Nucleotide sequence, genomic organization and synthesis of infectious transcripts from a full-length clone of artichoke mottle crinkle virus

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The complete nucleotide sequence of the genome of artichoke mottle crinkle virus (AMCV), a member of the tombusvirus group, has been determined. The genome is 4790 nucleotides (nt) in length. A full-length cDNA of the AMCV genome has been cloned in pUC9 downstream of the T7 RNA polymerase promoter. Transcripts were infective when inoculated onto Nicotiana clevelandii and N. benthamiana plants. The AMCV genome contains five open reading frames (ORFs). The first ORF from the 5' terminus (ORF1) encodes a protein with a predicted Mr of 33K. ORF2 extends through the amber termination codon of ORF1 to yield a polypeptide of predicted Mr 92K and which is the putative RNA-dependent RNA polymerase. ORF3 codes for the coat protein (41K). Two nested ORFs in different reading frames (ORFs 4 and 5) code for a 22K and a 19K polypeptide respectively. Sequence homologies suggest that the 22K protein could be involved in cell-to-cell movement of virus. ORFs 3, 4 and 5 are translated from two 3' coterminal subgenomic (sg) RNAs, the 5' termini of which have been mapped. The two sg RNAs are 2155 (sg1) and 934 (sg2) nt in length. ORF3 is expressed from sg1 RNA whereas ORFs 4 and 5 are potentially expressed from sg2 RNA. Time course experiments with Cynara scolymus protoplasts indicate that during AMCV infection both positive and negative strands of genomic and sg RNAs are produced and that sg2 RNA is produced before and at a higher level than sg1 RNA.

Introduction

Artichoke mottle crinkle virus (AMCV) is a member of the tombusvirus group (Martelli et al., 1988) which is found naturally only in the globe artichoke (Cynara scolymus L.) in which it causes large chlorotic to pale-green blotches, plant size reduction, leaf distortion and a decrease in the number of flower heads (Martelli, 1965; Quacquarelli & Martelli, 1966).

The tombusviruses have icosahedral particles of about 30 nm in diameter made up of 180 protein subunits each with an Mr of 40K to 43K (Martelli et al., 1988). Their genome is a single molecule of positive sense, single-stranded RNA of 4700 to 4800 nucleotides (nt) (Gallitelli et al., 1985). Most of the definitive members of the tombusvirus group are serologically related (Koenig & Gibbs, 1986; Martelli et al., 1988) with a few exceptions, one of which is cucumber necrosis virus (CNV) (Rochon & Tremaine, 1988). The determination of three tombusvirus genomic sequences, those of tomato bushy stunt virus-cherry strain (TBSV-cherry; Hearne et al., 1990), cymbidium ringspot virus (CyRSV; Grieco et al., 1989a) and CNV (Rochon & Tremaine, 1989), has been reported. The genomes of these three viruses contain five equally sized open reading frames (ORFs) similarly positioned on the genome. The presence of a sixth ORF downstream of ORFs 4 and 5 in tombusvirus genomes has been proposed (Boyko & Karasev, 1992), but Dalmay et al. (1993) clearly demonstrated that the product encoded by ORF6 is not required in CyRSV infection and that the conservation of nucleotide sequence in ORF6 is probably due to structural reasons.

Total RNA extracts from plants infected with TBSV, CNV and CyRSV show, in addition to genomic RNA, two 3' coterminal subgenomic (sg) RNAs of approximately 2.2 kb (sg1) and 0.9 kb (sg2) (Hillman et al., 1989; Rochon & Johnston, 1991; Russo et al., 1988). Translation of the genomic RNAs of these three tombusviruses yields a 33K polypeptide in each case (p33). A 92K polypeptide (p92) can be derived from readthrough of
the amber termination codon of ORF1. In vitro translation of TBSV genomic RNA in the presence of calf liver tRNA produced a protein of 90K (Hayes et al., 1988). The p92 of TBSV and CNV have been shown to share homology with putative and known replicases of animal and plant viruses (Rochon & Tremaine, 1989; Hearne et al., 1990). Recently, Dalmay et al. (1993) reported that both the 33K and the 92K proteins are necessary for CyRSV RNA replication. The coat protein (CP) gene (ORF3) is located downstream of ORF2 (p92) and is translated from sg1 RNA. ORF4 and ORF5 are two nested ORFs, in different frames, which can be translated from sg2 RNA (Rochon & Johnston, 1991) to yield two polypeptides of 22K and 19K respectively. Data on CyRSV indicate that the 22K protein is involved in the cell-to-cell movement of virus (Dalmay et al., 1993).

