Purification of Recombinant Budgerigar Fledgling Disease Virus VP1 Capsid Protein and Its Ability for In Vitro Capsid Assembly†

REBECCA E. D. RODGERS,¹ DECHING CHANG,² XIAOYIN CAI,¹ AND RICHARD A. CONSIGLI^{1*}

*Division of Biology, Section of Virology and Oncology, Kansas State University, Manhattan, Kansas 66506,¹ and
Department of Microbiology, Chung Shan Medical and Dental College, Taichung, Taiwan, Republic of China²*

Received 20 December 1993/Accepted 10 February 1994

A recombinant system for the major capsid VP1 protein of budgerigar fledgling disease virus has been established. The VP1 gene was inserted into a truncated form of the pFlag-1 vector and expressed in *Escherichia coli*. The budgerigar fledgling disease virus VP1 protein was purified to near homogeneity by immunoaffinity chromatography. Fractions containing highly purified VP1 were pooled and found to constitute 3.3% of the original *E. coli*-expressed VP1 protein. Electron microscopy revealed that the VP1 protein was isolated as pentameric capsomeres. Electron microscopy also revealed that capsid-like particles were formed in vitro from purified VP1 capsomeres with the addition of Ca^{2+} ions and the removal of chelating and reducing agents.

Budgerigar fledgling disease virus (BFDV), the first avian member of the polyomavirus family, was found to cause an acute disease in fledgling budgerigars (3, 5, 23, 24). BFDV is classified as a polyomavirus on the basis of its morphology, its resistance to organic solvents, and the facts that its virions have a buoyant density of 1.34 g/ml and its DNA is supercoiled and double stranded with a molecular weight of 330,000 (5, 16, 28). BFDV also has the capability to transform nonpermissive primary hamster embryo fibroblasts, an ability common to polyomaviruses (28).

Many similarities were found when the genome of BFDV, composed of 4,980 bp (42), was compared with the genomes of both murine polyomavirus and simian virus 40 (SV40) (38). The genomes of these three viruses can be divided into early and late coding regions. The early region of BFDV codes for large and small T antigens similar to those of SV40 and murine polyomavirus. Murine polyomavirus also codes for a middle T antigen, while BFDV and SV40 do not (38, 47). The late region in all three viruses codes for the three structural proteins VP1, VP2, and VP3. In BFDV the molecular masses of these structural proteins are 42, 39, and 29 kDa, respectively (22). Previous studies have shown that the major capsid protein VP1, in both murine polyomavirus and SV40, can be differentially modified during translation in several ways (31, 32), including phosphorylation (1, 2, 4, 18, 19, 29, 39). Recently it was reported that the VP1 protein of BFDV can also be posttranslationally modified and that the VP1 protein consists of five distinct subspecies, with two being phosphorylated (22). Schmidt et al. (45) demonstrated that the minor capsid protein VP2 was modified by myristoylation.

A recombinant system has been established for the major capsid protein VP1 of murine polyomavirus (27). This recombinant system has generated large quantities of the VP1 protein, which have been used in numerous studies (10, 11, 21, 36, 37, 43, 44). The current study used a similar strategy for the

construction of a BFDV VP1 recombinant system. The recombinant VP1 protein produced was purified by immunoaffinity chromatography, isolated as pentameric capsomeres, and further analyzed for the ability to self-assemble into capsid-like particles.

Primary cultures of chicken embryo cells, from 9- to 11-day-old embryonated hen eggs, were infected with BFDV as described previously (34). Infected cultures were maintained in serum-free Dulbecco's modified Eagle's medium at 39°C. BFDV virions were purified by CsCl isopycnic equilibrium centrifugation (22). Polyclonal antibodies to BFDV were prepared in New Zealand White rabbits by injection of CsCl-purified BFDV virions as previously described for the production of murine polyomavirus polyclonal antibodies (33). Monoclonal antibodies to BFDV-VP1 (3G10G5) were produced as previously described by Fattaey et al. (17).

Recombinant VP1 proteins were constructed and expressed in the prokaryotic expression vector Δ pFlag. Δ pFlag was created by digestion of the prokaryotic expression vector pFlag-1 (IBI, New Haven, Conn.) with restriction enzymes *Nde*I and *Sal*I to delete 114 bp from nucleotides 101 to 214, as described by Chang et al. (10). This was done to allow expression of an authentic VP1 protein.

