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Synthesis of the Putative Red Clover Necrotic Mosaic Virus RNA Polymerase by Ribosomal Frameshifting *in Vitro*

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The red clover necrotic mosaic virus (RCNMV) genome is split between two single-stranded RNA species termed RNA-1 and RNA-2. RNA-1 directs the synthesis of 88-kDa (p88), 57-kDa (p57), 37-kDa (p37), and 27-kDa (p27) polypeptides and RNA-2 a 35-kDa (p35) polypeptide *in vitro*. The coding order of the RNA-1 products was determined to be 5′-p27-p57-p37-3′. Antibodies to synthetic peptides representing the carboxyl terminal portions of p27 and p57 immunoprecipitated their respective polypeptides in addition to p88, suggesting that p88 is a fusion protein. A frameshift heptanucleotide sequence element has been identified in RCNMV RNA-1. In addition, a stable stem–loop secondary structure adjacent to the heptanucleotide sequence is predicted. Together, these sequence elements suggest that a ribosomal frameshifting event occurs which allows translational readthrough of the p27 open reading frame into the p57 open reading frame, generating the observed p88 product. An RNA-1 expression construct fusing the p57 and the CP open reading frame was engineered to investigate the ribosomal frameshifting event. CP antibodies immunoprecipitated a fusion protein of the predicted size containing the carboxyl portion of CP. Site-directed mutagenesis of the frameshift element indicates that *in vitro*, p88 can also be expressed alternatively by suppression of an amber termination codon. Based on these data, we propose that the putative RCNMV RNA polymerase is an 88-kDa polypeptide expressed by a ribosomal frameshifting mechanism similar to those utilized by retroviruses.

INTRODUCTION

Red clover necrotic mosaic virus (RCNMV), a member of the dianthovirus group, is characterized by a genome split between two single-stranded RNAs of 3.9 kb (RNA-1) and 1.45 kb (RNA-2). The RNAs are encapsidated together in 30–32 nm icosahedral virions by 180 copies of a 37-kDa capsid protein (CP) (Hollings and Stone, 1977).

The monocistronic RNA-2 contains a single open reading frame (ORF) capable of encoding a 35-kDa polypeptide which has been observed both *in vitro* (Lommel *et al.*, 1988) and *in vivo* (Osman and Buck, 1991). The RCNMV cell-to-cell movement activity has been genetically mapped to RNA-2 (Osman and Buck, 1987; Paje-Manalo and Lommel, 1989).

The polycistronic RNA-1 contains three ORFs capable of encoding polypeptides of 27, 57, and 37 kDa (Xiong and Lommel, 1989) (Fig. 1). Capsid protein synthesis and replication functions have been genetically mapped to RNA-1 (Osman and Buck, 1987; Paje-Manalo and Lommel, 1989). Amino acid sequence alignment with a number of related spherical RNA plant viruses within the tombus-, carmo-, and luteovirus groups and identification of conserved RNA-dependent RNA polymerase motifs suggest that the polypeptides encoded by the RNA-1 p27 and p57 ORFs constitute the viral polymerase (Koonin, 1991). Most of these related plant viruses have polycistronic genomic RNAs and, with the notable exception of barley yellow dwarf luteovirus (BYDV), the 5′ proximally located 88–92 kDa polymerase ORF is interrupted by an inframe amber termination codon (Beier *et al.*, 1984; Carrington *et al.*, 1989; Guilley *et al.*, 1985; Hearne *et al.*, 1990; Miller *et al.*, 1988; Nutter *et al.*, 1989). Periodic suppression of the amber termination codon with an amber suppressor tRNA during translation results in the attenuated expression of the polymerase fusion protein (Beier *et al.*, 1984). Unlike these other viruses, the polymerases of RCNMV, BYDV (Brault and Miller, 1992), and potato leafroll luteovirus (PLRV) appear to be encoded by two adjacent out of frame ORFs (Mayo *et al.*, 1989).

Previously, Morris-Krsinich *et al.* (1983), reported that the capsid protein and a 36-kDa polypeptide were synthesized *in vitro* from RNA-1 and a 34-kDa polypeptide from RNA-2. The number and size of RNA-1-programmed proteins do not account for the RNA-1 coding capacity. Consequently, we have reinvestigated the *in vitro* expression of RCNMV RNA-1. In this report, we present the *in vitro* translation profile of the RCNMV genome and relate it to the genetic map of the virus.

Retrovirus RNA genomes are also polycistronic and the internally located polymerase (*pol*) ORF is expressed as a fusion protein with *gag* or *pro* (Varmus,

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1988). Retrovirus-like frameshifting requires a heptanucleotide sequence element which is often adjacent to a secondary structure which may involve pseudoknot formation (Atkins *et al.*, 1990). We have identified a nucleotide sequence element and a potential secondary structure at the termination of the RNA-1 5′ proximal p27 ORF which is predicted to facilitate ribosomal frameshifting. Furthermore, we present *in vitro* translation studies that illustrate that RCNMV produces a putative polymerase of 88 kDa by a −1 ribosomal frameshifting event mechanistically similar to retrovirus and coronavirus polymerase expression.

