

2- Results

2.1- Suppression of HBV replication following cytokine gene transfer

Adenoviral vectors carrying a cassette, which contains mIFN γ and mIFN β genes driven by CMV promoter in an E1A/E1B deletion (fig.4) were generated and employed in this study. As shown fig.4, a GFP reporter gene was also expressed under control of a second CMV promoter in AdGmIFN β , but not in AdmIFN γ . In order to apply cytokine gene therapy to HBV infection, the aim in this first section 2.1.1 was to show that cytokine genes were expressed and secreted. Their biologic functions were also tested in mouse hepatocytes culture and in mouse, if it is possible. In the second section 2.1.2, the effect of mIFN γ and mIFN β expression on HBV replication in mouse hepatocytes culture was determined, and followed by the characterization of this inhibiting effect in HBV transgenic mice in section 2.1.3.

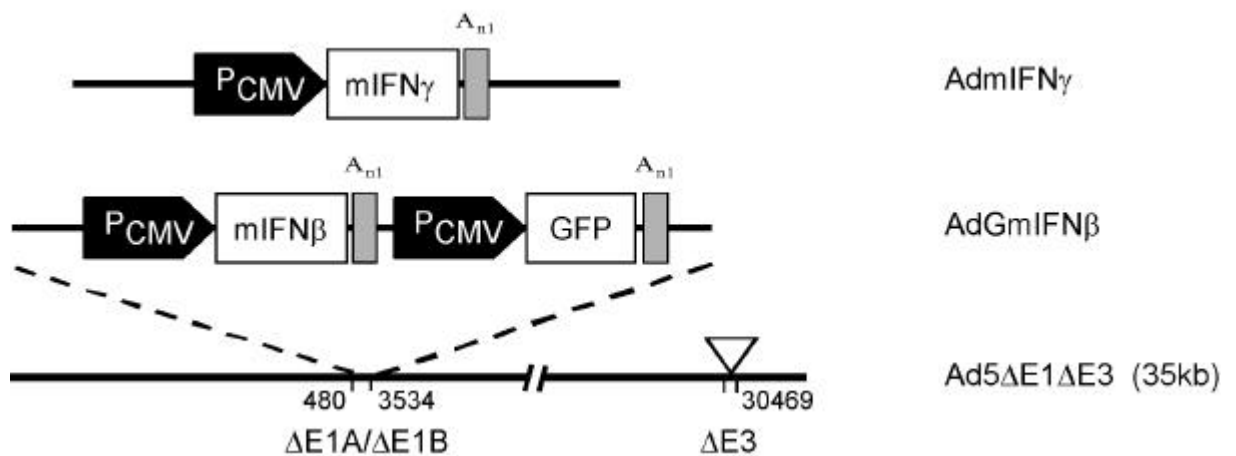


fig. 4: schematic presentation of adenoviral vectors for constitutive expression of type I and II interferon. Genes coding for mouse interferon gamma (mIFN γ) or interferon beta (mIFN β) driven by a cytomegalovirus promoter (P_{CMV}) were inserted into E1A/E1B deletion of human adenoviral type 5 genome. An1 corresponds to the SV40 polyadenylation site. In construct AdGmIFN β a second cassette for GFP expression upstream from gene coding for mIFN β was inserted. AdmIFN γ was kindly provided by T. Ritter, Charité, Berlin.

2.1.1- Expression and secretion of cytokine following adenoviral transduction

2.1.1.1- Detection of secretion of mIFN γ using mIFN γ -specific ELISA

Secretion of mIFN γ from mouse hepatocytes transduced with AdmIFN γ

Type II interferon is normally not expressed in hepatocytes, but in immune cells such as macrophages, T-lymphocytes and B-lymphocytes. To determine the secretion and functional activity of mIFN γ , mouse hepatocytes AML12 (Wu et al., 1993) were infected with AdmIFN γ at moi of 10 i.u./cell. The supernatant was analyzed by an established enzyme-linked immunosorbent assay (ELISA) specific for mIFN γ (table 3). A strong secretion of mIFN γ was monitored up to 3 days p.i. at a level of 219-444 I.U./L from hepatocytes transduced with AdmIFN γ but not from hepatocytes transduced with mock adenoviral vectors, AdGH1.3 and AdGFP. Attempts to detect secreted mIFN γ in mouse serum following i.v. injection of 1×10^9 i.u. of AdmIFN γ were not successful. The level was below the detection limit (40 pg/ml) of the assay (data not shown). Short half life time of IFN γ made it impossible to establish a direct quantitative approach for determining the secretion of mIFN γ *in vivo*.

	Secretion of mIFN γ (IU/liter)		
	AdmIFN γ	control	
		AdGH1.3	AdGFP
day 2 p.i.	3	0	0
day 3 p.i.	219	0	0
day 4 p.i.	444	0	0

Table 3: Secretion of mouse IFN γ by transduced AML12 cells with AdmIFN γ .

ELISA analysis of cell culture supernatants for interferon gamma. The amount of mIFN γ (I.U./ml) was determined relative to standard with recombinant mIFN γ .

Comparison of secretion level of constitutive expression and tet-inducible expression of mIFN γ *in vitro*.

The mouse AML12 cells were infected with AdmIFN γ at increased moi and the titer of secreted mIFN γ was measured 2 days p.i. in supernatant. Human hepatoma cells expressing transactivator, which was described in section 2.2.1.1.2, were transduced with an adenovirus, which mediated tet-inducible mIFN γ expression, at a moi of 5 i.u. per cell. After 2 days induction of mIFN γ expression, the level of secreted mIFN γ in

supernatant was measured in the same ELISA assay. The level of mIFN γ from transduced AML12 cells with AdmIFN γ at moi of 5 i.u. per cell corresponded to the level of fully induced mIFN γ expression in transduced human hepatoma cells.

2.1.1.2- Indirect detection of secretion of mIFN α and mIFN β by induction of 2',5'-oligoadenylate synthetase (2'5'OAS) transcription

To prove that type I interferons were secreted and functional active, no established ELISA for mouse IFN β and IFN α were available. Protein 2',5'-oligoadenylate synthetases (2'5'OAS) are a family of interferon-induced enzymes, which are responsible for the antiviral states established in cells in response to viral infection (Chebath et al., 1987; Sen and Ransohoff, 1993). Using Northern blot analysis, induction of 2'5'OAS transcription by mIFN α and mIFN β was detectable.

Induction of 2'5'OAS transcription by secreted mIFN β provided from transduced mouse hepatocytes with AdGmIFN β

As show the fig.5a, the transduction of AML12 cells with AdGmIFN β induced a strong transcription of 2'5'OAS after 4 days, which was not detected in cells transduced with AdGFP or AdvIL10. Incubation of AML12 cells with supernatant containing mIFN β also activated transcription of 2'5'OAS unlike supernatants called vIL10, GFP or just medium (fig.5a).

Induction of 2'5'OAS transcription in mouse hepatocytes by secreted mIFN α and mIFN β following a full tet-induction of mIFN α and mIFN β expression.

HuH7 cells expressing a transactivator, which was described in section 2.2.1.1.2, were transduced with recombinant adenoviruses, which mediated tet-inducible mIFN α , mIFN β or GFP expression (see section 2.2.1 for description of these vectors). Following a full tet-induction of gene expression, the supernatant was transferred onto mouse AML12 cells. As shown the fig.5b, a strong transcription of 2'5'OAS was observed in AML12 cells treated with supernatants containing secreted mIFN α or mIFN β and weakly by cells treated supernatant provided from cells with tet-induced GFP expression, called in fig.5b GFP. No definite explanation for the latter unexpected observation was found. Because the homology of mouse IFN α or IFN β to human

interferon is only 40%, no induction of 2'5'OAS transcript was observed in transduced HuH7 cells expressing a transactivator.

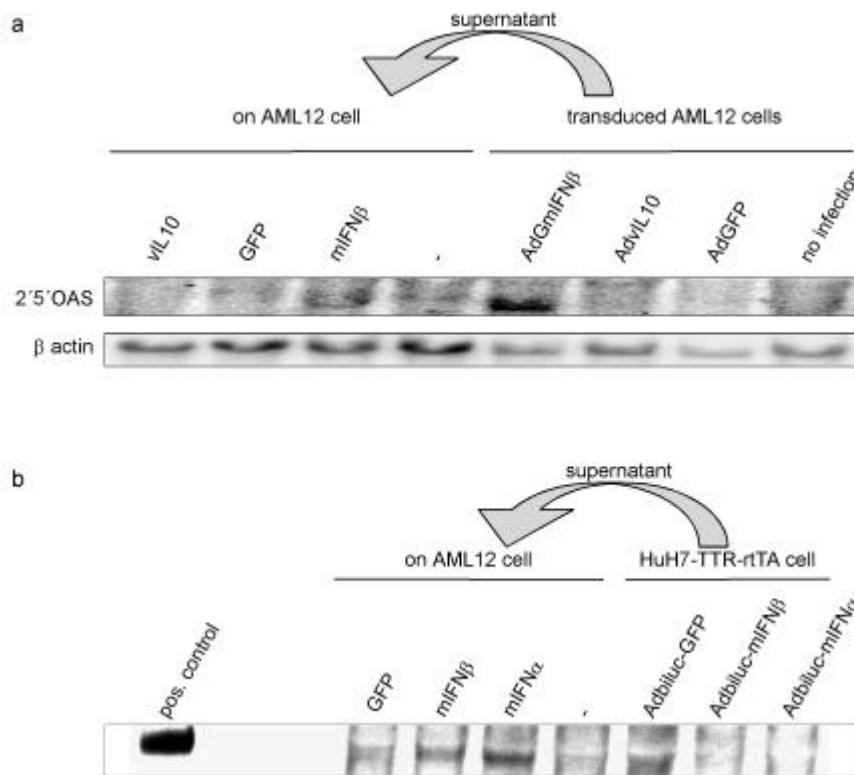


fig. 5: Induction of 2'5'OAS transcript by incubation with cell culture medium containing secreted cytokines.

(a) AML12 cells were infected with recombinant adenoviruses (AdGFP, AdvIL10, AdGmIFN β) at moi of 10 i.u./cell. The cell culture medium, called here supernatant, was collected each days and transferred onto new AML12 cells. At day 4 p.i., total RNA was extracted from cell lysate and used for Northern blot analysis with $\alpha^{32}\text{P}$ labeled probe specific for mouse 2'5'OAS. To normalize the amount of loaded RNA, the membrane was hybridized with $\alpha^{32}\text{P}$ labeled probe specific for mouse β actin.

(b) HuH7 cells expressing a transactivator, called HuH7-TTR-rtTA cell (see section 2.2.1.1.2), were infected with indicated recombinant adenovirus (Adbiluc-GFP, Adbiluc-mIFN α and Adbiluc-mIFN β see section 2.2.1) at moi of 10 i.u./cell. Following full tet-induction of gene expression, the supernatant was collected each day and was transferred onto mouse AML12 cells. At day 4 p.i., total RNA was extracted from cell lysate and used for Northern blot analysis with $\alpha^{32}\text{P}$ labeled probe specific for mouse 2'5'OAS.

2.1.1.3- Indirect measurement of secretion of interferon by detection of induction of IP10 transcription *in vitro* and *in vivo*.

Since measurement of mIFN γ secretion in sera or in liver homogenate of mice transduced with AdmIFN γ was not possible using mIFN γ specific ELISA, a indirect detection was employed using real-time RT PCR method with liver homogenate. It is

shown, that IFN γ mediates induction of CXX chemokine IFN γ -inducible protein-10 (IP-10/CXCL10, abbreviated here as IP10) (Luster et al.,1993; Soejima and Rollins, 2001). In collaboration with T. Giese (Department of Immunology, University of Heidelberg, Germany), the induction of IP10 transcription was quantified in IP10 mRNA copies per ml of cell or liver homogenate normalized to GAPDH and LHD gene transcription.

Induction of IP10 transcription by secreted mIFN β and mIFN γ in cell culture

To prove that cytokines were secreted and functional active, primary mouse hepatocytes (PMH) were prepared from NMRI mouse. The PMH were infected with adenoviruses

Table 4: Induction of interferon-inducible gene IP10 in primary mouse hepatocytes (PMH) culture transduced with AdmIFN γ or AdGmIFN β .

	IP10 mRNA copies per copies of GAPDH gene
transduced PMH with	
adenovirus control	4538
AdGmIFN β	19840
AdmIFN γ	105873
incubation with supernatant containing:	
control	2350
mIFN β	1488
mIFN γ	67517

Primary mouse hepatocytes (PMH) isolated from liver of MNRi mouse were infected with recombinant adenovirus (AdGmIFN β , AdmIFN γ or AdShuttle as control) at moi of 10 i.u. per cell. The cell culture medium, called supernatant, was collected each day and transferred into PMH cells from the same preparation. At day 4 p.i. mRNA was extracted from cell lysate and used for real-time RT-PCR detection of increase in IP10 mRNA per ml cell lysate normalized to GAPDH and LDH RNA copies is shown. (the analysis was kindly performed by T. Giese, Dept. of Immunology, University of Heidelberg).

AdmIFN γ , AdGmIFN β or AdShuttle as control. The corresponding cell culture medium was transferred onto non-transduced PMH. As shown in table 4, an empty adenovirus vector, AdShuttle, induced IP10 transcription 2-fold higher as in non-transduced cells. A 4- to 23-fold increase of IP10 transcripts was observed in cells transduced with AdGmIFN β and AdmIFN γ respectively. The effect of supernatant containing mIFN β on

IP10 transcription was minor (1,5-fold), whereas secreted mIFN γ mediated a strong induction (45-fold).

Induction of IP10 transcription by secreted mIFN α , mIFN β and mIFN γ *in vivo*.

To control the activity of secreted cytokine *in vivo*, transgenic mice were injected with 2×10^8 i.u. of recombinant adenovirus. These adenoviral vectors mediated gene transfer into hepatocytes of cytokine gene and reporter gene for luciferase under the control of a tet-regulated bidirectional promoter (see section 2.2.1 and fig.13a for more description of

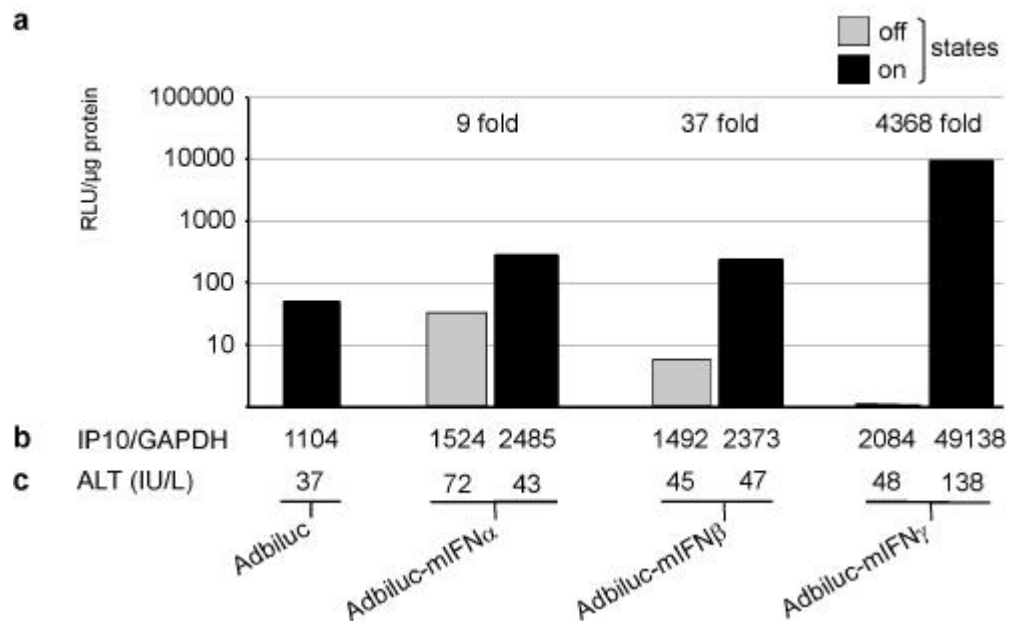


fig. 6: Induction of IP10 transcription and luciferase activity in liver of transgenic mice following full induction of cytokine expression.

