ジャガイモXウイルス接種タバコプロトプラストから分離した粗膜画分におけるウイルスRNAのinvitro合成

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In Vitro Viral RNA Synthesis in a Crude Membrane Fraction from Tobacco Protoplasts Inoculated with Potato Virus X

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Abstract

A membrane fraction was isolated by differential centrifugation from PVX-inoculated tobacco protoplasts to investigate *in vitro* viral RNA synthesis. This fraction was capable of catalyzing the *in vitro* synthesis of a genomic-length (6.4 kb) and two subgenomic-length RNAs (2.1 and 0.9 kb). The different sensitivities of the RNAs to S1 nuclease at high and low salt concentrations suggested that the synthesized genomic-length RNA was mainly the replicative intermediate RNA (RI) and that the two subgenomic-length RNAs were single-stranded. Most of the synthesized RNAs were shown to be of a positive polarity based on ribonuclease protection assay. Dependent on the exogenous template, the fraction treated with micrococcal nuclease synthesized only the genomic-length RNA but not the subgenomic RNAs. Treating the membrane fraction with protease abolished synthesis of the subgenomic-length RNAs, but not that of genomic-length RNA. The synthesizing activity for the subgenomic-length RNAs was released into the soluble fraction after treatment with the detergent Brij 58. These results suggested that genomic and subgenomic RNAs are replicated at different sites in the membrane; genomic RNA synthesis occurs inside the membranes, whereas subgenomic RNA synthesis occurs on their surface.

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Key words: potato virus X, tobacco protoplasts, RNA polymerase, genomic and subgenomic RNA.

INTRODUCTION

Various studies with plus-strand RNA viruses indicated that viral RNA replication requires both virus-encoded replicase subunits and host components, and that these components are membrane-associated[6,7,9,14]. Despite much effort, most of the viral replicases completely or partially lose their activity during purification. Even if the activity is retained, except for a few viruses[8], most replicases can only synthesize replicative intermediates (RI) from plus-strand RNA template. Therefore, the mechanisms of viral RNA replication are still unclear.

Potato virus X (PVX) is the type member of the potexvirus group. Its genomic RNA is a single-stranded, positive-sense RNA, which has a cap structure at the 5′ terminus and a poly (A) tract at the 3′ end[3]. The complete nucleotide sequence of PVX has already been determined[10]. The genomic RNA is about 6.4 kb in length and contains at least five open reading frames (ORFs) coding for proteins of 165 kDa (ORF 1), 24 kDa (ORF 2), 12 kDa (ORF 3), 8 kDa (ORF 4) and 25 kDa (ORF 5, coat protein cistron). The 165 kDa protein is presumed to be the viral RNA replicase as it is homologous to the RNA polymerase of other viruses. ORFs 2, 3 and 4 partially overlap and are referred to as the triple gene block. The triple gene block products are involved in cell-to-cell movement[2]. The 25 kDa protein encapsidates viral RNA and is necessary for the spread of the virus throughout a plant[1,5]. In infected tissues, two major subgenomic RNAs of 0.9 kb and 2.1 kb are present as well as the genomic RNA[11].

Although PVX has been well characterized at the molecular level in genome structure and gene expression, much remains unknown about the replication of PVX RNA in the host cells. In this paper, we describe *in vitro* viral RNA synthesis in a crude membrane fraction from PVX-inoculated tobacco protoplasts, as well as some characteristics of viral RNA polymerase activity in the membranes, to gain clues to understanding the precise replication mechanism of PVX-RNA.

MATERIALS AND METHODS

Virus and cells PVX common strain (PVX-O)[16] was used throughout the experiments. Virions and viral RNA were prepared as described by Huisman *et al.* (1988)[10]. Tobacco culture cells (BY-2) were kept as a suspension[17]. Protoplasts were prepared from BY-2 cells

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by incubation in 1% Cellulase Onozuka RS (Yakult Honsha Co.), 1% Driselase (Kyowa Hakko Kogyo Co.), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co.), and 0.4 M D-mannitol (pH 5.5) at 28°C for 2 hr. The protoplasts were rinsed with three changes of 0.4 M mannitol.

