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ORIGINAL RESEARCH PAPER

A new idea for simple and rapid monitoring of gene expression: requirement of nucleotide sequences encoding an *N*-terminal HA tag in the T7 promoter-driven expression in *E. coli*

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Abstract Mammalian expression vectors are used to overexpress genes of interest in mammalian cells. High temperature requirement protein A1 (HtrA1), used as a specific target, was expressed from the pHA-M-HtrA1 plasmid in HEK293T cells, inducing cell death. Expression of HtrA1 was driven by the pHA-M-HtrA1 mammalian expression vector in *E. coli* resulting in growth suppression of *E. coli* in an HtrA1 serine protease-dependent manner. By using various combinations of promoters, target genes and *N*-terminal tags, the T7 promoter and *N*-terminal HA tag in the mammalian expression vector were shown to be responsible for expression of target genes in *E. coli*. Thus the pHA-M-HtrA1 plasmid can be used as a novel, rapid pre-test system for expression and cytotoxicity of the specific target gene in *E. coli* before assessing its functions in mammalian cells.

Keywords HA tag · High temperature requirement protein A1 (HtrA1) · Mammalian expression vector system · T7

J.M Moon and G.Y Kim contributed equally to this study.

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Introduction

Among the vectors used for molecular cloning, the pcDNA and pCS2 + mammalian expression vectors contain the immediate-early cytomegalovirus (CMV) and bacteriophage promoters to drive transcription of genes in mammalian cells and in vitro, respectively (Melton et al. 1984; Kim et al. 2011; Sousa and Mukherjee 2003). Because *E. coli* RNA polymerase (RNAP) has little specificity on these bacteriophage T7/SP6 promoters (Paschal et al. 2008; Studier and Moffatt 1986), T7/SP6 promoter-driven gene transcription is known to rarely occur in *E. coli* strains lacking bacteriophage RNAP, including DH5α and TOP10 (http://openwetware.org/wiki/E._coli_genotypes) (Paschal et al. 2008).

High temperature requirement protein A1 (HtrA1), a member of the HtrA serine protease family, is highly conserved from bacteria to humans (Clausen et al. 2011). The HtrA1 serine protease matures to an active form through processing of its *N*-terminal region (Hou et al. 2005; Chien et al. 2006), and its

overexpression in mammalian cells induces cell death in a serine protease-dependent manner (Chien et al. 2004; Kim et al. 2011). We fortuitously found that growth of *E. coli* harboring the pHA-M-HtrA1 plasmid encoding the active HtrA1 protein in mammalian cells is suppressed during the bacterial culture as the first step in obtaining the plasmid DNA. As discovered in our study, this growth suppression phenomenon of *E. coli* occurs due to the expression of HtrA1 driven by the T7 promoter. Additionally, we show that the nucleotide sequences encoding an *N*-terminal HA tag in the mammalian expression vectors is an essential element for the T7 promoter-driven expression in *E. coli* even without trans-acting T7 RNAP. We report here that the pHA-M-HtrA1 plasmid can be replaced by any gene of interest and used as a vector system to pre-test gene expression and cytotoxicity in *E. coli* before applying it to mammalian cells.

Materials and methods

Plasmid construction

The pHA-M-HtrA1 plasmid was generated by digesting the pcDNA3-HA and pBS-M-HtrA1 plasmids with *Eco*RI and *Xho*I (Park et al. 2009; Kim et al. 2011). To construct the pM-HtrA1 plasmid, PCR amplification was performed with the pHA-M-HtrA1 plasmid as a template and the specific primers pair (Supplementary Table 1). The amplified M-HtrA1 cDNA fragment was digested with *Eco*RI and *Xho*I, and the resulting product was inserted into the pcDNA3.0 plasmid. Information for all plasmids described in this paper is given Supplementary Table 2 or is available upon request.

E. coli growth analysis

E. coli TOP10 harboring the indicated plasmid was seeded in liquid Luria–Bertani broth with 50 µg ampicillin/ml (LB-Amp) and incubated at 37 °C. Growth was monitored from the OD_{600} values. A growth curve graph was constructed by Sigma Plot program version 9.0. Error bars represent the standard error of the mean (SEM) of three independent experiments. Levels of statistical significance were assessed by one-way ANOVA and the Tukey post hoc comparison of means test.

