CTMI (2005) 287:133–159

Coronavirus Reverse Genetics by Targeted RNA Recombination

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Abstract Targeted RNA recombination was the first reverse genetics system devised for coronaviruses at a time when it was not clear whether the construction of full-length infectious cDNA clones would become possible. In its current state targeted RNA recombination offers a versatile and powerful method for the site-directed mutagenesis of the downstream third of the coronavirus genome, which encodes all the viral structural proteins. The development of this system is described, with an emphasis on recent improvements, and multiple applications of this technique to the study of coronavirus molecular biology and pathogenesis are reviewed. Additionally, the relative strengths and limitations of targeted RNA recombination and infectious cDNA systems are contrasted.

1 Introduction

Targeted RNA recombination was developed to address the need for a reverse genetic system for coronaviruses at a time when it was uncertain whether the construction of full-length infectious cDNA clones was technically feasible or, indeed, even possible. As detailed elsewhere in this volume, this goal has now been realized, largely through the tenacity and ingenuity of a handful of investigators. Concurrently, the ensuing decade since its origination has allowed targeted recombination to evolve into a productive methodology that, across the boundaries of multiple laboratories and viral species, has enabled coronavirus studies to take advantage of the opportunities offered by reverse genetics.

In this chapter we begin with a brief background on the prominence of recombination in coronavirus RNA synthesis and then detail how this property has been exploited for the purposes of site-directed mutagenesis of the coronavirus genome. We describe the scientific problems to which targeted recombination has been successfully applied, and finally we comment on the future prospects for this technique. Throughout our discussion emphasis is placed on new developments in the field since the last time this subject was reviewed (Masters 1999).

2 Coronavirus RNA Recombination

RNA recombination is a well-established phenomenon among animal, plant, and bacterial RNA viruses (reviewed in Lai 1992; Nagy and Simon 1997). As a mechanism of genetic exchange, it provides these viruses with a powerful evolutionary attribute. Recombination is concomitant with viral RNA replication. The consensus model for its occurrence is that the viral RNA polymerase, with a nascent RNA strand attached to it, dissociates from its template and resumes RNA synthesis after it has bound elsewhere to the same or to another template. This "copy-choice" or template-switching mechanism was originally established for polioviruses (Kirkegaard and Baltimore 1986), the viral species for which RNA recombination was first demonstrated (Ledinko 1963), but it seems to be generally applicable.

Homologous RNA recombination takes place when there is a switch of templates between regions of high sequence similarity. This particular form of recombination had only been observed for—and was thus be-

lieved to be restricted to—positive-strand RNA viruses, but it has recently also been demonstrated for a minus-strand RNA virus (Plyusnin et al. 2002). Homologous RNA recombination occurs at a remarkably high rate among coronaviruses (Lai 1992, 1996). Their huge genome size and particular mode of replication, employing a discontinuous mode of transcription, may favor polymerase template switching (Brian and Spaan 1996). Accordingly, the phenomenon also has been observed for other nidoviruses, particularly the arteriviruses (Li et al. 1999; Yuan et al. 1999; van Vugt et al. 2001).

Experimental evidence for RNA recombination in coronaviruses has rapidly accumulated, ever since its first description in the mid-1980s (Lai et al. 1985). Essentially all of the early work was done with mouse hepatitis virus (MHV) by taking skillful advantage of the availability of distinctive natural viral strains and classic mutants generated in the laboratory. Initially, through the analysis of progeny obtained from coinfection of culture cells or mouse brains with different MHV variants and application of different selection principles (e.g., temperature sensitivity, cell fusion ability, sensitivity to neutralization by specific antibodies), many of the fundamental features of coronavirus recombination were elucidated (Lai et al. 1985; Keck et al. 1987, 1988a,b; Makino et al. 1987). Sequence analyses revealed that recombination can happen virtually anywhere along the genome but that particular virus combinations show preferred crossover regions, probably owing to selective pressure (Banner et al. 1990). Many MHV recombinants were found to have multiple crossovers, consistent with an exceptionally high frequency of recombination. The overall frequency per passage was estimated at approximately 1% per 1,300 nucleotides (or 25% over the entire genome) by long-range mapping using temperature-sensitive mutants (Baric et al. 1990). Similar studies subsequently demonstrated that, within a relatively short interval, the recombination frequency is uniform (Banner and Lai 1991) but it increases progressively from the 5′ to the 3′ end of the MHV genome, presumably because of participation of subgenomic (sg) RNAs (Fu and Baric 1994). Although homologous RNA recombination has been less extensively studied in other viral species, the experimental demonstration of this phenomenon has not been limited to the group 2 coronavirus MHV. It has been shown as well for the group 3 coronavirus infectious bronchitis virus (IBV) (Kottier et al. 1995) and the group 1 coronavirus transmissible gastroenteritis virus (TGEV) (Sánchez et al. 1999), for the former by coinfection of viruses into embryonated eggs and for the latter by electroporation of defective RNA into infected cells in tissue culture.

Recombination of coronaviruses appears to be a process of significant importance in the wild. Its occurrence has been shown to contribute to the natural evolution of IBV. This highly contagious virus comprises many different serotypes, and new ones emerge regularly, with the result that these viruses escape from host immunity and cause new outbreaks. Although many of the new variants arise by genetic drift as a result of subtle mutations in the spike protein (S) gene, similar to the changes that lead to antigenic drift in influenza viruses, new serotypes apparently also originate from genetic exchange of S gene sequences between different viruses through homologous RNA recombination (Kusters et al. 1990; Cavanagh et al. 1990; Wang et al. 1993; Jia et al. 1995). Of considerable impact on these evolutionary processes is the veterinary practice of vaccination. Large-scale application of (combinations of) live attenuated vaccine viruses drastically enhances the opportunities for recombination. The identification of vaccine-derived sequences in field isolates is therefore not surprising (Kusters et al. 1990; Wang et al. 1993; Lee and Jackwood 2001). Rather, these events actually seem to occur at high frequency and are not restricted to the S gene region, as attested by the complex genetic makeup of IBV strains that carry the footprints of multiple independent recombinations (Jia et al. 1995; Lee and Jackwood 2000).

