Cloning, Expressing and Purification of the Recombinant Polyomavirus VP1 and Characterization of its DNA Binding Domain

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The polyomavirus major capsid protein VP1 was cloned into a truncated prokaryotic expression vector △pFlag and expressed in *E. coli* (RK1448). The recombinant VP1 protein was expressed at high (9%) level when compared to host cell proteins. The expressed VP1 protein was purified to near homogeneity by immunoaffinity chromatography. Electron microscopy of the isolated VP1 protein was observed to have the capsomere-like structure. The recombinant VP1 capsomeres were found to assemble into capsid-like particles in the presence of exogenous calcium. Wild type recombinant VP1 expressed in *E. coli* (RK 1448) was shown to have DNA binding activity and the synthetic peptide (Ala^1-Cys^{11}) of the VP1 N-terminus was also shown to have high affinity for DNA binding. All of the three positively charged amino acids (Lys^3-Arg-Lys^5) within the DNA binding domain of VP1 appear to be essential for the DNA binding activity in this study.

Key words: DNA tumor virus; gene cloning; immunoaffinity chromatography; dot blot.

Introduction

The polyomavirus is a DNA tumor virus and its genome encodes three structural proteins VP1, VP2 and VP3 with molecular weights of 45, 35, and 23 kd respectively[(1)]. The 45 kd VP1 molecule is the major capsid protein of polyoma, comprising approximately 70-75% of the capsid shell protein. VP1 of polyoma is not a single protein entity but separable into distinct species when subjected to isoelectric focusing[(2)]. Six species can reproducibly be identified by two-dimensional

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gel electrophoresis of polyoma VP1, designated as species A to F in order of decreasing isoelectric point[3].

Polyoma VP1 isospecies D, E and F are phosphorylated[2]. There is no evidence of sequence differences between the 6 isospecies of VP1, as evident from identical peptide maps after trypsin, chymotrypsin, and V8 protease digestion[4]. Comparison of the phosphorylation site of *in vivo* ^{32}p-labeled D, E and F by peptide mapping has shown that each species contained a single major phosphopeptide. The major phosphorylated amino acids were threonine (70%) and serine (30%). By using the chemical cleavage methods to generate the VP1 fragments, the major phosphorylation site was localized to a central region of VP1 in the 29K fragment region[5]. Fattaety and Consigli[6] demonstrated that all species of VP1 are present in the cytoplasm of infected cells early in infection. The species were also present in the nucleus of the cells. This suggests that the modifications which give rise to the 6 isospecies of VP1 occur in the cytoplasm of infected cells prior to their transport into the mucleus. More recently, Li and Garcea[7] have shown that threonine-63 and threonine-156 of VP1 were the major phosphorylated amino acids and the threonine-to-alanine change at residue 156 was inefficient in assembly of 240S viral particles. in addition, Salunke et al. [8] have demonstrated that the VP1 cloned into pALVP1tac plasmid and expressed at approximately 2-3% in *E. coli* RB791 was able to form capsid-like structure *in vitro.*

In this study, we have cloned the VP1 into a prokaryotic expressing vector△pFlag and expressed in *E. coli* RK1448 at high level (9%) when compared to the host cell proteins. In addition, the high efficient immunoaffinity chromatography was employed to purify the recombinant VP1 from the *E. coli* lysate. The purified VP1 was also to from capsid structure after adding Ca^{+2} ions. Therefore, it will facilitate our study on the mechanism(s) of the virion assembly. Furthermore, the biochemical approach using the synthetic peptide containing the first eleven amino acids of the VP1 for DNA binding assay also substantiated our previous genetic findings[11] which have shown that the DNA binding domain was located at the N-terminus of the VP1 protein.

Materials and Methods

Recombinant VP1 construct

Recombinant VP1 was constructed and expressed in the modified prokaryotic expression vector pFLAG-1 (IBI, New Haven, Conn.). pFLAG-1 was digested with enzymes NdeI and SalI to delete 114 bp from nucleotides 101 to 214. The deleted region contains an OmpA signal peptide the FLAG peptide, and five cloning sites. Mung bean nuclease digestion of the pFLAG plasmid was followed by blunt-end DNA ligation with T4 DNA ligase. The original pFLAG-1 plasmid was altered to allow expression of authentic VP1. The new expression construct was designated as △pFlag (Fig. 1).

