Translation of cucumber necrosis virus RNA in vitro

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The in vitro translation products directed by cucumber necrosis virus (CNV) RNA were analysed in both rabbit reticulocyte lysate and wheatgerm extract cell-free translation systems. In rabbit reticulocyte lysates, one major protein of approximate Mr 34.6K was produced. In wheatgerm extracts, four proteins of approximate Mr values 41-6K, 34.6K, 24K and 20K were produced. The genomic locations of the CNV in vitro translation products were determined using several experimental approaches including, first, hybrid-arrested translation using negative-sense RNA corresponding to selected regions of the CNV genome, second, in vitro translation of synthetic positive-sense CNV transcripts and third, in vitro translation of CNV virion RNA fractionated according to size. Together these experiments demonstrated that the protein of Mr 34.6K is derived from the 5'-proximal coding region, the 41-6K protein is derived from an internal coding region, and that at least one but probably both the 24K and 20K proteins are derived from the 3'-terminal coding region. In addition, immunoprecipitation of in vitro translation products using anti-CNV polyclonal serum demonstrated that the 41.6K protein is the coat protein. The templates for the expression of CNV cistrons were investigated by in vitro translation of sucrose gradient-fractionated CNV virion RNA as well as in vitro translation of positive-sense synthetic transcripts.

Introduction

Cucumber necrosis virus (CNV), a member of the tombusvirus group (Rochon & Tremaine, 1988), is an isometric plant virus with a 30 nm particle composed of 180 copies of a single capsid protein with an approximate Mr of 41K (Tremaine, 1972). The CNV genome is monopartite and consists of single-stranded, positive-sense RNA of approximately 4.7 kb (Tremaine, 1972; Rochon & Tremaine, 1989). Systemic infection by CNV is limited to cucumber in nature (McKeen, 1959) and natural spread of the virus is facilitated by zoospores of the fungus, Olpidium radicale (Dias, 1970).

The complete nucleotide sequence of the CNV genome has been determined and from this the genome organization was deduced (Rochon & Tremaine, 1989). The proposed organization is in agreement with that suggested recently for cymbidium ringspot virus (CyRSV), a tombusvirus (Russo et al., 1988; Grieco et al., 1989), and also tomato bushy stunt virus-cherry (TBSV-cherry), a close relative of the type member of the tombusvirus group (Morris & Carrington, 1988; Hillman et al., 1989). The genome of CNV contains five long open reading frames (ORFs) with the capacity to encode proteins of 33K, 92K 41K, 21K and 20K. The 92K protein, if produced, would arise from readthrough of the 33K amber termination codon. The 92K protein is implicated as the viral replicase on the basis of amino acid sequence similarity with the putative replicases of other RNA viruses. The ORF for the 41K protein located immediately downstream of the 92K ORF is suggested to encode the viral coat protein since its amino acid sequence closely resembles that of other tombusviruses (Hillman et al., 1989; Riviere et al., 1989; Rochon & Tremaine, 1989). The 3' terminus of the CNV genome contains an ORF for a 20K protein which is completely nested within an ORF for a 21K protein.

We show here that the translation strategy of CNV involves the formation of subgenomic RNA species which are 3'-coterminal with genomic RNA. A subgenomic strategy has been shown for other tombusviruses such as TBSV (Henriquez et al., 1978; Henriquez & Morris, 1979; Morris, 1983; Hayes et al., 1984, 1988) and CyRSV (Gallitelli & Hull, 1985; Burgyan et al., 1986).

Methods

Virus purification and antibody production. CNV was propagated in Nicotiana clevelandii and isolated by a modification of the pH 5 method of Tremaine et al. (1983). Polyclonal antiserum to intact CNV particles was prepared by intramuscular injection of a rabbit at 1 week intervals.

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for 4 weeks using 1 mg of purified virus emulsified in Freund’s incomplete adjuvant (Gibco). IgG was purified from the antiserum by affinity chromatography using Protein A-Sepharose CL-4B (Pharmacia).

**Virus RNA extraction.** CNV RNA was extracted from purified virus by swelling the virus particles in the presence of 10 mM-EDTA for 10 min at 4 °C followed by extraction with phenol–chloroform–SDS at pH 8-9.

