Biol. Chem., Vol. 380, pp. 397 – 401, March 1999

Short Communication

Site-Specific Fluorescence Labelling of Recombinant Polyomavirus-Like Particles

Uli Schmidt*, Janet Kenklies, Rainer Rudolph and Gerald Böhm

Institut für Biotechnologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 3, D-06120 Halle (Saale), Germany

* Corresponding author

For the development of gene therapy protocols based on polyomavirus-like particles, we describe a method for fluorescence labelling of virions in order to study virus-cell interactions preceding gene delivery. Site-specific fluorescence labelling of polyomavirus-like particles is achieved via a single cysteine residue and maleimide conjugates of fluorescence dyes (fluorescein, Texas Red). Polyomavirus-like particles can be assembled *in vitro* from recombinant capsomers produced in *E. coli*. Since the assembly process is independent of disulfide bond formation, all cysteine residues of the wild-type protein were replaced by serines, and a new unique cysteine residue was introduced for the attachment of the fluorescence marker.

Key words: Fluorescence marker / Gene therapy / *In vitro* assembly / Polyomavirus / VP1.

Viral vectors for gene transfer experiments are widely used and several gene delivery systems are now in clinical testing. However, none of these systems had notable clinical successes so far, in contrast to expectations and theoretical possibilities (Anderson, 1998). This may be partly attributed to insufficient *in vivo* analysis of vector delivery (Bartlett and Samulski, 1998). Currently, preclinical assays for gene delivery such as the polymerase chain reaction (PCR) and histological staining are used to assess gene transfer, but they provide little insight into the steps preceding gene expression. Recently, new technologies emerged, using fluorescently labelled viruses to study virus-cell interactions and the subsequent steps for gene expression (Leopold *et al*., 1998). These techniques have several advantages: they are safe and inexpensive compared to radioactivity and electron microscopy, other commonly used methods to investigate viral entry pathways, and experiments can be performed with living cells and animals (Bartlett and Samulski, 1998).

Fluorescence labelling techniques applied so far for viral shells used amine-coupling of cyanine dyes (Leopold *et al*., 1998). Since amino groups are widely distributed on protein surfaces, the labels are attached unspecifically to the virion surface. This may possibly interfere with functional or structural properties, e.g. receptor binding sites. Therefore, a site-specific labelling method was established in this work in order to circumvent any interference of the dye with functional analyses. Thiol groups of cysteine residues are far less frequent in proteins than amino groups, and there are several reagents available for thiol-specific modifications, e.g. iodoacetamides or maleimides.

In order to establish a model system for investigation of gene transfer by fluorescence techniques, polyomavirus-like particles consisting of polyomavirus protein 1 (VP1) were used, which is an already well-characterised system (Salunke *et al*., 1986; Eckhart, 1991; Schmidt *et al*., manuscript in preparation). Murine polyomavirus is a subspecies of the papovaviridae family which are non-enveloped, double-stranded DNA viruses with circular genomes of 5.3 kb in size. They can induce tumors in laboratory animals and transform cells in culture. The icosahedral polyomavirus shell (triangulation number T=7d) has a diameter of approximately 45 nm and consists mainly of a major coat protein, VP1 (360 copies per capsid), and the minor coat proteins VP2 and VP3 (Eckhart, 1991). The crystal structures of VP1 in the virus shell as well as a proteolytically truncated form of pentameric VP1 have been reported recently (Stehle *et al*., 1994; Stehle and Harrison, 1997). *In vitro* studies demonstrated that purified VP1 can form virus-like particles consisting of 72 pentamers (Salunke *et al*., 1986). This feature makes the protein attractive for *in vitro* packaging of DNA (Slilaty *et al*., 1982) and gene transfer experiments (Forstova *et al*., 1995). The capsomer VP1 can be produced in recombinant form in *Escherichia coli* cells. Recently, a novel purification procedure for the recombinant VP1 protein was developed using an auto-proteolytically removable affinity tag. Highly purified capsomers are obtained in a single step (Schmidt *et al*., manuscript in preparation).

