Virogenomics: a novel approach to antiviral drug discovery

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Target discovery in virology has been limited to the few open-reading frames encoded by viral genomes. However, several recent examples show that inhibiting host-cell proteins can prevent viral infection. The human genome sequence should, therefore, contain many more genes that are essential for viral propagation than viral genomes. A systematic approach to find these potential cellular antiviral targets is global host gene expression analysis using DNA microarrays. Several recent studies reveal both unique and common strategies by which viruses change the gene expression profile of the host cell. Moreover, work in progress shows that some of the host pathways discovered by expression profiling are important for viral replication. Thus, human genomics tools have the potential to deliver novel antiviral drugs.

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▼ The completion of the human genome sequence reveals for the first time a full picture of all possible drug targets[1,2]. Therefore, most areas of drug discovery will profit from genomic as well as post-genomic research[3]. One of the less obvious areas to benefit from the completion of the human genome is antiviral-drug discovery because most current antiviral efforts target proteins encoded by the viral genome rather than the host genome[4].

The reasons for targeting the virus rather than the host are self-evident, because inhibiting viral functions will generally be less toxic to the host. However, the number of potential targets encoded in viral genomes is rather limited. This is particularly true for small viruses such as HIV, hepatitis C virus (HCV) and human papillomavirus (HPV), each of which encode less than 10 gene products. Moreover, many of the viral genes do not encode proteins with functions that can be easily targeted by small molecules.

An alternative strategy is to inhibit the function of host proteins that are essential for the virus to complete its replicative cycle. As obligatory parasitic life-forms, viruses depend on the host-cell proteins during their entire life cycle. It is, therefore, reasonable to assume that the number of targets in the host cell genome by far exceeds the number of targets in the viral genome. The challenge for drug discovery is: first, to identify cellular components essential for virus propagation; and second, to weed out those cellular targets that are also essential functions for the host. Thus, targeting host-cell functions for antiviral therapy will encounter the same toxicity obstacles as other areas of drug discovery. However, from these other areas of drug discovery we also know that many host functions can be blocked by small molecules without any obvious toxic effects on the host.

Host-cell pathways essential for viral infection

Several recent examples demonstrate that each step of the viral life cycle is susceptible to inhibiting host cell functions. At the level of viral entry, a prominent example is the co-receptor for HIV, CCR5 (reviewed in Refs 5,6). This cellular protein is required for membrane fusion during entry of HIV into the host cell, and individuals lacking CCR5 are more resistant to HIV infection. By blocking this host protein it is possible to inhibit HIV entry into cells. Consequently, compounds blocking CCR5 are currently under development for the treatment of AIDS. Further, the nuclear import of HIV particles can be inhibited by small molecules that interfere with the interaction of the matrix protein and the nuclear

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import machinery[7]. Such compounds should not affect the host because they mimic the viral protein.

Viral gene expression and replication depends on a plethora of host-cell factors and, therefore, many potential cellular targets are to be found at this level. One area of intervention is signal transduction. For instance, human cytomegalovirus (HCMV) replication can be inhibited by p38 inhibitors that block the mitogen-activated protein-kinase (MAPK) signaling pathway[8] and Kaposi sarcoma-associated virus (KSHV)-infected cells undergo apoptosis upon inhibition of the transcriptional activator, nuclear factor-κB (NFκB) (Ref. 9). These results are unexpected because both targets are part of the cellular pro-inflammatory response and thus one would expect these signaling molecules to have an antiviral effect, rather than being required by the virus. A similarly unexpected finding was that an avian adenovirus depends on cellular heat-shock proteins for its replication. Expression of the viral anti-apoptotic protein gam-1 induced heat-shock protein 40 (hsp40) expression. Gam-1 deleted viruses are unable to replicate unless cells were heat shocked or hsp40 was overexpressed[10]. Again, one would have expected that heat-shock proteins represent the cellular antiviral defense.

