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Adenovirus Early Region 1A Modulation of Interferon Antiviral Activity

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Human adenovirus type 5 (Ad5) is a DNA virus which replicates as efficiently in human A549 cells treated with human interferon-α_2 (IFN) as in untreated cells. Vesicular stomatitis virus (VSV), on the other hand, is a negative-strand RNA virus which is very sensitive to the effects of IFN treatment in A549 cells. The IFN-mediated inhibition of VSV replication was not observed in cells coinfected with Ad5. Abrogation of IFN-mediated antiviral activity was maximal when Ad5 infection preceded VSV infection by at least 36 h, but did not require adenovirus DNA synthesis for manifestation. Coinfection experiments with VSV and deletion variants of adenovirus demonstrated that neither virus-associated RNA synthesis nor expression of adenovirus early regions E1B, E2A, E3, or E4 are required for abrogation of IFN-mediated inhibition of VSV replication. However, expression of early region E1A was essential, suggesting that E1A products can modulate, either directly or indirectly, IFN activity in adenovirus-infected cells.

Interferons are a class of proteins which exhibit potent antiviral, immunomodulatory, and other biological activities. Exposure of cells to interferon can alter cellular metabolism so that replication of a wide variety of subsequently infecting viruses is inhibited. Viral RNA synthesis, viral protein synthesis, and virus maturation can be inhibited in interferon-treated host cells. The rate-limiting step for virus replication in interferon-treated cells therefore depends on both the host cell response to interferon treatment and the mode of replication of the virus used for infection (for review, see references 15, 38, and 41).

Replication of most viruses can be inhibited to some extent by interferon treatment of host cells, but the degree of inhibition can vary dramatically for different viruses. Vesicular stomatitis virus (VSV) is an enveloped negative-strand RNA virus of the *Rhabdoviridae* family that is extremely sensitive to the effects of interferon treatment (41). In some cell lines this reduction is due primarily to an inhibition of VSV protein synthesis (21, 22), although effects on primary viral RNA transcription (19, 20; L. Belkowski and G. Sen, personal communication) and virion maturation (18) have also been reported. In contrast, replication of many DNA viruses (e.g., adenoviruses and vaccinia virus) is only marginally inhibited in cells treated with interferon (41).

Elucidation of the mechanisms by which interferon-resistant viruses escape the effects of interferon treatment may provide important insight into the basic processes leading to inhibition of sensitive viruses as well as an understanding of how those processes might be modulated. Studies with vaccinia virus have demonstrated that a virally induced or encoded product can inactivate the double-stranded RNA-dependent protein kinase induced by interferon treatment (27, 34, 50, 51). Inactivation of this kinase correlates with the ability of vaccinia virus to rescue VSV replication in interferon-treated cells (50). Unfortunately, the complexity of the vaccinia virus genome (~250 kilobase pairs) has precluded identification of the responsible vaccinia virus product.

Adenovirus type 5 (Ad5), like vaccinia virus, is a DNA-containing virus which is relatively insensitive to the antiviral effects of interferon treatment. This communication reports the identification of an activity in Ad5-infected cells that prevents human interferon-α_2 (IFN)-mediated inhibition of VSV replication in coinfected cells. The smaller (36 kilobase pairs), well-characterized genome, and the availability of Ad5 mutants have allowed tentative characterization of this activity. It is an adenovirus early region E1A-encoded or -induced product which does not require adenovirus DNA synthesis or expression of early regions E1B, E2A, E3, or E4 for manifestation. The activity is distinct from that of the virus-associated (VA) RNA_I of Ad5 which has previously been shown to enhance adenovirus resistance to IFN treatment by inactivation of the IFN-induced double-stranded RNA-dependent protein kinase (13, 26, 37).

MATERIALS AND METHODS

Cells and virus. All cell lines used were propagated as monolayer cultures in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin sulfate (50 μg/ml). Cells were mycoplasma-free after cultivation in antibiotic-free medium, as judged by biochemical and culture techniques.

VSV serotype Indiana was plaque purified three times, and a stock was prepared in A549 (human lung carcinoma) cells at a multiplicity of infection of 0.01 PFU/cell. Plaque-purified human Ad5 and the early region E3 deletion mutant Ad5Δ(78.9-84.3) (3) were propagated in monolayer cultures of A549 cells. The Ad5 deletion variant Ad5*dl*802 lacks sequences encoding a portion of the adenovirus DNA-binding protein (DBP) (65.4 to 66.1 map units) and was propagated in a human cell line (21F1) expressing the early region 2A product under the control of the mouse mammary tumor virus long terminal repeat (35). Mutants Ad5*dl*1312 (11, 12), Ad5*dl*337 (30), Ad5*dl*338 (31), and Ad5*dl*434 (9, 14), which lack different sequences encoding portions of early regions 1A and 1B, were propagated on human 293 cells (a cell line expressing the left-hand 11% of the adenovirus genome [8]). The Ad5 VA RNA_I-deficient mutant Ad5*dl* 331 (46) was propagated in KB cells. The early region 4 deletion mutant Ad2*dl*808 (47) was propagated in W162 cells (transformed Vero cells which constitutively express E4 prod-

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ucts). Virus titers were determined in the cells used for propagation.

IFN. Recombinant DNA-derived human IFN-α2 (previously referrred to as interferon-αA) was purified from plasmid-transformed *Escherichia coli* as described previously (7, 49) and was greater than 95% pure as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The specific activity of the preparation was approximately 10^8 IU/mg of protein as determined by a cytopathic effect inhibition assay with VSV and Madin-Darby bovine kidney cells (5). Assay results were standardized against a human leukocyte interferon standard (no. Ga23-902-530, National Institutes of Health).

