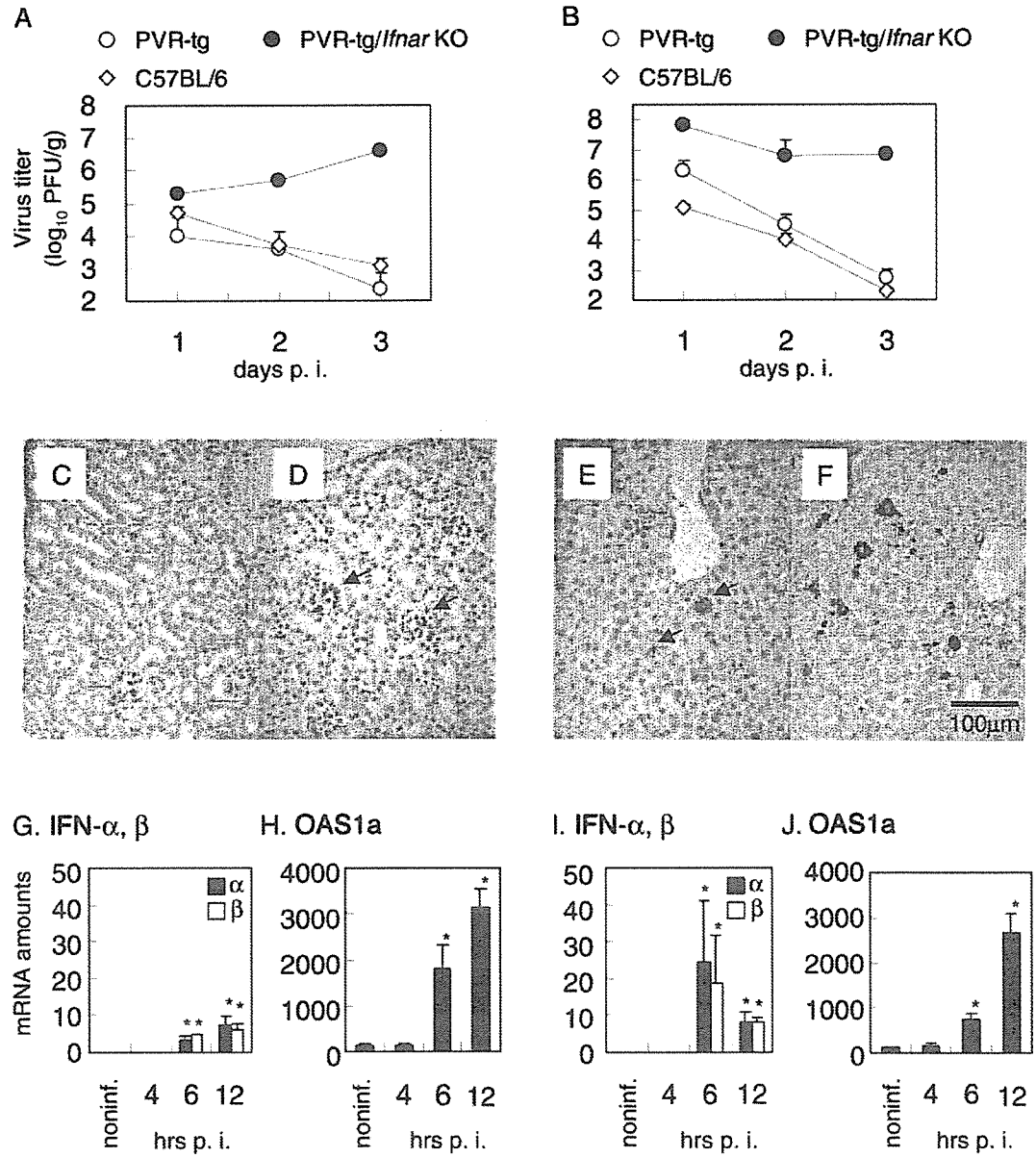


FIG. 1. PV replication and IFN response in vivo. (A and B) PV replication in mouse kidneys (A) and liver (B). C57BL/6 mice, PVR-tg mice, and PVR-tg/*Ifnar* knockout (KO) mice were inoculated intravenously with 10^7 PFU of the Mahoney strain. Mice were sacrificed daily. The mice were perfused with 20 ml of phosphate-buffered saline, and the virus titer in the kidneys was determined by plaque assay. The error bars indicate standard errors of the means (SEM). (C to F) Immunohistochemical detection of PV antigens. PVR-tg mice (C and E) and PVR-tg/*Ifnar* knockout mice (D and F) were intravenously inoculated with 10^8 PFU of PV, and the kidneys and livers were examined 1 day p.i. PV antigens were detected in a few cells on two glomeruli of the kidneys (arrows) of the PVR-tg/*Ifnar* knockout mice (D), but not in the wild-type PVR-tg mice (C). Only a few PV antigen-positive cells were detected in the livers of the PVR-tg mice (E), but a large number of PV antigen-positive cells were detected in the livers of PVR-tg/*Ifnar* knockout mice (F). (G to J) IFN response in vivo. PVR-tg mice were inoculated with 10^8 PFU of PV. The total RNAs of the kidneys or livers of the uninfected (noninf.) and infected mice were isolated. IFN-α/β (G) and OAS1a (H) mRNA levels in the kidneys and IFN-α/β (I) and OAS1a (J) mRNA levels in the liver were determined by quantitative real-time PCR analysis. The copy numbers of the mRNA per 10^7 copies of rRNA are shown. The values represent the averages (± SEM) of three mice. The asterisks indicate a significant difference ($P < 0.05$; Student's t test) in comparison with the uninfected samples.

possible that the amounts of IFN-α and IFN-β mRNAs detected at 6 h p.i. were not sufficient to bring about ISG induction. Alternatively, it is also likely that the IFN mRNA detected at 6 h p.i. and later was not translated or secreted as IFN protein, since the shutoff of host protein synthesis begins to inhibit the translation of IFN mRNAs approximately 2 h after PV infection (9) and the PV 3A protein disrupts normal ER-Golgi trafficking, preventing the secretion of cytokines (4, 6).

