoadenylate derivative, was much lower in RK6 cells than in RK8 cells. In accord with this observation, the introduction of 2′-5′-oligoadenylates into cells inhibited protein synthesis much less strongly in RK6 cells than in RK8 cells. These results are consistent with the notion that the 2′-5′-oligoadenylate-dependent RNase L may be a mediator of the inhibition of EMCV replication by interferons.

Among the many biological activities of interferons (IFNs) are their antiviral actions (2, 20, 21, 27, 31). Although these actions have been studied extensively, the biochemical mechanisms by which IFNs inhibit the replication of particular viruses remain unclear. The products of various IFN-inducible genes (28) clearly take part in these processes, but their exact roles remain to be elucidated. Only the murine IFN-inducible gene Mx has been proven to be a mediator of IFN action against influenza virus (34), and a human 2′-5′-oligoadenylate [2-5(A)] synthetase isoenzyme (of 40 kilodaltons [kDa]) has been shown to be a mediator of IFN action against mengovirus. [This isoenzyme is one of four 2-5(A) synthetase isoenzymes identified in human cells. The isoenzymes are of 40, 46, 67, and 100 kDa and differ in intracellular location (6).]

The inhibition of the replication of different viruses by IFNs is mediated by different biochemical pathways. The most convincing evidence in support of this diversity of antiviral actions comes from the studies with cell lines which are partially responsive to IFNs (1, 3, 7, 8, 11, 13, 16, 17, 22, 26, 32, 35). In these lines, IFNs inhibit the replication of only some of the viruses whose replication they inhibit in cells fully responsive to IFNs. Such partially responsive lines have been identified by screening cell lines for antiviral responses to IFNs. In this communication we report the successful use of a selection procedure for the isolation from a cell line of clonal derivatives which differ from the parental line of their antiviral responses.

The effects of IFNs on encephalomyocarditis virus (EMCV) replication in many cell lines have been extensively investigated (2, 20). EMCV replication and protein synthesis are strongly inhibited in most IFN-treated cells. As noted above, there is experimental proof for a possible role of a 2-5(A) synthetase in the inhibition of the replication by IFN of EMCV and of mengovirus, a related picornavirus: the replication of both viruses is inhibited (even without IFN treatment) in cells expressing high levels of 2-5(A) synthetase constitutively. Such cells were obtained by transfection with an expression vector containing a 2-5(A) synthetase isoenzyme cDNA under the control of a constitutive promoter (6).

At present, the only known function of the products of 2-5(A) synthetase [i.e., 2-5(A)] is the activation of latent RNase L, the second enzyme of the 2-5(A) synthetase-RNase L pathway (21). There are data indicating that one or both of the enzymes of this pathway are defective in various IFN-treated cells in which EMCV replication in not impaired (8, 13, 29, 32, 33). Moreover, it has been shown that the replicative-intermediate form of EMCV RNA can activate 2-5(A) synthetase (24), that 2-5(A) is accumulated in some IFN-treated, EMCV-infected cells (14), and that rRNA cleavage (thought to be catalyzed by activated RNase L) occurs in some IFN-treated EMCV-infected cells (37).

In this report we provide further data in line with the involvement of RNase L in the anti-EMCV action of IFN. The data are based on experiments with several novel cloned cell lines which differ in the pattern of antiviral response to IFN.

MATERIALS AND METHODS

We have described in previous publications the source of IFN (3); the procedure for culturing JLSV-11 cells (3); methods for the EMCV and vesicular stomatitis virus (VSV) yield reduction assay (11, 32); methods for measuring retrovirus production (11, 32); methods for the 2-5(A) synthetase assay (32); methods for isolating total cytoplasmic RNA and Northern (RNA) analyses (18); characteristics of the 2-5(A) synthetase cDNA probe used (19); the method for synthe-

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sizing (2'-5')pppApApApA[^{32}P]pCp (9), which was purified by high-pressure liquid chromatography (12); methods for binding and cross-linking (2'-5')ppp(A)$_4$[^{32}P]pCp to proteins in cell extracts (9, 10); and the method for introducing 2-5(A) into cells (33). The 2-5(A) trimer 5'-triphosphate was purchased from Pharmacia, Inc., Piscataway, N.J. Rabbit antibody to EMCV and mouse IFN-β (specific activity, 2×10^8 U/mg of protein) were purchased from Lee Biomolecular Research, San Diego, Calif.