Infectious virus yield reduction assay for IFN activity. IFN activity against viruses was quantitated by a single-cycle infectious virus yield reduction assay. Different concentrations of IFN ranging from 1 to 10,000 IU/ml were applied to cells 24 h prior to infection with virus at a multiplicity of infection of 10 PFU/cell. Cells for assay of VSV replication were harvested 24 h after infection, and cells for assay of adenovirus replication were harvested 48 h after infection. Infected cells in equivalent volumes of medium were frozen and thawed, and lysates were diluted serially in 10-fold increments. Appropriate dilutions were then used to infect confluent monolayer cultures of A549 cells. After a 1-h adsorption period, cells were overlaid with DMEM containing 0.7% agar, 2% fetal bovine serum, and 25 mM $MgCl_2$. VSV titration preparations were fixed in a 10% (wt/vol) Formalin solution after 24 h and stained with crystal violet. Ad5 plaques were visible beginning 5 days after infection and were monitored daily for 10 days or until no new plaques appeared. Adenovirus titration preparations received fresh agar overlay medium every 3 or 4 days.

Viral protein synthesis. Viral protein synthesis was monitored by [^{35}S]methionine labeling of infected cell cultures. Infected cultures were labeled from 5 to 6 h following VSV infection or from 47 to 48 h following Ad5 infection in the presence of methionine-free, serum-free DMEM containing 50 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham Corp.) per ml. Labeling of VSV proteins in coinfected cells was done from 5 to 6 h after VSV infection. Mock-infected, Ad5-infected, and interferon-treated control cells were propagated in parallel and labeled at equivalent times. Immediately after labeling, monolayer cultures were rinsed twice with ice-cold phosphate-buffered saline and lysed at room temperature in buffer containing 50 mM Tris, pH 8.3, 1% SDS, 10% glycerol, 0.02% bromphenylblue, and 0.01 M dithiothreitol. After the lysates were heated to 100°C for 3 min, equal portions of each sample were fractionated by electrophoresis in 12% polyacrylamide gels containing 0.1% SDS. The gels were dried and exposed to X-ray film without the use of scintillation enhancement.

Ad5 DNA synthesis. Ad5 DNA synthesis was monitored by the procedure described by Rice and Klessig (35). Infected cells were labeled for 4 h in the presence of 100 μCi of [^{3}H]thymidine per ml at either 24 or 48 h following Ad5 infection, and low-molecular-weight DNA was isolated. The DNA was digested with restriction endonuclease *Kpn*I (New England Biolabs) and fractionated by electrophoresis in 0.7% agarose gels. Ad5-specific DNA fragments were detected by flourography of dried gels following treatment with En^{3}Hance (New England Nuclear Corp.).

VA RNA synthesis. Ad5- and mutant adenovirus-infected cells were labeled from 1 to 48 h after infection with 10 μCi of $^{32}P_i$ (>400 Ci/mmol; Amersham) per ml. Nucleic acids were extracted from 0.65% Nonidet P-40 supernatant fractions with phenol and chloroform immediately after labeling and fractionated by electrophoresis on denaturing 8.0% polyacrylamide gels containing 4 M urea. The top 1 cm of the gel was removed prior to autoradiography to eliminate overexposure of film due to labeled high-molecular-weight products.

RESULTS

Differential inhibition of Ad5 and VSV replication in IFN-treated A549 cells. The effects of IFN treatment on VSV and Ad5 replication in A549 cells were compared by a single-cycle infectious virus yield reduction assay (Fig. 1A). A549 cells were exposed to various IFN concentrations (0 to 10,000 IU/ml) for 24 h and then infected with either VSV or Ad5. VSV-infected cells were harvested at 24 h postinfection (hpi), and infectious virus present in cell extracts was quantitated by plaque assay on A549 cells. Quantitation of infectious Ad5 virus yield was identical, except that Ad5-infected cells were harvested at 48 hpi because this virus has a longer replication cycle than VSV.

Infectious VSV yield in IFN-treated cells was reduced in a dose-dependent manner. Treatment with 100 IU of IFN per ml reduced infectious VSV yield to only 2% of the level found in untreated cells, and treatment with 10,000 IU of IFN reduced infectious VSV yield more than 1,000-fold. In contrast, infectious AD5 yield in A549 cells was not significantly reduced by IFN treatment at concentrations as high as 10,000 IU/ml.

Similar results were observed in a parallel experiment in which viral protein synthesis was monitored as a measure of virus replication (Fig. 1B). In this experiment, cells which were IFN-treated and infected in parallel with those used for the infectious virus yield reduction assay were labeled for 1 h with [^{35}S]methionine at a time corresponding to optimal viral protein synthesis (5 to 6 hpi for VSV and 47 to 48 hpi for Ad5). Protein extracts from these cells were fractionated on SDS-polyacrylamide gels, and viral protein synthesis was monitored by direct autoradiography of the dried gel. At 5 to 6 hpi synthesis of all major VSV proteins (L, G, N, NS, and M) was significantly inhibited when cells were treated with IFN (100 IU/ml). Synthesis of VSV proteins could not be detected in cells treated with ≥1,000 IU of IFN per ml. Inhibition of VSV-induced shutoff of host cell protein synthesis paralleled the inhibition of VSV protein synthesis. Synthesis of Ad5 proteins, however, was only slightly inhibited even after treatment with as much as 10,000 IU of IFN per ml.

In the experiments discussed above, IFN was present in the medium of treated cells only until the time of virus adsorption. When IFN was also included in the overlay medium following virus adsorption, identical results were obtained in both the virus yield reduction and the protein synthesis assays (data not shown).