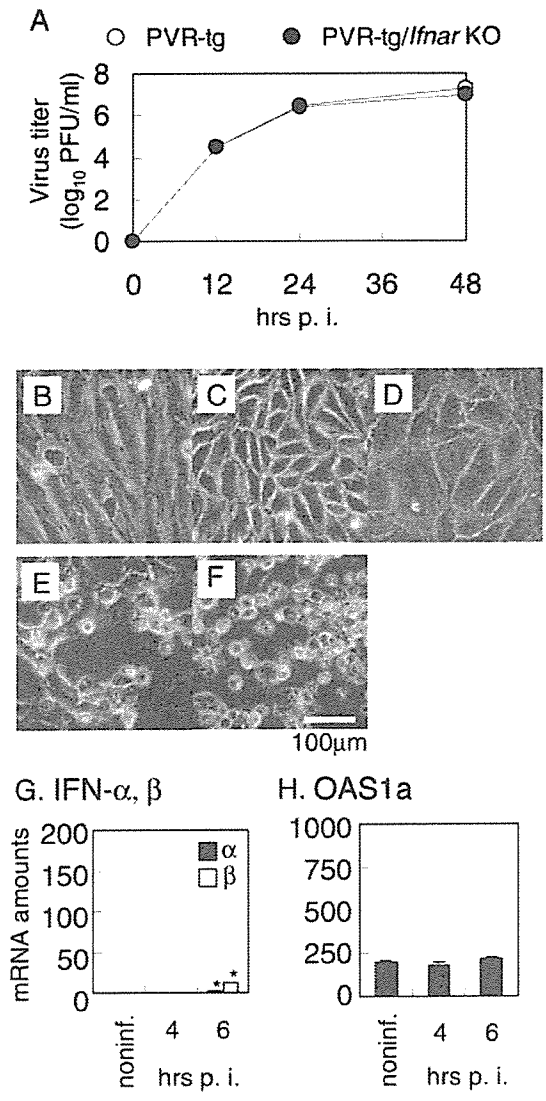


FIG. 2. PV replication and IFN response in cultured kidney cells. (A) Time course of PV titers in cultured kidney cells. Primary cultured kidney cells from PVR-tg mice and PVR-tg/*Ifnar* knockout (KO) mice were infected with PV at an MOI of 0.001. At each time point, the cells were disrupted by three cycles of freezing and thawing, and virus titers were determined by plaque assay. The PV propagation profiles in the two types of cell were indistinguishable. (B to D) Morphology of uninfected primary cultured kidney cells. At least three different kinds of cells were present in the uninfected culture. (E and F) Kidney cells from PVR-tg mice (E) and from PVR-tg/*Ifnar* knockout mice (F) were infected with PV at an MOI of 1.0. CPE was observed at 24 h p.i. (G and H) IFN response in cultured kidney cells from PVR-tg mice. The cells were infected with PV at an MOI of 10. Total RNA of the cells was isolated at the indicated times p.i., and the IFN-α/β (G) and OAS1a (H) mRNA levels were determined by quantitative real-time PCR analysis. The asterisks indicate a significant difference ($P < 0.05$; Student's t test) in comparison with the uninfected (noninf.) samples. The error bars indicate SEM. Note that the vertical scales in panels G and H are different from those in Fig. 1G to J.

Thus, they did not contribute to establishing the antiviral state. We therefore postulate that the acquisition of PV susceptibility in the kidney cells after cultivation is due to the loss of IFN response during the process of cultivation.

PV susceptibility in liver cells in vitro. We then prepared liver cells in culture by collagenase treatment. We obtained 1×10^7 viable liver cells per mouse and plated them onto plastic plates at a density of 1.25×10^5 cells/cm^2. The live cells readily attached to the plates and could be maintained for several days, after which the cells were deformed and died. Based on the morphology, most of the cells were hepatocytes, and other cells were not included in the culture (Fig. 3B and D). We then compared the PV susceptibilities of liver cells derived from PVR-tg mice and of those derived from PVR-tg/*Ifnar* knockout mice. The cells from wild-type PVR-tg mice also acquired susceptibility to PV but, unexpectedly, were not fully susceptible. After PV inoculation at an MOI of 0.001, the titer in the liver cells derived from wild-type PVR-tg mice increased much more slowly than in cultured kidney cells and other cell lines (compare Fig. 2A, 3A, and 4A). Cells with CPE were rarely observed at 24 h p.i. (Fig. 3C). The PV titer continued to increase until 72 h p.i. We stopped observation at that time, since the cells were starting to deteriorate, and therefore, it was difficult to distinguish cellular damage from CPE caused by PV infection. On the other hand, PV replication in the cells from PVR-tg/*Ifnar* knockout mice was as efficient as in the cultured kidney cells and other cell lines (Fig. 2A, 3A, and 4A). The infected cells showed CPE with shrinkage and loss of the nuclear membrane at 24 h p.i. (Fig. 3E). When the cells of PVR-tg mice were infected with PV at an MOI of 50, the titer reached a plateau at 6 h p.i., but it was approximately 10 times lower than that in liver cells of PVR-tg/*Ifnar* knockout mice (data not shown). These results indicate that the liver cells derived from wild-type PVR-tg mice are partially resistant to PV.

We then examined the IFN response in primary cultured liver cells of PVR-tg mice infected with PV at an MOI of 10. Significant levels of IFN-α and IFN-β mRNAs were observed at 4 h p.i., and induction of OAS1a mRNA was observed at 6 h p.i. (Fig. 3F and 3G). It is worth noting that IFN response occurred in liver cells as early as 6 h p.i. These results suggest that the primary cultured liver cells derived from PVR-tg mice still retain the capability for a rapid IFN response, as they did in vivo. Although the amounts of IFN mRNAs detected in the liver cells in culture are larger than those detected in the liver in vivo (Fig. 1I), this does not necessarily mean that IFN inducibility in the cultured cells is higher than that in the liver in vivo. Since the cells were infected with PV at an MOI of 10 in the in vitro experiment, the IFN mRNAs produced per infected cell may be lower than those in vivo. It is possible that this early response contributes to the partial resistance of the liver cells to PV infection.

IFN induction in primate cells after PV infection. The above-mentioned results suggested that primary cultured mouse cells were susceptible to PV infection when they could not establish the antiviral state mediated by rapid IFN response. We therefore investigated whether this is true for the cultured AGMK cells and several other cell lines. AGMK cells at the third to fifth passages from the primary culture were used in the experiments. JVK-03 is a cell line spontaneously immortalized from a primary culture of AGMK cells (30).

