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Expression of potyvirus coat protein in *Escherichia coli* and yeast and its assembly into virus-like particles

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When the full-length coat protein (CP) of the potyvirus, Johnsongrass mosaic virus (JGMV), was expressed in *Escherichia coli* or yeast, it assembled to form potyvirus-like particles. The particles were heterogeneous in length with a stacked-ring appearance and resembled JGMV particles in their flexuous morphology and width. This cell-free assembly system should permit analysis of the mechanisms of particle assembly and genome encapsidation. Two mutant forms of CP produced by site-directed mutagenesis failed to assemble into virus-like particles.

Introduction

The potyvirus group consists of at least 175 members and represents the largest and economically most important of the 34 plant virus groups (Shukla & Ward, 1989*a*, *b*). Potyviruses have long, flexuous rod-shaped particles, 700 to 900 nm long and 11 nm wide (Hollings & Brunt, 1981), which contain one positive-sense, single-stranded, polyadenylated RNA of about 10 kb, encapsidated by approximately 2000 copies of a single coat protein (CP) with M_rs ranging from 30K to 37K (Hollings & Brunt, 1981; Shukla & Ward, 1989*a*). It has been shown for many potyviruses that the genome encodes a large precursor polyprotein which undergoes proteolytic cleavage to yield at least eight proteins (Dougherty & Carrington, 1988; Shukla & Ward, 1989*a*). Of these, only the VPg, which is attached to the 5′ end of the genome, and the CP are present in mature virus particles.

In vitro studies have shown that purified particles of potato virus Y, the type member of the potyvirus group, can be dissociated into monomers by treatment with a high salt concentration (e.g. 0·5 M-NaCl), or at a pH below 6 or above 9. They can then be reassociated (by adjusting the salt concentration or pH) into long flexuous rods, in the presence or absence of the viral RNA (McDonald *et al.*, 1976; McDonald & Bancroft, 1977). It has been proposed that seven to eight CP monomers form a ring-like structure and that several of these rings assemble in either the presence or absence of the RNA genome to form full-length virus particles or stacked-ring particles, respectively (McDonald & Bancroft, 1977).

Although virus particles purified from infected plant tissues can be used to isolate CP monomers for *in vitro* assembly studies under various physiological conditions, the range of investigations that can be undertaken is limited. A microbial expression system, in which the CPs were expressed and assembled into virus particles would provide a useful way both to identify those CP regions crucial for particle assembly (by site-directed mutagenesis methods) and to study the mechanisms of genome encapsidation.

In this paper we report the use of *Escherichia coli* and *Saccharomyces cerevisiae* as hosts for the synthesis of the coat protein of Johnsongrass mosaic virus (JGMV).

Methods

Strains, media and growth. E. coli strains DH1 (F⁻ *recA1 gyrA96 thi7 hsdR17 supE44* λ^-) and TG1 (*lac–pro supE thi7 hsdD/F′ traD36 proAB lacI*^{q} *lacZ M15*) were used for maintenance and purification of *E. coli* and *E. coli*/yeast shuttle vectors. *E. coli* DH1 was also used for expression of the CP. RZ1032 Dut⁻ Ung⁻ and BMH71-18 *mutL* Dut⁺ Ung⁺ strains were used in oligonucleotide mutagenesis experiments. Materials and methods for growing *E. coli*, transformation and isolation of plasma DNA are those routinely used (Maniatis *et al.*, 1982). *S. cerevisiae* strain JHRY1-5D (*α his4-519 ura3-52 leu2-3 112 trp1 pep4-3*; J. Rothman, U.S.A.) used for expression of the CP was grown at 30 °C in YEPD or minimal medium (Sherman *et al.*, 1983) with or without the appropriate amino acids or nucleotides. The procedure described by Ito *et al.* (1983) was used for yeast transformation.

Source of DNA encoding JGMV CP. A cDNA fragment encoding the full-length CP and C-terminal part of the adjacent nuclear inclusion protein (NIb) has previously been cloned and sequenced (Gough *et al.*, 1987). *A BglII–ScaI* fragment (Fig. 1) encoding this sequence was cloned into pT3T7 18U (Pharmacia) to give pT3T718U : *BglII–ScaI*.

