Interferon Induction by Adenovirus Type 12: Stimulatory Function of Early Region 1A

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Adenoviruses are generally weak interferon inducers, triggering chicken embryo fibroblast cells by a UV-resistant viral component, probably the capsid or capsid elements, to produce 50 to 100 IU of interferon per ml. Adenovirus types 12, 18, and 31, however, can induce by a UV-sensitive mechanism 10 to 20 times more interferon than other types do. By using mutant and recombinant adenoviruses, we demonstrated that early region 1A was responsible for the enhanced interferon production of chicken cells infected with adenovirus type 12.

Interferons (IFN) are a group of proteins with diverse biological activities, whose production can be triggered by different inducers in cells (23). Among inducers, the most often used are viruses. Although some studies provide support for viral components or products involved in IFN induction (hemagglutinin-neuraminidase glycoprotein and double-stranded RNA; 4, 9, 10, 16), the mechanism of inducer action has not been clarified yet.

Human adenoviruses induce IFN in nonpermissive chicken embryo fibroblast (CEF) cells (1, 26, 27). IFN is not produced in adenovirus-infected human, monkey, mouse, and hamster cells (7). Most of the adenovirus types investigated are weak IFN inducers in CEF cells (adenovirus type 2 [Ad2], Ad3, Ad4, Ad5, Ad6, Ad9, Ad15, and Ad19), resulting in IFN of low titer, i.e., 50 to 100 IU/ml (26). We have analyzed the inducing properties of Ad2 in detail to identify the viral component(s) required for IFN induction (26, 27).

Since the IFN-inducing ability of Ad2 virions is relatively UV resistant and empty capsids lacking DNA are as effective inducers as complete virions are, it seems that the capsid or a capsid element(s) is responsible for activating the IFN gene in CEF cells infected by Ad2 and probably by other weak adenovirus inducers (26, 27).

Some types (Ad12, Ad18, and Ad31) are potent inducers, since they induce IFN up to a few thousand international units per milliliter in CEF cells (26). The IFN-inducing ability of these potent inducers is largely UV sensitive (27). Purified Ad12 empty virions do not induce IFN of high titer, but incomplete particles carrying only 10 to 20% of the left part of the genome trigger cells to produce IFN of high titer (27). These results suggest that the E1 region, which occupies the left part of the viral genome (21), might have some role in the process of IFN induction by Ad12. Using mutant and recombinant adenoviruses (Table 1), we demonstrated that the E1A region was indeed responsible for the enhanced IFN-inducing ability of Ad12.

Cells and viruses. HEp-2 and 293 cells were used for propagating wild-type (WT) and mutant viruses. CEF cells were used for production and assay of IFN. Cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Ad2 WT, Ad5 WT, Ad12 WT, Ad5 d1312 (gift of T. Shenk; 12), Ad5 rc-1 (22), Ad12 in203S

(18), Ad12 d1203E10, and Ad12 d1203 (K. Shiroki, unpublished results) were used (Table 1). Adenoviruses were purified by CsCl density gradient centrifugation as described previously (28). Multiplicity of infection was calculated by using the formula 1 optical-density unit of purified virus at 280 nm is equal to 10^{12} particles. Vesicular stomatitis virus Indiana was used as the challenge virus to evaluate the titer of IFN.

IFN induction and assay. Induction and assay of IFN were performed as described previously (1, 26, 27), with minor modifications. Briefly, 2-day old primary CEF cells (10^7 cells in 9.6-cm² petri dishes) were infected with 1,000 virus particles per cell in a 0.2-ml volume. At 1 h later, 2 ml of medium supplemented with 5% fetal calf serum was added and cultures were incubated for various periods (usually for 48 h), at which time IFN titers were determined. Interval production of IFN was performed by incubating the induced cells until the times indicated below, when the medium was harvested for assay of IFN and replaced with fresh medium until the next interval, at which time this process was repeated. IFN titers were expressed in international units per milliliter compared with a reference standard chicken IFN preparation (MRC research standard A, 62/4; London, United Kingdom). For UV irradiation, 10^{10} virus particles in a drop of 0.1 ml of phosphate-buffered saline were placed into a plastic petri dish and treated for 15 min in a darkroom for a total dose of 17,500 ergs/mm².