In previous work we have reported both the nucleotide sequence and the localization of the AMCV CP gene (Tavazza et al., 1989). Furthermore, Grieco & Gallitelli (1990) have shown that the 3'-terminal region of the AMCV genome contains two nested ORFs similar to those found in the TBSV, CyRSV and CNV genomes.

In this paper we report the complete nucleotide sequence of the AMCV genome and its expression strategy. Furthermore, we demonstrate that in vitro transcripts synthesized from a full-length cDNA clone of the AMCV genome are infectious.

**Methods**

**Propagatation and purification of virus.** The original source of AMCV was kindly provided by Dr D. Gallitelli (Department of Plant Pathology, University of Bari, Italy). AMCV was propagated in Nicotiana clevelandii or N. benthamiana plants. The virus particles and viral RNA were purified as described by Gallitelli et al. (1985) and by Hari et al. (1979) respectively.

cDNA cloning and sequencing. The synthesis and cloning of the 3' terminus was performed as follows. Ten µg of genomic RNA was polyadenylated with poly(A) polymerase (Amersham) as described by the supplier. One µg of polyadenylated genomic RNA was used as the template for oligo(dT)-primed first-strand synthesis of cDNA using the cDNA Synthesis System Plus kit (Amersham). Blunt-ended cDNA fragments were inserted in the Smal site of pUC9. Several clones containing about 2 kb from the 3' terminus of the genomic RNA were obtained. Two cDNA clones (pAMCV11 and pAMCV17) covering the remaining portion of the AMCV genome were produced as described previously by Tavazza et al. (1989). Double-stranded plasmid sequencing was performed by the dideoxynucleotide chain termination method using Sequenase (USB).

**RNA sequencing.** One µg of viral RNA was treated with methylmercuric hydroxide as described by Toole et al. (1984) and sequenced using the primer 5' AGGAGAGAAATCCCTACGCAAAGC 3' which is complementary to nt 61 to 85 of AMCV genomic RNA, using the GemSeq Transcript Sequencing System (Promega) as described by the supplier.

**Total RNA extraction from leaves and protoplasts.** Total RNA extracts from leaves were produced as described by Chomczynski & Sacchi (1987) with some modifications. Leaves were homogenized in three volumes of a buffer containing 100 mM-sodium acetate, 4 M-guanidine isothiocyanate, 10 mM-EDTA, 0.5% (w/v) sodium lauryl sarcosinate and 0.7% 2-mercaptoethanol, extracted with two volumes of acid phenol-chloroform (3:1) and precipitated with one volume of isopropanol. The nucleic acids were resuspended in 200 µl of 0.1% (w/v) SDS per gram of starting material and 1/50 volume of 5 M-NaCl was added. The sample was mixed by vortexing and was incubated on ice for 10 min. After phenol-chloroform (1:1) extraction, the RNA was precipitated with one volume of isopropanol.

Total RNA extraction from protoplasts was performed as described above, with the exception of the first step. In this case 0.5 ml of the above buffer was added to 100 µl of frozen protoplasts and mixed by vortexing prior to incubation at 65 °C for 10 min and phenol-chloroform extraction.

**Primer extension.** AMCV-infected N. clevelandii RNA extract was sucrose gradient-fractionated (Brakke & van Pelt, 1970) and fractions enriched for the 2.2 kb and 0.9 kb sg RNAs were treated with methylmercuric hydroxide prior to annealing with the 5' 32P-labelled oligonucleotides PEX1 (5' ACTGCTTCGTGCTAACTGG 3') and PEX2 (5' GAAAGAAGGTTGCTAATCGG 3') respectively. PEX1 and PEX2 are complementary to nt 2707 to 2725 and 3977 to 3993 of the AMCV genomic RNA, respectively. The same oligonucleotides were used in parallel dideoxynucleotide sequencing reactions with pUC9 subclones of pAMCV17 and pAMCV97 (see Tavazza et al., 1989).

The annealing reactions were performed under the same conditions used for RNA sequencing. After annealing, dNTPs (1 mM each) and avian myeloblastosis virus reverse transcriptase (Promega) were added and the samples were incubated at 42 °C for 1 h.

