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# Alpha interferon-induced antiviral response noncytolytically reduces replication defective adenovirus DNA in MDBK cells

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**Abstract**

Although alpha interferon (IFN-α) is of benefit in the treatment of viral hepatitis B, HBV replication has been refractory to the cytokine in commonly used hepatocyte-derived cell lines. In search for a cell culture system to study the mechanism by which IFN-α inhibits HBV replication, we infected a variety of cell lines with an adenoviral vector containing a replication competent 1.3-fold genome length HBV DNA (AdHBV) and followed by incubation with IFN-α. We found that IFN-α efficiently decreased the level of HBV DNA replicative intermediates in AdHBV infected Madin–Darby bovine kidney (MDBK) cells. Further analysis revealed, surprisingly, that IFN-α did not directly inhibit HBV replication, rather the amount of adenovirus DNA in the nuclei of MDBK cells was reduced. As a consequence, HBV RNA transcription and DNA replication were inhibited. Experiments with adenoviral vector expressing a green fluorescent protein (GFP) further supported the notion that IFN-α treatment noncytolytically eliminated adenovirus DNA, but did not kill the vector infected MDBK cells. Our data suggest that IFN-α-induced antiviral program is able to discriminate host cellular DNA from episomal viral DNA and might represent a novel pathway of interferon mediate innate defense against DNA virus infections.


*Keywords:* Interferon; Hepatitis B; Adenovirus; Vectors

## 1. Introduction

Type I interferons (IFNs) are a family of cytokines produced by many types of somatic cells in response to virus infections and coordinate host innate and adaptive immunity to viruses. They are essential for vertebrates to defend against virus infections. This is best illustrated in mice in which the IFN receptor gene is "knocked out" and their key signaling components, such as STAT1 and STAT2 are impaired. Virus infections of those mice lead to overwhelming virus propagation and rapid death (Sen, 2001; Stark et al., 1998; Stetson and Medzhitov, 2006).

Previous studies have shown that the interferon-induced host antiviral program plays an important role in controlling hepatitis B virus (HBV) infections (Guidotti et al., 1999, 2002; Wieland et al., 2004). IFN-α has historically been the standard of care for chronic hepatitis B and its therapeutic efficacy has been improved by the recent introduction of pegylated interferon (Gish, 2005). Forty-eight-week treatment of peginterferon alpha 2a can result in HBeAg serum conversion and reduction of viral load in 30–40% of the patients tested (Janssen et al., 2005; Lau et al., 2005). In comparing with HBV DNA polymerase inhibitors, IFN-α therapy is able to lead to HBsAg serum conversion in a small (8%), but significant fraction of treated patients, which is an indication of the clinical cure of chronic HBV infection (Lau et al., 2005; Wursthorn et al., 2006).

However, much about the molecular basis of interferon's efficacy with HBV infection remains a mystery. It is also not yet known why some patients respond to the therapy, but others do not (Gish, 2005). Detailed knowledge of the molecular mechanisms by which interferon inhibit HBV replication would help in understanding the pathogenesis of HBV infection and

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establishing the foundation for the improvement of interferon therapeutic efficacy. Unfortunately, the insensitivity of HBV replication to interferon treatment in the most commonly used cell lines, such as Huh7 and HepG2, has impeded such mechanistic studies (Hayashi and Koike, 1989; Ladner et al., 1997; Tur-Kaspa et al., 1990).

To search for cell lines in which HBV replication is sensitive to IFN-α, we took advantage of broad cell tropism and high efficiency of gene delivery of adenovirus vectors. A 1.3-fold genome length HBV DNA was inserted into a replication defective adenoviral vector (Ad5 ΔE1/ΔE3). The resultant recombinant virus, designated as AdHBV, is used to delivery HBV genome into a variety of cells to initiate HBV replication. In the course of evaluating the effects of IFN-α on HBV DNA replication in AdHBV infected cells, we observed that IFN-α profoundly reduced HBV DNA in MDBK cells. Further mechanistic analysis revealed that the observed reduction of HBV DNA replication is secondary to the elimination of adenovirus vector DNA from the nuclei of infected cells. Thus, our data suggest, for the first time, that an IFN-α-induced antiviral program is able to discriminate host cellular DNA from extra-chromosomal viral DNA. This might represent a novel mechanism by which IFN-induced cellular antiviral program controls DNA virus infections.

## 2. Materials and methods

### 2.1. Construction of recombinant adenoviruses

Replication deficient adenovirus Ad-HBV and Ad-GFP were constructed using Stratagene's AdEasy-XL kit. Briefly, a CMV-IE driven HBV ayw subtype (Fallows and Goff, 1995) or GFP expression cassette (He et al., 1998) was inserted into the multiple cloning site of pShuttle vector, digested with PmeI followed by transformation of BJ-5183-Ad-1 cell that harbors pAdEasy-1 vector. pAdEasy-1 is an ampicillin resistant 33.4 kb plasmid that contains all genes of adenovirus serotype 5 (Ad5) but E1 and E3. Recombinants of pAdEasy-HBV or pAdEasy-GFP were selected through kanamycin resistance and were linearized with Pac I. Five micrograms of linearized DNA were used to transfect HEK293 cell with Lipofectamine reagents (Invitrogen) in a T25 flask. Culture medium was replaced every 3 days, and viral plaques were observed 10–14 days post-transfection. Cells were then collected with 1 ml culture medium and experienced three times freeze and thaw in methanol/dry ice bath. Cellular debris was removed by microcentrifugation at 12,000 × *g* for 10 min and 5 μl of supernatant were used to inoculate HEK293 cells in T75 flask for further amplification of adenoviruses.