MATERIALS AND METHODS

Genomic RNA isolation

RCNMV (Australian isolate) was propagated and maintained in *Nicotiana clevelandii*. Virus was purified and virion RNA phenol extracted as previously described (Lommel *et al.*, 1988). Preparative separation of the two genomic RNA components was achieved by electrophoresis of heat denatured virion RNA in 1% agarose gels containing 1× TBE buffer (89 m*M* Tris–borate, 89 m*M* boric acid, and 2 m*M* EDTA, pH 8.0) for 1 hr at 60 V. Ethidium bromide-stained RNA bands were visualized by long-wave uv transillumination and excised from the gel. RNA was electroeluted from the agarose into a 5 *M* NaCl salt bridge at 100 V for 2 hr in sterile 0.5× TBE. RNA was ethanol-precipitated from the salt bridge solution and resuspended in sterile water. Electroelution was repeated to eliminate trace contamination of the RNA with the other component.

Construction of RNA-1 3′ co-terminal deletions and CP fusions

The synthesis of the full-length RNA-1 (pRC1) and RNA-2 (pRC2) cDNA and cloning within the transcription vector, pBS(+) (Stratagene) have been described previously (Xiong and Lommel, 1989, 1991). The numerical designations for specific nucleotides were assigned according to Lommel *et al.* (1988) for RNA-2 and Xiong and Lommel (1989) for RNA-1. A series of 5′ terminal deletion subclones were constructed by selective restriction of pRC1 at unique restriction sites except for pRC1*Ba* which required a partial digestion with *Bam*HI. After digestion, overhangs were filled in with DNA polymerase I Klenow fragment followed by recircularization of the plasmid by blunt-end ligation (Maniatis *et al.*, 1982).

CP polymerase fusion constructs were prepared by restriction fragment deletion. An internal *Bam*HI fragment was deleted from pRC1 to generate a fusion between the amino-half of p57 and the carboxyl-half of the CP.

Mutagenesis of the ribosomal frameshifting element

Several point and frameshift mutations in and around the RCNMV ribosomal frameshift site were constructed by site-specific mutagenesis of ssDNA templates using synthetic oligonucleotides as described by Kunkel *et al.* (1987). The RCNMV frameshift heptanucleotide element was changed to a BYDV frameshift heptanucleotide element using the oligonucleotide 5′-CCCTTGAGGGTTTTAGGCG-3′ in the clone pRC1BYDV. The frameshift region was also changed to an amber termination codon by addition of a G residue after the p27 ORF termination codon using the oligonucleotide 5′-ATTTTTAGGGCGGCCCACTC-3′ forming pRC1Amb. A single p88 ORF was generated by changing the amber termination codon to a tyrosine residue using the oligonucleotide 5′-ATTTTTACGGCGGCCCACTC-3′ (pRC1p88). The putative p57 ORF initiation codon was destroyed with 5′-CCAGTAGAGTCGACAAAAGTAAG-3′ (pRC1Met1) and a second downstream inframe methionine codon was eliminated with 5′-ACATAATATTATAGTTAGT-3′ (pRC1Met2). Two mutations were constructed that were predicted to prevent p88 expression. The frameshift heptanucleotide was destroyed with 5′-AATCCCTTGAGGACTTCTAGGCGG-3′ (pRC1NoFS) and a termination codon was incorporated in the −1 frame after the frameshift heptanucleotide sequence with 5′-CTTGAGGATTTTTAGTAGGCTAACTCAGCTTTCCGGT-3′ (pRC1-1Stop). All site-directed mutants were confirmed by DNA sequence analysis.

In vitro transcription, translation, and immunoprecipitation

Positive polarity run-off transcripts of RCNMV RNA-1 cDNA clones were synthesized using T7 RNA polymerase following linearization of pBS(+) plasmids with *Hin*dIII or *Sph*I (Xiong and Lommel, 1991). Transcription reactions were carried out according to the manufacturer's instructions (BRL). RNA was ethanol-precipitated together with the templates and translated *in vitro*.

Rabbit reticulocyte lysates (Green Hectares, Oregon Wisconsin) were used to translate viral RNAs and *in vitro* transcripts as described by Lommel *et al.* (1988). The [^{35}S]methionine-labeled translation products were analyzed by discontinuous SDS 12.5%–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). After electrophoresis, gels were fixed and processed by fluorography. Immunoprecipitation of *in vitro* translation products was carried out as described by Hiebert and Purcifull (1981) with *Staphylococcus aureus* (Cowan strain) cell walls and either non-immune or immune serum.