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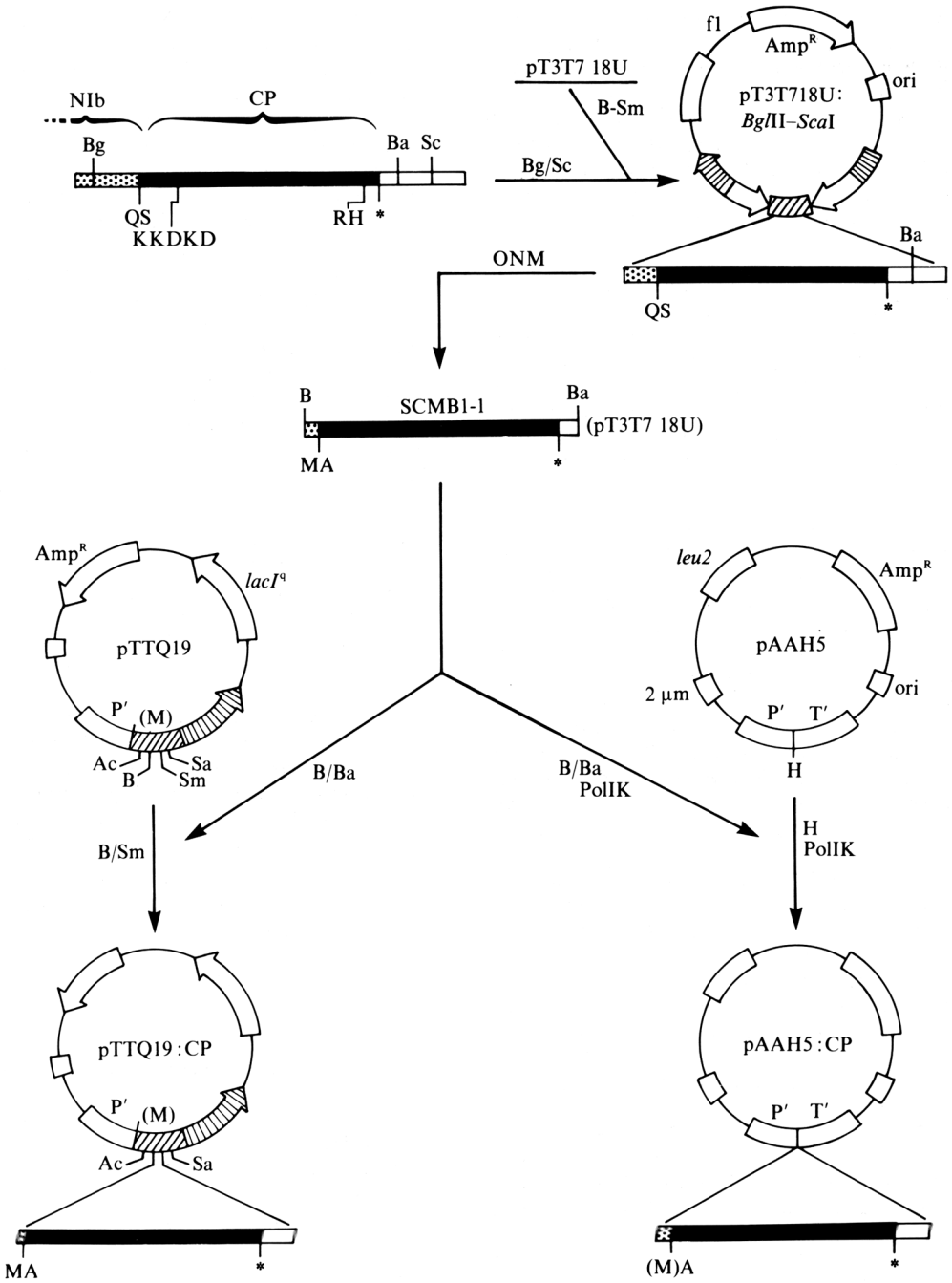

Fig. 1. Schematic representation of the construction of vectors for expression in *E. coli* and *S. cerevisiae*. In the DNA fragment shown at the top left, the open box indicates the untranslated region of the genome. In pTTQ19, cross-hatched and hatched boxes represent the regions containing multiple cloning sites and the *lacZ* gene, respectively. ONM, oligonucleotide mutagenesis; Ac, *Acc*I; B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; H, *Hin*dIII; Sa, *Sac*I; Sc, *Sca*I; Sm, *Sma*I. Capital letters shown below the CP-encoding region represent the amino acid residues; M, likely translation initiation codon; *, stop codon; P′, promoter; T′, terminator.

For many potyviruses it has been established that the proteolytic processing of the polyprotein occurs at Gln-Ser, Gln-Gly or Gln-Ala sites to separate CP from NIb. To express a clone containing only the CP sequences, the glutamine codon (CAG) was changed to a methionine codon (ATG) by oligonucleotide mutagenesis. The 43-mer primer (5′ GAAGATGTGGTGGATCCAGAAAATATGGCAGGCATTGAGGATG 3′) was designed to introduce simultaneously a *Bam*HI restriction endonuclease cleavage site (−8 to −13), base G at the +4 position and base A at the −3 position. DNA sequence analysis confirmed all the designed changes plus an unintended G to A substitution at +8 changing a glycine codon to an aspartic acid codon. This construct was named SCMB1-1.

Expression vectors. The vectors pTTQ19 (Amersham) and pAAH5 (Ammerer, 1983) were used for expression in *E. coli* and yeast, respectively. pTTQ19 contains the IPTG-inducible *tac*$^-$ promoter, and the ADC1 promoter present in pAAH5 is constitutively expressed. The *Bam*HI–*Bal*I fragment encoding the full-length CP was isolated from SCMB1-1 and was either cloned directly into pTTQ19 restricted with *Bam*HI and *Sma*I or was filled in by PolIK (Klenow fragment of DNA polymerase I) and cloned into the PolIK-filled *Hin*dIII site of pAAH5, to generate vectors pTTQ19:CP and pAAH5:CP, respectively (Fig. 1).

