CHAPTER III

HSF1 regulates both HSP40 promoter and HIV-1 LTR driven gene expression during HIV-1 infection.
3.1 INTRODUCTION

Cellular heat shock proteins (HSPs) are molecular chaperones involved in protein folding, transport and assembly. In addition some of these proteins are specifically induced during stress conditions like heat shock, UV irradiation, and even microbial or viral infection (Creagh et al., 2000; Brenner et al., 2001). Recent studies have revealed that they are also involved in apoptosis and immune response (Takayama et al., 2003; van Eden et al., 2005). Viruses modulate expression of many cellular proteins for their successful replication within the host cell and induction of HSPs has been reported as one of the earliest change in cellular gene expression following viral infection (Asea et al., 2000). HIV-1 infection leads to significant increase in level of HSP27 and HSP70 during early stage of infection prior to viral mRNA synthesis (Wainberg et al., 1997).

Nef, a 27–30 kDa myristoylated phosphoprotein, encoded by HIV-1 is thought to contribute in viral pathogenesis by modulating cellular gene expression and signaling pathways (Saksela, K., 1997; Simmons et al., 2001). Nef-deleted viruses fail to replicate efficiently in vivo, and do not develop symptoms of acquired immunodeficiency syndrome (AIDS) (Geyer et al., 2001; Greene et al., 2002). It helps the virus to evade immune response by downregulating CD4 and MHC I surface molecules preventing superinfection. Nef has been also implicated in the activation of T cells, making the cells permissible to the virus (Doms et al., 2000; Fackler et al., 2002). Nef was previously reported as a negative factor for viral replication in T- cell lines (Arora et al., 2002; Niederman et al., 1989) but later reports have demonstrated Nef as an enhancer of viral replication (Glushakova et al., 1999; Papkalla et al., 2002; Lundquist et al., 2004; Joseph et al., 2003; 2005). However, the molecular
mechanism of this positive effect remains to be clearly understood. In our efforts to identify HIV-1 Nef interacting host cellular factors, we have shown earlier that Nef interacts with a human DnaJ homologue, HSP40, and this interaction was required for increased viral gene expression. Furthermore, we also showed that HSP40 expression was increased in NL4-3 transfected cells in a Nef-dependent manner and this up-regulation was necessary for increased viral gene expression and replication (Kumar et al., 2005). However the mechanism of HSP40 up-regulation during HIV-1 infection remains to be elucidated.

The inducible expression of HSPs is regulated by heat shock transcription factors (HSFs). Vertebrate HSF family has four members (murine and human HSF1, 2 and 4 and a unique avian HSF3), and of these HSF1 and HSF2 are ubiquitously expressed and conserved (Pirkkala et al., 2001; McMillan et al., 1998). In contrast to HSF1, HSF2 is refractory to classical stress stimuli. Neither HSF2 nor any other HSF is able to substitute for HSF1 or to rescue stress response (Xiao et al., 1999; Amin et al., 1988). HSF1 is the major transcription factor that regulates the transcription of HSP genes in response to stress. It binds to conserved regulatory sequences in the HSP promoter known as heat shock elements (HSE). HSE is represented by two or three inverted repeats of the sequence nGAAAn (Xiao et al., 1988; Wu, C., 1995). The activity of HSF1 is regulated at two distinct levels i.e. oligomerization and transcriptional competence. Under normal conditions, HSF1 is predominantly present in a cytoplasmic monomeric inactive form; however, upon stress it gets homo-trimerized and translocated to nucleus, where it acquires high affinity binding activity to HSE and transcription enhancing activity. Recent studies have indicated that phosphorylation also plays a major role in regulation of HSF1 activity. Specifically, Ser residues i.e. Ser\textsuperscript{230} and Ser\textsuperscript{326} are
inducibly phosphorylated during stress, which enhances its transcriptional activity (Hietakangas et al., 2006; Guettouche et al., 2005; Holmberg et al., 2001). Several studies have suggested that heat shock could activate the transcription of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) in cells (Hashimoto et al., 1996; Re et al., 1989) however, heat shock induced transcriptional activation of the HIV-1 LTR have not been clearly understood (Kretz-Remy et al., 1994; 2001). HIV-1 LTR promoter regulates the transcription of HIV-1 provirus (Gaynor, R., 1992). It contains binding sites for many cellular transcription factors such as NF-kB, Sp1, USF and AP1 etc., and therefore can be activated by many stimuli (Steffy et al., 1991; Garcia et al., 1994). The response of HIV-1 to various stress proteins, including heat shock proteins, could also lead to modulation of HIV-1 LTR mediated gene expression (Stanley et al., 1990).

In the present chapter we have analyzed the role of HSF1 in HIV-1 gene expression and replication. Our results clearly show that HSF1 positively regulates HIV-1 gene expression and replication by two distinct pathways. Firstly it induces HSP40 expression in association with viral protein Nef, both of which has been earlier shown to be required for increased viral gene expression. Secondly, activated HSF1 directly interacts with HIV-1 LTR promoter to induce viral gene expression and replication.

3.2 MATERIALS AND METHODS

3.2.1 Cell Lines, Plasmids and reagents

HIV-1 NL4-3 Nef cloned in pcDNA (pcDNA-Nef) was a kind gift from Dr. M. Federico (Olivetta et al., 2006) and NL4-3 Nef and its point mutant tagged with HA (HA-Nef) plasmids were obtained from Dr. W.C. Greene (Geleziunas et al., 2001). pCMV HSF1-Flag was a kind gift from Dr. Joseph
Goldman which was further subcloned in pET-28a(+) vector. The NL4-3 molecular clone (pNL4-3) was obtained from the National Institutes of Health AIDS repository (Adachi et al., 1986). The nef-deleted NL4-3 molecular clone (pNL4-3ΔNef) was obtained from Dr. J. C. Guatelli (Chowers et al., 1994). HIV-1 LTR and its deletion mutant-luciferase constructs were obtained from Dr. Takashi Okamoto (Takada et al., 2002). pGVB-HSP40 promoter luciferase construct was a kind gift by Dr. Kenzo Ohtsuka (Hata et al., 1998) which was further subcloned in pGL3 luciferase vector. HEK 293T (human embryonic kidney cell line) and Jurkat cells were obtained from the NCCS Cell Repository, India. CEM-GFP, a CD4+ human T cell line, was obtained from the National Institutes of Health AIDS repository (Gervaix et al., 1992). Rabbit polyclonal anti-Nef sera was obtained from Dr. Shahid Jameel, ICGEB (Chaudhry et al., 2005) and monoclonal and polyclonal Nef antibodies were obtained from Chemicon and National Institute of Health AIDS Repository respectively. Antibodies against HSP40, HA tag and phosphorylated HSF1 were obtained from Santa Cruz Biotechnology, USA. Monoclonal and polyclonal HSF1 antibodies were also obtained from Chemicon, USA and Cell Signaling, USA respectively. HSF1 Ab-4 (Clones 4B4 + 10H4 + 10H8 Rat monoclonal antibody) was obtained from Thermo Fisher Scientific, USA. Mouse monoclonal Flag antibody was obtained from Sigma, USA. Control siRNA and Sp1 siRNA were obtained from Santa Cruz Biotechnology, USA and HSF1 siRNA was from Dharmacon, USA.

3.2.2 Transient Transfection and Luciferase Assay

HEK-293T cells were transfected with different luciferase (pLTR-luc and HSP40 luc) constructs along with other expression vectors using calcium phosphate precipitation method (Kingston et al., 2003) and harvested 36h post-transfection for luciferase assay. For siRNA experiments, cells were first
transfected with siRNA using lipofectamine 2000, followed by another transfection with reporter plasmid and expression vectors with a gap period of 24h. The cells were then lysed in cell lysis reagent (Promega), and were analysed for luciferase activity using Luclite substrate (PerkinElmer Life Sciences, USA). Luciferase assays were performed using Top Count luminometer (Packard life Sciences, USA). The results were normalized to GFP expression.

