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• BRIEF REPORTS •

Cloning of HBsAg-encoded genes in different vectors and their expression in eukaryotic cells

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Abstract

AIM: To compare the efficiency of different plasmids as DNA vectors by cloning three HBsAg-encoded genes into two eukaryotic expression vectors, pRc/CMV and pSG5UTPL/Flag, and to express HBsAg S, MS, and LS proteins in SP2/0 cells, and to establish monoclone SP2/0 cell strains that are capable of expressing S or S2S proteins stably.

METHODS: Segments of S, preS2-S, preS1-preS2-S genes of Hepatitis B virus were amplified by routine PCR and preS1-S fragment was amplified by Over-Lap Extension PCR. The amplified segments were cleaved with restricted endonuclease Hind III/Not I followed by ligation with pRc/CMV, or BamHI/EcoR I followed by ligation with pSG5UTPL/Flag. After the plasmid vectors were cleaved with the correspond enzymes, the amplified segments were inserted into pRc/CMV or pSG5UTPL/Flag plasmid vectors with T4 DNA ligase. KOZAK sequence was added before the initial ATG code of each fragment using specific primer. The inserted segments in the recombinant plasmids were sequenced after subcloning. BALB/c mice myeloma cells (SP2/0 cell line) were transfected with the recombinant plasmids. The expressions of the different recombinants were compared by Western-blot, using a monoclonal anti-HBs antibody as the primary antibody and peroxidase-labeled multi-linker as the secondary. Stable SP2/0-pRc/CMV-S or SP2/0- pRc/CMV-MS clones were established through clone screening with G418.

RESULTS: Fragments with anticipated size were harvested after PCR. After recombination and screening, the sequences of the inserted segments in the recombinants were confirmed to be S, preS2S, preS1-preS2S and preS1S encoding genes, determined by sequencing. The results of Western-blot hybridization were positive for the anticipated proteins. Among them, pRc/CMV-S or pRc/CMV-MS demonstrated the highest expressing their respective antigen.

CONCLUSION: Eight recombinant plasmids expressing S, M, L or preS1S proteins are obtained. For hepatitis surface antigen expression in eukaryotic cells, the vector pRc/CMV is superior to pSG5UTPL/Flag, and pRc/CMV-S and pRc/CMV-MS are the most efficient in the pRc/CMV clones. SP2/0 cells stably expressing HBsAg are established, and may be used as target cells for evaluating the CTL activity of a DNA vaccine in vitro.

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INTRODUCTION

Hepatitis B virus (HBV) infection is epidemic worldwide[1-3]. Unfortunately, there are no satisfactory drugs to cure HBV-related diseases, and the only way to control the epidemic is through vaccination[4-6]. Great efforts have been made to develop more successful vaccines than those currently available to prevent or to treat HBV infection. The newest approach is genetic immunization. This involves the transfer of a viral gene into host somatic cells by a plasmid vector, with subsequent endogenous production and intracellular processing of the virus' structural proteins into small peptides. The processed peptides eventually induce a broad-based immune response[7-11]. Hepatitis B virus surface antigen consists of S (S, small surface), MS (medium surface, S+preS$_2$), and LS (large surface, S+preS$_1$+preS$_2$) HBsAg molecules. We have reported that DNA vaccine expressing HBsAg S molecule induced humoral and cellular immune responses against HBsAg[12,13]. Because the preS antigen is necessary for HBV to penetrate the cell membrane of the host, vaccines containing the preS antigen are more effective. Hence, we constructed a series of plasmids encoding small (S), medium (M), or large (L) envelope proteins of HBsAg utilizing different promoters. In addition, the expression levels of these recombinant plasmids were evaluated, in order to choose a better vector for designing future DNA vaccines.

MATERIALS AND METHODS

Gene fragments expressing S, MS, LS, and preS1S proteins

Plasmid pBHB$_4$ (containing the *adr* subtype of HBV genome DNA) was used as a template to amplify S, preS$_2$ and preS$_1$ fragments. The following primers were used: the forward primers: SF (nt 28-41), 5' - GCG AAT TCT AGC TTA TCG ATC ACC *ATG GAG AAC ACA AC*, complementary to the S gene; S$_2$F (nt 3077-3090), 5' - GCG AAT TCA AGC TTA TCG ATC ACC *ATG CAG TGG AAC ACA TC*, complementary to the preS$_2$ gene; and S$_1$F (nt 2179-2733), 5' - GCG AAT TCA AGC TTA TCG ATC ACC *ATG GGA GGT TGG TC*, complementary to the preS$_1$ gene. The same reverse primer was used for all reactions: SR (nt 705-693): GCG CGG CCG CTT AGG ATC CAA TCG *ATA CCC AA*. The primers contain a flanking sequence having endonuclease enzyme sequence

for the convenience of clone manipulation.

