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THE MODULATION OF HTLV-1 GENE EXPRESSION FOLLOWING INDUCTION OF THE CELLULAR STRESS RESPONSE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

By

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*****

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ABSTRACT

Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiologic agent of a clinically aggressive form of adult T-cell leukemia/lymphoma and is associated with a variety of lymphoproliferative disorders. HTLV-1 infection is typically associated with long incubation periods between exposure to the virus and disease manifestation. Viral protein expression is postulated to play a critical role in the pathogenesis of HTLV-1-associated diseases, however limited information is known regarding host cell mechanisms that control viral gene expression. Therefore, knowledge of the cellular events which initiate or enhance viral gene expression are important in understanding the mechanism of HTLV-1 induced disease. The first objective was to evaluate the modulation of HTLV-1 gene expression following induction of the cellular stress response in HTLV-1 infected lymphocytes. The cellular stress response was elicited by treatment with either Na arsenite or thermal stress and was monitored by demonstrating increased expression of heat shock protein (hsp) 72, the major inducible member of the hsp 70 family. Induction of the cellular stress response in HTLV-1-infected lymphocytes resulted in significantly increased HTLV-1-mediated syncytia formation. Intracellular viral proteins and released capsid (p24) protein were increased in stressed infected lymphocytes as compared to nonstressed infected lymphocytes. HTLV-1 RNA accumulation by slot blot
northern blot analysis of infected lymphocytes suggested minimal changes in RNA accumulation following stress induction.

Transient transfection assays are commonly used to analyze transcriptional modulation of gene expression. Interpretation of these studies is dependent on knowing the current physiologic state of the transfected cell. We examined four different transfection methods (DEAE-dextran, electroporation, calcium phosphate, and liposome-mediated) for their ability to induce the stress response by measuring the expression of hsp 72 following transfection. We demonstrate that electroporation increases expression of hsp 72 in a suspension cell line, HUT 78. Additionally, DEAE-dextran and liposome-mediated transfection resulted in increased hsp 72 expression in an adherent cell line (Hela). Liposome-mediated transfection differentially induced cell stress, dependent on the transfection time in serum-free culture conditions. We examined the stress responsiveness of two viral promoters, the human T-cell lymphotropic virus type 1 long terminal repeat (HTLV-1-LTR) and human cytomegalovirus immediate early transcriptional unit (CMV-IE). By examining the changes in reporter gene activity of these viral gene constructs, we found the maximal stress-mediated enhancement of transcription with both promoters did not occur until the cells recovered for 24 hr following transfection. In summary, we report that commonly used methods of transient transfection induce the cellular stress response and a recovery period is required following transfection when analyzing cellular stress responsive genes.

We next examined the modulation of transcription of the HTLV-1 long terminal repeat (LTR) following induction of the cellular stress response. We demonstrate with
transient transfection assays, that physiologic stress enhances basal transcription from the HTLV-1 LTR and is independent of Tax. By using deletion mutant constructs of the viral LTR, we demonstrate that the stress responsive region is localized between -52 and +157 of the U3/R region. We also show that whole cell extracts generated from stressed HTLV-1 infected cell lines direct more transcription from the wild type HTLV-1 LTR as assayed by in vitro transcription as compared to extracts from nonstressed cells. We were unable to abolish this increase in transcriptional activity of the stressed extracts with low concentrations of α-amanitin, suggesting the effect is mediated through the overlapping transcriptional promoter of the LTR. This study describes a novel mechanism in which a basic physiologic response to cellular stress enhances HTLV-1 gene expression via a core promoter element.

Although western blot analysis revealed increased intracellular viral env protein (gp68) expression following induction of the stress response, northern blot and slot blot analysis demonstrated minimal differences in env mRNA accumulation in response to cellular stress and the differences did not compare to the magnitude of intracellular env protein expression and syncytia formation. Therefore, suggesting that in HTLV-1 infected cell lines the accumulation of viral env protein in response to cellular stress is a result of post-transcriptional events. We show that the increase in HTLV-1-mediated syncytia formation following stress induction is a result of increase cell surface expression of gp46. Cellular stress in MT 2.6 cells did not alter the turnover of intracellular gp68 as no changes in viral protein half-life were demonstrated as compared to nonstressed cells. However, env expression in stressed cells treated with a protein
synthesis inhibitor (cycloheximide) indicate the effect is mediated through increase protein synthesis.

In conclusion, this work demonstrates how the cellular stress response can act as an important cofactor, allowing virus to escape from latent states of transcription and enhancing cell-to-cell transmission of HTLV-1. The cellular stress response is induced by a variety of biologic stimuli including inflammation, hypoxia, and febrile episodes. The cellular stress response that is potentially associated with opportunistic infections or other concurrent diseases in HTLV-1 infected people may enhance virus expression and promote progression of viral-associated disease.
ACKNOWLEDGMENTS

I wish to express sincere gratitude to my adviser, Michael Lairmore, for his initial faith in my capability by accepting someone with no prior research experience as a graduate student in his laboratory. I also want to thank him for his scientific guidance, encouragement, support, and personal interest in my well being.

Garret Newbound is sincerely thanked for his scientific input, especially critical evaluation of data and whose enthusiasm for research really helped to enhance my interest at an important stage in my career.

I am grateful to my lab mates Nate Collins and Deb Guyot for their moral support and friendship.

I want to thank Mike Oglesbee for his sustained commitment to my project and scientific input. I would also like to thank my committee members, Larry Mathes and Dan Sedmak for their complete cooperation.

I want to thank all personnel of biomedical media, especially Tim Vogt for the production of graphic illustrations of only the highest quality.

I want to thank the office staff, Lisa Wilkosz, Sally Murray, Georgia Porcelli and Elaine Bletz for their help and Kim Sagartz for computer support. Lastly, I want to acknowledge my companion, Darren, who made sacrifices in his own career to allow me
to pursue mine. I could never if succeeded without his constant support and encouragement to finish.

This work was supported by a National Institutes of Health, Physician Scientist Award.
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CHAPTER 1

LITERATURE REVIEW

HTLV-1 AND THE CELLULAR STRESS RESPONSE

History and Epidemiology of HTLV-1

Detection of type C retroviral particles from cultured lymphocytes of human patients with cutaneous T-cell lymphoma (115) and adult T-cell leukemia (90, 58) led to the discovery of the first human retrovirus, human T-cell lymphotropic virus type 1 (HTLV-1). HTLV-1 has subsequently been shown to be the etiologic agent for adult T-cell leukemia/lymphoma (142), an aggressive, fatal T-cell malignancy first described by Tasatsuki et al. (132) and a chronic, degenerate myelopathy referred to as HTLV-1 associated myelopathy or tropical spastic paraparesis (HAM/TSP) (42, 113). More recently, other diseases associated with HTLV-1 include uveitis (91), arthropathy (101, 74), infectious dermatitis (79), and polymyositis (139, 93).

Epidemiological studies have found that HTLV-1 infection is endemic in numerous parts of the world. Areas endemic for HTLV-1 include southwest Japan (59), Central and South America (117, 87, 89), the Caribbean Islands (15), Africa (29, 120) Melanesia (5) and among certain risk groups in the United States (71, 140). HTLV-1 infection is recognized as an important public health problem and currently all blood donated for
transfusion in the United States in screened for HTLV-1 (2). The highest seroprevalence in the United States is among blacks and Hispanics (71) with the infection more common among intravenous drug users.

HTLV-1 is a highly cell-associated virus, therefore the mode of transmission is through infected cells and not cell-free infectious virus (110, 140). Several modes of transmission for HTLV-1 exist and include from mother to child, sexual transmission (97), blood transfusion (110), and sharing of needles between IV drug abusers (71). Breast milk is the major route of HTLV-1 transmission from carrier mothers to children (73, 57). Sexual transmission is primarily from male to female with lower incidence in male to male and female to male exposures (9, 95).

Molecular Biology of HTLV-1

HTLV-1 targets predominantly CD4+ lymphocytes and randomly integrates into the host cellular genome. The HTLV-1 proviral genome which is 9032 nucleotides in length (124) encodes group specific antigen (Gag), protease (Prt), polymerase (Pol), and envelope (Env) gene products. Long terminal repeats (LTR) which contain cis-acting regulatory sequences are located at the 5’ and 3’ ends of the viral genome. A unique region termed the pX region is located at the 3’ end of the viral genome. Four open reading frames (I thru IV) in the pX region encode the proteins Tax (40 kD), Rex (27 kD), Tof (30 kD), and Rof (12 kD) (34).

Tax functions as a potent transactivator of transcription of the integrated provirus (126, 38). Tax localizes to the nucleus but does not bind directly to DNA enhancer or promoter regions. Instead, Tax enhances the binding of cellular transcriptional factors to
activate transcription through distinct regulatory elements termed Tax-responsive elements 1 and 2 (TRE-1 and TRE-2). Tax functions not only as a potent transcription transactivator of the viral LTR but also activates expression of other cellular genes. Some of these include the interleukin-2 (IL-2) receptor alpha subunit (27, 125), IL-2 (60, 125), granulocyte macrophage colony-stimulating factor (100), c-fos (36), c-jun (37) and HIV-1 (16). Tax transactivation of these genes is mediated through NF-κB sites (82, 6) and serum response element sites (37, 130). Tax also represses the expression of the DNA polymerase gene (62). Tax not only is a transcriptional activator of RNA polymerase II dependent cellular genes, but has been shown to transcriptionally activate RNA polymerase III-dependent genes by accelerating the rate of transcription initiation complex assembly (46).