The 5.3-kb polyomavirus genome was digested with EcoRI and cloned into the EcoRI site of pUC12 to create pPURAR. This construct was amplified in *E. coli* JM109 and was used as the template for amplifying VP1 gene by the polymerase chain reaction. The amplified DNA fragments were digested with BglII, purified by agarose gel electrophoresis and Magic PCR Preps columns (Promega, Madison, Wis.), and cloned into BglII-digested prokaryotic expression vector △pFlag (Fig. 1). The construct was then used to transform *E. coli* RK1448 cells.

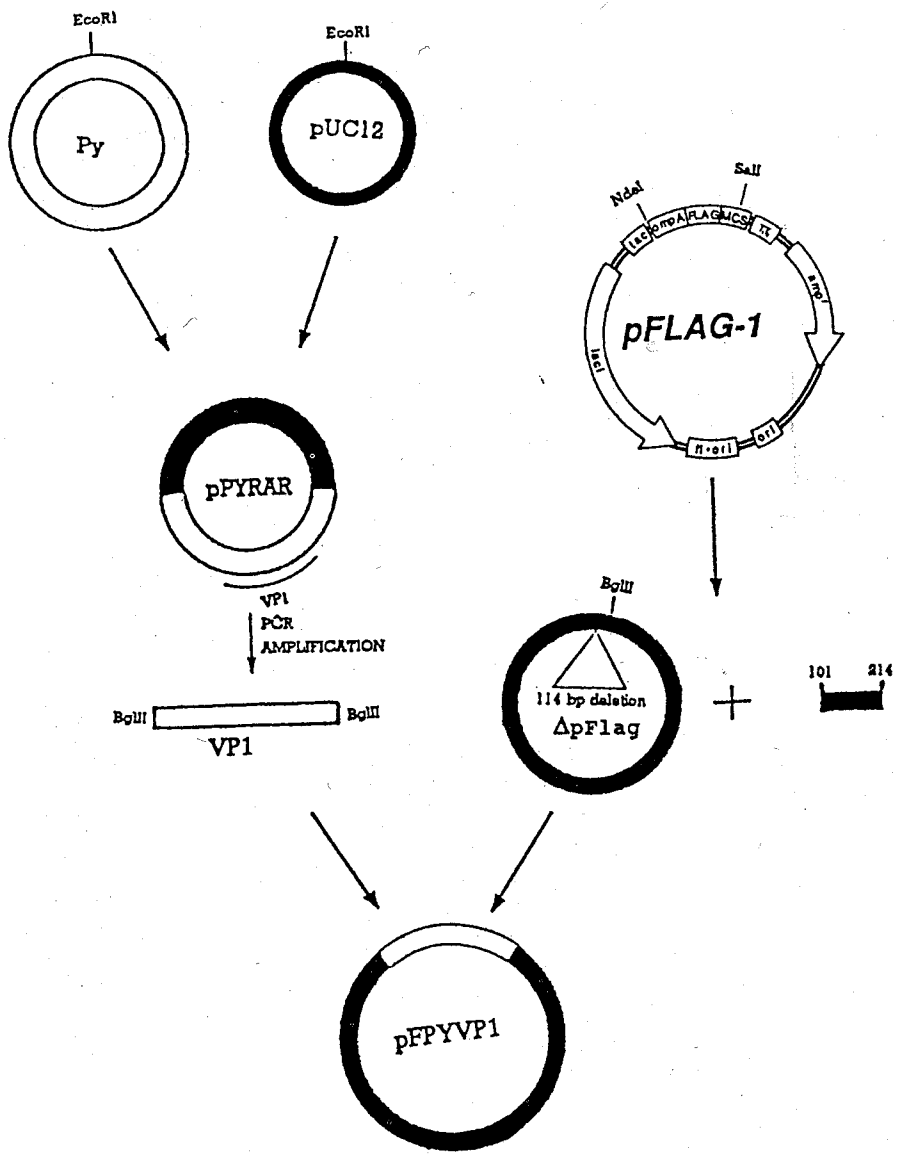

Figure 1. Construction of Wild Type and 5'end Truncated VP1 Expression Vector. Polyoma genomic DNA was cloned into pUC12 plasmid at Eco RI site to generate pPYRAR as a PCR template. The wild type VP1 DNA fragments with Bgl II restriction linkers was amplified by PCR from pPYRAR as a PCR template. The wild type VP1 DNA fragments with Bgl II restriction linkers was amplified by PCR from pPYRAR template and was subcloned in the proper orientation into the unique Bgl II site of the prokaryotic expression vector, △pFLAG to generate pFPYVP1 and express authentic VP1. △pFlag was derived from pFLAG-1 after deletion of the fusion signal peptide encoding region by Nde 1 and Sal 1. The new expression constructs was transformed into *E. coli* RK 1448 for VP1 protein expression. Details of the construction of VP 1 DNA fragments and plasmids were fully described in Material and Methods.

Recombinant VP1 expression and purification

VP1 was expressed from plasmid △pFlag grown in *E. coli* RK1448 at 37℃. Protein expression was induced at early logarithmic phase by the addition of 1.7 mM (final concentration) isopropyl-β-D-thiogalactopyranoside, and the expressed polyomavirus protein was purified by immunoaffinity chromatography.

