Ambisense coding strategy of the rice stripe virus genome: in vitro translation studies

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Rice stripe virus (RSV), the type species of the tenuivirus group, contains four RNA segments as its genome. Sequence analyses of the three smaller segments indicated that all of them have ambisense coding strategies. To examine the ambisense nature of the genomic RNAs, we synthesized open reading frames (ORFs) by transcribing cDNA clones for RNA segments 2, 3 and 4 in both directions using T7 RNA polymerase and translated each RNA in vitro using two systems: reticulocyte lysates and wheat-germ extracts. We detected the proteins encoded by the ORFs present in the 5′-proximal regions of both viral RNAs (vRNAs) and their complementary RNAs (cRNAs). Translation in vitro of total vRNA generated proteins encoded by the ORFs present in the 5′ regions of vRNAs. The overall results are consistent with the prediction that RSV RNAs, at least up to segment 2, are ambisense in their coding strategy.

Introduction

Rice stripe virus (RSV) is transmitted by planthoppers and replicates in both plant and insect hosts (for reviews, see Toriyama, 1986a; Francki et al., 1991). RSV is the type species of the tenuivirus group of plant RNA viruses, which includes maize stripe virus (MStpV), rice grassy stunt virus and rice hoja blanca virus (RHBV) (Gingery et al., 1981; Hibino et al., 1985; Morales & Niessen, 1983). Four (RSV and RHBV) or five (MStpV) segments each of both single-stranded [henceforth referred to as viral RNA (vRNA) in this report] and double-stranded RNAs [duplex of vRNA and its complementary RNA (cRNA)] (Falk & Tsai, 1984; Ishikawa et al., 1989; Toriyama & Watanabe, 1989) can be isolated from purified particles of this group of viruses.

The 5′- and 3′-terminal sequences of single-stranded vRNA from RSV are conserved among the four segments and are complementary to each other (Takahashi et al., 1990), indicating that vRNA is potentially able to form an intramolecular secondary structure in the shape of a panhandle, as found for the genomic RNA of negative-strand animal RNA viruses (Honda et al., 1990; Hsu et al., 1987). Such a unique secondary structure is believed to be important for specific recognition by vRNA-dependent RNA polymerase (reviewed in Ishihama & Nagata, 1988). The nucleotide sequences have been determined for vRNA segments 3 and 4 from two RSV strains (Kakutani et al., 1990, 1991; Zhu et al., 1991, 1992). Recently, we cloned and sequenced the cDNA for RNA segment 2 (Takahashi et al., 1993). The overall results indicate that all the RNA segments sequenced so far have ambisense coding strategies, each containing at least one open reading frame (ORF) at the 5′-terminal region on both the vRNA and cRNA strands (see Fig. 1). In agreement with this coding strategy, the RNA-dependent RNA polymerase is associated with virions (Toriyama, 1986b) and the RNA polymerase solubilized from RSV virions recognizes the 3′-terminal conserved sequence of vRNA as the promoter for transcription (Barbier et al., 1992).

The ambisense coding strategy of RSV RNA, however, has not been proven hitherto. Here we describe the results of in vitro translation of RNAs transcribed from cDNA clones each containing a specific ORF predicted from the cDNA sequence and of total vRNA isolated from virions, which indicate that RNA segments 4 and 3 (and possibly 2) are indeed ambisense in their coding strategy.

Methods

Construction of cDNA plasmids. Full-sized cDNAs for RNA segments 3 and 4 were prepared as described previously (Zhu et al., 1991, 1992). Fragments including the complete ORFs located at both ends of each cDNA were isolated and inserted into an expression vector, pET3b, between XbaI and BamHI sites, to generate the expression plasmids pRSV4v, pRSV4c, pRSV3v and pRSV3c (Fig. 1). For the construction of pRSV3c, pRSV3v and pRSV4v, the 5′ termini of cDNAs were directly ligated to the XbaI site of pET3b whereas the 3′ termini were ligated to the vector after blunting. For the construction of pRSV4c, the 3′ terminus of the cDNA fragment was converted to a
Fig. 1. Construction of expression plasmids for RSV-encoded proteins. Major ORFs on vRNA and cRNA of RSV segments 2, 3 and 4 are shown by the hatched bar. For construction of the plasmids expressing these ORFs, cDNA clones were cleaved with the appropriate restriction enzymes indicated on each cDNA fragment and inserted into the expression plasmid pET3b.