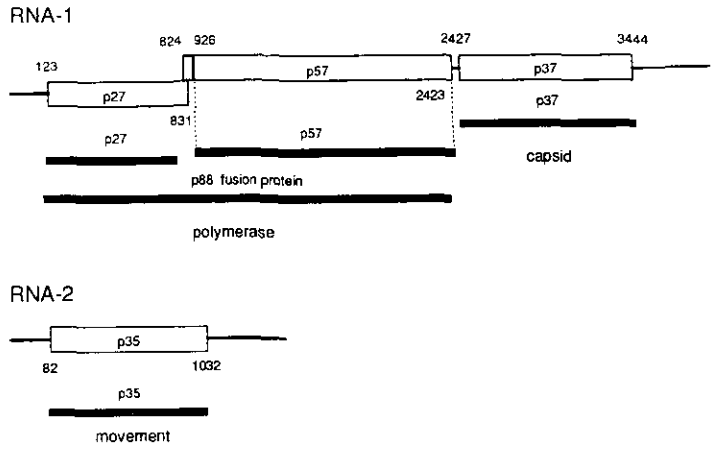

FIG. 1. Genome organization of RCNMV and *in vitro* translation products. Each rectangle in the schematic of the RCNMV genome organization represents an open reading frame defined by an initiation and termination codon whose nucleotide position is labeled either above or below the border of the open reading frame. Open reading frames are labeled according to the size of the predicted polypeptide that each encodes. Black bars represent proteins synthesized *in vitro* relative to the region of genomic RNA which encodes it. Fluorograph of SDS–PAGE separated [^{35}S]methionine labeled *in vitro* translation products directed by RCNMV RNA-1 and RNA-2. The protein standard (Prot. std.) lane contains [^{14}C]-labeled myosin, 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; α-chymotrypsinogen, 25.7 kDa; β-lactoglobulin, 18.4 kDa; and lysozyme, 14.3 kDa.

Antibody preparation

Antibodies to CP were raised in rabbits by injecting SDS–PAGE-purified RCNMV CP subunits intramuscularly. Two synthetic oligopeptides, IRENKAVAGFKSLEDF representing the carboxyl terminus of p27 and PTHYSRIHKDLIKAR representing the carboxyl terminus of p57 and p88, were synthesized (Xiong and Lommel, 1989). For each peptide an additional amino-terminal non-viral cysteine residue was included to facilitate coupling to keyhole limpet hemocyanin (KLH) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester linkage (Harlow and Lane, 1988). Oligopeptides coupled to KLH were then injected intramuscularly into rabbits. Antibodies were purified from antisera by affinity column chromatography with epoxy-activated Sepharose 6B (Pharmacia) coupled with the corresponding synthetic oligopeptide.

RESULTS

In vitro translation analysis of the RCNMV genome

Electrophoretically separated RCNMV RNA-1 directed the synthesis of four polypeptides, p88, p57, p37, and p27, in addition to several minor components ranging in size from 20 to 23 kDa, in a rabbit reticulocyte *in vitro* translation system (Fig. 1). The p27 protein was the major RNA-1 translation product while p57 and p37 were produced in significant but lower quantities. The p88 was synthesized at barely detectable levels in comparison to p27 or the RNA-2 encoded p35. Consequently, the detection of p57 and p88 required overexposure of the fluorographs relative to p27 and p35. No amino acid sequence similarity was observed between p27 and p57 by V8 protease cleavage analysis (data not shown). The low level of p88 synthesis *in vitro* prevented protease cleavage comparisons with p27 and p57. Translation of separated RNA-2 yielded a single major polypeptide, p35 (Fig. 1). A few minor low molecular weight protein bands, presumably products of degradation, internal initiation, or premature termination were also visible.

Translational mapping of RNA-1 encoded products

A series of 3′ co-terminal subclones were generated from pRC1 by incremental 5′ deletions of approximately 500 nucleotides using convenient restriction sites (Fig. 2). Uncapped, positive polarity run-off transcripts from each deletion clone were synthesized and equimolar amounts translated *in vitro*. Transcripts from the two smallest RNA-1 deletion constructs, pRC1*Acl* (0.3 kb) and pRC1*Ba* (1.1 kb), did not direct the synthesis of a detectable protein (Fig. 2, left panel). The next largest construct, pRC1*Xb* (1.5 kb), produced a large quantity of p37 CP relative to the other transcripts and viral RNA-1. Transcripts from construct pRC1*Hi* (2.6 kb) directed the synthesis of a reduced amount of p37 relative to pRC1*Xb*, but no larger polypeptides. In addition to p37, pRC1*Ps* (2.8 kb) yielded polypeptide species similar in mobility to p57. The pRC1*Ac* transcript, lacking approximately the 5′ terminal 400 bases of RNA-1, directed the synthesis of proteins comigrating with viral RNA-1 encoded p57 and p37. The p27 major