3.2.3 HIV-1 Infection and Virus Quantitation

CEM-GFP or Jurkat cells (5x10⁶) were infected with HIV-1 NL4-3 virus at 0.1 MOI in the presence of Polybrene (1µg/ml) as described earlier (Liu et al., 1991). Peripheral blood was collected from normal seronegative donors and PBMCs were isolated by Ficoll-Hyphaque (Amersham Biosciences) gradient centrifugation. Cells were activated with 5µg/ml phytohemagglutinin (Sigma) for 36-48h. 5x10⁶ activated PBMCs were infected with 0.5 MOI of NL4-3 virus for 4 h in the presence of Polybrene (1µg/ml) with intermittent mixing as described earlier (Liu et al., 1991). After washing, the cells were plated in complete medium supplemented with human interleukin-2 (Roche Applied Bioscience) at 20 units/ml concentrations and incubated at 37 °C in a humidified CO₂ incubator. The culture supernatants of infected and pNL4-3 transfected cells were used to determine virus production by p24 antigen capture ELISA (Perkin Elmer Life Sciences, USA).

3.2.4 Cloning, expression and purification of His-HSF1

Human HSF1 cDNA was PCR amplified from pCMV5-HSF1-Flag vector using Accuprime Taq from Invitrogen, USA. The PCR product was then cloned into pET 28a+ vector at HindIII and XhoI sites using primers listed below.
Forward Primer (DM 733) - 5’ TCAAGCTTGCATGGATCTGCCCGTGG 3’
Reverse Primer (DM 734) - 5’ TACTCGAGCTAGGAGACAGTGGGGT 3’

Following confirmation by both restriction digestion and DNA sequencing this vector was used for expressing His-HSF1 protein by transforming Escherichia coli BL21 (DE3) competent cells. These cells were induced at 37°C with 1 mM isopropyl β-D-thiogalactoside to induce the expression of His-HSF1 protein. Following induction the cells were resuspended in binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole and 0.1% Tween-20, pH 8.0) and lysis was done by sonication and lysozyme treatment of the cell suspension. The clear lysate was separated from the cellular debris by centrifugation at 12000 rpm/4°C/10min. His-HSF1 was purified from the lysate by incubating the lysate with equilibrated Ni-NTA beads (Qiagen, Germany) in binding buffer for 2h at 4°C followed by washing of beads with binding buffer containing 20 mM imidazole and elution of His-HSF1 from beads using the same binding buffer containing 250 mM imidazole.

3.2.5 Immunoprecipitation, His Pull-down, and Immunoblotting

HEK-293T cells overexpressing HA-Nef and Flag-HSF1 were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.12 M NaCl, 0.5% NP40, 0.5 mM NaF, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) on ice for 45 min. Clarified lysates were incubated with monoclonal Flag antibody and the antigen-antibody complex was pulled down by an equal mixture of protein A and protein G agarose beads (Invitrogen, USA) followed by resolution on 12% SDS-PAGE. The proteins from gel were transferred on to PVDF membrane and the membrane was probed with HA antibody. The blots were developed by the ECL Plus system (Amersham Biosciences, USA). Similar co-immunoprecipitation experiment was performed with uninfected and
infected CEM-GFP cells. The clarified lysates of these cells were immunoprecipitated with Nef polyclonal antibody and were immunoblotted with monoclonal HSF1 antibody.

Transfected 293T cells overexpressing wild type and different point mutants of Nef were lysed in cold lysis buffer (25 mM HEPES, pH 7.3, 0.1 M NaCl, 5 mM EDTA, 0.5% TritonX100 and 1 mM DTT) with protease inhibitor cocktail (Roche Applied Bioscience, Germany). The clarified lysates were incubated with His-HSF1 protein in binding buffer pH 8.0 (50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, 10 mM Imidazole, and 0.1% Tween20) overnight at 4°C followed by pull down with Ni-NTA beads. These bead bound complexes were subjected to three washes with binding buffer having 20 mM imidazole. The complexes were then resuspended in Laemmli’s buffer, boiled and resolved on 12% SDS-PAGE. Proteins were transferred onto PVDF membrane and the membrane was probed with polyclonal HA antibody. Furthermore, HIV-1 infected CEM-GFP and Jurkat cell lysates were run on SDS-PAGE, followed by immunoblotting for HSP40 and HSF1 respectively.

3.2.6 Reverse Transcription-PCR

RNA was prepared from 2x10\(^6\) HIV-1 NL4-3 infected and uninfected CEM-GFP cells using TRIzol Reagent (Invitrogen, USA). The cDNA was made using Moloney murine leukemia virus reverse transcriptase (Invitrogen) followed by amplification by PCR for HSP40 and human β-Actin with Taq polymerase (Invitrogen) using standard conditions and gene-specific oligonucleotide primers as listed below:

human β Actin: forward, 5’ TGACGGGGTCAACCACACTGTCCTGCTCTG3’ and reverse, 5’CTAGAAGCATTTGCGGTGGACGATGGAGGG3’, human HSP40: forward, 5’CAGGATCCATTTTGCCTGAGCAGGACGGG3’, and reverse, 5’ GTGGAAGAGAATGAAGTGAGG3’.
3.2.7 Cloning of HSP40 promoter in pGL3 vector and construction of point mutants by site-directed mutagenesis

pGVB HSP40 promoter luc construct (gifted by Dr. Kenzo Ohtsuka) was used to amplify wild type HSP40 promoter sequence (-277 to +1) and a deletion mutant of HSP40 promoter from -246 to +1 position using primers shown in Table 3.1, having KpnI (forward primer) and XhoI (Reverse primer) restriction sites. These PCR products were first cloned into pGEMT-Easy cloning vector (Promega, USA). The pGEMT HSP40 promoter fragments were then subcloned into pGL3 basic luciferase vector (Promega, USA) at KpnI and XhoI sites.

<table>
<thead>
<tr>
<th>Primer Details</th>
<th>Primer Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP40 promoter wild type (-277) FW</td>
<td>TAGGTACCCGATTGGCTCGTCC</td>
</tr>
<tr>
<td>HSP40 promoter deletion mutant (-246) FW</td>
<td>GTGGTACCCTCCTCCGACCTGTG</td>
</tr>
<tr>
<td>HSP40 promoter (+1) Rev</td>
<td>TACTCGAGACCCCCTCCTGCG</td>
</tr>
</tbody>
</table>

Table 3.1 Primers used in PCR amplification of wild type HSP40 promoter and its deletion mutant.

HSP40 promoter point mutants were made by Site-directed mutagenesis technique using in vitro PCR site-directed mutagenesis kit (Stratagene USA). This PCR based site-directed mutagenesis method incorporated site-specific mutations as shown in Fig.3.3A in double stranded pGEMT-HSP40 construct (-246 to +1). Amplification was performed using Pfu Turbo DNA polymerase and mutant oligonucleotide primers as shown in Table 3.2 in a thermal cycler following manufacturer’s instructions. These oligonucleotide primers, each
complementary to opposite strands of the vector, were extended during thermal cycling by Pfu Turbo DNA polymerase which replicates both plasmid strands with high fidelity without displacing the mutant oligonucleotide primer. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. These PCR products were then subjected to digestion with Dpn I endonuclease (target sequence: 5´-GmATC-3´) which is specific for methylated and hemi-methylated DNA and was therefore used to digest the parental DNA template and to select for mutation-containing newly synthesized DNA. The nicked vector DNA containing the desired mutations was then used to transform XL1-Blue ultra competent cells. The entire process was performed following manufacturer’s instructions (Stratagene, USA).

Table 3.2 List of primers used in mutagenesis

<table>
<thead>
<tr>
<th>Mutation site</th>
<th>Primer used in mutagenesis</th>
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<tbody>
<tr>
<td>SP1 site</td>
<td>5’GCCGAGGGCGGAGCGTTATTTGCTGGGCCCCCGCGC3’</td>
</tr>
<tr>
<td>CAAT BOX</td>
<td>5’GAAAGGGCGGCGGCGTTCAGGCCGGCGGCCGGG 3’</td>
</tr>
<tr>
<td>1st HSE</td>
<td>5’GGGCGGGCGGCGGCGGTCTGGTCTGGAGGGCTGG3’</td>
</tr>
<tr>
<td>2nd HSE</td>
<td>5’GGGCTGGCGGCTGCTGTGCTGCTGCCGGACGGG 3’</td>
</tr>
</tbody>
</table>

pGEMT-HSP40 promoter point mutants were confirmed by DNA sequencing and were further subcloned into pGL3 Basic vector at KpnI and XhoI restriction sites.

