Journal of Animal and Veterinary Advances 11 (9): 1316-1321, 2012
ISSN: 1680-5593

Development and Identification of Stably BHK-21 Cell Lines Expressing T7 RNA Polymerase

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Abstract: A full-length of cDNA copy of the T7 RNA polymerase was cloned into the EcoR I and Sal I sites of PCI-neo vector under the control of the human Cytomegalovirus (CMV) immediate-early enhancer/promoter and designated PCI-T7. BHK-21 cell lines expressing PCI-T7 were developed using conditional medium containing Geneticin (G418) for 2 weeks. A helper DNA plasmid, named as pT7 HN was co-transfected into VT7 cell and detected by Western blot. These results showed that the T7 RNA polymerase of the vaccine virus strain VTF7-3 was expressed in stable recombinant cell lines and VT7 cell line expressing T7 RNA polymerase stably at least 30 passages. The developed system would be offer an attractive and safe alternative to other inducible eukaryotic expression systems which provides an important platform for study of the rescue and function of NDV strain NA-1.

Key words: T7 RNA polymerase, VT7 cell line, virus rescue, vaccine, geneticin, plasmid

INTRODUCTION

Vaccinia Virus (VACV or VV) is a large, complex and enveloped virus belonging to the poxvirus family that it has a linear, double-stranded DNA genome approximately 190 kb in length that encodes for approximately 250 genes (Ryan and Ray, 2004). Meanwhile, vaccinia virus is well known for its role as a vaccine that eradicated the smallpox disease, making it the first human disease to be successfully eradicated by science (Li *et al*., 2011; Castillo-Olivares *et al*., 2011; Czub *et al*., 2005). Recently many studies have been shown that recombinants DNA plasmid contains T7 MVA expressing genes of target expresses target gene only in stably co-transfected cells (Kovacs *et al*., 2003; Kusov *et al*., 2002). However, the highest expression levels have been achieved when the gene encoding the RNA polymerase from the bacteriophage T7 was integrated into the VACV genome and the gene of interest was under bacteriophage T7 promoter (Mohamed and Niles, 2004).

Newcastle Disease Virus (NDV) also known as Avian Paramyxovirus type 1 (AMPV-1) cause an infectious viral disease of birds named as Newcastle Disease (ND) which leads to severe economic losses in the poultry industry worldwide (Yin *et al*., 2010; Saif *et al*., 2008). Due to the current rise in NDV-caused economic losses in the poultry industry and to develop a better method to rescue Newcastle disease virus strain NA-1 by reverse genetic, a stable Baby Hamster Kidney 21 (BHK-21) cell lines expressing constitutively cytoplasmic T7 RNA polymerase were necessary developed. In order to establish both a stable BHK-21 cell line expressing T7 RNA polymerase and a helper virus-free reverse genetic system, the retroviral gene transfer technology was used. The NDV strain *NA-1 HN* gene which under the control of T7 promoter was expressed in stable VT7 cell lines of over 30 passages. Since, it only direct transfect a help-plasmid, named as a pT7 HN plasmid that under the control of T7 promoter, into the stable VT7 cell lines, it would be provide vital methods for further studies about the rescue system as well as function of NDV strain NA-1.

MATERIALS AND METHODS

Virus and cells: The vaccinia virus strain VTF7-3 which was presented as a gift from Dr. Cheng at the Department of Agriculture, National Poultry Disease Research Center, China. The eukaryotic expression plasmid pT7 HN contains both T7 promoter and HN of NDV was constructed by PCR. The Vaccinia virus was propagated in BHK-21 cells and used for transfection. BHK-21 cells were grown in Dulbecco's Modified Eagle's Medium

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(DMEM, Invitrogen) supplemented with 8% heated-inactivated Fetal Bovine Serum (FBS), 2 Mm L-glutamine, 100 μg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin at 37°C and 5% CO_2.

Amplification of *T7 RNA* polymerase genes: To amplify *T7 RNA* polymerase genes, one pairs of PCR primers were designed based on the sequences published in GenBank (Accession No.: U67175, AY305005). For amplifying *T7 RNA* genes, T7F1: 5'-ATAGAATTCTCGC GCTGCACTGGG-3',T7R1:5'-ATAGTCGACGGCCACTCT TGCGAAT-3'. PCR amplification in the reaction buffer containing 10× LA Taq Buffer 5 μL, dNTP (2.5 mmol L^{-1}) 4 μL, upper and lower primers (10 pmol μL^{-1}) 2 μL, LA Taq polymerase (1 U μL^{-1})1 μL, VTF7-3 3 μL, ddH_2O 35 μL, to a final volume of 50 μL. PCR reaction was performed in the thermocycler with the following program: denaturation at 95°C for 3 min, 30 cycles consisted of denaturation at 95°C for 40 sec, annealing at 67°C for 40 sec and extension at 72°C for 3 min and was ended with the final extension at 72°C for 10 min. The amplified PCR products were visualized after electrophoresis on a 2% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen Corp., Carlsbad, CA, USA).

