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Author(s)	Manzoor, Rashid; Kuroda, Kazumichi; Yoshida, Reiko; Tsuda, Yoshimi; Fujikura, Daisuke; Miyamoto, Hiroko; Kajihara, Masahiro; Kida, Hiroshi; Takada, Ayato
Citation	Journal of Biological Chemistry, 289(11), 7599-7614 https://doi.org/10.1074/jbc.M113.507798
Issue Date	2014-03-14
Doc URL	http://hdl.handle.net/2115/56577
Rights	This research was originally published in Journal of Biological Chemistry. Manzoor, Rashid; Kuroda, Kazumichi; Yoshida, Reiko; Tsuda, Yoshimi; Fujikura, Daisuke; Miyamoto, Hiroko; Kajihara, Masahiro; Kida, Hiroshi; Takada, Ayato. Heat Shock Protein 70 Modulates Influenza A Virus Polymerase Activity. 2014. vol.289 pp.7599-7614. © the American Society for Biochemistry and Molecular Biology.
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Heat Shock Protein 70 Modulates Influenza A Virus Polymerase Activity

Rashid Manzoor¹, Kazumichi Kuroda², Reiko Yoshida¹, Yoshimi Tsuda^{1,*}, Daisuke Fujikura³, Hiroko Miyamoto¹, Masahiro Kajihara¹, Hiroshi Kida⁴, Ayato Takada^{1,5}

¹From the Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Sapporo, 001-0020, Japan.

²Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine, Tokyo, 173-8610, Japan.

³Division of Infection and Immunity, Research Center for Zoonosis Control, Hokkaido University, Sapporo, 001-0020, Japan.

⁴Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, Japan,

⁵School of Veterinary Medicine, the University of Zambia, P.O. Box 32379, Lusaka, Zambia.

*Current address: Department of Microbiology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan.

Running title: Hsp70 enhances influenza A virus polymerase activity

To whom Correspondence should be addressed: Ayato Takada, Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Kita 20 Nishi 10, Kita-Ku, Sapporo, 001-0020, Japan. Tel: +81-11-706-9502. Fax: +81-11-706-7310. E-mail: atakada@czc.hokudai.ac.jp.

Keywords: RNA viruses, Influenza virus, viral polymerase, cell fractionation, NF-kappa B (NF-KB), viral replication, heat shock protein 70, heat shock

Background: It has been shown that heat shock protein 70 (Hsp70) plays a role in influenza A virus replication.

Results: A correlation between viral replication/transcription activities and nuclear/cytoplasmic shuttling of Hsp70 was observed.

Conclusion: Hsp70 modulates the influenza A virus polymerase activity.

Significance: This study, for the first time, suggests that Hsp70 may actually assist in influenza A virus replication.

ABSTRACT:

The role of heat shock protein 70 (Hsp70) in virus replication has been discussed for many viruses. The known suppressive role of Hsp70 in influenza virus replication is based on studies conducted in cells with various Hsp70 expression levels. In this study, we determined the role of Hsp70 in influenza virus replication in HeLa and HEK293T cells, which express Hsp70 constitutively. Co-immunoprecipitation and immunofluorescence studies revealed that Hsp70 interacted with PB2 or PB1 monomers and PB2/PB1 heterodimer but not with the PB1/PA heterodimer or PB2/PB1/PA heterotrimer, and translocated into the nucleus with PB2 monomers or PB2/PB1 heterodimers. Knocking down Hsp70 resulted in reduced virus transcription and replication activities. Reporter gene assay, immunofluorescence

assay and western blot analysis of nuclear and cytoplasmic fractions from infected cells demonstrated that the increase in viral polymerase activity during the heat shock phase was accompanied with an increase in Hsp70 and viral polymerases levels in the nuclei, where influenza virus replication takes place; whereas a reduction in viral polymerase activity was accompanied with an increase in cytoplasmic relocation of Hsp70 along with viral polymerases. Moreover, significantly higher levels of vRNA were observed during the heat shock phase than during the recovery phase. Overall, for the first time, these findings suggest that Hsp70 may act as a chaperon for influenza virus polymerase, and the modulatory effect of Hsp70 appears to be a sequel of shuttling of Hsp70 between nuclear and cytoplasmic compartments.

INTRODUCTION:

Influenza A viruses are pleomorphic, enveloped RNA viruses belonging to the family *Orthomyxoviridae*. The genome of influenza A viruses consists of eight single stranded RNA segments of negative polarity having partially complementary ends that form a closed structure. In a virus particle, each viral RNA (vRNA) segment exists in association with multiple monomers of nucleoprotein (NP) and a single copy of a polymerase heterotrimer consisting of a polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic

protein (PA); thereby constituting a viral ribonucleoprotein complex (vRNP). These vRNPs are independent functional units capable of transcription and replication (1,2). The PB1, which has motifs for binding to the vRNA and cRNA promoters, is the core subunit for RNA synthesis and responsible for the assembly of PB2 and PA into a multifunctional enzyme complex (3-5). The PB2 subunit is responsible for cap snatching from the host pre-mRNAs and initiation of transcription by providing these 5'-capped RNA fragments to copy the template (6-8). Multiple functions have been proposed for the PA subunit such as protease activity, endonuclease activity, promoter binding activity, and assembly of polymerase subunits into functional polymerase complex (9-13).

Upon infection, virus enters the cell by receptor-mediated endocytosis; the vRNP complex is released into the cytoplasm and thence transported into the nucleus. Since virus transcription and replication take place in the nucleus and viral protein synthesis and virus assembly take place in the cytoplasm, the viral proteins have to be shuttled between the nucleus and cytoplasm. This entire cascade of events requires interaction of viral proteins with host cellular factors. Some of these host factors participate in the virus replication and transcription. Ran binding protein 5 was reported to interact with PB1 alone or with PB1/PA heterodimers and transfer them into the nucleus (14). Alpha-importins have been reported to

interact with influenza virus PB2 and NP in host-dependent manner (15,16). Interaction of heat shock protein 90 (Hsp90) with PB2 has been reported to stimulate the viral polymerase activity (17). Hsp90 is also known to participate in assembly and nuclear transport of viral polymerase proteins by binding with PB2 monomers or PB2/PB1 heterodimers (18). Recently, host chaperonin CCT has been proposed to act as a molecular chaperone for PB2 protein by assisting its folding and incorporation into the heterotrimeric polymerase complex (19).

The heat shock protein 70 family comprises of highly related, stress-inducible or constitutively expressed, cytosolic or compartment-specific, 66 kDa to 78 kDa isoforms (20,21). Of these, heat shock protein 70 (Hsp70) and heat shock cognate 70 protein (Hsc70) are two major cytoplasmic isoforms. All heat shock proteins share a highly conserved domain structure comprising (i) an N-terminal ATPase domain, (ii) a middle region with protease sensitive site, (iii) C-terminal substrate binding domain, and (iv) a G/P-rich C-terminal region containing an EEVD-motif enabling it to bind co-chaperones and other heat shock proteins. Many housekeeping roles have been attributed to Hsp70 such as protein folding and prevention of aggregation, refolding of misfolded and aggregated proteins, signal transduction by controlling the conformation of proteins, anti-apoptotic effect, in tumorigenesis indicated by higher expression levels in many tumor cells,

and membrane translocation of organellar and secretory proteins (20-25).

The role of Hsp70 in virus replication is quite broad, as it inhibits the replication of some viruses (26-28) and helps the replication of other viruses (29-33). Studies conducted to elucidate the role of Hsp70 in influenza virus replication have suggested that Hsp70 inhibits influenza virus replication either by preventing the nuclear export of the RNP complex (34) or by disrupting the binding of viral polymerase with viral RNA (35).

Generally, in non-transformed cells under normal conditions, Hsc70 is expressed abundantly whereas Hsp70 is expressed at relatively low levels, but its expression increases considerably under various types of stress. Interestingly, elevated levels of Hsp70 have been reported in many tumors and transformed cell lines (36-38). Theodorakis and Morimoto (39) reported that HeLa and 293 cells constitutively expressed high levels of Hsp70. Many host factors interacting with influenza virus proteins have been identified in HEK293T and HeLa cells (16,18,40,41). If Hsp70 had an inhibitory effect on influenza virus replication, it would have made 293T or HeLa cells unsuitable for influenza virus study rather than facilitating their large-scale application. Keeping this assumption in mind, we decided to study the role of Hsp70 in cells constitutively expressing it.

In this study we found that Hsp70 interacted and translocated into the nucleus with

PB2 monomers or PB2/PB1 heterodimers, presumably assisting the assembly of the viral polymerase complex. Knockdown of Hsp70 resulted in significant reductions in both virus transcription and replication. Moreover, an increase in viral polymerase activity was observed during the heat shock phase compared to the recovery phase, coinciding with the subcellular movement of Hsp70, suggesting that Hsp70 acted as chaperone for the viral polymerase complex.

EXPERIMENTAL PROCEDURES

Cells, virus and reagents— Human embryonic kidney 293T cells (HEK293T) and HeLa cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and antibiotics at 37 °C in a 5% CO₂ atmosphere. Influenza virus strain, A/Puerto Rico/8/1934 (H1N1) (PR8) was obtained from the virus repository of our laboratory.

Primary monoclonal antibodies (mAbs) used in western blot analysis and immunofluorescence assays included anti-PB2 (143/3), anti-PB1 (81/2) and anti-PA (58/1 and 65/4) (42-44) anti-FLAG® M5 (F4042, Sigma), anti-HA tag (12CA5, ab16918), anti-Hsp70 (C92F3A-5), anti-α-tubulin (DM1A, ab7291), anti-Hsp90 (16F1, ab13494), anti-β-actin (ab6276) and polyclonal anti-Lamin B1 (ab16048). All primary antibodies used were of mouse origin except anti-Lamin B1 and anti-Hsp90, which were rabbit polyclonal and rat

monoclonal antibodies, respectively. HRP-conjugated anti-mouse, anti-rabbit and anti-rat mAbs were used as secondary antibodies (Jackson ImmunoResearch) in western blot analysis, while Alexa Fluor-488® goat anti-mouse IgG and Alexa Fluor-405® goat anti-mouse IgG antibodies (Invitrogen) were employed in immunofluorescence assays.

Prostaglandin A1 (PGA1) and tumor necrosis factor alpha (TNF α) were purchased from Cayman Chemicals, USA and Wako, Japan, respectively.

