The 12 kDa protein of potato virus M displays properties of a nucleic acid-binding regulatory protein

Andrea Gramstat, Angelos Courtpozanis and Wolfgang Rohde

Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, FRG

Received 27 August 1990; revised version received 5 October 1990

The 3′ terminal 1.4 kb segment of potato virus M (PVM) genomic RNA was cloned and sequenced. This part of the viral genome encodes the capsid protein CP as well as a 12 kDa protein of as yet unknown function. Both proteins were expressed in bacteria and their nucleic acid-binding properties studied. The 12 kDa protein (pr12), but not the capsid protein bound single- and double-stranded nucleic acids. This property of pr12 in conjunction with a zinc finger motif located adjacent to a basic region of the 12 kDa protein suggests that it may act as a regulatory factor during virus replication.

Potato virus M; Protein, 12 kDa; Nucleic acid-binding capacity; Regulatory factor

1. INTRODUCTION

Potato virus M (PVM) is a member of the carlavirus group [4] with a single-stranded, polyadenylated, positive-sense genomic RNA approximately 8.5 kb in size. The 3′ terminal sequence of some 3.6 kb has been determined recently for a Russian PVM wild strain [18]. The genome organization of this part of the viral genome was shown to contain the 'triple gene block' present in plant RNA viruses belonging to diverse groups [14,15]. This conserved gene cluster is separated by a small intergenic region from the two 3′ proximal genes encoding the capsid protein CP and a 12 kDa protein (pr12) of unknown function.

As was noticed previously [18], pr12 contains a zinc finger motif that is characteristic of a variety of eukaryotic regulatory proteins with DNA-binding properties [22]. Such motifs are also part of proteins encoded by plant RNA viruses as, for example, in the capsid proteins of tobacco streak and alfalfa mosaic virus [21] or in the helper component of potato virus Y [16]. In an attempt to study the functional role of pr12 and its mode of expression during PVM replication, we have cloned this gene and expressed the pr12 protein in a bacterial expression vector. Here we report that PVM pr12 binds to single- and double-stranded RNA or DNA, while the PVM capsid protein CP fails to do so.

2. MATERIALS AND METHODS

2.1. *Construction and characterization of PVM-specific cDNA clones*

Polyadenylated RNA was isolated from tomato plants infected with a German PVM wild strain (supplied by Dr Sarkar, Hohenheim) using repeated oligo(dT) cellulose affinity chromatography. cDNA libraries were constructed in the *Lambda* insertion vector NM1149 according to previously published protocols [20]. PVM-specific cDNA clones were identified by molecular hybridization with 5′-^{32}P-labeled dTTTCTTCGTTGAATCTCCCATACTCACTATTTC, an oligodeoxynucleotide complementary to the N-terminal sequence of the PVM CP gene [18] (Fig. 1). Forty-eight of the cDNA clones giving hybridization signals were chosen at random for further analysis. One of them (pvmB) was shown to contain two *Eco*RI fragments of 1.1 and 0.25 kb, respectively, that were subcloned into pUC19 and sequenced by the chain-termination as well as by the chemical degradation method [11,19]. The sequence across the internal *Eco*RI site was established on the subcloned PCR2 product (see below; Fig. 1) using primer no. 1 for direct plasmid sequencing by the chain-termination method.

2.2. *Expression of PVM proteins in bacteria*

Sequences encoding the CP gene or the 12 kDa protein were amplified from *Lambda* clone pvmB by the polymerase chain reaction (PCR) using appropriate pairs of oligodeoxynucleotide primers (CP: no. 1 + no. 4; pr12: no. 3 + no. 5; see Fig. 1). The PCR products were purified by separation on 1.3% agarose gels and subcloned into the bacterial expression vector pEA305 as modified by John et al. [7]. Clones positive for the expression of the expected fusion proteins between the N-terminal 17 kDa of the *Lambda* repressor c_I and the corresponding PVM protein were selected for use in the nucleic acid-binding experiments.

2.3. *Nucleic acid-binding experiments*

Total protein extracts from colonies expressing c_I/PVM fusion proteins of CP and pr12, respectively, as well as from various control colonies were separated on 10% polyacrylamide gels [9] and electroblotted onto nitrocellulose membranes. Membranes were washed twice for 30 min each with an aqueous solution of 6 M urea/0.1% NP-40. Membrane-bound proteins were renatured in buffer A containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 50 mM NaCl, 0.2 g/l BSA, 0.2 g/l Ficoll/0.2 g/l polyvinylpyrrolidone for 1 h with 2 changes of buffer. After renaturation the membranes were incubated with radioactive-labeled nucleic acids (40 000 dpm/ml) in 50 ml of buffer A.

