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Cloning and expression analysis of multiple proteins encoding P gene of Newcastle disease virus

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Viral gene oncotherapy is emerging as a biotherapeutic cancer treatment modality based on targeted killing of cancer cells by viral genes. Newcastle disease virus (NDV) has the property to cause selective oncolysis of tumor cells sparing normal cells. NDV has a single stranded negative sense RNA genome, which is 15,186 nucleotide long and consists of six genes, which codes for eight proteins. NDV like other paramyxoviruses has the ability to generate multiple proteins from the P gene. P protein is encoded by an unedited transcript of the P gene, whereas the V and W protein are the results of RNA editing event in which one and two G residues are inserted at a conserved editing site within the P gene mRNA resulting in V and W transcripts, respectively. Although NDV is known to cause oncolysis by triggering apoptosis, the role of different viral proteins in selective oncolysis is still unclear. P gene edited products are known for its anti-apoptotic property in homologous host. In the present study, NDV P gene and its RNA edited products were amplified, cloned, sequenced and in vitro expression was done in HeLa cells. Further constructs were assayed for their apoptosis inducing ability in HeLa cells. Preliminary study suggested that P, V and W proteins are not apoptotic to HeLa cells.

Keywords: Annexin V assay, *In vitro* expression, Newcastle disease virus, P gene, RNA editing, Viral gene oncotherapy

Newcastle disease (ND) is a highly contagious disease of almost all avian species that has caused substantial loss to the industry around the world[1]. The disease has world wide distribution and poses a major threat to poultry industry. NDV has been classified as a member of the newly defined genus *Avulavirus* in the family *Paramyxoviridae*[2]. NDV has a single stranded negative-sense RNA genome, which is 15,186 nucleotides long[3,4]. The genomic RNA contains six genes encoding at least eight proteins[5]. Among these, nucleoprotein (NP), the phosphoprotein (P), and the large polymerase protein (L) forms the nucleocapsid. NDV like other Paramyxoviruses has the ability to generate multiple proteins from the P gene. P protein is encoded by an unedited transcript of the P gene, whereas the V and W proteins are the result of an RNA-editing event in which one and two G residues are inserted at a specific position within the P gene mRNA resulting in V and W transcripts, respectively[6]. P protein is encoded by P mRNA transcript of 1451 nucleotides. The protein comprises of 395 amino acid residues[7]. The P gene ORF encodes an unedited version of mRNA, whereas addition of one and two non-template dependent G residues at position 484 in the sequence 476-UAAAAAGGGCCCA-489 yields mRNAs that encodes for V and W protein, respectively[8]. Paramyxovirus V proteins are readily identifiable by a highly conserved cysteine-rich domain at their C-termini derived from the overlapping ORF[8,9]. This conserved C-terminal domain (CTD) is approximately 50% identical among all paramyxovirus V proteins and contains seven invariant cysteine residues. It has been shown that V protein plays an important role in preventing IFN responses and apoptosis in chicken cells, but not in human cells. These species specific effects of V make it a determinant of host range restriction[10]. The V and W share their amino (N) - terminal domains with P protein and vary at their carboxy (C) termini. NDV has the property to cause selective oncolysis of tumor cells sparing normal cells[11]. It exploits the defects in interferon signaling pathway to selectively kill the

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cells[12], however, newer mechanisms have also been suggested[13]. NDV is one of the widely studied viruses for virotherapy. Viral gene oncotherapy is an emerging biotherapeutic cancer treatment modality which is based on selective infection/killing of cancers cells by viral genes. Although NDV is known to cause oncolysis by triggering apoptosis, the role of different proteins of NDV in selective oncolysis is still unclear. It is also not known that edited products of P gene i.e. V and W proteins have apoptotic and/or, antiapoptotic properties in heterologous hosts. In the present study, NDV P gene and its RNA edited products were amplified, cloned, sequenced and analyzed for in vitro expression to be used as oncolytic agents in future studies.

