

# Critical role of segment-specific packaging signals in genetic reassortment of influenza A viruses

Boris Essere<sup>a,1</sup>, Matthieu Yver<sup>a,1</sup>, Cyrille Gavazzi<sup>b</sup>, Olivier Terrier<sup>a</sup>, Catherine Isel<sup>b</sup>, Emilie Fournier<sup>b</sup>, Fabienne Giroux<sup>a</sup>, Julien Textoris<sup>c</sup>, Thomas Julien<sup>a,d</sup>, Cléo Socratous<sup>a</sup>, Manuel Rosa-Calatrava<sup>a,d</sup>, Bruno Lina<sup>a,e</sup>, Roland Marquet<sup>b,2</sup>, and Vincent Moules<sup>a,d,2</sup>

<sup>a</sup>Virologie et Pathologie Humaine and <sup>d</sup>VirNext, Faculté de Médecine RTH Laennec, Université Lyon 1, EA 4610, 69372 Lyon Cedex 08, France; <sup>b</sup>Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 67084 Strasbourg, France; <sup>c</sup>Unité de Recherche sur les Maladies Infectieuses et Emergentes, Centre National de la Recherche Scientifique Unité Mixte de Recherche 7278, Institut National de la Santé et de la Recherche Médicale U1095, Faculté de Médecine Timone, Université Aix-Marseille, 13385 Marseille Cedex 5, France; and <sup>e</sup>Laboratoire de Virologie Est, Centre de Biologie et de Pathologie Est, Hospices Civils de Lyon, 69677 Bron Cedex, France

Edited by Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN, and approved August 14, 2013 (received for review May 7, 2013)

**The fragmented nature of the influenza A genome allows the exchange of gene segments when two or more influenza viruses infect the same cell, but little is known about the rules underlying this process. Here, we studied genetic reassortment between the A/Moscow/10/99 (H3N2, MO) virus originally isolated from human and the avian A/Finch/England/2051/91 (H5N2, EN) virus and found that this process is strongly biased. Importantly, the avian HA segment never entered the MO genetic background alone but always was accompanied by the avian PA and M fragments. Introduction of the 5' and 3' packaging sequences of HA<sub>MO</sub> into an otherwise HA<sub>EN</sub> backbone allowed efficient incorporation of the chimerical viral RNA (vRNA) into the MO genetic background. Furthermore, forcing the incorporation of the avian M segment or introducing five silent mutations into the human M segment was sufficient to drive coinorporation of the avian HA segment into the MO genetic background. These silent mutations also strongly affected the genotype of reassortant viruses. Taken together, our results indicate that packaging signals are crucial for genetic reassortment and that suboptimal compatibility between the vRNA packaging signals, which are detected only when vRNAs compete for packaging, limit this process.**

hemagglutinin | RNA packaging

The mechanisms by which animal viruses are introduced into and are disseminated through the human population remain to be addressed. In particular, emerging pathogenic influenza viruses, such as the highly pathogenic avian H5N1 virus and the 2009 “swine” H1N1 virus (H1N1pdm2009), pose major public health and scientific challenges (1, 2). Even though the natural reservoirs of influenza A viruses are wild aquatic birds, influenza A viruses exhibit a broad host range and a wide antigenic diversity, represented by combinations of 17 hemagglutinin (HA) and nine neuraminidase (NA) subtypes (3). Two subtypes of influenza A viruses, H1N1 and H3N2, currently are circulating in the human population.

The genome of influenza A viruses is composed of eight single-stranded, negative-sense viral RNA (vRNA) segments. Each segment is associated with the heterotrimeric polymerase complex consisting of polymerase basic proteins 1 and 2 and polymeric acid (PB1/PB2/PA) and is covered by the viral nucleoprotein (NP) to form a viral ribonucleoparticle (vRNP). The fragmented nature of the genome allows the exchange of gene segments when two or more influenza viruses coinfect the same cell, in a process named “genetic reassortment” (4). Genetic reassortment is a major feature of influenza evolution and cross-species transmission and also is important for the generation of antigenically novel isolates by introducing novel HA segments in compatible genetic backgrounds (5–7). Future pandemic viruses most likely will carry different HA genes to which human populations are immunologically naive. The strains giving rise to the 1918 Spanish, 1957 Asian, and 1968 Hong Kong influenza pandemics all harbored

an HA segment derived from an avian virus. The avian viruses circulating in the waterfowl are the source of the HA genes most likely to be introduced into the human population (8). Phylogenetic, epidemic, epizootic, and virology studies suggest that swine serve as “mixing vessels” for the generation of human–avian–swine reassortant viruses.

When the reassortment process takes place between a human and an avian influenza virus, there are in theory 127 possible reassortant viruses harboring the avian HA segment. Two studies used forced reverse genetics (i.e., a minimal set of reverse genetic plasmids allowing no competition between segments) to generate all 127 reassortant viruses carrying the HA segment from an avian H5N1 virus in the genetic background of a human H3N2 or the HA segment from an avian H9N2 virus in the genetic background of the human 2009 pandemic H1N1 virus (9, 10). They showed that 49% (H5N1/H3N2) and 58% (H9N2/H1N1) of these reassortant viruses replicated efficiently in Madin–Darby canine kidney (MDCK) cells (9, 10). However, several reports indicated that the number of observed natural or experimental reassortant viruses is much smaller than 127, suggesting that reassortment is somehow restricted (4, 11, 12). When analyzing viruses from the nasal secretions of ferrets coinfecting with human H3N2 and avian H5N1 viruses, Jackson et al. (13) observed that only 3.1% were reassortant viruses

## Significance

**Genetic reassortment is one of the main mechanisms by which pandemic viruses emerge during influenza A coinfection, but little is known about the molecular mechanisms affecting this process. Here, we studied genetic reassortment between a human and an avian influenza A strain, focusing on the generation of reassortant viruses containing the avian HA gene, which have pandemic potential. We found that this genetic process was strongly biased, and we show that packaging signals are crucial for genetic reassortment and that suboptimal compatibility between the segment-specific packaging signals of the two parental viruses limits the emergence of reassortant viruses.**

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<sup>1</sup>B.E. and M.Y. contributed equally to this work.

