A synthetic gene coding for the coat protein of tobacco mosaic virus (TMVCP) was expressed in *E. coli* under the direction of the lacUV5 promoter. Modification of the 3' end of the TMVCP gene by insertion of a region coding for an antigenic epitope from poliovirus type 3 resulted in the production of a hybrid TMVCP (TMVCP-polio 3). Both the *E. coli*-produced TMVCP and TMVCP-polio 3 were shown to assemble into virus-like rods under acidic conditions in *E. coli* extracts. Their purification was accomplished in a single step by chromatography on Sepharose 6B. TMVCP-polio 3 induced the formation of poliovirus neutralizing antibodies following injection into rats. The level of immune response was related to the degree of polymerization of the TMVCP-polio 3 preparations.

**RESULTS**

**Synthesis of the TMV coat protein gene.** By synthetizing DNA containing the TMVCP coding sequence it was possible to produce a gene having both a convenient arrangement of restriction enzyme cleavage sites and an "optimized" set of codons for efficient translation in a prokaryotic system. The synthetic coding sequence specified the synthesis of a peptide having a C-terminal attachment site for the assembly of peptides linked to the virus-like rod in vitro. This hybrid molecule induces the production of anti-poliovirus neutralizing antibodies following injection into rats.

**FIGURE 1** Sequence of the synthetic TMVCP coding region. The synthetic TMVCP coding sequence was designed to contain predominantly preferred codons for translation in a prokaryotic host while at the same time coding for the authentic amino acid sequence of the coat protein from TMV Vulgaris. The regions marked 5' and 3' were separately synthesized and cloned. It should be noted that the Gla site indicated at the proximal end of the 5' region is destroyed after ligation to the translation initiation linker in the final expression construct (see Fig. 2).
ifying the coat protein of TMV Vulgare$^{15}$, is shown in Figure 1, and was synthesized as two halves, which were separately cloned for propagation in bacteria.

The insertion of the 5' and 3' halves of the TMVCP coding sequence into the final bacterial expression vector is shown in Figure 2. This construction utilized two double stranded linkers which adapted the restriction ends and to supply the remaining nucleotide sequences needed for the N-terminal and C-terminal amino acids. The final construct (pTMVCP) contained the entire TMVCP coding sequence located downstream from the lacUV5 promoter$^{14}$.

**Insertion of the poliovirus type 3 epitope.** The synthetic TMVCP gene was designed to facilitate 3' end modifications to produce TMVCP's with predetermined C-terminal extensions. Evans et al.$^{15}$ described the location of an epitope on the VP1 of poliovirus type 3 consisting of eight amino acids. To express this epitope on the C-terminus of the TMV coat protein, the pTMVCP expression plasmid was cleaved with Apal and BamHI to remove the small fragment coding for the two C-terminal amino acids (Fig. 2). This fragment was replaced with a double stranded Apal-BamHI linker coding for the two C-terminal amino acids, plus two glycine residues to serve as a spacer, plus the eight amino acids of the poliovirus epitope (Glu Gin Pro Thr Thr Arg Ala Gin). Thus the final construct, pTMVCP-polio 3, specified the expression of a modified TMV coat protein molecule containing an additional 10 residues at its C-terminus.

**Expression of TMVCP and TMVCP-polio 3 in E. coli.** Western blot analysis demonstrated expression of TMVCP and TMVCP-polio 3. Bacterial lysates of cultures containing either the pTMVCP or pTMVCP-polio 3 expression plasmids were electrophoresed on an SDS-polyacrylamide gel (SDS-PAGE). Proteins were transferred to nitrocellulose and incubated sequentially with anti-TMV antiserum and radiolabeled protein-A. Autoradiography revealed a product reacting with anti-TMV antiserum and radiolabeled protein-A. This product was cleaved with Apal and BamHI to remove the small fragment coding for the two C-terminal amino acids (Fig. 2). This fragment was replaced with a double stranded Apal-BamHI linker coding for the two C-terminal amino acids, plus two glycine residues to serve as a spacer, plus the eight amino acids of the poliovirus epitope (Glu Gin Pro Thr Thr Arg Ala Gin). Thus the final construct, pTMVCP-polio 3, specified the expression of a modified TMV coat protein molecule containing an additional 10 residues at its C-terminus.