The 4.9-kb BFDV genome was digested with *Eco*RI and cloned into the *Eco*RI site of pGEM-7 to create pGBFDV. This construct was amplified in *Escherichia coli* JM109 and was used as the template for amplifying the VP1 gene by PCR. For amplification of VP1, the sense primer 5'-CACTTGAGATC TATGTCCCAAAAAGGA-3' and the antisense primer 3'-GGTTTCGAGGGGCGATTTCTAGATGTACA-5' were used. The primers were constructed with *Bgl*II sites immediately 5' to the start or stop codon to facilitate subsequent manipulations. The amplified DNA fragments were digested with *Bgl*II, purified by agarose gel electrophoresis and Magic PCR Preps columns (Promega, Madison, Wis.), and cloned into *Bgl*II-digested prokaryotic expression vector Δ pFlag. The constructs were then used to transform *E. coli* RK1448 cells. BFDV VP1 was expressed from the plasmid Δ pFlag grown in *E. coli* RK1448 for 7 h at 37°C. Maximum protein expression was induced at early logarithmic phase by the addition of 1.7 mM (final concentration) isopropyl- β -D-thiogalactopyranoside.

* Corresponding author. Mailing address: Division of Biology-Ackert Hall, Section of Virology and Oncology, Kansas State University, Manhattan, KS 66506. Phone: (913) 532-6625. Fax: (913) 532-6653.

† Contribution 94-296-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan.

FIG. 1. Various stages in the purification process of recombinant BFDV VP1 protein. *E. coli* RK1448 cells, expressing BFDV VP1 proteins, were pelleted by centrifugation at $4,000 \times g$ for 10 min. The pellet was resuspended in H_2O (pH 8.0), and lysozyme (Calbiochem Corporation, La Jolla, Calif.) was added to a final concentration of 1 mg/ml. After 20 min of incubation at $4^\circ C$, 0.06% sodium deoxycholate (Sigma, St. Louis, Mo.) was added. This was followed by the addition of protease inhibitors (aprotinin, *N*-tosyl-L-lysine chloromethyl ketone, *N*-tosyl-L-phenylalanine chloromethyl ketone, and phenylmethylsulfonyl fluoride; final concentration of each, 10 $\mu g/ml$), after which the cells were sonicated (five to six 30-s bursts). The total lysate was centrifuged at $16,500 \times g$ for 20 min. The resulting pellet was resuspended in H_2O (pH 8.0). The supernatant, which contained the soluble form of the VP1 protein, was loaded onto the immunoaffinity column. VP1 protein was eluted with various concentrations of $MgCl_2$ (see Fig. 2). Purified VP1 eluted with 2 M $MgCl_2$ was pooled. Aliquots were taken at various stages during purification, analyzed by SDS-12.5% PAGE, and stained with Coomassie blue. Lanes: 1, molecular weight markers (in thousands); 2, total cell lysate; 3, resuspended cell pellet; 4, supernatant; 5, pooled purified VP1 protein eluted with 2 M $MgCl_2$ from the immunoaffinity column. Arrows denote VP1 protein.

The *E. coli* RK1448 cells expressing the BFDV VP1 protein were harvested and lysed to obtain a total cell lysate. The recombinant cell lysate was immunoprecipitated with a polyclonal BFDV antibody and analyzed by sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) to verify that the VP1 protein was being expressed by the *E. coli* RK1448 cells (data not shown).

Experiments were performed to determine the optimum environment to solubilize the recombinant VP1 protein. Buffers with various NaCl concentrations (250 mM to 1 M) were used, and it was found that the solubility of the VP1 protein increased as the NaCl concentration decreased (data not shown). RIPA buffer (20 mM morpholinopropane sulfonic acid [pH 7.0], 150 mM NaCl, 1% [wt/vol] deoxycholate, 1% [vol/vol] Nonidet P-40, 0.1% SDS, 2 mM EDTA), Tris-buffered saline, and H_2O (pH 8.0) were also compared for their ability to solubilize the recombinant VP1 protein. It was found that RIPA buffer and H_2O (pH 8.0) gave optimum solubilization (data not shown). Since RIPA buffer contains several detergents which could be detrimental to the protein, it was decided that solubilization with water would be the most beneficial in studies to isolate BFDV VP1.