<sup>2</sup>To whom correspondence may be addressed. E-mail: [r.marquet@ibmc-cnrs.unistra.fr](mailto:r.marquet@ibmc-cnrs.unistra.fr) or [vincent.moules@univ-lyon1.fr](mailto:vincent.moules@univ-lyon1.fr).

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possessing the HA H5 gene, and they corresponded to only five distinct genotypes. Genetic reassortment between human H3N2 and an equine H7N7 virus has been studied using cotransfection (4). Only 1.6% of purified viruses, corresponding to two genotypes, were reassortant viruses possessing the HA H7 gene (4). In contrast, a high frequency of genetic reassortment was observed recently between swine-origin H1N1 and avian H5N1 viruses: 64% of purified viruses, corresponding to 20 different genotypes, were reassortant viruses possessing the HA H5 gene (14). In this case, the high reassortment rate was attributed to the triple reassortant internal gene cassette, consisting of the avian PA and PB2 genes, the nonstructural (NS), NP, and matrix (M) swine genes, and the human PB1 gene (15).

The low number of reassortant genotypes usually generated from genetically diverse influenza viruses suggests incompatibilities at the protein and/or genomic level. Accumulating evidence indicates that protein incompatibility among the vRNP components is a limiting factor for reassortment between two viruses (4, 16–18), but little is known about genetic incompatibilities between the vRNA segments. Although incompatibility between proteins is expected to have similar effects in cotransfection or coinfection experiments and in forced reverse genetic experiments, genomic incompatibilities may have several possible effects, especially at the level of the vRNA-packaging signals. Some incompatibilities between packaging signals might reduce viral replication in the absence of competition (absolute incompatibility), whereas more subtle ones might be revealed only when vRNA segments from the two parental viruses compete for packaging (suboptimal compatibility). Reverse genetics-derived reassortant viruses (RGd-RV) that possess the H5N1 (H5) HA in an otherwise H3N2 genetic background show high replicative capacities in MDCK cells (10). Similarly, RGd-RV with the HA gene from H5N1 virus in the H1N1pdm2009 genetic background replicated efficiently in primary human respiratory epithelial cells and caused 100% mortality in mice (19). However, phylogenetic analyses of natural or experimental reassortant viruses have shown that the HA segment from avian, swine, or equine viruses was never incorporated alone in the genetic background of a human virus (13, 14, 20): The HA segment is packaged with additional groups of gene segments depending on the viral subtypes involved in the coinfection process (13, 14). The inability to obtain a virus containing a nonhuman HA gene in an otherwise human genetic background, in contrast with the ability to produce “7+1” RGd-RV with a high yield of replication, suggests that the reassortment process might be restricted by suboptimal compatibility between the vRNA-packaging signals (10).

To predict how pandemic influenza viruses can emerge, the complex molecular mechanisms limiting or facilitating genetic reassortment must be deciphered. Using reverse genetics, *cis*-packaging signals of the human H1N1 WSN and PR8 strains were found to reside at both ends of each vRNA, including the UTRs, along with up to 80 bases of adjacent coding sequences (21–28). In this study, we generated reassortant viruses in vitro from avian H5N2 and human H3N2 viruses to identify incompatibilities between the two parental viruses arising at the vRNA level. Our experiments focusing on the generation of reassortant viruses containing the HA H5 gene segment in an H3N2 genetic background indicate that genomic suboptimal compatibility driven by the selective packaging mechanism limits the generation of HA H5 reassortant viruses in vitro.

## Results

### Genetic Reassortment Between Avian H5N2 and Human H3N2 Viruses.

To explore the molecular basis of the restriction of genetic reassortment among influenza A viruses, we studied genetic reassortment between two genetically divergent influenza A viruses: A/Moscow/10/99 (MO, H3N2) originally isolated from human and the avian virus A/Finch/England/2051/91 (EN, H5N2).

The MO and EN viruses are genetically different in each segment (with 73.8–89.0% identity between the internal gene segments at the nucleotide level).

We first generated reassortant viruses by cotransfecting 293T cells with two complete sets of reverse genetics plasmids encoding the viral RNAs of MO and EN viruses as previously described (4, 29). The supernatants of plasmid-transfected cells then were used for plaque assays on MDCK cells. A total of 100 plaque-purified viruses from two independent experiments were genotyped by using sets of virus-specific primers. Because the reassortant viruses incorporating the H3 HA<sub>MO</sub> segment lack pandemic potential because of widespread immunity in the human population, viruses containing the HA<sub>MO</sub> segment were not analyzed further. We isolated five viruses corresponding to the WT EN virus and 21 reassortant viruses containing the H5 HA<sub>EN</sub> segment and at least one MO segment that corresponded to six different genotypes designated R1 to R6 (Table 1). Within these reassortant viruses, 62.5% of the gene segments analyzed by RT-PCR were of EN origin (Table 1). Remarkably, all reassortant viruses contained the PA<sub>EN</sub> and M<sub>EN</sub> segments, and two genotypes, R1 and R2, were present at high frequencies.

In a complementary approach, we generated reassortant viruses by coinfecting MDCK cells with recombinant MO and EN viruses produced by reverse genetics as previously described by Octaviani et al. (14) (Table 2). Because these two viruses replicated at similar levels up to 48 h postinfection (h.p.i.) (Fig. S14), MDCK cells were coinfecting for 10 h with the MO and EN viruses at the same multiplicity of infection (m.o.i.), 2.5. A total of 100 plaques were isolated from two independent coinfection experiments and were amplified in MDCK cells. Of the 100 plaque-purified viruses, we isolated 15 viruses corresponding to WT EN virus and 23 HA<sub>EN</sub>-containing reassortant viruses corresponding to seven different genotypes, designated X1 to X7 (Table 2). Within these reassortant viruses, 58% (107/184) of all gene segments analyzed by RT-PCR were of EN origin (Table 2). As observed in the transfection experiments, all reassortant viruses incorporated the PA<sub>EN</sub> and M<sub>EN</sub> genes, in addition to HA<sub>EN</sub> (Table 2). One genotype, X1, was present at high frequency.