In vitro translation. In vitro translation in the presence of [14C]methionine (New England Nuclear; specific activity approx. 1100 Ci/mmole) was carried out using both rabbit reticulocyte lysate and wheatgerm extract cell-free translation systems (Promega Biotec). Translation reactions were programmed with between 120 and 180 µg/ml CNV RNA and incubated for 2 h at 22 to 25 °C. In one set of experiments translation reactions were supplemented with between 0.5 and 2 mg/ml calf liver tRNA (Boehringer Mannheim), yeast tRNA (Sigma) or an enriched source of N. clevelandii tRNA obtained from the 2 M-lithium chloride-soluble fraction of nucleic acid isolated from uninfected leaves (Diaz-Ruiz & Kaper, 1978). Translation products were analyzed by SDS-PAGE in 12% or 15% polyacrylamide gels and 2 mg/ml calf liver tRNA (Boehringer Mannheim), yeast tRNA (Sigma) or an enriched source of N. clevelandii tRNA obtained from the 2 M-lithium chloride-soluble fraction of nucleic acid isolated from uninfected leaves (Diaz-Ruiz & Kaper, 1978). Translation products were analyzed by SDS-PAGE in 12% or 15% polyacrylamide gels (Laemmli, 1970) and subsequent fluorography using Entensify (New England Nuclear). The sizes of CNV in vitro translation products were estimated by comparison with [14C]-methylated proteins (Amersham) and the published sizes of brome mosaic virus (BMV) translation products (Ahlquist et al., 1981).

**Immunoprecipitation.** Immunoprecipitation of the CNV coat protein was carried out according to a modification of the procedure of Oliver & Boyd (1984). Fifty µl of a 10% (w/v) solution of protein A–Sepharose CL-4B (Pharmacia) was pre-incubated with 1·5 ml normal serum for 1·5 h at room temperature, washed three times and resuspended in NET buffer (150 mM-NaCl, 5 mM-EDTA, 50 mM-Tris–HCl pH 7·4) containing 0·65% NP40. This suspension was used to preclcar wheatgerm extract translation reactions (25 µl) previously programmed with CNV virion RNA. A 1:1 mixture of the suspension and the translation reaction was incubated for 30 min and then briefly centrifuged in an Eppendorf microfuge. The supernatant was transferred to another tube and the pellet was discarded. The supernatant was then preclcarised again as described above and the pellet discarded. The translation reaction supernatant was then incubated with 300 µl anti-CNVT IgG (1·5 mg/ml) which had previously been bound to Protein A–Sepharose CL-4B (Pharmacia) in the manner described above. The mixture was incubated overnight at 4 °C with slow shaking, briefly centrifuged in an Eppendorf microfuge and the pellet washed four times with NET containing NP40. The pellet was finally resuspended in a 1 : 1 mixture of NET containing NP40 and 2X Laemmli sample mix and the solution was heated to 90 °C for 2 min before being analysed by SDS-PAGE and fluorography.

**Production of synthetic transcripts.** Synthetic transcripts of either positive (+) or negative (−) polarity were produced in vitro using either T7 or T3 RNA polymerase [Bethesda Research Laboratories (BRL)] and Bluestreak or Bluescribe phagemids (Stratagene) containing CNV cDNA inserts corresponding to different regions of the CNV genome (see Fig. 1). In vitro transcription reactions were carried out in the presence of RNAse (Promega Biotec) using 40 µg/ml restriction enzyme-linearized template DNA and transcription conditions recommended by BRL. The reaction mixtures were incubated at 37 °C for 45 min after which 0·2 unit RNase-free DNase I (Stratagene) and an additional 10 units of RNase A were added. The mixtures were then incubated at 37 °C for a further 15 min. The amount of RNA transcript produced was determined either spectrophotometrically or by comparison with CNV virion RNA after agarose gel electrophoresis and ethidium bromide staining. The synthesis of a full-length infectious CNV cDNA clone (pK2) will be described elsewhere.