3.2.8 Preparation of Nuclear and Cytoplasmic Fraction

Uninfected and HIV-1 infected Jurkat cells (10⁷) were pelleted down and washed with ice cold phosphate-buffered saline. Cells were resuspended in
400 µl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM p-aminobenzoic acid, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 50 µg/ml aprotinin) and kept on ice for 15 min to swell. After that 25 µl of 10% Nonidet P40 was added to the cells and vortexed for 10 sec. Then samples were centrifuged at 4°C for 30 sec to remove the cytoplasmic fraction. The nuclear fraction was prepared by resuspending the remaining pellet in 200 µl of ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM p-aminobenzoic acid, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 50 µg/ml aprotinin) and kept on ice. These nuclear extract were used to study the phosphorylation of HSF1 by immunoblotting using polyclonal pHSF1 (Ser230) antibody.

3.2.9 Chemical crosslinking by EGS (N-hydroxyl succinimidester)

Uninfected and infected CEM-GFP cells (5x10⁶) were washed with ice cold phosphate-buffered saline. The cell pellet was resuspended in 100-200 µl cold lysis buffer (25 mM HEPES pH 7.3, 100 mM NaCl, 5 mM EDTA, 0.5% Triton-X100, protease inhibitor cocktail at 2X concentration, 0.5 mM PMSF and 1 mM DTT) followed by lysis on ice for 30 min. The cell lysate was spun at 12000 rpm for 10 min at 4°C. The supernatant was used as lysate for crosslinking. 100 µg lysate was diluted with cold lysis buffer to make a final volume of 28.5 µl. To this, 2 mM EGS (1.5 µl of 40 mM EGS) was added and kept at room temperature for 15 min. This was followed by quenching with 100 mM glycine (3 µl of 1M Glycine) for 5 min at room temperature. To this 10 µl of 3X sample buffer was added and boiled at 95°C for 5 min. The lysate was run on 5% SDS PAGE (Westerheide et al., 2009). Similar crosslinking studies were also done by lysing the uninfected and infected cells in lysis buffer (25 mM
HEPES, pH 7.3, 0.1 M NaCl, 5 mM EDTA, 0.5% TritonX100 and 1 mM DTT) having 1 mM DTT and running the lyate under non reducing condition by using sample buffer without β-mercaptoethanol.

3.2.10 Electrophoretic mobility shift assay (EMSA)

Synthetic oligonucleotides spanning the NF-κB - Sp1 region of LTR were labelled using [γ-32P] dATP (BRIT, INDIA) by T4 polynucleotide kinase (NEB, USA) for 30 min at 37°C and were purified by Probequant G-50 columns (Amersham, USA). Binding reactions were set with both recombinant His-HSF1 protein and nuclear extract from uninfected and HIV-1 infected Jurkat cells. Binding reaction mixture was incubated at 37°C for 15 min and loaded on 6% non-reducing polyacrylamide gel. Oligonucleotide sequences for P1, P2, P3 and P4 with their position in LTR are given in Table 3.3.

Table 3.3 Oligonucleotide sequences of EMSA probes.

<table>
<thead>
<tr>
<th>Probes (Position in HIV-1 LTR)</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (-105 to -82) Both NF-κB sites of LTR</td>
<td>GGGACTTTCCGCTGGGGACTTTCC</td>
</tr>
<tr>
<td>P2 (91 to -68) 1st NF-κB and 3rd Sp1 site</td>
<td>GGGACTTTCCAGGGAGTG GTGGC</td>
</tr>
<tr>
<td>P3 (-67 to -46) 1st and 2nd Sp1 site</td>
<td>TGGGCGGGACTGGGGAGTGGCG</td>
</tr>
<tr>
<td>P4 (-80 to -57) 2nd and 3rd Sp1 site</td>
<td>GGGAGGTGTGGGCTGGGGAC</td>
</tr>
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3.2.11 Chromatin Immunoprecipitation

CEM-GFP cells (2x10⁷) were infected with NL4-3 virus at 0.1 MOI. On day 5 post infection, cells were harvested and fixed with 1% formaldehyde.
Following fixing, ChIP was performed by performing primary immunoprecipitation with Nef polyclonal antibody using ChIP assay kit (Upstate Biotechnology, USA) according to manufacturer’s instructions. In sequential ChIP experiments, the eluate which came after first immunoprecipitation with polyclonal anti Nef antibody was reincubated with second antibody i.e. rat monoclonal HSF1 antibody (Chemicon, USA) or rabbit anti Sp1 antibody (Santa Cruz, USA) overnight at 4°C. The occupancy on HSP40 promoter was checked using different primers as schematically presented in Figure 3.6A. The sequences for the primers used for PCR amplification is give in Table 3.4 below.

**Table 3.4 Primer sequences used in HSP40 promoter ChIP**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (Fwd)</td>
<td>5’ GTGGTACCTCCTCCGACCTGTG3’</td>
</tr>
<tr>
<td>F2 (Fwd)</td>
<td>5’ TAGGTACGGGCGGAAGGTCTCT3’</td>
</tr>
<tr>
<td>R1 (Rev)</td>
<td>5’ TACTCGAGACCCCCTCCTGCG3’</td>
</tr>
<tr>
<td>R2 (Rev)</td>
<td>5’ TACTCGAGCGCCGCTCCTTT3’</td>
</tr>
</tbody>
</table>

Similarly, HIV-1 NL4-3 infected Jurkat cells were harvested and ChIP analysis was done for LTR promoter as described above. Here the immunoprecipitation was done using HSF1 antibody and the recruitment of HSF1 on LTR promoter was checked using different primers as represented in Figure 3.12A and the sequences of primers used for PCR in the ChIP assay are given in Table 3.5 below.
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Table 3.5 Oligonucleotide primer sequences used in HIV-1 LTR ChIP

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (Fwd)</td>
<td>GGAGTACTACAAAGACTGCT</td>
</tr>
<tr>
<td>F2 (Fwd)</td>
<td>TGGGGACTTTCCAGGGAG</td>
</tr>
<tr>
<td>F3 (Fwd)</td>
<td>GCGGGGACTGGGGAGTGG</td>
</tr>
<tr>
<td>R (Rev)</td>
<td>TAACCAGAGAGACCAGTA</td>
</tr>
</tbody>
</table>

3.2.12 Immunofluorescence Microscopy

HEK-293T cells grown on coverslips were transfected with both pcDNA-Nef and pCMV-HSF1 by Lipofectamine 2000 and cells were harvested 24h post-transfection, for immunofluorescence studies. Paraformaldehyde-fixed and permeabilized cells were blocked with 10% FCS and stained with polyclonal HSF1 and monoclonal Nef antibody. The secondary antibodies used for HSF1 and Nef were Cy3-conjugated goat anti-rabbit IgG (Chemicon) and Cy2-conjugated goat anti-mouse IgG (Chemicon), respectively. After washing, cells were counterstained with DAPI present in the mounting media. Thereafter mounted slides were analyzed with a confocal microscope (Zeiss LSM 510, Germany).

Uninfected and NL4-3 infected (Day 5 post infection) Jurkat cells were fixed with 2% paraformaldehyde and permeabilized. These cells were spun on a glass slide in cytopsin at 1000 rpm for 7 min to obtain a monolayer of cells. Then this monolayer of cells was stained with polyclonal HSF1 antibody or pHSF1 (Ser230) antibody. The secondary antibody used for pHSF1 and HSF1 was Cy3-conjugated Goat anti-rabbit IgG (Chemicon, USA) and was counterstained with DAPI (1μg/ml) present in the mounting media. Thereafter
after samples were analyzed with a confocal microscope (Zeiss LSM 510 META, Germany).

3.2.13 Quantitation of HSF1 Expression by Real-time PCR

HSF1 expression level was analyzed in HIV-1 infected cells by quantitative real-time RT-PCR in a 10 µl reaction mixture containing SYBR Green iQ supermix (Bio-Rad) and 10 pmol concentration of each of the human GAPDH and HSF1 primer pairs as listed here: human GAPDH forward: 5’ GAAGGTGAAGGTCGGAGTC 3’ and reverse: 5’GAAGATGGTGATGGGAT TTC 3’, human HSF1 forward: 5’ GCCTTCCTGACCAAGCTGT 3’ and reverse: 5’ AAGTACTTGGGCACGCACCTC 3’ by using the realplex4 Mastercycler (Eppendorf, USA). The amplification was performed using one cycle of 95°C for 2 min and 40 cycles of 94°C for 1 min, 60°C for 30 sec, and 68°C for 1 min followed by melting curve analysis. The changes in the threshold cycle (Cₜ) values were calculated by the equation \( \Delta C_T = C_{T,target} - C_{T,input} \). The fold difference was calculated as follows: fold difference = \( 2^{-(\Delta \Delta CT)} \).