Rescue of VSV replication in IFN-treated cells by coinfection with Ad5. One possible explanation for the insensitivity of Ad5 replication to IFN treatment is that Ad5 can either encode or induce a product which prevents manifestation of the antiviral activity induced by IFN treatment. If this hypothesis is correct, VSV replication should not be inhibited by IFN treatment in cells coinfected with Ad5. To test this possibility, the effects of Ad5 coinfection on VSV replication in IFN-treated and control cells were investigated. In untreated A549 cells, Ad5 coinfection did not significantly affect the replication of VSV if Ad5 infection preceded VSV infection by 24 h or less. However, Ad5

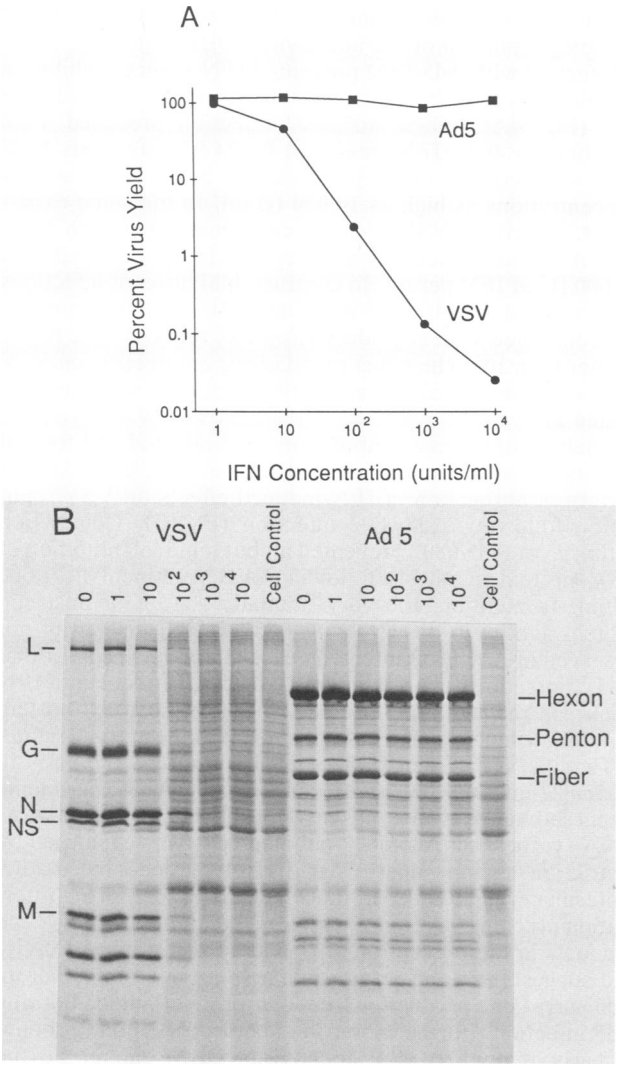

FIG. 1. Differential inhibition of Ad5 and VSV replication in IFN-treated A549 cells. Subconfluent monolayers of A549 cells were incubated in the presence of indicated concentrations of IFN for 24 h and then infected with either Ad5 or VSV. (A) Infectious virus yield was determined by plaque assay. Results are expressed as the percent of virus yield in cells not treated with IFN. The titer of infectious VSV and Ad5 from untreated cells was 6.7×10^8 and 2.7×10^8 PFU/ml, respectively. (B) Viral protein synthesis was monitored in a parallel experiment. Shown is an autoradiograph of labeled proteins fractionated in a denaturing polyacrylamide gel. Captions at top of autoradiograph indicate infecting virus and amount of IFN used for pretreatment of cells. L, G, N, NS, and M indicate the positions of the major VSV proteins.

infection at 36 or 48 h prior to VSV infection reduced VSV yield by a factor of 5 to 10 (Table 1). This inhibition was modest compared with the effects of IFN treatment on VSV replication (reduction of infectious virus yield by >1,000-fold at 10^4 IU/ml) and did not preclude interpretation of the effects of Ad5 coinfection on IFN inhibition of VSV replication.

In IFN-treated cells, infection with Ad5 36 to 48 h prior to VSV infection (12 to 24 h prior to IFN treatment) resulted in almost complete abrogation of IFN-induced inhibition of VSV replication, as measured by infectious VSV yield reduction (Fig. 2). Coinfection with Ad5 at the same time as

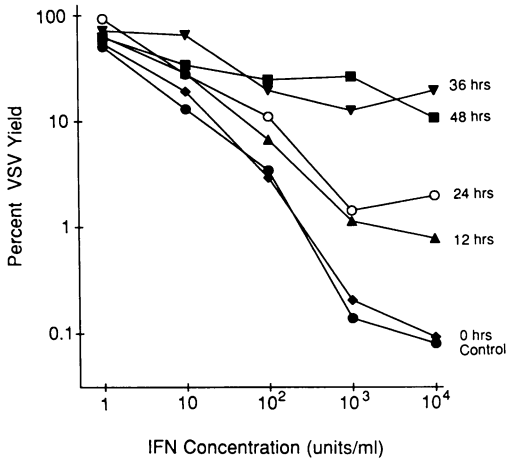

FIG. 2. Abrogation of IFN-induced reduction of infectious VSV yield by coinfection with Ad5. A549 cells were infected with Ad5 at the indicated times prior to VSV infection, and infectious VSV yield was determined by plaque assay (see Materials and Methods). All cells were incubated for 24 h prior to VSV infection with the indicated concentrations of IFN. Results are expressed as the percent of VSV yield in the absence of IFN treatment for each coinfection. VSV titers in untreated control cells for each coinfection are shown in Table 1.

VSV infection, however, had virtually no effect on the inhibition of VSV replication by IFN treatment. Ad5 infection prior to IFN treatment was not required for abrogation of IFN-induced antiviral activity, as Ad5 infection 12 or 24 h prior to VSV infection (12 h after or at the same time as IFN treatment) resulted in substantial reduction of the IFN-induced inhibition of VSV replication.

IFN-mediated inhibition of VSV protein synthesis was also monitored in coinfected cells that were IFN treated and infected in parallel with those used for the virus yield reduction assay discussed above. Trends similar to those in the virus yield reduction experiments were observed (Fig. 3). Coinfection with Ad5 48 h prior to VSV infection in the absence of IFN treatment resulted in a modest reduction of VSV protein synthesis. The magnitude of this reduction was comparable to that observed for reduction of virus yield in an Ad5 coinfection (5- to 10-fold difference, Table 1). Synthesis of Ad5 proteins was also marginally reduced in coinfections. When cells were exposed to IFN (1,000 IU/ml) 24 h prior to VSV infection (24 h after Ad5 infection), only a modest reduction in VSV protein synthesis relative to un-

TABLE 1. Effect of Ad5 coinfection on infectious VSV yield

Time of Ad5 coinfection (h before VSV infection)[a]	Infectious VSV yield (PFU/ml)	Relative VSV yield (% of control)
Control[b]	1.2×10^9	100
0	1.5×10^9	125
12	1.2×10^9	100
24	8.9×10^8	74
36	1.8×10^8	15
48	1.8×10^8	15

[a] Ad5 and VSV infections were performed at a multiplicity of infection of 10 PFU/cell. At 24 h after VSV infection, cells were frozen and thawed, and infectious VSV was quantitated by plaque assay on A549 cells.
[b] Control cells were infected with VSV only.

