Interferon-Regulated Pathways That Control Hepatitis B Virus Replication in Transgenic Mice†

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We previously showed that the intrahepatic induction of cytokines such as alpha/beta interferon (IFN-α/β) and gamma interferon (IFN-γ) inhibits hepatitis B virus (HBV) replication noncytopathically in the livers of transgenic mice. The intracellular pathway(s) responsible for this effect is still poorly understood. To identify interferon (IFN)-inducible intracellular genes that could play a role in our system, we crossed HBV transgenic mice with mice deficient in IFN regulatory factor 1 (IRF-1), the double-stranded RNA-activated protein kinase (PKR), or RNase L (RNase L) (IRF-1−/−, PKR−/−, or RNase L−/− mice, respectively), three well-characterized IFN-inducible genes that mediate antiviral activity. We showed that unmanipulated IRF-1−/− or PKR−/− transgenic mice replicate HBV in the liver at slightly higher levels than the respective controls, suggesting that both IRF-1 and PKR individually appear to mediate signals that modulate HBV replication under basal conditions. These same animals were responsive to the antiviral effects of the IFN-α/β inducer poly(I-C) or recombiant murine IFN-γ, suggesting that under these conditions, either the IRF-1 or the PKR genes can mediate the antiviral activity of the IFNs or other IFN-inducible genes mediate the antiviral effects. Finally, RNase L−/− transgenic mice were undistinguishable from controls under basal conditions and after poly(I-C) or IFN-γ administration, suggesting that RNase L does not modulate HBV replication in this model.

Hepatitis B virus (HBV) is a noncytopathic, enveloped virus that causes acute and chronic hepatitis and hepatocellular carcinoma (4). It was previously shown that the intrahepatic induction of alpha/beta interferon (IFN-α/β) that occurs in the livers of HBV transgenic mice after injection of poly(I-C) or infection with unrelated hepatotropic viruses, such as lymphocytic choriomeningitis virus and adenovirus (9, 21), downregulates HBV replication noncytopathically (21). The contribution of IFN-α/β to this process was demonstrated by showing that antiviral activity is completely blocked in HBV transgenic mice that were either genetically deficient for the IFN-α/β receptor (21) or treated with antibodies to IFN-α/β (10). Recent studies have shown that the mechanism whereby IFN-α/β inhibits HBV replication in the transgenic mouse liver relies on the inhibition of formation and/or the destabilization of immature HBV RNA-containing capsids (29).

The intrahepatic induction of gamma interferon (IFN-γ) also inhibits HBV replication noncytopathically; this effect is achieved by injecting HBV transgenic mice with HBV-specific cytotoxic T lymphocytes (10, 21) or interleukin 12 (3) or by infecting them with mouse cytomegalovirus (2). Experiments with antibodies to IFN-γ or mice genetically deficient for IFN-γ have demonstrated the importance of this cytokine as a mediator for the antiviral activity of these stimuli.

Upon binding to specific surface receptors, IFN-α/β and IFN-γ activate a variety of IFN-inducible genes, some of which trigger common intracellular antiviral pathways (25). A large variety of IFN-inducible genes have been identified to date; most of these are activated by the JAK-STAT signal transduction cascade (6, 7, 14, 25, 34). However, how these genes exert their intracellular antiviral activities is still poorly understood. Among the interferon (IFN)-inducible genes, those for the IFN regulatory factor 1 (IRF-1), RNase L, and double-stranded RNA-activated protein kinase (PKR) systems are some of the best characterized. IRF-1 is an IFN-inducible transcription factor that regulates nitric oxide production (1, 19) and cytokine signaling (22) and mediates antiviral activities against several viruses, including coxsackievirus (19). RNase L, a cellular RNase activated by 2’,5’-oligoadenylates produced by IFN-induced, double-stranded RNA-dependent synthetase (2’,5’-OAS), degrades viral and cellular RNAs (5, 15, 31). This pathway has been shown to selectively reduce the intracellular RNA content of viruses such as human immunodeficiency virus (20), encephalomyocardarditis virus (EMCV) (18), and vaccinia virus (8). The inhibition of viral protein synthesis initiation by IFN-inducible PKR has been shown to suppress certain viral infections, including those with EMCV (14) and reovirus (23). Recently, the interaction between the hepatitis C virus NS5 and E2 proteins and PKR was suggested to reduce the sensitivity of this virus to IFN-α/β (13, 26, 28).

