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SEQUENCE ANALYSIS AND EXPRESSION OF VP3 GENE OF CHICKEN ANEMIA VIRUS

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SUMMARY

Chicken anemia virus (CAV) isolate BL-5 VP3 gene was amplified, sequenced and expressed in *E. coli* as a fusion protein together with a six-histidine tag. The deduced amino acid sequence of VP3 was identical to other CAV isolates Cux-1, A2 and CIA-1 except for a single amino acid substitution at position 12. SDS-PAGE and Western blot analysis indicated that the expressed protein is insoluble and was found primarily from the cell lysate fraction. Expression of the protein was detected as early as 1 h, with maximum expression (~12% of the total protein) at 6 h post induction with IPTG. This study indicates that VP3 protein is highly expressed in insoluble form in *E. coli.* however, the biological function of the protein remains to be studied.

Key words: chicken anemia virus, VP3, expression, fusion protein

INTRODUCTION

Chicken anemia virus (CAV) is an important viral agent that causes subclinical and clinical immunosuppressive disease in chicken (McNulty, 1991). CAV is classified under a newly recognised animal virus family, the *Circoviridae* (Lukert *et al.*, 1995). The virus is small, non-enveloped, spherical, 18 to 23 nm in diameter, containing a circular single-stranded DNA genome of 2.3 kb (McNulty, 1991; Noteborn *et al.*, 1991).

The CAV genome has three partially overlapping major open reading frames coding for proteins of 52 (VP1), 24 (VP2) and 14 (VP3) kDa (Claessens *et al.*, 1991; Meehan *et al.*, 1992). VP1 encodes for capsid protein which plays an important role in virus spread and cell tropism (Renshaw *et al.*, 1996). However, both VP1 and VP2 are essential in inducing protective neutralising antibodies (Koch *et al.*, 1995). VP3 gene product, which is also known as apoptin, is involved in the induction of apoptosis and is associated with viral pathogenicity (Jeurissen *et al.*, 1992; Noteborn *et al.*, 1994). In addition, apoptin also exhibits oncolytic properties on human malignant cell lines (Danen-Van *et al.*, 1997). Being the smallest, non-glycosylated and proline-rich among the three CAV proteins, the VP3 gene has been cloned and expressed using different prokaryotic systems by several investigators (Cunningham *et al.*, 2001; Pallister *et al.*, 1994). In this study, we determined the sequence and immunogenicity of VP3 of a local CAV isolate BL-5.

MATERIALS AND METHODS

Virus

The CAV BL-5 isolate considered in this study has been described previously by Chowdhury *et al.* (2002a). The virus was propagated in MSB-1 cells and viral DNA was extracted following methods described previously by Chowdhury *et al.* (2002a).

Polymerase chain reaction

VP3 gene was amplified using forward primer (nts 481-497, 5'-GGAATTCAAATGAACGCTCTC-3') and reverse primer (nts 841-856, 5'-GGGATCCATCTTACAGTCTTA-3') which were designed based on the sequence of a reference CAV isolate, Cux-1 (Noteborn *et al.*, 1991). Polymerase chain reaction (PCR) was carried out in a 50 µl PCR reaction mixture with the following thermal cycling profiles: an initial incubation at 94°C, followed by 30 cycles of 94°C for 1 min, 60°C for 1.5 min, 72°C for 2 min, a final incubation at 72°C for 10 min and cooling at 4°C. The PCR products were run on 1% agarose gel electrophoresis and purified by Geneclean kit (BIO101, USA) and cloned into TOPO TA Cloning® system (Invitrogen, USA) following the supplier's instructions.

