

## The NTP-binding motif in cowpea mosaic virus B polyprotein is essential for viral replication

Sander A. Peters,<sup>1</sup>† Jan Verver,<sup>1</sup> Ellen A. A. Nollen,<sup>1</sup> Jan W. M. van Lent,<sup>2</sup> Joan Wellink<sup>1\*</sup> and Ab van Kammen<sup>1</sup>

Departments of <sup>1</sup>Molecular Biology and <sup>2</sup>Virology, Wageningen Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

We have assessed the functional importance of the NTP-binding motif (NTBM) in the cowpea mosaic virus (CPMV) B-RNA-encoded 58K domain by changing two conserved amino acids within the consensus A and B sites (GKSRTGK500S and MDD545, respectively). Both Lys-500 to Thr and Asp-545 to Pro substitutions are lethal as mutant B-RNAs were no longer replicated in cowpea protoplasts. Transiently produced mutant proteins were not able to support trans-replication of CPMV M-RNA in cowpea protoplasts in contrast to transiently produced wild-type B proteins. Therefore loss of viral RNA synthesis was a result of a protein defect rather than an RNA template defect. Mutant B polyproteins were correctly processed *in vitro* and *in vivo* and the regulatory function of the 32K protein on processing of B proteins was not affected by these

mutations. Since regulation of processing by the 32K protein depends on interaction with the 58K domain, the mutations in the NTBM apparently do not interfere with this interaction. The Asp-545 to Pro substitution left intact the binding properties of the 84K precursor of the 58K protein, with respect to ATP-agarose, whereas the Lys-500 to Thr substitution decreased the binding capacity of the 84K protein, suggesting that the Lys-500 residue is directly involved in ATP binding. The Lys-500 to Thr substitution in the 58K domain resulted in an altered distribution of viral proteins, which failed to aggregate into large cytopathic structures as observed in protoplasts infected with wild-type B-RNA. However viral proteins containing the Asp-545 to Pro substitution showed a normal distribution in protoplasts.

### Introduction

The genome of cowpea mosaic virus (CPMV) consists of two plus-strand RNAs, B-RNA and M-RNA, that are translated into large polyproteins. The B-RNA-encoded 200K polyprotein is proteolytically processed at specific Gln/Ser, Gln/Met and Gln/Gly sites by the virus-encoded 24K proteinase to yield mature functional proteins (Wellink *et al.*, 1986; Garcia *et al.*, 1987; Vos *et al.*, 1988). A detailed processing scheme for the 200K polyprotein is shown in Fig. 1. Proteolytic processing has been shown to be regulated by the B-RNA-encoded 32K protein that forms a complex with the 170K protein (Peters *et al.*, 1992). The B-RNA and its encoded enzyme activities constitute an autonomous RNA replicon, since the B-RNA has been shown to replicate independently from M-RNA in isolated plant cells (Goldbach *et al.*, 1980). On the other hand B-RNA is dependent on M-RNA-encoded activities for movement from cell to cell (Wellink & van Kammen, 1989). The action of each

individual protein in the replicative machinery is not fully understood. Sequence homology studies have shown a conservation between comovirus and corresponding picorna-, nepo-, poty-, bymo- and calicivirus non-structural proteins (Franssen *et al.*, 1984a; Gorbalenya *et al.*, 1989a, 1990; Neill, 1990; King *et al.*, 1991). These include proteins containing a consensus sequence corresponding to the so-called NTP-binding motif (NTBM). This is a characteristic amino acid sequence motif for ribonucleotide-utilizing proteins, consisting of two separate parts referred to as the A and B sites (Walker *et al.*, 1982). Available experimental data suggests that NTBM-containing viral proteins might in fact be NTPases that are involved in processes such as duplex unwinding during RNA replication, transcription, mRNA translation, signal transduction, and membrane transport (Walker *et al.*, 1982; Gorbalenya & Koonin, 1989; Gorbalenya *et al.*, 1989b; and references therein).

In the CPMV B polyprotein an NTBM is located in the N-terminal part of the 58K domain (Dever *et al.*, 1987; Gorbalenya & Koonin, 1989) and is contained within different processing products (see Fig. 1). Several

† Present address: Department of Virology, Institute of Medical Microbiology, State University of Leiden, P.O. Box 320, 2300 AH Leiden, The Netherlands

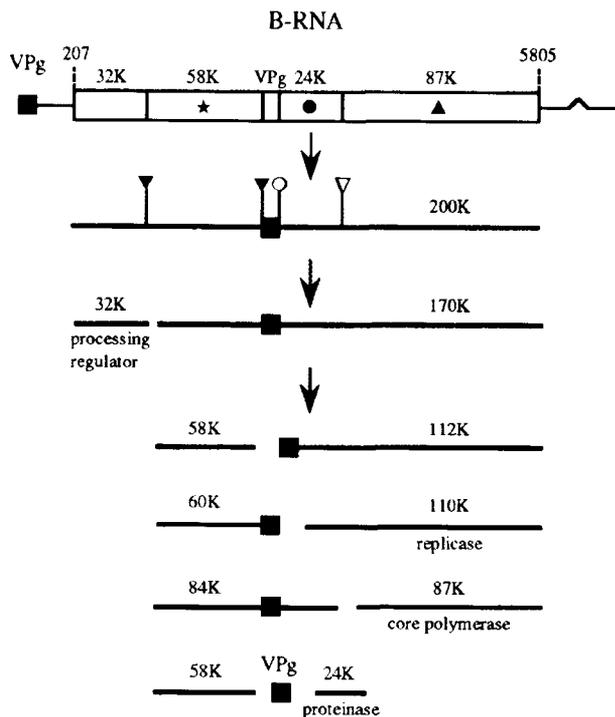


Fig. 1. Expression and genetic organization of B-RNA from CPMV. The RNA contains a long open reading frame (shown by the open bar). The positions of the start and stop codon are indicated. Cleavage sites are indicated by (O) Gln/Met, (∇) Gln/Gly, and (▼) Gln/Ser. B-RNA encodes a 200K polyprotein which is processed into 32K and 170K proteins. The 170K is processed at three different sites into three sets of proteins of 58K + 112K, 60K + 110K, and 84K + 87K. VPg can be generated via cleavage of the 112K protein or 84K protein. The 110K and 60K have been shown to be stable cleavage products. Symbols within the B-RNA represent conserved domains: ★, nucleotide binding domain; ●, proteinase domain; ▲, polymerase domain.

lines of evidence support a role for the NTBM-containing 60K protein in viral RNA replication. Chemical cross-linking studies have shown that the 60K and 84K (60K + 24K) proteins specifically bind ATP suggesting that the NTBM is functionally important (S. Peters, unpublished results). The B-RNA-encoded 60K protein is involved in the induction of vesicular structures in insect cells (van Bokhoven *et al.*, 1992). Such cytopathic structures are also observed in CPMV B-RNA-infected cowpea mesophyll protoplasts and have been shown to be the site of viral RNA replication (Assink *et al.*, 1973; de Zoeten *et al.*, 1974; Rezelman *et al.*, 1982). Furthermore it has been suggested that the 60K protein has a role in anchoring the viral replication complex to these vesicular structures (Eggen & van Kammen, 1988). Next to the vesicles amorphous structures are present that appear as electron-dense material upon electron microscopic analysis and were shown to contain B-RNA-encoded proteins (Wellink *et al.*, 1988). The induction of these cytopathic structures is a property of B-RNA-encoded proteins and has been shown to be independent

of plant factors and viral replication (van Bokhoven *et al.*, 1992).

