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Protein P4 of Double-Stranded RNA Bacteriophage φ6 Is Accessible on the Nucleocapsid Surface: Epitope Mapping and Orientation of the Protein

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Protein P4, an early protein of double-stranded RNA bacteriophage φ6, is a component of the virion-associated RNA polymerase complex and possesses a nucleoside triphosphate (NTP) phosphohydrolase activity. We have produced and characterized a panel of 20 P4-specific monoclonal antibodies. Epitope mapping using truncated molecules of recombinant P4 revealed seven linear epitopes. The accessibility of the epitopes on the φ6 nucleocapsid (NC) surface showed that at least the C terminus and an internal domain, containing the consensus sequence for NTP binding, protrude the NC shell. Four of the NC-binding antibodies distorted the integrity of the NC by releasing protein P4 and the major NC surface protein P8. This finding suggests a close contact between these two proteins. The dissociation of the NC led to the activation of the virion-associated RNA polymerase. The multimeric status of the recombinant P4 was similar to that of the virion-associated P4, indicating that no accessory virus proteins are needed for its multimerization.

Bacteriophage φ6 is an enveloped double-stranded RNA (dsRNA) virus of *Pseudomonas syringae* pv. phaseolicola (53). The envelope contains five virus-specific proteins (3, 25, 35, 51). Inside the envelope resides the polyhedral nucleocapsid (NC) (3, 31, 32, 51), which encapsidates three dsRNA genome segments designated L, M, and S (45, 52). The virus particle contains one copy of each genomic segment (10).

The NC is composed of an inner dodecahedral particle consisting of early proteins P1, P2, P4, and P7 and the late, major surface protein P8 (32). NC disruption studies have revealed that Ca^{2+} ions are important for the integrity of the P8 shell around the NC (27, 37, 40). The early proteins are encoded by the genomic L segment (9, 34). A cloned cDNA copy of the L segment directs the synthesis and assembly of an empty procapsid structure in *Escherichia coli* or *P. syringae* pv. phaseolicola (16). The procapsids package the positive-sense viral RNA segments and synthesize the minus strands inside the particle (14, 17, 19). The filled procapsids are capable of plus-strand synthesis (20), which proceeds via a semiconservative mechanism (13, 49, 50). A particle structurally similar to the filled procapsid can be prepared in vitro by removing the P8 from the NC surface (40). The P8-free particle is analogous to the entering viral particle (42). It has been designated the NC core to distinguish it from the assembly intermediate, the filled procapsid (40). The generic name "polymerase complex" will be used to describe the particle containing the proteins P1, P2, P4, and P7 when no distinction between the different particle stages appearing during infection is needed. The purified NCs have previously been shown to infect host cell spheroplasts, and protein P8 was found to be necessary for NC entry (36). The cDNA-derived procapsids that have synthesized minus strands can be coated with purified protein P8 to form infectious NCs (39), which indicates that procapsids produced in *E. coli* are identical to viral procapsids.

The suggested stoichiometry, based on biochemical data, of the protein composition of the NC particle is 120 P1, 120 P4, 100 P7, 20 P2, and >1,000 P8 (32). Protein P1 is the primary structural component of the procapsid, forming a cagelike structure (27, 37). Protein P2 appears to be essential for RNA synthesis (20). Sequence alignments with known RNA-dependent RNA polymerases from other dsRNA and positive-strand RNA viruses and φ6 P2 show similarity (5, 26). The NC has recently been shown to possess a nucleoside triphosphate (NTP)-binding site with associated NTP phosphohydrolase activity (18). This activity has been assigned to protein P4. The molecular size of P4 is 35.0 kDa, as calculated from the sequence (34). The protein is released as a multimer from the NC by EDTA treatment (37).

A virion-associated RNA polymerase activity is common for all dsRNA viruses. The φ6 NC exhibits an enhanced RNA polymerase activity after the P8 shell is removed in vitro by chelating Ca^{2+} ions (40). The resulting NC core is thus analogous with the single-shelled subviral particles of the reoviruses and rotaviruses. The rotavirus transcriptase is also activated in vitro upon removal of calcium ions from the virus particle, which leads to dissociation of the outer shell (7, 8, 30). Reovirus uncoating prior to transcriptase activation involves proteolytic digestion of the outer capsid followed by a second endoproteolytic cleavage (4, 44, 47). Conversion of the bluetongue virus to a transcriptionally active particle also proceeds via two uncoating steps (23).

In this work, we have studied protein P4, one of the φ6 polymerase complex major protein species, in more detail. We have produced and characterized a panel of monoclonal antibodies (MAbs) against P4. The approximate locations of the epitopes were determined by using C-terminal truncations, and the epitopes which are exposed at the NC surface were identified. Binding of the MAbs with respect to the structural integrity of the NC particle was analyzed. The

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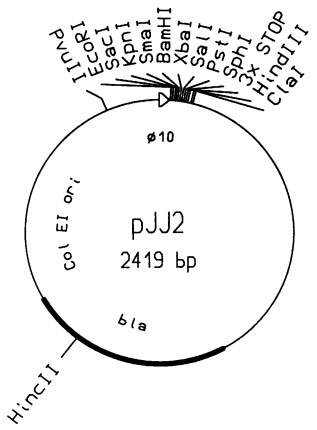

FIG. 1. Plasmid pJJ2, used for subcloning the bacteriophage ϕ6 gene 4 and preparing its C-terminal truncations. The plasmid contains an ampicillin resistance gene (*bla*) and the pUC18 polylinker site with a stop codon in three reading frames. ϕ10 is the T7-specific RNA polymerase promoter.

multimeric status of recombinant P4 and its truncations was determined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM109 containing plasmid pLM574 was used to obtain incomplete procapsid particles. pLM574 contains a cDNA copy of a deleted genomic L segment, which produces proteins P1, P2, and P4 (20). *E. coli* HMS174(DE3) (46) was used as the cloning and expression host. For the cloning and exonuclease III deletions of gene 4, coding for protein P4, a plasmid was constructed by replacing the pUC8 polycloning site in plasmid pT7-5 (later version of pT7-1 [48]) with the pUC18 polycloning site. Stop codons in three reading frames (GCATGCTAAGTAAGTAAGCTT) were also added between *Sph*I and *Hin*dIII sites to obtain plasmid pJJ2 (Fig. 1).