Cell culture and transfection

HEK293T cells were grown in DMEM supplemented with 8.5 % (v/v) heat-inactivated fetal bovine serum. HEK293T cells (2×10^5 cells/well) in 6-well plates were transfected with 1 µg plasmid DNA (0.9 µg target plasmid and 0.1 µg pCS-EGFP plasmid) and Fugene HD transfection reagent according to the manufacturer's instruction. At 12 h post-transfection, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI), and GFP-positive cells were analyzed for monitoring condensed or fragmented apoptotic nuclei under fluorescence microscopy.

Immunoblot (IB) analysis

E. coli culture ($OD_{600} = 0.6$ or 10^8 cells/ml) was harvested by centrifugation at 16,000×*g* for 1 min. Cells were lysed in 80 µl of 1× SDS loading buffer [144 mM *β*-mercaptoethanol, 2 % v/v SDS, 5 % v/v glycerol, 0.004 % Bromophenol Blue, 60 mM Tris/HCl (pH 6.8) 0.05 mM EDTA] and boiled for 3 min. Transfected HEK293T cells were lysed for 30 min on ice in radioimmune precipitation assay buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 1 % sodium deoxycholate] containing protease inhibitors, 10 µg aprotinin/ml, 10 µg leupeptin/ml, and 1 mM PMSF. Proteins from *E. coli* (1.8×10^7 cells, 14 µg) and HEK293T cell lysates (20 µg) were resolved by 13 % SDS-PAGE and transferred to a nitrocellulose membrane. Antibodies (Abs) used for IB assays were anti-FLAG mAb, anti-HA pAb and anti-HtrA1 pAb (Kim et al. 2011). Proteins were detected with the HRP-conjugated secondary Abs and ECL immunoblotting system kit. The densities of target protein bands were measured by Multi Gauge V3.1 software.

Results and discussion

Expression of HtrA1 driven by the pHA-M-HtrA1 mammalian expression vector in *E. coli*

For efficient functional studies of mature-HtrA1 (M-HtrA1), we generated various M-HtrA1 constructs using the pcDNA3.0 and pCS2 + vectors (Fig. 1a). HtrA1 was introduced in-frame with the *C*-terminal FLAG epitope tag to effectively and specifically detect