Selection procedure for clones sensitive to anti-EMCV action of IFN. JLSV-11 cells were plated at a density of approximately 10^6 cells per 100-mm-diameter dish. The cells were treated with 100 U of IFN per ml for 18 h and then infected with EMCV at a multiplicity of infection of 0.1. At 10 h postinfection, the medium was removed, and cultures were washed with fresh medium and refed with medium containing anti-EMCV antibody (1:100). Medium with antibody was changed every third day to remove the dead cells. After 1 week several cell colonies started growing. They were trypsinized, pooled, and cultured in the presence of anti-EMCV antibody for an additional 3 weeks. Once again, most of the cells died, and only eight colonies grew; they were cultured in the presence of antibody for further 2 weeks. Antibody was removed from the medium, and cells were maintained for 2 weeks and then cloned by being plated at very low density. Well-separated colonies were trypsinized in cloning cylinders and propagated further.

Analysis of EMCV-specific protein synthesis. Subconfluent monolayers of cells were treated with IFN for 16 h when required. Viral protein synthesis was measured by pulse-labeling cells for 0.5 h at desired times postinfection with methionine-free minimal essential medium supplemented with 100 μCi of [^{35}S]methionine per ml. After being radiolabeled, the monolayers were washed three times with a cold buffer containing 35 mM Tris hydrochloride (pH 7.5) and 146 mM NaCl. Cells were lysed by the addition of 0.2 ml of lysis buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$, 0.2% Triton X-100) to the dishes. They were kept on ice for 3 to 5 min, scraped, and centrifuged for 10 min at 1,500 × *g* to remove the nuclei. The supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% acrylamide).

RESULTS

Isolation of variant clones. We reported earlier that IFN has differential antiviral effects in the mouse JLSV-11 cell line (32). VSV replication is strongly inhibited by IFN, whereas EMCV replication is only marginally affected, and the production of retroviruses is impaired to an intermediate degree. In this communication we report the isolation of clonal derivatives from this cell line in which the antiviral effects of IFN differ.

We isolated many clones by plating the cells at a low density and picking colonies as they grew. Each colony was grown individually, and the effects of IFN on the replication of VSV and EMCV in these colonies were tested. The colonies of interest were subcloned and retested. In the experiments described in this report we have used two clones isolated by these methods. In clone RK402 the replication of neither virus was inhibited by IFN, whereas in clone RK6 the replication of VSV was sensitive to IFN but that of EMCV was totally insensitive.

The third type of variant used in our studies is represented by the clone RK8. In this clone the replication of both VSV and EMCV was inhibited by IFN. We did not obtain any clone of this class by dilution cloning, presumably because cells with this phenotype were very rare in the original population. Adequate selection procedures were necessary for isolating these clones.

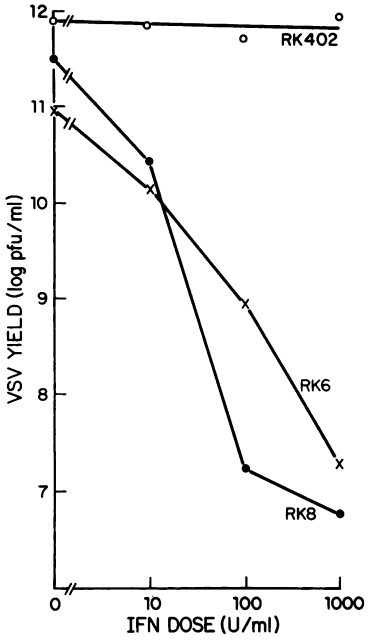

FIG. 1. IFN dose response of VSV replication in RK8, RK6, and RK402 cell lines. Cells were treated with increasing doses of IFN for 16 h and subsequently infected with VSV at a multiplicity of infection of 10. Virus yields were determined by a plaque assay on L929 cells.

