

An advantage of this method is that different lectins can be used to capture proteins with selected glycans; moreover, the noncovalently bound sugar chains can be eluted with appropriate monosaccharides for analysis. Even though hydrolysis of the PNGase F-generated glycosylamine also tags the free oligosaccharides with ^{18}O , they cannot be traced to their parent peptides.

The approach does have several limitations. First, using a lectin column to select glycopeptides only binds a subset of N-glycans. Most known *C. elegans* glycoproteins are expected to bind, but this is not true for those from more complex organisms. Peptides derived from very abundant proteins (e.g., histones) will also contaminate the bound glycopeptides. *C. elegans* also contains PNGase F-resistant N-glycans, so these will not be counted. This may be a more significant problem in the analysis of glycosylation in lower organisms where N-glycan structure, and thus PNGase F-resistance, is mostly unexplored. ^{18}O -tagging during the cleavage is important to avoid false positives because nonenzymatic deamidation of asparagine to aspartic acid does occur and is indistinguishable from the PNGase F-catalyzed reaction. Careful comparisons of ^{16}O and ^{18}O -labeled peptides can be done by high-resolution MALDI-MS to address this issue, but for some laboratories the cost of H_2^{18}O may be prohibitive.

Notwithstanding their limitations, these methods should make it possible for the first time to analyze complex protein mixtures to determine whether N-glycosylation sites of specific proteins are equally occupied under different physiological states or in different tissues. For instance, site occupancy in DNase I varies, and perhaps this is true of many proteins. Asn-Asp-Ser and Asn-Glu-Ser sequences are thought to be inefficiently N-glycosylated⁹. Critical receptors such as glycine receptor, sodium channel protein, glutamate receptor 3, NMDAR1, metabotropic glutamate receptor 1 beta, neurexin 4, brevinan, T-cell receptor beta chain and protocadherin gamma all contain such sequences. Although clearly speculation at this point, variable glycosylation might influence ligand binding or formation of signaling complexes in the membrane and this now can be investigated. Sugar and peptide specialists alike can exploit the methods and are likely to find that the peptides surrounding some glycosylation sites have additional modifications. For instance, multiple tyrosine-sulfate residues are required for efficient

carbohydrate-dependent binding of P-selectin glycoprotein ligand-1 to P-selectin¹⁰. The real ligand is a fucosylated sugar chain embedded in a patch of modified amino acids. The sensitive tools are now at hand to investigate many important questions regarding the glycosylation status of complex protein samples.

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RNAi puts a lid on virus replication

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Effective inhibition of hepatitis B virus replication in mice by RNA interference suggests an antiviral treatment strategy.

In recent years, we have witnessed the discovery of a major and universal mechanism of gene regulation called RNA silencing or RNA interference (RNAi). Although the mechanism by which RNAi controls gene regulation is only partly understood (Fig. 1a), considerable interest is focusing on the putative therapeutic applications of these molecules, particularly for fighting viral infections. In this issue, McCaffrey *et al.*¹ demonstrate that short hairpin RNAs (shRNAs) block replication of hepatitis B virus (HBV) *in vivo*, bringing us one step closer to the use of RNAi as an antiviral therapy.

The idea that RNAi could be applied in antiviral therapies was first suggested by studies in plants, where RNAi appears to function as a major natural antiviral mechanism. Several lines of evidence support this contention: infection by plant viruses elicits strong gene silencing; plant viruses encode a variety of inhibitors of the RNA silencing machinery; and mutations in genes that encode for the RNA silencing machinery result in enhanced susceptibility to virus infection^{2,3}. Viral replication can also be efficiently suppressed by experimentally induced RNA silencing. In animal cells, initial experiments first focused on the simple issue of whether mammalian viruses are susceptible to RNAi or not. Although any RNA can potentially be subject to degradation by the RNAi machinery, it was not clear whether viral RNAs would

also be effectively targeted. Virus genomes are often protected by a proteinaceous structure or through their association with cellular membranes during replication; although many different types of viruses have been shown to be susceptible to RNAi in tissue culture, whether it would be possible to induce an effective antiviral RNAi response *in vivo* in the context of multiple systemic regulatory systems, such as the immune system, was not known.

McCaffrey *et al.* employ a technique called hydrodynamic transfection to introduce plasmids encoding the HBV genome together with HBV-specific shRNAs into mouse liver. This technique allows efficient gene transfer by rapidly injecting a large volume of DNA solution into mice via the tail vein (Fig. 1b). Among the organs transfected with the exogenous HBV DNA (liver, spleen, kidney and pancreas), the liver showed the highest levels of gene expression with approximately 40% of hepatocytes expressing the transgenes. The authors show that the HBV-specific shRNAs significantly reduce viral mRNAs and protein expression, thus inhibiting virus replication in hepatocytes of HB-infected mice. These results, obtained using transient *in vivo* cotransfection of plasmids, provide an important proof of principle that antiviral activity by RNAi can be achieved in animals. They also open the door for what promises to be a very exciting and prolific new field of antiviral therapeutics.