Construction of plasmids- The plasmids containing influenza virus polymerase and NP genes of A/Hong Kong/483/1997 (H5N1) (HK483) (pCAGGS-HK483PB2, -PB1, -PA and -NP), PR8 (pCAGGS-PR8-PB2, -PB1, -PA and -NP) and A/Aichi/2/1968 (H3N2) (Aichi) (pHH21-AichiPB2, -PB1, -PA and -NP) were kindly provided by Dr. Yoshihiro Kawaoka, Institute of Medical Science, University of Tokyo, Japan. For constructing plasmids expressing full length PB2 and PB1 proteins of the HK483 influenza virus with N-terminal FLAG-tags (FLAG-HK483PB2 and FLAG-HK483PB1), PCR products were amplified from pCAGGS-HK483PB2 and pCAGGS-HK483PB1 as templates using following primer pairs: FLAG-PB2-F/*SacI*

(5'-TATTGAGCTCATGGATTACAAGGATGAC GACGATAAGGGCGGCATGGAAAGAATAAA AGAACTACG-3') and PB2-R/*XhoI*

(5'-ATATCTCGAGTCACTAATTGATGCCATCCGAA TTC-3') for FLAG-HK483PB2 and FLAG-PB1-F/*Acc65I* (5'-TATTGGTACCCATGGATTACAAGGATGAC GACGATAAGGGCGGCATGGATGTCAATCC GACTTTAC-3') and PB1-R/*SphI* (5'-ATATGCATGCTCACTACTTCCCTTGCCG TCCGAGCTC-3') for FLAG-HK483PB1. Then, amplified FLAG-HK483PB2 and FLAG-HK483PB1 PCR products were digested with *SacI/XhoI* and *Acc65I/SphI* restriction enzymes, respectively, and cloned into the respective restriction sites of pCAGGS plasmid. The ORFs of PB2, PB1 and PA genes of Aichi were amplified from pHH21-AichiPB2, -PB1 and -PA as templates using following primer pairs: PB2-1F/*KpnI*

(5'-GGGGTACCCATGGAAAGAATAAAAGAAC TACG-3') and PB2-R/*XhoI* (5'-ATCTCGAGTTAATTGATGCCATCCGAA TTC-3') for Aichi-PB2, PB1-1F/*KpnI* (5'-GGGGTACCCATGGATGTCAATCCGAC-3') and PB1-R/*XhoI* (5'-ATCTCGAGTCACTATTTTGCCTCTG-3') for Aichi-PB1 and PA-1F/*KpnI* (5'-GGGGTACCCATGGAAGATTGTAC-3') and PA-R/*XhoI* (5'-ATCTCGAGCTATCTTAATGCATGTG-3') for Aichi-PB1. The amplified PCR products were cloned into the respective restriction sites of pCAGGS plasmid.

For cloning human Hsp70 (NM-005345), total RNA was extracted from

HEK293T cells using an RNeasy mini kit (Qiagen) and reverse transcribed using oligo(dT)₂₀ primer to generate cDNAs. These cDNAs were used to amplify the Hsp70 coding region using primer pair hHsp70-F/*KpnI* (5'-ATAAGGTACCATGGCCAAAGCCGCGGC GATC-3') and hHsp70-R/*XhoI* (5'-ATATCTCGAGTCACTAATCTACCTCCTC AATGG-3'). The amplified PCR product was digested with *KpnI* and *XhoI* restriction enzymes and cloned into the corresponding restriction sites of the pCAGGS expression plasmid producing a pCAGGS-Hsp70 plasmid. Hemagglutinin-tag (HA-tag) was inserted at the N-terminus of the Hsp70 coding region by PCR using pCAGGS-Hsp70 as the template and primer pair HA-hHsp70-F/*KpnI* (5'-GGGGTACCACCATGGACTACCCATACG ATGTTCCAGATTACGCTGCCAAAGCCGCG GCGATCGGCATCGAC-3') and hHsp70-R/*XhoI*. The amplified PCR product was digested with *KpnI* and *XhoI* restriction enzymes and cloned into the respective sites of the pCAGGS expression plasmid yielding pCAGGS-HA-Hsp70.

The reporter plasmid pHW72-Luc2CP was constructed by substituting the open reading frame of enhanced green fluorescent protein (EGFP) in pHW72-EGFP plasmid (kindly provided by R. Webby at St. Jude Children Research Hospital) with firefly luciferase gene (45).

Co-immunoprecipitation assays— HEK293T cells, grown in 10 cm tissue culture plates, were transfected with the plasmids indicated in the figures, using TransIT[®]-LT1 (Mirus, USA). At 48 hours post-transfection, cells were washed twice with cold PBS and collected by centrifugation. Cell pellets were resuspended in lysis buffer (50mM Tris HCl, 280mM NaCl, 0.5% Triton-X 100, 0.2mM EDTA, 2mM EGTA, 10% glycerol and 1mM DTT; supplemented with a protease inhibitor mixture, Complete Mini EDTA free, [Roche Applied Sciences]), subjected to sonication and clarified by centrifugation at 14000 g for 10 minutes. Then anti-FLAG affinity gel (anti-FLAG[®] M2-agarose gel, Sigma), washed three times with lysis buffer, was incubated with whole cell extracts overnight at 4°C with gentle rotation. The gel was washed 5 times with lysis buffer and the bound proteins were eluted using 3x FLAG peptide (F4799, Sigma) according to the manufacturer's instructions. The immunoprecipitated proteins were identified by western blotting using protein specific antibodies.

Reciprocal immunoprecipitation was carried out by transfecting HEK293T cells with HA-Hsp70 and viral polymerase expression plasmids as indicated in the figures. The immunoprecipitation procedure was the same as that described above except that whole cell extracts were mixed with anti-HA affinity gel (EZviewTM Red Anti-HA affinity gel, Sigma) and bound proteins were eluted using influenza HA

peptide (12149, Sigma) according to the manufacturer's instructions..

Cell Fractionation— HEK293T cells were fractionated into cytosolic and nuclear fractions, with little modification, as described by Suzuki *et al* (46). Briefly, cells grown in 10 cm tissue culture plates were infected with PR8 influenza virus at a multiplicity of infection (MOI) of 1 or mock infected. After 12 h of incubation, the cells were subjected to heat shock or allowed to recover for indicated time points (Fig. 5). The monolayers and resuspended cells were washed twice with ice-cold PBS. Then pelleted cells were resuspended in ice-cold 0.1% NP40-PBS and lysed by pipetting up and down several times. A portion of the cell suspension was kept as "whole cell lysate (WCL)". The cell lysates were centrifuged at 14000 x g for 1 min and the supernatants were collected as "cytosolic fraction" while the pellets (nuclei) were washed twice with ice-cold 0.1% NP40-PBS. The harvested pellets were resuspended in Laemlli sample buffer, sonicated for 30 seconds, and collected as "nuclear fraction". Equivalent proportions of two fractions were analyzed by SDS-PAGE and western blotting. The purity of the fractions was assessed by detecting specific subcellular marker proteins such as α -tubulin as cytoplasmic protein and Lamin B1 as nuclear protein.

siRNA design and knockdown of Hsp70— The small interfering RNA (siRNA) targeting Hsp70

(siHsp70-1) was purchased from Santa Cruz Biotechnology Inc. (sc-29352). An additional Hsp70-specific siRNA (siHsp70-2: 5'-CGGUGGGUGCAGUCGGACAUGA-3') was designed using an online siRNA designing tool: Design for Small Interfering RNA (DSIR) (47). The siHsp70-2 and a non-silencing control siRNA (5'-AAUUCUCCGAACGUGUCACGU-3') (48) were purchased from Sigma Genosys siRNA Service, Japan. HEK293T and HeLa cells were transfected twice (first reverse transfection and second forward transfection) on alternative days with Hsp70-specific siRNA, control siRNA and transfection buffer alone (Mock) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendations. HEK293T and HeLa cells were transfected with final concentrations of 80 nM and 40 nM for siHsp70-1, respectively, and 80 nM for siHsp70-2. Control siRNA concentrations were kept similar to those of Hsp70-specific siRNAs. Twenty-four hours after Hsp70 knockdown, cells were either infected with PR8 influenza virus at a MOI of 0.1 or transfected with HK483 or PR8 RNP expression plasmids for further analysis.

Luciferase reporter assays— Luciferase (*Firefly* and *Renilla* luciferase) activities were measured with GloMax96 microplate Luminometer (Promega) using the Dual-Luciferase® assay system (Promega). All transfections in HEK293T

or HeLa cells were performed using TransIT[®]-LT1 (Mirus) according to the manufacturer's recommendations. Luciferase activities were measured at 24 hours post-transfection, except where otherwise indicated. *Firefly* luciferase activities were normalized to the transfection control *Renilla* luciferase activities and were expressed relative to that of mock-treated cells, which were set to 1.

Influenza virus polymerase-driven luciferase activities (Viral polymerase activities) were measured by transfecting cells with the indicated vRNP expression plasmids, pCAGGS-NP (400 ng), -PB2, PB1 and -PA (200 ng each), the pHW72-Luc2CP *Firefly* luciferase reporter plasmid (100 ng) and pRL-CMV (Promega) *Renilla* luciferase transfection control reporter plasmid (50 ng), except where otherwise indicated.

To assess the effect of heat shock, PGA1 and plasmid-mediated overexpressed Hsp70 on NF-κB promoter activity, HEK293T and HeLa cells were transfected with pNFκB-Luc, an NF-κB promoter-dependent *Firefly* luciferase reporter plasmid (Agilent Technologies), and pRL-CMV, a transfection control reporter plasmid alone or with pH-A-Hsp70. At 24-hours post-transfection, cells were treated with PGA1, heat shock, or TNFα alone or combination of them as indicated in figures.

Over-expression of Hsp70 and viral transcription/replication activity— Hsp70

overexpression was achieved either by transfecting cells with the Hsp70 expression plasmid, by treating them with PGA1 or by subjecting them to heat shock.

Plasmid-mediated Hsp70 overexpression was achieved by transfecting HeLa and HEK293T cells with increasing doses of the HA-Hsp70 expression plasmid along with HK483 RNP expression plasmids and reporter plasmids as indicated before. The total plasmid concentration was adjusted using the empty pCAGGS plasmid.

In order to induce Hsp70 overexpression by PGA1, cells transfected with viral RNPs and reporter plasmids were treated with PGA1 (20 µg/ml) for 3 hours, and then growth medium containing PGA1 was replaced with fresh growth medium. Four hours later cell lysates were prepared and luciferase activities were measured.

We also ascertained the relation between the subcellular localization of Hsp70 during different phases of the heat shock response and influenza virus transcription and replication activities either by transfecting cells with vRNP expression and reporter plasmids or by infecting cells with PR8 influenza virus at a MOI of 1. The schematic diagram of the experiment is shown in Fig. 4A. An additional group of cells transfected with all sets of plasmids except pCAGGS-PB1 was included as a background control. The heat shock response was divided into pre-heat shock, heat shock and recovery (post-heat shock) phases.

At 24 hours post-transfection or 12 hours post-infection (p.i), cells in one group were subjected to heat shock at 42°C for 3 hours, representing the heat shock phase. Another group of cells was subjected to heat shock at 42°C for 3 hours and then allowed to recover at 37°C for 4 hours. This group represented the recovery phase. Cells maintained at 37°C throughout the course of the experiment served as untreated controls and represented the pre-heat shock phase. Polymerase activities were measured by dual luciferase reporter assay while viral replication was determined by measuring viral NP gene vRNA, mRNA and cRNA levels. We also determined the viral polymerase activities in mock, control siRNA and Hsp70-specific siRNA treated HeLa cells during the pre-heat shock, heat shock and recovery phases at indicated time points. The schematic diagram of the experiment is shown in Fig. 6A. The values of control siRNA and siHsp70 treated cells were expressed relative to those of mock-treated cells of the same group at that particular time point.