To prepare the afinity column, 20 ml of rabbit anti-polyomavirus serum was precipitated by addition of sodium sulfate to 18% final concentration. The precipitate was resolublized in 5 ml of 20 mM Tris buffer (pH 8.0) containing 28 mM NaCl and dialyzed against this same buffer overnight. IgG was purified by passage of the antiserum through a protein A column (BIO- RAD, CA). The purified IgG was then coupled to 10 ml of Affi-Gel HZ hydrazide gel (BIO-RAD, CA). Purification and coupling of the antibody were performed according to the company's specifications. The bacterial cell extract was applied to the affinity column at a flow rate of 2 ml/hr. The column was then washed with 0.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.2). VP1 was subsequently eluted using $MgCl_2$ gradient (0 M to 3.5 M) in 10 mM phosphate buffer (pH 7.2).

The eluate was dialyzed overnight against 0.15 M NaCl in 10 mM sodium phosphate buffer (pH 7.4) and concentrated to 0.1-0.5 mg/ml using an Amicon concentrator, Centricon-10 (Amicon Co., MA). After affinity chromatography, the purity of VP1 was evaluated by 12.5% SDS-PAGE and Western blot.

Capsomere and capsid formation of recombinant VP1

The purified VP1 protein was subsequently dialyzed against capsomere buffer which contains 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 15 mM 2- mercaptoethanol and 5% glycerol. The pentameric capsomere-like structure of VP1 was observed by electron microscope. The capsid-like structure of VP1 was formed by the addition 0.5 mM $CaCl_2$ and removal of the chelator and reducing agent by dialysis.

Peptide Synthesis

Five peptides were synthesized (Fig. 4) using the Merrifield method of solid phase synthesis[9]: wild-type VP1-NLS (wtNLS): Ala^{1}-Pro-Lys- Arg-Lys-Ser-Gly-Val-Ser-Lys-Cys^{11}; nonNLS: Leu^{260}-Thr-Thr-Val-Leu-Leu-Asp-Glu-Asn-Cys^{269}; VP1 altered sequence 3-NLS (AS3-NLS): Ala-Pro-*Thr-Arg-Lys-Ser-Gly-Val-Ser-Lys-Cys; VP1 AS4-NLS: Ala-Pro-Lys-*Thr-Lys-Ser-Gly-Val-Ser-Lys-Cys; and VP1 AS5- NLS: Ala-Pro-Lys-Arg-*Thr-Ser-Gly-Val-Ser-Lys-Cys.

