Chapter 3

MOLECULAR
CHARACTERIZATION OF
NP-CYTOSKELETON
INTERACTIONS
3.1. INTRODUCTION

Nucleoprotein is a major structural protein of the influenza virus which has multiple functions in the virus life cycle. It is the main antigenic determinant amongst the influenza viral proteins. The influenza viral NP (Type A, strain PR/8/34) is coded by RNA genome segment five, a 1565 nucleotides long sequence of which 1494 nucleotides code for 498 amino acid residues long polypeptide. It is a basic protein rich in arginine with a net positive charge of +14 at pH 6.5 (Lamb, 1989). In vitro, it binds to RNA nonspecifically, but in vivo NP binds to complete cRNA (plus polarity) and vRNA (minus polarity), forming cRNP and vRNP, respectively (Krug et al., 1989). The RNA binding site in NP polypeptide has been mapped between the amino acid residues 91 and 188 (Kobayashi et al., 1994). NP has karyophilic signal(s) for nuclear translocation (Neumann et al., 1997; Wang et al., 1997) and along with the polymerase complex plays a critical role in nuclear translocation of vRNP after uncoating of the infectious virus.

NP and perhaps M1 protein of influenza A virus interact with the actin cytoskeleton of the virus-infected MDCK cells (Husain & Gupta, 1997). The interaction of NP with actin cytoskeleton seems to be direct but the M1-cytoskeleton interactions appear to be mediated by some other viral component(s) probably NP or NS1 (Zhang & Lamb, 1996; Avalose et al., 1997). The present study was undertaken to further understand the specificity of the influenza viral NP interaction with the host cell cytoskeleton. Earlier, it has been shown that in case of the human papilloma virus type 16 the C-terminus of E1-E4 proteins is dispensable for their association with the keratin filaments (Roberts et al., 1997). In addition, hepatitis C viral ORF3 protein looses its interaction with the cytoskeleton upon deleting the 32 amino acid residues from the N-terminal end (Zaffrullah et al., 1997). The present study involves the construction of a series of mutants with a deletion of amino acid residues from N-terminal, C-terminal, at both the N- and C-terminal ends as well as from the central region of the NP
polypeptide. All the mutants were expressed in COS1 cells and their interactions with the actin cytoskeleton were analysed.

3.2. MATERIALS AND METHODS

3.2.1. Luria- Bertini (LB) Medium

- Bacto Tryptone - 1 %
- Bacto Yeast extract - 0.5 %
- Sodium chloride - 1 %

- pH of the medium was adjusted to 7.0 with sodium hydroxide (1N).
- For LB agar medium 1.5 % Bacto agar was added to the above solution.
- Volume of the solution was adjusted with double distilled water (DDW) and sterilized by autoclaving at 121°C and 15 lb for 15 min.

3.2.2. Cells and Plasmids

MDCK, CV1, and COS1 cells were grown and maintained in Eagle’s minimum essential medium (MEM; Gibco BRL,USA) supplemented with 10% FCS (Sigma, USA) and 1X antibiotic-antimycotic solution (100X; Gibco BRL). Cells were routinely grown in 25 cm² culture flasks at 37°C under 5% CO₂ and subcultured in a ratio of 1:2. Plasmid CMV-NP was a kind gift from Dr. Peter Palese (Mount Sinai School of Medicine, New York,USA). Wild type NP gene and its deletion mutant constructs were cloned in pcDNA3 (Invitrogen, Inc,USA). Plasmid pGEM-T easy was obtained from Promega Corp. USA and used for the direct cloning of the PCR generated DNA fragments. E. coli DH5α {F’/endA1 hsdR17 (r−m+) supE44 thi-1 recA1 gyrA (NalI) relA1 Δ(lacZYA-argF) U169 deoR [φ80lac Δ(lacZ) M15] } was used as bacterial host cells.

3.2.3. Preparation of Competent Cells

Reagents: Autoclaved as above.

- Calcium chloride - 100 mM
A single colony of *E. coli* DHsa was inoculated in 5 ml LB medium (without ampicilline) and grown overnight at 37°C, 200 rpm to prepare the primary inoculum.

0.5 ml of this primary inoculum was added in the 50 ml of LB medium and grown for 1.5-2 h at 37°C and 200 rpm to reach the optical density (OD) of the culture 0.25-0.3 at 650 nm.

Culture was chilled to 0°C by keeping in the ice and centrifuged at 5000 xg, 4°C for 8 min.

The supernatant was discarded and the pellet was resuspended very gently in 16 ml ice cold calcium chloride.

Cells were incubated on ice for 20 min. and centrifuged as above.

Supernatant was discarded and pellet was resuspended in 8 ml ice cold calcium chloride very gently.

Ice cold glycerol was added to the suspension at a final concentration of 15%.

The resulting suspension was distributed in the aliquots of 0.25 ml and stored at -70°C which was stable for about six months.

**3.2.4. Transformation of *E. coli* DH<sub>5a</sub>**

The aliquot of the competent cells was thawed on ice and distributed in the fractions of 100 μl each.

Atleast 100 ng plasmid DNA was mixed with the cells and incubated on ice for 25 min.

Cells were given a heat shock at 42°C for 2 min. and immediately kept in ice for further 2 min.

The volume of the suspension was made upto 1 ml with LB medium and incubated at 37°C for atleast 1 h.

Cells were pelleted down by centrifugation at 12,000 xg for 30 sec.

The 0.8 ml supernatant was removed leaving behind 0.2 ml medium in the pellet.
• Cells were suspended in the remaining 0.2 ml medium and the suspension was spread on the LB agar plates supplemented with 100 μg/ml ampicilline (10 mg/ml stock prepared in 50 % ethanol).
• Plates were kept at the room temperature for 10 min. and then incubated at 37°C, overnight in an inverted position.

