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Review

Expression strategies of ambisense viruses

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Abstract

Among the negative RNA viruses, ambisense RNA viruses or 'ambisense viruses' occupy a distinct niche. Ambisense viruses contain at least one ambisense RNA segment, i.e. an RNA that is in part of positive and in part of negative polarity. Because of this unique gene organization, one might expect ambisense RNA viruses to borrow expression strategies from both positive and negative RNA viruses. However, they have little in common with positive RNA viruses, but possess many features of negative RNA viruses. Transcription and/or replication of their RNAs appear generally to be coupled to translation. Such coupling might be important to ensure temporal control of gene expression, allowing the two genes of an ambisense RNA segment to be differently regulated. Ambisense viruses can infect one host asymptomatically and in certain cases, they can lethally infect two hosts of a different kingdom. A possible model to explain the differential behavior of a given virus in different hosts could be that perturbation of the translation machinery would lead to differences in the severity of symptoms.

Keywords: Ambisense; RNA; Virus; Replication; Transcription; Translation

1. Introduction

Single-stranded (ss) RNA viruses possess either positive or negative sense RNA genomes. Those with negative RNA genomes are themselves divided into segmented and non-segmented viruses. Among the segmented negative RNA viruses, not all viruses contain a strictly negative genome; several viruses possess (an) ambisense RNA segment(s) and therefore could constitute a distinct sub-category of multipartite ssRNA viruses: the ambisense RNA viruses (or ambisense viruses).

The genome of positive RNA viruses is of the same sense as mRNAs and thus, can serve directly as template for translation. Therefore, viral proteins are expressed upon entrance of the viral genomic (g) RNA into the cell.

In contrast, the genome of negative RNA viruses cannot be translated; a transcription step giving rise to positive, i.e. sense mRNAs is mandatory before expression of viral proteins is possible. To achieve this obligatory transcription step, negative RNA viruses encapsidate their RNA-dependent RNA polymerase (RdRp).

Ambisense RNA(s) of ambisense viruses are partly of positive and partly of negative polarity. The 5′ part of an ambisense RNA is of positive polarity containing an open reading frame (ORF) that can theoretically be directly translated. The 3′ part of this same RNA is of negative polarity. It contains an ORF, but in the complementary strand (Fig. 1). Indeed, this second part of the ambisense RNA must be transcribed prior to translation. To express this second gene, ambisense viruses also encapsidate their RdRp. This approach to stocking genetic information is designated the ambisense coding strategy (Ihara et al., 1984). The two coding regions of an ambisense RNA do not overlap, but are separated by a non-coding intergenic region (IR). Although ambisense viruses are considered ssRNA viruses, both strands of an ambisense RNA segment are usually found in the virion in unequal amounts (Fig. 1; reviewed in Ramirez and Haenni, 1994; Schmaljohn, 1996; Southern, 1996). To circumvent the designation positive or negative RNA, that would be inadequate, the

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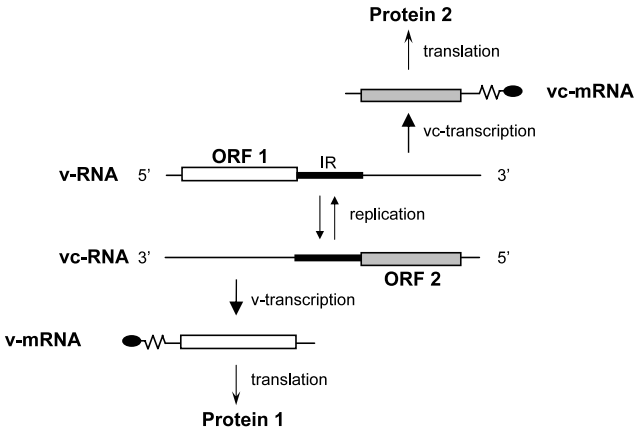

Fig. 1. Schematic representation of the two strands of a model ambisense RNA with the transcription, translation and replication steps. v, Viral strand; vc, viral complementary strand; the intergenic region (IR) is indicated by a thick line; ●, cap structure; ⌇⌇, non viral nucleotides.

term viral (v) is used to refer to the more abundant RNA strand present in the virus and viral complementary (vc) for the complementary strand (reviewed in Ramirez and Haenni, 1994; Schmaljohn, 1996; Southern, 1996).

Based on these observations, ambisense viruses could theoretically be expected to possess some characteristics of positive RNA viruses concerning their expression strategies and of negative RNA viruses in their transcription behavior. These unique features make ambisense viruses a remarkable 'group' of viruses. Furthermore, most ambisense viruses are not strictly ambisense, but contain negative as well as ambisense RNA segments and, to express their ambisense RNA segments, they must resort to similar strategies as 'strict' ambisense viruses.

