(**Fig. 1a**). These reporters incorporate a phosphorylation sequence specific for a particular kinase and a phosphopeptide binding domain. Phosphorylation induces an intramolecular interaction between the domain and the phosphorylation site. The resulting conformational change brings two GFP variants in closer proximity, thus increasing the degree of FRET. A drawback of these constructs is that the change of fluorescence after phosphorylation is modest, and consequently, kinase activity has to be high to be detected. There is also potential for the reporter to be phosphorylated by kinases other than the one intended that have overlapping specificity.

An alternative strategy is the generation of stand-alone synthetic peptide substrates that undergo changes in fluorescence upon phosphorylation (**Fig. 1b**). The Lawrence group has reported the design of environment-sensitive fluorophores that respond to phosphorylation of an adjacent serine residue in a peptide substrate, but the change in fluorescence is still rather small[6]. Larger differences in fluorescence have been attained by appending a metal-chelating fluorophore to a peptide substrate[7]. Phosphorylation of the substrate introduces an additional metal-coordinating group, which causes a large increase in affinity for calcium ion, thereby enhancing fluorescence at a fixed calcium concentration. The versatility of these probes is limited, however, by a requirement that the fluorophore and phosphorylation site be closely positioned, which may interfere with recognition by some kinases.

Imperiali and coworkers have built on this concept of using a metal chelation–sensitive fluorophore. Their approach was to incorporate this sensor function in the peptide substrate as an unnatural amino acid (Sox)[8]. By also including an intervening β-turn sequence in the peptide substrate, one can place this unnatural amino acid further away from the phosphorylation site. This additional spacing alleviates interference with the enzyme. In the presence of magnesium ion concentrations typically used in kinase assays, the phosphorylated peptides undergo changes in fluorescence up to threefold greater than any previously reported substrate (**Fig. 1c**). Additionally, because the position of the fluorophore can be varied, these peptides offer more flexibility in design, which should allow accommodation of a wider range of kinases.

By running assays in microtiter plates, crude cell lysates can be divided and the activity of multiple kinases examined simultaneously in a sensitive, continuous assay format. The use of crude systems has the potential for phosphorylation of the peptides by unintended kinases with similar phosphorylation specificity, but also allows the addition of inhibitors and use of assay conditions that favor the kinase of interest. By doing so, the group showed that they could assay three enzymes (PKA, MK2 and Akt) specifically and with similar results as are obtained from conventional, more labor-intensive radiolabel assays with purified kinases conducted on a much larger scale.

Theoretically, fluorescent substrates can now be made to order for any kinase of interest for which a peptide substrate is known, though careful validation will be required with any new substrate to ensure that the observed activity is indeed due to the intended kinase rather than to nonspecific phosphorylation. The convenient assay format will make this approach an appealing substitute for radiolabel-based kinase assays, which cannot be run in crude lysates owing to high levels of background phosphorylation.

Future improvements to their design should allow the scope of these reagents to widen. Reporters with slightly higher affinity for magnesium, for example, could be conjugated to cell-penetrating peptide sequences and used to monitor kinase activity in living cells with unparalleled sensitivity. In addition, modified Sox residues with distinct excitation and emission wavelengths could allow dynamics of several kinases to be followed simultaneously in the same cell, allowing researchers to more fully elucidate the intricate details of complex signaling pathways.

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Herpesviruses throw a curve ball: new insights into microRNA biogenesis and evolution

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Validation and comparison of previously unknown microRNA genes in related herpesviruses yielded several surprises, most notably in regard to viral evolution and microRNA biogenesis. An explosion of literature has recently appeared describing the identification and mechanism of action of microRNAs (miRNAs)—small RNA regulators of gene expression in plant and animal cells.

In this issue of *Nature Methods*, Pfeffer *et al.* computationally and experimentally examine several mammalian RNA and DNA viruses for the presence of miRNA genes[1]. This study is an important step toward understanding whether regulatory pathways mediated by small RNAs in animal cells are used by viral pathogens for their own gene regulation or to subvert cellular defense mechanisms (**Fig. 1**).