Two transgenic mice expressing the rtTA (see section 2.2.1) per group were infected i.v. with 2×10^8 i.u. of recombinant adenoviruses, which mediated transfer of tet-inducible cytokine and luciferase genes co-expression (Adbiluc, Adbiluc-mIFN α , Adbiluc-mIFN β or Adbiluc-mIFN γ). Drinking water supplemented with 2 mg/ml doxycycline inducing the expression of both transgenes (on-state: black bars) At day 3 p.i. the mice were sacrificed. (a) Luciferase activity was performed in homogenate of liver. (b) Total RNA was extracted and employed for real-time RT-PCR detection of IP10 mRNA copies per ml of liver homogenate normalized to GAPDH and LDH transcripts (analysis was performed by T. Giese, Department of Immunology, University of Heidelberg). (c) ALT values in 32 μ l serum of each mouse at time point of sacrifice was indicated.

Adbiluc-mIFN γ , Adbiluc-mIFN β , Adbiluc-mIFN α and Adbiluc as control). As shown the fig.6, cytokine genes were expressed in the on-state via induction of luciferase activity (fig.6a) in liver of mice treated with drug. A strong induction (19-fold) of IP10 transcript and 4368-fold increased of luciferase activity were also detected in liver of the mouse infected with Adbiluc-mIFN γ in the on-state (fig.6a and fig.6b). No significant induction of IP10 transcription was observed, when these mice were infected with control adenovirus Adbiluc or with Adbiluc-mIFN α and Adbiluc-mIFN β in the on-state. The cut-off of IP10 RNA detection was 1100 copies/ml.

In summary, the real-time RT-PCR specific for IP10 proved sufficient for monitoring indirectly the secretion of IFN γ *in vitro* and *in vivo*, as well as of type I interferons by a weaker induction factor than IFN γ . However, a signal amplification by activation of macrophage like Kupffer cells present in primary culture or in liver tissue could not be excluded. No significant elevation of ALT values in sera was observed, except in the mouse with full induction of mIFN γ expression.

2.1.2- Characterization of antiviral effect of cytokine expression on HBV replication in transduced mouse hepatocytes line

At first, the efficiency of adenoviral gene transfer into mouse AML12 cells has to be determined before to characterize any effect of gene expression.

Transduction efficiency onto mouse AML12 cells with adenovirus

Since the ability to transfer gene into mouse hepatocytes with human adenoviral vector type 5 was not as efficient as with HuH7 or HepG2 cells (personal communication Klöcker U.), the conditions for the adenoviral infection on AML12 cells were performed by a consecutive medium reduction, supplementation of 1,7% DMSO in medium and overnight incubation. The AML12 cells were infected with reporter adenovirus AdGFP at increasing moi's, and as shown fig.7, the percentage of cells expressing GFP was determined by FACS analysis. Infection with AdGFP at moi of 50

i.u./cell targeted about 85% of AML12 cells, almost all the cells were transduced at moi of 100 i.u./cell. Using these conditions AML12 cells were infected with AdGH1.3 L⁻ (Sprinzl M.F. et al., 2001), and the intracellular HBV DNA replication was established 3 days p.i.. HBV DNA content in transduced AML12 cells was estimated at 10-fold lower than in HuH7 cells at the same transduction efficiency (data not shown).

Inhibiting effect of mIFN γ and mIFN β expression on the HBV replication in transduced AML12 cells

To show which effect has cytokine expression on the HBV replication, AML12 cells were co-infected with AdGH1.3L⁻ and AdmIFN γ , AdGmIFN β or AdGFP as control. Seven days post infection, total DNA was extracted from the cell lysate and subjected to dot blot analysis using $\alpha^{32}\text{P}$ -labeled HBV-DNA probe (fig.8a). The dot signal was quantified and was shown as HBV DNA copies per transduced cells (fig.8b). Co-infection with reporter adenovirus AdGFP reduced intracellular HBV DNA (1,5-fold). This effect was 5-to 10-fold by co-infection with AdmIFN γ and up to 5-fold with AdGmIFN β in a dosis dependent

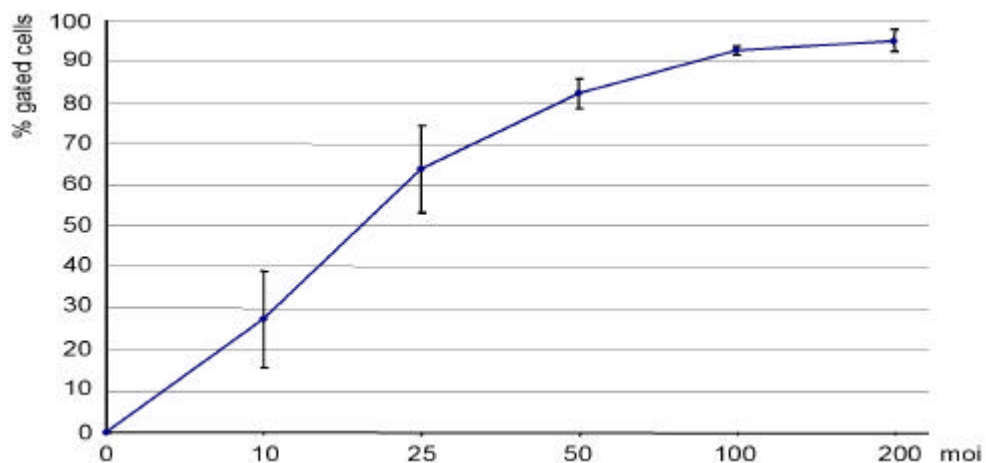


fig. 7: A multiplicity of infection of 100 i.u. of AdGFP per cell was necessary to target almost all AML12 cells.

AML12 cells were infected with AdGFP at indicated moi's. At day 3 p.i., cells were resuspended in PBS for FACS analysis to gated cells for GFP. Percentage of GFP expressing cells (mean \pm standard deviation) in four independent experiments were determined.

manner (fig.8b). An 2-to 5-fold lower amount of HBV DNA was detected in cells treated with supernatant containing secreted mIFN γ or mIFN β . If a second infection with AdGmIFN β 2 days after transduction with AdGH1.3L $^{-}$ was performed, the inhibiting effect of mIFN β on HBV replication was comparable, but not that of mIFN γ (data not shown). Probably, mIFN γ has an antiviral effect only during establishment of HBV replication, and mIFN β activated antiviral pathways in cell acting also on established HBV replication.

To get more closely to an *in vivo* approach, primary mouse hepatocytes (PMH) were prepared and the same experimental setting was employed. As shown fig.9, the strong

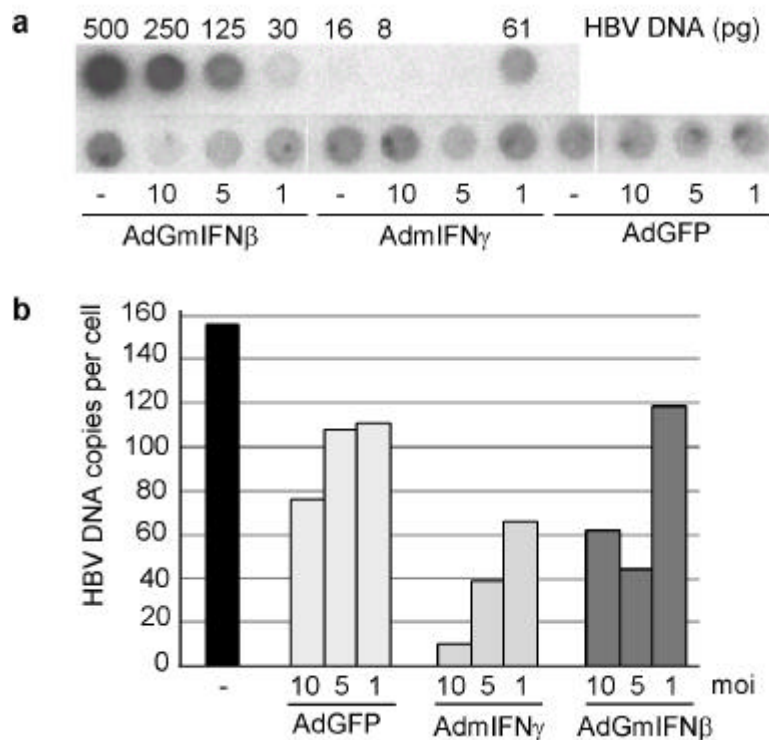


fig. 8: mIFN γ and mIFN β decreased intracellular HBV DNA in transduced AML12 cells in dose dependent fashion.

(a) AML12 cells were co-infected with AdGH1.3L $^{-}$ at moi of 50 i.u./cell and second adenovirus (AdGFP, AdmIFN γ or AdGmIFN β) at moi of 1 to 10 i.u./cell. 80-85% of cells expressed GFP indicating transduction with AdGH1.3L $^{-}$. At day 7 p.i., cells from five independent experiments were lysed and intracellular DNA was extracted from pooled cell lysates. Dot blot analysis was performed using $\alpha^{32}\text{P}$ labeled HBV-specific DNA probe.

(b) Signals of dot were quantified by phospho-imaging. Each bars corresponds to value of HBV DNA copies per transduced cells of five independent experiments at the same condition with unique virus stock preparation.

inhibiting effect of mIFN γ was reproducible with a 10-fold decrease of HBV DNA copies per cell relative to the control set up with AdGFP. A 3- to 5-fold decrease of HBV DNA copies per cell was observed in cell culture co-infected with AdGmIFN β . In this system, any amplification of the antiviral effect by stimulated macrophages present in the liver and primary culture such as Kupffer cells was not observed. In primary cells, a stronger suppression of HBV DNA copies was observed in PMH co-infected with AdGFP (1,5-fold versus 3-fold).

Cytotoxicity assays were performed by crystal violet staining in order to estimate the effect of adenovirus infection on AML12 cells at increasing moi and the action of strong secretion of cytokine under CMV promoter. No significant cytotoxic effects (<10%) were observed in conditions of the experiments in AML12 cells. Purification of adenoviral stock preparations could reduce the cytotoxic effect (data not shown).

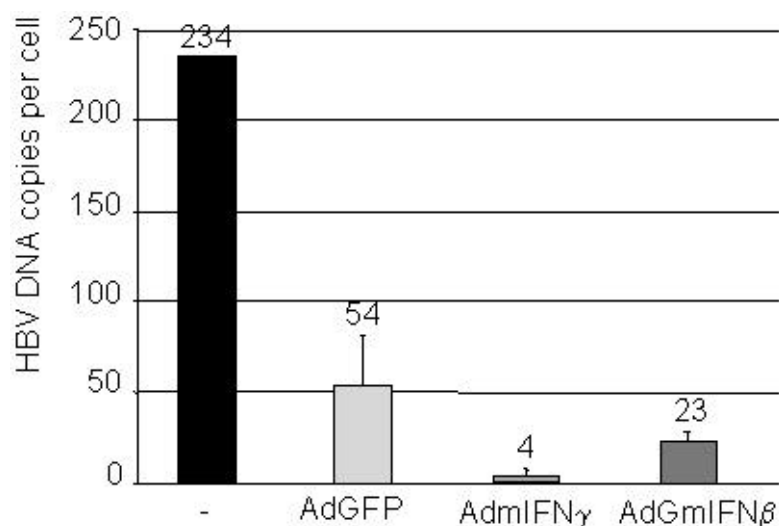


fig. 9: Expression of mIFN γ and mIFN β decreased intracellular HBV DNA in transduced primary mouse hepatocytes (PMH).

PMH were co-infected with AdGH1.3L $\bar{}$ at moi of 50 i.u./cell and with second adenovirus (AdGFP, AdmIFN γ or AdGmIFN β) at moi of 5 i.u./cell. 80% of cells were transduced with AdGH1.3 L $\bar{}$. At day 7 p.i., total intracellular DNA was extracted for dot blot analysis with a $\alpha^{32}\text{P}$ labeled HBV-specific DNA probe. Following quantification by phospho-imaging, each bars corresponded to mean values of HBV DNA copies per cells in two independent experiments with different preparations of PMH.

Taken together, these results show that the genes coding for mIFN γ or mIFN β were expressed and their gene products were secreted and were functionally active. mIFN β and mIFN γ have an antiviral effect on the establishment of HBV infection in mouse hepatocytes cultures (fig.8 and 9). In this *in vitro* model, an inhibitory effect was

observed by the expression of mIFN γ during establishment of HBV replication. In contrast, mIFN β expression also reduced intracellular HBV DNA mainly following establishment of HBV replication. No amplification of antiviral effect of mIFN γ or mIFN β by stimulated Kupffer cells was detected in primary cell culture.

2.1.3- Characterization of the antiviral effect of cytokine expression on HBV replication in HBV transgenic mice

To compare the results obtained in tissue culture with them in mouse, the efficiency of gene transfer into the liver of mice injected i.v. with given dosis of adenoviral vector has to be characterized. It is shown, that 95% of adenovirus reach the liver following i.v. injection (Prevec L. et al., 1989; Jaffe HA. et al., 1992). To characterize this adenoviral gene transfer, increasing doses of adenovirus reporter AdGFP (from 10^8 to 10^9 i.u.) were injected i.v. and the mice were sacrificed at 14 days p.i. The percentage of GFP-expressing liver cells was determined in 12 μ m liver sections. Injection with 10^8 i.u. AdGFP targeted about 10% of the liver cells in comparison with a higher dose of 10^9 i.u. AdGFP, which reached almost all liver cells (data not shown). Thus, increase of the dose of injected adenovirus influenced the efficiency by which liver cells are transduced. In addition, the host immune response to the vector backbone is dependent on the dose of vector used.

After the efficiency of the adenoviral gene transfer into the liver has been determined, the antiviral effect of mIFN γ and mIFN β expression on HBV replication observed in mouse hepatocytes cultures has to be shown and characterized in HBV transgenic mice. To determine which dose of recombinant adenovirus was necessary to repress efficiently hepatic HBV replication, mice were injected i.v. with increased dose of both adenoviral vectors AdmIFN γ and AdGmIFN β in the section 2.1.3.1. Using these acquired data, the time course of the antiviral effect of cytokine expression was shown in section 2.1.3.2 and even the kinetic of mIFN γ secretion indirectly via the detection of induction of IP10 transcription in the liver in the section 1.3.3 of this study.

2.1.3.1- Antiviral effect on HBV replication dependent on the dose of the adenoviral vector

The aim was to investigate an antiviral effect of cytokine expression on HBV replication in homozygous HBV 1.3 fsX(-)3'5' transgenic mice (Weber O. et al., 2002). Nine groups (three mice per group) of age-, sex-, and serum HBeAg-matched transgenic

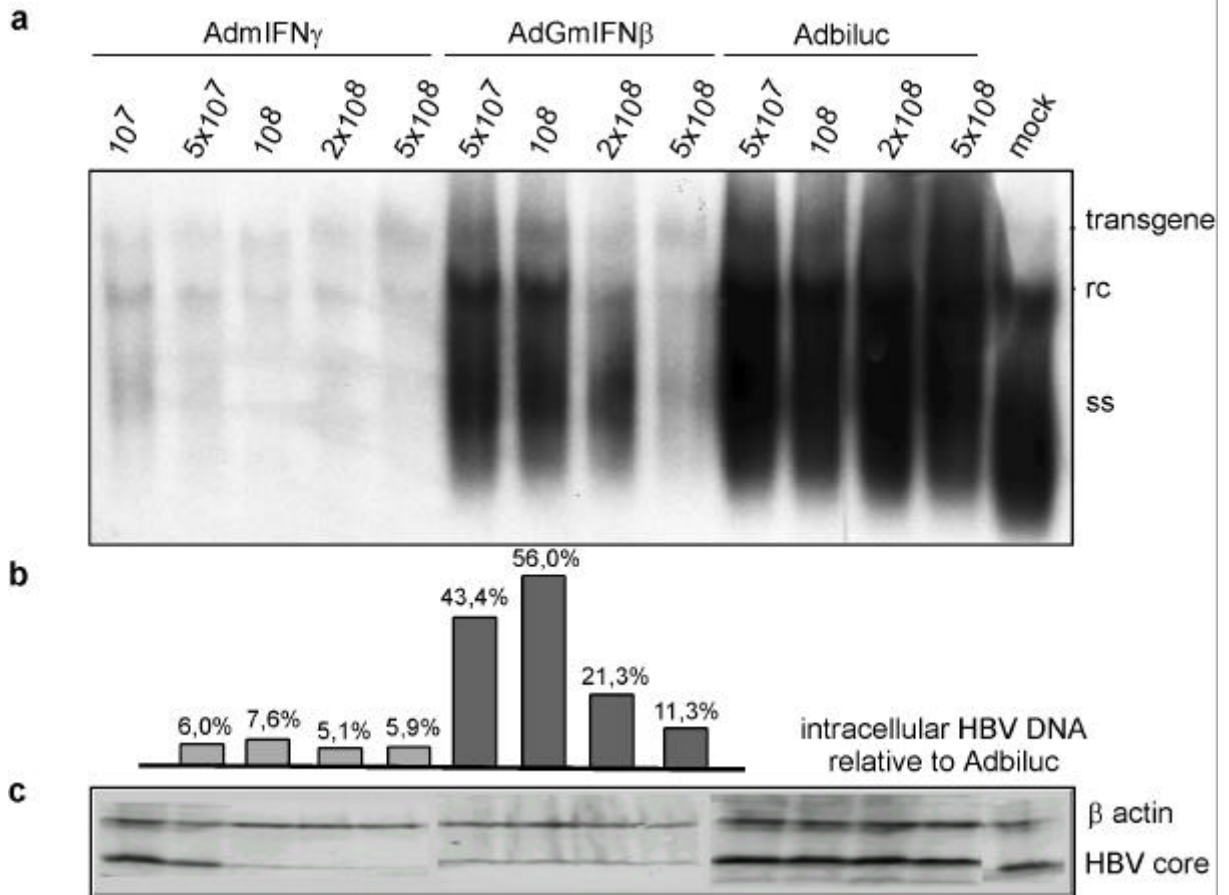


fig. 10: HBV replication is blocked by expression of interferon *in vivo*.