Sequence analysis

Two positive colonies were selected and subjected to sequencing using ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin Elmer, USA) in an automated DNA sequencer (ABI PRISM® 377 DNA Sequencer) following method described by Chowdhury *et al.* (2002b). Both clones were sequenced in both directions. The sequence data were analysed using BLASTN 2.0.11 program of National Institute of Biotechnology Information (NCBI). The isolates, GenBank Accession Numbers and references of sequences used for sequence alignment were as follows : SMSC-1 (AF285882), 3-1 (AF390038) (Chowdhury *et al.*, 2003), Cux-1 (M55918) (Noteborn *et al.*, 1991), Cloned isolate 10 (U66304) (Meehan *et al.*, 1997), CAU269/7 (AF227982) (Brown *et al.*, 2000), 704 (U65414) (Connor *et al.*, 1991), A2 (AB031296)

(Yamaguchi *et al.*, 2001) and CIA-1 (L14767) (Renshaw *et al.*, 1996). The sequences were aligned using the ClustalW multiple alignment program under the "BioEdit" software package based on the nucleotide and amino acid sequences. The nucleotide sequences have been submitted to GenBank database under the accession number AF527037.

Expression of VP3 protein

The VP3 gene was subcloned into pRSET A (Invitrogen, USA), downstream and in-frame with the N-terminal fusion peptide by using specific primers. The forward primer (5'-GGGGATCCATGAACGCTCTCT-CCAAGAAGATA-3') and reverse primer (5'-GGGAATTCTTACAGTCTTATACACC TTCTT-3') were designed with *EcoR*I and *BamH*I sites, respectively. The PCR reaction mixture and thermal cycling profiles were performed as described previously. The recombinant plasmid was transformed into *E. coli* strain BL21(DE3) pLysS as the host for expression. Protein expression was induced by the addition of isopropyl-1-thio-B-D-galactosidase (IPTG) to 1 mM and the cells were incubated at 37°C for up to 8 h. The bacterial cells were collected by centrifugation and the cell pellets were dissolved in 1 ml of phosphate buffer saline (PBS). The cells were sonicated and centrifuged at 14,000 x g for 15 min. The supernatant was removed to a fresh tube whereas the cell lysates were resuspended with the same volume of PBS as the supernatant part. The cell lysates and supernatant fractions from each sample of induced and non-induced with IPTG were prepared according to the method of Laemmli (1970) with slight modification. The samples were resuspended in sample buffer [0.5 M Tris-HCl (pH 6.8), 10% (w/v) SDS, 1% (w/v) bromophenol blue, glycerol and 2-mercaptoethanol] and boiled for 5 min before loading into the stacking gel. Sample preparations were electrophoresed in a 12.5% (w/v) discontinuous acrylamide slab gel separated by a 4% (w/v) stacking gel. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue or transferred onto a PVDF membrane (Immun-Blot™, BIO-RAD) for Western blot analysis. The membrane was incubated in 1% (w/v) blocking buffer (KPL, USA) at room temperature for 2 h followed by incubation with primary antibody of anti-HisG monoclonal antibody (mab) (Invitrogen, USA) diluted 1:5000 or mab to VP3 (Mab51.3) (Chandratilleke *et al.*, 1991) diluted 1:2000 for 1 to 2 h. The membrane was then washed 3 times in the wash buffer (KPL, USA) and incubated in secondary antibody solution of alkaline phosphatase conjugated goat anti-mouse (KPL, USA) diluted 1:2500 for 2 h and in BCIP/NBT phosphatase substrate system (KPL, USA) for detection of the desired protein. The concentration of the fusion protein was estimated using Gene Snap Software of Syngene documentation system (Syngene, UK) by dividing the intensity of the VP3 band over the intensity of the total protein bands observed at each line after induction with IPTG.

RESULTS

Analysis by agarose gel electrophoresis indicated a single DNA fragment of expected size of approximately 400 bp from PCR amplified products (data not shown). The complete open reading frame of VP3 of BL-5 is 366 bp in length which expected to encode for a protein of 121 amino acids. Nucleotide sequence comparison showed that BL-5 showed more than 99% homology to CAV isolates SMSC-1, 3-1, A2 and CIA-1 with only one substitution. This substitution resulted in a single amino acid substitution from proline to glutamine at position 12 (Fig. 1). On the other hand, BL-5 showed the lowest homology, 98% to the Australian isolate CAU269/7 with 6 substitutions (data not shown). These substitutions resulted in 5 amino acid substitutions (Fig. 1).

