

The putative protein 6 of the severe acute respiratory syndrome-associated coronavirus: Expression and functional characterization

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Abstract The SARS-CoV open reading frame 6 (ORF6) is transcribed into mRNA6 and encodes a putative 7.5 kDa accessory protein, SARS 6, with unknown function. In this study, we have confirmed the SARS 6 protein expression in lung and intestine tissues of the SARS patients and in SARS-CoV infected Vero E6 cells by immunohistochemistry. Further studies by immunoblot and confocal microscopy analyses revealed the expression and the endoplasmic reticulum (ER) localization of the recombinant SARS 6 protein in mammalian cells. Expression of SARS 6 protein in mammalian cells elicits biological activity of stimulating cellular DNA synthesis.

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1. Introduction

The severe acute respiratory syndrome (SARS) is an infectious disease with mortality rate approximately 10% of the cases and had spread over 30 countries in 2003 [1]. The pathogenic mechanisms of the causative agent, SARS-CoV, are not well characterized.

The SARS-CoV genome analyses predicted fourteen ORFs. ORFs 1a and 1b constitute the replicase genes required for viral RNA synthesis. The remaining 12 ORFs encode the four structural proteins, Spike (S), Membrane (M), Nucleocapsid (N) and Envelope (E), as well as eight putative accessory proteins varying in size and position in the genome [2,3]. The eight putative accessory proteins share no homology to known proteins.

The uniqueness of the SARS putative accessory proteins to other known coronavirus might be crucial to the clinical manifestation of SARS. However, the functions of SARS-CoV hypothetical proteins are poorly understood. Recently, acces-

sory protein SARS 3a was found to induce apoptosis [4]. In addition, the hypothetical protein 7a was shown to interact with SARS 3a and the structural proteins in Vero E6 cells [5,6]. These findings highlighted the potential functions of these hypothetical proteins in the pathogenesis of SARS.

The SARS 6 protein is 63 amino acids in length, also known as the X3 protein. A minimal transcription regulatory sequence is located upstream of the gene (ORF6) and its mRNA (mRNA6) exists in SARS-CoV infected Vero cells [7,8]. In this study, the expression and possible function of the SARS 6 protein were investigated.

2. Materials and methods

2.1. Cell culture

Vero E6 (African green monkey kidney fibroblasts) and CHO (Chinese hamster ovary) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in high glucose DMEM (Gibco BRL Life Technologies Inc., Carlsbad, CA, USA) containing 3.7 g/l sodium bicarbonate, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% CO₂.

2.2. Selection of clinical specimens

Samples were selected retrospectively following the guidelines of local ethical committee [9]. SARS was diagnosed according to previously established criteria of the World Health Organization (www.who.int/csr/sars/en/). The outbreak in the Prince of Wales Hospital, Hong Kong, and the sequences of events leading to the world epidemics has been reported [10,11]. Pathology of all 8 autopsies has been previously described [12]. The control autopsies were chosen randomly at around the same period of time (March–November) in preceding years. The diagnosis included lung and non-lung pathology. These controls were not age-matched and they had no SARS-CoV infection as confirmed by in situ hybridization and five different antibodies to SARS-CoV N, S, M and 3a proteins. For cases with pulmonary infection, routine cultures for common virus and bacteria had been performed. Coronaviral infections of any kind were not suspected clinically in these cases.

2.3. SARS-CoV infection of Vero E6 cells

The CUHK-W1 strain of SARS-CoV (GenBank Accession No. AY278554) was grown and assayed in the cells [13,14]. Briefly, SARS-CoV was used to infect Vero E6 cells maintained in DMEM with 10% FBS at 60–70% confluence, and with a multiplicity of infection (MOI) of 10. Subsequently, cells were harvested at the desired cytopathic effect (CPE) for immunohistochemistry detection [9].

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2.4. Generation of SARS 6 protein antipeptide antibody

Rabbit anti-SARS 6 antibody (designated PUP3) was produced by Abgent Inc. (San Diego, CA, USA) and Century Biotech Limited (Hong Kong, China). The peptide sequence for antibody production was TKKNYSELDDDEEPMELDYP [9].