Construction of the recombinant plasmid pCI-T7: The *T7 RNA* polymerase gene sequence containing EcoR/sal I digestion site was gained by PCR as well as the recombinant DNA plasmid expressing T7 RNA polymerase was construct under the pCI-neo as a backbone.

Development of identification of stable BHK-21 cell lines expressing T7 RNA polymerase: About 1 day before the transfection, 2.5×10^4 BHK-21 cells per well were seeded into 6 well cell culture plate (cells approximately reached 70-80% confluence). The recombinant plasmid pCI-T7 was transfected into BHK-21 cells by Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA, USA). After 48 h of post-transfection, the transfected cells were planted with the medium containing 1.0 g L^{-1} of G418 and incubated in 37°C, 5% of the CO_2 in the continued culture for 2 weeks. The stable cell lines expressing T7 RNA polymerase was named as VT7 cell lines. At the same time, the pCI-neo plasmid and BHK-21 cell were set as negative and blank control.

Expression of the *T7 RNA* polymerase genes transcripts mRNA in the VT7 cell lines over serial passage 30 times was analyzed by RT-PCR.

Functional identification of the VT7 cell lines: BHK-21 cell lines expressing T7 RNA polymerase were transfected with a plasmid pT7 HN expressing the *HN* gene of NDV strain NA-1 under the control of the bacteriophage T7 promoter using the Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA, USA). After 48 h of post-transfection, the expression of *HN* gene in transfected BHK-21 cell lines was observed by Western blot.

The transfected cells lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The BHK-21 cells cell were used as negative control. Membrane was placed in blocking solution (5% fat-free milk in TBST buffer) at 4°C overnight and incubated with anti-HN IgG at the dilution of 1/300 in TBST buffer for 2 h at room temperature, followed by incubation for 1 h with second antibody conjugated with horseradish peroxidase at the dilution of 1/1000 in TBST buffer, followed by visualizing with NBT/BCIP substrates (Promega, USA).

RESULTS

To gain of T7 RNA polymerase gene sequence: *T7 RNA* polymerase gene sequence was gained using specific primers by PCR. As shown in Fig. 1, the size of PCR product was consistent with the expected.

Identification of the recombinant plasmids pCI-T7: The recombinant plasmids were identified by both EcoR I and Sal I double enzymes digestion system. Then, the digested products were analyzed on a 2% agarose gel under 120 V for 40 running time, the results indicated that T7 RNA polymerase gene and pCI-neo expression vectors were 2,877 and 5,453 bp, respectively (Fig. 2).

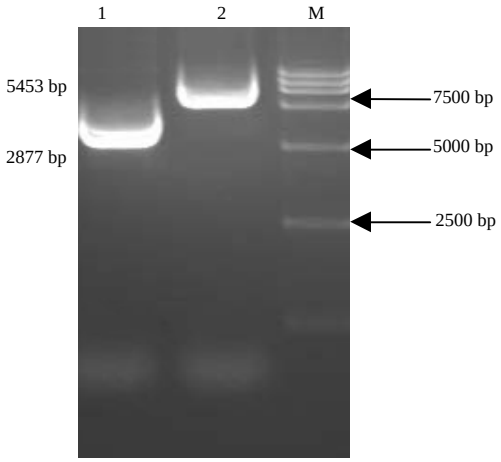

Fig. 1: The results of electrophoretic indentification for 1 and 2 through 0.8% agarose gels. The PCR products amplified T7 RNA pol with primers T7F1 and T7R1 were designated as lane 1. pCI-neo expression vectors was in lane 2; DL15000 Marker fragment (TaKaRa) was in lane M