Inoculation of protoplasts with viral RNA Tobacco protoplasts (2×10^6) prepared from the BY-2 cells were suspended in 0.8 ml of ice-cold cell suspension solution[17] and then mixed with 50 μg PVX-RNA. Thereafter, electroporation was carried out according to Watanabe *et al.* (1987)[18]. The protoplasts were washed three times with a 0.4 M mannitol solution to remove unincorporated PVX-RNA, then suspended in a 10 ml culture medium and incubated at 28°C.

Fractionation of the extracts from PVX-inoculated tobacco protoplasts PVX-RNA- or mock-inoculated tobacco BY-2 protoplasts (2×10^6) were homogenized in 0.5 ml of buffer A [50 mM Tris-HCl, 15 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM EGTA, 0.1 mM phenylmethyl sulphonylfluoride and 1 μg/ml leupeptin]. The homogenate was centrifuged at $5000\times g$ for 10 min, and the supernatant was centrifuged at $30,000\times g$ for 30 min. The resulting pellet was resuspended in 25 μl of buffer B [50 mM Tris-HCl, 50 mM KCl, 10 mM DTT, 20% glycerol and 1 mM EGTA, (pH 8.0)]. This fraction (P_3 fraction) was used for the RNA polymerase activity assay.

RNA polymerase assays and analysis of in vitro synthesized RNA The standard RNA polymerase assay mixture contained 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 5 mM DTT, 5% glycerol, 1 mM of ATP, GTP, and CTP, 100 μM UTP, 5 μCi [α-^{32}P]UTP(400-800 Ci/mmol), 2 units/ml RNase inhibitor, 25 μg/ml of actinomycin D (AMD) and 20 μl of the P_3 fraction in a total volume of 200 μl. The reaction mixture was incubated at 28°C for 60 min, and the reaction was terminated by the addition of 100 μl of buffer C [50 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 0.25% SDS, 1% sodium dodecyl-*N*-sarcosinate, 0.4% sodium deoxycholate]. After incubation for another 15 min, the synthesized products in the reaction mixture were extracted with phenol/chloroform/isoamylalcohol (v/v: 25/24/1), precipitated with ethanol, then analyzed by 1.2% agarose gel electrophoresis under non-denaturing or denaturing (formaldehyde) conditions, followed by autoradiography. PVX-virion RNA and λ-DNA digested with *Hin*dIII or Perfect RNA™ Markers (Novagen) were co-electrophoresed as markers.

Treatment of RNA polymerase reaction products with nuclease S1 Total RNA products generated by the P_3 fraction were resuspended in S1 buffer [280 mM NaCl, 50 mM sodium acetate (pH 4.5), 4.5 mM $ZnSO_4$, 20 μg/ml of denatured salmon sperm DNA]. S1 nuclease was added to make a final concentration of 5000 units/ml. Samples were incubated at 37°C for 60 min, and analyzed by 1.2% denaturing agarose gel electrophoresis, followed by autoradiography.

Construction of recombinant plasmid and in vitro transcription cDNA corresponding to the 3′ terminal half of PVX RNA was synthesized by the method of Gubler and Hoffman (1983) using a modified kit (Amersham) and cloned into pUC18 to give plasmid pXT165. cDNA fragments corresponding to two regions of nucleotides 3461-4058 (ORF 1) and 5717-6066 (ORF 5) of the PVX genome were excised from plasmid pXT165 with *Hin*dIII/*Eco*RI sites for ORF 1 and the *Pst*I site for ORF 5, respectively, and further subcloned into the *Hin*dIII and *Eco*RI sites and the *Pst*I site of plasmid pSPT18 (Novagen), a transcription vector, respectively, which contains the two promoters of T7 RNA polymerase and SP6 RNA polymerase. The resultant plasmids were referred to as pSPT 165-1 for ORF 1 and pSPT165-2 for ORF 5.

Plasmids pSPT 165-1 and pSPT 165-2 were transcribed with T7 RNA polymerase or SP6 polymerase after linearization with *Hin*dIII or *Eco*RI, to give plus-strand or minus-strand RNA transcripts, respectively. The transcription reaction was performed using a Boehringer Mannheim Transcription Kit.