HBV1.3 transgenic mice were injected with AdmIFN γ , AdGmIFN β and a control vector, Adbiluc (see section 2.2.1) or saline. Three animals per group matched for sex, age and level of HBeAg were used and at day 14 p.i. liver tissues were analyzed.

(a) Southern-Blot was performed with 20 μ g of total liver DNA digested with *Hind III* isolated from pooled livers of each group of mice. Bands corresponding to the integrated transgene (**transgene**), relaxed circular (**rc**) and single-stranded (**ss**) linear HBV DNA replicative forms are indicated. The membrane was hybridized with α^{32} P-labeled HBV-specific DNA probe. HBV DNA replicative intermediate were quantified by phospho-imaging (**b**) in comparison to control group infected with Adbiluc. The integrated transgene was used to normalize the amount of DNA analyzed.

(c) Western-Blot was performed with a homogenate of pooled livers from each group of mice (14% SDS PAGE). Polyclonal antibody H801 and a monoclonal anti β actin were used to detected HBV core protein and β actin as a normalization standard.

mice received a single i.v. injection of recombinant adenovirus at doses ranging from 10^7 to 5×10^8 i.u. per mouse. Mice were bled and sacrificed 14 days later. Livers were harvested for DNA-, RNA-extraction and intracellular viral protein analysis. Diagnostic analysis with blood serum was performed and HBV DNA copies from secreted viral particles were counted by real-time PCR specific for HBV (table 5). Three mice were injected with saline solution, were sacrificed at the same time point and represent the mock group. Four groups of three mice were injected with control adenovirus, called Adbiluc (see section 2.2.1) at doses ranging from 5×10^7 to 5×10^8 i.u. per mouse.

As shown in the Southern blot analysis of fig.10a, no change in HBV replicative intermediates was observed in mice injected with Adbiluc. HBeAg-titers were constant 14 days after injection of Adbiluc (table 5). Only a slight decrease (2-fold) of HBV DNA copies in the serum was detected relative to the value of the mock group (table 5). No liver damage was observed 2 weeks after injection in respect to normal transaminase (ALT) values (45-70 IU/liter) and histologic analysis with slide of fixed liver tissue provided in department of pathology (DKFZ, Heidelberg) (data not shown).

Injection of even the lowest dose (10^7 i.u.) of AdmIFN γ strongly repressed intracellular HBV DNA replication (fig.10a). A 95% inhibition was observed in mice injected with higher doses (fig.10b). The amount of intracellular viral protein estimated from Western Blot as HBV core decreased proportionally to the adenoviral titer employed (fig.10c). No effect on mRNA isolated from total RNA of pooled liver was observed (data not shown). Although HBeAg titers in blood remained unchanged (table 5), the secreted particles containing HBV DNA decreased by 25- to 50-fold relative to mock treated animals. A 2,9-fold increase of the ALT value in the group of mice infected with the highest titer of 5×10^8 i.u. of AdmIFN γ monitored moderate liver damage. This might be caused by activation of specific cytotoxic lymphocytes (CTL) mediated by the release of mIFN γ .

The HBV DNA replicative intermediates decreased in a dose dependent fashion relative to the injected AdGmIFN β (fig.10a). HBV DNA replicative intermediates were reduced at 50-88% relative to the corresponding control group (fig.10b). The effect on intracellular viral core protein was not completely understood because the amount of core protein decreased to 50% relative to mock-treated mice independently of the

adenovirus dose used. It seems that protein synthesis, as represented by detection of β actin, (fig.10c) was repressed by secreted mIFN β . HBeAg titers and HBV DNA copies in serum of mice remained unchanged in comparison with data of mice infected with Adbiluc. Solely, the highest dose of 5×10^8 i.u. of AdGmIFN β induced a mild liver disease represented by a 5-fold increase of ALT values (table 5).

Table 5: HBV DNA copies, HBeAg and ALT levels in serum of HBV transgenic mice injected with AdmIFN γ , AdGmIFN β or a control vector (Adbiluc).

saline solution (mock)						
HBeAg (S/Co)	before infection	168				
	post infection (p.i.)	103				
ALT (IU/liter)		68				
HBV DNA copies per μ l serum		1,1E+05				
		dosis (infectious unit) 1×10^7 5×10^7 1×10^8 2×10^8 5×10^8				
Adbiluc						
HBeAg (S/Co)	before infection		158	161	154	158
	post infection (p.i.)		116	129	147	167
ALT (IU/liter)			78	44	72	60
HBV DNA copies per μ l serum			1,0E+05	4,1E+04	4,9E+04	5,5E+04
AdmIFN γ						
HBeAg (S/Co)	before infection	161	175	122	130	114
	post infection (p.i.)	107	114	138	126	142
ALT (IU/liter)		56	112	102	175	195
HBV DNA copies per μ l serum		2,1E+03	2,6E+03	3,5E+03	8,1E+03	1,4E+04
AdmIFN β						
HBeAg (S/Co)	before infection		133	132	108	133
	post infection (p.i.)		107	99	132	144
ALT (IU/liter)			49		46	325
HBV DNA copies per μ l serum			4,1E+04	2,2E+05	1,5E+03	2,3E+05

In summary, these results indicate that locally secreted mIFN γ from 10% of transduced liver cells (corresponding to an i.v. infection of 10^8 i.u. of adenovirus) was already sufficient to suppress 95% of intracellular HBV DNA replication. A 90% decrease of HBV replication was observed with secreted mIFN β , when 5-fold higher dose of AdGmIFN β was injected.

2.1.3.2- Kinetics of an antiviral effect on HBV replication

According to the results in the previous section, a therapeutic dose of a mixture of 10^8 i.u. of each adenovirus, AdmIFN γ and AdGmIFN β , seems to be promising. Using this gene therapy, the kinetics of the antiviral effect on HBV replication of mIFN γ and mIFN β was followed. To characterize the time course of an antiviral effect of mIFN γ and mIFN β expression on hepatic HBV replication in HBV transgenic mice, matched groups of five mice were infected i.v. with a mixture of 10^8 i.u. of AdmIFN γ and 10^8 i.u. of AdGmIFN β . Their livers were harvested on days 1, 3, 7, 14, 28 and 42 postinfection. Control mice received injection i.v. with 2×10^8 i.u. of Adbiluc, and their livers were harvested on the same day.

As shown in fig.11a, hepatic HBV DNA replicative forms were significantly reduced at the first day after adenovirus infection in the absence of biochemical evidence of liver disease, because ALT activity was slightly elevated to 98 to 143 I.U./liter. The adenovirus-mediated effect was prolonged until day 3 p.i. At day 7 p.i. the steady-state of HBV replication was restored in livers of control mice. In contrast to previous publications, no second wave (Cavanaugh et al., 1998) was observed at a dose of 2×10^8 i.u. of the adenovirus preparation, suggesting that no or low influx of T cells occurred in the liver.

The levels of intrahepatic HBV DNA replicative forms in mice infected with AdmIFN γ plus AdGmIFN β was comparable to the control mice during the first three days (fig.11a). From day 3 p.i. mIFN γ and mIFN β production mediated a 4- to 6-fold decrease of HBV DNA replicative intermediates relative to the control mice until 14 days p.i. (fig.11b). Hepatic level of HBV DNA was restored to steady-state after 28 days. The ALT value was slightly elevated like in the sera of control mice (table 6). This reflects the onset of a weak inflammatory liver disease after four weeks of a local secretion of cytokines.

The time course of the intrahepatic viral core protein in control mice was similar to the kinetics of HBV DNA replicative forms with a reduction at three days p.i. followed by a restoration of steady-state content (fig.11c). Determination of the distribution of hepatitis B core antigen between nucleus and cytoplasm was not done in this study, but it is known that intranuclear capsid antigen content is unaffected in adenovirus-infected

mice, so that variation of amount of HBV nucleocapsid particles in the cytoplasm was not easily detectable (Wieland S. personal communication). In mice infected with AdmIFN γ plus

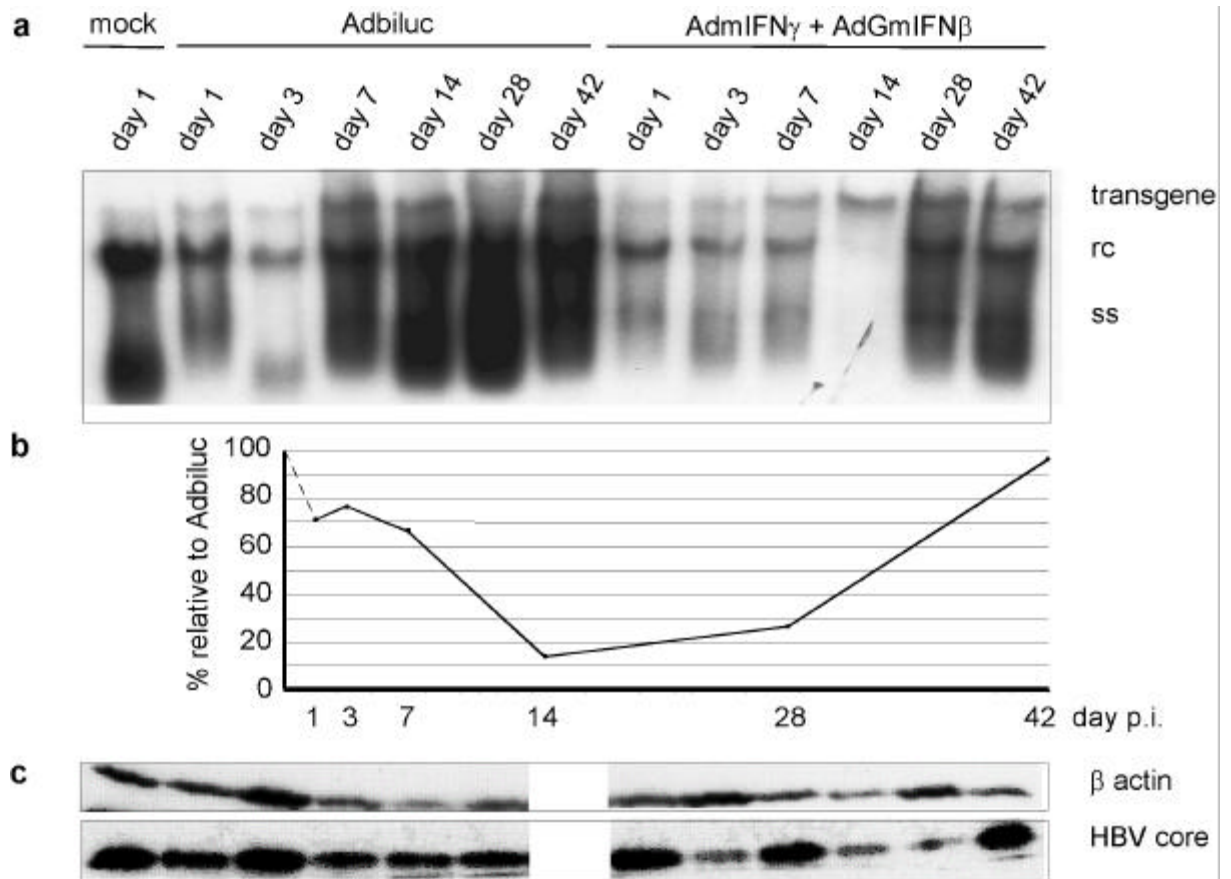


fig. 11: HBV replication is transiently blocked by expression of interferon.

HBV1.3 transgenic mice were injected with 10^8 i.u. of each AdmIFN γ and AdGmIFN β or 2×10^8 i.u. of control vector Adbiluc (see section 2.2.1) or saline. Five animals per group matched for sex, age and level of HBeAg were used and liver tissues were analyzed at different days p.i. as indicated.

(a) Southern-Blot was performed with 20 μ g of total liver DNA digested with *Hind III* isolated from each group of mice. The membrane was hybridized with a 32 P-labeled HBV-specific DNA probe. (b) HBV DNA replicative intermediate were quantified by phospho-imaging and presented in comparison to control group of mice infected with Adbiluc.

(c) Western-Blot was performed for liver homogenate of each group of mice (14% SDS PAGE). Polyclonal antibody H801 and a monoclonal anti β actin were used to detected HBV core protein and β actin as a normalization standard.

AdGmIFN β , hepatic viral core protein decreased at day 14 p.i.. only about 2-fold relative to control mice, when the signal of the Western blot (fig.11c) was quantified by

fluor-imaging after reaction with the ECFTM substrate. However at day 42 p.i., the steady-state level of nucleocapsid protein was restored.

HBeAg titer in blood of both groups of mice remained unchanged (table 6). The same biphasic time course observed on level of hepatic HBV DNA intermediate forms was reproducible by the content of HBV DNA copies in serum of mice infected with AdmIFN γ and AdGmIFN β : the first wave on day 1 p.i. was characterized by a decrease of 4-fold in comparison with serum of control mice and the second phase occurred at day 7 p.i. at 25- and then 6-fold reduction of HBV DNA copies per μ l serum respectively at day 14 p.i. and at day 28 p.i. copies relative to serum of the control mice. The level of steady-states was restored at day 42 p.i.

Table 6: HBV DNA copies, HBeAg and ALT levels in serum of HBV transgenic mice infected with AdmIFN γ and AdGmIFN β or a control vector (Adbiluc) from 7 to 28 days p.i. relative to mice infected with Adbiluc.

	day 1	day 3	day 7	day 14	day 28	day 42
saline solution (mock)						
HBeAg (S/Co) p.i.	135					
ALT (IU/liter)	137					
HBV DNA copies per μ l serum	7,9E+04					
Adbiluc						
HBeAg (S/Co) p.i.	115	111		149	222	174
ALT (IU/liter)	98	86	46	317	375	
HBV DNA copies per μ l serum	1,1E+05	1,3E+04	3,2E+04	6,2E+04	4,8E+05	1,9E+05
AdmIFNγ + AdGmIFNβ						
HBeAg (S/Co) p.i.	124	105		63	152	132
ALT (IU/liter)	143	63	83	134	115	
HBV DNA copies per μ l serum	2,9E+04	1,7E+04	1,8E+04	2,3E+03	8,0E+04	2,7E+05

No change was observed at the level of the 3,5kb pregenomic and 2,1kb envelope HBV mRNAs in the liver of control mice or interferon treated mice (data not shown).

The results indicate that HBV nucleocapsids and replicative intermediates are more susceptible to cytokine-induced inhibition than HBV RNA and that suppression of HBV replication is not due to a decrease of transcriptional templates. However, the inhibitory

effect on HBV replication was transient. This transient effect is not due to the replacement of transduced hepatocytes, because the adenovirus genome was detectable in the liver by PCR at all time points of the study (data not shown).

2.1.3.3- Time course of constitutive expression of cytokine *in vivo*

To understand this transient antiviral effect on HBV replication, the kinetics of secretion of cytokine has to be followed *in vivo*. Groups of three matched HBV transgenic mice for sex, age and levels of HBeAg in serum were injected i.v. with 2×10^8 i.u. of AdmIFN γ and their livers were harvested on days 3, 7, 14, 21, 28 and 42 after infection.