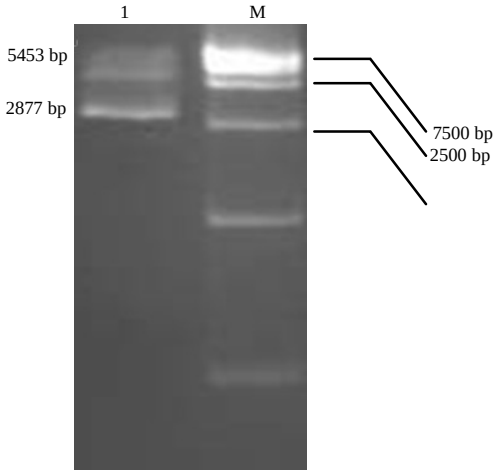

Fig. 2: The results of double digestion indentification for the recombinant plasmids through 0.8% agarose gels. The PCR products amplified T7 RNA pol were designated as lane 1. pCI-neo expression vectors was in lane 2; DL15000 DNA Marker in lane M

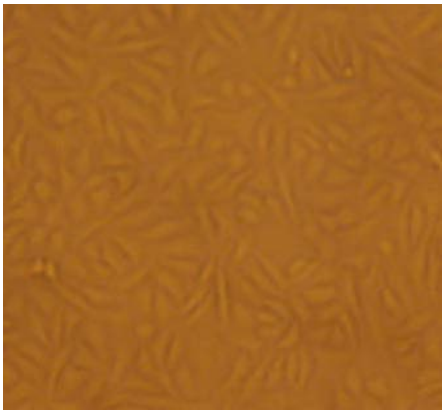

Fig. 3: VT7 cell morphology

Morphology of VT7 cell lines at 30th generation: Cell morphology at the continuous passage of 30th VT7 cell generation was observed under microscope. As shown in Fig. 3, the 30th VT7 mono-cultural cell layer shows with spindle-shaped.

Identification of BHK-21 stable cells expressing T7 RNA polymerase: To determine whether the *T7 RNA* polymerase gene was integrated stably into the chromosome of BHK-21 cell, *T7 RNA* polymerase gene present in BHK T7 cells was analyzed by RT-PCR. The results showed that PCR fragments about 2877 bp were detected (Fig. 4) in BHK T7 cells at the 30th passage (VT7 F30). The sequencing data aslo showed the expression of *T7 RNA* polymerase gene from 30th passaged VT7 cell lines are 100%. These data

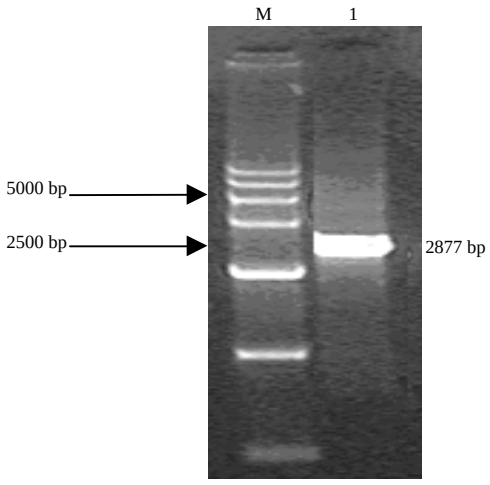

Fig. 4: RT-PCR products from VT7 cell lines at the 30th passages were electrophoresed through 0.8% agarose gels. The *T7* polymerase gene amplification products fragments about 2877 bp were detected in lane 1. DL 15000 Marker fragment was in lane M

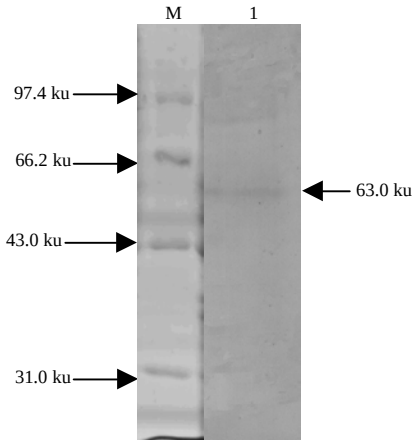

Fig. 5: Western blotting analysis of the NDV NA-1 strain HN protein expressed in table VT7 cell lines. VT7 positive cell lines were transfected with validation plasmid pT7-HN containing the NDV NA-1 strain HN protein coding sequence under the control of the bacteriophage T7 promoter. Lane 1, HN proteins; M, protein marker. Arrow at the right indicates the position of HN proteins

demonstrated that *T7 RNA* polymerase gene has been integrated stably into the chromosome of VT7 cell lines.