Figure 1 shows the various steps in the purification of recombinant BFDV VP1 protein. The *E. coli* RK1448 paste expressing the BFDV VP1 protein was resuspended in H_2O (pH 8.0) and sonicated to produce a total cell lysate (Fig. 1,

lane 2). The sonicate was centrifuged to pellet large *E. coli* debris (Fig. 1, lane 3), and the supernatant was found to contain the soluble VP1 protein (lane 4). Aliquots of the above-mentioned supernatant were analyzed by SDS-PAGE; arrows in Fig. 1 denote the position of VP1. The supernatant, containing VP1 protein, was then loaded onto the immunoaffinity column.

Various concentrations of $MgCl_2$ in 10 mM phosphate buffer were used to elute the VP1 protein from the immunoaffinity column. Figure 2A shows the proteins eluted from the immunoaffinity column with 0.5 M $MgCl_2$. The majority of the proteins eluted were *E. coli* proteins. Some VP1 protein can be detected migrating as a minor band at 42 kDa in lanes 4 through 9 of Fig. 2A. The proteins that were eluted with 2 M $MgCl_2$ can be seen in Fig. 2B. A single, major band of VP1 protein can be observed migrating at 42 kDa in Fig. 2B, lanes 2 through 7. The contaminating *E. coli* proteins present in the gel shown in Fig. 2A are noticeably absent in the 2 M $MgCl_2$ fractions (Fig. 2B). The 36-kDa band observed beneath the 42-kDa band (Fig. 2B) is likely a breakdown product of the VP1 protein. Western blot (immunoblot) analysis of the 2 M $MgCl_2$ fractions (the same fractions as those shown in Fig. 2B) was able to recognize both the VP1 protein migrating at 42 kDa and the protein migrating at 36 kDa (Fig. 2C).

Table 1 summarizes the isolation of the VP1 protein through the various stages of the purification process. The percentage of VP1 protein, found throughout the purification procedure, was determined by densitometry. The supernatant fraction was found to contain a total of 4.8 mg of VP1 protein, which constitutes 80% of the VP1 found in the *E. coli* lysate. When the total amount of VP1 in the supernatant (4.8 mg) was compared with the total amount of VP1 purified (0.2 mg), a significant decrease in the quantity of VP1 was observed. This decrease in VP1 protein was attributed to the fact that when the soluble VP1 was eluted from the immunoaffinity chromatography column with 0.5 M $MgCl_2$, some of the VP1 eluted with contaminating *E. coli* proteins (Fig. 2A). Therefore, these fractions could not be used for further studies. However, when the VP1 protein was eluted with 2 M $MgCl_2$, it was found to be in a highly purified form (Fig. 2B). The VP1 proteins which eluted with 2 M $MgCl_2$ were pooled and analyzed by SDS-PAGE (Fig. 1, lane 5). This pooled fraction of VP1 was found to constitute 3.3% of the original *E. coli*-expressed VP1 protein.

This highly purified preparation of VP1 was dialyzed to remove the $MgCl_2$ and then analyzed by electron microscopy, which revealed that the VP1 protein was isolated as pentameric capsomeres (Fig. 3A). Capsomeres were measured, and the average diameter was found to be 7.5 nm. This value was similar to the average diameter (7.4 nm) observed in native capsomeres from BFDV virion preparations. Similarly, when the recombinant VP1 protein from murine polyomavirus was isolated, it was also found to exist as pentameric capsomeres (43, 44). This ability to isolate capsomere-like structures from purified VP1 protein was originally observed by Brady and Consigli (6) when murine polyoma virion proteins were solubilized by guanidine-HCl and separated by Sepharose chromatography.

