VIRUSES AND INTERFERON: A FIGHT FOR SUPREMACY

Michael G. Katze, Yupeng He*§ and Michael Gale Jr‡*

The action of interferons (IFNs) on virus-infected cells and surrounding tissues elicits an antiviral state that is characterized by the expression and antiviral activity of IFN-stimulated genes. In turn, viruses encode mechanisms to counteract the host response and support efficient viral replication, thereby minimizing the therapeutic antiviral power of IFNs. In this review, we discuss the interplay between the IFN system and four medically important and challenging viruses — influenza, hepatitis C, herpes simplex and vaccinia — to highlight the diversity of viral strategies. Understanding the complex network of cellular antiviral processes and virus–host interactions should aid in identifying new and common targets for the therapeutic intervention of virus infection. This effort must take advantage of the recent developments in functional genomics, bioinformatics and other emerging technologies.

**Department of Microbiology, University of Washington, Seattle, Washington 98195-8070, USA.*
‡Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9048, USA.
§Y.H. is currently at Antiviral Research, Abbott Laboratories, Abbott Park, Illinois 60064-6217, USA.
Correspondence to M.G.K.
e-mail: honey@u.washington.edu
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Interferons (IFNs), although best known for their antiviral properties[1,2], are potent regulators of cell growth[3] and have immunomodulatory activity[4]. Indeed, an emerging theme is that these cytokines are important regulators of innate and adaptive immune responses. Furthermore, studies now highlight the importance of cross-talk between cellular regulatory pathways that control IFN signalling, apoptosis, inflammation and the stress response (BOX 1). There are two main types of IFN, type I and type II. Type I or 'viral' IFNs include IFN-α, IFN-β, IFN-ω and IFN-τ; type II IFN is IFN-γ. Most types of cell can produce IFN-α and IFN-β, which are the best-characterized type I IFNs, whereas IFN-γ is produced only by certain cells of the immune system, including natural killer (NK) cells, $CD4^+$ T helper 1 (T_H1) cells and $CD8^+$ cytotoxic T cells. There are 14 different IFN-α genes, but only one IFN-β and one IFN-γ gene. IFNs mediate their effects through interactions with type-specific receptors, which are different and non-redundant for the type I and type II IFNs[5]. IFNα/β-receptor-knockout mice (as well as IFN-γ-receptor knockouts) cannot mount effective antiviral responses[6,7]. The IFN receptors do not have enzymatic activity, but they set in motion a complex signalling pathway that ultimately results in the transcription of hundreds of IFN-stimulated genes (ISGs) (FIG. 1 and BOX 2). It is now clear that although IFN levels markedly increase in response to virus infection, the sequence of events, types of IFN that are produced and ISGs that are targeted have an important effect on the outcome.

The regulation of IFN-β synthesis is well characterized and requires the participation of several transcription-factor complexes, such as nuclear factor-κB (NF-κB), ATF/JUN and, in particular, the IFN-regulatory factors (IRFs)[5,8]. These factors are often activated by phosphorylation on serine residues. However, the crucial feature is that activation of the IRFs is triggered by virus infection, probably through the production of viral double-stranded (ds)RNA and other virus-specific signals. Reflecting the intimate relationship between viruses and their host, host cells have evolved signalling mechanisms to sense and respond to virus infection. As described below, such mechanisms involve cross-talk between different cellular pathways to modulate the expression and antiviral function of IFNs and specific IFN-induced gene products. Similarly to IFN-β, the IFN-α genes are also activated in response to virus infection and the induced serine phosphorylation of specific transcription factors. However, the IFN-α and IFN-β genes are not expressed at the same level or with the same kinetics after virus infection. There seems to be a crucial positive-feedback loop that depends on

Box 1 | **Cross-talk between IFN-regulated pathways**

An emerging theme in the interferon (IFN) field is the cross-talk that occurs between the main cellular regulatory pathways. One of the most exciting new directions arises from the observation that Toll-like receptors (TLRs) and their adaptor proteins — such as myeloid differentiation primary response gene 88 (MYD88) and Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP; see below) — have links to the IFN pathways and, in some cases, are actually themselves induced by IFNs[122–124]. TLRs are a family of innate immune-recognition receptors that recognize molecular patterns that are associated with microbial pathogens, and they induce anti-microbial immune responses[125]. The link between TLRs, IFNs and viruses became evident from the report that TLR3 recognizes double-stranded (ds)RNA (which is often a by-product of virus replication)[126]. This interaction results in the activation of nuclear factor-κB (NF-κB) and the production of type I IFNs. Double-stranded RNA also induces the expression of IFNs by TLR-independent mechanisms and activates several of the IFN-induced proteins and enzymes. It is not yet clear whether TLR3 is a bona fide receptor that induces IFN production in response to viruses, but it is intriguing that mice that have deletions in the protein kinase *PKR*, RNase L and myxovirus-resistance (*Mx*) genes can still respond to dsRNA or viral infection. This indicates that there are further pathways (possibly through TLRs) for viral recognition by host cells[127]. It will be interesting to monitor the infection of TLR3-deficient mice with various viral pathogens. A recent report concerning TLR4 is also of note. Horng *et al.* have identified and characterized TIRAP, which controls the activation of MYD88-independent signalling pathways downstream of TLR4 (REF. 128). Importantly, these investigators found that PKR is a component of both the TIRAP- and MYD88-dependent signalling pathways. Even more remarkably, P58^{IPK} — the influenza-virus-activated cellular inhibitor of PKR — was also found in a complex with TIRAP and PKR, which indicates that PKR and its regulators (including the PKR activator PACT) might be downstream targets that are activated by TIRAP.

many IRFs to control IFN expression, although recent *in vivo* experiments indicate that there is an even more complex system of regulation[9]. The expression of IFN-β and IFN-α4 seems to be induced early through the action of IRF3 (REF. 10). However, the other IFN-α genes require that IRF7 is synthesized and activated for their expression[11,12]. Unlike IRF3, IRF7 is not constitutively expressed and it needs to be transcriptionally activated through the IFN receptor/Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway, as do most IFN-induced genes (BOX 2). IFN-β and IFN-α4 provide the initial signal that allows IRF7 to be produced, thereby leading to the expression of the full spectrum of IFNs and ISGs.

Interferon-stimulated genes

The workhorses of the type I IFN system are the many ISGs[1,13] (FIG. 1). Until the advent of DNA microarrays, it was thought that there were perhaps 30–40 ISGs, a small number of which were thought to have antiviral properties. Now, we know that there are hundreds of IFN-regulated genes, many of which are repressed or downregulated by IFN[14,15] (G. Geiss *et al.*, unpublished observations). We must, therefore, completely re-evaluate our thoughts on how IFNs interfere with virus infection and how, in turn, viruses fight back. Clearly, the host repertoire that is involved in host defence is much more extensive than was previously thought.