Unbiased genetic reassortment should produce 99.2% reassortant viruses with (at least) the HA<sub>EN</sub> gene segment (127/128). Here, our cotransfection and coinfection experiments yielded only 21% and 23% reassortant viruses containing the HA<sub>EN</sub> gene, respectively. The reassortant virus containing only the HA<sub>EN</sub> gene segment in an otherwise MO genetic background (MO-HA<sub>EN</sub>) was never detected: the M<sub>EN</sub> and PA<sub>EN</sub> gene segments were consistently associated with the HA<sub>EN</sub> gene segment in all reassortant viruses analyzed. The probability that in cotransfection and coinfection experiments all reassortant viruses contain the PA<sub>EN</sub> and M<sub>EN</sub> segments purely by chance is  $2.2 \times 10^{-13}$  and  $1.4 \times 10^{-14}$ , respectively. Taken together, our data indicate that genetic reassortment between the MO and EN viruses is strongly biased.

### Incorporation of the HA<sub>EN</sub> Segment into the MO Genetic Background.

The fact that we did not recover the MO-HA<sub>EN</sub> reassortant virus in the previous experiments could indicate that this virus is nonviable or noncompetitive. However, the number of reassortant viruses analyzed was not sufficient to recover it with a high probability. To distinguish between these explanations, we first attempted to produce this virus by cotransfecting 293T cells with the eight corresponding reverse genetics plasmids and found that this virus was efficiently rescued and generated infectious particles that replicated similarly to the MO virus in MDCK cells (Fig. S14 and Table 3).

Next, to assess whether the HA<sub>EN</sub> segment could be incorporated alone into the MO genetic background when it competes with its HA<sub>MO</sub> counterpart, we analyzed the rescue of reassortant viruses using nine plasmids: the eight required to produce

**Table 1. Genotypes of reassortant viruses obtained by cotransfection of EN (H5N2) and MO (H3N2) viruses**

Genotype	Gene origin								Frequency (no. viruses)
	PB1	PB2	PA	HA	NP	NA	M	NS	
R1	MO	EN	EN	EN	EN	MO	EN	MO	8
R2	MO	MO	EN	EN	MO	MO	EN	MO	5
R3	EN	EN	EN	EN	EN	MO	EN	MO	3
R4	EN	EN	EN	EN	EN	MO	EN	EN	3
R5	EN	MO	EN	EN	EN	EN	EN	MO	1
R6	MO	MO	EN	EN	MO	EN	EN	EN	1
No of reassortant viruses possessing the respective EN segment/total no. of HA <sub>EN</sub> viruses	7/21 (33%)	14/21 (67%)	21/21 (100%)	21/21 (100%)	15/21 (71%)	2/21 (9%)	21/21 (100%)	4/21 (19%)	

293T cells were transfected with 16 reverse genetics plasmids corresponding to the eight gene segments of the MO and EN viruses. The cotransfection supernatants were plaque purified on MDCK cells. The genotypes of the plaque-purified viruses were determined by strain-specific PCR.

the MO virus, plus the plasmid corresponding to HA<sub>EN</sub> (Fig. S2). The cotransfection of HA<sub>MO</sub> and HA<sub>EN</sub> plasmids causes competition between the vRNAs they express for incorporation into virions (30). 293T cells were transfected with equal amounts (1 µg) of the nine plasmids. To define the rate of incorporation of the HA<sub>EN</sub> vRNP into infectious viral particles, plaque purification was carried out from the supernatant of the transfected cells. We analyzed a total of 96 plaque-purified viruses from two independent experiments using HA-specific RT-PCR (C1; Table 4). Interestingly, all 96 plaque-purified viruses contained the HA<sub>MO</sub> gene segment, confirming the preferential incorporation of the HA<sub>MO</sub> gene segment into the MO genetic background (C1; Table 4). As a control, we performed the same gene-competition experiment with a twofold lower amount of HA<sub>MO</sub> plasmid; all 48 of the plaque-purified viruses we analyzed also contained the HA<sub>MO</sub> gene segment.

Similarly, we performed the rescue of reassortant viruses using the eight plasmids corresponding to the MO virus, plus the plasmid corresponding to NA<sub>EN</sub> (C2; Table 4). The introduction of segment 6 of the two genes causes competition between the corresponding vRNAs for incorporation into the MO genetic background (30). After 293T cells were cotransfected with the nine plasmids, viruses were plaque purified and analyzed as above. We also checked that incorporation of the NA<sub>EN</sub> gene segment into the MO genetic background enabled production of infectious particles (virus MO-NA<sub>EN</sub>; Fig. S1B and Table 3). We analyzed a total of 96 plaque-purified viruses from two

independent experiments using NA-specific RT-PCR; 20.8% of plaque-purified viruses contained the NA<sub>EN</sub> gene (C2; Table 4).

Thus, although the MO-HA<sub>EN</sub> reassortant virus produced by forced reverse genetics has high replicative capacities, there is a preferential incorporation of the HA<sub>MO</sub> gene rather than the HA<sub>EN</sub> gene in the MO genetic background. Interestingly, NA<sub>MO</sub> and NA<sub>EN</sub> genes exhibit 82.6% of identity at the nucleotide level, whereas HA<sub>MO</sub> and HA<sub>EN</sub> genes show only 49.18% identity.

#### Packaging Regions Are Involved in the Generation of Reassortant Viruses.

The incorporation of the avian NA<sub>EN</sub> gene into the MO genetic background suggests a link between the nucleotide identity of the segments involved in the competition and their capacity to be incorporated into infectious particles. It was shown previously that foreign genes can be packaged into influenza particles by adding flanking segment-specific packaging sequences (24, 27, 28, 31–33). In the case of the human H1N1 WSN and PR8 viruses, the ends of the coding regions are important for the incorporation of each vRNA in virions, although the relative contributions of the 3' and 5' ends differ among the vRNA segments (28). The packaging signals of the HA vRNA from the WSN H1N1 strain of influenza A virus include both ends of the coding sequence (in antisense orientation), namely nine nucleotides at the 3' end and 80 nucleotides at the 5' end of the HA vRNA (24, 27).