There are three TRE-1 sites located in the 5' U3 region of the HTLV-1 LTR which consist of 21 base pair repeats which are each divided into regions, A, B, and C. The B motif contains core nucleotide sequences (TGACG) with homology to the palindromic cyclic AMP (cAMP) response element (CRE). This serves as a recognition site for members of the cAMP responsive element binding proteins and activating transcription factor (CREB/ATF) family which are leucine zipper (bZIP) transcription factors. Several members of the CREB/ATF family have been reported to bind to the TRE-1 sites (105, 106, 12, 144, 4, 35, 137, 149) which include CREB1 (35, 150, 148), ATF-1 (149), ATF-2 (35) and CREM (130). The exact mechanism in which Tax transcriptionally transactivates through the CREB/ATF proteins is not completely understood. Tax may enhance the DNA binding activity of these proteins (150, 4, 35, 3,
148) by promoting bZIP dimerization (137, 3). A recent study demonstrated that Tax increased both the stability of the bZIP dimer and DNA affinity of the dimer by associating with the basic segment of the bZIP protein (8). Tax also recruits the transcriptional co-activator, CREB binding protein (CRB) and in a manner independent of CREB phosphorylation (78).

The TRE-2 site is located between the two proximal TRE-1 sites and has been shown to be a binding site for SP1 (105), TIF-1 (88), Ets 1 (18, 45), Est 2 (18, 122), Elf-1 (24), NF-κB (104) and Myb (19).

Sequences downstream of the RNA initiation site have also been shown to be important for HTLV-1 basal gene expression. A 45-nucleotide sequence between +195 to +240 was shown to be required for HTLV-1 basal gene expression (67). This region was later shown to bind the transcriptional factor, YB-1 (68). A sequence in the R region (+205 to +240) of the LTR was found to suppress transcription associated with a CREB binding factor (141). In addition, the LTR contains an overlapping transcriptional promoter that is not transcribed by pol II but an intermediate pol II/pol III complex which utilizes the same transcriptional initiation site (114, 30).

The viral transregulatory protein Rex (27 kDa) functions at the posttranscriptional level to regulate viral gene expression. Rex localizes to the nucleolus to positive regulate the expression of the mRNA species that encode the gag/pol (unspliced) and env (singly spliced) gene transcripts and negative regulates the doubly spliced mRNA species of the pX region. (54). Rex mediates this effect by binding directly to an 255 nucleotide RNA stem-loop structure in the 3' end of the viral LTR (R/U3) termed the Rex-responsive
element (REX RE) (49). This stem-loop structure also plays an important role in the polyadenylation of HTLV-1 RNA transcripts (1). Rex has also been shown to stabilize the α-chain mRNA of the IL-2 receptor (65).

Open reading frames I and II of the pX region encode recently described gene products termed p12/tof and p30/rof respectively. The function of these proteins are not completely understood, however a recent report as demonstrated that p12/tof decreases the surface expression the β and γc chain of the IL-2R (94). Therefore, suggesting that p12/tof may be involved in the dysregulation of IL-2 signaling pathways commonly seen in HTLV-1 transformed cells (94).

Pathogenesis of HTLV-1

ATLL is an aggressive, fatal malignancy of mature CD4+ lymphocytes and is characterized by a long incubation period of 20 to 30 years (133). Studies have shown a lifetime risk of an asymptomatic carrier developing ATLL to be 1-4% (131, 76). The clinical course of ATLL is progressive and divided into several clinicopathological patterns which include: preleukemia, crisis, smoldering, chronic, and acute (70, 72). The neoplastic lymphocytes in the peripheral blood are often referred to as "flower cells" because of their characteristic large cleaved nuclei (132). The HTLV-1 provirus in monoclonally integrated in the leukemic T-cells (143) and commonly the IL-2Rα is overexpressed (28).

The mechanism of oncogenesis is unknown, however it is felt that viral protein expression, in particular Tax play an important role in T-cell transformation. Tax may promote transformation by inappropriately deregulating the expression of cellular genes involved in cell growth and differentiation. When the pX coding sequences were
integrated into a transformation-defective herpesvirus, it was able to transform T-cells (48). In combination with the ras oncogene, Tax was able to transform fibroblasts in vitro (116). Studies in transgenic mice expressing the Tax gene have not developed lymphoid tumors but have developed mesenchymal tumors (98) and multicentric neurofibromas (56).

Tropical spastic paraparesis (TSP) was first associated with HTLV-1 infection in patients in Columbia (42). At the same time a similar disease was described in Japan associated with HTLV-1 referred to as HTLV-1 associated myelopathy (HAM) (113). It was later discovered that TSP and HAM were the same diseases, therefore the term HAM/TSP is utilized (118). HAM/TSP is a chronic, slowly progressive neurologic condition. The lifetime risk of developing HAM/TSP is .25% (66). The incubation period between exposure and onset of neurologic disease is more rapid in individuals infected by blood transfusion (47) with a history of previous blood transfusion found in 13%-20% of the reported cases of HAM/TSP in Japan (112). The provirus is polyclonally integrated in lymphocytes with a high viral load determined to be 3-15% in one study (44) or 1 of every 100 to 5000 peripheral blood mononuclear cells (PBMC) (43, 11). Lesions in the spinal cord are characterized by demyelination with inflammatory infiltrates consisting primarily of lymphocytes and macrophages. The lymphocytes are primarily of the CD8+ phenotype (92). Patients also have been shown to have higher levels of cytotoxic T-cells which were CD8+ and HLA class I-restricted and predominantly recognized HTLV-1 Tax (61). he pathogenesis of disease in unclear, however it is hypothesized that it is an immunologic-mediated disease as a result of increased viral gene expression (7).
As eluded to, the pathogenesis of HTLV-1-associated diseases is unknown however virus gene expression is considered essential in initiation of the multi-step process of leukemogenesis or the immune activation of HAM/TSP. Enhancement of viral gene expression from latent states of transcription relies on the intracellular environment, therefore cellular physiologic events which alter gene expression are important in understanding disease pathogenesis. Little is known about host cell physiologic events which modulate HTLV-1 gene expression.

**Cellular Stress Response**

The heat shock response or cellular stress response is a defense mechanism against conditions that are damaging to the cell. This defense response preserves vital cellular components in a protected state until the stress is removed and provides for reactivation of cellular functions as quickly as possible following stress (75). In response to stress the cell produces a characteristic set of proteins termed heat shock proteins (hsp) or cellular stress proteins. These proteins are produced by both prokaryotic and eukaryotic cells and have remained highly conserved in evolution (39, 53, 63). Hsp include constitutive as well as stress-inducible members. Proteins encoded by genes that have one or more heat shock consensus sequences and are induced by stress are defined as heat shock proteins (121). Stress proteins are grouped according to their molecular weight into 6 families: hsp 100, hsp 90, hsp 70, hsp 60, small hsp, and ubiquitin. Cellular stress is induced by a variety of stimuli such as changes in the physiologic state which include, fever, hypertrophy, inflammation, mitogens, viral infection, and oxidant damage (102). Environmental insults
such as heat shock, amino acid analogs, and heavy metals induce the stress response.

Multiple chemical stressors have been recognized which include Na arsenite and ethanol (102).

The hsp 70 family is the most abundant and best-studied family of stress proteins in eukaryotes and is composed of constitutively expressed member and stress-inducible members (hsp 72). The constitutive members are hsp 73, the glucose-regulated protein (grp) 78, binding protein (BiP), and grp 75. Hsp 72 is also produced constitutively in primate cells (75). All hsp 70 proteins bind and hydrolyze ATP (10). The function of hsp 70 family members are diverse and include promotion and/or correction of protein folding and assembly (10, 33) and cellular trafficking of proteins during normal protein synthesis or following cellular stress (22). Proteins which perform these functions are commonly referred to as chaperone proteins. Members of the hsp 70 group have been shown to interact with several different proteins. For example, hsp 72/73 facilitate the uncoating and release of clathrin triskelions from clathrin coated vesicles (135, 21). Hsp 70 have been shown to associate with the steroid hormone receptor and may target the activated receptor to the nucleus (32). BiP, is localized in the lumen of the endoplasmic reticulum and binds to immunoglobulin heavy chains during the course of their assembly with immunoglobulin light chains (17).

There are three members of the hsp 90 family, two forms of 90 designated α and β, and grp 94. Hsp 90 proteins have been shown to stabilize multiple proteins in an inactive state (83). Hsp 90 has been shown to interact with steroid hormone receptors (estrogen, progesterone) and keep these receptors in an inactive state until stimulation by
a ligand to cause dissociation (83). Hsp 90 associates with the tyrosine kinase, pp60src following synthesis and transports the protein to the plasma membrane where is in inserted as an active kinase (111).

Hsp 60 is a mitochondrial protein in eukaryotic cells and functions similar to hsp 70 to promote protein folding and assembly of mitochondrial proteins (50). Hsp 60 has close homology to the Escherichia coli protein GroEL (63).