Perhaps the most intensely studied type I IFN-induced gene is the dsRNA-activated serine/threonine protein kinase, which is now known as PKR[16]. Activated PKR can negatively affect cell-regulatory pathways, primarily messenger-RNA translation and transcriptional events. As for IFN itself, viral-specific RNAs can activate PKR, which inhibits virus replication and the production of virion progeny. Nearly all viruses have developed strategies to downregulate the activity of PKR so that virus replication is not compromised[17]. Moreover, there are several cellular regulators, both inhibitors and activators, of PKR. Another crucial pathway involves the IFN-mediated response that is responsible for mRNA degradation, which comprises two enzymes — 2′,5′-oligoadenylate synthetase (OAS) and RNase L[18,19]. This pathway also seems to be activated by dsRNA. Originally, it was thought that this pathway was focused only on the degradation of viral RNAs, as part of the IFN-mediated antiviral artillery. Clearly, however, cellular RNAs are also targets of this pathway, which indicates that it has an important cell-regulatory role. Both knockout mice[20] and convincing *in vitro* experiments show that this pathway has an important antiviral role. The myxovirus-resistance (Mx) proteins were among the first IFN-induced proteins to be studied in the context of a virus infection[21]. Mx proteins are IFN-inducible GTPases; their antiviral activity requires enzymatic function. The function of the Mx proteins was determined primarily in the influenza- and Thogoto-virus systems. A recent study has shown that MxA binds to the nucleocapsid proteins of bunyaviruses and causes the redistribution of viral capsid proteins as a mechanism to inhibit bunyavirus replication[22]. This turns out to be a highly complicated story because of the differences between human and mouse Mx proteins, the differences between nuclear and cytoplasmic forms of Mx and the spectrum of viruses that are negatively affected by Mx proteins. A recently discovered IFN-induced gene is the RNA-specific adenosine deaminase ADAR[23], although its potential antiviral function requires characterization. ADAR is involved in RNA editing by virtue of its ability to deaminate adenosine to yield inosine, which provides a mechanism to alter the functional activity of viral and cellular RNAs. Such RNA editing occurs on viral RNAs, particularly NEGATIVE-STRAND RNA GENOMES. These modifications might relate to the persistence of infection and/or be a mechanism by which mRNAs are inactivated late in virus infection.

As mentioned earlier, IFNs have potent immunomodulatory properties. It is probable that the complete IFN response involves both innate and adaptive immune responses[4,24]. MHC class I and II molecules present antigenic peptides, derived from the degradation of viral proteins, to CD8$^+$ T cells and CD4$^+$ T cells, respectively. Both class-I-restricted CD8$^+$ T cells and class-II-restricted CD4$^+$ T cells are activated during viral infection[25]. So, it is no accident that IFNs also upregulate the expression of MHC class I and II, thereby enhancing the cellular immune response to virus infection *in vivo*. This might be a later event in the host-response repertoire, primarily contributing to recovery from infection, rather than being an initial host defence.

NEGATIVE STRAND RNA GENOME
Genomic viral RNA that is complementary to the messenger RNA that is produced during infection.

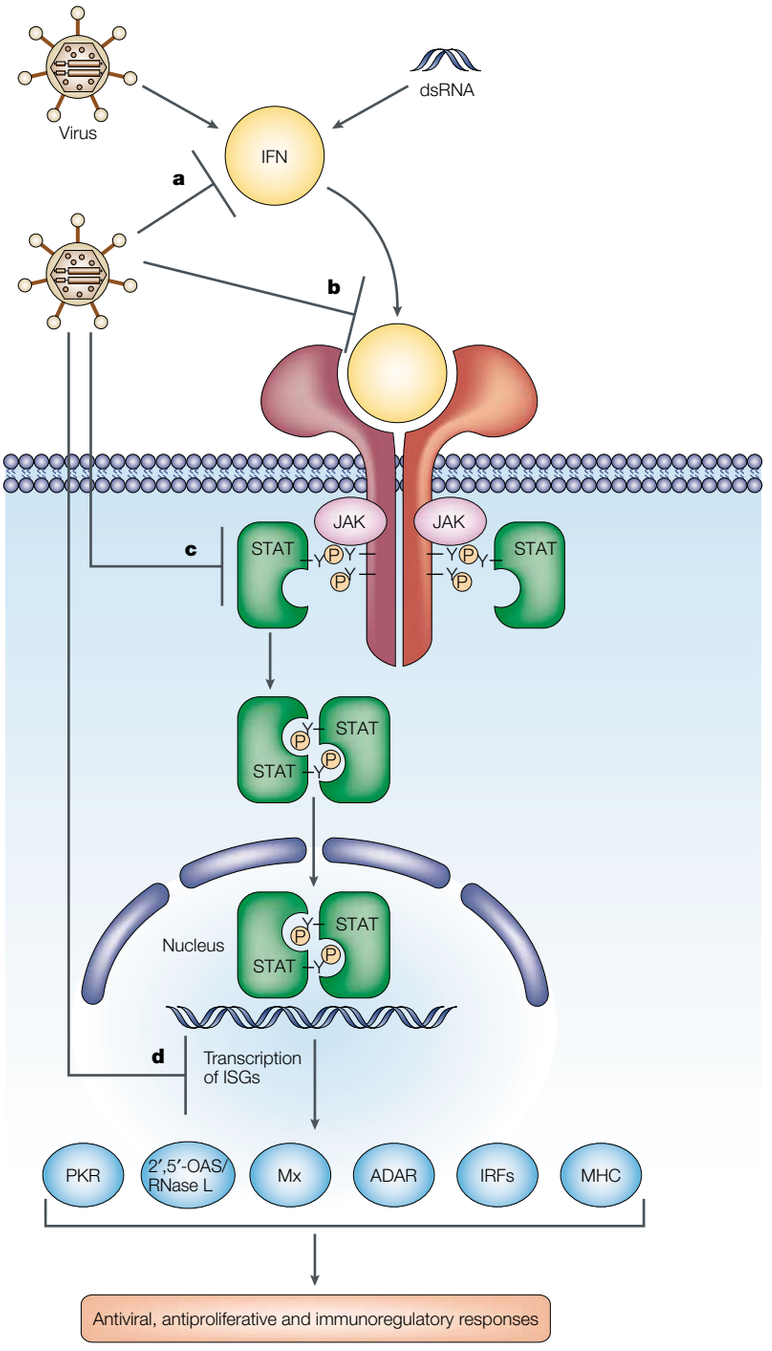


Figure 1 | **Overview of the IFN pathway and viral-counteracting strategies.** Type I interferons (IFNs) are a group of antiviral cytokines that are induced during viral infection by viral-replication products, such as double-stranded (ds)RNA. IFNs exert their biological functions by binding to specific cell-surface receptors. In turn, this triggers the intracellular IFN signalling pathway — mainly the JAK–STAT pathway (see BOX 2 figure) — which eventually induces the expression of a large number of IFN-stimulated genes (ISGs). The ISGs, the workhorses of the IFN response, set up an antiviral, antiproliferative and immunoregulatory state in the host cells. However, most, if not all, viruses have evolved a broad spectrum of strategies to block and interfere with the IFN pathway. Common viral strategies include **a** | blocking of IFN induction/expression **b** | intercepting receptor binding of IFNs through viral decoy IFN receptors **c** | perturbation of the intracellular IFN signalling pathway and **d** | directly downregulating the level of expression of ISGs. ADAR, RNA-specific adenosine deaminase; IRF, IFN-regulatory factor; JAK, Janus kinase; Mx, myxovirus-resistance proteins; OAS, oligoadenylate synthetase; PKR, protein kinase; STAT, signal transducer and activator of transcription.

As a protein family, the IRFs have received much attention for their roles in regulating the host response to virus infection[26]. The first IRFs — IRF1 and IRF2 (we are up to IRF10 at the last count[27]) — were identified originally as a transcriptional activator and repressor, respectively. The IRFs are extremely important during virus infection and the host response[8], and they are targeted by viruses for regulation during infection. Some viruses, such as human herpes virus 8 (HHV8) or Kaposi's sarcoma-associated herpesvirus (KSHV), encode IRF homologues to act as decoys and thereby evade host IFN-mediated defence[28]. At least four members of the IRF family — IRF1, IRF3, IRF5 and IRF7 — act as transducers of virus signalling. In response to infection, these transcription factors are phosphorylated on serine residues and transported to the nucleus, where they can activate or repress the transcription of either IFNs themselves or IFN-regulated genes.

Viruses fight back

Viruses have been reported to block nearly all aspects of the IFN regulatory pathway[2,29–31]. This includes the disruption of dsRNA and IFN receptor/JAK–STAT signalling events, the inhibition of IRF and NF-κB functions, and other mechanisms that target the antiviral actions of specific ISG products. As there have been several reviews published on this subject recently[2,29–31], we do not attempt to cover the entire topic, but focus on four medically important virus systems.