Materials and Methods

Cells and viruses—Chicken embryonic fibroblast (CEF) primary cell culture derived from 9 days old embryonated chicken eggs was used for replication of NDV (velogenic Bareilly strain) available in our laboratory. Before actual experiment, NDV was passed to 4 times in CEF cells. The infected CEF cells were harvested after 96 h and cells were lysed by subjecting to three cycles of freeze (-20 °C) and thaw (37 °C). Small aliquots of the CEF cell lysate containing viral particles were stored in sterile tubes at -20 °C until use. For experiment, overnight grown CEF (70-80% confluency) was infected with NDV (100µL of CEF cell lysate containing NDV). After 36 h of infection, cell monolayer was used for total RNA isolation.

RNA extraction and cDNA preparation—Total RNA was extracted by using Trizol reagent (Invitrogen, USA) as per the manufacturer's protocol. Briefly, after 36 h of NDV infection to CEF chicken embryonic fibroblasts (CEFs), cells were processed for RNA extraction. Culture supernantant was discarded carefully without dislodging the monolayer. Trizol reagent (1 mL) was added to the monolayer and cell membrane was broken by multiple pippeting at room temperature (RT). Chloroform (200 µL) was added, mixed and centrifuged at 10,000 *g* for 10 min at RT and aqueous phase containing total RNA was precipitated with 500 µL of isopropanol. RNA pellet was washed with 70% ethanol, air dried, and resuspended in 25 µL of DEPC treated water. cDNA was synthesized in 50 µL reaction volume. Total RNA (1.5 µg) was mixed with 50 ng random hexamer primers, incubated at 70 °C for 10 min followed by 5 min incubation at 25 °C. Then 10 µL of 5 X RT buffer, 80 units of RNase inhibitor, 2 µL of 10 mM dNTP mix and 400 units of revert Aid reverse transcriptase (MBI, Fermentas) were added and incubated at 42 °C for 1 h followed by heat inactivation (15 min at 75 °C) of the enzyme. cDNA thus formed was stored at -20 °C till further use.

Primer designing—Primers were designed for NDV P gene using Lasergene software (DNASTAR, Inc. Madison, WI, USA), from the sequences available at GenBank. Specificity of primers was checked by NCBI blast programme (http://www.ncbi.nlm.nih.gov/BLAST). Details of primers used in this study are given in Table 1.

Amplification strategies for P gene and its RNA edited products—NDV P and V genes' were amplified using gene specific primers in a single step PCR and W gene was amplified by overlapping PCR. PCR reaction was performed in 50 µL volumes containing 5 µL of 10 X PCR buffer, 1.5 mM $MgCl_2$, 10 pmol of each forward and reverse primer, 200 µmol of dNTP mix, 2.5 U of DNA polymerase (Taq DNA polymerase and DeepVent™ DNA polymerase in 12:1 ratio) and 5 µL of cDNA. After initial denaturation at 95 °C for 5 min, the reaction was carried out in thermal cycler (T personal, Biometra, U.K.) using following conditions: 35 cycles

Table 1—Primers used for cloning and characterization of P, V and W genes (Nucleotide residues inserted in primer sequences are indicated in capital letters)

Gene	Primers	Product length	References
P and V	F 5' cgg gta gaa gag aga cat cca gag ac 3'	1297 bp	MBL/DBT120
	R 5' gga gcc tgt tat gag ttg tga tg 3'		MBL/DBT118
W	F 5' cgg gta gaa gag aga cat cca gag ac 3'	487 bp	MBL/DBT124
	R 5' tgg gCC ccc ttt tta gca ttg gac ga 3'	314 bp	MBL/DBT125
	F 5' caa tgc taa aaa ggg GGc cca tgg tcg ggt cc 3'		MBL/DBT126
	R 5' gcc tcc atc ata gac atc atc g 3'		MBL/DBT127
P/v/w nested	F 5' tcaagaccggagcaagcaact 3'	348 bp	MBL/DBT130
	R 5' gcgcaaagtcggcaggtag 3'		MBL/DBT131
GAPDH	F 5' gaa ggt gaa ggt cgg agt c 3'	225bp	Kalali et al., 2008
	R 5'gaa gat ggt gat ggg att tc 3'		

each of denaturation (95 °C for 30 sec), annealing (48 °C for 45 sec) and extension (72 °C for 1 min). Final extension was done at 72 °C for 5 min. The PCR amplicon was analysed in 1.5 % agarose gel containing 0.5 µg/mL ethidium bromide at 70 volts for 1 hour and visualized on ultraviolet transilluminator.