Comparison of the nucleotide sequence of the HA packaging regions of the MO and EN viruses revealed 55.4% and 30.0%

**Table 2. Genotypes of reassortant viruses obtained by coinfection of MDCK cells with the MO and EN viruses**

Genotype	Gene origin								Frequency (no. viruses)
	PB1	PB2	PA	HA	NP	NA	M	NS	
X1 (= R2)	MO	MO	EN	EN	MO	MO	EN	MO	8
X2	EN	EN	EN	EN	MO	EN	EN	MO	4
X3	EN	EN	EN	EN	EN	MO	EN	EN	3
X4	MO	MO	EN	EN	EN	EN	EN	MO	3
X5	EN	MO	EN	EN	MO	MO	EN	MO	2
X6	EN	MO	EN	EN	MO	EN	EN	MO	2
X7	EN	MO	EN	EN	EN	MO	EN	MO	1
No of reassortant viruses possessing the respective EN segment/total no. of HA <sub>EN</sub> viruses	12/23 (52%)	7/23 (30%)	23/23 (100%)	23/23 (100%)	7/23 (30%)	9/23 (39%)	23/23 (100%)	3/23 (13%)	

MDCK cells were coinfecting with EN and MO viruses at an m.o.i. of 2.5 each for 10 h. The supernatants were plaque purified on MDCK cells, and the genotypes of the plaque-purified viruses were determined by strain-specific PCR.

**Table 3. Genomic composition and viral titer of recombinant viruses**

Virus	Gene origin								Viral titer log <sub>10</sub> TCID <sub>50</sub> /50 μL
	PB2	PB1	PA	HA	NP	NA	M	NS	
MO	MO	MO	MO	MO	MO	MO	MO	MO	7.0
EN	EN	EN	EN	EN	EN	EN	EN	EN	7.3
MO-HA <sub>EN</sub>	MO	MO	MO	EN	MO	MO	MO	MO	6.8
MO-NA <sub>EN</sub>	MO	MO	MO	MO	MO	EN	MO	MO	6.2
MO-HA <sub>CEN</sub>	MO	MO	MO	HA <sub>CEN</sub>	MO	MO	MO	MO	7.0
MO-PA <sub>EN</sub>	MO	MO	EN	MO	MO	MO	MO	MO	0
MO-HA <sub>EN</sub> M <sub>EN</sub>	MO	MO	MO	EN	MO	MO	EN	MO	6.3
MO-HA <sub>EN</sub> PA <sub>EN</sub>	MO	MO	EN	EN	MO	MO	MO	MO	6.5
MO-M <sub>EN</sub>	MO	MO	MO	MO	MO	MO	EN	MO	7.0
MO-M <sub>C5MO</sub>	MO	MO	MO	MO	MO	MO	M <sub>C5MO</sub>	MO	6.3
MO-HA <sub>EN</sub> M <sub>C5MO</sub>	MO	MO	MO	EN	MO	MO	M <sub>C5MO</sub>	MO	6.8
MO-M <sub>C3MO</sub>	MO	MO	MO	MO	MO	MO	M <sub>C3MO</sub>	MO	6.3
MO-HA <sub>EN</sub> M <sub>C3MO</sub>	MO	MO	MO	EN	MO	MO	M <sub>C3MO</sub>	MO	6.3

Viral titers (log<sub>10</sub> TCID<sub>50</sub>/50 μL) were determined on MDCK cells at 48 h.p.i. using the Reed and Muench statistical method.

identity in the 3' (9 nt) and 5' (80 nt) regions, respectively. We hypothesized that the low level of identity between the HA<sub>MO</sub> and HA<sub>EN</sub> packaging regions may be responsible for the absence of incorporation of the HA<sub>EN</sub> gene into the MO genetic background. To test this hypothesis, we produced a chimerical HA segment (HA<sub>CEN</sub>) containing the HA packaging sequences of the HA<sub>MO</sub> segment by modifying the 3' and 5' extremities of HA<sub>EN</sub> (Fig. 1A). This chimerical HA segment codes for a protein that differs from the WT HA<sub>EN</sub> protein by 23 amino acids. Therefore, we checked that incorporation of the chimerical HA<sub>CEN</sub> segment into the MO genetic background (virus MO-HA<sub>CEN</sub>; Table 3) allowed the production of infectious particles in the same manner as MO and MO-HA<sub>EN</sub> viruses (virus MO-HA<sub>CEN</sub>; Fig. S1C). To determine the rate of incorporation of the chimerical HA<sub>CEN</sub> vRNA segment into infectious viral particles, we studied the rescue of reassortant viruses using the eight plasmids corresponding to the MO virus plus the plasmid corresponding to chimerical HA<sub>CEN</sub> segment (C3; Table 4). The analysis of 96 plaque-purified viruses from two independent experiments showed that the chimerical HA<sub>CEN</sub> vRNA was incorporated into 44% of the virions (C3; Table 4).

Thus, introduction of the MO HA packaging signals in an otherwise EN HA segment increased its capacity to be incorporated into the MO genetic background. Our data suggest that the weak homology between the HA packaging regions of the HA<sub>EN</sub> and HA<sub>MO</sub> segments might be responsible for the preferential incorporation of the HA<sub>MO</sub> gene into the MO genetic background.

**The 3' End of the M<sub>EN</sub> Coding Region Is Involved in the Incorporation of the HA<sub>EN</sub> Segment into the MO Genetic Background.** We observed that the M<sub>EN</sub> and PA<sub>EN</sub> gene segments segregated systematically with the HA<sub>EN</sub> gene segment in all the reassortant viruses we analyzed. We thus planned to estimate the contribution of the PA<sub>EN</sub> and/or M<sub>EN</sub> segments to the preferential incorporation of the HA<sub>EN</sub> segment into the MO genetic background.