Preparation of Peptide-Protein Conjugates

Synthetic peptides were conjugated to BSA (Pentex Co. IL) using the heterobifunctional crosslinking agent, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Pierce Chemical Co. IL) as described previously[10]. Briefly, 10 mg of BSA was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) to which 0.5 mg MBS dissolved in dimethylformamide (10 mg/ml) was slowly added. This reaction of MBS with e-amino groups of lysine residues in BSA was allowed to proceed for 30 min at room temperature with inverted mixing. Unreacted MBS was then removed by gel filtration through a 5 ml Sephadex G-50 column (Pharmacia Inc., NJ) using same buffer. BSA derivatized with MBS was combined with 2.9 mg of the appropriate synthetic peptide in 50 mM sodium phosphate buffer (pH 7.0) for 2 h at room temperature with inverted mixing. This reaction involved crosslinking of the synthetic peptide to the MBS-protein complex through a thioether formed with the free

sulfhydryl group on the carboxy-terminal cysteine of the peptide. Unconjugated peptide was removed by overnight dialysis against 50 mM sodium phosphate buffer (pH 7.0) at 4°C. The coupling ratio was based on examination of the conjugates separated by 12.5% SDS-PAGE.

Nick-translated 32p Labeled DNA

pGEM-7 plasmid DNA was obtained from Promega company. One ug of pGEM-7 DNA was nick-translated using 120 pmoles of α [^{32}P] dCTP (Du Pont, Boston, MA) as described previously (1). The radiolabeled DNA was purified by Clean-up column (Promega). The specific activity of radiolabeled DNA was 1.8 x 10^7 cpm/ug.

DNA Binding Assay

For nondenaturing dot blot assay, proteins or peptide conjugates (0.1 ug) were directly spotted onto nitrocellulose. The nitrocellulose was probed with ^{32}P labeled pGEM-7 DNA (1×10^5 cpm/ml). The protocols of DNA blotting were the same as described earlier for Southwestern blotting[11].

Results

Purification

Polyomavirus VP1 gene was cloned into truncated prokaryotic expression vector △pFlag to form pFPYVP1. The genetic alteration of △pFlag and insertion VP1 have been con-