To investigate further the role of the NTBM in viral proliferation we have introduced site-specific mutations in the NTBM-coding sequence and have tested the effect on viral RNA replication, translation and polyprotein processing, and cellular localization of viral proteins. The results obtained show that the NTBM is essential for viral RNA replication.

## Methods

**Oligonucleotide-directed site-specific mutagenesis.** To introduce mutations into the 58K-coding region, plasmid pTB1G (Eggen *et al.*, 1989) was used as the starting material. Plasmid pTB1G contains the full-length cDNA of CPMV B-RNA, which is cloned behind a bacteriophage T7 RNA polymerase promoter. RNA transcripts derived from pTB1G have been shown to be infectious in cowpea mesophyll protoplasts (Eggen *et al.*, 1989). The positions of restriction sites and nucleotides refer to the position in the B-RNA sequence determined by Lomonosoff & Shanks (1983). A *NsiI-SstI* fragment from pTB1G (positions 1393 to 2301) was inserted into the *PstI/SstI* site of M13mp19. The phosphorylated oligonucleotides (A) 5'-CCTGACTCATCAGCAAAC**TGGTACCAGT**GCGTGACTTTCC-3', complementary to nucleotides (nt) 1687 to 1726 of the 58K coding region, and (B) 5'-CGGCGGCAAA**AGGATCCATCAGCAC**-3', complementary to nt 1827 to 1851, were used for site-directed mutagenesis according to the method described by Kunkel (1985). The nucleotides that differ from the wild-type sequence are shown in bold. Using oligonucleotide A two nucleotide substitutions at position 1705 (A to C) and 1706 (A to C) create an additional *KpnI* site (underlined), whereas in oligonucleotide B two nucleotide substitutions at position 1839 (G to C) and 1840 (A to C) generate an additional *BamHI* site (underlined). Oligodeoxynucleotides were synthesized with a cyclone DNA synthesizer (Biosearch). Standard recombinant DNA techniques were used for transformation in competent *Escherichia coli* DH5aF' as described (Sambrook *et al.*, 1989). Recombinant clones M13mp19K500T and M13mp19D545P were analysed by restriction enzyme mapping and nucleotide sequence analysis as previously described (Sanger *et al.*, 1977; Korneluk *et al.*, 1985).

**Construction of plasmids.** The *PvuII-SstI* fragments from M13mp19K500T and M13mp19D545P were reinserted in the *PvuII/SstI* site (positions 1625 to 2301) of pTB1G and generated mutant transcription vectors pTBK500T and pTBD545P. To produce B proteins in cowpea mesophyll protoplasts that contain amino acid substitutions in the NTBM, pMB200 (van Bokhoven *et al.*, 1993) and pMB116 were used. pMB116 was generated by inserting a 3857 bp *BgIII-BamHI* fragment from pMB200 into the *BgIII/BamHI*-digested vector pMON999 (van Bokhoven *et al.*, 1993). pMB200 and pMB116 direct the transient expression of the 200K polyprotein and the 116K protein (32K + 60K + 24K) respectively in cowpea mesophyll protoplasts under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The *PvuII-SstI* fragments (positions 1625 to 2301) of these expression vectors were exchanged with the *PvuII-SstI* fragments from M13mp19K500T and M13mp19D545P and generated the mutant transient expression vectors pMB200K500T, pMB200D545P, pMB116K500T and pMB116D545P respectively.

**In vitro transcription and translation.** The procedures for *in vitro* transcription and translation of RNA templates have been described recently (Peters *et al.*, 1992). In the present experiments aliquots of transcription mixtures were translated in rabbit reticulocyte lysate from Promega Biotec.

**Transfection of protoplasts, transient expression and preparation of subcellular fractions.** Cowpea mesophyll protoplasts were transfected with CPMV RNA, B-RNA or transient expression vectors or with both transient expression vectors and M-RNA as described by van Bokhoven *et al.* (1993). Protoplasts were harvested 20 h post-transfection and subjected to fractionation by centrifugation at 30000 *g* for 30 min. Extracts were prepared as described by Franssen *et al.* (1982).

**Northern blot analysis.** Total nucleic acids from infected and uninfected cowpea mesophyll protoplasts were extracted 40 h post-transfection. Approximately  $1 \times 10^6$  protoplasts were mixed with 200  $\mu$ l 100 mM-Tris-HCl pH 7.5, 10 mM-EDTA and 1% (w/v) SDS and 200  $\mu$ l phenol and vortexed for 2 min at room temperature. After addition of 200  $\mu$ l chloroform the suspension was vortexed for 5 min followed by 10 min centrifugation at 16000 *g*. The aqueous phase was transferred to a fresh tube and extracted with 1 ml chloroform. The nucleic acid was recovered by precipitation with ethanol and sodium acetate and separated on a 1% denaturing agarose gel as described by McMaster & Carmichael (1977). The RNA was transferred from the gel to GeneScreene filter (NEN Research Products) and prehybridized as recommended by the manufacturer. Hybridization overnight was performed under the same conditions with  $9 \times 10^6$  c.p.m. of a B-RNA- or M-RNA-specific probe generated by random primer labelling as described (Feinberg & Vogelstein, 1983), using a *Sst*I-*Bam*HI fragment (positions 2301 to 3857) from pTB1G or a *Bgl*III-*Nco*I fragment (positions 189 to 3068) from pTM1G. After hybridization the blot was washed twice with  $2 \times$  SSC, 0.5% SDS at 42 °C for 30 min, followed by autoradiography.

**Affinity chromatography on 5'ATP-agarose.** A frozen pellet of approximately  $1.5 \times 10^6$  protoplasts was mixed with 100  $\mu$ l in binding buffer (20 mM-Tris-HCl pH 7.5, 25 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 5 mM-DTT, 0.5 mM-PMSF and 1 mM-EDTA). Supernatant fractions prepared by centrifugation at 30000 *g* for 30 min were mixed with 50  $\mu$ l 5'ATP-agarose (Sigma; the ligand is coupled via an 11 atom spacer to the agarose) in an Eppendorf tube and incubated for 30 min at 4 °C. In addition, fractions were chromatographed in binding buffer in presence of 2.5 mM-ATP to examine the binding specificity. The supernatant fraction was removed after the agarose was spun down for 30 s at low speed. The agarose was washed three times with 1 ml binding buffer and then boiled in 50  $\mu$ l protein sample buffer for 3 min to release the bound proteins from the agarose. Proteins were then fractionated in an SDS-polyacrylamide gel and identified by immunoblotting.

**Immunological detection of CPMV B proteins and gel electrophoresis.** Protein samples were heated in sample buffer (10% glycerol, 5%  $\beta$ -mercaptoethanol, 2% SDS, 0.01% bromophenol blue, 75 mM-Tris-HCl pH 6.8) for 3 min at 100 °C. Proteins were subjected to fractionation in a 7.5% SDS-polyacrylamide gel according to Laemmli (1970). Gels were either dried and autoradiographed with Kodak X-Omat film or analysed by immunoblotting using anti-VPg (Eggen *et al.*, 1988) or anti-110K serum (van Bokhoven *et al.*, 1992) as a primary antibody and anti-rabbit IgG alkaline phosphatase (Promega Biotec) as a second antibody as described (Blake *et al.*, 1984). Immunofluorescent detection of CPMV B proteins in infected cowpea mesophyll protoplasts was performed with the same antisera and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Nordic) as described by van Lent *et al.* (1991).