2.5. Immunohistochemical studies

Paraffin blocks were prepared from infected or control Vero cells fixed with 10% formalin. Autopsy specimens were fixed with 10% formalin and embedded in paraffin blocks and standard avidin-biotin method was used for immunohistochemical studies on 4- μ m sections. PUP3 antibodies (1:100 dilution) and StreptABCComplex/HRP Duet Reagent Set (DAKO, Glostrup, Denmark) were used with 3,3'-diaminobenzidine tetrahydro-chloride as the chromogen. Antigen retrieval was performed by microwave pretreatment twice in 10 mM citrate buffer, pH 6.0, or 0.1 M EDTA buffer, pH 8.0, with preliminary heating at 780 W for 3 min followed by 480 W for 10 min.

2.6. Construction of SARS 6 protein expression vectors

The SARS 6 cDNA (CUHK-Su10, GenBank Accession No. AY282752) [3] was subcloned into pEGFP-N1 (BD Bioscience, Clontech, Palo Alto, CA, USA) and pcDNA3.1 (Invitrogen, Life Technologies, Carlsbad, CA, USA) vectors. For cloning the SARS 6-EGFP (Enhanced green fluorescent protein) fusion protein, the SARS 6 cDNA was PCR amplified using primer set SARS 6-GFP-F (5'-GGCGCCGAGCTCATGTTTCATCTTGTTGAC-3') and SARS 6-GFP-R (5'-CCGGGGTACCTCTGGATAATCTAACTCCAT-3'), incorporating *SacI* and *KpnI* sites for ligation into the pEGFP-N1 vector. To generate the untagged protein, the SARS 6 cDNA was PCR amplified using forward primer SARS 6-F (5'-GGCGCCGTTACCATGTTTCATCTTGTTGAC-3') and reverse primer containing the

translation stop codon, SARS 6-R (5'-GGCGCCTCTAGATTATGGATAATCTAACTC-3') incorporating *KpnI* and *XbaI* sites for ligation into the pcDNA3.1 vector. These constructs were sequenced to confirm the correct identity and the orientation of the subcloned fragments.

2.7. Expression of SARS 6 protein in Vero E6 and CHO cells

Plasmids were introduced into cells by Lipofectamine™ 2000 reagent (Invitrogen, Life Technologies). To establish the polyclonal cell lines with stable recombinant protein expression, cells harboring pEGFP-N1-based constructs were exposed to 1000 μ g/ml G418 (Sigma, St. Louis, MO, USA) whereas cells harboring pcDNA3.1-based constructs were exposed to 120–200 μ g/ml hygromycin B (Merck & Co. Inc., NJ, USA) for a one-month selection period.

2.8. Immunoblot assay

Cells were suspended in PBS and lysed by sonication on ice. Cell proteins were separated by SDS-PAGE and transferred onto a Hybond membrane. The membrane was blocked with 5% non-fat dry milk for 1 h and then incubated with primary antibody for 1.5 h at room temperature. The membrane was then washed and incubated with an appropriate secondary antibody for 1 h at room temperature. The detection steps were performed using the enhanced chemiluminescent ECL kit (Amersham Biosciences, Buckinghamshire, UK).

2.9. Subcellular localization of SARS 6 protein

Cells were seeded on sterile coverslips in 12-well plates then transfected with SARS 6-EGFP fusion constructs and/or pDsRed2-ER marker plasmids (BD Bioscience, Clontech). 30 h posttransfection, cells were washed and observed under a laser scanning confocal microscope (Leica Model TCS-NT, Leica Microsystems, Heidelberg, Germany).

