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Construction and Eukaryotic Expression of Recombinant Large Hepatitis Delta Antigen

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Abstract

Background: Hepatitis delta virus (HDV) is a subviral human pathogen that exploits host RNA editing activity to produce two essential forms of the sole viral protein, hepatitis delta antigen (HDAg). Editing at the amber/W site of HDV antigenomic RNA leads to the production of the large form (L-HDAg), which is required for RNA packaging.

Methods: In this study, PCR-based site-directed mutagenesis by the overlap extension method was used to create the point mutation converting the small-HDAg (S-HDAg) stop codon to a tryptophan codon through three stages.

Results: Sequencing confirmed the desirable mutation and integrity of the L-HDAg open reading frame. The amplicon was ligated into pcDNA3.1 and transfected to Huh7 and HEK 293 cell lines. Western blot analysis using enhanced chemiluminescence confirmed L-HDAg expression. The recombinant L-HDAg localized within the nuclei of cells as determined by immunofluorescence and confocal microscopy.

Conclusion: Because L-HDAg requires extensive post-translational modifications, the recombinant protein expressed in a mammalian system might be fully functional and applicable as a tool in HDV molecular studies, as well as in future vaccine research.

Keywords: Hepatitis Delta Virus, L-HDAg, SOEing-PCR

Introduction

Hepatitis delta virus (HDV) is a satellite of hepatitis B virus (HBV) (1-2). The HDV virion contains an inner core structure (1, 3-4) containing a small, single-stranded, circular RNA molecule of about 1.7 kb that is associated with the only known virus-encoded protein, hepatitis delta antigen (HDAg), and an outer envelope of hepatitis B surface antigen (1-2, 5). HDV RNA replication proceeds via a double rolling-circle mechanism in which the genomic and antigenomic full-length species serve as the respective templates for the synthesis of the other strand (3-4, 6-7).

There are two forms of the HDAg; the first, the small delta antigen (S-HDAg), a 195 amino acid polypeptide, is essential for replication of the RNA genome; the second, the large delta antigen (L-HDAg), 19 amino acids longer than S-HDAg at its C-terminus, arises as a consequence of post-transcriptional RNA-editing (8-9).

Editing at the amber/W site of antigenomic HDV RNA extends the S-HDAg open reading frame (ORF) by 19 amino acids (aas), inducing the generation of the large antigen (L-HDAg). Editing of HDV RNA is highly specific for the amber/W site and mainly occurs late in the viral infection cycle (10-12). These two isotypes of HDAgs are identical for the first 195 aas from the N-terminus, so they share some features. One important common feature is a

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coiled-coil domain. This domain plays a critical rule in activities of L-HDAg and S-HDAg. L-HDAg is a dominant inhibitor of HDV replication and is required for assembly of the HDV particle. L-HDAg can package into pseudo-viral particles with hepatitis B surface antigens in the absence of both HDV RNA and S-HDAg (13). Interestingly, L-HDAg is modified by an isoprenoid lipid on a cysteine residue located near its carboxyl terminus (13), and this modification is necessary for HDAg to facilitate HDV assembly.

Gene splicing by overlap extension provides a powerful method of site-directed mutagenesis without dependence on restriction sites or ligase, and a simple, generally applicable way of using a polymerase reaction to perform site-directed mutagenesis in vitro. Overlap extension PCR (SOEing-PCR) has three main advantages over traditional methods; these are 1) recombination and mutagenesis can be performed simultaneously, 2) all molecular products are mutated, and 3) it is an in vitro process that does not need a plasmid or phage for multiplication of the gene and can be used directly in experiments (14).

In the present study, the L-HDAg ORF was constructed by changing the amber codon to a tryptophan codon via SOEing-PCR, and cloned for expression in a mammalian cell system. L-HDAg expression was assessed by Western blotting, immunofluorescence, and confocal microscopy.