3.2.5. Minipreparation of Plasmid DNA from the Transformants

Reagents: Autoclaved.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEG lysis buffer</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>Tric-HCl (pH 8.0)</td>
<td>25 mM</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>10 N</td>
</tr>
<tr>
<td>SDS</td>
<td>10 %</td>
</tr>
<tr>
<td>Sodium acetate, (pH 5.2)</td>
<td>3 M</td>
</tr>
<tr>
<td>TE</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (pH 8.0)</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

• A single colony of the transformants was inoculated in 5 ml LB medium supplemented with 50 μg/ml ampicilline and grown at 37°C for atleast 6-7 h.
• Cells were pelleted down by centrifugation at 12,000 xg for 20 sec at 4°C in a microfuge tube and supernatant was discarded.
• The cell pellet was resuspended completely in 100 μl of ice cold TEG buffer by vortexing.
• 200 μl of freshly prepared SDS (1%, v/v) and sodium hydroxide (0.2N) solution was added to the bacterial suspension.
• The contents were mixed gently by inverting the tube to allow the complete lysis of the cells and incubated at room temperature for 5 min.
150 µl of ice cold sodium acetate solution was added to the suspension, mixed gently and incubated on ice for 10 min.

Centrifuged at 12,000 xg, 4°C for 10 min.

Supernatant was collected into the fresh microfuge tube and mixed with equal volume of phenol : chloroform : isoamylalcohol (25:24:1) mixture by vortexing.

Centrifuged at 12,000 xg for 5 min. at room temperature and aqueous phase was collected into fresh tubes.

Two volumes of absolute ethanol was added to it, mixed gently and incubated at -70°C for 30 min.

Centrifuged at 12,000 xg for 15 min. at 4°C.

Supernatant was removed, pellet was washed with 70 % ethanol and centrifuged as above.

The DNA pellet obtained was dried at 37°C for 5 min. and resuspended in TE.

3.2.6. Purification of Plasmid DNA Using Qiagen Kit

A primary culture of the bacterial cells harbouring the desired plasmid was prepared in 5 ml medium.

LB medium (250 ml) was inoculated with this culture (10 %) and grown at 37°C and at 200 rpm for atleast 12 h.

The culture was centrifuged and cell pellet collected, which was completely suspended in 10 ml of Qiagen buffer P1 by vortexing.

10 ml of Qiagen buffer P2 was added to this suspension and mixed gently.

Incubated at room temperature for 5 min.

Finally, 10 ml of Qiagen buffer P3 (chilled) was added to the suspension and mixed gently.

Incubated on ice for 20 min. and centrifuged at 12,000 xg for 30 min. at 4°C.

The supernatant was collected into a fresh tube and recentrifuged as above for 15 min. to clear the small protein particles.

In the mean time Qiagen Tip-500 column was equilibrated with 10 ml of buffer QBT.
After second centrifugation the supernatant was collected and loaded on the column. 
Column was washed two times with 30 ml buffer QC each time. 
Finally, DNA was eluted from the column by 15 ml buffer QF. 
The DNA was precipitated with 10.5 ml isopropanol (0.7 volume) and centrifuged at 12,000 xg for 30 min. at 4°C. 
DNA pellet was washed with 70 % ethanol and recentrifuged as above for 15 min. 
Pellet was dried at 37°C for 5-8 min. and resuspended in 0.5 ml TE. 
1 μl of this suspension was electrophoresed on 0.8 % agarose gel. 
The Qiagen column purified DNA was used in the transfection of mammalian cells.

3.2.7. Agarose Gel Electrophoresis

Reagents:
Running buffer: TBE (20X);
- Tris buffer (w/v) - 21.6%
- Boric acid (w/v) - 11%
- EDTA (v/v; 0.5 M, pH 8.0) - 8%

TAE (50X);
- Tris buffer (w/v) - 24.2%
- Acetic acid (v/v) - 5.71%
- EDTA (v/v; 0.5M, pH 8.0) - 10%

0.8-1.0 % agarose was boiled in TAE or TBE buffer (1X) and cooled to 45°C, ethidium bromide added to a final concentration of 0.5 μg/ml and poured into the gel plate to polymerize for 15-20 min.

Upon complete polymerization the gel was put in the apparatus and submerged into the running buffer.
• Samples were mixed with the tracking dye (generally bromophenol blue) and loaded in the wells.
• Gel was run at 100 V at room temperature.
• DNA bands were visualized in the UV transilluminator.

3.2.8. Restriction Digestion of DNA
The restriction enzymes were procured from the standard companies like, Promega, Boehringer Mannheim, New England Biolabs and stored at -20°C.

Reaction mixture: The reaction mix was prepared on ice.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1 µl (&lt;1 µg)</td>
</tr>
<tr>
<td>Enzyme buffer (10X)</td>
<td>2 µl (1X)</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>1 µl (10 units)</td>
</tr>
<tr>
<td>Water (sterile)</td>
<td>16 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The contents were mixed and incubated at 37°C for 2-12h according to the requirement. The digested DNA was resolved on the agarose gel.

3.2.9. Elution of DNA from the Agarose Gel
• Portion of the agarose gel containing the desired band of DNA was excised and chopped into small pieces.
• A tiny hole was made at the bottom of the micrufuge tube and blocked with a plug of glass wool.
• The agarose pieces were kept in the tube placed in another tube and centrifuged at 10,000 xg for 10 min. at room temperature.
• The eluate was collected from the lower tube and precipitated with absolute ethanol as described in previous section.
• The DNA pellet was dried and suspended in TE and 1 µl of it was checked on agarose gel.
3.2.10. Ligation Reaction

The DNA ligase was procured from Boehringer Mannheim and stored at -20°C. The reaction mixture was prepared on ice. Usually, for ligation reaction an insert to vector ratio of 3 : 1 was used.