**BamHI** site by adding a **BamHI** linker and then ligated into pET3b, the **XhoI** site of which had been converted to a **BamHI** site by adding the linker.

Full-sized cDNA for RNA segment 2 was prepared by ligation of two cDNA fragments (Takahashi et al., 1993). A **BamHI**–**KpnI** fragment including the entire ORF in the 5'-terminal region of vRNA 2 was inserted into pET3b between **BamHI** and **XhoI** sites to generate pRSV2v. Likewise, a **BamHI**–**KpnI** fragment encoding the amino-terminal 754 amino acid residues of the ORF in the 5'-proximal region of cRNA 2 (the complete p2c ORF is composed of 834 codons) was inserted into the expression plasmid pET3b between **BamHI** and **XhoI** sites, leading to formation of the recombinant plasmid pRSV2c.

**Transcription in vitro of cDNA and translation in vitro**. vRNAs and cRNAs were prepared by transcribing cDNAs using T7 RNA polymerase (Takara). For this purpose, the expression plasmids were cleaved at either **XhoI** or **ClaI** sites within the vector, and the linearized plasmids were transcribed in vitro by T7 RNA polymerase. Since the sequences between the T7 promoter in the expression plasmids and the cDNA inserts, ligated at the **XhoI** site, did not contain an ATG codon in phase with the viral ORFs, we could expect the synthesis of full-sized intact viral proteins without any extra N-terminal sequences encoded by the vector. After in vitro transcription under standard reaction conditions in the presence of 0.5 mM of cap monomer (m7GpppG), the RNA products were isolated by treatment with phenol–chloroform.

Transcripts thus obtained were translated in vitro using two cell-free translation kits, prepared from reticulocyte lysates (Wako) and wheatgerm lysates (Amersham). Protein synthesis in vitro was carried out at either 30 °C (reticulocyte system) or 25 °C (wheatgerm system) for 60 min as recommended by the manufacturers. Products labelled with either [35S]methionine (Amersham) or [3H]leucine (Amersham) were analysed by gel electrophoresis and fluorography.

**Results**

**Translation in vitro in a reticulocyte lysate**

Transcripts derived in vitro from cDNA clones were translated in vitro using a reticulocyte lysate. Fig. 2 shows an example of autoradiograms of the translation products directed by two different concentrations of RNA (0.02 and 0.10 mg/ml). Viral RNAs corresponding...
Table 1. In vitro translation products of RSV RNAs

<table>
<thead>
<tr>
<th>RNA</th>
<th>Protein</th>
<th>Predicted*</th>
<th>Reticulocyte</th>
<th>Wheatgerm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA2c</td>
<td>p2c†</td>
<td>85.3 (754 aa)</td>
<td>(37), 33, 22</td>
<td>54, 41, (33), 26</td>
</tr>
<tr>
<td>RNA2v</td>
<td>p2v</td>
<td>22.8 (199 aa)</td>
<td>26, 25, 24</td>
<td>26</td>
</tr>
<tr>
<td>RNA3c</td>
<td>p3c</td>
<td>35.1 (322 aa)</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>RNA3v</td>
<td>p3v</td>
<td>23.9 (211 aa)</td>
<td>27, 26</td>
<td>27, 26</td>
</tr>
<tr>
<td>RNA4c</td>
<td>p4c</td>
<td>32.4 (286 aa)</td>
<td>32, (29)</td>
<td>32, 29</td>
</tr>
<tr>
<td>RNA4v</td>
<td>p4v</td>
<td>20.5 (178 aa)</td>
<td>22</td>
<td>22</td>
</tr>
</tbody>
</table>

* The $M_r$ of the protein encoded by the 5'-proximal major ORF of each RNA was estimated from the published RNA sequences: RNA2c and RNA2v (Takahashi et al., 1993); RNA3c and RNA3v (Zhu et al., 1991); RNA4c and RNA4v (Zhu et al., 1992). aa, Amino acids.
† p2c (85-3K) is an incomplete polypeptide of ORF p2c (94K), estimated from the pRSV-2c clone used for the synthesis of RNA2c. 
‡ Major translation products in each reaction are underlined; proteins in parentheses are products detected in some experiments.

to segments 3 and 4 (henceforth referred to as RNA3v and RNA4v), prepared by transcribing pRSV3v and pRSV4v, respectively, were efficiently translated (lanes 8 and 9, and 12 and 13), yielding products, p3v and p4v, of apparent $M_r$s of 27K and 22K respectively, as measured by electrophoretic mobility. The sizes of these products are close to those expected from the ORFs (p3v, 23-9K; p4v, 20-5K) located on the 5'-proximal regions of vRNA 3 and 4 (Table 1).