Mutant coat protein constructs. Site-directed amino acid substitutions were made to change Trp^{130}-Tyr^{131} to Gly-Pro and Arg^{194}-Gln^{195} to Asp-Leu. The primers 5′ AATTCCAGTTCGGGCCCAACAGAGTCAA 3′ and 5′ AAACCAACATTAGATCTGTGCATGATGCAT 3′, also designed to create *Apa*I and *Bgl*II sites at amino acid positions 130 to 131 and 194 to 195, respectively, were used. The mutated constructs were screened by restriction enzyme analysis and, where required, the sequences of the clones were confirmed by DNA sequencing. The constructs were designated pTTQ19:CP^{W*Y*} and pTTQ19:CP^{R*Q*}.

Analysis of expressed proteins in E. coli *and yeast.* Cultures of *E. coli* DH1 grown overnight at 37 °C in LB plus ampicillin were diluted 1:50 in fresh medium, grown for 60 to 90 min at 37 °C, induced by the addition of 500 μM-IPTG (final concentration) and further incubated at 37 °C for 90 to 120 min. One ml cultures (approx. OD_{600} of 0·52) were pelleted and resuspended in 100 μl of loading buffer (60 mM-Tris–HCl pH 7·5, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0·001% bromophenol blue) and boiled for 3 min. Samples (10 to 20 μl) were used for SDS–PAGE analysis. For purification of CP or potyvirus-like particles (PVLPs) from *E. coli*, cells from 100 ml of induced culture were resuspended in 4 ml of mix I (20% sucrose, 100 mM-Tris–HCl pH 8, 10 mM-EDTA), 84 μl of 5 mg/ml lysozyme was added and the reaction mixture was incubated for 5 to 10 min at room temperature followed by 15 min on ice. The spheroplasts were resuspended in 1 ml of sterile water and used for Sepharose S-1000 column chromatography. Alternatively, the spheroplasts were resuspended in 0·4 to 0·8 ml of mix II (100 mM-Tris–HCl pH 8, 20% sucrose, 10 mM-$MgCl_2$, 1 μg/ml each of DNase and RNase) and further diluted with water to a final volume of 2 ml for direct immune electron microscopy or for column separation. The fractions with the maximum A_{280} were collected by centrifugation at 50000 r.p.m. for 80 min in a Beckman SW28 rotor and the pellet fraction was analysed by immunoblotting techniques (Laemmli, 1970; Harlow & Lane, 1988).

For analysis of CP expressed in yeast, 10 to 100 ml of early to mid-logarithmic phase (OD_{600} of 0·2 to 1·5) cells were pelleted, washed in 1·2 M-sorbitol and resuspended in 1 to 10 ml of solution I [0·9 M-sorbitol, 0·1 M-EDTA pH 8·0, 14 mM-2-mercaptoethanol and 100 μg/ml zymolyase 100T (Sakagu Kogono Company)], and incubated at 37 °C for 30 to 45 min to generate spheroplasts. The spheroplasts were washed in the above solution without zymolyase and lysed by resuspension in 0·1 to 1 ml of sterile water for direct immune electron microscopy or in loading buffer for SDS–PAGE analysis.

Sucrose density gradient purification of CP. The methods for sucrose density gradient centrifugation used were essentially as described by Adams *et al.* (1987) and Muller *et al.* (1987). To purify CP which had been expressed in *E. coli*, the cells were collected from 0·1 to 1 litre of induced cultures, treated with lysozyme, and the resulting spheroplasts were suspended in 1 to 2 ml of 100 mM-Tris–HCl pH 8, 10 mM-$MgCl_2$, 1 μg/ml each of RNase and DNase, and were briefly sonicated prior to centrifugation. To purify CP which had been expressed in yeast, cells from a litre culture were washed in 10 ml of 1 × TEN (10 mM-Tris–HCl pH 7·5, 2 mM-EDTA, 100 mM-NaCl), and resuspended in 2 ml of fresh 1 × TEN buffer. Phenylmethylsulphonyl fluoride (60 μl of 0·2 M) and 4 g of 0·45 μM glass beads were added and the cells were disrupted in a Braun homogenizer (B. Braun, Melsungen AG) at 0 °C, for five 20 s intervals with a 20 s break between. The suspension, in which 90% of the cells had been broken, was centrifuged at 3000 r.p.m. for 5 min. The bead pellet plus the interphase containing cell debris were further extracted with 2 ml of fresh 1 × TEN by brief vortexing and centrifugation. The two supernatant fractions were pooled and ultracentrifuged at 55000 r.p.m. at 4 °C in a Beckman TLA100 ultracentrifuge using a TLA100-2 rotor for 1 h and the pellet was resuspended in a total volume of 2 ml of 1 × TEN. The cell extracts from *E. coli* or yeast were analysed by sucrose density gradient (10 to 40%) centrifugation in an SW28 rotor at 26000 r.p.m. for 3 h at 15 °C. One ml fractions were collected and the samples shown to contain CP by immunoblotting were pooled and dialysed against 10 mM-phosphate buffer pH 7·2 containing 100 mM-NaCl at 4 °C for 48 to 72 h. For further concentration of the sample, 100 to 500 μl samples were centrifuged at 100000 r.p.m. for 8 min at 4 °C in a Beckman TL100 ultracentrifuge. The pellet was resuspended in the loading buffer for SDS–PAGE analysis or in the phosphate or TEN buffer for electron microscopic analysis.