Another area of intervention is viral cell-cycle control. Human cytomegalovirus (HCMV) arrests cells in their G1 phase. This is accompanied by an increase in the cell-cycle-dependent kinase (cdk)-2–cyclin E complex. Interestingly, roscovitine, an inhibitor of cdk-1, 2 and 5, but not cdk-4 and 6, inhibited HCMV replication. By contrast, other cell-cycle inhibitors had no effect[11]. Moreover, herpes simplex virus (HSV) 1 and 2 were also inhibited by roscovitine as well as olomoucine, another cdk inhibitor[12]. This result emphasizes the fact that it is possible that different viruses depend on the same cellular pathways thus enabling a cross-species inhibition. Further support for this concept comes from recent observations that budding of the retroviruses HIV and Rous sarcoma virus, and also the filovirus Ebola, was dependent on the same intracellular pathway[13–16]. The gag proteins of these viruses contain so-called L domains that mediate the ubiquitination of gag. This step seems to be essential for virus release because decreasing the intracellular concentration of ubiquitin with inhibitors of the proteasome results in an accumulation, but not release, of virus particles at the plasma membrane. Another inhibitory strategy was used in the case of HCMV, whereby a protein-inhibitor of furin, a cellular enzyme required for processing of the essential glycoprotein B (gB), resulted in a dramatic decrease in the release of infectious virus[17]. Although this summary is far from complete, it demonstrates the tremendous opportunity for exploiting host-cell targets in antiviral drug discovery.

DNA microarray analysis to monitor host–virus interaction

Novel host-protein targets can be found by many different approaches. However, the advent of post-genomic tools represents an unprecedented opportunity to identify potential targets by global, systematic approaches. To date, the most successful functional genomics tool is global gene-expression analysis using DNA microarrays[18]. High-density DNA arrays are generated by spotting DNA fragments, usually derived by PCR, or synthetic oligonucleotides onto solid surfaces such as glass slides or filter membranes[19]. Alternatively, oligonucleotide probes can be directly synthesized on glass wafers using photolithography[20]. Because these techniques allow the deposition of thousands of probes on solid-support surfaces, it is now possible to simultaneously monitor the expression levels of the corresponding messenger RNAs isolated from various sources. The advantages and disadvantages of this technology have been extensively reviewed[21,22]. The main advantage of this technology is that many samples can be tested because sample-throughput can be scaled-up easily. High-density DNA microarrays have already been used successfully to analyze the global responses of cells to external changes, such as serum addition to fibroblasts[23], growth-factor signalling[24], or intracellular changes such as malignant transformation[25]. Thus, it is no longer an emerging technology, but a widely applicable, robust new tool for target discovery on a global scale.

This technology is particularly useful for the analysis of host–pathogen interactions[26] because well-controlled experiments are possible by comparing the mRNA populations of infected cells with non-infected cells. Also, the genetic manipulation of infectious organisms allows the comparison of profiles obtained from wildtype or mutant forms. Moreover, changes in cellular gene-expression levels in response to pathogen insult can be assessed in models of infectious disease by specifically inhibiting host gene-products to determine their significance in the virus life cycle. Initially, access to this new tool has been rather restricted because of the high costs associated with either purchasing commercially available microarrays or with the generation of the infrastructure for manufacturing microarrays. As a consequence, results of studies using microarrays to examine virus–host relationships are just beginning to emerge in print. A summary of studies using DNA microarrays to examine virus–host interactions is shown in Table 1.

Common host-cell pathways affected by different viruses

These studies reveal certain characteristic changes that are shared by several different viruses or patterns that are