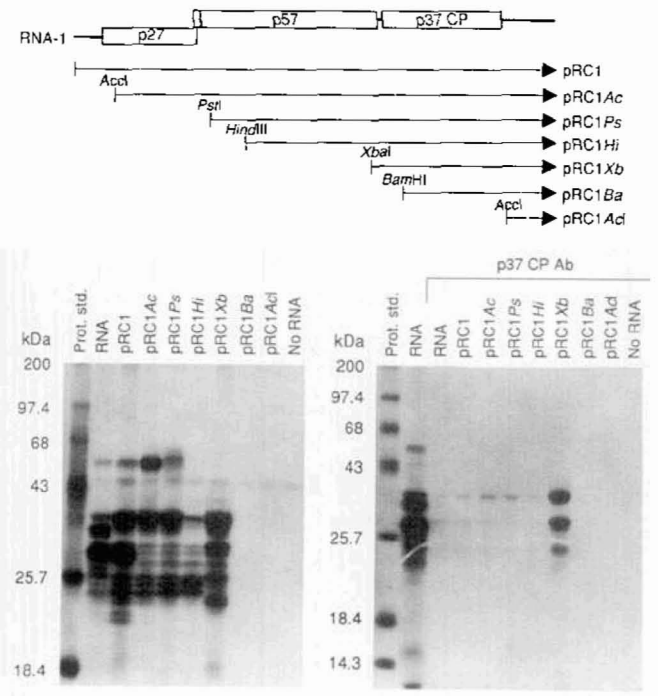

FIG. 2. SDS–PAGE and capsid protein antiserum immunoprecipitation analysis of *in vitro* translation products directed by a series of RCNMV RNA-1 deletion transcripts. Schematic of RNA-1, the RNA-1 clone, pRC1, and transcripts from deletion constructs showing the restriction site defining the 5′ terminus relative to genomic RNA-1. RCNMV virion RNA and runoff transcripts from *Hin*dIII or *Sph*I linearized plasmids were translated *in vitro* in the presence of [^{35}S]-methionine and products separated by SDS–PAGE and visualized by fluorography. The right panel contains translation products immunoprecipitated by RCNMV capsid protein antiserum and assayed by SDS–PAGE and fluorography. [^{14}C]-Labeled protein molecular weight standards are those listed in Fig. 1.

translation product from viral RNA-1 was not synthesized from the pRC1*Ac* transcript. However, the transcript from the full-length clone, pRC1, produced a large amount of p27, as well as small amounts of p57 and p37 *in vitro*. The synthesis of p88 directed by pRC1 transcripts was not observed at the exposure illustrated (Fig. 2), but was detected upon longer exposure (data not shown). Thus, the genome organization of RNA-1 is from 5′ to 3′: p27, p57, and p37 CP, in agreement with the nucleotide sequence of RNA-1 (Xiong and Lommel, 1989).

Immunoprecipitation of RCNMV translation products

RCNMV CP antiserum immunoprecipitated p37 as well as a 28-kDa polypeptide and several minor smaller polypeptides establishing p37 as CP (Fig. 2, right panel, and Fig. 3). The 28-kDa product observed *in vitro* is most likely the result of internal initiation from one of several in frame methionine codons present within the 5′ terminal quarter of the CP gene. No RNA-2 directed translation products were immunoprecipitated by CP antiserum. Carboxyl-terminal oligopeptide antibodies were generated to both the predicted p27 and p57/p88 non-structural proteins. The p27 antibody immunoprecipitated p27 as well as p88 (Fig. 3). In addition, the p27 antibody immunoprecipitated the series of minor bands ranging in size from 20 to 23 kDa, establishing them as subsets of p27. The p88 antibody immunoprecipitated p57, p88, and smaller amounts of several minor proteins, presumably p57 and or p88 degradation products. This result clearly showed that p88 is composed of both p27 and p57 sequences. The p35, as well as all the other smaller products encoded by RNA-2, were only immunoprecipitated by the p35 carboxyl-terminal oligopeptide antibody (Fig. 3).

CP reporter system to observe ribosomal frameshifting

Nucleotide sequence analysis suggested that p88 may arise by a ribosomal frameshifting event near the