Based on the SDS-PAGE (Fig. 2) and Western blotting (Fig. 3), the band of the expressed VP3 protein was found between 15 to 20 kDa primarily from cell lysate samples by 1 h post-induction and the intensity of the bands increased significantly after 4 h post-induction (Fig. 2). The concentration of the protein was 0.6% of the total *E. coli* protein after 1 h which increased to 5% after 4 h post-induction. The highest expression of 12% was at 6 h post-induction. In contrast, the expression was undetectable from the supernatant samples (Fig. 2). However, a faint band of the expected size was observed following Western blotting with antiHisG mab (Fig. 3, lane 2) indicating that the level of expression in the supernatant samples was very low. A faint band was also observed from the cell lysate samples of the induced recombinant plasmid following Western blotting using mab to VP3 (Fig. 3, lane 4).

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                    10        20        30        40        50
                    ....|....| ....|....| ....|....| ....|....| ....|....|
Consensus           MNALQEDTPP GPSTVFRPPT SSRPLETPHC REIRIGIAGI TITLSLCGCA
BL-5                .......... .Q........ .......... .......... ..........
Cux-1               .......... .......... .......... .......... ..........
SMSC-1              .......... .......... .......... .......... ..........
3-1                 .......... .......... .......... .......... ..........
CAU269/7            ...H...... .......... .......... .......... .V........
Isolate 704         .......... .......... .......... .......... ..........
A2                  .......... .......... .......... .......... ..........
CIA-1               .......... .......... .......... .......... ..........
C.Isolate10         .......... .......... .......... ........A. ..........

                    60        70        80        90        100
                    ....|....| ....|....| ....|....| ....|....| ....|....|
Consensus           NARAPTLRSA TADNSESTGF KNVPDLRTDQ PKPPSKKRSC DPSEYRVSEL
BL-5P               .......... .......... .......... .......... ..........
Cux-1               .......... .......... .......... .......... ..........
SMSC-1              .......... .......... .......... .......... ..........
3-1                 .......... .......... .......... .......... ..........
CAU269/7            ...V...... ......N... .......... .......... ..........
Isolate 704         .......... .......... .......... A......... ..........
A2                  .......... .......... .......... .......... ..........
CIA-1               .......... .......... .......... .......... ..........
C.Isolate10         .......... .......... .......... .......... ..........