The BFDV capsomeres were further investigated to determine their ability to form capsid-like particles. The capsomeres were dialyzed to remove the chelating and reducing agents and to introduce $CaCl_2$ into the reaction mixture. The preparations were then analyzed by electron microscopy to determine whether capsid-like particle assembly occurred. It was found that capsid-like particles were assembled in the presence of 0.5 mM $CaCl_2$. Diameter measurements of these capsid-like par-

FIG. 2. Purification of BFDV VP1 protein by immunoaffinity chromatography. The immunoaffinity column was prepared from an anti-BFDV VP1 monoclonal antibody (3G10G5), which was purified from mouse ascites fluid by the protocol described for the Affi-Gel Protein A MAPS II kit (Bio-Rad Laboratories, Hercules, Calif.), and coupled to a CNBr-activated Sepharose 4B column as described by the manufacturer of the CNBr-activated Sepharose 4B kit (Pharmacia, Piscataway, N.J.). The supernatant, containing the recombinant BFDV VP1 protein, was loaded onto the column, and the VP1 proteins were eluted by stepwise addition of $MgCl_2$ (0.5, 2, and 3.5 M) in 10 mM phosphate buffer (pH 7.2). Fractions (1 ml) were collected in tubes containing 5% glycerol (to help stabilize the protein) and assayed for protein content at an absorbance of 280 nm. Fractions containing protein were dialyzed against a solution containing 10 mM Tris (pH 7.2), 1 mM EDTA, 150 mM NaCl, 15 mM β -mercaptoethanol, and 5% glycerol to remove the $MgCl_2$ and also to prevent aggregation, as described by Salunke et al. (44). (A) Proteins eluted with 0.5 M $MgCl_2$ were analyzed by SDS-12% PAGE and stained with Coomassie blue. Proteins eluted with 2 M $MgCl_2$ were also analyzed by SDS-12% PAGE and either stained with Coomassie blue (B) or subjected to Western blot analysis with an anti-BFDV polyclonal antibody and ^{125}I -labeled *Staphylococcus aureus* protein A. Radioactive bands were visualized by autoradiography (C). Numbers on the left show molecular weight (in thousands).

TABLE 1. Summary of purification (from 1.6 g of cells)

Purification stage	Vol (ml)	Total ^a protein (mg)	Total ^a VP1 protein (mg)	% VP1 by densitometry ^b	Yield (%)
Lysate	15.0	150.0	6.0	4.0	100.0
Pellet	14.0	32.2	0.8	2.4	13.0
Supernatant	14.0	110.0	4.8	4.4	81.0
Purified VP1 (pooled)	3.6	0.2	0.2	83.0	3.3

^a Determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

^b Proteins that were separated by SDS-PAGE and stained with Coomassie blue were scanned with a Kontes Fiber Optic Scanner (model 800). The relative amount of protein in the various bands was determined by calculating the area under each peak. The percentage of VP1 protein was found by comparing its area with the total area of all proteins within a given sample.

ticles were found to range from 52 to 58 nm (Fig. 3B). When these capsid-like particles were compared with native BFDV virions (diameter, 42 to 56 nm), they were found to be quite similar in size and shape (Fig. 3C). Other laboratories have also reported variations in the diameters of purified BFDV virions, ranging from 42 to 55 nm (3, 14, 15, 40).

The importance of calcium in maintaining the structural integrity of the murine polyomavirus was originally demonstrated by Brady et al. (8, 9). They were able to dissociate virions into capsomere subunits and a 48S DNA-protein complex by chelating virion-associated Ca^{2+} with EGTA [ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid] in the presence of a reducing agent. They later reported that the removal of the chelating and reducing agents with the reintroduction of Ca^{2+} ions into the virion dissociation mixture of capsomeres and DNA-protein complexes allowed the reassembly of the dissociation products into virion-like particles (7, 48). Preliminary studies of the dissociation of BFDV virions have also demonstrated that Ca^{2+} ions are an integral part of the virion structure and are responsible for maintaining the integrity of the native BFDV virions (unpublished data). In SV40, the chelation of Ca^{2+} ions with sodium carbonate resulted in the dissociation of virions also (12). Recombinant murine polyomavirus VP1 proteins located in the cytoplasm of Sf9 cells were observed to form capsid-like particles with the addition of the calcium ionophore ionomycin to the cytoplasm of these cells (35). In addition, recombinant papillomavirus L1 protein was also reported to self-assemble into capsid-like particles in baculovirus (25, 41) and vaccinia virus (20) expression systems. Salunke et al. (43, 44) reported the role of Ca^{2+} in the in vitro assembly of murine polyomavirus recombinant VP1 capsomeres into capsid-like particles, and now we have reported a similar mechanism of Ca^{2+} dependency for the avian polyomavirus. Involvement of Ca^{2+} ions in maintaining the integrity of virions as well as in capsid assembly appears to be a universal feature for the papovaviruses.