Cloning and truncation of gene 4. Gene 4 was subcloned from plasmid pLM500 as an *Nci*I-*Pst*I fragment (nucleotides [nt] 2882 to 4034 from the L segment [34]) to the *Sma*I site in pJJ2 to obtain pJTJ7. Plasmid pLM500, obtained from L. Mindich, contains nt 1 to 4034 from the L segment cloned in pLM254 (33) as a *Pst*I fragment. Exonuclease III deletions of pJTJ7 to produce C-terminal P4 truncations were made according to Sambrook et al. (43) except that after the S1 treatment, the samples were phenol extracted and precipitated prior to ligation. The deletion plasmids were screened according to size, and the clones of appropriate size were tested for expression of P4 fragments. The exact sizes of the selected clones were determined by DNA sequencing. In several clones, S1 nuclease and Klenow treatments had removed nucleotides from the stop cassette in the vector (Fig. 1), which led either to termination without any additional amino acids or to a tail coded from the vector. The clones chosen for the deletion series of gene 4 are listed in Table 1.

Growth and purification of the phage. Bacteriophage ϕ6 (wild type) was grown on *P. syringae* pv. phaseolicola HB10Y (53) as described previously (36, 38) except that the isolation was performed in 20 mM Tris (pH 7.5). ^{14}C-labeling of the virus proteins was performed as described by Ojala et al. (36). After purification, the radioactively labeled phage preparation was immediately treated with butylated hydroxytoluene to remove the ϕ6 spike protein (2, 25) and stored at −70°C.

Isolation of ϕ6 subviral particles. NC isolation was done in principle as previously described (36) except that Triton X-114 was prepared in 10 mM Tris (pH 7.5)–150 mM NaCl, and the butylated hydroxytoluene-treated phage was extracted first with approximately 3% and then with 1% (wt/vol) Triton X-114. The NC used in RNA polymerase reactions was always isolated from newly prepared fresh virus. The preparation was layered on the top of a 5 to 20% sucrose gradient in 20 mM Tris (pH 8.0)–1 mM $MgCl_2$–200 mM KCl and centrifuged in a Sorvall TH641 rotor (30,000 rpm at 15°C for 50 min). The light-scattering zone containing the NC was collected. In vitro uncoating of the NC to obtain core particles was performed by chelating Ca^{2+} ions essentially according to Olkkonen et al. (40). The freshly prepared NCs were first sedimented through a 20% sucrose cushion in 20 mM Tris (pH 7.5) (Sorvall T-865 rotor; 40,000 rpm at 10°C

TABLE 1. C-terminal deletions of P4 used for epitope mapping

Construct	No. of aa from P4	No. of extra aa	Total no. of aa	Last aa from P4	Additional aa
pJTJ7	332	0	332	Leu	
pJTJ7.3/3	319	2	321	Asp	Ser Lys
pJTJ7.3/7	311	0	311	Glu	
pJTJ7.4/6	301	3	304	His	Cys Ser Lys
pJTJ7.5/7	289	0	289	Ser	
pJTJ7.6/7	247	17	264	Val	Ser Lys Leu Ile Asp Lys Leu Ser Asn Met Arg Ile Lys Ser Ile
pJTJ7.5/11	243	2	245	Val	Ser Lys
pJTJ7.6/8	230	1	231	Val	Glu
pJTJ7.7/5	212	2	214	Leu	Cys Lys
pJTJ7.8/6	201	1	202	Ser	Lys
pJTJ7.8/5	182	3	185	Phe	Leu Ser Lys
pJTJ7.9/5	170	17	187	Met	Val Ser Lys Leu Ile Asp Lys Leu Ser Asn Met Arg Ile Lys Ser Ile
pJTJ7.8/3	162	16	178	Val	Ser Lys Leu Ile Asp Lys Leu Ser Asn Met Arg Ile Lys Ser Ile
pJTJ7.10/7	152	2	154	Ala	Gly Lys
pJTJ7.9/4	142	1	143	Pro	Lys
pJTJ7.10/4	111	2	113	Arg	Ser Lys
pJTJ7.12/8	90	16	106	Val	Ser Lys Leu Ile Asp Lys Leu Ser Asn Met Arg Ile Lys Ser Ile
pJTJ7.12/7	81	0	81	Ala	
pJTJ7.14/6	73	0	73	Thr	

for 3 h), then resuspended in 20 mM Tris (pH 7.4), and adjusted to a final protein concentration of 300 μg/ml for uncoating.

Preparation of incomplete procapsid particles. Incomplete procapsid particles containing proteins P1, P2, and P4 were produced in *E. coli* containing plasmid pLM574. They were purified according to Gottlieb et al. (20) except that the cells were resuspended in 10 mM potassium phosphate (pH 7.2)–1 mM $MgCl_2$–5% glycerol–100 mM KCl–5 mM phenylmethylsulfonyl fluoride before passage through a French pressure cell. Cell debris was removed by centrifugation, and the supernatant was applied on a 5 to 20% sucrose gradient containing 10 mM potassium phosphate (pH 7.2), 1 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride and centrifuged (27,000 rpm at 15°C for 2 h) in a Beckman SW41 rotor. The fractions were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as previously described (38). The material in the peak fractions was collected by centrifugation (Sorvall T-865 rotor; 37,000 rpm at 5°C for 2.5 h). The pellet was suspended in 10 mM potassium phosphate–1 mM $MgCl_2$–150 mM NaCl and stored at −70°C.

Production of MAbs against protein P4. BALB/c mice were immunized subcutaneously with P1, P2, and P4 containing incomplete procapsid particles emulsified with complete Freund's adjuvant. The first booster was given subcutaneously 3 weeks after the primary immunization. The second booster was given 2 months later intraperitoneally. For both immunizations and boosters, mice received 200 to 250 μg of the antigen. Splenic lymphocytes were fused with P3-NS1-Ag4-1 cells (29) essentially as described by Gefter et al. (15). Hybridomas were grown and isolated as described by Olkkonen et al. (41). Culture supernatants were tested for antibody production by enzyme-linked immunosorbent assay (ELISA) using purified NCs as the antigen. The positive cultures were cloned twice by limiting dilution and passaged in pristane-primed BALB/c mice for production of ascites fluid.