Cellular Stress Response and Disease

Cellular stress proteins not only have essential roles in normal physiologic reactions of the cell, but have been implicated in a number of disease states. Hsp have been implicated in certain immune responses such as antigen processing/presentation, autoimmune diseases, and tumor rejection. Hsp appear to be dominant antigens during infection with bacteria or parasites (146). This likely occurs because microbes that enter the host express elevated levels of hsp (20, 23) and as a result hsp serve as a target for the immune system. Hsp-derived peptides have been shown to be associated with MHC class I or II molecules for antigen presentation (99). Multiple infectious agents evoke an immune response to hsp, examples include Mycobacterium tuberculosis (123, 145), Treponema (55), and Plasmodium (14).

T-cell crossreactivity to the these highly conserved hsp’s of infectious agents has implicated the stress response in the development of autoimmune diseases. These include insulin-dependent diabetes (31, 25) and rheumatoid arthritis (136, 25). The increase expression of hsp in cells of these inflammatory diseases further support their potential involvement in autoimmune diseases (69).
Increased expression of stress proteins (hsp 70 and hsp 90) have been observed in transformed cells (86, 13). Immunization of mice with hsp 70 or 90 isolated from tumors caused an immunologic response with lack of development of tumors in challenged animals (127, 134).

Although unclear, hsp's may also have a role in antigen presentation. Hsp 70 has been shown to bind a peptide fragment of cytochrome c that is a major antigenic determinant for mouse T cells (80). Two hsp 70 genes are located in the human major histocompatability complex (119). It is suggested that stress proteins may promote peptide transport to the cell surface during antigen presentation and facilitate interaction of antigenic peptides with the T-cell receptor (145).

Recent focus has been placed on the potential role of the cellular stress response in modulating the outcome of virus infection within mammalian hosts. Cellular stress factors may be important in enhanced expression of viral genes and promotion of viral escape from latent states of transcription. In E. coli, the bacterial analogue of hsp 72 enhances the transcription of lambda phage core particles (103). Induction of the cellular stress response by both heat shock and Na arsenite initiated transcription of the major intermediate early transcription unit of human cytomegalovirus (41). Heat shock (40, 128, 77), oxidative stress (81), heavy metals (40), and Na arsenite (40) also has been demonstrated to induce HIV-1 gene expression. This effect is dependent on the NF-κB region of the HIV-LTR (128), however one study was unable to detect the binding of any protein to the NF-κB element following stress induction, indicating that this site is not directly involved (77). Increased light nucleocapsid expression in canine distemper virus
has been demonstrated following stress response induction and is associated with increases in viral cytopathic effects (109). The significance of this observation is that persistent infections can be reactivated following stress response induction through hsp-mediated changes in viral RNA metabolism (108).

There is evidence to support that stress proteins may be involved in the assembly of viral particles. One report found hsp 70 associated with the capsid precursor of poliovirus and coxsackievirus B1 (85). The adenovirus type 5 fiber protein has been shown to associated with hsp 70 (84). Vaccinia virus infection not only induced the stress response but complexing of hsp 70 with viral proteins was observed (64). Hsp 60 was found associated with the HTLV-1 Tax protein by coimmunoprecipitation assays (96). These type of interactions of chaperone proteins with viral proteins may; promote viral assembly and release, stabilize viral proteins, facilitate or target transport of viral proteins through cellular organelles.

The relationship of the cellular stress response and HTLV-1 replication has not been reported. The elucidation of mechanisms that control HTLV-1 replication during the period of clinical latency are important in understanding the pathogenesis of HTLV-I-associated diseases. Hsp play diverse and essential cellular physiological roles. Heat shock proteins can be induced by many different stress conditions which include inflammation, hypoxia, and febrile episodes. The stimuli that may induce or augment expression of HTLV-1 proteins play an important role in the process of cellular transformation or contribute to neurologic disease.
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CHAPTER 2
ENHANCED HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 EXPRESSION FOLLOWING INDUCTION OF THE CELLULAR STRESS RESPONSE

INTRODUCTION

Human T-cell lymphotrophic virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL) (47, 50, 19) and is associated with a chronic, degenerative neurologic disease known as HTLV-1-associated myelopathy or tropical spastic paraparesis (HAM/TSP) (42, 13). In addition, HTLV-1 infection has been associated with certain forms of polymyositis (33), polyarthritis (37), dermatitis (22), and uveitis (29). Because of the long incubation period between exposure and disease manifestation, the pathogenesis of HTLV-1 has not been fully elucidated. However, it is clear that host cell control of HTLV-1 replication is a primary determinant of virus expression and subsequent disease. In HTLV-1 infected, asymptomatic individuals the integrated provirus is maintained in a transcriptionally attenuated state with minimal to no expression of viral RNA and protein. In contrast, a dramatic increase in the number of circulating infected cells is observed in HAM/TSP patients, with one study demonstrating \textit{in vivo} HTLV-1 RNA expression in 1 of every 5000 PBMC's (14, 15). These observations suggest that viral replication may be related to disease development.
There is limited understanding of the cellular mechanisms that regulate HTLV-1 transcription and determine viral protein expression. Transcription of the HTLV-1 proviral genome is highly regulated by the viral proteins, Tax and Rex. Tax forms complexes with cellular factors to enhance transcription of the integrated provirus and activates expression of other host cell genes associated with lymphocyte proliferation, including interleukin 2 (IL-2) (16), IL-2 receptor alpha subunit (20), granulocyte-monocyte colony stimulating factor (28), and the proto-oncogene c-fos (9). Rex promotes cytoplasmic expression of gag/pol and env RNA transcripts, thereby increasing the expression of viral structural proteins (18). Host cell factors that promote transcription of HTLV-1 from latent states of gene expression are likely to be important in understanding the mechanisms of HTLV-1-associated lymphoproliferative diseases.

The cellular stress response is a basic cellular reaction to adverse changes in the cellular environment. Cellular stress can be induced by a variety of stimuli which include increased temperature, Na arsenite, ethanol, mitogens, cytokines, hypoxia, and viral infection (49, 31). The cellular response to stress results in production of a characteristic set of proteins referred to as heat shock proteins (hsp). The functions of hsp are diverse and include promotion or correction of protein folding and assembly (7), as well as mediation or facilitation of cellular trafficking of proteins (1, 5).

The cellular stress response modulates the transcription of a variety of persistent viruses. Heat shock initiates transcription of a cytomegalovirus (CMV) major intermediate early transcription unit following transfection into Rat-9G cells (11). In addition, canine distemper virus (CDV) RNA production increases following induction of the cellular stress
response in persistently infected mink lung cells (41). Heat treatment induces human immunodeficiency virus (HIV) expression in chronically infected promonocytic and T-lymphocytic cell lines, which is most likely mediated through the NF-κB-like enhancer region of the HIV-1 LTR (46, 12). This study was designed to determine the role of the cellular stress response in modulating HTLV-1 expression in persistently infected lymphocytes. We monitored expression of the 72 kd heat shock protein (hsp 72) to demonstrate optimal induction of the cellular stress response in HTLV-1-infected lymphocytes. Induction of the stress response by Na arsenite or thermal treatment resulted in enhanced HTLV-1-mediated syncytia formation mediated by increased HTLV-1 envelope expression. Both cell-associated viral proteins and released soluble capsid protein (p24) were increased in infected cell lines as compared to controls following stress induction. In addition, stressed lymphocytes supported enhanced HTLV-1 LTR promoter activity in transient transfection assays. These data have important implications for understanding how a highly conserved and ubiquitous cellular response to stress can modulate HTLV-1 expression and alter biologic activity.

MATERIALS AND METHODS

Cell lines. MT 2 (27) and HUT 102 (10) are persistently infected HTLV-1 transformed T-lymphocytes derived from patients with ATLL. MT 2, clone 6 (MT 2.6) was derived by limited dilution cloning of the parent strain MT 2. HUT 78 is a virus negative transformed T-lymphocyte cell line (10). All of the above cell lines were maintained at 37°C in complete RPMI 1640 [10% fetal calf serum (FCS), 1.2 mM glutamine, 60 units/ml penicillin, and 60 μg/ml penicillin, and 60 μg/ml streptomycin].
The human osteosarcoma cell line (HOS, American Type Culture Collection) was used as indicator cells in the syncytia formation and inhibition assay and maintained at 37°C in Dulbecco's modified Eagle's media supplemented with 10% FCS, 1.2 mM glutamine, 60 units/ml penicillin, and 60 μg/ml streptomycin.