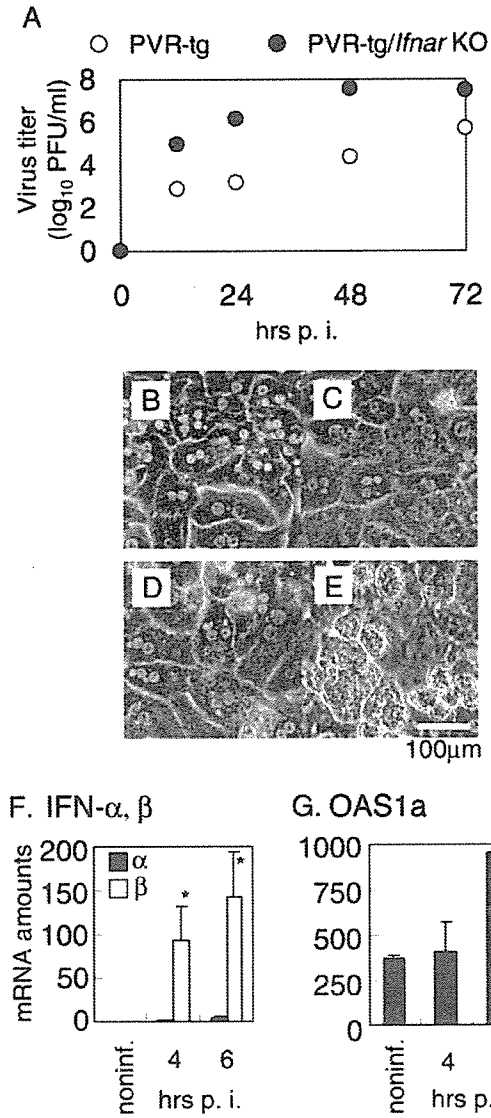


FIG. 3. PV replication and IFN response in cultured liver cells. (A) Time course of PV titers in cultured liver cells. Primary cultured liver cells from PVR-tg mice and PVR-tg/*Ifnar* knockout (KO) mice were infected with PV at an MOI of 0.001. At each time point, PV titers were determined by plaque assay. Note that the titer in the cells from PVR-tg mice increased much more slowly than that in cells from PVR-tg/*Ifnar* knockout mice. (B to E) Morphology of primary cultured liver cells. Liver cells from PVR-tg mice (B and C) and from PVR-tg/*Ifnar* knockout mice (D and E). Uninfected liver cells are monotonic (B and D). The cells were infected with PV at an MOI of 1.0 and observed at 24 h p.i. (C and E). The CPE was rarely observed in the liver cells from PVR-tg mice (C) but was clearly observed in liver cells from PVR-tg/*Ifnar* knockout mice (E). (F and G) IFN response in liver cells from PVR-tg mice. The cells were infected with PV at an MOI of 10. Total RNA of the cells was isolated, and the IFN-α/β (F) and OAS1a (G) mRNA levels were determined by quantitative real-time PCR analysis. The asterisks indicate a significant difference ($P < 0.05$; Student's t test) in comparison with the uninfected (noninf.) samples. The SEM are indicated by vertical bars. Note that the vertical scales in panels F and G are different from those in Fig. 1G to 1J.

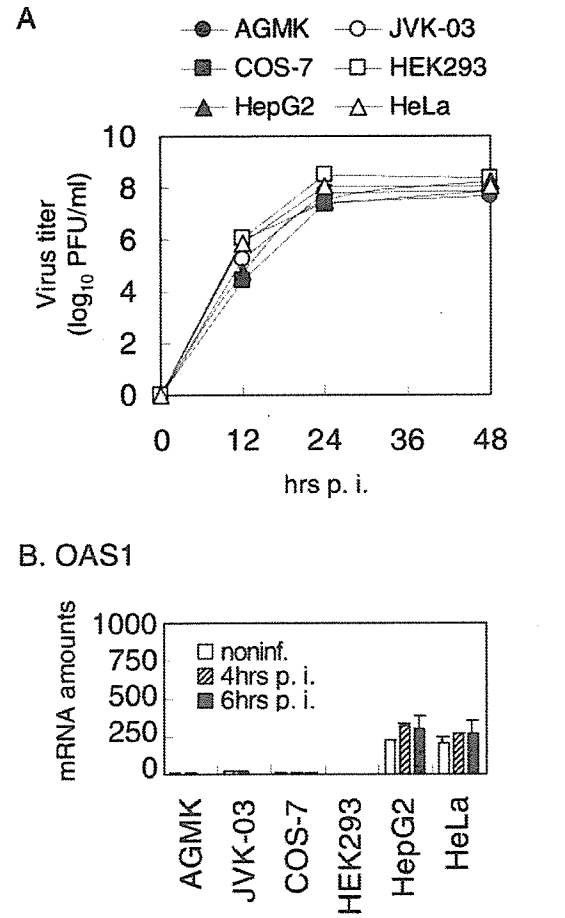


FIG. 4. PV replication and IFN response in cultured cells. (A) Time course of PV titers in AGMK cells and primate cell lines. AGMK cells, JVK-03 cells, COS-7 cells, HEK293 cells, HepG2 cells, and HeLa cells were inoculated with PV at an MOI of 0.001. The cells were disrupted at the indicated time points, and PV titers were determined. (B) Expression of OAS1 mRNA in infected cells. Cells were infected with PV at an MOI of 10. At 4 and 6 h p.i., total RNA was isolated from the cells, and mRNA levels were determined. Note that OAS1 mRNA levels did not change during the course of infection in all cells. The SEM are indicated by vertical bars. noninf., uninfected.

AGMK cells, JVK-03 cells, HEK293 cells, COS-7 cells, HepG2 cells, and HeLa cells were tested for PV propagation and IFN response. All the cells were highly susceptible, and PV infection spread easily when the cells were infected at an MOI of 0.001 (Fig. 4A). Almost no IFN-α or IFN-β mRNA expression was detected within 6 h after PV infection at MOIs of 0.001, 0.1, and 1 (data not shown). An increase in OAS1 mRNA levels was not observed upon PV infection at an MOI of 10 (Fig. 4B) or at the different MOIs (data not shown). The results suggest that these cells do not respond to PV rapidly or do not respond at all.

Reduced expression levels of ISGs in kidney cells. We hypothesized that kidney cells have lost the rapid inducibility of IFNs because of the decrease in the expression levels of genes involved in IFN production. We compared the expression levels of various genes in the kidneys in vivo and kidney cells in