Insert DNA - 7 µl (~30ng)
Vector DNA - 1 µl (~10ng)
Ligase buffer (10X) - 1 µl (1X)
DNA Ligase - 1 µl (1 unit)
Total - 10 µl

The contents were mixed and incubated at 20°C for 8-10 h. The ligation mixture was transformed into the *E. coli* cells and plated on LB plates as described above.

3.2.11. DNA Probe Preparation by Random Priming Method

- Approximately 50 ng of DNA was taken and its volume was made up to 30.5 µl with water.
- DNA was denatured at 100°C for 5 min. and immediately kept on ice.
- The reaction mixture was prepared as follows:
  Denatured DNA - 30.5 µl
  Labeling buffer (5X) - 10 µl
  Primer (10X) - 5 µl
  αdCTP [³²P] - 3 µl
  Klenow enzyme - 1.5 µl (1.5 unit)
- The contents were mixed and incubated at 37°C for 30 min.
- The volume of the mixture was made up to 100 µl with 50 µl TE.
- A 1 ml spin column of Sephadex G-50 was packed in a 1 ml syringe and loaded with the reaction mixture.
- Centrifuged at 3000 xg for 4 min. and eluate was collected in a microfuge tube.
The purified probe was stored at -20°C.

3.2.12. Dot Blot

Reagents:
- Sodium hydroxide: 0.5N
- Dot blot solution:
  - Ammonium acetate: 1M
  - Sodium hydroxide: 0.02M
- Ammonium acetate: 5M

The cells were lysed in 400 μl of 0.5N sodium hydroxide and then mixed with 40 μl of ammonium acetate solution.

Two Whatmann papers (3mm) and nitrocellulose membrane were cut of the size 11.5 x 8 cm.

The membrane was soaked in warm water for a while and then in dot blot solution.

For blotting, the wet papers were placed over the dot blot apparatus followed by the membrane.

The apparatus was clamped and connected to the vacuum pump.

Different dilutions of the samples were added into the wells of the apparatus and vacuum was applied.

The membrane was removed and the blotted surface was marked with a pencil.

The membrane was baked for 2h at 80°C to crosslink the DNA.

3.2.13. Hybridization

SSC (20X)
- Prehybridization solution: 10 ml
- SSC (20X): 2.5 ml
- Denhardts reagent (50X): 1 ml
- SDS (10 %): 0.5 ml
Salmon sperm DNA - 10 μl
Water - 6 ml

• The baked nitrocellulose membrane was soaked in 2X SSC for 2 min.
• It was placed in a plastic bag and the prehybridization solution was added to the bag.
• The bag was sealed after removing the air bubbles and incubating at 56°C for 4h with constant shaking.
• The radioactive DNA probe was denatured at 100°C for 5-10 min. and added into the plastic bag.
• Incubated overnight at 56°C with constant shaking.
• Next day, the membrane was removed and washed with 2X SSC at room temperature for 5 min. two times.
• Next washing was done with 0.2X SSC for 15 min. at 56°C.
• Finally, the membrane was washed with 0.2X SSC + 0.1% SDS for 15 min. at 65°C.
• The membrane was wrapped in the Saran wrap and exposed to x-ray film. The x-ray film was developed as described in the previous chapter.

3.2.14. Polymerase Chain Reaction (PCR)

All the reagents and Taq polymerase enzyme for the amplification of the DNA fragments were procured from Promega Corp. USA. The oligonucleotide primers were designed using the computer software programme Lasergene Navigator and later the initiation codon, termination codon and required restriction sites were added in the sequence. A Kozak sequence was also added upstream to the initiation codon in the upstream primers. The primers were obtained from Rama biotech, India as a lyophilized powder between the OD 6.0-8.0 at 260 nm. The amount of DNA in the primers was calculated using the formula:

\[ 1.0 \text{ A}_{260} \text{ unit ssDNA} = 33 \mu g/ml \]
The lyophilized primers were reconstituted in sterile water at a concentration of 1 μg/μl and stored in small aliquots at -20°C. For a typical PCR reaction the reaction mixture was prepared in a PCR tube (0.5 ml) as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium chloride (25 mM)</td>
<td>- 1.5 mM</td>
</tr>
<tr>
<td>Polymerase buffer (10X)</td>
<td>- 1X</td>
</tr>
<tr>
<td>dNTP mix (10 mM)</td>
<td>- 200 μM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>- 1 ng</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>- 1 unit</td>
</tr>
<tr>
<td>Primer 1</td>
<td>- 0.5 μg</td>
</tr>
<tr>
<td>Primer 2</td>
<td>- 0.5 μg</td>
</tr>
<tr>
<td>Water</td>
<td>- remaining volume</td>
</tr>
<tr>
<td>Total</td>
<td>- 50 μl</td>
</tr>
</tbody>
</table>

The contents were mixed thoroughly and overlaid with three drops of mineral oil. Reaction conditions were set in the PCR machine and tubes were kept in the reaction block.

**Reaction conditions**: Total 35 cycles, 10 cycles 25 cycles
- Denaturing temperature: 94°C, 1 min. 94°C, 1 min.
- Annealing temperature: 42-47°C, 1 min. 50-55°C, 1 min.
- Primer extension temperature: 72°C, 2.5-3 min. 72°C, 2.5-3 min.