To that aim, we first checked the viability of MO-PA<sub>EN</sub>, MO-M<sub>EN</sub>, MO-HA<sub>EN</sub>PA<sub>EN</sub>, and MO-HA<sub>EN</sub>M<sub>EN</sub> viruses (Table 3) produced by reverse genetics. Viral titers were determined in MDCK cells 48 h.p.i. Surprisingly, we failed to rescue the MO-PA<sub>EN</sub> virus in three individual experiments (Table 3), whereas the viral titers for MO-M<sub>EN</sub>, MO-HA<sub>EN</sub>M<sub>EN</sub>, and MO-HA<sub>EN</sub>PA<sub>EN</sub> viruses at 48 h.p.i. were 7.0, 6.3, and 6.5 log 50% tissue infective culture dose (TCID<sub>50</sub>)/50 μL, respectively (Table 3 and Fig. S1D). Because the MO-HA<sub>EN</sub>, MO-M<sub>EN</sub>, and MO-HA<sub>EN</sub>M<sub>EN</sub> viruses could all be rescued, we focused on the M<sub>EN</sub> segment, and we hypothesized that substituting the M<sub>EN</sub> segment for the M<sub>MO</sub> segment could increase the probability of incorporating the HA<sub>EN</sub> segment into the MO background. We rescued reassortant viruses using the plasmids corresponding to the MO virus minus the M<sub>MO</sub> plasmid, plus the M<sub>EN</sub> and HA<sub>EN</sub> plasmids (C4; Table 4). RT-PCR analyses of a total of 96 plaques from two independent experiments showed that 60% of the viruses contained the avian HA<sub>EN</sub> gene (C4; Table 4). Notably, recombinant MO-M<sub>EN</sub> and MO-HA<sub>EN</sub>M<sub>EN</sub> viruses exhibited replication capacities similar to those of MO and MO-HA<sub>EN</sub> viruses, respectively (viruses MO-M<sub>EN</sub> and MO-HA<sub>EN</sub>M<sub>EN</sub>; Fig. S1D).

**Table 4. Competitive reverse genetics experiments**

Competitive reverse genetic experiments	Origin of reverse genetic plasmid								No. of purified viruses analyzed	Incorporation rate of EN gene (%)
	PB1	PB2	PA	HA	NP	NA	M	NS		
C1	MO	MO	MO	MO/EN	MO	MO	MO	MO	96	0
C2	MO	MO	MO	MO	MO	MO/EN	MO	MO	96	20.8
C3	MO	MO	MO	MO/HA <sub>CEN</sub>	MO	MO	MO	MO	96	44.0
C4	MO	MO	MO	MO/EN	MO	MO	EN	MO	96	60.0
C5	MO	MO	MO	MO/EN	MO	MO	M <sub>C5MO</sub>	MO	96	0
C6	MO	MO	MO	MO/EN	MO	MO	M <sub>C3MO</sub>	MO	96	56.0

293T cells were transfected with plasmids for the generation of vRNAs and viral proteins of the gene segments indicated in the table. These plasmid combinations allowed competition between the HA<sub>MO</sub> and HA<sub>EN</sub> vRNAs in the presence of the M<sub>MO</sub>, M<sub>EN</sub>, M<sub>C5MO</sub>, or M<sub>C3MO</sub> vRNAs (rows C1, C4, C5, and C6, respectively), competition between the NA<sub>MO</sub> and NA<sub>EN</sub> vRNAs in the presence of the HA<sub>MO</sub> segment (row C2), or competition between the HA<sub>MO</sub> and HA<sub>CEN</sub> vRNAs in the presence of the M<sub>MO</sub> vRNA (row C3). Genotypes of the plaque-purified viruses were determined by strain-specific PCR.



signals located at both ends of the coding region of the  $M_{EN}$  segment could be responsible for the incorporation of the  $HA_{EN}$  segment into the MO genetic background by constructing chimerical M segments. The chimerical  $M_{C5MO}$  contains the nine point substitutions corresponding to the 5' region of  $M_{EN}$  in an otherwise  $M_{MO}$  segment (Fig. 1B). This 9-nt substitution translates into five amino acid changes in the M2 protein (Fig. 1B). We next determined that these substitutions in the M2 protein did not affect replication of infectious particles containing the  $HA_{MO}$  or the  $HA_{EN}$  segments (viruses MO- $M_{C5MO}$  and MO- $HA_{EN}M_{C5MO}$ ; Fig. S1E and Table 3). Similarly, we produced a chimerical  $M_{C3MO}$  vRNA containing the five substitutions specific to the 3' end of the  $M_{EN}$  vRNA in an otherwise  $M_{MO}$  segment (Fig. 1B). These five substitutions did not change the amino acid sequence of the MO M1 protein. However, one of the substitutions (at position 55) might affect M mRNA splicing and thus expression of the M2 protein (36). To verify this point, we compared the M2 protein levels in MDCK cells infected with MO, MO- $M_{C3MO}$ , or MO- $HA_{EN}M_{C3MO}$  viruses by Western blot (Fig. S3). Our data indicate that the M2 level was not affected significantly in the MO- $M_{C3MO}$  virus as compared with the MO and MO- $HA_{EN}M_{C3MO}$  viruses, suggesting that the point substitution at position 55 does not affect splicing. We next determined that incorporation of the chimerical  $M_{C3MO}$  vRNA into the MO and MO- $HA_{EN}$  genetic backgrounds did not affect production of infectious particles (viruses MO- $M_{C3MO}$  and MO- $HA_{EN}M_{C3MO}$ ; Fig. S1F and Table 3).

Then we analyzed reassortant viruses rescued using the plasmids corresponding to the MO virus minus the  $M_{MO}$  segment, plus the chimerical  $M_{C5MO}$  and the  $HA_{EN}$  plasmids (C5; Table 4). None of the 96 purified viruses possessed the  $HA_{EN}$  segment, indicating that the nine point substitutions in the 5' region of the chimerical  $M_{C5MO}$  segment were unable to drive the incorporation of the  $HA_{EN}$  segment into the MO genetic background (compare rows C1, C4, and C5 in Table 4). Finally, we analyzed reassortant viruses rescued using plasmids corresponding to the MO virus, minus the  $M_{MO}$  segment, plus the chimerical  $M_{C3MO}$  and  $HA_{EN}$  reverse genetics plasmids (C6; Table 4). Importantly, 56% of the 96 plaque-purified viruses that we analyzed incorporated the  $HA_{EN}$  vRNA.