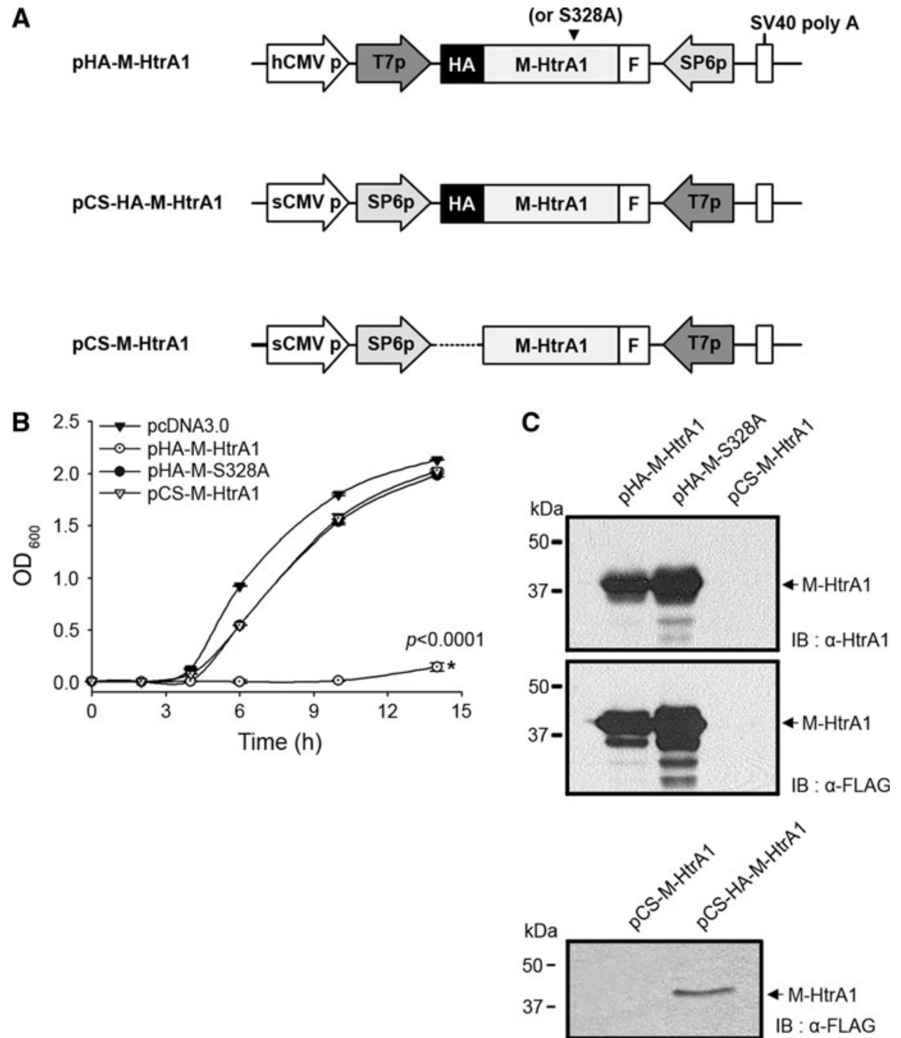

Fig. 1 M-HtrA1 is expressed from the mammalian pHA-M-HtrA1 plasmid in *E. coli*, resulting in growth suppression of *E. coli*. **a** Schematic representation of the mammalian expression vectors encoding mature HtrA1 (M-HtrA1). M-HtrA1 is a putative mature form (amino acid 150–480) (Kim et al. 2011). S328A indicates a proteolytically inactive HtrA1 mutant generated by replacing the catalytic 328-serine residue with alanine. Abbreviations: *h*, *s*, *p* and *GST* denote human, simian, promoter and glutathione *S*-transferase, respectively. **b** Growth curves of *E. coli* cultures. *Error bars* represent the standard error of the mean (SEM) of three independent experiments. **c** Expression of M-HtrA1 driven by mammalian expression vectors in *E. coli*

the M-HtrA1 protein expressed in mammalian cells. Additionally, the pHA-M-HtrA1 plasmid encoding HtrA1 fused in-frame with an *N*-terminal HA tag was constructed, because the exact cleavage site for maturation of HtrA1 has not yet been determined (Chien et al. 2004, 2006). During bacterial culture for preparation of plasmid DNA, we fortuitously found that the growth of *E. coli* harboring the pHA-M-HtrA1 plasmid is largely suppressed compared with *E. coli* harboring other plasmids (Fig. 1b). The *E. coli* growth rate was assessed by measuring the OD_{600} of *E. coli* harboring the indicated plasmids at 2 h intervals. In the case of the control (pcDNA3.0) and other plasmids, *E. coli* cells were in the lag phase for approx. 4 h ($OD_{600} = 0.1$), followed by exponential proliferation of the cells ($OD_{600} = 1.8$ at 10 h incubation), and the bacterial culture finally reached the stationary phase (Roszak and Colwell 1987).

In contrast, the *E. coli* harboring the pHA-M-HtrA1 plasmid maintained the lag phase for 12 h ($OD_{600} = 0.1$) and thereafter began to grow gradually. When compared with the 6 h cultures, the cell number of *E. coli* harboring the pHA-M-HtrA1 plasmid was approx. 0.02 % of that of the control *E. coli* ($OD_{600} = 1$ or 8×10^8 cells/ml). Consistent with our previous study and others that HtrA1 induces cell death in a serine protease-dependent manner in mammalian cells (Chien et al. 2004; Kim et al. 2011), the growth suppression function of the pHA-M-HtrA1 plasmid was completely abolished in the pHA-M-S328A plasmid encoding the catalytically inactive HtrA1 mutant. Despite the presence of active HtrA1,

the pCS-M-HtrA1 plasmid did not exhibit growth suppression of *E. coli*.