Studies with the murine polyomavirus system have localized the Ca^{2+} -binding domain to a 16-kDa fragment of VP1 which was generated by formic acid cleavage of VP1. This fragment, which extended from Pro-232 to Asp-364, was shown to have in vitro Ca^{2+} -binding capabilities (30). This 16-kDa fragment was also found to have a sequence of 12 amino acids (Asp-266 to Glu-277) that was quite similar to the 12-amino-acid loops which make up part of the EF hand structures found in many calcium-binding proteins, including parvalbumin, calmodulin, thrombospondin, and troponin C (13, 26, 46). To study this Ca^{2+} -binding domain, Haynes et al. (21) utilized single-site point mutations within this domain, as well as a deletion

FIG. 3. Electron microscopy of purified BFDV capsomeres and capsid-like particles. (A) Electron microscopy of purified recombinant BFDV VP1 capsomeres. (B) Electron microscopy of capsid-like particles assembled in vitro from purified recombinant BFDV VP1 capsomeres. CaCl_2 was added to a sample of pooled BFDV VP1 protein, eluted from the immunoaffinity column by 2 M MgCl_2 , to give a final concentration of 0.5 mM, and the mixture was allowed to incubate for 10 min. Dimethyl sulfoxide (DMSO) (Fisher Chemical, Fair Lawn, N.J.) was added to the sample to a final concentration of 20%, and the mixture was incubated for 30 min (7). The samples were then dialyzed in reassembly buffer containing 10 mM Tris (pH 7.2), 150 mM NaCl, 0.5 mM CaCl_2 , 5% glycerol, and 5% DMSO. (C) Purified native BFDV virions. Electron microscopy samples were prepared as described previously (21). Bar (shown in panel B but applies to all panels), 100 nm.

mutation which eliminated the putative Ca^{2+} -binding domain from the VP1 protein. Both mutations resulted in the inability to form infective viral progeny in cells transfected with the murine polyomavirus DNA coding for the respective mutations. On purification, recombinant VP1 proteins with the point mutations were found to exist as pentameric capsomeres but were unable to form capsid-like particles in the presence of calcium (21).

It is interesting to note that a comparison of the amino acid sequences of the murine polyomavirus VP1 and the BFDV VP1 (42) reveals a region in the BFDV VP1 protein (237-DENGVGPLCKGD-248) that is virtually identical to the Ca^{2+} -binding domain (266-DENGVGPLCKGE-277) in the murine polyomavirus VP1 protein. It seems probable that this analogous amino acid sequence is the Ca^{2+} -binding domain in the BFDV VP1 protein. Confirmation that this conserved domain in the BFDV VP1 is actually the Ca^{2+} -binding domain is currently being sought in our laboratory.

This investigation was supported by Public Health Service grant CA07319 from the National Cancer Institute, by NASA NAGW no. 1197 and 2328, and by the Kansas Health Foundation of Wichita.

We thank Avelina Paulsen for assistance with electron microscopy and Kevin Mapes, Scott Smiley, and LaDonna Grenz for technical assistance.