Homologous RNA recombination also plays an important role in the evolution of feline coronaviruses. These viruses fall into two serotypes, with type I viruses being the most prevalent. Unlike type I viruses, the type II viruses cross-react with canine coronavirus (CCoV) in virus neutralization assays, and sequence analysis of their S genes indeed confirms this relatedness: Serotype II viruses appear to be derived from recombination between type I feline coronaviruses and CCoV (Motokawa et al. 1995; Vennema et al. 1995; Herrewegh et al. 1995). Detailed analyses of two type II strains revealed that each actually resulted from double recombination, with crossover points located both upstream and downstream of the S gene (Herrewegh et al. 1998). Importantly, all of the crossover points were unique, and subsequent sequencing of the 3′ genomic region of two additional type II strains showed that the template switches in this region had occurred at different sites in all four viruses: two each in the envelope protein (E) and the membrane protein (M) genes (Vennema 1999). Obviously, these viruses must have arisen from independent recombination events. Although it is not known in which host species the coinfection of feline and canine coronaviruses takes place, these observations suggest that such occurrences are not overly rare.

More generally, RNA recombination is also believed to have been instrumental in the emergence of the three coronavirus groups. Viruses from these groups characteristically differ in the identities and genomic locations of their nonessential genes. These group-specific genes are presumed to have been acquired by recombination, in this case nonhomologous, with cellular or heterologous viral RNAs. A case in point is the hemagglutinin-esterase (HE) gene found in several group 2 coronaviruses as well as in toroviruses. This gene was presumably derived from recombination between an ancestral coronavirus and influenza C virus, as is suggested by its remarkable sequence similarity to the corresponding orthomyxoviral HE gene (Luytjes et al. 1988). Apart from still-undefined roles in interactions with their respective hosts (de Haan et al. 2002a), the functions and possible origins of the other group-specific genes remain elusive.

3
Targeted RNA Recombination: Methodology and Technical Issues

3.1
Original Development of the System

Targeted RNA recombination was devised as a means of introducing specified changes into the coronavirus genome through recombination between a donor synthetic RNA and a recipient parent virus possessing some characteristic that allows it to be counterselected. The genomic changes to be introduced are first generated in a cDNA transcription vector, and donor RNA is transcribed in vitro from this plasmid. After RNA recombination in infected cells, viral progeny bearing the desired alterations are selected on the basis of their possession of a phenotypic property not found in the original recipient virus.

The earliest scheme for targeted RNA recombination came about by the fortunate confluence of a number of separate discoveries. First, as outlined in the previous section, an abundance of experimental work, primarily with MHV, had demonstrated that RNA recombination is a frequent event in the coronavirus infectious cycle. Second, it had recently been shown that each coronavirus sgRNA possesses a negative-strand counterpart (Sethna et al. 1989). Although the original proposal that sgRNAs function as replicons has not proved correct, this key finding made clear that the positive-strand sgRNAs serve as substrates for the viral polymerase, thus rendering them likely participants in polymerase-

mediated recombination. Finally, an MHV mutant was found that had the ideal properties for the recipient parent virus. This mutant, Alb4, was among a collection of classic, random mutants isolated on the basis of production of an atypical cytopathic effect at the nonpermissive temperature (39°C) (Sturman et al. 1987). Alb4 is temperature sensitive, but it is not an absolute conditional-lethal mutant, in that it produces plaques at the nonpermissive temperature that are tiny by comparison with the wild type. Additionally, virions of Alb4 are thermolabile, exhibiting a drop in infectious titer of two to three orders of magnitude when held at the nonpermissive temperature for 24 h, a treatment that only minimally affects the viability of the wild type. The lesion in Alb4 was found to reside in the nucleocapsid (N) gene, the gene closest to the 3′ untranslated region (3′ UTR) of the genome, and consists of an 87-nt (in frame) deletion (Koetzner et al. 1992) that removes a 29-amino acid linker connecting two functional domains of the N protein (Parker and Masters 1990).

The experiment establishing the principle of targeted RNA recombination, then, was carried out by cotransfection of mouse cells with the purified genome of Alb4 and a synthetic copy of sgRNA7, which is the smallest of the MHV sgRNAs and serves as the mRNA for N protein (Fig. 1) (Koetzner et al. 1992). The synthetic donor RNA contained the wild-type (undeleted) version of the N gene and was tagged with a presumed nondeleterious 5-nt insertion in the 3′ UTR. The precarious nature of this latter assumption was only revealed years later, when it was found that the insertion had been made in a mutable loop of an RNA secondary structure that is absolutely essential for MHV replication (Hsue and Masters 1997). The viral progeny resulting from the cotransfection were subjected to a heat-killing step, so as to greatly reduce the background of Alb4 parent virus, and candidate recombinants were identified as viruses forming large (i.e., wild-type sized) plaques at the nonpermissive temperature. The presence in the putative recombinants of both the region that is deleted in Alb4 and the 5-nt tag was verified by size or restriction fragment polymorphisms in RT-PCR products from genomic RNA that had been isolated from purified virions. Additionally, the 5-nt tag, which is present in neither wild-type MHV nor the Alb4 mutant, was demonstrated by direct sequencing of genomic RNA of the recombinants. These viruses were thus the first engineered site-specific mutants of a member of the coronavirus family.