RNA3c and RNA4c (transcripts from pRSV3c and pRSV4c) contain the ORFs of the 5'-proximal region of cRNAs 3 and 4, respectively. Translation products from these RNAs each gave a single major band (lanes 6 and 7, and 10 and 11) of $M_r$s 34K (p3c) and 32K (p4c) expected from the corresponding ORF (p3c, 35-1K; p4c, 32-4K; Table 1), indicating that these ORFs on cRNAs 3 and 4 can be translated into proteins. Even after correction for the methionine content (10, 4, 7 and 5 for p3c, p3v, p4c and p4v, respectively), the level of protein synthesis was the highest for RNA4v, followed by RNA3v and RNA4c, and the lowest for RNA3c.

In contrast, no discrete band of translation products was detected for RNA2c and RNA2v at the same concentrations used for translation of segment 3 and 4 RNAs. When the RNA concentration was increased to 0.4 mg/ml or the same amount (0.02 and 0.10 mg/ml) of RNA was translated using a wheatgerm extract in place of reticulocyte lysate, translation products were detected for both RNA2c and RNA2v (see Fig. 4), suggesting that the efficiency of translation in the reticulocyte lysate is very low for transcripts from segment 2 cDNAs.

Translation in wheatgerm extract

The same set of RNAs was translated in an extract from wheatgerm, derived from one of the host plants, and the results of gel electrophoresis are shown in Fig. 3. The $M_r$ values of the major translation products from RNA3v, RNA4v, RNA3c and RNA4c were apparently identical.
Fig. 4. Comparison of translation products in reticulocyte lysate and wheatgerm extract. Translations in vitro of RSV vRNA and cRNA were carried out as described in Fig. 2 and 3 using two different systems, a reticulocyte lysate and a wheatgerm extract. Approximately the same amounts (on the basis of total radioactivity) of labelled products were analysed in parallel (lanes 1, 3, 5, 7, 9, 11 and 13, reticulocyte system; lanes 2, 4, 6, 8, 10 and 12, wheatgerm system) using an SDS-15% polyacrylamide gel. Lanes 1 and 2, no RNA template; lanes 3 and 4, RNA2c; lanes 5 and 6, RNA2v; lanes 7 and 8, RNA3c; lanes 9 and 10, RNA3v; lanes 11 and 12, RNA4c; lanes 13 and 14, RNA4v.

with those made in the reticulocyte lysate, but their levels differed significantly between the two translation systems. In the wheatgerm extract, the efficiency of translation was highest for RNA4v, whereas that of RNA4c was markedly reduced. In addition, several discrete bands of smaller polypeptide products were identified for RNA4c (lanes 10 and 11).

Translation of RNA2c and RNA2v in the wheatgerm extract gave several discrete translation products. A single major polypeptide ($M_r$, 26K) was detected for RNA2v (lanes 4 and 5), which is close to the $M_r$, 22-7K expected from the RNA sequence. On the other hand, several bands were detected for translation products of RNA2c (lanes 2 and 3), with $M_r$ values of 54K, 41K and 26K. The pRSV2c used for preparation of RNA was expected to generate a polypeptide of 754 amino acid residues ($M_r$, 85K). These translation products may represent partial N-terminal fragments of the ORF in the 5'-proximal region of RNA2c.

**Comparison of products between two translation systems**

To compare directly the size and the relative level of translation products made in the reticulocyte lysate and the wheatgerm extract, we performed gel electrophoretic analysis of the translation products in parallel (Fig. 4).

The mobilities of the major products from RNA3c (lanes 7 and 8), RNA3v (lanes 9 and 10), RNA4c (lanes 11 and 12) and RNA4v (lanes 13 and 14) were identical, within the range of resolution, indicating that the 5'-proximal major ORFs from both vRNA and cRNA of the genome segments 3 and 4 were translated in both the reticulocyte and wheatgerm extract although the relative efficiencies of translation varied with the system and the RNA species. In a high resolution gel system, p3v was separated into two components ($M_r$, 27K and 26K).