Table 1. DNA microarray studies of viral infection

Virus	Experiment	Array	Cells	Cutoff points	Changes in expression	Remarks
HCMV (Ref. 22)	Infection 40 min, 8 h, 24 h	6600 Oligo human Affymetrix	Human foreskin fibroblast (HFF)	4.0×	258	Interferon-stimulated genes
HCMV (Ref. 31)	gB, 2–24 h	8942 (est.) human in-house	HFF	2.3×	441	Interferon stimulated genes
Influenza (Ref. 43)	Virus infection	15,000 human in-house	HeLa	1.5× >750	4h = 61 8h = 329	Cytokines (IL-6) Gene-expression ubiquitin
Influenza (Ref. 43)	Heat-inactivated virus	15,000 human in-house	HeLa	1.5× >750	4h = 84 8h = 13	Metallothionin Cell-cycle ubiquitin
HSV-1 (Ref. 35)	Adenovirus-expressing ICP0	>7500 human Incyte[b]	Human embryonic lung (HEL)	2.0×	13	Not shown
HSV-1 d106 (Ref. 35)	Infection ICP0 +++ ICP27 deleted	>7500 human Incyte	HEL	2.0×	427	Not shown
HSV-1, UV treated (Ref. 34)	Infection 24 h	19,000 (est.) human in-house	HEL	2.0×	32	Interferon-stimulated genes
HSV-1 KM110 (Ref. 34)	Infection Mutation in VP16/ICP0	19,000 (est.) human in-house	HEL	2.0×	33	Interferon-stimulated genes
KSHV (Ref. 38)	Transfection LANA	~6000 (est.) human spotted	BJAB Burkitt lymphoma	2.0×	15	Interferon-stimulated genes
HIV-1 (Ref. 44)	Infection 2 and 3 days	1506 (est.) human in-house	CEM $CD4^+$ T cells	1.5×	20	T cell traffic Transcription
Polio (Ref. 36)	Infection	7000 human in-house	HeLa	1.7×	18 up-regulated 12 down-regulated	Polysomal-associated RNAs
HPV31 (Ref. 41)	Transfected viral genome	7075 (est.) UniGEM Incyte	Human foreskin keratinocytes	2.3×	153	Repression of Stat 1 Interferon-stimulated genes
CVB3 (Ref. 46)	Mouse infection Day 3 and 9	7000 (est.) rat	Myocardium	1.8×	619	Different cellular pathways
MDV (Ref. 37)	Infection 48 h, 96 h	1126 (est.) chicken	Chicken embryo fibroblasts	2.0×	21, 22, 13 overlap	Interferon-stimulated genes
HCV (Ref. 45)	Transfection NS region Days 5 and 10	6416 oligo human Affymetrix	HepG2	2.0×	9 (day 5) 15 (day 10), no overlap	Various genes

Abbreviations: HCMV, human cytomegalovirus; HSV-1, herpes simplex virus-1; UV, ultraviolet; KSHV, Kaposi sarcoma-associated virus; LANA, latency-associated transcript; HPV-31, human papillomavirus-31; HCV, hepatitis C virus; MDV, Marek's disease virus; CVB3, coxsackievirus-3; ICP0, infected cell protein 0; ICP27, infected cell protein 27; gB, glycoprotein B; ISG, interferon-stimulated gene; IL-6, interleukin-6. [a]Affymetrix, Palo Alto, CA, USA. [b]Incyte, Palo Alto, CA, USA.

differential (Fig. 1). The use of DNA microarrays to study the host response to viral infection was pioneered by Thomas Shenk's laboratory (Princeton University, Princeton, NJ, USA) who followed the gene-expression profile of fibroblasts infected with HCMV over a 48 h period[27]. Using the human GeneChip™ (Affymetrix, Santa Clara, CA, USA), which displays oligonucleotides corresponding to >6,500 known human genes, it was observed that the expression of 258 mRNAs changed more than the arbitrarily set cut-off point of fourfold. Interestingly, the most dramatic changes