The structure of the VP1 capsomer reveals a large hole with a diameter of approx. 16 Å in the centre of the pentamer, between the monomer subunits (Figure 1A). In the native virus this hole is partially occupied by a loop from the minor coat proteins VP2 or VP3 (Chen *et al*., 1998), but it is empty in virus-like particles consisting only of VP1. For fluorescence labelling residue 248 was chosen which is a threonine in the wild-type protein. This residue is located in a loop segment at the inner edge of the central hole of the pentamer (Figure 1A). This position is not close

Fig. 1 Position of Cysteine Residues in the Mutant VP1-CallS and Wild-type VP1.
(A) Structure of the VP1 pentamer (core), viewed from the top. The position of T248C is marked by a sphere; the shielded site is located within the central hole of the molecule and is inaccessible for any bulky molecule, but well accessible for the fluorescence dyes used in this work. (B) Positions of four of the six cysteines resolved in the 3.6 Å crystal structure (Stehle *et al*., 1994), and one cysteine from a neighbouring subunit within the pentamer, forming a possible disulfide bond; C11 and C15 are not resolved. Methods: Figures were generated by MolScript (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).

to the oligosaccharide receptor binding site, making an interference of the label with receptor binding unlikely. Furthermore, the mainly hydrophobic labels are shielded within the protein, thus preventing aggregation due to decreased solubility. However, this site is accessible for larger molecules such as peptides, and protein modelling studies suggested that there is sufficient space to label all subunits in a pentamer.

First, all six cysteine residues present in the wild-type protein were replaced by serines (mutant VP1-CallS) in order to avoid modification of these residues. Recently, we could demonstrate that in contrast to several other members of the papovavirus family (Stehle *et al*., 1996; Li *et al*., 1998) the *in vitro* assembly process of murine polyomavirus-like particles is independent of disulfide bond formation (Schmidt *et al*., manuscript in preparation), although intrapentamer disulfide bonds are observed in the crystal structures between residues C19 and C114′ of neighbouring monomers; these are, however, only partially oxidised (Figure 1B; Stehle *et al*., 1994). Replacement of all cysteines by serines had no effect on the stability or *in vitro* assembly properties of the protein (Schmidt *et al*., manuscript in preparation). In a second site-directed mutagenesis step, threonine 248 was replaced by cysteine, now allowing covalent attachment of the label (VP1-CallS-T248C).

In order to establish a well-characterised labelling system for future investigations of gene transfer protocols, fluorescein- and Texas Red-C_5-maleimides were tested for thiol-specific labelling at position 248 of VP1-CallS-T248C. Maleimides form stable thioether bonds with thiol groups by nucleophilic addition. Amino groups, on the other hand, should not react when they are protonated. Therefore, site-specificity is pH dependent. To ensure correct conditions, especially pH, labelling results of the mutants VP1-CallS (no cysteine) and VP1-CallS-T248C (five cysteines per pentamer) were compared. Reactions were carried out at pH 7.2 for both variants using similar protein and dye concentrations. Samples were analysed by SDS-PAGE which separates protein from excess dye. Labelled protein can be detected by UV light, both unlabelled and labelled protein can be visualised by conventional Coomassie staining. As shown in Figure 2A, the cysteine-free mutant VP1-CallS remains completely unlabelled, whereas mutant VP1-CallS-T248C shows a strong Texas Red fluorescence. This demonstrates that labelling of thiol groups via maleimides at the unique cysteine 248 in VP1-CallS-T248C is completely site-specific at these conditions.

For thiol-specific labelling it is also essential that all thiol groups are in a reduced state. The distances of cysteine residues at positions 248 in the capsomer are large enough to prevent intrapentamer disulfide bond formation, and the shielded position of these thiol groups within the central hole does not allow the formation of interpentamer disulfide bonds. In agreement with these predictions there was no difference whether a reduction step prior to labelling was applied or not (data not shown).