Limited proteolysis of intact virus and E. coli*/yeast extracts.* Purified preparations of JGMV were treated with lysyl endopeptidase (Wako Chemicals) at a concentration of 6 μg per mg of virus in sterile water for 30 min at room temperature (Shukla *et al.*, 1989*a*). Treated samples were centrifuged in the TLA100-2 rotor as described above. The pellet was resuspended in 0·05 M-sodium phosphate buffer pH 7·2 or deionized sterile water. Aliquots (100 μl) of *E. coli* or yeast spheroplasts in sterile water were incubated at room temperature for 30 min with or without lysyl endopeptidase and centrifuged at 100000 r.p.m. at 4 °C for 8 min. The supernatant fractions were transferred to fresh tubes and the pellets were resuspended in 100 μl of water. Aliquots (15 μl) of the resuspended pellet or supernatant fractions were each mixed with 5 μl of the loading buffer and analysed by SDS–PAGE.

Immunoblotting. Proteins from *E. coli* and yeast were electrophoretically transferred to nitrocellulose membranes, immunoblotted as described by Harlow & Lane (1988) and probed with rabbit polyclonal antisera. Rabbit polyclonal antisera raised against purified JGMV particles (JG:AS) and purified, denatured, truncated CP cores of trypsin-treated JGMV particles (JG:Core AS) were used as described by Shukla *et al.* (1989*b*).

Electron microscopy. Samples of virus or extracts from *E. coli* or yeast were mounted on Formvar carbon-coated, glow-discharged grids. Immune electron microscopy and antiserum decoration of cell extracts were carried out using polyclonal antiserum JG:AS at a dilution of 1:500. The grids were negatively stained with 1% uranyl acetate pH 4 and examined in a JEOL 100B electron microscope at 80 kV and 100 kV and 100000 × magnification.

Results

Expression and polymerization of CP in E. coli *and yeast*

In order to establish a cell-free system for the synthesis and assembly of potyvirus CP, expression vectors pTTQ19 and pAAH5 containing the CP-encoding region from JGMV (Fig. 1) were transformed into *E. coli* and yeast, respectively. In *E. coli* the full-length protein expressed from pTTQ19:CP construct should contain an additional 16 amino acids at its N terminus, with 12 generated from the multiple cloning sites present

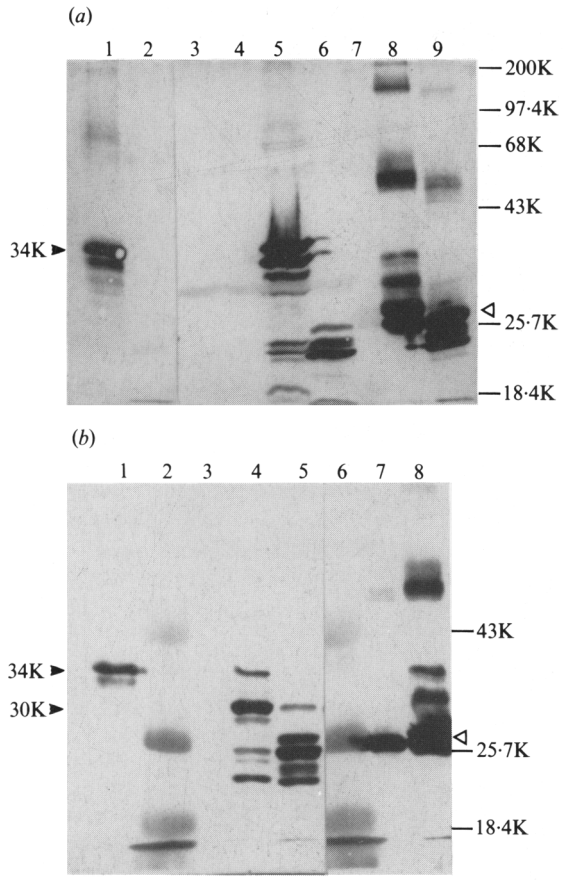

Fig. 2. Immunoblot analysis of CP expressed in *E. coli* (*a*) and *S. cerevisiae* (*b*) probed with the polyclonal antiserum JG : Core AS raised against purified, denatured, truncated CP cores of trypsin-treated JGMV particles. The bands were visualized by a horseradish peroxidase reaction. (*a*) Lanes 1 and 2, freeze-dried CP purified from JGMV; lane 3, *E. coli* DH1; lane 4, DH1/pTTQ19; lanes 5 and 6, DH1/pTTQ19 : CP; lane 7, M_r standards; lanes 8 and 9, purified JGMV samples stored at 4 °C. Lanes 2, 6 and 9 contain extracts treated with lysyl endopeptidase. Filled and open arrowheads indicate the full-length CP and the CP without the N terminus, respectively. (*b*) Lane 1, freeze-dried CP; lanes 2 and 6, M_r standards; lane 3, JHRY1-5D/pAAH5; lanes 4 and 5, JHRY1-5D/pAAH5 : CP; lanes 7 and 8, JGMV stored at 4 °C. Lanes 5 and 7 contain samples treated with lysyl endopeptidase.