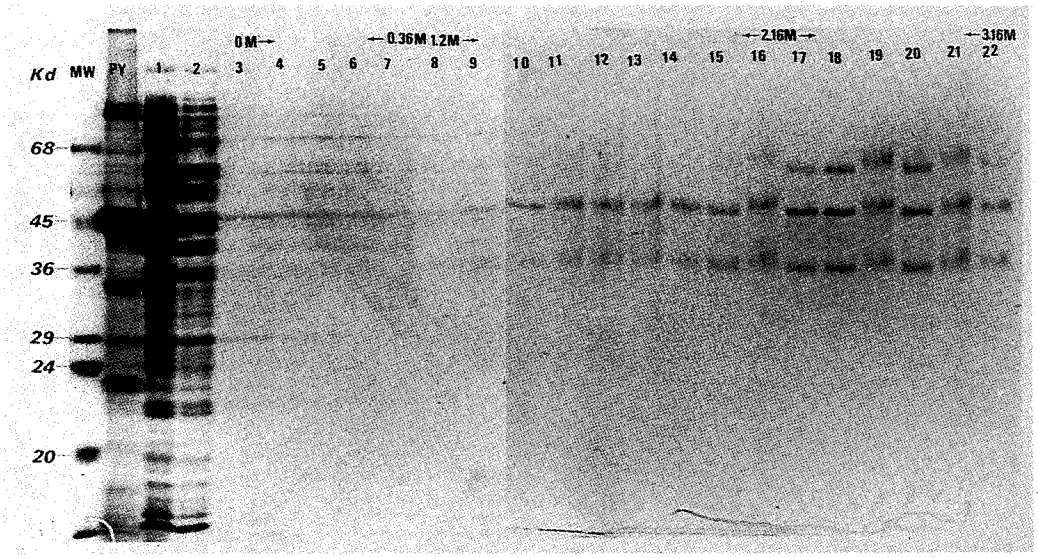

Figure 2. Immunoaffinity isolation of recombinant VP1. The total *E. coli* lysate containing recombinant VP1 was loaded onto the anti-polyoma agarose column. VP1 was eluted by $MgCl_2$ gradient (0 M to 3.5 M) and detected by OD 280 nm. The three OD 280 nm absorption peak areas, (0 M-0.36 M, 1.2 M-2.16 M, and 2.16 M-3.16M) obtained from $MgCl_2$ elution were pooled and assayed by SDS- PAGE with Coomassie blue staining. Lane MW: molecular weight markers. Lane PY: polyoma virion. Lane 1: total lysate of *E. coli* with VP1 expression. Lane 2: follow-through of the affinity column. Lane 3 to 7: fractions collected between 0 M to 0.36 M of $MgCl_2$. Lane 8 and 16: fractions collected between 1.2 M and 2.16 M of $MgCl_2$. Lane 17 to 22: fractions collected between 2.16 M and 3.16 M of $MgCl_2$.

firmed by DNA sequencing (not shown). After induction of IPTG, VP1 protein was expressed at about 9% of total proteins according to densitometry of Coomassie blue staining of SDS-PAGE (Fig. 2, lane 1). VP1 protein was purified by immunoaffinity chromatography. $MgCl_2$ gradient from 0 to 3.5 M was used to elute VP1 protein from the affinity column. Three absorbance peaks were detected at OD 280 nm when the $MgCl_2$ gradient were within 0-0.36 M, 1.20-2.16 M and 2.16-3.16 M respectively. The fractions within the peak areas were collected and evaluated by SDS-PAGE. SDS-PAGE revealed that fractions within 0 to 3.6 M of $MgCl_2$ contained many *E. coli* proteins (Fig. 2, lane 8 to 16). The fractions within 2.16 to 3.16 M of $MgCl_2$ contained VP1 and a 60 kd protein (Fig. 2, lane 17 to 22). The 36 kd protein (Fig. 2, lane 9 to 22) was a fragment of VP1 protein and the 60 kd protein (Fig. 2, lane 17 to 22) was a *E. coli* protein as confirmed by Western blot (data not shown). The VP1 protein at the second peak area within 1.2 to 2.16 M of $MgCl_2$ were collected and dialyzed against Tris buffer to remove $MgCl_2$.

Capsomere and capsid-like structure formation

To form capsomere structure, the pruified VP1 protein was subsequently dialyzed against capsomere buffer which contains 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 15 mM 2-mercaptoethanol and 5% glycerol, pH 7.4 for overnight at room temperature. The pentameric capsomere-like structure of VP1 was observed by electron microscope (Fig. 3A). The capsid-like structure of VP1 was formed by the ad-