Immunofluorescence assay- All monoclonal antibodies, used for the colocalization studies of Hsp70 and polymerase subunits were of mouse origin. Therefore, double staining was achieved by labeling mouse anti-PB2, -PB1 and -PA mAbs with Alexa Fluor 488 or Alexa Fluor 594 dyes using a Zenon® Alexa Fluor® 488 labeling kit (Z25002) and Zenon® Alexa Fluor® 594 labeling kit (Z25007). Prior to labeling, all primary

antibodies were affinity purified using an Affi-Prep MAPS II Kit (Bio Rad).

HEK293T cells were grown in 8-well Lab-Tek™ chamber slides and were transfected with the indicated expression plasmids as shown in the figures. The slides were treated with poly-L-lysine (Cultrex) prior to the cell propagation according to the manufacturer's instructions. At 24 hours post-transfection, cells were washed with sterile ice-cold PBS, fixed and permeabilized with ice-cold methanol and blocked with 1.0% bovine serum albumin in PBS for 30 minutes. Later, cells were incubated for 30 minutes with primary antibodies (1:1000 diluted in PBS-BT; PBS containing 1% BSA and 0.05% Tween-20) specific for Hsp70 or HA-tag. After incubation with primary antibodies, cells were washed with PBS and incubated with indicated Alexa Fluor-conjugated secondary antibodies (1:1000 diluted in PBS-BT) for further 30 minutes and then again washed with PBS.

Single staining of the polymerase subunits was achieved by adding Alexa Fluor 488-or Alexa Fluor 594-labeled primary antibodies specific for PB2, PB1 or PA proteins. Double staining was achieved by adding the combination of Alexa Fluor 488- and Alexa Fluor 594-labeled primary antibodies specific for the PB2, PB1 or PA proteins. The unbound antibodies were removed by washing the cells with PBS. A second fixation was done by treating cells with 4% formaldehyde in PBS for 15 minutes. The cells were washed with PBS and their nuclei were

stained with DAPI.

After the final washing, mounting medium (Vectashield, Vector Laboratories) was added and cover slips were applied to the slide. The cells were examined using the LSM780 confocal microscope (Carl Zeiss) and pictures were taken using Zen 2010D (Carl Zeiss) software.

RNA extraction, reverse transcription (RT) and real-time PCR– Total RNA was extracted from infected cells using Isogen (Nippon Gene, Japan) following the manufacturer's protocol. Reverse transcription (RT) was carried out using Superscript III reverse transcriptase kit (Invitrogen) in a 20 µl reaction mixture containing 300 ng of total RNA and the NP gene and sense-specific primers for influenza virus vRNA

(5'-AGTAGAAACAAAGGTATTTTC-3'),

cRNA

(5'-AGCAAAAGCAGGGTAGATAATCACTCA C-3') and mRNA [oligo(dT)₂₀]. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primer was also included in the RT reaction mixtures for vRNA and cRNA estimation. RT reaction was carried out according to the manufacturer's protocol. One microliter of RT mixture was used in real-time PCR using SYBR[®] Premix Ex Taq[™] II (Tli RNase H Plus) kit (Takara, Japan) and gene specific primers. The reaction was performed at 95°C for 10 s followed by 40 cycles at 95°C for 5 s and at 60 °C for 30 s.

All the reactions were carried out in replicates of three. The specificity of the primers was assessed by dissociation (melting) curve analysis. The levels of PCR products were monitored and analyzed with a CFX96[™] Real-Time PCR Detection System (BioRad). The relative amounts of viral RNAs (mRNA, cRNA and vRNA), expressed as threshold cycle (C_T) values, were normalized by the amount of GAPDH mRNA and expressed relative to an arbitrary value of 1 (49). Sequences of the gene-specific primers were

NP-1186F:	5'-
ACCAATCAACAGAGGGCATC-3'	and
NP-1333R:	5'-
TGATTCGGCCTCATGTCA-3';	
Hsp70-1933F:	5'-
GAGGCAGACAAGAAGAAGGT-3'	and
Hsp70-2067R:	5'-
GCTGATGATGGGTTACACA-3';	and
housekeeping gene GAPDH-556F:	
5'-TGCACCACCAACTGCTTAGC-3'	and
GAPDH-642R:	
5'-GGCATGGACTGTGGTCATGAG-3'.	

RESULTS:

Hsp70 interacts with influenza virus PB2 and PB1 monomers as well as PB2/PB1 heterodimers but not with PB2/PB1/PA heterotrimers– Iwai *et al.* (50) reported that addition of FLAG- or HA-tags to the N-termini of influenza virus polymerase subunits did not interfere with their assembly into functional heterotrimeric polymerase complex. Therefore, to investigate

the interaction of influenza virus RNA polymerase with Hsp70, we constructed expression plasmids encoding N-terminal FLAG-tagged viral polymerase proteins (FLAG-HK483PB2, FLAG-HK483PB1) and N-terminal HA-tagged Hsp70 (HA-Hsp70). Co-immunoprecipitation experiments carried out using FLAG-HK483PB2 and HA-Hsp70 showed that Hsp70 was successfully precipitated with PB2, and vice versa, indicating that addition of FLAG-tag to the PB2 subunit or HA-tag to Hsp70 did not affect the interaction between them (Fig. 1 A and B).

It was shown that endogenous Hsp70 interacted with PB2 and PB1 monomers, (35) but it was not clear if Hsp70 interacted with viral polymerase heterodimers or heterotrimers. HEK293T cells transfected with FLAG-HK483PB2 or FLAG-HK483PB1 expression plasmids alone or in combination with other polymerase subunit expression plasmids were analyzed by immunoprecipitation studies as shown in Fig. 1 (C, D). We found that endogenous Hsp70 was co-immunoprecipitated with both FLAG-HK483PB2 and FLAG-HK483PB1 monomers as well as FLAG-HK483PB2/PB1 and FLAG-HK483PB1/PB2 heterodimers but not with FLAG-HK483PB1/PA heterodimer and FLAG-HK483PB2/PB1/PA or FLAG-HK483PB1/PB2/PA heterotrimers (Fig. 1 C, D). Moreover, reciprocal immunoprecipitation using HA-Hsp70 and PR8 polymerase subunits

confirmed that exogenous HA-Hsp70 also interacted mainly with the PB2 and PB1 monomers, and to a lesser extent with the PA monomer (Fig. 1E). Similar results were obtained with Aichi (H3N2) polymerase subunit (data not shown), indicating that this interaction was not influenza virus strain-specific. These findings suggested that Hsp70 seemed to act as chaperone for PB2 and PB1 proteins and appeared to assemble PB2/PB1 dimer and was released from the complex upon introduction of the PA subunit.

Hsp70 translocates and colocalizes with influenza virus PB2 protein- Although, it has been shown that Hsp70 translocated into the nucleus upon infection with influenza virus (35), it was not clear whether Hsp70 translocated into the nucleus with the vRNP complex or with individual polymerase subunits. In order to address this issue, the subcellular localization of Hsp70 with each of the polymerase subunits was studied in HEK293T cells transfected with individual polymerase subunit expression plasmids. As already known, PB2 protein was mainly present in the nuclei of cells while PB1 and PA proteins were present in both the cytoplasm and the nucleus (18). In mock-transfected cells, endogenous Hsp70 was mainly located in the cytoplasm (Fig. 2A, a), and it only translocated into the nuclei of the cells expressing PB2 protein, but not in the cells expressing PB1 or PA (Fig. 2A, b-d). Similarly, Hsp70 translocated into the nuclei with only PB2

protein of PR8 influenza virus (data not shown). Since viral replication takes place in the nucleus, the polymerase subunits have to be transported into it to make a functional polymerase complex, which consists of PB2, PB1, and PA subunits; PB1 being in the center having binding sites for PB2 and PA subunits whereas PB2 and PA subunits do not interact directly (51). In order to explain the assembly of viral polymerase subunits into a functional polymerase complex while keeping in view the results for co-immunoprecipitation obtained in the present study, we transfected HEK293T cells with plasmids expressing HK483PB2/PB1, PB1/PA and PB2/PA heterodimers and studied their colocalization patterns as well as effects on the localization of Hsp70. It was found that Hsp70 and PB1 translocated into the nuclei of the cells expressing PB2/PB1 heterodimers, suggesting that co-expression of PB2 with PB1 facilitated the nuclear translocation of the PB1 subunit (Fig. 2B, a). Although, trace amount of PB1 could be seen in the cytoplasm, its amount was quite small compared to the predominant cytoplasmic presence when cells expressed PB1 alone (Fig. 2A, c). In the cells expressing PB1/PA heterodimers, both PB1 and PA were mainly present in the nuclei of the cells with little cytoplasmic presence and these cells showed little nuclear accumulation of Hsp70 (Fig. 2B, b). In the cells expressing PB2/PA heterodimers, the PB2 subunit localized in the nucleus along with Hsp70, whereas PA predominantly remained in

the cytoplasm indicating that there was no direct interaction between PB2 and PA subunits (Fig. 2B, c).

Knockdown of Hsp70 in HEK293T and HeLa cells resulted in reduced viral transcription and replication activity— Since both HEK293T and HeLa cells express Hsp70 constitutively, we assessed the role of endogenous Hsp70 in influenza virus transcription and replication by knocking-down it using Hsp70-specific siRNAs (siHsp70-1 and siHsp70-2). First, we determined the role of Hsp70 in virus transcription by luciferase reporter assay using siHsp70-1 siRNA in HEK293T and HeLa cells. The results showed 48% and 66% reduction in influenza virus polymerase activity in both cell lines (Fig. 3B and D). Western blot analysis also confirmed 58% and more than 80% reductions in Hsp70 level in HEK293T and HeLa cells, respectively (Fig. 3A, C). It has been shown that siRNAs can produce unexpected or divergent results partially due to off-target effects (52,53). Therefore, another siRNA (siHsp70-2) was designed. Hsp70 knockdown by siHsp70-2 also resulted in 53% and 46% reductions in the polymerase activities of both HK483 and PR8 vRNPs in HeLa cells, respectively (Fig. 3E and F). We then determined the effect of Hsp70 knockdown on influenza virus replication. Hsp70 was knocked down in HeLa cells using siHsp70-2 as before. Then, the cells were infected with the PR8 influenza virus at a MOI of 0.1. At eight hours p.i., total RNA

was isolated from the cells and the mRNA, cRNA, and vRNA levels of the NP gene were quantified by real time RT-PCR. The results showed a significant reduction in all viral RNA species tested compared to those in control siRNA-treated cells (Fig. 3G). Consistently, protein levels of PB2, PB1, and NP also decreased as determined by western blotting (Fig. 3H and I). These results indicated the requirement of Hsp70 for efficient virus transcription and replication in HEK293T and HeLa cells.