For this purpose JLSV-11 cells were treated with IFN and infected with EMCV at a low multiplicity of infection. At 10 h later the medium was removed and replaced with medium containing anti-EMCV antibody. Although most of the cells died, several cell colonies were visible after 1 week of culturing. They were trypsinized, pooled, and cultured for another 3 weeks in the presence of anti-EMCV antibody. Again, only eight colonies of cells grew; they were pooled and cultured in the presence of antibody for another 2 weeks. At this time antibody was removed for 2 weeks and the cells were cloned by being plated at low density. Colonies were picked and expanded, and their antiviral responses were tested. The clone RK8 was chosen for further studies because VSV and EMCV replication in this clone was very sensitive to IFN.

Antiviral effects of IFN. A detailed investigation of IFN action was carried out with the three representative clones, RK8, RK6 and RK402, at IFN concentrations up to 1,000 U/ml. VSV replication was inhibited by IFN, in a dose-dependent manner, in both RK8 and RK6 clones, but not in RK402 (Fig. 1). In contrast, EMCV replication was inhibited by IFN only in clone RK8 (Fig. 2). Production of the resident retrovirus, Moloney murine leukemia virus, was also inhibited strongly by IFN in RK8 but not in the other two clones (Fig. 3). These results clearly support our earlier conclusions that anti-VSV, anti-EMCV, and anti-retrovirus effects of IFN can be dissociated in suitable cell lines.

IFN inhibits EMCV protein synthesis in RK8 cells but not in RK6 cells. To explore the mechanism of anti-EMCV action of IFN in RK8 cells, we examined the effects of IFN on viral protein synthesis in the two lines RK8 and RK6 (Fig. 4).

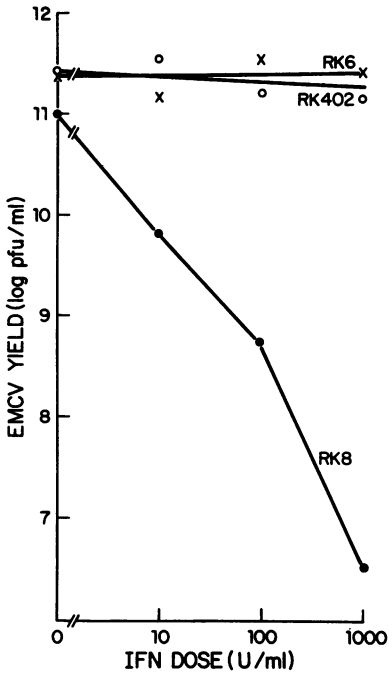

FIG. 2. IFN dose response of EMCV replication in RK8, RK6, and RK402 cell lines. Cells were treated with increasing doses of IFN for 16 h and subsequently infected with EMCV at a multiplicity of infection of 10. Virus yields were determined by a plaque assay on L929 cells.