Also, the optimum reaction time and required dye excess for maximum labelling was determined. Reaction times between 2 and 60 min were screened, combined with varying dye concentrations in the range of 2 to 20-fold molar excess. The results are shown in Figures 2B and 2C.

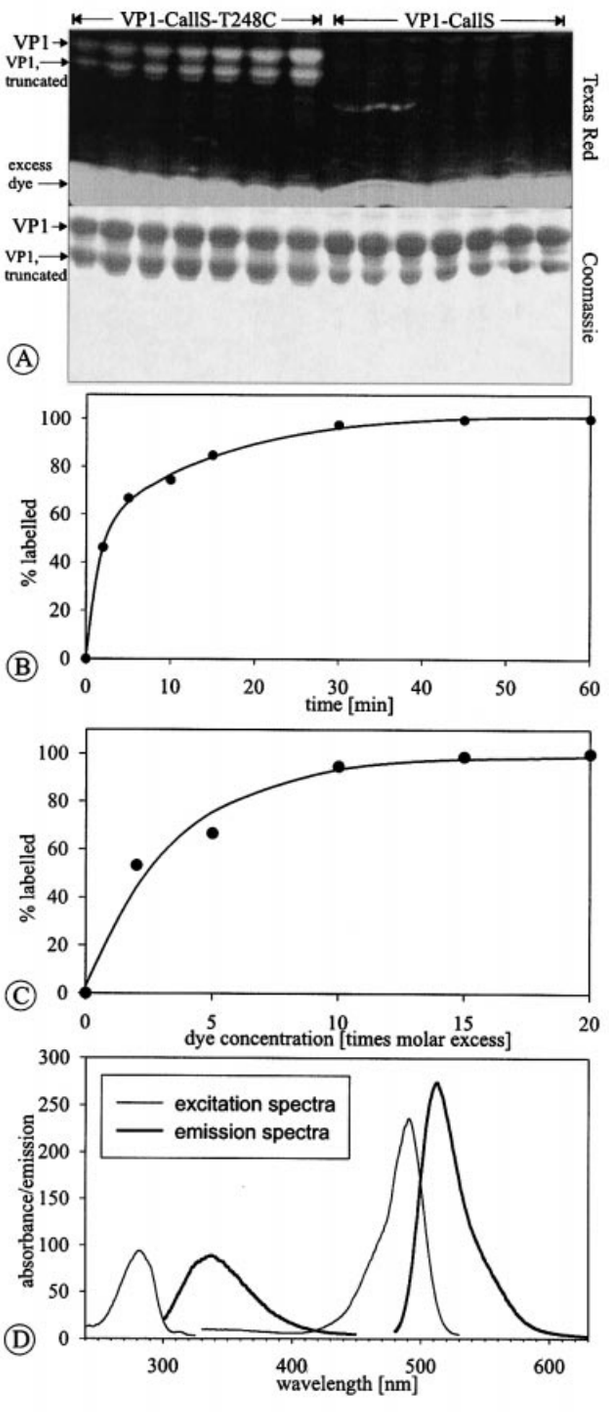

Fig. 2 Labelling and Fluorescence Analysis of VP1 Variants. (A) Labelling of VP1-CallS-T248C compared to VP1-CallS. Labelling was carried out with a 10-fold Texas Red-C_5-maleimide excess with varying times (2, 5, 10, 15, 30, 45, 60 min; lanes 1 to 7: VP1CallS-T248C; lanes 8 to 14: VP1-CallS). Represented are Texas Red fluorescence and Coomassie stained images of the same gel. In all samples a double band of VP1 appears which is due to partial proteolytic degradation. (B) Time dependence of the labelling reaction. (C) Dependence of the labelling reaction on the dye concentration. The maximum values were set to 100%, although less than 5 dye molecules were bound per pentamer as determined by absorbance measurements (see text). (D) Fluorescence spectra of VP1-CallS-T248C-fluorescein after removing excess dye by dialysis. Methods: Purified protein samples with concentrations of 0.3 to 0.5 mg/ml were dialysed against labelling buffer [20 mM HEPES, 200 mM NaCl, 1 mM EDTA, 5% (w/v) glycerol, pH 7.2]. A 2 to 20-fold molar excess of fluorescein- or Texas Red-C_5-maleimide (Molecular Probes) was added. The reaction mixture was stirred at room temperature for 2 to 60 min. Then the reactions were stopped by adding a 100-fold excess of reduced DTT and the mixtures were stirred for additional 30 min. Samples were directly used for SDS-PAGE to separate proteins from excess dye. Gels were quantified densitometrically using the software Phoretix 1D (Phoretix). Both fluorescence and Coomassie Brilliant Blue staining was measured. Amounts of labelled protein were normalised to total protein concentration. Fit functions were determined with Table Curve 2D (Jandel Scientific). Fluorescence spectra were recorded at 20 °C on a Hitachi F-4500 fluorescence spectrometer. Prior to the measurements labelled protein samples (10-fold molar dye excess, 60 min at room temperature) were dialysed at least four times against labelling buffer (300-fold excess of volume) in order to remove excess dye.

The reaction follows a hyperbolic function; it proceeds very fast in the beginning and gradually reaches a maximum after 30 min. A 10-fold molar excess of the dye was sufficient for maximum labelling.