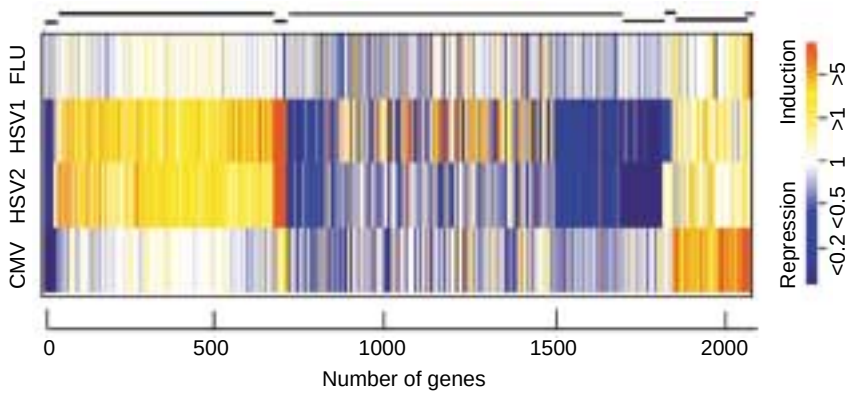

Figure 1. Cellular genes changing in response to viral infection. Human primary fibroblasts were infected with either human cytomegalovirus, herpes simplex virus-1, herpes simplex virus-2 or influenza virus. RNA was isolated from infected cells and used to probe human cDNA arrays covering ~10,000 human cDNA clones. Results are shown for those genes changing at least twofold (either up or down) in expression for at least one of the infected cells compared with mock-treated cells. The ratios compared with mock-treated cells are color-coded as shown. Results were clustered according to their expression profiles across the different infections. The number of clusters is shown at the right. Each horizontal line represents the ratio for a given gene in each of the four infections. The clustering reveals both common and different patterns of host-cell gene expression upon viral infection.

were observed for genes known to be regulated by interferon.

This observation confirmed earlier studies by the same group using differential display analysis of HCMV-infected and non-infected cells[28]. Differential display analysis[29], as well as microarray studies[30], of cells treated with interferons further indicated that many more genes were induced or repressed by interferons than previously assumed. This raised the possibility that most, if not all, genes regulated by HCMV belonged to the interferon pathway. To determine the extent of overlap between the expression profiles, the hybridization patterns of mRNA isolated from cells treated with interferon were compared with that of cells infected with HCMV using cDNA microarrays displaying a random collection of >9000 genes. Surprisingly, both expression profiles were virtually identical when compared with each other, except that the interferon response genes (ISGs) were induced later by HCMV, compared with interferon-treated cells[31]. This finding is even more remarkable considering that HCMV represents the most complex human virus genome, encoding more than 200 viral genes, yet we were unable to detect other strongly induced groups of genes, at least during the early stages of infection examined in this study.

It would appear that gB is predominantly responsible for the induction of the interferon-response genes, because purified gB alone was able to trigger the IFN response as well[31]. Therefore, it seems that the initial interaction of HCMV with its (as yet unknown) cell-surface receptor triggers a signaling cascade resulting in induction of the interferon-responsive genes. Importantly, this cascade does not involve signaling through the interferon receptor itself. The cellular response to HCMV infection could either represent a cellular innate immune-defense mechanism, a neutral side effect of viral infection, or it could be beneficial for the virus. Some recent observations (discussed later) suggest that at least some of the HCMV-induced genes are required for viral replication. By contrast, some of the ISGs are well-known antiviral proteins. Moreover, HCMV is known to interfere with the induction of ISGs via the interferon-receptor pathway at later stages of infection, suggesting that some of the interferon-gene products are unwanted[32,33].

Interestingly, HSV was recently also shown to induce interferon responsive genes[34]. However, in contrast to HCMV, such induction was not observed in cells infected with wildtype virus, but only in cells infected with either UV-irradiated virus or with viral mutants that lack both major transactivators required for HSV gene-expression, VP16 and ICP0 (infected cell protein 0). Because wildtype HSV destroys most, if not all, cellular mRNAs and interferes with host mRNA splicing, it was concluded that the induction of ISG is normally prevented by the host cell shut-off function, as well as additional genes that require VP16 or ICP0 for their expression. Thus, HSV first triggers, then disarms, this cellular response.

Another HSV-mutant that was subjected to DNA microarray analysis was d106, which exclusively expressed the major immediate-early (IE) transcription factor ICP0[35]. Many cellular genes were found to change in d106-infected cells. By contrast, ICP0 expression from adenovirus had only a moderate effect on cellular gene-expression, but was still able to complement IE-deleted virus thus indicating that cellular genes activated by ICP0 might not be important for viral gene activation. The role of host-cell genes for HSV gene expression and replication, therefore, still needs to be elucidated. An interesting possibility would be that some antiviral host-cell genes have evolved to escape the HSV host-cell shut-off function. Evidence for such a selection comes from studying RNA-virus infected cells. Many RNA viruses inhibit the initiation of host-cell

translation by degrading essential translation factors. By contrast, viral translation occurs in the absence of these factors via internal ribosomal entry sites. Remarkably, microarray analysis of poliovirus-infected cells revealed a subset of cellular RNAs that are also translated from internal entry-sites upon inhibition of cap-dependent translation[36]. Some of these genes might play a role in antiviral defense.