**Hybrid-arrested translation (HART).** The procedure used was similar to that described by Paterson et al. (1977) except that negative-sense RNA rather than cDNA was used. RNA–RNA hybrids were formed using a threefold molar excess of synthetic negative-sense RNA over CNV virion RNA. The hybridization was carried out in a 50 µl volume containing 1 to 6 µg CNV RNA, 3 to 18 µg negative-sense RNA, 20% deionized formamide (BRL), 10 mM-Tris–HCl pH 7·5 and 400 mM NaCl and then incubated for 2 h at 48 °C.

**Sucrose density gradients.** Linear–log sucrose density gradients were prepared according to Brakke & van Pelt (1970) and used to fractionate CNV virion RNA according to size. The gradient was collected from top to bottom using a Buchler Auto Densi-Flow IIC gradient collector and a Gilson microfractionator.

**Northern blots.** RNA was electrophoresed through 1% agarose gels containing 5 mM-methylmercuric hydroxide (Bailey & Davidson, 1976), transferred to Zeta-Probe (Bio-Rad) under alkaline conditions (Vrati et al., 1987) and hybridized to 32P-labelled nick-translated cloned CNV cDNA probes (Rigby et al., 1977).

**Results**

**Comparison of CNV translation products in rabbit reticulocyte lysates and wheatgerm extracts**

In vitro translation products directed by CNV RNA were analysed in both rabbit reticulocyte lysates and wheatgerm extract cell-free translation systems. Fig. 2 shows that in rabbit reticulocyte lysates, one major protein was produced, approximately 34·6K (lane 3), whereas in wheatgerm extracts, four major proteins of approximately 41·6K, 34·6K, 24K and 20K were produced (lane 4). The possibility will be discussed that the different number of translation products produced in the twore systems reflects a differential selection of AUG initiation codons by the ribosomes of plants and animals (Kozak, 1986; Lütcke et al., 1987). The wheatgerm system was chosen for most of the remaining studies since the sizes and numbers of the proteins produced from CNV RNA are similar to those predicted from the nucleotide sequence (Rochon & Tremaine, 1989).

**Genomic location and identification of CNV in vitro translation products**

(i) **Identification of CNV-specific in vitro translation products**

To determine whether all of the CNV in vitro translation products are viral RNA-specific, a full-length negative-sense CNV transcript, [CNVTx 18/4701(−); see Fig. 1] was synthesized and incubated under hybridization conditions with CNV virion RNA. Translation of the resulting hybrids demonstrated that the full-length negative-sense transcript arrested the synthesis of all proteins produced by CNV virion RNA in wheatgerm extracts (Fig. 3, lane 6). The arrest was specific because...
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(i) Synthesis of CNV proteins in vitro

The synthesis of proteins by BMV RNA was not altered by the CNV negative-sense transcript (compare lanes 1 and 4). Also the arrest was not a result of degradation of CNV virion RNA during incubation because mock-hybridized CNV RNA produced the same proteins that were produced by untreated CNV RNA (compare lanes 2 and 5). These results indicate that negative-sense RNA can be used to arrest protein synthesis specifically. In addition, they verify that the four major proteins synthesized from CNV virion RNA in wheat germ extracts are CNV-specific, i.e., none of the protein products is a result of translation of encapsidated host RNA as suggested for another tombusvirus (Hayes et al., 1988). The results of translation of a full-length positive-sense transcript representing the CNV genome will be described below.

(ii) Genomic location of the 34-6K protein coding region

To determine which proteins arise from the 5'-proximal region of the CNV genome, a synthetic negative-sense transcript corresponding to this region [CNVTx 18/910(−); see Fig. 1] was used in HART studies. In vitro translation of CNV virion RNA which had been hybridized with CNVTx 18/910(−) resulted in a dramatic reduction in the synthesis of the 34-6K polypeptide but had little or no affect on other CNV proteins (Fig. 3, lane 8). Translation of a positive-sense transcript covering the 5' region of CNV RNA, [CNVTx 18/1090(+); Fig. 1], resulted in the synthesis of one protein which comigrated with the CNV 34-6K translation product as well as another protein which had a slightly faster mobility corresponding to approximately 31K (Fig. 3, lane 9). The synthesis of a 34-6K protein from this template suggests again that the 34-6K protein is derived from the 5'-proximal region of the CNV genome. One possible origin of the smaller product was suggested through the translation of another positive-sense transcript [CNVTx 260/2156(+); see Fig. 1] whose coding region overlaps that of CNVTx 18/1090(+) but which is truncated at its 5' terminus. Translation of
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Fig. 2. Comparison of CNV RNA in vitro translation products in rabbit reticulocyte lysates (lanes 1 to 3) and wheat germ extracts (lanes 4 to 6). Products were analysed by SDS-PAGE (12% gel) and autoradiography. Lanes 1 and 6, BMV RNA (20 μg/ml); lanes 2 and 5, no added RNA; lanes 3 and 4, CNV RNA (160 μg/ml). The M<sub>v</sub> values of CNV in vitro translation products are indicated on the left of the gel.