**Purification of TMVCP and TMVCP-polio 3 from E. coli.** Purification of TMVCP and TMVCP-polio 3 from E. coli. Purification of TMVCP and TMVCP-polio 3 was based on their self-assembling properties. Sonicated lysates of both pTMVCP and pTMVCP-polio 3 containing bacteria were dialyzed overnight (pH 5.0) to induce polymerization of the TMVCP products. Following concentration by ultrafiltration, the samples were each chromatographed on Sepharose 6B and the fractions analyzed by SDS-PAGE. The peaks at the void volume represented the TMVCP product while the cross indicates the TMVCP-polio 3 product. The two bands located at the extreme top and bottom of lanes 3-6 represent E. coli products which nonspecifically react with the radiolabeled protein-A.

**Characterization of the polymeric TMVCP products from E. coli.** Electron micrographs of TMVCP and TMVCP-polio 3 (Fig. 4) demonstrate the presence of rods and disks of the type previously observed for natural TMVCP$^{10,17}$. The TMVCP-polio 3 appeared to polymer-
Electron microscopic analysis of pH 5.0 assembled TMVCP and TMVCP-polio 3. Samples of TMVCP and TMVCP-polio 3 purified from a pH 5.0 Sepharose 6B column were concentrated to 0.1 mg per ml, for electron microscopic analysis. Magnifications were at ×176,000. Micrograph A, TMVCP; micrograph B, TMVCP-polio 3.

Immunogenicity studies. An initial immunization experiment indicated that the TMVCP-polio 3 product could indeed induce an anti-poliovirus neutralizing response after injection into rats (data not shown). However, these data did not indicate whether the observed response was directed against the aggregated form of the TMVCP-polio 3 or against material which had disaggregated soon after injection. To investigate this point, a second immunization was performed in which we compared the immune responses to three forms of TMVCP-polio 3: pH 5.0 polymerized, pH 8.0 disaggregated, and material assembled in the presence of TMV genomic RNA at neutral pH. The latter product was produced by reacting TMVCP-polio 3 with TMV genomic RNA in vitro to form virus-like rods stable over a broad pH range. This reaction was monitored by electron microscopy (data not shown).

Table 1a presents the results of this experiment showing that the pH 5.0 assembled TMVCP-polio 3 elicited a higher neutralizing response than the pH 8.0 disassembled sample. Moreover, the pH stable, RNA-assembled TMVCP-polio 3 did not induce a greater response indicating that the pH 5.0 sample remained aggregated after injection.

The difference in the levels of neutralizing antibody induced by the pH 5.0 and pH 8.0 TMVCP-polio 3 samples was shown to be reproducible in a further immunization study employing five rats per group (Table 1b).

### Table 1 Immunogenicity testing.

| Inoculum               | Rat | Neutralizing Antibody Titers (Log₂) |
|------------------------|-----|------------------------------------|
|                        |     | 1 week after 2nd injection | 1 week after 3rd injection | 1 week after 4th injection | 3 weeks after 4th injection |
| TMVCP-polio 3          |     | 1 | 7 | 9 | 10 | 11 |
| pH 5.0                 |     | 2 | 3 | 6 | 8 | 8 |
| CFA                    |     | 3 | — | 3 | 3 | — |
| TMVCP-polio 3          |     | 4 | — | 2 | 4 | 1 |
| pH 8.0                 |     | 5 | 3 | 4 | 6 | 4 |
| CFA                    |     | 6 | — | — | dead | dead |
| RNA-assembled          |     | 7 | 2 | 7 | 4 | 9 |
| CFA                    |     | 8 | — | 1 | 6 | |
| RNA-assembled          |     | 9 | 2 | 2 | 5 | 5 |