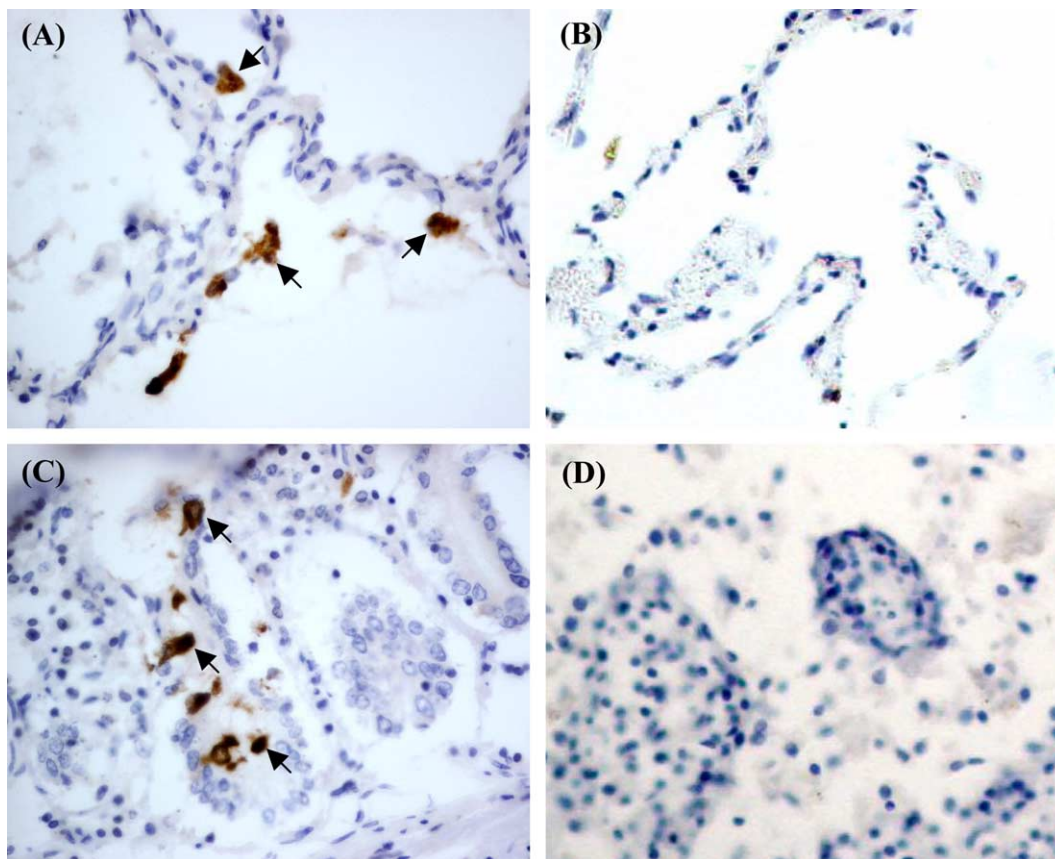


Fig. 1. Immunohistochemical staining of SARS 6 protein using anti-SARS 6 antibody (PUP3). Lung sections of (A) SARS patient and (B) Non-SARS patient. Terminal ileum sections of (C) SARS patient and (D) Non-SARS patient. The arrowheads indicate the positive signals.

2.10. DNA synthesis analysis

Cells were plated in 24-well plates (0.7×10^5 cells/well) and synchronized for 48 h in DMEM containing 0.1% FBS. DNA synthesis was then performed by incubating cells in medium containing 10% FBS and $1 \mu\text{Ci/ml}$ [^3H]-thymidine (Amersham Biosciences) for 24 h at 37 °C. At the end of incubation, media were removed and cells were washed with ice-cold PBS. Cellular macromolecules were then fixed with ice-cold 10% trichloroacetic acid (TCA). Unincorporated [^3H]-thymidine was removed by further washing steps. TCA precipitates were dissolved by 1% SDS and subjected to scintillation counting [15].

3. Results and discussion

3.1. Detection of SARS 6 protein in SARS-CoV infected patients and cells

By immunohistochemical staining, SARS 6 protein was detected in pneumocytes of the lungs and surface enterocytes of terminal ileum from eight SARS autopsy cases (representative data shown in Fig. 1A and C). Immunohistochemical signals were positive only in those tissues in which virus could be isolated [9]. No positive signals were detected in the corresponding tissues from ten non-SARS cases (Fig. 1B and D). It is known that lung and small intestine are the primary targets of SARS-CoV infection [10]. Our results confirmed that the hypothetical SARS 6 protein indeed existed and expressed in lung and intestine tissues of SARS-CoV infected clinical patients.

To further confirm the expression of SARS 6 protein in SARS-CoV infected cells, Vero E6 cells were infected with SARS-CoV CUHK-W1 strain (GenBank Accession No. AY278554) followed by immunohistochemical staining. The results showed that SARS 6 protein was detected in SARS-CoV infected cells but not in non-infected control cells. Fig. 2A and 2B provides photographic details consistent with previously described observations [9].