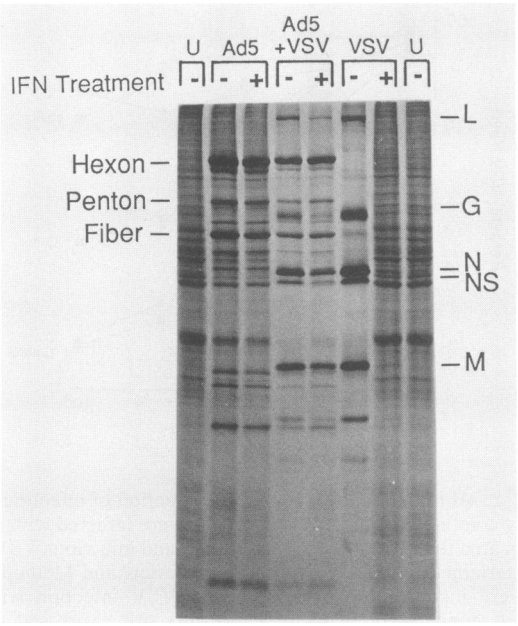

FIG. 3. Abrogation of IFN-induced inhibition of VSV protein synthesis by coinfection with Ad5. A549 cells infected with Ad5 48 h prior to VSV infection and incubated in the presence or absence of 1000 IU of IFN per ml were labeled from 5 to 6 h after VSV infection with [^{35}S]methionine. Shown is an autoradiograph of a denaturing polyacrylamide gel indicating positions of major VSV and Ad5 proteins. Notations at top of gel indicate infecting viruses and IFN treatment; U, uninfected cells. Cells used for labeling of proteins were propagated, infected, and IFN treated in parallel with those used for determination of infectious virus yield in Fig. 2. Hexon, penton, and fiber are three major Ad5 structural proteins.

treated coinfected cells was observed. This difference was again comparable to that observed for reduction of infectious virus yield under the same conditions (1,000 IU of IFN per ml reduced infectious VSV yield by 64%; see Fig. 2). This modest reduction contrasts sharply with the nearly complete inhibition of VSV protein synthesis observed in the absence of Ad5 coinfection. IFN-mediated inhibition of VSV protein synthesis was not affected in cells infected with Ad5 at the same time as VSV infection, but was progressively abolished as the time of Ad5 infection relative to VSV infection was increased (data not shown). Quantitation in all cases was comparable to that observed for reduction of infectious VSV yield in the same cells.

Abrogation of IFN-induced antiviral activity in the absence of Ad5 DNA synthesis. Adenovirus proteins have been classified as early and late according to the kinetics of their appearance following infection of permissive cells and their dependence on viral DNA synthesis for optimal expression. The results of the experiments discussed above suggest that the activity responsible for abrogation of IFN-induced antiviral activity in Ad5-infected cells might be a late Ad5 product expressing optimal activity at least 36 hpi. However, many early Ad5 products continue to be expressed and can accumulate at late times after infection. To determine whether Ad5 DNA synthesis is required for abrogation of IFN-induced antiviral activity, a deletion variant of Ad5 which is defective for viral DNA synthesis was compared with wild-type Ad5 for its ability to prevent IFN-induced inhibition of VSV replication. Ad5*dl*802 is completely defective for viral DNA synthesis and late gene expression in normally permissive cell lines as a result of a 242-base-pair deletion of genomic sequences (65.4 to 66.1 map units) encoding a portion of the adenovirus DBP (35).

Infection with Ad5*dl*802 prevented IFN-induced inhibition of infectious VSV yield at least as efficiently as wild-type Ad5 (Fig. 4A). When Ad5*dl*802 infection preceded VSV infection by 48 h (IFN treatment by 24 h), infectious VSV yield was reduced only by a factor of 2 to 3 even at IFN concentrations as high as 10,000 IU/ml. In the same experiment, infectious VSV yield in cells coinfected with wild-type Ad5 was reduced by a factor of 5 in cells treated with 10,000 IU of IFN per ml. In contrast, inhibition of infectious VSV yield in cells treated with 10,000 IU of IFN per ml but not coinfected with any adenovirus was reduced by a factor greater than 10^4. The effect of Ad5*dl*802 coinfection on VSV replication in the absence of IFN treatment was minimal (Table 2).

Analysis of protein synthesis in cells infected and treated in parallel with those described above revealed a similar pattern of abrogation of IFN-induced effects on VSV replication following Ad5*dl*802 infection (Fig. 5). Coinfection with Ad5 or Ad5*dl*802 prevented all but minimal inhibition of VSV protein synthesis following IFN treatment at 1,000 IU/ml. In addition, the very dramatic shutoff of host cell protein synthesis observed in Ad5-infected cells was not observed in Ad5*dl*802-infected cells. These data suggest that Ad5 coinfection does not modulate the antiviral effect of IFN treatment through a nonspecific shutoff of host cell protein synthesis which prevents establishment of the antiviral state.

The absence of detectable late Ad5 protein synthesis in Ad5*dl*802-infected cells was confirmed in the experiments discussed above. The absence of detectable adenovirus DNA synthesis in Ad5*dl*802-infected cells was confirmed by restriction enzyme analysis of [^{3}H]thymidine-labeled low-molecular-weight DNA in these cells (Fig. 6A). This information precludes a requirement for any adenovirus late gene products in the prevention of IFN-induced antiviral activity and suggests that the responsible activity must be either an Ad5 early gene product or a cellular product induced during Ad5 infection. Furthermore, the major polypeptide product of early region 2A (the adenovirus DBP) cannot be the responsible early gene product, since a deletion mutation in the gene encoding this protein had no effect on the abrogation of IFN-induced antiviral activity.