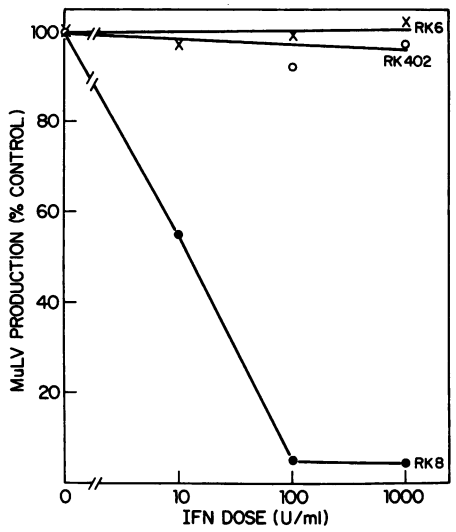

FIG. 3. IFN dose response of murine leukemia virus (MuLV) production by RK8, RK6, and RK402 cell lines. Nearly confluent cultures in 100-mm plates were treated with the indicated doses of IFN for 18 h, and the medium was changed and then collected after another 6 h. Murine leukemia virus virions present in the culture medium were pelleted, suspended, and assayed for associated reverse transcriptase activity as described earlier (11, 32). The amounts of virus produced by different cell lines were different. The ratio of virus production by control RK8, RK402, and RK6 cells was 7:94:243. For each cell line the amount produced by the control culture was taken as 100%.

IFN-treated and control cells were infected with EMCV, and proteins were pulse-labeled metabolically at various times after infection. In both cell lines, with progressive infection, synthesis of several virus-specific proteins was apparent. All these proteins are presumably the cleavage products of the viral polyprotein translated from the viral mRNA. Concomitant with the enhanced synthesis of the viral proteins, synthesis of many cellular proteins was severely inhibited in virus-infected cells. IFN treatment had different effects on viral protein synthesis in RK8 and RK6 cells. In IFN-treated RK8 cells, the viral proteins were undetectable. However, the viral proteins were synthesized equally in IFN-treated and control RK6 cells, although the onset of viral protein synthesis was delayed in the IFN-treated cells. IFN treatment of RK8 cells not only inhibited viral protein synthesis, but also alleviated the shutoff of host protein synthesis. Surprisingly, although IFN did not inhibit viral protein synthesis in RK6 cells, it still relieved the shutoff of host protein synthesis almost completely. This observation suggests that an enhanced viral protein synthesis does not necessarily cause a shutoff of host protein synthesis.

Induction of 2-5(A) synthetase. The observed inhibition of viral protein synthesis in IFN-treated RK8 cells could be a consequence of viral mRNA degradation brought about by an activation of the 2-5(A) synthetase-RNase L pathway. If this were the case, one would expect to observe a difference in the status of the enzymes of this pathway between IFN-treated RK6 and RK8 cells. We therefore tested whether 2-5(A) synthetase is induced equally well by IFN in the two cell lines.

The 1.7-kilobase mRNA for 2-5(A) synthetase was present in approximately the same abundance in IFN-treated RK8 and RK6 cells (Fig. 5). However, the level of this mRNA in untreated RK8 cells was higher than that in untreated RK6 cells. In RK402 cells the level of this mRNA was very low, irrespective of IFN treatment. The induction patterns of another IFN-inducible mRNA, 202 (30), in these three lines were very similar to the induction pattern of 2-5(A) synthetase mRNA (data not shown).

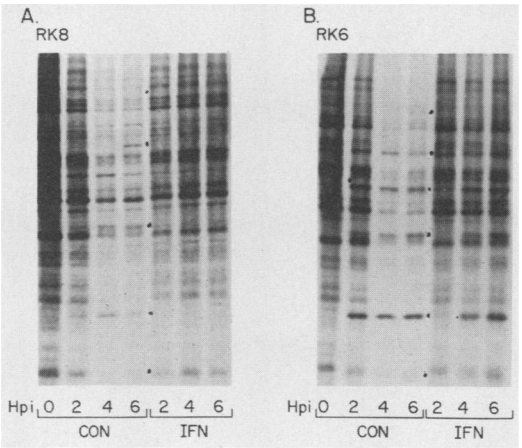

FIG. 4. Rate of protein synthesis in control and IFN-treated EMCV-infected RK8 and RK6 cell lines. Cells were mock or IFN treated (500 U/ml) for 16 h before infection (10 PFU per cell). At the indicated hours after EMCV infection (Hpi), individual cultures were pulse-labeled with [^{35}S]methionine for 30 min, and cell extracts were prepared as described in Materials and Methods. Equal volumes of labeled cell extracts were analyzed. A fluorograph of the dried gel is shown. Numbers below the tracks indicate the time after infection (hours) when the pulse-labeling was started. The major EMCV polypeptide bands are indicated by dots. Six such bands were observed in the extracts of RK6 cells, and five were observed in extracts of RK8 cells.