To obtain a unique fragment containing the S and $preS_1$ gene, overlap extension PCR was used. The forward primer (S_1-SF) is complementary to the beginning of the S gene and to the end of preS1 gene, while the reverse primer (S-S_1R) is complementary to the end of the $preS_1$ gene and the beginning of the S gene (Figure 1). Using $pBHB_4$ as a template, and the S_1-SF/SR and S_1F/S-S_1R primers, the resultant PCR product contained the 3' end of $preS_1$ gene followed by the complete S gene sequences that the two PCR fragments contained complementary ends. When these fragments were consequently used as templates and S_1F/SR primers, only the $preS_1$-S was amplified.

S_1-SF (5')	*CAT CCT CAG GCC*	ATG GAG AAC ACA
	preS1 (nt3065-3076)	S(nt28-39)
S-S1R (5')	*TGT GTT CTC CAT*	GGC CTG AGG ATG
	S(nt39-28)	preS1(nt3076-3065)

Figure 1 Primers designed for Overlap-Extension PCR.

Recombinant plasmids construction

The PCR fragments and plasmid vector pRc/CMV (containing the CMV promoter, Invitrogen, USA) were cleaved by HindIII/NotI (Bio-Lab, USA), while the PCR fragments and plasmid vector pSG5UTPL/Flag (containing SV40 promoter) were cleaved by BamHI/EcoR I (Bio-Lab, USA). The cleaved products were ligated using T_4 DNA ligase. *E.coli* XLBlue1 was transformed using the ligated products and screened for the positive clones containing the inserted fragments, which was performed by colony-PCR techniques. The positive colonies were amplified, extracted, purified, and identified by endo-nuclease cleavage. The sequences of the inserted fragments were confirmed by sequencing with Nucleic Acid Auto-Analysis (TYPE 373A).

Transient expression in vitro

The extracted recombinant plasmids were purified by CsCl/ethidium bromide equilibrium centrifugation. SP2/0 cells (myeloma cells from inbred BALB/c mice) were transfected with purified plasmids by an amended calcium chloride method as described elsewhere[14]. Two days later, the cells were collected in LAC buffer (20 mmol· L^{-1} Hepes pH 7.9, 400 mmol· L^{-1} NaCl, 1 mmol· L^{-1} EDTA, 9 mmol· L^{-1} CHAPS, 250 ml· L^{-1} glycerol) containing 1/200V 0.5 mol· L^{-1} DTT, 2.5 mmol· L^{-1} EDTA, 5 g· L^{-1} Apotinine, 5 g· L^{-1} Leupetine and 0.2 mol· L^{-1} PMSF, and lysed by sonication (Ubra-Cell, Sonics & Materials, USA). Supernatants were collected and analyzed as follows: proteins were separated by 120 g· L^{-1} SDS-polyacrylamide gel, electrophoresis, and transferred to PVDF membranes (Millipore, USA), followed by blocking for 2 h in PBS containing 50 g· L^{-1} nonfat dry-milk at room temperature. To detect HBsAg, the membranes were first incubated with mono-clone anti-HBs antibody (BioGenex, USA) at 1:1 000 dilution and conjugated antibodies were detected with horseradish peroxidase-labeled multi-linker antibodies (BioGenex, USA) at a dilution of 1:300. The blots were visualized by chemiluminescence reactions (Luminol and Iodophenol, Fluka).

Long-term transfected cell strains

After the transient in vitro expression, the plasmids demonstrating a high level of expression were chosen to permanently transfect SP2/0 cells. After 2 d of transfection, fresh cultural medium containing 200 mg· L^{-1} G418 (at this concentration, there were no viable SP2/0 cells after 2 w in preliminary studies) was added in order to grow the cells under G418 pressure. The media was refreshed every 3 to 5 d. 2w later, cells forming colonies were chosen using a light microscope, and subcultured under G418 pressure. After 30 generations, the DNA was extracted following the protocols described elsewhere[15] and detected the HBV S gene by dot-blot hybridization (Dig-probe of HBV S was prepared in our laboratory, Dig-labeling Kit from Roche Company). HBsAg in the culture media and supernatant of cell lyses were detected by ELISA (Kits from SABC), HBsAg in the cells by immunochemistry. Presence of the plasmid was detected by *in situ* hybridization with the corresponding probe (labeled in our laboratory, Dig-label kit from Roche Company). The cells containing the HBV S gene and the HBsAg were chosen as long-term expression cell strains and stored in liquid nitrogen for further use as the target cells for evaluation of CTL activity after DNA immunization.