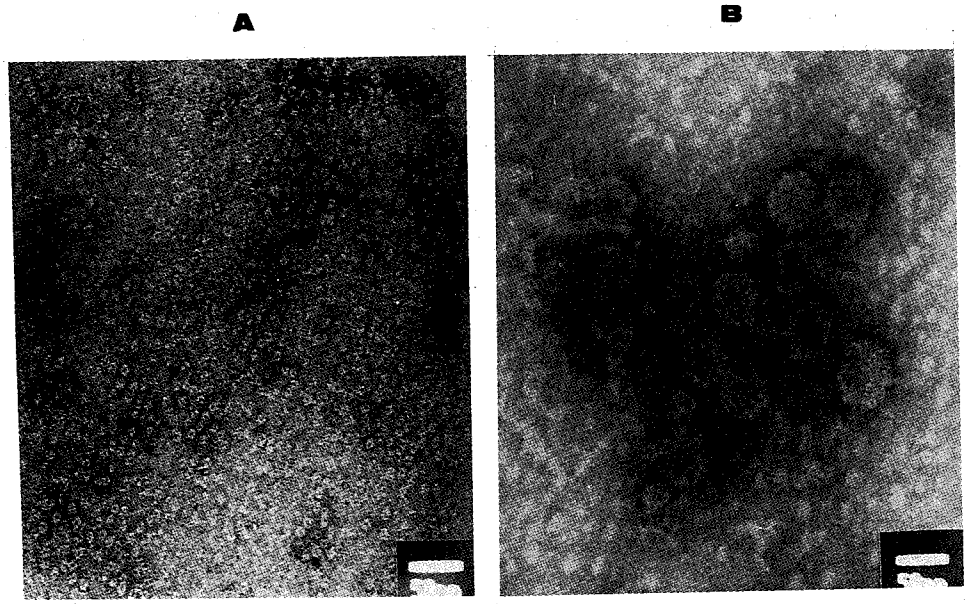

Figure 3. Electron microscopic analysis of purified recominant polyomavirus VP1 protein under differnet buffer conditions. (A) VP1 in capsomere buffer containing EDTA and β-mercaptoethanol but lacking calcium. (B) VP1 in capsid buffer containing $CaCl_2$ after removal of chelator and reducing agent.

dition of 0.5 mM of $CaCl_2$ and removal of the chelator and reducing agent (Fig. 3B). The average size of the capsomere-like and capsid - like structures were about 10 nm and 42 nm respectively.

DNA Binding to Synthetic Peptides

In order to substantiate the results from the genetic subtractive experiments which showed the first eleven amino acids to be the DNA binding domain of VP1[11] and to determine the importance of the positively charged amino acids (Lys^3-Arg-Lys^5) within the DNA binding domain of VP1, five synthetic peptides were synthesized (Fig. 4) and crosslinked to BSA for DNA binding assay. The nondenatured affinity purified recombinant VP1 was used as the positive control in the dot blot analysis. The VP1 and different peptide-BSA conjugates (0.1 ug) were spotted onto nitrocellulose and probed with nick-translated ^{32}P labeled pGEM-7 DNA. It was found that only recombinant VP1 (Fig. 5, spot 1), and the NLS peptide-BSA conjugate (Fig. 5, spot 3) were able to bind DNA. These results were consistent with our previous genetic subtractive findings which showed that the NLS region was the DNA binding domain of VP1[11]. BSA (Fig. 5, spot 2), non-NLS-BSA (Fig. 5, spot 4), AS3-NLS-BSA (Fig. 5, spot 5), AS4-NLS-BSA (Fig. 5, spot 6), and AS5-NLS-BSA (Fig. 5, spot 7) all failed to bind the ^{32}P labeled DNA. The dot blot DNA binding assay demonstrated that the first eleven amino acids was able to binding DNA and all of the three positively charged amino acids within the DNA binding domain of VP1 are required for the DNA binding activity.

Discussion

We have cloned the polyomavirus major capsid protein VP1 into a truncated prokaryotic expression vector △pFlag and expressed in *E. coli* (RK 1448). The VP1 protein, was expressed at high (9%) level when compared

Peptides	Amino acid sequences
wtNLS	Ala-Pro-Lys-Arg-Lys-Ser-Gly-Val-Ser-Lys-Cys
AS3-NLS	Ala-Pro-*Thr-Arg-Lys-Ser-Gly-Val-Ser-Lys-Cys
AS4-NLS	Ala-Pro-Lys-*Thr-Lys-Ser-Gly-Val-Ser-Lys-Cys
AS5-NLS	Ala-Pro-Lys-Arg-*Thr-Ser-Gly-Val-Ser-Lys-Cys
nonNLS	Leu-Thr-Thr-Val-Leu-Leu-Asp-Glu-Asn-Cys

Figure 4. Amino acid sequence of synthetic peptides. Wild-type synthetic peptide (wtNLS) represents the N-terminal sequence (Ala^1- Cys^{11}) of polyomavirus VP1. The altered sequence peptides represent changes in the underlined basic amino acid sequence (Lys^3-Arg-Lys^5): AS3-NLS has lysine-3 substituted with threonine; AS4-NLS has arginine-4 substituted with threonine; AS5-NLS has lysine-5 substituted with threonine. These amino acid substitutions are denoted with an asterisk (*). The control peptide (nonNLS) represents amino acids Leu^{260}-Cys^{269} of polyomavirus VP1.