Induction of interferon-responsive genes has also been observed for the chicken herpesvirus, Marek's disease virus[37]. Moreover, the major latency-associated transcript of the human tumor virus, Kaposi-associated herpesvirus (KSHV), was also found to induce predominantly interferon-regulated genes[38]. This observation is in line with our observation that human endothelial cells infected with latent KSHV display ISG upregulation[39]. Upon induction of the lytic cycle, ISG expression is downregulated, most likely by a viral homolog of interferon response factor (IRF), a transactivator required for ISG induction. The viral IRF is known to interfere with ISG induction[40].

Thus, α- β- and γ-herpesviruses have been observed to induce the interferon response at some stage of their life cycle, only to then interfere with this response later. These results also suggest that different stimuli, such as glycoproteins or nuclear proteins, are able to stimulate interferon-responsive genes. Thus, transcription of this group of genes occurs not only after stimulation with interferons but by many different pathways.

In contrast to herpesviruses, DNA microarray analysis of HPV-infected cells revealed a significant repression of interferon-regulated genes in human keratinocytes[41]. In addition to downregulating the basal level of interferon genes, an inhibition of interferon-induced gene transcription was also observed when interferon was added exogenously. The viral proteins that seem to be responsible for the repression of ISGs are the oncoproteins E6 and E7 as suggested in a recent study[42]. In E6-transfected fibroblasts, a repression of endogenous ISG expression, as well as of the interferon-genes themselves, was observed. Similar to whole virus, ISG induction by exogenously added IFN was also inhibited. In contrast to the IFN-response genes, a strong induction of pro-inflammatory genes was observed.

A similar dichotomous behavior of ISG and inflammatory genes in KSHV-infected cells has been observed, with ISGs being progressively downregulated during lytic replication and inflammatory genes being upregulated[39]. The dramatic induction or repression of interferon-stimulated genes, as well as the large percentage of the host-cell response dedicated to this pathway, emphasizes the importance of interferon-regulated genes for the interplay between virus and host.

Virus-specific host-cell pathways

Many viruses induce and repress cellular genes in addition to ISGs and pro-inflammatory genes. Influenza-infected cells have been shown to upregulate only a few genes, whereas many more host genes are downregulated[43]. Similar to HSV, influenza degrades host-cell RNAs so that it still needs to be demonstrated whether the observed lower hybridization intensity was caused by active downregulation or by degradation. Strikingly, several groups of host genes were induced by heat-inactivated virus, suggesting that replication-independent events can trigger host-gene expression. This observation is also consistent with host-gene induction by UV-inactivated HCMV[28] or purified gB[31]. These data, therefore, suggest that the interaction between virus and host-cell receptor might play a role beyond attachment by triggering intracellular signaling cascades.

Infection of human $CD4^+$ T cells by HIV showed a clear regulation of several cellular genes not associated with interferon induction[44]. From 1506 genes arrayed on a glass support, 20 genes were differentially expressed above background level. The HIV-regulated genes function in a variety of cellular processes such as signaling, regulation of protein expression or trafficking, as well as different metabolic pathways. Certainly, many more genes will be revealed to be affected by HIV once DNA-arrays are used that cover a larger percentage of all human open-reading frames.

DNA-microarray analysis of HCV-infected cells has been hindered by the lack of a suitable tissue-culture model. Therefore, cells that were transfected with a partial genome encoding HCV non-structural genes were used for DNA microarray analysis[45]. Of the 6416 genes analyzed, the expression of only nine was altered more than twofold at day five after transfection, and the expression of a different set of 15 genes was changed at day 10. No induction ratio of more than fourfold was observed. In the absence of an independent quantification it cannot be excluded that these results are false-positives. However, the non-structural genes of HCV do not seem to have a great impact on host-cell gene expression. Whether this will also hold true in the context of the whole virus remains to be seen.