Of the two (or three) bands made in the reticulocyte lysate from RNA2v, the slowly migrating 26K component coincided in mobility with the product made in the wheatgerm extract (lanes 5 and 6). Translation of RNA2c was more efficient in the wheatgerm extract than in the reticulocyte lysate, producing several large polypeptides (41K, 54K, 75K and about 90K), suggesting that a small amount of the full-length product (85K protein) was synthesized in the wheatgerm extract.

**Identification of genes for the coat protein and the major non-structural (NS) protein**

Two viral proteins, the coat protein of 35K (the major structural component) and the putative RNA polymerase protein of 230K, have been identified in purified RSV.
Ambisense genome of RSV

1129

(a)

(b)

Fig. 5. Immunoprecipitation of translation products. Translation in vitro was carried out using a reticulocyte lysate and [35S]methionine, and labelled products were immunoprecipitated using antibodies against RSV virion or NS4 proteins. Immunoprecipitation was carried out according to the method of Yasuda et al. (unpublished results).

(a) Mixture of translation products of RNA3c and RNA3v; (b) mixture of translation products of RNA4c and -4v. Lanes 1, translation products; lanes 2, preimmune serum; lanes 3, anti-virion (almost equivalent to anti-coat protein) serum; lanes 4, anti-NS4 serum.

particles. In RSV-infected plants, a major NS protein of 21K accumulates at a high level. We prepared antibodies in rabbits against these three proteins, purified by gel electrophoresis, as well as against whole virions. Mixtures of the translation products were incubated with these antibodies, and immunoprecipitates were analysed by gel electrophoresis and fluorography.

When each translation product was tested separately, the anti-coat protein antiserum (and the anti-virion antiserum) cross-reacted only with p3c. When a mixture of p3v and p3c was analysed, only p3c was precipitated (Fig. 5a, lane 3). Likewise, the anti-major NS protein serum cross-reacted only with p4v, and precipitated a single component from a mixture of p4c and p4v (Fig. 5b, lane 4). Thus, we concluded that (i) the coat protein is coded for by cRNA of segment 3; and (ii) the major NS protein of 23K is encoded by a gene located in the 5'-proximal region of vRNA segment 4 (hence we designated this major NS protein NS4). These results agree with the coding strategy predicted based from the sequence analyses. However we could not detect any protein cross-reacting with the anti-230K protein, the putative RNA polymerase, present in virus particles (data not shown). This is consistent with the prediction, deduced from comparison of the size of protein and RNA, that the 230K protein is encoded by the largest RNA segment 1 (Takahashi et al., 1993).

Translation in vitro of virion RNA

Finally, in vitro translation of RNA from purified virus particles was carried out. Since the content of dsRNA is less than 10% the level of single-stranded vRNA in the virus preparations used, the major products of in vitro translation might represent proteins encoded by the 5'-proximal regions of vRNA segments. Total RNA extracted from purified virions was translated using the reticulocyte lysate. As shown in Fig. 6(a) (lanes 2 to 5), three major products were identified with apparent M, values of 39K, 27K and 22K, respectively. The mobility of the smallest component agreed well with that of the NS4 protein encoded by RNA4v (p4v). The most abundant product of 25K was considered to be the product of vRNA segment 3 (p3v), based on its electrophoretic mobility. To confirm this prediction, we isolated RNA segments 3 and 4 by gel electrophoresis and carried out in vitro translations separately. The result, shown in Fig. 6(b), clearly indicated that vRNAs 3 and 4 encode 27K/26K (p3v) and 22K (p4v) products, respectively.

The origin of the most slowly migrating component (M, 39K) is not known yet; it might represent the putative product of either vRNA 2 (p2v) or vRNA 1 (p1v). Sequence analysis, however, indicates that vRNA 2 codes for a protein of 22.7K (Takahashi et al., 1993). We could not detect a band corresponding to the coat protein, as expected because it is encoded by the complementary strand of single-strand vRNA segment 3. To detect translation products from cRNA, we then tried in vitro translation after heat-denaturation of total viral RNA. However, we still failed to detect coat protein synthesis, presumably because the amount of cRNA in the viral RNA preparation is too low for detection. Likewise, we could not detect the putative RNA polymerase protein of 230K. One possibility is that the RNA polymerase is encoded by cRNA of segment 1. This possibility is being examined by making a full-length cDNA copy of RNA segment 1.