### 2.2. Cell culture and virus infection

African green monkey kidney cell line CV1, human cervical cancer cell line (HeLa), immortalized mouse hepatocytes AML12 and Madin–Darby bovine kidney (MDBK) cells were cultured with DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum, 1× nonessential amino acids (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. Lamivudine was obtained from Glaxo Smith Kline. IFN-α1b was obtained from PBL Inc. To infect MDBK cells with recombinant adenovirus, $10^6$ cells were suspended in 1 ml medium and 50 μl of adenovirus stock (containing $10^6$ pfu) were added. Cells were then seeded into six-well plates and cultured for 12 h. Culture media were then changed and cells were harvested at indicated time points.

### 2.3. Nucleic acid analysis

Intracellular HBV core DNA was extracted as described previously (Zhou et al., 2006). Briefly, cells from one 35 mm dish were lysed with 1 ml of lysis buffer containing 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% NP40 and 2% sucrose at 37 °C for 10 min. Cell lysate was centrifugated at 5000 × *g* for 5 min. The pellet was used to extracted nuclei DNA. The supernatant was mixed with 250 μl of 35% PEG-8000 containing 1.5 M NaCl. After 1 h incubation in ice, viral nucleocapsids were precipitated by centrifugation at 12,000 × *g* for 10 min at 4 °C, followed by 1 h digestion at 37 °C in 400 μl of digestion buffer containing 0.5 mg/ml pronase (Calbiochem), 0.5% SDS, 150 mM NaCl, 25 mM Tris–HCl (pH 8.0) and 10 mM EDTA. The digestion mixture was extracted twice with phenol and DNA was precipitated with ethanol, dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). To extract nuclei DNA, the cell pellet was resolved in 1 ml of lysis buffer containing 0.5 mg/ml pronase (Calbiochem), 0.5% SDS, 150 mM NaCl, 25 mM Tris–HCl (pH 8.0) and 10 mM EDTA, and incubate at 37 °C overnight. The digestion mixture was extracted twice with phenol and DNA was precipitated with ethanol, dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). One half of the DNA sample from each plate was digested with Hind III and resolved by electrophoresis into a 1.5% agarose gel. Gel was then subjected to denaturalization in a solution containing 0.5 M NaOH and 1.5 M NaCl, followed by neutralization in a buffer containing 1 M Tris–HCl (pH 7.4) and 1.5 M NaCl. DNA was then blotted onto Hybond-XL membrane (GE Health care) in 20× SSC buffer (3 M sodium chloride, 0.3 M sodium citrate [pH 7.0]).

Total cellular RNA was extracted with TRIzol reagents (Invitrogen), by following the instructions of the manufacturer. Ten microgram of total RNA was resolved in 1.2% agarose gel containing 2.2 M formadelhyde and transferred onto Hybond-XL membrane in 20× SSC buffer.

For the detection of HBV DNA and RNA, membranes were probed with either an α-$^{32}$P-UTP (800 Ci/mmol, Perkin-Elmer) labeled minus or plus strand specific full-length HBV riboprobe, respectively. Hybridization was carried out in 5 ml EKONO hybridization buffer (Genotech) with 1 h pre-hybridization at 65 °C and overnight hybridization at 65 °C followed by a 1 h wash with 0.1× SSC and 0.1% SDS at 65 °C. The membrane was exposed to a phosphoimager screen and hybridization signals were quantified with QuantityOne software (Bio-Rad).

To produce riboprobes for the detection of AdGFP DNA and GFP mRNA, pEGFP-C1 vector (Genebank accession no:

U55763) was used as template for PCR amplification of EGFP open reading frame with forward primer 5′-GCTAGCGCTA CCGGTCGCCA C-3′ (nt 591–611) and reverse primer 5′-ACTTGTACAG CTCGTCCATG C-3′ (nt 1330–1310). A 740 bp fragment was then cloned into pGEM-T Easy vector (Promega) and linearized with Sph I for SP6 driven in vitro transcription.

### 2.4. Cell transfection and reporter assay

MDBK cells were trypsinized and washed with complete DMEM medium once and serum-free DMEM/F12 medium trice. Cells were then suspended in serum-free DMEM/F12 medium in a concentration of $10^7$ cells/ml. To 200 μl of cell suspensions in an electroporation cuvette (0.2-cm gap; BTX, San Diego), 20 μg of plasmid pCMVHBV were added. The cells were immediately electroporated with an ECM 630 apparatus (BTX) set to 200 V and 1000 μF. After electroporation the cell suspension was kept for 5 min at room temperature and then diluted into DMEM supplemented with 10% fetal bovine serum and nonessential amino acids and seeded into two 60-mm diameter petri dishes (Guo et al., 2001). After 24 h culture, media were changed and cells were left untreated or treated with 1000 IU/ml of IFN-α. Cells were harvested at the indicated times before and after treatment. The levels of input plasmid DNA and HBV mRNAs were determined by Southern and Northern blot hybridization, respectively.

For reporter assay, MDBK cells were seeded into 24-well plates at a density of $1 \times 10^5$ cells per well and 24 h later, cells were transfected with 0.8 μg of plasmid pCMV-Rluc (Promega) per well with lipofectamine 2000 by following the manufacturer's directions. Transfected cells were treated with indicated concentrations of IFN-α for 24 h and cells were then lysed and Renilla luciferase activities in the cell lysates were determined with a luciferase assay kit (Promega).

### 2.5. FACS analysis

Cells were seeded into six-well plates at a density of $10^6$ cells per well and infected with AdGFP at m.o.i. of one. Cells were left untreated or treated with 1000 IU/ml of IFN-α starting at the time of infection or 1 day post-infection. Cells were trypsinized daily post-infection, washed with PBS and fixed with 2% paraformaldehyde. Intracellular GFP expression was analyzed by FACS analysis (FACScan).