## Results

### Mutations in the NTBM-coding region are detrimental to viral RNA replication

The 58K domain of the B polyprotein contains two

highly conserved amino acid sequences, GKSRTG-K500S and MDD545 respectively, that correspond to the consensus A and B sites found in proteins utilizing NTP. (Walker *et al.*, 1982; Dever *et al.*, 1987; Gorbalenya & Koonin, 1989; Gorbalenya *et al.*, 1990). To assess the functional importance of these two conserved elements, a single amino acid substitution was introduced in each element and the effect on virus viability was analysed. Translation of the BK500T-RNA will result in a polyprotein, in which the conserved Lys-500 (AAG) residue in the A site of the NTBM has been replaced by a Thr (ACC) residue. In BD545P-RNA the coding sequence for the conserved Asp-545 (GAU) residue in the B site of the NTBM has been changed into a Pro (CCU) residue. Upon transfection of cowpea mesophyll protoplasts with BK500T-RNA or BD545P-RNA no accumulation of viral proteins was detected using an immunofluorescence assay, whereas in B-RNA- or CPMV RNA-transfected protoplasts viral proteins were produced (data not shown). This suggests that BK500T-RNA and BD545P-RNA cannot replicate in cowpea protoplasts. This was confirmed by a Northern blot analysis. The hybridization signal indicated an efficient replication of B-RNA and CPMV RNA (Fig. 2, lanes 1 and 3), whereas hybridization could not be detected with RNA extracted from protoplasts transfected with BK500T-RNA, BD545P-RNA or mock-inoculated protoplasts (Fig. 2, lanes 2, 3 and 5). Apparently either

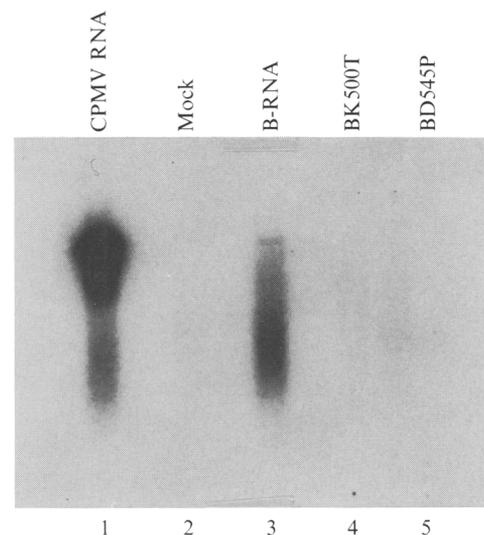


Fig. 2. Synthesis of CPMV B-RNA in transfected cowpea mesophyll protoplasts. Protoplasts were transfected with CPMV RNA (lane 1), mock-inoculated (lane 2), wild-type B-RNA (lane 3), BK500T-RNA (lane 4) or BD545P-RNA (lane 5). After 40 h of incubation total RNA was extracted from protoplasts. RNA products were separated, transferred onto membrane filter and hybridized with a B-RNA-specific <sup>32</sup>P-labelled probe.

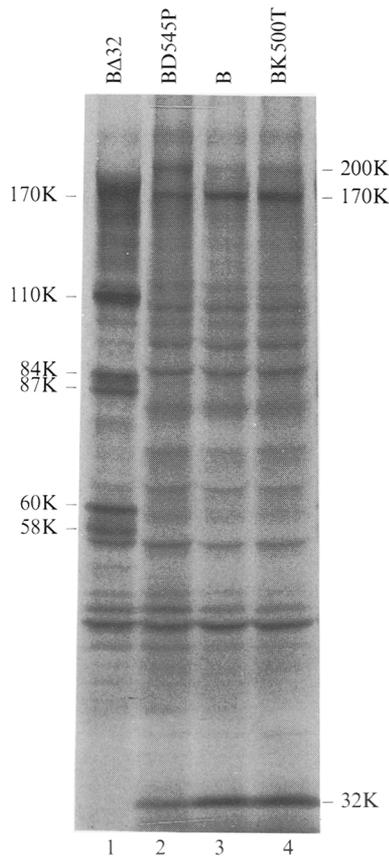


Fig. 3. Proteolytic processing of translation products containing amino acid substitutions in the NTBM consensus sequence located in the 58K domain of the CPMV B-polyprotein. BD545P-RNA (lane 2), B-RNA (lane 3) and BK500T-RNA (lane 4) were translated in presence of [<sup>35</sup>S]methionine for 4 h. The products were separated on a 7.5% SDS-polyacrylamide gel and their positions are indicated at the right side. At the left side positions of marker proteins obtained from a BA32-RNA translation (Peters *et al.*, 1992) (lane 1) are indicated.

the NTBM or the coding sequence for the NTBM is crucial for viral replication.

#### *In vitro* processing of mutant B-RNA translation products

To determine the integrity of mutant B-RNAs and to study the effect of the Lys-500 to Thr and Asp-545 to Pro substitutions on proteolytic processing *in vitro*, mutant B transcripts were translated in rabbit reticulocyte lysate and the translation products were compared to those of wild-type B-RNA. Similar to wild-type B-RNA, BK500T-RNA and BD545P-RNA were also translated into 200K polyproteins (Fig. 3, lanes 2 to 4). This indicates that the large open reading frame of both mutant RNAs was intact. Processing of the mutant 200K polyproteins generated the 170K and the 32K proteins that comigrated with the wild-type primary processing products. This suggests that the mutant polyproteins are

correctly processed. The primary processing of the BK500T polyprotein is indistinguishable from wild-type whereas the primary processing of the BD545P polyprotein was less efficient and after 4 h of incubation resulted in smaller amounts of the 170K and 32K primary cleavage products, as compared to the amount of wild-type processing products (Fig. 3, lanes 3 to 4). Possibly as a result of the Asp-545 to Pro substitution, the conformation of the nascent polypeptide chain has changed such that intramolecular cleavage at the Gln/Ser site is less favourable. Processing at secondary cleavage sites in the mutant 170K proteins was not observed (Fig. 3, lanes 2 and 4). Therefore the Lys-500 to Thr and Asp-545 to Pro substitutions appear to have no influence on the regulatory role of the 32K protein in processing of the 170K protein (see discussion).