                    110       120
                    ....|....| ....|....|.
Consensus           KESLITTTPS RPRTARRCIRL
BL-5P               .......... ..........
Cux-1               .......... .....K.R...
SMSC-1              .......... ..........
3-1                 .......... ..........
CAU269/7            .......... ..........
Isolate 704         .......... ..........
A2                  .......... ..........
CIA-1               .......... ..........
C.Isolate10         .......... .......R...
```

Fig. 1. Alignment of deduced amino acid sequences of VP3 from different isolates of CAV. The deduced amino acid of BL-5 was identical to other isolates, SMSC-1, 3-1, A2 and CIA-1 except for a single amino acid substitution at position 12.

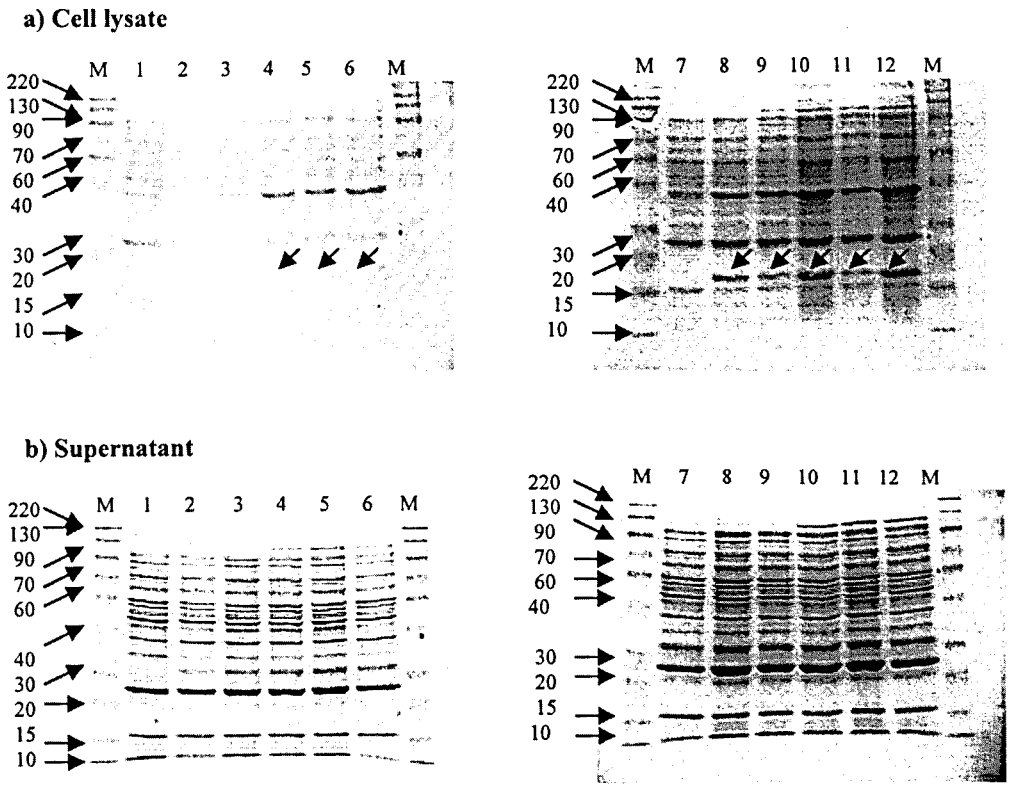

Fig. 2. Expression of VP3 fusion protein from (a) cell lysate and (b) supernatant samples
Lane 1- BL21(DE3)pLysS host cells; Lane 2- non-recombinant plasmid; Lane 3- uninduced recombinant plasmid (0 h); Lane 4- induced recombinant plasmid (1 h); Lane 5- induced recombinant plasmid (2 h); Lane 6- induced recombinant plasmid (3 h); Lane 7- uninduced non-recombinant plasmid; Lane 8- induced recombinant plasmid (4 h); Lane 9- induced recombinant plasmid (5 h); Lane 10- induced recombinant plasmid (6 h); Lane 11- induced recombinant plasmid (7 h); Lane 12- induced recombinant plasmid (8 h); Lane M- Benchmark™ Prestained Protein Ladder (Invitrogen, USA). The arrows show the bands of approximately 18 kDa that represent the expressed VP3 fusion protein.

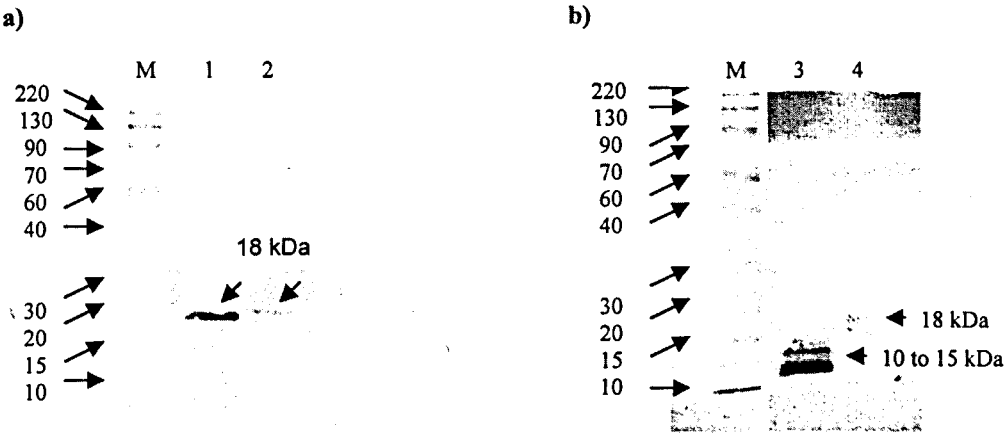

Fig. 3. Western blot analysis on expressed VP3 fusion protein detected by (a) Anti-HisG (Invitrogen, USA) and (b) monoclonal antibody against VP3 (Mab51.3)
Lane 1 – cell lysate sample of 4 h induced recombinant plasmid; Lane 2 – supernatant sample of 4 h induced recombinant plasmid; Lane 3 – cell lysate sample of infected MSB1 cells; Lane 4 – cell lysate sample of 4 h induced recombinant plasmid. Lane M - Benchmark™ Prestained Protein Ladder.

DISCUSSION

In this study, we analysed the nucleotide sequence of VP3 of a local CAV isolate BL-5. The size of the VP3 open reading frame is similar to those of other CAV isolates identified in previous studies (Brown *et al.*, 2000; Chowdhury *et al.*, 2003; Noteborn *et al.*, 1991; Pallister *et al.*, 1994). The results also showed that the VP3 gene was well conserved and a very low variation was found in both nucleotide and amino acid sequences. The low variation of VP3 gene probably reflects its conserved role in viral pathogenicity (Noteborn *et al.*, 1994). However, the unusually high amino acid substitution between BL-5 and CAU296/7 deserve further studies.