## 3. Results

### 3.1. HBV core associated DNA is reduced in AdHBV infected cells incubated with IFN-α

The molecular pathway of HBV replication initiated by the infection of adenoviral vector (Ad5 ΔE1/ΔE3) containing replication component 1.3-fold genome length HBV DNA (AdHBV) is illustrated in Fig. 1. Upon infection of cells, AdHBV DNA is delivered into nucleus and serves as a transcription template for HBV pregenomic (pg) RNA and subgenomic RNAs under the direction of CMV IE promoter and authentic HBV L, M/S and X promoters, respectively. The viral pgRNA is translated to produce both the core protein and the DNA polymerase. The DNA polymerase binds to the epsilon sequence within the pgRNA to prime viral DNA synthesis and initiate nucleocapsid assembly. Subsequently the viral polymerase converts the pgRNA into relaxed circular (rc) DNA. The nucleocapsids mature as rcDNA is formed and can be enveloped and secreted out of cells (Seeger and Mason, 2000).

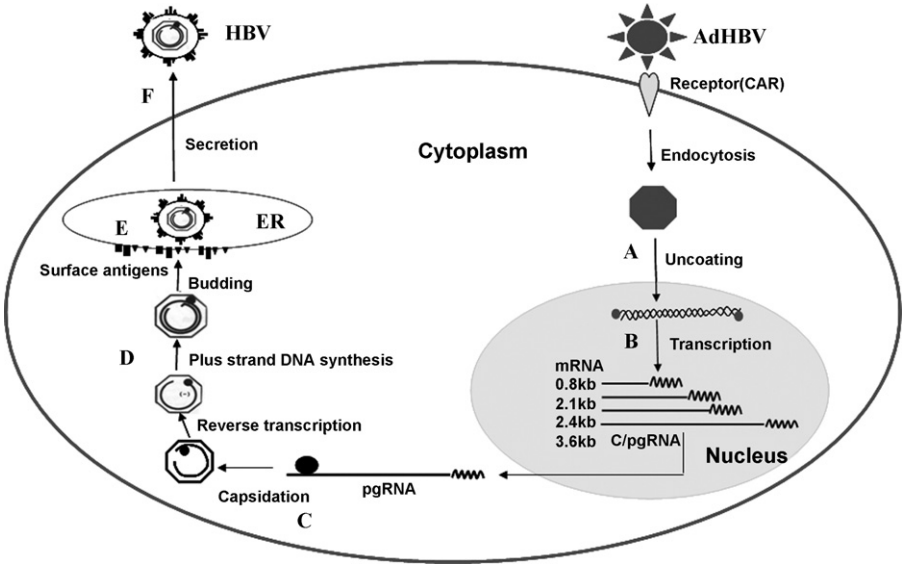


Fig. 1. Schematical representations of recombinant adenovirus vector infection and HBV replication cycle. Replication defective recombinant adenovirus AdHBV infects cells and delivers its genomic DNA into nucleus (A). AdHBV genome serves as a transcription template to transcribe HBV pgRNA and subgenomic RNA L, M/S under the direction of CMV IE and authentic HBV L, M/S promoters (B). HBV RNAs are transported into cytoplasma and synthesize viral proteins. The DNA polymerase then binds to pgRNA to initiate HBV nucleocapsid (core) assembly (C). Subsequently the viral polymerase converts the pgRNA into relaxed circular (rc) DNA (D). The nucleocapsids mature as rcDNA is formed and can be enveloped (E) and secreted out of cells (F).

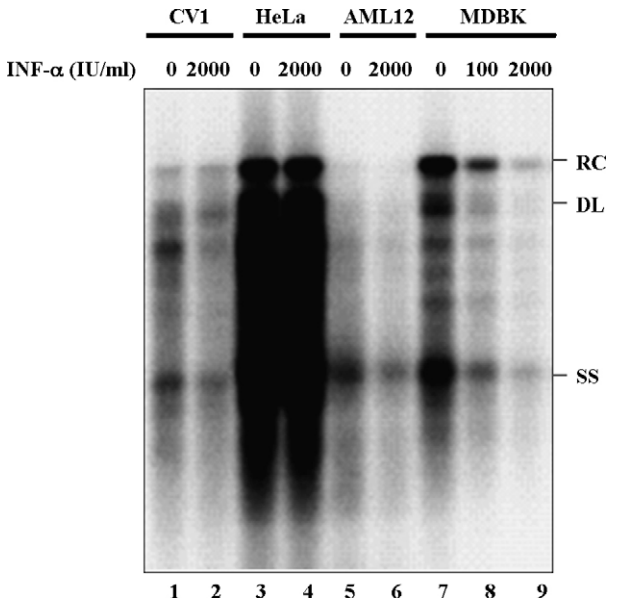


Fig. 2. IFN-α treatment reduced the level of HBV DNA replication intermediates in AdHBV infected MDBK cells. CV-1, HeLa, AML-12 and MDBK cells were seeded in to six-well plates at a density of $10^6$ cells per well and cells were infected with AdHBV. Twenty-four hours after infection, cells were left untreated or treated with indicated concentration of IFN-α for 2 days. The level of intracellular HBV core DNA was determined by Southern blot hybridization. RC, relaxed circular DNA. DL, double-stranded linear DNA; ss, single-stranded DNA.

With an adenovirus vector that could deliver a replication competent HBV genome into different cell types, African green monkey kidney cell line CV1, human cervical cancer cell line (HeLa), immortalized mouse hepatocytes AML12 and Madin–Darby bovine kidney (MDBK) cells were infected and the degree to which HBV replication was inhibited by IFN-α was determined. Although AdHBV vector could infect all four cell lines and initiate HBV DNA replication, albeit, at a different efficiency, IFN-α could only efficiently reduce the level of HBV DNA replicative intermediates in AdHBV infected MDBK cells (Fig. 2).