Cloning and sequence analysis—The PCR product was gel purified using MiniElute gel extraction kit (Qiagen, Germany) as per manufacturer's instruction. DNA eluted with 25 µL of elution buffer was checked for purity on 1.5 % agarose gel visualized using ethidium bromide staining. Final amplicons were gel purified and cloned in pTZ57R/T cloning vector (InsTAclone PCR cloning Kit, MBI, Fermentas). White colonies were selected for screening of recombinant clones. Orientation of recombinant plasmids was confirmed by colony PCR using T7 promoter sequencing primer and gene specific forward primers and product size was confirmed by RE digestion. P, V and W genes were sub-cloned into pcDNA 3.1 + mammalian expression vector and were sequenced at "DNA sequencing facility" chromous Biotech, Bangalore. Nucleotide sequences were checked using blast program (NCBI) and compared with the reported sequences using Lasergene software (DNASTAR Inc. USA).

Confirmation of P, W and V genes' expression in mammalian cell culture—For expression studies HeLa cells were obtained from NCCS, Pune and maintained in DMEM supplemented with 10 % foetal calf serum (FCS) and gentamycin (50 µg/mL). Cells were transfected with recombinant genes using Lipofectamine 2000 (Invitrogen) transfecting reagent in six well plate following manufacturer instructions. Briefly, 4.0 µg of recombinant plasmid DNA was diluted with 250 µL of DMEM (serum and antibiotic free) and 10 µL Lipofectamine 2000 was diluted with 250 µL of DMEM (serum and antibiotic free). Both tubes were incubated at room temperature for 5 min afterwards contents of both tubes were mixed and incubated for 30 min at 37 °C. DMEM (0.6 mL, serum and antibiotics free) was added to the mixture and layered over cells grown to 60-70 % confluency and incubated at 37 °C and 5% CO_2. After 5 hours, culture media was replaced with complete growth media (DMEM with 10 % FCS).

i) *RT-PCR analysis*: Cells were harvested after 48 hours post transfection, total RNA was extracted, cDNA synthesized as described earlier and RT-PCR was performed using nested set of primers (MBL/DBT/130 and 131) specific for P gene (Table 1). GAPDH gene specific primer was used as negative/internal control.

ii) *Indirect Fluorescent antibody test (IFAT)*: For IFAT, transfected cells (after 72 hours of incubation) were processed by method described elsewhere[14]. Briefly, cells were washed three times with phosphate buffer saline containing 1mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS) and fixed in 3.0 % paraformaldehyde (sigma) for 20 minute. Afterwards, cells were washed thrice with PBS and permeabilized with 0.2% triton ×-100 for 5 min. Cells were then incubated in blocking solution (PBS containing 2 % BSA) for one hour, after which they were incubated with primary antibody (anti-NDV, polyclonal, dilution 1:200) harvested from chickens challenged for NDV velogenic strain. After washing thrice with PBS, cells were incubated with FITC labeled secondary antibody (anti-chicken, KOMABIOTECH, South Korea) for two hours. After washing with PBS five times, cells were observed with fluorescent microscopy (Nikon inverted microscope, ECLIPSE Ti) and photographs were captured under 40X objective lens.

iii) *Western Blot Analysis*—Cell lysates from vector-P-,V-and W-transfected HeLa cells at 72h were separated on 15 % denaturing polyacrylamide gel (SDS-PAGE) and expression of P, V and W proteins were detected by western blotting as described elsewhere[15].

Role of P, V, and W proteins in apoptosis: Annexin-V Binding assay—Phosphatidylserine translocation to the outer leaflet of plasma membrane was detected by annexin V staining of the transfected cells. Experiment was performed using the Vybrant Apoptosis assay kit, (Invitrogen, USA). Briefly, cells were harvested by mild trypsinization at different time intervals and resuspended in 1X annexin-binding buffer to a density of 1 x 10^6 cells /ml. 5µl of Alexa flour® 488 annexin V was added to 100 µl of cell suspension and cells were incubated at room temperature for 15 min. Afterwards, 400 µl of 1× annexin-binding buffer was added and cells were kept on ice and analyzed by flow cytometry (FACS Calibur, Beckton Dickinson, USA) using FL1 (530nm) and FL3 (575nm) bandpass filters.