upstream of the *Bam*HI–*Sma*I sites used for cloning the CP gene and four from the C-terminal region of the NIb gene (Fig. 1). The vector pTTQ19 carries an efficient promoter (*tac*) and a consensus Shine–Dalgarno sequence for efficient expression. In the yeast construct, pAAH5 : CP, translation initiation would be expected to occur at the first AUG of the CP mRNA to result in the synthesis of a full-length, unfused CP. To serve this purpose, the codon (CAG) encoding the glutamine in the Gln-Ser proteolytic cleavage site at the junction of the NIb and CP-encoding regions of the polyprotein (Fig. 1) was replaced with an initiation codon (AUG) by oligonucleotide mutagenesis. In addition, nucleotide A was substituted for the C residue at the −3 position to promote more efficient translation initiation in yeast (Cigan & Donahue, 1987).

Immunoblot analyses of protein extracts from *E. coli* DH1/pTTQ19 : CP and *S. cerevisiae* strains JHRY1-5D/pAAH5 : CP showed that CP was synthesized in both microbial hosts (Fig. 2*a*, *b*). In *E. coli* there were two predominant bands which reacted with the polyclonal antiserum raised against the core portion of the coat protein (JG : Core AS). The larger of the two migrated slightly more slowly than the native CP (34K), probably due to the additional 16 amino acids at the N terminus. The smaller one could either be a product of internal initiation at the newly introduced methionine codon (Fig. 1) or a proteolytic derivative of the larger polypeptide. In yeast, most of the CP synthesized seemed to undergo partial proteolysis to give rise to a band of about 30K (Fig. 2*b*). Nevertheless, a band of 34K corresponding to the full-length CP was detected. The cleavage of the full-length CP appeared to occur when the proteins were extracted from zymolyase-treated spheroplasts and the correct size protein could be isolated when proteins were extracted by homogenizing cells with glass beads (Fig. 3). In extracts of both *E. coli* and yeast, however, there were many other CP-specific bands smaller than 34K which were presumably generated by non-specific degradation of the full-length CP as seen in protein preparations from viruses propagated in plants (Shukla *et al.*, 1988). The smaller proteins could also have been generated by internal initiation or premature termination of translation, during gene expression. CP-specific bands were absent in host strains containing the expression vectors without the CP-encoding region (Fig. 2).

Lysyl endopeptidase treatment of CP synthesized in E. coli *and yeast*

To gain an insight into the integrity of folding and particle assembly of the CP expressed in *E. coli* and yeast, the protein extracts were subjected to mild lysyl endopeptidase treatment. Lysyl endopeptidase selectively removes the surface-exposed N terminus of JGMV particles, by cleaving at Lys^{68} to Lys^{70}, leaving the rest of the particle intact (Shukla *et al.*, 1988, 1989*a*). Immunoblot analysis indicated that a portion of the expressed form of CP was resistant to enzyme cleavage (Fig. 2*a*, *b*). The apparent M_r of this band was 26K and it comigrated with the polypeptide formed when purified JGMV particles were treated with this enzyme. This suggests that the CP expressed in *E. coli* and yeast