Preparation and blot analysis of mRNA. A 10-μg sample of total cytoplasmic RNA (13) was applied to a 0.7% formaldehyde agarose gel (15), and RNA was blotted onto nitrocellulose (15). Prehybridization and hybridization were carried out by the method of Wahl et al. (30). The 1,046-base-pair *KpnI*-*AccI* DNA fragment (588 to 1,634) of the cloned Ad12 *EcoRI* C fragment was labeled with ³²P by nick translation (15) and used as a probe.

IFN induction by Ad2 WT, Ad5 WT, and Ad12 WT. Early regions of Ad5 WT and Ad12 WT are expressed in nonpermissive CEF cells (25; see below) in parallel with the appearance of IFN upon infection. Some of the previous results obtained from induction experiments with adenoviruses are summarized in Fig. 1A, lanes 1 to 3. Supernatant of CEF cells infected with Ad12 WT contained 10 to 20 times more IFN than that of Ad2 WT- or Ad5 WT-induced cells. In all experiments, 1,000 viral particles per cell were used for induction, which proved to be optimal for all types investi-

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TABLE 1. Viruses used for IFN induction

Virus	Virus genome contains:				Reference
	Ad5 E1A	Ad5 E1B	Ad12 E1A	Ad12 E1B	
Ad5 WT	+	+			
Ad5 <i>dl312</i>	—	+			12
Ad5 <i>rc-1</i>	—	+			22
Ad12 WT			+	+	
Ad12 <i>dl203E10</i>			—	—	Unpublished
Ad12 <i>dl203</i>			—	+	Unpublished
Ad12 <i>in203S</i>			+	+	18

^a The E1B-coding region is under the control of the E1A promoter.

^b A 6-base-pair insertion in the E1A 13S mRNA-coding region and the 12S mRNA intron.

gated (26). The IFN-inducing capacity of Ad12 WT but not that of Ad2 WT and Ad5 WT was reduced drastically by 17,500 ergs of UV treatment per mm² (Fig. 1A, lanes 1 to 3). This UV dose resulted in more than a 100-fold reduction in infective titers of the viruses (26). The UV treatment eliminated the difference in the IFN-inducing ability between Ad12 WT and Ad2 (Ad5) WT. Further UV treatment slowly reduced the IFN-inducing capacity of all three types similarly (data not shown). The difference in the IFN-inducing ability between Ad2 (Ad5) WT and Ad12 WT might be simply explained by presuming the presence of an inhibitor in Ad2 (Ad5) WT-infected CEF cells, preventing high IFN production. However, this explanation is not likely, since in coinfection experiments, Ad5 WT did not inhibit the IFN-inducing ability of Ad12 WT, even when infection with Ad5 WT preceded Ad12 WT infection by 2 h (Fig. 1B, lanes 1 to 5). Consequently, Ad5 WT did not have or express the viral function resulting in a high IFN yield in CEF cells.

Role of E1A in the IFN induction of Ad12. By using incomplete particles, we have previously shown (27) that the left part of the Ad12 WT genome (E1 region) might play a role in the high-IFN-inducing ability of this virus. To determine whether the Ad12 E1 region is in fact responsible for the high IFN yield, CEF cells were infected with Ad12 *dl203E10*, which is a deletion mutant of the E1 region (0.5 to 10 map units; K. Shiroki, unpublished results), and IFN titers were determined. Infected cells produced IFN in low titer, and the inducing ability of the mutant virus was UV resistant (Fig. 1A, lane 4); thus, as a consequence of the loss of the E1 region, Ad12 became a weak inducer. The IFN-inducing ability of Ad12 *dl203*, a deletion mutant of the E1A region (1.4 to 4.2 map units; K. Shiroki, unpublished results) was identical to that of Ad12 *dl203E10* (Fig. 1A, lane 5). Furthermore, recombinant adenovirus Ad5 *rc-1* (22), whose genome has a deletion between 79.9 and 82.5 map units of Ad5 *dl312* (12) DNA with an insertion of complete E1A (0.1 to 5.5 map units) of Ad12 DNA at the deletion site, induced IFN in high titer (Fig. 1A, lane 6). More than 90% of the IFN-inducing ability of Ad12 *rc-1*, similar to that of Ad12 WT, was destroyed by UV treatment (Fig. 1A, lane 6). The IFN-inducing ability of Ad5 *dl312* was identical to that of Ad5 WT (Fig. 1A, lane 7), indicating that the Ad5 E1A region was not required for IFN induction by Ad5 WT.