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The small RNA species known as miRNAs were first recognized in the nematode *Caenorhabditis elegans* and were later found to be common throughout the animal and plant kingdoms[2,3]. By pairing with messenger RNA, these regulators can negatively regulate the expression of certain genes. Perfect complementarity to a target mRNA results in cleavage of the target, whereas imperfect complementarity results in translational repression by an as-yet-unknown mechanism. In general, most animal miRNAs function by the latter mechanism. The presence of five distinct miRNAs in the human Epstein-Barr virus has been demonstrated previously[4]. Implementing a new predictive computer algorithm and an improved small-RNA cloning technique, Pfeffer *et al.* have extended this analysis to survey a broad range of mammalian viruses. Genomes of viruses from the relatively simple nonpathogenic adeno-associated virus to the complex DNA viruses of the poxvirus family (including smallpox virus), and the herpesvirus family (associated with encephalitis and cancer) were probabilistically analyzed for their likelihood of containing miRNA genes and then examined for the presence of characteristic miRNA precursor structures. This computational survey of 29 viruses, together with a detailed validation of predicted miRNAs in herpesviruses, one of the viral families surveyed, has identified 33 previously unknown viral miRNAs, providing an opportunity to study viral miRNA conservation and evolution.

What is the level of conservation among viral miRNAs? Quite strikingly, the viral miRNA genes predicted and experimentally validated by Pfeffer *et al.* are conserved neither among the viruses examined nor within the genomes of their mammalian hosts. Even more intriguingly, not only are the sequences not conserved, the positions within the viral genomes do not seem to be conserved either. The study of one particular subfamily of herpesvirus (the γ-herpesviridae) reveals that relative positions of miRNAs in the viral genomes can be correlated to the evolutionary distance of each virus from a common ancestor[5]. In these viruses, conserved 'core' genes are often centrally located, whereas more recently acquired genes are clustered toward the genomic termini. Phylogenetic analysis has suggested that different members of the family are evolving at substantially different rates[4]. The miRNA cluster in the rapidly evolving mouse herpesvirus MHV68 maps near nonconserved genes at a single genomic terminus, whereas the miRNAs in the more slowly evolving Epstein-Barr virus are interspersed among core genes in two distinct regions[4]. In Kaposi sarcoma virus, another member of the same family that is evolving at an intermediate rate, the miRNAs map at the boundary of core and other genes in this virus. When taken together with the lack of sequence conservation, the lack of positional conservation of miRNA genes within this family of viruses suggests that these miRNAs are not likely to be part of an ancient system dictating direct regulation of essential viral genes.

If viral miRNAs are not involved in core replication functions, what exactly are they doing? Although it remains possible that the seemingly unrelated miRNAs recognize separate sequence motifs within a common target, a more likely explanation is that the miRNAs in each virus have a highly specific function. The herpesviruses have evolved such that they can latently persist in a unique cell type, causing only minor pathogenicity to the host. It is thus conceivable that viral miRNAs might initiate and maintain a program of gene expression that is uniquely tailored to propagate latency in a distinct cell lineage. Indeed, recent genomic studies suggest that individual miRNAs can effect quite broad changes in gene expression[6,7]. In contrast, some RNA viruses, such as poliovirus and the DNA poxviruses, do not have a latent state and actively replicate in the cytoplasm. Even if these viruses did encode miRNAs, it is unclear how their gene products would access the cellular machinery in the nucleus that processes primary miRNA transcripts.

The computational predictions are not entirely consistent with this speculation because miRNAs were predicted to be absent in three of the herpesviruses analyzed (HHV3, HHV6 and HHV7) that have the capacity to remain latent in their host and to be present in two poxviruses (variola virus and vaccinia virus) that replicate in the cytoplasm. These predictions, however, were not experimentally verified in these five viruses. Thus, the question of whether miRNAs serve similar functions within or among these families remains to be rigorously addressed.