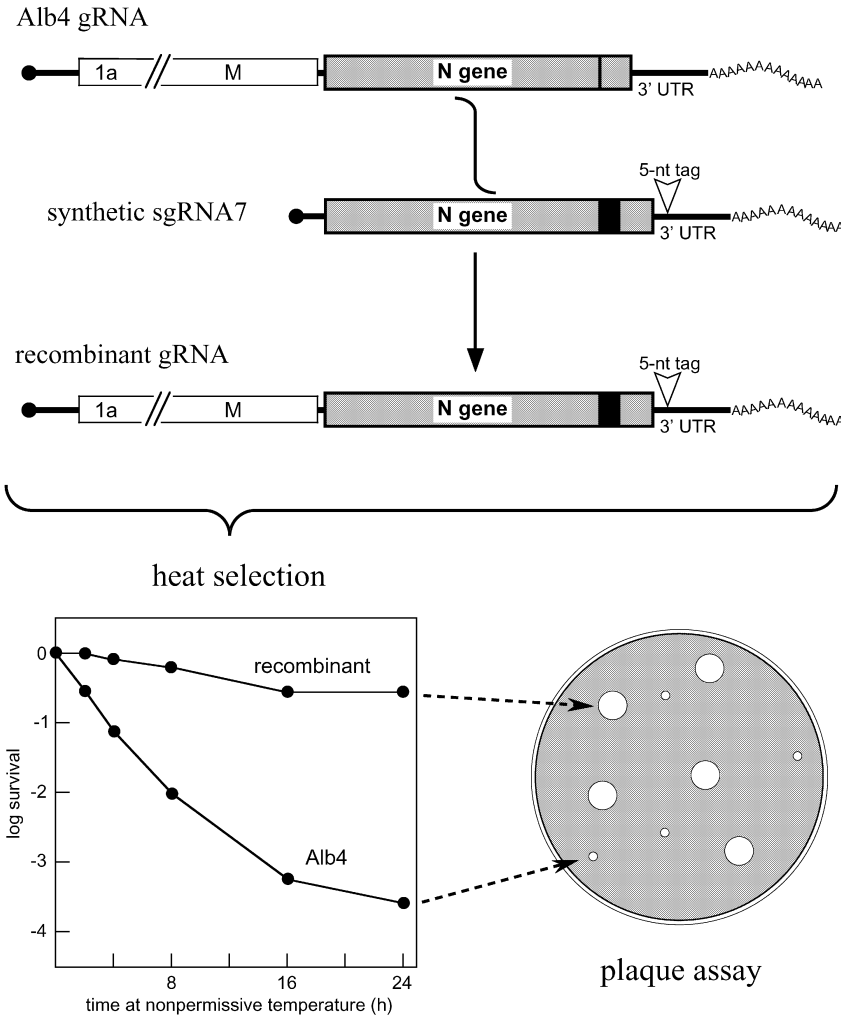

Fig. 1. Earliest implementation of targeted RNA recombination. Genomic RNA (*gRNA*) from the Alb4 mutant and synthetic donor RNA were cotransfected into cells. The donor RNA contained the wild-type N gene, including the 87-nt region that is deleted in Alb4 (*black rectangle*) and was also tagged with a 5-nt insertion in the 3′ UTR. After harvest of progeny virus, the Alb4 parent was selectively killed by heat treatment, and recombinants were identified as viruses forming large (wild-type size) plaques at the nonpermissive temperature

3.2
Improving the Donor RNA: DI and Pseudo-DI RNAs

The initial demonstration of targeted RNA recombination was soon followed by a report of the incorporation of genetic markers into the MHV genome by using a defective interfering (DI) RNA, MIDI-C, as the donor RNA (Fig. 2) (van der Most et al. 1992). In this case, a coding-silent marker tagging the region of the Alb4 N gene deletion was successfully transferred from the DI RNA to Alb4 recipient virus. Additionally, it was shown that recombinants bearing MIDI-C-derived markers that had been transduced into gene 1 of wild-type MHV could be isolated by screening, without any prior selection. Most importantly, the efficiency of obtaining recombinants with this DI RNA as the donor appeared to

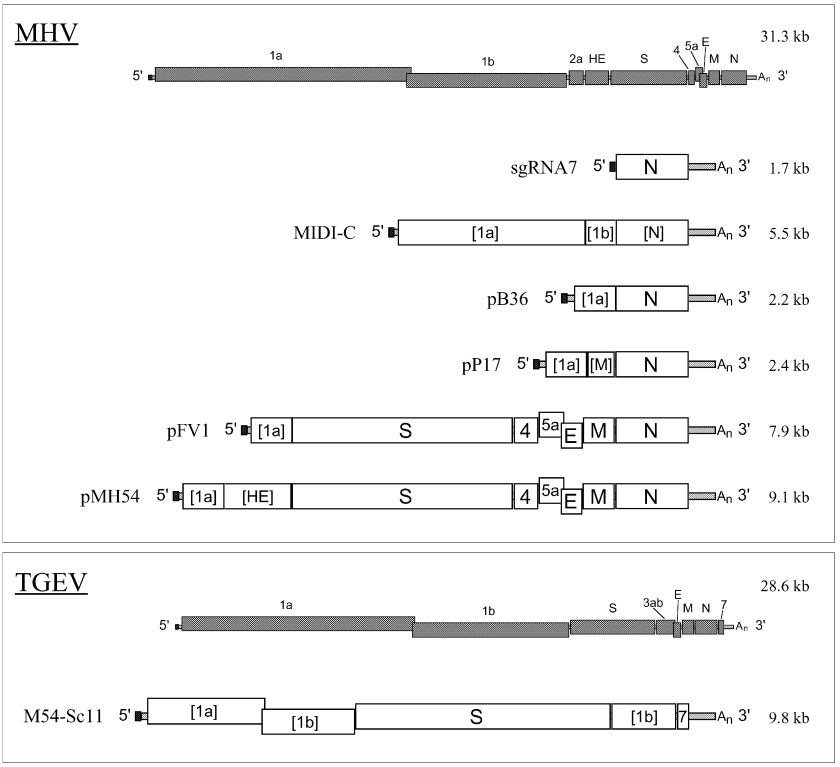

Fig. 2. DI and pseudo-DI donor RNAs used in targeted recombination studies. Shown at the *top* of each panel is the genome from which the donor RNAs are derived. *Brackets* indicate fragments of full-length genes

be significantly higher than had been achieved with sgRNA7 as the donor. Coronavirus DI RNAs are parasitic elements that arise through the accumulation of extensive deletions, which eliminate most of the coding capacity of the genome while retaining *cis*-acting elements essential for viral polymerase recognition. They are therefore not independently viable, but they replicate by feeding into the RNA synthesis machinery provided by a helper virus.

