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Development of A Monkey Kidney Cell Line Which Expresses Poliovirus Capsid Protein

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=Abstract=

The RNA genome of poliovirus encodes a long polyprotein precursor and this polyprotein is cleaved proteolytically by viral protease to yield mature proteins. The mature proteins derived from the P1 polyprotein precursor are the component of capsids. To further delineate the process of capsid assembly and encapsidation, in a first attempt, a cell line which expresses the authentic P1 polyprotein was established. CV-1 cells were transfected with the pRCRSVS1P1 plasmid DNA which contains 5'ncr sequences, whole authentic capsid gene of poliovirus and neomycin resistance gene. These cells were treated with G418 for 3 months, and eventually G418 resistant cells were selected and formed colonies. Each colony was picked and grown in the media containing G418. DNA analysis indicated that 1 of 13 neomycin resistant cell lines (R2-18) contains whole poliovirus P1 capsid gene segment which was incorporated into the genome. Immuneprecipitation of cell lysates with sera from rabbit immunized with inactivateded Sabin type 1 particles demonstrated the constitutive expression of the poliovirus P1 capsid protein from R2-18.

Key Words: Poliovirus, Capsids, 5'ncr, neomycin resistance, G418

INTRODUCTION

Poliovirus is taxanomically classified as a member of the family Picornaviridae. The family Picornaviridae consists of four subfamilial generas. Poliovirus is included in the subfamilial genus Enterovirus [8]. Poliovirus is serologically divided into 3 different serotypes. Nucleotide sequence homology among 3 serotypes is 71%. The homology in the amino acid sequences among 3 serotypes is 88% [8]. Poliovirus is a small (about 27nm in diameter), and non-enveloped virus. Poliovirus virion consists of 60 copies of each of the capsid proteins (VP1, VP2, VP3, and VP4), and one copy of single stranded genomic RNA [7]. The RNA genome of poliovirus contains a single open reading frame which encodes a long polyprotein. This polyprotein precursor is cleaved proteolytically to yield mature products. The initial cleavage of a Tyr-Gly pair by 2A protease separates the P1 structural protein from the P2-P3 non-structural protein precursor [16]. Most of the other cleavages occur between Gln-Gly pairs by viral protease $3C^{pro}$ or $3CD^{pro}$ [6]. The mature proteins derived from the P1 capsid protein precursor are the structural com-

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ponents of the viral capsid.

Poliovirus RNA has unusually long 5' noncoding sequences (ncr), consisting of about 740 nucleotides. The 5'-terminus of poliovirus mRNA is not capped. One of the features of poliovirus infection is the inhibition of cap dependent cellular translation [4]. However, poliovirus mediate cap-independent, internal initiation of translation by using an internal ribosome entry site (IRES) which is located between 140 and 630 of poliovirus 5'ncr [12, 13].

The availability of poliovirus cDNA has provided the opportunity to use molecular genetics to investigate the capsid assembly and encapsidation. Studies of poliovirus morphogenesis suggest that the assembly process is initiated by the orderly activation of latent assembly domains in the polypeptide P1. Cleavage of two bonds within P1 by $3C^{pro}$ or $3CD^{pro}$, followed by structural rearrangements, yields a 5S capsid protomer. Association of five 5S protomers forms 14S pentamer and, then 14S pentamers are further assembled into either a RNA containing provirions or empty capsid particles [14]. Whereas much have been identified concerning the capsid assembly and encapsidation, the minimum requirement and the actual mechanism of viral RNA encapsidation remains to be determined.

To gain better understanding of the process of capsid assembly and encapsidation, as a first step, a cell line which expresses the authentic P1 polyprotein was established. This cell line was characterized in terms of DNA incorporation into host genome, and P1 capsid protein expression.