3.3 RESULTS

3.3.1 HIV-1 Nef induces HSP40 expression by activating the HSP40 promoter

It was shown earlier from our lab that HSP40 is induced in HIV-1 infected cells (Kumar et al., 2005). Furthermore, it was also shown that HSP40 interacts with HIV-1 Nef and is induced in Nef dependent manner in HIV-1 NL4-3 transfected HEK-293T cells (Kumar et al., 2005). In order to confirm this finding in HIV-1 infected cells, we have now infected human CD4+ T cell line, CEM-GFP with HIV-1 NL4-3 wild type and Nef deleted virus and analyzed HSP40 expression on different days post infection by both RT-PCR and immunoblotting. As shown in Fig. 3.1A and 3.1B, HSP40 was induced in NL4-
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3 infected cells in a time dependent manner. However, this increase was not observed in Nef deleted NL4-3 infected CEM-GFP cells (Fig. 3.1C and 3.1D) confirming that HSP40 up-regulation during HIV-1 infection is dependent on the Nef protein.

Figure 3.1: HSP40 is upregulated in HIV-1 infected T cells in a Nef dependent manner. (A, B) HSP40 is up-regulated in HIV-1 NL4-3 infected CEM-GFP cells. HIV-1 NL4-3 infected CEM-GFP cells were harvested on day 1, 3, 5 and 7 post-infection for RT PCR and on day 3, 5 and 7 equal amounts of protein from uninfected and infected cells was used for immunoblotting of HSP40. (C, D) Nef is required for upregulation of HSP40 expression in HIV-1 infected T cells. CEMGFP cells were infected with NL4-3ΔNef virus and cells were harvested for RT-PCR and immunoblotting of HSP40.

3.3.2 Mechanism of Nef mediated induction of HSP40

In order to understand the mechanism of Nef mediated induction of HSP40, we have analyzed the sequence of HSP40 promoter as presented in Fig. 3.2A; (Hata et al., 1998) and have used HSP40 promoter-luciferase reporter vector for promoter activity assay. 293T cells were co-transfected with HSP40 promoter-Luciferase construct along with wild type or NL4-3ΔNef molecular
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close followed by analysis of reporter activity. The results show that HSP40 promoter activity was induced significantly by wild type NL4-3 but not with NL4-3ΔNef (Fig. 3.2B) suggesting thereby that Nef probably induces HSP40 promoter activity. In order to confirm this observation, we then co-transfected individual HIV-1 protein expressing vectors like Tat, Nef and Rev along with HSP40 promoter luciferase construct in 293T cells and the results clearly show that Nef specifically induces HSP40 promoter driven luciferase activity whereas Tat and Rev did not show any significant effect (Fig. 3.2C).

Figure 3.2: Nef induces HSP40 expression by activating the HSP40 promoter. (A) DNA sequence of the HSP40 promoter used in the present work. The Figure shows the position of various transcription factors (bold) on HSP40 promoter. The schematic diagram
on top shows the transcription factor binding sites in the promoter. (B) 293T cells were co-transfected with HSP40-luc and HIV-1 wild type NL4-3 or Nef deleted NL4-3 molecular clone. 48h post transfection, cells were lysed and luciferase assay was performed. (C) 293T cells were transfected with HSP40 luc and different expression vectors for Tat, Nef and Rev followed by luciferase assay as described in the text.

We then wanted to identify the region of HSP40 promoter that could be involved in Nef mediated activation of the promoter, for which we created one deletion mutant (Fig.3.3B) and several point mutants (Fig. 3.3D) of the HSP40 promoter-luciferase construct as described in materials and methods. The mutants are diagrammatically represented in Fig. 3.3A. PCR amplification was performed with one pGEMT HSP40 promoter deletion mutant (-246 to +1) vector as template, for introducing point mutations at various transcription factor binding sites as shown in Fig.3.3C. Subcloning of these point mutated HSP40 promoter fragments was done in pGL3 basic reporter vector and was confirmed by both restriction digestion (Fig.3.3D) and DNA sequencing.
**Figure 3.3: Cloning of HSP40 promoter mutants** (A) Schematic representation of HSP40 promoter deletion and point mutants (B) Restriction digestion confirmation of pGL3-HSP40 promoter wild type (-277 to +1) and deletion mutant (-246 to +1) reporter vectors. (C) PCR amplification for HSP40 promoter point mutants. Each arrow indicates the position of PCR product for different point mutants of HSP40 promoter having point mutation for a specific transcription factor binding site by site directed mutagenesis. (D) Restriction digestion confirmation of pGL3-HSP40 promoter point mutants constructs. Arrow indicates the position of mutated HSP40 promoter fragment released upon digestion.

These mutant HSP40 promoter constructs (Fig. 3.4A) were then used in co-transfection assay along with Nef in 293T cells followed by analysis of luciferase activity, the results of which are shown in Figure 3.4B.
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Figure 3.4: HSE elements are required for HIV-1 Nef dependent upregulation of HSP40 gene expression. (A) Schematic representation of HSP40 promoter mutants, star marked site indicates the mutated site. (B) Nef induces HSP40 promoter through HSE element. 293T cells were co-transfected with each of the HSP40 promoter mutant luc construct along with Nef expression vector as indicated in the Figure. 48h post transfection, cells were lysed and luciferase assay was performed as described in the text. Expression of Nef is shown by western blotting. The data presented is the mean of three independent experiments with error bars representing standard error of mean (SEM). Asterisk represents statistically significant results (p< 0.05).

There was no significant change in the activity of -246 deletion mutant luc along with Nef as compared to the wild type HSP40 (-277) promoter construct. Therefore rest of the point mutants were made on the -246 HSP40 promoter sequence. The results show that HSE elements in the HSP40 promoter were definitely involved in Nef mediated up-regulation of promoter activity, whereas CAAT box did not seem to play any role in this induction (Fig. 3.4B). Sp1 sites seem to play some role in Nef mediated up-regulation of HSP40 promoter activity; however, this effect was not as significant as observed with HSE elements (Fig. 3.4B).
3.3.3 HSF1 is required for Nef mediated up-regulation of HSP40 in HIV-1 infected cells

In order to analyze whether both Sp1 and HSF1 are required for Nef mediated induction of HSP40 promoter, endogenous HSF1 and Sp1 expression was silenced by gene specific siRNA transfection in 293T cells (Fig. 3.5A inset). These cells were then co-transfected with Nef and HSP40 promoter-luciferase construct followed by quantitation of luciferase activity. Our results show that silencing of Sp1 by specific siRNA did not significantly reduce the induction of HSP40 promoter by Nef as the promoter activity was almost similar to that of control siRNA transfected cells (Fig. 3.5A). However, HSF1 knockdown resulted in significant reduction in promoter activity compared with control siRNA transfected cells (Fig. 3.5A). When both Sp1 and HSF1 were silenced, the reduction in Nef mediated induction of HSP40 promoter did not change significantly as compared to that observed with HSF1 alone silenced cells. This result further shows that HSF1 plays an important role in Nef induced HSP40 expression, and the role of Sp1 was not found to be significant.
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Figure 3.5: HSF1 is necessary for Nef mediated up-regulation of HSP40 promoter activity (A) 293T cells were first transfected with 100 nM HSF1 smart pool siRNA, Sp1 siRNA or control siRNA and 24h post siRNA transfection cell were again transfected with HSP40 luc and Nef expression vector. 48h post transfection, cells were lysed and luciferase assay was performed as described in the text. The gene
silencing efficiency of both the siRNAs are shown as inset of Fig 5A. (B) 293T cells were co-transfected with HSP40 promoter luc construct along with either Nef or HSF1 expression vector alone or Nef and HSF1 together as indicated in the figure. 48h post transfection cells were lysed and luciferase assay was performed as described earlier. The data presented is the mean of three independent experiments and error bars are indicative of SEM.

As HSF1 is known to be the major transcription factor involved in regulation of Heat shock promoters by interacting with HSE enhancer sequences and Nef was also observed to mediate its effects through HSE elements, we then co-transfected HSF1 and Nef along with HSP40 promoter-luciferase reporter construct to analyze the promoter activity. The results indicate that although Nef or HSF1 alone can induce the promoter activity significantly but when expressed together they further induce the activity to a higher level (Fig. 3.5B).