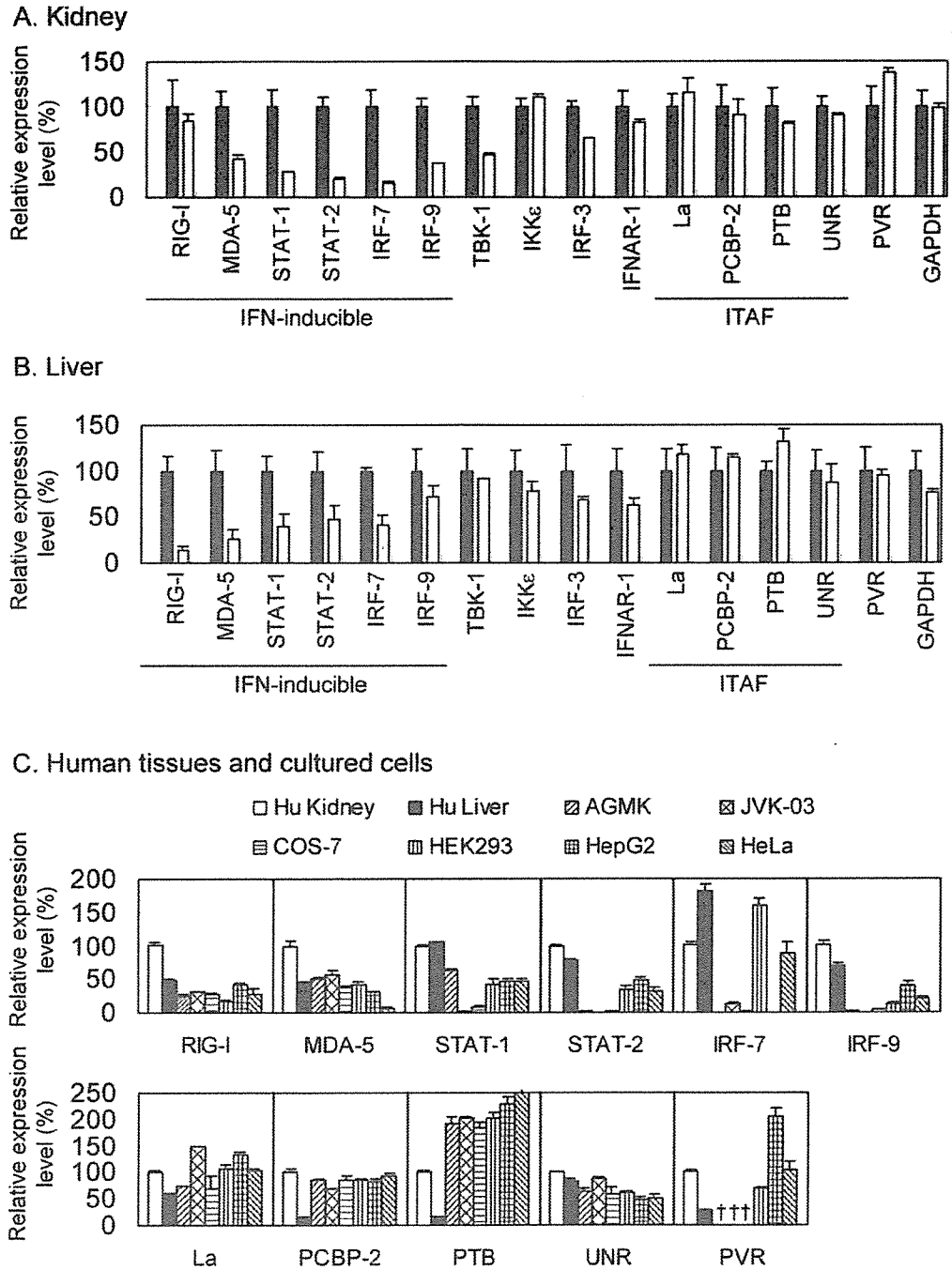
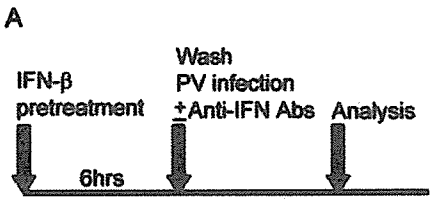


FIG. 5. Comparison of expression levels of ISGs, ITAFs, and PVR mRNAs. (A) Levels of mRNAs in kidney cells in vivo (closed bars) and in vitro (open bars). RNA levels were determined by quantitative real-time PCR. The level of expression of each gene in the kidneys in vivo is represented as 100%. (B) Levels of mRNAs in liver cells in vivo (closed bars) and in vitro (open bars). The level of expression of each gene in the liver in vivo is represented as 100%. (C) mRNA levels involved in the IFN response (top) and mRNA levels of ITAFs and PVR (bottom) in human (Hu) and monkey cells. The level of expression of each gene in the human kidney in vivo is represented as 100%. †, PVR mRNAs in the monkey cells could not be measured by the TaqMan probe for human PVR because of the sequence difference in the probed region. The SEM are indicated by vertical bars.

vitro by RT-PCR. RIG-I, MDA-5, STAT-1, STAT-2, IRF-7, and IRF-9 are involved in the IFN response and are induced by IFNs (25, 33, 36, 54, 65). As shown in Fig. 5A, the levels of MDA-5, STAT-1, STAT-2, IRF-7, and IRF-9 mRNAs were reduced to less than 50% of their original levels after cultivation. TBK-1, IKKε, IRF-3, and IFNAR-1 are also involved in the IFN response (41, 57, 66) but are not IFN inducible. Their expression levels, and that of a housekeeping gene, the

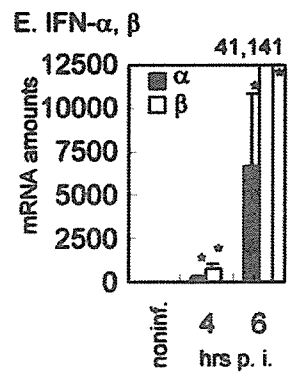


D

	Induction level ± SEM
RIG-I	15.8 ± 2.5
MDA-5	23.9 ± 4.3
STAT-1	16.6 ± 2.7
STAT-2	18.5 ± 1.3
IRF-7	129.1 ± 12.1
IRF-9	15.0 ± 0.7

B

IFN-β pretreatment (IU/ml)	IFN activities in the culture medium ± SEM (IU/ml)
10.0	31.3 ± 20.2
3.2	16.5 ± 6.6
1.0	20.1 ± 0.5
0.3	8.8 ± 0.1
0.1	5.6 ± 0.4
0	<3
noninf.	<3



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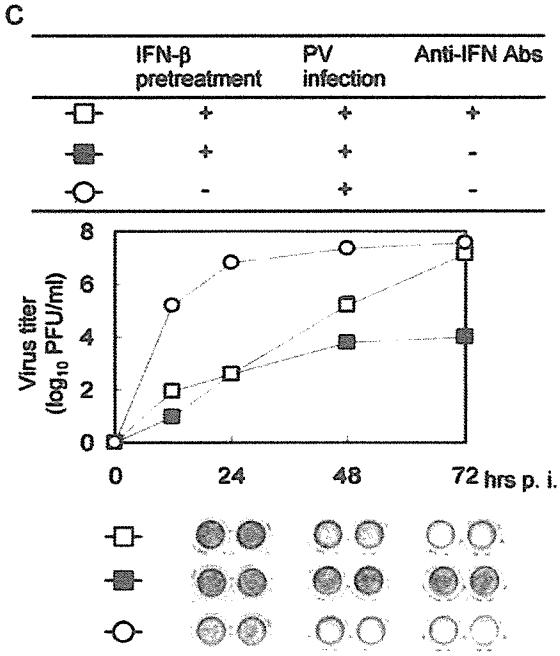

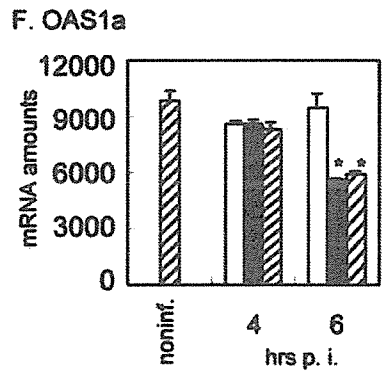

FIG. 6. Effect of priming on the susceptibility of cultured mouse kidney cells. (A) Experimental procedure. Kidney cells were pretreated with IFN-β at the indicated doses for 6 h. The cells were washed with MEM three times and challenged with PV with or without anti-IFN-α and -β antibodies (Abs) to neutralize the effects of newly synthesized IFNs. The cells were tested for survival, PV titers, and IFN response at the indicated time points. (B) Resistance of mouse kidney cells to PV infection after pretreatment with IFN-β. The cells in duplicate were treated with IFN-β at the indicated doses (left column). The cells were then infected with PV at an MOI of 0.1 for 3 days, and surviving cells were visualized by staining them with Amido black 10B. The IFN activities in the culture medium at 72 h after challenge were measured by the CPE dye uptake method (right). Mean values (± SEM) of two wells are indicated. Note that IFNs were induced only in the pretreated cells. noninf., uninfected. (C) Time course of PV replication in pretreated kidney cells. The cells pretreated with 1 IU/ml of IFN-β were infected with PV at an MOI of 0.001 in the presence (+) or absence (−) of anti-IFN antibodies, and the PV titer was determined at each time point. Open circles indicate the PV titer of untreated kidney cells infected at the same MOI. Note that the PV titer did not increase after 48 h p.i. if the anti-IFN antibodies

GAPDH gene, did not show considerable change. Notably, a decrease in the level of IRF-7 after cultivation was most evident in the kidneys. Similarly, in the liver, the expression levels of these IFN-inducible mRNAs also decreased (Fig. 5B). RIG-I, MDA-5, STAT-1, STAT-2, and IRF-7 mRNA levels decreased to less than 50% after cultivation. The decrease in the expression levels of these genes may be responsible for the acquisition of susceptibility.