Materials and Methods

Primers design

For SOEing-PCR, two pairs of external and internal primers were designed. Sequence, polarity, and position of the primer pairs, as well as restriction enzyme cutting sites are presented in Table 1. The external forward and reverse primers, h1 and h4, respectively, were designed from the HDAg ORF at locations 1598 (start codon) and 954 (stop codon) according to Kuo numbering (15). NehI and BamHI restriction sites and additional nucleotides on either side were created in primers h1 and h4 respectively, conforming to our cloning strategies. The internal primers h2 and h3, in addition to providing the desired mutation, contained restriction sites for NcoI. This site was used to confirm ligation of fragments and site-directed mutations.

Table 1. Sequences of primers used to amplify a 675-nucleotide (nt) product from cDNA by SOEing-PCR. Primers h2 and h3 overlap and contain the mutation. Primers h1 and h4 are external primers and start from nucleotides 1594 and 938, respectively (L-HDAg length); numbering is according to Kuo et al.

Primer designation	Sequence (5' to 3')	Position & Polarity	Amino acid substitutions
h1	5' cgc cgc tag ccg aga tga g 3'	1594-1612 Sense	-
h2	5' gaa gag tat atc cca tg*g aaa tcc c 3'	999-1023 Antisense	amber/w
h3	5' ggg att tcc* atg gga tat act ctt c 3'	999-1023 Sense	amber/w
h4	5' tgg gat ccc gct tta ttc act gg 3'	938-960 Antisense	-

Underlined: restriction sites; *: codon changes in contrast to the wild type sequence.

SOEing-PCR

SOEing-PCR was performed in three stages. In the first and second stages, pCDNA3.1D2, a clone containing two tandem repeats of complete HDV cDNA, was used as the template (16). PCRs were carried out in reaction volumes of 25 µl containing 1X Pfu buffer (20 mM Tris-HCl pH 8.8), 10 mM $(NH4)_2SO_4$, 10 mM KCl, 0.1% (v/v) Triton X-100, 0.1 mg/ml of bovine serum albumin (BSA), 2 mM $MgSO_4$, 0.2 mM of each dNTP, 10 pmol of each forward and reverse primer, 50-100 ng of template DNA, and 1 U of Pfu DNA polymerase (Fermentas, Lithuania).

The first PCR stage used primers h1 and h2 as shown schematically in Fig. 1 under the following conditions: initial denaturation at 94 ºC for 3 min followed by 34 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 55 ºC for 30 sec, and extension at 72 ºC for 1 min, followed by a final extension step at 72 ºC for 10 min. The second PCR stage used primers h3 and h4 in the above PCR mixture under the following conditions: initial denaturation at 94 ºC for 3 min followed by 34 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 55 ºC for 30 sec, and extension at 72 ºC for 20 sec, followed by a final extension step at 72 ºC for 10 min. The third PCR stage used a 1:100 dilution of the

amplicons obtained in the first and second stages together as the template.

In the third PCR stage, to produce and amplify the full-length L-HDAg ORF cDNA, primers h1 and h4 were used in the reaction mixture under the following thermal cycling profile: initial denaturation at 94 ºC for 3 min followed by 34 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 55 ºC for 30 sec, and extension at 72 ºC for 1 min, followed by a final extension step at 72 ºC for 10 min.

The first and second PCR stages used *Pfu* and the third stage used *Taq* DNA polymerase (Fermentas).

Plasmid and cloning

The PCR product was gel-purified and cloned into the pTZ57R/TA vector (Fermentas). Following blue-white screening, the recombinant pTZ57R/T-LHDAg vectors were selected. The orientation of the inserted fragment was verified by restriction analysis. To verify the desired mutations, the recombinant vector was sequenced bidirectionally using M13/pUC forward and reverse sequencing primers. pcDNA3.1(-) and pIRES2-EGFP were used as expression plasmids in mammalian cells.

The L-HDAg fragment was digested with NheI and BamHI and subcloned into the pcDNA3.1(-) and pIRES2-EGFP vectors that were linearized with the same restriction endonucleases. The new recombinant vectors were restriction-digested to confirm cloning fidelity. All DNA manipulations including restriction digestion, T4 ligation, and agarose gel electrophoresis were carried out as described by Sambrook and Russell (17).