CNVTx 260/2156(+) (data not shown) produced a protein that comigrated with the 31K protein produced by CNVTx 18/1090(+). The 31K protein might arise from aberrant (leaky) translation (Kozak, 1981) from AUG codons which are downstream from the AUG codon of the 33K ORF. Since analysis of the nucleotide sequence revealed that the 5' region has the capacity to encode a protein no larger than 33K (Rochon & Tremaine, 1989), it is proposed that the 34.6K CNV in vitro translation product represents the 33K protein predicted from the nucleotide sequence.

(iii) Genomic location of the 41.6K protein coding region
As it seemed reasonable that the 41.6K in vitro translation product might arise from the 41K CNV ORF, a synthetic negative-sense transcript [CNVTx 2566/3234(−); see Fig. 1] covering the 41K ORF was synthesized and used in HART studies with CNV virion RNA. Fig. 3 (lane 10) shows that this transcript arrested the translation of the 41.6K protein suggesting that this CNV in vitro translation product corresponds to the 41K ORF predicted from the nucleotide sequence. The in vitro translation products of a positive-sense transcript encompassing the 41K ORF [CNVTx 2566/4116(+) ; see

Fig. 3. HART studies. Wheat germ extracts were programmed with: 20 μg/ml BMV RNA (lane 1); 120 μg/ml CNV RNA (lane 2); no added RNA (lane 3); 20 μg/ml BMV RNA plus 60 μg/ml CNVTx 18/4701(−) (lane 4); 120 μg/ml mock-hybridized CNV RNA (lane 5); 120 μg/ml CNV RNA plus 360 μg/ml CNVTx 18/4701(−) (lane 6); 240 μg/ml pk2 transcript (lane 7); 120 μg/ml CNV RNA plus 80 μg/ml CNVTx 18/910(−) (lane 8); 240 μg/ml CNVTx 18/1090(+) (lane 9); 120 μg/ml CNV RNA plus 50 μg/ml CNVTx 2566/3234(−) (lane 10); 240 μg/ml CNVTx 2566/4116(+) (lane 11); 120 μg/ml CNV RNA plus 80 μg/ml CNVTx 3634/4639(−) (lane 12) or 240 μg/ml CNVTx 3634/4639(+) (lane 13). Hybrid arrests were done as described in Methods. All negative-sense transcripts were tested for translation and were found to give insignificant protein synthesis. In vitro translation products were analysed by SDS-PAGE (15% gel) and autoradiography. The M<sub>v</sub> values of CNV in vitro translation products are indicated on the left of the gel.
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Fig. 1, are shown in Fig. 3 (lane 11). A protein that comigrated with the CNV 41.6K product can be seen; however the protein is faint indicating that this transcript is not an efficient template. The weak mRNA activity of CNVTx 2566/4116(+) is likely to be because the transcript contained 5’-proximal AUG triplets upstream from the 41K ORF which were followed shortly by in-frame termination codons (particularly since the short reading frame set by one AUG triplet terminated after the start of the 41K ORF). The presence of upstream AUG triplets has been demonstrated to reduce protein synthesis significantly from downstream coding regions (Kozak, 1984a).