3.2. Expression of recombinant SARS 6 protein

The expression of the SARS 6–EGFP fusion protein was determined by immunoblot assays using anti-GFP or anti-SARS 6 (PUP3) primary antibodies (Fig. 3A and B). The molecular weight of EGFP and SARS 6 protein are 27 and 7.5 kDa, respectively. In both Vero E6 and CHO cells, the anti-GFP antibody recognized the expressed SARS 6–EGFP fusion protein showing the anticipated ~ 34.5 kDa band (Fig. 3A, lanes 2 and 4). The PUP3 antibody also recognized a single band with the expected molecular size (Fig. 3B). No signals were detected in Vero cells transfected with SARS 3b–EGFP fusion constructs or pEGFP-N1 control vectors (Fig. 3B, lanes 2 and 3), indicating the specificity of the antibody against the SARS 6 protein. Using the same antibody, expression of the untagged SARS 6 protein in Vero E6 cells was also confirmed (Fig. 3C). Interestingly, given the same amount of sample loading, the expression of the untagged SARS 6 protein was weaker relative to the

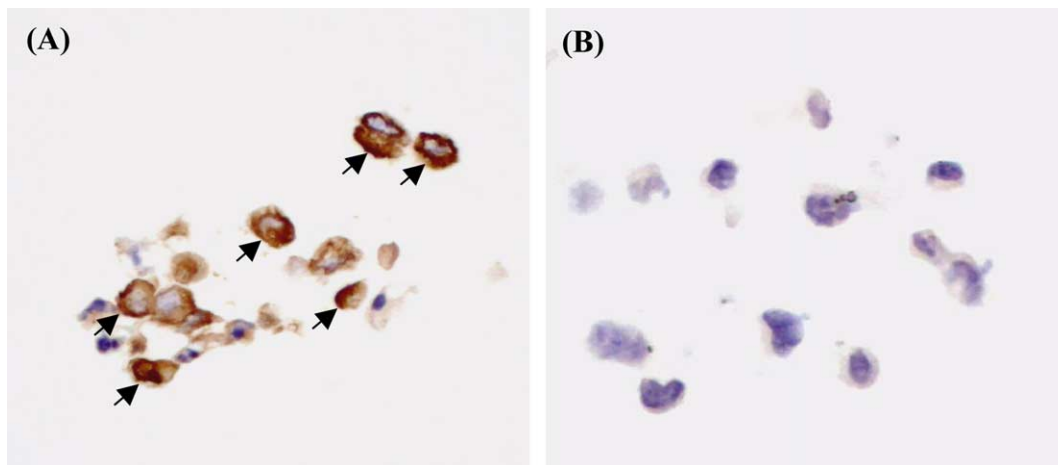


Fig. 2. Immunohistochemical staining of SARS 6 protein in Vero E6 cells using anti-SARS 6 antibody (PUP3): (A) SARS-CoV infected cells; (B) non-infected cells. The arrowheads indicate the positive signals.

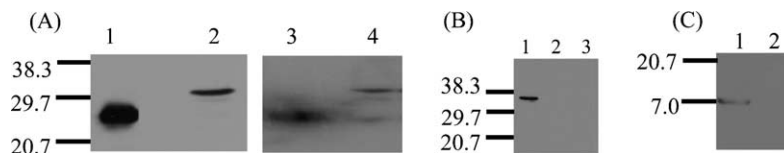


Fig. 3. Expression of recombinant SARS 6 protein. (A) SARS 6–EGFP fusion protein-expressing Vero E6 cells and CHO cells were probed with anti-GFP antibody (1:3000 dilution). Lane 1–50 μg protein from pEGFP-N1 vector transfected Vero E6 cells. Lane 2–50 μg protein from SARS 6–EGFP fusion protein-expressing Vero E6 cells. Lane 3–20 μg protein from pEGFP-N1 vector transfected CHO cells. Lane 4–20 μg protein from SARS 6–EGFP fusion protein-expressing CHO cells. (B) SARS 6–EGFP fusion protein-expressing Vero E6 cells probed with anti-SARS 6 antibody (PUP3) (1:3000 dilution). Lane 1–50 μg protein from SARS 6–EGFP fusion protein-expressing cells. Lane 2–50 μg protein from SARS-CoV SARS 3b–EGFP fusion protein-expressing cells. Lane 3–50 μg protein from pEGFP-N1 vector transfected cells. (C) Untagged SARS 6 protein-expressing Vero E6 cells probed with anti-SARS 6 antibody (PUP3) (1:3000). Lane 1–50 μg protein from untagged SARS 6 protein-expressing cells. Lane 2–50 μg protein from pcDNA3.1 vector transfected cells.