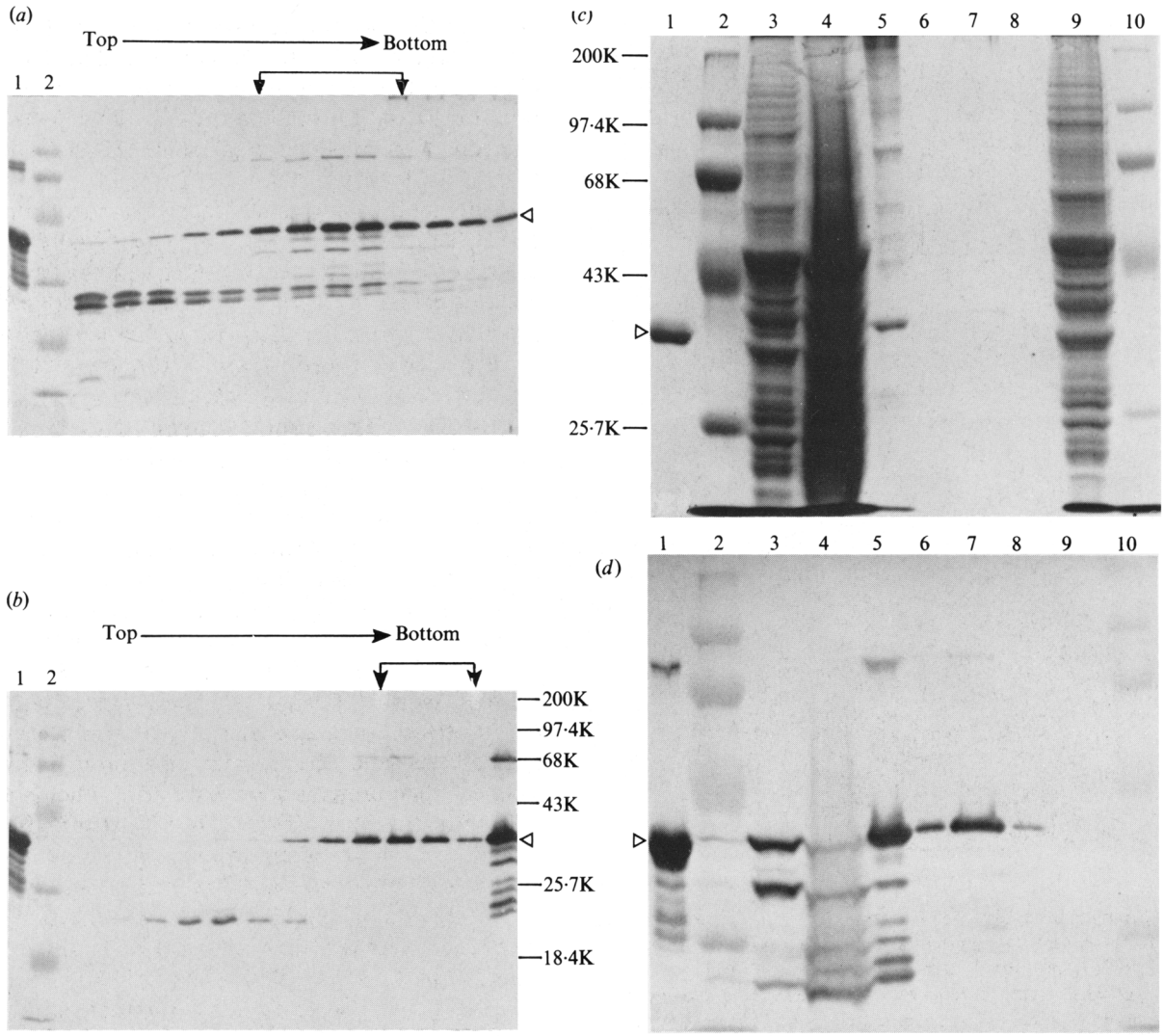

Fig. 3. Sedimentation in sucrose density gradients of CP material from *S. cerevisiae* JHRY1-5D/pAAH5 : CP. (*a* and *b*) Aliquots from every third fraction were analysed by immunoblotting. Fractions indicated by the line between the vertical arrows from the first sucrose gradient (*a*) were pooled and centrifuged in a second gradient (*b*). Fractions indicated by the line between the arrows were pooled from the second gradient and dialysed. Lane 1, JGMV; lane 2, M_r standards. The last lane in (*b*) contains a sample aliquot of the pellet formed by centrifuging the pooled samples from the first gradient at 55000 r.p.m. for 1 h. (*c*) Coomassie blue-stained gel and (*d*) immunoblot of several representative fractions obtained during purification. Lane 1, JGMV particle protein; lanes 2 and 10, M_r standards; lane 3, total extract; lane 4, supernatant fraction after centrifugation at 3000 r.p.m.; lane 5, as right-hand lane of (*b*); lane 6, pooled fraction from the second gradient; lanes 7 and 8, pellet fraction and supernatant fraction, respectively, following centrifugation of pooled fractions at 100000 r.p.m. from the second gradient; lane 9, total extracts from JHRY1-5D/pAAH5. Open arrowheads indicate the full-length CP band.

assumed a structure similar to that in JGMV particles. It is also possible that the CP monomers and rings are folded in such a way that the N-terminal region is exposed and readily accessible to proteolytic attack. CP preparations that had been denatured in formic acid, dialysed and freeze-dried were degraded completely when treated with lysyl endopeptidase (Fig. 2*a*).

Transmission electron microscopy

Protein extracts from *E. coli* DH1/pTTQ19 : CP and yeast JHRY1-5D/pAAH5 : CP were analysed by column chromatography or more routinely by sucrose gradient (10 to 40%) centrifugation. After two gradient centrifugations, relatively pure fractions containing full-length CP were obtained (Fig. 3*a* to *d*). The smaller proteins