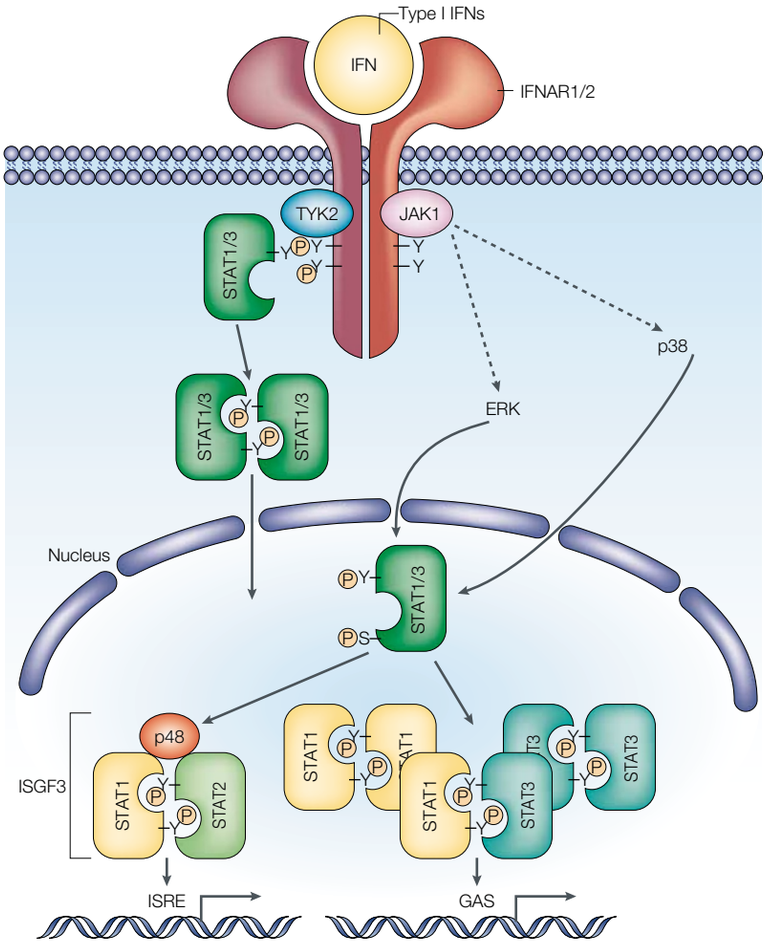
Influenza virus

Influenza virus, an orthomyxovirus that has a segmented negative-strand RNA genome, has a prominent role in the history of IFNs. After all, type I IFN was first discovered using heat-inactivated influenza-virus-infected chick cells[32]. Moreover, the antiviral Mx proteins inhibit influenza-virus replication at many levels, and this was one of the early prototype systems to study the antiviral effects of IFN[33]. Curiously, there are no reports of strategies of influenza virus to negate the various effects of Mx proteins, probably because no one has looked. There are, however, many reports of viral strategies to evade other aspects of the IFN response — in particular, the role of the viral *NS1* gene product in disarming the host innate-defence system and blocking PKR activity[34,35]. Recently, Garcia-Sastre[36] has reviewed these influenza-virus strategies; therefore, we discuss only the poorly understood, but emerging, theme that viruses can usurp a cellular stress response to fight the innate IFN response (in other words, viruses can turn the host on itself); and the anti-IFN effector non-structural protein NS1, the pandemic of 1918–1919 (BOX 3) and mechanisms of pathogenesis.

Stressed out. P58^{IPK} is a cellular molecular co-chaperone and member of the heat-shock 40 DnaJ family of proteins[37]. P52^{RIPK} is a cellular protein that has homology to the heat-shock protein 90 kD (HSP90) family of HSPs[38]. P58^{IPK} is known to interact with HSP40 and HSP70, and it can stimulate the ATPase activity of the latter[39]. So, what relevance does this have for influenza virus and the

Box 2 | **The IFN receptors and JAK–STAT signalling**

The primary players in the interferon (IFN) signalling pathways are the signal transducers and activators of transcription (STATs) and Janus kinases (JAKs)[129,130] (see figure). STATs are latent transcription factors that become tyrosine phosphorylated by the JAKs. There are many members of the JAK–STAT family of proteins that have a role in various combinations in regulating the synthesis of both IFNα/β- and IFN-γ-induced genes[131]. Type I IFNs bind to their cell-surface specific receptors (IFNAR1/2) and activate the intracellular IFN signalling pathway, which involves mainly JAK1 and TYK2, and STAT1, STAT2 and STAT3. The JAKs phosphorylate and activate the STATs, which homo- or heterodimerize and translocate to the nucleus to induce the expression of the IFN-stimulated genes (ISGs). The STAT1–STAT2 heterodimer complexes with nuclear protein p48, and this complex binds to the IFN-stimulated response element (ISRE) sequences in the promoters of ISGs. STAT1 and STAT3 homo- and heterodimers bind to γ-IFN-activated sequence (GAS) elements. In addition, the mitogen-activated protein kinase (MAPK) pathways (involving extracellular-signal-regulated kinase (ERK) and p38 MAPK) have a role in IFN signalling, by phosphorylating serine residues of STAT1 and STAT3 and further enhancing their transcriptional activity. Type II IFN signalling follows a similar, but distinct, pattern. The events that are responsible for driving transcription from the ISRE — the *cis*-acting DNA element that is found in IFN-α/β-inducible genes — are important for virus regulation. For IFN-α/β, phosphorylated STAT1–STAT2 heterodimers are recruited to ISRE sequences together with IFN-regulatory factor 9 (IRF9) or p48 to form ISGF3, which is a multi-component transcription complex. This regulation is distinct from that of the IFN-γ pathway, which relies on STAT1 homodimers for activation through the GAS element. Most of the components of these complex signalling pathways have been targeted for disruption in experiments using knockout mice[132,133]. These mouse experiments have validated the importance of these host pathways in fighting virus infection.



IFN response? P58^{IPK} is a cellular inhibitor of PKR that is activated by influenza-virus infection[40]. P58^{IPK}-mediated inhibition of PKR ensures that viral mRNA translation is not compromised due to excessive phosphorylation of eukaryotic initiation factor 2, α-subunit (eIF-2α). P52^{RIPK} is a cellular inhibitor of P58^{IPK} that indirectly potentiates PKR activity through its ability to regulate the function of P58^{IPK} (REF. 38). HSP40 is also a negative regulator of P58^{IPK}, keeping it in an inactive complex until it is required. Furthermore, we found recently that the gene that encodes P58^{IPK} has an endoplasmic-reticulum stress element (ERSE) in its promoter region (W. Yan *et al.*, unpublished observations). This promoter is activated during the stress of the unfolded protein response (UPR). Moreover, it now seems that P58^{IPK} can interact with and inhibit the eIF-2α kinase PERK, which controls protein synthesis during the stress of the UPR (W. Yan *et al.*, unpublished observations). This can result in the translation of specific mRNAs, such as the transcription factor C-EBP-homologous protein (CHOP)[41]. So, it seems that P58^{IPK} is at the centre of a cellular stress pathway that is related to the IFN response. What is truly remarkable from the point of view of the virus is that it outsmarts the host at its own game. The host cell has set up several sensors to deal with stresses of all kinds (including virus infection and the UPR). The P58^{IPK} stress-regulatory pathway has been taken over by the virus to downregulate the host-defence IFN system (FIG. 2). The end result is that the virus does not need to devote important coding capacity to the regulation of the host response.