Increase in viral polymerase activity during the heat shock phase is correlated with increased intra-nuclear accumulation of Hsp70 and viral polymerases— It is well known that following heat shock, there is a marked increase in the production and nuclear localization of Hsp70, which slowly returns to the cytosol during the recovery phase (54,55). We also confirmed by immunofluorescence assay that Hsp70, after 3 h of heat shock at 42°C, mainly localized in the nucleus and most of it relocated into the cytoplasm after 4 h of recovery at 37°C (Suppl. Fig.1). We hypothesized that if Hsp70 had an inhibitory effect on viral polymerase activity, it should have been more pronounced during the heat shock phase than the recovery phase, coinciding with the movement of Hsp70 into the nucleus. Moreover, increase or decrease in viral polymerase activities might be related to increase or decrease in viral polymerase proteins and Hsp70 levels during these phases of heat shock

response. To test these hypotheses, we measured the viral transcription and replication activities as well as viral polymerase proteins and Hsp70 levels in infected cells by western blotting.

Oza *et al* (56) studied the influence of neuronal cell differentiation on the heat shock gene expression. They transfected the cells with Hsp70 promoter-dependent luciferase reporter plasmid along with Renilla expressing internal control plasmid, subjected the cells to heat shock for different durations and determined the luciferase activities; therefore suggesting the applicability of luciferase reporter assay in this study. HeLa and HEK293T cells were transfected with the indicated plasmids. Cell lysates were prepared and luciferase activities were measured during the pre-heat shock, heat shock and recovery phases as shown in Fig 4A. Interestingly, the viral polymerase-driven luciferase activities of HK483 and PR8 vRNPs in both cell lines were significantly higher in cell lysates prepared immediately after heat shock than in cell lysates prepared after 4 hours of recovery, thus coinciding with the movement of Hsp70 inside the cells (Fig. 4B-E).

We also evaluated the relations among the pre-heat shock, heat shock and recovery phases and the relative quantities of mRNA, cRNA and vRNA of the NP gene of PR8 influenza virus in infected 293T and HeLa cells. At 12 hours p.i., the infected cells were subjected to heat shock as described above and total cellular RNA was isolated, reverse transcribed and

relative quantities of m-, c- and vRNA of NP gene were determined. We found more than two-fold increase in the mRNA levels of the NP gene in HEK293T cells during the heat shock and recovery phases compared to the pre-heat shock phase. There was about 44% reduction in vRNA level during the recovery phase whereas vRNA levels remained unchanged during the pre-heat shock and heat shock phases. Interestingly, cRNA levels also remained unchanged during the pre-heat shock, heat shock and recovery phases (Fig. 4F). In HeLa cells, there was about 40% reduction in cRNA, 33% reduction in vRNA and a slight but non-significant reduction in mRNA levels of the NP gene during the heat shock phase compared to the pre-heat shock phase. During the recovery phase, there was about 71% and 56% reduction in vRNA level compared to the pre-heat shock and heat shock phases, respectively (Fig. 4G). Although cRNA level was reduced to 56% during the recovery phase in comparison to the pre-heat shock phase, there was no significant difference in cRNA and mRNA levels between the heat shock and recovery phases. It is noteworthy that there were differences in the levels of RNA species of NP gene between HEK293T and HeLa cells. A significant reduction in vRNA level was observed in HeLa cells during the heat shock phase whereas it remained unchanged in HEK293T cells. Similarly, cRNA levels were also reduced significantly in HeLa cells during the heat shock and recovery phases whereas they remained unchanged in HEK293T

cells.

Later, we measured viral polymerase proteins and Hsp70 quantities in the subcellular fractions during different phases of heat shock response to ascertain the possible relation with viral polymerase activities. HEK293T cells were infected with PR8 influenza virus at a MOI of 1. After 12 h, cells were treated as in Fig. 4A, and an additional 24 h recovery phase sample was also collected. Nuclear and cytoplasmic fractions were analyzed by western blotting (Fig. 5A). It was quite interesting to find that there was 3-4 fold increase in the viral PB2, PB1 and PA proteins and 1.5 fold increase in NP protein levels in the nuclear fractions with reduction in their cytoplasmic levels during the heat shock phase. In case of Hsp70, about 12-fold increases in the nuclear fraction and slight reduction in the cytoplasmic fraction was seen. During the recovery phase at 4 h post-heat shock, a reduction in PB2, PB1, PA and NP protein levels in the nuclear fractions with corresponding increase in cytosolic fractions was observed; and this nuclear to cytoplasmic shift continued even at 24 h post-heat shock. A similar but less pronounced reduction of Hsp70 levels in nuclear fractions and increase in cytosolic fractions was observed (Fig. 5B). These findings suggested a possible correlation between Hsp70 assisted increase in viral polymerase proteins levels in the nucleus resulting in increased viral polymerase activities. Interestingly, we did not detect Hsp90 in the nuclear fractions prepared from PR8 infected

cells. Western blot analysis of whole cell lysates (WCL) from “A” showed a reduction an overall quantity of viral proteins (Fig. 5C).

Heat shock restores viral polymerase activity in Hsp70 knocked-down cells– We hypothesized that if Hsp70 had inhibitory effect on influenza virus polymerases, it should be observed in the Hsp70 knocked down cells during the heat shock phase, when Hsp70 expression is induced and Hsp70 translocates into the nucleus. Therefore, we determined the viral polymerase activities in mock, control siRNA and siHsp70 treated HeLa cells at 30, 90 and 180 minutes after heat shock as well as in the pre-heat shock and recovery (4 h and 24 h post-heat shock) phases (Fig. 6A). Interestingly, an increase in the viral polymerase activity with increase in heat shock duration (heat shock phase) and subsequent reduction in viral polymerase activity with increase in recovery time (recovery phase) were observed (Fig. 6B). Western blot analysis showed an increase in the Hsp70 levels in Hsp70 knocked-down cells during the heat shock and recovery phases (Fig. 6C). These findings also confirmed that during the heat shock phase, Hsp70 translocated into the nucleus and increased the viral polymerase activity while during the recovery phase, although Hsp70 levels increased, Hsp70 moved out into the nucleus leading to reduction in viral polymerase activity.

Plasmid mediated overexpression of Hsp70

reduces the viral polymerase activity– The knockdown of Hsp70 not only reduced the polymerase activity in Hsp70-depleted HEK293T and HeLa cells but also reduced the virus replication in infected cells. From these experiments it was clear that Hsp70 at normal levels (refers to Hsp70 level in cells not treated to induce Hsp70 production) was required for influenza virus replication in both cell lines. However, what would be the effect on virus polymerase activities if Hsp70 is overexpressed in HEK293T and HeLa cells? To determine this, Hsp70 overexpression was achieved by transfecting cells with increasing doses of the Hsp70 expression plasmid.

Both HEK293T and HeLa cells were transfected with HK483 RNP expression plasmids, reporter plasmids and the HA-Hsp70 expression plasmid in doses of 100, 200, 400 and 800 ng or empty pCAGGS to adjust the total amount of transfected DNA. Cell lysates were prepared 24 hours post-transfection and viral polymerase activities were measured. Compared to mock-treated cells, a dose-dependent reduction in polymerase activity in both cell lines was observed (Fig. 7A). Interestingly, western blot analyses showed no significant changes in the amounts of PB2 or PB1 proteins in mock- and HA-Hsp70 transfected cells (Fig. 7B). Immunofluorescence staining of HA-tag revealed that HA-Hsp70, like endogenous Hsp70, remained mainly in the cytoplasm of mock-transfected cells or cells expressing PB1

and PA proteins (data not shown) but translocated into the nuclei of the cells expressing PB2 protein (Fig. 7C).

Hsp70 over-expression suppresses the NF-κB activity— So far, the results obtained showed that suppression of viral polymerase activity by plasmid-mediated Hsp70 overexpression or by heat shock during the recovery phase varied in terms of degree of suppression and viral proteins expression. The results also suggested that these treatments might be affecting virus replication through different mechanisms. One of the common key factors affected is NF-κB. Heat shock, PGA1 and Hsp70 overexpression (57-60) have been shown to inhibit NF-κB activity in a variety of cells, which is required for efficient influenza virus replication (61). Therefore, we also determined the effect of heat shock, PGA1 and plasmid mediated Hsp70 overexpression on NF-κB activity in HEK293T and HeLa cells. The cells were transfected with the pNF-κB-luc reporter plasmid (which expresses the firefly luciferase protein under the influence of the NF-κB promoter) alone or with pHA-Hsp70 (800 ng), and pRL-CMV plasmid as a transfection control. We found that TNF α , (10 ng/ml for 3 hours) significantly increased the luciferase activity compared to the mock control indicating the activation of NF-κB promoter. Both PGA1 and heat shock treatments significantly inhibited the NF-κB promoter activity, as indicated by a more than 80% reduction in luciferase activity.

Plasmid mediated Hsp70 overexpression caused only about 25% suppression in NF-κB promoter activity. (Fig. 8A-D).

DISCUSSION:

In this study, we investigated the role of Hsp70 in influenza virus transcription and replication in cells constitutively expressing Hsp70. Although previous studies (34,35) have shown that Hsp70 inhibits influenza virus replication, those studies were conducted in cells either expressing low levels of Hsp70 or higher levels of Hsp70 were induced by PGA1 or heat shock treatment. However, other cellular events contingent upon PGA1 or heat shock treatment were over-looked in those studies. In the present study we found that Hsp70 had modulatory effect on influenza virus replication, presumably by facilitating the viral polymerase activity when present at normal levels and by suppressing the polymerase activity when its levels were increased above normal levels. Previously Li *et al.* (35) demonstrated that Hsp70 interacted with PB2 and PB1 proteins and translocated into the nuclei of A549 cells upon infection with influenza virus. We also found that Hsp70 interacted with the PB2 and PB1 monomers. Additionally, we found that Hsp70 coprecipitated with the PB2/PB1 heterodimer and inclusion of the PA subunit in the PB1/PA heterodimer or PB2/PB1/PA heterotrimer resulted in separation of Hsp70 as indicated by its absence in immunoprecipitates (Fig. 1). Naito *et al.* (18)

reported that Hsp90 α interacted with influenza virus PB2, PB1 monomers or PB2/PB1 heterodimers and translocated into the nucleus with PB2 monomers or PB1/PB2 heterodimers. They suggested that PB2 helped in the nuclear translocation of PB1. Hemerka *et al.* (62) also demonstrated the nuclear accumulation of PB1/PB2 heterodimers using bimolecular fluorescence complementation assay. In contrast, Fodor and Smith (63) have reported that the PB1 and PA subunits interact in the cytoplasm and are transported into the nucleus as a heterodimer while PB2 is transported into the nucleus as a monomer. It is also reported that PB2 and PB1 are present in the nucleus and PA remains in the cytoplasm during the early phase of infection (64). In general, current findings (Figs. 2) suggest that Hsp70 acted as a chaperon and translocated into the nucleus with PB2/PB1 dimer and that polymerase subunits assemble into a functional complex heterotrimeric complex in the nucleus.