**Cellular stress response induction.** The cellular stress response was induced in the cell lines using either Na arsenite or thermal treatment. Induction protocols were optimized by exposing each of the cell lines to one of several concentrations of Na arsenite (80μM, 160μM, and 240μM) for 1.5 h or by exposure to 41°C, 43°C, 44°C, or 45°C for 1 h while the cells were in a logarithmic phase of growth. Cells were placed in 15 ml conical centrifuge tubes and heated in a Precision Scientific shaking water bath with a digital readout. Temperatures were further monitored by using a Cole-Parmer thermometer with an accuracy of ±0.2°C. Induction of the stress response was determined by indirect immunofluorescence staining of $1 \times 10^6$ cells for hsp 72, the major inducible member of the hsp 70 family. The primary antibody was a 1:100 dilution of a murine MAb against hsp 72 (C92F3A-5, StressGen, Victoria, BC). The secondary antibody (1:20) was a rabbit anti-mouse IgG phycoerythrin-conjugate (Sigma, St. Louis, MO). Specific staining ($n=10,000$ events) was measured by flow cytofluorometric analysis using an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL). Isotypic phycoerythrin labeled MAb controls (mouse IgG, Sigma) were included in each trial to define background. The immunofluorescent flow cytometric data was analyzed by an immunofluorescence analysis program (Easy II, Coulter Corp.). Optimal stress induction was defined as the maximum expression of hsp 72 with ≤20% decrease in cell viability based on trypan blue dye exclusion.
Radioimmunoprecipitation assay. Radioimmunoprecipitation assay (RIPA) was performed as previously described (17). Briefly, MT 2.6 cells were metabolically labeled with $[^{35}\text{S}]$cysteine and $[^{35}\text{S}]$methionine (Trans$^{35}\text{S}$-label, ICN, Irvine, CA), disrupted with RIPA lysing buffer (0.2 M NaH$_2$PO$_4$, 0.15 M NaCl, 1% phenylmethysulfonyl fluoride, 100 U of aprotinin), and clarified for 45 min at 45,000 rpm (Beckman, SW 55). Supernatants were collected and pre-cleared with protein A-Sepharose beads (CL-4B, Sigma) for 60 min and centrifuged at 16,000 g for 5 min. The lysates were incubated with a MAb against hsp 72 (C92F3A-5) for 18 h at 4°C. Immune complexes were precipitated with protein A-Sepharose and the bound immune complexes were washed with RIPA buffer and eluted by boiling. Samples were electrophoretically resolved in an SDS/10% polyacrylamide gel and visualized by autoradiography.

Syncytia formation and inhibition assay. An HTLV-1-mediated cell fusion assay was performed as described (34). Human osteosarcoma cells (HOS) were used as a positive indicator of HTLV-1-mediated cell fusion. The HOS cells were seeded at $6 \times 10^4$ cells/well in Chamber Slide™ culture chambers (Nunc, Naperville, IL). Twenty-four hours post induction of the stress response with Na arsenite or thermal treatment, stressed and nonstressed HUT 102 cells were seeded at $4.5 \times 10^4$ cells/well with the HOS cells. Stressed and nonstressed HUT 78 cells were cocultivated with HOS cells to serve as a virus negative control. All cocultures were done in quadruplicate. After 24 h the slides were rinsed in phosphate-buffered saline, fixed in methanol, and stained with Wright-Giemsa. The number of syncytia (>4 nuclei) were counted in four 10x objective fields by light microscopy. Inhibition of syncytia formation
was evaluated by adding HTLV-1 human antiserum (Scripps Institute, La Jolla, CA), or 2.75 μg/ml of a human MAb against HTLV-1 envelope gp46 (WA04/2B10/2D3, courtesy of Dr. Steven Foung, Stanford University, Palo Alto, CA), or HTLV-1 negative human serum.

**Antigen capture assay for HTLV-1 p24 capsid protein.** Cell culture supernatants were evaluated for HTLV-1 p24 capsid protein using a commercially available antigen capture assay kit (HTLV-I, II Ag Assay, Coulter Corp). This assay will detect as little as 15 pg/ml of HTLV-1 p24 protein (48). Cell culture supernatants were collected at 24 and 48 h following induction of the stress response by Na arsenite or thermal treatment. Viable cell counts were determined by trypan blue dye exclusion at the time of collection and the amount of HTLV-1 p24 calculated on a per cell basis. Results were compared with nonstressed cells.

**Western blot analysis.** Western blot analysis was performed to determine the expression of cell-associated viral proteins as described (23). Briefly, cell lysates from 1 x 10^7 HUT 102, MT 2.6, and HUT 78 cells were prepared at 24 and 48 h post cellular stress induction. Protein concentrations within the lysates were determined by the Micro BCA Protein Assay (Pierce, Rockford, IL). Twenty μg of lysate were resolved electrophoretically through a SDS/12% polyacrylamide gel. The resolved proteins were transferred to nitrocellulose and the membranes blocked with a 5% non-fat dry milk solution for 4h. The membranes were incubated for 15-18 h with HTLV-1 human antiserum (Scripps), or previously optimized concentrations of MAbs directed against HTLV-1 gp46 (clone IC11) or hsp 72 (StressGen). Bound primary antibody was visualized by an avitin-biotin-horseradish peroxidase procedure (Vectastain ABC,
Vector Laboratories, Burlingame, CA) Densitometric analysis of specific signal was performed with a laser densitometer (Molecular Dynamics ImageQuant™). Results were compared with nonstressed cells.

**Transfections.** HUT 78 cells were transfected by electroporation with an HTLV-1 LTR-CAT plasmid (pU3R-I-CAT, 44) or an HTLV-I LTR-luciferase reporter plasmid (LTR-luciferase). The HTLV-1 LTR-luciferase plasmid which contains the LTR cloned upstream of a luciferase gene has been previously described (36). Cotransfections were performed in HUT 78 cells with the LTR-CAT or luciferase construct and an HTLV-1 LTR-Tax plasmid (pHTLV I-Tax, 35). Electroporated but nontransfected (mock) cells and cells transfected only with reporter constructs were included in each trial as controls. The cells were washed and resuspended at 2 x 10⁷ cells/ml in complete RPMI. Fifteen μg of the LTR-luciferase or 7.5 μg of the pU3R-I-CAT singly or with 2 μg of the LTR-Tax plasmid was added to a 250 μl aliquot of cells and placed in an electroporation cuvette. To control for transfection efficiency, the aliquots were combined and cultured in complete RPMI for 24 h after electroporation. Cells were then separated into two groups and one group was subjected to cellular stress by 80 μM of Na arsenite. Cell lysates were harvested 24 h following induction of the cellular stress response by a freeze-thaw technique in 0.5M Tris-HCl buffer (pH=8.0). The cellular extracts were assayed for CAT activity by phase extraction and liquid scintillation counting (CAT Enzyme Assay, Promega, Madison, WI) or luciferase activity by liquid scintillation counting (Luciferase Assay System, Promega).
Northern blot analysis. Total RNA was extracted from MT 2.6 and HUT 102 cells by a guanidinium isothiocyanate, phenol-chloroform method (2) at 6, 12, and 24 h after induction of the cellular stress response. The RNA (20 μg) was electrophoretically fractionated in a 1.2% agarose/formaldehyde gel and transferred to a nylon membrane (Nytran, Scheicher and Schuell, Keene, NH) by capillary transfer. The membrane was prehybridized at 42°C for 4-6 h in prehybridization solution (25 mM KPH₄, 5X SSC, 5X Denhardt's solution, 50 μg/ml salmon sperm DNA, 50% formamide) and hybridized with a viral gene specific radiolabeled probe in the same solution with 10% dextran sulfate for 18 h at 42°C. Viral gene specific probes were generated by restriction enzyme digestion (XhoI/EcoR1) of an HTLV-1 LTR-CAT plasmid (pU3R-I-CAT). The cDNA probes were labeled with 32P-CTP by random base pair priming (DECAprime™, Ambion, Austin, TX). The membranes were washed with 2X SSC for 30 min at 42°C followed by .1X SSC for 30 min at 42°C and specific signals were visualized by autoradiography.

Slot Blot Analysis. Total RNA was extracted as above from 2 x 10⁴ cells at 6, 12, and 24 h post induction of the cellular stress response and serially diluted five-fold for slot blot analysis. The RNA was loaded onto a nitrocellulose membrane using a slot blot manifold as described (44). As above, the filters were prehybridized at 42°C for 4-6 h, hybridized for 18 h at 42°C, washed under stringent conditions, and analyzed by autoradiography. Autoradiographic signal intensities were quantitated by laser densitometry. Results were compared with nonstressed cells.
RESULTS

Induction of the cellular stress response in HTLV-1 infected cell lines by thermal and Na arsenite treatments. To determine successful induction of the cellular stress response, the expression of hsp 72, the major inducible member of the hsp 70 family and a sensitive indicator of the stress response, was evaluated by flow cytometric analysis 24 h following stress. Sodium arsenite treatment induced expression of hsp 72 in a dose dependent fashion in MT 2.6 (Fig. 2.1) and HUT 102 cells (data not shown) when exposed to 80 μM, 160 μM, and 240 μM concentrations. Eighty μM of Na arsenite consistently induced hsp 72 expression with minimal cell death (<20%) in both cell lines. The degree of cell death was greater with 160 μM and 240 μM, varying from a 30 to 80% decrease in cell viability as compared to nonstressed controls. Similarly, thermal treatment of MT-2.6 and HUT 102 cells with 41°C, 43°C, 44°C, and 45°C induced hsp 72 expression (11.2-46.6% positive). However, 44°C and 45°C resulted in ≥20% cell death by 24 h. Due to minimal effects on cell viability, 80 μM of Na arsenite and 43°C were selected as treatment protocols to further evaluate HTLV-1 replication following induction of the cellular stress response. The induction of hsp 72 (following Na arsenite) in MT 2.6 cells was further demonstrated by both western blot analysis (Fig 2.2A) and radioimmunoprecipitation analysis of cell lysates post cellular stress (Fig. 2.2B).