expression of the SARS 6–EGFP fusion protein. The CMV promoter drives protein expression in both pcDNA3.1 and pEGFP-N1 vectors, the possibility that the SARS 6–EGFP fusion protein has higher stability as compared to the un-

tagged SARS 6 protein cannot be excluded. The difference in expression may also be due to the differences in the temporal expression or transcription kinetics of the two plasmids.

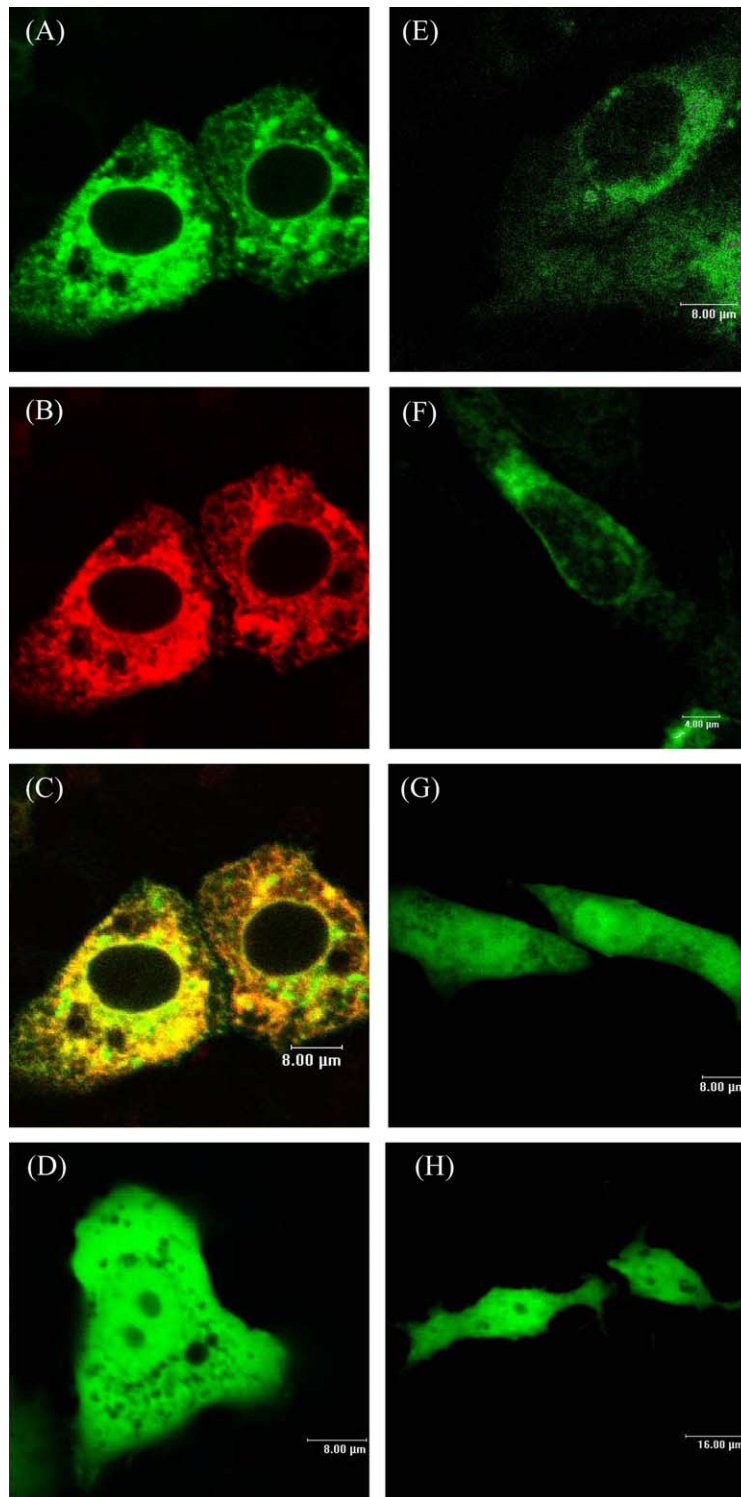


Fig. 4. Subcellular localization of recombinant SARS 6 protein by confocal microscopy. (A)–(C) Vero E6 cells co-transfected with SARS 6–EGFP fusion constructs and pDsRed2-ER marker plasmids showing (A) perinuclear localization of SARS 6–EGFP fusion protein, (B) ER compartment, and (C) Merged image of colocalization of SARS 6 with the ER marker protein. (D) Subcellular localization of EGFP in Vero E6 cells. (E, F) Subcellular localization of SARS 6–EGFP fusion protein expressed in CHO cells. (G, H) Subcellular localization of EGFP expressed in CHO cells.

3.3. Subcellular localization of SARS 6 protein

The SARS 6 protein is expressed both in lung and intestine of SARS patients and SARS-CoV infected Vero E6 cells by immunohistochemical staining as shown in Figs. 1 and 2. Although the specific subcellular localization of SARS 6 protein cannot be determined in these immunohistochemical results, notice the positive signals detected outside the nuclei of the SARS-CoV infected Vero E6 cells (Fig. 2). The subcellular localization of the SARS 6 protein in mammalian cells was further determined by expressing the SARS 6–EGFP fusion constructs in Vero E6 and CHO cells. Confocal microscopy showed that SARS 6–EGFP protein was localized to the perinuclear region and colocalized with ER marker (Fig. 4A–C) in Vero E6 cells. A similar localization pattern of the SARS 6–EGFP was obtained in CHO cells (Fig. 4E and F). In contrast, the control EGFP protein was distributed throughout the cells (Fig. 4D, G and H) as expected [16]. EGFP fusion protein has been a commonly used marker to determine subcellular localization of proteins, i.e., the HIV accessory protein Vpr, to circumvent the need for immunofluorescence staining [16]. This approach is particularly suitable for the studies to which the antibody supply is limited (i.e., the current