### 3.2. Steady-state levels of HBV mRNA are reduced by IFN-α in AdHBV infected MDBK cells

The observed reduction of HBV DNA in IFN-α treated MDBK cells could be due to either direct inhibition of HBV replication or inhibition of viral RNA transcription from recombinant adenovirus genome by IFN-α-induced cellular antiviral response. To distinguish between these possibilities, AdHBV-infected MDBK cells were left untreated or treated with IFN-α and the HBV DNA polymerase inhibitor lamivudine either alone or in combination for 2 days. Cytosolic capsid associated HBV DNA and viral mRNA were analyzed by Southern and Northern blot hybridization, respectively. As expected and shown in Fig. 3, lamivudine inhibits HBV DNA replication in dose-dependent manner, but leaves HBV mRNA transcription not detectably affected (lanes 3–5).

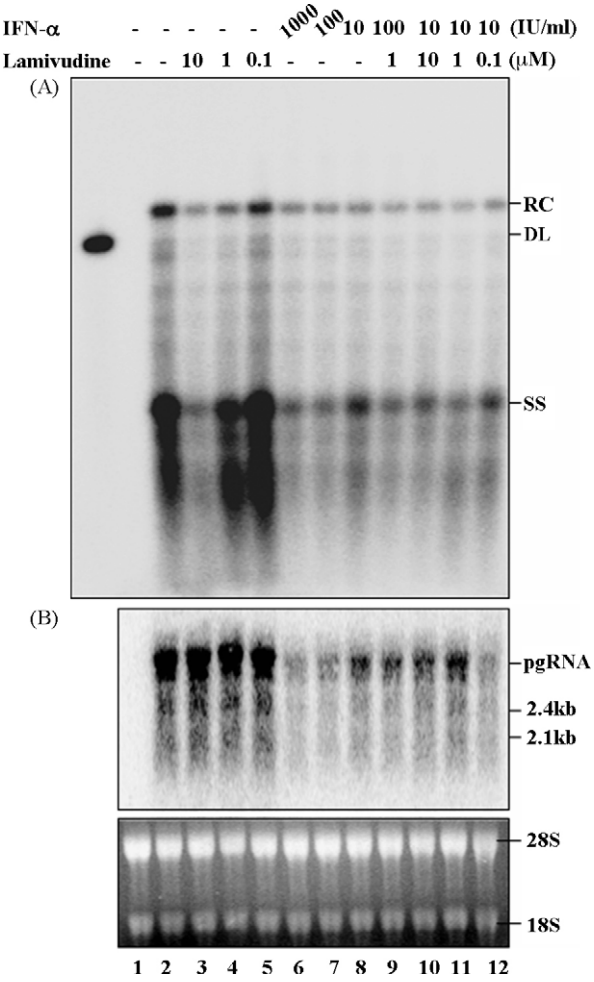


Fig. 3. IFN-α reduced the amount of HBV RNA in AdHBV infected MDBK cells. MDBK cells were seeded in to six-well plates at a density of $10^6$ cells per well and cells were infected with AdHBV. Twenty-four hours after infection, cells were left untreated or treated with indicated concentration of IFN-α, lamivudine or in combination for 2 days. The levels of intracellular HBV core DNA (A) and HBV RNA (B) was determined by Southern and Northern blots hybridization, respectively. RC, relaxed circular DNA. DL, double-stranded linear DNA; ss, single-stranded DNA. Ribosomal RNA served as loading control. Lane 1 is loaded with DNA or RNA extracted from uninfected MDBK cells.

Consistent with previous results, IFN-α efficiently reduced HBV DNA levels. However, HBV RNA levels also decreased in comparison with untreated control (lanes 6–8). Under the combination treatment of lamivudine and IFN-α (lanes 9–12), levels of both HBV DNA and RNA were reduced. Interestingly, the reductions of HBV mRNA levels in the cells under the combination treatment are less profound than that in the cells under IFN-α treatment alone. This result is consistent with our previous finding that lamivudine treatment delays HBV RNA decay in HepAD38 cells (Zhou et al., 2006). The underlined mechanism of this observation is not yet known. Nevertheless, these results implied that the inhibition of HBV DNA replication in AdHBV infected MDBK cells by IFN-α is secondary to the effects of reduced viral RNA transcription.

### 3.3. IFN-α does not inhibit the transcription activity of CMV IE promoter

HBV pregenomic RNA (3.6 kb) transcription from the recombinant adenovirus genome is under the control of a CMV IE promoter. The subgenomic viral RNAs (2.4 and 2.1 kb) for HBV envelope proteins are regulated by authentic HBV L and M/S promoters. To determine if IFN-α could directly inhibit the transcription of CMV IE and HBV L and M/S promoters, MDBK cells were transfected with a plasmid pCMVHBV and 24 h later, left either untreated or treated with IFN-α for an additional 72 h. Intracellular plasmid DNA and HBV transcripts were measured by Southern and Northern blot hybridization. The results demonstrated that, even in the absence of IFN-α, the transfected plasmid DNA was eliminated from cells very quickly and consequently, the steady-state levels of HBV RNA transcripts also declined rapidly. Interestingly, IFN-α treatment seemed to neither accelerate plasmid DNA degradation nor inhibit HBV RNA transcription (Fig. 4A and B).

To further confirm these observations, MDBK cells were transfected with plasmid pCMV-Rluc (Promega), which expresses Renilla luciferase regulated by a CMV IE promoter. Twenty-four hours post-transfection, cells were left untreated or treated with 10–1000 IU/ml of IFN-α for 24 h and luciferase activity in cell lysates was measured. The results again showed that IFN-α did not significantly inhibit the transcription activity of CMV IE promoter (Fig. 4C).