**Northern blot analysis.** RNA was electrophoresed through a 1% agarose gel containing formaldehyde according to the method of Sambrook et al. (1989), blotted to Hybond-N membrane (Amersham) and hybridized with 32P-labelled in vitro transcripts as described by the manufacture. After hybridization, the filters were treated with RNase A and washed as described by the supplier, with the addition of final extensive washing in 0.1 x SSPE and 0.1% (w/v) SDS at 65 °C. Specific fragments of pAMCV11, pAMCV17 and pAMCV97 were subcloned in pSP65 in order to obtain synthetic transcripts of either positive (+) or negative (−) polarity. The genomic locations of the transcripts are shown in Fig. 1(a). Autoradiograms were scanned in a Beckman DU-70 spectrophotometer equipped with a gel-scanning device and an automatic area integrator and the areas beneath the peaks were calculated.

**Protoplast isolation and infection.** C. scolymus L. protoplasts were isolated from a 10-day-old suspension culture as previously described (Ordas et al., 1991), except that after the last wash the protoplasts were resuspended in a 0.4 m-mannitol solution. Polyethylene glycol-mediated RNA inoculation was carried out essentially as described by Maule (1983) with minor modifications. Twenty µg of AMCV RNA was used to inoculate 2 x 10^6 protoplasts; after the inoculation, the mixture was diluted with the washing medium (Ordas et al., 1991) and centrifuged at 200 g for 3 min. After two additional washes the protoplasts were resuspended (10^6/ml) in liquid MS medium (Murashige & Skoog, 1962) in which the NH_4NO_3 was replaced by casein hydrolysate (1 g/l) and to which 0.5 M-mannitol was added, containing 0.5 mg/l 6-benzylaminopurine, 2 mg/l naphthaleneacetic acid and 2 mg/l 2,4-dichlorophenoxyacetic acid pH 5.6. The protoplasts were then incubated in a growth cabinet at 25 °C under continuous light (1000 lx). After incubation the protoplasts were diluted with washing medium, centrifuged at 1000 g for 3 min and the pellet was then frozen in liquid nitrogen and stored at −80 °C.

**Construction of a full-length cDNA clone.** The 5' terminal region of the AMCV genome was synthesized using the GeneAmp RNA PCR kit.
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Fig. 1. (a) AMCV cDNA clones encompassing the entire genome; pAMCVIT is one of the six 3' clones obtained by priming first-strand synthesis with oligo(dT) on polyadenylated AMCV RNA. The regions of pAMCV11, pAMCV17 and pAMCV97 that were subcloned to obtain radiolabelled transcripts are delimited by the restriction enzyme sites used: B, BamHI; E, EcoRI; P, PstI; A, AvaI; H, HinclI. The polarity and localization of the probes are indicated by arrows. (b) Northern blot analysis of RNA extracted from: lanes 1, AMCV virions; lanes 2, AMCV-infected N. clevelandii plants; lanes 3, healthy N. clevelandii plants. The probe used is indicated above each panel. Arrows indicate the position of genomic (g) and sg (sg1 and sg2) RNAs.

Fig. 2. Schematic representation of the construction of the AMCV full-length clone pAM-G. Solid lines indicate vector sequences, shaded boxes indicate viral sequences and the open box indicates the T7 promoter; different shading of boxes represents the different sources of fragments, as described in the text. Restriction enzymes used are indicated.

In vitro transcription and plant inoculation. The full-length clone pAM-G was linearized with SacII and the 3' protruding ends were blunt-ended with T4 DNA polymerase (Amersham) according to Sambrook et al. (1989). RNA transcripts were obtained using T7 RNA polymerase (Promega) used according to the supplier's instructions. After the reaction, transcripts were mixed with one volume of inoculation buffer (Heaton et al., 1989) and inoculated onto N. clevelandii and N. benthamiana plants (1 to 2 µg/leaf).

Computer analysis. Nucleotide and amino acid sequence data were analysed on a DEC/VAX 8800 computer using the software developed by the Genetics Computer Group (GCG) (1991). Sequence database screening was performed using the FASTA program. Multiple sequence
Fig. 3. For legend see opposite.
alignments were generated using the PILEUP program. Pairwise sequence comparisons were done by the GAP program using the randomizations command line option to calculate the adjusted alignments. Generally AS values greater than seven can be observed alignment score, S r is the mean score obtained upon 25 random simulations of the alignment procedure and is the standard deviation of the score. Generally AS values greater than seven can be considered a firm indication of a genuine relationship between two sequences (Koonin et al., 1991).