B. Comparison of immune responses to TMVCP-polio 3 at pH 5.0 and pH 8.0.

| Inoculum               | Rat | Neutralizing Antibody Titers (Log₂) |
|------------------------|-----|------------------------------------|
|                        |     | 1 week after 2nd injection | 1 week after 3rd injection |
| TMVCP-polio 3          |     | 1 | 7 | 7 |
| pH 5.0                 |     | 2 | 6 | 7 |
| CFA                    |     | 3 | 1 | 3 |
|                        |     | 4 | 2 | 3 |
|                        |     | 5 | — | 3 |
| TMVCP-polio 3          |     | 1 | 1 | 4 |
| pH 8.0                 |     | 2 | 1 | 3 |
| CFA                    |     | 3 | — | 3 |
|                        |     | 4 | 2 | 1 |
|                        |     | 5 | — | — |

—indicates no significant response. CFA, complete Freund's adjuvant.
These immunization data support the idea that the molecular weight of the TMVCP-polio 3 antigen has a definite influence on the efficiency of the immune response.

It should be noted that significant variations were observed in the levels of the immune responses between individual rats in a given injection group. We attribute this variation to the immunization regime employed (see Experimental Protocol). Rats injected with inactivated Salk vaccine in a similar manner usually show significant variations in their immune responses (data not shown).

DISCUSSION

We have described the development of a genetically engineered, self-assembling, peptide-carrier vaccine system combining the tobacco mosaic mosaic virus coat protein with a poliovirus epitope. It is difficult to compare the immunization results for the TMVCP-polio 3 with those previously reported for the same epitope attached to bovine thyroglobulin. This is due to differences in the animal species used for immunization and in the actual amounts of antigenic peptide injected per animal. In the present studies, rats injected with 200 µg of TMVCP-polio 3 received approximately 10 µg of epitope peptide, while in the Salk-vaccinated control rats the experimental error was equivalent of several hundred micromgrams of peptide was injected per animal. Thus, the data do not allow a valid comparison of the immune responses for the two systems.

It should be noted that the levels of neutralizing antibody shown in Table 1 were dependent upon the use of complete Freund's adjuvant (CFA). With the use of adjuvant for four injections, it was possible to achieve a neutralizing antibody titer, in some rats, as high as that observed from a single injection of Salk vaccine. Using aluminum phosphate as an adjuvant, the TMVCP-polio 3 still induced neutralizing responses in some rats but the levels were significantly reduced (data not shown). We have recently shown that multiple TMVCPs, containing different epitopes, can be copolymerized in vitro to produce multispecific heteropolymers. It will be interesting to determine if heteropolymers containing multiple epitopes from poliovirus type 3 show enhanced immunogenicity.

A related system has recently been described by Valenzuela et al., in which they reported the use of the hepatitis B surface antigen (HBsAg) as a carrier of antigenic epitopes. A recombinant HBsAg gene containing a herpes simplex glycoprotein D coding segment in which they reported the use of the diphtheria toxoid as a carrier for the capsular polysaccharide of Haemophilus influenzae type B was shown to have a stimulatory effect. In immunizations using the polysaccharide antigen coupled to diphtheria toxoid, it was found that preimmunization with the toxoid alone significantly enhanced the subsequent immune response to the conjugated hapten. On the other hand, a recent report describing the use of the tetanus toxoid as a carrier has shown that preimmunity to the carrier molecule has a suppressive effect on the subsequent immune response to coupled peptides. Detailed immunization experiments will have to be performed for all carriers in order to determine their potential usefulness for vaccine production.

EXPERIMENTAL PROTOCOL

Synthesis of the TMVCP gene. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and were purified by electrophoresis on preparative DNA sequencing gels. Bands were visualized by UV imaging. The region of the TMVCP coding sequence in Figure 1 marked 5' was assembled from a collection of 13 overlapping oligonucleotides whereas the region marked 3' was assembled from a collection of 17 oligonucleotides. The ligations were performed in two stages in which groups of 4 to 5 complementary oligonucleotides were initially annealed and ligated. The products of these reactions were pooled for the final ligations yielding the 198 bp 5' (Clal-AcI) and the approximate 370 bp 3' fragment (AcI-Apal). The 5' and 3' fragments were separately cloned into pBR322 for propagation in E. coli. Assembly of the pTMVCP and pTMVCP-polio 3 expression plasmids is described in the text.