Enhancement of HTLV-1-mediated syncytia formation following induction of the cellular stress response. A syncytia formation assay was performed to determine if the cellular stress response altered HTLV-1-mediated cytopathic effects. Twenty-four hours after
induction of the cellular stress response with Na arsenite or thermal treatment, HUT 102 cells were cocultivated with HOS syncytia indicator cells for an additional 24 h. HOS cells incubated with stressed HUT 102 cells contained approximately a mean two-fold increase in syncytia (Fig. 2.3 and 2.4) when compared to nonstressed controls. A four-fold increase in the number of syncytia containing >20 nuclei was observed in the stressed cells as compared to nonstressed controls (data not shown). We did not observe the formation of syncytia when stressed HUT 78 cells were cocultured with HOS cells. To evaluate whether HTLV-1 envelope proteins mediated syncytia formation, the assay was repeated in the presence of HTLV-1 human antiserum or human MAb directed against HTLV-1 envelope (gp46). HTLV-1 mediated syncytia formation was inhibited by greater than 90% by each antibody (Fig 2.4). No inhibition of syncytia formation was observed in the presence of serum negative for HTLV-1 antibodies (data not shown).

Released viral p24 capsid protein and intracellular viral proteins were increased in HTLV-1 infected cell cultures following cellular stress. To determine the effects of the cellular stress response on viral protein expression both released viral p24 capsid protein and intracellular viral protein expression was determined after induction of the stress response. HTLV-1 p24 capsid protein was significantly (p<0.05) increased (1.8-3.5 mean fold increase) in cell culture supernatants of HUT 102 and MT 2.6 cells at 24 and 48 h following stress induction by either Na arsenite or thermal treatment (Fig. 2.5) when compared to nonstressed controls. In addition, western blot analysis of HUT 102 and MT 2.6 cell lysates at 24 and 48 h after induction of the cellular stress response indicated increased (1.7-3.6 fold) cell-associated
viral proteins (p19, p24, p28, gp68, gp61) when compared to non-stressed HTLV-1-infected cell lysates (Fig. 2.6).

**Enhanced LTR activity is observed in stressed Hut 78 cells transfected with LTR reporter- and Tax- expressing plasmids.** Transient transfections in HUT 78 cells were performed to evaluate HTLV-1 LTR promoter activity following cellular stress induction. HUT 78 cells were transfected with an LTR CAT or luciferase reporter plasmid, physiologic stress induced 24 h later, and reporter gene activity measured after an additional 24 h. In stressed HUT 78 cells singly transfected with the LTR-luciferase or CAT reporter construct an increase of 1.9 to 3.6 fold respectively of reporter gene activity was observed as compared to nonstressed controls (Fig. 2.7). Stressed HUT 78 cells cotransfected with the LTR-luciferase or CAT reporter construct and an LTR-Tax expressing plasmid, demonstrated a 3.0 to 6.6 fold increase in reporter gene activity respectively, over nonstressed controls (Fig. 2.7)

**Modulation of HTLV-1 RNA expression following the cellular stress response.** To evaluate changes in RNA accumulation following induction of the cellular stress response, slot blot analysis was performed on RNA isolated from equal numbers of MT 2.6 and HUT 102 cells. The degree of HTLV-1 RNA accumulation varied in different trials with a range of 0-2 fold increase in autoradiographic intensity when compared to nonstressed controls at 6, 12 and 24 h post induction of the stress response (Fig. 2.8). Northern blot analysis of HTLV-1 RNA was performed at 6, 12, and 24 h following induction of the cellular stress response. The membranes were probed with a full length LTR probe which recognizes all species of HTLV-1
RNA. Northern blot analysis demonstrated no alterations in the pattern of expression of HTLV-1 RNA species in either MT 2.6 or HUT 102 following stress induction (Fig. 2.9). The LTR radiolabeled probe did not bind RNA isolated from the viral negative control HUT 78.

DISCUSSION

In this study, we have demonstrated that a highly conserved cellular response to stress alters HTLV-1-mediated cytopathic effects and increases expression of viral proteins from HTLV-1 infected cell lines. Specifically, our data demonstrates a significant increase in HTLV-1-mediated syncytia formation in stressed HUT 102 cells following induction of the cellular stress response with either Na arsenite or thermal treatment. Inhibition of syncytia by anti-HTLV-1 antibody demonstrated that both basal and stress-induced syncytia formation is mediated by enhanced HTLV-1 envelope glycoprotein (gp46) expression. Induction of the cellular stress response with either Na arsenite or thermal treatment resulted in a significant increase in released p24 capsid protein and intracellular HTLV-I protein expression as compared to nonstressed controls. Taken together, these data indicate that enhancement of viral protein expression following cellular stress mediates HTLV-1-cell fusion. Using two mechanistically distinct modes of stress response induction, supports that the observed viral phenotypic changes are due to induction of the cellular stress response and not merely related to pleiotropic effects such as heat-associated increased membrane fluidity. These findings parallel observations of Oglesbee et al. (41) who reported that hyperthermic or Na arsenite shock resulted in an increase of total viral protein expression and cell-free infectious progeny.
release in a stable persistent infection model of CDV. Stress response induction was associated with an increase in viral fusion protein (F) which correlated to a dramatic induction of viral induced syncytia. Our data has important implications for cell-to-cell transmission of HTLV-1. This highly cell-associated virus is primarily transmitted between virally infected cells through membrane fusion. In vitro, inflammatory cytokines (8, 30) and oxygen free radicals (6) have been shown to promote the cellular stress response. Therefore, disease states potentially associated with hsp induction, for example febrile episodes associated with opportunistic infections, may enhance HTLV-1 distribution within an infected person. The potential for increased virus expression during other disease states may influence the degree of viral-mediated lymphoproliferative disease in HTLV-1 infected people. In this regard, several recent reports have shown a progression from a healthy carrier state to ATLL in individuals with a history of opportunistic infections (3, 43).

Increased HTLV-1 protein expression following induction of the cellular stress response may, in part, be mediated by enhanced transcriptional activation of the LTR promoter. Transfection of HUT 78 cells with an HTLV-1 LTR reporter plasmid resulted in a 1.9 to 3.6 fold increase in basal transcription 24 h after induction of the cellular stress response with Na arsenite. To determine if cellular stress could modulate Tax activity to further enhance transcriptional activity, cotransfections with the LTR reporter plasmid and a Tax expressing plasmid were performed. There was enhancement of transcriptional activity in the presence of Tax of 1.5 to 1.8 fold over basal transcription, however the degree is less than expected with a Tax mediated transcription event. Potential mechanisms to explain the increased HTLV-1 promoter activity following cellular stress induction includes stabilization of host cell
transcriptional factors known to bind the LTR. Regulation of HTLV-1 expression is mediated through the viral LTR which contains promoter and enhancer regions [(e.g. Tax responsive regions 1 and 2 (TRE-1 and TRE-2)]. Tax does not bind to these regions directly but cooperates with cellular transcriptional factors to upregulate transcription. A number of cellular transcriptional factors have been described that bind to both TRE-1 and TRE-2, including c-myb (4) and an NF-κB-like factor (38). Interestingly, an increase in the half-life of c-myb has been documented after heat treatment in a lymphoma cell line (24). In addition, NF-κB has been incriminated in enhancement of HIV-1 gene expression by demonstrating reduced heat stress responses in reporter gene constructs with mutated NF-κB sites (46). The cellular stress response has been shown to modulate transcription of persistent infections of other viral families. In a stable persistent infection model of CDV, an increase in viral RNA production was demonstrated following Na arsenite and thermal stress, both in cell free transcription assays and within infected cells (41). Furthermore, Geelen et al. (11) demonstrated transcriptional activation of a major immediate early transcription unit of the human cytomegalovirus in a stable transfection of Rat-9G cells following heat or Na arsenite treatment.

There was a difference between the magnitude of stress-induced change in HTLV-I infected transformed cell lines in that the amount of protein expressed was generally 2-3 fold greater than the increase in viral transcripts suggesting a posttranscriptional event. However, transfection of noninfected cell lines suggested transcriptional upregulation from the viral LTR following induction of the cellular stress response. There are a number of reasons that may account for this potential discrepancy. First, the HTLV-1 transformed
cells lines are high producers of viral transcripts. Therefore, demonstrating further transcriptional activation of these genes may be difficult. The HTLV-1 transcripts may contain sequences, not present in reporter gene transcripts, that influence the levels of translation. Finally, the use of two unique systems of evaluating viral expression, transient transfections of reporter constructs versus HTLV-1 infected transformed cell lines precludes direct correlation of these data. More studies are required will further delineate HTLV-1 transcriptional activation and viral protein stabilization following induction of the cellular stress response.

Heat shock proteins and viral proteins may interact to promote viral assembly and release, stabilize viral proteins, and facilitate or target transport of viral proteins through cellular organelles. Other in vitro viral model systems have shown an association of hsp 70 with viral proteins. Oglesbee et al. (39, 40) demonstrated that hsp 70, induced by in vivo CDV infection, was extensively colocalized with the CDV major core protein. Infection of human macrophages by vaccinia virus caused increased hsp 70 production, with a large fraction of the hsp 70 associated with viral proteins, suggesting their involvement in viral protein assembly (21). In addition, hsp 70 coimmunoprecipitates with capsid precursors of poliovirus and coxsackievirus B1 (25), and with a fiber protein of adenovirus (26). Interestingly, hsp 60 was recently demonstrated to be associated with the HTLV-1 Tax protein by coimmunoprecipitation assays (33).