**Results and Discussion**

**Analysis of viral RNAs**

RNA extracted from AMCV particles and from both AMCV-infected and mock-inoculated *N. clevelandii* plants was analysed by Northern blotting. Filters were probed (Fig. 1b) with three different 32P-labelled transcripts of negative polarity a(−), b(−) and c(−), obtained from subclones of the three cDNA clones pAMCV11, pAMCV17 and pAMCV97 (Tavazza et al., 1989) (Fig. 1a).

Probe a(−) is the transcript of the *BamHI*—EcoRI fragment of pAMCV11 and spans nt 1339 to nt 2185. It hybridizes only with genomic length RNA in both virion- and infected plant-derived RNA. Probe b(−) (the *PstI*—*AvaiI* fragment of pAMCV17, nt 2765 to 3535) hybridizes to genomic length RNA and also to a virus-specific RNA of about 2.2 kb. Probe c(−) (the *HincII*—*HincII* fragment of pAMCV97 nt 3850 to 4614) revealed two strong stop points at the 3′ end of the run-off extension analysis in the presence of dideoxynucleotides. The nucleotide sequence of the 3′ terminus found by sequencing the 5′ terminus of AMCV RNA as a template (Fig. 1a). The genome was sequenced from both strands and each position was sequenced an average of four times, including the previously sequenced regions of clone pAMCV17 (Tavazza et al., 1989). The sequence of the region spanning nt 2800 to 3100 on the minus strand revealed the presence of three G residues at positions 2910 to 2912 which were not detected on the plus strand in previous studies (Tavazza et al., 1989).

The sequence of the 5′ terminus of the viral RNA was obtained using an oligonucleotide complementary to nt 61 to 85 of the AMCV genomic RNA and primer extension analysis in the presence of dideoxynucleotides. Two strong stop points at the 3′ end of the run-off transcripts were found by sequencing the 5′ terminus of AMCV genomic RNA (not shown). Similar results were found by Ahlquist & Janda (1984) and Gupta & Kingsbury (1984) when sequencing capped RNAs. These authors concluded that reverse transcriptase adds a nucleotide to the reverse transcript of a capped RNA molecule, to generate the slower migrating run-off product, and that the faster migrating species corresponds to the presence of reverse transcription at the preceding nucleotide, which initiates the RNA. The results on the infectivity of capped and uncapped RNA transcripts (see later), together with analogous results obtained for CyRSV, CNV and TBSV (Burgyan et al., 1990; Rochon & Johnston, 1991; Hearne et al., 1990),

sg RNAs. The same situation was observed for virions derived from plants infected with infectious transcripts (not shown).

**Nucleotide sequence strategy**

The nucleotide sequence of the AMCV genome shown in Fig. 3 was obtained (except for a few nucleotides at the 5′ terminus) from clones pAMCV11, pAMCV17 and pAMCV97 (see Tavazza et al., 1989) and from six cDNA clones obtained using synthetically polyadenylated AMCV RNA as a template (Fig. 1a).
and ORF2 are necessary for the replication of CyRSV both the p33 and the p92 readthrough products of ORF 1 mutagenesis analysis of CyRSV RNA has illustrated that proteins are highly conserved (Table 1). Recently, the p92 proteins of tombusviruses reveal that both Amino acid sequence comparisons among the p33 and extends from nt 3904 to nt 4422 and potentially encodes two nested ORFs the AMCV genome terminates with a polypeptide of Mr 19320 (p19). Downstream of the ORF4 and ORF5 ORF3 runs from nt 2668 to nt 3834 and encodes the CP, intergenic region of 30 nt separates ORF2 from ORF3. would extend the ORF to a UGA termination codon at 5' terminus, followed by the first AUG codon which initiates ORF1. ORF1 spans from the AUG at nt 181 to an amber termination codon at nt 1071, encoding a polypeptide with a predicted Mr of 33221 (p33). Readthrough of the amber termination codon of ORF1 would extend the ORF to a UGA termination codon at nt 2637, resulting in ORF2. The predicted Mr of the readthrough product of ORF2 is 92046 (p92). An intergenic region of 30 nt separates ORF2 from ORF3. ORF3 runs from nt 2668 to nt 3834 and encodes the CP, having a deduced Mr of 41163 (p41). ORF4 and ORF5 are two nested ORFs, in different frames, following the CP gene from which they are separated by a 37 nt and a 69 nt intergenic region, respectively. ORF4 is the larger of the two, starting at nt 3872 and terminating at nt 4441 and encoding a polypeptide of Mr 21610 (p22). ORF5 extends from nt 3904 to nt 4422 and potentially encodes a polypeptide of Mr 19320 (p19). Downstream of the two nested ORFs the AMCV genome terminates with a 349 nt non-coding region.