Transfection of Huh7 and HEK293 cells

One day prior to transfections, 5 x 10^5 cells were plated into 35 mm 6-well plates and cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum, 100 IU/mL of penicillin, 100 mg/mL of streptomycin, and 2 mM L-glutamate. Following overnight incubation (18–20 h), when the confluency reached 70%, cells were transfected with pcDNA3.1-LHDAg, pIRES2-EGFP-LHDAg, pcDNA3.1D2 as a positive control, and pcDNA3.1(–) as a negative control using 4 µg of plasmid and 10 µl of lipofectamine (Invitrogen), according to the manufacturer's protocol. The cells were harvested 72 h after transfection.

Immunocytochemistry

Huh7 and HEK293 cells were seeded into wells of 24-well culture plates. The transfections were performed as above. 72 h post-transfection, cells on the plate were washed with cold phosphate-buffered saline (PBS) and fixed in 3-4% paraformaldehyde for 15 min at room temperature. The cells were again washed with cold PBS and incubated for 10 min in PBS containing 0.25% Triton X-100. The cells were washed with cold PBS and incubated with 1% BSA in PBS-Tween 20 (PBST) for 30 min to block non-specific antibody binding. Then the samples were incubated for 1 h at room temperature with the appropriate dilution of a human high-titer anti-delta antiserum as the primary antibody. After washing, the samples were incubated with a 1:25 dilution of anti-human IgG conjugated with fluorescein isothiocyanate (FITC) (Razi Fara Teb, Iran) for 60 min at 37 °C. Antibody-labeled cells were observed by both fluorescent (Olympus IF Microscope) and confocal microscopy (Leica Microsystems).

Western blotting

Delta antigen was detected by Western blot analysis of cell lysates 2 days post-transfection. Cells were lysed directly with 400 µl of sample buffer (80 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.0015% bromophenol blue) per well and boiled for 5 min. 30 µl of the sample solutions were separated by 12.5% SDS-polyacrylamide minigels, along with molecular weight protein marker (Fermentas, Lithuania) and electrophoretically transferred to nitrocellulose filters. The filters were blocked with 3% skimmed milk and incubated overnight with a 1:100 dilution of a human high-titer anti-delta antiserum. After washing, filters were incubated with a 1:1000 dilution of goat anti-human IgG conjugated with HRP (Razi Fra Teb, Iran) for 1 h. Bands were detected by enhanced chemiluminescence (ECL).

