Arch Virol (1994) [Suppl] 9: 213–220

Archives of Virology

© Springer-Verlag 1994

Recombination between Sindbis virus RNAs

S. Schlesinger and **B. G. Weiss**

Department of Molecular Microbiology, Washington University Medical School, St. Louis, Missouri, U.S.A.

Summary. The Sindbis virus RNA genome is divided into two modules – one coding for the nonstructural protein genes and the other coding for the structural protein genes. In our studies of recombination, the two parental RNAs were defective in different modules. Analysis of the recombinant RNAs demonstrated that the parental RNAs each contributed its intact module and that the crossovers occurred within the defective modules. The recombinational events giving rise to infectious virion RNAs could create deletions, rearrangements or insertions as long as they occurred outside of the functional module. These crossovers produced RNA genomes that contained two functional subgenomic RNA promoters.

Introduction

The evolution of viruses in the natural world can be an important factor in the appearance of new human and animal diseases. Recombination between viruses is one mechanism by which viruses can evolve and is now a well recognized phenomenon among positive strand RNA viruses. Recombination between RNA viruses which contain nonsegmented genomes was first reported about 30 years ago with mutants of poliovirus [7]. Since then recombination has also been described for apthoviruses [11], coronaviruses [14] and several plant viruses including brome mosaic virus [2] cowpea chlorotic mottle virus [1] and barley stripe mosaic virus [3]. We recently reported that the alphavirus, Sindbis virus, undergoes recombination in cultured cells [22] and there is also evidence that alphaviruses can undergo recombination in nature. Based on a comparison of the sequence of Sindbis virus and that of two other alphaviruses, eastern equine encephalitis virus (EEEV) and western equine encephalitis virus (WEEV), Hahn et al. proposed that WEEV arose by recombination between a Sindbis-like virus and an EEEV-like virus [6].

Mechanisms of recombination between RNA viruses have not been investigated in detail, but template-switching is considered to be the most likely means by which genetic exchange between nonsegmented RNA genomes can occur [10]. Studies with poliovirus have provided evidence that indicates the viral polymerase switches templates during the synthesis of the negative strand [12]. In a recent review, Lai distinguished three types of RNA recombination [13]. The first type, homologous recombination, describes recombinational events in which the crossover is precise. There is, as yet, no evidence for any enzymatic mechanism to achieve this precision. In some crosses a precise crossover may be the only means of maintaining a functional gene and it would be the only way to obtain viable recombinant progeny. The second type is termed aberrant homologous recombination; although the two RNAs undergoing recombination are homologous, the crossover is not precise. This type of recombination can be expected during template switching if there are regions of the genome, either within coding sequences or in noncoding regions, which can tolerate some imprecision at the crossover point. It is the type of recombination we describe here for Sindbis virus. It has also been found with polioviruses and bromoviruses. The third type of recombination is nonhomologous or illegitimate, as the RNAs have no obvious homology. This type of recombination has been described for RNA viruses. We isolated defective interfering (DI) RNAs of Sindbis virus in which the 5′ terminus was replaced by a cellular $tRNA^{Asp}$ [18]. Cellular sequences have been identified in other viral RNAs; a ubiquitin-coding sequence was found in a bovine diarrhea virus [17] and a sequence from 28S rRNA was inserted into the hemagglutinin gene of influenza virus [9]. Recombination also may have occurred between a coronavirus and influenza C virus: the hemagglutinin-esterase gene found in some strains of coronaviruses shares 30% sequence homology with the hemagglutinin gene of influenza C virus [16].