Discussion

In this study, we presented the first experimental evidence indicating that at least the three smaller segments (vRNAs 2, 3 and 4) of the RSV RNA genome are ambisense in coding strategy. The translation product derived from vRNA 2 corresponded in size with the 5'-
Fig. 6. Translation in vitro of virion RNA. (a) Translation in vitro was carried out in reticulocyte lysate using untreated (lanes 2 and 4) or heat-denatured (90 °C for 5 min) virion RNA (lanes 3 and 5). Products labelled with [35S]methionine were electrophoresed in a 15% polyacrylamide gel together with the translation products of in vitro transcripts. Lane 1, no RNA template; lanes 2 and 3, 36 µg virion RNA; lanes 4 and 5, 0.36 µg virion RNA; lanes 6 to 11, RNA2c, RNA2v, RNA3c, RNA3v, RNA4c and RNA4v in that order.

(b) Translation in vitro was carried out in wheatgerm lysate using [3H]leucine as a labelled substrate and in the absence (lane 1) or presence of total virion RNA (lanes 2 and 3), isolated RNA 3 (lanes 4 and 6) or RNA 4 (lanes 5 and 7). RNA segments 3 and 4 were isolated by electrophoresis on a 2.4% gel followed by elution with 0.5 N-ammonium acetate/1 mM-EDTA/0.1% SDS solution. In reactions 3, 6 and 7, total wheatgerm tRNA (5 µg) was included. Products were electrophoresed in a 10% gel.

proximal ORF on vRNA, whereas the size of the protein product from the 5' region of cRNA 2 is not clear yet because the cDNA clone used in this study lacked the 3'-proximal portion of the ORF on cRNA 2. Nevertheless, it is clear that the complementary strand of vRNA 2 can be translated into proteins(s). In the case of MStpV, the ambisense coding strategy was shown for vRNA segments 3 and 4 by analysis of subgenomic RNA from infected plants (Huiet et al., 1991, 1992) or in vitro translation of transcripts from cDNA (Huiet et al., 1991). In vitro translation of RHBV vRNA indicated that its coat protein is not coded for by vRNA (Ramirez et al., 1992).

In the case of ambisense viral genome, it is reasonable to expect that the proteins translated directly from vRNA, such as the NS4 protein, are components involved in the early phase of the virus replication cycle. On the other hand, other proteins, such as the coat protein, which could be synthesized only after synthesis of cRNA, might be required in the late stages of the virus replication cycle. This consideration is consistent with our recent finding that p2c (the putative product of RNA2c) has sequence stretches similar to parts of the surface glycoproteins of some animal RNA viruses (Takahashi et al., 1993).

From the RNA sequence comparisons, it was suggested that RSV shares a low but significant level of similarity with animal bunyaviruses (Kakutani et al., 1991). On this basis, it is proposed that plant tenuiviruses share the same origin as animal bunyaviruses. Most animal bunyaviruses, however, carry negative-strand RNA segments in their genome, and only a few virus species belonging to this family (the Phlebovirus and Uukuvirus genera) carry one ambisense RNA among their three genomic RNA segments (Elliott, 1990). Recently, tomato spotted wilt virus (Kormelink et al., 1992) and impatiens necrotic spot virus (Law et al., 1992) belonging to the Tospovirus genus of the plant Bunyaviridae family were found to carry two ambisense RNA among their three genome RNA segments. On the other hand, some animal arenaviruses carry two ambisense RNA segments (Bishop & Auperin, 1987). However, the overall composition of the genome varies between these viruses and plant tenuiviruses. For instance, tenuiviruses contain four (RSV, RHBV) or five (MStpV) RNA segments as the genome, among which at least three (RSV) or two (MStpV) small segments have been suggested to have an ambisense coding strategy (Takahashi et al., 1993; and other work cited there).

We thank M. Takahashi for preparation of RSV, M. Yamagishi and N. Fujita for technical advice and discussion, and R. S. Hayward for critical reading of the manuscript. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan, and the Ministry of Agriculture, Forestry and Fisheries of Japan.
Japan, the Joint Research Program of The Graduate University for Advanced Studies, and the NIG Cooperative Research Programmes.

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(Received 19 November 1992; Accepted 1 February 1993)