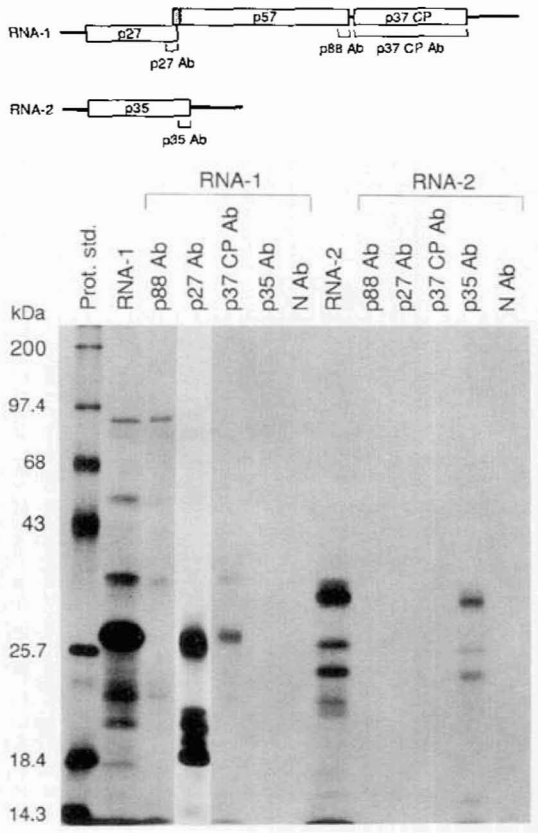

FIG. 3. Immunoprecipitation analysis of RCNMV *in vitro* translation products. RNA-1 and RNA-2 *in vitro* translation products immunoprecipitated with capsid protein antibodies (p37 CP Ab), non-immune antibodies (N Ab), or with carboxyl-terminal oligopeptide antibodies to p88 (p88 Ab), p27 (p27 Ab), or p35 (p35 Ab). The portion of the viral protein that was used as an immunogen is identified as brackets on the RCNMV genome map. RNA was translated in a rabbit reticulocyte lysate amended with [^{35}S]methionine. Products immunoprecipitated with the antibodies were separated by SDS–PAGE and fluorographed. [^{14}C]Labeled protein molecular weight standards are those listed in Fig. 1.

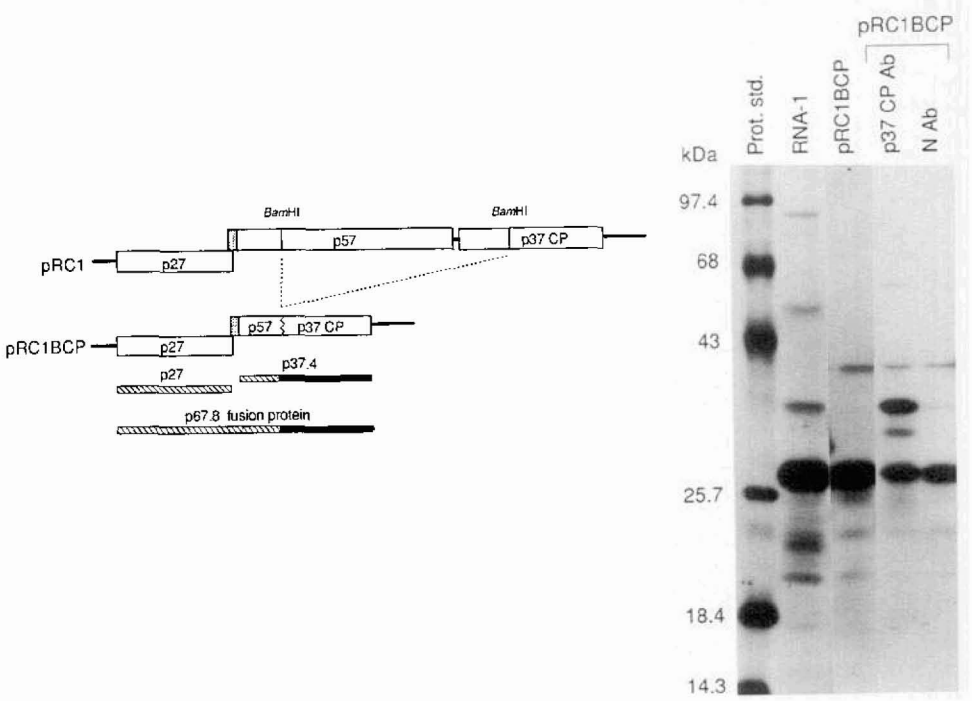

FIG. 4. *Schematic and in vitro translation and immunoprecipitation analysis of peptides directed by the RCNMV p57 capsid protein open* reading frame fusion construct, pRC1BCP. Proteins predicted to be synthesized from the transcripts are depicted as solid and hatched bars. Runoff transcripts were generated and translated *in vitro*. pRCIBCP translation products were immunoprecipitated with capsid protein (p37 CP Ab) and normal (N Ab) antisera. Immunoprecipitations were performed with an excess of translation product resulting in the presence of p27 in the N Ab lane. [^{14}C]-Labeled protein molecular weight standards are those listed in Fig. 1.

termination codon of the p27 ORF, allowing translation to continue into and through the p57 ORF (Xiong and Lommel, 1989). To investigate this possibility, a p57–CP fusion construct was made. Deletion of an internal *Bam*HI fragment from pRC1 generates an inframe fusion between the amino-terminal portion of p57 and the carboxyl-terminal portion of p37 CP. The construct (pRC1BCP) was predicted to encode a CP fusion peptide of 37.4 kDa, and assuming ribosomal frameshifting, a 67-kDa fusion polypeptide (Fig. 4). Run-off RNA transcripts were prepared from pRC1BCP after *Sma*I linearization. Analysis of the *in vitro* translation products directed by the pRC1BCP transcript showed that it directed the synthesis of a major 27-kDa product with a small amount of a 37.4-kDa polypeptide (Fig. 4). The predicted 68-kDa fusion polypeptide was produced at an extremely low level and was only detectable after concentration by immunoprecipitation with CP antiserum (Fig. 4). In addition to the 68-kDa polypeptide, the 37.4-kDa polypeptide was also immunoprecipitated, as was expected, since both of these polypeptides were composed of the carboxyl-terminal portion of CP.