- A final cycle was done at 72°C for 5 min. to extend all the incompletely amplified DNA fragments.
- 5 μl of it was checked on agarose gel to confirm the amplification of the desired fragment.

The amplified PCR fragments were purified on the gel using the procedure described above and subjected to the restriction digestion. The digested fragments were again precipitated with absolute ethanol to remove the enzyme proteins, resuspended in TE or water and used as insert in the cloning.
3.2.15. Transfection and Metabolic Labeling of the Cellular Proteins

MDCK, CV1 and COS1 cells were grown in 35 mm well size culture plates at 50-80% confluency. Cells were initially washed two times with serum-free MEM and transfected using lipofectin-mediated transfection procedure. Briefly, DNA and 15 µg lipofectin (10 mg/ml stock; Gibco BRL, USA) were diluted separately in 100 µl serum-free MEM and incubated at room temperature for 30 min. Both the solutions were mixed together and incubated further at room temperature for 15 min. The volume of the DNA-lipofectin mixture was adjusted to 1 ml by adding 0.8 ml serum-free MEM. The complex was added to the cells and the mixture incubated for 5 h at 37°C. The DNA-lipofectin complex was then replaced with 2 ml of fresh MEM supplemented with 10 % FCS and the cells were further incubated at 37°C for desired periods of time.

For 35S-methionine labeling of the cellular proteins in the transfected cells at the desired period of time, the old medium was removed and the cells washed two times with methionine-free MEM and then 1 ml methionine-free MEM supplemented with 100 µCi/ml L-[35S]-methionine (1175 Ci/m mole, Dupont NEN) was added. Cells were incubated with the labeling mixture for 2 h at 37°C and harvested for further analysis.

3.2.16. Immunoprecipitation and SDS-PAGE

35S-labeled cells were harvested and resuspended in 1 ml cold phosphate-buffered saline (PBS, pH 7.5). Cells were centrifuged at 5,000 xg for 30 sec at 4°C. Supernatant was discarded and the cells were lysed in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-Cl (pH 7.5); 150 mM NaCl; 1% TritonX-100; 0.1% sodium dodecyl sulfate (SDS); 0.5% sodium deoxycholate; 1 mM phenylmethylsulphonylflouride (PMSF) and protease inhibitor cocktail (Boehringer Mannheim, Germany)]. The cell lysate was incubated on ice for 10 min. Cellular debris was cleared by centrifugation at 12,000 xg for 10 min. at 4°C. Supernatant was collected and used for immunoprecipitation. For
immunoprecipitation, 5 μl polyclonal rabbit anti-NP (1:100 dilution), which was prepared as described earlier (Husain & Gupta, 1997), was added to the lysate. The mixture was incubated on ice for 2 h. 7.5 mg Protein A-sepharose (Amersham-Pharmacia biotech, England) was added to the suspension and the mixture incubated at 4°C for 4 h under gentle shaking. The sepharose beads containing the immunoprecipitate were pelleted by centrifugation at 5,000 xg for 5 min. at 4°C. Supernatant was discarded and sepharose beads were washed two times with 0.5 ml RIPA buffer containing 5 mg/ml bovine serum albumin followed by another wash with RIPA buffer alone. The beads were mixed with equal volume of 2X SDS-sample buffer, heated at 100°C for 10-15 min. and centrifuged at 12,000 xg, 4°C for 5 min. The supernatant containing the proteins was analysed by 10 % SDS-PAGE.

To study the interaction of NP and its mutants with the cytoskeleton, 35S-labeled cells were harvested, washed with PBS and fractionated into triton-soluble and triton-insoluble fractions as described in the previous chapter. Triton-insoluble proteins were solubilized in SDS-buffer. Volume of both triton-soluble and insoluble fractions was raised to 0.5 ml by adding RIPA buffer. NP polypeptides were immunoprecipitated from both the fractions as above and resolved on the SDS-PAGE.

Protein bands were visualized by fluorography followed by autoradiography. Each gel was stained with Coomassie blue prior to fluorography to monitor the presence of equal amount of proteins in each lane. Densitometric scanning of the radioautograms was carried out on Pharmacia Ultrascan XL. Peak area of each band was determined and used for the comparative analysis of the NP-cytoskeleton interactions.
3.3. RESULTS

3.3.1. Cloning of NP Gene in Plasmid pcDNA3

Plasmid pcDNA3 was used as an expression vector for cloning and expression of influenza viral NP gene and its deletion mutants in COS1 cells (Fig. 3.1). For cloning the NP gene, plasmid CMV-NP which contained the influenza virus type A/PR8/34 NP gene cloned in plasmid pcDNA1 was digested with EcoRI and XhoI to release the 1.5 kb gene insert (Fig. 3.2), and then the NP gene ligated with plasmid pcDNA3 previously digested with EcoRI/XhoI and transformed into E. coli cells. The recombinant plasmid pMHNP (pcDNA3 with NP) was isolated from the transformants and checked for the presence of NP insert by restriction digestion (Fig. 3.8).

3.3.2. Cloning of NP Gene in Plasmid pGEM

To use the T7 promoter sequence present in the plasmid pGEM (a linearized plasmid backbone obtained after release of the PCR fragment cloned in plasmid pGEM-T easy by EcoRI/XbaI digestion) as an upstream primer to create the C-terminal deletions in NP gene by PCR, NP gene was cloned in plasmid pGEM. This plasmid was first digested with EcoRI followed by Nsil to create the EcoRI and NsiI overhangs. The NP gene was released from the plasmid CMV-NP by sequential digestion with the same enzymes. Both the DNAs were ligated and transformed into E. coli cells. The recombinant plasmid pGNP (pGEM with NP) was checked by restriction analysis (Fig. 3.3).