These results raise the possibility that expression of the M-HtrA1 protein may be directed from the pHA-M-HtrA1 plasmid in *E. coli*, hence HtrA1 proteolytic activity may play a key role in the growth suppression of *E. coli*. To investigate this possibility, the expression of HtrA1 was examined by IB assay (Fig. 1c). The 39 kDa M-HtrA1 protein band was detected from *E. coli* harboring the pHA-M-HtrA1 and pHA-M-S328A plasmids, but not from that harboring the pCS-M-HtrA1 plasmid. Collectively, the results demonstrate that expression of the M-HtrA1 protein in *E. coli* is driven by the pHA-M-HtrA1 mammalian expression vector and induces the growth suppression of *E. coli* in an HtrA1 serine protease-dependent manner.

T7 promoter and nucleotide sequences encoding an *N*-terminal HA tag are required for the target gene expression in *E. coli*

The discrepancy between M-HtrA1 protein expressions of two mammalian expression vectors, pHA-M-HtrA1 and pCS-M-HtrA1, raises the possibility that certain cis-acting elements responsible for the protein expression exist within the pHA-M-HtrA1 plasmid. First, in order to investigate whether this phenomenon is specific to HtrA1, different target genes were replaced with HtrA1 in the pHA-M-HtrA1 plasmid and the expression of target genes in *E. coli* was determined by IB assay (Fig. 2a). Like the HtrA1 results, X-linked inhibitor of apoptosis protein (XIAP) and macrophage migration inhibitory factor (MIF) with an *N*-terminal HA tag were expressed in *E. coli*, whereas SOD1 was not expressed despite the existence of the T7 promoter in the same vector backbone. Although protein expression was not observed from the pCS-M-HtrA1 plasmid, which contains the SP6 promoter, lower levels of the M-HtrA1 expression could be detected from the pCS-HA-M-HtrA1 containing the *N*-terminal HA tag, exhibiting approx. 5 % of expression levels driven by the pHA-M-HtrA1 plasmid (Fig. 1c). Subsequently, to substantiate the above hypothesis, expression of Parkin with a different *N*-terminal tag, which is driven by the same pcDNA3.0 vector backbone, was assessed by IB assay (Fig. 2b). Strong expression of Parkin was observed in the HA-tagged vector, but not in the MYC-tagged vector.

Taken together, the results indicate that the T7 promoter and nucleotide sequences encoding an *N*-terminal HA tag are essential cis-acting elements that can direct the expression of target genes from the mammalian expression vector system in *E. coli*. Although the exact action mechanism of this phenomenon has not yet been elucidated, biochemical and molecular studies, such as mutation and transcriptional analyses, will provide conclusive evidence as to whether these nucleotide sequences serve as the exact determinants for transcriptional regulation directed by endogenous RNAP in *E. coli* (Prosen and Cech 1986).

The pHA-M-HtrA1 mammalian expression vector is a valuable system for evaluating the expression and cytotoxicity of a human target gene in *E. coli*

Generally, it takes at least 1 week to investigate expression directed from the mammalian expression vector and cellular toxicity of the target genes in mammalian cells after molecular cloning (Sambrook and Russell 2001). We obtained unexpected and interesting results that the mammalian expression vector system can be used to direct expression of the target protein in *E. coli*; thus, many human genes can be diagnosed in *E. coli* prior to examining their cytotoxicity in mammalian cells. To test this possibility, the expression and cytotoxicity of HtrA1 were comparatively analyzed in both *E. coli* (Fig. 3) and mammalian cells (Fig. 4). Consistent with the results observed above, expression of HtrA1 in *E. coli* was directed from the pHA-M-HtrA1 plasmid, but not from the pM-HtrA1 plasmid generated by removal of an *N*-terminal HA tag (Fig. 3a, b). In parallel, the effect of the serine protease HtrA1 on *E. coli* growth corresponded to the HtrA1 expression in *E. coli*. As these two plasmids are mammalian expression vectors, expression of the 37- and 39-kDa HtrA1 proteins was clearly detected in HEK293T cells transfected with either the pM-HtrA1 or pHA-M-HtrA1 plasmid (Fig 4a). Consistent with the expression of HtrA1 in HEK293T cells, cell death was similarly induced by HtrA1 directed from both plasmids in a serine protease-dependent manner (Fig. 4b) (Chien et al. 2006; Chien et al. 2004; Kim et al. 2011). These results suggest that HtrA1, a highly conserved human ortholog of bacterial HtrA, may degrade proteins that play a key role in the survival of *E. coli*.