Western blotting of E. coli produced TMVCP products. Confirmation of the presence of TMVCP products in E. coli clones containing the pTMVCP or the pTMVCP-polio 3 plasmids was obtained by Western blot analysis. Cultures of E. coli strain JM103 containing the respective plasmids were inoculated 1:100 into L-broth containing 1 mM isopropyl-β-D-thiogalactoside (IPTG) and 50 µg ampicillin per ml. Cultures were grown to an OD675 of 1.0 after which 0.25 ml of each culture was centrifuged and resuspended in SDS-PAGE sample buffer and lysed by incubation in a boiling water bath for 5 minutes. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel after which the proteins were transferred to nitrocellulose. Nitrocellulose blots were reacted with either anti-TMV antisera or anti-poliovirus type 3 vaccine antiserum diluted 1:100 in incubation buffer.

Electron microscopy of TMVCP-epitope products. TMVCP preparations were carried out by growing 8 liters cultures of pTMVCP or pTMVCP-polio 3 containing bacteria as described in the previous section. At an OD675 of 0.5 the bacteria were chilled on ice and harvested by centrifugation. Bacteria were resuspended in 60 ml of 0.5 M sucrose, 0.2 M NaCl, 0.1 M tris, pH 7.9, 50 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride and sonicated twice at an amplitude of 18 microns for 50 seconds. A 1.0-2.0 pause between sonications (MSE Soniprep 150). After removal of cell debris by centrifugation at 10,000 RPM in a Sorvall SS34 rotor the supernatant was dialyzed overnight at room temperature against 0.1 M sodium acetate, pH 5.0, to induce TMVCP aggregation. Following dialysis the precipitate was reprecipitated as described above and the supernatant was concentrated to a volume of 50 ml using an Amicon ultrafiltration membrane with a molecular weight cutoff of 10,000 daltons. The concentrate was reprecipitated 2 times and rechromatographed on a S-500 column (5 x 30 cm) in 0.1 M sodium acetate, pH 5.0. Fractions containing TMVCP products were identified by PAGE analysis.

Electron microscopy of TMVCP-epitope products. TMVCP and TMVCP-polio 3 preparations were prepared for electron microscopy by applying a drop to formvar coated grids and blotting off excess sample after 30–60 seconds. Grids were negatively stained with 2% phosphotungstic acid for 30–60 seconds after which the excess was removed by blotting and the grids were allowed to dry. Samples were examined on a Phillips 200 electron microscope.

Immunogenicity testing. TMVCP-polio 3 was tested for immunogenicity using coronavirus free, Wistar rats as suggested by
van Steenis et al. 20 Antigens, at a concentration of 0.2 mg per ml, was mixed with an equal volume of complete Freund's adjuvant prior to each injection. The first and second injections were spaced three weeks apart while the remaining injections were two weeks apart. For a given injection, each rat received 1 ml i.p. and 1 ml i.d. (multiple sites). Rats were bled seven days following each injection. Sera were tested for neutralizing antibodies by a neutralization assay. A pH of less than 7.2 indicated cell death and residual virus activity due to a lack of complete virus neutralization. The pH 5.0 TMVC-polio 3 samples (0.1 sodium acetate) were obtained from the void fractions of the preparative Sepha­rose 6B column runs (see above). The pH 8.0 TMVC-polio samples were obtained by dialyzing the pH 5.0 samples against 30 mM Tris-HCl, pH 8.0, overnight at 4°C. RNA-assembled TMVC-polio 3 used in the second immunization experiment was prepared by dialyzing a preparation of TMVC-polio 3 at 0.4 mg/ml against 0.1 M sodium pyrophosphate (pH 7.0) for two days at room temperature in order to induce disk formation. Assembly was accomplished by adding dithiothreitol to 1 mM and placent al ribonuclease inhibitor to 75 units per ml after which a 1.3 fold stoichiometric excess of TMV genomic RNA was added. Incubation was continued for 18 hours at 25°C after which sucrose assembly was assayed by electron microscopy as described above.

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