Adenovirus early region E1A expression required for abrogation of IFN-mediated antiviral activity. In the absence of viral DNA synthesis, six major adenovirus early regions are expressed, which have been referred to as E1A, E1B, E2A, E2B, E3, and E4 (4, 42). In the previous section, experiments with the deletion mutant Ad5*dl*802 showed that expression of early region E2A was not required for abrogation of IFN-mediated inhibition of VSV replication. To determine the requirement for expression of other early region genes in this process, several additional adenovirus mutants with defects in expression of other major early region genes were tested for their ability to prevent IFN-induced inhibition of VSV replication. Coinfection experiments with the deletion variant Ad5Δ(78.9-84.3), which lacks most of the protein-encoding sequences of early region 3 (3), revealed a pattern of rescue of VSV replication in IFN-treated cells similar to that of wild-type Ad5 (Table 2, Fig. 4A). Similarly, the deletion variant Ad2*dl*808, which lacks most of the protein-encoding sequences of early region 4 (47), prevented IFN inhibition of VSV replication just as efficiently as the parental wild-type, Ad2 (Fig. 4B). This experiment also demonstrated that Ad2 can abrogate IFN-mediated antiviral activity in a manner similar to that of Ad5. Side-by-side

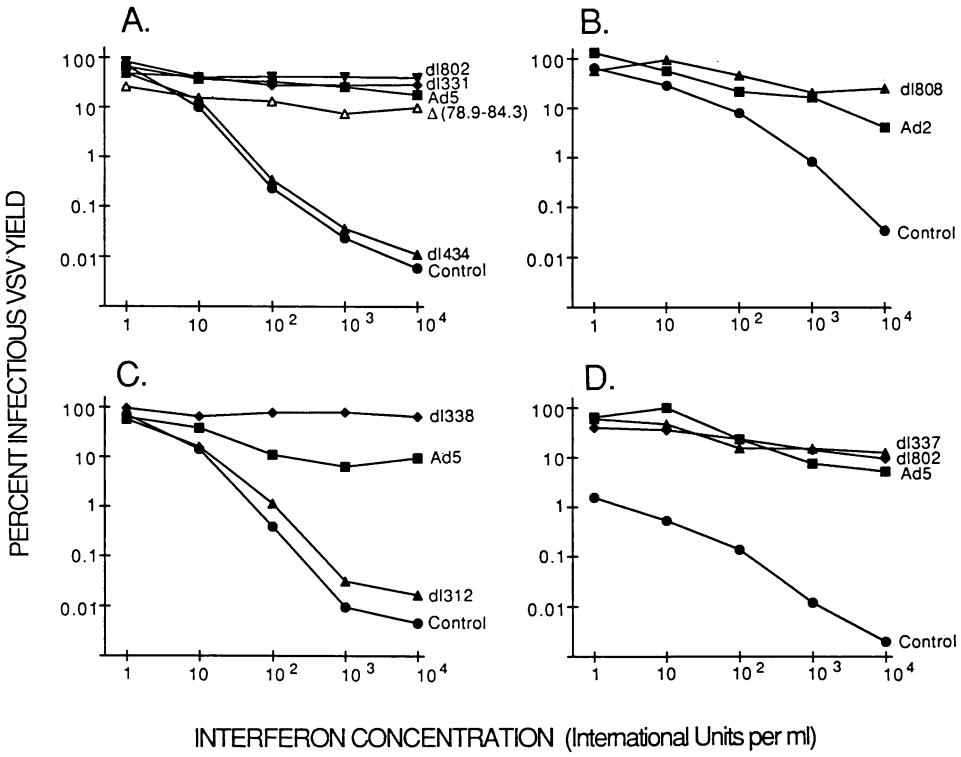

FIG. 4. Abrogation of IFN-induced inhibition of infectious VSV yield in cells coinfected with adenovirus deletion variants. A549 cells were incubated in the presence of indicated concentrations of IFN for 24 h prior to VSV infection and coinfected with the indicated adenovirus deletion variants 48 h prior to VSV infection, except in panel D, where adenovirus infection preceded VSV infection by 24 h (see text). Infectious VSV yield was determined by plaque assay and is expressed as the percentage of VSV yield in cells not treated with IFN for each coinfection. Each panel represents the results of independent experiments. VSV titers from control untreated cells for each coinfection are presented in Table 2, along with the genomic coordinates of deleted sequences and the affected genes of each adenovirus mutant.

TABLE 2. Effects of coinfection with adenovirus deletion variants on infectious VSV yield

Expt	Virus[a]	Deleted sequence (map units)	Defective gene(s) or product(s)	VSV yield (PFU/ml)	Relative VSV yield (% of control)
A	Control			1.7×10^9	100
	Ad5	None	None	1.3×10^8	8
	Ad5*dl*802	65.4–66.1	E2A	5.0×10^8	29
	Ad5*dl*434	2.6–8.7	E1A, E1B	1.9×10^9	112
	Ad5*dl*331	29.5–29.6	VA RNA_1	2.9×10^7	2
	Ad5Δ(78.9-84.3)	78.9–84.3	E3	6.2×10^8	36
B	Control			1.2×10^9	100
	Ad2	None	None	4.2×10^7	4
	Ad2*dl*808	91.7-97.4	E4	7.3×10^7	6
C	Control			1.7×10^9	100
	Ad5	None	None	1.9×10^8	11
	Ad5*dl*312	1.5–4.5	E1A	2.3×10^9	135
	Ad5*dl*338	7.9–9.4	E1B 55K and 17K	1.0×10^7	1
D	Control			2.6×10^9	100
	Ad5	None	None	2.0×10^8	8
	Ad5*dl*802	65.4–66.1	E2A	2.4×10^9	92
	Ad5*dl*337	5.0–5.4	E1B 21K	3.0×10^7	1

[a] Cells were infected with adenovirus 48 h (experiments A, B, C) or 24 h (experiment D) prior to VSV infection. Infectious VSV yield 24 h after VSV infection was determined by plaque assay on A549 cells. Control cells were mock infected 48 or 24 h prior to VSV infection.

comparisons of Ad2 and Ad5 in coinfection experiments revealed indistinguishable effects (data not shown). Infectious VSV yield reduction assays (Fig. 4B) and VSV protein synthesis assays (data not shown) gave similar results for both mutants. Therefore, expression of early regions E3 and E4, like that of early region E2A, is not required for abrogation of IFN-induced antiviral activity.