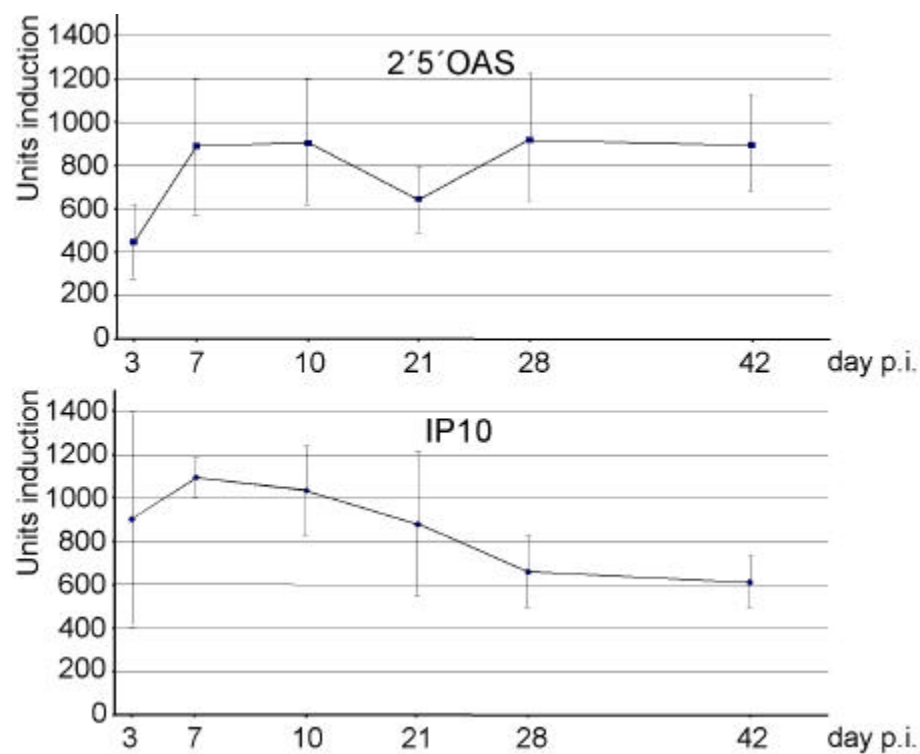


fig. 12: Time course of 2'5'OAS and IP10 induction in HBV transgenic mice infected with AdmIFN γ

Heterozygous HBV transgenic mice were injected i.v. with 2×10^8 i.u. of AdmIFN γ . Three animals per group matched for sex, age and HBeAg level were sacrificed at different days p.i. as indicated and their livers were analyzed. Total RNA was extracted and employed for real time RT PCR detection of IP10 and 2'5'OAS mRNA in units induction, which corresponds to mRNA copies of IP10 or 2'5'OAS per ml liver homogenate normalized relative to copies of GADPH and LDH RNA (analysis was performed by T. Giese, Department of Immunology, University of Heidelberg). ALT values at time point of sacrifice is indicated. AdmIFN γ was kindly provided by T. Ritter, Charité, Berlin.

The mRNA was extracted from total RNA in homogenate of liver of each mouse, and investigated by real-time RT-PCR detection of 2'5'OAS and IP10 RNA relative to housekeeping genes.

The expression and secretion of mIFN γ in the liver of mice was monitored by an induction of IP10, which reached a maximum level at day 7 p.i.. Then the IP10 induction decrease progressively to steady state of 600 units (fig.12) at day 42 p.i., which corresponds to basal induction of IP10 in liver cell. 2'5'OAS was also induced in the first seven days to a maximal level of 900 units, and this induction statement was maintained constant in the liver until the end of the experiment. However it is know, that IFN γ mediate indirectly induction of IFN α/β , which specifically mediated 2'5'OAS induction response (Muller M. et al., 1993; Stark G.R. et al., 1998). 2'5'OAS induction was not a sensitive marker for mIFN γ expression. IP10 induction is much more specific. However, why the induction level of IP10 and 2'5'OAS was so low, is not clear. No control of the adenovirus AdmIFN γ stock was recently performed to determine the infectivity of the adenovirus and/or the secretion of mIFN γ in cell culture. Loss of material during the RNA extraction and by RT reaction could be an other explanation. In fact, a decrease of mIFN γ expression was monitored from day 14 p.i. to day 42 p.i..

In summary of these experiments in mice, both cytokines, mIFN γ and mIFN β , has an inhibiting effect on HBV replication in livers of HBV transgenic mice. The effect of expression of mIFN γ was specially effective and induced elevation of ALT level in sera of the mice by strong expression level, which was only expected in these experiments. Using a low or intermediate dose of recombinant adenoviral vector to inject into the mice, the host-immune response was significantly reduced during the first three days p.i. According the gene transfer efficiency in the used mice, 10% transduced hepatocytes of the liver with 10^8 i.u. of each recombinant adenovirus AdmIFN γ and AdGmIFN β , which were sufficient to suppress 95% HBV replication. It will correspond to an estimated moi of 1 i.u. per transduced cell or moi of 0,1 i.u. per hepatocytes in the liver of mouse, when 10^9 hepatocytes were constitute the 50 g liver of the mouse. In comparison with *in vitro* data, the antiviral effect on HBV replication was amplified about 2-fold in the liver of mice. However, this inhibiting effect was shown as transient;

therefore, mIFN γ secretion in the liver of transduced mice with AdmIFN γ decreased continually after 7 days p.i..

2.2- Effect on HBV replication following gene transfer for tet-regulatable and liver specific cytokine expression.

Results from the first part of this study showed that adenoviral vectors efficiently targeted the liver but liver specific gene expression was not assured. Cytokine expression under the control of early cytomegalovirus promoter (P_{CMV}) is constitutive and at high level. This may interfere with signaling systems in the cell and could lead to cellular toxicity or cause non-physiological perturbations of regulatory pathways.

To resolve these problems, a cassette containing tetracycline-inducible gene expression system based on the Tet-On system (Gossen M. et al., 1995) was transferred into the hepatocytes via adenoviral vector. To monitor quantitatively the tet-inducible cytokine expression, a tetracycline-regulated bidirectional promoter was used for the co-expression of cytokine and the luciferase reporter genes. This model using adenoviral vector and tet-inducible co-expression of gene of interest, such cytokines, and the luciferase gene was established *in vitro* (section 2.2.1.1) and in mouse (section 2.2.1.2). This set of adenoviral vectors was called the Adbiluc system. Following its establishment in mouse, it should be possible to separate the effect of cytokine expression on HBV replication from the effect of the adenoviral gene transfer itself in HBV transgenic mice (section 2.2.2).

2.2.1- Establishment of a system allowing regulatable, liver specific gene expression using adenoviral vectors

To establish a tetracycline-dependent gene expression system *in vitro* or in mouse, two components has to be present in the cell: the tetracycline-dependent transcription activator (tTA) or the reverse tet-transactivator (rtTA) and a tTA/rtTA-responsive promoter element. As tTA/rtTA-responsive promoter element, the bidirectional promoter P_{bi-1} (Baron U. et al., 1995) was used in this study for the co-regulation of two genes expression: the luciferase as reporter gene and the transgene. As shown fig.13a, the cassette containing P_{bi-1} fused with luciferase gene and a multiple cloning site (MCS) in opposite site, was included in the E1 deletion region of human adenoviral

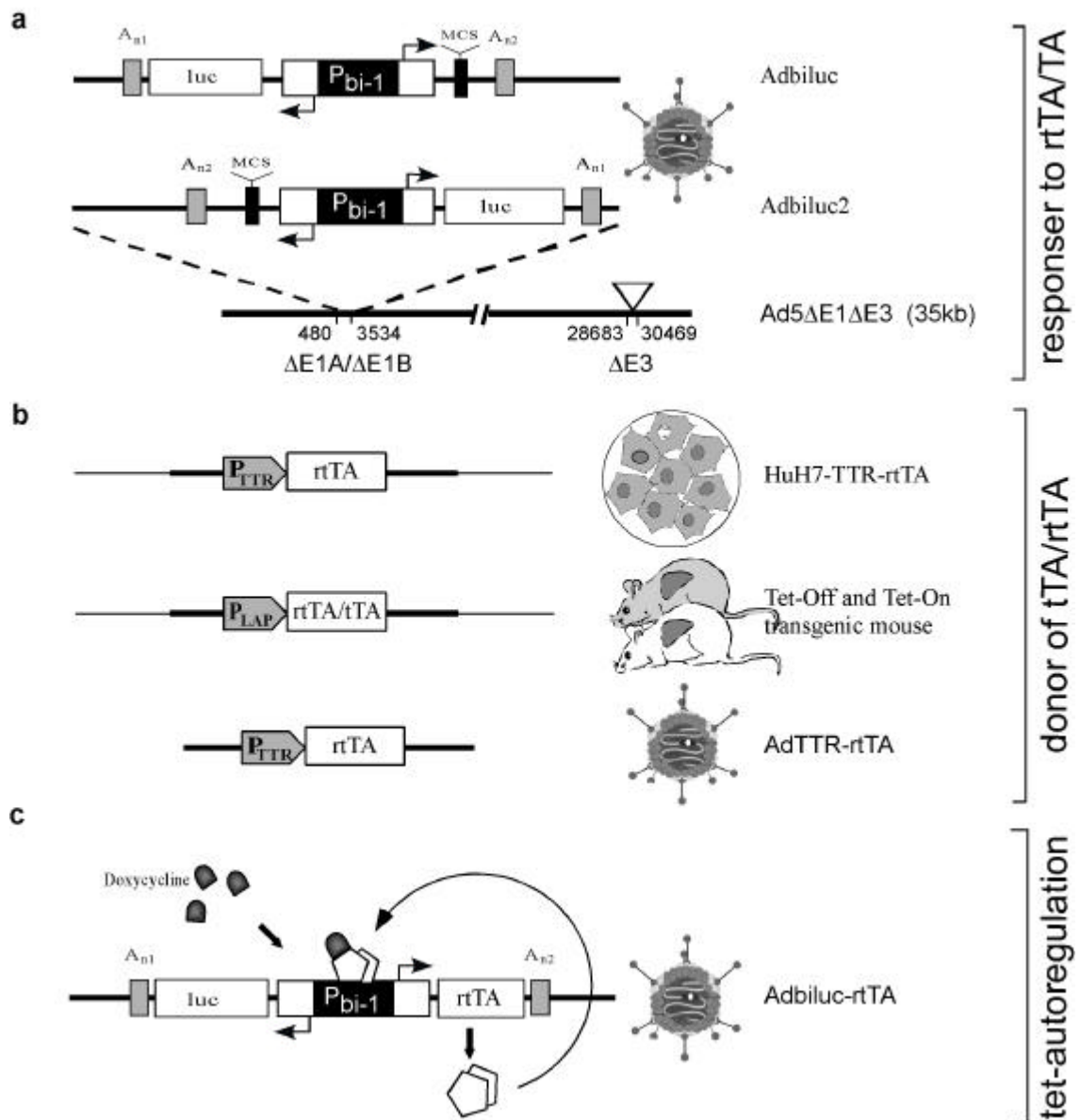


fig. 13: Schematic presentation of tetracycline-regulatable gene expression: Adbiluc system.

(a) The rtTA/tTA-responsive bidirectional promoter P_{bi-1} (Baron et al., 1995) was included in the E1A/E1B deleted region (480-3534 bp) of the human adenovirus genome type 5. P_{bi-1} allows co-expression of firefly luciferase (**luc**) and the transgene of interest, which could be cloned into the multiple cloning site (**MCS**). Depending on the orientation of insertion of fragment luc- P_{bi-1} -MCS relative to adenovirus genome, Adbiluc and Adbiluc2 were generated.

(b) The tTA and rtTA genes were expressed under the control of liver specific promoter, P_{TTR} or P_{LAP} . This cassette was whether integrated in chromosomes of HuH7 cells (**HuH7-TTR-rtTA**) and in genome of mice (**Tet-Off and Tet-On transgenic mice**) or in the adenovirus genome (**AdTTR-rtTA**). These three models corresponds to donor of rtTA or tTA *in vitro* and *in vivo*.

(c) The rtTA and the P_{bi-1} promoter were carried in the same adenoviral vector type of Adbiluc (**Adbiluc-rtTA**). The basal expression was sufficient to express rtTA, which recognized its specific DNA target sequence on P_{bi-1} in presence of doxycycline. A tet-autoregulation was monitored via luciferase co-expression.

A_{n1} corresponds to the SV40 late polyadenylation site whereas A_{n2} comprises the intron/polyA signal of the rabbit β -globin gene.

type 5 genome. This MCS was used for insertion of cytokine or a second reporter gene like GFP. Depending of the orientation of the inserted cassette relative to adenovirus genome, two vectors was constructed: Adbiluc and Adbiluc2 (fig.13a).

To induce gene expression from Adbiluc or Adbiluc2 vectors, tTA or rtTA has to be produced. As donor model for tTA or rtTA expression under the control of a liver specific promoter, three alternatives were established in this study (fig.13b). The first, an *in vitro* model was the establishment of HuH7 cell line expressing rtTA under the control of transthyretin promoter (P_{TTR}) (Fung w. et al., 1988; Qian X. et al., 1995). These cells called HuH7-TTR-rtTA were described in section 2.1.1.2. The *in vivo* models were already generated mice producing tTA (Kistner A. et al., 1996) or reverse tet-transactivator (rtTA) (Schönig K. et al., 2002) under the control of liver specific P_{LAP} promoter (Talbot, D. et al., 1994). The last alternative should concern tissue culture and experiment with mice: a second adenoviral vector was generated to transfer the rtTA gene driven under the P_{TTR} promoter, the AdTTR-rtTA vector.

An other model using one-component adenoviral vector was also developed in this study. As shown fig.13c, the rtTA gene was inserted in MCS of Adbiluc and Adbiluc2 construct (not presented). Using the basal expression level, rtTA was expressed and induced its expression in presence of doxycycline. This tet-autoregulation was monitored via the co-expression of luciferase, as presented in section 2.1.1.4.

The establishment of the tetracycline-dependent gene expression was presented in the three *in vitro* systems in section 2.2.1.1, and then in mice in section 2.2.1.2. Following transduction with the vectors of type Adbiluc, the induction factors, kinetics, tissue specificity of expression of transgene were characterized via luciferase activity. The GFP gene as second reporter gene was also cloned into the MCS of Adbiluc vector to demonstrate the co-expression of both genes *in vitro* and *in vivo*.

2.2.1.1- Establishment of the Adbiluc system *in vitro*

As presented above, two adenoviral vectors, Adbiluc and Adbiluc2, were constructed dependent of the orientation of the cassette containing P_{bi-1} promoter relative to adenovirus genome (fig. 13a). To determine, at first, which orientation of this cassette is

more favorable to obtain a tight expression of gene of interest inserted into the MCS, primary human hepatocytes were transduced with both vectors and luciferase activity was assayed. Therefore, HuH7 cells and generated HuH7 cells expressing rtTA (fig.13b) were transduced with both adenoviral vectors to characterize the induction factor of gene expression via luciferase activity and the basal expression levels in off-state. According these results, Adbiluc vector was identified as a good candidate for a tight expression of transgene, and GFP or cytokine genes were cloned in Adbiluc construct. In section 2.2.1.1.3, the second alternative model was characterized following a double infection of HuH7 cells with adenoviral vector AdTTR-rtTA (fig.13b), which mediated rtTA gene transfer as donor for rtTA, and an vector type of Adbiluc carried GFP reporter gene, Adbiluc-GFP. The later vector corresponded to acceptor element for rtTA. And finally in section 2.2.1.1.4, the tetracycline-dependent gene expression was established in HuH7 cells transduced with the one-component adenoviral vector carried rtTA. This vector, called Adbiluc-rtTA, was described in fig13c.

2.2.1.1.1- Basal expression of luciferase by Adbiluc in primary human hepatocytes

To determine the effect of the adenoviral cis-acting element on the basal induction of gene expression by monitoring luciferase activity in off-states, the cassette containing the tTA responsive bidirectional promoter cassette was inserted into the E1 deletion

Table 7: Higher background expression of luciferase in Adbiluc construct than Adbiluc2 in primary human hepatocytes culture.

Adbiluc	1 moi	5 moi	10 moi
RLU/ μ g protein (mean)	55	120	84
standard deviation	+/- 28	+/- 73	+/- 15

Adbiluc2	1 moi	5 moi	10 moi
RLU/ μ g protein (mean)	4	20	38
standard deviation	+/- 1	+/- 7	+/- 20

Primary human hepatocytes (PHH) (kindly provided by A. Untergasser) were infected with Adbiluc or Adbiluc2 at increasing moi from 1 to 10 i.u./cell. At day 5 p.i. the cells were lysed for luciferase assay. The mean value and standard deviation (SD) of luciferase activity from three independent experiments is shown.