### 3.4. INF-α treatment of AdHBV infected MDBK cells reduces adenovirus DNA in dose- and time-dependent manners

The results presented above implied that the inhibition of IFN-α on HBV mRNA transcription in AdHBV-infected MDBK cells could be due to a change of nuclear structure that renders adenovirus vector genomes unsuitable as transcription templates. Alternatively, adenovirus genomic DNA could have been eliminated from the nuclei of infected cells. To distinguish between these two possibilities, MDBK cells were infected with AdHBV and 40 h after infection, cells were treated with 1000, 100, 10 IU/ml of IFN-α and 10, 1 μM lamivudine, respectively, for 48 h. The levels of nuclear adenovirus DNA, cytoplasmic HBV DNA and HBV RNA were determined by hybridization analysis. Consistent with the results presented above, lamivudine treatment affected neither adenovirus DNA nor HBV RNA transcription, but did effectively inhibit HBV DNA replication (Fig. 5). In marked contrast, IFN-α indeed reduced the levels of nuclear and cytoplasmic adenovirus DNA (Fig. 5A upper bands and Fig. 5B), and HBV RNA and HBV DNA (Fig. 5A and C).

To further characterize the effects of IFN-α on AdHBV DNA, a time course study was performed. MDBK cells were infected with AdHBV and IFN-α treatments were started at 1 day before infection, at the same time of infection, at 1 day or 2 days after infection, respectively. Cells were harvested at the indicated time points post-infection and the amount of adenovirus DNA, HBV RNA transcription and DNA replicative intermediates were determined as described above. As shown in Fig. 6, we found that without IFN-α treatment, HBV RNA transcription and DNA replication increased over 3 days, and adenovirus DNA could be detected in both cytoplasm and nuclei. On the other hand, incubation with IFN-α before or at the time of infection completely prevented HBV RNA transcription and DNA replication, and nuclear adenovirus DNA was drastically decreased. IFN-α treatment since 1 or 2 days after AdHBV infection prevented further accumulation of HBV DNA in infected cells, and efficiently reduce adenovirus DNA and viral mRNA (Fig. 6). This phenomenon is best illustrated in cells treated with IFN-α since 2 days after AdHBV infection. As shown in Fig. 6, while the levels of both HBV RNA and DNA are increased from days 2–3 after AdHBV infection (comparing lanes 2 and 3 in Fig. 6A and C) and nuclear AdHBV vector DNA remains at similar levels (comparing lanes 2 and 3 in Fig. 6B) in untreated cells, IFN-

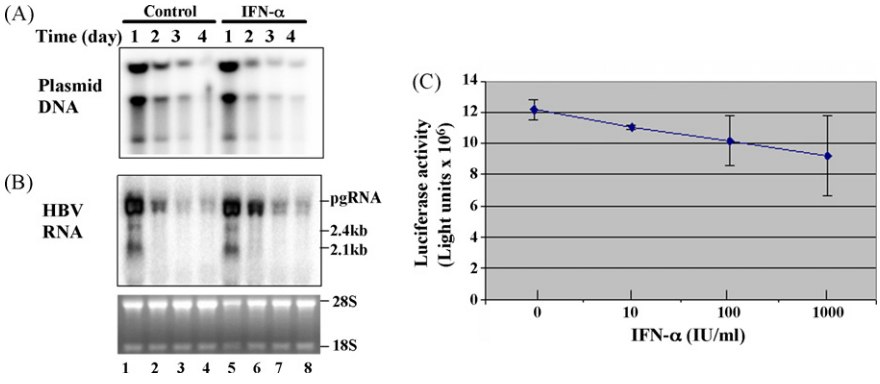


Fig. 4. IFN-α did not inhibit CMV IE promoter directed transcription in MDBK cells. MDBK cells were transfected with plasmid pCMVHBV and 24 h post-transfection, cells were left untreated or treated with 1000 IU/ml of IFN-α. Cells were harvested at the indicated times before and after treatment. The levels of input plasmid DNA (A) and HBV mRNAs (B) were determined by Southern and Northern blot hybridization. Ribosomal RNA served as loading control. (C) MDBK cells were transfected with plasmid pCMVRluc and 24 h post-transfection, cells were left untreated or treated with the indicated concentration of IFN-α for additional 24 h. Cells were harvested and luciferase activity in cell lysates was determined. The average light units from three individual samples and standard derivation were plotted.