Thus, the five point substitutions in the 3' region of the coding region of the chimerical  $M_{C3MO}$  segment play a crucial role in the incorporation of the  $HA_{EN}$  segment in the MO genetic background. Because they do not affect the sequence of the M1 protein, these substitutions must exert their effect at the RNA level.

**Genetic Reassortment Between EN and Recombinant MO- $M_{C3MO}$  Viruses.** To confirm, in a viral context, that the 3' terminal region of the M coding sequence is involved in the incorporation of the  $HA_{EN}$  segment into the MO genetic background, we analyzed  $HA_{EN}$ -containing reassortant viruses produced by coinfecting MDCK cells with recombinant MO- $M_{C3MO}$  and EN viruses, each at a 2.5 m.o.i. Eighty-five single plaques were isolated from two independent coinfection experiments and subsequently were amplified in MDCK cells. Among these, 27 (32%) reassortant viruses that incorporated the  $HA_{EN}$  segment could be classified into six different genotypes designated V1 to V6 (Table 5). Interestingly, the fraction of plaque-purified reassortant viruses obtained by coinfecting the EN virus with the MO- $M_{C3MO}$  virus was 9% higher than when coinfecting EN and MO viruses (32% versus 23%). The chimerical  $M_{C3MO}$  vRNA was present in 82% (22/27) of the reassortant viruses (Table 5), whereas the  $M_{MO}$  vRNA was never recovered in reassortant viruses generated by coinfecting EN and MO viruses (Table 2). Comparison of the V1–V6 and X1–X7 genotypes (Tables 2 and 5) showed that reassortant viruses obtained by coinfecting EN and MO- $M_{C3MO}$  viruses contained fewer EN segments than those obtained by coinfection of EN and MO viruses [58/216 (27%) versus 107/184 (58%),  $P = 2.2 \times 10^{-10}$ ]. Significantly, 75, 4, and 0% of the reassortant viruses possessed the  $PA_{EN}$ ,  $PB1_{EN}$ , and  $PB2_{EN}$  gene segments, respectively (Table 5), whereas 100, 52, and 30% of reassortant viruses from the EN/MO coinfection possessed these EN genes (Table 2). Similarly, for other internal gene segments, only 4, 4, and 11% of reassortant viruses obtained by coinfecting EN and MO- $M_{C3MO}$  viruses possessed the  $NP_{EN}$ ,  $NS_{EN}$ , and  $NA_{EN}$  gene segments, respectively.

In conclusion, we have demonstrated that five point substitutions at the 3' end of the  $M_{MO}$  coding region that do not affect the sequence of the M1 protein are sufficient to modify completely the genetic composition of reassortant viruses obtained from a EN/MO- $M_{C3MO}$  coinfection. The percentage of EN genes contained in reassortant viruses was smaller than those obtained from the EN/MO coinfection, whereas the fraction of reassortant viruses was 39% higher. Importantly, 25% of the reassortant viruses contained only the  $HA_{EN}$  segment (and the  $M_{C3MO}$  segment) in an otherwise MO genetic background (Table 5), but in the absence of these five mutations the  $HA_{EN}$  segment never was incorporated alone in the MO genetic background.

## Discussion

The segmented nature of the genome of influenza A viruses allows reassortment of gene segments between viruses coinfecting a single host cell. Reassortant viruses carrying an HA gene differing from the H1 and H3 lineages currently circulating in the

**Table 5. Genotypes of reassortant viruses obtained by coinfection of MDCK cells with the EN and MO- $M_{C3MO}$  viruses**

Genotype	Gene origin								Frequency (no. viruses)
	PB1	PB2	PA	HA	NP	NA	M	NS	
V1	MO	MO	EN	EN	MO	MO	$M_{C3MO}$	MO	11
V2	MO	MO	MO	EN	MO	MO	$M_{C3MO}$	MO	7
V3	MO	MO	EN	EN	MO	MO	EN	MO	5
V4	MO	MO	EN	EN	MO	EN	$M_{C3MO}$	MO	2
V5	EN	MO	EN	EN	MO	EN	$M_{C3MO}$	MO	1
V6	MO	MO	EN	EN	EN	MO	$M_{C3MO}$	EN	1
No. Of $HA_{EN}$ viruses possessing the respective EN segment/total no. of $HA_{EN}$ viruses	1/27 (4%)	0/27 (0%)	20/27 (74%)	27/27 (100%)	1/27 (4%)	3/27 (11%)	5/27 (18%)	1/27 (4%)	

MDCK cells were coinfecting with EN and MO- $M_{C3MO}$  viruses at an m.o.i. of 2.5 each for 10 h. The coinfection supernatants were plaque-purified on MDCK cells. The genotypes of the plaque-purified viruses were determined by strain-specific PCR.

human population have a high pandemic potential and therefore are of special interest.

Previous *in vivo* and *in vitro* coinfection or cotransfection experiments showed that genetic reassortment between distantly related influenza viruses is strongly biased and generated limited number of reassortant genotypes (4, 11–13, 17). In line with these results, we observed genetic reassortment rates of 21 and 23% between the EN and MO viruses in cotransfection and coinfection experiments, respectively; these rates are much lower than the 99.2% rate expected for unbiased genetic reassortment (Tables 1 and 2). In addition, the R1 and X1 genotypes were recovered in 8 of 21 and 8 of 23 reassortant viruses, respectively (Tables 1 and 2). The probabilities of recovering any particular genotypes eight times within 21 or 23 reassortant viruses purely by chance are  $2.7 \times 10^{-12}$  and  $6.4 \times 10^{-12}$ , respectively, indicating that genetic reassortment between the EN and MO viruses is strongly biased. In comparison, a recent study of the genetic reassortment of influenza viruses differing only by two or three silent mutations in the middle of the gene segments demonstrated a high reassortment rate (88.4%), and the 123 different reassortant genotypes obtained from 241 purified viruses revealed no particular association between gene segments (37).