3.3.3. Construction of Deletion Mutants of NP

All the deletion mutants of influenza viral NP were constructed using the conveniently available EcoRI and XbaI sites of pcDNA3 except the mutant NPΔM85 for which the EcoRI and XhoI sites of pcDNA3 were used. To construct this mutant plasmid CMV-NP was digested with BglII (at nucleotide positions 807 and 1062) and religated. This deleted NP gene was then subcloned into pcDNA3 at the EcoRI/XhoI sites, the resulting plasmid was denoted as pNPBgl. Mutant
Fig. 3.1. Mammalian expression vector pcDNA3.
Fig. 3.2. Plasmid CMV-NP containing NP gene of influenza virus A/PR8/34. Lane 1, λ/HindIII marker; Lane 2, plasmid CMV-NP; Lane 3, CMV-NP digested with EcoRI/Xhol.
Fig. 3.3. Restriction analysis of recombinant plasmid pGNP. Lane 1, 1 kb DNA ladder; Lane 2, pGNP digested with EcoRI; Lane 3, pGNP digested with Nsil; Lane 4, pGNP digested with EcoRI/XhoI; Lane 5, pGNP digested with HindIII/XhoI; Lane 6, intact pGNP.
NPΔC165 was constructed using single SphI site present in NP gene. The CMV-NP was digested with SphI at the nucleotide position 1043 in the NP gene and at another SphI site present at the 3' end in the multiple cloning sites (MCS) of the vector. The digested CMV-NP was religated and the deleted NP gene was subcloned into pcDNA3 at EcoRI/XbaI sites. The resulting plasmid designated as pNPS1.

The remaining NP deletion mutants used in this study were generated by PCR amplification (Fig. 3.4; Table 3.1). The DNA fragments amplified by PCR were checked on 0.8 % agarose gel (Fig. 3.5 A, B, C). For generating the fragments (c), (d) and (e) plasmid pGNP was used as template and for rest of the fragments CMV-NP was used as template. The PCR generated fragments (c), (d) and (e) were double digested with EcoRI and XbaI and cloned directly into EcoRI/XbaI sites of pcDNA3 (Fig. 3.6) and the recombinant plasmids designated as pNPC1, pNPC2, pNPC3, respectively. The PCR generated fragments (a), (b), (f), (g) and (h) were cloned first in plasmid pGEM-T easy. Some polymerases, e.g. Taq polymerase, add one extra adenine nucleotide at the 3' end of the amplified DNA fragment leading to a 3'-A overhang. Plasmid pGEM-T has 3'-T overhang which helps in the direct ligation of PCR generated fragment with 3'-A overhang. It also offers a choice of common restriction sites in the MCS to subclone the PCR product in another vector. So each fragment cloned in this way in pGEM-T easy was released by double digestion with EcoRI/XbaI and the resulting restriction fragments were subcloned into pcDNA3 (Fig. 3.7). The recombinant plasmids containing the fragments (a), (b), (f), (g) and (h) were called as pNPN1, pNPN2, pNPN1C1, pNPN1C2 and pNPN2C1, respectively. The right orientation of each clone was confirmed by restriction analysis (Fig. 3.8) and/or automated DNA sequencing (ABI 37718 system).

3.3.4. Expression of Wild type NP

Wild type nucleoprotein (NP) gene of influenza A/PR8/34 virus was subcloned in mammalian expression vector pcDNA3 and expressed under CMV

![Image](attachment:image.png)
Fig 3.4. Amplification of DNA sequences of the deletion mutants by PCR.
Table 3.1. Sequences of oligonucleotide primers used to create the deletion constructs of NP by PCR.

<table>
<thead>
<tr>
<th>Sequences Amplified by PCR</th>
<th>Upstream Primers</th>
<th>Downstream Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>145-1542 NP AN33</td>
<td>5'-TGC ATGCAT C ATG GGT GGA ATT GGA CGA TTC-3'</td>
<td>5'-TGC TCTAGA TTA ATT ATC GTA TTC CTC TGC-3'</td>
</tr>
<tr>
<td>169-1542 NP AN41</td>
<td>5'-TGC ATGCAT C ATG CAA ATG TGC GAA CTC AAA CTC-3'</td>
<td>5'-TGC TCTAGA TTA ATT ATC GTA TTC CTC TGC-3'</td>
</tr>
<tr>
<td>46-1491 NP AC16</td>
<td>5'-GTA ATA CGA CTC ACT ATA GGG C-3'</td>
<td>5'-TGC TCTAGA TTA ACT CAT GTC AAA GGA AGG CAC GAT-3'</td>
</tr>
<tr>
<td>46-1428 NP AC37</td>
<td>5'-GTA ATA CGA CTC ACT ATA GGG C-3'</td>
<td>5'-TGC TCTAGA TTA CCG CCC CTG GAA AGA CAC AT-3'</td>
</tr>
<tr>
<td>46-1404 NP AC45</td>
<td>5'-GTA ATA CGA CTC ACT ATA GGG C-3'</td>
<td>5'-TGC TCTAGA TTA TGG TCT TGC ACT TTC CAT CAT C-3'</td>
</tr>
<tr>
<td>145-1491 NP AN33C16</td>
<td>5'-TGC ATGCAT C ATG GGT GGA ATT GGA CGA TTC-3'</td>
<td>5'-TGC TCTAGA TTA ACT CAT GTC AAA GGA AGG CAC GAT-3'</td>
</tr>
<tr>
<td>145-1428 NP AN33C37</td>
<td>5'-TGC ATGCAT C ATG GGT GGA ATT GGA CGA TTC-3'</td>
<td>5'-TGC TCTAGA TTA CCG CCC CTG GAA AGA CAC AT-3'</td>
</tr>
<tr>
<td>169-1491 NP AN41C16</td>
<td>5'-TGC ATGCAT C ATG CAA ATG TGC GAA CTC AAA CTC-3'</td>
<td>5'-TGC TCTAGA TTA ACT CAT GTC AAA GGA AGG CAC GAT-3'</td>
</tr>
</tbody>
</table>
Fig. 3.5 A. PCR amplified DNA fragments a (lane 2), b (lane 3). Lane 1, 1 kb DNA ladder.
Fig. 3.5 B. PCR amplified DNA fragments c (lane 2), d (lane 3), e (lane 4). Lane 1, 1 kb DNA ladder.
Fig. 3.5 C. PCR amplified DNA fragments f (lane 2), g (lane 3), h (lane 4). Lane 1, 1 kb DNA ladder.
Fig. 3.6. Cloning strategy of PCR generated DNA fragments c, d and e.
NP gene