Labeled single-stranded RNAs were synthesized by SP6 RNA polymerase in the presence of [α-^{32}P]CTP (110 TBq/mmol) from linearized pSP65 templates containing the PVM 1.1 kb *Eco*RI fragment of clone pvmB (Fig. 1, coordinates 264–1400) in sense or antisense orientation. Double-stranded RNA was prepared from these

Correspondence address: W. Rohde, Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, FRG

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

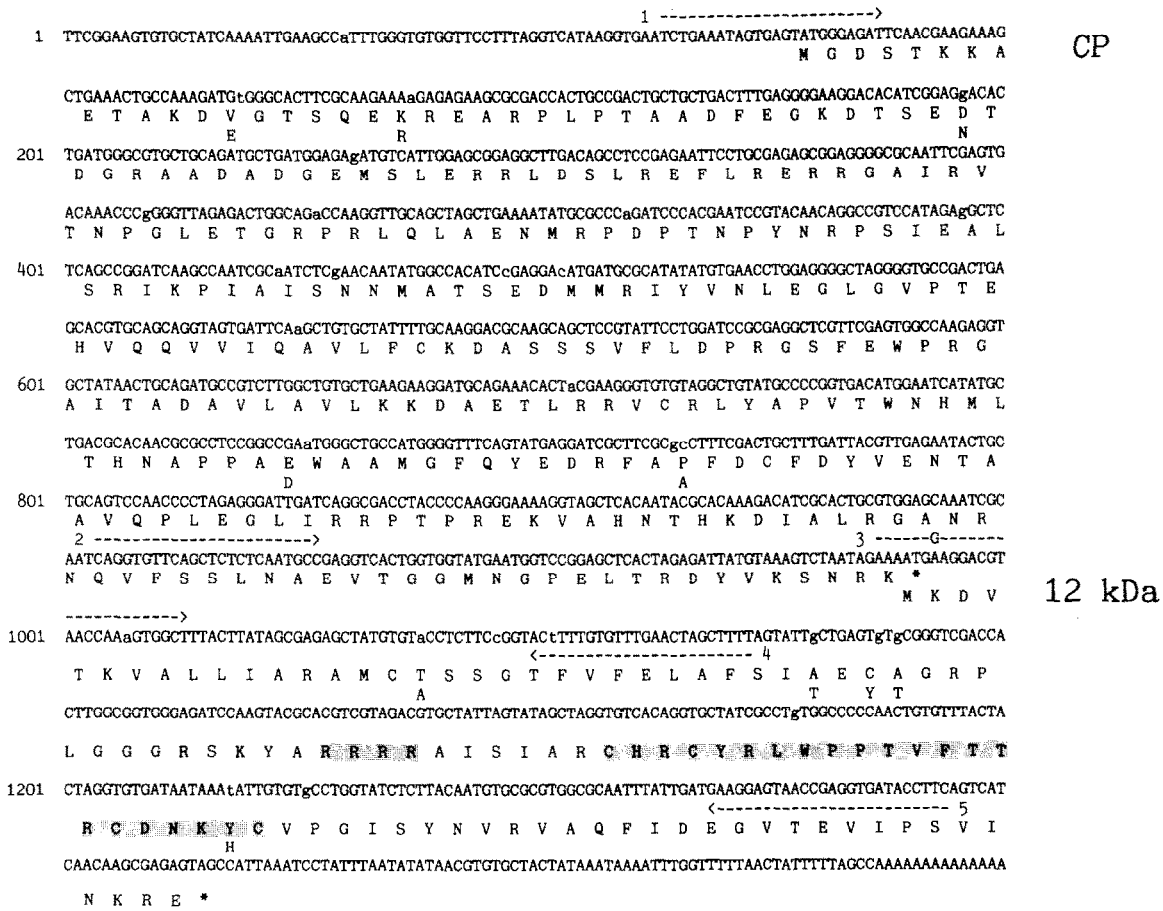

Fig. 1. Nucleotide sequence of the 3′ terminal 1.4 kb of PVM genomic RNA (German isolate). Positions in the nucleotide sequence different from the sequence of a Russian isolate [18] are shown in small case letters. Protein sequences for CP and pr12 are given in the one-letter amino acid code, and amino acid exchanges in comparison to the previously published PVM sequence [18] are presented at the appropriate positions. Numbered arrows indicate the oligodeoxynucleotide primers used in the PCR amplification of corresponding DNA sequences. Shaded areas point out a highly basic region and the C-proximally located zinc finger domain. CP, ORF for the capsid protein; 12 kDa, ORF for pr12.