Ambisense viruses comprise four genera (*Arenavirus*, *Phlebovirus*, *Tospovirus* and *Tenuivirus*), distributed between Arenaviridae and Bunyaviridae families and a small nonclassified genus of plant viruses, the tenuiviruses (Table 1). It has been proposed that tenuiviruses be included in the Bunyaviridae family (reviewed in Ramirez and Haenni, 1994). Arenaviruses and phleboviruses are animal or human-infecting viruses, whereas tospoviruses and tenuiviruses are plant-infecting viruses.

Ambisense viruses are found in very different eukaryotic systems. On the one hand, they infect humans and cause hemorrhagic fevers, such as Lassa and Rift Valley fevers (reviewed in Gonzalez-Scarano and Nathanson, 1996; Peters et al., 1996). On the other hand, they lead to economically important losses in animals (reviewed in Gonzalez-Scarano and Nathanson, 1996; Peters et al., 1996) and plants (reviewed in Gingery, 1988; Goldbach and Peters, 1994).

Ambisense viruses replicate in one or two hosts. The primary host is an insect (phleboviruses, tospoviruses and tenuiviruses) or a rodent (arenaviruses), which is a designated vector or reservoir of these viruses. Viral multiplication in these hosts is generally persistent and is asymptomatic, except in rare cases (reviewed in Ramirez and Haenni, 1994; Gonzalez-Scarano and Nathanson, 1996; Peters et al., 1996) or when induced in laboratory conditions. However, these viruses can be transmitted to a second host, either another animal or a plant host. It is often via infection with this second host that the most severe aspects of viral multiplication are observed, sometimes resulting in death of the infected organism. Some arenaviruses, such as TCRV arenavirus (see Table 1 for abbreviations), solely infect rodents and transmission to other animals or to humans has never been observed, whereas the LCMV arenavirus is a common human pathogen that has been extensively studied for immunological purposes (reviewed in Peters et al., 1996).

This review highlights the similarities found in all ambisense viruses whether or not they are 'strict' ambisense viruses and their close relationship with negative RNA viruses. No review describing ambisense viruses in this broad sense has appeared since the review by Bishop (1986). The review focuses on the transcription, replication and to a certain extent, on the translation strategies of ambisense viruses. Complementary information related to specific families and/or genera can be found elsewhere (reviewed in Salvato, 1993; Ramirez and Haenni, 1994; Elliott, 1996a; Peters et al., 1996; Schmaljohn, 1996; Southern, 1996; Lee and de la Torre, 2002; Meyer et al., 2002).

Viruses of the Arenaviridae and Bunyaviridae families are enveloped (reviewed in Southern, 1996; Schmaljohn, 1996). Tenuiviruses are considered non-enveloped viruses. However, sequence data indicate that they possess glycoproteins homologous to those of the envelope proteins of phleboviruses (de Miranda et al., 1996; Estabrook et al., 1996; Toriyama et al., 1998).

The Arenaviridae family is composed of one genus, the *Arenavirus* whose genome contains two ambisense RNA segments designated L (large) and S (small; Table 1; reviewed in Southern, 1996).

The Bunyaviridae family contains five genera, the *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (Table 1; reviewed in Schmaljohn, 1996). The genome of viruses of this family is composed of three RNA segments referred to as L, M (medium) and S. These segments are of negative polarity in bunyaviruses, hantaviruses and nairoviruses (Table 1). The S and/or M genome segments of the phleboviruses and tospoviruses are ambisense, whereas the L and/or M segments are negative (Table 1).

Tenuiviruses resort to similar coding strategies as ambisense viruses of the Bunyaviridae, but their genome is composed of four to six RNA segments of ambisense and/or negative polarities designated 1–6 in order of

Table 1
Genome organization of viruses of the Arenaviridae and Bunyaviridae families and of the *Tenuivirus* genus