#### *Transient expression of mutant B-RNA-coding sequences*

To study the effect on proteolytic processing *in vivo*, CPMV B-RNA sequences carrying mutations in the NTBM-coding region were transiently expressed in protoplasts using pMB200K500T and pMB200D545P. The processing products of the wild-type 200K protein produced by pMB200 and mutant 200K proteins from pMB200K500T and pMB200D545P were indistinguishable from the viral proteins found in CPMV RNA-inoculated protoplasts upon analysis with anti-110K serum (Fig. 4, lanes 1 to 4) or anti-VPg serum (Fig. 4, lanes 5 to 14). This indicates faithful processing of the transiently produced wild-type and mutant proteins *in vivo*. *In vivo* processing of the mutant 200KD545P proteins appeared to be equally efficient to the wild-type 200K protein (Fig. 4, lanes 2, 3, 7 and 9), in contrast with *in vitro* processing, which showed a decrease in efficiency for the 200KD545P polyprotein processing (see Fig. 3, lane 2). Protoplasts transfected with pMBK500T produced a lower amount of viral proteins than pMB200- or pMBD545P-transfected protoplasts. The lower level was most pronounced for the 170K, 110K and 87K proteins (see Fig. 4, lanes 2 and 4). In protoplasts transfected with pMBD545P the amount of 60K protein seems to be higher than in pMB200-transfected protoplasts. Currently we have no explanation for this observation.

Cleavage of the 116K protein (32K + 60K + 24K) produced by pMB116 generated 84K and 32K proteins (the latter protein not shown) (Fig. 4, lane 12), but further processing of the 84K protein into 60K and 24K was not observed. This is consistent with earlier *in vitro* results and again confirms our notion that the 32K protein can arrest the proteolytic processing of B proteins (Peters *et al.*, 1992). With respect to cleavage patterns observed for the mutant 116K and the wild-type 116K

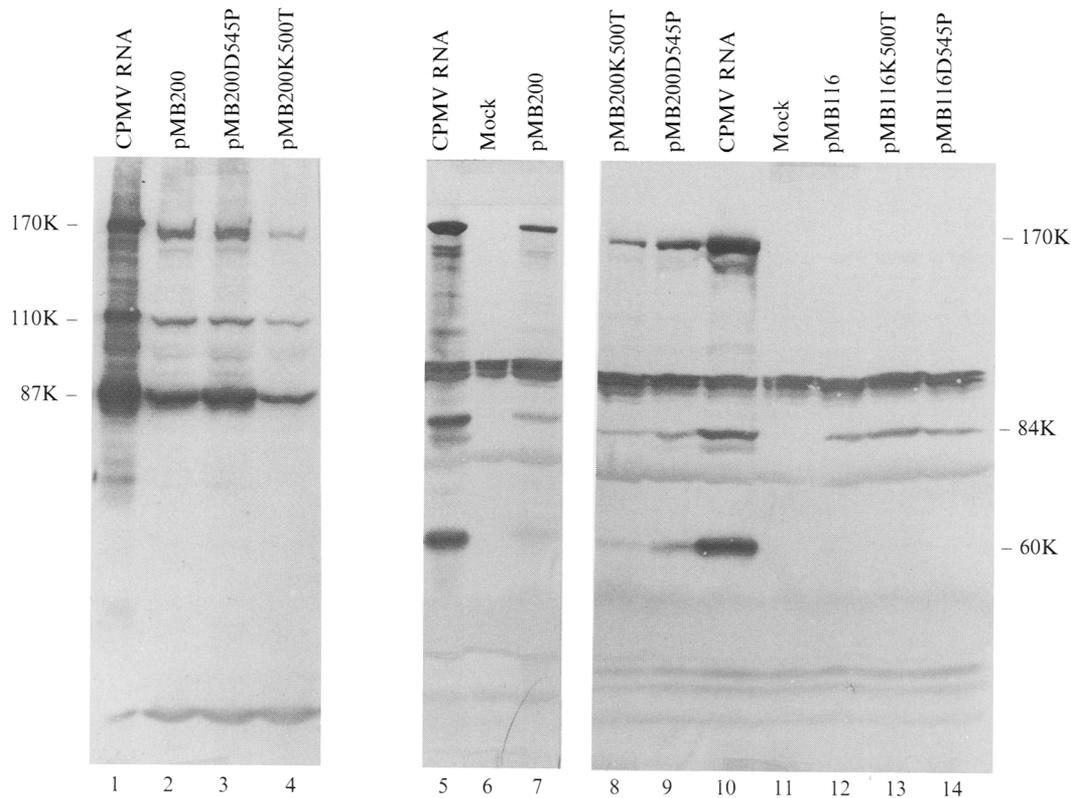


Fig. 4. Immunoblot analysis of proteins transiently produced in cowpea protoplasts. For detection of proteins anti-110K (lanes 1 to 4) and anti-VPg (lanes 5 to 14) sera were used as primary antibodies and anti-rabbit alkaline phosphatase conjugate as the second antibody. Viral proteins from CPMV RNA-inoculated protoplasts (lanes 1 and 5) served as marker proteins and are indicated on the right and the left side. The nomenclature of the constructs that direct transient expression of B-RNA sequences, shown on top of the immunoblot, is described in Methods.

proteins, differences were not found either (Fig. 4, lanes 12 to 14). Apparently the non-viability of the mutant transcripts BK500T and BD545P is not caused by interference of the Lys-500 to Thr and Asp-545 to Pro substitutions with translation or proteolytic processing of viral proteins.

The CPMV B proteins transiently produced by pMB200 are active in trans-replication of M-RNA in protoplasts (van Bokhoven *et al.*, 1993). In contrast to wild-type B proteins the mutant proteins from pMB200K500T and pMB200D545P were not able to support the replication of M-RNA (see Fig. 5, lanes 2 and 3). This indicates that a protein function essential to viral RNA replication has been disrupted by the amino acid replacements.

#### Chromatography of CPMV B proteins on 5' ATP-agarose

To investigate whether introduced amino acid substitutions in the NTBM disturbed a ribonucleotide-binding activity, extracts prepared from transfected protoplasts

were incubated with ATP-agarose. Bound viral proteins were subsequently eluted from the absorbent with protein sample buffer and subjected to an immunoblot analysis. In extracts prepared from CPMV-infected protoplasts the 170K, 112K, 110K, 87K, 84K and 60K proteins, which were detected with anti-110K and anti-VPg serum, had bound to ATP-agarose (Fig. 6, lane 3 and Fig. 7, lane 6). The origin of the band just above the 60K protein in lane 3 is unknown. The binding of 170K, 112K, 110K, 87K, 84K and 60K proteins was inhibited in the presence of 2.5 mM-ATP (Fig. 6, lane 5). The competition for binding by ATP indicates the specificity of binding of the aforementioned proteins. The ATP-binding sites are probably located in the 87K core-polymerase domain and in the 58K domain. Since B-RNA-encoded proteins are mainly associated in a complex (Franssen *et al.*, 1984b; Eggen & van Kammen, 1988; Peters *et al.*, 1992) the effect of the mutations in the NTBM on ATP-binding should be studied in absence of the 87K domain, to exclude retention of B proteins that are complexed with the core-polymerase but do not bind ATP. Indeed, binding was observed for the wild-type

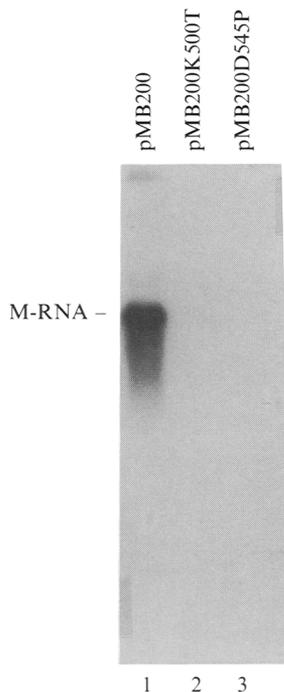


Fig. 5. Synthesis of CPMV M-RNA by B proteins transiently expressed in protoplasts. Protoplasts were transfected with pMB200 (lane 1), pMB200K500T (lane 2) or pMB200D545P (lane 3) together with M-RNA. Total RNA extracted at 40 h post-transfection was separated in a 1% denaturing agarose gel followed by transfer of the RNAs onto GeneScreen. The blot was hybridized with a probe specific for M-RNA.