As the inducible effect of Nef on HSP40 promoter seems to be associated with HSF1, we then looked at the recruitment of Nef on HSP40 promoter at HSE element in vivo (Fig.3.6A) by chromatin immunoprecipitation assay. Cross-linked chromatin from HIV-1 NL4-3 infected and uninfected CEM-GFP cells was pulled down with Nef antibody followed by PCR amplification using primers F2 and R1 (Fig. 3.6A) spanning HSE region of HSP40 promoter. Strong PCR signal was observed in infected cells (Fig. 3.6B, lane 3) indicating recruitment of Nef on HSP40 promoter HSE elements. Since HSE is the consensus site for HSF1, we further checked the co-occupancy of Nef and HSF1 on this HSE element by sequential ChIP assay followed by PCR amplification using primers F2 and R1. Again co-occupancy of Nef and HSF1 was observed in infected cells (Fig. 3.6C, lane 3).
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Figure 3.6: Nef and HSF1 are recruited on HSP40 promoter in HIV-1 infected cells in vivo. (A) Schematic representation of HSP40 promoter showing the position of primers used in ChIP analysis. (B) Nef is recruited on HSP40 promoter during HIV-1 infection. CEMGFP cells were infected at 0.1 MOI and on day 5, cells were harvested for ChIP and sequential ChIP analysis. Cells lysates from both uninfected and infected formaldehyde fixed cells were immunoprecipitated with Nef antibody. The chromatin was used to check recruitment on HSP40 promoter by PCR amplification using HSE region spanning primers as described in the text. IgG control antibody was also used for immunoprecipitation with infected cell lysate. (C, D, E) Uninfected and infected CEMGFP cells were used for sequential ChIP analysis by doing primary immunoprecipitation with anti-Nef and secondary
immunoprecipitaion with HSF1 or Sp1 antibody. Chromatin from both uninfected and infected cells was used for PCR amplification with HSP40 promoter specific primers as described in the text.

Under the same condition no detectable amplification was observed in PCR using F1 and R2 primers for region upstream to HSE (Fig. 3.6D, lane 2) whereas strong PCR signal was observed in both, the broader region encompassing HSE (F1 and R1 primers, lane 1) as well as the narrow region containing just HSE elements (F2 and R1 primers, lane 3) indicating specific recruitment of HSF1 and Nef at this site. We have also looked at the co-occupancy of Nef and Sp1 on HSP40 promoter at HSE region (Fig. 3.6E) by sequential ChIP followed by PCR amplification using primers F2 and R1 (Fig. 3.6A). No PCR amplification signal was detected with Nef and Sp1 (Fig. 3.6E, lane 2) whereas under similar conditions we could detect the recruitment of Nef and HSF1 (lane 3). These results further validate our earlier finding that HSF1 is required for Nef induced HSP40 gene expression.

3.3.4 Nef interacts with Human HSF1 both in vitro and in vivo

To test whether the recruitment of Nef and HSF1 on HSP40 promoter requires their physical interaction, we performed co-immunoprecipitation with lysate from 293T cells expressing Flag-HSF1 and HA-Nef. The immunoprecipitation was performed with monoclonal Flag antibody followed by immunoblotting with polyclonal HA antibody. Nef protein co-immunoprecipitated with human HSF1 (Fig. 3.7A, lane 3) in cells expressing both the proteins. Similar co-immunoprecipitation experiments with anti-Nef antibody from the lysates of uninfected or HIV-1 NL4-3 virus-infected CEM-GFP cells also showed interaction of HSF1 (lane 2) with Nef in infected CEM-GFP cell lysate (Fig. 3.7B). Thus both HSF1 and Nef interact in HIV-1 infected cells and might exist
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as a complex on HSP40 promoter for its induction. To further identify the HSF1 interacting domain of Nef, purified His-HSF1 was incubated with Nef (wild type or point mutants) overexpressing 293T cell lysates followed by pull down of complex by immobilization on Ni-NTA beads (Fig. 3.7C). We observed that Nef protein was specifically pulled down by His-HSF1 (lane 1-4) however mutation in proline rich motif of Nef between amino acids 69-78 completely abolished its interaction with HSF1 (lane 5). This result further confirms that Nef and HSF1 interact with each other and proline rich motif of Nef seems to play an important role in this interaction.
Figure 3.7: HIV-1 Nef physically interacts with HSF1, both in vitro and in vivo (A) HSF1 and Nef coimmunoprecipitates from the transfected cell lysate. 293T cells were transfected with the HA-Nef and FLAG-HSF1 expression vector. After 48h, the cells were lysed, immunoprecipitated with FLAG antibody followed by immunoblotting with HA antibody as indicated in the figure. (B) Nef coimmunoprecipitates with HSF1 in HIV-1-infected CEM-GFP cell lysate. CEM-GFP cells were infected with HIV-1 NL4-3 virus. The cells were lysed on day 5 post infection and were immunoprecipitated with Nef antibody followed by
immunoblotting with HSF1 antibody. (C) Poly proline motif of Nef is required for the interaction of Nef with HSF1. 293T cells were transfected with different HA tagged point mutants of Nef. HSF1 pulls down Nef from lysates of all point mutants except the one having mutation in proline rich motif (aa 69-78) as depicted in lane 6.

3.3.5 Nef and HSF1 co-localize in the nucleus of transfected and infected cells

HSF1 has been reported to localize in the cytoplasm before heat shock and translocate into the nucleus after heat shock (Pirkkala et al., 2001; Wu et al., 1995; Hietakangas et al., 2006), whereas Nef has been found to be predominantly a cytoplasmic protein, but also reported to be present in the nucleus of HIV-1 infected and transfected cells (Ovod et al., 1992; Kienzle et al., 1992; Murti et al., 1993). As these proteins were found to interact, it would be interesting to know if these two proteins co-localize in the cell. Therefore, we performed immunofluorescence staining for both HSF1 and Nef in co-transfected 293T cells and also in pNL4-3 transfected 293T cells. As shown in Fig. 3.8A and 3.8B, confocal microscopic images of immunostained cells clearly indicate that both proteins co-localize only in the nucleus of both Nef and HSF1 overexpressing cells (Fig. 3.8A) and pNL4-3 transfected cells (Fig. 3.8B). We then further analyzed the localization of these proteins by looking at HIV-1 infected Jurkat cells. There also we observed co-localization of Nef and HSF1 proteins only in the nucleus of infected cells (Fig. 3.8C). These results further confirm that Nef and HSF1 not only interact with each other but they also co-localize in nucleus during HIV-1 infection.
Figure 3.8: Nef and HSF1 co-localize in Nef and HSF1 expressing and HIV-1 infected cells. (A) Nef and HSF1 co-localize in Nef and HSF1 overexpressing cells. 293T cells were transfected with HA-Nef and HSF1 expression vector. 24h post transfection cells were harvested for indirect immunofluorescence studies using Nef antibody (Chemicon) and HSF1 antibody (Cell Signalling). (B) Nef and HSF1 co-localization in HIV-1 pNL4-3 transfected 293T cells. 293T cells were grown on coverslip and transfected with pNL4-3. 24-36h post transfection, transfected cells were stained with monoclonal Nef antibody and polyclonal HSF1 antibody as described before. The indirect immunofluorescence was visualized by confocal microscopy. (C) Nef and HSF1 colocalization in HIV-1 infected Jurkat cells. Jurkat cells were infected at 0.5 MOI and on day 3 post infection, cells were stained for Nef and HSF1 expression and were analyzed by confocal microscopy.

3.3.6 HSF1 is induced in HIV-1 infected cells

Although modulation of HSPs have been reported during HIV-1 infection, but HSF1 expression levels have not been studied during HIV-1 infection.
Therefore, we assessed HSF1 expression levels in HIV-1 infected Jurkat cells by immunoblotting and immunofluorescence staining for HSF1. The results show that HSF1 was up-regulated in HIV-1 infected Jurkat cells (Fig. 3.9A and Fig. 3.9B).