Family/genus	Examples of viruses	RNA genome	Polarity	Size (kb)	Encoded proteins
Arenaviridae[a]		2 segments:			
Arenavirus	JUNV, LCMV, LASV, TCRV, PICV	L	Ambisense	7.2	Z (v)+L (vc)
		S	Ambisense	3.4	GP (v)+NP (vc)
Bunyaviridae		3 segments:			
Bunyavirus[b]	BUNV, GERV, LACV	L	(−)	6.0–7.5	L
		M	(−)	4.3–4.8	G2–NSm–G1
		S	(−)	0.8–1.0	N/NSs
Hantavirus[b]	HTNV, SNV, SEOV	L	(−)	6.5	L
		M	(−)	3.6–3.7	G1–G2
		S	(−)	1.6–2.1	N
Nairovirus[b]	DUGV	L	(−)	12–14	L
		M	(−)	4.3–6.5	G1–G2
		S	(−)	1.7–2.0	N
Phlebovirus[c,d]	PTV, RVFV, SFSV, TCSV, UUKV	L	(−)	6.4	L
		M	(−)	3.2–4.9	[NSm]*–G1 (or G2)–G2 (or G1)
		S	Ambisense	1.7–1.9	NSs (v)+N (vc)
Tospovirus[c,e]	INSV, TSWV	L	(−)	8.8–9	L
		M	Ambisense	4.8–5	NSm (v)+G2–G1 (vc)
		S	Ambisense	2.9–3	NSs (v)+N (vc)
Tenuivirus[c,f,g]	MSpV, RHBV, RSV	4 (or 5) segments:			
		1	(−)	9	Pol
		2	Ambisense	3.3–3.5	pv2 (v)+pc2 (vc)
		3	Ambisense	2.3–2.5	NS3 (v)+NC (vc)
		4	Ambisense	1.9–2.2	NS4 (v)+NSvc4 (vc)
		5[†]	(−)	1.3	NS5
	RGSV[‡]	6 segments	Ambisense	2.6–9.8	

Complementary references are indicated in the text. Viruses abbreviations: BUNV, Bunyamwera; DUGV, Dugbe; GERV, Germiston; HTNV, Hantaan; INSV, Impatiens necrotic spot; JUNV, Junín; LACV, La Crosse; LASV, Lassa; LCMV, lymphocyte choriomeningitis; MSpV, maize stripe; PICV, Pichinde; PTV, Punta Toro; RGSV, rice grassy stunt; RHBV, rice hoja blanca; RSV, rice stripe; RVFV, Rift Valley fever; SEOV, Seoul; SFSV, Sandfly fever Sicilian; SNV, Sin Nombre; TCRV, Tacaribe; TOSV, Toscana; TSWV, tomato spotted wilt; UUKV, Uukuniemi. Proteins abbreviations: L, RdRp; Pol, putative RdRp, G1 and G2, glycoproteins; pv2 and pc2, putative glycoproteins; NP, N or NC, nucleoprotein, for the sake of clarity, the nucleoprotein is referred to as NP in the text; NS, non-structural proteins; Z, RING type protein.

[a] Viruses with ambisense RNAs. Reviewed in Salvato (1993) and Southern (1996).

[b] Viruses with negative RNAs, reviewed in Schmaljohn (1996).

[c] Viruses with negative and/or ambisense RNAs.

[d] Reviewed in Giorgi (1996).

[e] Reviewed in Goldbach and Peters (1996).

[f] Reviewed in Ramirez and Haenni (1994).

[g] Reviewed in Toriyama et al. (1998).

* Protein NSm is absent in phleboviruses of the Uukuniemi group.

[†] RNA segment 5 is not present in RStV and RHBV.

[‡] Although RGSV is considered a tenuivirus, due to differences in genome sequences, it has recently been proposed to be the type species of a new genus related to the tenuiviruses (Toriyama et al., 1998).

their decreasing size (Table 1; reviewed in Ramirez and Haenni, 1994; Toriyama et al., 1998).

Although ambisense viruses are found in distinct families and infect either plants or animals, they nevertheless share many characteristics. Some of these features are the following: (i) they possess a multipartite single-stranded RNA genome; (ii) at least one RNA segment is ambisense; (iii) the RNAs are encapsidated by the nucleocapsid protein to form ribonucleoproteins (RNPs); (iv) a viral RdRp is associated with the RNPs; (v) the largest RNA segment encodes the RdRp; (vi) the RNPs are enveloped by glycoproteins (except for the tenuiviruses for which no envelope has been detected); (vii) the 5′ and 3′ ends of each genome segment are conserved and can base-pair to form a panhandle structure; (viii) gRNAs of both polarities are found in the RNPs, the v-gRNAs being more abundant than the vc-gRNAs; (ix) they produce subgenomic RNAs (mRNAs) that contain non-viral nucleotides (nt) and a cap structure at their 5′ end, indicative of a cap-snatching mechanism for synthesis of the viral mRNAs; (x) the 3′ end of their mRNAs is not polyadenylated; and (xi) transcription and replication presumably occur in the cytoplasm.

Most of the ambisense virus features are also shared by multipartite negative RNA viruses, such as viruses within the *Bunyavirus, Hantavirus* and *Nairovirus* genera of the Bunyaviridae family (features i, iii–vii and ix–xi; reviewed in Schmaljohn, 1996) and viruses of the Orthomyxoviridae family (features i, iii–vii and ix; reviewed in Lamb and Krug, 1996).