NS1. The influenza-virus-encoded non-structural NS1 protein is a viral gene product that seems to do everything (reminiscent of the simian virus 40 (SV40) T antigen) — such as controlling mRNA transport, splicing, polyadenylation and translation, to mention only a few of its ascribed functions[36]. So, one would never predict (from the literature at least) that a virus that lacks the complete *NS1* gene could ever be viable. However, the only 'real' phenotype seems to be that the *NS1*-deleted virus is exquisitely sensitive to the antiviral effects of IFN. The deleted virus can replicate effectively only in knockout cells that lack key components of the IFN regulatory pathway[35]. So, NS1 has been identified as an important viral component of the influenza-virus armament against IFN. Indeed, our recent microarray study[42] showed that during influenza-A/PR/8/34-virus infection of human lung epithelial cells, deletion of the *NS1* gene increased the number and level of expression of cellular genes that are implicated in the IFN pathway and other antiviral pathways. Specifically, viruses that were deleted of the *NS1* gene induced a general increase in the transcription of ISGs and NF-κB-mediated gene expression compared with wild-type virus, and were observed to differentially regulate genes of the suppressor of cytokine signalling (SOCS) family, which modulate cytokine and growth-factor signalling in a classic negative-feedback loop[43]. The complete mechanisms by which NS1 disrupts the IFN response are not known yet, although clearly, the inhibition of PKR (in addition to cellular P58^{IPK}) and regulation of the IRFs are involved[36].

Box 3 | **NS1 and the great influenza pandemic of 1918**

Of the influenza viruses, type A viruses cause the most illness and have caused three important worldwide outbreaks during the past century[134]. The influenza pandemic of 1918 was remarkable for many reasons. Twenty-eight per cent of the world's population (500 million people) were potentially infected during the pandemic. Estimates of the total mortality that resulted from this pandemic range from 20 million to 40 million people[135]. It was proposed — in retrospect, in a somewhat naive way — that the ability of viral non-structural protein NS1 to combat the interferon (IFN) response was the main determinant of virus pathogenicity in 1918–1919. Although it is still too early to completely discount this theory, it seems that the pandemic-type NS1 (in a WSN-influenza-virus background) actually attenuates virulence *in vivo*[136]. However, the studies were carried out in mice and the WSN virus that was used in these studies was mouse adapted. In addition, our recent microarray study[42] showed that an influenza-A/PR/8/34 virus that contained the *NS1* gene of the 1918 flu pandemic was better than the parental virus at blocking the expression of IFN-regulated genes in human lung epithelial cells. In this study, WSN virus induced the expression of some IFN-stimulated genes (ISGs) to high levels. By contrast, infection with recombinant WSN virus carrying the pandemic flu *NS1* gene failed to significantly induce the expression of any ISGs, and the levels of the myxovirus-resistance protein MxA and ISG15 were markedly reduced. Studies using a better animal model (preferably a non-human primate such as the Asian macaque) are required to fully understand the role of NS1 in pandemic influenza. Therefore, the hunt continues for the cause of this great pandemic, looking at both viral and host factors that might have affected virus replication and virulence[137].

Hepatitis C virus

No other virus has received more attention than hepatitis C virus (HCV) with regard to IFNs[44–46]. The reason for this is simple — type I IFN is used as the main antiviral therapeutic against HCV in humans. At present, IFN is prescribed in combination with ribavirin (a guanine nucleotide analogue. This virus is interesting because: HCV infects 2–4% of the world population; IFN therapy is a billion-dollar industry; IFN is ineffective in most treated individuals, the explanations for which are controversial; nearly every large pharmaceutical and biotechnology company has a programme to develop more-effective HCV antiviral therapeutics; and there is no robust animal or tissue-culture model to study the natural history of HCV infection, replication and pathogenesis[47,48].

HCV and IFN therapy. HCV resistance to IFN therapy is defined loosely as the continued presence of HCV RNA in patient serum after treatment[49]. As with many RNA viruses, HCV circulates in the host as a population of QUASISPECIES, most probably selected from mutations that have accumulated in the HCV genome due to the infidelity of the NS5B RNA polymerase during viral replication[50]. Different HCV isolates or genotypes have different levels of sensitivity to IFN treatment. In particular, HCV genotypes 1 and 4 are less sensitive to type I IFN therapy than are HCV genotypes 2 and 3; the latter genotypes have a response to pegylated IFN and ribavirin combination therapy in up to 85% of individuals, whereas the former genotypes show IFN resistance in most patients. This is a problem in the United States, where HCV genotype 1 is the predominant form. As differences in the HCV genome might affect the structure and function of viral genome and proteins, these modifications might, in turn, affect interactions with many host-cell functions, including those that are involved in the antiviral actions of IFNs in infected cells. To elucidate the mechanisms by which selected HCV variants escape the antiviral effects of type I IFN, Enomoto and colleagues compared full-length sequences of IFN-α-responsive and -unresponsive viruses from HCV-infected patients[51,52]. Genotype-1b-HCV-infected patients who had a sustained response to type I IFN therapy were found to carry isolates that contain many recurring mutations in a region of 40 amino acids in the carboxyl half of NS5A, which corresponds to residues 2209 to 2248 of the HCV polyprotein. These observations indicate that this NS5A region, termed the IFN-sensitivity-determining region (ISDR), might have a role in HCV resistance to IFN treatment (FIG. 3).

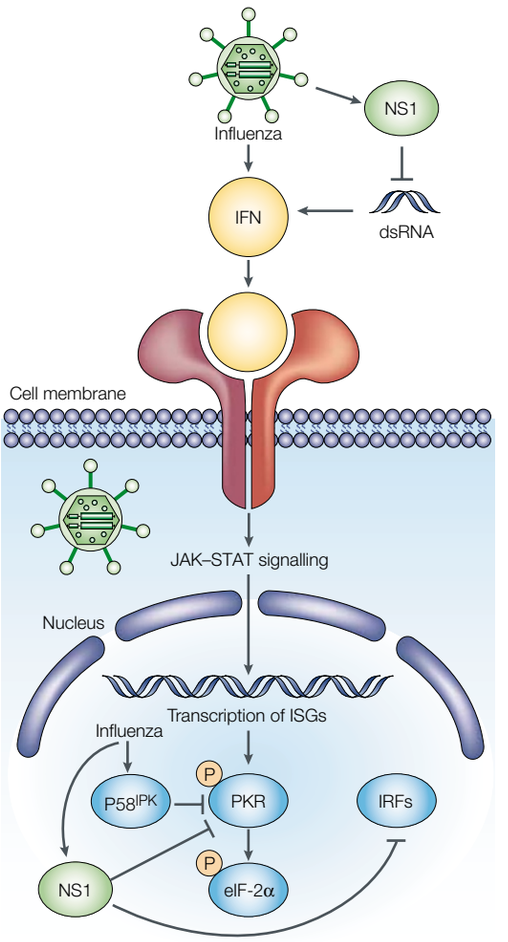


Figure 2 | **Interplay between the type I IFN pathway and influenza virus.** Influenza virus activates the P58^{IPK}-mediated host-cell stress-response pathway, and uses the P58^{IPK} pathway against the host cell by directing P58^{IPK} to block the activation of the protein kinase PKR. The influenza-virus non-structural protein NS1 — a double-stranded (ds)RNA-binding protein — blocks the dsRNA-dependent pathways, including the induction of type I interferon (IFN) expression. In addition, NS1 interacts directly with PKR and inhibits its kinase function. NS1 might also interfere with the function of IFN-regulatory factors (IRFs). eIF-2α, eukaryotic initiation factor 2, α-subunit; ISGs, IFN-stimulated genes; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

QUASISPECIES
A family of closely related, but slightly different, viral genomes. Viral genetics variants, derived from the original infecting virus, that are present during an infection.

