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through the use of an animal model that allows extensive collection of lymphoid tissues throughout the body at defined time points after infection. The simian immunodeficiency virus (SIV)–macaque model is the most appropriate in which to perform these studies owing to the extensive similarities between the immune systems of macaques and humans and their respective immunodeficiency viruses.

The convincing demonstration of rapid and selective depletion of CD4+ T cells in the intestinal mucosa is an important first step that raises many questions, several of which were touched on in our publication and by Kraehenbuhl. Among these are mechanistic questions of how selective CD4+ T-cell depletion occurs, including the provocative possibility of altered cell trafficking. Several related questions are also raised. For example, does CD4 depletion occur throughout the mucosal immune system or is it limited to the intestinal mucosa? Would CD4+ T-cell depletion in the intestinal mucosa occur with the same speed after intra-rectal inoculation or after intra-vaginal inoculation? How does this rapid and profound depletion of CD4+ T cells in the intestinal mucosa affect the systemic immune system?

These and related questions raised by our recent publication have clearly increased awareness of the potential importance of the mucosal immune system in the pathogenesis of AIDS.

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Getting a handle on RNA virus recombination

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Most viruses have RNA genomes. We must understand how they generate diversity so we can anticipate and respond to the arrival of new strains or entirely new viruses. Owing to the high error rate of RNA-dependent replication, RNA viruses exist as heterogeneous populations of molecules known as quasispecies1. The advantage of quasispecies to the virus is that, as selection pressures change, a fit genome might already exist in the quasispecies population or can evolve rapidly. The disadvantage of the high error rate is that accumulation of too many mutations is damaging1. However, these genetic defects can be rescued by recombination with other molecules in the quasispecies that have functional genes2. Furthermore, by recombination, a virus can suddenly acquire entirely new traits (whole genes) in one step.

Recombination is common in RNA viruses3,2 and it might be more important than accumulation of point mutations for significant evolutionary change and speciation of RNA viruses. Viruses in groups as diverse as the alphaviruses2, coronaviruses3 and luteoviruses4 contain genes closely related to those of other groups, indicating recent recombination events in their evolution.

Understanding viral recombination is important for the safe deployment of transgenic, virus-resistant plants. Dozens of crop species have been engineered to express viral genes. This confers resistance to the virus from which the transgene was derived5. However, recombination between an invading virus and the transgenic viral RNA can yield viable progeny6. To assess the risks of new, virulent viruses arising from such events, a better understanding of RNA recombination mechanisms is essential.

Recombination has been well studied in poliovirus3, coronaviruses2, brome mosaic virus (BMV)3, turnip crinkle virus (TCV)8 and others. In some cases, recombination has been detected by the use of different selectable markers on different portions of each parental genome. The occurrence of both markers on the same genome demonstrates that replication has occurred5. This method detects rare events but generally gives a low-resolution view of the junctions of the recombinated genomes. For higher resolution, recombination is analyzed by sequencing across the junctions of parental and progeny RNAs.

RNA virus recombination events have been classified into homologous, aberrant homologous and nonhomologous recombination9. However, Nagy and Simon10 have suggested revising the classification into sequence similarity-essential, similarity-assisted and similarity-nonessential recombination. Most evidence supports a copy-choice mechanism8,10, in which...
the replicase switches from copying one RNA (donor template) to another (acceptor template) without releasing the nascent strand (Fig. 1).

**Recombination in vitro**

The details of viral recombination mechanisms have remained a mystery owing to the dependence on in vivo systems (cell culture or whole organism). In vivo assays require the progeny RNA to be able to replicate and survive in the cell independently of the actual recombination event. In vitro, the recombination event cannot be separated into its individual biochemical steps: (1) generation of the nascent (primer) strand on the donor template, (2) transfer of the primer strand and replicase to the acceptor template, and (3) elongation on the acceptor template. Moreover, if there is sequence identity across the recombination junction, the precise site of recombination cannot be determined by sequencing.

A recent paper from Simon’s research group describes a cell-free replication system that overcomes these obstacles and opens the way for detailed biochemical analysis of RNA recombination. This system examines recombination between 194-nt satellite RNA D (sat-RNA D) and 356-nt satellite RNA C of TCV (Fig. 1). Sat-RNA C is a chimera of sat-RNA D and a TCV defective interfering (DI) RNA. DI RNAs are efficient replicons comprising small portions of a viral genome from which most of the coding regions have been deleted by recombination. Sat-RNAs have no homology to their helper viruses. Their origins are unknown. Both sat- and DI RNAs contain cis-acting signals for replication by the replicase of C and sat-RNA D as a single RNA molecule. The primer and acceptor strands were connected by a large loop (Fig. 1), resulting in a partially

**Fig. 1.** Structures involved in recombination between turnip crinkle virus (TCV) satellite RNA D (sat-RNA D) and sat-RNA C. (a) Maps of template (negative [−]) strands of 194-nt sat-RNA D and 356-nt sat-RNA C are shown. The light blue portion of sat-RNA C has 88% homology to sat-RNA D (dark blue). The portion of sat-RNA C that is derived from the TCV genome is shown in red. The light blue and red arrow indicates strand switching and the direction of movement of replicase during positive [+] strand synthesis. The dark blue arrow shows the nascent strand from the donor template; the dashed blue line indicates the portion of sat-RNA C that is homologous to sat-RNA D. (b) The structure used for primer extension assays by Nagy et al. Primer and acceptor strands are connected by a large loop (green). The dashed red arrow indicates RNA synthesis, which includes the bulged stem–loop portion of the template, in the in vitro assay. Modified with permission from Ref. 10.