84K protein from pMB116, although not all of the protein was retained by the ATP-agarose (Fig. 7, lanes 7 to 9). This is not due to limiting amounts of ATP-agarose, since a molar excess of B proteins from the CPMV RNA fraction over that of the 84K protein from pMB116-transfected protoplasts was bound (Fig. 7, lanes 6 and 9). Probably a small part of the 84K proteins is misfolded upon translation of MB116-RNA such that they are not able to bind ATP. The ATP-binding capacity of the 84K protein with the Lys-500 to Thr replacement is clearly reduced as the major part of this protein is present in the eluent fraction (Fig. 7, lanes 10 to 12). This difference in ratio of bound and unbound mutant 84K protein compared to that observed for wild-type 84K protein, suggests that the Lys-500 residue in the A site of the NTBM is involved in the binding of ATP. However the finding that a minor part of these mutant proteins was retained suggests that the binding capacity was not completely lost. The Asp-545 to Pro mutation did not markedly decrease the ATP-binding capacity of the 84K protein (Fig. 7, lanes 13 to 15). The Asp-545 amino acid residue is probably not directly involved in the binding of ATP.

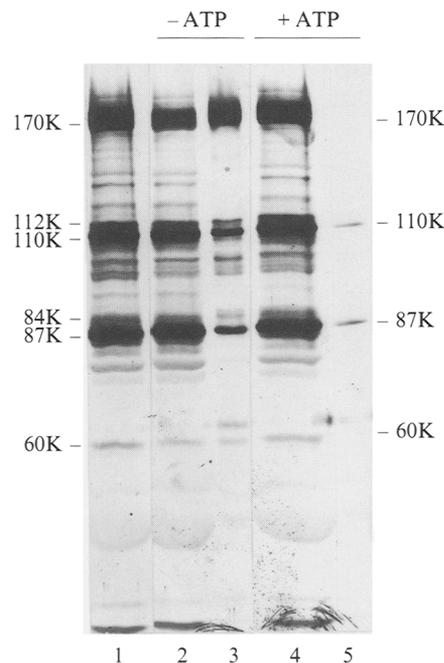


Fig. 6. Competition of binding of CPMV B proteins to 5'ATP-agarose by ATP. Extracts prepared from  $1.5 \times 10^6$  protoplasts infected with CPMV RNA were incubated with ATP-agarose either in the absence of ATP (lanes 2 and 3) or in presence of 2.5 mM-ATP (lanes 4 and 5). After incubation unbound proteins (lanes 2 and 4) were separated from the ATP-agarose by centrifugation and immobilized proteins were then eluted from the absorbent by boiling in sample buffer (lanes 3 and 5). Proteins were fractionated in an SDS-polyacrylamide gel. Detection of viral proteins was performed with both anti-VPg and anti-110K serum. Marker proteins (lane 1) are indicated at the left side.

#### *Distribution of CPMV B polyproteins in cowpea protoplasts*

To localize CPMV B proteins in cowpea protoplasts in which 200K proteins have been transiently produced, an immunofluorescent staining with anti-VPg and anti-110K serum and FITC-conjugated goat anti-rabbit IgG was performed. Using anti-VPg serum the immunofluorescent label in protoplasts transfected with either CPMV RNA, pMB200 or pMB200D545P is concentrated in distinct areas in the cytoplasm of the protoplast (Fig. 8*a, b, c*). A similar labelling is obtained with anti-110K serum (data not shown), whereas such structures have not been detected in mock-inoculated protoplasts (data not shown). A striking difference in the immunofluorescent staining pattern was observed in protoplasts which were transfected with pMB200K500T. After treatment with anti-VPg and anti-110K serum the immunofluorescent label appeared to be distributed over the entire protoplast (Fig. 8*d*, and data not shown) and was not concentrated in specific areas of the cytoplasm, indicating that the Lys-500 to Thr substitution has resulted in an altered localization of viral proteins.

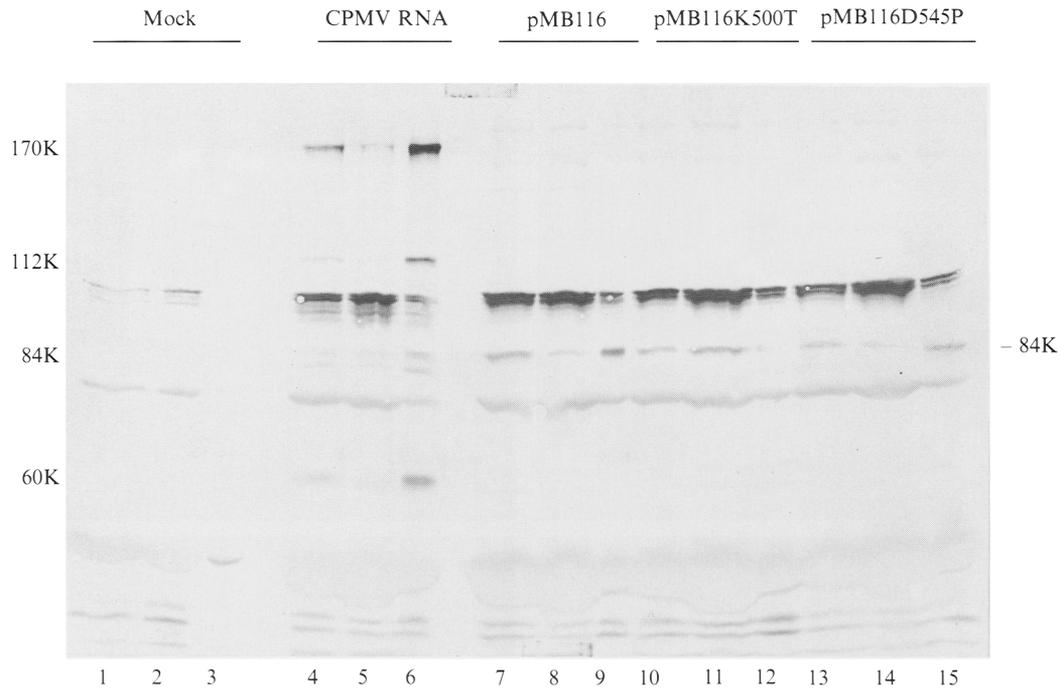


Fig. 7. Isolation of CPMV B proteins by affinity chromatography on 5'ATP-agarose. Extracts prepared from  $1.5 \times 10^6$  mock-inoculated protoplasts (lanes 1 to 3) or protoplasts transfected with CPMV RNA (lanes 4 to 6), pMB116 (lanes 7 to 9), pMB116K500T (lanes 10 to 12) or pMB116D545P (lanes 13 to 15) were incubated with 5'ATP-agarose. After incubation unbound proteins in the supernatant (lanes 2, 5, 8, 11 and 14) were separated from the ATP-agarose and immobilized proteins were then eluted from the absorbent (lanes 3, 6, 9, 12 and 15). Lanes 1, 4, 7, 10 and 13 contain untreated samples prepared from  $0.5 \times 10^6$  protoplasts. Proteins were fractionated in an SDS-polyacrylamide gel. To identify the viral proteins an immunoblot analysis using anti-VPg serum was performed.