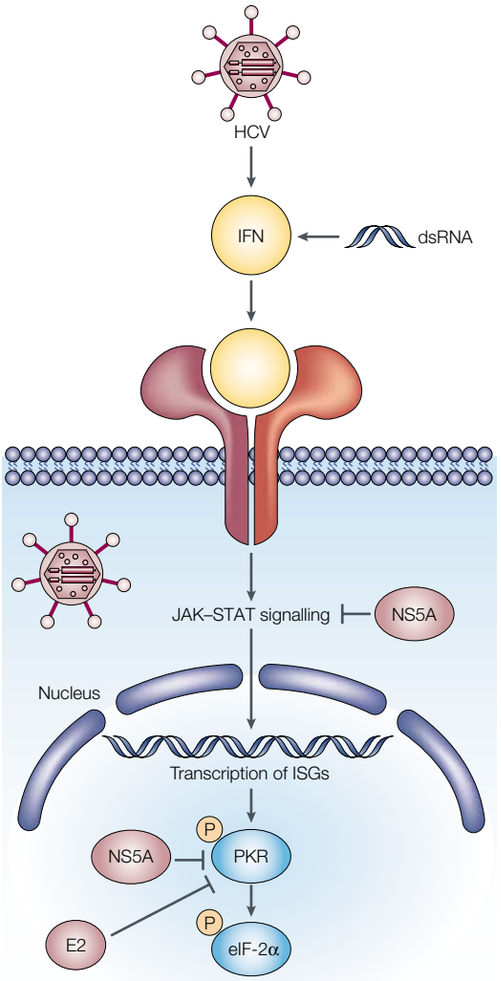


Figure 3 | **Interplay between the type I IFN pathway and HCV.** Both hepatitis C virus (HCV) NS5A and E2 proteins have been shown to interact with and inhibit the function of the protein kinase PKR. Our recent results indicate that NS5A also directly targets the intracellular interferon (IFN) signalling pathway by disrupting the cross-talk between the mitogen-activated protein kinase (MAPK) and JAK–STAT pathways[44]. dsRNA, double-stranded RNA; eIF-2α, eukaryotic initiation factor 2, α-subunit; ISGs, IFN-stimulated genes; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

However, the predictive value of the ISDR in determining the outcome of IFN therapy, particularly for European and North American HCV isolates, has been questioned by other studies[53]. Nevertheless, a recent study based on a new statistical META-ANALYSIS of a database of 675 published individual ISDR sequences strongly indicates that there is a significant correlation between the NS5A ISDR and the IFN response[54]. Despite this controversy, NS5A and the ISDR provide the first hint of a potential molecular mechanism by which specific HCV genotypes might escape the IFN response. Although the correlation is not universally accepted, it is clear that NS5A is important in conferring IFN resistance to HCV.

META-ANALYSIS
A large-scale comparison of NS5A sequences isolated from IFN-resistant or IFN-sensitive HIV-infected patients.

HCV adapts to pressure imposed by the cell. HCV cannot be propagated *in vitro*. This limitation has been overcome partially by the development of the HCV replicon, which is an autonomously replicating subgenomic viral RNA that encodes a drug-selectable marker and the viral components that are required for authentic HCV RNA replication[55,56]. This system allows the selection of stable cell lines that support HCV RNA replication. Recent *in vitro* studies using the HCV replicon system are beginning to shed some light on how HCV interacts with the host cell and the IFN system. Sequence analysis of stable HCV-replicon populations has shown that the process of viral RNA replication in culture involves selection for adaptive mutations, many of which occur in the NS5A coding region[47]. Evidence indicates that such mutations do not arise stochastically, but are, in part, determined by specific antiviral pressures from the host cell that are induced as a result of viral RNA replication. Although initial reports concluded that the HCV replicon is simply sensitive to IFN[56,57], we now know that the actual picture is not so simple. Type I IFN treatment results in a marked reduction in viral RNA levels, but extremely high and prolonged doses of IFN are required to completely ablate replicon replication[58]. These observations raise two main questions: is IFN resistance of HCV an acquired trait; and what are the molecular mechanisms by which IFN suppresses HCV replication? The second question is most relevant considering that type I IFN has been approved as a drug to treat HCV infection, yet the actual mechanisms of drug action are unknown. To address these issues, we have isolated several distinct replicon quasispecies that have been selected in IFN-treated cells[59]. These HCV replicons have incorporated further mutations, including many in NS5A, that confer increased resistance to the IFN-induced antiviral response when passaged in fresh cells that have not previously been exposed to IFN. Comparison of the phenotypic properties of such IFN-adapted replicons and those replicons isolated in the absence of IFN has shed light on specific stages of HCV replication that are likely to be targeted by IFN action, including, most importantly, the assembly of the viral replicase complex on the substrate RNA. What is now required is a genetic dissection of the viral sequences that confer IFN resistance and a comprehensive identification of host ISGs that function to limit HCV replication. Such an approach will help to define the molecular mechanisms of the action of IFN on HCV replication, and might lead to improved therapies for the millions of HCV-infected individuals worldwide.

Herpes simplex virus

Herpes simplex virus type 1 (HSV1) is a neurotropic DNA virus that infects a large proportion of the human population[60]. Direct contact of HSV1 with host mucosal tissues results in primary infection and eventual dissemination of progeny virions from the site of infection to neuronal tissue, where they persist in a latent state in the sensory ganglia. This latent state is punctuated by sporadic reactivation of virus replication in peripheral

mucosal tissue that is innervated by the infected ganglia. Genetic studies have defined various virus–host interactions that induce or counteract the cellular antiviral state to affect HSV replication and latency. These studies show that viral modulation of the IFN system, and PKR in particular, is the main basis for neurovirulence and pathogenesis associated with HSV1 infection[61].

HSV1 triggers the IFN response. The virion structure of HSV1 includes a lipid envelope that contains 11 viral-encoded glycoproteins, at least a subset of which are thought to facilitate interaction with and attachment to the host cell[62]. The infection of cultured human cells with HSV1 induces the production and secretion of IFN-α, which is attributed to specific interactions between the virus and cell-surface proteins. In particular, IFN-α production can be induced in human mononuclear cells by culturing cells in the presence of purified, recombinant HSV1 glycoprotein D (gD), perhaps through a mechanism that involves gD-mediated stimulation of intracellular signalling through engagement of CC-chemokine receptor 3 (CCR3) or CXC-chemokine receptor 4 (CXCR4)[63]. So, it seems that host–viral-glycoprotein interactions might induce cellular signalling events that culminate in IFN production. The mechanisms by which chemokine receptors might signal the host antiviral response and induce IFN production remain unclear, but in a similar manner to tumour-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and TNF signalling[64], this might well be attributable to receptor cross-talk with IFN signalling pathways. Relevant to this idea, the interactions of HSV1 with host cells have now been shown to activate IRF3 and to trigger the production of IFN in cultured human fibroblasts[65]. IRF3 activation was attributed to the (gD-dependent) viral-glycoprotein-mediated entry of HSV1 into the host cell, possibly through chemokine-receptor interactions with the viral glycoproteins. The potential involvement of CCR3 and CXCR4 in the host response to HSV1 is intriguing, as both types of receptor are present on the surface of the dendritic-cell-like major IFN-producing cells. So, one might propose that as the primary host for HSV infection, humans have evolved cellular mechanisms to 'sense' and rapidly respond to HSV before it gains a foothold in the cell. This would indicate that viral-glycoprotein interactions might be a potential cellular sensing mechanism that signals the presence of HSV to induce the rapid production of IFN and the expression of ISGs, thereby preparing the cell to counter a possible HSV infection. Collectively, these results raise the questions of how IFN affects HSV replication and how the virus counteracts the potentially deleterious effects of IFN to persist in the infected cell.