Based on the aforementioned studies, it is possible that the IRF-1, RNase L, or PKR genes represent intracellular candidate genes that mediate the antiviral activities of IFN-α/β and/or IFN-γ in our system. To test this hypothesis, we crossed transgenic mice that replicate HBV with mice that are genetically deficient for IRF-1, RNase L, or PKR (IRF-1−/−, RNase L−/−, or PKR−/− mice, respectively), and we monitored the contributions of these gene products to the antiviral effects of the systemic administration of poly(I-C) or IFN-γ.
MATERIALS AND METHODS

Mice. The HBV transgenic mouse lineage 1.3.32 (inbred C57BL/6) used in this study (official designation, Tg[HBV 1.3 genome][Chi32]) was described previously (11). These mice replicate HBV at high levels in the liver without any evidence of cytopathology. Lineage 1.3.32 was crossed with IRF-1+/−, RNase L−/−, or PKR−/− (30) mice. IRF-1+/− mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Heterozygous mice from lineage 1.3.32 were repeatedly backcrossed with homozygous mice from each of the three knockout lineages to yield progeny that were screened for hepatitis B e antigen (HBcAg) in the serum (by using a commercially available kit from Abbott Laboratories, Abbott Park, Ill.) to confirm the absence of HBV infection. HBcAg-positive progeny were screened for homozgyosity of the null mutations by PCR as described previously (16, 30, 32). Mice found either homozygous or heterozygous for the null mutation were sacrificed 24 h later. Their livers were processed for histological analysis or snap frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses (see below).

Biochemical and histological analyses. The extent of hepatocellular injury was monitored by measuring serum alanine aminotransferase (sALT) activity at multiple time points after treatment with saline, poly(I-C), or IFN-γ. sALT activity was measured with a Paramax chemical analyzer (Baxter Diagnostics, Inc., McGaw Park, Ill.) exactly as previously described (10). For histological analysis, liver tissue samples were fixed in 10% zinc-buffered formalin (Anatech, Battle Creek, Mich.), embedded in paraffin, sectioned (3 μm), and stained with hematoxylin and eosin exactly as described elsewhere (10).

Statistical analysis. A two-tailed nonparametric Wilcoxon test was used to assess the statistical significance of the experimental variation in the intrahepatic content of HBV replicative forms among transgenic mice that were either heterozygous or homozygous for the IRF-1 or PKR null mutation. A P value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Higher levels of HBV replication in the livers of IRF-1+/− or PKR−/− mice. HBV transgenic mice from lineage 1.3.32 were crossed with IRF-1+/−, RNase L−/−, or PKR−/− mice. Groups (six mice per group) of age (8 to 10 weeks), sex (male), and serum HBcAg-matched animals that were either heterozygous (plus/minus) or homozygous (minus/minus) for the respective null mutation were sacrificed, and their livers were harvested. Following extraction, total hepatic DNAs were pooled for each group and analyzed for HBV replication by Southern blot analysis.

As shown in Fig. 1, IRF-1+/− or PKR−/− mice replicated HBV in the liver at levels about 2.2- or 1.5-fold higher than the respective heterozygous control littermates or RNase L−/− mice (as measured by phosphorimaging analysis with the transgene band for normalization). As shown in Table 1, the experimental variation in the levels of HBV replication between individual IRF+/− and IRF−/− mice and individual PKR+/− and PKR−/− mice was statistically significant (P values of 0.035 and 0.030, respectively), while no significant difference was detected between RNase L−/− and RNase L+/− mice (data not shown). The levels of HBV replication observed in all groups of heterozygous control mice were comparable to those observed in wild-type mice from lineage 1.3.32 (data not shown).

![Image of Table 1](http://jvi.asm.org/)
RNase L−/− or PKR−/− animals, groups of age-, sex-, and serum HBeAg-matched transgenic mice that were either heterozygous or homozygous for the respective null mutation were injected intravenously with a single dose of poly(I-C) (200 μg/mouse) and sacrificed 24 h later. Total hepatic DNA was analyzed for HBV replication by Southern blot analysis. Bands corresponding to integrated transgene (Trans.), relaxed circular (RC), and single-stranded (SS) linear HBV DNA replicative forms are indicated. The integrated transgene can be used to normalize the amount of DNA bound to the membrane. Total hepatic RNA was analyzed for the expression of cytokine- and 2′,5′-OAS-specific transcripts by an RPA. TNF, tumor necrosis factor; IL, interleukin. The RNA encoding ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane. The results were compared with those observed for livers pooled from 10 age-, sex-, and serum HBeAg-matched transgenic littermates injected with saline (NaCl). The mean sALT activity, measured at the time of autopsy, is indicated for each group and is expressed in units per liter.