complementary RNAs by liquid hybridization, mung bean digestion of residual single strands and gel filtration. For DNA-binding experiments, the 1.1 kb PVM *Eco*RI cDNA fragment was endlabeled by Klenow repair in the presence of [α-^{32}P]dATP (110 TBq/mmol). This double-stranded DNA was either used directly for binding experiments or after alkaline denaturation for the production of single-stranded DNA. After incubation with the respective radioactive nucleic acids, membranes were washed with buffer B containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 200 mM NaCl for 1 h with two changes of wash solution. Radioactive bands were identified by autoradiography.

3. RESULTS

3.1. *Sequence analysis of the PVM 3′ terminus*

The sequence determined for the PVM-specific cDNA clone pvmB comprises approximately 1400 nucleotides and contains the 3′ proximal open reading frames (ORFs) of PVM genomic RNA encoding the capsid protein CP and a 12 kDa protein (pr12) followed by 70 nucleotides of 3′ untranslated region and a short polyA tract (Fig. 1). When compared with the sequence of the Russian PVM strain [18], the German isolate shows 26 point mutations in the coding region giving rise to 5 amino acid exchanges each for CP and pr12. While some of these are conservative exchanges (D→E, R→K), others introduce a structure-breaking amino acid (A→P), an uncharged residue (E→V) or even change charges (N→D). An additional guanosine residue was also found in the sequence after position 1225 changing the open reading frame with respect to the sequence of the Russian PVM strain to encode a viral protein of M_w 12 100 Da (108 amino acids). A similar observation has been made on the original sequence by Rupasov et al. ([18], Zavriev, personal communication).

In the putative zinc finger motif of the 12 kDa protein (Fig. 1), the histidine residue of the Russian PVM pr12 is replaced by tyrosine, which would make the adjacent cysteine the candidate for being involved in a Cys-Cys....Cys-Cys metal ion binding domain (Fig. 2) [8]. The identical arrangement of four cysteines has been recently reported for the 16 kDa protein of the lily symptomless carlavirus (LSV) [13] as well as for the potato virus S (PVS) 11 kDa protein [10]. Most notable is the existence of a highly conserved region in pr12

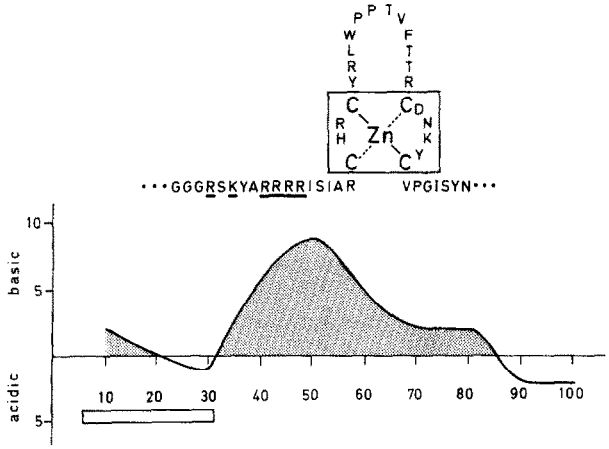

Fig. 2. Charge distribution for the PVM 12 kDa protein. Charge distribution was calculated for 10 consecutive amino acid residues each. The zinc finger motif and its approximate location in the pr12 sequence (residues 57 through 78, Fig. 1) adjacent to a cluster of basic amino acids (bars) is indicated. The open box refers to a hydrophobic region [18].

(Fig. 3, position 41–81 of the PVM pr12 amino acid sequence) which comprises the basic region and the zinc finger motif (Fig. 2). Twenty-three out of 41 amino acid residues are identical for the corresponding proteins of PVM, LSV and PVS pointing to an important functional role of these two regions. Similarly the 3′ untranslated region of 70 nucleotides is totally conserved between the Russian and the German isolate, while other plant virus strains may differ considerably in this region as e.g. noticed for potato virus Y strains [26]. In addition the AT-rich stretch of some 35 nucleotides preceding the polyA tail is highly conserved for the three carlaviruses. This sequence conservation argues for the importance of this region during PVM replication, possibly in the binding by proteins of the viral replicase complex.