(480-3534 bp) in two opposite orientations: Adbiluc and Adbiluc2 adenoviruses were generated (fig.13). Primary human hepatocytes (PHH) were infected with increased moi of adenovirus, and luciferase activity was measured five days after infection (table 7). In comparison to the luciferase activity in transduced HuH7 cells (fig.14), the activity was one log higher in PHH. Basal luciferase activity of Adbiluc in transduced PHH was 5-fold higher than PHH cells infected with Adbiluc2. According these results, the open reading frame coding for mIFN α , mIFN β and mIFN γ were cloned in the shuttle plasmid pAdbiluc and not in pAdbiluc2.

2.2.1.1.2- Gene expression in hepatoma HuH7 cells expressing the reverse tet-transactivator

A human liver cell line constitutively synthesizing the better reverse tet-transactivator rtTA2^S-S2 (Urlinger S. et al, 2000) under the liver specific transthyretin promoter (P_{TTR}) (Qian X. et al., 1995) was generated by cotransfecting HuH7 cells with the plasmid pUHD-0,6-TTR-rtTA(FFF) (constructed by R. Löw, 1999) and pSV2neo. Forty clones resistant to G418 (500 μ g/ml) were isolated and assayed for constitutive production of rtTA2^S-S2 by transient transfection with luciferase reporter plasmid pBi-2 (Baron et al., 1995). After screening for resistant clones with high doxycycline-dependent induction of luciferase expression and low background level, six clones were selected to generate the stable expressing rtTA cell line HuH7-TTR-rtTA. Subclone#3 was selected and employed in this work. To determine how many cells could express reverse tet-transactivator (rtTA) after 10 passages, HuH7-TTR-rtTA were infected with reporter adenovirus Adbiluc-GFP at increasing moi ranging from 1 to 30 i.u./cell in the off- or the on-state. As shown in fig.15, 80-85% of seeded cells expressed GFP when more than one adenoviral genome was transferred in each cell corresponding to a moi of 3 i.u./cell. The remaining 10-15% cells did not express GFP; this may be, because no rtTA was expressed or because the cells were not transduced with Adbiluc-GFP.

To monitor basal expression of luciferase in a hepatoma cell line, HuH7 cells were infected with Adbiluc or Adbiluc2 at increasing moi ranging from 0,5 to 10 i.u./cell in the off/on-state. 24 hours after induction, no luciferase activity was detected in transduced HuH7 lysate (fig.14), because no tet-transactivator was expressed in the

HuH7 cell line. These results were also reproducible in HeLa cells, which correspond to standard *in vitro* system established earlier (Gossen M. et al., 1995). The activation of luciferase expression occurred in a tTA-dependent fashion.

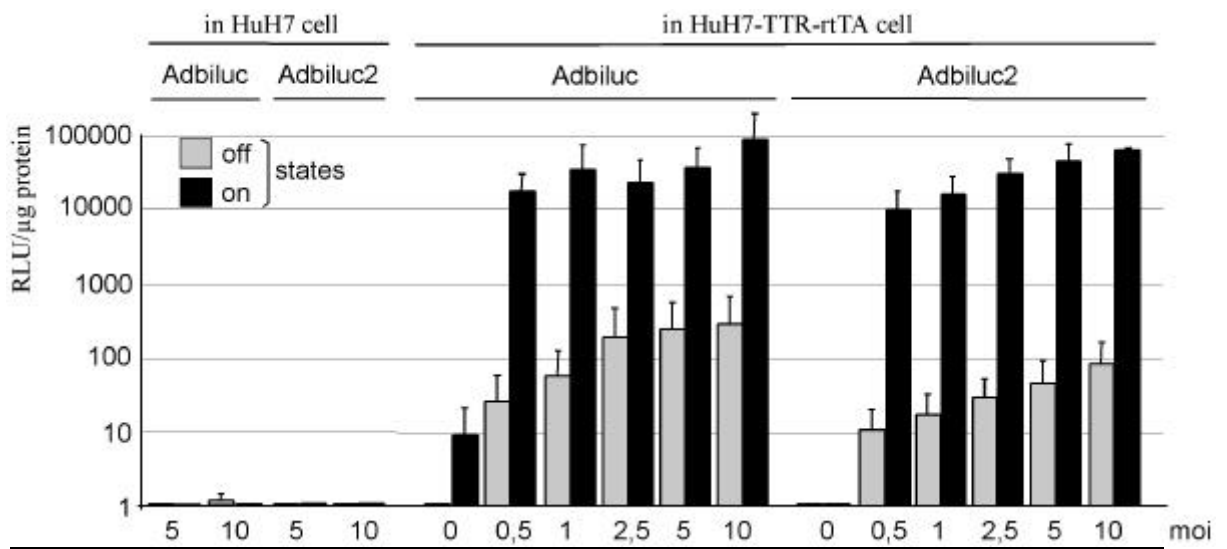


fig. 14: Tet-inducible Luciferase expression in HuH7 cell.

Hepatoma cell lines HuH7 with or without the reverse tet-transactivator (rtTA) were infected with Adbiluc or Adbiluc2 at increasing moi's. To induce luciferase expression, 1μg doxycycline per ml medium was added. Luciferase activity and protein content were determined in cell lysates after 24 hours. Each bar represents the mean luciferase activity normalized to the protein content in three independent assays. Black bars corresponded to on-states and light bars to luciferase activity values in off-state.

In addition, the rtTA-expressing human hepatoma cells (HuH7-TTR-rtTA) were infected at increasing moi ranging from 0,5 to 10 i.u./cell with Adbiluc or Adbiluc2. 24 hours after addition of 1μg/ml doxycycline, a significant induction of luciferase activity was reported at factor 100 in lysate of transduced cells with Adbiluc or Adbiluc2 (fig.15). Basal expression of luciferase activity was increased proportional to the used moi. As previously described, basal expression of luciferase in PHH cells or HuH7-TTR-rtTA cells transduced with Adbiluc was 5-fold higher than cells transduced with Adbiluc2. The cut-off of this system *in vitro* was at 100 relative light units (RLU) per μg protein.

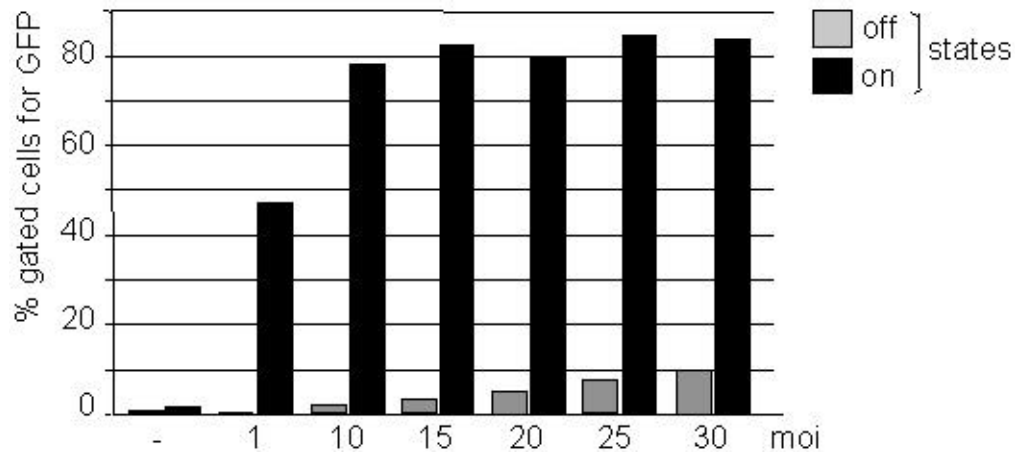


fig. 15: GFP is expressed in max. 80% of HuH7-TTR-rtTA cells following Adbiluc-GFP transduction.

HuH7-TTR-rtTA cells were infected with reporter adenovirus Adbiluc-GFP at increasing moi's. 24 hours after addition of 1 μ g/ml doxycycline, cells were resuspended in PBS for FACS analysis to account GFP expressing cells. Black bars corresponded to cell suspension in on-state and lightly bars to cells in off-state.

2.2.1.1.3- Adenovirus mediated rtTA expression

Instead of using hepatoma cell line stable expressing rtTA, a double adenovirus infection strategy was established: one adenovirus AdTTR-rtTA, that was needed to mediate the expression of rtTA under liver specific promoter for transthyretin (P_{TTR}) and second adenovirus of type Adbiluc. To compare expression level of rtTA in HuH7-TTR-rtTA cells and in transduced HuH7 cells with AdTTR-rtTA, total RNA was extracted from cell lysate and Northern Blot analysis was performed with the total amount of extracted RNA using $\alpha^{32}P$ labeled rtTA-specific DNA probe. The signal intensity of the 1,1kb transcript extracted from HuH7-TTR-rtTA cells was 2-fold higher than the signal from AdTTR-rtTA transduced HuH7 cells (fig.16a). No RNA specific to rtTA was detected in HuH7 cell lysate. However, the normalization of amount of RNA was performed only by using the account of cells. Thus, the expression of rtTA was confirmed following AdTTR-rtTA infection.

To establish a tet-induction and tet-regulation of luciferase expression, HuH7 cells were co-infected with AdTTR-rtTA and Adbiluc-GFP at increasing ratio from 1:1 to 1:1000. Cells were grown and maintained with or without doxycycline in medium (fig.16b). No significant difference was observed in an other experiment performed with an inverted ratio (data not shown). As shown in fig.16b, luciferase activity was strongly induced (800000 RLU/ μ g protein), when cells were co-infected at a ratio of 1:1. Despite only

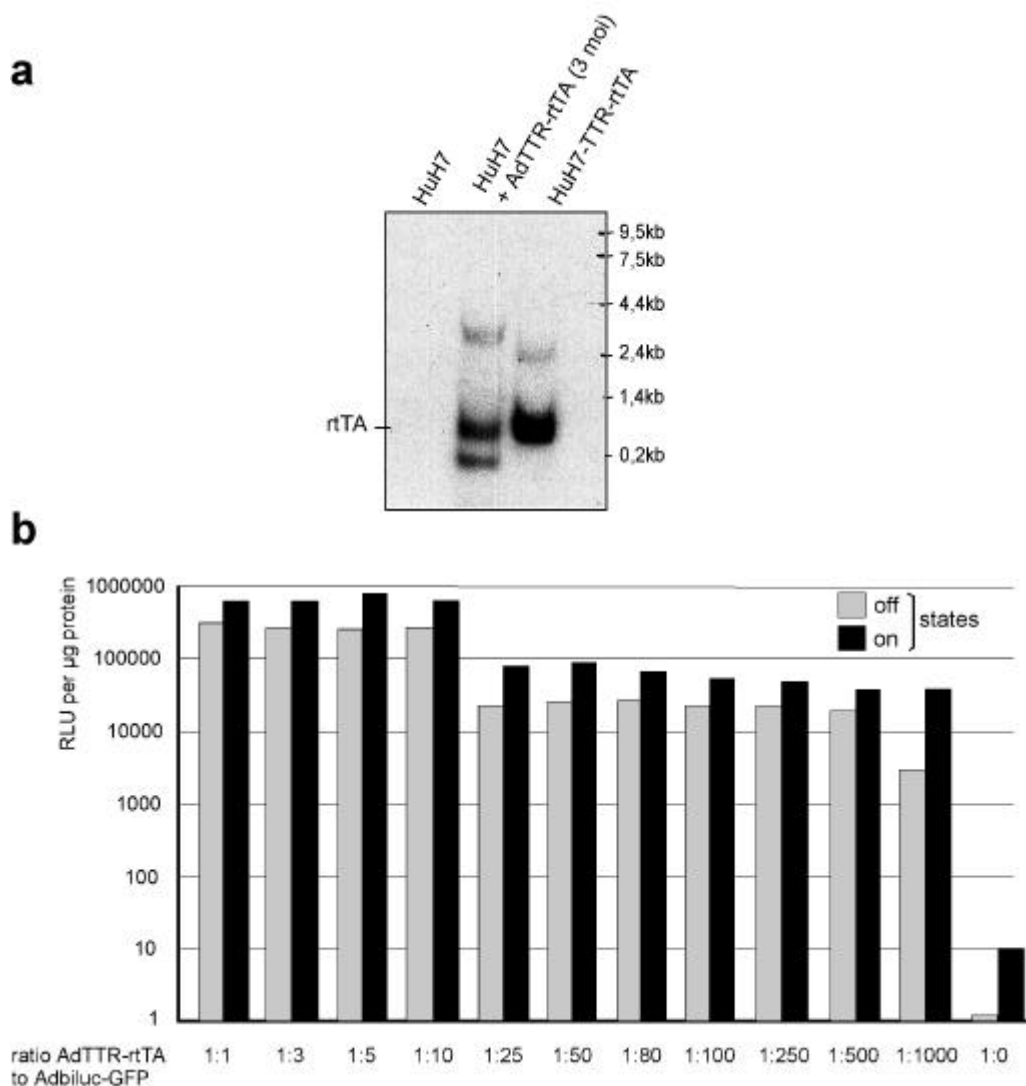


fig. 16: Strong induction of luciferase expression in HuH7 cells co-transduced with AdTTR-rtTA and Adbiluc-GFP in a doxycycline independent fashion.

(a) Transcription of rtTA in transduced HuH7 cells with AdTTR-rtTA in comparison to transcription in HuH7-TTR-rtTA cells. Northern Blot was performed with total RNA extracted from transduced HuH7 with AdTTR-rtTA at moi of 3 i.u./cell or from HuH7-TTR-rtTA cells. RNA extracted from HuH7 cells represented negative control. The membrane was hybridized with $\alpha^{32}\text{P}$ -labelled rtTA-specific DNA probe.

(b) HuH7 cells were co-infected with AdTTR-rtTA at moi of 1 i.u./cell and Adbiluc-GFP at increasing moi from 1 to 1000 i.u./cell. Induction of system occurred by addition of 1 μg doxycycline per ml medium. 90% of the cells expressed GFP in the on-state. At 24 hours p.i. the cells were lysed for luciferase assay. The black bars represented the luciferase activity values in on-states and the lightly bars to values to the off-state. These data provided from a single experiment.

one third of the cells was expected to be transduced with AdTTR-rtTA at a moi of 1 i.u./cell, the level of activity was 10-fold higher than in experiment performed in HuH7-TTR-rtTA cells transduced with Adbiluc at an moi of 10 i.u./cell (fig.15). By increasing

the moi of Adbiluc-GFP, expressed rtTA was saturated. Luciferase expression was not regulated.

Because the ratio between expressed transactivator and transactivator responsive promoter element may be not the most important point, induction of luciferase activity was checked by transfection in HuH7 cells with the plasmids used to generate AdTTR-rtTA. After homologous recombination in BJ5183 *E.coli*, transfected cells were infected with Adbiluc-GFP in either on- or off-state and luciferase activity was measured. In all fifty clones screened, luciferase activity was strongly induced independent of doxycycline (data not shown). Because sequencing of the adenoviral plasmids failed, point mutations, which may change conformation of rtTA molecules were not excluded; thus, the dimer rtTA could bind to promoter P_{bi-1} without affinity for doxycycline. Double infection by i.v. injection with 5×10^8 i.u. of AdTTR-rtTA and 5×10^8 i.u. of Adbiluc-GFP failed in C57B/6 mice: no luciferase activity and no GFP expression were detected in the liver of these mice after one week induction with 2 mg/ml doxycycline in drinking water, although luciferase and GFP were expressed *in vitro* using the same viral stock.

2.2.1.1.4- Tet-inducible luciferase expression using an one-component vector system

A one-vector system, which mediates tet-inducible luciferase expression, was generated as following: the rtTA gene was inserted in the multiple cloning site of our shuttle plasmid pAdbiluc or pAdbiluc2, and the corresponding adenoviruses Adbiluc-rtTA and Adbiluc2-rtTA were produced. It was expected, that basal expression of rtTA was efficient to induce its own expression in an autoregulatory fashion. To determine the time course of tet-induction of the luciferase expression in cell culture, HuH7 cells were infected at a moi of 10 i.u./cell with Adbiluc-rtTA (and Adbiluc2-rtTA will be later analyzed). The cells were grown with or without doxycycline in medium and luciferase activity was measured in cell lysate at different time points after infection. Using Adbiluc-rtTA, basal luciferase expression was 300 RLU/ μ g protein, and luciferase activity was induced 100- to 260-fold after 4 days p.i., when doxycycline was added (fig.17).