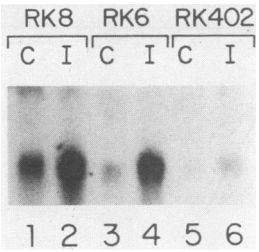

FIG. 5. Induction of (2-5)A synthetase mRNA in RK8, RK6, and RK402 cell lines. Cells were treated with 500 U of IFN per ml for 15 h. Total cytoplasmic RNA was isolated, and 20 μg of RNA was denatured in the presence of formaldehyde and electrophoresed for Northern analysis. The blot was hybridized with a human 2-5(A) synthetase cDNA probe (19). Lanes: C, control; I, IFN treated.

When we measured the level of 2-5(A) synthetase activity in extracts made from the three cell lines, the results were in agreement with the mRNA data (Fig. 6). The enzyme level was relatively high in untreated RK8 cells, but increased considerably when the cells were treated with increasing doses of IFN. RK6 cells contained much less enzyme constitutively, but the enzyme levels were comparable in IFN-treated RK6 and RK8 cells. The induction was very poor in RK402 cells, although some enzyme activity was present in these cells after treatment with high doses of IFN.

Levels of RNase L. Since we observed little difference in the levels of 2-5(A) synthetase between IFN-treated RK8 and RK6 cells, it became important to measure the levels of RNase L, the second type of enzyme of the same pathway. We were unable to measure this enzymatic activity, in a reproducible fashion, in extracts from IFN-treated RK8 and RK6 cells (data not shown). The main difficulty in these measurements was the presence in these extracts of a high level of 2-5(A)-independent RNase activity. To circumvent the problem of measuring RNase L levels by assaying for RNase activity, we determined the 2-5(A)-binding capacity of these extracts. It has been shown that such binding

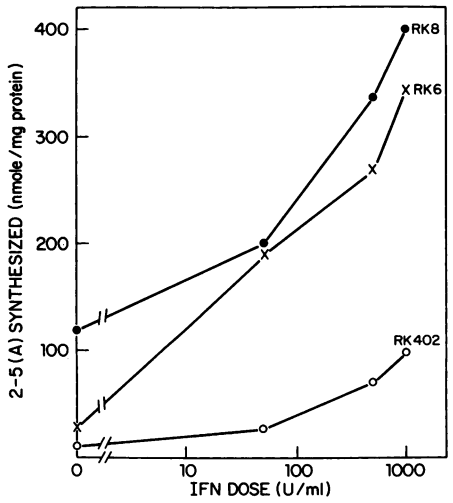

FIG. 6. Effect of increasing doses of IFN on 2-5(A) synthetase activity in RK8, RK6, and RK402 cell lines. Cells were treated with various doses of IFN for 18 h where indicated. Detergent extracts were made and 2-5(A) synthetase activities were measured as described in Materials and Methods. Amounts of 2-5(A) synthesized are expressed as nanomoles of AMP polymerized per milligram of protein, with the assumption that the products were all trimers.