**Figure 3.9: HSF1 is up-regulated in HIV-1 infected cells.** HSF1 is up-regulated in HIV-1 infected Jurkat cells. (A) Lysates from uninfected and NL4-3 infected Jurkat cells on day 5 post infection were used for immunoblotting with anti HSF1 antibody. (B) HSF1 expression is induced in HIV-1 infected Jurkat cells. Uninfected and Infected Jurkat cells were immunostained for HSF1 and analysed by confocal microscopy (C) HSF1 is upregulated in HIV-1 infected Jurkat cells as analyzed by RT-PCR. RNA was prepared from uninfected and NL4-3 virus infected Jurkat cells.
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on day 5 post infection followed by quantitative real time PCR for HSF1 using gene specific primers, as listed under “Materials and Methods”. GAPDH was used as internal control for normalization. (D) HSF1 expression is also upregulated in HIV-1 NL4-3 infected peripheral blood mononuclear cells. RNA was prepared from uninfected and NL4-3 virus infected PBMCs on day 5 post infection followed by quantitative real time PCR for HSF1 using gene specific primers as described before.

We further confirmed this finding by quantitative real-time PCR with RNA prepared from uninfected and HIV-1 infected (day 5) Jurkat cells and human PBMCs. As shown in Figure 3.9C and 3.9D, HSF1 expression was induced in HIV-1 infected Jurkat and PBMCs. Taken together all these data clearly indicate up-regulation of HSF1 in HIV-1 infected cells.

3.3.7 HSF1 enhances HIV-1 LTR driven gene expression

We have shown above that HSF1 expression is induced during HIV-1 infection (Fig. 3.9). We then wanted to examine whether HSF1 plays a direct role on HIV-1 gene expression. As HSF1 is a well characterized transcription factor regulating not only expression of HSPs but also other promoters, we have analyzed its effect on HIV-1 LTR (Fig. 3.10A) mediated gene expression if any. Interestingly, HSF1 overexpression resulted in significant increase in LTR driven gene expression in presence of Tat alone or Tat and Nef together (Fig. 3.10B).
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Figure 3.10: HSF1 modulates HIV-1 LTR driven gene expression in Nef independent manner. (A) Schematic representation of HIV-1 LTR and the deletion mutants used in the present study. (B) HSF1 enhances HIV-1 LTR driven gene expression. 293T cells were co-transfected with LTR luc and HSF1 expression vector alone or along with Nef and Tat expression vectors followed by luciferase assay as described in the text. (C) HSF1 induces HIV-1 LTR independent of Nef. 293T cells were transfected with wild type or Nef deleted NL4-3 molecular clone and different expression vectors along with the LTR-luc reporter as indicated in the Figure. The cells were lysed and luciferase assay was performed. (D) HSF1 induces HIV-1 LTR promoter activity through its enhancer region. 293T cells were transfected with different LTR luc mutants as shown in Fig.A above along with HSF1 and pNL4-3. 48h post transfection cells were harvested for luciferase assay. The data presented
above are mean of three independent experiments with error bars indicating SEM. Asterisk indicates statistically significant changes (p< 0.05).

Our earlier observation that Nef interacts with HSF1 to augment HSP40 gene expression during HIV-1 infection led us to investigate whether HSF1 mediated induction of LTR driven gene expression is also Nef dependent. We thus, co-transfected HEK-293T cells with HIV-1 NL4-3 wild type or nef-deleted NL4-3 clone along with LTR-luciferase vector and HSF1. As shown in Fig. 3.10C, HSF1 induced LTR driven gene expression with both wild type and nef-deleted NL4-3, indicating thereby that activity of HSF1 on HIV-1 LTR is Nef independent. As HIV-1 LTR is known to have binding sites for a large number of cellular transcription factors (Pereira et al., 2000), we then wanted to identify the region of LTR promoter which could be involved in HSF1 mediated induction of LTR activity. For this we used different deletion mutants of LTR promoter (schematically described in Fig. 3.10A) for co-transfection with HSF1 and pNL4-3 in 293T cells followed by reporter assay, the results of which are shown in Fig. 3.10D. HSF1 overexpression with CD12 luc (wild type) and CD23 luc (-117 luc), which includes NFκB and its downstream elements, showed almost similar induction of LTR activity. Further deletion in the LTR present in CD52 luc (-65 luc) and CD54 luc (-48 luc) resulted in absence of any induction with HSF1. Thus our results indicate that region downstream of -117 encompassing NFκB and Sp1 elements is involved in HSF1 mediated induction of LTR activity whereas, sites upstream of NFκB like AP1 and Myb seem to play no role.

3.3.8 Cloning, expression and purification of human HSF1 protein

Human HSF1 cDNA was PCR amplified from pCMV5-HSF1-Flag vector. The PCR amplification is shown in Fig. 3.11A below.
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Figure 3.11: Cloning, induction and purification of HSF1 in pET28a+ vector
(A) The PCR amplification of human HSF1. (B) Restriction digestion for confirmation of pET- His-HSF1 after the PCR product was cloned into pET28a+ vector at HindIII and XhoI sites. Arrow indicates the position of insert released upon digestion. (C) Induction of His-HSF1 expression. Uninduced and induced lysates were loaded at different volume (4, 6, 8 and 10 µl) to see the induction with 1 mM IPTG. (D) Purification of His-HSF1 protein. His-HSF1 was purified using Ni-NTA beads as described in the text and the purified protein was loaded at different volume (2, 4, 6, and 8 µl) in SDS-PAGE.
The PCR product was then cloned in pET 28a+ vector at HindIII and XhoI sites and cloning was confirmed by restriction digestion (Fig.3.11B). After further confirming the positive clone by DNA sequencing, this vector was used for expressing His-HSF1 protein by transforming into BL21 (DE3) competent host cells. The induction and the purification of this protein are described in materials and methods and the results are represented in Fig. 3.11C and Fig.3.11D respectively. The results clearly show induction of His-HSF1 (Fig.3.11C) on IPTG treatment and purification of His-HSF1 to near homogeneity (Fig.3.11D).

3.3.9 HSF1 induces HIV-1 gene expression by interacting with enhancer region of the LTR

Using deletion mutants of HIV-1 LTR, we have shown above that HSF1 mediated induction of HIV-1 promoter is dependent upon the enhancer region. HSF1 normally functions by interacting with HSE (heat shock elements) present on HSP promoters, which is represented by two or three inverted repeats of the sequence nGAAAn (Wu et al., 1995; Hietakangas et al., 2006) and are present in HSP promoters. In order to identify the HSF1 binding sequence in HIV-1 LTR, we performed bioinformatic analysis of the HIV-1 LTR nucleotide sequence using TFSEARCH software (Yutaka Akiyama, 1995), which revealed a putative HSE element (86% similarity to consensus HSE element) in LTR enhancer region. Based on this analysis, we made 4 oligonucleotide probes P1, P2, P3 and P4 encompassing the enhancer region of LTR as shown in Fig. 3.12A. We then evaluated HSF1 binding to these sequences in vitro by Electrophoretic mobility shift assay (EMSA). Recombinant His-HSF1 protein was used in EMSA with all four probes, mentioned above along with consensus HSE probe as positive control. Interestingly, probes P1, P3 and P4 failed to form any complex whereas P2
formed nucleoprotein complex with His-HSF1 (Figure 3.12B). P2 comprises of nucleotide sequence from position -69 to -91 (GGACCTTTCCCAGGGAGGTGTGGC) which includes first NF-κB and third Sp1 site (from the TATA box) of HIV-1 LTR promoter. To further confirm the specificity of HSF1 binding to P2, we analyzed P2 binding with increasing amount of His-HSF1 protein, a dose dependent increase in complex formation was observed (Fig. 3.12C). Binding specificity of P2 for HSF1 was also confirmed by competition experiments showing loss of binding with the addition of 50, 100 and 150 fold excess of cold P2 (lanes 3, 4 and 5) whereas cold P1 (lane 6) taken as negative control did not show any reduction in binding (Fig. 3.12D). In order to see the binding with cellular HSF1, we then prepared the nuclear extract from HIV-1 infected and uninfected Jurkat cells. Furthermore when uninfected and infected cells nuclear extracts were used, increased binding of P2 was observed with infected nuclear extract as compared to uninfected nuclear extract (Fig.3.12F) which could be explained by increased HSF1 expression during HIV-1 infection (Fig. 3.9). P2 binding specificity for infected nuclear extract was further checked by competition experiment that showed loss of binding with excess of cold oligo but not with non-specific oligonucleotide (Fig. 3.12F). The binding specificity of cellular HSF1 was further confirmed by pre and post incubation of HSF1 antibody in the binding reaction (lane 3 and 4). Loss of P2 binding was observed in pre-incubation with anti HSF1 antibody however, post-incubation with anti HSF1 did not show any effect on binding (Fig.3.12G). This shows that HSF1 antibody specifically blocks the binding between P2 and HSF1. Then we wanted to see HSF1 recruitment in vivo on HIV-1 promoter by ChIP assay. Cross-linked chromatin from HIV-1 infected and uninfected Jurkat cells was pulled down with HSF1 antibody and PCR amplification for recruitment on LTR promoter was performed using forward primers (F1, F2, and F3) and reverse primer (R) spanning
enhancer region as shown in Fig. 3.12A. Strong signal in PCR with primers F1/R and F2/R was obtained, which includes a region downstream to NF-AT1 from -144 to +12 and -93 to +12 (Fig. 3.12H). However, in the same experiment no PCR signal was obtained with F3/R primer set encompassing a region downstream to third Sp1 site from TATA box between -75 to +12 (Fig. 3.12H), suggesting the recruitment between -93 to -75 LTR sequence which encompasses the putative HSF1 binding site identified earlier in EMSA studies. Thus our in vitro and in vivo studies on LTR promoter have shown for the first time, the binding and recruitment of HSF1 on the newly identified HSE element on HIV-1 LTR.
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Figure 3.12: HSF1 interacts with putative HSE element on HIV-1 LTR both in vitro and in vivo (A) Schematic representation of HIV-1 LTR showing the positions of enhancer region for oligonucleotide probes (P1, P2, P3 and P4) were used in
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EMSA and the primers (F1, F2, F3 and R) used in ChIP analysis respectively. (B) His-HSF1 specifically binds to probe, P2. His-HSF1 was incubated with probes P1, P2, P3 and P4 as shown in Fig. 3.12B and their position is described in the text. HSE element consensus was taken as positive control probe. (C) Specificity of binding was studied by incubating P2 with increasing amount of HSF1 protein. (D) His-HSF1 binding was competitively inhibited by cold P2 oligonucleotide. P1 oligonucleotide was taken as nonspecific negative control. (E) Interaction of P2 and increasing concentration of uninfected and infected Jurkat nuclear extract. (F) Competitive inhibition of P2 binding with HIV-1 infected Jurkat nuclear extract using increasing doses of cold oligo. (G) Preincubation with HSF1 antibody blocks HSF1 binding on P2. Specificity of binding was checked by pre-incubating HSF1 with HSF1 antibody. (H) HSF1 binds to putative HSE element on HIV-1 LTR in vivo. HIV-1 infected Jurkat cells on day 5 post infection were used for chromatin immunoprecipitation with monoclonal HSF1 antibody. Chromatin obtained following immunoprecipitation was used for PCR amplification using primer F1, F2, F3 and R as described in the text.