In discussing strategies and when no clear data exist for ambisense viruses of the Bunyaviridae, we refer to the data obtained for the negative RNA viruses of this family, since many features of viruses of this family are believed to be shared by its various genera (reviewed in Elliott, 1996a; Schmaljohn, 1996).

2. Expression strategies

Since ambisense RNAs are of positive and negative polarities, the ORF residing in the v-gRNA could theoretically be translated, as are the ORFs of viral RNAs of positive polarity. However, gene expression strategies of ambisense viruses are generally believed to consist of a transcription step, giving rise to capped mRNAs before translation occurs, similarly to the requirement of fully negative RNA viruses. Indeed, an RdRp activity linked to purified RNPs or virions was detected in vitro for arenaviruses (Carter et al., 1974; Lopez et al., 1995), phleboviruses (Ranki and Pettersson, 1975), tospoviruses (Adkins et al., 1995) and tenuiviruses (Toriyama, 1986, 1987; Nguyen et al., 1997), indicating that ambisense viruses are basically negative RNA viruses. In addition, the first in vitro attempts to translate total RNAs extracted from ambisense virions were unsuccessful (Leung et al., 1977).

More recent evidence obtained with tenuiviruses (Falk et al., 1987; Ramirez et al., 1992; Hamamatsu et al., 1993; Nguyen et al., 1997) and for TSWV tospovirus (Nguyen, 1998) showed that total RNAs extracted from purified RNPs or virions, were able to direct protein synthesis in an in vitro translation system. This raised the interesting question of whether the gRNAs of ambisense viruses could also be translated in vivo. Were this to be the case, how would one distinguish translation of gRNAs from translation of mRNAs? As opposed to mRNAs, gRNAs are not capped and are encapsidated. Numerous positive RNA viruses have uncapped and encapsidated gRNA that are efficiently translated. These viruses usually resort to various strategies to replace the cap-poly(A) usually required for translation in the host cell, such as an internal ribosome entry site, a VPg protein, different translational enhancer elements at the 5′ or 3′ end or both of the gRNAs (for reviews, see Fields et al., 2001). None of these features have been described for the gRNAs of ambisense viruses. However, the non-translatability of gRNAs of ambisense viruses has, to date, not been demonstrated. Therefore, the hypothetical role of gRNAs in translation remains unclear since, if it does occur, translation of gRNAs might interfere with translation of mRNAs. However, such a situation might be important in terms of regulation of protein expression as discussed below.

3. Transcription

The mRNAs of ambisense viruses contain non-viral nucleotides at their 5′ end. These extensions are from 0 to 7 nt in arenaviruses (reviewed in Meyer et al., 2002), 7–25 nt in phleboviruses (Ihara et al., 1985; Collett, 1986; Simons and Pettersson, 1991; Grò et al., 1992) and tospoviruses (Kormelink et al., 1992b; Duijsings et al., 1999) and 10–23 nt in tenuiviruses (Huiet et al., 1993; Ramirez et al., 1995; Shimizu et al., 1996; Nguyen et al., 1997; Estabrook et al., 1998).

In addition to non-viral extensions, the mRNAs of TCRV arenavirus (Garcin and Kolakofsky, 1990), bunyaviruses (reviewed in Schmaljohn, 1996) and RHBV tenuivirus (Ramirez et al., 1995) also contain a cap structure. Co-infection of a tospovirus and an RNA virus serving as a cap donor, provided evidence that cap structures in addition to RNA extensions are present at the 5′ end of tospoviruses mRNAs (Duijsings et al., 1999).

In summary, for all ambisense viruses, synthesis of mRNAs presumably occurs via a cap-snatching mechanism similar to that of influenza virus (reviewed in Lamb and Krug, 1996), i.e. the viral RdRp 'steals' the cap structure and several nucleotides from host mRNAs by endonucleolytic cleavage and uses them as primer to synthesize its own mRNAs. However, contrary to the situation in influenza virus, cap-snatching and transcription of ambisense viruses occurs in the cytoplasm (see below).