RCNMV frameshift element mutations

A frameshift heptanucleotide defined by the ability to facilitate ribosomal frameshifting in accordance with the −1 simultaneous slippage model (Jacks *et al.*, 1988a) was identified in RNA-1 immediately 5′ to the p27 ORF amber termination codon (see below). To determine the role of this potential frameshifting element in p88 synthesis, several point and frameshift mutations were introduced into this region of RNA-1 (Fig. 5). Nucleotide 826 was changed from an A to a G residue generating the frameshift heptanucleotide sequence GGGUUUU predicted in BYDV (pRC1BYDV). Insertion of a G residue after the p27 ORF termination codon (mutant pRC1Amb) shifts the downstream −1 frame into the same frame as the p27 ORF generating a p88 ORF punctuated by an in-frame amber termination codon. An additional mutation in pRC1Amb, changing nucleotide number 833 from a G to a C residue altered the p27 ORF amber termination codon to a tyrosine encoding codon. This mutant, termed pRC1p88, generated a single uninterrupted p88 ORF, eliminating the p27 ORF. Two mutants were generated that were predicted to prevent p88 expression *in vitro*. Mutant pRC1-1Stop incorporated a termination codon immediately 3′ of the predicted frameshift heptanucleotide. Mutant pRC1NoFS destroyed the frameshift heptanucleotide preventing ribosomal frameshifting in accordance with the simultaneous slippage model.

All the mutant *in vitro* transcripts except pRC1p88 directed the synthesis of a large quantity of p27 in addition to smaller quantities of p37 CP (Fig. 5). The p88 fusion protein from pRC1BYDV and pRC1Amb was synthesized in approximately the same quantity as from

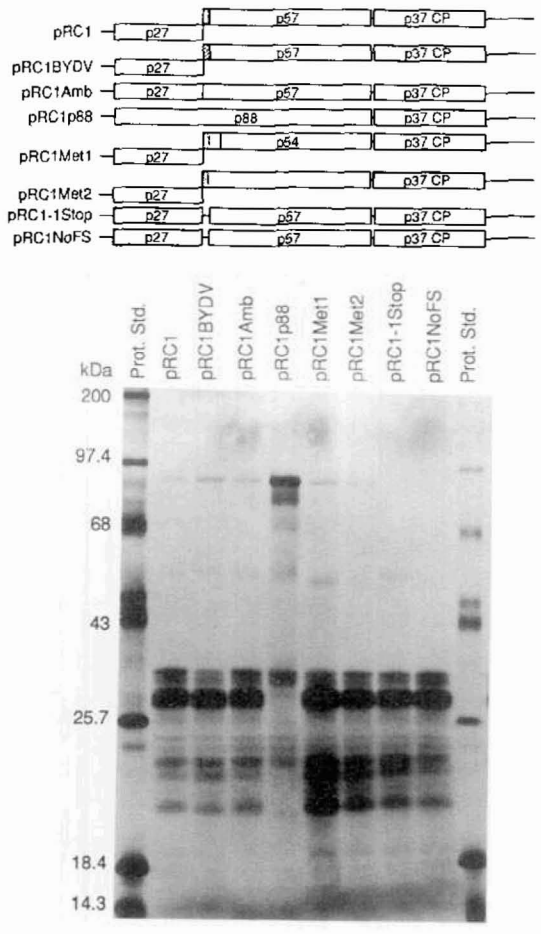

FIG. 5. Schematic of RCNMV RNA-1 constructs with site-directed mutations in and around the predicted ribosomal frameshift site affecting the expression of p27, p57, and p88. pRC1, wild-type RCNMV RNA-1 transcript. pRC1BYDV is a site-directed mutant changing the RCNMV frameshift heptanucleotide to a BYDV frameshift heptanucleotide. pRC1Amb is a single nucleotide insertion generating a frameshift mutant resulting in a p88 open reading frame interrupted by an inframe amber termination codon. pRC1p88 is an additional point mutation of pRC1Amb changing the in-frame amber termination codon to a tyrosine codon resulting in a single p88 open reading frame. pRC1Met1 is a point mutation eliminating the methionine codon defining the beginning of the p57 open reading frame. pRC1Met2 is an additional point mutation of pRC1Met1 changing the second methionine codon at nucleotide 1007 to an isoleucine codon. pRC1-1Stop is a point mutation incorporating a termination codon in the −1 frame following the frameshift heptanucleotide sequence. pRC1NoFS has several point mutations destroying the frameshift heptanucleotide sequence. Fluorograph of *in vitro* translation products of the mutated RNA-1 transcripts separated by SDS–PAGE. [^{14}C]-Labeled protein molecular weight markers are those listed in Fig. 1.

the wild-type transcript pRC1. Synthesis of p88 from pRC1Amb indicated that an amber terminator readthrough event occurred. The major translation product from the pRC1p88 transcript was p88, with smaller amounts of p37 CP and p57 and no p27 protein synthesis.