PCR amplification

Amplified DNA Fragment with 3'-A overhang

pGEM-T easy with cloned PCR fragment

Linearized plasmid pGEM-T easy with 3'-T overhangs

Double digestion with EcoR1 and Xba1

+ insert DNA with 5'-overhangs

Recombinant plasmid with desired deletion in NP gene

Fig. 3.7. Cloning strategy of PCR generated DNA fragments a, b, f, g and h.
Fig. 3.8. Restriction digestion of all recombinant clones.
promoter in mammalian cells. For the expression of NP gene, the plasmid pMHNP which contains the NP gene cloned in pcDNA3 was transfected into MDCK cells using Lipofectin. After 24 h and 48 h of the transfection cells were pulse labeled, harvested and an aliquot of the cells was saved and the cell pellet was stored at -20°C. Rest of the cells were lysed, NP was immunoprecipitated and analysed by SDS-PAGE. The aliquoted cells were lysed and checked for the presence of the plasmid DNA in the cells by dot blot hybridization. Different dilutions of the cell lysate were blotted on the nitrocellulose membrane which was incubated with 32P-labeled NP probe. Upon autoradiography it was observed that an insignificant amount of the plasmid DNA could be transfected into MDCK cells, and with this DNA expression NP polypeptide could not be detected (data not shown). In the next experiment CV1 cells (African green monkey kidney cell line) were used for the expression of the gene. CV1 cells were transfected and analysed as above, and it was observed that the plasmid DNA was being delivered in the cells (Fig. 3.9) but surprisingly, no expression of NP could be detected. A possible explanation of it could be the incompatibility of CV1 cells machinery with the CMV promoter of the vector. Finally, the COS1 cells (SV40-transformed African green monkey kidney cell line) were used for the study. The COS1 cells were transfected initially using different concentrations of pMHNP DNA (2.5 μg, 5.0 μg, 10 μg) and the plasmid was checked by dot blot (Fig. 3.10). The transfected cells at 12 h posttransfection were pulse-labeled with 35S-methionine and NP was immunoprecipitated from the cell lysate using anti-NP antibodies. Proteins were resolved on the SDS-PAGE. Expression of NP was observed in COS1 cells which was maximum in the cells that were transfected with 2.5 μg of DNA, as compared to those transfected with 5 μg or 10 μg of DNA (Fig. 3.11). To determine the best time point after transfection for radiolabeling of the NP polypeptides in the transfected cells, the COS1 cells were transfected with 2 μg of DNA as described above and then pulse labeled at 6h, 12h, and 18h with 35S-methionine. NP polypeptides were immunoprecipitated from the cell lysates using anti-NP antibodies. The immunoprecipitate was resolved by electrophoresis on SDS-
Fig. 3.9. Dot Blot hybridization of transfected MDCK and CV1 cell lysates using NP probe.
Fig. 3.10. Dot Blot hybridization of transfected COS1 cell lysate using NP probe.
Fig. 3.11. Expression of NP in COS1 cells.
The maximum labeling of NP polypeptides was achieved at 18h post-transfection (Fig. 3.12). In all subsequent experiments 2.5 μg DNA for the transfection and 18h posttransfection time point for pulse labeling were used.

### 3.3.5. Interaction of NP with the Cytoskeleton

It has previously been shown that NP interacts with the host cytoskeleton (triton-insoluble proteins) during influenza virus infection in MDCK cells (Avalos et al., 1997; Husain & Gupta, 1997). To map the region(s) of NP polypeptide involved in this interaction, a series of N-terminal, C-terminal, and both N- and C-terminal deletion mutants as well as the central portion deleted mutant of NP were constructed in the mammalian expression vector pcDNA3 (Fig. 3.13). The cytoskeleton of the COS1 cells was isolated using the procedure described in the previous chapter. Cytoskeletal proteins were resolved on 10% SDS-PAGE and the bands were visualized by Coomassie blue staining. A prominent actin band (43 kDa) was observed in the triton-insoluble fraction (Fig. 3.14).