study). In many cases, the construction of a C-terminal fusion protein retains the normal biological activity of the heterologous partner [16–18]. Pewe et al., reported similar perinuclear subcellular localization of SARS 6 protein with HA tag on the background of the neurotropic strains of mouse hepatitis virus (MHV) in rodent cells [19]. The identification of the SARS 6 protein subcellular localization laid the ground for further understanding of its role in SARS-CoV biology. Nevertheless, whether endoplasmic reticulum localized SARS 6 protein elicits any biological activities by interacting with other viral or cellular proteins requires further investigation.

3.4. Thymidine incorporation studies

In the attempt to elucidate the biological function of the SARS 6 protein, [³H]-thymidine incorporation was measured in both CHO and Vero E6 cells expressing the untagged SARS 6 protein to evaluate its effects on DNA synthesis. In these experiments, since unincorporated [³H]-thymidine was removed following the fixation of macromolecules by TCA in the washing steps, the cell associated radioactive counts in these experiments reflect the actual DNA synthesis. Fig. 5 and Table 1 show that both transient and stable expression of the SARS 6 protein induced DNA synthesis in CHO cells (Fig. 5A) and in Vero E6 cells (Fig. 5B).

Viral accessory proteins have been shown to elicit function in cell growth and survival. The HIV accessory protein Vpr is not essential for HIV replication. However, Vpr induces cell cycle arrest and apoptosis when expressed in cultured mammalian cells [20,21]. In addition, human T-lymphotropic virus type 1 (HTLV-1) accessory protein p12¹ is localized to the endoplasmic reticulum and it can increase intracellular calcium and cell proliferation [22]. It is speculated that a protein localized to ER may modify intracellular signaling pathways by regulating Ca²⁺ homeostasis in the ER and thus affect cellular DNA synthesis. Similarly, SARS-CoV may also evolve various mechanisms to optimize cellular conditions to its own benefit. At the current stage, the significance of SARS 6-induced DNA synthesis in SARS-CoV biology remains elusive, since such observation is obtained independent of the context of the whole viral genome. Nevertheless, such biological activity