Results

We detected recombination between Sindbis virus RNAs in our studies to develop Sindbis virus as a vector for introducing foreign genes into cells [5, 22]. Sindbis virus is the prototype member of the *Alphavirus* genus of the *Togaviridae* family. The alphavirus genome is a nonsegmented RNA molecule containing approximately 12 kb plus a poly(A) tail [20, 21]. The 5′ two-thirds of the RNA codes for the proteins required for replication and transcription of the RNA. The 3′ one-third codes for the structural proteins – the capsid protein and the proteins that comprise the envelope of the virion. The nonstructural proteins are translated from genomic length mRNAs; the structural proteins are

translated from a subgenomic RNA (26S RNA) identical in sequence to the 3′ one-third of the genome. This subgenomic RNA is transcribed from the minus strand of genomic RNA by initiation from an internal promoter that spans the junction between the structural and nonstructural genes. The cDNA of the Sindbis virus genome has been cloned downstream of the bacteriophage SP6 DNA dependent RNA polymerase. RNA transcribed in vitro from the cDNA is competent to produce infectious virions when transfected into cultured cells [19]. The availability of cloned cDNAs of both Sindbis virus and the closely related Semliki Forest virus made it possible to create and analyze a wide range of mutations and deletions in their genomes. It also made feasible the use of alphavirus RNAs as vectors for introducing foreign genes into cultured cells [15, 23]. The first self-replicating vector contained the nonstructural protein genes and the cis-acting sequences of the alphavirus genome [23]. The structural protein genes were replaced by the bacterial gene coding for the bacterial enzyme chloramphenicol acetyltransferase. When the vector RNA was transfected into cells, both genomic and subgenomic RNAs were produced, but the genomic RNA was not packaged. The vector RNA was packaged when it was transfected into cells with a defective RNA that produced a subgenomic RNA coding for the viral structural proteins [5]. The defective RNA was also packaged and many particles contained both RNAs. In addition to complementation between the two genomes we sometimes observed recombination – the appearance of an infectious nonsegmented RNA. Both complementation and recombination are illustrated in Fig. 1. The pattern of viral RNAs synthesized in cells transfected with TRCAT and the defective RNA carrying the 26S sequences [DI(26S)] is seen in lane 1. These data show that some cells were transfected with both RNAs since the DI genomic RNA and its subgenomic 26S RNA are dependent for their synthesis on the presence of the nonstructural proteins which would be coded by TRCAT. Virus harvested from the transfected cells was used to infect new cells and the pattern of RNAs produced in those cells is shown in lane 2. A band migrating slower than TRCAT can be seen in this lane. This RNA was shown to be infectious after it was isolated from a gel without prior denaturation and transfected into new cells [22]. Recombinant viruses were then plaque-purified and lanes 3 and 4 represent the RNA patterns obtained from infections with two different plaque isolates.

We have carried out studies on recombination with a variety of Sindbis RNAs (Table 1). In most experiments one of the parental RNAs was the vector RNA TRCAT. The other parental RNA was either the defective-helping RNA DI(26S) or an RNA with a deletion in one of the nonstructural protein genes and an intact structural protein gene