To analyse the host cytoskeleton-NP interactions, the COS1 cells were transfected with the deletion mutants of NP along with the wild type NP. The transfected cells were radiolabeled with $^{35}$S-methionine and fractionated into triton-soluble and triton-insoluble fractions. NP polypeptides from both the fractions were immunoprecipitated using anti-NP antibodies. The immunoprecipitate was analysed on SDS-PAGE followed by autoradiography. Autoradiograms were scanned and the protein bands were quantitated by densitometric analysis. Fig. 3.15, shows that deletion of N-terminal amino acid (aa) residues of NP led to a significant decrease in its triton-insolubility. While about 50% NP was triton-insoluble in case of the wild type, only about 32 and 25% triton-insolubility was observed upon deleting 33 aa and 41 aa residues, respectively, from the N-terminal end of NP (Table 3.2). These results demonstrate that the N-terminus of NP is involved in binding with the cytoskeleton of the COS1 cells. To analyse the role of the C-terminus of the protein in binding with the cytoskeleton, C-terminus deleted mutants were constructed. Like the N-
Fig. 3.12. Time dependent expression of NP in COS1 cells.
Fig. 3.13. Digrammatic representation of different deletion mutants of NP constructed in plasmid pcDNA3.
Fig. 3.14. Triton-soluble (S) and triton-insoluble (I) proteins of COS1 cells.
Fig. 3.15. Effect of the N-terminal deletions on the triton-insolubility of NP. T, total cell lysate; S, triton-soluble fraction; I, triton-insoluble fraction.
Table 3.2. Effect of different amino acid residue deletions on the triton-solubility and triton-insolubility of NP.

<table>
<thead>
<tr>
<th>Deletion Mutants of NP</th>
<th>NP in TritonX-100 soluble fraction (% of total*)</th>
<th>NP in TritonX-100 insoluble fraction (% of total*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type NP</td>
<td>47.58 ± 6.31 (12)</td>
<td>52.40 ± 6.31 (12)</td>
</tr>
<tr>
<td>NP ΔN33</td>
<td>67.66 ± 8.48 (3)</td>
<td>32.33 ± 8.48 (3)</td>
</tr>
<tr>
<td>NP ΔN41</td>
<td>75.12 ± 5.59 (3)</td>
<td>24.86 ± 5.59 (3)</td>
</tr>
<tr>
<td>NP ΔC16</td>
<td>92.70 ± 1.58 (3)</td>
<td>7.29 ± 1.58 (3)</td>
</tr>
<tr>
<td>NP ΔC37</td>
<td>78.19 ± 4.44 (3)</td>
<td>21.79 ± 4.44 (3)</td>
</tr>
<tr>
<td>NP ΔC45</td>
<td>80.40 ± 2.30 (3)</td>
<td>19.58 ± 2.30 (3)</td>
</tr>
<tr>
<td>NP ΔC165</td>
<td>88.26 ± 1.25 (3)</td>
<td>11.72 ± 1.25 (3)</td>
</tr>
<tr>
<td>NP ΔN33C16</td>
<td>70.40 ± 5.46 (3)</td>
<td>29.58 ± 5.46 (3)</td>
</tr>
<tr>
<td>NP ΔN33C37</td>
<td>98.66 ± 1.89 (3)</td>
<td>1.33 ± 1.89 (3)</td>
</tr>
<tr>
<td>NP ΔN41C16</td>
<td>84.47 ± 2.65 (3)</td>
<td>15.51 ± 2.65 (3)</td>
</tr>
<tr>
<td>NP ΔM85</td>
<td>44.82 ± 1.4 (3)</td>
<td>55.17 ± 1.4 (3)</td>
</tr>
</tbody>
</table>
terminus, C-terminus deletions also led to considerable decrease in the triton-insolubility of NP (Fig. 3.16). As much as about 93% of NP became triton-soluble by deleting 16 aa residues from the C-terminus. However, this increased triton-solubility was slightly decreased by further deleting the C-terminal amino acid residues (Table 3.2).

It would appear that both the N- and C-terminal ends of NP are involved in its interactions with the host cell cytoskeleton. To analyse it further, mutants with deletions at both the N- and C-terminal ends of NP were constructed and then their interactions were examined with the triton-insoluble proteins. Fig. 3.17 shows that the interaction of NP with the cytoskeleton considerably decreased by using NPΔN33C16 and NPΔN41C16 mutants and virtually abolished by deleting 33 aa residues from the N-terminus and 37 aa residues from the C-terminus of NP (Table 3.2). These results clearly demonstrate that both the N- and C-termini of NP are involved in its interaction with the host cell cytoskeleton. This is further supported by the observation that deletion of 85 aa residues from the central region of the NP polypeptide did not affect its triton-insolubility as compared to the wild type (Fig. 3.18, Table 3.2).

In all the above experiments apart from the immunoprecipitation of NP by anti-NP antibodies, in most of the results an extra band (probably representing actin) was observed which was being coimmunoprecipitated with NP or its mutant polypeptides. To convincingly document the binding of NP with the actin filament components of the COS1 cytoskeleton, a coimmunoprecipitation experiment was carried out. For this, cell lysates of NP transfected and mock transfected COS1 cells were divided into two equal parts. One half was immunoprecipitated with anti-actin monoclonal antibodies (Sigma, USA) and the other half was immunoprecipitated with anti-NP antibodies. As shown in Fig. 3.19, anti-actin antibodies coimmunoprecipitated NP along with actin. Similarly, anti-NP antibodies coimmunoprecipitated NP and actin, indicating that NP directly interacted with actin cytoskeleton of COS1 cells.
Fig. 3.16. Effect of the C-terminal deletions on the triton-insolubility of NP. T, total cell lysate; S, triton-soluble fraction; I, triton-insoluble fraction.
Fig. 3.17. Effect of the N- and C-terminal deletions on the triton-insolubility of NP. T, total cell lysate; S, triton-soluble fraction; I, triton-insoluble fraction.
Fig. 3.18. Effect of the deletion of amino acid residues from the central region of NP on its triton-insolubility.
Fig. 3.19. Coimmunoprecipitation of NP as well as actin with anti-actin and anti-NP antibodies.
These results clearly demonstrate that influenza viral NP associates with the actin filament component of the cytoskeleton and that both the N-terminus and C-terminus of NP are involved in this interaction.

