Infectious RNA Produced by \textit{in vitro} Transcription of a Full-length Tobacco Rattle Virus RNA-1 cDNA

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\begin{abstract}
A near full-length cDNA clone of tobacco rattle virus (TRV) RNA-1 was constructed by joining together nine overlapping cDNA clones using restriction sites in the regions of overlap. At the 5' end of the cDNA, oligonucleotide mutagenesis was used to insert nucleotides which were missing from the cDNA placing the construct immediately on the 3' side of the P, promoter of phage \lambda, to create pTR7116. Extraneous non-viral nucleotides had been deleted from the 3' end of the TRV cDNA to create a unique \textit{SmaI} site in pTR7116 in which the nucleotides CCC were provided by the viral cDNA, and GGG by the vector. As a result, pTR7116 could be linearized with \textit{SmaI} and transcribed \textit{in vitro} to yield RNA molecules with 5' and 3' termini identical to those of natural TRV RNA-1. These transcripts were infectious when inoculated onto leaves of tobacco and produced the subgenomic RNA species typical of an infection with TRV RNA-1.
\end{abstract}

The tobravirus, tobacco rattle virus (TRV) has a bipartite RNA genome but is unusual in that it can cause infections in the absence of a gene for the capsid protein which is carried on the smaller component (RNA-2) of the genome (Harrison & Robinson, 1986). These infections, called NM-type infections, occur when RNA-2 is lost; those caused by the complete TRV genome are called M-type infections.

NM infections cause symptoms on the host plant which may be more severe than those of the corresponding M-type infection and involve both replication of the viral RNA and its slow spread from cell to cell (Cadman & Harrison, 1959; Harrison & Robinson, 1978). In the anticipation that features of NM infections will be common to those of infections caused by other plant RNA viruses in their non-encapsidated phase, we have initiated a detailed molecular analysis of the larger RNA (RNA-1) of TRV to understand its ability to cause disease in plants.

The analysis so far, based on the analysis of interviral homologies in the nucleotide sequence of RNA-1, has suggested particular functional significance for several coding and non-coding regions. For example, in a gene encoding a 194K protein there are three domains that show similarity to plant viruses outside the tobravirus group (Hamilton \textit{et al.}, 1987). Two of these domains have been assigned functions related to RNA replicase activity: one domain is thought to be involved in nucleotide binding and the other in RNA recognition. Both of these domains are found in genes of a diverse group of eukaryotic viruses, sometimes referred to as the Sindbis group (Goldbach, 1986).

There is also similarity between the 29K protein encoded by TRV RNA-1 and the 30K protein of tobacco mosaic virus (TMV) (Boccara \textit{et al.}, 1986). This region of similarity in the 29K protein includes a domain which, in the TMV 30K protein, is known to be involved with the cell-to-cell movement of the virus (Ohno \textit{et al.}, 1983; Zimmern & Hunter, 1983).

Other features of the TRV RNA-1 which have been revealed by nucleotide sequence analysis and which may have functional significance include a tRNA-like structure at the 3' end (Van

Fig. 1. Construction of the full-length TRV RNA-1 clone. (a) The eight overlapping cDNAs are shown relative to one another together with a map of the restriction enzyme sites used in the construction (excluding polylinker sites). The cDNAs were cloned into the PstI site of either pUC19 or pBR322 after G-C tailing. All intermediate clones leading to the formation of pTR7621 involved the ligation of at least two fragments with pUC19. The first clone, pTR18, contained the KpnI/XhoI fragment from cDNA 25B and the XhoI/PstI (polylinker) fragment from 13A. The cDNA 543B was found to extend the sequence further towards the viral 3' end and was therefore added to pTR18. This resulted in clone pTR752 which contained the KpnI/AvaI fragment from cDNA 25B and the XhoI/PstI (polylinker) fragment from 13A. The cDNA 543B was found to extend the sequence further towards the viral 3' end and was therefore added to pTR18. This resulted in clone pTR752 which contained the KpnI/AvaI fragment from pTR18 and the AvaI/PstI (polylinker) fragment from 543B. At the 5' end, pTR713 was formed by ligation of a HindIII/AccI fragment from 31B and an AccI/SstI fragment from 4A. A partial AccI digest was used to isolate both fragments. PTR722 was produced by ligation of these fragments: PstI (polylinker)/BglII fragment from 24B, a BglII/AccI (partial) fragment from 25A and an AccI (partial)/SstI fragment from pTR713. pTR741
Belkum et al., 1987) and repeated sequences at the 5' end, which are also present in RNA-2 (Hamilton et al., 1987).