RNase protection assay of RNA polymerase products PVX-related RNAs in the reaction mixture were characterized with a Ribonuclease Protection Assay Kit (Ambion) basically according to the manufacturer's instructions. RNAs extracted from the reaction mixture were annealed with minus- or plus-strand transcripts from pSPT165-1 or pSPT165-2. The single-stranded RNAs in the mixture were then removed by digestion with 0.25 units/ml of RNase A and 50 units/ml of RNase T_1. The protected double-stranded RNAs were recovered by phenol-chloroform extraction and ethanol precipitation, and analyzed by polyacrylamide gel electrophoresis, followed by autoradiography.

Treatment of the P_3 fraction with micrococcal nuclease The P_3 fraction (50 μl) was supplemented with calcium chloride (final concentration, 1 mM) prior to the addition of 20 units/ml of micrococcal nuclease (Pharmacia). The mixture was filled with sterilized deionized water to a final volume of 70 μl and incubated at 30°C for 30 min. Nuclease digestion was stopped by adding EGTA to a final concentration of 5 mM. The treated fraction was assayed for RNA polymerase activity under conditions similar to those already described.

Treatment of the P_3 fraction with protease The P_3 fraction (20 μl) was treated with bovine trypsin (0.25%) and/or V8 protease (0.1 mg/ml) from *Staphylococcus aureus* and incubated at 30°C for 3 hr. The treated P_3 fraction was assayed for RNA polymerase activity under the conditions already described. The reaction products were analyzed by 1.2% non-denaturing agarose gel electrophoresis.

Solubilization of viral RNA polymerase with detergents Detergents (Brij 58, CHAPS, NP-40, or Triton X-100) at a final concentration of 1% were added to the P_3 fraction. The mixture was stirred at 4°C for 2 hr, then centrifuged at $30,000\times g$ for 30 min. The resul-

tant pellet was resuspended in buffer B. Both the resuspended pellet and the supernatant were assayed for RNA polymerase activity under the conditions already described.

RESULTS

Detection of RNA polymerase activity in crude membrane fraction prepared from PVX-infected tobacco protoplasts and characterization of their products

The homogenate prepared from PVX-inoculated tobacco protoplasts was fractionated by differential centrifugation. Each fraction was assayed for RNA polymerase activity. The P_3 fraction showed RNA polymerase activity without the addition of the RNA template. When the reaction products were analyzed by non-denaturing agarose gel electrophoresis, they were separated into one broad, heterodispersed band that migrated behind the PVX-genomic RNA (6.4 kb) (referred to as genomic-length RNA) and two bands that migrated to positions corresponding to the 2.1 kb and 0.9 kb of subgenomic RNAs (referred to as subgenomic RNAs) found in PVX-infected plants (Fig. 1a). RNA polymerase activity was not detected in the P_3 fraction from mock-inoculated protoplasts.

The synthesized products were characterized by treatment with nuclease. Whereas they were insensitive to DNase treatment, no bands were detected after treatment with S1 nuclease at low salt concentration in agarose gel electrophoresis, suggesting that these products are RNA molecules. Furthermore, products treated with S1 nuclease at a high salt concentration completely lost the 2.1 kb and 0.9 kb subgenomic RNAs, but gained a clear-cut genomic-length RNA (Fig. 1b). These results indicated that the 2.1 kb and 0.9 kb subgenomic RNAs were single-stranded. The heterodispersed band of genomic length appeared to be composed of both the replicative form (RF) and the replicative intermediate (RI), because the cells supporting viral RNA replication usually contain RF and RI. Since only single-stranded tails of RI could be digested with S1 nuclease under high salt conditions, the resulting RNA molecules seemed to be fully double-stranded RNA of genome size, so that they migrated in agarose gels as a sharp band. The polarity of synthesized viral RNA was determined with the RNase protection assay. When the two probes for the plus-strand were used, two protected fragments were detected. However, the probes for the minus-strand formed no band. These results suggest that the reaction products have plus polarity (Fig. 2).