The immunoglobulin class and subclass of each antibody was determined by using a mouse hybridoma subtyping kit (Boehringer Mannheim). The MAbs were purified from the ascites fluid by affinity chromatography (GammaBind Plus Sepharose; Pharmacia) according to the manufacturer's instructions. The immunoglobulin concentration of purified antibody preparations was 200 to 500 μg/ml. $MgCl_2$ was added to obtain a final concentration of 5 mM prior to characterization of the purified MAbs.

Characterization of purified P4-specific MAbs. The specificity of each MAb was determined by immunoblotting (41) using peroxidase-conjugated horse anti-mouse immunoglobulin G (IgG; Vector Laboratories) as the secondary antibody. Radioimmunoprecipitation (RIP) was performed by using protein A-Sepharose CL4B (Pharmacia) as previously described (25) except that the antigen was freshly prepared radioactively labeled NC. The antigen was first treated with a purified antibody preparation at room temperature for 1.5 h, protein A-Sepharose was added, and the mixture was incubated at room temperature for 1 h, after which the protein A-Sepharose beads were separated by centrifugation. The protein A-associated radioactivity was analyzed by liquid scintillation counting and by autoradiography of the proteins separated by SDS-PAGE (16% polyacrylamide gel).

In the NC antibody sedimentation assay, a mixture of freshly prepared unlabeled and radioactively labeled NC particles was incubated with MAbs at room temperature for 1 h. The sedimentation analysis was carried out as described by Kenney et al. (25). The radioactivity in the fractions and in the pellet was measured by liquid scintillation counting. The gradient top and peak fractions were pooled; the proteins were precipitated with 10% (wt/vol) trichloroacetic acid (TCA) and analyzed by SDS-PAGE (16% polyacrylamide gel).

Preparation of Fab fragments. Purified MAbs (1S1, 4S4, 4S7, 4S10, 8K4, 8Q2, and 8Q3) were digested with papain (Sigma) essentially as described by Harlow and Lane (22). The optimal reaction mixture for IgG1 digestion contained 1.5 μg of papain per 100 μg of antibody, 1 mM EDTA, and 100 mM sodium acetate (pH 5.5). The optimal cysteine concentration in the reaction varied between individual antibodies: 10 mM cysteine for digestion of 4S7, 8K4, and 8Q3; 5 mM for 1S1 and 4S4; and 3 mM for 4S10. The digestion was performed at 37°C for 7 to 8 h. The optimal digestion of the IgG2A antibody 8Q2 was achieved with 1.5 μg of papain per 100 μg of antibody, 1 mM cysteine, 1 mM EDTA, and 100 mM sodium acetate (pH 5.5) at 37°C for 1.5 h.

The Fab fragments were purified by ion-exchange chromatography (Fractogel DEAE 650S; Merck) as described by Annunziato and Marciani (1). The Fab-containing fractions were analyzed by SDS-PAGE (12% polyacrylamide gel). The residual amounts of IgG or Fc were removed by a treatment with protein A-Sepharose beads to obtain pure Fab preparations.

RNA polymerase assay. Ten microliters of the NC or the core peak fraction from the sucrose gradient was incubated with 2 μl of purified MAbs or Fabs at room temperature for 15 min. The RNA polymerase reaction mixture contained 56 mM Tris-HCl (pH 8.0), 4.3 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM ammonium acetate, 66 mM KCl, 5 mM dithiothreitol, 1 mM each ATP, CTP, and GTP, 0.1 mM UTP, 5% polyethylene glycol 6000, 40 U of RNAsin (Promega), and 6 μCi of [α-^{32}P]UTP (Amersham PB 10203; 3,000 Ci/mmol). The final volume of the mixture was 33 μl containing 10 μl of the sample to be assayed. The reaction was allowed to proceed 1 h at 30°C. It was stopped by adding 10 μg of carrier tRNA and 1 ml of ice-cold 10% (wt/vol) TCA, kept on ice for 45 min, and transferred to Whatman GF/C filters. The filters were washed with 10% TCA (twice), 5% TCA (five times), absolute ethanol (twice), and acetone (once) and dried, and the radioactivity was measured by liquid scintillation counting.

Epitopic mapping. *E. coli* HMS174(DE3) cells harboring plasmids that code for P4 and its C-terminal truncations were grown to approximately 3×10^8 cells per ml and induced for 3 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The collected cells were suspended to 1/10 of the original culture volume in a 3× concentrate of sample buffer (38). The proteins were separated by SDS-PAGE (16% polyacrylamide gel), transferred to polyvinylidene difluoride membranes (Millipore), and reacted with MAbs as described by Olkkonen et al. (41). The MAbs were first reacted with the full-length P4 and three truncations to divide the protein into four quarters, after which fine mapping of the epitopes inside the quarters was performed. The protein sequence of P4 was analyzed by the Genetics Computer Group software package (11).

Analysis of the multimeric status of intact and truncated forms of P4. Cells producing full-length P4 and the truncated forms were grown as described above, harvested, and suspended to 1/10 of the original culture volume in 20 mm Tris (pH 7.5). Cells were lysed by passage through a French pressure cell, and cell debris was removed by centrifugation.

TABLE 2. Characterization of MAbs against protein P4

MAb[a]	Class or subclass	RIP[b]	Sedimentation class[c]	RNA polymerase activity[b]		Epitope location[e] (aa no.)
				NC	Core	
4H1	IgG1	+	I	0	0	320–332
4S1	IgG1	+	II	−	0	290–301
4S2	IgG1	+	II	−	−	290–301
4S3	IgG1	+	II	−	0	82–90
4S4	IgG1	+	II	−	−	290–301
4S5	IgG1	+D	II	0	−	290–301
4S6	IgG1	+	II	−	−	290–301
4S7	IgG1	−	I	0	0	74–81
4S8	IgG1	+	II	−	−	82–90
4S9	IgG1	+D	III	+	−	112–142
4S10	IgG1	+D	III	+	−	290–301
4S11	IgG1	−	I	0	0	143–152
4S12	IgG1	+	II	−	−	290–301
4S13	IgG1	+D	III	+	−	290–301
4R1	IgG1	+	II	−	−	290–301
4R2	IgG3	−	I	0	0	290–301
4R3	IgG1	+D	III	+	−	290–301
4R4	IgG1	+D	II	−	−	290–301
4R5	IgG3	+	I	−	−	290–301
4R6	IgG1	+D	II	0	0	248–289
1K5	IgG1	ND	I	0	0	
1S1	IgG1	−	I	0	0	
8K3	IgG1	+	I–II	0	0	
8Q2	IgG2A	+D	III	+	0	
3O4	IgG1	−	I	ND	ND	

[a] The first number indicates the specificity, the letter represents the fusion, and the last number indicates order.