The results obtained from immunoprecipitation and immunofluorescence studies suggested that Hsp70 played an important role in virus replication, and this role was confirmed by knocking down Hsp70 using Hsp70-specific siRNA. In contrast to Li *et al.* (35), our results suggested that knocking down Hsp70 caused a reduction in HK483 polymerase activity in HEK293T and HeLa cells (Fig. 3B and D). Since our findings were not in agreement with those of Li *et al.*, we thought that the reduction in polymerase activity could be an

off-target effect of siHsp70-1 siRNA. Therefore, another siRNA (siHsp70-2) targeting Hsp70 ORF was used. Interestingly, siHsp70-2 not only reduced the polymerase activities of both HK483 and PR8 RNPs but also reduced the virus replication as indicated by reductions in the mRNA, cRNA, and vRNA levels of the NP gene in infected cells (Fig. 3E-I).

Zeng *et al.* (55) demonstrated that there was a two-fold increase in the nuclear import and a three-fold decrease in the nuclear export of Hsp70 during the heat shock phase, resulting in the nuclear accumulation of Hsp70. In contrast, there was a decrease in the nuclear inflow and a marked increase in the nuclear outflow of Hsp70 during the recovery phase, resulting in relocation/accumulation of Hsp70 in the cytoplasm. They also found that Hsp70 not only interacted with protein aggregates but also with diffuse cytoplasmic and nucleoplasmic proteins, thereby extending its cytoprotective effect. In this study, viral polymerase-driven reporter assays and nuclear/cytoplasmic localization analyses suggested a correlation between the sub-cellular location of Hsp70 and viral polymerase activity. Possibly, during the heat shock phase, nuclear translocation of Hsp70 caused an increased inflow/accumulation of viral polymerase subunits into the nucleus which is the site of virus replication, and might facilitate their assembly into functional polymerase complexes. It is also conceivable that enhanced viral polymerase activity might be due to the increased chaperon

activity of Hsp70 translocated into the nucleus. Furthermore, during the recovery phase, increased nuclear outflow and reduced inflow of Hsp70 might reduce nuclear levels of viral polymerase subunits either by reducing their inflow, increasing their outflow or both leading to reduction in viral polymerase activity. These findings are in part supported by Lang *et al.* (65), who compared the effects of different incubation temperatures on the polymerase activities and growth of low pathogenic avian influenza viruses. They reported a significant increase in virus titers at 39°C as well as 20-60% increase in viral polymerase activities compared to at 35°C. An increase in the mRNA levels and reduction in vRNA and cRNA levels after heat shock are in-line with the findings of Dalton *et al.* who (66) reported that Hsp70 was not responsible for down-regulation of viral RNA synthesis. They also reported that higher temperature affected the vRNA levels more than the cRNA levels, while mRNA levels either increased or remained unchanged. Interestingly, there were differences in mRNA, cRNA and vRNA levels of the NP gene between HEK293T and HeLa cells during the pre-heat shock, heat shock and recovery phases. These difference could be due to differences in the response of cells to the heat shock and subsequent events leading to the recovery from heat shock, requiring further investigation.

Despite, increase in viral transcriptional activity and increased intra-nuclear accumulation

of viral proteins during the heat shock phase, a reduction in total amount of viral proteins, in agreement with previous finding (35), was observed in heat shocked or PGA1 treated cells (Fig. 5C and Suppl. Fig. 2). Moreover, in agreement with Li *et al.* (35), we also observed no change in viral protein contents in cells transfected with increasing doses of HA-Hsp70 expression plasmid. This could be due to difference in mechanism of virus suppression by heat shock, PGA1 and plasmid-mediated overexpressed Hsp70. Both heat shock and PGA1 are well-known stress response inducers to initiate global cell responses involving many cellular events such as induction of Hsp70 production, inhibition of the NF-κB activity (58,59,67), increase in the I-κB α production (68,69), increase in ubiquitin expression (70,71), translational and transcriptional arrest (72) and degradation of damaged or misfolded proteins (73), all of which possibly affect the virus transcription and replication through various mechanisms thereby causing a reduction in total viral proteins. On the other hand, so far reported spectrum of overexpressed Hsp70 to interfere with other cellular events is quite narrow such as inhibition of NF-κB (57,60) and protein kinase C activities (74) etc. In fact, Hsp70 has been shown to interact and coprecipitate with p65, c-Rel, p50 and IκB α inhibitory protein thus causing delay in activation of NF-κB complex (75).

NF-κB, despite acting as an immediate early mediator in the immune and inflammatory

responses, is also involved in promoting many pathologic events such as progression of AIDS by enhancing the transcription of human immunodeficiency virus-1 (76), increase in susceptibility of cells to influenza virus infection (61) and the efficiency of influenza virus production (77). Kumar *et al.* (78) showed that Knockdown of p65 of NF-κB significantly reduced the vRNA levels in infected cells and overexpression of p65 caused a significant increase in vRNA levels as determined by a cRNA-luciferase-based reporter assay. Considering these findings, we sought to clarify whether plasmid mediated Hsp70 overexpression, heat shock or PGA1 affected the NF-κB activity in cells constitutively expressing Hsp70. Our findings, in agreement with previous ones, showed that all these treatments suppressed the NF-κB activity (Fig. 8). Interestingly, there appeared to be a relation between the suppression of the virus polymerase activity and reduced NF-κB promoter activity. The inhibition of viral polymerase activity by plasmid-mediated overexpression of Hsp70 (approx. 35%) (Fig. 5) was about two-fold less than that achieved by

cells treated with heat shock and PGA1 (60-80%) (Suppl. Fig.2). Similarly, plasmid-mediated overexpression of Hsp70 caused about 25% suppression of the NF-κB promoter activity whereas the heat-shock or PGA1 treatment caused more than twice (>80%) reduction of the NF-κB promoter activity. However, despite these findings, the exact difference in mechanism of reduction in viral polymerase activity by plasmid-mediated Hsp70 overexpression, heat shock or PGA1 is not clearly known and requires further investigation.

In conclusion, the findings in the present study suggest that Hsp70, at normal levels, acts as a chaperone for viral polymerases in HEK293T and HeLa cells. The modulatory effect of Hsp70 on viral polymerase observed during different phases of heat-shock response appears to be a consequence of directional movement of Hsp70 between cytoplasmic and nuclear compartments, while some other cellular pathways stimulated by the heat-shock or PGA1 treatments might independently affect the viral polymerase activity and therefore obscure the effects of Hsp70.

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FOOTNOTES:

We are thankful to the Japan Society for the Promotion of Science (JSPS) for providing funding to Rashid Manzoor. This study was supported by the Strategic Research Base Development Program for Private Universities subsidized by MEXT (2010), a Grant-in-Aid for Scientific Research, JSPS, and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID).

The Abbreviations used are: Hsp70, heat shock protein 70; PR8, A/Puerto Rico/8/1934 (H1N1); HK483, A/Hong Kong/483/1997 (H5N1); Hsp90, heat shock protein 90; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2; PA, polymerase acidic protein; NP, nucleoprotein; vRNP, viral ribonucleoprotein; PGA1, prostaglandin A 1; MOI, multiplicity of infection.

FIGURE LEGENDS:

FIGURE 1. Hsp70 interacts with PB2, PB1 monomers and their dimers but not with PB2/PB1/PA heterotrimer. (A and B) Effects of addition of HA- and FLAG-tags on the interaction of Hsp70 with PB2 of HK483 influenza virus. HEK293T cells were transfected with indicated plasmids, and immunoprecipitated proteins were identified by western blotting using anti-HA tag, -FLAG tag, -Hsp70 and -PB2 mAbs. **(C)** HEK293T cells were transfected with plasmids encoding HK483-FLAGPB2 alone or in combination with plasmids expressing the indicated polymerase subunits of HK483 influenza virus. Proteins were immunoprecipitated using anti-FLAG affinity gel and analyzed by SDS-PAGE followed by western blotting. **(D)** Immunoprecipitation was carried out as in “C” except that HEK293T cells were transfected with plasmids encoding HK483-FLAGPB1 protein alone or in combination with plasmids expressing the indicated viral polymerase subunits. **(E)** HEK293T cells were transfected with a plasmid encoding HA-Hsp70 alone or in combination with plasmids encoding the indicated polymerase subunits of PR8 influenza virus. Proteins were immunoprecipitated using anti-HA affinity gel and analyzed by SDS-PAGE followed by western blotting.

FIGURE 2. Hsp70 translocates into the nucleus with PB2 monomer or PB2/PB1 heterodimer. Subcellular localization of Hsp70 with viral polymerase subunits was analyzed by confocal laser-scanning microscopy. **(A)** HEK293T cells were transfected with the indicated plasmids of HK483 influenza virus or with empty plasmid (Mock). At 24 h post-transfection, cells were fixed, blocked and stained with mouse anti-Hsp70 and Alexa Fluor 488[®] goat anti-mouse IgG mAbs. Staining of viral polymerase proteins was done by using anti-PB2, -PB1 and -PA mAbs labeled with Alexa Fluor[®]594 dye. Arrows in panel “b” indicate the nuclear localization of Hsp70. Cell nuclei were stained with DAPI. **(B)** HEK293T cells were cotransfected with plasmids encoding viral polymerases (PB2, PB1 and PA) of HK483 influenza virus. At 24 h post-transfection, cells were fixed, blocked and stained with mouse anti-Hsp70 and Alexa Fluor 405[®] goat anti-mouse IgG mAbs. Dual staining of polymerase proteins was achieved by labeling the anti-PB2, -PB1 and -PA mAbs with the Alexa Fluor[®] dyes (Red, Alexa Fluor[®]594 and Green, Alexa Fluor[®]488 dye). Arrows in panels “a” and “c” indicate the nuclear localization of Hsp70.

FIGURE 3. Knocking down Hsp70 decreases the virus transcription and replication. (A and C) Western blot analysis (bottom) and its quantification (top). HEK293T “A” and HeLa “C” cells were transfected twice on alternate days with transfection reagent only (Mock), control siRNA or Hsp70-specific siRNA (siHsp70-1). The Hsp70 levels were normalized to β-actin which served as loading control, and were expressed relative to the mock-treated cells. **(B and D)** Hsp70 knockdown was carried out as in “A and C”. Twenty-four hours after knocking down the Hsp70, HEK293T “B” and HeLa “D” cells were transfected with HK483 RNP expression plasmids pCAGGS-NP (200 ng), -PB2, -PB1 and -PA (100 ng each), pHW72-Luc2CP *Firefly* luciferase (50 ng) and pRL-CMV *Renilla* luciferase (25 ng) reporter plasmids. After 24 h, viral polymerase activities were measured. Non-silenced cells served as controls (Mock). Results are based on three independent experiments, each performed in duplicate or triplicate. **(E and F)** Polymerase activities of HK483 and PR8 influenza viruses were measured in HeLa cells as in “B and D”, except that knockdown of Hsp70 was carried out using siHsp70-2 siRNA. Results are based on three independent experiments, each performed in triplicate. **(G)** Hsp70 was knocked down as in “E and F”, and cells were infected with PR8 influenza virus at a MOI of 0.1. At 8 h p.i., m-, c- and vRNA levels of the NP gene were quantified by real-time PCR, normalized with GAPDH mRNA and expressed relative to those of control siRNA-treated cells. Results are based on three independent experiments. **(H and I)** Western blot analysis of viral proteins in Hsp70 knocked down HEK293T cells. Cells were infected as in “G”. At 12 h p.i., cell lysates were analyzed by western

blotting. Indicated protein levels were normalized to the β -actin which served as loading control, and were expressed relative to the mock-treated cells. Data were analyzed by student t-test. Error bars represent mean \pm SEM. * P<0.05, **P<0.01 and ***P<0.001.