A deletion variant of Ad5 (Ad5*dl*434) defective for expression of Ad5 early regions E1A and E1B (9, 14) was also tested for its ability to prevent IFN-mediated inhibition of VSV replication. Coinfection with Ad5*dl*434 had no effect on IFN-induced inhibition of either infectious VSV yield (Fig. 4A) or VSV protein synthesis (Fig. 5), suggesting that expression of one or both of these early regions is required for abrogation of IFN-induced antiviral activity.

Two Ad5 mutants with sequence deletions which prevent expression of E1B products (Ad5*dl*337 and Ad5*dl*338 [30, 31]) both prevented IFN-induced inhibition of VSV replication as well as wild-type Ad5 did in coinfection experiments (Fig. 4C and D). In the case of Ad5*dl*337, the accelerated cytopathic effect characteristic of the phenotype of this virus (30) precluded any meaningful interpretation of the effects of IFN treatment in the 48-h VSV coinfection assay used for other adenovirus mutants. Therefore, for this mutant a modified protocol was used in which cells were infected with Ad5*dl*337 just 24 h prior to VSV coinfection. IFN treatment was initiated immediately after virus adsorption. Under these conditions Ad5*dl*337 coinfection prevented IFN-mediated inhibition of VSV replication in a manner that was

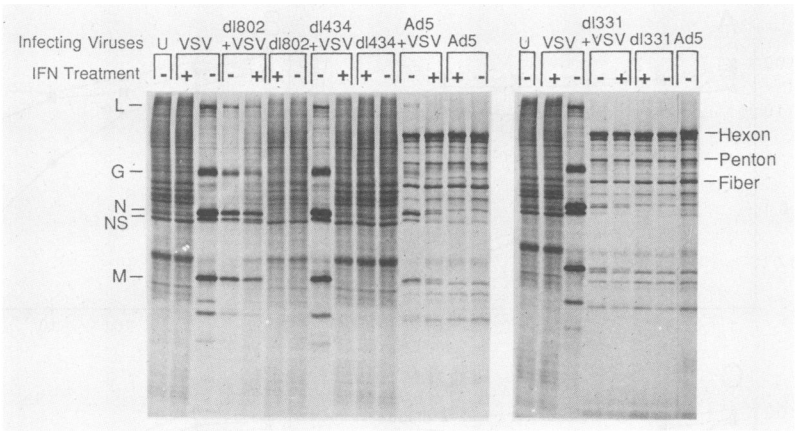

FIG. 5. IFN-induced inhibition of VSV protein synthesis in cells coinfected with Ad5 deletion variants. VSV protein synthesis was monitored in cells incubated in the presence of 1,000 IU of IFN per ml for 24 h prior to VSV infection and coinfected with different Ad5 deletion variants 48 h prior to VSV infection. Shown is an autoradiograph of proteins labeled with L-[^{35}S]methionine from 5 to 6 h after VSV infection and fractionated on a denaturing polyacrylamide gel. Propagation, infection, and IFN treatment of cells were done in parallel with those used for determination of infectious VSV yield in Fig. 4A. See the legend to Fig. 3 for details.

indistinguishable from wild-type Ad5 and Ad5*dl*802 viruses, included as controls (Fig. 4D). Ad5*dl*337 lacks 146 base pairs within the coding region for the 21,000-molecular-weight (21K) polypeptide product of E1B (30), and Ad5*dl*338 lacks 524 base pairs which code for portions of both the 55K and 17K polypeptide products of E1B (31). These two mutants together therefore demonstrate that the three major products encoded by early region E1B are not required for abrogation of IFN-induced antiviral activity.

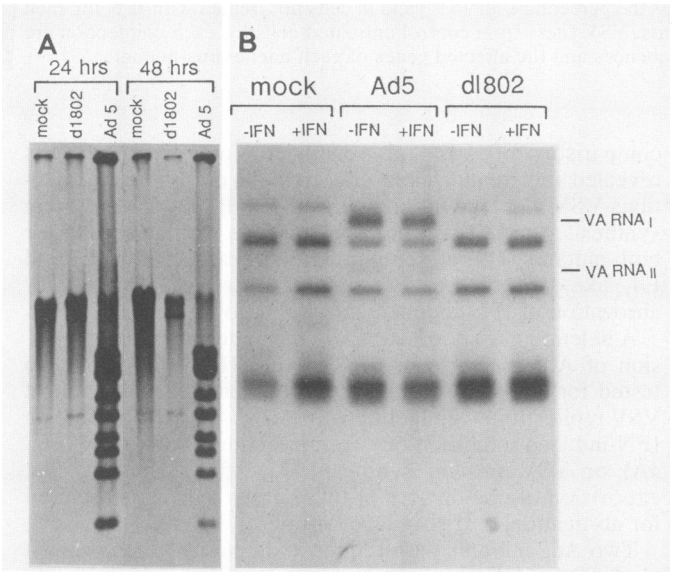

FIG. 6. Adenovirus DNA synthesis and VA RNA synthesis in A549 cells infected with the Ad5 deletion variant Ad5*dl*802. (A) Mock-infected, Ad5-infected, or Ad5*dl*802-infected A549 cells were labeled for 4 h with [^{3}H]thymidine at 24 and 48 hpi. Low-molecular-weight DNA was then isolated, digested with restriction endonuclease *Kpn*I, and fractionated on a 0.7% agarose gel. Shown is a flourograph of the gel, with infecting viruses and time of labeling indicated. (B) Mock-infected, Ad5-infected, or Ad5*dl*802-infected A549 cells were labeled from 1 to 48 hpi with $^{32}P_i$. Both untreated cells and cells incubated in the presence of IFN (1,000 IU/ml) beginning at 24 hpi were used. RNA from labeled cells was fractionated on a denaturing urea-polyacrylamide gel. Shown is an autoradiograph of a portion of the gel, indicating infecting viruses, IFN treatment, and positions of VA RNA_I and VA RNA_{II} bands.