The internal p57 ORF identified by sequence analysis is apparently expressed directly from RNA-1 *in vitro* (Fig. 1). To establish that the p57 ORF resulted in the expression of p57, the predicted p57 ORF initiation codon at nucleotide 926 was changed to an aspartic acid codon. This mutant, pRC1Met1, directed the synthesis of a protein slightly smaller than p57 (Fig. 5). The smaller protein was presumably produced by initiation at the second AUG codon, 80 nucleotides downstream from the first AUG codon in the p57 ORF. This second methionine codon was changed to an isoleucine codon in the mutant pRC1Met2. This mutant did not direct the expression of p57 or a smaller product *in vitro*.

DISCUSSION

RCNMV RNA-1 directs the synthesis of p27, p37 (CP), p57, and p88 in addition to a population of smaller species serologically related to p27 and p37 *in vitro*. The coding regions of the RNA-1 encoded polypeptides have been mapped. Excluding p88, the relative location and size of the observed RNA-1 directed *in vitro* products correlates with the three ORFs deduced from the nucleotide sequence analysis (Fig. 1). Only p37 is immunoprecipitated by CP antiserum (Figs. 2 and 3), indicating that the other observed products are not related to CP. No amino acid sequence similarity was observed between p27 and p57 by V8 protease cleavage map analysis (data not shown). Together the serological and mapping evidence suggest that the coding sequence for p88 overlaps and is composed of the p27 and p57 ORFs.

The p37 CP has been mapped to the 3′ proximal p37 ORF and appears to be relatively inaccessible for translation from full-length virion RNA as evidenced by its low level synthesis *in vitro* (Figs. 1 and 2). In contrast, a large amount of CP is synthesized from the 1.5-kb 3′ co-terminal *in vitro* transcript pRC1*Xb*, which has only 20 nucleotides 5′ to the CP initiation codon. These observations are consistent with the conclusion by Osman and Buck (1990) that the CP ORF is expressed *in vivo* from a 3′ co-terminal 1.5-kb subgenomic RNA.

RCNMV polymerase expression by ribosomal frameshifting

A small amount of an 88-kDa polypeptide which reacts to both p27 and p57 carboxyl-terminal peptide antibodies but not the capsid protein antibody is synthesized *in vitro* (Fig. 3). These data suggest that p88 is a fusion protein resulting from translational frameshift of the p27 ORF into the p57 ORF. It is unlikely that p88 is a cleavage precursor of p57 and p27 because p27 is produced in much greater quantities than p57. The fact that only a minor amount of p88 is produced *in vitro* is consistent with a readthrough event (Beier *et al.*, 1984) or a ribosomal frameshifting event (Atkins *et al.*, 1990; Jacks *et al.*, 1988a). The RCNMV RNA-1 nu-