FIGURE 4. Hsp70 enhances the viral polymerase activity during the heat shock phase.

(A) Schematic diagram illustrating the experiment layout. (B and C) HEK293T and (D and E) HeLa cells were transfected with HK483 and PR8 RNP expression plasmids along with reporter plasmids. After treating cells as in “A”, cell lysates were prepared and viral polymerase activities were measured. Cells maintained at 37°C (pre-heat shock) served as controls. Results are from three independent experiments, each performed in triplicate. (F and G) Viral RNA expression in HEK293T “F” and HeLa “G” cells infected with PR8 influenza virus. The cells were infected at a MOI of 1. After 12 h, cells were treated as in “A”, total RNA from cells was collected and m-, c- and v-RNA levels of the NP gene were quantified by real-time PCR. GAPDH was used as reference gene. Data are presented as fold change (C_T values) relative to cells infected at 37°C. Results are based on three independent experiments performed in duplicate or triplicate. Data were analyzed by student t-test. Error bars represent mean \pm SEM. * P<0.05, **P<0.01 and ***P<0.001.

FIGURE 5. Correlation between nuclear-cytoplasmic shuttling of Hsp70 and viral polymerase protein levels in subcellular fractions. (A) HEK293T cells were infected with PR8 influenza virus (MOI 1) or mock infected. At 12 h post-infection, cells were treated as in Fig. 4 A. An additional 24 h recovery phase sample was also included. The nuclear and cytoplasmic fractions from mock and PR8 infected-cells were prepared and analyzed by western blotting. Quality of fractions was assessed by blotting for Lamin B1 (nuclear fraction) and α -tubulin (cytoplasmic fraction). (B) Relative quantification of PB2, PB1, PA, NP and Hsp70 in nuclear and cytoplasmic fractions. The levels of PB2, PB1, PA, NP and Hsp70 in the nuclear fractions were normalized to the Lamin B1, while levels of PB2, PB1, PA, NP and Hsp70 in the cytoplasmic fraction were normalized to the α -tubulin and were expressed relative to those during pre-heat shock phase. The results are from two or three independent experiments. (C) Western blot (bottom) and relative quantities of PB2, PB1 (top) in WCL from “A”. The PB2 and PB1 levels were normalized to the β -actin and expressed relative to their pre-heat shock levels. The values are from three independent experiments. Error bars represent mean \pm SEM.

FIGURE 6. Hsp70 induction restores viral polymerase activity during the heat shock phase in Hsp70 knocked-down cells. (A). Schematic diagram illustrating the experiment layout. (B) HeLa cells

were treated with Hsp70 specific siRNA (siHsp70-2), control siRNA or transfection reagent only (Mock) twice at alternate days. At 24 h post-knock-down, cells were transfected with HK483 RNP expression plasmids along with reporter plasmids as indicated in Fig. 3. After 24 h, cells were treated as in “A”, cell lysates were prepared and viral polymerase activities were measured. Mock cells served as control. The values were normalized to the mock of each group at indicated time points. The results are based on three independent experiments, each performed in triplicate. Error bars indicate mean \pm SEM. (C) Western blot showing changes in Hsp70 levels in mock, control siRNA and siHsp70-2 treated cells at different time points. β -actin served as loading control.

FIGURE 7. Plasmid-mediated Hsp70 overexpression decreases the influenza virus polymerase activity. (A) HEK293T and HeLa cells were transfected with 100 ng, 200 ng, 400 ng and 800 ng of the HA-Hsp70 expression plasmid or empty vector (Mock) along with HK483 RNP expression plasmids and reporter plasmids. After 24 h, viral polymerase activities were measured. Results are based on three independent experiments, each performed in triplicate. The student t-test was used for statistical comparison of each of the HA-Hsp70 transfected group with mock. Error bars represent mean \pm SEM. * P < 0.05 and ** P < 0.01. (B) Representative western blot of cell lysates from “A”. (C) Immunofluorescence staining of HEK293T cells transfected with the HA-Hsp70 expression plasmid alone “A” or cotransfected with HK483 PB2 expression plasmid “B”. The expressed HA-Hsp70 was stained with anti HA-tag and Alexa Fluor 488[®] goat anti-mouse IgG (green) mAbs and PB2 with purified mouse anti-PB2 mAb labeled with Alexa Flour[®] 594 dye (red). Cell nuclei were stained with DAPI.

FIGURE 8. Heat shock, PGA1 and plasmid-mediated overexpressed Hsp70 reduces NF- κ B promoter activity. (A) HEK293T and (B and C) HeLa cells were transfected with pNF κ B-Luc (1 μ g) carrying an NF- κ B promoter-dependent luciferase reporter construct and pRL-CMV (100 ng), a transfection control reporter plasmid. (A and B) At 24 h post-transfection, cells were either treated with PGA1 (30 μ g/ml), TNF α (10 ng/ml) or first with PGA1 for 3 h and then with TNF α for 4 h. Untreated cells served as mock control. After the indicated treatments, NF- κ B promoter-driven luciferase activities were measured by dual luciferase reporter assay. (C) At 24 h post-transfection, cells were either subjected to heat shock for 3 h, treated with TNF α (10 ng/ml) for 4 h or first heat-shocked for 3 h and then treated with TNF α for 4 h. Untreated cells kept at 37°C served as mock control. Results are from three independent experiments performed in triplicate. Error bars represent mean \pm SEM. (D) HEK 293T cells were co-transfected with pNF κ B-Luc (100 ng), pRL-CMV (50 ng) and pHA-Hsp70 (800 ng).

In mock cells, empty pCAGGS plasmid was replaced with pH-A-Hsp70. After 24 h, cells were stimulated with TNF α (5 ng/ml) for 4 h and then NF- κ B promoter-driven luciferase activities were measured by dual luciferase reporter assay. Results are from two independent experiments, each performed in triplicate. Error bars represent mean \pm SEM.

Fig. 1

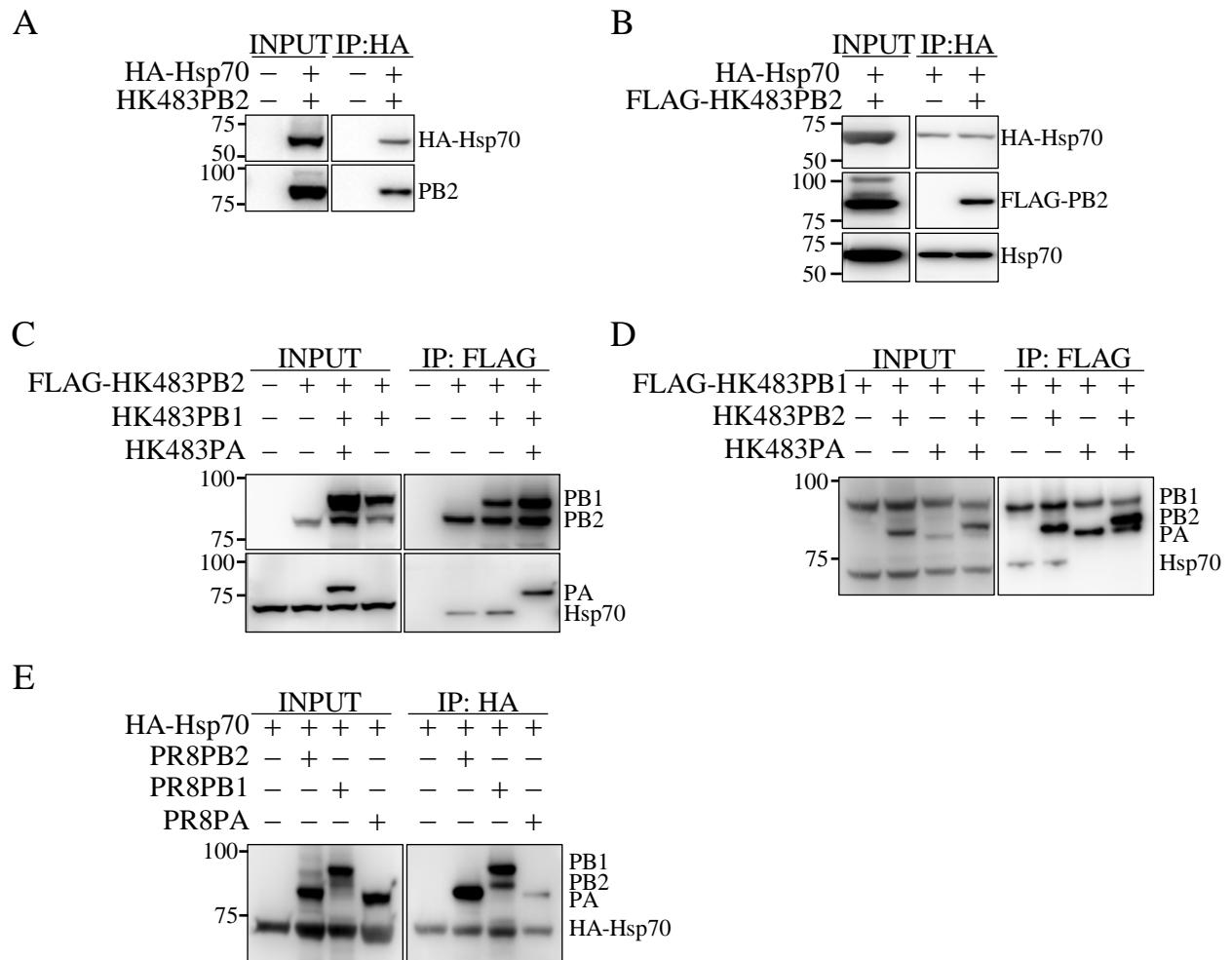
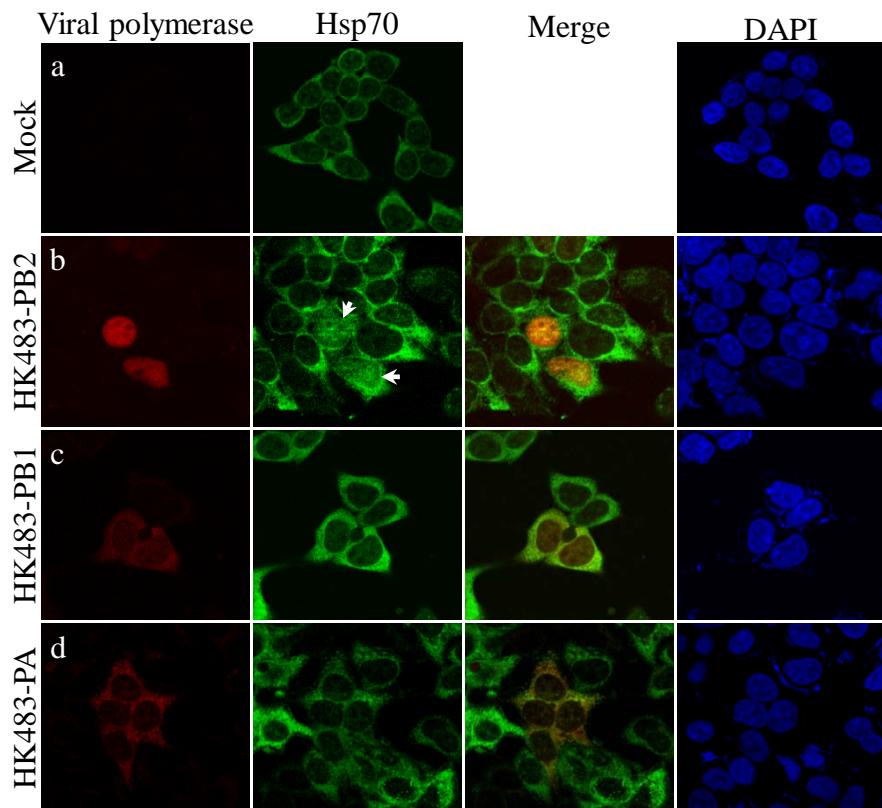