Previously, Simon’s lab identified a recombination hot spot between bases 173–179 of sat-RNA C and the 3′ end of sat-RNA D, which produces a hybrid RNA molecule. They found that a bulged stem-loop structure in the acceptor template strand is required for recombination in vitro (Fig. 1). This is a form of similarity-assisted recombination. The recent paper by Nagy et al. focuses on recognition of the acceptor template by the replicase and extension of the nascent strand, which acts as a primer. The latter function is of particular interest because TCV (and BMV) replicases cannot normally extend a primer annealed to single-stranded RNA. In contrast, poliovirus replicase can copy a template consisting only of poly(A) using either oligo(U) or its genome-linked protein (VPg) as a primer. However, the model for TCV recombination involves extension of the nascent strand in a way that is biochemically the same as primer-extended RNA synthesis (Fig. 1).

A primer and a ‘handle’

To test both the primer function and the requirements of the acceptor RNA to be recognized by the replicase, Nagy et al. have developed an elegant assay in which they have constructed the putative recombination complex of sat-RNA C and sat-RNA D as a single RNA molecule. The primer and acceptor strands were connected by a large loop (Fig. 1), resulting in a partially
duplex structure comprising this artificial stem-loop, and the upstream bulged stem-loop, which had been previously identified as being important for recombination. This bulge then extended the primer strand,copying the bulged stem-loop and the single-stranded 5′ end of the molecule. The small products were then rapidly quantified and sized. However, no productive primer extension was observed to interpret because of the high degree of secondary structure, which resisted denaturation.

Using an exhaustiv e set of point mutations, Nagy et al.\textsuperscript{15} went on to compare the effects of mutations on primer extension \textit{in vitro} with their effects on recombination \textit{in vivo} (in which case the bulged stem was located in the negative strand of sat-RNA\textsuperscript{13}). Importantly, there was a good correlation between \textit{in vitro} and \textit{in vivo} effects. Generally, mutations that reduced efficiency of primer extension to \textless 24% of that on the ‘wild-type’ molecule eliminated recombination \textit{in vivo}. Both stems in the bulged stem-loop upstream (in the template sense) of the acceptor strand were necessary for efficient primer extension, although their sequence was not as important. Deletion of bases in the bulge greatly reduced primer extension and eliminated recombination. Some specific bases in the terminal loop were also necessary.

The second feature shown to be necessary was the complementarity between the primer strand and the acceptor strand. The length, sequence, and 3′ position of the primer were not important in determining whether extension could take place. Less stable priming stems gave less efficient extension. Thus, primer binding to the acceptor serves only as an extendable nascent RNA strand and is not involved in specific recognition of the acceptor by the replicase. In support of the role of the bulged stem-loop in specific recognition by the replicase, RNA molecules containing only this region, without the primer strand, were found to inhibit primer extension at the position where the natural mutant bulged stem-loops did not inhibit primer extension significantly. Thus, two structures are necessary to attract the nascent RNA and the replicase to the acceptor strand. The region of complementarity attracts the nascent RNA strand, and the bulged stem-loop may be the ‘handle’ by which the replicase grabs the acceptor strand.

**Future questions**

The above model could not be supported without an \textit{in vitro} assay. Although Simon’s work is a promising step early in the dissection of recombination mechanisms, many questions remain. For example, what features on the donor template encourage dissociation of the replicase and nascent strand? Possibilities proposed for a variety of RNAs include a stable secondary structure, the 5′ end of the template itself or AU-rich regions that weaken the interaction between the nascent strand and the template. In coronaviruses, dissociation has been attributed to the nonprocessive nature of the replicase\textsuperscript{8}. In BMV, the replication complex is initially highly abortive but then undergoes a modification allowing efficient elongation\textsuperscript{11}. Perhaps, at some points of the RNA synthesis, the replicase complex can revert to its loosely bound abortive state and subsequently dissociate.

What is the nature of the protein that interacts with the bulged stem-loop? Presumably, it is a component of the viral replicase, but is it the virally encoded RNA-dependent RNA polymerase itself or a host factor that participates? How does acceptor recognition by the replicase machinery compare with the specific recognition of the origins of replication at the 3′ ends of positive and negative strands and internally at subgenomic RNA promoters?

More broadly speaking, how widely does this type of mechanism apply? Recognition of an internal structure on a negative strand, as described here, resembles subgenomic RNA synthesis. Subgenomic RNA synthesis may be primer dependent in coronaviruses\textsuperscript{12} but is primer independent in plant viruses\textsuperscript{14}. However, we propose that plant viral subgenomic RNA promoters can also be recognized by this donor-prime d mechanism in the recombination events that generate variation in luteoviruses\textsuperscript{5}. This may occur in other viruses that produce subgenomic mRNAs, including many plant viruses, coronaviruses and alphaviruses.

In contrast to the above viruses, recombination has been detected in poliovirus at hot spots for which no replication origin or subgenomic promoter would normally be predicted\textsuperscript{15}. What are the features of the RNA and replicase that promote picornaviral recombination at specific sites? The recent demonstration of poliovirus recombination in cell-free systems\textsuperscript{16} bodes well for elucidation of the mechanism in the near future. Now that recombination has been demonstrated in two such very different \textit{in vitro} systems, TCV and poliovirus, and numerous other cell-free replication systems are available, the next few years should yield a bounty of detailed analyses of a variety of different RNA recombination mechanisms.

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