A model for the initiation of mRNAs and gRNAs synthesis, designated 'prime-and-realign', has been proposed by the group of Kolakofsky for viruses of the Arenaviridae and Bunyaviridae families (reviewed in Garcin et al., 1995). In this model using the HTNV hantavirus genome (template v-RNA: $^{3'}$AUCAUCAUC...$^{5'}$), either a capped oligonucleotide ending with a 3′ G residue or a single GTP is used as primer, respectively for transcription or replication. In both cases, priming occurs at the C residue at position +3 from the 3′ end of the template RNA. Elongation of one or a few nucleotides would proceed before realignment on the template occurs. The G residue that served for priming thus extrudes. This residue corresponds to the non-templated nucleotide observed in arenavirus gRNAs. For viruses of the Bunyaviridae family, an endonuclease activity presumably removes this extra-nucleotide. Moreover, nucleotide sequence data ob-

tained for RVFV phlebovirus (Prehaud et al., 1997), TSWV tospovirus (Duijsings et al., 1999) and MSpV tenuivirus (Estabrook et al., 1998) also favors this model, indicating that all ambisense viruses can initiate viral RNA synthesis via a prime-and-realign mechanism.

Recently, following a study on the cap-snatching mechanism on TSWV tospovirus, Duijsings et al. (2001) observed that a single base complementarity is sufficient for priming. Initiation of transcription would occur at the 3′ ultimate residue rather than at the penultimate residue. Therefore, the prime-and-realign model appears not to adequately explain TSWV tospovirus transcription initiation. However, no alternative model has been proposed by the authors.

Transcription of an ambisense RNA is complicated by the necessity to synthesize two mRNAs, one of v and one of vc polarity. For the sake of clarity, a simplified scheme of ambisense RNA is presented in Fig. 1, in which v-RNA and vc-RNA contain, respectively, ORFs 1 and 2. Two types of transcription can be distinguished (Fig. 1), referred to as 'vc-transcription' (synthesis of vc-mRNAs) and 'v-transcription' (synthesis of v-mRNAs).

Since both strands of gRNAs are present in viral particles, v- and vc-transcriptions could occur upon entrance of the virus into the cell. However, v-gRNAs being more abundant than vc-gRNAs (reviewed in Ramirez and Haenni, 1994; Schmaljohn, 1996; Southern, 1996), to obtain abundant v-transcription, sufficient vc-gRNAs templates would be required and therefore, replication would need to have started.

For all ambisense viruses, both v- and vc-mRNAs can be detected in vivo, indicating that the proteins encoded by the two strands of an ambisense RNA segment are most likely expressed via these mRNAs (Parker et al., 1984; Ihara et al., 1985; Franze-Fernandez et al., 1987; Francis and Southern, 1988; Kormelink et al., 1992a; Ramirez et al., 1992; Nguyen et al., 1999).

Transcription termination of ambisense RNAs occurs in the IR. This region is usually rich in secondary structures that presumably stop elongation by the RdRp (Emery and Bishop, 1987). However, secondary structures are not present in the IR of the S RNA of SFSV, RVFV and TOSV phleboviruses (Giorgi et al., 1991). For these viruses, termination of transcription is most likely dictated by specific sequences recognized by the viral RdRp. Indeed, their IRs are rich in C tracts (v-RNA) or G tracts (vc-RNA). Conserved sequence motifs have also been observed within these IRs, but their potential role in transcription termination is unknown.

Another distinctive feature of viruses of the Arenaviridae and Bunyaviridae families and of tenuiviruses concerns the 3′ end of their mRNAs. Unsuccessful attempts to isolate mRNAs by oligodT column chromatography (reviewed in Ramirez and Haenni, 1994; Schmaljohn, 1996; Southern, 1996) indicate that the mRNAs of these viruses are not polyadenylated. Direct analyses of the 3′ ends of the mRNAs of LCMV arenavirus (Meyer and Southern, 1993) and of TOS phlebovirus (Grò et al., 1992) confirmed that the 3′ ends of these mRNAs are not polyadenylated and are heterogeneous in length.

4. Replication

In addition to the prime-and-realign model, another model for initiation of gRNA synthesis has recently been proposed (Meyer and Schmaljohn, 2000): the viral RdRp would cleave the 5′ end of gRNAs and use the resulting fragments as primers to initiate synthesis of full-length RNA by *cis*- or *trans*-priming in a way similar to the cap-snatching mechanism.

Similarly to negative RNA viruses, the 5′ and 3′ ends of the gRNAs of ambisense viruses are complementary over 10–25 nt and can base-pair to form panhandle structures. Corresponding circular structures were observed by electron microscopy (reviewed in Ramirez and Haenni, 1994; Schmaljohn, 1996; Southern, 1996). The 5′ and 3′ ends of the gRNAs are conserved between the segments of a given virus and between viruses belonging to a same viral genus in the case of all ambisense viral groups (reviewed in Ramirez and Haenni, 1994; Schmaljohn, 1996; Meyer et al., 2002).