In an attempt to systematically optimize the performance of targeted recombination, the efficiencies of the two types of donor RNA were directly compared (Masters et al. 1994). For this purpose, a nonnatural MHV DI RNA, designated pB36 RNA, was constructed to contain the entire N gene, mimicking the composition of a well-characterized, naturally arising DI RNA of bovine coronavirus (BCoV) (Chang et al. 1994). It was found that this donor DI RNA replicated abundantly and consistently yielded targeted recombinants with Alb4 at an efficiency on the order of 10^{-2}, some two to three orders of magnitude greater than that obtained with sgRNA7 donor RNA. This meant that candidate (large plaque) recombinants could generally be identified directly against the background of small plaques formed by the Alb4 parent, without the need for a heat-killing counterselection step. Because the mechanistic details of RNA recombination remain to be unraveled, it has not yet been resolved whether the increased donor efficiency of DI RNAs results from their replicative competence or from some other intrinsic property. It is possible that the critical feature of DI RNAs is not their ability to replicate per se, but, rather, that they possess some sequence or structural element that brings about their localization to the RNA synthesis compartment, or that facilitates their alignment with homologous regions of the acceptor genome template.

Despite our not understanding precisely why DI RNAs work so well, it was nevertheless straightforward to design additional donor RNAs based on the relatively simple composition of pB36 RNA, which comprises only the 5′- and 3′-terminal segments from the MHV genome, connected by a short heterologous linker (Masters et al. 1994). The inclusion of more material from the 3′ end of the genome resulted in progressively larger plasmid vectors for donor RNAs—pP17 (Fischer et al. 1997a), pFV1 (Fischer et al. 1997b), and pMH54 (Kuo et al. 2000) (Fig. 2)—which were collectively capable of transducing mutations into any of the genes downstream of gene 1, the viral replicase gene. The availability of these larger donor RNAs, termed pseudo-DI RNAs because it has never been directly determined whether they are replication competent, consequently places all of the MHV structural genes within

the reach of the targeted recombination method. Separately, a similar principle was applied to a different coronavirus species, TGEV, by the insertion of the S protein gene into a naturally occurring DI RNA of that virus (Méndez et al. 1996). However, in this case the donor RNA, M54-SC11 (Fig. 2), was not completely colinear with the 3′ end of the recipient genome, and thus the formation of the recombinants that were isolated was dependent upon two crossover events, one upstream and one downstream of the targeted region (Sánchez et al. 1999). Work has also been done toward using a modified naturally occurring DI RNA of IBV in targeted RNA recombination, but the recovery of viable recombinants from this system has not yet been reported (Neuman et al. 2001).

3.3
Improving the Recipient Virus: Host Range-Based Selection

Although the Alb4 mutant was invaluable in moving coronavirus genetics from classic to molecular capabilities, a fundamental limitation of the scheme described above is that selection against Alb4 makes sense only if the mutant being sought is more fit than Alb4 at the nonpermissive temperature. This precondition still allows the selection of a wide variety of mutants, but it places a restriction on the range of problems to which a genetic system could potentially be applied. Two studies made use of alternative strategies to circumvent this fitness precondition. In one study, an RT-PCR-based screen of large pools of candidate recombinant plaques was employed to identify clustered charged-to-alanine mutations made in the E gene (Fischer et al. 1998). The fact that the resulting E protein mutants were temperature sensitive and thermolabile explained why they could not be isolated by a heat-killing selection. A second means around the fitness requirement was to carry out a different type of selection. In this case, neutralization with monoclonal antibodies specific for the S protein of MHV strain A59 (the strain to which Alb4 belongs) was used to obtain recombinants that had incorporated the S gene of MHV strain 4 (Phillips et al. 1999). However, both of these alternatives had disadvantages. Mutant identification by screening is extremely labor intensive and of uncertain efficiency, and strain-specific monoclonal antibody selection is applicable only under special circumstances.

Superseding these two particular exceptions, a very powerful positive selection strategy was enabled by the creation of an interspecies chimeric mutant of MHV in which the ectodomain of the S protein was replaced with its counterpart from feline infectious peritonitis virus

(FIPV) (Kuo et al. 2000). This substitution had its foundation in work done with viruslike particles (VLPs), which had suggested that the determinants for functional S protein incorporation into virions reside solely in the transmembrane domain and the endodomain of the molecule (Godeke et al. 2000). Because both MHV and FIPV are stringently species specific in tissue culture, the interspecies chimeric mutant, named fMHV, was readily obtained by a targeted RNA recombination experiment that selected for a virus that had acquired the ability to grow in feline cells. It soon became apparent that the inverse of this selection would provide significantly greater flexibility in the construction of MHV mutants than the Alb4-based targeted recombination scheme. The use of fMHV as the recipient virus with donor RNA transcribed from a pMH54-derived vector, which would restore the region encoding the MHV S gene ectodomain, should, in principle, allow the selection of recombinants harboring any nonlethal MHV mutation (Fig. 3, top panel). No matter how fragile its phenotype, the constructed mutant should be identifiable on the basis of its having regained the ability to grow in murine cells, in contrast to the fMHV parent, which can only grow in feline cells. The feasibility and utility of this strategy have now been proven repeatedly in multiple laboratories.