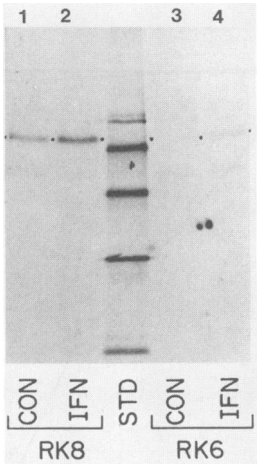

FIG. 7. Cross-linking of (2′-5′)ppp(A)$_4$[^{32}P]pCp to proteins in extracts of RK8 and RK6 cells. The preparation of extracts from RK8 and RK6 cells, and the conditions of binding and cross-linking of a 2-5(A) derivative by UV irradiation are described in Materials and Methods. Reaction mixtures containing 150 μg of cell extract proteins were incubated with (2′-5′)ppp(A)$_4$[^{32}P]pCp (10,000 cpm) at 4°C for 1 h and irradiated with UV at 36,000 J/m^2. The proteins were analyzed by gel electrophoresis. Lanes: 1 and 3, untreated RK8 and RK6 cells; 2 and 4, RK8 and RK6 after IFN treatment (500 U/ml for 16 h), respectively. ^{14}C size markers used (lane STD) were phosphorylase *b* (92,500 Da); bovine serum albumin (68,000 Da); ovalbumin (46,000 Da); carbonic anhydrase (30,000 Da); and lysozyme (14,300 Da). An autoradiograph of the dried gel is shown.

activity reflects the level of RNase L in a cell extract (9). A radiolabeled 2-5(A) derivative, (2′-5′)ppp(A)$_4$[^{32}P]pCp, was incubated with extracts and then cross-linked to the binding proteins. These proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. The most prominent labeled band was of 79 kDa, which corresponds to the molecular mass of RNase L (Fig. 7). Untreated RK8 cells contained substantial amounts of this protein; this level was further increased upon IFN treatment. In contrast, this radiolabeled band was undetectable in the lane with the control RK6 cell extract and was barely visible in the lane with the extracts from IFN-treated RK6 cells. In addition to the 79-kDa band, a faint band of 47 kDa was present in all lanes. Its intensity was not higher in the lanes with IFN-treated cells, and its identity remains to be established. The observed difference in the extent of labeling of the 79-kDa band in the extracts of RK8 and RK6 cells was not due to a differential rate of degradation of the radiolabeled ligand during incubation with extracts of the two cell lines; there was no significant cleavage of the ligand during incubation with any of the extracts, as determined by thin-layer chomatographic analyses (data not shown). It is also unlikely that the extracts of RK6 cells contained proteolytic activities which degraded the 2-5(A)-binding proteins present in them (25), because inhibitors of protease were included in the buffers used for making the cell extracts. Moreover, when an extract was made from a mixture of equal numbers of IFN-treated RK8 and RK6 cells, it contained the same level of 79-kDa binding protein as that in an extract made from the same number of IFN-treated RK8 cells (data not shown).

A filter-binding assay was performed to obtain a more quantitative estimation of RNase L in these extracts. The same amount of the labeled 2-5(A) derivative was incubated with various concentrations of cell extracts, and the amount

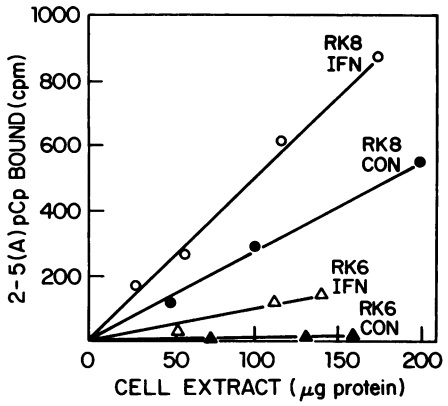

FIG. 8. Radiobinding assay for estimation of 2-5(A)-dependent RNase L levels. Different amounts of proteins in cell extracts from RK8 and RK6 cells were incubated with the same amount of (2'-5')ppp(A)$_4$[^{32}P]pCp (10,000 cpm) at 4°C for 1 h, and then the protein–2-5(A) complex was separated from unbound 2-5(A) by filtration through nitrocellulose filters. The filter-bound radioactivities were quantitated by scintillation spectrometry.

of protein-bound 2-5(A) was measured by its retention on nitrocellulose filters. This retention increased linearly with an increasing concentration of cell extracts (Fig. 8). The level of 2-5(A) retained in extracts from IFN-treated RK6 cells was at least five times lower than that in extracts from IFN-treated RK8 cells.