REFERENCES

- Anders, D. G., and R. A. Consigli. 1983. Chemical cleavage of polyomavirus major capsid structural protein VP1: identification of cleavage products and evidence that the receptor moiety resides in the carboxy-terminal region. *J. Virol.* 48:197-205.
- Anders, D. G., and R. A. Consigli. 1983. Comparison of non-phosphorylated and phosphorylated species of polyomavirus major capsid protein VP1 and identification of the major phosphorylation region. *J. Virol.* 48:206-217.
- Bernier, G., M. Morin, and G. Marsolais. 1981. A generalized inclusion body disease in the budgerigar (*Melopsittacus undulatus*) caused by a papovavirus-like agent. *Avian Dis.* 25:1083-1092.
- Bolen, J. B., D. G. Anders, J. Trempey, and R. A. Consigli. 1981. Differences in the subpopulations of the structural proteins of polyoma virions and capsids: biological function of the multiple VP1 species. *J. Virol.* 37:80-91.
- Bozeman, L. H., R. B. Davis, D. Gaudry, P. D. Lukert, O. J. Fletcher, and M. J. Dykstra. 1981. Characterization of a papovavirus isolated from fledgling budgerigars. *Avian Dis.* 25:972-980.
- Brady, J. N., and R. A. Consigli. 1978. Chromatographic separation of the polyoma virus proteins and renaturation of the isolated VP1 major capsid protein. *J. Virol.* 27:436-442.
- Brady, J. N., J. D. Kendall, and R. A. Consigli. 1979. *In vitro* reassembly of infectious polyoma virions. *J. Virol.* 32:640-647.
- Brady, J. N., V. D. Winston, and R. A. Consigli. 1977. Dissociation of polyoma virus by the chelation of calcium ions found associated with purified virions. *J. Virol.* 23:717-724.
- Brady, J. N., V. D. Winston, and R. A. Consigli. 1978. Characterization of a DNA-protein complex and capsomere subunits derived from polyoma virus by treatment with ethyleneglycol-bis- N,N' -tetraacetic acid and dithiothreitol. *J. Virol.* 27:193-204.
- Chang, D., X. Cai, and R. A. Consigli. 1993. Characterization of the DNA binding properties of polyomavirus capsid proteins. *J. Virol.* 67:6327-6331.
- Chang, D., J. I. Haynes, J. N. Brady, and R. A. Consigli. 1992. The use of additive and subtractive approaches to examine the nuclear localization sequence of the polyomavirus major capsid protein VP1. *Virology* 189:821-827.
- Christiansen, G., T. Landers, J. Griffith, and P. Berg. 1977. Characterization of components released by alkali disruption of simian virus 40. *J. Virol.* 21:1079-1084.
- da Silva, A. C. R., and F. C. Reinach. 1991. Calcium binding induces conformational changes in muscle regulatory proteins. *Trends Biochem. Sci.* 16:53-58.
- Davis, R. B., L. H. Bozeman, D. Gaudry, O. J. Fletcher, P. D. Lukert, and M. J. Dykstra. 1981. A viral disease of fledgling budgerigars. *Avian Dis.* 25:179-183.
- Dykstra, M. J., and L. H. Bozeman. 1982. A light and electron microscopic examination of budgerigar fledgling disease virus in tissue and in cell culture. *Avian Pathol.* 11:11-28.
- Dykstra, M. J., C. C. Dykstra, P. D. Lukert, and L. H. Bozeman. 1984. Investigations of budgerigar fledgling disease virus. *Am. J. Vet. Res.* 45:1883-1887.
- Fattaey, A., L. Lenz, and R. A. Consigli. 1992. Production and characterization of monoclonal antibodies to budgerigar fledgling disease virus major capsid protein VP1. *Avian Dis.* 36:543-553.
- Fattaey, A. R., and R. A. Consigli. 1989. Synthesis, posttranslational modifications, and nuclear transport of polyomavirus major capsid protein VP1. *J. Virol.* 63:3168-3175.
- Garcea, R. L., K. Ballmer-Hofer, and T. L. Benjamin. 1985. Virion assembly defect of polyomavirus *hr-t* mutants: underphosphorylation of major capsid protein VP1 before viral DNA encapsidation. *J. Virol.* 54:311-316.
- Hagensee, M. E., N. Yaegashi, and D. A. Galloway. 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J. Virol.* 67:315-322.
- Haynes, J. I., II, D. Chang, and R. A. Consigli. 1993. Mutations in the putative calcium-binding domain of polyomavirus VP1 affect capsid assembly. *J. Virol.* 67:2486-2495.
- Haynes, J. I., II, and R. A. Consigli. 1992. Phosphorylation of the budgerigar fledgling disease virus major capsid protein VP1. *J. Virol.* 66:4551-4555.
- Hunter, B., A. Gagnon, D. Onderka, J. Goltz, and B. Holmes. 1979. Viral hepatitis in southern Ontario. *Can. Vet. J.* 20:176-179.
- Kaletka, E. F., W. Herbst, F.-J. Kaup, R. Jank-Ludwig, H.-J. Marshall, W. Drommer, and M. Krautwald. 1984. Untersuchungen zur Virusätiologie einer mit Hepatitis und Befiederungsstörungen einhergehenden Krankheit bei Wellensittichen (*Melopsittacus undulatus*). *Zentralbl. Veterinärmed. Reihe B* 31:219-224.
- Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Dürst, L. Gissmann, D. R. Lowy, and J. T. Schiller. 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J. Virol.* 67:6929-6936.
- Lawler, J., and R. O. Hynes. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* 103:1635-1638.
- Leavitt, A. D., T. M. Roberts, and R. L. Garcea. 1985. Polyoma