Although the complete lack of sequence and positional conservation of viral miRNAs was unexpected enough, another surprise came from the examination of their biogenesis.

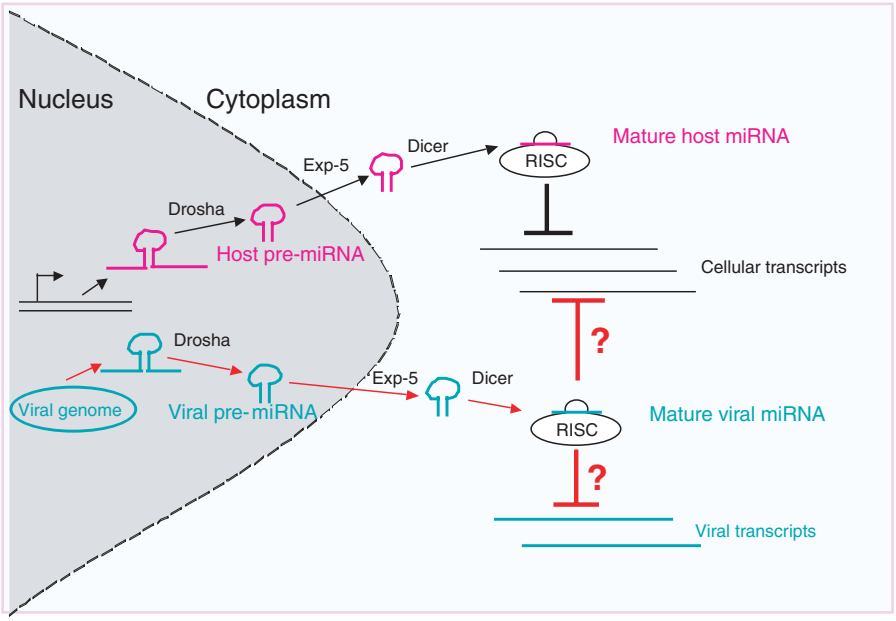

Figure 1 | A new study identifies miRNAs in several human viruses. Similar to the miRNAs of the host cell, they are thought to be processed in the nucleus to a precursor form by the enzyme Drosha and transported to the cytoplasm by exportin-5, where the precursor is processed by the enzyme Dicer and incorporated into the RNA-induced silencing complex (RISC). The targets of miRNAs on the viral or the host transcripts have yet to be identified.

The present model for mammalian cells is that most miRNAs are made from capped and polyadenylated RNA polymerase II transcripts, a feature which helps to explain the tissue-specific nature of miRNA expression[8,9]. Notably, analysis of the microRNAs of one mouse herpesvirus revealed that a subset of these gene products are produced under the control of RNA polymerase III as short hairpins fused to tRNA-like structures. RNA polymerase III is involved in the constitutive transcription of tRNAs and the 5S ribosomal RNA. Thus, these viral miRNAs would be expected to be constitutively expressed regardless of cell type. This is in stark contrast to the tissue specificity observed for many animal miRNAs, which are presumably expressed by the same RNA polymerase II–driven transcription, which controls such processes as cellular division and differentiation. It will be interesting to see whether this mode of miRNA biogenesis is observed for any animal miRNAs and, if so, what role these constitutively expressed regulators have in cell biology.

Much of the function of miRNAs in mammalian gene regulation remains unknown. The discovery and characterization of miRNAs in the *Herpesviridae* has already provided insights into miRNA evolution and biogenesis. Whether viral miRNAs act on viral or host genes remains to be addressed. The apparent restriction of viral miRNAs to complex DNA viruses may reflect an ability of these viruses to more elegantly manipulate host gene expression and defense mechanisms relative to their more stripped-down and efficient cousins. Elucidation of the function of these viral miRNAs in the host cell will no doubt shed light on whether these new regulators modulate pathogenesis and should yield important lessons on the role of host miRNAs in normal cellular processes.

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