These results showed that viruses containing the Ad12 E1A region were potent inducers, while viruses lacking this region were weak IFN inducers.

IFN induction by an Ad12 mutant defective in production of 13S E1A mRNA. Ad12 *in203S* produces normal 12S mRNA but contains a 6-base-pair insertion coding two amino acids (Pro and Gly) in the 13S mRNA-coding region and the 12S mRNA intron (18). In Ad12 *in203S*-infected HeLa or KB

cells, 13S-dependent early-viral-gene expression is not detected; therefore, the altered protein production of 13S mRNA is nonfunctional (18). However, in CEF cells, the IFN-inducing ability of Ad12 *in203S* was identical to that of Ad12 WT at multiplicities of infection of 1,000 (Fig. 1A, lane 8), 500, 250, and 100 particles per cell (data not shown).

Transcription of Ad12 E1A in CEF cells. Since the expression of Ad12 early genes has not been investigated in CEF cells, we analyzed the kinetics of IFN production in parallel with the appearance of E1A mRNAs in these cells. IFN production and the presence of transcripts were not detectable at 5 h postinfection (data not shown). Ad12 E1A mRNAs were present at 12 h; they reached the maximum level at 24 h, and they were barely detected at 36 and 48 h postinfection (Fig. 2A). These results indicate that the expression of the E1A gene in nonpermissive CEF cells was delayed compared with its activation in permissive cells (14,

FIG. 1. (A) IFN-inducing ability of Ad12 WT (lane 1), Ad2 WT (lane 2), Ad5 WT (lane 3), Ad12 *dl203E10* (lane 4), Ad12 *dl203* (lane 5), Ad5 *rc-1* (lane 6), Ad5 *dl312* (lane 7), and Ad12 *in203S* (lane 8). CEF cells were infected by intact virions (□) or by UV-treated virions (▨), and IFN samples were collected 48 h after infection. Numbers on the columns show the percent survival of the IFN-inducing ability of viruses after UV irradiation with 17,500 ergs/mm². (B) IFN production of CEF cells infected with Ad5 WT (lane 1), Ad12 WT (lane 2), and Ad5 WT and Ad12 WT together (lane 3). Ad5 WT infection preceded infection with Ad12 WT by 2 h (lane 4), and Ad12 WT infection was followed by Ad5 WT infection 2 h later (lane 5).

19). IFN synthesis correlated well with the expression of the E1A region (Fig. 2B, interval production); however, synthesis of IFN continued for at least 12 h following the decay of the E1A mRNA level. Cumulative IFN production showed no significant decrease in IFN yield during incubation (Fig. 2B). The steady-state levels of Ad12-specific E1A mRNAs and the yields of IFN produced in CEF cells infected with mutant and recombinant adenoviruses at 24 h after induction are shown in Fig. 3. Infection of cells with the Ad12 *dl203E10* E1 deletion mutant did not result in the appearance of E1A transcripts, and the mutant induced IFN of low titer (Fig. 3A and B, lane 2). In contrast, Ad12 WT, Ad5 *rc-1*, and Ad12 *in203S* infections triggered the cells to produce IFN of high titer, and Ad12 E1A mRNAs were observed in all cases (Fig. 3A and B, lanes 1, 3, and 5). In Ad12 *in203S*-infected CEF cells, similar to KB cells (18), the level of E1A mRNA was much lower than it was in Ad12 WT-infected CEF cells (Fig. 3A, lanes 1 and 5). The kinetics of appearance of Ad12 E1A mRNA were similar in WT- and mutant-infected CEF cells (data not shown). It seems that the low level of Ad12 E1A mRNA is sufficient for maximal IFN production in CEF cells. The low level of E1A mRNA in Ad12 *in203S*-infected CEF cells is probably a consequence of the lack of an active

FIG. 2. (A) Levels of Ad12 E1A transcripts. (B) Interval (□) and cumulative (▨) IFN production of Ad12 WT-infected CEF cells at 0 (lane 1), 12 (lane 2), 24 (lane 3), 36 (lane 4), and 48 (lane 5) h after induction.