Statistical analysis:—Data are reported as mean value ± SEM of three independent observations. The multiple comparisons between groups were done by using a SAS macro program in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). P values of <0.05 were considered statistically significant.

Results and Discussion

NDV induced oncolysis is achieved by intra-tumoral viral replication mediated cell lysis. NDV is not pathogenic to humans and has been extensively studied as an oncolytic agent in several different human tumor cell lines and tumor models[16,17]. The effects of individual gene of NDV on oncolysis are not yet clear. To establish the role of P gene and its RNA edited products V and W, in NDV induced pathogenesis, the mRNA transcripts were amplified, cloned in pcDNA 3.1+, eukaryotic expression vector, and characterized in this study. CEF culture infected with NDV, 36 hours p.i. when cytopathic effect (CPE) started to appear (Fig. 1a), was selected for total RNA isolation and amplification of P and V transcripts. In our cloning strategies, P and V mRNAs have been amplified by simple PCR (Fig. 1b), and W mRNA was amplified by overlapping PCR. W transcript was relatively a rare mRNA and could not be amplified by simple PCR, therefore, to amplify W mRNA overlapping PCR was done. The W specific primers were designed by adding two C and two G nucleotide residues to the primers MBL/DBT/125 and MBL/DBT/126, respectively (Table 1). The PCR was performed in three steps. First step PCR