A functional assay is required to test the significance of the various features of TRV RNA-1. Recently, two approaches have proved useful as functional assays of plant virus genomes. One involves the expression of viral genes from the genome of a transgenic plant to complement mutations in the viral genome (Deom et al., 1987). The second approach involves the creation of mutations in the viral genome at the DNA level. The mutated viral sequence is then inoculated onto plants either as DNA or after in vitro transcription for viruses with RNA genomes (Meshi et al., 1987; Saito et al., 1987; Knorr & Dawson, 1988; Meshe et al., 1988). We are following the second approach and in this communication we describe the construction of a full-length clone of TRV RNA-1 and its transcription in vitro into infectious RNA.

The promoter used for in vitro transcription of the TRV RNA-1 cDNA was derived from plasmid pPM1 (Ahlquist & Janda, 1984). This promoter, rather than the T3, T7 or SP6 promoters, was selected because the final constructs would allow the transcription of an RNA molecule with a 5' A residue identical to that of natural TRV RNA-1. Transcripts obtained using the other promoters would contain a 5' G residue derived from the promoter sequence itself which extends into the transcribed region.

Attempts to synthesize full length cDNA of TRV RNA-1 were not successful, so the full-length cDNA was reconstructed from a series of shorter overlapping clones. These clones were derived from purified TRV (strain SYM) RNA as described previously (Bocchara et al., 1986) and have been sequenced over the entire length of the viral RNA (Hamilton et al., 1987). Construction of the full-length cDNA is shown in Fig. 1. Firstly isolated fragments were ligated in a stepwise manner to form a near full-length cDNA (pTR7621), a clone which was missing 45 bases at the 5' terminus and 96 at the 3' terminus compared with the viral sequence. Then a