The amount of labelled thiol groups per pentamer for maximum labelling was determined by absorbance measurement. 1.5 dye molecules were attached per pentamer, indicating that the maximum of five dye molecules per pentamer is not reached. This is most likely due to steric constraints. Excess dye was removed either by dialysis or size-exclusion chromatography. In addition to the intrinsic protein fluorescence, these samples showed signals according to the emission maxima of fluorescein or Texas Red, respectively, indicating covalent attachment of the dyes (Figure 2D).

The functional integrity of fluorescence-labelled VP1 capsomers was tested by

(i) analysis of *in vitro* assembly and

(ii) uptake experiments into eukaryotic cells.

For fluorescence-labelled VP1, the same assembly conditions were applied as described for mutant VP1-CallS (Schmidt *et al*., manuscript in preparation) which is a simplified procedure of the method originally reported for the wild-type protein (Salunke *et al*., 1986, 1989). Electron micrographs of these samples showed a population of 45 nm-particles (Figure 3A) in analogy to the (unlabelled) wild-type protein. The assembly can be monitored by size-exclusion chromatography using an analytical HPLC column TSK-PW6000XL with a column volume of 14 ml. This technique is suitable to separate virus-like particles from capsomers which are eluting at approximately 9 ml and 12 ml, respectively, (Schmidt *et al*., manuscript in preparation). To exclude the possibility of protein loss due to unspecific aggregation or inefficient assembly of fluorescence-labelled virions, assembly of VP1-CallS-T248C-dye was quantified by size-exclusion chromatography. Chromatograms were recorded by simultaneous absorbance measurement at 280 nm and 582 nm (Texas Red) or 492 nm (fluorescein, data not shown). In the elution

profile only two peaks were observed (Figure 3B): one at 8.9 ml representing a population of virus-like particles and another at 12.3 ml representing remaining capsomers. Integration of peak areas showed an assembly efficiency of 75% (Figure 3B) which is similar to the unlabelled protein (Schmidt *et al*., manuscript in preparation); there were no larger aggregates detectable. Therefore, fluorescence labels at position 248 do not significantly alter the protein conformation and do not interfere with the assembly process.

