ISSN 1009 - 3095 *Journal of Zhejiang University(SCIENCE)* V.2, No.4, P.445 - 447, Oct. - Dec., 2001
http://www.periodicals.com.cn; http://www.zju.edu.cn;
http://lib.zju.edu.cn/English; http://www.zjupress.com

CONSTRUCTION AND IDENTIFICATION OF THE RECOMBINANT OF THE AAV VECTOR AND HUMAN INTERFERON-GAMMA*

ZHU Hai-hong(朱海红)[1], CHEN Zhi(陈 智)[1],
ZHANG Ming-tai(章明太)[1], DING Lie-ming(丁列明)[2]

([1] *Institute of Infectious Diseases, College of Midicine, Zhejiang University, Hangzhou 310003, China*)

([2] *Department of Medicine, University Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA*)

Received Jan.6, 2001; revision accepted Apr.18, 2001

Abstract: To construct and identify further a recombinant of Adeno-associated virus and interferon-gamma for gene therapy, the full-length IFN-γcDNA containing signal peptide was amplified by PCR, and then cloned into the pUC18. After screening, the fragment from the positive clone was then subcloned into pwp19. After the correct recombinant was identified by digestion with SacI and BamHI, it was transfected into lymphocyte cell line H9 mediated by calcium phosphate, and the expression of IFN-γ was detected by RT-PCR and ELISA. The result showed that the IFN-γ were expressed in the H9 cells transfected with pwp/IFN-γ. The so constructed recombinant plasmid pwp19/IFN-γ containing the full-length IFN-γ gene was expressed in mammalian cells.

Key words: Adeno-associated virus, gene therapy, interferon-gamma
Document code: A **CLC number:** R51

INTRODUCTION

An Adeno-associated virus(AAV) particle is composed of three structural proteins and a linear, single-stranded DNA(ssDNA) genome of approximately 4.7 kb. AAV is a defective human parvovirus that normally replicates productively only in the presence of helper functions provided by a co-infecting adenovirus or herpesvirus. AAV has not so far been implicated as the etiologic agent of any disease, and the viral DNA integrates into chromosome 19 at a site previously localized to position q13.3-qter(Samulski et al., 1989). So the site-specific integration of an AAV provirus has no apparent affect on cell growth, morphology, or differentiation. These properties of AAV suggested that it could be useful as a gene transfer vector; so a recombinant plasmid constructed to facilitate genetic analysis of the virus and the introduction of exogenous genes into a variety of cells (Cottard et al., 2000, Bjorklund et al., 2000).

Human interferon-γ(IFN-γ) has some biological activities such as anti-virus, anti-tumor and anti-liver fibrosis. IFN-γ expressed in eukaryotic cell by the method of gene therapy may make up for the disadvantages of clinical application of IFN-γ expressed in prokaryotic cell.

In this work, we constructed and identified a recombinant plasmid pwp19/ IFN-γ.

MATERIALS AND METHODS

Plasmids, bacterial strains and cells

pwp19 contain inverted terminal repeats of AAV and can be expressed in eukaryotic cells (Nahreini et al., 1993). They were provided by the Arkansas University for Medical Sciences and the John L. Mcllellan VA Medical Center.

pGEM-1/IFN-γ plasmid containing signal peptide served as a PCR template of the full-length IFN-γ CDNA provided by the Academy of Military Medical Sciences.

pUC18 plasmid was purchased from Roche Diagnostics Ltd. (Sambrok et al., 1989)

* Project(39570653) supported by NFSC.

Plasmids mentioned above were amplified in *E. coli* strain DH5α.

H9 cells make up an immortalized human lymphocyte line.

cDNA synthesis

pGEM-1/IFN-γ plasmid was prepared from E. coli strain DH5α by the alkaline method(Sambrok et al., 1989). Then after 1:1000 dilution, the plasmid was used as PCR template. The upstream primer and downstream primer used were 5′-GCA TGA AAT ATA CAA GTT ATA TCT TGG-3′,5′-ATT TAC TGG GAT GCT CTT CGA CCT C-3′, respectively(Devos. et al., 1982). To 50 μL solution, 5 μL of 10 × PCR Buffer, 3 μL of 50mmol / L $MgCl_2$, 2.5 μL of 1% W-1, 4 μL of dNTPs (2 mmol/L), 1 μL of upstream primer (20 pmol), 1 μL of downstream primer (20 pmol), 23.5 μL of H_2O, 10 μL of denatured template, and 1U Taq DNA polymerase (Gibco BRL) were added。 After PCR through 35 cycles (94 ℃ for 1 minute, 55 ℃ for 40 seconds, 72 ℃ for 40 seconds), the PCR products were detected by 1.5% agarose gel containing 0.5 μg/mL EB.