The strength of the host range-based selection has been most dramatically demonstrated by its ability to recover a mutant with a two-residue truncation of the carboxy terminus of the M protein (Kuo and Masters 2002) and a mutant with the critical E gene entirely deleted (Kuo and Masters 2003). Both of these mutants are severely impaired, forming tiny plaques at all temperatures and yielding infectious titers that are, at most, orders of magnitude lower than those of the wild type. In the initial selection of these and a number of other highly defective mutants, the recombinants being sought were identified as tiny plaques among a mixture of tiny and wild-type-sized plaques (Kuo and Masters 2002, 2003). Analysis of the latter showed that they were reconstructed wild-type viruses, which had arisen via a second crossover event occurring downstream of the restored MHV S gene, but upstream of the mutation of interest (Fig. 3, top panel). Although double crossovers occur with lower frequency than a single crossover, they can constitute a significant fraction of the initial recombinants in cases in which the wild type has a marked growth advantage over the constructed mutant. To preclude the possibility of the second crossover event, a variant of fMHV, designated fMHV.v2, has been constructed in which the gene order downstream of the S gene has been rearranged (Fig. 3, middle panel). The use of fMHV.v2 eliminates the background of progeny recombinants generated

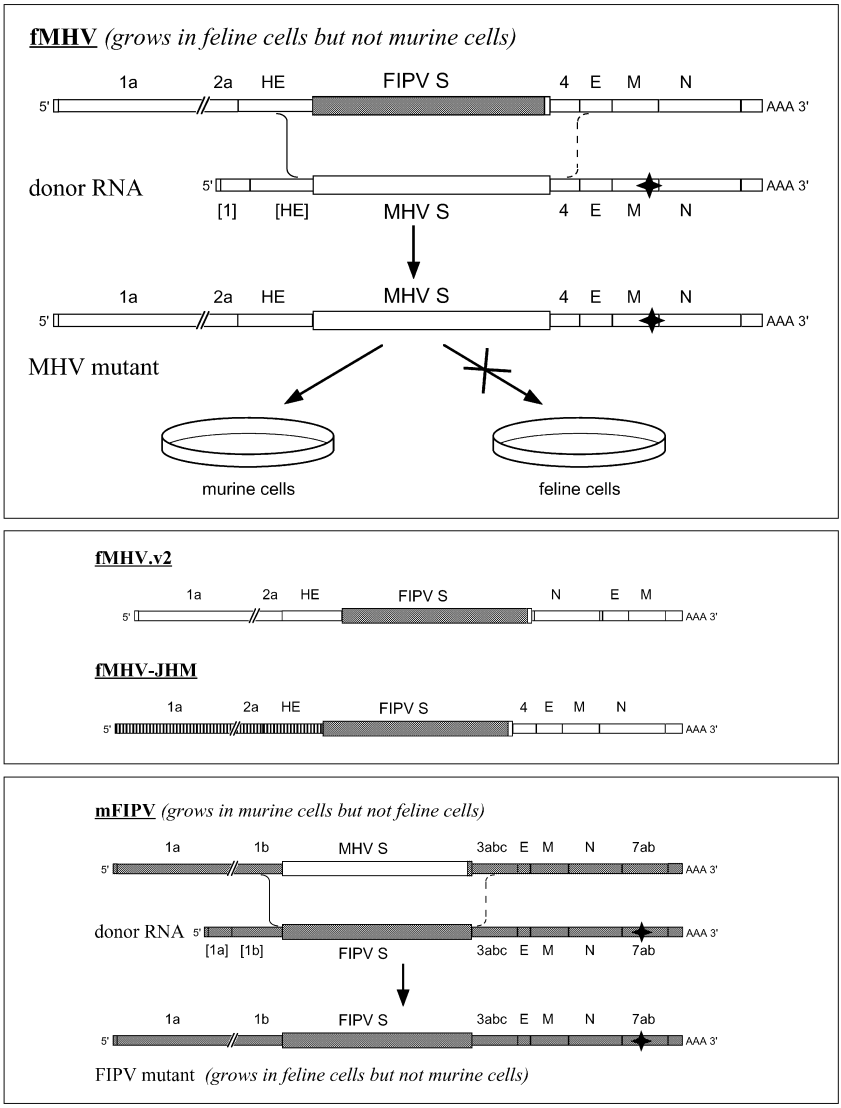

Fig. 3. Host range-based selection. *Top panel*: Selection strategy with the interspecies chimeric coronavirus fMHV, which contains the portion of the S gene encoding the ectodomain of the spike protein of FIPV (*shaded rectangle*) but is otherwise identical to MHV. fMHV is able to grow in feline cells but cannot grow in murine cells. In targeted recombination with donor RNA that restores the MHV S ectodomain, a single crossover (*solid line*), within the HE gene, can generate a recombinant that has reacquired the ability to grow in murine cells and has also incorporated an engi-

by second crossovers, and it is of particular utility in the recovery of unselected markers that are debilitating or that are located far downstream of the S gene (Goebel et al. 2004).

In addition to depth, the host range-based selection system has been shown to have breadth. The fMHV structural genes have been incorporated into the JHM strain of MHV, resulting in fMHV-JHM (Fig. 3, middle panel) (Ontiveros et al. 2001). This chimeric virus, in conjunction with the appropriate JHM strain counterpart of the pMH54 vector, has been used to construct site-directed mutants in MHV-JHM, thereby providing proof of principle for the applicability of this system to MHV strains other than strain A59. A more far-reaching extension of the method has been achieved by the construction of mFIPV, an interspecies chimeric mutant of FIPV in which the ectodomain of the S protein has been replaced with that of MHV (Haijema et al. 2003). This virus, which, as expected, has a host cell species permissivity exactly the converse of that of fMHV, provides the starting point for construction of site-directed mutations in the structural and nonstructural genes of FIPV (Fig. 3, bottom panel). These results establish host range-based selection as a general blueprint for the carrying out of reverse genetics in all coronaviruses, or at least in those that exhibit some level of host range restriction in tissue culture.

4
Targeted RNA Recombination: Spectrum of Applications

The impact of reverse genetic systems on progress in virology has been overwhelmingly demonstrated for most families of viruses over the last two decades, much to the frustration of many a coronavirologist. The new availability of multiple systems for engineering coronaviral genomes suddenly provides these investigators with unexpected opportunities, requiring choices to be made. These choices will be guided by the

neered mutation (*star*). A potential second crossover (*broken line*) would regenerate a wild-type recombinant lacking the mutation. *Middle panel*: Variant interspecies recipient viruses fMHV.v2, which greatly reduces the probability of the undesired downstream second crossover, and fMHV-JHM, which can be used to construct mutants of the JHM strain. *Bottom panel*: Selection strategy with mFIPV, entirely analogous to the fMHV scheme (*top panel*)

particular research question and by the practical and theoretical limitations of the various engineering systems. Because of the restrictions inherent in its selection principle, targeted RNA recombination in its present format will retain its greatest value in the study and manipulation of functions specified by the genomic regions downstream of the polymerase gene. The vast potential of this technology for coronavirus research can perhaps best be envisaged just by looking back at the first ten years of its existence. What follows is a brief survey of important contributions that the targeted recombination approach has made in the different areas of its application. Unless otherwise specified, the work discussed refers to MHV.