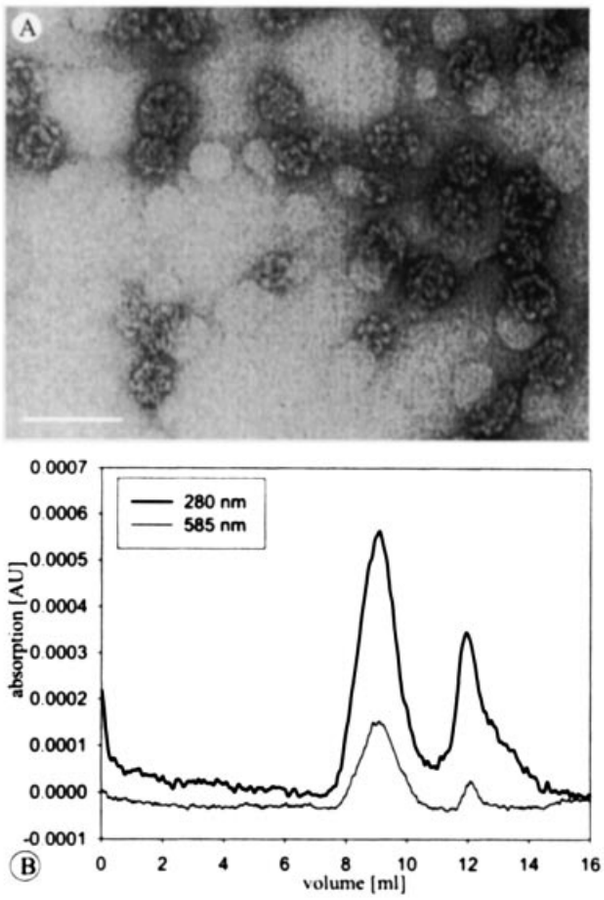

Fig. 3 *In Vitro* Assembly of Fluorescence-Labelled VP1.
(A) Electron micrograph of virus-like particles assembled from Texas Red-labelled capsomers. Fluorescence-labelled polyomavirus-like particles are indistinguishable from unlabelled cysteine-free and wild-type capsids. Scale bar = 100 nm. (B) Size-exclusion chromatography of *in vitro* assembled Texas Red-labelled VP1CallS-T248C. The elution profile shows peaks of virus-like particles at 8.9 ml and of free capsomers at 12.3 ml, similar to the wild-type protein (Schmidt *et al*., manuscript in preparation). Methods: For assembly into 45 nm-virus-like particles protein samples with concentrations between 0.3 to 0.5 mg/ml were dialysed for 2 days at room temperature. Starting conditions were 10 mM HEPES, 1 mM EDTA, 1 M NaCl, 5% (w/v) glycerol, pH 7.2, then changed to 10 mM HEPES, 0.5 mM $CaCl_2$, 150 mM NaCl, 5% (w/v) glycerol, pH 7.2 and finally to 10 mM HEPES, 0.5 mM $CaCl_2$, 5% (w/v) glycerol, pH 7.2. For electron microscopy studies, a BS-500 instrument from Tesla, Brnu (Czech Republic) was used at 90 kV with a magnification factor of 44 000. Staining of the specimen was performed with uranyl acetate on bacitracin-incubated (0.1 mg/ml, 1 min) copper-carbon-grids following standard protocols. Analytical size-exclusion chromatography was performed with a Biocad Vision HPLC/FPLC-system (Perseptive Biosystems) and an analytical TSK-PW6000XL HPLC column (Toso Haas) with a column volume of 14 ml. 50 μl of a sample prepared as described above were applied to the HPLC column equilibrated with a buffer consisting of 10 mM HEPES, 0.5 mM $CaCl_2$, 200 mM NaCl, 5% (w/v) glycerol, pH 7.2 with a flow rate of 1 ml/min at room temperature.

Fluorescence-labelled polyomavirus-like particles will be used for investigating virus-cell interactions. Therefore, it is necessary that the particles besides there structural integrity also keep their receptor-binding capability which is essential for entering eukaryotic cells. In order to test the ability of labelled polyomavirus-like particles to 'infect' eukaryotic cells and to exclude the possibility of blocking receptor-binding with the fluorescence label, cultures of C2C12 mouse muscle cells were incubated with different concentrations of Texas Red-labelled virus-like particles for 10 to 60 minutes. By confocal laser-scanning microscopy red fluorescent spots of accumulated labelled protein were detected within the cells (Figure 4) which increased in intensity with particle concentration and incubation time, thus indicating an efficient uptake of the particles. Unspecific attachment of the labelled particles to