Cloning of the IFN-γ gene

Via T-A cloning, the PCR product was ligated with pUC18 vector digested beforehand by SmaI and had thymine added to its 3′end. After screening on LB agar plate containing ampicillin/X-gal/IPTG in JM109, the recombinants were identified. Then the positive colonies containing the IFN-γ gene were verified by PCR and restriction analysis with SacI and BamHI. One of them with correct inserting direction was further obtained by digestion with XbaI and MvaI.

The recombinant plasmid pwp19/IFN-γ was then constructed by insertion of IFN-γ into the SacI and BamHI site of pwp19. After screening by ampicilline resistance, PCR and digestion with SacI and BamHI, the positive recombinant was proved correct. The cloning strategy is shown in Fig1.

Transfection

H9 cells were cultured with RPMI1640 containing 10% fetal calf serum. Large-scale prepared pwp19/IFN-γ recombinant was transfected into H9 cell mediated by calcium phosphate coprecipitation. Cells transfected with pwp19 vector, cultured

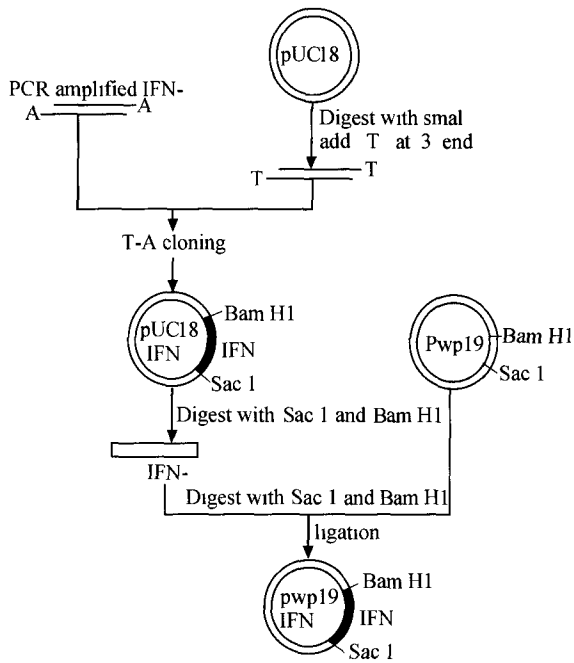

Fig.1 Strategy for cloning recombinant pwp19/IFN-γ

with calcium phosphate without plasmid , and cultured without any manipulations served as controls. Sixty hours after transfection, the supernatants of cultures were collected and the cells were harvested after washing twice with PBS.

Expression of IFN-γ

The total RNA, and protein were successively extracted with Trizol Reagent (Gibco BRL, U.S.A).

After digestion with DNaseI (RNase free), the total RNA from the cells were utilized for RT-PCR, using the oligo dT_{15} (20 pmol), 5 μL of 5 × first strand buffer, 2 μL of 100 mmol/L DTT, 2 μL of 2 mmol/L $dNTP_S$, 100 u SuperScript II RT(Gibco BRL), 5 μL of sample RNA (pretreated in 70 ℃ bath for 10 minutes and then chilled on ice)。 The mixture was incubated at 37 ℃ for 1 hour. The RNA-DNA hybrid was used for PCR amplification using the primer mentioned above and the same procedure.

The supernatants of cell cultures and the proteins extracted from cells were measured for IFN-r using ELISA kit (Genzyme, U.S.A).

RESULTS

cDNA synthesis:

After PCR reaction, the 504 bp DNA band

(Fig. 2) was detected from plasmid pGEM-1/IFN-γ by the agarose gel.