(iv) Genomic location of the 24K and 20K protein coding regions

HART was also tested as a means of determining which in vitro translation products arise from the 3’-terminal region of the CNV genome. Experiments using a synthetic negative-sense transcript [CNVTx 3634/4639(–); see Fig. 1] corresponding to this region showed that this transcript consistently diminished the translation of all CNV proteins (Fig. 3, lane 12). However, the arrest was not non-specific since it did not reduce translation of BMV RNA (data not shown). The reason this transcript reduces all CNV in vitro translation products is not understood at present. It is possible that the 3’-terminal region of the CNV genome has a regulatory role in translation which the negative-sense RNA used in this experiment prevented. A regulatory role for the 3’ terminus has been suggested for carnation mottle virus RNA (Saloman et al., 1978). Alternatively the binding of synthetic transcripts to CNV virion RNA might affect the RNA secondary structure at sites other than those which have hybridized and, in turn, affect the efficiency of translation from these regions. The involvement of secondary structure in the regulation of translation by antisense RNA at sites away from those which are directly affected has been demonstrated in prokaryotic systems (for a review, see Inouye, 1988).

Translation of a synthetic positive-sense transcript [CNVTx 3634/4639(+); see Fig. 1] corresponding to the 3’ terminus of the CNV genome resulted in the synthesis of proteins that comigrated with the 24K and 20K protein produced by CNV virion RNA in wheatgerm extracts (Fig. 3, lane 13). This result suggests very strongly that the 24K and 20K proteins are derived from the 3’-terminal coding region of CNV RNA. Analysis of the nucleotide sequence from which CNVTx 3634/4639(+) was transcribed shows that it does not have the capacity to encode a protein of 24K. This protein is therefore proposed to be the 21K protein predicted from the CNV sequence. In addition, it is proposed that both the 21K and 20K proteins are translated in vitro from the same RNA derived from the 3’ terminus of the CNV genome. In a separate paper, we will demonstrate the bifunctional nature of the subgenomic RNA which encodes these proteins (D. M. Rochon & J. C. Johnston, unpublished results).

(v) Immunoprecipitation of the CNV coat protein

Comparison of the amino acid sequence of the CNV 41K encoding ORF with the coat proteins of several tombus-viruses indicates that it encodes the CNV coat protein (Hillman et al., 1989; Rochon & Tremaine, 1989). To determine whether the 41-6K in vitro translation product is the coat protein, CNV in vitro translation products were immunoprecipitated with CNV IgG (prepared against intact viral particles) bound to Protein A-Sepharose CL-4B (Pharmacia). Fig. 4 shows that the
41.6K protein is precipitated (lane 3). Bands corresponding to other CNV translation products are also precipitated but not as efficiently as the 41.6K protein. In control experiments (data not shown), a small but significant amount of non-specific binding of CNV in vitro translation products to both CNV antiserum and Protein A-Sepharose CL-4B (Pharmacia) occurred. The immunoprecipitation results suggest that the CNV 41.6K protein is the CNV coat protein and together with the HART results and those for translation of positivesense transcripts indicate that the CNV coat protein is derived from the 41K-encoding ORF of the CNV genome.

Natural template(s) for the expression of CNV proteins

(i) Northern blot analysis of fractionated CNV RNA
To determine whether CNV generates subgenomic RNAs during infection, Northern blots of RNA extracted from CNV-infected leaves were prepared and probed with a cloned CNV segment corresponding to the 3' terminus of the genome. Fig. 5 shows the result of an experiment in which an infectious CNV transcript (pK2; unpublished results) was used as inoculum. In addition to genomic-length RNA several subgenomic-length RNAs are visible with estimated sizes of 3-2, 2-3, 1-5 and 1.0 kb (lane 3). It should be noted that when our laboratory strain of CNV was used as an inoculum the primary hybridizing species in infected leaf extracts was approximately 0.5 kb and only very minor amounts of other viral RNA species were detected. We have recently found that this RNA species is a defective interfering RNA similar to that first described by Hillman et al. (1987; D. M. Rochon & J. C. Johnston, unpublished results). To determine whether CNV subgenomic RNAs are encapsidated and therefore present in virion RNA preparations, RNA purified from virus particles was fractionated according to size on a linear-log sucrose gradient and samples from selected fractions were similarly analysed by Northern blots using a 3'-terminal probe (Fig. 6). Results of this study show that CNV virion RNA preparations contain, in addition to genomic-sized RNA, several 3'-coterminus RNA species of subgenomic size (approx. 2-3, 1-5 and 1-0 kb). We did not observe encapsidation of the 3-2 kb RNA species detected in CNV-infected leaves nor did we observe efficient encapsidation of the 0.5 kb defective interfering RNA [which is visible only after enrichment by sucrose gradient fractionation (compare lanes 1 and 9, Fig. 6)].