With regard to the ITAFs and PVR, we directly measured the mRNA levels of PTB, La, PCBP-2, UNR, and PVR and compared the expression levels in the kidneys and livers of PVR-tg mice in vivo and in vitro (Fig. 5A and B). The expression levels of these mRNAs in vivo and in vitro did not differ significantly. In situ hybridization and immunofluorescence studies revealed that PVR mRNA is not expressed in all cells in the kidneys of PVR-tg mice in vivo (23, 50). However, all the primary cultured cells are susceptible to PV infection. We therefore could not exclude the possibility that additional expression of PVR in the kidney cells that had no PVR expression in the kidneys in vivo also occurred during the cultivation process.

In addition, we determined the levels of ISGs involved in the IFN response in primate cells. The human TaqMan probes worked sufficiently in measuring mRNA for these genes of the African green monkey. Since it was not possible to compare the expression levels of ISGs before and after cultivation directly, we used human kidney and liver RNAs that were commercially available as controls. The mRNA levels for each ISG in AGMK cells, JVK-03 cells, COS-7 cells, and HepG2 cells were different from cell to cell. However, in general, they were lower than those of the human kidney and liver in vivo (Fig. 5C). In the HEK293 cells and HeLa cells, IRF-7 mRNA levels were much higher than or similar to those in human kidneys. In these cells, however, the levels of other ISG mRNAs tested were lower than those in the human kidneys. The fact that HEK293 cells and HeLa cells failed to induce rapid IFN response upon PV infection suggested that high-level expression of IRF-7 alone was insufficient for rapid IFN response. Rapid and robust IFN response was not achieved unless a positive-feedback loop of IFN response was formed (60). This idea is supported by the experimental results showing that depletion of the gene products involved in the IFN response using RNA interference or dominant-negative techniques resulted in a decrease in IFN response (5, 64). Our results also suggest that the shortage of one of the ISGs may be rate limiting for the whole IFN response.

Expression levels of ITAF mRNAs in cultured human and monkey cells were also determined. The expression levels of ITAF mRNAs were not greatly different from cell to cell (Fig. 5C). The expression levels of La autoantigen, PCBP-2, and UNR mRNAs were almost the same as those in human kidneys in vivo. The levels of PTB mRNA were approximately twofold higher than those in human kidneys in vivo. However, the absolute values for PTB mRNA levels were the same as those for PTB mRNA levels in mouse kidneys and livers in vivo (4,000 to 10,000 copies/10^7 copies of 18S rRNA). Thus, the PTB levels observed in the cultured cells were not exceptionally high. We therefore consider that the changes in mRNA levels for La, PCBP-2, PTB, and UNR do not contribute to the acquisition of susceptibility to PV infection in these cells in culture. We could not measure the PVR mRNA levels for monkey cells by the TaqMan probe designed for the human PVR gene. In the HEK293, HepG2, and HeLa cells, the PVR mRNA levels were not significantly higher than those in the human kidney or liver. Thus, the acquisition of PV susceptibility is associated with a decrease in ISG expression levels. These results strongly suggest that the kidney cells in culture, as well as many cell lines of primate origin, have lost rapid IFN response due to the loss of factors that regulate this response.

Effect of priming of mouse kidney cells with IFN. We tested if pretreatment of mouse kidney cells with a small amount of IFN reconverted the cells to PV resistance by acquisition of rapid IFN inducibility. The experimental procedures are shown in Fig. 6A. Kidney cells were treated with various concentrations of recombinant mouse IFN-β for 6 h. They were then infected with PV at an MOI of 0.1. The culture medium was removed for IFN assay, and the cells were fixed with 4% paraformaldehyde at 3 days p.i. and stained with Amido black 10B. The cells pretreated with more than 0.3 IU of IFN-β were protected from PV infection (Fig. 6B). Consistent with the protection phenomenon, IFN activity was observed in the culture medium of the protected cells (Fig. 6B). Similarly, cells were protected when they were infected with PV at an MOI of 1 (data not shown). The infected cells had been alive for more than a week on the day uninfected control cells died because of overgrowth. However, the cells were killed by PV when infected at an MOI of 10 (data not shown). The cells pretreated with IFNs became resistant to PV infection, except when they were infected at a very high MOI.

We compared the virus growth in the pretreated (1 IU/ml for 6 h) and untreated kidney cells after PV infection at an MOI of 0.001 (Fig. 6C), and the viabilities of the cells were monitored by Amido black 10B staining (Fig. 6C, bottom). PV replication was greatly inhibited in the pretreated cells compared to the untreated cells. Notably, the increase in PV titer was not observed after 48 to 72 h p.i. (Fig. 6C, middle), despite the presence of many uninfected cells (Fig. 6C, bottom). This indicated that the infection did not spread after 48 h p.i.,

were not added, despite the presence of viable cells in the wells. At the bottom, survival of the cells at indicated time points was displayed by Amido black 10B staining. (D) ISG levels in pretreated cells. Kidney cells were pretreated with 1 IU/ml of IFN-β for 6 h. mRNA levels of each gene relative to the untreated cells are indicated. (E) IFN-α and -β mRNA induction in pretreated cells. The cells were pretreated with 1 IU/ml of IFN-β as described above and infected with PV at an MOI of 10. The total RNA was isolated at the indicated time points, and the IFN-α/β mRNA levels were determined. The asterisks indicate a significant difference ($P < 0.05$; Student's t test) in comparison with the uninfected samples. The error bars indicate SEM. Note that the vertical scale is different from those in Fig. 1G and I, 2G, and 3F and that very large amounts of IFN mRNAs are expressed in the primed cells. (F) OAS1a levels in pretreated cells. The pretreated cells (1 IU/ml of IFN-β) were infected with PV at an MOI of 10 in the absence (open bars) or in the presence (solid bars) of anti-IFN antibodies or were kept uninfected (hatched bars). The asterisks indicate a significant difference ($P < 0.05$; Student's t test) in comparison with the infected cells (open bars) at each time point.