Since the 5′ and 3′ ends of gRNAs are well conserved, promoter regions for transcription and/or replication of ambisense viruses are most likely located within these regions.

For arenaviruses and phleboviruses, using reverse genetics systems, it was shown that the 5′ untranslated region (UTR) and 3′ UTR are necessary for transcription and replication of a reporter gene by the L and NP proteins (Lopez et al., 1995; Lee et al., 2000; Accardi et al., 2001; Flick and Pettersson, 2001; Lopez et al., 2002). Prehaud et al. (1997) further characterized the promoter region indicating that the region required for transcription resides within the first 13 nt of the 3′ end in the v or vc strand of the S RNA of RVFV phlebovirus. For the RSV tenuivirus, Barbier et al. (1992) showed that purified viral RdRp could synthesize short synthetic RNAs ($\sim$40 nt) containing solely the 3′ conserved region of the gRNAs, indicating that this region probably contains the promoter for transcription.

Recent studies, using reverse genetics, showed that both the 5′ and 3′ UTR of the M RNA of the UUKV phlebovirus constitute the promoter region recognized by the viral RdRp (Flick et al., 2002). Conservation of the panhandle structure itself and most importantly of the sequence of two proximal sites in this region is essential for recognition of the promoter by the RdRp.

During in vivo replication, ambisense viruses produce mainly v-gRNAs (Ihara et al., 1985; Franze-Fernandez et al., 1987; Francis and Southern, 1988; Fuller-Pace and Southern, 1988; Simons et al., 1990; Kormelink et al., 1992a; Nguyen et al., 1997, 1999; Nguyen, 1998; Flick et al., 2002). This is similar to what is observed for other multipartite negative RNA viruses (reviewed in Lamb and Krug, 1996). Therefore, the observed predominance of v-gRNAs in viral particles is most likely due to regulation of the synthesis of gRNAs at the replication rather than at the encapsidation step.

Termination of replication is presumably due to the detachment of the RdRp as it reaches the end of the template RNA. Alternatively, and as for influenza virus, the RdRp could remain attached to the 5′ end of the template RNA and the elongating RNA would slip through the RdRp as a thread through the hole of a needle (reviewed in Lamb and Krug, 1996).

5. RNA synthesis and translation

One of the peculiarities of ambisense viruses is that RNA synthesis appears to be somehow linked to translation. For LACV and GERV bunyaviruses, in vitro transcription as well as replication were reported to require ongoing protein synthesis, also designated 'translational requirement' (reviewed in Kolakofsky and Hacker, 1991; Vialat and Bouloy, 1992). Translation was proposed to be required because scanning of the viral RNA by the translation machinery would destabilize secondary structures allowing the RdRp to proceed without risk of premature transcription termination. Moreover, this scanning, if it occurs, would subvert part of the translation machinery and would hinder the cell translation process. Whether scanning of the gRNA by the translation machinery would lead to translation of the gRNA is unknown, however, it would be a means for the virus to control at the same time the host translation machinery and the expression of its own viral genes.

In the case of TCRV and JUNV arenaviruses (Franze-Fernandez et al., 1987; Tortorici et al., 2001) and PTV phlebovirus (Ihara et al., 1985), studies of infected cells showed that an inhibitor of protein synthesis could inhibit replication and v-transcription, but not vc-transcription of S RNA. Induction of these steps most likely requires de novo synthesis of viral and/or cellular factors. Recently, the NP was proposed to be one of the proteins involved because of its putative transcriptional antiterminator activity (Tortorici et al., 2001). Another possibility is that translation is required for the induction of replication and v-transcription, such as described above for LACV and GERV bunyaviruses (reviewed in Kolakofsky and Hacker, 1991; Vialat and Bouloy, 1992).

Studies of the in vitro RdRp activity of TSWV tospovirus revealed only replication (Adkins et al., 1995; Nguyen, 1998). No subgenomic-sized mRNAs were detected, suggesting that translational requirement might be necessary for the synthesis of mRNAs in vitro (Nguyen, 1998).

When the RdRp activity of the RHBV tenuivirus was examined in vitro, abundant vc-transcription of RNAs 3 and 4 of RHBV was observed, whereas v-transcription was barely detected (Nguyen et al., 1997) and replication yielded mainly v-gRNAs 3 and 4. v-Replication and vc-transcription were observed whether the reaction was coupled or not to translation. Intriguingly, direct translation of newly synthesized RNAs that were phenol-extracted yielded only v-encoded proteins in levels similar to direct translation of total gRNA, whereas when the same newly synthesized RNAs were analyzed by coupling translation to transcription, abundant vc-encoded proteins were synthesized. Inclusion of inhibitors of protein synthesis affected neither transcription nor replication, indicating that in vitro RHBV vc-transcription and v-replication do not require translation. Although such coupling was not necessary for RNA synthesis, it led to increased translation of vc-encoded proteins, suggesting a means for the virus to regulate protein expression of v-RNA versus vc-RNA genes.