Omission of one or all of the three unlabeled ribonucleotides ATP, GTP and CTP from the reaction mixture prevented [α-^{32}P] UTP incorporation into PVX-specific RNA bands, indicating that the synthesized RNAs did not result from the terminal labeling by terminal transferase (data not shown). Mg^{2+} ion was required for PVX-RNA polymerase activity; its optimal concentration was in the range of 5 to 10 mM. Higher concentrations of Mg^{2+} caused a gradual

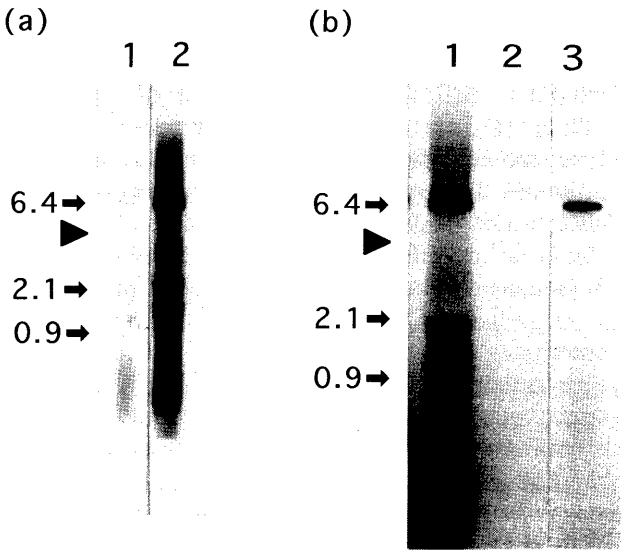

Fig. 1. Analysis of RNA products synthesized *in vitro* by the P_3 fraction prepared from PVX-inoculated tobacco protoplasts. (a) RNA products synthesized *in vitro* by P_3 fraction. Lane 1, mock-inoculated protoplasts; lane 2, PVX-inoculated protoplasts. (b) RNA product digested with S1 nuclease prior to electrophoresis. Lane 1, RNA products without S1 nuclease treatment; lane 2, RNA products treated with S1 nuclease at a low concentration of NaCl (0.1 M); lane 3, RNA products treated with a high concentration of NaCl (0.4 M). In (a) and (b), positions of 6.4 kbp RNA, 2.1 kb RNA, and 0.9 kb RNA are shown at the left. The arrowheads mark the positions of PVX virion RNA.

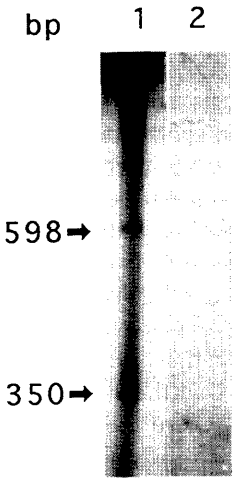

Fig. 2. RNase protection assay of RNA products synthesized *in vitro* by the P_3 fraction prepared from PVX-infected tobacco protoplasts. Minus-strand (lane 1) and plus-strand transcripts (lane 2) were used for assay as described in Materials and Methods. Positions of the protected fragments are indicated at the left.

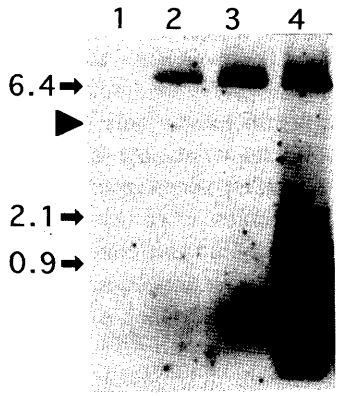

Fig. 3. Analysis of RNA products synthesized *in vitro* by micrococcal nuclease-treated P_3 fraction with or without the addition of exogenous PVX-RNA template. Lane 1, no exogenous template; lanes 2 and 3, 1 and 5 μg/ml PVX-RNA template, respectively; lane 4, without micrococcal nuclease treatment. Positions of 6.4 kbp RNA, 2.1 kb RNA and 0.9 kb RNA are shown at the left. The arrowhead marks the position of PVX virion RNA.

decrease in enzyme activity (data not shown).

Topology of viral RNA synthesis sites in membrane

Electron microscopy of the P_3 fraction revealed many membranous vesicles of various sizes (data not shown); therefore, the RNA polymerase in this fraction was presumed to be associated with membranes. In addition, viral RNA was synthesized in the P_3 fraction without exogenous template RNA. Thus, the viral polymerase, RNA template and possibly host proteins were assumed to form a complex capable of catalyzing the synthesis of viral RNA in membranes. We attempted to determine the topology of the RNA synthesis sites in membranes by treating the P_3 fraction with micrococcal nuclease or protease.