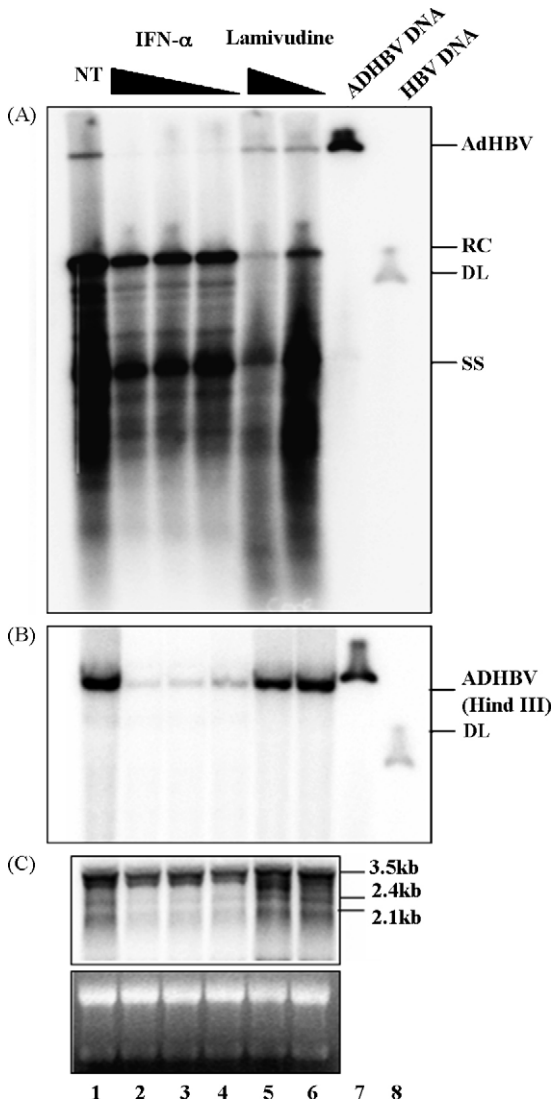


Fig. 5. IFN-α treatment efficiently reduced the levels of AdHBV genomic DNA in MDBK cells. MDBK cells were seeded in to six-well plates at a density of $10^6$ cells per well and cells were infected with AdHBV. Forty hours after infection, cells were left untreated (NT, lane 1) or treated with 1000 μM (lane 2), 100 μM (lane 3), 10 IU/ml (lane 4) of IFN-α and 10 (lane 5), 1 μM (lane 6) lamivudine, respectively, for 48 h. The levels of nuclear AdHBV DNA (A), intracellular HBV core DNA (B) and HBV RNA (C) was determined by Southern and Northern blots hybridization, respectively. Purified AdHBV DNA without (lane 7, panel A) or with HindIII digestion (lane 7, panel B) and 50 pg of unit length HBV DNA (lane 8) served as controls. RC, relaxed circular DNA; DL, double-stranded linear DNA; ss, single-stranded DNA; Ribosomal RNA served as loading control.

α treatment profoundly reduces the levels of nuclear AdHBV DNA (comparing lanes 2 and 12 in Fig. 6B). Meanwhile, the levels of HBV replicative intermediates and mRNA are not significantly changed (comparing lanes 2 and 12 in Fig. 6A and C).

Taken together, the results presented above clearly demonstrated that the primary target of IFN-α-induced antiviral program in AdHBV infected MDBK cells is AdHBV genomic DNA. Upon reductions of cell nuclear AdHBV DNA, HBV mRNA transcription and DNA replication are consequently declined.

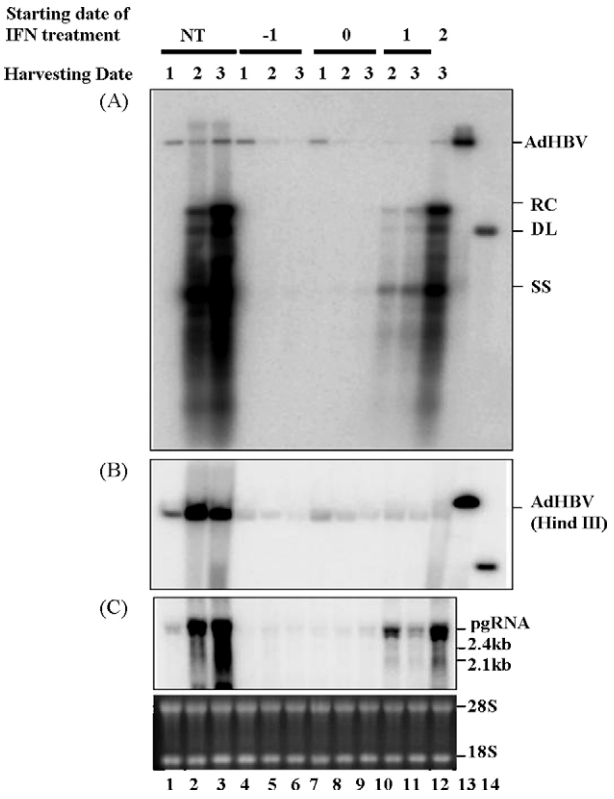


Fig. 6. Time course study of IFN-α-induced reductions of AdHBV DNA. MDBK cells were infected with AdHBV an left untreated (NT, lanes 1–3) or treated with 1000 IU/ml of IFN-α starting at 1 day before infection (lanes 4–6), at the time of infection (lanes 7–9), at 1 day (lanes 10 and 11) and 2 days (lane 12) post-infection. Cells were harvested at indicated time points after infection. The levels of nuclei AdHBV DNA (A), intracellular HBV core DNA (B) and HBV RNA (C) was determined by Southern and Northern blot hybridization, respectively. Purified AdHBV DNA without (lane 13, panel A) or with HindIII digestion (lane 13, panel B) and 50 pg of unit length HBV DNA (lane 14) served as controls. RC, relaxed circular DNA. DL, double-stranded linear DNA; ss, single-stranded DNA. Ribosomal RNA served as loading control.

### *3.5. IFN-α reduces adenovirus vector DNA by noncytolytic mechanisms*

An important question concerning the mechanisms by which IFN-α reduces adenovirus vector DNA is whether IFN-α induces death of AdHBV infected cells. In the previous experiments, we did not observe a significant death of AdHBV infected cells under either untreated or IFN-α treated conditions, as determined by microscopy and cell counting. The total cell numbers and viability of the cells recovered from each time points following adenovirus infection are similar between IFN-α treated and untreated conditions (data not shown). Furthermore, we observed that under IFN-α treatment, the decline of AdHBV DNA was usually faster and more profound than that of HBV core DNA and viral mRNA (Fig. 6, comparing lanes 2 and 12). Because they should reside in same cells, it thus suggests a noncytolytic mechanism of AdHBV DNA elimination by IFN-α.