Fig. 2

A



B

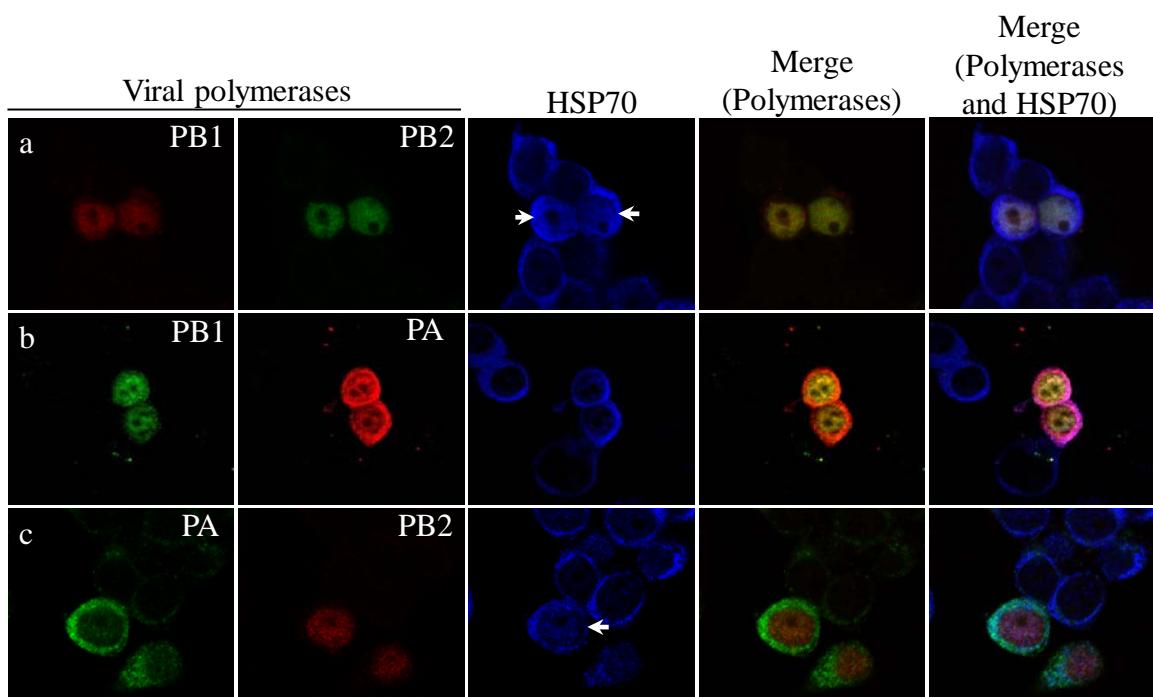


Fig. 3

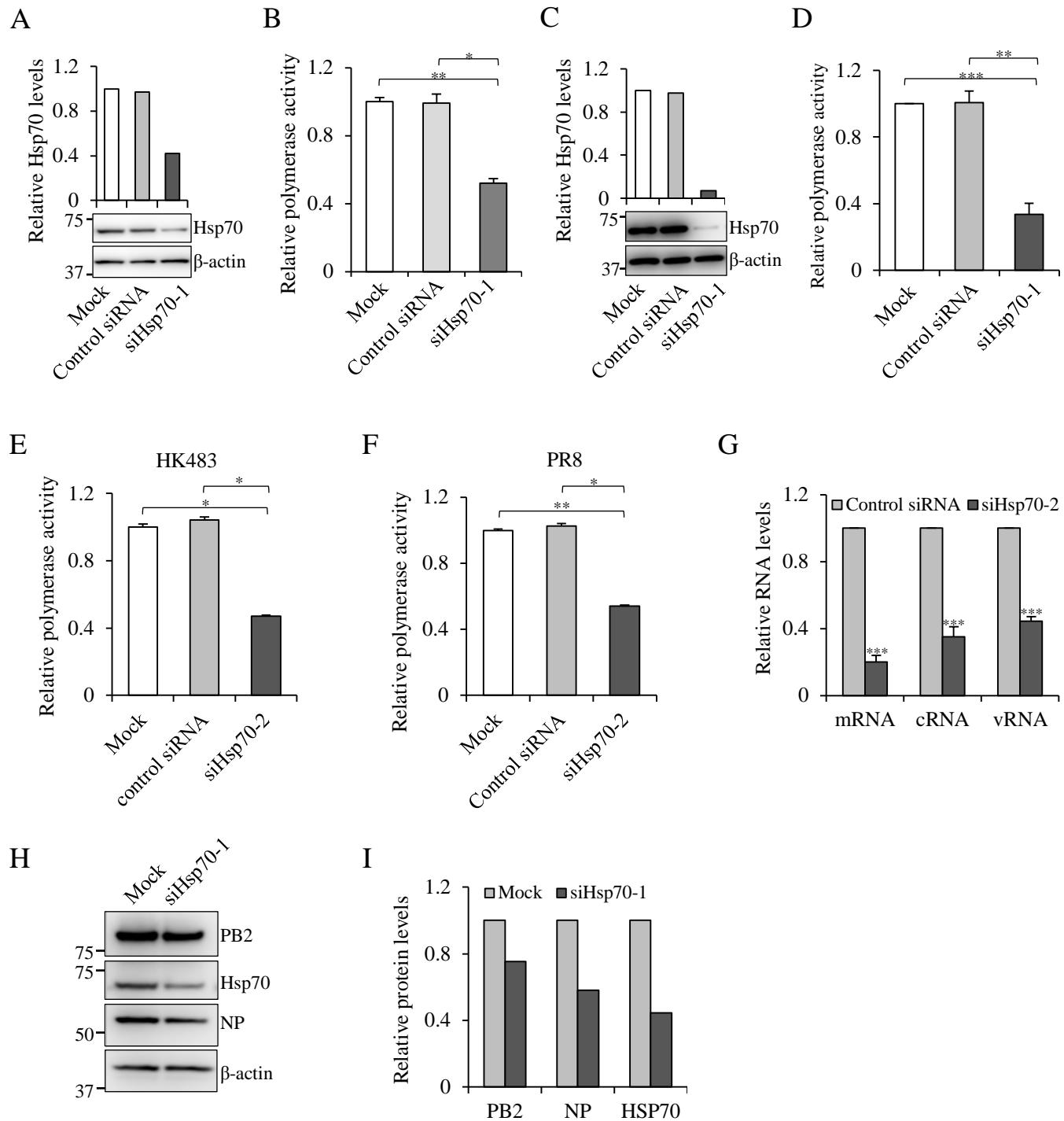
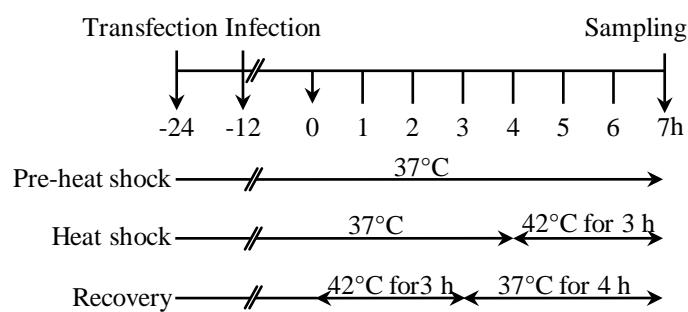
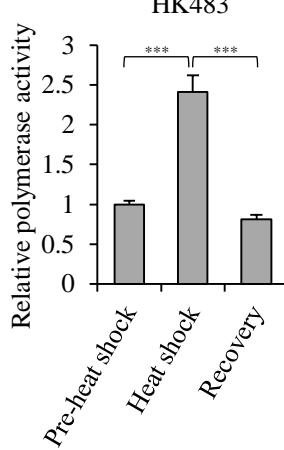


Fig. 4

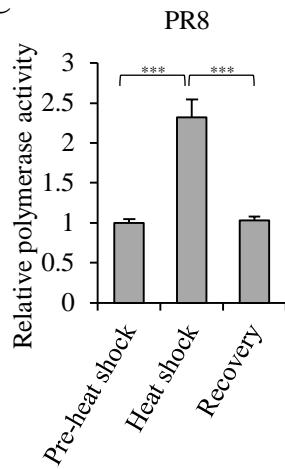
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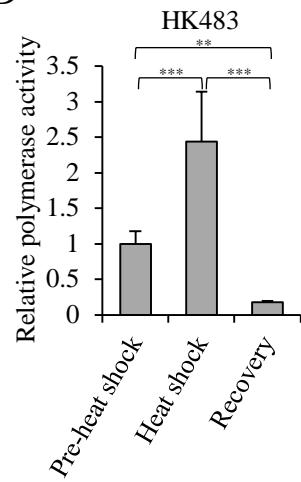
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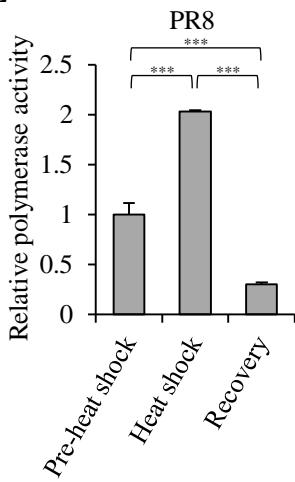
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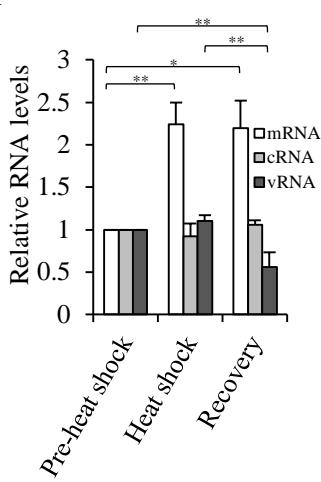
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E