Amino acid sequence comparisons (i) ORF1 and ORF2
Amino acid sequence comparisons among the p33 and the p92 proteins of tombusviruses reveal that both proteins are highly conserved (Table 1). Recently, mutagenesis analysis of CyRSV RNA has illustrated that both the p33 and the p92 readthrough products of ORF1 and ORF2 are necessary for the replication of CyRSV RNA (Dalmay et al., 1993). This result confirms the involvement of tombusvirus p92 in RNA replication as previously suggested by Rochon & Tremaine (1989) and Hearne et al. (1990) on the basis of the presence of the GDD motif (Kamer & Argos, 1984).

Amino acid sequence comparisons of the AMCV p33 with sequences contained in the Swissprot database were performed using the FASTA program (UWGCG) and the best alignments were further analysed with the GAP program (UWGCG). The only significant homology (besides that with other tombusvirus p33s) was found with the p22 pre-readthrough portion of the putative RNA-dependent RNA polymerase of tomato necrosis virus strain D (TNV-D; Coutts et al., 1991) (AS = 9) but not TNV-A (Meulewaeter et al., 1990) (AS ≈ 0). It is noteworthy that AMCV p33 is more closely related to TNV-D p22 (AS = 9) than TNV-D p22 is to TNV-A p22 (AS = 3). Both TNV-D and TNV-A have an overall genomic organization similar to that of the carmovirus group. The genomic organization of the tombusvirus AMCV, the carmovirus turnip crinkle virus (TCV; Hacker et al., 1992) and TNV-D is shown in Fig. 4.

Amino acid sequence comparison of AMCV p92 with the Swissprot database revealed that the closest similarity (besides that with the p92s of other tombusviruses) was found with p82 of TNV-D (the putative RNA-dependent RNA polymerase) which showed high identity (41-6%) throughout the sequence. Moreover, AMCV p92 showed homology with TNV-A p22 and with putative viral RNA-dependent RNA polymerases belonging to supergroup II as defined by Koonin (1991), but this homology was limited to the readthrough portion of the protein only. Tombusvirus p92 did not contain the purine NTP-binding domain as defined by Hodgman (1988) and Gorbalenya et al. (1988).

These similarity studies on the pre-readthrough and on the readthrough portions of tombusvirus p92 revealed that TNV-D might represent a link between the tombusvirus and carmovirus groups. In fact the 5' region of the TNV-D genome is closely related to tombusviruses whereas its 3' region has an organization typical of carmovirus genomes (Fig. 4), suggesting that gene exchange mediated by recombination (Bujarski & Kaesberg, 1986) may have had a role in the evolution of these viruses.

(ii) ORF3
AMCV ORF3 is translated from sgl RNA and codes for a 41K protein which has been identified previously as the CP (Tavazza et al., 1989), on the basis of its homology with the TBSV CP (Hopper et al., 1984). Furthermore, polyclonal antibodies raised against AMCV virions react with the 41K protein produced in infected plants (not...
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AMCV p22 52 SGGYTMRIRIKIPLVS RKAQVSGKLY LRIDSD
TBSV p22 52 SGGYTMRIRIKIPLVS RKAQVSGKLY LRIDSD
CNV p22 52 SGGYTMRIRIKIPLVS RKAQVSGKLY LRIDSD
CyRSV p22 52 SGGYTMRIRIKIPLVS RKAQVSGKLY LRIDSD
CMV p32 84 GINSVFQVL CAVTRTVS TDAEGSLKIY LADLDG

Fig. 5. Multiple sequence alignment of the tombusvirus p22 region spanning amino acids 52 to 86 with the region of CMV movement protein (p32) spanning positions 84 to 116. In the consensus sequence (Con.), asterisks indicate residues with similar chemical properties shared by all sequences, Δ indicates the amino acids which constitute the central motif of family II virus movement proteins (Koonin et al., 1991).