FIG. 3. (A) Levels of E1A mRNAs. (B) IFN production of CEF cells infected with Ad12 WT (lane 1), Ad12 *dl203E10* (lane 2), Ad5 *rc-1* (lane 3), no virus (lane 4), and Ad12 *in203S* (lane 5) at 24 h after infection.

13S mRNA product, which has a positive regulatory function on E1A expression (18). In human cells, the transcription of the Ad12 *in203S* E1B region is also reduced markedly because of the lack of an active E1A 13S mRNA product (18). This effect could not be studied in CEF cells by Northern analysis, because the level of E1B-specific transcripts was extremely low even in Ad12 WT-infected cells.

Discussion. The results presented here show that an adenovirus was a potent IFN inducer if its genome contained the E1A region of Ad12 but that viruses containing the E1A region of Ad5 or no E1A region were weak inducers (Fig. 1A). Thus, Ad12 WT was a potent IFN inducer, but its E1 and E1A deletion mutants (Ad12 *dl203E10* and Ad12 *dl203*) were weak inducers (Fig. 1A, lanes 1, 4, and 5). On the other hand, the weak IFN inducer Ad5 *dl312* became a potent inducer when the Ad12 E1A region was inserted into its genome (Ad5 *rc-1*; Fig. 1A, lanes 6 and 7). These findings are in complete agreement with the results of UV experiments. By using 17,500 ergs of UV per mm², 87 to 93% of the IFN-inducing ability of the potent inducers was destroyed, and the difference in the IFN-inducing ability of potent and

FIG. 4. Model for induction of IFN by Ad12 and Ad2 (Ad5) in CEF cells.

weak inducers was eliminated (Fig. 1A), suggesting a UV-resistant component responsible for a low level of IFN production in adenovirus-infected CEF cells and a UV-sensitive viral component or mechanism responsible for enhanced IFN production in CEF cells infected with potent inducers.

On the basis of these results and previous observations (27), we propose a model of how Ad12 and either Ad2 or Ad5 induce IFN in CEF cells (Fig. 4). According to this model, adenoviruses are generally weak IFN inducers, triggering CEF cells to produce IFN by their capsid or capsid elements. However, if the virus genome carries an Ad12 E1a region, the virus behaves as a potent inducer, because an E1a product(s) could stimulate capsid-induced IFN production. It is well demonstrated that E1a proteins have *trans*-acting function, turning on or stimulating viral (2, 11), transferred (6, 8, 24), and endogenous (17) genes. Other members of subgroup A (Ad18 and Ad31) are also potent inducers, probably because they carry E1a regions similar to that of Ad12.

IFN production of Ad12 *in203S*-infected cells was similar to that of Ad12 WT-infected cells (Fig. 1A, lanes 1 and 8). Since the 13S mRNA product of the mutant virus is functionally defective (18), it seems that the 12S mRNA or the altered 13S mRNA product is sufficient for stimulating IFN production. Recently, we have found that the 13S mRNA product alone is effective in the stimulation of IFN production, since an Ad12 virus carrying the cDNA of the 13S mRNA induced IFN at the WT level (unpublished results).

An interesting observation is that Ad12 and Ad5 E1a products behaved differently with respect to the stimulatory effect on IFN production in adenovirus-infected CEF cells. Similarly, expression of the class I major histocompatibility complex (MHC) antigens has been shown to be changed in Ad12-transformed cells as compared with cells transformed by Ad5 (3, 20, 29). The suppression of MHC expression is due to an active switching-off process. This might also be the case with the IFN gene in CEF cells, if the gene is under negative regulation by a *trans*-acting repressor(s), as supposed for the human IFN- β gene (5).

We thank Erzsebet Kusz, Katalin Kovacs, and Katalin Horvath for excellent technical assistance.

ADDENDUM

Recently, we have found that the E1A 12S mRNA product of Ad12 is not effective in the stimulation of IFN production, because an Ad12 virus carrying the cDNA of the 12S mRNA induced IFN at a low level and its IFN-inducing ability was UV resistant, similar to that of Ad12 *dl203*.

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