Functional identification of the VT7 cell lines: To check whether the *HN* gene expressed in VT7 cell or not, the HN protein in whole cell protein extract was detected by Western blotting. As shown Fig. 5, HN protein with

molecular weights of 63 ku was detected only in transfected cell lines, not in either control cell lines or black plasmid cell lines. The data demonstrated that both HN proteins from NDV NA-1 strain and T7 RNA polymerase were expressed in VT7 positive cell lines (Fig. 5).

DISCUSSION

A single strand negative chain RNA viruses salvation process is usually construct of genome-wide cDNA cloning and auxiliary plasmid carrying can stabilize a common expression T7 RNA polymerase lines (or carrying in advance with restructuring poxvirus infected cells). In auxiliary plasmid provide relevant enzymes, under the action of cDNA cloning for transcription and each gene expression, eventually assemble infectious viral particles (Mohamed and Niles, 2004). In reverse genetics technologies to produce a decade or 2 years, the initial negative chain RNA viruses salvation generally adopted by reorganization poxvirus infected cells to provide T7 RNA Polymerase Method, this method to save the efficiency is not high even save the virus also need to the complicated procedure from vaccinia virus isolated. Vaccinia virus in auxiliary save process may cause RNA restructuring and produce a cell pathological changes will affect the newborn save virus proliferation.

Schnell *et al*. (1994) first used could express T7 RNA polymerase restructuring poxvirus VTF7-3 strains carrying cell auxiliary infection of infectious save out of the rabies virus particles but this VV/T7 has system save is very inefficient, usually around from 107 cells can only get a infection units.

Its reason is VV infections Produce Cytopathic Effect (PCE) will eventually kill load cells thus, affecting the purpose protein expression and save virus proliferation, VV copy can interfere with the save the virus produced even save out infectious viral particles but often far less than the quantity, the virus purification VV process trival, complex.

Although, later based on VV/T7 has system derived a series of alternative systems such as FPV/T7 has system, performing/T7 has system and Bac/T7 has system, etc. but are used in other expression system replace VV carrying cell line to produce T7 has infected RNA polymerase, reduce CPE production to ensure the smooth save virus but still cannot guarantee the virus efficiency (Czub *et al*., 2005; Kusov *et al*., 2002; Okeke *et al*., 2009; Delaney *et al*., 2010; Hodge *et al*., 2003; Goonetilleke *et al*., 2003). Obviously, build a plant independent stable expression T7 RNA polymerase lines, solve the above problem is undoubtedly the best method.

Buchholz *et al*. (1999) with BHK-21 cells-T7 has constructed aiming lines/five successful save a respiratory syncytial virus That same year, Romer-Oberdorfer *et al*. (1999), etc. with this cell lines save out of the NDV Clone-30 plants. Then, overseas scholars and successively constructed the other can stable expression T7 has RNA polymerase of cell lines such as 293-3-4 6, SK-6T7 (Van Gennip *et al*., 1999), etc. Liu suggested that the BHKT7 cell line was stable even after multiple passages, FMD virus was rescued efficiently from the BHKT7 cells, expressing sufficient T7 RNAP to support *ex vivo* transcription and efficient rescue. These lines construction not only greatly improved the virus to save the efficiency but also has promoted the RNA viruses reverse genetics study (Kobayashi *et al*., 2007; Massin *et al*., 2005; Huang *et al*., 2004; Walpita and Flick, 2005).

This room prophase constructs save goose source vice sticky virus making-1 strain of three necessary auxiliary plasmid and combining domestic and foreign successful experience for establishing a goose source vice sticky virus making-1 plant efficient reverse genetic operation platform, this test will T7 has *RNA* polymerase gene into BHK-21 cells, the G418 gradually dominant selection, on screening of survival training, continuous preach cell expand 30 generations, cellular form has not seen the abnormal, followed by a cells extracted total RNA RT-PCR, the Western blot identification, Confirm the VT7 lines can construct expressed efficiently T7 RNA polymerases (Huang *et al*., 2000; Chang *et al*., 1999; Place *et al*., 1999).

CONCLUSION

The results of this study shows that the retroviruses gene transfer technology established express T7 RNA polymerase of cell lines may be more stable but can stable expression T7 RNA polymerase 30 generations of cell lines already enough virus salvation. Stable expression T7 RNA polymerase VT7 lines established for the next step of constructing and establishment of miniature genome effective virus save system to lay the foundation.

ACKNOWLEDGEMENT

This research was supported by Scientifical and Technological Project of Guangdong province of China (No.: 2008A020100020).

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