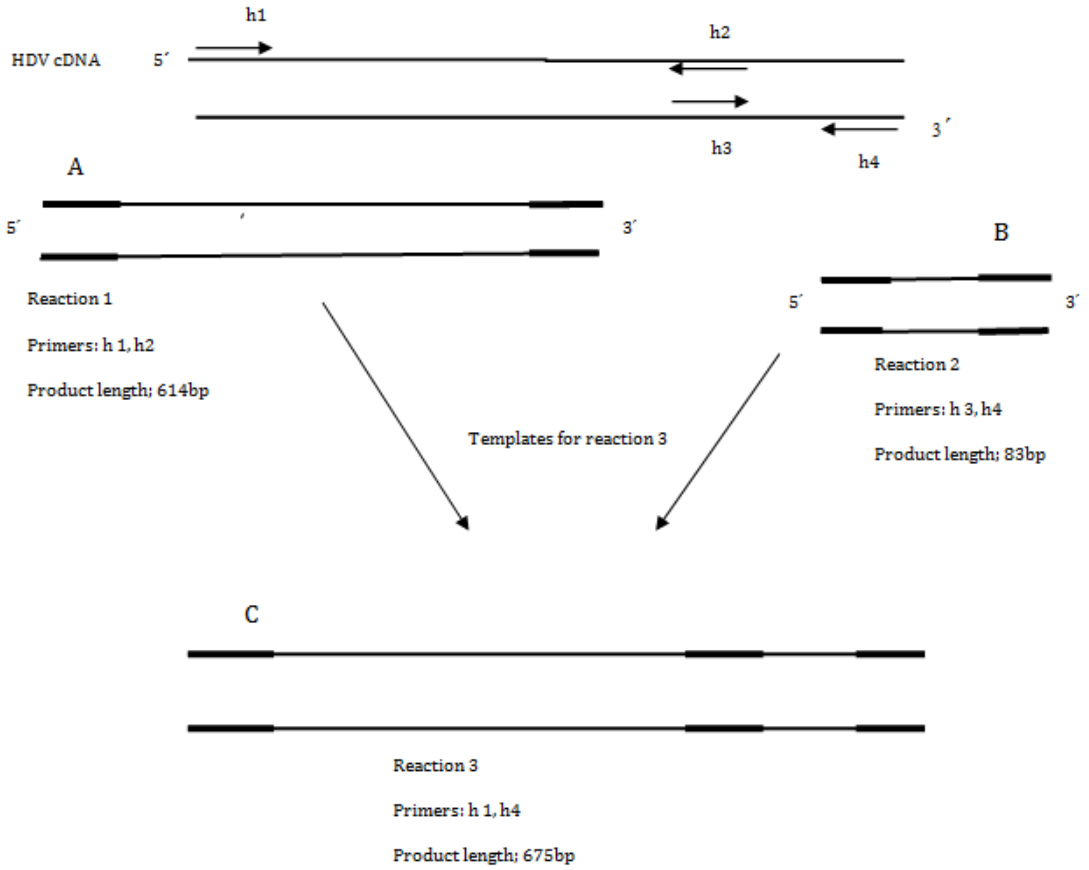

Fig. 1. Schematic diagram of SOEing-PCR. Region A was amplified using the outer forward and inner reverse primers h1 and h2. Region B was amplified using the inner forward and outer reverse primers h3 and h4. Regions A and B were fused by SOEing-PCR (C). Primers h2 and h3 are complementary and have the same nucleotide modifications.

Results

The L-HDAg ORF was constructed and amplified by SOEing PCR (Fig. 2). Agarose gel electrophoresis of NcoI-digested L-HDAg amplicons confirmed the desired mutation in the final PCR products. Additionally, the pTZ57R/TL-HDAg ligation product (pTZ57R/T LHDAg) sequence verified the fidelity of the site-directed mutagenesis procedure to generate the L-HDAg ORF.

Restriction analysis confirmed the identity of thepcDNA3.1-L-HDAg and pIRES2-EGFP-L-HDAg ligation products (pcDNA3.1-LHDAg and pIRES2-EGFP-LHDAG, respectively) (Fig. 3). pIRES2-EGFP-LHDAg and pcDNA3.1-LHDAg were lipo-transfected into HEK293 and Huh7 cells to compare the transfection efficiency of these two clones in each cell line. pIRES2-EGFP contains the internal ribosome entry site (IRES) between the cloning site and the enhanced green fluorescent protein (EGFP) coding region. This permits both L-HDAg and EGFP to be translated from a single bicistronic mRNA and the protein products to be visualized by fluorescent microscopy (results not shown).

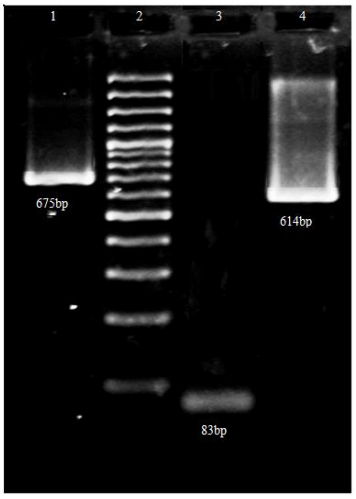

Fig. 2. Agarose gel electrophoresis of PCR products. Lanes 1, 3, and 4 are PCR products obtained from stages 1-3, respectively. Lane2: Molecular weight marker: GeneRuler 100 bp DNA ladder (Fermentas).

To test whether pcDNA3.1-LHDAg can drive HDAg expression, the construct was transfected into HEK293 and Huh7 cells and the cells were analyzed by immunocytochemistry.

Both pcDNA3.1-LHDAg and pIRES2-EGFP-LHDAg expressed L-HDAg in both cell lines, although after trying several times, each time the transfection efficiency of Huh7 cells by Lipofectamin was lower than that observed in HEK293 cells.