Even though cotransfections and coinfections generated similar reassortant genotypes, only one genotype was common to the two experiments (R1 = X2) (Tables 1 and 2). Although surprising, this result was not completely unexpected. Indeed, Kawaoka and coworkers who used the two experimental approaches to study genetic reassortment between seasonal and pandemic H1N1 viruses also obtained significantly different results (4, 14, 17). As discussed by these authors, the ratios of the vRNAs competing for packaging are not the same in the two experimental set-ups because of the differing replicative abilities of the two test viruses. In addition, these two types of experiments were performed using different cell types, and the possibility that cell factors affect genetic reassortment cannot be excluded.

In the following part of the study, we focused on the systematic segregation of the PA<sub>EN</sub> and M<sub>EN</sub> segments with HA<sub>EN</sub> (Tables 1 and 2). This observation was made both in cotransfection and coinfection experiments and thus is independent of the experimental approach. During coinfection *in vivo*, the avian H5N1 HA, PB2, and PA segments were found to cosegregate within a human H3N2 genetic background (13), whereas *in vitro* the NP, PB2, and HA H5N1 segments cosegregated in the human H1N1 genetic background in most reassortant viruses (14). In another study, the HA and M H7N7 segments cosegregated in the H3N2 genetic background (4). Thus, the identity of the segments that cosegregate with HA segment depends on the viral subtypes that are involved in the reassortment process.

In line with previous studies on genetic reassortment between human and avian, swine, or equine viruses (4, 9, 10, 13) and our cotransfection and coinfection studies (Tables 1 and 2), the HA<sub>EN</sub> segment never was incorporated into the MO genetic background in competition experiments using nine reverse genetics plasmids (Table 4), even though the MO-HA<sub>EN</sub> virus produced by forced reverse genetics replicated efficiently in MDCK cells (Table 3 and Fig. S1). Incompatibilities between proteins of the parental viruses should affect the replication of the viruses rescued using eight plasmids, because incompatibilities between the polymerase subunits do affect replication (4, 14, 16–18, 38, 39). We thus suspected that suboptimal compatibility between the packaging signals of the vRNAs restricted incorporation of the HA<sub>EN</sub> vRNA in the MO genetic background in competition experiments. Previous work on the H1N1 WSN and PR8 strains localized the packaging signals of the HA segment at both ends of the coding region and in the flanking UTRs (24, 26–28). This localization allowed us to construct a chimerical segment in which the central part of the HA<sub>EN</sub> segment was flanked by the packaging signals of the HA<sub>MO</sub> segment (Fig. 1). This

construct revealed that the packaging regions of the HA vRNA are important for its incorporation into the MO genetic background (Table 4).

To understand the origin of the cosegregation of the HA<sub>EN</sub>, PA<sub>EN</sub>, and M<sub>EN</sub> segments into the MO background during genetic reassortment, we planned several competition experiments using nine reverse genetics plasmids and virus rescue using eight plasmids. Surprisingly, we were unable to rescue the MO-PA<sub>EN</sub> virus, whereas the MO-PA<sub>EN</sub>HA<sub>EN</sub> virus was rescued efficiently (Table 3). Because HA<sub>EN</sub> proteins have not been shown to interact functionally with PA<sub>EN</sub> proteins, we suggest that absolute incompatibility between the packaging signals of the heterologous HA and PA vRNAs prevents rescue of the MO-PA<sub>EN</sub> virus. In contrast, the MO-M<sub>EN</sub> and MO-HA<sub>EN</sub>M<sub>EN</sub> viruses could be rescued and replicated efficiently. Competition experiments then showed that the M<sub>EN</sub> segment plays a crucial role in the preferential incorporation of the HA<sub>EN</sub> segment into the MO genetic background (Table 4). This specific role of the M segment in the incorporation of the HA segment in a heterogeneous genetic context is consistent with its central role in vRNA packaging in the homogenous PR8 genetic context (40). It also fits with the observation that the M segment segregates with the HA H7N7 segment in reassortment experiments with a H3N2 virus (4). Maines et al. (41) suggested that the infectivity of reassortant viruses bearing the HA and NA genes of the H5N1 virus may be enhanced by the presence of the H5N1 M gene, in line with the observation that the replication of reassortant viruses bearing an HA segment from an avian virus and an M segment from a human virus is compromised substantially (42). Octaviani et al. (17) showed that viruses containing a combination of seasonal H1N1 HA and swine-origin H1N1 NA and M genes have enhanced growth properties. They suggested that the predominance of quasi-spherical virus particles associated with this genotype increased viral production. However, in our study, the MO-HA<sub>EN</sub>M<sub>EN</sub> and MO-HA<sub>EN</sub> viruses exhibited replication capacities similar to those of MO and EN viruses, suggesting that packaging-signal contributions outweigh the benefits of a possible spherical budding phenotype. In addition, electron tomography did not reveal any significant morphological difference between the parental MO and EN viruses (43, 44).

It has been reported that both ends of the coding regions of segment M from the H1N1 WSN virus are required for efficient packaging of M vRNA into virions (26, 28). However, we found that only the 3' packaging region of the M vRNA is able to drive the incorporation of the HA<sub>EN</sub> segment into the MO genetic background (Table 4). Significantly, the five silent point substitutions we introduced in the M<sub>C3MO</sub> segment also had a general effect on genetic reassortment between MO and EN viruses (Table 5). Of note, the MO-M<sub>C3MO</sub> virus replicated efficiently (Table 3 and Fig. S1F), indicating that the five substitutions present in this virus do not have a deleterious effect on the MO-M<sub>C3MO</sub> vRNA structure. Our study directly demonstrates that heterosubtypic genetic reassortment is limited not only by incompatibilities between proteins but also at the level of the vRNAs themselves. This restriction is mediated by suboptimal compatibility between divergent packaging signals and is a direct consequence of the segment-selective packaging mechanism of influenza A viruses.