In contrast, an adenovirus mutant (Ad5*dl*312) which lacks 1,030 base pairs of genomic sequence encoding only E1A products (11, 12) was incapable of preventing IFN-mediated antiviral activity. Ad5*dl*312 coinfection failed to prevent IFN-mediated reduction of infectious VSV yield (Fig. 4C) or VSV protein synthesis (data not shown), demonstrating that adenovirus E1A expression, but not E1B, E2A, E3, or E4 expression, is required for abrogation of IFN-induced antiviral activity.

VA RNA not required for modulation of IFN-induced antiviral activity. VA RNA_I and VA RNA_{II} are small adenovirus-encoded RNAs transcribed by RNA polymerase III (32, 39, 48). Expression of VA RNA genes, like many other adenovirus genes, is dependent on *trans* activation as a result of adenovirus E1A expression (1, 6, 10). Furthermore, VA RNA_I has been shown to play a role in enhancing adenovirus late protein synthesis, apparently by preventing activation of a cellular double-stranded RNA-dependent protein kinase (13, 26, 37, 46). This kinase can be induced by interferon treatment in some cells and has been shown to play a major role in preventing replication of many viruses in interferon-treated cells by inhibiting translation of mRNA (15). In addition, Kitajewski et al. (13) have suggested that disruption of the Ad5 VA RNA_I gene may result in enhanced susceptibility of adenovirus to IFN pretreatment. Because of the role the double-stranded RNA-dependent kinase has been reported to play in the inhibition of viral protein synthesis in interferon-treated cells and the ability of VA RNA_I to inhibit its activities, the effects of VA RNA_I on adenovirus abrogation of IFN-induced antiviral activity were investigated.

A deletion variant of Ad5 which does not synthesize VA RNA_I (Ad5*dl*331) was still competent in its ability to prevent IFN-induced inhibition of infectious VSV yield (Fig. 4A). It is interesting, however, that infectious VSV yield in cells coinfected with Ad5*dl*331 but not treated with IFN was only 2% of that in cells infected with VSV alone (Table 2). This VSV yield was reduced by a factor of 4 compared with that observed in cells coinfected with wild-type Ad5 (8% of control VSV-infected cells in this experiment). VSV protein synthesis was also reduced in cells coinfected with Ad5*dl*331

compared with those coinfected with wild-type Ad5. However, neither infectious VSV yield nor VSV protein synthesis (Fig. 5) was reduced significantly further by treatment with IFN in cells coinfected with Ad5*dl*331 and VSV.

These results imply that VA RNA_I is not required for abrogation of IFN-mediated inhibition of VSV replication by Ad5. This result was confirmed and extended to include VA RNA_{II} by demonstrating that neither VA RNA_I nor VA RNA_{II} synthesis could be detected in Ad5*dl*802-infected cells (Fig. 6B). As discussed above, Ad5*dl*802 can very efficiently prevent IFN-mediated inhibition of VSV replication.

DISCUSSION

VSV and Ad5 represent two classes of lytic viruses which differ in their ability to replicate in IFN-treated cells. IFN treatment of host A549 cells resulted in reduction of infectious VSV yield by a factor exceeding 10^3, and VSV protein synthesis was inhibited to below-detectable levels. In contrast, identical IFN treatment of host A549 cells had no significant effect on infectious Ad5 yield or Ad5 protein synthesis. The contrasting replication strategies of Ad5 and VSV provide many possibilities which could account for their different sensitivity to IFN treatment, but no conclusive demonstration of the mechanism by which Ad5 replication escapes inhibition in IFN-treated cells exists. Data presented in this communication demonstrate that a *trans*-acting activity present in Ad5-infected cells can prevent IFN-mediated inhibition of VSV replication. This activity may be at least partially responsible for the ability of Ad5 to replicate efficiently in IFN-treated host cells.

Results of coinfection experiments in IFN-treated A549 cells demonstrated the existence of this activity and established a temporal pattern for its expression. Ad5 infection 36 or 48 h prior to VSV infection completely abrogated the effects of a 24-h IFN treatment on VSV replication, and Ad5 infection 12 or 24 h prior to VSV infection substantially reduced IFN inhibition of VSV replication, although to a lesser extent. Simultaneous coinfection with Ad5 and VSV had no observable effect on IFN inhibition of VSV replication. Therefore, maximal expression of the IFN antagonistic activity ocurs late in the Ad5 replication cycle.

The results of coinfection experiments with adenovirus deletion variants suggest that the activity which prevents IFN-mediated inhibition of VSV replication is dependent on Ad5 early region E1A expression for manifestation, but does not require Ad5 DNA synthesis or late gene expression. The deletion variant Ad5*dl*802, which is defective for expression of early region E2A and hence for viral DNA synthesis and late gene expression, prevented IFN-mediated inhibition of VSV replication as well as wild-type Ad5 did. In contrast, two deletion mutants, Ad5*dl*312 and Ad5*dl*434, which are defective for expression of early region E1A did not prevent IFN-mediated inhibition of VSV replication in coinfection experiments. Since Ad5*dl*312 lacks sequences encoding only E1A products, a product of Ad5 early region E1A is either directly or indirectly involved in the abrogation of IFN-induced inhibition of VSV replication.