possibly because newly synthesized IFNs accumulated in the culture medium. Pretreated cells were also infected in the presence of anti-IFN antibodies. In the presence of anti-IFN antibodies, the PV titer increased over time, indicating that the PV infection could spread. In parallel with the increase in PV titer, protection was not observed after 48 h p.i. The results suggest that the pretreatment of IFN-β confers rapid IFN inducibility on the cells and that the antiviral state is maintained by the newly produced IFNs. We then determined the mRNA levels of the IFNs and ISGs after pretreatment with 1 IU of IFN-β and subsequent PV infection. As expected, all the ISG mRNA levels increased due to the pretreatment and became higher than the mRNA levels of ISGs in the kidney in vivo (Fig. 6D). Furthermore, induction of IFN-α and IFN-β mRNAs at high levels was detected as early as 4 h p.i. (Fig. 6E). Consistent with this result, IFN activity was detected in the supernatant of PV-infected cells at 6 h p.i. (data not shown). OAS1a mRNA was also induced at high levels by the pretreatment, and the levels were maintained during the infection period (Fig. 6F). OAS1a mRNA levels in the pretreated cells were not maintained unless IFN was continuously supplied. In fact, the levels decreased over time after removal of IFN-β without PV infection or after PV infection in the presence of anti-IFN antibodies (Fig. 6F). This suggests that the expression levels are maintained by newly synthesized IFNs during this period. All these results indicate that loss of rapid IFN response after cultivation in kidney cells is reversible and that primed kidney cells restore rapid IFN inducibility and resistance to PV infection.

DISCUSSION

In this study, we demonstrated that the change in susceptibility of cells during the process of cultivation in vitro is controlled most strongly by the level of IFN response. Kauder and Racaniello and Ren and Racaniello predicted that a factor(s) other than PVR is needed for the acquisition of PV susceptibility after cultivation. They also showed that the PV IRES is functional in the mouse kidney in vivo, suggesting that a factor(s) other than ITAFs is important for the change in susceptibility (26, 50). In the cases of kidney cells and primate cell lines, rapid IFN response is lost in association with a decrease in at least some ISG expression. Although it is obvious that the expression of PVR and ITAFs influences the pathogenicity of PV in vivo in some specific situations, it did not change significantly during the cultivation process, and thus, it may not have a strong influence on this phenomenon.

PV is a highly cytopathogenic virus, and the infected cells are destroyed by lytic replication of the virus. PV has several mechanisms to inhibit IFN action. The 2A protease cleaves eIF-4G, leading to the suppression of cap-dependent protein synthesis (9). Host proteins, including IFNs, cannot be translated after shutoff. This shutoff phenomenon occurs as early as approximately 2 h p.i. in HeLa cells. The 3C protease cleaves the p65-RelA subunit of the NF-κB complex (42). Since the IFN-β gene is also positively regulated by NF-κB through positive regulatory domain II on the IFN-β gene promoter (10, 34, 62), cleavage of p65-RelA suppresses the NF-κB-mediated expression of IFN-β. Cleavage of p65-RelA is observed 3 h p.i. in HeLa cells. The 3A gene product inhibits the trafficking of secreted proteins, including IFNs (4, 6). The PV-infected cells in monolayer cultures either do not produce a large amount of IFNs or do not produce them at all (59) (Fig. 2G), probably due to inhibition by these viral proteins. Because of these anti-IFN effects of PV, the effects of IFNs on susceptibility to PV have not been taken into consideration.

As the inhibition by PV proteins operates within a few hours p.i., host cell defense must operate rapidly. It is known that treatment with a small dose of IFNs can enhance the amount of IFN produced and bring about IFN production at an earlier time in many cell lines. This phenomenon is known as priming (59). Using embryonic fibroblasts, Hata et al., Sato et al., and Taniguchi and Takaoka have shown that cells expressing very low levels of IRF-7 cannot induce a rapid and strong IFN response upon virus infection, while the cells expressing certain levels of IRF-7, possibly together with other ISGs, by a spontaneous weak IFN stimulation are able to form a positive-feedback loop of IFN action and induce a rapid and strong IFN response (Fig. 7A and B) (16, 54, 60). The former and the latter states may correspond to the "unprimed state" and "primed state," respectively. If PV infection occurs in the unprimed cells, the viral proteins can accumulate to the threshold levels quickly, IFN action is strongly inhibited by the viral proteins, and the cells will not be able to establish the antiviral state (Fig. 7A). Conversely, if PV infection occurs in the primed cells, the ISGs have already accumulated above the threshold level before infection, and a rapid and strong IFN response will occur, strengthening the cellular antiviral state (Fig. 7B). Since both expression of viral proteins after PV infection and expression of ISG induction by IFNs occur rapidly, which of them gains the initiative first is very important in determining susceptibility. The cells must be primed to produce IFNs in response to PV infection (Fig. 6) (24). From this point of view, "primed or not primed" is a very important factor in determining the fate of the cells and tissues.

We previously showed that the cells in some extraneural tissues are normally protected by the IFN response (21). The cells in the extraneural tissues in vivo expressed ISGs to a certain degree before PV infection. Expression of OASs and PKR may inhibit viral replication. In addition, ISGs that are involved in IFN production, like RIG-I and IRF-7, support a rapid and strong IFN response. Therefore, it is possible to consider that cells in the extraneural tissues in vivo are normally in the "primed state." Indeed, constitutive expression of IFNs under physiological conditions is known in humans (13, 61). It is likely that the cells in the extraneural tissues may be exposed to the constitutive IFNs. This weak IFN signal may be important for maintaining the basal ISG levels (60). On the other hand, down-regulation of ISG expression in cultured cells occurred during the process of cultivation (Fig. 5). The acquisition of susceptibility was associated with this decrease in expression of ISGs. Priming of the kidney cells with a small amount of IFN increased the basal ISG levels (Fig. 6C). This, in turn, restored rapid IFN response and resistance to PV (Fig. 6). The results suggest that cultured cells in vitro do not receive constitutive IFNs or do not produce constitutive IFNs by themselves. Therefore, we consider that the acquisition of PV susceptibility by cells in vitro is due to the transition of the cells from the "primed state" to the "unprimed state."