[b] NC-antibody complexes precipitated with protein A-Sepharose CL4B beads (see Materials and Methods). +, recognition of the NC; −, no precipitation of the NC; D, release of NC-specific proteins; ND, not determined.

[c] See Materials and Methods and Fig. 2 for details of the sedimentation analysis.

[d] See Materials and Methods for details of the RNA polymerase assay. 0, no effect; −, more than twofod reduction of polymerase activity; +, more than twofold activation; ND, not determined.

[e] P4 region containing the epitope for each MAb (see Table 1 and Fig. 3).

A 0.2-ml sample of the preparation was layered on top of a 5 to 20% sucrose gradient (20 mM Tris [pH 7.5]) and centrifuged (38,000 rpm for 17 h at 5°C) in a Beckman SW50.1 rotor. The gradients were fractionated, and the proteins were precipitated with 10% TCA and analyzed by SDS-PAGE (16% polyacrylamide gel). Aldolase (161 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), and trypsin inhibitor (20 kDa) were used as molecular weight standards.

RESULTS

P4-specific MAbs. Incomplete procapsid particles, containing P1, P2, and P4, were used as an antigen to produce P4-specific MAbs. We isolated 20 hybridoma cell lines secreting antibodies against the NC proteins. The MAbs were all positive in Western immunoblots, suggesting that their antigenic determinants are linear. They also gave a good response in ELISA when NCs treated at 80°C were used as an antigen. The specificity of the antibodies was identified by immunoblotting (Table 2). Nineteen hybridoma lines showed a specificity for protein P4, and only one cell line (1S1) was P1 specific.

RIP. The 19 P4-specific MAbs obtained in this study and one isolated earlier (4H1 [41]) were tested for the ability to recognize intact ϕ6 NC particles by RIP. Seventeen of the P4-specific MAbs precipitated ϕ6 NC particles in the RIP tests. Three of twenty antibodies did not recognize NC particles (Table 2). The virus spike protein P3-specific 3O4 (25, 41) and the P1-specific 1S1 were used as negative controls. Two P8-specific antibodies (8K3 and 8Q2 [41]) were used as positive controls for NC recognition. The protein-A Sepharose-associated radioactivity originating from NC proteins was analyzed by SDS-PAGE. Two kinds of NC-recognizing P4 antibodies were observed (Table 2). One type precipitated more than 80% of the NC-associated radioactivity and showed normal ratios of NC proteins in the precipitate. The other type precipitated selectively more protein P4 than the other NC proteins, which were mainly found in the supernatant fraction. These MAbs are indicated by +D in Table 2.

Sedimentation analysis. To further examine the binding of the P4-specific MAbs to the NC particles, we carried out a sedimentation analysis. The sedimentation profile obtained with the control antibody against the ϕ6 spike protein P3 (3O4 [25, 41]) was used as a reference. One P1-specific antibody and two P8-specific MAbs were also included in the analysis. The P1-specific antibody (1S1) displayed a profile similar to that of the reference. The P8-specific 8K3 (41) formed two separate peaks in the sedimentation assay. The first peak contained single NC particles, whereas the faster-sedimenting fraction contained probably multimeric NC particles. The radioactivity sedimenting to the bottom of the tube was twice the amount found in the reference profile.

The P4-specific antibodies incubated with NCs could be divided into three classes according to their effect on the sedimentation behavior of the NC (Fig. 2; Table 2). The antibodies of sedimentation class I did not change the sedimentation of the NC in the 5 to 20% sucrose gradient in comparison with the reference. Sedimentation class II is composed of antibodies that aggregated NC particles. The antibodies of sedimentation class III dissociated the NC structure. The P8-specific 8Q2 (41) also interfered with the NC integrity by releasing about 80% of the protein P8 from the NC. The rest of the NC proteins sedimented as the RNA-filled core particles.

Antibodies (4S7, 4S11, and 4R2) that did not precipitate NC particles in the RIP test are members of sedimentation class I. The RIP-positive 4H1 and 4R5 displayed similar sedimentation profiles as well (Fig. 2A). The antibodies of sedimentation class II (11 of 20) recognized and aggregated NC particles strongly. More than 65% of the NC-associated radioactivity was found in the sediment (Fig. 2B). The sedimentation profiles and the SDS-PAGE analysis revealed that treating the NCs with the MAbs of sedimentation class III disrupts the NC structure (Fig. 2C). The gradient top fractions contained approximately 80% of the P8, 50 to 90% of the P4, and less than 10% of the P1, as estimated from the autoradiography. The remaining intact NC particles showed normal sedimentation behavior. The material sedimenting to the bottom contained mainly the P1 cagelike structures alone or associated with protein P4.

Effect of MAb treatments on RNA polymerase activity. The in vitro-isolated NC core particles display a strong RNA polymerase activity (plus-strand synthesis) compared with that of the intact NC. The activity of the core can be quenched by reassembling the P8 coat onto it (40). The RNA