An early region E1A product could function indirectly by regulating expression of other viral or cellular genes whose products could directly modulate IFN-induced antiviral activity. The 289-amino-acid polypeptide encoded by adenovirus early region E1A enhances expression of other adenovirus early genes as well as the major late transcriptional unit of adenovirus (2, 11, 16, 23, 24, 33). Several Ad5-encoded products could therefore be considered candidates for a role in the abrogation of IFN-induced antiviral activity. Analysis of additional adenovirus mutants in coinfection assays demonstrated that products of the other major early regions of adenovirus (E1B, E2A, E3, and E4) are not required for abrogation of IFN-mediated antiviral activity. However, not all E1A-modulated adenovirus genes have been examined. The adenovirus DNA polymerase and C-terminus proteins encoded by early region E2B, a small polypeptide of unknown function encoded by sequences within the "i" leader of the adenovirus major late transcriptional unit, the 52- and 55-kilodalton proteins of region L1, the PIVa polypeptide reputed to be involved in adenovirus virion maturation, and the pIX protein of the virion capsid are all adenovirus products which do not require viral DNA synthesis for expression and are dependent on E1A activity for optimal expression (4, 17, 28, 29, 43). The 289-amino-acid early region 1A product can also enhance expression of specific cellular genes (25, 40). It is therefore possible that prevention of IFN-induced antiviral activity may be mediated by an Ad5-induced cellular product.

VA RNA_I of adenovirus has been shown to prevent activation of the interferon-induced double-stranded RNA-dependent protein kinase, a protein which can inhibit viral protein synthesis in some interferon-treated cells by phosphorylating the alpha subunit of eucaryotic translation initiation factor 2 (eIF-2α [13, 26, 37]). In cells infected with the Ad5 deletion variant Ad5*dl*331 (which does not make VA RNA_I), adenovirus late protein synthesis is reduced compared with that in cells infected with wild-type Ad5, providing evidence that VA RNA_I does function in vivo (Fig. 5) (46). Furthermore, in contrast to wild-type Ad5, replication of Ad5*dl*331 in 293 cells is somewhat sensitive to the effects of IFN pretreatment (13). It seemed possible therefore that VA RNA_I might be responsible for preventing IFN-induced inhibition of VSV replication in Ad5-infected cells. However, when Ad5*dl*331 was tested in a VSV coinfection assay, Ad5*dl*331 prevented IFN inhibition of VSV replication just as efficiently as wild-type Ad5 did. This result clearly demonstrates that VA RNA_I does not play a significant role in this process. This finding was confirmed by data showing that neither VA RNA_I nor VA RNA_{II} synthesis could be detected in Ad5*dl*802-infected cells (Fig. 6B). Apparently, either viral DNA synthesis or early region 2A expression is required for VA RNA synthesis. While it is possible that VA RNA_I may play an auxiliary role in preventing IFN-induced antiviral activity, the abrogation of IFN-induced inhibition of VSV replication by two deletion variants of Ad5 (Ad5*dl*331 and Ad5*dl*802) which do not make detectable levels of VA RNA_I demonstrates that a different product present in Ad5-infected cells is responsible for preventing IFN-induced inhibition of VSV replication. This product is not VA RNA_{II}, since this RNA could not be detected in Ad5*dl*802-infected cells.

The mechanism by which Ad5 prevents IFN-induced inhibition of VSV replication in coinfected cells is unknown. One possibility is that Ad5 prevents establishment of an antiviral state following IFN treatment by a general shutoff of host cell macromolecular synthesis. However, much experimental evidence argues against this possibility. In Ad5*dl*802-infected cells, host cell protein synthesis was not significantly reduced, but very dramatic abrogation of IFN-induced antiviral activity was observed (Fig. 4 and 5). Furthermore, significant inhibition of IFN-induced antiviral activity was observed even when Ad5 infection occurred 12 h after IFN treatment (Fig. 2). In this system, IFN treatment

of cells for 12 h was sufficient for maximum inhibition of VSV replication (data not shown). The above considerations suggest therefore that an E1A-encoded product or a product whose expression is dependent on the presence of an E1A product can inhibit specific IFN-induced activities which prevent replication of VSV.

In human amnion U cells, inhibition of VSV replication following treatment with IFN is due primarily to specific inhibition of VSV protein synthesis which correlates with an induction of double-stranded RNA-dependent protein kinase activity (21, 22, 36). Furthermore, coinfection with vaccinia virus can abolish interferon-mediated inhibition of VSV replication in a manner similar to that observed for Ad5. This abolition correlates with an inhibition of the double-stranded RNA-dependent protein kinase (44, 45, 50, 51). Therefore, it is possible that an E1A-encoded or -induced product which inhibits the activity of double-stranded RNA-dependent protein kinase might be responsible for the observed rescue of VSV replication in IFN-treated coinfected cells. This product, however, must act independently of VA RNA_I and would be a much more potent inhibitor of kinase activity than VA RNA_I, since VA RNA_I is not required for adenovirus rescue of VSV replication in IFN-treated A549 cells.

It is possible that IFN-induced inhibition of VSV replication could also be abrogated in Ad5-infected cells by a different mechanism not involving the double-stranded RNA-dependent protein kinase. In human fibroblast GM2767 cells, IFN treatment does not induce the double-stranded RNA-dependent protein kinase, but nevertheless significantly inhibits VSV replication (36). Interferon-induced inhibition of primary VSV transcription (19, 20; L. Belkowski and G. Sen, personal communication) and virion maturation (18) have also been reported for different cells. Since the mechanism by which IFN inhibits VSV replication in A549 cells is presently unknown, an Ad5-induced or -encoded product could prevent IFN-mediated antiviral activity by antagonizing any one of these processes.

The ability of a virus to prevent interferon-mediated inhibition of its own replication has obvious evolutionary advantages. Such an ability would be even more advantageous and possibly even requisite for a virus with a relatively long replication cycle, such as Ad5. In Ad5 infections, induction of interferon synthesis following infection and manifestation of antiviral activity late in the infectious cycle may occur in the same cells. Ad5 has therefore apparently developed multiple mechanisms to prevent interferon-mediated effects late in the virus replication cycle. VA RNA_I has been shown by others to inhibit the activity of the interferon-induced double-stranded RNA dependent protein kinase (13, 26, 37), and experiments presented in this communication demonstrate the existence of a distinct E1A-encoded or -induced function which either is more efficient than VA RNA_I in inhibiting the activity of IFN-induced double-stranded RNA-dependent protein kinase or works via a different mechanism. Genetic and biochemical approaches should lead to identification of the responsible adenovirus gene product and elucidation of its mechanism of action.

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