For arenaviruses, phleboviruses and tenuiviruses, v- and vc-transcriptions appear to have different requirements: v-transcription appears to require either translation, the intervention of newly synthesized viral and/or cellular factors or that replication be initiated, whereas vc-transcription has no such requirements. Consequently, these two steps are probably regulated differently.

Coupling of translation to RNA synthesis indicates that transcription probably occurs in the cytoplasm. Indeed, transcription and replication of viruses of the Arenaviridae and Bunyaviridae families presumably take place in the cytoplasm (reviewed in Peters et al., 1996; Schmaljohn, 1996). Co-infection of either a tospovirus (Duijsings et al., 1999) or a tenuivirus (Estabrook et al., 1998) and an RNA virus known to solely multiply in the cytoplasm indicated that for tospoviruses and tenuiviruses, transcription and most likely replication, occur in the cytoplasm.

Recently, Cornu and de la Torre (2001) and Lopez et al. (2002) showed that the Z protein (see below) could inhibit transcription and replication of LCMV and TCRV arenaviruses respectively, but not encapsidation.

The Z protein (v-encoded protein) is a small RING type (cysteine-rich zinc binding) protein that is highly conserved among arenaviruses (Djavani et al., 1997). It is encoded by the L v-RNA and is present in virions (Salvato et al., 1992). It binds the promyelocytic leukemia protein and was proposed to act as a

molecular mimic of this protein to subvert the cellular machinery needed for viral replication (reviewed in Borden et al., 1998). The Z protein binds ribosomal proteins and eIF-4E (eukaryotic initiation factor 4E; Campbell Dwyer et al., 2000; Kentsis et al., 2001) and represses specific cell translation in a cell culture system. Additionally, eIF-4E can reduce Z translational repression, as supposedly does NP (Campbell Dwyer et al., 2000). Presumably, the balance between the levels of Z protein, eIF-4E and NP regulates the translation of viral mRNAs over cellular mRNAs. A zinc binding activity has been shown for the N protein of JUNV arenavirus (Tortorici et al., 2001) indicating that NP might balance the Z protein effect. In light of the translational requirement mentioned above, the action of the Z protein on translation could possibly be linked to its inhibitory effect on viral RNA synthesis (Lee et al., 2000; Cornu and de la Torre, 2001; Lopez et al., 2002).

The Z protein was previously reported to be required for TCRV arenavirus transcription and replication (Garcin et al., 1993), contrary to the recent observations on LCMV and TCRV arenaviruses mentioned above (Lee et al., 2000; Cornu and de la Torre, 2001; Lopez et al., 2002). This discrepancy could be due to the fact that the first experiments performed on TCRV arenavirus probably affected unknown cellular factors that interact with the Z protein and are required for viral RNA synthesis.

No equivalent of the Z protein has been reported for other ambisense viruses. Although the bunyaviral NSs protein has been shown to inhibit viral RNA synthesis (Weber et al., 2001), the phleboviral NSs protein appears not to be necessary for transcription/replication (Billecocq et al., 2000; Accardi et al., 2001; Flick and Pettersson, 2001). NSs exists only in three (*Bunyavirus*, *Phlebovirus* and *Tospovirus*) of the five genera of the Bunyaviridae. For bunyaviruses, the NSs and NP genes overlap, whereas for phleboviruses and tospoviruses these two genes are ambisense. Possibly, the NSs proteins of different genera have different functions. Although its role in transcription/replication is unclear, the NSs protein of both phleboviruses and bunyaviruses appear to be antagonist of the interferon response (Bouloy et al., 2001; Bridgen et al., 2001).

6. Evolutionary relationships

An interesting feature that might concern the ambisense strategy was reported for LACV bunyavirus. Although bunyaviruses are negative RNA viruses, for LACV virus, synthesis of pseudo 'v-mRNAs' was detected (Hacker et al., 1990). These RNAs, designated anti-mRNAs, are heterogeneous in length. They are synthesized at very low levels and have non-viral extensions and a cap structure, as do the LAC viral mRNAs. However, they contain no ORF and would be non-functional as mRNAs. Their putative function in the viral cycle is unknown, but their presence indicates that negative and ambisense species of the Bunyaviridae family might be more closely related than assumed. This probably reflects a common evolutionary ancestor. Bunyaviruses could have recently lost their ambisense coding strategy to become negative RNA viruses. Conversely, by convergent evolution, they might be in the process of evolving towards the ambisense strategy: a negative segment transforming itself into an ambisense RNA by acquiring a new gene in the opposite polarity (reviewed in Elliott, 1996b). Hence, ambisense viruses with their many features resembling those of negative RNA viruses might derive from negative RNA virus ancestors.