4.1 Virion Structure and Assembly

By their nature, the earliest versions of the targeted recombination method revolved around the N gene. They allowed the mapping of the extreme thermolability of virions of MHV-A59 mutants Alb4 and Alb1 to a deletion (Koetzner et al. 1992) and to a point mutation (Masters et al. 1994), respectively, in this gene. By an analysis of a panel of independently isolated revertant viruses this thermolability could, for Alb4, subsequently be attributed to a disturbed RNA binding capacity of the N protein. By the use of targeted recombination, critical evidence was obtained linking the restoration of the wild-type phenotype to a single reverting amino acid mutation, different for each revertant, in a domain of the N protein to which RNA binding had been previously mapped (Peng et al. 1995a). It was therefore somewhat surprising that major parts of this domain, as well as the segment that is deleted in Alb4, could be exchanged without penalty by the corresponding domain of the BCoV N protein (Peng et al. 1995b). The resulting MHV-BCoV chimeric viruses were viable and thermally stable. In contrast, for other regions of the N protein, such as the terminal domains, interspecies exchange was not tolerated, presumably because these regions are involved in protein-protein interactions that are specific for each virus.

Interactions between M molecules are thought to provide the major force for the assembly of the coronavirus envelope (Rottier 1995; Vennema et al. 1996). In a mutational study investigating the primary structural requirements of the M protein for assembly of VLPs from co-expressed M and E proteins, it was found that stringent structural conditions must be satisfied for envelope formation. In particular, the extreme carboxy terminus of M was shown to be crucial in this system (de Haan

et al. 1998). The mere deletion of the terminal residue (MΔ1) almost completely abolished assembly, whereas an M protein mutant additionally lacking the penultimate residue (MΔ2) was entirely assembly incompetent. By contrast, when these deletions and other mutations in the carboxy-terminal domain were transferred to the MHV genome by targeted recombination, the resulting effects were generally much less severe, or were even absent. The MΔ1 viral mutant, for instance, had no detectable defect. Apparently, in the context of the complete virion, changes that are devastating in the VLP system can be accommodated by other stabilizing interactions, most likely between the envelope and the nucleocapsid. Although the MΔ2 viral mutant could not be identified in this study and was thus considered nonviable (de Haan et al. 1998), the power of the host range-based selection system later enabled its isolation (Kuo and Masters 2002). The MΔ2 virus formed tiny plaques in tissue culture and grew extremely poorly, and on passage, revertants with strongly improved growth properties rapidly emerged. Genetic analysis of a large number of second-site revertants, combined with the targeted reintroduction of some of the reverting mutations back into the MΔ2 mutant genome, identified residues both in the M protein and in the N protein that could compensate for the two-residue deletion. This provided compelling evidence for a structural interaction between the carboxy termini of these two proteins in MHV.

Despite its minute abundance in virions, the E protein is a critical factor in the assembly of coronaviruses. Its function, however, is still unresolved. To study the role of E, clustered charged-to-alanine mutations were introduced into the protein through targeted recombination (Fischer et al. 1998). Three viable mutant viruses were obtained, two of which were temperature sensitive whereas the third had a wild-type phenotype. Both temperature-sensitive mutants were markedly thermolabile when grown at the permissive temperature. When virions of one of these E mutants were viewed by electron microscopy, particles with strikingly aberrant shapes were observed. These data indicated an important role for the E protein in virion morphogenesis and stability. Remarkably, however, it has recently become clear that this role is not essential. Again, because of the power of the host range-based selection system of targeted recombination, it has been possible to isolate a mutant of MHV from which the E gene is entirely deleted (Kuo and Masters 2003; de Haan and Rottier, unpublished results). Although the ΔE mutant produces tiny plaques with an unusual morphology, has a slow growth rate, and grows to low infectious titer, it is, nevertheless, completely viable. Curiously, the E protein appears to be an absolute requirement for the

group 1 coronavirus TGEV; growth of TGEV from which the E gene has been deleted is essentially dependent on *in trans* complementation by expressed E protein (Curtis et al. 2002; Ortego et al. 2002).

4.2 RNA Replication and Transcription

Because all intergenic regions, including their associated transcription-regulating sequences (TRSs), as well as the 3′ UTR, are accessible for manipulation by targeted RNA recombination, this methodology allows the study of many questions related to viral replication and transcription. An initial foray in this direction sought to define functionally equivalent segments of the 3′ UTRs of MHV and BCoV. This led to the identification of a conserved bulged stem-loop secondary structure at the upstream end of the 3′ UTR, adjacent to the stop codon of the N gene (Hsue and Masters 1997). The stem-loop was shown to be essential for virus viability as well as for DI RNA replication. More recent work (Hsue et al. 2000; Goebel et al. 2004), using mutational analysis combined with chemical and enzymatic probing, has refined the picture of this structure and has delineated its relationship with a downstream, and partially overlapping, RNA pseudoknot that was first discovered in BCoV (Williams et al. 1999). The mutually exclusive nature of the stem-loop and the pseudoknot suggests that they are components of a molecular switch, functioning to mediate some event during RNA synthesis.

Coronaviruses have a genome organization in which the order of the essential genes (5′-polymerase-S-E-M-N-3′) is strictly conserved, despite the high frequency of RNA recombination of these viruses. To find out whether this fixed gene order is in some way a vital property, deliberate rearrangements were introduced into the viral genome through targeted recombination. All attempted gene rearrangements were found to be tolerated, generally with surprisingly little effect on the growth characteristics of the recombinant viruses in cell culture or, for one virus tested, in the mouse host (de Haan et al. 2002b).