When the P_3 fraction was treated with micrococcal nuclease that digests both single-stranded and double-stranded RNAs, no synthesis of the labeled RNA could be detected, indicating that the endogenous template RNA is fully or partially exposed on the surface of the membranes (Fig. 3). To test the *in vitro* initiation of RNA synthesis by the P_3 fraction that was treated with micrococcal nuclease, PVX-RNA was added as an exogenous template prior to the reaction. Genomic-length RNA was synthesized, but not subgenomic RNAs (Fig. 3), indicating that the exogenous template was able to enter the sites where the endogenous template had been located prior to the nuclease treatment.

The P_3 fraction digested with either V8 protease or trypsin was centrifuged at 30,000$\times g$, then the pellet was assayed for RNA polymerase activity. The polymerase activity for genomic-length RNA remained unaltered, but that for subgenomic RNA disappeared. Two bands did migrate slightly faster than genomic-length RNA in extracts treated with trypsin; the identity of these

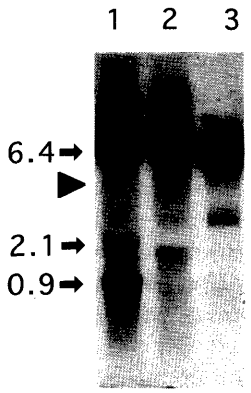

Fig. 4. RNA products synthesized *in vitro* by the P_3 fraction which had been treated with proteases. Lane 1, without protease; lanes 2 and 3, with V8 protease and trypsin, respectively. Positions of genomic 6.4 kbp RNA, 2.1 kb RNA, and 0.9 kb RNA are shown at the left. The arrowhead marks the position of PVX virion RNA.

products have not been verified (Fig. 4). RNA polymerase for genomic RNA, therefore, appears to be integrated into membranes, although that for subgenomic RNAs was exposed on the membranes.

Some proteins are reportedly bound to membranes via a glycosyl phosphatidyl inositol anchor (GPI-anchor), which can be cleaved with phospholipase C (PIPLC)[4]. To examine whether PVX-RNA polymerase was bound to membranes via this anchor, the P_3 fraction was treated with PIPLC and centrifuged at 30,000$\times g$. The resulting supernatant and pellet were tested for RNA polymerase activity. Activity was found in the pelleted membranes, but not in the supernatant (data not shown). Thus, the GPI-anchor does not seem to be involved in the association of RNA polymerase with membranes.

Treatment of membranes with a high salt solution

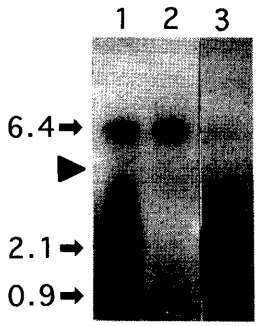

Fig. 5. Solubilization of RNA polymerase from the P_3 fraction with the detergent Brij 58. Lane 1, RNA products synthesized *in vitro* by the P_3 fraction without detergent treatment; lanes 2 and 3, RNA products synthesized *in vitro* by the pellet and supernatant, respectively, that were obtained by centrifugation after the P_3 fraction was treated with the detergents. Positions of genomic 6.4 kbp RNA, 2.1 kb RNA, and 0.9 kb RNA are shown at the left. The arrowhead marks the position of PVX virion RNA.

might wash off all proteins that were peripherally bound to membranes via an ionic interaction. To examine this possibility, the P_3 fraction was treated with 1 M KCl and centrifuged at 30,000×*g*. RNA polymerase activity was not found in the supernatant, and the salt treatment had no effect on RNA polymerase activity in the pellet (data not shown), indicating that the RNA polymerase was not ionically bound to membranes.

Solubilization of viral RNA polymerase

We attempted to solubilize the RNA polymerase from the membranes using several detergents (Brij 58, CHAPS, NP-40 or Triton X-100). Treatment of the P_3 fraction with Brij 58 released RNA polymerase capable of synthesizing two subgenomic RNAs under the direction of the endogenous template, in a form that was no longer pelleted by centrifugation. In this case, polymerase activity for the genomic-length RNA stayed in the pellet after centrifugation at 30,000×*g* without any loss in activity (Fig. 5). All other detergents failed to release the RNA polymerase activity (data not shown).