The ambisense coding strategy could have arisen from errors of the RdRp that would have switched template during replication using a copy-choice-type mechanism (reviewed in Lai, 1992). Since the 5′ and 3′ ends of ambisense RNAs are conserved and the 5′ ends of the corresponding mRNAs are also conserved, a homologous aberrant recombination event could have occurred.

Evidence of recombination in the NP ORF and 3′ UTR of the S segment of Tula hantavirus (Sibold et al., 1999) and in the 5′ end of the NP gene, the IR or 3′ end of the GP gene for Whitewater Arroyo arenavirus (Charrel et al., 2001) exists. Recently, recombination events have been obtained in Tula hantavirus in a transfection system; the recombination events described occurred mostly in frame, in the NP ORF of the S RNA (Plyusnin et al., 2002). Studies by Inoue-Nagata et al. (1998) on the formation of TSWV tospovirus defective interfering (DI) RNAs indicate that the DI RNAs result from a recombination event. In the same study, a DI RNA was shown to possess a dimeric structure; the linkage point between the two monomers of this dimeric DI RNA has the exact 3′ and 5′ ends of the L RNA sequence of TSWV tospovirus. A similar recombination event could have been at the origin of the association of two genes yielding an ambisense RNA. Such recombination events associating two genes presupposes that the IR created between the two genes resembles either the 5′ UTR of one strand or the 3′ UTR of the other strand or a combination of both depending on the position where the RdRp switched template. The 5′ UTR of bunyaviruses is highly structured and has been shown to trigger early termination of replication associated with translational requirement (reviewed in Kolakofsky and Hacker, 1991). If during recombination of two RNA segments, part of the 5′ UTR becomes the IR, it might be rich in secondary structures that could trigger transcription termination. Because not all ambisense viruses possess an IR rich in secondary structures, the IR might have been created mainly with part of the 3′ UTR. In this case, a replication termination signal might

have degenerated into a transcription termination signal. These two possibilities are not exclusive and the IR might be rich in secondary structures and contain a termination signal.

Another question that could be asked is why IRs exist at all. Recombination might have given rise to overlapping ORFs, as is the case for the S segment of bunyaviruses (see Table 1). Studies of DI RNAs of different viruses suggest that maintaining the ORFs is essential for survival of DI RNAs (Schmaljohn, 1996; Inoue-Nagata et al., 1998 and references therein). This indicates that, in some cases, there is a preferential selection of DI RNAs with functional ORFs. The formation and maintenance of ambisense RNAs might have had the same requirements. Therefore, the two ORFs of an ambisense RNA would not overlap and the existence of the IR would be a necessity.

7. Concluding remarks

The ambisense coding strategy is an unusual way of encoding genes that presumably allows the virus to temporally control expression of the viral proteins, in particular if, as observed for RHBV tenuivirus, coupling of translation to transcription enhances the level of vc-encoded versus v-encoded protein expression. In any event, translation itself and/or translational control appear to play an important role in regulation of gene expression of ambisense viruses.

Ambisense viruses have two hosts in which they can replicate. In their vector or reservoir host, infection is usually asymptomatic. However, in another host, multiplication of the virus can be lethal. A differential behavior has been observed for LACV bunyavirus: in mosquito cells, mRNA synthesis required no concurrent translation, whereas in baby hamster kidney cells, translation was necessary (Raju et al., 1989). Interestingly, ribavirin, a compound chemically related to the m^7G cap structure, is used to cure arenaviral infection, although its mechanism of action is unknown (reviewed in Southern, 1996). Moreover, RNA synthesis of ambisense viruses, such as that of arenaviruses replication and transcription and TSWV mRNA synthesis, appear to require translation. Taken together, these results suggest that the severity of symptoms could theoretically be linked to perturbation of the host cell translation machinery by the virus. Replication/transcription experiments in different host cell types would be helpful to shed further light on the differences observed in different hosts. At present, there are many complementary ways to study ambisense virus replication/transcription such as cell culture, in vitro assays and reverse genetics systems. Since ambisense viruses are the meeting point of different viral families and are able to replicate in different hosts whether plants or animals and have different behaviors depending on the host, it would be particularly important to better understand the complex replicative cycle of ambisense viruses, in order to find the means to alleviate the lethal aspects of these pathogens.

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