We and others hypothesized that selective packaging of the eight vRNAs into an influenza viral particle might involve direct base-pairing interactions between the packaging signals (22, 28, 43, 44). Recently, we showed that, *in vitro*, all MO genomic vRNA segments are involved in a single network of intermolecular interactions involving regions located at both ends of the vRNAs, suggesting that selective packaging of vRNPs is mediated by intermolecular RNA–RNA interactions (43). The EN vRNAs also form a single network of interactions *in vitro*, but, surprisingly, the networks formed by EN and MO vRNAs are not the same,

and most vRNA–vRNA interactions are not conserved between these viruses (43, 44). Thus, if the same interactions take place in vivo, the lack of conservation of the vRNA–vRNA interactions might contribute to limit genetic reassortment between divergent viruses. Importantly, we showed that the 3' region of the M<sub>MO</sub> vRNA interacts with the HA<sub>MO</sub> vRNA (43). Combined with our finding that five silent mutations in the 3' region of the M<sub>MO</sub> segments modulate the incorporation of the HA<sub>EN</sub> segment into the MO genetic background, these data support the idea that vRNA–vRNA interactions are involved in the genetic reassortment process. Because multiple reassortment events involving several viral subtypes usually are observed in the generation of reassortant viruses in vivo, the role of interactions between heterosubtypic vRNAs for the production of infectious reassortant viruses deserves further investigation.

## Materials and Methods

**Cells and Viruses.** MDCK cells (American Type Culture Collection CCL34) were purchased from Cambrex Bioscience. Cells were passaged twice weekly in serum-free Ultra-MDCK medium (Cambrex Bioscience) supplemented with 2 mM L-glutamine (Sigma Aldrich), penicillin (225 U/mL), and streptomycin (225 µg/mL) (Cambrex Bioscience). 293T HEK cells were maintained in DMEM supplemented with 10% (vol/vol) FCS and 2 mM L-glutamine (Sigma Aldrich), penicillin (225 U/mL), and streptomycin (225 µg/mL) (Cambrex Bioscience). MDCK and 293T cells were maintained at 37 °C with 5% CO<sub>2</sub>.

**Reverse Genetic Systems.** All MO genes were previously cloned into the pHW2000 vector to generate viruses by reverse genetics (43, 45). The HA, NA, M, NP, NS, PA, PB1, PB2, and EN genes (A/Finch/England/2051/91 virus) also were cloned into the pHW2000 vector. To do so, viral RNA was extracted from the infected MDCK cell-culture supernatant by using the QIAamp viral RNA minikit (Qiagen) according to the manufacturer's instructions. A two-step RT-PCR was carried out for full-length amplification of each viral RNA gene segment, by using the influenza A universal RT primer 5'-AGC AAA AGC AGG-3' (Eurogentec) (46). The cDNAs obtained from the different genes were cloned into the pHW2000 vector (47) allowing expression of both negative- and positive-strand RNAs and viral proteins from two different promoters. All constructs were verified by sequencing. Reassortant viruses were generated by reverse genetics as previously described (45, 47). Plasmids were mixed (1 µg per plasmid) in 100 µL Opti-MEM (Gibco-BRL) with 2.5 µL of TransIT-LT1 reagent (Mirus) per microgram of DNA, according to the manufacturer's instructions, and were added to 293T cells in six-well tissue-culture plates (48). At 48 h posttransfection, supernatants were harvested and used to infect MDCK with Eagle's Minimum Essential Medium

supplemented with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone trypsin (1 µg/mL) (45).

**Chimerical Gene Segments.** The wild-type HA<sub>EN</sub> ORF was amplified by PCR. The 3' region was generated using annealed primers including the 3' packaging region from the HA<sub>MO</sub> segment. After ligation of the HA<sub>EN</sub> ORF and the HA<sub>MO</sub> 3' regions, resulting DNA were amplified using specific PCR primers (5'-TATTCA CTGCG TCAGG GAGCAG AAGCAG GGG-3' and 5'-AACTCG CCACTG TTGAAT AAATTG-3'). The 5' packaging region of HA<sub>MO</sub> including the eighty 5' nucleotides of the HA<sub>MO</sub> ORF was amplified by PCR from the HA<sub>MO</sub> segment. The intermediate PCR product was ligated, and the resulting product was amplified and selected by specific primers (5'-TATTCA CTGCG TCAGGG AGCAGA AGCAGG GG-3' and 5'-ATATCA CTGCG TCGTAT TAGTAG AAACAA GGGTGT-3'). The recombinant HA<sub>CEN</sub> gene also was cloned into the pHW2000 reverse genetic vector (47).

The M<sub>C3MO</sub> gene was obtained by gene synthesis (Eurogentec) and was cloned into the pHW2000 vector (47). Five different site-directed mutagenesis experiments were performed on the M<sub>MO</sub> gene previously cloned in pHW2000 plasmid to obtain the M<sub>C3MO</sub> gene by using mismatched PCR primers (Table S1) and PfuUltra II DNA polymerase (Agilent Technologies). All plasmid sequences were verified by sequencing.

**PCR Screening of Gene Origins.** After amplification on MDCK cells in 48-well tissue-culture plates, vRNA was extracted from the culture supernatant using the RNeasy Mini kit (Qiagen). DNase treatments (RNase-Free DNase Set; Qiagen) were realized during vRNA extraction according to the manufacturer's instructions. RT-PCR started with the denaturation of 8.5 µL of vRNA in the presence of 200 ng of the Uni12 primer (5'-AGC AAA AGC AGG-3') for 5 min at 70 °C. Then cDNA strands were synthesized with 10 units of M-MuLV RT (Euromedex) and 20 mmol of dNTP (Euromedex) in a final volume of 20 µL for 1 h at 42 °C. To identify gene origin (MO and EN), PCR was performed on 5 µL of cDNA in the presence of 200 ng of each primer, 20 mmol of dNTP (Euromedex), and 1.25 units of GoTaq polymerase (Promega) for 30 cycles of 1 min at 94 °C, 1 min at 58 °C or 60 °C, and 1 min at 72 °C (Table S2). The primers were designed to be specific for a targeted origin of HA, PB1, NP, NA, and NS gene with PfuUltra II DNA polymerase (Agilent Technologies) and enable amplification by PCR followed by direct identification of gene origin by 0.8% agarose gel electrophoresis.

The origin of PB2, M, and PA genes was determined by NcoI or MunI digestion (37 °C for 60 min) after PCR amplification of cDNAs (30 cycles of 1 min at 94 °C, 1 min at 58 °C or 60 °C, and 1 min at 72 °C). M<sub>MO</sub>, M<sub>C3MO</sub>, and M<sub>C5MO</sub> screenings were realized by sequencing.

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