DISCUSSION

Viral RNA polymerase activity was found in the P_3 fraction prepared from PVX-infected tobacco protoplasts, suggesting an association with membranes, similar to the features of other positive single-stranded RNA viruses[7]. The RNA polymerase found in the P_3 fraction can synthesize not only genomic, but also two subgenomic RNAs found in the infected plants. Treating the P_3 fraction with protease and solubilizing RNA polymerase activity with the detergent Brij 58 suggested that genomic-length RNA and subgenomic RNAs are synthesized at different sites in the membranes, and that the polymerase for genomic RNA may be integrated within the membranes. In contrast, a portion of the polymerase for subgenomic RNAs appeared to be exposed on the outer surface of the membranes, although we cannot rule out the possibility that protease or detergent treatment affected the membranes, thereby altering the location of the viral RNA polymerase.

Although synthesis of genomic RNA and subgenomic RNA *in vitro* by RNA polymerase from virus-infected plants has been reported in several viruses[7-9,12], this is the first suggestion that they are synthesized at different sites in the membranes. The separation of the polymerase for genomic RNA from that for subgenomic RNAs should facilitate our understanding the molecular mechanisms of replication of both RNAs in the PVX life cycle.

Removal of the endogenous template with micrococcal nuclease showed that the template was localized on the surface of the membranes. This result contrasts to RNA polymerase preparations of foxtail mosaic virus, a member of the potexvirus, where the endogenous RNA-template in crude membrane fractions is resistant to micrococcal nuclease treatment[15], but is in accord with that of brome mosaic virus (BMV)[13] and cucumber mosaic virus[8]. When template viral RNA was added to the nuclease-treated P_3 fraction, only genomic-length RNA was synthesized, whereas subgenomic RNA was not. Two possibilities are considered for this result. The P_3 fraction could initiate the synthesis of minus-strand RNA on plus-strand exogenous template RNA, but could not synthesize the plus-strand RNA on the synthesized minus-strand RNA. The subgenomic RNA of BMV is synthesized by internal initiation of plus-strand RNA synthesis on a minus-strand template[12]. Although whether this is also the case for the synthesis of PVX subgenomic RNAs is unclear, they would not be synthesized in the case where the plus-strand RNA could not synthesize on the minus-strand RNA. Another possibility is that the polymerase for subgenomic RNAs was dissociated by micrococcal nuclease digestion of the endogenous template RNA. Further experimentation will be necessary to address their question.

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和 文 摘 要

闕 暁楓・高松則之・細川大二郎：ジャガイモXウイルス接種タバコプロトプラストから分離した粗膜画分におけるウイルスRNAの *in vitro* 合成

ジャガイモXウイルス接種タバコ培養細胞プロトプラストの磨砕液より，内在する鋳型RNAをもとに *in vitro* においてウイルスゲノムRNA (6.4 kb) と二種類のサブゲノムRNA (2.1 kb と 0.9 kb) を合成する粗膜画分を得た。これらのRNA合成産物はRNaseに対する感受性からゲノム長RNAの多くは複製中間体であり，サブゲノム長RNAは一本鎖であることが分かった。また，リボヌクレアーゼプロテクション実験により，これらの合成RNAはほとんどが(+)鎖RNAであった。さらに，本膜画分をmicrococcal nucleaseで処理すると，内在性鋳型RNAは消化され，RNA合成産物はみられなくなったが，PVX-RNAを鋳型として添加するとゲノム長RNAの合成のみが認められ，二種類のサブゲノム長RNAの合成はみられなかった。また，本膜画分をプロテナーゼ (V8プロテナーゼおよびトリプシン) で処理するとゲノム長RNAの合成は影響を受けなかったが，二種類のサブゲノム長RNAの合成活性は消失した。さらに，界面活性剤 (Brij 58) を用いて可溶化を行ったところ，サブゲノム長RNAの合成活性は本膜画分から可溶化されたが，ゲノム長RNAの合成活性は可溶化されなかった。以上の結果から，PVXのゲノムRNAとサブゲノムRNAは膜の異なる部位において合成され，前者は膜の内部に局在するのに対し，後者は膜の表面近くに存在すると推測された。