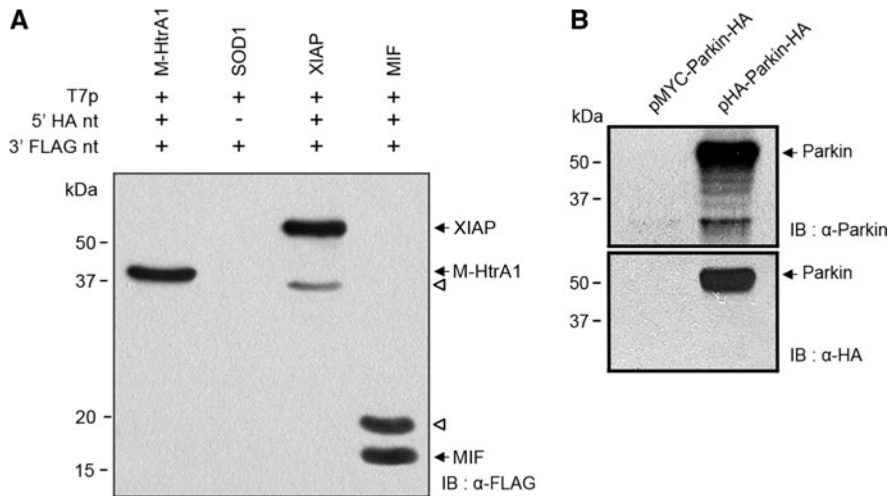

Fig. 2 The T7 promoter and nucleotide sequences encoding an *N*-terminal HA tag in mammalian expression vectors are essential for expression of target genes in *E. coli*. **a** Expression of different human genes driven by mammalian expression vector system in *E. coli* was evaluated. *Open arrowheads* indicate non-specific bands. *Signatures* of + and − indicate existence of tagging system and T7 promoter. **b** Specificity of an *N*-terminal HA tag for expression of Parkin in *E. coli*

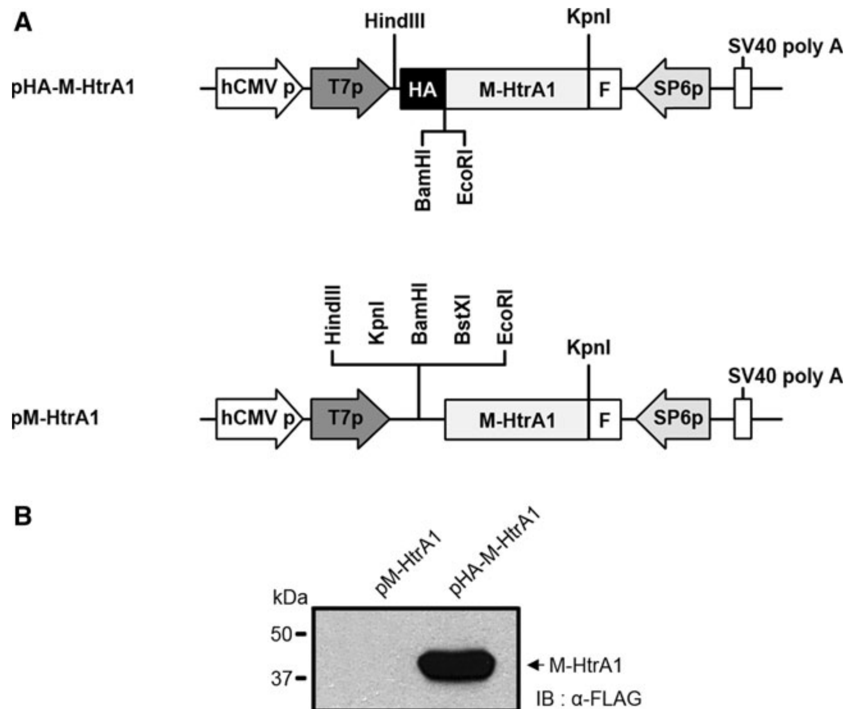

Fig. 3 A useful vector system for rapid evaluation of expression and cytotoxicity of human target genes in *E. coli*. **a** Schematic diagrams of main regions for cloning in the indicated expression vector system. The nucleotide sequence between HA tag and M-HtrA1 is GGATCC CCG *GAATTC* (BamHI and *EcoRI* restriction enzyme sites in bold and in italic, respectively). The M-HtrA1 in the provided vectors can be replaced with other genes. **b** Expression pattern of M-HtrA1 in *E. coli*