As shown in Fig. 3, the inability to detect a strong signal despite the high expression was probably associated with the poor presentation of the epitopes due to the insoluble form of the protein. Alternatively, probably the mab is specific against conformational epitope that is missing in the protein. Another possible explanation, is to the size of the protein, since most proteins with small molecular mass are usually poor immunogens (Hey, *et al.*, 1994). In a recent study by Cunningham *et al.* (2001), it has been shown that VP3 protein which was expressed as a fusion protein with gluthathione *S*-transferase and a six-histidine tag was mostly insoluble. The inability to express the VP3 in soluble form was probably associated with the overall amino acid sequences of the protein. Amino acid sequence analysis using the ProtParam tool software (http://www.expasy.com) predicated that VP3 protein is unstable and may be less water soluble based on the high instability index of 65.75, which exceeded the minimum value of a stable protein with instability index 40. However, Cunningham *et al.* (2001) indicated that a very low level of antibody to VP3 is induced in response to CAV infection in chickens suggesting that the protein can be developed into a diagnostic reagent from the diagnosis of CAV. Probably the use of mammalian expression systems such as baculovirus, adenovirus and yeast may be able to produce VP3 protein with diagnostic characteristics.

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RINGKASAN

ANALISIS JUJUKAN DAN EKSPRESI GEN VP3 VIRUS ANEMIA AYAM

Gen VP3 virus anemia ayam (CAV) isolat BL-5 diamplifikasi, dijujuk dan diekspres dalam *E. coli* sebagai protein gabungan dengan tag histidina enam. Jujukan asid amino VP3 adalah serupa dengan isolat CAV lain, Cux-1, A2 dan CIA-1 kecuali bagi satu perubahan asid amino pada kedudukan 12. Analisis SDS-PAGE dan Sap Western menunjukan protein yang terekspress adalah tidak larut air dan dijumpai kebanyakanya daripada bahagian lisat sel. Ekspresi protein boleh dikesan seawal 1 jam dengan ekspres maksima (~12% dari jumlah protein) pada 6 jam selepas diaruh dengan IPTG. Kajian ini menunjukan VP3 boleh diekspres dengan banyak dalam *E. coli* walau bagaimanapun, fungsi biologi protein tersebut perlu dikaji semula.