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The fragment was then cloned between the SphI (polylinker)/SstI and 17A SstI/KpnI fragments. Finally pTR7621 was constructed using the pTR741 SphI (poly linker)/KpnI and pTR752 KpnI/HindIII (poly linker) fragments. (b) Clones pTR1 were constructed by subcloning into Bluescribe+ a Pr1/EcoRI fragment containing the Pr promoter isolated from plasmid pPM1 (Ahlquist & Janda, 1984). The 5' 446 base fragment from pTR721, resulting from Smal (poly linker)/EcoRI digestion, was cloned into pBP1 to produce pTRP1. (2) pTRP1 was mutagenized using an 89-mer nucleotide (A. Northrop, Microchemical Facility, I.A.P. Babraham, U.K.) to remove the G-C tail and poly linker sequences and insert the missing 5' bases determined previously (Hamilton et al., 1987). The clone was transformed into JM101 which was subsequently superinfected with phage VCS-M13 (Stratagene) and ssDNA was prepared. The oligonucleotide contained 23 bases partly complementary to the Pr promoter. The variant nucleotide converted the three 3'-terminal bases (part of the introduced Smal site) back to the original λ P sequence. The oligonucleotide also contained 21 bases complementary to the 5' end of cDNA 24B. The oligonucleotide was treated with kinase and together with normal M13 forward 17-mer sequencing primer was annealed to the ssDNA template (Zoller & Smith, 1987). Primer extension was carried out using both ligase and Klenow enzymes for approximately 5 h at 16 °C. The DNA was then transformed into JM101 and resultant colonies screened by colony hybridization using the 89-mer as a probe. Of the colonies, 16% hybridized stably to the 89-mer and one clone (pTR722) was confirmed by sequencing. (3) A fragment from pTR22 containing the P promotor and the complete 5' terminus of TRV RNA-1 was cloned into pUC19 to remove various flanking restriction sites. This was carried out by digesting pTR22 with PstI, filling in with Klenow polymerase and releasing the fragment with EcoRI. The fragment was then cloned between the Smal and EcoRI sites of pUC19 to create pTRP33. (4) The cDNA pTRMA61 extends the viral sequences contained in pTR7116 to the genuine viral 3' end. Deletion of the poly(A) tail and creation of the Smal site in pTRMA61 was carried out by Bal 31 deletion from the poly linker SstI site. After Bal 31 digestion the insert was released with PstI and cloned into pUC19. Colonies were screened for the presence of a Smal site and positive ones further screened by sequencing. (5) The insert from one clone was isolated using KpnI/HindIII digestion and cloned into Bluescript SK+ by ligating with HindIII sites. (6) The insert fragment from pTR33 was released by EcoRI/BamHI digestion and cloned into pTR785 to produce pTR791. (7) The fragment (II) from pTR721 was then cloned into pTR791 to form pTR7102. (8) The final step involved cloning the pTR7621 Smal/StuI fragment (III) into pTR7102 to form the complete construct pTR7116. Sequences are indicated as follows: open boxes, TRV sequence; hatched boxes, Pr/promoter sequence; straight lines, pUC19; dotted lines, Bluescribe+; sawtooth lines, Bluescript SK+. Heavy lines at box ends indicate G-C tails. The gene organization of TRV RNA-1 is shown at the bottom.
plasmid was constructed which contained the Pm promoter derived from the plasmid pPM1 (Ahlquist & Janda, 1984) and the 5' 446 bases of pTR7621. A synthetic oligonucleotide was used to insert the missing 5' bases, delete the non-viral sequence between the promoter and the TRV sequence, and change the three 3' bases of the Pm promoter back to the original λ P, promoter sequence (see Fig. 1 legend). A cDNA containing the viral 3'-terminal sequences was treated with Bal 31 to remove the homopolymer tail acquired during the cloning procedure. The 3'-terminal viral sequence ends in -CCC_{OH} (Minson & Darby, 1973). Deletion of the homopolymer tail and subsequent ligation into a Smal-cut vector created a unique Smal site. Use of Smal would thus allow the production of transcripts with 3' termini identical to the viral RNA. The completed 5' and 3' ends were joined into one construct and the remaining TRV RNA-1 cDNA sequence inserted. The last part required a two-step ligation as it was not possible to insert the remaining part of the cDNA as one fragment. All junction points were verified by sequencing subclones containing the junction regions as previously described (Hamilton et al., 1987).

The final outcome of this construction was a plasmid, pTR7116, which could be transcribed in vitro to produce RNA molecules which are identical at both the 5' and 3' termini to the natural viral RNA-1.

Transcription reactions were based on those described by Ahlquist & Janda (1984). RNA polymerase was obtained from four independent companies; reactions using lots from New England Biolabs and Boehringer Mannheim contained full-length transcripts, although it also became apparent that different batches from these companies gave different amounts. As the viral RNA is most probably capped (AbouHaidar & Hirth, 1977), the cap analogue m'G(5')ppp(5')A (Pharmacia) was also introduced into the transcription reaction. The standard reaction contained 2 μg of Smal-digested pTR7116, 25 mM-Tris–HCl (pH 8.0), 5 mM-MgCl₂, 150 mM-NaCl, 1 mM-dithiothreitol, 200 μM-rGTP, -rCTP and -rUTP, 50 μM-rATP, 0.5 mM of the cap analogue, 30 units RNAsin (Anglian Biotech.) and 2 units RNA polymerase in a total volume of 20 μl. The reaction was carried out at 37 °C for 30 min. Two μl of 500 μM-rATP was then added and the reaction continued at 37 °C for a further 30 min. Thirty μl of 50 mM-EDTA was added to stop the reaction. Approximately 1-2 μg of RNA transcripts was produced from each reaction and estimations based on Northern blot analysis and densitometer scans of autoradiograms indicated that between 40 and 50% of them were full-length transcripts.