PV exhibits a paradoxical tropism. The replication sites of

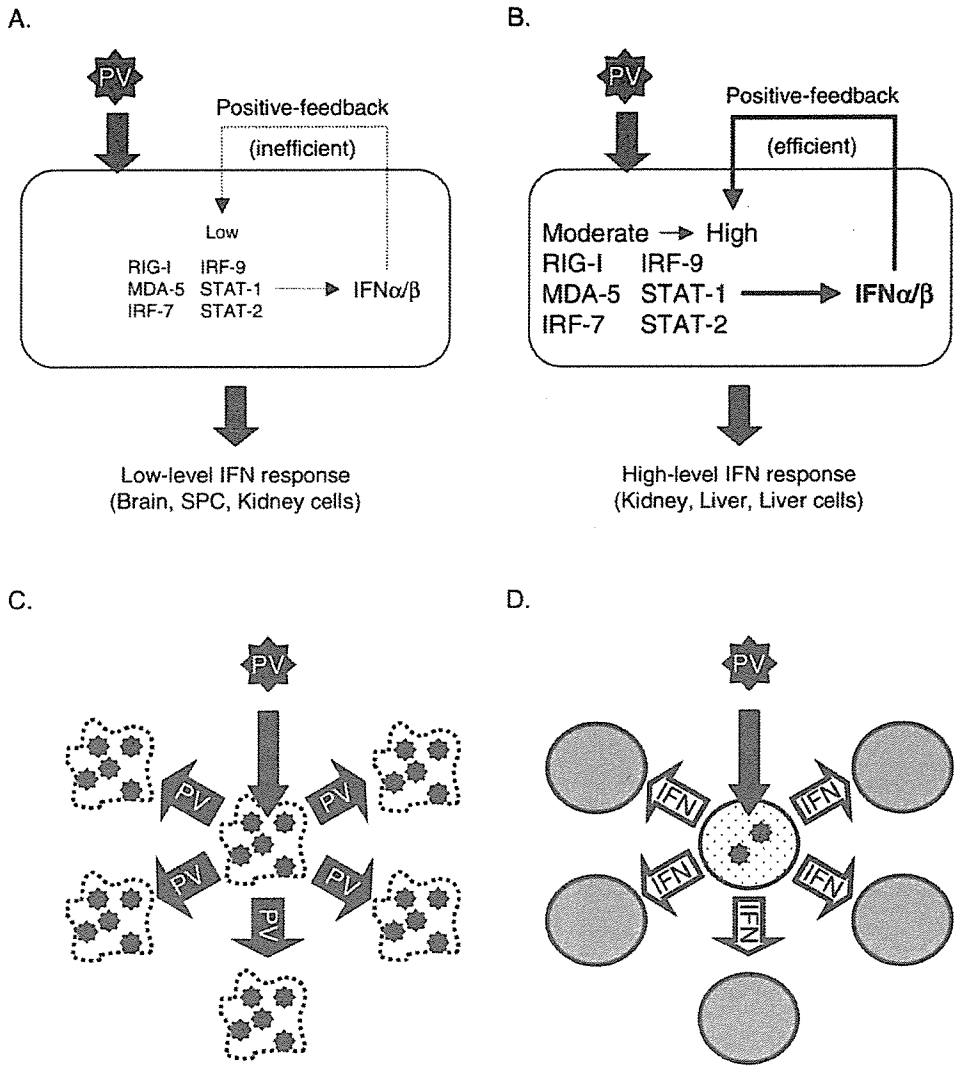

FIG. 7. Schematic diagram of PV susceptibility as controlled by IFN response. (A and B) Difference between IFN responses in unprimed and primed cells. In the unprimed cells (A), ISGs are expressed at very low levels. Upon PV infection, PV can replicate without antiviral action by the cell. The accumulated viral proteins inhibit IFN action, and ISGs cannot be expressed at sufficient levels to form a positive-feedback loop of IFN action. Thus, only a poor IFN response occurs. In the primed cells (B), ISGs are expressed before PV infection and they contribute to rapid induction of IFN by forming a positive-feedback loop. Induced ISGs further strengthen the antiviral state. (C and D) PV replication in the unprimed (susceptible) tissue or cells in culture and in the primed (nonsusceptible) tissue or cells in culture. In the susceptible tissues or cell cultures (C), PV can replicate efficiently in the initial infected cells (center) and spread easily to the surrounding cells. Pathological lesions are produced when this chain of events occurs. In the nonsusceptible tissue or cell cultures (D), a small number of infected cells (center), which have been primed, act as victims to produce IFN rapidly. The infected cells supply IFN to the surrounding cells before they release a large amount of progeny virus. The antiviral state in the surrounding cells (gray) is strengthened by the IFN. The chain of infection does not occur in the primed tissues and cells.

PV in vivo are restricted to a few tissues, including the CNS. However, PV is able to propagate in monolayer cultures derived from almost any tissue of primates. By taking the IFN response of the cells and tissues into consideration, it is now possible to explain this situation. The behavior of the cells and tissues after PV infection is largely dependent on the expression levels of ISGs, namely, the degree of priming. Infection in the tissues occurs at a very low MOI in natural infection in humans and experimental infection in tg mice in vivo. Therefore, initial infection in the tissues occurs in only a small number of cells (Fig. 1B). In the tissues where the cells are primed, these small numbers of infected cells become victims to produce IFNs rapidly (Fig. 1G and H). PV cannot spread easily, and severe pathological lesions are not produced, since the surrounding cells receive IFNs and strengthen the antiviral state before they are exposed to PV (Fig. 7D). Thus, the IFN response prevents the chain of events leading to massive infection. This was also true for the primed kidney cells in vitro (Fig. 6). On the other hand, in susceptible tissues, PV will spread from the initial infectious center to surrounding cells

because the IFN response is not sufficient (Fig. 7C). Similarly, PV can spread very easily in cultured cells because they are not in the primed state (Fig. 2). Therefore, the IFN response has a strong influence on the susceptibility of the cells both in vivo and in vitro. The paradox is now elucidated, since both tissue and cell specificities of PV infection are regulated, at least in part, by the same mechanism mediated by the IFN response.

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Rapid Genome Sequencing of RNA Viruses

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We developed a system for rapid determination of viral RNA sequences whereby genomic sequence is obtained from cultured virus isolates without subcloning into plasmid vectors. This method affords new opportunities to address the challenges of unknown or untypeable emerging viruses.

Over the past few years, global migration has led to emerging infectious diseases that pose substantial risks to public health. To prevent potential outbreaks, early detection of infectious pathogens is necessary. In particular, the recent outbreak of severe acute respiratory syndrome (SARS) provided important lessons on how unknown viruses should be detected rapidly. Thus, a standardized and qualified system is required for rapid nucleic acid sequence determination for newly emerging viruses.

Recently, we developed a new method for detecting RNA viruses. This method, based on cDNA representational difference analysis (cDNA RDA), uses 96 hexanucleotides that are not suitable for priming ribosomal RNAs but that normally prime most of the genome of an RNA virus as primers for reverse transcription in cDNA RDA (*1*). However, the RDA method with a cloning step requires at least 1 week for the determination of the nucleic acid sequence.

The Method

Our new system for rapid determination of viral RNA sequence (RDV) uses whole-genome amplification and direct sequencing techniques (Figure 1). The RDV method comprises 6 procedures: 1) effective destruction of cellular RNA and DNA for semipurification of viral particles, 2) effective elimination of DNA fragments by using a prefiltration column system and elution of small amounts of RNA, 3) effective synthesis of first- and second-strand cDNAs, 4) construction and amplification of a cDNA library, 5) construction of a second cDNA library, and 6) direct sequencing using optimized primers. The RDV method enables a broad range of partial nucleotide sequences within the entire viral RNA genome to be obtained within 2 days without cloning into plasmids.

To eliminate contaminating cellular RNA and DNA from the samples, 0.001 μg of RNase A (Qiagen, Hilden, Germany) and 1 μL (2 U) of Turbo DNA-free DNase I (Ambion, Austin, TX, USA) with 1× Turbo DNA-free buffer were incubated at 37°C for 30 min under conditions that prevented destruction of viral RNA in the viral particles. The RNA in the viral particles was then extracted within 30 min by using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). We confirmed that DNA was effectively eliminated by this RNA extraction kit.