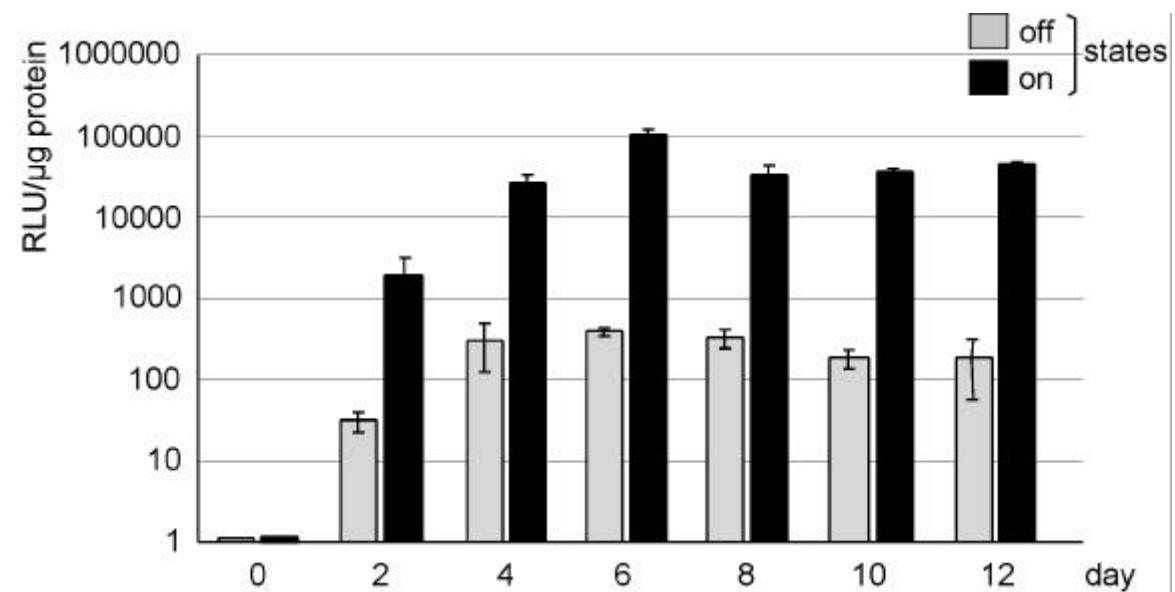


fig. 17: Kinetic of induction of luciferase expression in HuH7 cells transduced with Adbiluc-rtTA.

HuH7 cells were infected with Adbiluc-rtTA at a moi of 10 i.u./cell. To induce luciferase expression, 1 μ g doxycycline per ml medium was added (on-state). Luciferase activity and protein content were determined in cell lysate at different time points. Each bar represents the mean value of luciferase activity normalized to the protein content in four independent assays. Black bars corresponds to the on-state and lightly bars the off-state.

2.2.1.2- Establishment of the Adbiluc system *in vivo*:

2.2.1.2.1- Induction of luciferase expression in the liver of tTA transgenic mice

To confirm the strong induction of luciferase activity observed in the liver cell line, sixteen tTA transgenic mice (called Tet-Off mice) were injected i.v. with 2.5×10^9 i.u. of control adenovirus Adbiluc or reporter adenovirus Adbiluc-GFP (fig.13). As previously reported, single injection i.v. with 2.5×10^9 i.u. of adenovirus allowed almost all hepatocytes to be targeted. 2 mg/ml doxycycline was added to the drinking water of a group of seven mice to

require the off-state. The remaining mice did not receive this drug and were thus kept in the on-state. Mice were sacrificed at day 2 p.i.. Liver, kidney, spleen, heart, leg muscles, lung and brains were harvested to assay luciferase activity. In the off-state, between 5 and 50 RLU per μ g protein were measured in the liver of mice infected with Adbiluc (fig.18a) The cut-off of the luciferase assay in liver homogenate is 1 RLU per μ g protein. Basal expression of luciferase in livers of mice infected with Adbiluc-GFP or

Adbiluc2 was about 5 RLU/ μ g protein. An induction (factor 1000 to 5000) of luciferase activity was validated in livers of mice in the on-state (fig 18a).

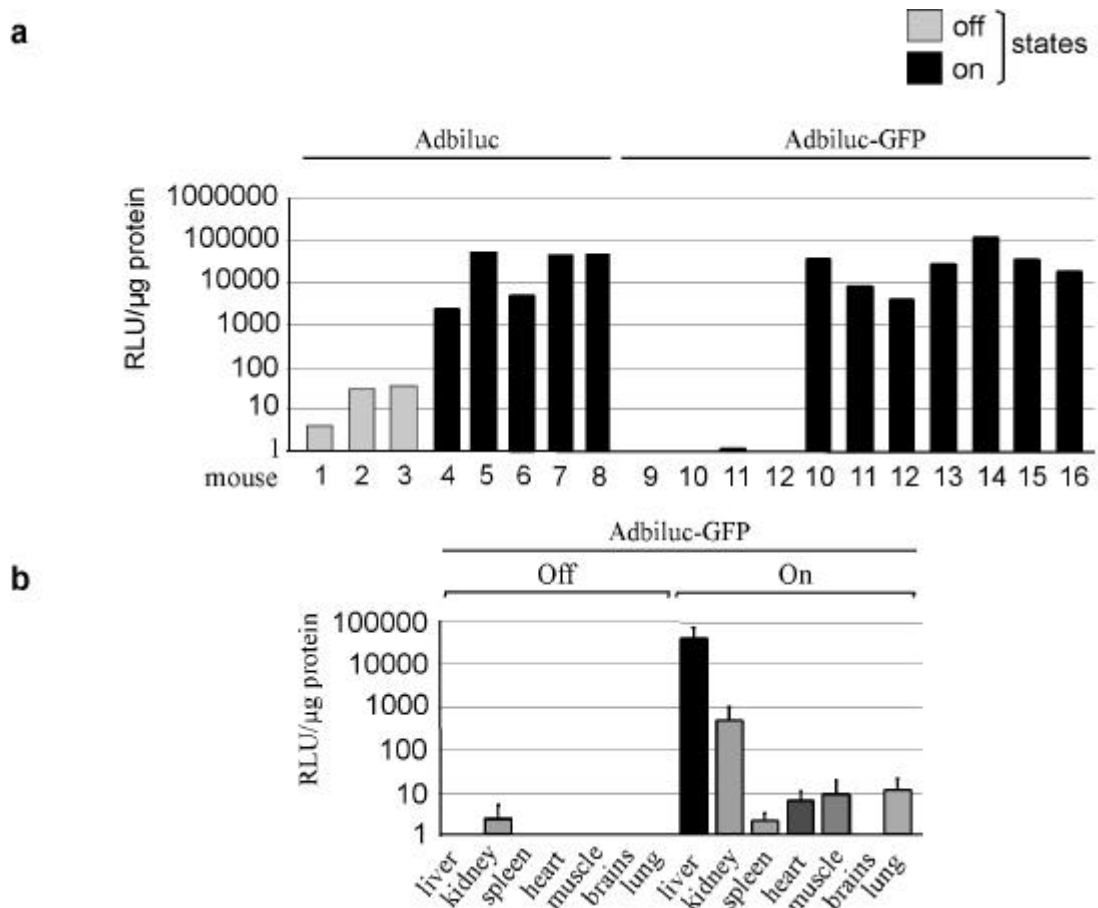


fig. 18: Tet-inducible Luciferase expression in tTA transgenic mice

(a) Induction of luciferase activity in liver of tTA transgenic mice infected with adenoviruses Adbiluc or Adbiluc-GFP. Sixteen tTA transgenic mice were infected i.v. with adenovirus at high dosis of $2,5 \times 10^9$ i.u.. To avoid any induction of luciferase or GFP expression, one group of mice received drinking water supplemented with 2 mg/ml doxycycline two days before injection and was prolonged during the experiment. Two days p.i. the mice were sacrificed, liver and other organs were collected. Each bar represents luciferase activity normalized to protein content in tissue from each individual animal. Black bars corresponded to on-states and light bars to luciferase activity values in the off-state.

(b) Liver specific co-expression luciferase and GFP in tTA transgenic mice following adenovirus injection with Adbiluc-GFP at dosis of $2,5 \times 10^9$ i.u.. Each bar represented mean value of luciferase activity normalized to protein content in different tissues of four animals selected in the on-state.

To study tissue-specificity of gene expression, luciferase activity was measured in all collected tissues. Less than 10 RLU/ μ g protein was determined in the on-state group of mice except from liver (34285 RLU/ μ g protein) and kidney (418 RLU/ μ g protein) (fig.18b). In the rtTA transgenic mice used it is reported, that tTA expression is liver specific, but a remaining expression occurs in the kidney (Kistner A. et al., 1996). The Adbiluc allows a liver specific and regulated gene expression *in vivo*.

2.2.1.2.2- Kinetics of luciferase activity in living tTA or rtTA transgenic mice

To study the time course of luciferase induction in tTA or rtTA transgenic mice, that were injected with $2,5 \times 10^9$ i.u. of Adbiluc-GFP, noninvasive bioluminescence imaging was performed using an intensified charged coupled photon counting device camera. A serial bioluminescence imaging examination was performed at 1, 3 and 4,5 hours p.i. with two infected tTA transgenic mice. At time point 4,5 hours p.i., beginning luciferase activity was detected in the liver by a low emission of light. Full induction was achieved at 24 hours p.i.(fig.19a). Treatment with 2 mg/ml doxycycline in drinking water for two days repressed the induction of luciferase activity. To re-induce gene expression, the drug was removed from the drinking water. Seven days are required to completely eliminate the doxycycline from the circulation. At day 10 p.i. a strong signal in the liver of both mice was detected, which persisted until day 31 p.i.. In contrast to the strong signal at day 24 p.i. light emission in both mice almost reduced to the low level of day 31 (4161 RLU/ μ g and 5 RLU/ μ g respectively).

This time course of turning on/off of luciferase expression was reproducible in the Tet-On system using the rtTA transgenic mice (Schönig K. et al., 2002). A strong bioluminescence signal was detected in liver tissues of all five rtTA transgenic mice treated with doxycycline (fig.19b). After removal of doxycycline, no luciferase activity in the liver was anymore. The re-induction of gene expression was occurred by supplementation of doxycycline: strong luciferase activity was observed on day 10 p.i. At day 15 p.i., the luciferase activity was 4721 and 141642 RLU/ μ g protein respectively, in the livers of the two mice in the on-state, demonstrating the individual variability.

In summary, these results have shown that i.v. injection of adenovirus type Adbiluc in tTA/rtTA transgenic mice was sufficient to establish luciferase expression dependent on

doxycycline in drinking water. Expression was first detected 4,5 hours p.i and could be switched on and off during a period of four weeks. Normal values of ALT activity were measured in these infected mice, indicating no liver inflammation during the strong expression of luciferase.

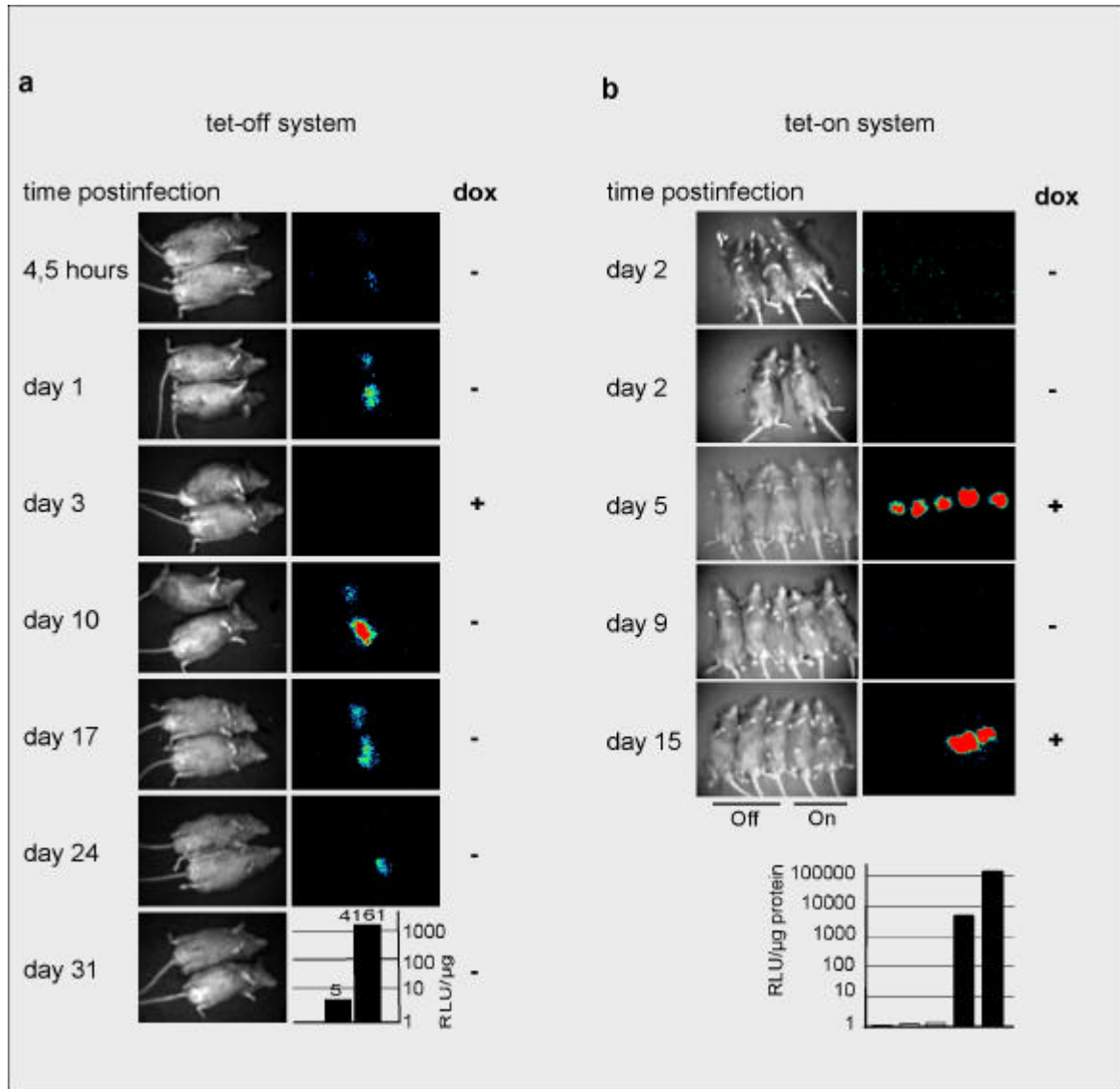


Fig. 19: bioluminescence imaging of luciferase activity in living mice

Bioluminescence imaging of luciferase was performed after intraperitoneal injection of avertin and then 150μg D-luciferin (AppliChem, Germany) as substrate for the enzymatic reaction in anesthetized mice. Images are representative of two tTA transgenic mice (tet-off **a**: male, 12 months old) and five rtTA transgenic mice (tet-on **b**: male, 3 months old) infected with $2,5 \times 10^9$ i.u. of Adbiluc-GFP. 2 mg/ml doxycycline was added to drinking water. Following sacrifice of mice, luciferase activity was measured in homogenate of liver or kidney of each animals. Note the log-scale for graphs of luciferase assay.

2.2.1.2.3- Co-expression of two marker genes in liver cells

With the tTA-responsive bidirectional promoter P_{bi-1} it was possible to monitor the expression of cytokine using the co-expression of luciferase gene. Co-expression of both genes was shown in transfected HeLa cells (Baron U. et al., 1995), but never following adenovirus transduction.