Young Nicotiana tabacum var. Samsun NN plants were inoculated at the four to five leaf stage by manually inoculating the transcription reactions, diluted to 20 mM-sodium phosphate (pH 7.0) and mixed with bentonite (5 mg/ml), onto carborundum-dusted leaves (Lister & Hadidi, 1971) which were then rinsed with water not more than 15 min post-inoculation. Plants were scored for visual symptoms and total RNA was extracted using the proteinase K–phenol method (Baulcombe & Buffard, 1983) and analysed by Northern blotting.

The Northern blots of viral RNA extracted from inoculated leaves showed that there were no detectable differences at the RNA level between natural NM infections and the infections produced by pTR7116 RNA (Fig. 2). The genomic RNA-1 was detected as were the two subgenomic RNA species, 1a and 1b, which encode the 29K and 16K proteins, respectively (Robinson et al., 1983; Boccara et al., 1986). Much less RNA-1, -1a and -1b were found in leaves inoculated with transcripts produced in the absence of the cap analogue. No viral RNA was found in leaves inoculated with linearized, non-transcribed pTR7116 and these leaves were indistinguishable from healthy leaves. Leaves inoculated with uncapped transcripts showed very small lesions 3 days after inoculation which became large necrotic areas after a further 3 days. However, consistent with the Northern blot analysis, inoculation with the capped transcripts produced more and larger necrotic lesions after 3 days. After 6 days the leaves died. On some plants, the necrotic symptoms moved down the petiole of the inoculated leaves and could be seen eventually as a necrotic band extending up the main stem, ultimately causing the whole plant to die. This spread accounts for the detection of TRV RNA in the 'systemically' infected leaves of plants (Fig. 2, lane 7). The lesions formed by inoculation with uncapped transcripts did not spread to the same extent (Fig. 2, lane 6). These symptoms were similar to those of natural TRV NM-type infections in the production of necrotic lesions and the ability of the infection to spread from cell to cell (Harrison & Robinson, 1981). However, inocula of
Fig. 2. Northern blot of RNA from pTR7116-infected leaves. Lane 1, an aliquot of capped RNA transcripts from pTR7116 that was used for inoculation (equivalent to 0.1 μg of the template DNA). Other lanes represent RNA prepared from healthy, buffer-inoculated plants (lane 2), from leaves inoculated (lane 3) or systemically infected (lane 4) with linearized plasmid, from leaves inoculated with transcripts (lane 5) or systemically infected by transcripts produced without (lane 6) or with (lane 7) cap analogue, and from NM-infected (lane 8) and M-infected (lane 9) plants. The genomic TRV RNA-1 (1) and TRV RNA-2 (2) are indicated together with the associated subgenomic species (1a, 1b and 2a). In addition the full-length pTR7116 transport is arrowed. Approximately 7 μg of total RNA was run in each lane and the blot was probed with a randomly primed BstEII/SmaI fragment from pTR7116, corresponding to the 3'-terminal 1.44 kb of TRV RNA-1. RNA was prepared 14 days post-inoculation.

transcripts induced lesions that were more uniform and more intensely necrotic than leaves inoculated with total RNA prepared from NM (strain SYM)-infected plants. Induction of these slightly different symptoms may have resulted from one of the cDNAs used in the construction of pTR7116 representing a natural variant RNA species or from sequence differences introduced during the cloning procedure. On N. clevelandii the symptoms induced by the pTR7116 transcripts and NM RNA were very similar except that the pTR7116 infection was slightly more vigorous.

We have described in this communication the construction of a full-length clone of TRV RNA-1 from multiple cDNA fragments and have demonstrated the infectivity of in vitro derived transcripts. This validates the biological activity of the previously reported sequence (Hamilton et al., 1987). Clone pTR7116 will therefore be extremely useful in the analysis of the basic characteristics of NM-type infections and will form the basis for future mutagenesis and gene replacement experiments.

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REFERENCES


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