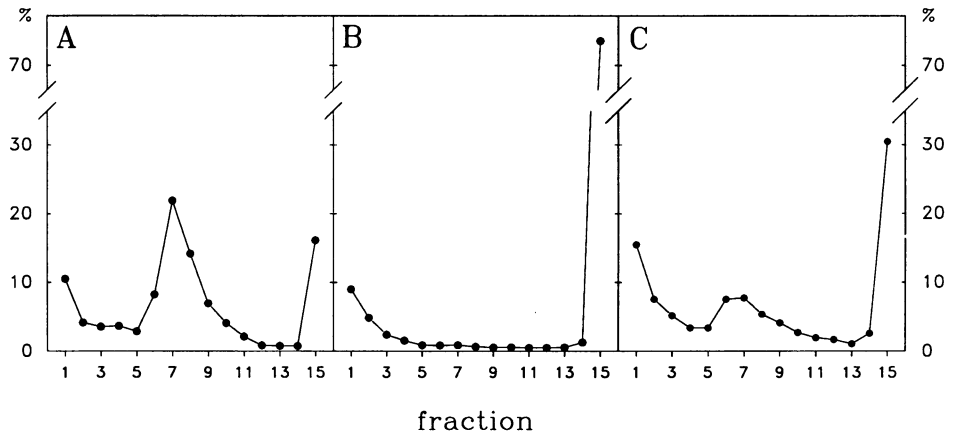

FIG. 2. Sedimentation profiles of the NC particles treated with P4-specific MAbs. A mixture of unlabeled and radioactively labeled NCs was treated with MAbs at room temperature for 45 min before being loaded on top of a 5 to 20% sucrose gradient. Fractions were collected, and radioactivity was measured. The *y* axis shows the relative counts-per-minute value of each fraction. Sedimentation is from top (fraction 1) to bottom (fraction 15 is the pellet). (A) Sedimentation class I, which includes the NCs treated with P4-specific MAbs (4H1, 4S7, 4S11, 4R2, and 4R5) that did not change the sedimentation of the NC compared with the reference (NCs treated with a nonspecific MAb 304). Similar profiles were obtained with NC-specific antibodies, 1K5, 1S1, and 8K3. (B) Sedimentation class II, which includes NCs treated with NC-recognizing P4-specific MAbs (4S1, 4S2, 4S3, 4S4, 4S5, 4S6, 4S8, 4S12, 4R1, 4R4, and 4R6) that aggregate the NC particles. (C) Sedimentation class III, which includes NCs treated with NC-disrupting P4-specific MAbs (4S9, 4S10, 4S13, and 4R3).

polymerase activity of the NCs and the core particles pretreated with the P4-specific MAbs was determined (Table 2). The P4-specific MAbs either enhanced or had no or little inhibitory effect on the plus-strand synthesis of the NC. On the other hand, the strong polymerase activity associated with the core particle either decreased or remained unaltered after antibody treatments. Treatment with P1-specific MAbs (1S1 and 1K5 [41]) or with P8-specific 8K3 had no influence on either the NC or the core.

The low polymerase activity of the NC was enhanced 10- to 15-fold after treatment with the antibodies disrupting the NC structure (sedimentation class III). The enhancing effect was even stronger when NCs were treated with 8Q2 or its Fab fragments. The NC-recognizing and -aggregating P4 antibodies of sedimentation class II (except 4S5 and 4R6) suppressed the residual RNA polymerase activity found in the NC preparation. The rest of the P4 MAbs had no effect on the NC in this assay.

The antibodies enhancing the plus-strand synthesis of the NC displayed only marginal inactivation of the core-associated RNA polymerase activity. The antibodies of sedimentation class II (except 4S1 and 4S3) reduced the activity of

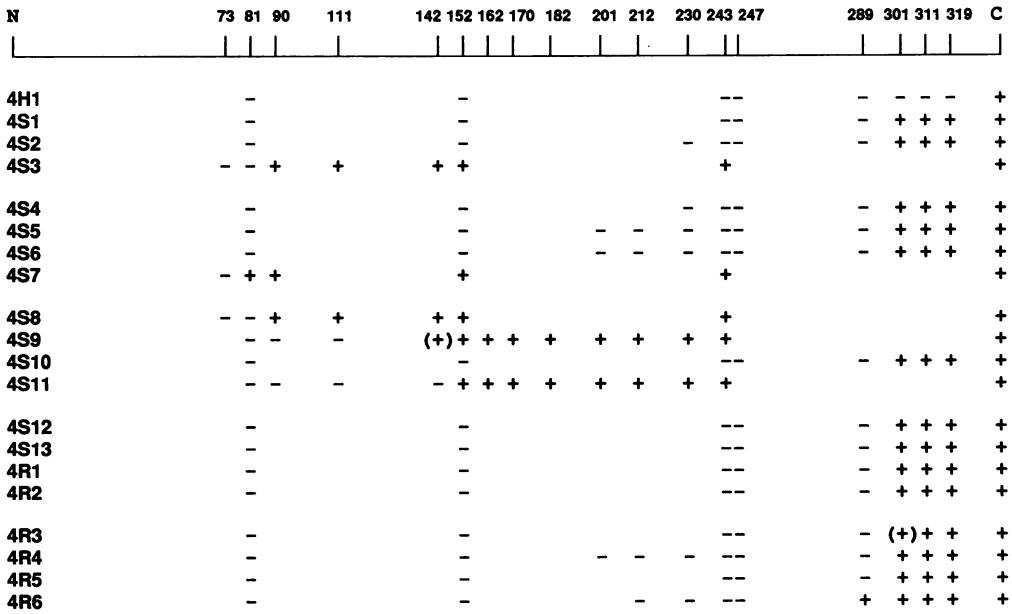

FIG. 3. Binding of the P4-specific MAbs to the C-terminally truncated forms of protein P4 in a Western blot analysis. The relative locations and lengths of the truncations are indicated at the top (see Table 1 for more details). +, positive reactivity in the blot; (+), weaker reactivity than +; −, negative reaction.