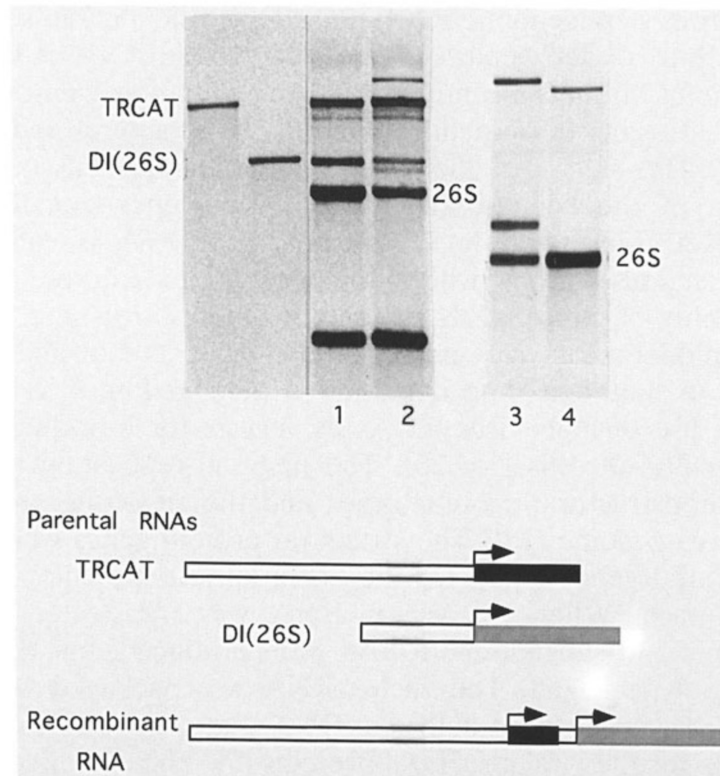

Fig. 1. Complementation and recombination between Sindbis virus RNAs. The upper panel shows the autoradiogram of the ^{3}H-uridine labeled RNA synthesized in cells transfected with Sindbis virus RNAs or infected with Sindbis virus. *1* is the pattern obtained from cells transfected with *TRCAT* and *DI(26S)* RNA; *2* is from cells infected with the virus harvested from the transfected cells; *3* and *4* each represent the pattern obtained from cells infected with a different plaque-purified recombinant virus. The first two unmarked lanes are ^{3}H-uridine labeled transcripts included as markers. The fastest migrating RNAs seen in *1* and *2* are the subgenomic RNAs derived from *TRCAT*. The diagrams below illustrate the parental and recombinant RNAs. The open boxes are either the intact four nonstructural protein genes (*TRCAT* and the recombinant RNA) or a deleted and rearranged version of them *DI(26S)*. The filled in black box represents CAT sequences and Sindbis virus sequences downstream of the start of the subgenomic RNA promoter and at the 3′ terminus. The complete *26S* sequence is indicated by the gray box

module. Recombinant viruses from the different crosses listed in Table 1 were isolated by plaque purification. Many of them had two unusual properties: the genomic RNAs were larger than the wild type Sindbis genomic RNA and cells infected with a recombinant virus synthesized an additional RNA species that migrated in an agarose gel between the genomic and subgenomic RNAs. Figure 2 shows the results of a cross between TRCAT and CR3.4, an RNA with a deletion in the nsP3

Table 1. Crosses between Sindbis RNAs that gave rise to infectious recombinants with two subgenomic RNA promoters

Parental RNA with defect in structural protein genes	Parental RNA with defect in nonstructural protein (nsP) gene
TRCAT-structural genes have been replaced by the CAT gene	DI(26S) – 5′terminal region comes from a DI RNA
TRCAT	CR1.8 – in frame deletion in nsP1 gene
TRCAT	CR3.4 – in frame deletion in nsP3 gene
$E2C_{415}C_{416}$ – two cysteines in the structural gene, PE2, were changed: one to serine; one to alanine [4]	DI(26S)
$E2C_{415}C_{416}$	CR2.4 – in frame deletion in nsP2 gene