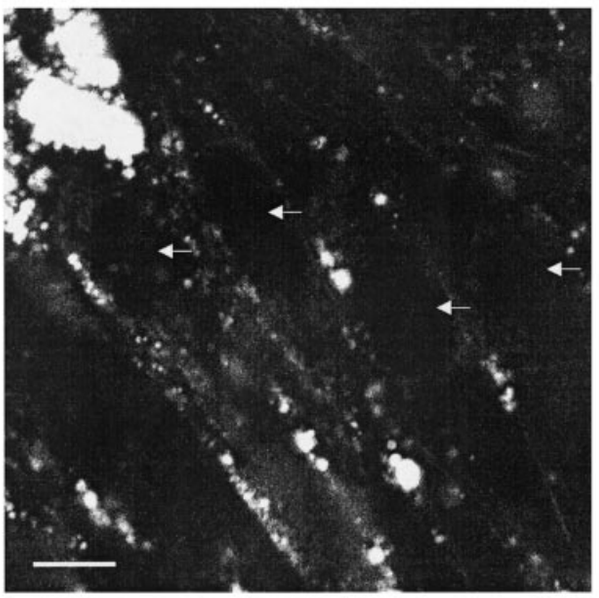

Fig. 4 Fluorescence micrograph of mouse myoblasts incubated with Texas Red labelled polyomavirus-like particles.
The nuclei are not accessible to the particles and are therefore visible as dark unstained spheres, marked by arrows. Scale bar = 10 μm. Methods: Texas Red-labelled polyomavirus-like particles were prepared as described above. The mouse muscle cell line C2C12 was cultivated in 8-well glass cover slips (Nunc) with 400 μl medium per well. After four days of growth, before reaching confluence, 2 to 50 ng labelled virus-like particles were added to the medium and incubated for 10 to 60 min. The cultures were subsequently washed twice with PBS and new medium was added. Immediately, images were recorded with a ZEISS confocal laser scanning microscope, Model 410, equipped with a 63-fold water immersion objective.

the cells could be excluded by competition with unlabelled VP1; addition of a twenty-fold molar excess of unlabelled protein blocked the uptake of labelled VP1 so that no fluorescence could be detected in the cells (data not shown). These results indicate that fluorescence labels at position 248 do not alter the functional integrity of the virus-like particles; the labelled VP1-mutant has unchanged receptor binding characteristics and uses the same cellular entry-pathway than the wild-type protein. However, these experiments also show that virus-like particles consisting only of VP1 do not enter the nucleus, as has been demonstrated for the complete virus by electron microscopy studies and Western blotting (Mackay and Consigli, 1976). Earlier studies suggested that the minor structural coat proteins VP2 and VP3 are necessary for endosomal escape and nuclear targeting (Chen *et al*., 1998).

Here, site-specific labelling of a virus shell with fluorescence dyes is reported for the first time. The labelling is achieved in a simple single-step procedure using mild conditions. It is possible to optimise the amount of attached labels by varying reaction time and dye concentration. Fluorescent VP1 capsomers keep their full biological activity regarding *in vitro* assembly and receptor binding. Uptake of fluorescent polyomavirus-like particles in eukaryotic cells can be detected either by conventional or confocal fluorescence microscopy demonstrating the usefulness of our approach for detailed studies of virion-cell interactions and intracellular localisation by fluorescence techniques. An analysis of these properties is very useful for the development of a gene therapy approach based on polyomavirus-like particles because bottlenecks for gene transfer, like insufficient endosomal escape, can be detected. Site-specific maleimide labelling also rises the possibility to couple synthetic peptides either for receptor and cell targeting in gene therapy applications or foreign epitopes for vaccine development.