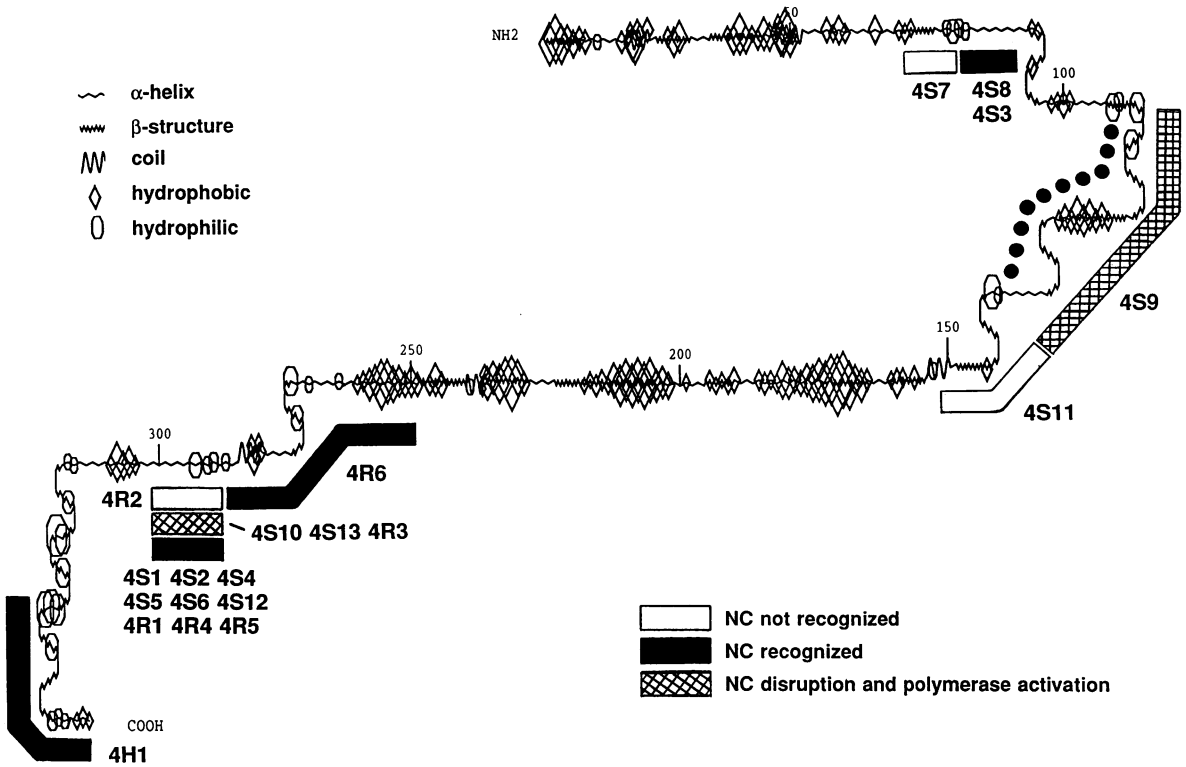

FIG. 4. Approximate locations of the epitopes on protein P4 (see Fig. 3). The protein secondary structure is based on a Chou-Fasman prediction (6). The hydrophobicity is derived by the method of Kyte and Doolittle (28). Black circles indicate the location of the proposed nucleotide-binding site (18, 54). The total length of P4 is 332 aa.

the core particle considerably. The polymerase activity of the core remained unaltered after treatment with antibodies not recognizing intact NCs in the RIP (4S7, 4S11, and 4R2).

The results obtained for the available Fab fragments were consistent with the results for the corresponding MAbs except the Fab fragment of 4S10. The residual polymerase activity of the NC was inhibited, not activated as with the bivalent 4S10. On the contrary, the inhibitory effect of the 4S10 Fab on the core-associated activity was even stronger than with the corresponding MAb.

Epitope mapping. Localization of the distinct antigenic determinants on protein P4 was carried out by a Western blot analysis of the C-terminal truncations of protein P4 expressed in *E. coli* (Table 1). Recognition of the truncated proteins by the antibodies is presented in Fig. 3. We were able to determine the approximate locations of seven different SDS-stable (linear) epitopes. The C-terminal end, consisting of amino acids (aa) 320 to 332, was recognized by 4H1, an NC-recognizing MAb that belongs to sedimentation class I. Most of the antibodies (13 of 20) were specific for the epitope(s) located between amino acids (aa) 290 and 301. The antibodies mapped to this region represent all three sedimentation classes. However, all but one (4R2) are NC-recognizing antibodies in the RIP. The epitope for 4R6, an NC-recognizing antibody of sedimentation class II, was mapped to between aa 248 and 289. The region between aa 112 and 142 was recognized by an NC-disrupting antibody, 4S9. MAbs 4S3 and 4S8 shared a common epitope located between aa 82 and 90. The epitopes for which 4S11 and 4S7 are specific reside between aa 143 and 152 and aa 74 and 81, respectively. These epitopes are not recognized in the RIP of intact NCs.

Multimerization of the full-length P4 and its C-terminal truncations. The previous NC disruption studies have shown that P4 is released as a multimer with a molecular size of about 160 kDa, as determined by sucrose gradient centrifugation (37). The molecular weights of the recombinant P4 and its C-terminal truncations were determined by rate zonal centrifugation. The full-length P4 was expressed mainly in a soluble form and sedimented as an approximately 140-kDa protein, in accordance with the results obtained by dissociating the P4 from the NC. The truncated forms of P4 sedimented as multimers up to the truncation (pJTJ7.5/7) lacking the last 43 C-terminal amino acids. Truncations shorter than that were no longer soluble and thus not available for this analysis.

DISCUSSION

The results of this study, summarized in Fig. 4, indicate that at least seven distinct regions of protein P4 are involved in the formation of SDS-stable (linear) epitopes. Protein P4 is known to interact and form a stable particle with the dodecahedral P1 cage (16, 27, 37). In this report, we have shown that P4 protrudes through the NC surface, indicating that the P8 shell does not completely cover the underlying core particle. P4 released from the NC appears as a multimer in the gradient analysis (37). Our results reveal that the recombinant P4, overexpressed in *E. coli*, is also assembled to a multimer in the absence of other viral proteins. The

multimer sedimented as a 140-kDa particle, corresponding to a tetramer (4 × 35 kDa).