Although the mechanism by which HTLV-1 promotes lymphoproliferative disorders is unknown, expression of viral proteins, in particular Tax, is an important factor in the proposed multi-step process of HTLV-1 disease states. Our data suggest that the
cellular stress response can act as an important cofactor, allowing virus to escape from latent states of transcription and enhancing cell-to-cell transmission of HTLV-1. The cellular stress response is induced by a variety of biologic stimuli including inflammation, hypoxia, and febrile episodes. The cellular stress response that is potentially associated with opportunistic infections or other concurrent diseases in HTLV-1 infected people may enhance virus expression and promote progression of viral-associated disease. This study provides an important model system of a basic cellular response that modulates HTLV-1 replication, providing the opportunity to enhance knowledge regarding the relationship between virus expression and lymphocyte proliferation.

REFERENCES


Figure 2.1: Dose dependent increase in hsp 72 expression in MT 2.6 cells following Na arsenite treatment. Specific staining for hsp 72 was measured by flow cytometry.
Figure 2.2: Western blot analysis and RIPA of hsp 72 expression in MT 2.6 cells following induction of the cellular stress response. (A) Western blot analysis of stressed (S) and nonstressed (NS) cell lysates from MT 2.6 cells at 24 h following stress induction. (B) RIPA of stressed (S) and nonstressed (NS) cells lysates from MT 2.6 cells at 6 hr following stress induction.
Figure 2.3: Syncytia formation of HOS cells following cocultivation with nonstressed and stressed HUT 102 cells. The number of syncytia were counted by light microscopy in both groups. NS=nonstressed; S=stressed
Figure 2.4: The effect of induction of the cellular stress response on HTLV-1-mediated syncytia formation of HOS cells. Stressed HUT 102 cells demonstrate a significant increase in the number of syncytia when compared to nonstressed controls. There is >90% inhibition of syncytia formation in the presence of HTLV-1 polyclonal sera and a Mab against viral envelope in both the stressed and nonstressed cocultivated cells.

(*mean±SEM of 4 trials. P<.05)
Figure 2.5: Quantitation of HTLV-1 p24 capsid protein in cell culture supernatants following induction of the cellular stress response in HUT 102 and MT 2.6 cells. The cellular stress response was induced by both Na arsenite and thermal treatment. Supernatants were collected and the quantity of p24 determined. A significant increase in p24 expression was also observed at 24 h. (mean±SD of 5 trials. P<.05)
Figure 2.6: Western blot analysis of HTLV-1 intracellular viral proteins 24 and 48 h following induction of the cellular stress response. Nonstressed (NS) and stressed (S) cell lysates from MT 2.6 and HUT 102 cells probed with HTLV-1 polyclonal sera.
Figure 2.7: Evaluation of stress mediated modulation of HTLV-1 promoter activity following induction of the cellular stress response in HUT 78 cells. Increased reporter gene activity (CAT and luciferase) was observed in single and cotransfected HUT 78 cells with a LTR-reporter construct and LTR-Tax expressing plasmid.
Figure 2.8: Slot blot analysis of HTLV-1 RNA isolated from MT 2.6 cells at 6 hr post induction of the cellular stress response. Total RNA was serially diluted (1-3) from nonstressed (NS) and stressed (S) MT 2.6 cells. There is a two fold increase in autoradiographic intensity from stressed cells when compared to nonstressed controls as determined by laser densitometry.

Figure 2.9: Northern blot analysis of HTLV-1 RNA isolated from MT 2.6 cells at 12 and 24 h following induction of the cellular stress response. The radiolabeled cDNA probe recognizes all species of HTLV-1 transcripts. There are no changes in the pattern of HTLV-1 RNA species expression. The same results were observed 6 hr post stress. This data represents at least three independent trials. US=unsliced, SS=singly sliced, DS=doubly spliced.
CHAPTER 3
TRANSCRIPTIONAL MODULATION OF VIRAL REPORTER GENE
CONSTRUCTS FOLLOWING INDUCTION OF THE CELLULAR STRESS
RESPONSE

INTRODUCTION

A variety of physiologic events modulate gene transcription in eukaryotic cells including cellular differentiation and response to cell activation signals (18). These events ultimately enhance or suppress transcription by activating factors which subsequently bind to response elements of the gene. Transient transfection assays are an important tool for investigating transcription, however changes in the physiologic state of the cell may influence or mask transcription of a particular gene. Several methods of transient transfection assays are available. Knowledge of the physiologic state of cells following the application of these techniques facilitates accurate interpretation of data from such studies.

In this study, we addressed the modulation of viral promoter expression following induction of the cellular stress response, a physiologic response to adverse changes in the cellular environment. Cells can initiate a stress response when exposed to a variety of metabolic and chemical insults, including hyperthermia, oxidizing agents, transition series metals, drugs effecting membrane structures (e.g. ethanol), serum stimulation, mitogens,
and lymphokines (13). The cellular stress response enhances the transcription of a variety of viruses, which include human immunodeficiency virus (HIV-1) (7, 16, 8), canine distemper virus (14), and cytomegalovirus (6). We have previously shown stress-mediated enhancement of human T-cell lymphotrophic virus type 1 (HTLV-1) transcription following cellular stress (1, 2). Studies to demonstrate modulation of viral gene transcription following cellular stress often utilize transient transfection assays. However, it is unclear if commonly used methods of transfection can induce the cellular stress response, thus affecting the results of transcription assays. To address this problem, we evaluated four methods of transient transfections for their ability to induce the cellular stress response during the delivery of viral reporter gene constructs. Calcium phosphate, liposome-mediated, and DEAE-dextran methods were examined using an adherent epithelial cell line (Hela). Electroporation was tested using a suspension cell line (HUT 78). Three of the four transfection methods (electroporation, DEAE-dextran, and liposome-mediated) induced the stress response as demonstrated by increased expression of the inducible hsp 72. Additionally, we demonstrate that liposome-mediated transfection differentially induces cell stress, dependent upon the amount of time cells were in serum-free medium before serum stimulation.

We determined if cells must undergo a recovery period following transfections, by analyzing the stress-mediated modulation of transcription of two viral promoters [the HTLV-1 long terminal repeat (HTLV-1-LTR) and human cytomegalovirus immediate early transcriptional unit (CMV-IE)]. Optimal stressed-mediated enhancement of transcription with each promoter did not occur until cells recovered for 24 hr following
transfection. Our data indicates that commonly used methods of transient transfections induce cell stress and that a recovery period is required following transfection to analyze stress responsive genes.

MATERIALS AND METHODS

Cell lines and stress response induction. Hela cells obtained from the American Type Culture Collection were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% FCS, 1.2 mM glutamine, 60 units/ml penicillin, and 60 µg/ml streptomycin (cMEM). HUT 78 cells (5) were maintained in RPMI 1640 supplemented as MEM above.

The cellular stress response was induced with Na arsenite or thermal treatment as previously described (2). Briefly, the cells were exposed to varied concentrations of Na arsenite (80 µM to 640 µM) for 1.5 hr and different temperatures (42, 43, 44, 45°C) for 1 hr. The optimal induction was defined as the maximum expression of the highly inducible hsp 72 protein with less than a 20% loss in cell viability as determined by trypan blue dye exclusion. Twenty-four hr following stress induction the expression of hsp 72 was determined by indirect immunofluorescence staining. The primary antibody was a 1:100 dilution of a murine MAb against hsp 72 (C92F3A-5, StressGen, Victoria, BC). The secondary antibody (1:20) was a rabbit anti-mouse IgG FITC-conjugate (Sigma, St. Louis, MO). Specific staining was measured by flow cytofluorometric analysis using a Coulter Elite cytometer. Isotypic FITC labeled MAb controls were included in each trial to define...
background. The immunofluorescent flow cytometric data was analyzed by an Immuno-4 analysis program (Coulter). Data was analyzed from three independent trials.

**Plasmid constructs.** To initially establish transfection efficiency with each method a luciferase reporter construct under control of the SV40 promoter was utilized (pGL2-Control, Promega, Madison WI). The HTLV-1-LTR luciferase plasmid which contains the LTR derived from pU3R-I (15) cloned upstream of a luciferase gene has been previously described (12). The CMV-IE luciferase plasmid contains the immediate early gene cloned into the XhoI/SalI site of a luciferase reporter plasmid (pGL2-Basic).

**Transient-transfection assays.** Hela cells (5 x 10⁵) were seeded into 60 mm culture dishes 24 hr before transfections to obtain a cell confluency of 50-70%. Twenty-four hr following transfection, expression of hsp72 by immunofluorescence staining was determined and compared to non-transfected controls.

Cells were transfected by liposome-mediated transfer using a commercially available product (Transfectam®, Promega, Madison WI). Transfectam and DNA concentrations were titered to establish the optimal ratio for maximal transfection efficiency. A lipid:DNA ratio of 3:1 was utilized in all promoter experiments. Initially, Hela cells were transfected for 3, 6, 12, or 24 hr following the recommended assay protocol for media (MEM) without serum by the manufacturer. Following transfection, media was removed and replaced with cMEM. Cells were transfected by calcium phosphate mediated transfer (5 Prime→3 Prime, Inc., Boulder CO) or DEAE-dextran...
mediated transfer (Promega, Madison WI) using the recommended assay protocol by the manufacturer. HUT 78 cells were transfected by electroporation as previously described (2). Transfections were harvested at the indicated times following cellular stress using Cell Culture Lysis 1X Reagent (Promega). Luciferase activity was measured by liquid scintillation counting following manufacturers recommendations (Promega).