RESULTS

Construction of recombinant plasmids

DNA fragments of 0.72kb, 0.88kb, and 1.24kb were generated by PCR methods ($pBHB_4$ template and SF/SR, S_2F/SR, S_1F/SR primers). The length of these fragments corresponded to the length of S, MS, and LS genes. When the PCR product primed by S_1F/S-S_1R and S_1-SF/SR was used as templates (overlap extension PCR), a 1.02kb DNA fragment, equal to the length of $preS_1$S was obtained. After cleavage by Hind III/Not I, S, MS, LS and $preS_1$S genes were inserted into a multiple cloning site downstream of the CMV promoter in pRc/CMV, while the fragments cleaved by Bam HI/EcoR I were inserted into multiple cloning site downstream of the SV40 promoter in pSG5UTPL/Flag. *E.Coli.* XLBlue1 cells were transformed separately with the series 8 recombinant plasmids, and cultured in LB containing ampicillin. PCR was performed on colonies, and 0.72kb, 0.88kb, 1.24kb, and 1.02kb fragments were obtained. When these PCR products were cleaved by restriction enzymes Hind III/Not I (pRc/CMV as vector) or Bam HI/EcoRI (pSG5UTPL/Flag as vector), the anticipated DNA bands were obtained. We have constructed CMV promoter controlled recombinant plasmids: pCMV-S, pCMV-MS, pCMV-LS, pCMV-S_1S; and SV40 promoter controlled recombinant plasmids: pSG5Flag-S, pSG5Flag-MS, pSG5Flag-LS, pSG5Flag-S_1S. KOZAK sequences (CACC) were placed before the initiation code of all recombinant plasmids to enhance the efficiency of protein expression. The sequences of the inserted fragments of all 8 plasmids were identical to HBV-S, MS, LS and $preS_1$S genes, confirmed by following sequencing.

Transient expression

After SP2/0 cells were transfected with recombinant plasmids above, the supernatants of lysed cells were run on SDS-PAGE and Western-Blot, all were positive for the anticipated bands, while the SP2/0 cells not transfected with the recombinants were negative for any HBsAg antigen. Among them, pRc/CMV-S and pRc/CMV-S2S demonstrated the highest expression.

Construction of long-term expression cell strains

pRc/CMV-S and pRc/CMV-S_2S were used to transfect SP2/0 cells. The transfected cells grew in clusters under the selection pressure of G418. After 2 mo, 10 strains of SP2/0-pRc/CMV-S and 16 strains of SP2/0-pRc/CMV-S_2S continued to grow well in G418-containing medium, while SP2/0 cells not transfected with recombinant plamid died within 2 wk. Of the G418 resistant monoclone cells, 3 strains of SP2/0-pRc/CMV-S and 4 strains of SP2/0-pRc/CMV-S_2S were positive for HBsAg in the supernatants of sonicated cells. The HBsAg was detected by ELISA. They were also positive for the protein

bands when detected by Western-Blot. Cytosolic HBsAg was detected by immunochemistry. The 7 cell strains above were also positive for pRc/CMV-S or pRc/CMV-S_2S when detected by *in situ* hybridization.

DISCUSSION

DNA immunization involves the recombination of the genes of interest with the selected eukaryotic expression plasmid/vector and the transfer of the recombinant plasmid into muscle or skin cells of the host, with a subsequent induction of specific immune responses[16-20]. DNA immunization is attractive for its advantages over traditional vaccines, and is discussed in elsewhere[21-24]. We have reported that HBV DNA vaccine NV-HB/s could express HBsAg in muscle cells after injecting into mouse muscle, followed by the induction of an immune response, including the switchover of anti-HBs production in peripheral blood[12,13].

HBsAg-encoded genes include S, preS_1, and preS_2. All the proteins expressed by these genes are antigenic. The elicited response is strongest with preS_1 and preS_2. The titer of preS antibody in rabbits immunized with preS protein is 400 times greater than that of S antibody in rabbits immunized with S protein. In addition, some mice are non-responsive to S protein of HBV, which is determined by an allele of H_2 gene. Vaccines containing HBV preS_1 and/or preS_2 antigen may alter this inherited non-responsive state, inducing anti-HBs in mice which were previously immunized with vaccines only containing S. Moreover, preS proteins are necessary in order for HBV to infect hepatocytes. preS_1 protein can bind hepatocytes specifically while preS_2 can bind hepatocytes through PHSA (polymerase human serum albumin). Because of the importance of HBV preS proteins, we constructed plasmids, which can be used for DNA vaccines containing the preS_1 and/or preS_2 genes. Several plasmids were constructed by different promoters in order to compare their expression proficiency. All these recombinant plasmids demonstrated expression in SP2/0 cells, while the vector pRc/CMV was the most effective, with the highest expression demonstrated as pRc/CMV-S and pRc/CMV-S_2S. pRc/CMV-S and pRc/CMV-S_2S may be used as good candidates for DNA immunization. Their efficacy in inducing immune responses is still in experimental process.

Long-term expression cell strains transfected with pRc/CMV-S or pRc/CMV-S_2S were established in our experiment through selection with G418 more than 30 generations. The cell lines SP2/0-pRc/CMV-S and SP2/0-pRc/CMV-S_2S can express HBsAg S protein or S_2S protein effectively and may be used as target cells for CTL test in the study of DNA immunization for HBV in the near future.

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