(iii) ORF4

AMCV ORF4 encodes a 22K protein (p22) which is translated from sg2 RNA. Recently, data obtained on mutagenized CyRSV RNA by Dalmay et al. (1993) have shown that the p22 of CyRSV is involved in the cell-to-cell movement of virus. The high degree of similarity between the CyRSV and AMCV p22 proteins (Table 1) strongly suggests an analogous function for the two proteins. Furthermore, computer-assisted amino acid sequence comparisons between the tombusvirus p22 and sequences contained in the Swissprot database revealed significant homology between a 35 amino acid region of tombusvirus p22 and a region of the cucumber mosaic virus (CMV) 32K protein (p32) (Davies & Symons, 1988). This region of the CMV p32 contains the conserved motif characteristic of putative and known viral movement proteins belonging to family II as defined by Koonin et al. (1991). The multiple sequence alignment of the tombusvirus p22 and CMV p32 regions containing this conserved motif is shown in Fig. 5.

(iv) ORF5

AMCV ORF5 is contained within ORF4 and is positioned as a −1 frameshift with respect to ORF4. ORF5 is translated from sg2 RNA (see below) and potentially encodes a 19K protein (p19) which is conserved among the tombusviruses (Table 1); this suggests that there is an important role for this protein in infection. However, available data concerning the function of tombusvirus p19, obtained from ORF5-mutagenized transcripts of CNV (Rochon & Johnston, 1991) and CyRSV (Dalmay et al., 1993) suggest that p19 is dispensable for both virus replication and cell-to-cell movement. No significant homologies were found between Swissprot database proteins and p19, either as an entire protein or in those regions showing the highest conservation among the tombusviruses.

Mapping of the 5′ ends of sg RNAs

In order to map precisely the 5′ termini of the sg RNAs, primer extension analysis was performed on sucrose gradient-fractionated RNA extracted from AMCV-infected N. clevelandii leaves. The results shown in Fig. 6 show that in both primer extension analyses only a single prominent product was obtained. The 5′ termini of sg1 and sg2 mapped at nt 2636 and nt 3857 of the AMCV genome, being 2155 nt and 934 nt in length, respectively. These data indicate that both p22 and p19 are translated from the same sg RNA (sg2) and are in agreement with previous results obtained for TBSV (Hillman et al., 1989), CyRSV (Grieco et al., 1989b) and CNV (Rochon & Johnston, 1991). Furthermore, as previously shown for CNV (Rochon & Johnston, 1991), AMCV, TBSV and CyRSV the p22 AUG is in a sub-optimal context (Kozak, 1989) whereas the p19 one is in the optimal context, suggesting that a ‘leaky scanning’
Fig. 6. Primer extension analysis of AMCV sg RNAs: sgl (a, lane E) and sg2 (b, lane E). To the right of the extensions is a set of sequencing reactions performed on pAMCV17 (a) and on pAMCV97 (b) primed with the same oligonucleotides used to initiate the reverse transcription reactions.

mechanism operates in the expression of ORF5 in the tombusviruses.

**Time course of AMCV infection in protoplasts**

In order to study the kinetics of AMCV genome expression, a time course experiment was performed using *C. scolymus* L. protoplasts. Three aliquots of $2 \times 10^6$ protoplasts were each infected with 20 µg AMCV RNA, then the aliquots were pooled and cultured in liquid medium. Protoplast aliquots ($1 \times 10^6$) were collected at 4 h intervals for 24 h, frozen in liquid nitrogen and stored at $-80^\circ C$. Total RNA extracted from each protoplast sample was divided into two identical aliquots, to duplicate samples for Northern analysis. $^{32}P$-labelled transcripts of negative c(−) and positive polarity c(+) (Fig. 1a) were used as probes. The results of probing RNA from virus-infected protoplasts with c(−) are shown in Fig. 7. Four h post-inoculation (p.i.) the genomic and the two positive sense sg RNAs were already present, albeit at different concentrations, sgl being the least abundant. From 12 to 16 h p.i. all three positive sense viral RNAs were significantly increased in amount before reaching a plateau at 20 h p.i. Furthermore, sg2 RNA, which is the template for the synthesis of p22 and p19, was present at higher levels than sgl RNA (the template for the CP) at any time after infection. The observed pattern of expression of the sg RNAs might account for the temporal regulation of the expression of the 22K and 19K polypeptides and of the CP.