## Discussion

In this paper we have presented the first evidence that the B-RNA-encoded NTBM plays an essential role in CPMV RNA replication. Using site-directed mutagenesis, Lys-500 to Thr and Asp-545 to Pro substitutions were introduced in the consensus A and B sites of the NTBM. These mutations severely impair RNA replication as demonstrated by the absence of detectable RNA synthesis. The mutations may even be lethal to the virus, but we cannot entirely exclude the possibility of greatly reduced levels of RNA synthesis that are too low to be detected in a Northern blot analysis. Such a low level of replication might result in the occurrence of wild-type revertants. In the case of the Thr-500 and Pro-545 mutants reversion to wild-type would require two nucleotide changes. Albeit at a very low frequency restoration of two introduced nucleotide changes has occasionally been observed for mutants in the poliovirus 2C protein which has been classified in the same superfamily of NTBM-containing proteins as the CPMV B-RNA-encoded 58K protein (Gorbalenya *et al.*, 1990). For mutant polioviruses carrying a Lys-135 to Ser or a Asp-177 to Gly substitution in the A and B sites of the NTBM respectively, revertants were isolated (Teterina *et*

*al.*, 1992), whereas Lys-135 to Gln and Asp-177 to Leu substitutions did not lead to reversion (Mirzayan & Wimmer, 1992).

The introduced nucleotide changes might either result in a defective RNA template or a defective protein. To address this we examined whether M-RNA replication in cowpea protoplasts could be supported by transiently produced proteins. Since the mutant proteins were not able to support the replication of M-RNA in contrast to CPMV B proteins transiently expressed from pMB200, it can be concluded that a protein defect underlies the non-viability of these mutants.

To investigate at which level the virus multiplication was blocked we have studied the effect of these mutations on translation and polyprotein processing. Primary processing of the 32K protein occurs already on the nascent polypeptide chain by an *in cis* cleavage (Franssen *et al.*, 1984c) and probably depends on the folding of the growing polypeptide chain that brings the primary cleavage site in proximity to the 24K proteinase (Peters *et al.*, 1992). *In vitro* primary processing of the B polyprotein with a Asp-545 to Pro replacement was delayed, but not inhibited. The Asp-545 to Pro substitution probably affects the local flexibility of the polypeptide chain such that the required folding for *in cis*

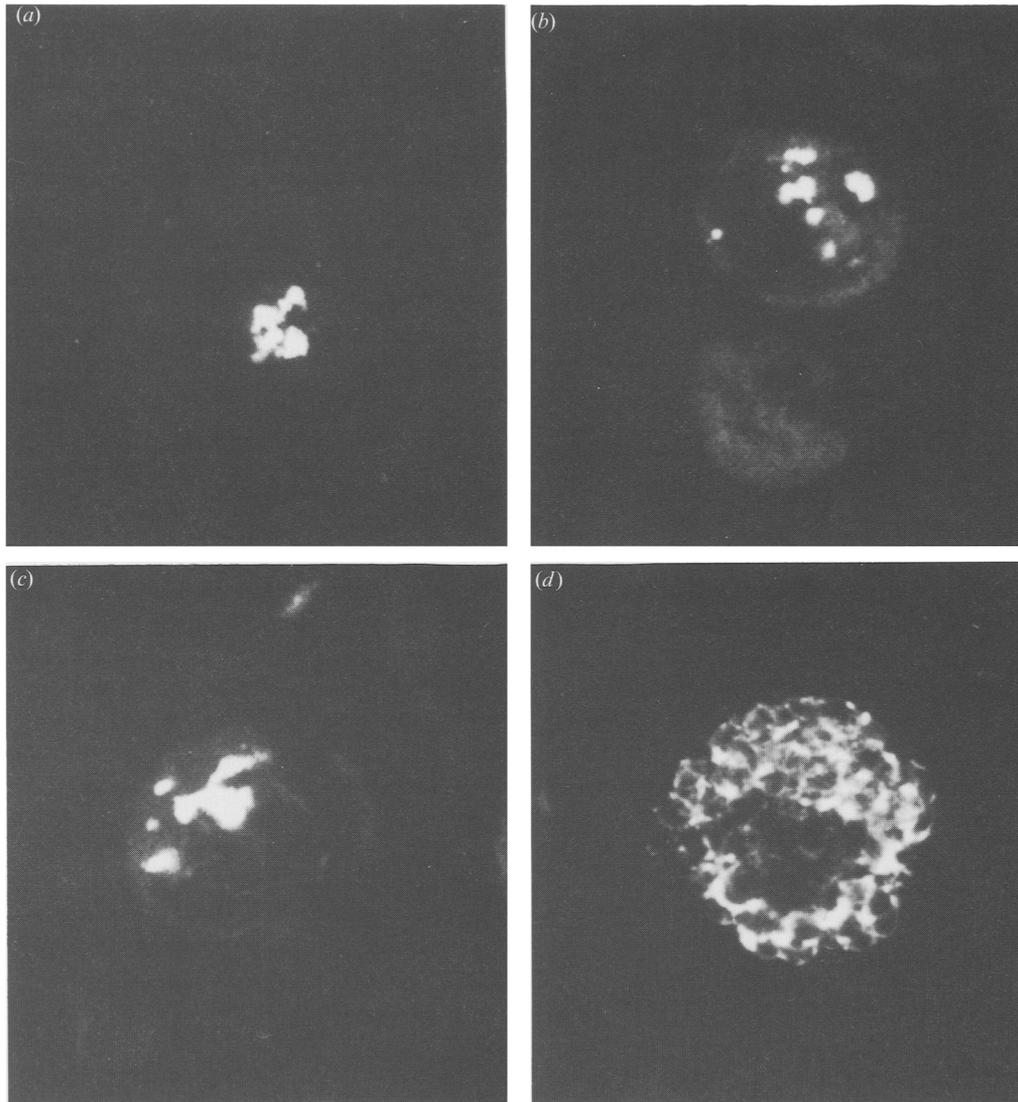


Fig. 8. Distribution of CPMV proteins in cowpea protoplasts. The immunofluorescent images represent typical examples of fluorescent staining of protoplasts transfected with CPMV RNA (a), pMB200 (b), pMB200D545P (c) or pMB200K500T (d). Detection of viral proteins was performed with anti-VPg serum goat-anti-rabbit IgG conjugated with FITC.

cleavage becomes more difficult. When the mutant B-RNA sequences were transiently expressed in protoplasts, 200K products were not detected. Apparently incubation over a longer period is sufficient for complete primary processing even for the 200K protein carrying the Asp-545 to Pro mutation. Moreover, no abnormal differences in cleavage patterns compared to the expression of wild-type B-RNA were observed, suggesting the precise processing of the mutant polyproteins. Previous results have pointed out the inhibitory function of the 32K protein on processing of the 84K protein *in vitro* which depends on the interaction with the 58K domain (Peters *et al.*, 1992). Apparently the 32K protein can also arrest the proteolytic processing of both wild-type and mutant 84K proteins in cowpea protoplasts, indicating that this function is not impeded by amino

acid substitutions within the NTBM. Therefore the NTBM has no crucial role in the interdomain communication with the 32K protein nor in translation and polyprotein processing. A similar conclusion was drawn from a genetic analysis on the polioviral NTBM (Mirzayan & Wimmer, 1992; Teterina *et al.*, 1992).