- virus major capsid protein VP1: purification after high level expression in *Escherichia coli*. J. Biol. Chem. **260**:12803–12809.
28. **Lehn, H., and H. Müller.** 1986. Cloning and characterization of budgerigar fledgling disease virus, an avian polyomavirus. Virology **151**:362–370.
 29. **Ludlow, J. W., and R. A. Consigli.** 1987. Differences in biological activity and structural protein VP1 phosphorylation of polyomavirus progeny resulting from infection of primary mouse kidney and primary mouse embryo cell cultures. J. Virol. **61**:509–517.
 30. **Ludlow, J. W., and R. A. Consigli.** 1987. Localization of calcium on the polyomavirus VP1 capsid protein. J. Virol. **61**:2934–2937.
 31. **Ludlow, J. W., and R. A. Consigli.** 1987. Polyomavirus major capsid protein VP1 is modified by tyrosine sulfuration. J. Virol. **61**:1708–1711.
 32. **Ludlow, J. W., and R. A. Consigli.** 1989. Hydroxyproline in the major capsid protein VP1 of polyomavirus. J. Virol. **63**:2881–2884.
 33. **McMillen, J., and R. A. Consigli.** 1977. Immunological reactivity of antisera to sodium dodecyl sulfate-derived polypeptides of polyoma virions. J. Virol. **21**:1113–1120.
 34. **Minocha, H. C., R. A. Consigli, and A. Eisenstark.** 1968. Concentration and purification of Newcastle disease virus. Am. J. Vet. Res. **29**:877–882.
 35. **Montross, L., S. Watkins, R. B. Moreland, H. Mamon, D. L. D. Caspar, and R. L. Garcea.** 1991. Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1. J. Virol. **65**:4991–4998.
 36. **Moreland, R. B., and R. L. Garcea.** 1991. Characterization of a nuclear localization sequence in the polyomavirus capsid protein VP1. Virology **185**:513–518.
 37. **Moreland, R. B., L. Montross, and R. L. Garcea.** 1991. Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1. J. Virol. **65**:1168–1176.
 38. **Pipas, J. M.** 1992. Common and unique features of T antigens encoded by the polyomavirus group. J. Virol. **66**:3979–3985.
 39. **Ponder, B. A., A. K. Robbins, and L. V. Crawford.** 1977. Phosphorylation of polyoma and SV40 virus proteins. J. Gen. Virol. **37**:75–83.
 40. **Randall, C. J., S. Lees, and D. M. Inglis.** 1987. Papovavirus-like infection in budgerigars (*Melopsittacus undulatus*). Avian Pathol. **16**:623–633.
 41. **Rose, R. C., W. Bonnez, R. C. Reichman, and R. L. Garcea.** 1993. Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike particles. J. Virol. **67**:1936–1944.
 42. **Rott, O., M. Kroger, H. Müller, and G. Hobom.** 1988. The genome of budgerigar fledgling disease virus, an avian polyomavirus. Virology **165**:74–86.
 43. **Salunke, D. M., D. L. D. Caspar, and R. L. Garcea.** 1986. Self-assembly of purified polyomavirus capsid protein VP1. Cell **46**:895–904.
 44. **Salunke, D. M., D. L. D. Caspar, and R. L. Garcea.** 1989. Polymorphism in the assembly of polyomavirus capsid protein VP1. Biophys. J. **56**:887–900.
 45. **Schmidt, M., H. Müller, M. F. G. Schmidt, and R. Rott.** 1989. Myristoylation of budgerigar fledgling disease virus capsid protein VP2. J. Virol. **63**:429–431.
 46. **Strynadk, N. C. J., and M. N. G. James.** 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. Annu. Rev. Biochem. **58**:951–998.
 47. **Tooze, J. (ed.).** 1980. DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 48. **Yuen, L. K. C., and R. A. Consigli.** 1982. Improved infectivity of reassembled polyomavirus. J. Virol. **43**:337–341.