3.3.10 HSF1 is functionally activated during HIV-1 viral infection

Earlier reports have shown induction of HSF1 transactivation activity by its phosphorylation, trimerization and nuclear translocation (Hietakangas et al., 2006; Guettouche et al., 2005; Holmberg et al., 2001). In order to understand the molecular basis of HSF1 regulated HIV-1 gene expression and replication, we have studied its localization, phosphorylation and trimerization during HIV-1 infection.

We have looked at the trimerization of HSF1 by chemical crosslinking using EGS and by non-reducing native-PAGE, followed by immunoblotting with monoclonal HSF1 antibody (Fig. 3.13A). HSF1 was predominantly present as monomer in uninfected cells (left panel lane 1), however, upon infection it...
was predominantly observed as a trimer (left panel lane 2). Similar results were also obtained with non-reducing native PAGE.

Thereafter we have studied the localization of HSF1 during HIV-1 infection. For this, Jurkat cells were infected with HIV-1 NL4-3 at 0.5 MOI and on day 3 post-infection both uninfected and infected cells were used for preparing nuclear extract as well as indirect immunofluorescence studies. Immunostaining of uninfected and HIV-1 infected Jurkat cells with phospho-HSF1 (S230) antibody showed cytoplasmic localization of pHSF1 in uninfected cells however, following infection phospho-HSF1 was predominantly localized in the nucleus. Notably, co-localization of pHSF1 with DAPI was distinctly visible in HIV-1 infected Jurkat cells (Fig. 3.13B). This was further confirmed by performing immunoblotting of nuclear and cytoplasmic fraction of uninfected and HIV-1 infected Jurkat cells using polyclonal pHSF1(S230) antibody. pHSF1 expression was predominantly found in the cytoplasmic fraction of uninfected cells, however, in infected cells it was predominantly present in the nuclear fraction (Fig. 3.13C). Functionally, these data clearly suggest that HSF1 is activated during HIV-1 infection by its trimerization, enhanced phosphorylation and nuclear translocation and thereby regulates viral gene expression and replication.
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Figure 3.13: HSF1 gets functionally activated by trimerization and phosphorylation upon HIV-1 infection. (A) HSF1 trimerization increases during HIV-1 infection. Uninfected and infected CEM-GFP cells lysates were used for
crosslinking by EGS as described in text. Same lysates were also used to study HSF1 trimerization under non-reducing condition (second panel). Trimerization was detected using Rat monoclonal HSF1 clone Ab-4 antibody. (B) Phosphorylated HSF1 is translocated to the nucleus of HIV-1 infected cells. Jurkat cells were infected by NL4-3 virus and on 3rd day post infection, cells were stained using polyclonal pHSF1(Ser230) antibody as described in the text. The indirect immunofluorescence was analyzed by confocal microscopy. (C) Detection of phosphorylated HSF1 in uninfected and infected Jurkat cells by western blotting. Uninfected and Infected Jurkat cells were used to prepare nuclear and cytoplasmic extract and expression of pHSF1 was analyzed by immunoblotting with pHSF1(Ser230) antibody.

3.3.11 HSF1 regulates HIV-1 gene expression and Replication

Our observation of HSF1 induced LTR-mediated gene expression led us to further investigate whether this enhanced LTR-mediated gene expression leads to increased virus production. We then performed a single cycle replication study in 293T cells by co-transfecting pNL4-3 along with HSF1 and virus production was analyzed by p24 antigen capture assay. As shown in Fig. 3.14A, HSF1 overexpression resulted in enhanced virus production as compared to control cells. Similar result was obtained with Jurkat cells, transfected with HSF1 first, followed by infection with NL4-3 virus (Fig. 3.14B) suggesting clearly that HSF1 overexpression leads to increased virus production. This finding was further validated by looking at the effect of HSF1 silencing on virus production. HSF1 was silenced in 293T and Jurkat cells using HSF1 specific siRNA. Silencing was followed by transfection with pNL4-3 in 293T cells and NL4-3 infection in Jurkat cells. Virus production was analyzed with p24 ELISA assay. HSF1 silencing in both 293T and Jurkat cells leads to significant reduction in virus production (Fig 3.14C, 3.14D), clearly indicating the importance of HSF1 in the viral life cycle.
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Figure 3.14: HSF1 overexpression leads to increase in HIV-1 replication whereas silencing of HSF1 leads to inhibition of HIV-1 replication. (A) HSF1 overexpression increases virus production. 293T cells were transfected with pNL4-3 vector along with HSF1 expression vector. 48h post transfection, culture supernatants were collected and virus production was determined by using p24 antigen capture ELISA. HSF1 expression was checked by RT-PCR and shown in inset (B) HSF1 overexpression increases virus production in HIV-1 infected Jurkat cells. Jurkat cells were transfected with HSF1 expression vector or control vector. 24h post transfection, cells were infected with NL4-3 virus as described in the text. 48h post infection, cells were harvested for RT PCR analysis and culture supernatant was used for p24 ELISA. (C) HSF1 downregulation reduces virus production in 293T cells. 293T cells were co-transfected with pNL4-3 and 50 nM, 100 nM, 150 nM HSF1 SMART pool siRNA or control siRNA duplex. 48h post transfection, culture
supernatant was collected for p24 ELISA. Efficiency of gene silencing was checked by RT-PCR and is shown in inset (D) Jurkat cells were transfected with 150 nM HSF1 siRNA or Control siRNA. 24h post transfection cells were infected with HIV-1 NL4-3 virus. 48h post infection, cells were harvested for RT PCR analysis and culture supernatant was used for p24 ELISA. The efficiency of knockdown is shown in the inset.

Taken together, all these results indicate that HSF1 regulates HIV-1 gene expression and replication by two distinct pathways. Firstly, it induces HSP40 promoter activity along with Nef and secondly, it directly interacts with HIV-1 LTR to induce viral gene expression and replication.