Experimental data from other characterized ATP/GTP-utilizing proteins have shown that the conserved positively charged lysine residue in the A site of the NTBM of these proteins interacts with the  $\alpha$ -phosphate group of the ribonucleotide (Walker *et al.*, 1982; Möller & Amons, 1985; Bradley *et al.*, 1987; De Vos *et al.*, 1988). The negatively charged aspartic acid residues in the B site form a salt bridge with the  $Mg^{2+}$  ion which, in turn, interacts with the negatively charged phosphate groups of the ribonucleotide and has been proposed to

have a catalytic function in NTP hydrolysis (La Cour *et al.*, 1985; Fry *et al.*, 1986; Jurnak, 1988; Weiner & Bradley, 1991). Recent data have indicated that the 60K and 84K proteins can most likely bind ATP directly to the NTBM in the 58K domain of these proteins (S. Peters, unpublished results). Both Lys-500 to Thr and Asp-545 to Pro substitutions might have interfered with an ATP-consuming function that somehow is essential for successful replication, since the charge, hydrogen-bonding ability and hydrophobicity are altered by these replacements. To assess the functional importance of these amino acids in ribonucleotide binding, the mutant proteins were tested for their ability to bind ATP by affinity chromatography. The results do not provide a clearcut answer as the observed differences in ATP-binding are marginal. Nonetheless, the reduced ATP-binding capacity for the 84K protein with a Thr-500 residue in the A site of the NTBM was reproduced in several independent experiments. This effect could either be the result of an altered conformation of the ATP-binding pocket or because the Lys-500 is directly involved in binding. Apparently the Asp-545 to Pro substitution near the A site can be tolerated without disturbing the ATP-binding properties. This supports the specific effect observed for the A site mutant and points towards a direct involvement of the Lys-500 residue in ATP-binding. Similar results have been reported for the large T antigen of simian virus 40 that, along with the 'picornavirus-like' NTBM-containing proteins, has been classified in the same superfamily of NTBM proteins (Gorbalenya *et al.*, 1990). Glu-473-Asp-474 to Ala-Ala amino acid substitutions in the Mg-binding B site of the large T antigen do not influence the ATP-binding properties but render a protein that is defective in ATP hydrolysis (Weiner & Bradley, 1991). By analogy, the Asp-545 to Pro substitution might have produced a mutant 84K protein that has preserved its binding capacity but might be defective in ATP hydrolysis.

Recently Mirzayan & Wimmer (1994) have provided evidence that the 2C protein of poliovirus expressed in insect cells contains ATPase activity, whereas a 2C protein with a mutation in the NTBM A site did not exhibit such activity.

In protoplasts transfected with pMB200 the immunofluorescent label was localized in distinct areas in the cytoplasm which is similar to that observed in CPMV RNA-infected protoplasts. These structures probably represent the cytopathic structures that are seen as electron-dense material upon electron microscopic analysis (Wellink *et al.*, 1988). Surprisingly, the viral proteins containing a single Lys-500 to Thr amino acid substitution fail to aggregate into such structures and were found dispersed over the entire protoplast, whereas

such an effect was not observed for the Asp-545 to Pro substitution. The altered localization coincided with a lower amount of mainly 170K, 110K and 87K proteins in BK500T-RNA-transfected protoplasts as compared to B-RNA- or BD545P-RNA-transfected protoplasts. Probably these unaggregated viral B proteins are more susceptible to proteolytic attack which might account for the lower expression level. The importance of the electron-dense structures in the viral life cycle remains unclear but it has been proposed that these structures represent sites where B proteins are maintained in an active conformation for replication (van Bokhoven *et al.*, 1993). The results described in this paper have indicated the NTBM is not involved in the assembly of the 32K/170K complex, but they may point towards the involvement in intermolecular contact that leads to multimerization of 32K/170K complexes. Whether this is promoted by binding of ATP, resulting in a conformation that allows the assembly into higher ordered complexes, remains unclear. Additional mutations in the NTBM will be required to investigate further the role of ATP-binding in protein-protein interactions.

We thank Hans van Bokhoven for construction of pMB116 and Nicole ter Maten for photography. This work was supported by the Netherlands foundation for Chemical Research (SON) with financial aid from the Netherlands Foundation for Scientific Research (NWO).

## References

- ASSINK, A. M., SWAANS, H. & VAN KAMMEN, A. (1973). The localization of virus-specific double-stranded RNA of cowpea mosaic virus in subcellular fractions of infected *Vigna* leaves. *Virology* **53**, 341-355.
- BLAKE, M. S., JOHNSTON, K. H., RUSSEL-JONES, G. J. & GOTSCHLICH, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphate-conjugated antibody on Western blots. *Analytical Biochemistry* **136**, 175-179.
- BRADLEY, M. K., SMITH, T. F., LATHROP, R. H., LIVINGSTON, D. M. & WEBSTER, T. A. (1987). Consensus topography in the ATP binding site of the simian virus 40 and polyoma virus large tumor antigens. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 4026-4030.
- DEVER, T. E., GLYNIAS, M. J. & MERRICK, W. C. (1987). GTP-binding domain: three consensus sequence elements. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 1814-1818.
- DE VOS, A. M., TONG, L., MILBURN, M. V., MATIAS, P. M., JANCARIK, J., NOGUCHI, S., NISHIMURA, S., MIURA, K., OHTSUKA, E. & KIM, S. H. (1988). Three-dimensional structure of an oncogene protein: catalytic domain of human c-H-ras p21. *Science* **239**, 888-893.
- DE ZOETEN, G. A., ASSINK, A. M., VAN KAMMEN, A. & ZABEL, P. (1974). Association of cowpea mosaic virus-induced double-stranded RNA with a cytopathological structure in infected cells. *Virology* **59**, 341-355.
- EGGEN, R. & VAN KAMMEN, A. (1988). RNA replication in comoviruses. In *RNA Genetics*, vol. 1, pp. 49-69. Edited by P. Ahlquist, J. Holland & E. Domingo. Boca Raton: CRC Press.
- EGGEN, R., KAAAN, A., GOLDBACH, R. & VAN KAMMEN, A. (1988). Cowpea mosaic virus RNA replication in crude membrane fractions from infected cowpea and *Chenopodium amaranticolor*. *Journal of General Virology* **69**, 2711-2720.
- EGGEN, R., VERVER, J., WELLINK, J., DE JONG, A., GOLDBACH, R. & VAN KAMMEN, A. (1989). Improvements of the infectivity of *in vitro* transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences. *Virology* **173**, 447-455.