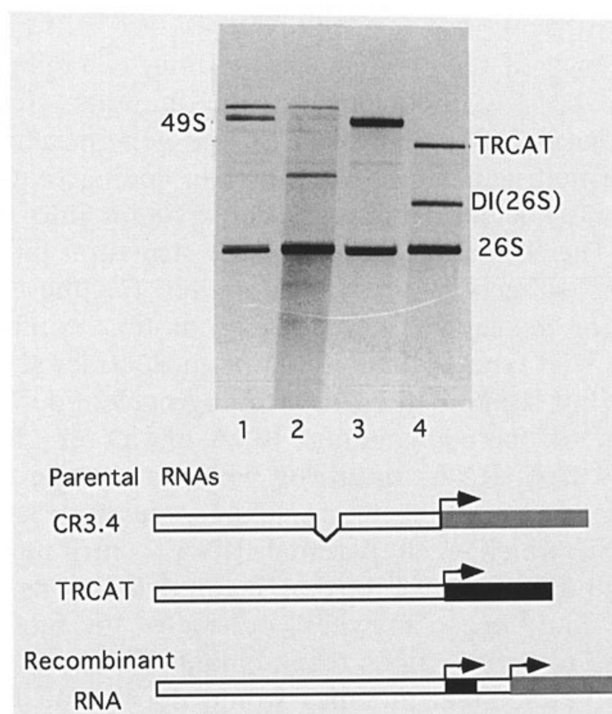

Fig. 2. Recombinant RNA profiles from a cross between TRCAT and CR3.4. The upper panel shows the autoradiogram of the ^{3}H-uridine labeled RNA synthesized in cells infected with Sindbis virus stocks obtained from independent plaques (*1* and *2*). *3* is the pattern of wild type Sindbis virus RNAs and *4* shows the markers. The lower diagrams illustrate the parental and recombinant RNAs. The symbols are identical to those described in Fig. 1

gene. Lanes 1 and 2 show the RNA patterns from cells infected with independent plaque isolates. Both show an RNA species larger than the wild type genomic (49S) RNA (lane 3) as well as an additional RNA species migrating between the genomic and subgenomic RNAs. The new RNA species differed in size from each other and from the new band seen in Fig. 1, lane 3.

Recombinant RNAs from the different crosses were sequenced in the region surrounding the subgenomic RNA promoter revealing that they contained two subgenomic RNA promoters [22]. This finding explains the larger size of the RNAs; they contain additional nucleotides. It explains the new species of RNA; two subgenomic RNAs are synthesized. The diagrams in Figs. 1 and 2 illustrate the structure of two of the recombinant RNAs. Insertions in the different recombinants varied in size ranging from 47 nucleotides to more than 800 nucleotides, indicating that the nucleotides directly surrounding the subgenomic promoter are not a "hotspot" for recombination.

Discussion

The high frequency of recombinants containing two subgenomic RNA promoters may be a consequence of the modular structure of the Sindbis virus genome. The 5′ module of the genome encompasses the nonstructural protein genes but the complete coding sequence of these genes extend one nucleotide plus the stop codon into the 26S RNA sequence [21]. The 3′ module codes for the structural proteins. It must also include the subgenomic RNA promoter for these genes to be expressed, making the subgenomic RNA promoter a component of both modules. In the wild type genomic RNA 48 nucleotides separate the two coding regions, but some of these noncoding nucleotides are important for the activity of the subgenomic RNA promoter. Recombination between Sindbis virus RNAs occurring within a module would have to be precise to conserve coding or cis-acting regulatory sequences. In a recombination in which each parental RNA contributed one of the modules, recombinational events that created deletions, insertions or rearrangements could occur anywhere outside of the functional module and still give rise to an infectious recombinant. The length of the region between the two functional modules would depend on the location at which recombination had occurred and on the stability of the recombinant RNA. The recombinant Sindbis virus genomes containing two subgenomic RNA promoters were larger than the 49S RNA, but they were unstable and eventually evolved to be the same size as wild type RNA. This is an important point; all of the recombinants were analyzed after plaque purification. Several produced the normal pattern of Sindbis

virus RNAs (Fig. 1, lane 4) and showed no evidence of any insert in the region of the subgenomic RNA promoter. These recombinants could be ones that had already undergone evolution by the time of analysis.

We have not yet investigated questions about the frequency of recombination between Sindbis virus RNAs or about the influence of sequence homology and structure on the site of crossover. Answers to such questions depend on being able to determine the total number of recombinational events taking place, not just those which lead to the selection of viable progeny. The recent success of Jarvis and Kirkegaard, using polymerase chain reaction to detect recombinant RNA molecules in poliovirus infected cells, makes these studies now seem feasible [8]. A better understanding of the factors involved in RNA recombination may shed further light on the evolution of these viruses.