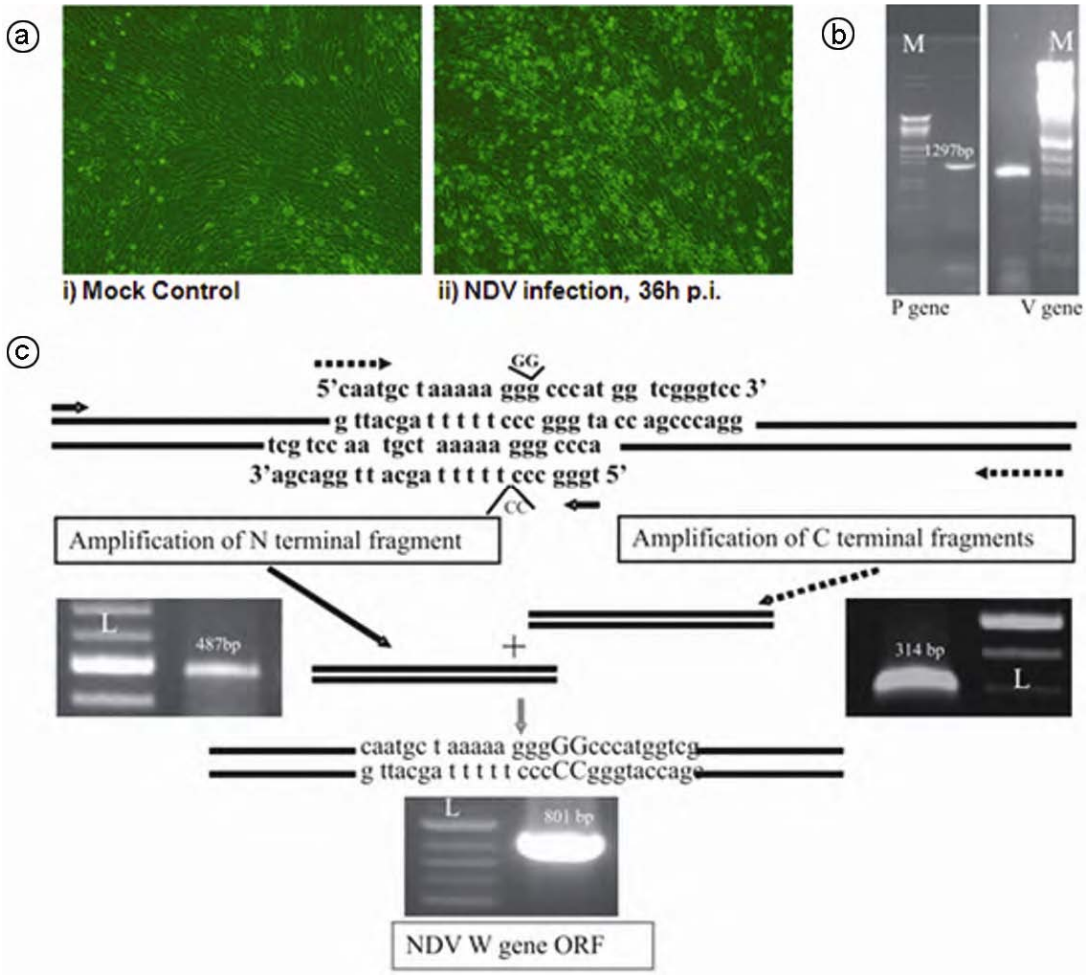

Fig. 1—Strategies for amplification of P, V and W ORFs: a) Phase contrast microscopic view of NDV induced cytopathic effect on CEF cells, 36h post infection (pi) (i) Mock control; (ii) NDV infected cells, (400X) b) amplification of NDV P and V ORFs, M indicates double digest DNA marker, c) Schematic overview of PCR for amplification of W ORF. In the first and second step of PCR N terminal and C terminal fragments of the W ORF were amplified using total RNA extracted from NDV infected CEF cells (36 hours (pi). In the third step of PCR, full length W ORF was amplified (using gel purified products of PCR templates generated during first and second steps mixed in 1:1 ratio), L indicates 100 bp DNA ladder.

was performed with primers MBL/DBT/124 & MBL/DBT/125; second step PCR was performed with primers MBL/DBT/126 & MBL/DBT/127 and the final step PCR was performed with gel purified PCR products of first and second step PCRs (in 1:1 ratio) as template with MBL/DBT/124 & MBL/DBT/127 primer pairs (Fig. 1c). Similar strategy was described for cloning of V gene by previous workers[18,19]. However, in our study, V gene was amplified naturally with simple PCR and overlapping PCR amplification strategy was used for amplification of W gene. The final amplified PCR products (P, V & W) were gel purified and cloned in pTZ57R/T cloning vector. The recombinant plasmids were checked by restriction endonuclease (RE) digestion and confirmed for orientation by colony PCR. These genes were cloned into pcDNA 3.1 + vector, by ligating the release from the reverse orientation clones of pTZ.ndv.p/v/w using EcoRI and Hind III REs. Initially, the recombinant pcDNA 3.1 + plasmids were checked by RE digestion. Further, the orientation of the amplicons in recombinant plasmids was confirmed by, PCR using T7 promoter specific primer and gene specific reverse primer and sequencing (Fig. 2). The recombinant plasmids of P, V and W genes were named as pcDNA.ndv.p, pcDNA.ndv.v and pcDNA.ndv.w, respectively. The sequences of P, V and W mRNA transcripts were submitted to GenBank data with accession numbers HQ589258, FJ644944 and HQ589256, respectively. All three sequences were analyzed for insertion of G residue(s) at conserved edit site (Fig. 3a). Chromatograms confirmed the insertion of one and two Gs at editing site (Fig. 3b) for the V and W transcripts, respectively. These insertions resulted in shorter ORFs encoding V and W proteins. *In silico* sequence analysis revealed ORFs of 1188 bp, 720 bp and 684 bp encoding P, V and W protein with 395, 239 and 227 amino acids, respectively. The V and W proteins share N terminal domain with P protein and vary at their C-termini (Fig. 3c). Several previous studies revealed the importance of P gene and its RNA edited products of paramyxoviruses[10,20,23] including NDV[24] in host-pathogen interaction.