The factors that determine the relative efficiencies of synthesis of coronavirus sgRNAs are as yet poorly understood. Evidence indicates that the identity of the TRS, its sequence context, and its genomic position can all contribute to the process. The fortuitous effect of one or more of three nucleotide changes introduced into a donor vector for targeted recombination (pMH54, Fig. 2), to create a convenient restriction site upstream of the gene 4 TRS, illustrates the importance of the TRS sequence context (Ontiveros et al. 2001; de Haan et al. 2002a). For unknown rea-

sons this modification led to a dramatic (at least sevenfold) upregulation of sgRNA4 synthesis. Other examples of unexpected context effects were observed in some genomically rearranged viruses (de Haan et al. 2002b). For instance, relocation of the gene 4–5a/E-M cluster to a location between the polymerase and S genes (mutant MHV-EMSmN) resulted in a strong increase in the level of synthesis of the (now) largest sgRNA, by comparison with its wild-type counterpart. The opposite was observed after relocation of the M gene to a position immediately downstream of the S gene (mutant MHV-SMEN): The sgRNA specifying the M protein was hardly detectable. Obviously, much more systematic work will need to be done to provide clear insights into these complex issues. To explore other questions related to coronavirus RNA synthesis, targeted RNA recombination has also been employed for the insertion of a new transcription unit into the MHV genome (Hsue and Masters 1999), as well as for the embedding of a high-affinity binding site for a putative host transcriptional factor (Shen and Masters 2001).

4.3 Pathogenesis

The ability to study the effect of targeted mutations in the viral genome on the course of a natural infection represents an essential tool with which to rigorously address the interplay between host and virus. This is illustrated most impressively by a series of studies on the role of the MHV S protein in viral pathogenesis. Modifications ranging from single amino acid changes to complete spike replacements were applied. In the first category the simple substitution Q159L in the receptor binding domain of MHV-A59 S protein significantly reduced viral virulence; replication in the liver and, consequently, the extent of viral hepatitis were strongly decreased (Leparc-Goffart et al. 1998). Similarly, amino acid substitutions at the S1/S2 cleavage site indicated that efficient cleavage and cell-cell fusion are not necessary for virulence (Hingley et al. 2002). In the category of large-scale modifications, the replacement of the entire S gene of MHV-A59, a moderately neurovirulent virus, by that of MHV-4, which is highly neurovirulent, resulted in a chimeric virus with dramatically increased neurovirulence. Although replication in the brain was not elevated, viral antigen staining and inflammation in the central nervous system were increased (Phillips et al. 1999). The acquired spike apparently conveys to the chimeric virus most of the pathogenic properties of its cognate virus. This interpretation was confirmed in an analogous exchange involving the MHV-2 S gene. In this case, the non-demy-

elinating phenotype of the latter virus was passed on to MHV-A59, a demyelinating strain (Das Sarma et al. 2000). Still further support for the role of S as the primary determinant of pathogenicity was provided by an analysis of the chimeric viruses for their ability to induce hepatitis after intrahepatic inoculation (Navas et al. 2001). The level of replication in the liver and the extent of hepatocellular damage paralleled those of the virus from which the spike had been obtained, that is, MHV-A59 carrying an MHV-4, MHV-A59, or MHV-2 spike exhibited low, moderate, or high replication and pathology, respectively. Finally, a series of chimeric viruses containing intramolecularly recombined MHV-4/MHV-A59 S genes in the MHV-A59 background was tested to further explore the determinants of neurovirulence within the MHV-4 spike (Phillips et al. 2001, 2002). Reciprocal exchanges of the S1 and S2 subunits, and of parts of the hypervariable region of S1, yielded viruses that replicated well in vitro but were generally severely attenuated in mice. These results suggest that such modifications disturb interactions within the S protein that are important for efficient infection in the mouse brain.

A critical role of the S protein in pathogenesis was also demonstrated for TGEV. By replacement, through targeted recombination, of most of the S gene of a respiratory TGEV isolate by that of a virus with enteric tropism, recombinants were obtained that had acquired the latter property (Sánchez et al. 1999). These recombinant viruses thereby also gained the ability to replicate to high titers in the porcine enteric tract, as well as the marked virulence that is the distinguishing trait of the enteropathogenic parent virus.

In addition to the genes encoding the polymerase and canonical structural proteins coronaviruses have a number of other genes, forming characteristic sets in each coronavirus group, the functions of which are as yet unknown. None of these genes is essential for replication, as was demonstrated by targeted recombination for MHV (Fischer et al. 1997a; Ontiveros et al. 2001; de Haan et al. 2002a) and for FIPV (Haijema et al. 2003). Targeted inactivation of gene 4 in MHV-JHM did not affect the virulence of this virus, whether it was inoculated intracranially or intranasally, nor were the pathological effects in the central nervous system any different from those of the wild type (Ontiveros et al. 2001). More drastic genetic changes of group-specific genes in the MHV-A59 background, however, were clearly attenuating. In this situation, viruses were constructed deleting genes 4 and 5a, genes 2a and HE, or all four genes, the latter deletions creating a “minimal” coronavirus. Removal of genes 4 and 5a, but not that of genes 2a and HE, reduced viral growth in cell culture slightly yet significantly. In intracranially inoculated mice,

however, the virulence of all three deletion mutants was clearly reduced (de Haan et al. 2002a). For FIPV, the deletion of the group-specific genes 3a, 3b, and 3c or genes 7a and 7b did not substantially influence in vitro growth properties; in contrast, the "minimal" virus lacking all five of these genes was strongly impaired. The oronasal inoculation of cats with these deletion viruses, at a dose confirmed to be lethal for wild-type FIPV, remained without clinical consequences. That the animals had actually been infected was clear from their development of virus-neutralizing antibodies (Haijema et al. 2003). These deletion studies suggest that the nonessential genes encode functions important for host-virus interactions.