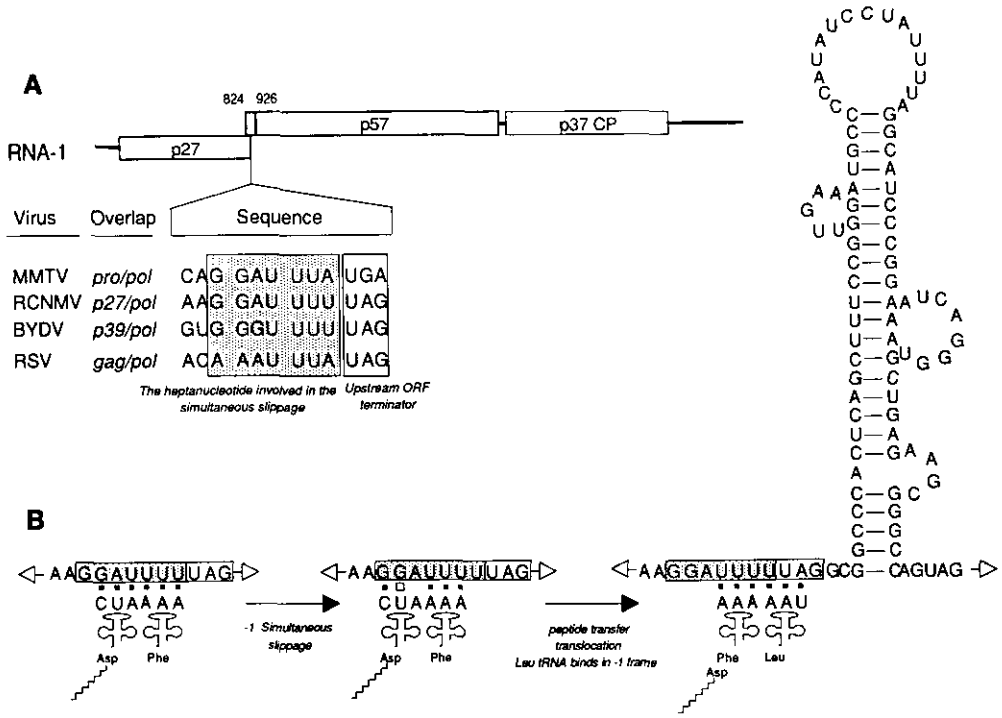

FIG. 6. Location within RCNMV RNA-1 of a frameshift heptanucleotide defined by the −1 simultaneous slippage model and a predicted stem–loop structure. (A) Comparison of the predicted RCNMV and BYDV frameshift heptanucleotide with those from mouse mammary tumor virus (MMTV) and Rous sarcoma virus (RSV). Heptanucleotide sequences listed are immediately adjacent to upstream open reading frame termination codon. (B) Schematic of −1 simultaneous slippage within the RCNMV frameshift heptanucleotide. Solid square denotes codon anticodon Watson Crick base pairing and hollow square non-Watson Crick codon anticodon base pairing after the ribosome has slipped one nucleotide in the −1 direction along viral RNA-1. Potential secondary structure adjacent and downstream from the RCNMV frameshift heptanucleotide is also depicted.

cleotide sequence reveals that the p27 ORF and p57 ORF are in different reading frames (Xiong and Lommel, 1989), excluding the possibility of a readthrough, and further suggesting the role of a ribosomal frameshifting event in the synthesis of p88.

For −1 ribosomal frameshifting in most eukaryotic genes studied to date, a heptanucleotide sequence is required, as defined by the ability to facilitate ribosomal frameshifting in accordance with the simultaneous slippage model. A frameshift heptanucleotide sequence element has been identified in RCNMV. This element is immediately upstream from the p27 ORF termination codon and is very similar to those of mouse mammary tumor virus (MMTV), RSV, and BYDV (Fig. 6A) as well as to those of the coronaviruses (Brierley *et al.*, 1987). The simultaneous slippage model can be applied to the RCNMV frameshift heptanucleotide (Fig. 6B). $tRNA^{Asp}$ with attached nearly complete p27 and $tRNA^{Phe}$ can base pair to the last two p27 ORF codons GAU and UUU in the ribosome P and A sites, respectively. Simultaneous slippage of the ribosome tRNA complex one nucleotide in the 5′ (−1) direction results in their complexing with the p27 ORF −1 frame codons, GGA and UUU. This results in incomplete codon anticodon base pairing in the first and second position of $tRNA^{Asp}$ and complete base pairing with $tRNA^{Phe}$. Normal peptide transfer and three nucleotide translocation bring the codon UUA in the p57 ORF into the A site where it pairs with $tRNA^{Leu}$.

In addition to the heptanucleotide sequence, stable stem-loop structures often involving pseudoknot formation are thought to facilitate the ribosomal frameshifting event in eukaryotic systems (Jacks *et al.*, 1988a,b; Hizi *et al.*, 1987; Le *et al.*, 1989; Ten Dam *et al.*, 1990). These secondary structures are typically found adjacent to the frameshift heptanucleotide. A stable stem–loop structure is predicted immediately downstream of the putative frameshifting heptanucleotide sequence in RCNMV (Zuker and Stiegler, 1981; Fig. 6B). The proposed RCNMV stem–loop structure is similar to the established structures in human immunodeficiency virus (HIV) (Jacks *et al.*, 1988b), RSV (Jacks *et al.*, 1988a) and to the predicted stem-loop structure of BYDV (Brault and Miller, 1992) and PLRV (Prüfer *et al.*, 1992).

Other viral translational readthrough elements facilitate RCNMV p88 expression *in vitro*

The RCNMV p88 is predicted to be the viral polymerase based on its amino acid sequence similarity to the putative carmo-, tombus-, and luteovirus polymerases

(Koonin, 1991). For the carmo- and tombusviruses the greatest sequence similarity occurs in the region of the polymerase expressed as an amber terminator readthrough product. By amino acid sequence alignment, it appears that RCNMV utilizes a ribosomal frameshifting event in place of an amber terminator readthrough to achieve the modulated expression of the polymerase. This situation is parallel to the retroviruses, where some produce *gag–pol* fusions by an amber terminator readthrough mechanism while most use a ribosomal frameshift mechanism (Yoshinaka *et al.*, 1985a,b).

We have mutated the predicted RCNMV RNA-1 frameshift element such that p88 expression would require amber termination codon readthrough. This construct yielded a limited amount of p88 *in vitro* (Fig. 5), illustrating that p88 is expressed regardless of whether a frameshift element or an inframe amber terminator is inserted within the RCNMV polymerase gene. This result suggests that the two polymerase expression modulation mechanisms are interchangeable, at least *in vitro* within the context of the RCNMV genome.

The suspected BYDV frameshift heptanucleotide sequence differs in one nucleotide from that of RCNMV. According to the simultaneous slippage model, the BYDV frameshift heptanucleotide should form a more stable codon anticodon base pairing when shifted to the −1 position. Given this, we predicted that mutation of the RCNMV frameshift heptanucleotide to a BYDV heptanucleotide would increase the efficiency of p88 synthesis *in vitro*. However, no striking difference in p88 accumulation was observed. Factors determining frameshifting efficiency are likely to be more complex than codon–anticodon base-pairing.

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