References

1. Allison R, Thompson C, Ahlquist P (1990) Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. Proc Natl Acad Sci USA 87: 1820–1824
2. Bujarski JJ, Kaesberg P (1986) Genetic recombination between RNA components of a multipartite plant virus. Nature 321: 528–531
3. Edwards MC, Petty ITD, Jackson AO (1992) RNA recombination in the genome of barley stripe mosaic virus. Virology 189: 389–392
4. Gaedigk-Nitschko K, Schlesinger MJ (1991) Site-directed mutations in Sindbis virus E2 glycoprotein's cytoplasmic domain and the 6K protein lead to similar defects in virus assembly and budding. Virology 183: 206–214
5. Geigenmüller-Gnirke U, Weiss B, Wright R, Schlesinger S (1991) Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome. Proc Natl Acad Sci USA 88: 3253–3257
6. Hahn CS, Lustig S, Strauss EG, Strauss JH (1988) Western equine encephalitis virus is a recombinant virus. Proc Natl Acad Sci USA 85: 5997–6001
7. Hirst GK (1962) Genetic recombination with Newcastle disease virus, polioviruses, and influenza. Cold Spring Harbor Symp Quant Biol 27: 303–308
8. Jarvis TC, Kirkegaard K (1992) Poliovirus RNA recombination: mechanistic studies in the absence of selection. EMBO J 11: 3135–3145
9. Khatchikian D, Orlich M, Rott R (1989) Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature 340: 156–157
10. King AMQ (1988) Genetic recombination in positive strand RNA viruses. In: Domingo E, Holland JJ, Ahlquist P (eds) RNA genetics, vol 2. CRC Press, Boca Raton, pp 150–185
11. King AMQ, McCahon D, Slade WR, Newman JWI (1982) Recombination in RNA. Cell 29: 921–928
12. Kirkegaard K, Baltimore D (1986) The mechanism of RNA recombination in poliovirus. Cell 47: 433–443
13. Lai MMC (1992) RNA recombination in animal and plant viruses. Microbiol Rev 56: 61–79

14. Lai MMC, Baric RS, Makino S, Keck J, Egbert J, Leibowitz JL, Stohlman SS (1985) Recombination between nonsegmented RNA genomes of murine coronaviruses. J Virol 56: 449–456
15. Liljeström P, Garoff H (1991) A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Bio/Technology 9: 1356–1361
16. Luytjes W, Bredenbeek PJ, Noten AF, Horzinek MC, Spaan WJ (1988) Sequence of mouse hepatitis virus A59 mRNA 2: indications for RNA-recombination between coronavirus and influenza C virus. Virology 166: 415–422
17. Meyers G, Tautz N, Dubovi EJ, Thiel H-J (1991) Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. Virology 180: 602–616
18. Monroe SS, Schlesinger S (1983) RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5′ ends. Proc Natl Acad Sci USA 80: 3279–3283
19. Rice CM, Levis R, Strauss JH, Huang HV (1987) Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J Virol 61: 3809–3819
20. Schlesinger S, Schlesinger M (1990) Replication of togaviridae and flaviviridae. In: Fields BN, Knipe DM (eds) Virology. Raven Press, New York, pp 697–711
21. Strauss EG, Strauss JH (1986) Structure and replication of the alphavirus genome. In: Schlesinger S, Schlesinger MJ (eds) The togaviridae and flaviviridae. Plenum Press, New York, pp 35–82
22. Weiss BG, Schlesinger S (1991) Recombination between Sindbis virus RNAs. J Virol 65: 4017–4025
23. Xiong C, Levis R, Shen P, Schlesinger S, Rice CM, Huang HV (1989) Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Science 243: 1188–1191

Authors' address: Dr. S. Schlesinger, Department of Molecular Microbiology, Washington University Medical School, St. Louis, MO 63110-1093. U.S.A.