4.4 Coronavirus Vaccines and Vectors

The technology of targeted recombination has already displayed a number of features that will be essential for the development of coronaviruses as vectors for vaccination and therapy. One is the capability of rendering these viruses avirulent by the deletion of particular genes, as demonstrated for MHV and FIPV. In the latter case, viruses lacking either the 3abc or the 7ab gene cluster were indeed shown to serve as live-attenuated vaccine candidates, because cats infected with these mutants were protected against subsequent challenge with an otherwise lethal dose of virulent FIPV (Haijema et al. 2003). Second, the ability to genetically rearrange coronavirus genomes provides a critical safety asset, because it will allow the construction of vaccine or vector viruses that, because of judiciously modified gene orders, should have vanishingly small probabilities of generating viable progeny through recombination with coronaviruses in the wild. Third, the potential to retarget coronaviruses by modification of their S proteins, on which the current host range selection system for recombinant viruses is also based, constitutes another important feature that might be further developed to enable the directing of vectors to predefined cellular surface antigens.

Finally, for their use both as vectors and as carrier vaccines, the demonstrated ability of coronaviruses to incorporate and express foreign genes is obviously essential. Green fluorescent protein (GFP) was the first nonviral protein to be expressed by a coronavirus (Fischer et al. 1997b). The recombinant MHV containing the GFP gene inserted in place of gene 4 grew as well as the wild type did, but its level of GFP expression was poor. A slightly different construct containing the "enhanced" GFP gene, again replacing gene 4 but also in the context of the

upregulated TRS4 of pMH54, yielded a virus that replicated as well as wild-type virus both in vitro and in the mouse central nervous system (Das Sarma et al. 2002). This virus produced fluorescence during infection in vitro and in mouse brain, and GFP expression was stably maintained through at least six passages in tissue culture. In another study luciferase gene expression cassettes were inserted at various positions in the MHV genome. Whereas the *Renilla* luciferase gene remained stable over eight passages, irrespective of its location, the firefly luciferase gene was lost quite rapidly as a result of the acquisition of deletions. Luciferase expression levels appeared to increase when the gene was positioned closer to the 3′ end of the genome (de Haan et al. 2003). Moreover, the simultaneous synthesis of both luciferase activities from a single engineered virus demonstrated the potential for the use of coronaviruses as multivalent expression vectors.

5 Conclusions and Future Prospects

Within the span of nearly a decade, targeted recombination has established itself as a powerful and versatile technique for the reverse genetics of the 3′ third of the coronavirus genome, which encompasses the region encoding all of the structural genes. The past two years, however, have seen the opening of a new frontier in coronavirus reverse genetics, with reports of the assembly of infectious cDNAs for TGEV (Almazán et al. 2000; Yount et al. 2000), HCoV-229E (Thiel et al. 2001), IBV (Casais et al. 2001), MHV (Yount et al. 2002), and SARS-CoV (Yount et al. 2003). These recent developments raise the question of whether targeted RNA recombination will retain interest only as an historic relic. We think that this is unlikely to be the case. It is more probable that each reverse genetic system will have its own specific advantages under a particular set of experimental circumstances. At this moment, one can only tentatively comment on the relative strengths and limitations of targeted RNA recombination and infectious cDNAs for coronavirus reverse genetics. The targeted recombination system is at a fairly mature stage of development. By contrast, work with the infectious clone systems is sufficiently early in exploring their potentiality that it is not clear how hardy or manipulable these systems may become.

The capability of paramount value that is provided by the infectious clones, no matter what the burden in experimental labor, is access to gene 1. The capacity to site-specifically mutagenize the exceedingly large

viral RNA polymerase gene will undoubtedly play a major role in the acquisition of an understanding of the workings of this complex machinery. Except for its periphery, gene 1 is effectively out of the range of targeted RNA recombination, because the construction of donor RNA vectors entering this region is hindered by precisely the same technical problems that made the assembly of infectious cDNAs so formidable a task. A second unique characteristic of the infectious clones is their potential to provide the means by which the "passage zero" situation can be examined for intentionally lethal mutant constructs. This property has been elegantly and forcefully exploited with the infectious clone of equine arterivirus in the study of nidovirus RNA synthesis (van Dinten et al. 1997; Tijms et al. 2001; Pasternak et al. 2001). However, in order for similar studies to be executed with coronavirus infectious clones, platforms need to be devised that can produce ample amounts of viral genome (and its resulting gene 1 translation product) in the initial round of launch. This must be done without the generation of significant levels of other RNA species that have the propensity to confound analysis or interfere with RNA synthesis. As of this writing, the reported infectious clone systems are not yet sufficiently robust to enable these types of experiments.

For work involving coronavirus structural genes, targeted RNA recombination is likely to remain the method of choice for many studies. One reason for this is its relative ease of manipulation. The largest of the donor RNA vectors are still threefold smaller than an entire genome. Thus mutagenesis at the DNA level can generally be carried out without subcloning steps. A second strong asset of targeted recombination is that the host range-based selection system has demonstrated both its efficiency, in straightforward isolation of desired mutants, and its power, in recovery of extremely defective mutants such as the M protein truncation and the E gene deletion (Kuo and Masters 2002, 2003). Finally, targeted recombination lends itself well to studies involving domain exchange between different proteins (Peng et al. 1995b), the exchange of genomic elements (Hsue and Masters 1997), or the creation of mutants containing multiple mutations. In these cases the system, through its own selection of allowable crossover sites, can reveal which substitutions retain functionality and which are lethal. Related to this, the targeted recombination system establishes a stringent criterion for the lethality of a given mutation. If markers, silent or otherwise, upstream and downstream of the mutation in question can be transferred from a single donor RNA to progeny recombinants, while the mutation itself is excluded by multiple crossover events, then this argues strongly that the mutation

produces a lethal phenotype (de Haan et al. 1998; Hsue et al. 2000). In this situation the donor RNA provides its own internal control. As mentioned above, a similarly convincing standard of lethality for the infectious clones will require a more vigorous RNA production at passage zero. The sum of these considerations makes it likely that targeted recombination will serve a useful role in coronavirus genetics for some time to come.

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