Although there is strong experimental evidence suggesting that the 79-kDa 2-5(A)-binding protein is RNase L, the formal demonstration of its identity awaits complete purification and characterization of this enzyme activity. To examine whether the observed difference in the levels of 79-kDa protein is accompanied by a parallel difference in 2-5(A)-dependent RNase activity in vivo, we performed the experiment whose results are shown in Fig. 9. We introduced increasing concentrations of 2-5(A) into the cell by transfection and measured their effect on cellular protein

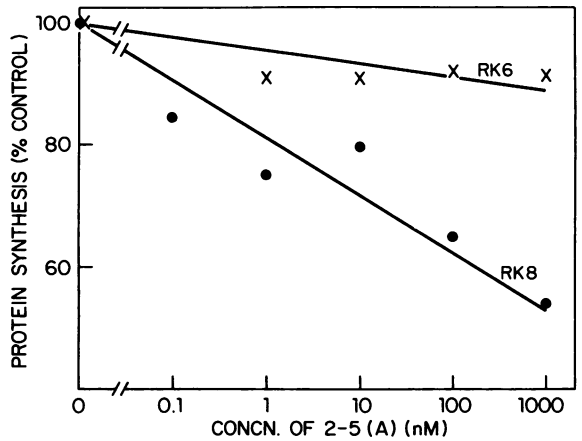

FIG. 9. Effects on protein synthesis of (2'-5')ppp(A)$_3$ introduction into RK8 and RK6 cells. Cells were treated with 500 U of IFN per ml for 16 h. The concentrations of 2-5(A) as indicated in the figure were those of the dilutions before being added to the culture medium. The control samples (100% protein synthesis) received similar treatments, except that no 2-5(A) was added. The transfection of 2-5(A) and pulse-labeling with [^{35}S]methionine were done as described in Materials and Methods.

synthesis by pulse-labeling the cells with [^{35}S]methionine. 2-5(A) inhibited protein synthesis in IFN-treated RK8 cells in a dose-dependent fashion, whereas in IFN-treated RK6 cells the inhibition was marginal. The observed difference in 2-5(A)-mediated inhibition of in vivo protein synthesis between the two cell lines might reflect the difference in their RNase L contents (21).

DISCUSSION

In our earlier studies, the antiviral effects of IFN in the JLSV-11 cell line were assessed (32). We observed that although replication of VSV is strongly inhibited, EMCV replication is barely sensitive to IFN in these cells. Biochemical studies of the status of 2-5(A) synthetase and RNase L revealed that although the former enzyme was strongly induced by IFN in these cells, the latter enzyme was undetectable (32). Hence, the conclusion was that the pathway is defective overall, and the lack of anti-EMCV response was tentatively attributed to this defect. The results presented in this report, dealing with newly isolated novel cell lines, provide further support of the above conclusions. We have shown that the appearance of the anti-EMCV phenotype parallels the appearance of a detectable level of RNase L in the cells.

Much support was collected favoring a role for RNase L in the anti-EMCV action of IFNs. This included the observation that 2-5(A) accumulates in IFN-treated EMCV-infected cells (14), that RNase L is activated in such cells (37), that the inhibition can be partially reversed by analogs of 2-5(A) (36), and that IFN treatment protects RNase L from inactivation by EMCV infection (4). Moreover, in many cell lines, including NIH MOL, RD-114, and NIH 3T3, in which the pathway involving RNase L is defective, EMCV replication is not sensitive to IFN (8, 25, 29, 32, 33).