to host cell proteins. The large quantities of VP1 protein generated by *E. coli* can be used to study the inter-and intra-molecular characteristics of VP1 molecules and interactions between VP1 and the biologically relevant molecules such as cellular receptors and hemagglutination domains. In addition, Bolen et al. have previously shown that VP1 consists of six species[2], with different pIs. The difference in pIs of the VP1 species conceivably resulted from different degrees and types of posttranslational modifications of VP1. Previously it has been found that VP1 is modified by sulfation, hydroxylation [12,13], and phosphorylation[2]. In addition, Brady et al.[1,14] have shown that chelation of calcium with ethyleneglycol-N', N'-tetraacetic acid (EGTA), in conjunction with disulfide bond disruption with the reducing agent dithiothreitol (DTT), resulted in the breakdown of the virion into its capsomere subunits and a DNA-protein complex. Interestingly, addition of exogenous $CaCl_2$ to this dissociated mixture permitted reassembly into intact virions which partially regained both infectivity and hemagglutination activity[15,16]. Polyomavirus VP1 which was expressed in *E. coli*, and purified in the presence of EDTA and β-mercaptoethanol was isolated as pentameric structures that resembled viral capsomeres[8]. Removal of the chelator and reducing agent together with addition of $CaCl_2$ resulted in assembly of these capsomeres into structures which resembled viral capsid[17]. Here, we have purified VP1 protein to near homogeneity by immunoaffinity chromatography. The purified VP1 protein was also able to form capsomere-like and capsid-like structures under specific conditions as mentioned before. Recently, we have also found that polyomavirus VP1 was the only capsid protein having DNA binding activity[11]. Although it is still not clear how the capsid proteins pack the viral minichromosomes, VP1 must play an important role in the mechanism(s) of virion assembly. Our cloned recombinant VP1 can also be used to elucidate the possible relationship among VP1 and other minor capsid proteins, VP2 and VP3, and viral DNA during late phase of lytic infection. Furthermore, the recombinant capsids have significant po-

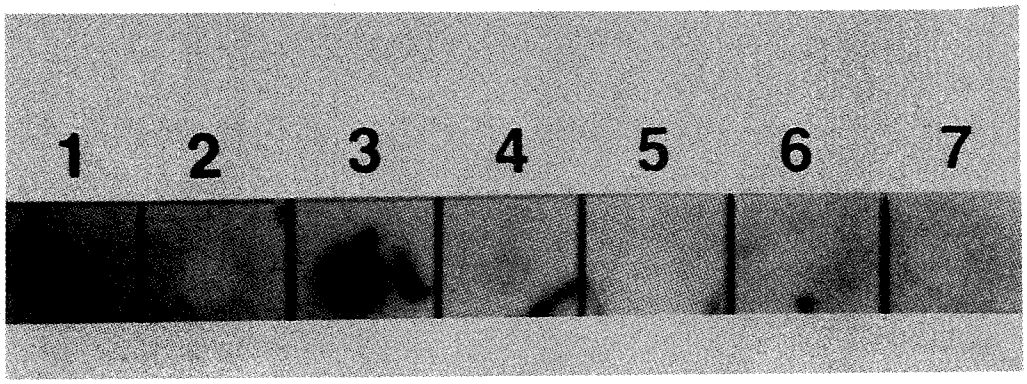

Figure 5. DNA binding to VP1 and synthetic peptide-protein conjugates. Spot 1: recombinant VP1, spot 2: BSA, spot 3: wtNLS-BSA, spot 4: non-NLS-BSA, spot 5: AS3-NLS-BSA, spot 6: AS4-NLS-BSA, spot 7: AS5-NLS-BSA. All spots were probed with nick-translated ^{32}P labeled pGEM-7 DNA. Details are described in Materials and Methods.

tential for use in targeted gene or drug delivery. Under the right conditions, virus capsids can be formed to encapsulate a pharmaceutical or gene vector. These special capsids might then serve as a carrier to deliver the gene of drug to a specific tissue. As the virus capsids "infect" the tissue, the contents will be introduced directly into the interior of the cells. Thus, virus capsids may have application for the treatment of many disease including cancer and genetic disorders.