As shown in Fig. 2, the hepatic content of HBV DNA replicative forms was profoundly reduced in all groups of IRF-1, RNase L, or PKR heterozygous or homozygous transgenic mice that were either heterozygous or homozygous for the respective null mutation were injected intravenously with a single dose of poly(I-C) (200 μg/mouse). Mice were bled and sacrificed, and livers were harvested 24 h later, when the antiviral activity of poly(I-C) is maximal (21, 29). Results were compared with those observed for livers pooled from six age-, sex-, and serum HBeAg-matched transgenic controls that were sacrificed 24 h after injection with saline.

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Role of IRF-1, RNase L, or PKR in antiviral activity induced by IFN-γ. The intrahepatic induction of IFN-γ inhibits HBV replication in transgenic mice (3, 10), and this effect occurs independently of IFN-α/β (21). IFN-γ and IFN-α/β bind to distinct surface receptors and activate multiple intracellular antiviral pathways, some of which are common and involve IRF-1, RNase L, or PKR (25). To monitor the role of IRF-1, RNase L, or PKR in the direct antiviral activity of IFN-γ, groups of animals (four mice per group) from the same lin-
eases as those used in the experiment shown in Fig. 1 were injected intravenously with a single dose of murine recombinant IFN-γ (200,000 U/mouse) and sacrificed 24 h after injection.

As shown in Fig. 3, HBV DNA replication was profoundly inhibited in the different groups of IRF-1, RNase L, or PKR heterozygous or homozygous transgenic mice after IFN-γ injection compared to the respective saline-injected controls (as measured by phosphorimaging analysis, the average reduction of HBV DNA replicative forms was about 10-fold in all groups of mice, with the exception of RNase L−/−, in which the reduction was about 6-fold). As in the experiment with poly(I-C), the inhibitory effect of IFN-γ on HBV replication was associated with the intrahepatic induction of 2′,5′-OAS RNA (Fig. 3), although this effect was less pronounced than that observed after poly(I-C) injection (Fig. 2), particularly for PKR−/− mice (Fig. 3). The notion that IFN-γ induces lower levels of 2′,5′-OAS RNA than IFN-α/β was previously demonstrated (24). Moreover, it was previously shown that the lack of the PKR gene results in a defect in IFN-γ-dependent signaling (17). Again, little or no liver disease was observed either histologically (data not shown) or biochemically (Fig. 3, bottom).

In summary, the results reported here showed that unmanipulated RNase L−/− transgenic mice replicate HBV in the liver at levels similar to those in the respective controls. This result, coupled with the fact that the deletion of RNase L activity does not block the antiviral effect of poly(I-C) or IFN-γ, suggests that RNase L is not likely to mediate the ability to inhibit HBV replication. The results also showed that unmanipulated IRF-1−/− or PKR−/− transgenic mice replicate HBV in the liver at levels slightly higher than those in the respective controls, suggesting that, under basal conditions, IRF-1 or PKR appears to mediate signals that modulate HBV replication. Follow-up experiments showed that the antiviral effect of poly(I-C) or IFN-γ was fully operative in the absence of either IRF-1 or PKR. Although there was a defect in IFN-γ induction of 2′,5′-OAS in the PKR−/− mice, the antiviral activity of IFN-γ was intact. Since 2′,5′-OAS is upstream of RNase L, these results are in accord with the lack of constitutive or induced anti-HBV activity in the RNase L−/− mice. Collectively, the data suggest either that high local concentrations of IFN-α/β or IFN-γ inhibit HBV replication by activating IRF-1 or PKR or that other IFN-inducible pathways mediate their antiviral effects. Future experiments with both IRF-1−/− and PKR−/− HBV transgenic mice will attempt to discriminate between these two hypotheses.

In keeping with the possibility that other IFN-inducible genes are involved, it is noteworthy that the advent of oligonucleotide arrays has enabled investigators to identify many novel genes that are induced or repressed by IFN-γ or IFN-α/β (6, 7). Furthermore, it was recently shown that although EMCV is susceptible to the antiviral activity of either RNase L or PKR, the simultaneous disruption of both gene products is...
still associated with residual IFN-dependent antiviral activity (33); this result indicates that as-yet-undefined IFN-inducible antiviral pathways are operative in the control of EMV. It is also worth mentioning that the Mx protein, an IFN-induced GTPase that selectively inhibits influenza viruses and bunyaviruses (12), is not involved in our system, since the genetic backgrounds (C57BL/6 and 129/Sv) of the mice used here are deficient for this particular protein (25). Future research aimed at further defining IFN-induced intracellular molecular events that control HBV is clearly warranted.

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