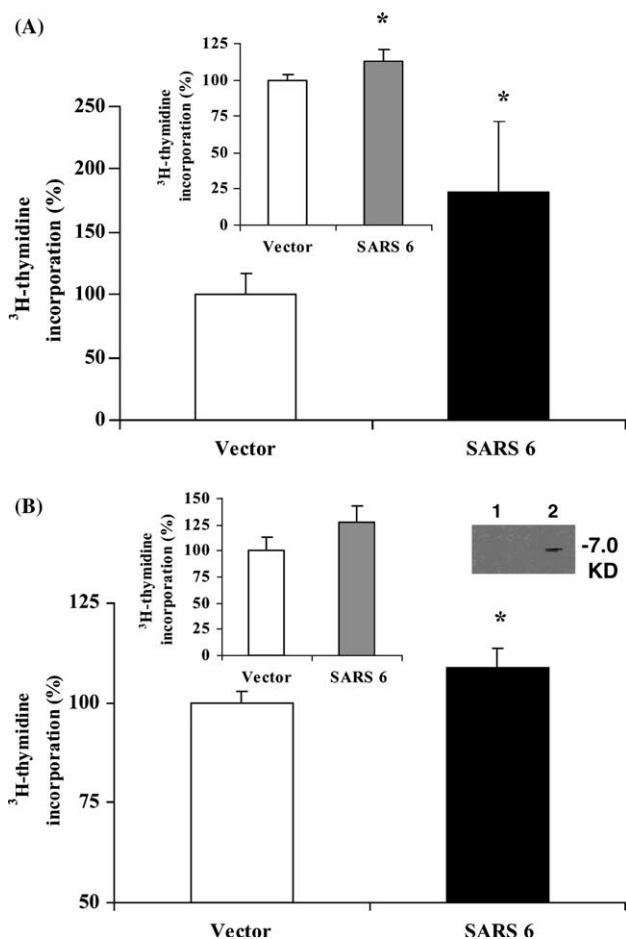


Fig. 5. SARS-CoV SARS 6 expression stimulates DNA synthesis. ³H-thymidine incorporation assays were performed on vector control and SARS 6-expressing cells. (A) Stable CHO cell lines. Inset, transiently transfected CHO cells (B). Stable Vero E6 cell lines. Inset (bar chart), transiently transfected Vero E6 cells. Inset (gel photo): Immunoblot confirmation of stable expression of the SARS 6 protein in Vero E6 cells using the anti-SARS 6 antibody (PUP3). Each lane contained 50 μ g total cell protein. Lane 1 – pcDNA3.1 vector transfected Vero E6 cells (vector control). Lane 2 – SARS 6-expressing Vero E6 cells. Each bar represents the means \pm S.D. of three to six experiments in triplicate or quadruplicate set up and the difference between control and SARS 6-expressing cells was determined by Student's *t* test with a significance determined at $p < 0.05$ level.

further substantiates the expression of the SARS 6 protein in the cells and implies its interaction with cellular components.

In conclusion, the evidence provided here indicates that the SARS-CoV ORF6 encodes a viral protein which is expressed

Table 1
Expression of SARS 6 stimulates DNA synthesis in several stable cell lines derived from independent transfection

Cell line	³ H-thymidine incorporation	
	Vector control cells (means \pm S.D. in DPM)	SARS 6-expressing cells (means \pm S.D. in DPM)
CHO 1	6107 \pm 1601.5	8428 \pm 1000.7*
CHO 2 ^a	5741 \pm 963.3	10451 \pm 3194.2*
CHO 3	6431.5 \pm 1312.7	10126 \pm 2437.5*
Vero E6 ^a	126622 \pm 3775.3	137486 \pm 6266.7*

^aData presented in Fig. 5.

*Significant difference between SARS 6-expressing and vector control cell lines detected by Student's *t* test ($p < 0.05$).

both in lung and intestine of SARS patients and SARS-CoV infected Vero E6 cells. Recombinant expression of the SARS 6 protein revealed its ER localization and biological activity of stimulating cellular DNA synthesis.

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