L-HDAg localized to the nuclei of transfected HEK293 cells (Fig. 4). Data for Huh7 cell line were not shown.

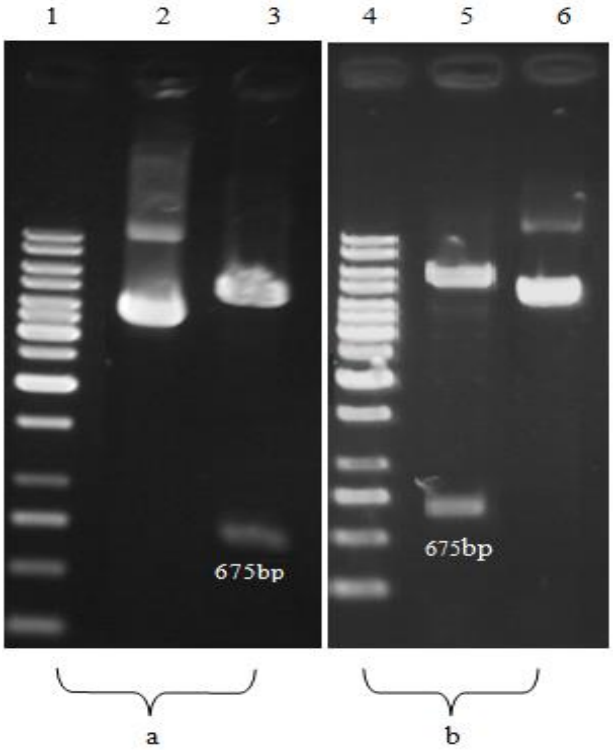

Fig. 3. pCDNA3.1(-) and pIRES2-EGFP clones were digested with NheI and BamHI and analyzed by agarose gel electrophoresis. Panel a: lane 2: undigested pIRES2-EGFP-LHDAg; lane 3: pIRES2-EGFP-LHDAg digested with NheI and BamHI. Panel b: lane 5: pcDNA3.1-LHDAg digested with NheI and BamHI; Lane 6: undigestedpcDNA3.1-LHDAg. Lanes 1 and 4: molecular weight markers: GeneRuler 1Kb DNA ladder (Fermentas).

Western blotting with ECL confirmed L-HDAg expression in the host cells (Fig. 5). Human high-titer anti-delta antiserum recognized a protein of 27 kDa in total cell lysates from cells transfected with pcDNA3.1-LHDAg. Data for Huh7 cell line not shown.

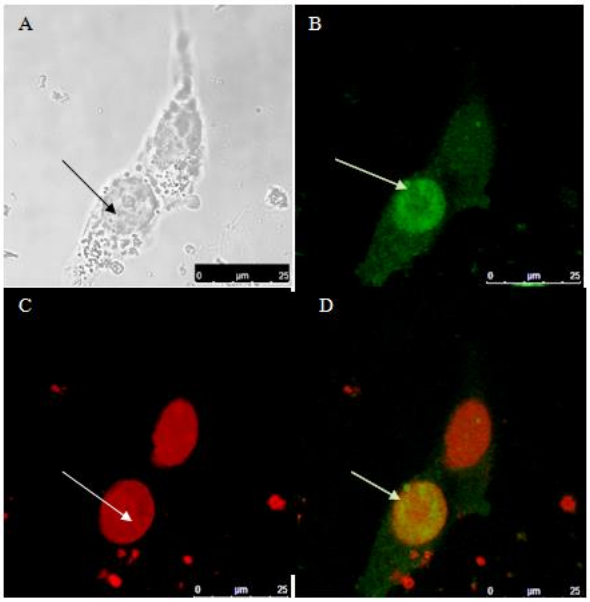

Fig. 4. Nuclear localization of recombinant L-HDAg in HEK293 cells by fluorescence immunostaining. HEK 293 cells were transfected with pcDNA3.1-LHDAg for 72 h and incubated with a 1:100 dilution of human high-titer anti-delta antiserum followed by incubation with a 1:25 dilution of anti-human IgG conjugated with fluorescein isothiocyanate (FITC) (Green, B). Nuclei were stained with propidium iodide (Red, C) and photomicrographs were captured using a Leica SP5 confocal microscope with a 63X objective. (A) Differential interference contrast (DIC) image of the HEK293 cells, (B) localization of L-HDAg in the nucleus of the transfected HEK293 cells, (C) DNA staining by propidium iodide, (D) overlay of B and C.