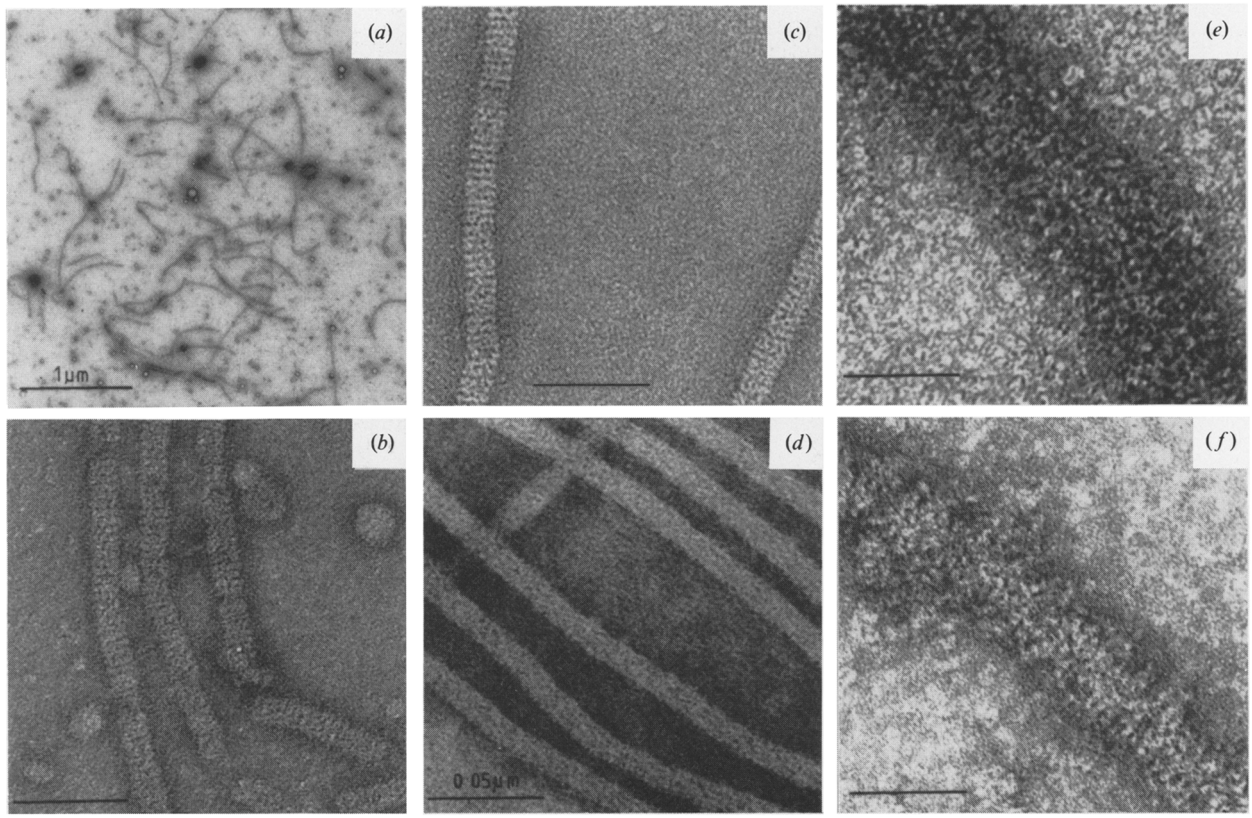

Fig. 4. Electron micrographs of PVLPs from *E. coli* (*a*, *b* and *e*) and *S. cerevisiae* (*c*). JGMV particles purified from plants are shown in (*d*). PVLPs from *E. coli* and PVLPs [reassembled following formic acid (60%) denaturation of JGMV followed by dialysis against 10 mM-phosphate buffer pH 7·2 containing 100 mM-NaCl at 4 °C] decorated with JG : AS are shown in (*e*) and (*f*), respectively. In (*a*) bar represents 1 μm and (*b*) to (*f*) bars represent 0·05 μm.

present in the upper fractions represent truncated forms of the CP and were not used in the present study. Only the fractions enriched for full-length CP were pooled for dialysis and subjected to electron microscopy. Electron microscopic analysis of purified fractions from *E. coli* as well as yeast extracts indicated the presence of PVLPs. The particles resembled those of JGMV in their flexuous morphology and width but were highly heterogeneous in length (Fig. 4*a* to *c*). The particles had a stacked-ring structure similar to that of potyvirus CP assembled without RNA (McDonald & Bancroft, 1977). Immune electron microscopy of crude extracts from *E. coli* and yeast transformants indicated the presence of PVLPs similar to those observed in purified fractions. No such structures were found in protein extracts from strains containing the expression vectors without the CP-encoding region. Finally, in antiserum decoration tests, particles derived from both *E. coli* (Fig. 4*e*) and yeast (not shown) as well as particles derived from the reassembly of dissociated JGMV CP monomers were heavily decorated with JG : AS (Fig. 4*e*, *f*).

Expression of mutant CP

A tertiary structure proposed for potyvirus CP monomers (Shukla *et al.*, 1988; Shukla & Ward, 1989*a*) is composed of seven helices and three loops with N and C termini projecting towards the surface (Fig. 5*a*). Epitope mapping by the use of antisera raised against intact and trypsin-treated potyvirus particles is consistent with this structural model (Shukla *et al.*, 1989*b*). Using this model as a guide we chose two regions (third and fifth helices, Fig. 5*a*) to study the effect on particle assembly of substituting amino acids which are highly conserved in potyviruses.

Expression of the mutant CP constructs pTTQ19 : CP^{W*Y*} (Trp^{130}–Tyr^{131} to Gly^{130}–Pro^{131}) and pTTQ19 : CP^{R*Q*} (Arg^{194}–Gln^{195} to Asp^{194}–Leu^{195}) in *E. coli* was tested by immunoblotting (Fig. 5*b*). The M_rs of the predominant bands which reacted with JG : Core AS in these cells extracts were similar to those of pTTQ19 : CP. Although the amounts of CP expressed in pTTQ19 : CP and pTTQ19 : CP^{R*Q*} were of similar levels, at least twofold less CP was expressed from