The results of probing RNA from virus-infected protoplasts with transcript c(+) are also shown in Fig. 7. It is not possible to distinguish whether the detected negative polarity molecules are in a free or duplex form; however, the specificity of the probe c(+) for negative strand sequences was confirmed by the hybridization results obtained with RNA purified from virus particles (Fig. 7b, lane V). In fact the positive sense probe failed to hybridize with the plus sense genomic-length RNA (compare a, lanes V and V' with b, lane V).

At 4 h p.i. the amount of negative sense sg2 was similar to the amount of genomic negative strand RNA whereas sgl RNA was undetectable. From 8 to 16 h p.i. the amounts of the genomic and the two sg RNAs increased, reaching a plateau which was stable until 24 h

![Fig. 7. Time course of AMCV infection in *C. scolymus* protoplasts. RNA extracted from mock-inoculated protoplasts (lane C) and from virus-infected protoplasts collected 4, 8, 12, 16, 20 and 24 h.p.i. (lanes 4, 8, 12, 16, 20, 24) was hybridized with probe c(−) (panel a) or c(+) (panel b) (see Fig. 1a). Lane V contains 200 ng of purified virus RNA; lane V' is the same as V, with a shorter exposure time. Arrows indicate the migration of genomic and sg RNAs.](image-url)
p.i. Interestingly, at any time p.i. the amounts of the two negative sg RNAs were similar to the amount of the negative strand of the replicating genomic RNA. This result is similar to the result obtained with TNV-A (Meulewaeter et al., 1992) and significantly different from the results obtained with plant viruses belonging to the tobamovirus, ilarvirus and tymovirus groups (Ishikawa et al., 1991; Van der Kuyl et al., 1991; Gargouri et al., 1989) for which negative sense sg RNAs were undetectable, or to bromoviruses (Marsh et al., 1991) in which levels of the negative sense sg RNA4 are very low compared with those of the genomic negative sense RNAs-1,-2 and -3.

The presence of such elevated levels of both sg2 and sg1 negative strand RNAs in the early stages of AMCV infection (at 4 and 8 h p.i. respectively) may suggest that the synthesis of AMCV sg RNAs is achieved by premature termination of minus strand synthesis followed by autonomous replication of sg RNAs.

**Infectivity of transcripts derived from a full-length cDNA clone**

The RNA sequencing of the 5' end of the AMCV genome did not allow us to identify the first two nucleotides. We decided to consider these nucleotides as two Gs on the basis of the T7 promoter consensus sequence (which extends into the transcript; Dunn & Studier, 1983) and on the basis of the results on the infectivity of TBSV and CNV synthetic transcripts (Hearne et al., 1990; Rochon & Johnston, 1991).

Attempts to synthesize an infectious full-length AMCV cDNA clone in one step, priming first-strand cDNA synthesis with the oligonucleotide INF3 and second-strand synthesis with the oligonucleotide INF5, were not successful. The infectious full-length cDNA clone pAM-G was obtained in a multi-step procedure as described above and shown diagrammatically in Fig. 2. The intermediate clone pAM-5' which was obtained by PCR amplification was sequenced to verify that no mutations had occurred.

Transcription of SacII-linearized pAM-G produces RNA transcripts identical to the AMCV genome, without additional nucleotides at the extremities, with the only uncertainty being that regarding the identity of the two 5' nucleotides. The infectivity of such run-off transcripts was assayed by inoculating 2 μg RNA per leaf to two systemic hosts for AMCV, *N. benthamiana* and *N. clevelandii*. Typical chlorotic lesions developed on the inoculated leaves 4 to 7 days after inoculation as occurred in plants inoculated with AMCV RNA extracted from virions. Both transcript- and AMCV virion RNA-inoculated plants developed systemic symptoms within 7 to 12 days after inoculation, with apical necrosis 2 weeks after infection. Total RNA extracted from both transcript- and AMCV RNA-inoculated plants showed an identical hybridization pattern after probing with the 3' specific transcript c(−) (data not shown).

A comparison of the infectivity of transcripts synthesized with or without the cap analogue 7mGpppG did not reveal any significant difference based on the number of chlorotic lesions observed on inoculated *N. clevelandii* leaves.

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