The locations of the antibody-binding sites and the effects of binding (Fig. 4) allow us to conclude the following. (i) P4 contains a hydrophilic C-terminal tail (approximately 70 aa), which contains three regions recognized by P4-specific MAbs. The majority of the MAbs (13 of 14) recognizing the tail also recognize the intact NC particles, indicating that the hydrophilic C-terminal end of the protein is exposed at the NC surface. The MAbs binding to the region between aa 290 and 301 (Fig. 4) displayed three different reactions with respect to NC recognition and disruption. It is possible that this binding site, composed of 11 aa, contains three separate epitopes. (ii) The second, mainly hydrophilic domain is located between aa 82 and 142. This region encloses two epitopes recognized at the NC surface: one by MAbs 4S3 and 4S8, and a second, which includes a consensus sequence characteristic of nucleotide-binding sites (18, 54), by MAb 4S9. This finding is consistent with a previous observation that intact NCs exhibit an NTP phosphohydrolase activity (18). We thus conclude that also this domain of P4 is accessible from outside the NC. On the other hand, these results do not exclude the possibility that more of P4 than these two regions would be exterior to P8. (iii) There are two mainly hydrophobic regions in protein P4, located approximately between aa 1 and 73 and aa 153 and 247. None of our MAbs reacted with these parts of the protein. However, the epitopes of MAbs 4S7 and 4S11 were mapped to the regions flanking the mainly hydrophilic domain that contains the proposed NTP-binding site. These epitopes are surface inaccessible, since the MAbs do not react with intact NCs. We suggest that these antigenic determinants reside inside the protein and/or at the interface of the multimer.

The consequences of P4-specific antibody binding for the NC integrity were analyzed by sedimentation analysis (Fig. 2) and by the RNA polymerase assay (Table 2). Two epitopes located between aa 112 and 142 and aa 290 and 301 were recognized by MAbs involved in disrupting the structural integrity of the NC (Fig. 2C). The binding of these MAbs (4S9, 4S10, 4S13, and 4R3) releases mainly proteins P8 and P4 from the NC and leads to the activation of the particle-associated RNA polymerase. On the basis of this finding, we suggest that there is a specific interaction between proteins P4 and P8 in the NC. A similar P4-P8 connection has been observed earlier by chemical crosslinking of the NC proteins (21).

The epitopes identified on protein P4 were compared with areas of antigenicity and surface probability predicted by computer algorithms (11). The hydrophilic domains containing the surface-accessible epitopes (Fig. 4) reside mostly in the C-terminal end of P4 but also in the region between aa 82 and 142. The predictions for both antigenicity (24) and surface probability (12) are in fairly good agreement with the results obtained in this study (data not shown). The two mainly hydrophobic domains of P4 (aa 1 to 73 and aa 153 to 247) were not recognized by our P4-specific MAbs, suggesting that they are surface inaccessible. This finding also correlates with the predicted antigenicity and surface probability.

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REFERENCES

1. **Annunziato, M. E., and D. J. Marciani.** 1987. Efficient purification of mouse monoclonal antibodies from ascites fluid by medium-performance anion exchange chromatography. Gene Anal. Tech. **4**:1–4.
2. **Bamford, D. H.** 1981. Lipid-containing bacterial viruses: disruption studies on φ6, p. 477–489. *In* M. S. DuBow (ed.), Bacteriophage assembly. Alan R. Liss, Inc., New York.
3. **Bamford, D. H., and E. T. Palva.** 1980. Structure of the lipid-containing bacteriophage φ6: disruption by Triton X-100 treatment. Biochim. Biophys. Acta **601**:245–259.
4. **Borsa, J., M. D. Sargent, P. A. Lievaart, and T. P. Copps.** 1981. Reovirus: evidence for a second step in the intracellular uncoating and transcriptase activation process. Virology **111**:191–200.
5. **Bruenn, J. A.** 1991. Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. Nucleic Acids Res. **19**:217–226.
6. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47**:45–148.
7. **Cohen, J.** 1977. Ribonucleic acid polymerase activity associated with purified calf rotavirus. J. Gen. Virol. **36**:395–402.
8. **Cohen, J., J. Laporte, A. Charpilienne, and R. Scherrer.** 1979. Activation of rotavirus RNA polymerase by calcium chelation. Arch. Virol. **60**:177–186.
9. **Cuppels, D. A., J. L. Van Etten, D. E. Burbank, L. C. Lane, and A. K. Vidaver.** 1980. In vitro translation of the three bacteriophage φ6 RNAs. J. Virol. **35**:249–251.
10. **Day, L. A., and L. Mindich.** 1980. The molecular weight of bacteriophage φ6 and its nucleocapsid. Virology **103**:376–385.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
12. **Emini, E. A., J. V. Hughes, D. S. Perlow, and J. Boger.** 1985. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J. Virol. **55**:836–839.
13. **Emori, Y., H. Iba, and Y. Okada.** 1980. Semi-conservative transcription of double-stranded RNA catalyzed by bacteriophage φ6 RNA polymerase. J. Biochem. **88**:1569–1575.
14. **Frilander, M., P. Gottlieb, J. Strassman, D. H. Bamford, and L. Mindich.** 1992. Dependence of minus-strand synthesis on complete genomic packaging in the double-stranded RNA bacteriophage φ6. J. Virol. **66**:5013–5017.
15. **Gefter, M. L., D. H. Margulies, and M. D. Scharff.** 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genet. **3**:231–236.
16. **Gottlieb, P., J. Strassman, D. H. Bamford, and L. Mindich.** 1988. Production of a polyhedral particle in *Escherichia coli* from a cDNA copy of the large genomic segment of bacteriophage φ6. J. Virol. **62**:181–187.
17. **Gottlieb, P., J. Strassman, A. Frucht, X. Qiao, and L. Mindich.** 1991. In vitro packaging of the bacteriophage φ6 ssRNA genomic precursors. Virology **181**:589–594.
18. **Gottlieb, P., J. Strassman, and L. Mindich.** 1992. Protein P4 of the bacteriophage φ6 procapsid has a nucleoside triphosphate-binding site with associated nucleoside triphosphate phosphohydrolase activity. J. Virol. **66**:6220–6222.
19. **Gottlieb, P., J. Strassman, X. Qiao, M. Frilander, A. Frucht, and L. Mindich.** 1992. In vitro packaging and replication of individual genomic segments of bacteriophage φ6 RNA. J. Virol. **66**:2611–2616.
20. **Gottlieb, P., J. Strassman, X. Qiao, A. Frucht, and L. Mindich.** 1990. In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage φ6: studies with procapsids assembled from plasmid-encoded proteins. J. Bacteriol. **172**:5774–5782.
21. **Hantula, J., and D. H. Bamford.** 1988. Chemical crosslinking of bacteriophage φ6 nucleocapsid proteins. Virology **165**:482–488.
22. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