HSV–IFN interactions. Through the functions of various viral proteins and host interactions, HSV1 can regulate the host response at many levels (FIG. 4). The processes of cellular exposure to HSV1 and viral binding or entry stimulate the production of IFN and expression of ISGs, perhaps through the activation of IRF3 (REF. 65). This poses an early threat to viral replication, which is, in part, countered by infected cell protein 0 (ICP0). ICP0 can interact with the host proteasome-mediated degradation pathway to alter the stability of certain ISG products[66], and the temporal expression of ICP0 correlates with a block in host JAK–STAT signalling processes[67,68]. However, ICP0-mediated regulation of ISG expression is not sufficient to counter the antiviral actions of PKR, the activity of which is probably induced by viral dsRNA products of the HSV1 transcriptome[67]. Unless the virus counteracts PKR function, the host cell will undergo a block in protein synthesis due to the high levels of eIF-2α phosphorylation that are catalysed by

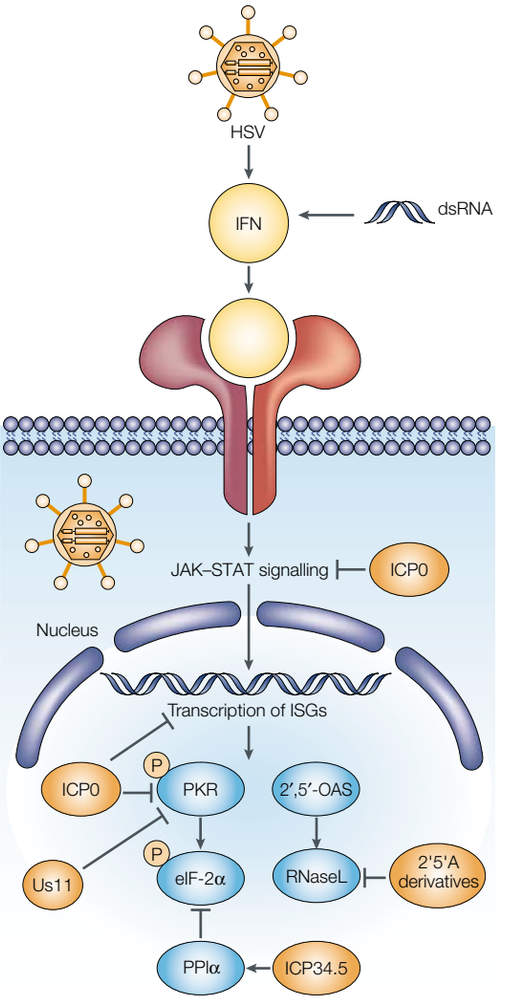


Figure 4 | **Interplay between the type I IFN pathway and HSV.** The herpes simplex virus (HSV) ICP0 protein disrupts the interferon (IFN) response by both blocking the JAK–STAT pathway and directly downregulating the level of expression of IFN-stimulated genes (ISGs). Also, HSV Us11 is an inhibitor of the protein kinase PKR. Interestingly, HCV ICP34.5 bypasses the effect of PKR on translational control by recruiting cellular protein phosphatase 1α (PP1α) to dephosphorylate eukaryotic initiation factor 2, α-subunit (eIF-2α). HSV also encodes 2′,5′adenosine (A) derivatives to block the 2′,5′-oligoadenylate synthetase (OAS)–RNase L pathway. dsRNA, double-stranded RNA; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

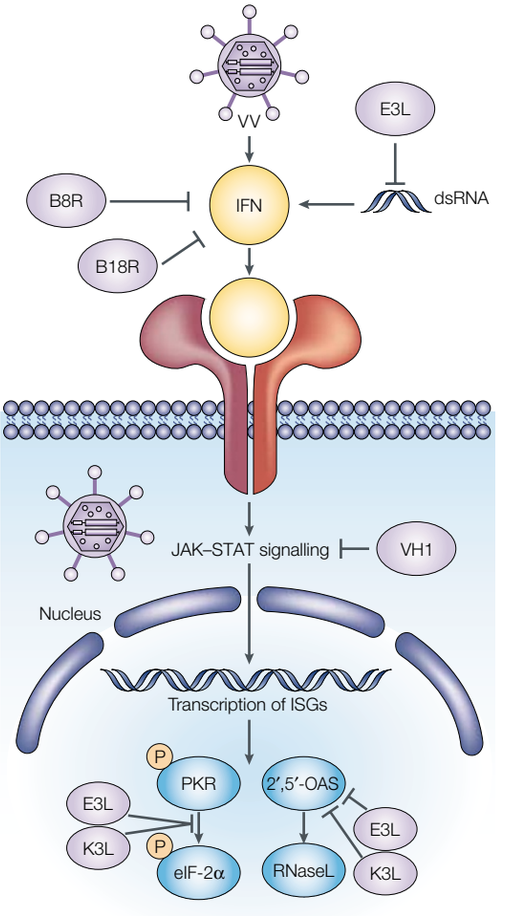


Figure 5 | **Interplay between the IFN pathways and vaccinia virus.** Vaccinia virus and other poxviruses encode soluble interferon (IFN) receptors (B8R and B18R) that block the binding of IFNs to their cell-surface receptors. The vaccinia virus *E3L* gene product is a double-stranded (ds)RNA-binding protein that inhibits activation of the protein kinase PKR and blocks IFN responses by sequestering dsRNA molecules. The vaccinia virus *K3L* gene encodes a eukaryotic initiation factor 2, α-subunit (eIF-2α) homologue that interferes with PKR function by acting as a pseudosubstrate. Both *E3L* and *K3L* gene products have also been proposed to block the IFN-induced 2′,5′-oligoadenylate synthetase (OAS) antiviral pathway. The vaccinia virus VH1 phosphatase, a virion component, intercepts the IFN signalling pathway through dephosphorylation of signal transducer and activator of transcription 1 (STAT1). JAK, Janus kinase.

active PKR. The primary mechanism by which HSV1 circumvents PKR action might be through the recruitment of protein phosphatase 1α (PP1α) by viral ICP34.5 into a high-molecular-weight complex that efficiently dephosphorylates eIF-2 (REF. 69), or through a direct interaction with PKR itself[70]. It is this ability to functionally counteract the effects of PKR that gives HSV1 its neurovirulent phenotype and allows virus growth in neuronal cells[61,71]. Leib *et al.*[71] have elegantly shown that a virus that is attenuated by deletion of *ICP34.5* has wild-type replication capacity and virulence in a host from which the *PKR* gene has been deleted, which provides a formal genetic test for identifying the *in vivo* mechanisms and targets of microbial virulence genes. Perhaps an evolutionarily more ancient system for inhibiting PKR resides in the Us11 coding region. In suppressor mutants of *ICP34.5*-deleted viruses, Us11 protein expression occurs early after infection[72]. This new expression pattern allows Us11 to inhibit PKR function, thereby supporting the growth of mutant HSV1 and partially restoring its neurovirulence. Overall, these results form an intriguing model to indicate that ICP34.5 and PKR are the key players in regulating HSV growth and pathogenesis. However, viral proteins are often multifunctional, and it is probable that ICP34.5 has other functions that contribute to HSV1 neurovirulence. In addition, such a model does not consider the contributions of other HSV1 proteins, which might function alone or cooperatively to interact with host antiviral systems. What other viral proteins might interact with the IFN system; do HSV gene products target specific ISGs for regulation; and what other ISGs might specifically affect HSV1 replication? Until the complex and pleiotropic actions of IFN on the host cell are completely understood, our understanding of the actions of IFN on viral replication and pathogenesis will remain incomplete.