(ii) In vitro translation of size-fractionated CNV RNA
To determine whether the subgenomic RNAs act as templates for the expression of CNV in vitro translation products, samples from selected sucrose gradient fractions were translated in wheatgerm extracts. Fraction 32, which contained RNA species of approximately 0.5 kb, did not have mRNA activity (Fig. 7, lane 4). As mentioned previously, the 0.5 kb RNA species is a defective interfering RNA and therefore should not have coding potential. Fractions 38 and 44, which contained RNA of 1.0 kb (approx.) produced proteins of 20K and 24K (lanes 5 and 6). Thus, it appears that the 1.0 kb subgenomic RNA serves as the template for the 24K and 20K proteins. Fractions 50 to 74 contained, in addition to 1-0 kb RNA molecules, species of approximately 1.5 and 2-3 kb. These fractions gave rise to proteins of 24K and 20K as well as a 41-6K protein (lanes 7 to 11). The 41.6K protein is most likely the product of the 2-3 kb subgenomic RNA since the 1-5 kb RNA would not be long enough to encompass the 41K ORF. It is therefore concluded that the 2-3 kb subgenomic RNA serves as the template for the 41-6K in vitro translation product. The 1-5 kb RNA could be a specific degradation product or another defective interfering RNA. The only fraction which contained an efficient template for the synthesis of the 34-6K product was fraction 74 (lane 11) which contained full-length genomic RNA (4.7 kb). However, as a sucrose gradient fraction containing exclusively genomic-length RNA was not obtained, a full-length synthetic positive-sense transcript, pK2, was translated.
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Fig. 7. In vitro translation products of selected sucrose gradient-fractionated CNV virion RNA samples. Wheatgerm extracts were programmed with BMV RNA (lane 1), CNV virion RNA (160 μg/ml) (lane 2), no added RNA (lane 3) or equal volumes of RNA from sucrose gradient fractions 32, 38, 44, 50, 56, 62, 68 and 74 (lanes 4 to 11, respectively). SDS–PAGE was in 15% acrylamide. The Mr values of CNV in vitro translation products are indicated on the right of the gel.

Discussion

In vitro translation of CNV virion RNA resulted in the synthesis of a single protein of 34.6K in rabbit reticulocyte lysates whereas four proteins of 34.6K, 41.6K, 24K and 20K were produced in wheatgerm extracts. Using the HART procedure and translation of positive-sense synthetic transcripts in wheatgerm extracts, the 34.6K coding region was mapped to the 5’ terminus of CNV RNA and the 41.6K, 24K and 20K proteins were mapped to downstream regions. In wheatgerm extracts, the 34.6K protein was translated from full-length genomic RNA whereas the 41.6K, 24K and 20K proteins were produced from subgenomic RNAs.

A number of explanations could account for the observed differences in translation of CNV virion RNA in wheatgerm extracts and rabbit reticulocyte lysates (Fig. 2). First, the presence of a low level endogenous capping activity present in wheatgerm extracts (Paterson & Rosenberg, 1979) but not in rabbit reticulocytes might influence the efficiency of translation of CNV RNA in vitro. It is not known whether CNV genomic or subgenomic RNAs are capped; however, an absence of a cap on CNV subgenomic RNAs might explain why these RNAs are not translated in rabbit reticulocyte lysates but are in wheatgerm extracts. Second, differences in optimal translation conditions (e.g. K⁺ or Mg²⁺ concentration, or template concentration) that exist between the rabbit reticulocyte lysate and wheatgerm extract systems might account for the observed differences in the number of translation products that are produced. The small amount of subgenomic RNA in CNV virion RNA preparations does not appear to be an important factor accounting for the lack of synthesis of the subgenomic RNA-directed 41.6K, 24K and 20K proteins in rabbit reticulocyte lysates since translation reactions programmed with microgram quantities of sucrose gradient-fractionated subgenomic mRNAs still did not result in detectable protein synthesis (data not shown). Third, a differential ability of the ribosomes of wheatgerm extracts and rabbit reticulocyte lysates to translate through regions of high secondary structure could explain the observed differences in the translation products synthesized. However this seems unlikely
Table 1. Sequences surrounding putative AUG initiation codons of CNV ORFs