Co-expression in HuH7-TTR-rtTA cells following infection with Adbiluc-mIFN γ

To determine if both genes were simultaneously expressed, the adenovirus Adbiluc-mIFN γ was employed. The HuH7-TTR-rtTA cells were infected with Adbiluc-mIFN γ at

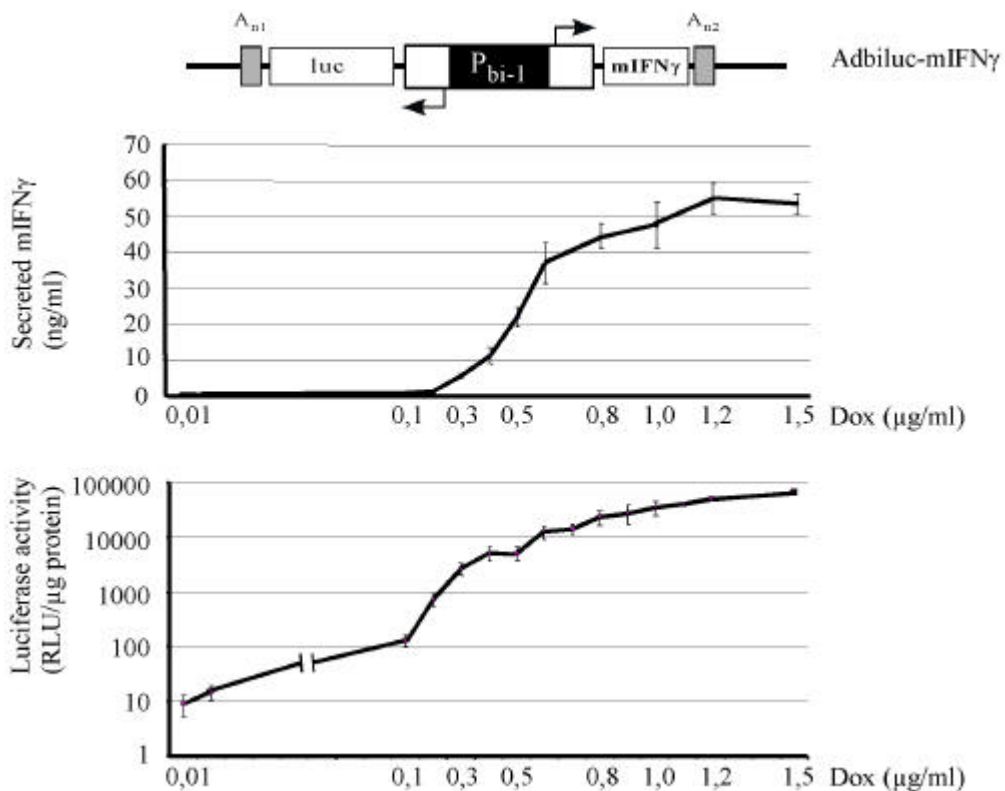


fig. 20: simultaneous expression of luciferase and mIFN γ in doxycycline concentration dependent fashion

The hepatoma cells HuH7-TTR-rtTA were infected with Adbiluc-mIFN γ , at a moi of 10 i.u./cell. The transduced cells were incubated in 1 ml medium complemented with increasing dosis of doxycycline from 0,01 to 1,5 μ g. Two days later the secreted cytokine mIFN γ in supernatant was quantified using ELISA assay specific to mIFN γ and luciferase activity was determined in cell lysate. Each point represents the mean value of secreted cytokine or luciferase activity normalized to protein content in three independent experiments.

different moi's and doxycycline was added at increasing concentration from 0,1 to 1,5 μ g/ml. 48 hours after induction, cell culture medium was collected and secreted mIFN γ

was quantified by ELISA. Luciferase activity was measured in cell lysates. As shown in fig.20, addition of increasing amounts of doxycycline lead to proportionally increasing luciferase activity and secretion of mIFN γ : the maximal level of luciferase activity (500061 RLU/ μ g protein) or mIFN γ secretion (55 ng/ml) were obtained at 1,0 to 1,2 μ g/ml doxycycline. The dose-response analysis showed that both luciferase activity and cytokine secretion showed comparable kinetics, demonstrating that both genes are co-regulated. A saturation at a concentrations of doxycycline of $\geq 1,0\mu$ g/ml in the cell culture model was observed. These data supported the strategy in this work to use luciferase gene as reporter for monitoring expression of cytokines *in vivo*, where no simple quantitative assays are available.

Co-expression in tTA transgenic mice following injection of Adbiluc-GFP

Sections at 12-15 μ m of organs from infected tTA transgenic mice with Adbiluc-GFP were produced by sliding microtomy following 4% paraformaldehyd fixation. By microscopy, almost all hepatocytes strongly expressed GFP in sections of the liver tissues of mice kept in the on-state (fig.21b). No GFP-expressing cells in the other collected tissues were observed. A luciferase activity of 100000 RLU/ μ g protein in the liver (fig 21a) correlated to GFP co-expression.

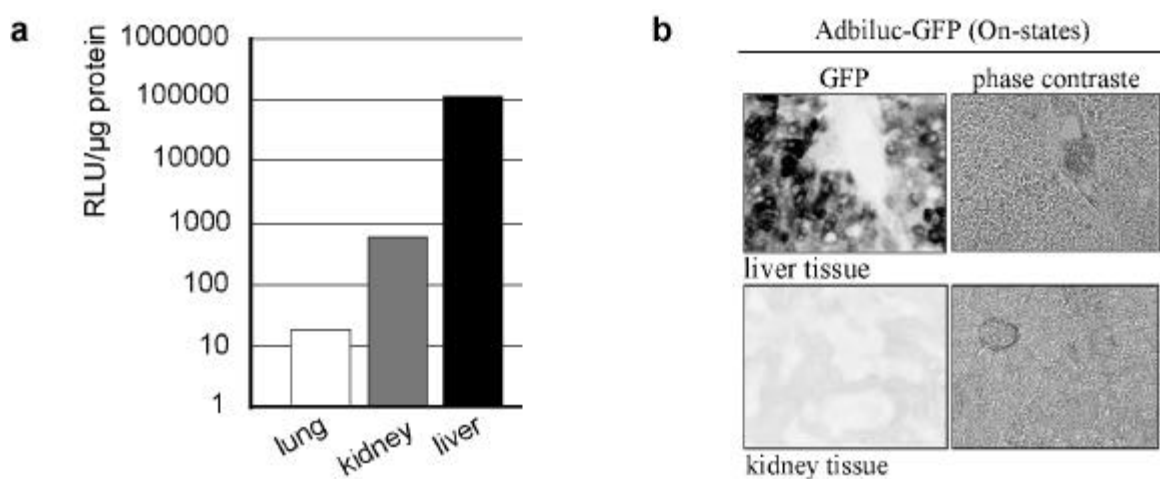


fig. 21: luciferase activity in liver and kidney in relation to GFP expression in tissue sections

(a) Each bar represented value of luciferase activity normalized to protein content luciferase activity in homogenate of liver or kidney of representative tTA transgenic mouse infected per i.v. with Adbiluc-GFP without supplementation of doxycycline in drinking water (on-states).

(b) By fluorescent microscopy, the proportion of GFP expressed cells was performed in liver or kidney section on 12 μ m of a same mouse.

Co-expression in rtTA transgenic mice following injection of Adbiluc-mIFN γ

Two rtTA transgenic mice were infected with 2×10^8 i.u. of Adbiluc-mIFN γ and one received doxycycline in drinking water. After sacrifice, the level of secreted mIFN γ in blood serum of these mice was below the detection limit of the employed ELISA assay (5pg/ml). It is known that gamma interferon mediates induction of CXX chemokine IFN γ -inducible protein-10 (IP10/CXCL10) (Luster et al., 1993). Thus, to monitor secretion of mIFN γ , an indirect quantification by a real-time RT-PCR assay was employed to determine the amount of IP10 mRNA normalized to housekeeping genes. As shown in fig.6a, the induction of the luciferase activity is more sensitive than the amplification of copies of IP10 mRNA normalized to GAPDH and LDH transcripts. The injection of 2×10^8 i.u. of Adbiluc-mIFN γ in rtTA transgenic mice targeted only 10% of liver cells. By addition of 2 mg/ml doxycycline in drinking water, luciferase activity was induced by a factor of 4368, but only by a factor of 19 for IP10 mRNA. Sensitivity and procedure of the assay supported the luciferase expression as quantitative marker for gene expression *in vivo*.

2.2.1.2.4- Time course of cytokine and luciferase expression following gene transfer in rtTA transgenic mice

To describe the kinetic of both genes, four groups of three heterozygous rtTA transgenic mice were each infected with 2×10^8 i.u. of Adbiluc-mIFN γ . 100 μ g/ml doxycycline was added in the drinking water at different time points post infection. All mice were sacrificed on day 24 p.i. following 3, 10, 17 and 24 days of doxycycline treatment. Luciferase assay and real time RT PCR for IP10 and 2'5'OAS mRNA normalized to housekeeping genes were performed with the liver homogenate of each mouse.

After 10 days in the on-state, luciferase activity decreased by 30% relative to the luciferase activity on day 3 p.i. (fig.22b); after 17 days of induction, luciferase activity fell to 14%. After 24 days induction, luciferase activity only corresponded to 1% of the activity on day 3 p.i.. However, the i.v. injection of only 2×10^8 i.u. of Adbiluc-mIFN γ per mouse and a moderate dose of 100 μ g/ml doxycycline added for 3 days to the drinking water induced a maximal luciferase activity of 139 RLU/ μ g protein (fig.22a) in the liver, which it was low in comparison to previously experiments. In the same way, after 10 days of induction the cytokine co-expression, monitored by IP10 mRNA level decrease to 30% of treatment after 3 days of induction (fig.22a). After 24

days of induction only 10% of induced IP10 was detected. The time course of cytokine-inducible 2'5'OAS transcription followed the IP10 and luciferase activity until 10 days of induction, and seemed to be restore to steady state after 24 days of induction. The standard deviations (SD) indicated in fig.22a of the mean value for luciferase activity, IP10 and 2'5'OAS induction were high, probably because of the use of heterologous transgenic mice.

These data argued for a downregulation of tet-inducing cytokine and luciferase co-expression in liver of rtTA transgenic mice.

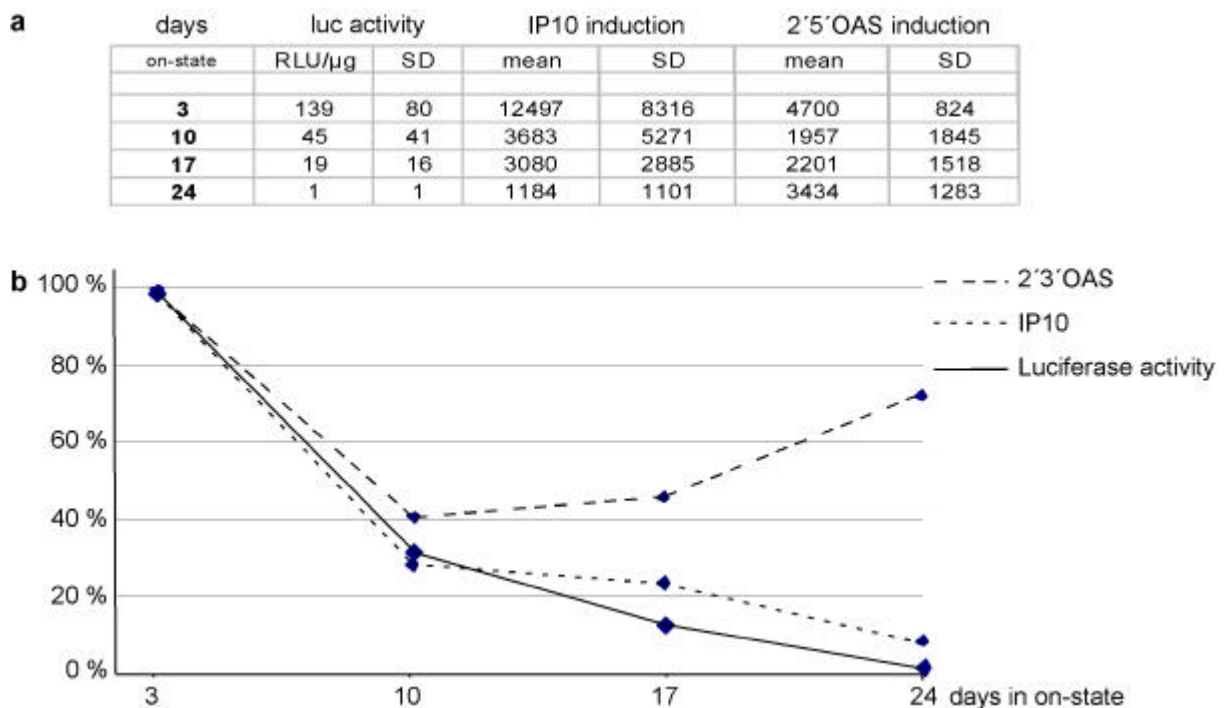


fig. 22: Downregulation of inducible luciferase, IP10 and 2'5'OAS expression during continuous doxycycline stimulation.

Three heterozygous rtTA transgenic mice per group were infected i.v. with 2×10^8 i.u. of Adbiluc-mIFN γ . Mice were sacrificed following doxycycline induction (on-state) as indicated, and their livers were analyzed. Total RNA was extracted from each liver and employed for real time RT PCR detection (**a**) of IP10 and 2'5'OAS mRNA copies normalized to housekeeping genes such as GAPDH and LDH (T. Giese, Department of Immunology, University of Heidelberg). Standard deviation was indicated (SD). (**b**) The time course of the mean values of luciferase activity, IP10- or 2'5'OAS-induction in the liver was graphical represented relative to their maximum values at day 3 post induction.

2.2.2- Antiviral effect of tet-inducible cytokine gene transfer on HBV replication in HBV transgenic mice, a model for chronic infection.

The disadvantage of constitutive cytokine expression described above could be overcome by applying Adbiluc to HBV transgenic mice. The Adbiluc system was established in tTA and rtTA transgenic mice as a tight gene transfer system to achieve a liver specific and tet-regulated gene expression. To use the system, HBV and rtTA had to be expressed. Double transgenic mice were generated and infected with Adbiluc vectors. Effects on HBV replication were characterized after induction of cytokine expression *in vivo* after the immune response to adenoviral particles 5-7 days p.i. had ceased .

2.2.2.1- H1.3/rtTA double transgenic mouse as model for chronic infection

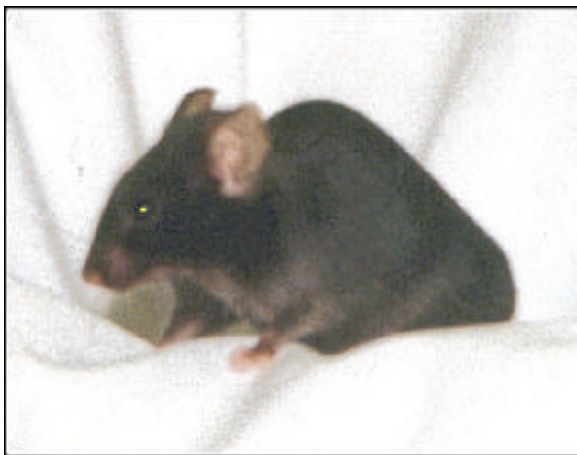


fig. 23: portrait of generated H1.3/rtTA double transgenic mouse as chronic HBV infection model.

To generate HBV transgenic mouse, which expressed rtTA essentially in liver, homozygous HBV1.3 fsX(-)3'5' transgenic mice were crossed with heterozygous rtTA transgenic mice. By PCR analysis, the F1 generation was screened using primers specific to sequence of rtTA (primer srtTA(+); primer srtTA(-)) to identify mice heterozygous for rtTA (fig.23). All mice had to be heterozygous for HBV.