An important question that has not yet been addressed is: how predictive are changes in the host-cell expression profiles observed *in vitro* for infections *in vivo*? Neighboring cells and, particularly immune cells, will certainly influence the expression profile of an infected cell *in vivo*. So far, only one study has been published that evaluated the gene-expression profile in coxsackievirus B3-infected mouse heart[46]. The high infection rate of myocytes allows the detection of coxsackievirus-induced genes *in vivo*. Using microarrays displaying ~7000 genes of a rat heart library the authors observed that the expression of ~10% of

the genes changed over the duration of infection. The host genes belonged to various pathways and the role of the different pathways for coxsackievirus pathogenesis is yet to be elucidated. Many other viruses do not infect tissues at such a high density. Recent advances in isolating individual cells by laser microdissection in combination with linear amplification of mRNA populations suggest that it will be possible to determine the expression profile in infected cells within tissues in these cases[47,48].

Analyzing viral gene expression

In addition to cellular RNAs, the expression pattern of viral RNAs can be monitored by microarrays. For the more complex viruses of the herpesvirus family this approach can be particularly helpful to determine the succession of gene expression in infected cells. Moreover, this global approach allows the analysis of the effect of mutagenizing viral genes on the expression of all other viral genes. Probes for each open reading frame of the entire genome of HCMV[49,50], HSV-1[51] and KSHV[52] were deposited on glass or filter carriers. Using these arrays it was possible to determine the kinetic class of each open-reading frame and compare the viral gene expression of wild-type with mutant viruses. Moreover, using DNA arrays, viral mRNAs were recently detected in viral particles of HCMV, which was a unexpected finding[50]. Thus, by combining viral arrays with cellular arrays it will be possible eventually to determine changes in the total mRNA content of a virally infected cell.

Functional consequences of pathways detected by microarrays

Changes in gene expression during viral infection are the result of either: (1) a cellular defense response, (2) a virally induced response that is beneficial or even essential for the virus, or (3) a side effect of either (1) or (2). For drug-discovery both (1) and (2) are interesting. Because viruses often develop countermeasures to (1), such as viral mechanisms to interfere with interferon signaling, it might be possible to interfere with these countermeasures. In the case of (2), it might be possible to target host-cell proteins directly. Of particular use are host-cell genes that are upregulated rather than repressed because it is generally easier to inhibit than to induce a given function.

In the next phase of virogenomics it will, therefore, be important to validate the importance of differentially expressed host genes for viral infection. At present, this work is still in progress in several laboratories and the results have not been published as yet, but were presented at scientific meetings[39,53,54]. In general, the strategy will be to use data obtained by microarrays to build novel hypotheses that can be tested experimentally. Preliminary findings have been obtained with HCMV. One of the pathways regulated in the context of the massive interferon-related changes occurring during HCMV infection is the regulation of prostaglandin synthesis[27]. As a consequence of HCMV-mediated upregulation of cyclo-oxygenase 2 (cox 2), increased release of prostaglandin E2 can be detected in HCMV-infected cells. Because inhibition of cox-2 prevents prostaglandin synthesis, the effect of various cox-2 inhibitors on HCMV replication was examined. Remarkably, a dose-dependent inhibition of HCMV growth was observed in tissue culture. Moreover, exogenous addition of prostaglandins restored HCMV growth suggesting an important role of this pathway for HCMV to complete its replicative cycle (J-P. Cong, H. Zhu, W. Bresnahan and T. Shenk, personal communication)[54]. Thus, DNA microarrays provided a new testable hypothesis that might not have been found with any other method. Because cox-2 inhibitors are already in clinical use, it is also possible to examine whether they ameliorate CMV disease or prevent HCMV reactivation.

In a similar approach, the role of gene products specifically induced by KSHV was evaluated. One of the gene products upregulated in KSHV-infected endothelial cells was the stem-cell growth-factor receptor, c-kit[39,53]. When c-kit signaling was inhibited, either by small molecules or by transfecting dominant-negative c-kit variants, a reversal of the KSHV-induced morphological changes, such as spindle-cell phenotypes, was observed. It is thus conceivable that inhibitors of c-kit signaling might be able to interfere with KSHV-induced tumor formation. Because receptor-tyrosine-kinase inhibitors that also inhibit c-kit signaling[55] are already in use for the treatment of myelogenic leukemias[56], such compounds can be evaluated for the treatment of Kaposi's sarcoma. These examples demonstrate the power of the functional genomics approach. Thus, by analyzing the global response to infection it is possible, at least in some cases, to find potential new treatments in remarkably few steps.

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