<table>
<thead>
<tr>
<th>CNV ORF</th>
<th>Consensus sequence for animals* (CACCAUGG)</th>
<th>Consensus sequence for plants† (AACAAUGGC)</th>
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</thead>
<tbody>
<tr>
<td>33K</td>
<td>* * *</td>
<td>CGACAUGG</td>
</tr>
<tr>
<td>41K</td>
<td>* * *</td>
<td>CACAAUGG</td>
</tr>
<tr>
<td>21K</td>
<td>* * *</td>
<td>AUUCAUGG</td>
</tr>
<tr>
<td>20K</td>
<td>* * *</td>
<td>AACCAUGG</td>
</tr>
</tbody>
</table>

* Indicates identity with the sequence surrounding the AUG codon for the indicated ORF with the consensus sequence for either animal or plant ribosomes.
† From Kozak (1986).
‡ From Lütcke et al. (1987).

because heating the RNA prior to translation had no effect on the translation products synthesized in either wheat-germ extracts or rabbit reticulocyte lysates (data not shown).

Last, a differential selection of AUG initiation codons by the ribosomes of plants and animals could account for the observed differences in translation of CNV. A comparison of the sequences surrounding the putative initiation codons for the ORFs encoding CNV 41, 33, 21, and 20K proteins with the consensus sequences established for either plant (Lütcke et al., 1987) or animal (Kozak, 1986) mRNAs (Table 1) shows that the sequences surrounding the putative AUG codons for the 41K- and 20K-encoding ORFs are in almost optimal context for translation by either plant or animal ribosomes. The sequence surrounding the putative 33K initiation codon is in a less optimal context for translation by animal ribosomes, although the nucleotide three positions upstream of the AUG codon [which is implicated as an important regulator of translational efficiency (Kozak, 1984b)] is a purine which is favoured over a pyrimidine in this position. The sequence surrounding the putative initiation codon for the 21K ORF is in poor context for translation by animal ribosomes and thus its synthesis might not be expected in rabbit reticulocyte lysates. The lack of translation of the 20K ORF in rabbit reticulocyte lysates could then be rationalized if its production were dependent upon prior translation of the upstream 21K ORF via leaky scanning (Kozak, 1981). The lack of synthesis of the 41K ORF in rabbit reticulocyte lysates cannot be correlated with the sequences surrounding the putative AUG initiation codon for this protein. This lack of correlation might indicate that sequences further upstream or downstream of the initiation codons might also modulate translational efficiency.

The 92K protein predicted from nucleotide sequence data is implicated as the CNV replicase on the basis of amino acid sequence similarity with other viral replicases (Rochon & Tremaine, 1989). The observed similarity is 3' of the 33K amber termination codon, in particular in a region surrounding and including a glycine–aspartate–aspartate tripeptide which has been found in the replicases and putative replicases of many plant and animal viruses. These observations suggest that the region downstream of the amber codon is translated in vitro. The most obvious means for expression of this portion of the genome is via readthrough translation since a subgenomic RNA corresponding to this region was not found (Fig. 6 and 7). Synthesis of the predicted 92K protein of CNV, however, was not observed in wheatgerm extracts even when exogenous calf liver tRNA, yeast tRNA or tRNA isolated from tobacco leaves was supplied (see Methods; data not shown). [Tyrosine-specific tRNAs from tobacco leaves have been shown to suppress termination at amber codons (Beier et al., 1984a, b.)] The lack of synthesis of the 92K protein might be due to a lack of the appropriate suppressor tRNA in the tRNA preparation used or, alternatively, the RNA sequence surrounding the termination codon (Pelham, 1978) might reduce the efficiency of readthrough of the CNV amber termination codon in vitro.

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References


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