The most direct evidence for the involvement of a component of the double-stranded RNA-dependent RNase pathway in IFN action against picornaviruses has come, as noted earlier, from very recent experiments (6). They have shown that the replication of mengovirus is impaired (even without IFN treatment) in transfected cell lines (see Introduction) which constitutively express a high level of the 2-5(A) synthetase isozyme of 40 kDa. Although these investigators did not measure the level of RNase L in these transfectants, presumably the constitutive level of this enzyme was high. Our results complement and support the above results. They demonstrate that the entire pathway has to be operative for anti-EMCV action. A high level of 2-5(A) synthetase without a sufficiently high level of RNase L is not enough. One can speculate, therefore, that a constitutive expression of 2-5(A) synthetase might be sufficient for the inhibition of EMCV replication in RK8 cells but not in RK6 cells. We have assumed in the above arguments that a five- to sixfold difference in the level of RNase L is critical. There could be additional factors, however, contributing to the difference in the phenotypes of RK8 and RK6 cells. The isozymes of 2-5(A) synthetase induced in the two cell lines could be different, and since the subcellular locations of these isozymes are different (5), their participation in the RNase L pathway could be different. This type of possibility could be ruled out by transfecting RK6 and RK8 cells with plasmids specifying the same 2-5(A) synthetase isozyme.

Although an active RNase L pathway can account for the anti-EMCV action of IFN in many cell lines, there are several reports in the literature which suggest that alternative pathways for this action may also exist. Recently

Mittnacht and Jacobsen (23) reported the isolation of cell variants from NIH 3T3 cell line in which EMCV replication was sensitive to IFN action. For the isolation of these cell variants they used a selection procedure similar to the one we used. They could not, however, detect any difference between the sensitive and the resistant clones with respect to 2-5(A) synthetase induction and RNase L levels. In the same vein, Krause et al. (15) did not observe any anti-EMCV effects of IFN in NIH 3T3 clone 1 cells under growth and IFN treatment conditions which increased the RNase L content of the cells. There was also no correlation between antimengovirus effects of IFN and the status of the enzymes of the RNase L pathway in several clonal derivatives of an Ltk^- cell line which have different spectra of responses to IFN (22).

Although the experiments described in this report were designed to investigate the mechanism of anti-EMCV action of IFN, they also shed some light on the action of IFN against VSV and retroviruses. Thus, in accord with conclusions drawn from our previous studies (17, 32), it is apparent that the anti-VSV action of IFN can be dissociated from its anti-EMCV action. The antiretroviral effects of IFN are apparently exhibited primarily at the level of virus morphogenesis. We have shown previously that the antiretroviral activity and the anti-EMCV and anti-VSV activities can be dissociated in certain cell lines such as RD-114 and NIH MOL,B (11, 32). A need for an active RNase L system for the antiretroviral action of IFN has not been demonstrated. It was therefore somewhat surprising, and it might be purely coincidental, that murine leukemia virus production by RK8 cells but not by RK6 cells was sensitive to IFN action.

The selection method used for isolating the RK8 clone can in principle be used for other cell lines and for other viruses. The best procedure, however, would be different in each case and must be developed empirically. The growth rates of the virus and of the cells are important parameters, as are the multiplicity of infection and the dose of IFN used. We had difficulty in adopting this procedure to cells such as RD-114, which tend not to grow at very low cell density.

It should be emphasized that the RK8-type cells were present originally in the JLSV-11 cell line and that no mutagenesis of the line was carried out before their isolation. This again exemplifies, as we have noted earlier (17, 32), the heterogeneity of cell lines in their IFN responses. The RK8 line is apparently cured of EMCV infection, since it has been maintained for a long time in regular medium without anti-EMCV antibody. The RK6 line seems to be representative of the majority of the cells in the parental JLSV-11 line. The effect of IFN in the RK402 line is absent or only very weak by all criteria tested so far. It is conceivable that this line is defective in a very early step of IFN action such as receptor binding.

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