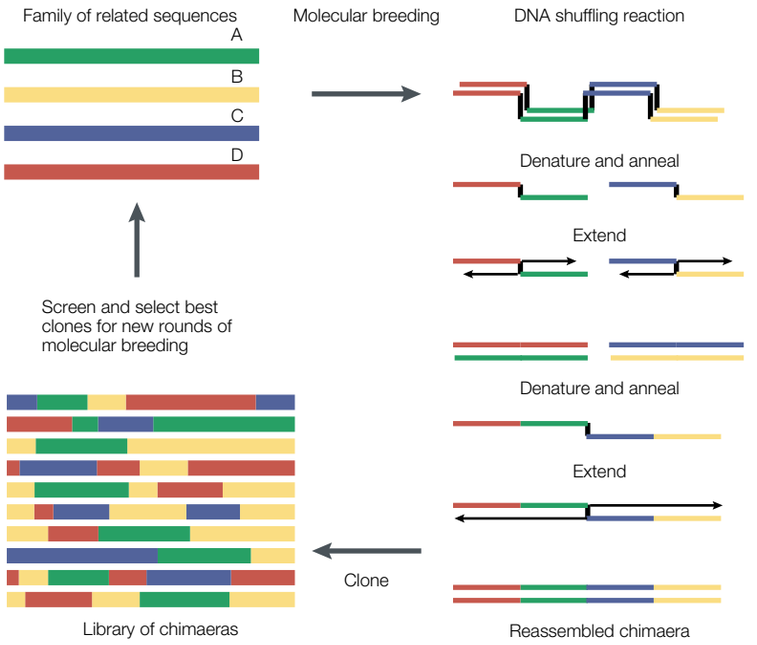
Vaccinia virus

Vaccinia virus is a member of the poxvirus family — a family of complex, large dsDNA viruses that replicate in the cytoplasm and have had important roles in the history of medicine[73,74]. Vaccinia virus was used as the vaccine to eliminate smallpox — a devastating disease caused by variola virus that is currently an important bioterrorism concern[75,76]. Poxviruses have evolved various mechanisms to interfere with the activity of host cytokines, including IFN[77–80] (FIG. 5). Uniquely, poxviruses encode soluble versions of cytokine receptors ('viroceptors') that intercept the normal activities of the target cytokines[81–83]. For example, the vaccinia-virus B8R protein binds soluble IFN-γ and prevents it from binding to cellular receptors[81]. This strategy to block the action of IFN-γ enables poxviruses to inhibit both the antiviral effects and, more importantly, the immune-regulatory functions of IFN-γ, simultaneously. The vaccinia-virus *B18R* open reading frame also encodes a soluble IFN-α/β receptor that blocks the binding of IFN-α/β to cell-surface receptors[84,85]. The IFN-γ receptor is highly conserved between members of the poxvirus family. Vaccinia virus that lacks the IFN-γ receptor is attenuated *in vivo*, although this deletion has no effect on virus replication *in vitro*[86,87].

Vaccinia virus uses at least two known functions to target PKR, which highlights the important role of this kinase in virus–host interactions. The vaccinia-virus proteins E3L[88–91] and K3L[91–93], which are conserved in variola virus[94,95], block IFN-mediated antiviral responses. E3L encodes a dsRNA-binding protein that is involved in the inhibition of PKR[89,90] by interfering with the binding of PKR to dsRNA[96]. E3L might also prevent PKR activation by masking the substrate-binding domain[97]. K3L, a vaccinia-virus-encoded eIF-2α homologue[92] that potentiates translation by inhibiting

Box 4 | **Molecular breeding**

DNA shuffling, pioneered by Maxygen, Inc., is a method for the permutation of natural diversity[138,139]. It is a method for quickly evolving genes, operons and whole viruses for the acquisition of any desired properties. This has been accomplished for the generation of new retroviruses that have altered tropisms[140], but more relevant to this review, also for the generation of new cytokines. DNA shuffling of a family of more than 20 interferon-α (IFN-α) genes was used to derive mutants or variants that have increased antiviral and antiproliferative activities in mouse cells[141]. Normally, the effects of IFN are species specific, such that human IFN works on human cells and mouse IFN works on mouse cells. There is minimal cross-species reactivity of these IFNs, except perhaps at very high concentrations. In the shuffling experiment, the most active human IFN clone was improved 285,000-fold relative to human IFN-α2a in mouse cells. Impressively, the three most active human clones were more active than native mouse IFN-α. Sequence analysis of the chimaeras showed that the sequence of the carboxy-terminal residues 121–125 correlates with the unusually high activity in mouse cells, which is consistent with previous site-directed mutagenesis studies[142]. On the basis of functional experiments and modelling, the residues in this region are proposed to interact with the mouse IFN-α receptor. The implications of this approach are profound. It will be of great interest to determine why the shuffled IFNs are so much more potent. Is it only due to enhanced receptor interactions? It will be interesting to carry out DNA-microarray analysis of cells that have been treated with these highly evolved cytokines. It will also be important to test whether these IFNs are active against hepatitis C virus in a replicon assay based on human liver Huh-7 cells, as these IFNs have only been tested so far against mouse encephalomyocarditis virus (EMCV), which is a particularly IFN-sensitive virus.



PKR and eIF-2α phosphorylation[93,98], acts by means of its homology to eIF-2α to interfere with the interaction of eIF-2α with PKR[96]. Deletion of the vaccinia-virus *K3L* gene reduced the ability of the virus to grow in IFN-treated cells[92], and vaccinia virus devoid of the *E3L* gene was also sensitive to the antiviral effects of IFN[91].

As a dsRNA-binding protein, vaccinia virus E3L can also block other dsRNA-mediated antiviral pathways, such as the IFN-induced OAS enzyme[99] and IRF3 and IRF7 phosphorylation[100], which indicates that there are other mechanisms for the anti-IFN effects of E3L that are distinct from its inhibition of PKR. Furthermore, E3L inhibits the adenosine-to-inosine editing activity of IFN-induced ADAR[101]. Although the amino-terminal domain of E3L is dispensable for infection of cells in culture, both the carboxy-terminal domain (which is required for IFN resistance, binds to dsRNA and inhibits PKR) and the amino-terminal domain of E3L were required for full pathogenesis in intranasal infections in a mouse model[102], which indicates the existence of dsRNA- and PKR-independent functions of E3L. Interestingly, vaccinia-virus virion-contained phosphatase (VH1) can bind to and dephosphorylate STAT1, which indicates a new mechanism by which vaccinia virus interferes with the onset of host immune responses by blocking the IFN signalling cascade through the dephosphorylating activity of the viral phosphatase VH1 (REF. 103).

IFN and functional genomics

The new technologies of functional genomics have had a marked impact on human biomedical research. In particular, global gene-expression analysis is now in widespread use in cancer and infectious-disease research, and it has become an integral part of the drug-discovery process[104–106]. Recent advances in proteomics have augmented this approach, making it possible to identify and quantify virtually all proteins that are present in a particular cell or tissue[107], and to characterize global protein–protein interaction networks in an organism[108]. Together, these technologies provide a global perspective on the complex interactions that occur between all levels of biological information ('systems biology'), from gene expression to protein production[109,110]. Making sense of the huge amounts of data that are generated by these approaches requires highly sophisticated information technologies, which is the domain of the relatively new discipline of bioinformatics. This confluence of genomic and information technologies brings a powerful new approach to the study of biological systems, as exemplified by recent studies[111]. The study of virus–host interactions and viral evasion of host defences will be revolutionized by these approaches. Reports from the Cleveland Clinic on DNA microchip analysis of IFN-treated cells have already changed the field[14,15]. Before this high-throughput analysis was possible, it was thought that there might be, at most, 30, 40 or perhaps 50 IFN-regulated genes. Now, their studies and our own (G. Geiss *et al.*, unpublished observations) show that there are probably hundreds of IFN-regulated genes, many of which are 'repressed' during IFN treatment. This must change the way that a virologist thinks about viral strategies to evade host defences.