MATERIALS AND METHODS

Construction of poliovirus P1 expression plasmids and sequencing

All DNA manipulations were performed according to standard procedures [15]. The poliovirus plasmids pS1T70 (gift from A. Nomoto, University of Tokyo) and pT7IC used for this study contain the complete infectious poliovirus Sabin type 1 cDNA or Mahoney type 1 cDNA [3, 9]. EcoR I/Nde I fragments of pSIT70 (3.4Kb) and pT7IC (5kb) were ligated and the resulting plasmid, pKC3S1cap contains the whole capsid genes of poliovirus Sabin type 1 and portions of type 1 Mahoney $3D^{pol}$. Hind III fragment of pKC3S1cap was subcloned into pRCRSV vector (Invitrogen corporation, San Diego, USA), resulting pRCRSVS1cap. pRCRSVS1cap plasmid DNA was digested with restriction enzyme Nde I and two synthetic oligonucleotids (Figure 2B) were annealed together and ligated into the Nde I digested pRCRSVcap, resulting pRCRSVS1P1 (Figure 1, Figure 2A). The plasmid pRCRSVS1P1 was sequenced to confirm that the inserted synthetic sequences placed two translation termination codons (TAG-TAG) immediately downstream of the codon for the authentic P1 carboxyterminal tyrosine residue (Figure 2A, Figure 3). The specific oligonucleotide (DG7: 5'-GAG GGC AGT GGC GTA CT-3') was used as primer for sequencing. The engineered poliovirus sequences pRCRSVS1P1 encode a 5'ncr region, and an authentic Sabin type 1 P1 polyprotein with a carboxy terminus identical to that generated when $2A^{pro}$ releases the P1 polyprotein from the nascent poliovirus polypeptide. For sequencing, DNA was prepared by using Wizard Minipreps DNA purification System (Promega) and sequenced by using Sequenase version 2.0 sequencing kit (Amersham/ Life Sciences).

Cells, transfection, and construction of transformed cell lines

All the cells used in this study were grown in Dulbecco's modified Eagle's medium (DMEM: Gibco) supplemented with 10% (vol/ vol) fetal calf serum. To generate poliovirus P1 protein expressing cell lines, pRCRSVP1S1 plasmid DNA (Figure 1) was transfected into CV-1 cells by calcium phosphate precipitation technique [5]. The transfected CV-1 cells were

cultured with medium containing G418 (250μg/ml: Gibco), and only cells that acquired neomycin resistance gene could grow and formed colonies. Individual colonies were picked for growth in the presence of the G418 and analyzed for the expression of poliovirus capsid P1.

Viruses

The stock of the poliovirus type 1 Sabin used in this study was derived from transfection of the infectious cDNA (S1T70).

Genomic DNA preparation

Genomic DNA was prepared from cultured cells by using Wizard genomic DNA purification system (Promega). The majority of DNA migrated at a position ≥50kb in a 0.4% agarose gel (data not shown).

Southern hybridization

Approximately 40μg of genomic DNA was digested with Hind III, separated by electrophoresis through a 0.8% agarose gel, and blotted on nitrocellulose filters as described [15]. DNA immobilized on filters was prehybridized under standard conditions (6 x SSC [1 x SSC is 0.15M NaCl, 17mM sodium acetate, pH 7.0], 10mM EDTA, 5 x Denhart's solution [1 x Denhart's reagent is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin], 0.5% SDS, and 100μg of salmone testis DNA per ml) for 4 h at 42℃. The prehybridization solution was replaced with 6 x SSC-0.5% SDS-50% formamide-5x Denhart's solution-10mM EDTA-100μg of salmone testis DNA per ml containing approximately 10^6 cpm of the riboprobe per ml. A [^{32}P] labeled 503-base riboprobe which is complementary to nucleotides 671 to 1174 of the poliovirus genome was used for hybridization [3]. Hybridization was performed at 42℃ for 16 h. Subsequent washing was done 2 times with 2 x SSC-0.5% SDS at room temperature for 5 min and then 0.1 x SSC-0.1% SDS at 65℃ for 2 h. The membrane was dried, and autoradiographed at -70℃.