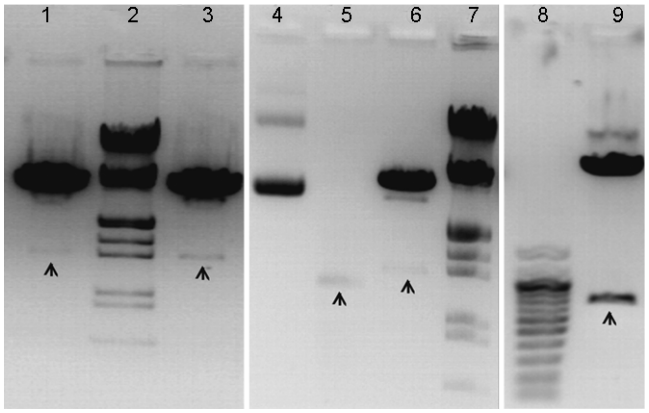

Fig. 2—Confirmation of recombinant plasmids: lane 1=RE digestion of pTZ.ndv.p; lane 2=double digest DNA marker; lane 3=RE digestion of pTZ.ndv.v; lane 4=uncut pcDNA.ndv.p; lane 5=colony PCR from pcDNA.ndv.p; lane 6=RE digestion of pcDNA.ndv.p; lane 7=Double digest DNA marker; lane 8=100 bp DNA ladder and lane 9=RE digestion of pcDNA.ndv.w (only representative gels are shown).

In vitro expression of P, V & W proteins was evaluated in HeLa cells transfected with the gene constructs-pcDNA.ndv.p, pcDNA.ndv.v and pcDNA.ndv.w, respectively. RT-PCR with nested primers common for P, V and W genes transcripts confirmed their expression in HeLa cells, 48 hour post transfection. While processing for total RNA extraction DNase (1 unit/µg of RNA) treatment was given to eliminate the possibility of plasmid contamination. GAPDH primers were used as internal control to assess the quality of cDNA. In mock (pcDNA 3.1 +) transfected cells no amplification was detected (Fig. 4a). Further, IFAT was done with polyclonal sera raised in a challenge study conducted with velogenic NDV in our laboratory. Intense fluorescence was observed in all cells transfected with gene constructs indicating the expression of P, V and W products. No fluorescence was observed in mock (vector control) transfected cells (Fig. 4b). Additionally, expression of P, V and W proteins were checked by western blot analysis using polyclonal antibody. Immunoblots analysis showed antigen-antibody complex of ~42-, ~26- and ~25- kDa in P, V, and W-transfected cells, which confirmed the expressions of these proteins in HeLa cells. Further apoptosis inducing ability of these constructs were studied in HeLa cells by annexin V-binding assay. Annexin V is known to have specific affinity with phosphatidylserine (PS)[25]. PS is usually located at inner leaflet of plasma membrane in normal cells which translocated to outer leaflet in early apoptotic cells, which could be detected with labeled annexin V. The percent annexin V positive cell populations in vehicle control, vector control, pCDNA.ndv.p, pCDNA.ndv.v, pCDNA.ndv.w and pCDNA.ndv.hn trasnfected cells are 17.42 ± 0.231, 14.96 ± 0.442, 8.54 ± 0.807, 7.42 ± 1.33, 4.26 ± 0.102 and 30.28 ± 3.208, respectively (Fig. 5). In present study, flow cytometric analysis revealed that there was a significant ($P \leq 0.05$) decrease in PS-positive cells in P,