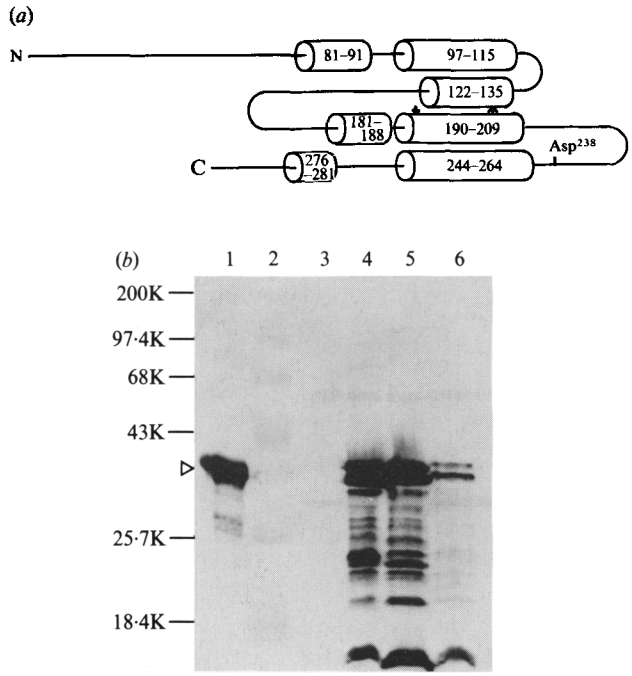

Fig. 5. (*a*) Schematic representation of the tertiary structure of the CP subunit proposed by Shukla & Ward (1989*a*). The cylinders represent the seven helices starting with the one nearest the N terminus. The numbers within each cylinder represent the first and the last amino acid residues of the corresponding helix. The arrows indicate the position of amino acid substitutions in the two mutant derivatives of pTTQ19 : CP. The position of amino acid Asp^{238} proposed to be involved in formation of a salt bridge with Arg^{194} (V. V. Dolja, personal communication) is also indicated. (*b*) Expression of mutant forms of CP in *E. coli*. The immunoblot was probed with JG : Core AS. The bands were visualized by an alkaline phosphatase reaction. Proteins are: lane 1, JGMV; lane 2, M_r standards; lane 3, *E. coli* DH1/pTTQ19; lane 4, DH1/pTTQ19 : CP; lane 5, DH1/pTTQ19 : CP^{R*Q*}; lane 6, DH1/pTTQ19 : CP^{W*Y*}.

pTTQ19 : CP^{W*Y*}. The pattern resulting from cleavage of the mutant proteins with lysyl endopeptidase was similar to that of full-length CP without the mutations suggesting that the N terminus is still exposed (data not shown). However, no PVLPs were detected by direct or immune electron microscopic analysis of extracts from *E. coli* expressing pTTQ19 : CP^{R*Q*} or pTTQ19 : CP^{W*Y*}.

Discussion

We have shown that when a cDNA encoding the full-length CP of a potyvirus is expressed in *E. coli* or *S. cerevisiae*, the resulting products readily assemble to form PVLPs. The PVLPs were similar in their morphology to those obtained by *in vitro* assembly of potyvirus CP monomers in the absence of RNA (McDonald & Bancroft, 1977). The PVLPs were flexuous, similar in width to that of JGMV particles but heterogeneous in length. It is unclear at this stage whether the CP monomers assembled to form PVLPs inside the cells and/or during processing of the cell extracts. Ultrathin sectioning of microbial cells expressing CP followed by electron microscopy should be able to distinguish these possibilities.

The microbial expression system for the assembly of potyvirus CP described here can be used to map the minimum region required for polymerization of CP into PVLPs. This can be achieved by engineering progressive deletions from the 5′ and 3′ ends into the CP gene, followed by their expression in *E. coli* or yeast. The mechanism of CP assembly and genome encapsidation can be investigated by synthesizing CP in various genetically engineered forms and allowing the purified monomers to assemble in the presence or absence of viral RNA. Furthermore, the full-length cDNA copies of the viral RNA could be modified and used in combination with the vectors containing strong promoters for the *in vitro* synthesis of RNA transcripts to map signals involved in functions such as the origin of assembly and genome attachment.

In this report we have described two mutant forms of CP where two amino acid residues in each mutant have been changed by site-directed mutagenesis. In the mutant pTTQ19 : CP^{W*Y*}, the expression levels of the CP were at least twofold less than the wild-type and this low concentration may have contributed to lack of assembly, especially if assembly is dependent on the concentration of CP monomers. However, the residues Trp^{130} and Tyr^{131} are conserved in 21 of 21 and 13 of 21 different potyviruses, respectively (Shukla & Ward, 1989*b*; Ward & Shukla, 1991) and thus may play a major role in the assembly process. In the mutant pTTQ19 : CP^{R*Q*}, despite levels of expression similar to those of pTTQ19 : CP, the mutant CP molecules were unable to form PVLPs. On the basis of amino acid sequence alignment of CP, the residues Arg^{194} and Gln^{195} are not only very highly conserved in potyviruses (21 out of 21 and 19 out of 21, respectively) but also in other flexuous viruses (V. V. Dolja, personal communication). It has been suggested that Arg^{194} might be involved in the formation of a salt bridge with the residue Asp^{238} which is critical for protein structure. Both these residues are invariant not only in potyviruses but also in 14 viruses from five different virus groups (V. V. Dolja, personal communication). The mutational change of Arg^{194} to Asp^{194} in the construct pTTQ19 : CP^{R*Q*} would result in two negatively charged residues which would disrupt formation of the salt bridge thought to be crucial for the assembly process.

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