Metabolic labeling and Radioimmunoprecipitation

Cells were washed once with PBS and incubated in DMEM without methionine/cysteine medium for 30 min, followed by an additional 3 h with DMEM without methionine/cysteine plus [^{35}S] methionine/cysteine (NEG expre^{35}S^{35}S-protein labeling mix) at 0.3mCi/ml final concentration. The cells were washed once with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Triton, 2.5mM Tris, 150mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate). The cellular DNA and debris were removed by centrifugation at 13,000 x g for 10 min at 4℃. A rabbit polyclonal antiserum raised against inactivated poliovirus particles (Sabin type 1) was used for immuneprecipitation of poliovirus capsid proteins (gift from R & D center, cheiljedang co., Korea). The antibodies were incubated with the extracts for 16 h at 4℃ with constant rocking. The immune-complexes were collected by a 1 h incubation with protein A-Sepharose (40μl of a 1:1 [wt/vol] mixture in RIPA) at room temperature with constant rocking. The beads were pelleted by centrifugation and washed three times with RIPA buffer. The bound proteins were eluted from the beads by boiling for 5 min in gel sample buffer (50mM Tris [pH 6.8], 5% SDS, 10% β-mercaptoethanol, 0.1% bromphenol blue). The proteins were electrophoresed through a polyacrylamide gel, fluorographed, dried, and exposed to X-ray film.

RESULTS

Construction of poliovirus capsid protein expression vector

The flow diagram for the construction of the poliovirus type 1 Sabin P1 protein expression vector used in this study is shown in Figure 1. Plasmid pRCRSV contains a β-lactamase gene for selective growth in ampicillin-containing

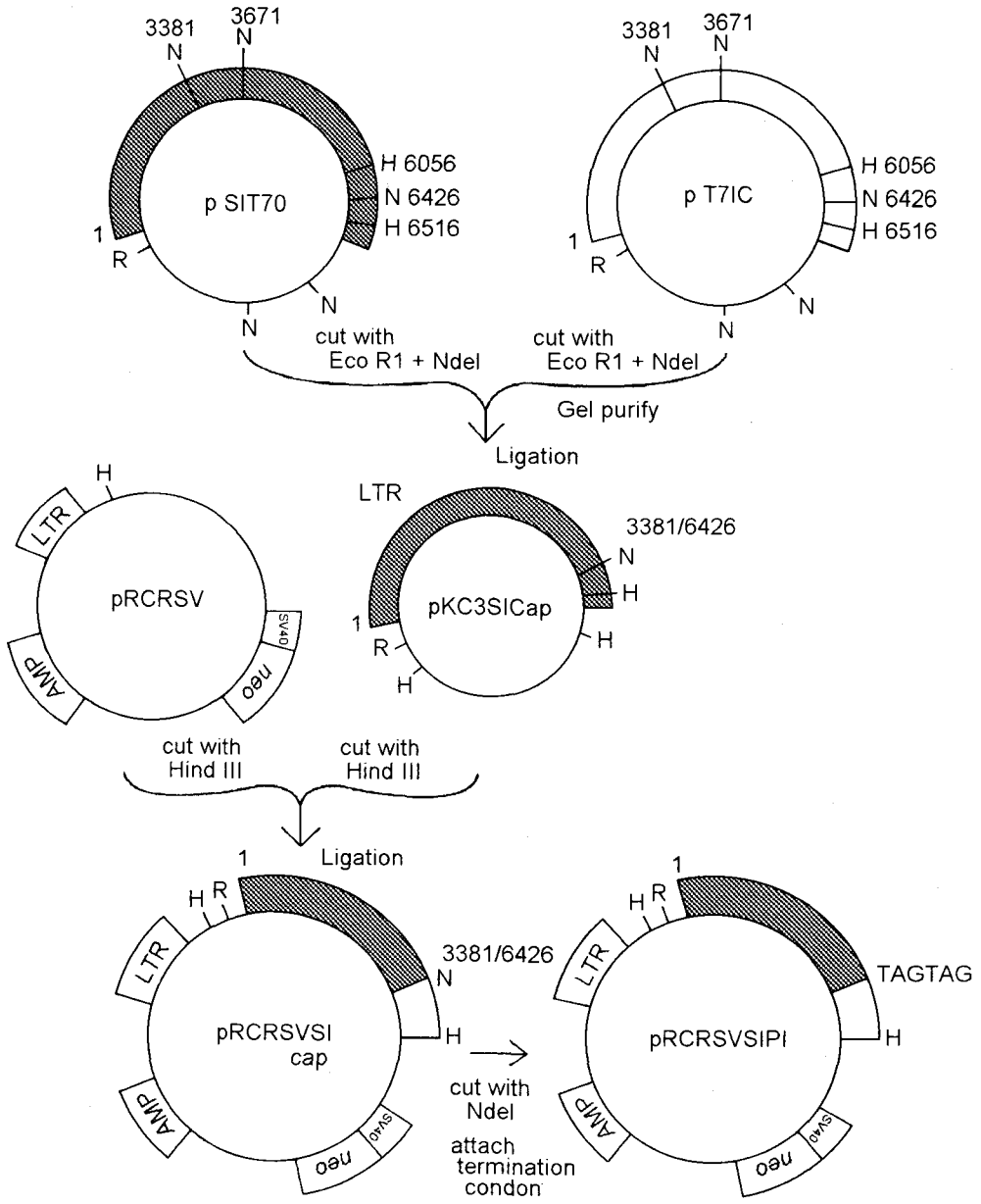
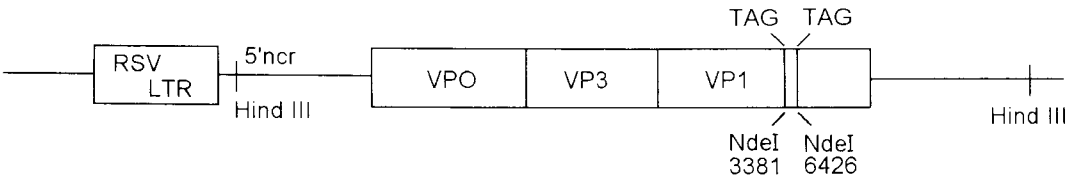