To further address this issue, we infected MDBK cells with adenovirus vector expressing green fluorescent protein (AdGFP) and left untreated and treated with IFN-α starting at the same

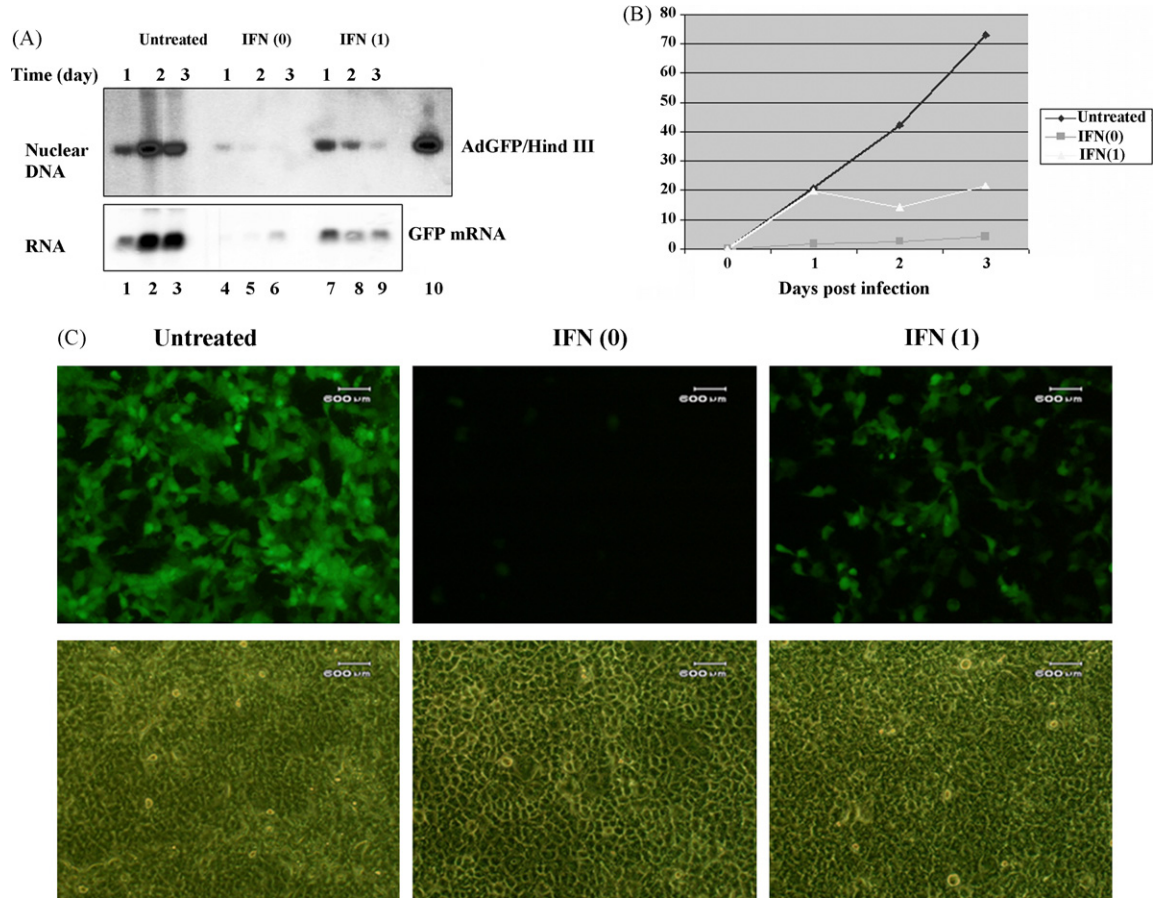


Fig. 7. Effects of IFN-α on AdGFP DNA and GFP expression in MDBK cells. Cells were seeded into six-well plates at a density of $10^6$ cells per well and infected with AdGFP. Cells were left untreated or treated with 1000 IU/ml of IFN-α starting at the time of infection [IFN(0)] or 1 day post-infection [IFN(1)]. Cells were harvested daily after the infection. The levels of nuclei AdGFP DNA, GFP mRNA were determined by Southern (upper panel) and Northern (lower panel) blots hybridization, respectively. HindIII digested AdGFP DNA served as control (lane 10). (B) Cells were trypsinized daily post-infection, washed with PBS and fixed with 2% paraformaldehyde. Intracellular GFP expression was analyzed by FACS analysis and plotted as percentage of GFP-positive cells. (C) Microscopic photographs of untreated and IFN-α treated cells were taken on day 3 after AdHBV infection.

time of infection or 1 day after infection, designated as IFN(0) or IFN(1), respectively. Cells were harvested at 1, 2 and 3 days after infection. Consistent with the previous findings, IFN-α treatment reduced AdGFP DNA and GFP mRNA (Fig. 7A). Photographs taken on day 3 after AdGFP infection clearly showed that IFN-α treatment starting at the same time of infection essentially prevented AdGFP infection and the number of GFP positive cells were reduced by IFN-α treatment starting at 1 day after infection. Importantly, cell morphology was similar between untreated and IFN-α treated cells (Fig. 7C). FACS analysis results showed that the fraction of GFP positive cells gradually increased in untreated cells from 20% at 1 day to 72% at 3 days post-infection. IFN-α treatment starting at the time of infection prevented AdGFP infection [IFN (0)]. Interestingly, IFN-α treatment starting at 1 day after infection [IFN (1)] only prevented the further increase of GFP positive cell numbers (Fig. 7B), suggesting that IFN-α treatment does not kill the AdGFP infected cells. Moreover, these results suggest that HBV proteins and replication do not play a role in IFN-α-induced reduction of adenovirus vector DNA in MDBK cell.

## 4. Discussion

The work presented in this report demonstrates that IFN-α treatment of adenovirus vector infected MDBK cells greatly reduces the input adenovirus vector genomes in a noncytopathic way. As a consequence, transgene expression is inhibited. Our data suggest that IFN-α-induced antiviral program is able to discriminate host cellular DNA from extra-chromosomal viral DNA and might represent a novel pathway of interferon mediate innate defense against DNA virus infections.