23. **Huismans, H., A. A. Van Dijk, and H. J. Els.** 1987. Uncoating of parental bluetongue virus to core and subcore particles in infected L cells. Virology **157:**180–188.
24. **Jameson, B. A., and H. Wolf.** 1988. The antigenic index: a novel algorithm for predicting antigenic determinants. Comput. Appl. Biosci. **4:**181–186.
25. **Kenney, J. M., J. Hantula, S. D. Fuller, L. Mindich, P. M. Ojala, and D. H. Bamford.** 1992. Bacteriophage ϕ6 envelope elucidated by chemical cross-linking, immunodetection, and cryoelectron microscopy. Virology **190:**635–644.
26. **Koonin, E. V., A. E. Gorbalenya, and K. M. Chumakov.** 1989. Tentative identification of RNA-dependent RNA polymerases of dsRNA viruses and their relationship to positive strand RNA viral polymerases. FEBS Lett. **252:**42–46.
27. **Ktistakis, N. T., and D. Lang.** 1987. The dodecahedral framework of the bacteriophage ϕ6 nucleocapsid is composed of protein P1. J. Virol. **61:**2621–2623.
28. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
29. **Köhler, G., S. C. Howe, and C. Milstein.** 1976. Fusion between immunoglobulin-secreting and non-secreting myeloma cell lines. Eur. J. Immunol. **6:**292–295.
30. **Ludert, J. E., F. Michelangeli, F. Gil, F. Liprandi, and J. Esparza.** 1987. Penetration and uncoating of rotaviruses in cultured cells. Intervirology **27:**95–101.
31. **Mindich, L.** 1988. Bacteriophage ϕ6: a unique virus having a lipid-containing membrane and a genome composed of three dsRNA segments. Adv. Virus Res. **35:**137–176.
32. **Mindich, L., and D. H. Bamford.** 1988. Lipid-containing bacteriophages, p. 475–520. *In* R. Calendar (ed.), The bacteriophages, vol. 2. Plenum Publishing Corp., New York.
33. **Mindich, L., G. MacKenzie, J. Strassman, T. MacGraw, S. Metzger, M. Romantschuk, and D. Bamford.** 1985. cDNA cloning of portions of the bacteriophage ϕ6 genome. J. Bacteriol. **162:**992–999.
34. **Mindich, L., I. Nemhauser, P. Gottlieb, M. Romantschuk, J. Carton, S. Frucht, J. Strassman, D. H. Bamford, and N. Kalkkinen.** 1988. Nucleotide sequence of the large double-stranded RNA segment of bacteriophage ϕ6: genes specifying the viral replicase and transcriptase. J. Virol. **62:**1180–1185.
35. **Mindich, L., J. F. Sinclair, and J. Cohen.** 1976. The morphogenesis of bacteriophage ϕ6: particles formed by nonsense mutants. Virology **75:**224–231.
36. **Ojala, P. M., M. Romantschuk, and D. H. Bamford.** 1990. Purified ϕ6 nucleocapsids are capable of productive infection of host cells with partially disrupted outer membranes. Virology **178:**364–372.
37. **Olkkonen, V. M., and D. H. Bamford.** 1987. The nucleocapsid of the lipid-containing double-stranded RNA bacteriophage ϕ6 contains a protein skeleton consisting of a single polypeptide species. J. Virol. **61:**2362–2367.
38. **Olkkonen, V. M., and D. H. Bamford.** 1989. Quantitation of the adsorption and penetration stages of bacteriophage ϕ6 infection. Virology **171:**229–238.
39. **Olkkonen, V. M., P. Gottlieb, J. Strassman, X. Qiao, D. H. Bamford, and L. Mindich.** 1990. In vitro assembly of infectious nucleocapsids of bacteriophage ϕ6: formation of a recombinant double-stranded RNA virus. Proc. Natl. Acad. Sci. USA **87:**9173–9177.
40. **Olkkonen, V. M., P. M. Ojala, and D. H. Bamford.** 1991. Generation of infectious nucleocapsids by in vitro assembly of the shell protein on to the polymerase complex of the dsRNA bacteriophage ϕ6. J. Mol. Biol. **218:**569–581.
41. **Olkkonen, V. M., P. M. Pekkala, and D. H. Bamford.** 1988. Monoclonal antibodies to the major structural proteins of bacteriophage ϕ6. Virology **165:**317–320.
42. **Romantschuk, M., V. M. Olkkonen, and D. H. Bamford.** 1988. The nucleocapsid of bacteriophage ϕ6 penetrates the host cytoplasmic membrane. EMBO J. **7:**1821–1829.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Sargent, M. D., D. G. Long, and J. Borsa.** 1977. Functional analysis of the interactions between reovirus particles and various proteases in vitro. Virology **78:**354–358.
45. **Semancik, J. S., A. K. Vidaver, and J. L. Van Etten.** 1973. Characterization of a segmented double-helical RNA from bacteriophage ϕ6. J. Mol. Biol. **78:**617–625.
46. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
47. **Sturzenbecker, L. J., M. Nibert, D. Furlong, and B. N. Fields.** 1987. Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle. J. Virol. **61:**2351–2361.
48. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
49. **Usala, S. J., B. H. Brownstein, and R. Haselkorn.** 1980. Displacement of parental RNA strands during in vitro transcription by bacteriophage ϕ6 nucleocapsids. Cell **19:**855–862.
50. **Van Etten, J. L., D. E. Burbank, D. A. Cuppels, L. C. Lane, and A. K. Vidaver.** 1980. Semiconservative synthesis of single-stranded RNA by bacteriophage ϕ6 RNA polymerase. J. Virol. **33:**769–773.
51. **Van Etten, J., L. Lane, C. Gonzalez, J. Partridge, and A. Vidaver.** 1976. Comparative properties of bacteriophage ϕ6 and ϕ6 nucleocapsid. J. Virol. **18:**652–658.
52. **Van Etten, J. L., A. K. Vidaver, R. K. Koski, and J. P. Burnett.** 1974. Base composition and hybridization studies of the three double-stranded RNA segments of bacteriophage ϕ6. J. Virol. **13:**1254–1262.
53. **Vidaver, A. K., R. K. Koski, and J. L. Van Etten.** 1973. Bacteriophage ϕ6: a lipid-containing virus of *Pseudomonas phaseolicola*. J. Virol. **11:**799–805.
54. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.