RESULTS

Induction of the cellular stress response in Hela cells by thermal and Na arsenite treatment. To determine successful induction of the cellular stress response, the expression of hsp 72, the major inducible member of the hsp 70 family and a sensitive indicator of the stress response, was evaluated by flow cytometric analysis. Increased expression of hsp 72 was observed when Hela cells were exposed to 160 to 320 μM of Na arsenite. The degree of expression (as compared to non-stressed cells) varied between experiments, however consistently the 320 μM concentration resulted in a marked expression (mean 71%) with <20% loss in cell viability (Fig. 3.1). Six hundred and forty μM of Na arsenite resulted in >80% loss in cell viability. Similarly, thermal treatment induced hsp 72 expression (means ranging from 18-52.5%, Fig. 3.1), however 45°C resulted in a >20% loss of cell viability as compared to 42, 43, and 44°C. Therefore, a concentration of 320 μM of Na arsenite or 44°C was used in all studies. The optimal concentration of Na arsenite and thermal induction was previously reported for the suspension cell line, HUT 78 (2).
Transient transfection methods differentially induce hsp 72. To determine if commonly used transfection methods induced the stress response, the expression of hsp 72 was evaluated by flow cytometry 24 hr following transfection. We determined hsp 72 expression following electroporation in HUT 78 cells because we had previously optimized electroporation with this cell line (2), and the relative ease of performing electroporation with a suspension culture compared to an adherent cell line. Transfection of HUT 78 cells by electroporation resulted in increased expression (41%) of hsp 72 (as compared to non-transfected, non-stressed controls) (Fig. 3.2A). In Hela cells, the serum-free conditions of liposome-mediated transfection resulted in an increased expression of hsp 72 which correlated with the amount of time the cells were cultured in serum-free medium (Fig. 3.2B). Serum-free conditions for 3 hr did not result in hsp 72 expression as determined by flow cytometry. Cells left in contact with serum-free medium for 6 and 24 hr had a mean expression (16% and 37%) of hsp 72 respectively (Fig. 3.2B). Calcium phosphate mediated transfections did not elicit increased expression of hsp 72 in Hela cells (Fig. 3.2A). DEAE-dextran mediated transfection resulted in a mean expression of 16% of hsp 72 in transfected cells as compared to non-transfected controls (Fig. 3.2A).

Transfection efficiency was the greatest with liposome-mediated DNA transfer. A luciferase reporter plasmid under control of the SV40 promoter was utilized to determine transfection efficiency among transfection methods. We first determined the optimal concentration of DNA at a fixed cell density in each method (data not shown). Because 3 hr liposome-mediated transfection did not induce the stress response, this transfection time
was used to compare with the other methods. We found that the liposome-mediated procedure resulted in greater reporter gene activity as compared to calcium phosphate and DEAE-dextran (32 fold and 230 fold higher respectively) (Fig. 3.3). Because of the marked induction of hsp 72 following electroporation, this technique was excluded from promoter analysis studies.

Maximal promoter activity was detected after the cells were allow to recover for 24 hr post-transfection. To test the influence of an optimized transfection method on promoters known to be responsive to cellular stress, we tested both the HTLV-1-LTR and CMV-IE reporter genes constructs. Three hour liposome-mediated transfection was found to have the maximum efficiency as compared to DEAE-dextran and calcium phosphate methods in Hela cells and did not elicit a stress response. While liposome-mediated transfection did not elicit a measurable cellular stress response, the method may induce tolerance to further induction of the stress response. Therefore, we determined the optimal recovery period for evaluation of the stress-mediated enhancement of our viral promoter constructs. The stress response was induced at 6 or 24 hr following transfection with both the HTLV-1-LTR construct and the CMV-IE construct. Luciferase activity was determined in cell extracts collected at 3, 6, 12, and 24 hr post stress and compared to non-stressed extracts. Both promoter constructs were nonresponsive to cell stress when only allowed a 6 hr recovery prior to induction of the cellular stress response by Na arsenite (Fig. 3.4A and 3.5A). However, when the cells remained in normal culture
conditions for 24 hr before induction of stress, an increase in promoter activity was observed at 3, 6, 12, and 24 hr following transfection (Fig. 3.4B and 3.5B).

DISCUSSION

To determine if the cellular stress response was induced following transient transfections, four transfection techniques were evaluated. These included liposome-mediated, DEAE-dextran, electroporation, and calcium phosphate transfections. We found that DEAE-dextran, electroporation, and liposome-mediated transfections increased the expression of hsp 72, which is a highly inducible member of the hsp 70 family and a sensitive indicator of stress response induction (19). While many chemical or physical agents are known to induce the stress response, the mechanisms in which they mediate the response with subsequent enhancement of hsp gene expression is complex and not completely understood (13). Electroporation mediates entry of plasmid DNA into cells by generating an electric field which creates cellular pores through which the DNA diffuses. However, the high-voltage electrical pulse can result in membrane disruption and alteration of protein structure (i.e. denaturation) (11) which are known to induce the stress response (4, 17, 9).

Serum-free conditions greatly enhance transfection efficiency when using liposome-mediated DNA delivery system (3). Liposome-mediated transfections in serum-free conditions are often performed overnight, however we found that the degree of expression of hsp 72 correlated with the amount of time the cells remain in serum-free conditions before returning to normal culture condition (i.e. serum-stimulation). Serum-
stimulation following serum-free conditions is a potent inducer of the stress response (20). Therefore, when analyzing stress responsive genes, long transfections times with this method may inhibit or mask a stress-mediated response.

Using an SV40-luciferase reporter vector, the liposome-mediated DNA delivery system resulted in the greatest transfection efficiency in Hela cells as compared to calcium phosphate and DEAE-dextran. Because of the pronounced induction of hsp 72 in HUT 78 cells following electroporation, we excluded this method from promoter analysis studies. A 3 hr transfection time with liposome-mediated transfer (i.e. serum-free conditions) did not result in increased expression of hsp 72 by flow cytometric analysis, therefore we chose this transfection protocol for subsequent kinetic experiments.

Even through no increase in expression of hsp72, as compared to controls, was found with a 3 hr transfection time, we wanted to establish if the cells still required a recovery period before stress induction. Our data indicates that maximal stress responsive gene activity with the HTLV-1-LTR and CMV-IE promoters is dependent on the cells being maintained in normal culture conditions for 24 hr before induction of the cellular stress response. When the stress response was induced at 6 hr following transfection minimal differences in reporter gene activity was observed with the HTLV-1-LTR and CMV-IE. The lack of promoter responsiveness with only a 6 hr recovery period may be related to multiple variables, including phase of cell cycle (e.g. G1/S block) or development of thermotolerance initiated by the serum-free to serum-stimulation conditions of the liposome-mediated transfections (20). Thus, the cells must be allowed to recover greater than 6 hr before maximal induction of the stress response can be elicited.
In conclusion, we found that certain transfection techniques do result in induction of the cellular stress response. Therefore, when analyzing stress mediated changes in gene expression by transient transfection methods, the technique itself may mask any observed changes. Additionally, we demonstrate that the maximal stress-mediated enhancement of transcription with both promoters did not occur until the cells recovered for 24 hr following transfection. In this study we used two viral promoters as a model system to study stress-mediated enhancement, however our data have important implications for investigations of any cellular stress responsive genes employing these commonly used transfection methods.

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Figure 3.1: Hsp 72 expression following induction of the cellular stress response with Na arsenite and thermal treatment in Hela cells. Hela cells were exposed to varied concentrations of Na arsenite and different temperatures. Immunofluorescent staining for hsp 72 was determined by flow cytometric analysis and data presented as percent expression of hsp 72 over non-stressed controls. \( \pm \) SEM of 3 trials.
Figure 3.2: Induction of hsp 72 following different methods of transient transfections. (A) The expression of hsp 72 was determined 24 hr following calcium phosphate and DEAE-dextran methods of transfection in HeLa cells. Percent expression of hsp 72 was determined in HUT 78 cells following electroporation. (B) Hsp 72 expression was determined following liposome-mediated transfection at indicated times in serum-free conditions. \( \overline{x} \pm SEM \) of 3 trials with liposome-mediated, calcium phosphate, DEAE-dextran
Figure 3.3: Comparison of transfection efficiency with DEAE-dextran, calcium phosphate and liposome-mediated transfer in Hela cells. Each method of transfection was first optimized in Hela cells for DNA concentration with a fixed cell density using an SV40 reporter construct. Transfection efficiency was then compared between techniques. Transfection efficiency was the greatest with liposome-mediated DNA delivery.
Figure 3.4: Comparison of stress-mediated modulation of HTLV-1-LTR activity at 6 and 24 hr recovery periods following transfections in HeLa cells. Maximal stress induced increase in promoter activity was detected after HeLa cells rested for 24 hr following liposome-mediated transfection. The stress response was induced at 6 (A) or 24 hr (B) following transfection with an HTLV-1-LTR reporter construct. Reporter gene activity was determined at 3, 6, 12, and 24 hr following stress induction. Luciferase activity of extracts from non-stressed transfected cells was assigned a value of 1.0. These data are presented as the fold increase in luciferase activity.
Figure 3.5: Comparison of stress-mediated modulation of CMV-IE activity at 6 and 24 hr recovery periods following transfections in Hela cells. Maximal stress induced increase in promoter activity was detected after Hela cells rested for 24 hr following liposome-mediated transfection. The stress response was induced at 6 (A) or 24 hr (B) following transfection with a CMV-IE reporter construct. Reporter gene activity was determined at 3, 6, 12, and 24 hr following stress induction. Luciferase activity of extracts from non-stressed transfected cells was assigned a value of 1.0. These data are presented as the fold increase in luciferase activity.
CHAPTER 4

THE CELLULAR STRESS RESPONSE ENHANCES HTLV-1 BASAL GENE EXPRESSION THROUGH THE CORE PROMOTER REGION OF THE LONG TERMINAL REPEAT

INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) infection is characterized by a long period of clinical latency during which viral gene expression is low to undetectable in lymphocytes (10). However, induced expression of viral proteins, particularly Tax appears to play a crucial role in the development of an aggressive leukemia of T-lymphocytes termed adult T-cell leukemia (31). Knowledge of the cellular events which initiate or enhance HTLV-1 transcription are critical in fully understanding the pathogenesis of disease resulting from altered viral gene expression.