3.2. *Nucleic acid-binding experiments*

Lambda clone pvmB DNA was restricted with *Hin*dIII or *Kpn*I and used as a template in the amplification of PVM sequences by the polymerase chain reaction (PCR). By this approach DNA fragments were obtained that contained the CP gene (primers 1 and 4: PCR2 product; Fig. 1), the genes for CP and 12 kDa (primers 1 and 5: PCR1 product) and the 12 kDa gene alone (primers 3 and 5: PCR3 product). PCR2 and PCR3 DNAs were subcloned into the modified expression vector pEA305 [7] and selected colonies were shown by polyacrylamide gel electrophoresis (PAGE) to express the capsid and the 12 kDa protein as fusion proteins (Fig. 4A). After blotting of proteins in nitrocellulose membranes and renaturation, filters were incubated with radioactively labeled nucleic acids. The results for one such experiment are depicted in Fig. 4B and demonstrate that pr12 binds to PVM sense RNA synthesized from the 1.1 kb *Eco*RI fragment of PVM cDNA clone pvmB (Fig. 1), while the capsid protein fails to do so under these conditions (Fig. 4B). The bound nucleic acids could not be removed by washing membranes in 4 M urea or heating in 0.1% SDS at 90°C for 30 min. Binding was also observed with single-stranded PVM RNA in the antisense orientation, with double-stranded RNA produced from the two complementary RNAs or with heterologous RNAs produced from a potato leafroll virus [23] or a barley A1 [17] cDNA clone (data not shown). In addition, when the labeled PVM-specific 1.1 kb DNA fragment was used in similar experiments, both single- and double-stranded DNAs were shown to bind, while the CP fusion protein did not bind nucleic acids in any of the experiments (data not shown).

```
PVM        GRSKYARRRRAISIARCHRCYRLWPPTVFTTRCDNKYCVPG
           *** *   *** ** ** **** ** *    *** * **
PVS        GRSTYSCKRRARSIGRCWRCYRVYPP VCNSKCDNRTCRPG
           *** *   ***  **** ******** ** ***** *****
LSV        GRSRYARRRRALQIGRCERCYRVYPP VCGSKCDNKTCRPG
           *** ******* * ** **** ** *    **** * **
PVM        GRSKYARRRRAISIARCHRCYRLWPPTVFTTRCDNKYCVPG

consensus  GRS.Y...RRA..I.RC.RCYR..PP.V....CDN..C.PG
```

Fig. 3. Protein sequence comparison of the basic region and the zinc finger motif of PVM pr12 to those of the PSV 11 kDa and LSV 16 kDa proteins. Asterisks denote identical amino acids, points represent any amino acid. The four cysteine residues assumed to form the zinc finger motif are boxed. The first glycine residue G corresponds to position 41 of the pr12 sequence.

4. DISCUSSION

Here we present evidence that the 12 kDa protein encoded by the 3′ terminal open reading frame of the PVM RNA genome is a nucleic acid-binding protein. This property is different from the single-strand nucleic acid-binding capacity of the P30 movement protein of tobacco mosaic virus (TMV) [2] in that PVM pr12 binds to both single- and double-stranded RNA or DNA. Secondly the 12 kDa protein does not exhibit any sequence similarities to TMV P30 or other putative transport proteins of plant viruses [12] which makes it unlikely that it has a physiological role in the spread of PVM infection from the original site of virus entry into the plant.

Although the exact function of pr12 during the PVM replication cycle remains obscure, certain indications for its possible role during virus replication can be derived from both structural and biochemical properties of this protein. For the carlaviruses PVS, LSV and PVM sequenced so far, amino acid sequence homology between the PVM 12 kDa protein and the corresponding proteins of PVS and LSV is located in the basic region and the adjacent zinc finger motif (Fig. 3).