2.2.2.2- Effect on HBV replication in H1.3/rtTA double transgenic mice

To separate the previously described effect of mIFN γ and mIFN β expression on HBV replication from an immune response to the adenoviral infection, four groups (eight mice per group) of H1.3/rtTA double transgenic mice were matched for age-, sex-, and HBeAg- level. These mice received a single i.v. injection of 2×10^8 i.u. of adenovirus type Adbiluc, which mediated tet-regulated mIFN α , mIFN β or mIFN γ expression. Four mice were injected with saline solution (mock control), were sacrificed at the same time

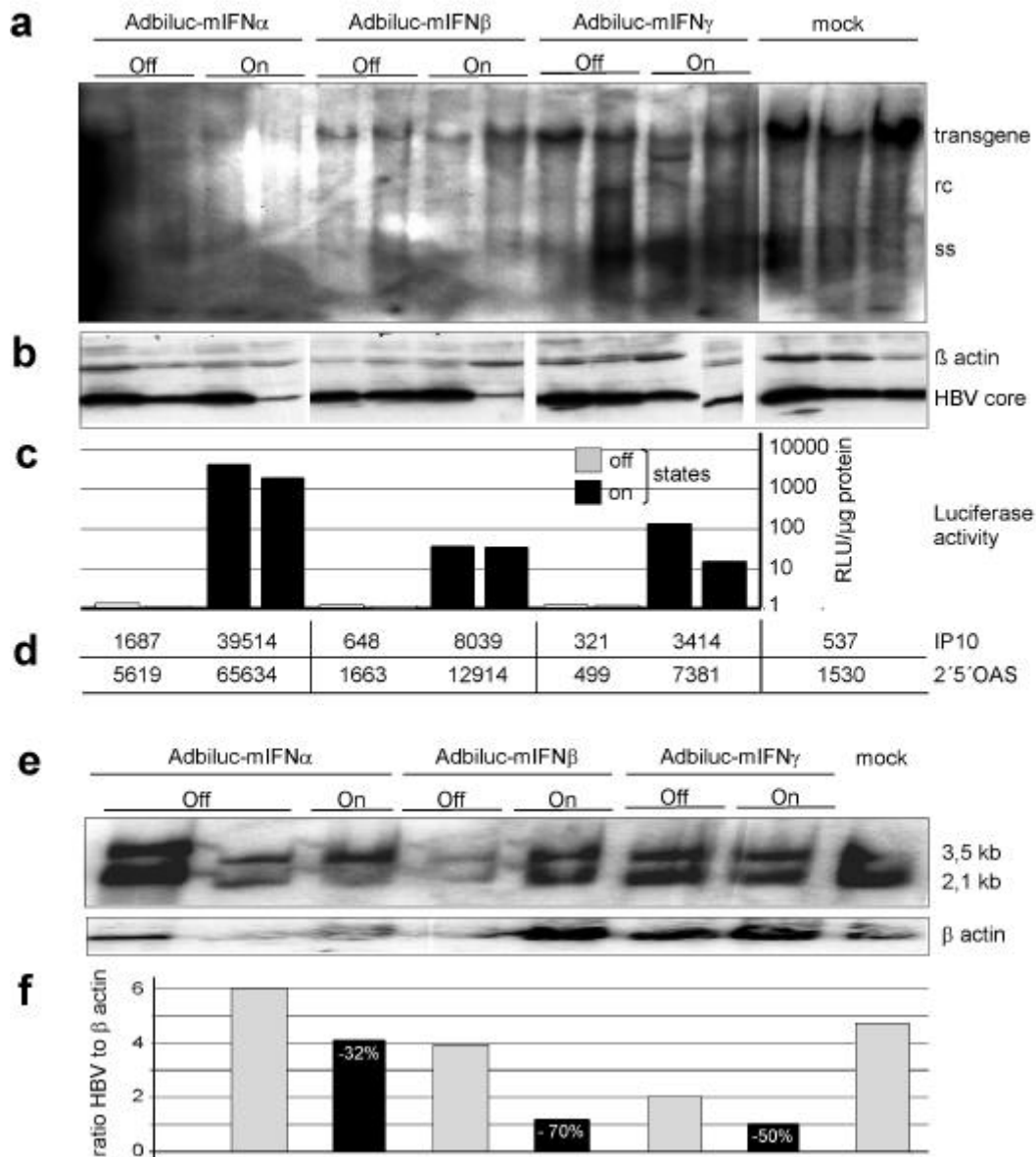


fig. 24: Induction of mIFN β and mIFN γ expression decreased HBV RNA transcripts, but no the HBV DNA and viral protein amount in the liver of H1.3/rtTA double transgenic mouse

Three groups of four H1.3/rtTA double transgenic mice matched for sex, age and level of HBeAg were injected with 2×10^8 i.u. of indicated adenovirus or saline (mock). At day 12 p.i. 100 μ g/ml doxycycline was added in drinking water for two mice per group. Mice were sacrificed at day 18 p.i. and livers were harvested.

- (a) Southern-Blot was performed with 20 μ g of total liver DNA digested with *Hind III* isolated from each mice. Bands corresponding to the integrated transgene (**transgene**), relaxed circular (**rc**) and single-stranded (**ss**) linear HBV DNA replicative forms are indicated. The membrane was hybridized with α^{32} P-labeled HBV-specific DNA probe.
- (b) Western-Blot was performed with homogenate of liver from each mouse (14% SDS PAGE). Polyclonal antibody H801 and monoclonal antibody anti β actin were used to detected HBV core protein and β actin as normalization standard.
- (c) Luciferase assay was performed with homogenate of each livers of mice in the off/on- state.

- (d) Total RNA was extracted from each liver of mice and employed for real time RT PCR detection of IP10 and 2'5'OAS mRNA copies normalized to housekeeping genes (analysis was performed by T. Giese, Department of Immunology, University of Heidelberg).
- (e) Northern Blot was performed with mRNA extracted from 200µg total RNA from pooled liver of each group of mice. Bands at 2.4- and 3,5-kb positions correspond to subgenomic and pregenomic HBV RNA forms. The membrane was hybridized with $\alpha^{32}\text{P}$ -labelled HBV- and then β actin-specific DNA probe. Both HBV RNA forms were quantified by phosphoimaging and represented (f) in ratio HBV RNA to β actin RNA. Indicated percent corresponds to decrease of HBV mRNA normalized to β actin mRNA of mice in the on-state relative to mice in the off-state for each type of cytokine.

point. At day 7 p.i., a dose of 2 mg/ml doxycycline was added to the drinking water for four mice per group (on-state). At day 11 p.i., an intraperitoneal injection of 2 mg doxycycline in 100µl PBS was performed in these mice. Unfortunately, all these mice died during the next 10 hours. However, mock treated mice, who received also an i.p. injection of doxycycline, survived. To continue the experiment, the remaining mice were separated at day 12 p.i. into subgroups of two mice each: one subgroup of mice received 100 µg/ml doxycycline in drinking water (on-state) the other remained in the off-state. Mice were sacrificed at day 18 p.i. and their livers and sera were harvested for analysis.

As shown in the Southern Blot in fig.25a, no change in HBV replicative intermediates was observed in mice injected with Adbiluc-mIFN γ relative to mock mice. The amount of intracellular viral protein measured as HBV core was also unchanged (fig.25b). However, luciferase expression in mice in the on-state was induced, but levels varied dependent on the adenovirus used. Mice infected with Adbiluc-mIFN α had a high luciferase activity of 5000-2000 RLU/µg protein and other mice infected with Adbiluc-mIFN β or Adbiluc-mIFN γ had 100- to 10-fold lower luciferase activity (fig.25c). However, no basal expression was detectable in the livers of all mice in the off-state.

The indirect monitoring of cytokine expression, with IP10 or 2'5'OAS induction, followed the same kinetics (fig.24d). Using the analysis of intrahepatic IP10 or 2'5'OAS induction, basal expression of IFN α could not be excluded: 3,5-fold basal induction of IP10 and 2'5'OAS was observed in mice infected with Adbiluc-mIFN α without doxycycline in drinking water. The amount of 3,5 kb and 2,1 kb mRNA's (fig.24e) seemed to be reduced in mice infected with Adbiluc-mIFN γ in the off-state although no cytokine was expressed

Table 8: HBV DNA copies, HBeAg, ALT levels in serum of heterozygous H1.3/rtTA double transgenic mice infected with Adbiluc-mIFN α , Adbiluc-mIFN β or Adbiluc-mIFN γ

saline solution (mock)	Adbiluc-mIFN α		Adbiluc-mIFN β		Adbiluc-mIFN γ		mock
	Off	On	Off	On	Off	On	On
HBeAg (S/Co) before injection	121	68	108	91	95	83	95
HBeAg (S/Co) day 5 p.i.	163	93	96	77	93	69	98
100 μ g/ml doxycycline day12 p.i.	-	+	-	+	-	+	-
HBeAg (S/Co) day 18 p.i.	135	139	74	52	69	50	78
ALT (IU/liter)	52	44	33	39	72	133	48
HBV DNA copies per μ l serum	6.355	1.805	2.845	1.336	15.025	7.480	10.631

according to IP10 or 2'5'OAS values (fig.24f). Induction of mIFN α expression had no effect on HBV mRNA's. Induction of mIFN β or mIFN γ reduced HBV mRNA's 70% and 50% , respectively (fig. 24f). No change on HBeAg titer in sera of mice was observed (table 8). Also, no elevation of the ALT value was observed, excepted a 2,8-fold increase in mice injected with Adbiluc-mIFN γ by induction of mIFN γ expression. A decrease of HBV DNA copies in serum (2- to 3,5-fold) was detected, when cytokine expression were induced in the liver (table 8). Liver histology (fig.25) showed a fatty degeneration of hepatocytes in mice injected with Adbiluc-mIFN α , which was enhanced after induction of cytokine expression. Only mild inflammation was observed here and in the livers of mice transduced with Adbiluc-mIFN β or Adbiluc-mIFN γ .

In summary induction of mIFN α , mIFN β or mIFN γ did not strongly repress HBV replication in this experiment.

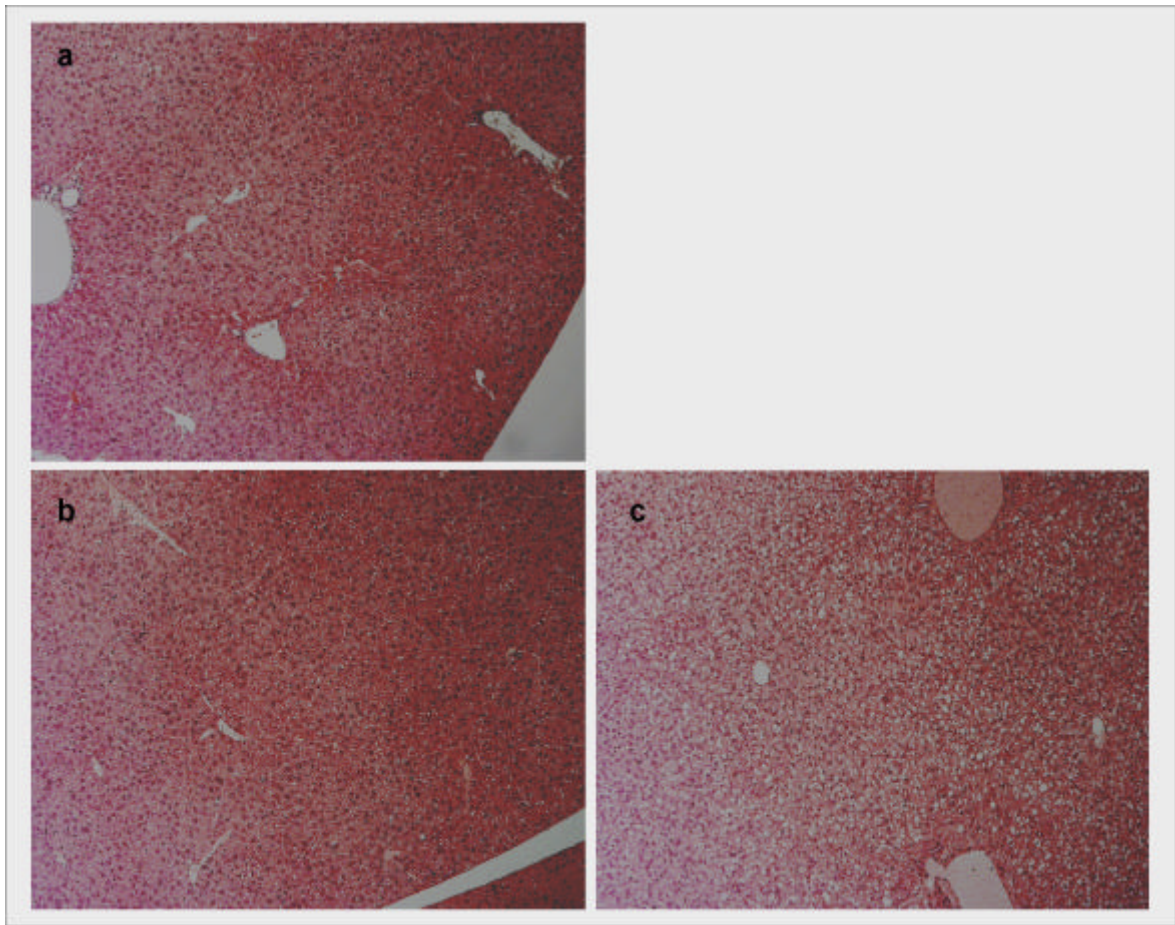


fig. 25: Fatty degeneration in livers of H1.3/rtTA double transgenic mice transduced with Adbiluc-mIFN α , Adbiluc-mIFN β or Adbiluc-mIFN γ in the on-state.

The liver of the previously used H1.3/rtTA double transgenic mice (fig. 24), which were injected with 2×10^8 i.u. of Adbiluc-mIFN α , with 100 $\mu\text{g}/\text{ml}$ doxycycline during 6 days (c) or without doxycycline (b). Mice no-infected with adenovirus represents mock (a). The livers were fixed in 4% paraformaldehyd and 10 μm sections were stained with hematoxylin and eosin (Department of Pathology, DKFZ, Heidelberg).

2.2.3- Tet-inducible cytokine expression in rtTA transgenic mice infected with AdH1.3, a model for acute HBV infection.

To determine the effect of cytokine expression on the immune response to an acute HBV infection, four groups (five mice per group) of heterozygous rtTA transgenic mice were matched for age-, sex- and HBeAg level. All mice received a single i.v. injection of a mixture of 10^9 i.u. of AdGH1.3, 10^8 i.u. of Adbiluc-mIFN γ and 10^8 i.u. of Adbiluc-mIFN α . 100 $\mu\text{g}/\text{ml}$ doxycycline was added to half of the animals at day 3 p.i. (on-state). The other mice remained in the off-state. Mice of each group were sacrificed at day 7 p.i. and at day 14 p.i.. Five mice were injected with only 10^9 i.u. of AdGH1.3 (positive control) and were sacrificed at day 14 p.i..

Table 9: HBV DNA copies, HBeAg, ALT levels and antibodies to HBsAg in serum and induction of IP10- and 2'5'OAS-RNA in the liver of mice.

in serum:	Adbiluc-mIFN α + Adbiluc-mIFN γ					
	AdGH1.3		Off-state		On-state	
	mean	SD	mean	SD	mean	SD
HBeAg (S/Co) before Dox	34	2,7	197	12,4	167	5,0
HBeAg (S/Co) day 7 p.i.	11	0,7	30	8,7	11	0,7
ALT (IU/liter)	303	110	256	96	200	92
HBV DNA copies per μ l serum	622	453	795	302	370	119
induction in the liver:						
IP10			40069	950	729040	23772
2'5'OAS			51223	1182	558014	15291
in serum:						
HBeAg (S/Co) before Dox	4	0,1	177	6,6	136	5,4
HBeAg (S/Co) day 14 p.i.	57	22,4	22	9,2	9	0,1
anti-HBs (mIU/ml) day 14 p.i.	7	3,6	4	2,5	4	3,5
ALT (IU/liter)	48	22	155	51	37	18
HBV DNA copies per μ l serum	-		567	112	538	95
induction in the liver:						
IP10	24627	581	11387	342	37972	2799
2'5'OAS	31495	703	32439	594	123266	4781

Heterozygous rtTA transgenic mice were infected with 10^9 i.u. of AdGH1.3 and/or 10^8 i.u. of Adbiluc-mIFN α + 10^8 i.u. of Adbiluc-mIFN γ . From day 3 p.i., 100 μ g/ml doxycycline was added to drinking water. Mice were sacrificed at day 7 p.i. or day 14 p.i..

Induction of IP10 and 2'5'OAS transcripts was observed in all mice at day 7 p.i. (table 9). This induction was reduced by day 14 p.i. and thus was probably induced by the AdH1.3 injection as expected. The elevated ALT level in serum of almost all injected mice also indicated a immuno-inflammation in the liver at day 7 p.i. ALT level was normalized again

at day 14 p.i.. By addition of 100 μ g/ml doxycycline to the drinking water, a further 18- and 10-fold induction of IP10 and 2'5'OAS transcription was observed at day 7 p.i.. The difference remained 3,5-fold at day 14 p.i.. A 2-fold reduction of the HBeAg titer in serum of mice injected with Adbiluc-mIFN α and Adbiluc-mIFN γ was observed, when cytokines were expressed (table 9). The HBV DNA copies in serum of mice injected with Adbiluc-mIFN α and Adbiluc-mIFN γ decreased 2-fold, when cytokine expression was induced (table 9). However, variation of HBeAg levels and HBV-DNA levels was high at day 7 and day 14 p.i.. Antibodies again the viral surface protein HBsAg were detectable at day 14 p.i.. The humoral response observed was still low at day 14 p.i. with or without induction of cytokine expression and closed to the detection limits of the diagnostic assay.

In summary, the data shown that mIFN α and mIFN γ were expressed in the livers of transduced mice. AdGH1.3 infection strongly induced IP10 and 2'5'OAS transcription during the first seven days, which interfered with the monitoring of cytokine expression. Minor changes in HBeAg titer and HBV DNA copies in sera of mice in the on-state were observed in this experiment.