An important question to be addressed is whether or not there is a functional interaction between the RNAi machinery and the mammalian immune system. This report compares the efficiency of RNA silencing in

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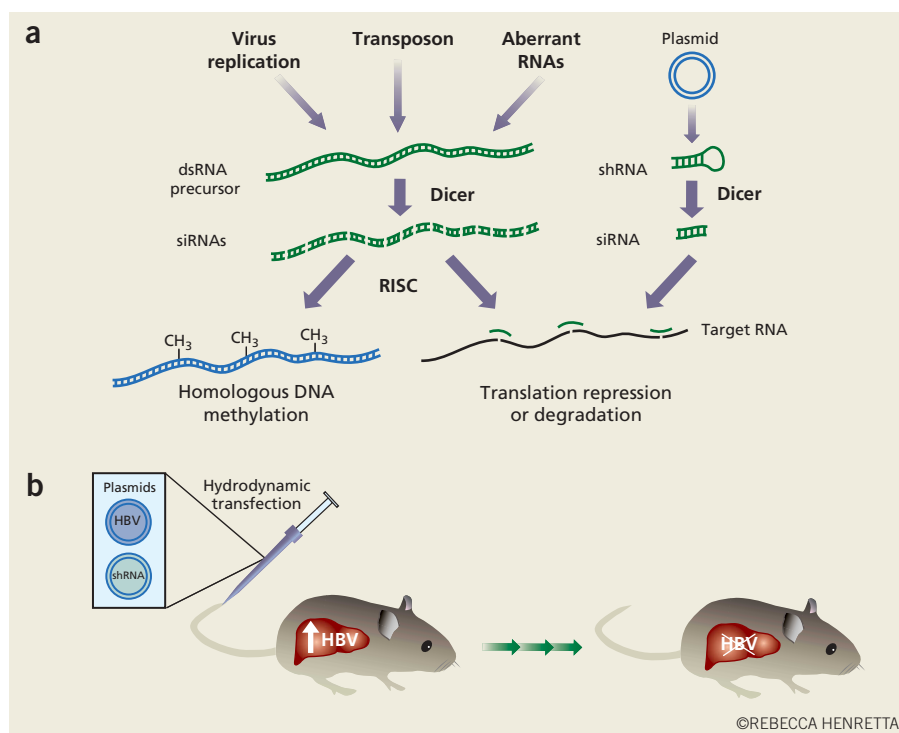


Figure 1 RNA silencing. (a) The central player in the RNA silencing pathways is double-stranded RNA, which acts as a guide to interfere, in a sequence-specific manner, with gene expression. RNA silencing can be initiated naturally by viruses or transposons through the generation of dsRNA during their replication, or artificially by the introduction of artificial dsRNA or plasmids encoding shRNAs. RNAi can silence gene expression in several different ways. It can inhibit transcription by inducing remodeling of the chromatin structure and can also act post-transcriptionally by targeting specific mRNAs for translation repression or RNA degradation. (b) Mouse tissues can be transfected with DNA plasmids encoding the HBV genome by hydrodynamic transfection, which artificially initiates virus replication. Cotransfection of plasmids encoding HBV-specific shRNAs results in inhibition of HBV replication.

normal and SCID (severe-combined immunodeficient) mice. Treatment with shRNAs resulted in inhibition of HBV replication in both immunodeficient and competent animal models. The SCID mice employed in this study are deficient in maturation of B and T cells, which are arrested at early stages of differentiation. In contrast, macrophages, dendritic cells and natural killer cells are unaffected. The data thus suggest that B and T cells are not required for the basic function of RNAi. However, it is still unclear whether some of the other unaffected lymphoid cells could be capable of regulating the RNAi activity. In particular, it would be interesting to determine whether the mammalian innate immune system interacts with the RNAi machinery.

The report by McCaffrey *et al.* employs an artificial method to initiate viral infection. The next challenge will be to determine whether RNAi can effectively hinder an authentic viral infection. To be effective,

most cells susceptible to virus infection should express the siRNA in a persistent manner. In this way, the RNAi strategy shares some similar benefits and limitations compared with other nucleic acid therapy approaches. Major obstacles include the efficient delivery of shRNA-expressing plasmids in large animals, including humans, and the problem of targeting specific cell types. Initial reports indicate that retroviral^{4,5} and adenoviral⁶ vectors are capable of carrying shRNAs and inducing RNA interference in targeted cells. It is possible to imagine that *ex vivo* introduction of DNA expressing shRNAs could yield cells 'immune' to infection that would subsequently be reintroduced into the individual. It would be particularly interesting to combine RNAi and stem cell technologies in a similar way to the recently reported approach⁷ to creating entire organs resistant to virus infection.

More importantly, and although several studies (including that of McCaffrey *et al.*)

have demonstrated that virus infection can be prevented by pretreatment or cotreatment with siRNAs, no study has yet shown that cells or tissues already infected with a virus can be cured. It is possible that animal viruses, like some plant viruses, inhibit the RNAi machinery shortly after the onset of replication, thus limiting the efficacy of the antiviral response. However, it is possible that in mammals the RNAi machinery would induce a systemic response, like in plants and worms, which may limit infection by preventing the spread of the virus to uninfected cells.

Another important practical issue relates to the virus' escape from RNAi. Viruses are likely to evade any given shRNA by mutation of the target sequences. Although, DNA viruses, which include HBV, are less likely to escape from small-interfering RNAs (siRNAs) because of their lower mutation frequency, it is important to know how well the RNAi machinery can accommodate mismatches. In this regard, poliovirus has been shown to escape RNAi inhibition by mutating a single nucleotide in the corresponding genomic region⁸. Alternatively, it may be possible to target cellular genes that are not subject to mutation. For example, depletion of CD4 using siRNAs can lead to a decrease in the infectivity of HIV⁹. The HIV coreceptor CCR5 has also been targeted, resulting in substantial protection of lymphocyte populations from HIV-1 infection¹⁰. Still, the biological plasticity of viruses may find ways to overcome even this strategy and thus, targeting a cellular factor may not guarantee complete protection from viral infection. Therefore, simultaneous targeting of several host cell factors involved in viral replication may be required. Given the rapid pace of the field, it seems likely that answers to many of these questions will be found soon, and the results of McCaffrey *et al.* add an important note of optimism to the feasibility of RNAi-based antiviral strategies.

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