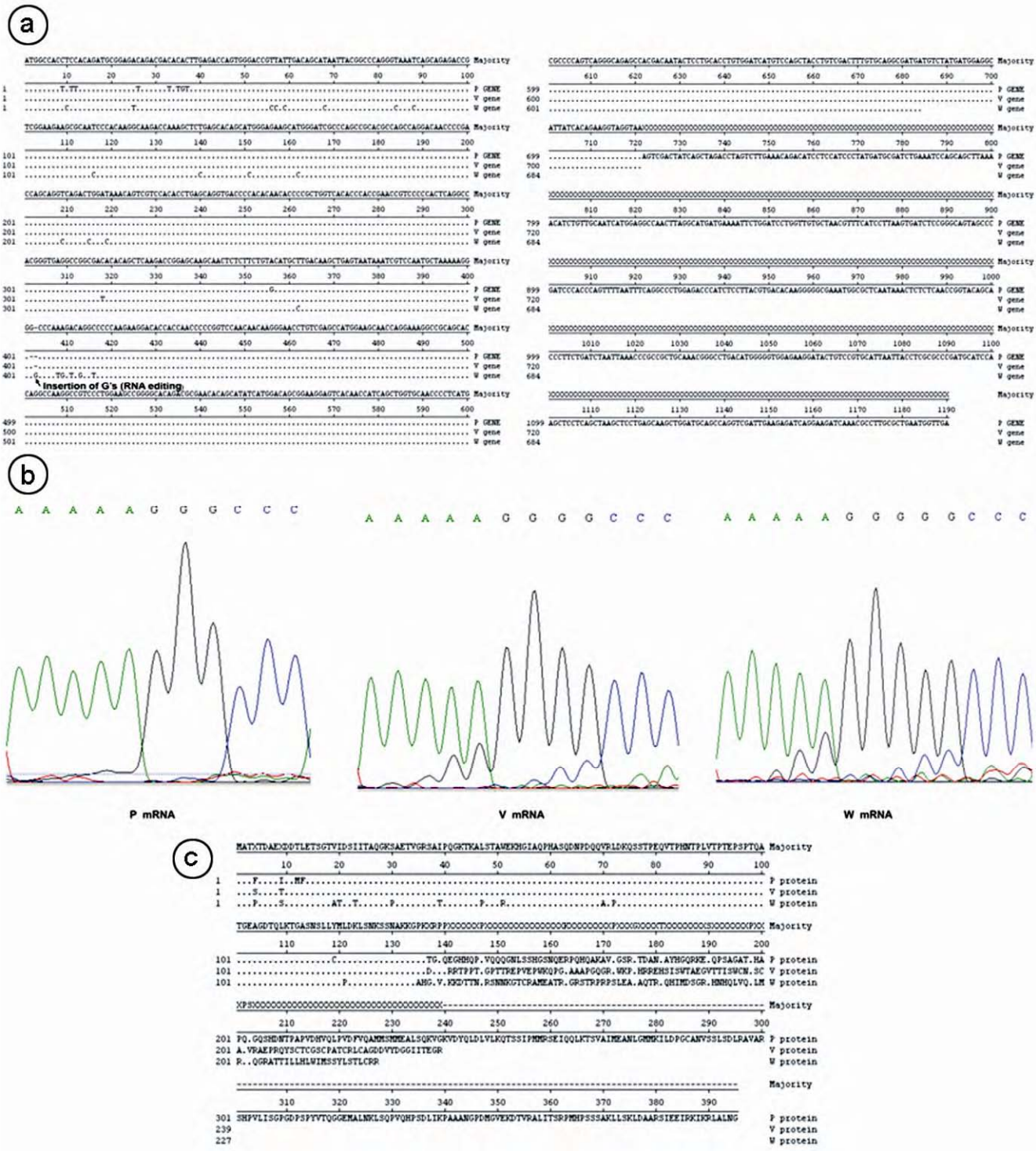

Fig. 3—Analysis of sequences of P, V and W ORFs: a) sequence alignment of P, V and W ORFs; insertion site for G residues are indicated b) chromatograms showing insertion of one and two G residues in V and W ORFs, respectively and c) alignment of amino acids of P, V and W ORFs.