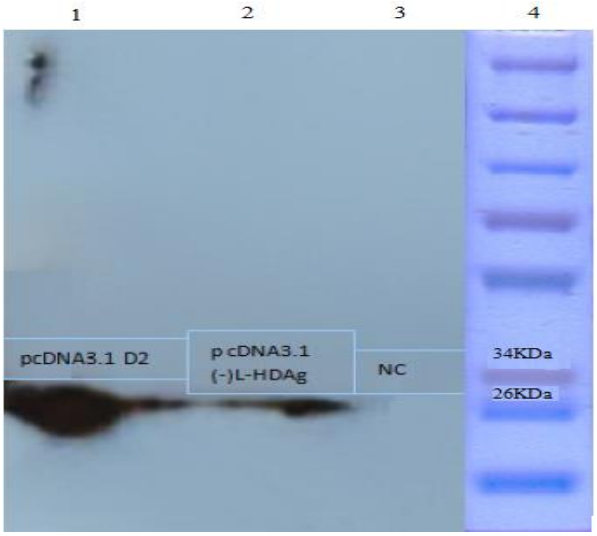

Fig. 5. Detection of recombinant pcDNA3.1-LHDAg by Western blotting using human anti-delta antiserum. Lane 1: lysate from HEK293 cells transfected with pcDNA3.1D2 as a positive control; lane 2: lysate from HEK293 cells transfected with pcDNA3.1-LHDAg; lane 3: lysate from untransfected HEK293 cells as a negative control.

Discussion

Cloning and sequencing of the HDV genome by Wang et al. showed that some clones contained a UAG (amber) stop codon at codon 196 of the HDAg ORF, whereas other clones had a UGG codon encoding tryptophan at this position, resulting in a protein of 214 amino acids (1989 Wang, Xia 1990). This observation led to the remarkable discovery of RNA editing, which happens during late stages of HDV replication. HDV RNA editing is a regulated process and L-HDAg ORF generation and accumulation occurs slowly and typically levels off for this reason we felt it would be more efficient to construct L-HDAg ORF in in vitro than to isolate it from patient's samples. Therefore, the L-HDAg gene was constructed using site-directed mutagenesis based on SOEing PCR. This method is faster and simpler than classic methods of site-directed mutagenesis and approaches 100% efficiency in the generation of the mutant product.

Transfection experiments with pcDNA3.1-LHDAg and pIRES2-EGFP-LHDAg in Huh7 and HEK293 cells resulted in L-HDAg production in both cell lines, although in our experience the transfection efficiency by Lipofectamin was lower in Huh7 than Hek 293 cells. The transfection efficiency is influenced by several parameters such as the vector construct, amount, purity, and quality of DNA used for transfection, and the transfection method. Considering that the above parameters were identical for our transfection experiments in the two cell lines, the observed difference in transfection efficiencies of Huh7 and HEK293 is likely due to inherent differences between the two cell lines in DNA uptake and endotoxin sensitivity.

In agreement with other studies, the recombinant L-HDAg accumulated in the nuclei of both cell types, with a nucleolar pattern. However, Bichko et al (7, 19-20) showed that HDAg can also accumulate in cytoplasmic vesicles of HuH7 cells, increasing with time after transfection. The biological significance of differential localization of HDAg, which seems to be cell-dependent, has not yet been established.

Because L-HDAg is extensively modified post-translationally, the recombinant protein expressed in mammalian systems may be fully functional and applicable as a platform in HDV molecular studies. The L-HDAg vectors constructed in the present work provide basic platforms for mutational analyses on L-HDAg, as well as the investigation of its possible interference in cell molecular pathways. Moreover, by improving the level of L-HDAg expression in such systems, and its production and purification in large scale, it could be used in future vaccine research.

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