F



G

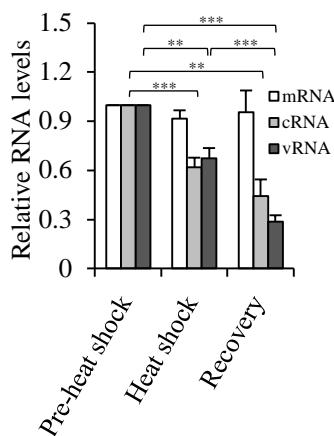


Fig. 5

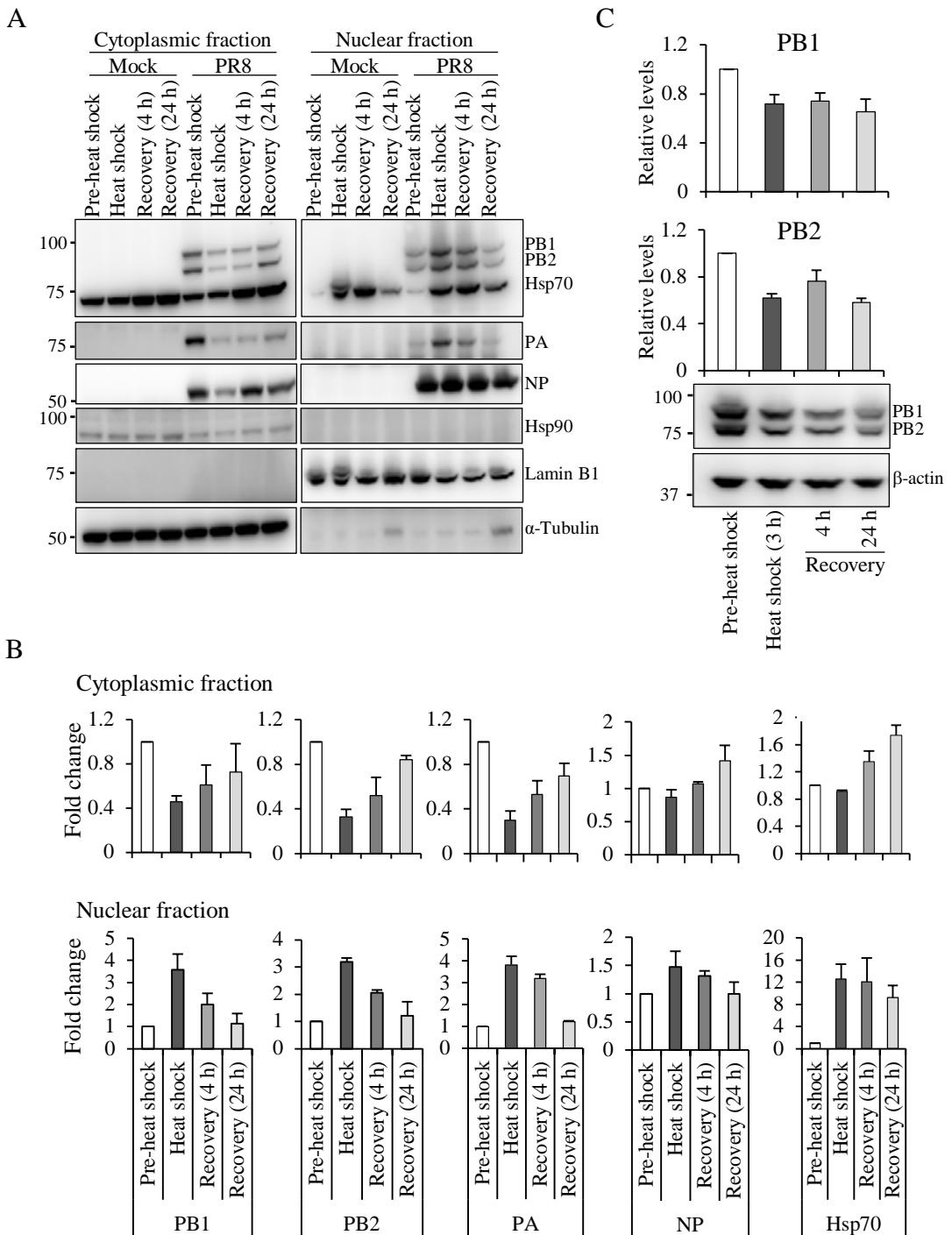


Fig. 6

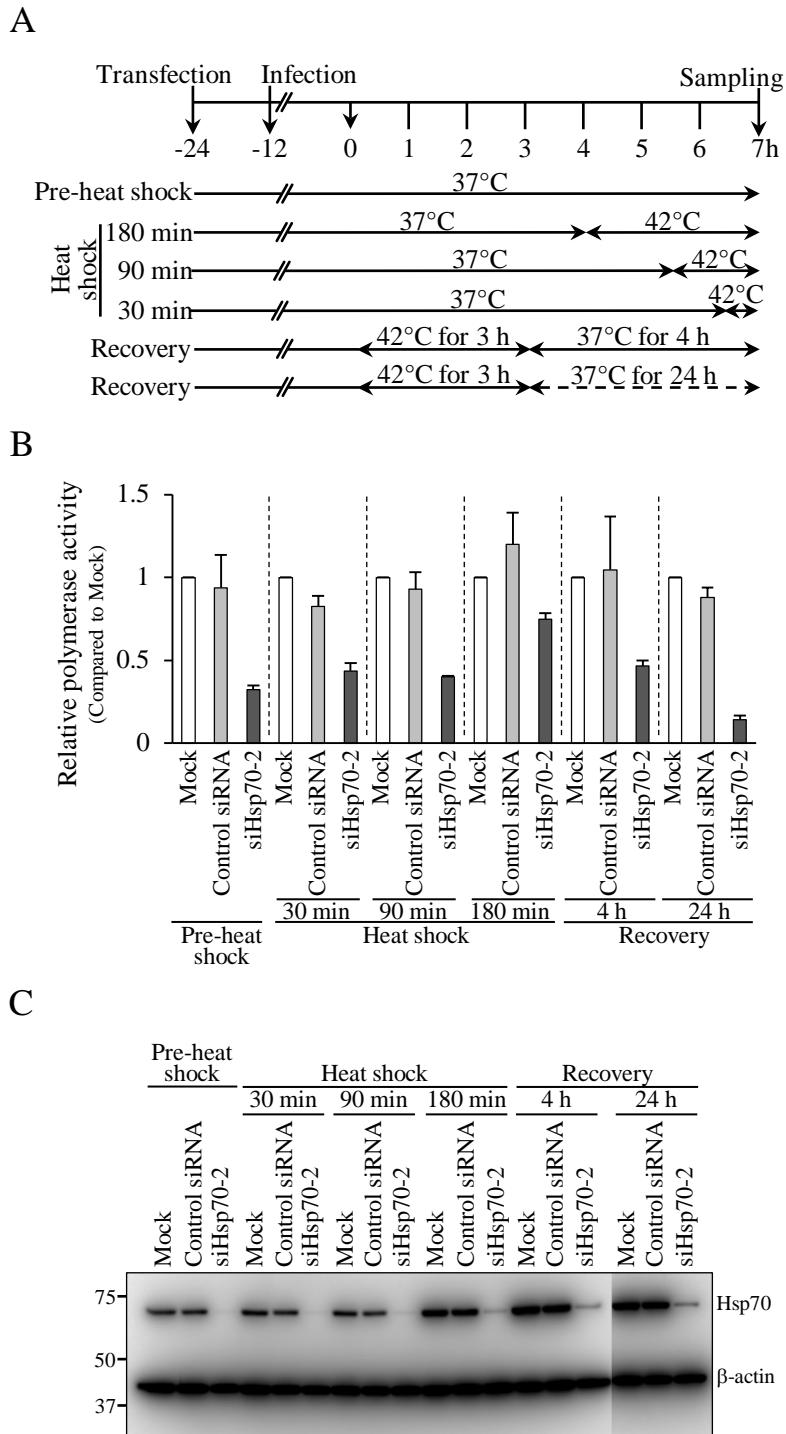
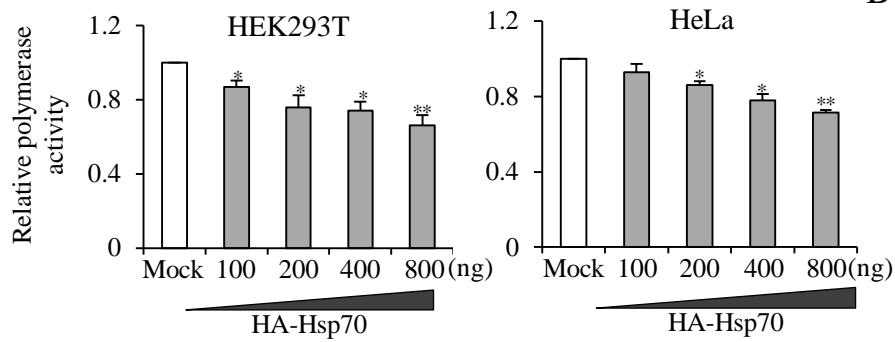
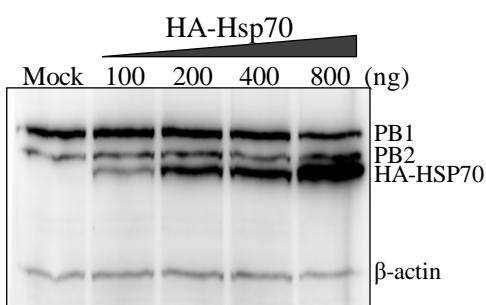


Fig. 7

A



B



C

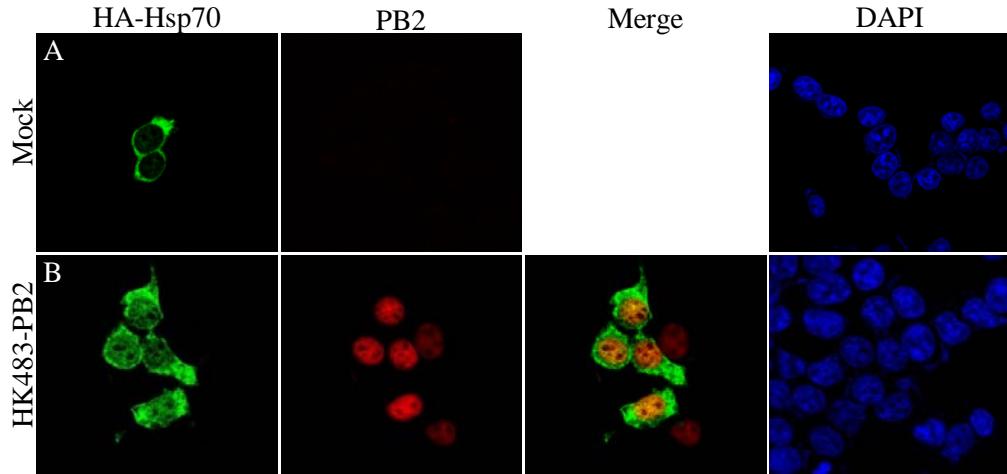


Fig. 8