Figure 1. Flow diagram for the construction of poliovirus capsid protein P1 expression plasmid, pRCRSVS1P1. H, Hind III; N, Nru I; R, Eco RI.

media in E. coli and neomycin resistance gene for selective growth in the media containing G418. In addition, the plasmid contains DNA sequences for autonomous replication in bacteria. Approximately 3.4kb Hind III fragment containing the 5'ncr sequence and P1 protein sequence was isolated from pKC3S1cap and was ligated with pRCRSV DNA which was digested with Hind III to form the plasmid pRCRSVS1cap. Restriction enzyme digestion and sequencing have shown that the orientation of the insert is correct (data not shown). To express the authentic P1 protein, two translation termination codon was introduced into

a.



b. 5 - TAT - TAG - TAG - ATC - TG - 3'

5 - T - ACA - GAT - CTA - CTA - A - 3'

Figure 2. A. Poliovirus sequences in pRCRSVS1P1 vector. **B.** Synthetic oligonucleotides used for creating two translation termination codons.

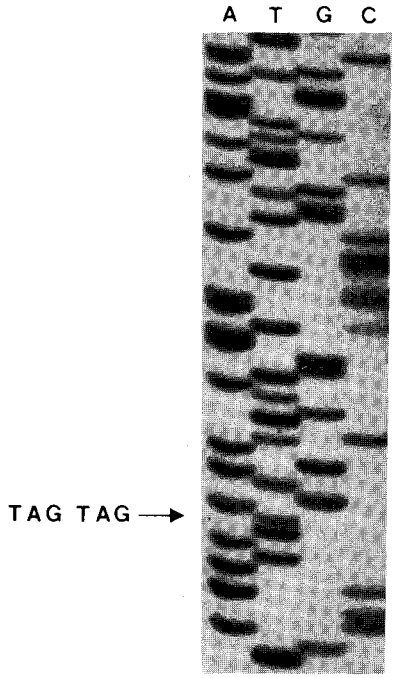


Figure 3. Nucleotide sequences of pRCRSVS1P1, which shows two translation termination codons (TAG-TAG) immediately downstream of the codon for the authentic P1 carboxyterminal tyrosine residue.

the carboxy terminal region of P1 open reading frame (Figure 2B). The proper insertion was confirmed by sequencing (Figure 3).