Cloning of the IFN-γ gene:

After ampicillin and PCR screening, the correct recombinant pwp19/IFN-$\gamma$was identified by digestion with SacI and BamHI. The fragment was 504bp in length.

Expression of IFN-γ:

After transfection, the 504 bp DNA band was detected from total RNA by RT-PCR from the transfected cells with the pwp19/IFN-γ recombinant, whereas no DNA bands were found from the control cells(shown in Fig2).

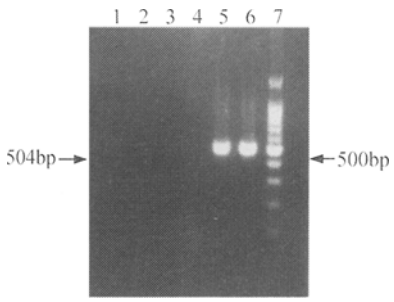

Fig.2 Products of total RNA from cells by RT-PCR
lane1: RNA from cells transfected with pwp19 vector;
lane2: RNA from cells cultured with calcium phosphate;
lane3: RNA from cells without any manipulation;
lane4: water;
lane5: RNA from cells transfected with pwp19/IFN – γ recombinant;
lane6: PCR product of pGEM – 1/IFN – γ plasmid;
lane7: 100bp ladder (Promega, U.S.A.)

The result showed that in the protein of the cells transfected with pwp19/IFN-γ recombinant, there were 17.2 pg IFN-γ in 3×10^6 cells, but no IFN-γ was found in the supernants of these cells; and IFN-γ was not detected in the supernants and the proteins of the control cells.

DISCUSSION

As the result showed, we successfully constructed the recombinant of pwp19/IFN-γ. In order to get it, we first constructed the recombinant of pUC18/IFN-γ. This was confirmed by PCR screening and restriction analysis. pUC18 plasmid take advantage of having a series of polylinkers containing a large number of unique sites for restriction endonuclease including SacI and BamHI, upstream and downstream of the site of SmaI which yield blunt ends after cleavage. This is useful because the terminals of the IFN-γ do not contain the two sites, and IFN-γ insert into pUC18 at the site of SmaI after cleavage via T-A cloning.

Then the interesting gene of IFN-γ with full-length cDNA was subcloned into AAV vector pwp19 so that a recombinant was constructed as confirmed by PCR product; and also corroborated by digestion with SacI and BamHI; and further proved by transient transfection into H9 cells. RT-PCR and ELISA results from RNA and protein, showed that IFN-γ was very weakly expressed in H9 cells, at yield of as low 17.2 pg IFN-γ in 3×10^6 cells. The failure to find IFN-γ in the supernatants of these cells in this study could possibly be due to the sensitivity of the ELISA used. As target cells for human gene therapy, lymphocytes have a lot of advantages: easy to obtain from body and manipulate *in vitro*; easy to culture and amplify *in vitro*; easy to be transducted with therapeutic genes effectively; able to endure the procedure of screening; easy to be transplanted into body. We utilized the H9 cells of a defective $CD4^+$ T lymphocyte cell line to observe the condition of transfection and expression of pwp19/IFN-γ recombinant.

References

Bjorklund, A., Kirik, D., Rosenblad, C. et al., 2000, Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res.*, **886**(1 – 2):82 – 98.

Cottard, V., Mulleman, D., Bouille, P. et al., 2000, Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis. *Gene Ther.*, **7**(22):1930 – 1939.

Devos, R., Cheroutre, H., Taya Y. T. et al., 1982, Molecular cloning of human immune interferon cDNA and its expression in eukaryotic cells. *Nucleic Acids Res.*, **10**(8):2487 – 501.

Nahreini, P., Woody, M.J., Zhou, S.Z. et al., 1993, Versatile adeno-associated virus 2-based vectors for constructing recombinant virions. *Gene*, **124**(2): 257 – 262.

Sambrok, J., Fristch, E.F., Maniatis, J., 1989. Moecular Cloning Laboratory Mannual. 2nd edition. Cold Spring Harbor, Cold Spring Harbor Laboratory Press. NY.

Samulski, R. J., Chang, L. S., Shenk T. et al., 1989, Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.*, **63**(9):3822 – 3828.