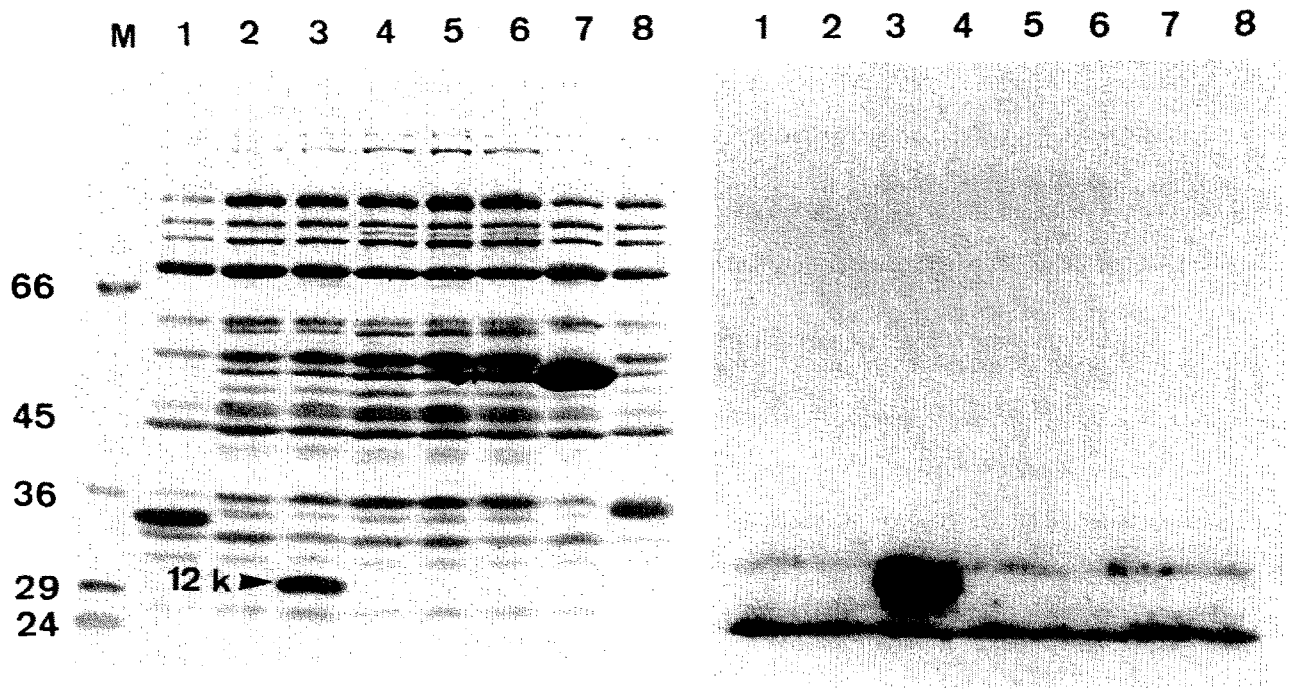

Fig. 4. Nucleic acid-binding capacity of PVM pr12. (A) Polyacrylamide gel electrophoresis of total bacterial extracts. Lanes 2, 4, 5, 6 represent colonies not expressing any fusion protein: (lane 1) PVM CP deletion fusion protein; (lane 3) PVM pr12 fusion protein; (lane 7) PVM CP fusion protein; (lane 8) potato leafroll virus 17 kDa fusion protein. M, protein molecular weight markers (numbers at the left margin indicate molecular weight in kDa). (B) Autoradiogram of (A) after blotting and incubation with ^{32}P-labeled PVM sense RNA.

Presumably it is this basic region that makes contact with nucleic acids and gives rise to the strong binding observed (Fig. 4B), and deletion mutation analysis of pr12 is under way to verify this assumption. The overall structure of pr12 together with its nucleic acid-binding property classifies it as a member of one class of eukaryotic transcriptional regulatory proteins characterized by a Cys-Cys zinc finger with an adjacent nucleic acid-binding domain [22].

Such plant virus proteins regulating transcription have been described for tobacco streak virus (TSV) as the type member of the ilarviruses and alfalfa mosaic virus (AlMV). The capsid proteins of these viruses are necessary for infection to occur and they operate via specific protein/RNA interactions [6,25] which depend on the presence of a basic domain as shown for AlMV CP [1]. In addition, the capsid proteins contain zinc finger motifs assumed to be involved in the process of activation of the replication complex [21]. The carlavirus genome possibly represents a variation on this theme. A 1.3 kb subgenomic RNA involved in the synthesis of capsid protein has been described recently for the carlavirus PVS [3]. In the case of PVM such subgenomic mRNA could give rise to translation of the PVM 12 kDa protein as a fusion protein with CP (Fig. 1) by a (−1) frameshift within the A-rich region AAUAGAAA**AUGA** preceding the pr12 AUG translational start codon (underlined) and representing the 3′ terminus of the CP coding region (UGA stop codon in bold letters).