Construction of cell lines with poliovirus capsid genes stably integrated

pRCRSVS1P1 DNA which encodes whole authentic Sabin type 1 capsid polyprotein P1 and neomycin resistance gene was transfected into CV-1 cell and treated with G418 (250μg/ml). Selection was carried out for 3 months and only G418 resistant cells were selected and formed colonies. Finally, 13 independent G418 resistant cell lines were harvested, and were examined for further analysis.

DNA analysis of transformed CV-1 cells

To determine whether transformed cells contain poliovirus sequences, the genomic DNAs from the cell lines were isolated, digested with Hind III, and analyzed by Southern hybridization (Figure 4). The probe employed is a riboprobe which is complementary to the portion of 5'ncr plus N-terminal 430 nucleotides of poliovirus P1 gene. A single hybridization band which corresponds to the band from pRCRSVS1P1 plasmid DNA (Figure 4 lane 4) at 3.4kb was observed from R2-18, indicating that sequences containing 5'ncr and P1 capsid region was incorporated into chromosome correctly (Figure 4). However, the other 12 cell lines examined showed no bands at all (Figure 4

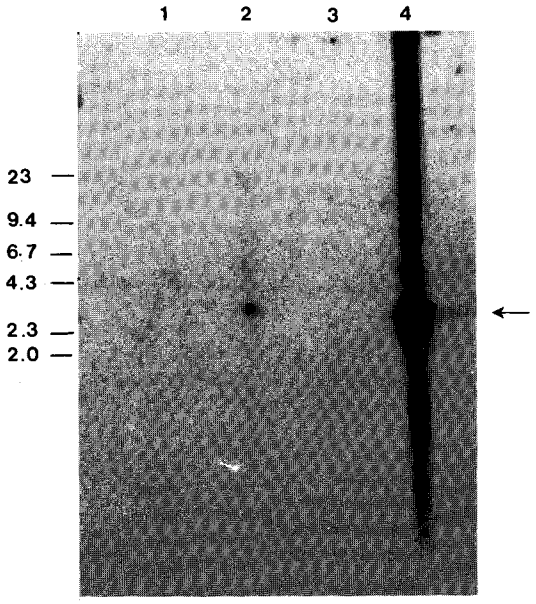


Figure 4. DNA analysis of pRCRSVS1P1-transformed CV-1 cells. Genomic DNAs (40μg) were digested with Hind III, electrophoresed on 0.8% agarose gels, blotted on nitrocellulose filters, and probed with 503 base riboprobe complementary to the nucleotides 671 to 1174 of the poliovirus genome. Fragments indicated by → corresponds to endogenous poliovirus P1 DNA fragments. Lane 1, CV-1 DNA; Lane 2, R2-18 DNA; Lane 3, R2-20; Lane 4, pRCRSVS1P1 plasmid DNA digested with Hind III. The positions and size of the molecular weight (in kb) are indicated.