- FEINBERG, A. P. & VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.
- FRANSSSEN, H., GOLDBACH, R., BROEKHUIJSEN, M., MOERMAN, M. & VAN KAMMEN, A. (1982). Expression of middle-component RNA of cowpea mosaic virus: *in vitro* generation of a precursor to both capsid proteins by a bottom-component RNA-encoded protease from infected cells. *Journal of Virology* **41**, 8–17.
- FRANSSSEN, H., LEUNISSEN, J., GOLDBACH, R., LOMONOSOFF, G. & ZIMMERN, D. (1984a). Homologous sequences in non-structural proteins from cowpea mosaic virus and picornavirus. *EMBO Journal* **3**, 855–861.
- FRANSSSEN, H., MOERMAN, M., REZELMAN, G. & GOLDBACH, R. (1984b). Evidence that the 32,000-Dalton protein encoded by bottom component RNA of cowpea mosaic virus is a proteolytic processing enzyme. *Journal of Virology* **50**, 183–190.
- FRANSSSEN, H., GOLDBACH, R. & VAN KAMMEN, A. (1984c). Translation of bottom component RNA of cowpea mosaic virus in reticulocyte lysate: faithful proteolytic processing of the primary translation product. *Virus Research* **1**, 39–49.
- FRY, D. C., KUBY, S. A. & MILDVAN, A. S. (1986). ATP-binding site of adenylate kinase; mechanistic implications of its homology with ras-encoded p21, F<sub>1</sub>-ATPase, and other nucleotide-binding proteins. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 907–911.
- GARCIA, J. A., SCHRIJVERS, L., TAN, A., VOS, P., WELLINK, J. & GOLDBACH, R. (1987). Proteolytic activity of cowpea mosaic virus encoded 24K protein synthesized in *Escherichia coli*. *Virology* **159**, 67–75.
- GOLDBACH, R., REZELMAN, G. & VAN KAMMEN, A. (1980). Independent replication and expression of B-component RNA of cowpea mosaic virus. *Nature, London* **286**, 297–300.
- GORBALENYA, A. E. & KOONIN, E. V. (1989). Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Research* **17**, 8413–8440.
- GORBALENYA, E. G., BLINOV, V. M., DONCHENKO, A. P. & KOONIN, E. V. (1989a). An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. *Journal of Molecular Evolution* **28**, 256–268.
- GORBALENYA, A. E., KOONIN, E. V., DONCHENKO, A. P. & BLINOV, V. M. (1989b). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Research* **17**, 4713–4730.
- GORBALENYA, A. E., KOONIN, E. V. & WOLF, Y. I. (1990). A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. *FEBS Letters* **262**, 145–148.
- JURNAK, F. (1988). The three-dimensional structure of c-H-ras p21: implications for oncogene and G protein studies. *Trends in Biochemical Sciences* **13**, 195–198.
- KING, A. M. Q., LOMONOSOFF, G. P. & RYAN, M. D. (1991). Picornaviruses and their relatives in the plant kingdom. *Seminars in Virology* **2**, 11–17.
- KORNELUK, R. G., QUAN, G. & GRAVEL, R. A. (1985). Rapid and reliable dideoxy sequencing of double-stranded DNA. *Gene* **40**, 317–323.
- KUNKEL, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proceedings of the National Academy of Sciences, U.S.A.* **82**, 488–492.
- LA COUR, T. F. M., NYBORG, J., THIRUP, S. & CLARK, B. F. C. (1985). Structural details of the binding of guanosine diphosphate to elongation factor Tu from *Escherichia coli* as studied by X-ray crystallography. *EMBO Journal* **4**, 2385–2388.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature, London* **227**, 680–685.
- LOMONOSOFF, G. P. & SHANKS, M. (1983). The nucleotide sequence of cowpea mosaic virus B RNA. *EMBO Journal* **2**, 2253–2258.
- MCMASTER, G. K. & CARMICHAEL, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 4835–4838.
- MIRZAYAN, C. & WIMMER, E. (1992). Genetic analysis of an NTP-binding motif in poliovirus polypeptide 2C. *Virology* **189**, 547–555.
- MIRZAYAN, C. & WIMMER, E. (1994). Biochemical studies on poliovirus polypeptide 2C. Evidence for ATPase activity. *Virology* **199**, 176–187.
- MÖLLER, W. & AMONS, R. (1985). Phosphate-binding sequences in nucleotide-binding proteins. *FEBS Letters* **186**, 1–7.
- NEILL, J. D. (1990). Nucleotide sequence of a region of the feline calicivirus genome which encodes picorna-like RNA-dependent RNA-polymerase, cysteine protease and 2C polypeptide. *Virus Research* **17**, 145–160.
- PETERS, S. A., VOORHORST, W. G. B., WERY, J., WELLINK, J. & VAN KAMMEN, A. (1992). A regulatory role for the 32K protein in proteolytic processing of cowpea mosaic virus polyproteins. *Virology* **191**, 81–89.
- REZELMAN, G., FRANSSSEN, H. J., GOLDBACH, R. W., IE, T. S. & VAN KAMMEN, A. (1982). Limits to the independence of bottom component RNA of cowpea mosaic virus. *Journal of General Virology* **60**, 335–342.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 5463–5467.
- TETERINA, N. L., KEAN, K. M., GORBALENYA, A. E., AGOL, V. I. & GIRARD, M. (1992). Analysis of the functional significance of amino acid residues in the putative NTP-binding pattern of the poliovirus 2C protein. *Journal of General Virology* **73**, 1977–1986.
- VAN BOKHOVEN, H., VAN LENT, J. W. M., CUSTERS, R., VLAK, J. M., WELLINK, J. & VAN KAMMEN, A. (1992). Synthesis of the complete 200K polyprotein encoded by cowpea mosaic virus B-RNA in insect cells. *Journal of General Virology* **73**, 2775–2784.
- VAN BOKHOVEN, H., VERVER, J., WELLINK, J. & VAN KAMMEN, A. (1993). Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA. *Journal of General Virology* **74**, 2233–2241.
- VAN LENT, J., STORMS, M., VAN DER MEER, F., WELLINK, J. & GOLDBACH, R. (1991). Tubular structures involved in movement of cowpea mosaic virus are also formed in infected cowpea protoplasts. *Journal of General Virology* **72**, 2615–2623.
- VOS, P., JAEGLE, M., WELLINK, J., VAN KAMMEN, A. & GOLDBACH, R. (1988). Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polyproteins. *Nucleic Acids Research* **16**, 1967–1985.
- WALKER, J. E., SARASTE, M., RUNSWICK, M. J. & GAY, N. J. (1982). Distantly related sequences in the  $\alpha$ - and  $\beta$ -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* **1**, 945–951.
- WEINER, B. M. & BRADLEY, M. K. (1991). Specific mutation of a regulatory site within the ATP-binding region of simian virus 40 large T antigen. *Journal of Virology* **65**, 4973–4984.
- WELLINK, J. & VAN KAMMEN, A. (1989). Cell-to-cell transport of cowpea mosaic virus requires both 58K/48K proteins and the capsid proteins. *Journal of General Virology* **70**, 2279–2286.
- WELLINK, J., REZELMAN, G., GOLDBACH, R. & BEYREUTHER, K. (1986). Determination of the proteolytic processing sites in the polyprotein encoded by the bottom-component RNA of cowpea mosaic virus. *Journal of Virology* **59**, 50–58.
- WELLINK, J., VAN LENT, J. & GOLDBACH, R. (1988). Detection of viral proteins in cytopathic structures in cowpea protoplasts infected with cowpea mosaic virus. *Journal of General Virology* **69**, 751–755.