### 3.4 DISCUSSION

Expression of several HSP family members is modulated during various disease conditions like cancer (Jatteela et al, 1999) and sepsis (Hashiguchi et al, 2001). Acute infection of cells with a variety of viruses also induces expression of stress proteins (Andrews et al., 1995; Jindal et al., 1994; Santoro et al., 1994). In case of acute HIV-1 infection, induction of HSP27 and HSP70 expression in CD4+ lymphocytic cells have been reported earlier (Wainberg et al., 1997), however, the molecular mechanism of these inductions during microbial infections is still obscure. We have shown earlier that expression of HSP40 increases during HIV-1 infection which leads to increased HIV-1 gene expression and replication in Nef dependent manner (Kumar et al., 2005). Extending our previous observation showing Nef dependent upregulation of HSP40 expression in pNL4-3 transfected cells (Kumar et al., 2005), now we have analyzed this phenomenon in HIV-1 infected T cells to decipher the mechanism of HSP40 up-regulation during HIV-1 infection. Our results clearly show that HSP40 is also up-regulated in a Nef dependent manner in HIV-1 infected T-cells.
Normally the regulation of induced HSP gene expression is dependent on the presence of HSE regulatory elements (Mirault, et al, 1982), an array of sequence element nGAAn or its variations on the promoter (Amin et al.1988; Xiao et al.1988) and heat shock factor (HSF) protein, which specifically bind to these HSE sequence and enhance HSP gene expression in response to stress (Baler et al., 1993; Sarge et al., 1993). In case of HIV-1 infection, there is only one report showing Vpr dependent modulation of HSP27 expression through HSF1 (Liang et al., 2007). We have shown here for the first time that HSF1, the transcription regulator of heat shock proteins, interacts with Nef. HSF1 is known to regulate HSP production by transcriptional regulation (Pirkkala et al., 2001; Trinklein et al., 2004), because of which we have studied the molecular mechanism of HSP40 modulation at the transcriptional level. Our reporter studies with HSP40 promoter clearly indicate that Nef induces HSP40 expression via its promoter. Nef, which was initially shown to be a transcriptional repressor of HIV-1 LTR (Ahmad et al., 1988; Niederman et al., 1989), has been recently shown to act as a positive modulator of HIV-1 LTR by different mechanisms (Witte et al., 2004). We have also previously shown that Nef positively regulates viral replication by interacting with Tat (Joseph et al., 2003). In our effort to understand mechanistic details of HSP40 modulation, we have found that positive effect of Nef on HSP40 expression is mediated by its interaction with HSF1. As HSF1 is known to regulate the HSP gene expression by binding to HSP gene promoter, we also investigated the recruitment of Nef on HSP40 promoter. From our ChIP and sequential ChIP analysis, Nef seems to be recruited at HSE element on HSP40 promoter along with HSF1. This finding was also supported by overexpression and gene silencing studies, where the overexpression of HSF1 enhances this effect; HSF1 silencing results in reduction of HSP40 promoter activity in presence of Nef. Our results imply that the co-occupancy of Nef and HSF1 on HSP40
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promoter activates the promoter to increase HSP40 expression in HIV-1 infected cells which in turn enhances viral gene expression and production as described earlier (Kumar et al., 2005).

HSF1 not only regulates the expression of HSP genes in response to elevated temperature but is also believed to be involved in developmental processes by regulating expression of other genes like inflammatory cytokines (Cahill et al, 1996). It can act both as transcriptional repressor as well as an activator depending on the presence of HSE element to which it binds and cohort of additional factors which it recruits (Xie et al., 2002; Susek et al., 1990). In view of the differential roles that HSF1 plays, for regulating the transcription of HSP or non HSP genes, in second part of our study we have intended to study a direct role of HSF1 in HIV-1 LTR driven gene expression. This study is supported by various previous observations where activation of LTR driven gene expression was reported under hyperthermic condition (Hashimoto et al., 1996; Re et al., 1989; Kretz-Remy et al., 1994, 2001; Steinhart et al., 1996). There was also a contradictory report showing suppression of LTR by a mutant heat shock factor (Ignatenko et al., 2003). However, the modulation of LTR by HSF1 remains unclear at the molecular level. In our single cycle replication studies, we have shown that HSF1 enhances Tat driven viral gene expression. Our reporter studies with mutant LTR luc constructs are indicative of positive regulation of HIV-1 LTR by HSF1 through the enhancer region. Computational analysis of HIV-1 LTR enhancer region has identified a putative HSE element in this region. There are very few studies showing the molecular mechanism of HSF1 regulated transcriptional activation of non HSP genes (Inouye et al, 2007).
In our effort to understand the molecular mechanism of HSF1 mediated positive regulation of HIV-1 LTR, we have clear evidences showing the DNA binding of HSF1 on HIV-1 LTR through sequence close to NF-κB and Sp1 site under both *in vitro* and *in vivo* conditions. Also, the enhanced expression of HSF1 in HIV-1 infected cells seems to further enhance this positive effect of HSF1 on HIV-1 LTR activity.

The transactivation potential of HSF1 is regulated by many factors like trimerization, phosphorylation and nuclear translocation (Wu et al., 1995). Monomeric state of HSF1 represents its inactive form which on exposure to stress, is rapidly converted into active form. The activation event is associated with transition of the monomer to trimer (Sarge et al., 1993). Activation of HSF1 is also marked by its concomitant post-translational modification like phosphorylation and translocation into the nucleus (Sarge et al., 1993). Therefore, to further explore the mechanism of HSF1 driven LTR gene expression, we have tried to correlate its DNA binding activity on LTR with its transcriptionally active state. The functional activation potential of HSF1 during HIV-1 infection was confirmed by studying various activation parameters of HSF1 activity like its trimeric state, localization and phosphorylation status. We observed that following HIV-1 infection, HSF1 shows increased trimerization. Immunofluorescence studies and immunoblotting studies have further shown increased phosphorylation and nuclear re-localization of HSF1 in HIV-1 infected cells, and thereby adding another important line of evidence for HSF1 transcriptional activation during HIV-1 infection. This functional activation seems to facilitate the binding of HSF1 on HIV-1 LTR to enhance viral gene expression.
However, it is still unclear, that how this binding activates LTR. This binding might induce some molecular and physical changes in HIV-1 LTR at the chromatin level that allow binding of some other activator molecules to mediate LTR activation as observed in case of IL-6 gene where the binding of HSF1 on its promoter led to opening of chromatin for binding of activator or repressor molecules (Inouye et al., 2007). Future studies in this area to understand the molecular details of this phenomenon at chromatin level may unravel novel pathways of gene regulation.

The activation of HSF1 mediated stress response in reaction to microbial infection is functionally important for elevated expression of HSPs which generally act as an alert signal for host to elicit anti-microbial immune responses (Di Cesare et al., 1992). We have also attempted here, to understand the functional relevance of HSF1 mediated HIV-1 LTR activation and found that HSF1 gets functionally activated to bind to HIV-1 LTR and positively regulate LTR driven gene expression as the overexpression of HSF1 enhances viral gene expression whereas HSF1 knockdown reduces virus production in both NL4-3 transfected 293T cells and HIV-1 infected Jurkat cells.

As regulation of HIV-1 gene expression involves interplay between viral and cellular host factors, our present study adds another complex layer of regulation in HIV-1 gene expression by HSF1. In summary, we conclude that HSF1 regulates viral replication by two distinct pathways. It interacts with Nef to enhance HSP40 expression in infected cells. HSP40 enhances viral replication by binding to Cdk9 and modulating the activity of P-TEFb (Kumar et al., 2005). Secondly, it also regulates the viral transcription via a DNA protein interaction. Enhanced expression, hyperphosphorylation and increased translocation of HSF1 in to the nucleus contribute to its binding on
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LTR via a putative HSE element identified for the first time in this study. So our present work provides a molecular basis for HSF1 mediated enhancement of viral gene expression and replication through a protein-protein and DNA-Protein interaction and also represents a strategy, by which the virus uses cellular proteins for its successful replication in the host (Fig. 3.15).

**Figure 3.15 HSF1 mediated regulation of HIV-1 gene expression and replication.** HSF1 regulates HIV-1 gene expression by two distinct pathways. First pathway involves a protein-protein interaction between Nef and HSF1 leading to activation of HSP40 promoter. The other pathway involves a DNA-protein interaction between HSF1 and HIV-1 LTR. Both these pathways lead to enhanced viral gene expression and replication.
3.5 REFERENCES


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