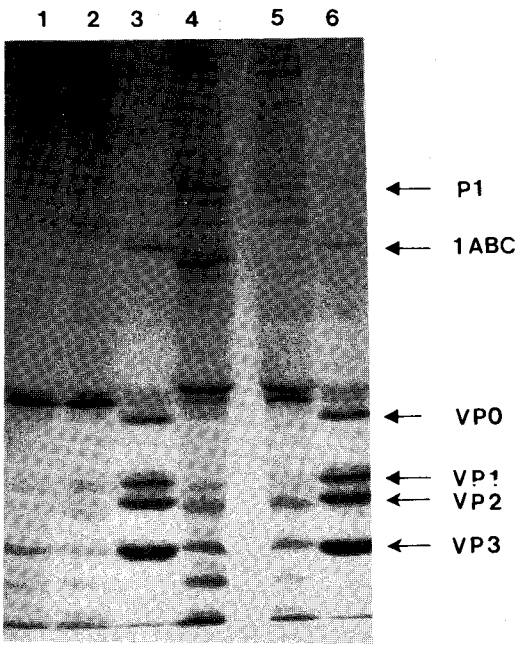


Figure 5. Expression of poliovirus P1 protein. Protein expression was analyzed by immunoprecipitation with sera from rabbit which was immunized with inactivated Sabin type 1 poliovirus. Lane 1 and 2, CV-1 cells as negative control; Lane 3 and 6, CV-1 cells which were infected with Sabin type 1 poliovirus; Lane 4 and 5, R2-18 cells. All the cells were starved with methionine/cysteine free DMEM for 30 min, and were labeled with [^{35}S] methionine/cysteine for 3 h.

lane 3, data not shown).

Expression of capsid protein P1

The expression of capsid protein P1 was determined by immunoprecipitation with rabbit antiserum using the lysates of cells metabolically labeled with [^{35}S]-methionine/cysteine (see Materials and Methods). This rabbit antisera immunoprecipitated a protein from R2-18, which corresponds to the P1 when analyzed in parallel with poliovirus protein immunoprecipitated from extracts of infected CV-1 cells (Figure 5, lane 4, 5). The rabbit antisera showed stronger reactivity with processed capsid protein than with the P1 capsid precursor (Figure 5, lane 3, 6). However, there was no expression of P1 from the other 12 cell lines examined (data not shown). These results indicate that a cell line R2-18 contains engineered poliovirus P1 sequences, which encodes an authentic P1 protein with a carboxy terminus identical to that generated when 2A^{pro} releases the P1 protein from the nascent poliovirus polypeptide.

DISCUSSION

In this study, establishment of CV-1 cell line which can express authentic P1 capsid protein was performed. CV-1 cells were transfected with the DNA which contains 5'ncr, whole authentic capsid sequences, and neomycin resistance gene. These cells were selected with G418. Of 13 neomycin resistant cells, genomic DNA only from R2-18 contains whole

region of capsid sequences (Figure 4) and expresses a P1 protein which corresponds to the P1 immunoprecipitated from extracts of infected cells (Figure 5). These results indicate that the genomic DNA from R2-18 encodes an authentic P1 gene and R2-18 cells constitutively express P1 protein with a carboxy terminus identical to that generated when $2A^{pro}$ cleaves the P1 from the nascent poliovirus polypeptide.

Most of the host cell protein synthesis is inhibited by infection with poliovirus [4]. This phenomenon is caused by alteration of the cap-binding protein complex (CBP), and the inactivation of the CBP complex is believed as the mechanism of specific inhibition of host cell translation [2, 10]. Poliovirus RNA is not capped, and its translation occurs by a cap-independent mechanism. Results from several experiments support the idea that the poliovirus protease $2A^{pro}$ is indirectly involved in cleavage of p220 [11], and that the IRES component of 5'ncr allows poliovirus translation by a cap-independent mechanism, and gives risistance to the host cell protein expression shut-off by poliovirus $2A^{pro}$ [12, 13]. Since the genomic DNA of R2-18 cells carries the IRES component, it is expected that R2-18 can express P1 protein even under the condition of inhibition by poliovirus $2A^{pro}$.

Previous studies have demonstrated that P1 is processed *in trans* by the $3CD^{pro}$ [17], and the expression of the two poliovirus proteins P1 and $3CD^{pro}$ in coinfected cells is sufficient for the correct processing of the P1 capsid protein as well as for the assembly of poliovirus empty capsid-like structures [1]. Therefore, it is probable that the P1 expressed from R2-18 is processed by the $3C^{pro}$ or $3CD^{pro}$ if supplied *in trans*, and the processed product can form an empty capsid-like structure in the cytoplasm. Further studies are going on to solve these questions.

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