Ribosome frameshifting around A- or U-rich sequences has been studied in detail for RNA tumor viruses [5]. For PVM this mechanism would produce in minor amounts a CP/12 kDa fusion protein that would closely resemble in structure (zinc finger motif; basic nucleic acid-binding domain) and putative function (activation of replication) the regulatory capsid proteins of TSV and AlMV. Stable potato transformation with vectors carrying this part of the PVM RNA genome as well as transient expression experiments using chimeric constructs of PVM sequences with the *E. coli* β-glucuronidase reporter gene as described for the study of regulatory sequences for translation in potato leafroll virus [24] will help in understanding PVM pr12 expression and function.

Acknowledgements: The excellent technical assistance of D. Becker and A. Kaufmann is gratefully acknowledged. This work was in part supported by a grant of EniMont America Biotechnology Laboratory (Princeton, USA).

REFERENCES

[1] Bol, J.F., Van Vloten-Doting, L. and Jaspars, E.M.J. (1971) Virology 46, 73–85.
[2] Citovsky, V., Knorr, D., Schuster, G. and Zambryski, P. (1990) Cell 60, 637–647.
[3] Foster, G.D. and Mills, P.R. (1990) J. Gen. Virol. 71, 1247–1249.
[4] Francki, R.I.B., Milne, R.G. and Hatta, T. (1985) Atlas of Plant Viruses, vol. II, CRC Press, Boca Raton.
[5] Hatfield, D.L., Smith, D.W.E., Lee, B.J., Worland, P.J. and Oroszlan, S. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 71–96.

[6] Jaspars, E.M.J. (1985) in: Molecular Plant Virology (Davies, J.W. ed.) pp. 155–221, CRC Press, Boca Raton.
[7] John, M., Schmidt, J., Wieneke, U., Kondorosi, E., Kondorosi, A. and Schell, F. (1985) EMBO J. 4, 2425–2430.
[8] Klug, A. and Rhodes, D. (1987) Trends Biochem. Sci. 12, 464–469.
[9] Laemmli, U.K. (1970) Nature 227, 681–685.
[10] MacKenzie, D.J., Tremaine, J.H. and Stace-Smith, R. (1989) J. Gen. Virol. 70, 1053–1063.
[11] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[12] Melcher, U. (1990) J. Gen. Virol. 71, 1009–1018.
[13] Memelink, J., Van der Vlugt, C.I.M., Linthorst, H.J.M., Derks, A.F.L.M., Asjes, C.J. and Bol, J.F. (1990) J. Gen. Virol. 71, 917–924.
[14] Morozov, S.Yu., Dolja, V.V. and Atabekov, J.G. (1989) J. Mol. Evol. 29, 52–62.
[15] Randles, J.W. and Rohde, W. (1990) J. Gen. Virol. 71, 1019–1027.
[16] Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S. and Casse-Delbart, F. (1989) J. Gen. Virol. 70, 935–947.
[17] Rohde, W., Marocco, A., Wissenbach, W., Barzen, E., Kristiansen, K. and Salamini, F. (1989) in: The Genetics of Flavonoids (Styles, D.E., Gavazzi, G.A. and Racchi, M.L. eds) pp. 79–95, Edizioni Unicopli, Milano.
[18] Rupasov, V.V., Morozov, S.Yu., Kanyuka, K.V. and Zavriev, S.K. (1989) J. Gen. Virol. 70, 1861–1869.
[19] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[20] Schwarz-Sommer, Zs., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., Mattes, M., Berndtgen, R., Peterson, P.A. and Saedler, H. (1985) EMBO J. 6, 287–294.
[21] Sehnke, P.C., Mason, A.M., Hood, S.J., Lister, R.M. and Johnson, J.E. (1989) Virology 168, 48–56.
[22] Struhl, K. (1989) Trends Biochem. Sci. 14, 137–140.
[23] Tacke, E., Sarkar, M., Salamini, F. and Rohde, W. (1989) Arch. Virol. 105, 153–163.
[24] Tacke, E., Prüfer, D., Salamini, F. and Rohde, W. (1990) J. Gen. Virol. 71 (in press).
[25] Van Vloten-Doting, L. (1975) Virology 65, 215–225.
[26] Wefels, E., Sommer, H., Salamini, F. and Rohde, W. (1989) Arch. Virol. 107, 123–134.