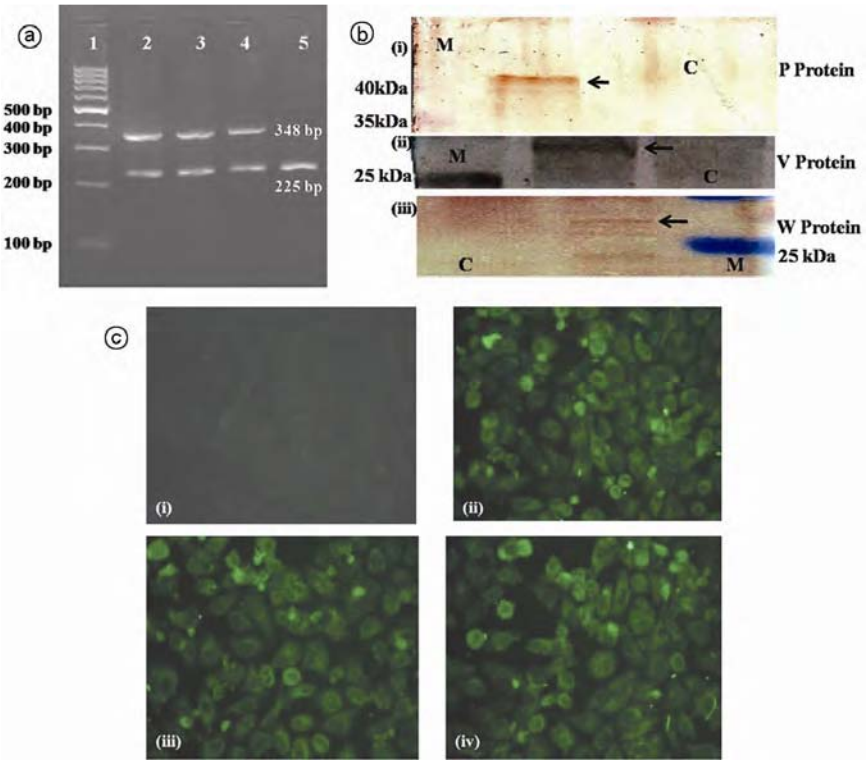

Fig. 4—*In vitro* expression of recombinant clones in HeLa cell lines: a) Reverse transcriptase PCR from transfected HeLa cells (48 hours post transfection). PCR products were resolved on 2.5% agarose gels and visualized with ethidium bromide staining under UV illuminator. lane 1=100 bp DNA ladder; lane 2=pcDNA.ndv.p transfection; lane 3=pcDNA.ndv.v transfection; lane 4=pcDNA.ndv.w transfection and lane 5=mock (pcDNA 3.1+ vector) transfection. b) Expression of NDV P, V and W proteins (72 post transfection); transfected HeLa cells were incubated with primary anti-NDV polyclonal antibody (raised from NDV challenged study in chickens) followed by FITC-conjugated anti-chicken IgG, i). cells transfected with vector (pcDNA 3.1+) alone plasmids did not react with the primary antibody, ii), iii) and iv) cells transfected with pcDNA.ndv.p, pcDNA.ndv.v and pcDNA.ndv.w recombinant plasmids, respectively. c) Western blot analysis of P, V and W proteins (72h post transfection); Abbreviations: C stands for mock (pcDNA 3.1 + vector) transfected lane, M stands for protein ladder, # SM1841, fermentas.

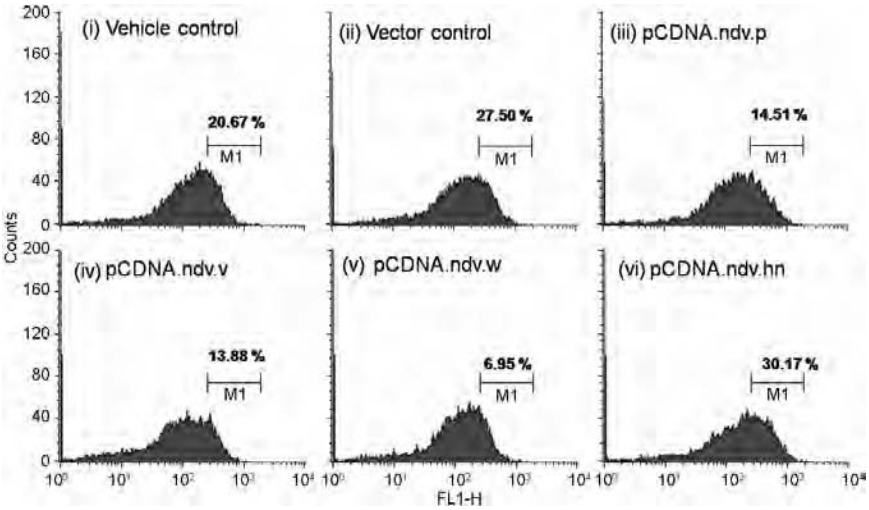

Fig. 5—Anexxin V binding assay: Histograms showing flow cytometric analysis of phosphatidylserine translocation in (i) vehicle control; (ii) vector control; (iii) pCDNA.ndv.p-; (iv) pcDNA.ndv.v-; (v) pcDNA.ndv.w- and (vi) pcDNA.ndv.hn-transfected HeLa cells after annexin V staining at 48h of transfection. Trasnsfecting reagent (lipofectamine 2000, Invitrogen, USA) treated cells, pcDNA 3.1 + -transfected cells and NDV HN gene construct (available in laboratory)-transfected cell were used as vehicle-, vector- and positive-control, respectively.

V and W transfected cells as compared with vector control. Results suggested that P, V and W proteins are not showing apoptotic effects in HeLa cells.

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