In conclusion, our data demonstrate for the first time that expression of the human target genes in *E. coli* is directed by the combination of both the T7 promoter and nucleotide sequences encoding an *N*-terminal HA tag. Notably, this mammalian expression vector system will be applied as a novel and rapid pre-test tool to evaluate whether the target gene is expressed, and the resulting protein has cellular toxicity in mammalian cells, because the expression of the target protein in and growth suppression of *E. coli* can be easily measured within 10–24 h. Furthermore, our study may provide new insight into

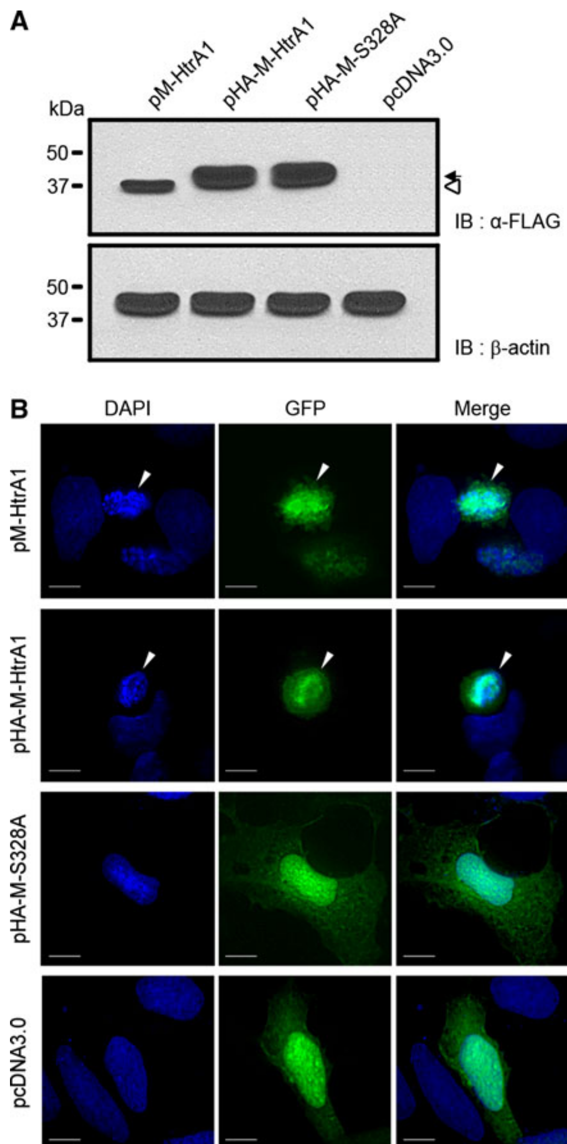

Fig. 4 Expression and cytotoxicity of M-HtrA1 in HEK293T cells. **a** The indicated plasmids were transfected into HEK293T cells, and HtrA1 expression was analyzed by IB with anti-FLAG Ab. *Arrowhead* and *arrow* indicate M-HtrA1 and HA-M-HtrA1, respectively. **b** HEK293T cells were transfected with plasmids encoding HtrA1 plus the pCS-EGFP plasmid at a 9:1 ratio. The nuclear morphology of GFP-positive cell (*green*) was analyzed by DAPI (*blue*) staining under fluorescence microscopy. *Scale bar* = 10 µm. *White arrowhead* indicates a typical phenotype of dead cells induced by HtrA1

the relationship between cellular toxicity and growth suppression of *E. coli* that can occur in molecular biology research, such as molecular cloning of the mammalian expression vector system.

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