3.4. DISCUSSION

Influenza A virus is an externally enveloped animal virus which buds out from the host cells after its encapsulation in their plasma membrane. The budding sites in the membrane are first decorated by specific viral glycoproteins mainly haemagglutinin (HA) and neuraminidase (NA), where ribonucleoprotein (RNP) particles traverse to affect the final virus assembly and subsequent budding (Petterson et al., 1988; Garoff et al., 1998). This process in principle may require an active involvement of the host cell cytoskeleton apparatus, as the various components of this apparatus are known to be required not only for guiding and movement of the intracellular material to be exported out of the cells, to the plasma membrane but also modifying the plasma membrane particle distribution and deformability properties (Geiger, 1983; Kelly, 1990). It may be speculated here that modification in the host cell plasma membrane bilayer-cytoskeleton interactions is caused by an interaction of influenza viral proteins with one or more protein components of the actin cytoskeleton.

Earlier studies have suggested that influenza viral NP and M1 protein interact with cytoskeletal actin in the influenza virus-infected Madin-Darby canine kidney cells (Husain & Gupta, 1997). Whereas M1 protein seems to indirectly interact with the host cell cytoskeleton (Zhang & Lamb, 1996; Avalose et al., 1997), direct interactions appear to occur between influenza viral NP and actin cytoskeleton (Husain & Gupta, 1997; Avalose et al., 1997). To further analyse the specificity of the NP-cytoskeleton interactions, several deletion mutants of this protein were created in this study and expressed in COS1 cells, then the association of these proteins with the triton-insoluble cytoskeletal proteins was analysed as described in Materials and Methods. Results of these studies further
demonstrate that the influenza viral NP directly interacted with the actin cytoskeleton of COS1 cells. This interaction of NP with the cytoskeleton was decreased by deleting the N-terminal or C-terminal amino acid (aa) residues of the protein, suggesting that both the ends of this protein are perhaps involved in its binding with the cytoskeletal components. This is further confirmed by the finding that in case of the NPΔN33C37 mutant, the binding was virtually abolished by deleting 33 aa and 37 aa residues from the N-terminal and C-terminal ends, respectively. However, the central portion of NP virtually played no role in its binding with the actin cytoskeleton, as no effect on triton-insolubility of NP was observed after deleting 85 aa residues from its central region in the mutant NPΔM85. It is intriguing to observe that the deletion beyond the sixteenth aa residue from the C-terminus of NP led to an increase in its triton-insolubility, as compared to NPΔC16 mutant. Similar trend was observed by further deleting 33 aa or 41 aa residues from the N-terminus of this mutant. It is therefore likely that simultaneous deletions at both the N- and C-terminal ends as well as aa deletions beyond the 16th aa residue from the C-terminus of NP could lead to the mutant proteins which have significantly different structures and consequently the different actin binding properties than the wild type NP.

It is likely that the phosphorylation/dephosphorylation plays a role in these interactions. NP is a phosphoprotein and phosphorylation/dephosphorylation is known to regulate many biological functions, including protein-protein interactions. Influenza A/PR8/34 has serine residues at both the N-terminal end (aa position 3) and C-terminal ends (aa position 473) which are known to be phosphorylated (Kistner et al., 1989). It is then possible that NP-cytoskeleton association may partly involve electrostatic interactions which is weakened upon deleting the above serine residue(s) This is well supported by our present finding that the mutant NPΔN33C37 which has a deletion of both the N- and C-terminal serine residues virtually failed to interact with cytoskeleton. Although, no direct evidence on the involvement of these serine residues in NP-cytoskeleton interactions is available,
but its direct importance may be ascertained from a recent report which describes the effects of replacement of these serine residues by alanine (Arrese & Portela, 1996).

The binding of NP with actin cytoskeleton should, in principle, modify the cytoskeleton structure by affecting the intermolecular interactions within the cytoskeletal matrix, which could, in turn, facilitate its dissociation from the overlying membrane bilayer (Geiger, 1983; Pumplin & Bloch, 1993). The thus-dissociated region of membrane bilayer may now serve as the budding sites in the plasma membrane. Similar function in case of paramyxoviruses, like Sendai virus (Giuffre et al., 1982; Senderson et al., 1995) and Newcastle disease virus (Higley & Way, 1997) may be served by the viral matrix protein. Also F17R protein of vaccinia and myxoma viruses (Reckmann et al., 1997) could play a role similar to that of the influenza viral NP in these infections. Further, the HIV Gag protein may at least partially fulfill its role in virus budding (Rey et al., 1996; Cudmore et al., 1997) by causing structural modification in the actin cytoskeleton.

Influenza virus NP is a 498 aa residues containing polypeptide which is quite rich in arginine residues (Huddleston & Brownlee, 1982). Most of these residues are, however, much away from both the N- and C-terminal ends. Since the actin cytoskeleton binding considerably decreased by separately deleting aa residues from the N- or C-terminus of NP and virtually abolished by deleting 33 aa residues from the N-terminal and 37 aa residues from the C-terminal ends which reduced the number of arginine residues only very marginally in the mutant proteins, it is inferred that the interactions between NP and actin cytoskeleton are not purely ionic in nature. It is suggested that after binding with cytoskeletal matrix at both its ends, the remaining portion of NP, which is quite rich in arginine residues, may offer additional binding site(s) for the other negatively charged viral components, e.g. vRNA and polymerase proteins. Further, nonspecific association of these binding sites on NP could also help in translocation of vRNP to the sites of budding in the host cell plasma membrane.