The ever-increasing amount of microarray data that has been generated by examining virus–host systems has consistently shown that the expression of IFN and IFN-induced genes is differentially regulated in many systems (and this phenomenon is not only limited to studies of virus–host interactions). Studies with different members of the herpesvirus family best exemplify this point. IFN-induced genes are transcriptionally activated during human cytomegalovirus (CMV) infection. Perhaps more interestingly, the addition of gB — the virion

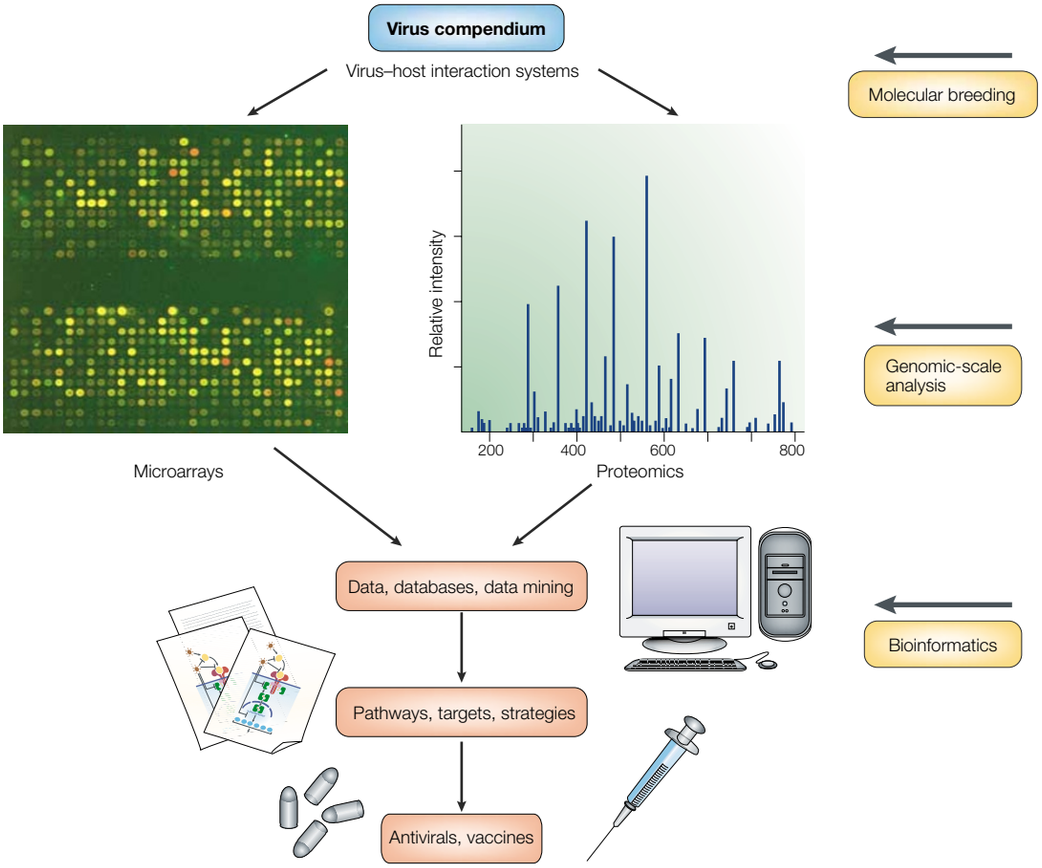


Figure 6 | **The virus compendium.** The virus compendium will use high-throughput, genomic-scale techniques to analyse various virus–host interaction systems, many of which could be improved or developed by molecular-evolution approaches, such as DNA shuffling (BOX 4). The collection of data sets will be integrated in compendium databases and will be subject to data mining, with the aid of bioinformatics and computational analysis. It is to be hoped that, as indicated by pilot studies, this effort will help us to understand consensus and pivotal pathways, targets and strategies during various virus–host interactions. This collection of data and knowledge will not only facilitate our search for new antiviral compounds and vaccines, but will also be useful in related areas, such as microbial pathogenesis, immunity and cell biology. This effort deserves the attention of the scientific community and requires proper cooperation and coordination of the research programmes at many institutions, both public and private, academic and industrial.

attachment protein — to cell cultures induces basically the same subset of genes that are induced by replication of the virus during a normal infection[112]. After infection with another herpesvirus, HSV, IFN-regulated genes were induced by a non-replicating mutant, but were inhibited by the wild-type virus, which indicates that virus replication is necessary to suppress the host-defence IFN response in this HSV system[113]. In related studies, we have found by microarray analysis that heat- or UV-inactivated influenza virus, which attaches to host cells, but does not replicate, also induces the expression of a substantial subset of the same genes that are dysregulated during a productive infection[114]. Interestingly, the cellular IFN response to influenza-virus infection is viral-strain specific. The PR8 strain induces the synthesis of several IFN-induced genes, whereas the WSN strain dysregulates very few IFN-induced genes. We speculate that this might account for the neurovirulence of WSN (a reduced IFN response allows increased viral replication), which might be due, in part, to the *NS1* gene product.

Little is known about how certain viruses trigger the IFN response or how this might occur as a result of viral attachment. It is widely thought that viral dsRNA intermediates that accumulate during the course of replication are the primary mediators that trigger IFN production. However, dsRNA is not present when inactivated virus or a viral attachment protein is used (unless contamination is a factor). We have carried out microarray analysis of dsRNA-treated cells that lack all type I IFN genes[115]. So, the possibility of gene induction by autocrine actions of IFN was eliminated. More than 175 genes were stimulated and nearly 100 genes were repressed — all in the absence of an IFN response. Different inflammatory cytokines and viruses also induced a subset of these dysregulated genes, which shows that there are interconnections between disparate pathways. Induction (and repression) of such a diverse family of genes has profound implications for virus–host interactions: this is a lot for the virus to cope with.

Another advantage of these high-throughput approaches is best illustrated by our work on HCV and microarrays. Many groups, including our own, have shown that the HCV NS5A protein confers type I IFN resistance, at least in part, through the inactivation of PKR[116–120]. To get a wider view, we carried out microarray analysis on cells that had been treated with type I IFN in the presence or absence of NS5A (or a mutant NS5A that is unable to bind PKR) (G. Geiss *et al.*, unpublished observations). Our goal was to define the molecular mechanisms that make HCV resistant to IFN treatment. At the same time, we were able to carry out a high-throughput analysis of the global effects of treating cells with IFN. Remarkably, we observed that a distinct subset of IFN-regulated genes were downregulated after treatment with NS5A, some of which were not downregulated by the NS5A mutant that is unable to bind PKR. Another microarray approach was used to examine transcriptional profiles in chimpanzees infected with HCV[121]. A progressive increase in the number of genes with altered expression profiles occurred until the peak in alanine transaminase (a liver enzyme, increased levels of which indicate liver damage/inflammation), at which time the expression of more than 180 genes was significantly altered. Prominent among these were many IFN-regulated genes, including STATs, IRFs, Mx proteins and OAS. Remarkably, the level of expression of some of these genes was altered nearly 100-fold. Therefore, it is not surprising that HCV must encode anti-IFN strategies.

Final thoughts toward a strategic plan

The greatest challenges for the future will involve designing and developing better antiviral therapeutics, perhaps in conjunction with an IFN that has been made more potent by molecular-breeding technologies (BOX 4). But, we desperately need a strategic plan. One possibility is to determine whether all viruses (or all RNA viruses, or all DNA viruses, or all respiratory viruses and so on) use common strategies to survive in the host and successfully replicate. We propose the establishment of a 'virus compendium' or a database that summarizes the events that occur during infection by all mammalian viruses (FIG. 6). This database should comprise both microarray transcriptional-profiling data and high-throughput proteomics information. Data should be assembled from various experimental systems, including *in vitro* infection systems, animal and human models, and surrogate systems such as the HCV replicon system. Using sophisticated software, such as Resolver™ and the proteomics software that has been developed at the Institute for Systems Biology, it should be possible to define common cellular pathways that are affected by virus infection or by overexpression of a viral protein. If specific genes/proteins are consistently up- or downregulated after infection with highly pathogenic viruses, we might be in a position to target these host-cell genes as a way to influence the outcome of a viral infection. Such an approach might lead us to the development of broadband antivirals — 'virus silver bullets' — which would be effective against a wide variety of viruses, just as antibiotics are effective against many different species of bacteria.

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DATABASES

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