The first eleven amino acids (Ala^{1}-Pro-Lys-Arg-Lys-Ser-Gly-Val-Ser- Lys-Cys[11] of the polyomavirus major capsid protein VP1 and the last twelve amino acids (Glu^{307}-Glu-Asp-Gly-Pro-Glu-Lys-Lys-Lys-Arg-Arg-Leu^{318}) of the minor capsid protein VP2/3 contain the similar basic amino acid stretches which were demonstrated to be responsible for nuclear localization of VP1[10] and VP2/3[18]. Although the first eleven amino acid region of VP1 was demonstrated to contain both the nuclear localization signal (NLS) and the DNA binding domain, they contain two separate functions. This observation is based on our previous microinjection studies, which showed that the synthetic NLS peptide was able to target non -nuclear proteins, (BSA; 68kd and IgG; 150 kd), to the nucleus[10]. These conjugates are too large to simply diffuse in through the nuclear pores and be retained in the nucleus by DNA binding. They apparently enter the small nuclear pore by a "nuclear-signal-mediated" transport mechanism. Therefore, it would seem logical that a nuclear retention process need not be invoked to explain the nuclear accumulation of these large molecules. In addition, amino acid substitution of lysine-3, arginine-4 and lysine-5 of wild type NLS with threonine abolished the DNA binding activity, but substitution of arginine-4 and lysine-5 of wild type NLS with threonine retained nuclear transport activity[10]. Furthermore, VP2 and VP3 of polyomavirus contain their own NLS at the C-terminus and are able to localized into the nucleus independently[5], but they do not have DNA binding activity[11].

The experimental evidence to date showed that VP1 is the only polyoma structural protein able to interact with DNA directly. Which raises a vital question of how could VP2 and VP3 be involved in virion assembly? We speculate that VP2 and VP3 may associate with VP1 before or after VP1 molecules attach to the viral DNA. Previous studies have shown that the virion dissociated capsomere subunits [1] and empty capsids[19] contain all of the three viral capsid proteins VP1, VP2, and VP 3. These findings indicated that there are possible interactions among VP1, VP2, and VP3. Our previous data[20] also indicated that when COS-7 cells were co-transfected with the NLS truncated VP1 and wild type VP2 genes the NLS truncated VP1 was transported to the nucleus which indicated that VP1 and VP2 may interact in the cytoplasm allowing the wild type VP2 to carry NLS truncated VP1 into the nucleus. In addition, we were able to co-immunoprecipitate the mixture of recombinant VP2 and VP1 with VP1 monoclonal antibody to show the interactions between VP1 and VP2[20]. Therefore, we assume that VP2 and VP3 may associate with VP1 to form capsomeres, and VP1 may play a central role in binding polyoma minichromosome in the nucleus through a sequential series of enents by gradual addition of capsid proteins around the polyoma chromatin to form a complete virion.

Obviously the exact mechanism of virion assembly is still unclear. The structural genes of polyoma VP1 and VP2 have been cloned and expressed in prokaryotic and eukaryotic expression plasmids[10,18] and are available in our laboratory. These clones may provide useful reagents for the future studies regarding

the biological functions and interactions of the structural proteins during virion assembly.

Acknowledgments

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多瘤性病毒VP1之基因選殖表達及純化以及其DNA結合區域之特性化

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多瘤性病毒主要殼體蛋白VP1基因已被我們選殖到原核表達載體△pFlag並且VP1蛋白也在大腸桿菌一（RK1448）內表達。與寄主細胞蛋白比較，VP1的表達量相當高（約佔總蛋白9%），VP1蛋白已被免疫親合性色層分析法純化出來，我們可利用適當的條件將此基因重組VP1形成次殼體（capsomere）及殼體（capsid）形狀的外型。此外，除了重組VP1可以結合DNA，生合成的VP1 N-端11個氨基酸也有DNA結合的能力，但是其間的3個鹼性氨基酸改變後便喪失了DNA結合能力，因而得知VP1 N-端11個氨基酸確為DNA結合的主要區域。

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