Wild-type adenoviruses are relatively resistant to IFN-α (Kajon and Spindler, 2000). This resistance is, at least in part, because viral E1A protein and VA RNA 1 can inhibit IFN signal transduction and PKR activity, respectively (Joseph and Look, 2001; Reich et al., 1988; Reichel et al., 1985). However, it was reported that IFN-α could inhibit DNA replication of the viruses in cultured cells (Doucas et al., 1996). Effects of the type I IFNs on adenovirus vectors have not been evaluated. It had been reported that IFN-γ and TNF-α inhibited transgene transcription of adenovirus vectors in cell cultures and mice in a virus-derived promoter-dependent manner (Kafri et al., 1998; Otake et al.,

1998; Sung et al., 2001). To our knowledge, data presented here provide the first direct evidence that IFN-α could inhibit transgene expression of adenovirus vector in certain type of cells, by reducing the amount of adenovirus vector genomes.

Ironically, adenovirus vectors had been used to express IFN-α, -β and -γ in cultured cells and in vivo for the treatment of viral diseases and cancer (Shin et al., 2005; Suzuki et al., 2003). Pharmacokinetic studies indicated that intravenous administration of adenovirus vectors containing human IFN-α2b gene directed by CMV immediate early promoter resulted in dose-dependent serum IFN-α concentrations that persisted 8–40 days with similar concentration–time profiles in rats, rabbits and chimpanzees. Furthermore, the persistence of serum IFN-α concentrations was over 200 days in beige/SCID immunodeficient mice (Demers et al., 2002). Adenovirus vectors mediated IFN-α gene therapy had been attempted to prevent and treat virus infections with limited success (Wu et al., 2003). For example, Aurisicchio and colleagues reported that infections of tamarins with a helper dependent adenovirus vector expressing tamarin IFN-α under the control of the tetracycline inducible transactivator rtTA2$^{s}$-S2 produced IFN-α upon administration of doxycycline and could delay, but not abrogate, the following infections of GB virus B (Aurisicchio et al., 2005).

The inhibition of transgene expression and elimination of adenovirus DNA by IFN-α was only observed in MDBK cells, and not in three other cell lines tested (Fig. 2 and data not shown). Due to the weak antiviral effects of human IFN-α2b in mouse cells, it is not clear at this time if mouse IFN-α could reduce adenovirus vector DNA in immortalized mouse hepatocytes (AML12 cells). To determine the effects of IFN-α on adenovirus DNA in human hepatocyte-derived cells, we have also tested the effects of IFN-α on AdHBV infection with human hepatoma cell line HepG2 and demonstrated that IFN-α does not reduce the levels of HBV RNA and DNA, and also has no effect on Ad vector DNA (data not shown). Despite our results seem indicating that elimination of adenovirus vector DNA from infected cells might represent a cell-type specific phenomenon, it does imply that under the selected conditions, interferon-induced antiviral programs are capable of purging latent viral DNA genome to cure infected cells.

It has been well documented that induction profile of IFN-stimulated genes (ISGs) by IFN-α is cell type specific (Schlaak et al., 2002). Furthermore, antiviral effects of IFN-α to a given virus, such as VSV, vary on different cell types (Keskinen et al., 1999). Hence, a reasonable explanation to our observations is that the cellular gene product(s) that mediate the reduction of Ad vector DNA are only induced in MDBK cells by IFN-α, but not other cells tested. It is of great interest to systematically compare the ISG expression profiles among those cells under the IFN-α treatment in the future studies to elucidate the underlined molecular mechanisms.

Interestingly, the work published by Chisari and colleagues indicated that HBV covalently closed circular (ccc) DNA, a stable replication intermediate of HBV replication and transcription template for viral RNA, could be noncytolytically eliminated from HBV infected hepatocytes during the clearances of transient HBV infections of chimpanzees, presumably mediated by interferons- and/or other cytokines-activated intracellular antiviral response (Wieland et al., 2004). Hence, it implies that nuclear eipsomal viral DNA could be targeted by intracellular antiviral programs.

An important question that remains to be answered is how IFN-α-induced antiviral response eliminates adenovirus vector DNA from the nuclei of MDBK cells. In this study, we noticed that transfected plasmid DNA in MDBK cells is unstable and its half-life is approximately 12 h (Fig. 4A and data not shown). In marked contrast, in the absence of IFN-α, AdHBV DNA, resulting from infection of the AdHBV vector, is apparently stable over 5-day period of time (data not shown). Furthermore, the amount of adenovirus vector DNA in the nuclei of infected cells were increased about four to eightfold in first 2 days and reached plateau at approximately 48 h post-infection (Figs. 6 and 7). Despite we could not rule out the possibility of a slow uncoating and/or nuclear translocation of adenovirus genomes in these cells, the replication of vector DNA remains a possibility. It has been shown that many cultured mammalian cells could support a limited degree of DNA replication of first generation adenovirus vector genomes (Nelson and Kay, 1997). However, Kay and colleagues demonstrated that persistence of adenovirus vector genome in mouse hepatocytes does not depend on viral DNA replication (Nelson and Kay, 1997). Furthermore, they showed that viral terminal protein (TP) prolongs Adenovirus vector persistence in cultured cells and in vivo (Lieber et al., 1997). Because adenovirus terminal protein (TP) mediates the nuclear matrix attachment and efficient transcription of Ad genome (Angeletti and Engler, 1998; Fredman and Engler, 1993; Schaack et al., 1990), it is, therefore, possible that IFN-α eliminates adenovirus vector DNA by interrupting the interaction of viral genome with nuclear matrix, which render the adenovirus vector DNA vulnerable to constitutive cellular mechanisms that degrade extra-chromosomal DNA, in much the same way the free plasmid DNA is degraded.

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