The techniques presented here are not restricted to polyomavirus-like particles and may be applied to other viral gene delivery systems and virus-like particles. Disulfide bonds are of general importance for protein structure and folding and in many cases may not be replaced by other residues without loss of functional integrity of the viral proteins. However, in many mature virions all essential cysteine residues will be oxidised or buried within the viral capsid. An alternative strategy would be the labelling of assembled and purified viral particles rather than capsomers after introducing a new cysteine residue by site-directed mutagenesis at a suitable site on the virion's surface. This would also allow a site-specific fluorescence labelling of a genuine infective virus. Hopefully, the application of fluorescence techniques will further enhance our understanding of virus-cell interactions.

Acknowledgements

This work was supported by a grant from Land Sachsen-Anhalt. We want to thank Dr. Robert Garcea, University of Colorado, USA., for providing a plasmid containing the original VP1 gene. We thank Dr. Thomas Nitzsche from our department for his help on electron microscopy and Dr. Martina Hause, Institut für Pflanzenbiochemie, Halle, for her assistance with confocal laser scanning microscopy. Finally, we are grateful to Ilka Knütter for excellent experimental assistance.

References

Anderson, W.F. (1998). Human gene therapy. Nature *392* Supp., 25 – 30.

Bartlett, J.S., and Samulski, R.J. (1998). Fluorescent viral vectors: A new technique for the pharmacological analysis of gene therapy. Nature Med. *4*, 635 – 637.

Chen, X.S., Stehle, T., and Harrison, S.C. (1998). Interaction of polyomavirus internal protein VP2 with the major capsid protein VP1 and implications for participation of VP2 in viral entry. EMBO J. *17*, 3233 – 3240.

Eckhart, W. (1991). Polyomaviridae and their replication. In: Fundamental Virology, 2nd ed. B.N. Fields and D.M. Knipe eds., (New York: Raven Press), pp. 727 – 741.

Forstova, J., Krauzewicz, N., Sandig, V., Elliott, J., Palkova, Z., Strauss, M., and Griffin, B.E. (1995). Polyomavirus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. Hum. Gene Ther. *3*, 297 – 306.

Kraulis, P.J. (1991). Molscript: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. *24*, 946 – 950.

Leopold, P.L., Ferris, B., Grinberg, I., Worgall, S., Hackett, N.R., and Crystal, R.G. (1998). Fluorescent virions: Dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. Hum. Gene Ther. *9*, 367 – 378.

Li, M., Beard, P., Estes, P.A., Lyon, M.K., and Garcea, R.L. (1998). Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. J. Virol. *72*, 2160 – 2167.

Mackay, R.L., and Consigli, R.A. (1976). Early events in polyomavirus infection: attachment, penetration and nuclear entry. J. Virol. *19*, 620 – 636.

Merritt, E.A., and Bacon, D.J. (1997). Raster3D: Photorealistic molecular graphics. Methods Enzymol *277*, 505 – 524.

Salunke, D.M., Caspar, D.L.D., and Garcea, R.L. (1986). Self-assembly of purified polyomavirus capsid protein VP1. Cell *46*, 895 – 904.

Salunke, D.M., Caspar, D.L., and Garcea, R.L. (1989). Polymorphism in the assembly of polyomavirus capsid protein VP1. Biophys J. *56*, 887 – 900.

Slilaty, S.N., Berns, K.I., and Aposhian, H.V. (1982). Polyoma-like particles: characterisation of the DNA encapsidated *in vitro* by polyoma empty capsids. J. Biol. Chem. *257*, 6571 – 6575.

Stehle, T., and Harrison, S.C. (1997). High-resolution structure of a polyomavirus VP1-oligosaccharide complex: implications for assembly and receptor binding. EMBO J. *16*, 5139 – 5148.

Stehle, T., Yan, Y., Benjamin, T.L., and Harrison, S.C. (1994). Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. Nature *369*, 160 – 163.

Stehle, T., Gamblin, S.J., Yan, Y., and Harrison, S.C. (1996). The structure of simian virus 40 refined at 3.1 Å resolution. Structure *4*, 165 – 182.

Received October 5,1998; accepted January 22,1999