In accordance with the Invitrogen manual, cDNA was synthesized, by using random hexamers (Takara Bio Inc., Kyoto, Japan) and Superscript III (Invitrogen, Carlsbad, CA, USA) lacking RNase H activity, at 50°C for 1 h. Then 60 U of RNase H (Takara Bio Inc.) added before synthesis of second-strand cDNA at 50°C for 1 h. In accordance with the manual, a whole genome amplification system (WGA; Sigma-Aldrich, Saint Louis, MO, USA), which was developed for amplification of genomic DNA, was used to amplify viral double-stranded cDNA. This process was

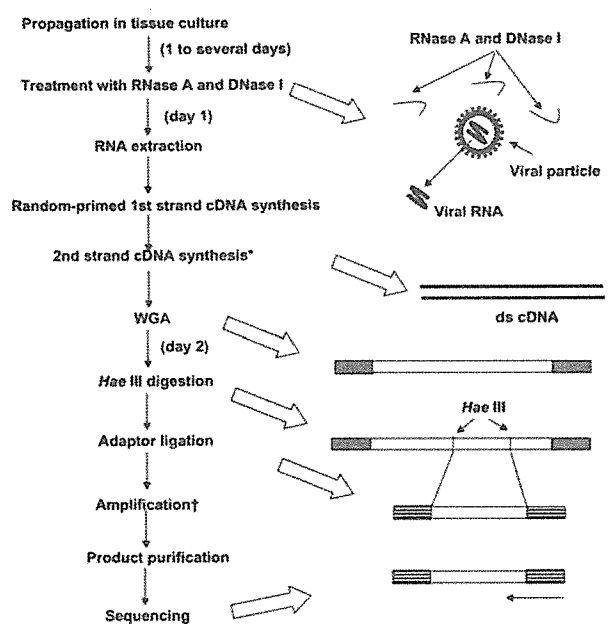

Figure 1. Overall scheme of the rapid determination of viral RNA sequence method. *By adding RNase H; WGA, whole genome amplification; †With specially designed primer sets as shown in Figure 2.

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performed within 90 min. Instead of the Taq polymerase recommended in the kit, we used 1.25 U of AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the PCR products. Primers were provided in the WGA kit, but no information regarding their sequences was obtained. The reaction mixture was heated at 95°C for 9 min (for activation of AmpliTaq Gold), followed by 70 cycles of amplification using Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 68°C for 1 min, primer extension at 72°C for 5 min, and denaturation at 94°C for 1 min.

The 1st cDNA library was digested with 40 U of *Hae*III (Takara Bio Inc.) at 37°C for 30 min. DNA was purified by using the MonoFas DNA isolation system (GL Science, Tokyo, Japan), and a blunt *EcoR*I-*Not*I-*Bam*HI adaptor (10 pmol; Takara Bio Inc.) was ligated at 16°C for 30 min by using DNA Ligation Kit, Mighty Mix (Takara Bio Inc.). The second cDNA library was amplified by PCR with specially designed primer sets in which 6 nucleotides composed of CC (*Hae*III-digested sequence) and 4 variable nucleotides were added to the 3′ end of the adaptor sequence (Figure 2). For example, 1 primer set was as follows: forward primer, H1–1: 5′-AATTCGGCGGCCGCG GATCC*CC*GGGG-3′; reverse primer H9–3: 5′-AATT CGGCGGCCGCGGATCC*CC*AGGA-3′ (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics) (Figure 2).

We always used >12 primer sets and 0.83 μmol of each primer per cDNA library. PCR was performed with AmpliTaq Gold Master Mix (Applied Biosystems). The reaction mixture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing and primer extension at 72°C for 30 s and denaturation at 94°C for 30 s. A single band was consistently obtained in ≈50% of the reactions. DNA was purified from the PCR by using MonoFas. Occasionally, we purified DNA fragments from the gels when >2 bands were detected. Direct sequencing was performed with the forward primer, reverse primer, or both.

When the number of viral particles in the sample was high, we omitted the RNase A and DNase I treatments and used the RNeasy Mini Kit (Qiagen) for RNA extraction. We occasionally used a whole transcriptome amplification kit (Rubicon Genomics Inc, Ann Arbor, MI, USA) instead of the WGA kit because both kits yielded similar amplification results.

In preliminary studies that used referential RNA viruses, we attempted to determine the nucleic acid sequences of SARS coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants (10–100 μL) by using the RDV method. The percentages of positive fragments (number of fragments containing viral nucleic acid/total number of

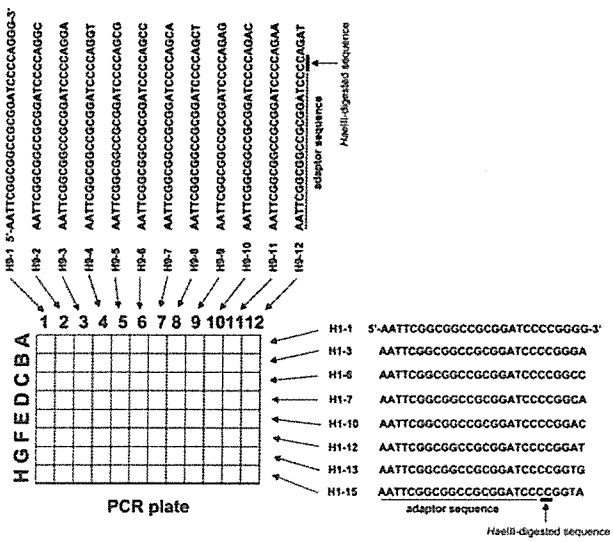

Figure 2. Primers used in rapid determination of viral RNA sequence method.

sequenced fragments) in the reactions for detection of these 5 viruses were 60% (3/5), 45% (5/11), 100% (12/12), 50% (5/10), and 40% (4/10), respectively. As a clinical application, a throat swab specimen from a patient with fever and upper respiratory infection was characterized. Although the specimen exhibited enterovirus-like cytopathic effect by inoculation into HEF and GMK cells when cell culture system for virus isolation was used (*2*), extracted RNA from the supernatant of the cells showed no amplification by reverse transcription–PCR (RT-PCR) when 1 of the conventional primer sets for human enteroviruses was used (*3*,*4*). In the cell culture supernatant analysis by the RDV method, the specimen exhibited amplification of the partial nucleotide sequences of coxsackie A14 virus (nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB275848–AB275853). Thus, the RDV method could detect unidentified cytopathic-effect agents such as enterovirus that could not be detected by RT-PCR when the conventional primer set for enteroviruses was used.

Conclusions

The RDV method is a rapid method for the direct determination of viral RNA sequences without using the cDNA cloning procedure. The limitations of the RDV method are the requirement for cell culture isolate and the large number of steps. However, RDV would be useful for species-independent detection of RNA viruses including unknown or untypeable emerging RNA viruses. Furthermore, with minor modifications, this method would also be applicable to the detection of DNA viruses and bacteria.

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