The cellular stress response is a physiologic response to adverse conditions in the cellular environment (29). Recent reports have implicated the stress response in the activation of latent viruses. For example, thermal and chemical stress initiated transcription of a cytomegalovirus (CMV) major immediate early transcription unit in stably transfected Rat-9G cells (6). Several reports have shown thermal stress to activate transcription of the HIV-1 long terminal repeat (LTR) (27, 7, 11). In addition, an increase
in viral RNA production was demonstrated following thermal stress or Na arsenite, in a persistent infection model of canine distemper virus (22). In these examples, the mechanism of activation of viral replication by cellular stress events is unclear. It is proposed that the activation of the HIV-1 LTR is mediated through the NF-κB region; however, one report was unable to detect the binding of any protein specifically to this region following heat stress, suggesting that this site is not directly involved (11).

We have previously reported that both thermal stress and Na arsenite increased HTLV-1 viral protein expression in persistently infected lymphocyte cell lines (1). Transient transfections with the HTLV-1 LTR located upstream of a reporter gene was stress inducible in HUT 78 cells, suggesting involvement of the LTR in stress mediated increases in HTLV-1 gene expression (1).

In this study, we investigated the degree in which physiologic stress increases HTLV-1 transcription via the viral protein Tax, and localized the responsive region in the viral LTR. We demonstrate by both in vitro transcription assays and transient transfections that induction of the stress response increases basal transcription from the LTR. Transient cotransfection assays indicate that stress induction of viral transcription is Tax independent. In addition, we provide evidence that the sequences responsible for the enhanced transcription are -52 through +157 of the U3/R region of the HTLV-1 LTR. Finally, our data suggest that the increase in transcription is mediated through an intermediate polymerase II/polymerase III transcriptional complex, demonstrated by the inability to abolish the effect with low concentrations of α-amanitin.
MATERIALS AND METHODS

Cell Lines and Stress Response Induction. MT-2 (16) and HUT 102 (5) are persistently infected HTLV-1 transformed T-lymphocytes derived from patients with ATLL. MT-2, clone 6 (MT 2.6) was derived by limited dilution cloning of the parent strain MT-2. All cells were maintained at 37°C in complete RPMI 1640 containing 10% fetal calf serum (FCS), 1.2 mM glutamine, 60 units/ml penicillin, and 60 μg/ml streptomycin. Hela cells were obtained from the American Type Cell Culture (Rockville, MD) and maintained in Eagle’s minimal essential medium supplemented with 10% FCS, 1.2 mM glutamine, 60 units/ml penicillin, and 60 μg/ml streptomycin.

The cellular stress response was induced in the cells lines using Na arsenite as previously described (1). The induction protocol was optimized for each cell line. Briefly, each cell line was exposed to a range of concentrations of Na arsenite (80 μM to 320 μM) for 1.5 hr. Induction of the stress response was determined by measuring cellular expression of hsp 72, the major inducible member of the hsp 70 family, at 24 hr following stress induction. Indirect immunofluorescence staining for hsp 72 was detected by flow cytofluorometric analysis. Optimal stress induction was defined as the maximum expression of hsp 72 with ≤20% decrease in cell viability based on trypan blue dye exclusion.
Promoter constructs. The HTLV-1 LTR luciferase plasmid which contains the LTR (wild type) derived from pU3R-I (26) cloned upstream of a luciferase gene has been previously described (19). The pU3Rcat dl6.2 plasmid is a deletion mutant of the wild type LTR promoter and lacks nucleotides -440 to -52 (2). The dl6.2 was digested with the restriction enzymes XbaI/Bg/II, the promoter fragment isolated by GENECLEAN II® (BIO 101, Inc., La Jolla, CA ), and cloned into the XbaI/Bg/II site of pGEM4Z (Promega, Madison, WI). The pGEM dl6.2 construct was digested with SacI/HindII, the promoter fragment isolated and cloned into the SmaI/SacI site of a luciferase reporter plasmid (pGL2-Basic, Promega, Madison, WI) and designated dl6.2 luciferase.

The pU3Rcat dl+157 plasmid is a downstream deletion mutant of the wild type LTR of pU3Rcat and lacks nucleotides +157 to +262 (9). The dl+157 was digested with Bg/II, the promoter fragment purified, cloned into the luciferase reporter plasmid pGL2-Basic, and designated dl+157 luciferase. Directionality was confirmed by restriction enzyme analysis.

The pHTLV-I Tax which contains the wild type LTR cloned upstream of the Tax gene has been described elsewhere (18).

Whole Cell Extracts and In Vitro Transcription. Whole cell extracts prepared as previously described (15) were collected at 6, 12, and 24 hr post induction of the cellular stress response from MT 2.6 and HUT 102 cells. In vitro transcription assays were
performed as described (24, 4) with modifications. Briefly, a 15 μl reaction volume consisted of 200-300 ng of plasmid DNA template, 3 μg of whole cell extract, 666 μM each of GTP, CTP, and ATP, 10 μCi of [32P]UTP (800 Ci/mmol), and 40 units of rRNasin (Promega, Madison, WI). Pre-initiation complexes were formed in the presence of DNA template, whole cell extract, and dialysis buffer [20 mM Hepes, pH 7.9, 100 mM KCl, 12.5 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 17% glycerol] at 30°C for 30 min. RNA synthesis was initiated with the addition of nucleotides and incubated for an additional 30 min at 30°C. Initially, the reactions were optimized for both template and extract concentration by performing titration experiments. Alpha-amanitin was added to the reaction cocktail at a final concentration of 6 μg/ml or 60 μg/ml where indicated (24). Reactions were terminated with stop buffer [18 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.2% SDS]. RNA was isolated by phenol-chloroform extraction, ethanol precipitated, and separated on a 4% polyacrylamide urea gel. Relative amounts of RNA were quantitated with a Molecular Dynamics PhosphorImager™ 445 SI (Molecular Dynamics, Inc., Sunnyvale, CA). All reactions were performed in triplicate in at least two independent experimental trials. A 32P-end-labeled fragment of DNA (616 bp) was added to each reaction at termination but prior to RNA extraction as an internal recovery control.

**Transient-transfection assays.** One day before transfections Hela cells were seeded into 60 mm culture dishes at 5 x 10^5 cells to obtain a cell confluency of 50 to 70%. The cells were transfected with plasmid constructs using a commercially available lipid-mediated
delivery system (Transfectam, Promega, Madison WI). Cells were transfected for 3 hr following the recommended assay protocol for media without serum by the manufacturer (Promega). Transfectam and DNA concentrations were titered to establish the optimal ratio for maximal transfection efficiency. A lipid:DNA ratio of 3:1 was utilized in all experiments. Twenty-four hr following transfection, the cellular stress response was induced with Na arsenite. Cells were harvested at 3, 6, 12, and 24 hr following physiologic stress using Cell Culture Lysis 1X Reagent (Promega). Luciferase activity was measured by liquid scintillation counting following manufacturers recommendations (Promega). All transfections were done in triplicate and repeated in at least 3 independent experiments. All data is expressed as the mean fold increase in luciferase activity in the transfected stressed cells as compared to the nonstressed controls.

RESULTS

Whole cell extracts from stressed cells direct more transcription from the wild type HTLV-1 LTR as compared to extracts from nonstressed cells. To measure the changes in transcriptional rates from the HTLV-1 LTR following cellular stress induction, in vitro transcription assays were performed. Physiologic stress was induced by Na arsenite in persistently infected HTLV-1 cell lines. Whole cell extracts were collected from stressed and nonstressed cells at 6, 12, and 24 hr following stress induction. An HTLV-1 promoter template was prepared by digesting the LTR luciferase plasmid with HindIII (Fig. 4.1). Whole cell extracts collected at 6 hr following stress directed more transcription (3-5 fold) from the HTLV-1 LTR as compared to non-stressed cells (Fig. 76).
Whole cell extracts collected at 12 and 24 hr post stress had an equivocal amount of transcriptional activity as the non-stressed cells (Fig. 4.2A). To determine if the increase in transcriptional activity from the 6 hr extracts was specific for the HTLV-1 LTR, we repeated the in vitro transcription assays with the adenovirus major late promoter (AdML). Transcription from the AdML directed a similar amount of transcription with the 6 hr stressed extracts as compared to nonstressed extracts (Fig. 4.2A). Therefore, this suggested that the increased transcriptional activity in the 6 hr stressed extracts was HTLV-1 LTR specific.

Whole cell extracts from stressed cells direct more transcription from the LTR in the absence of upstream regulatory regions. The in vitro transcription assays were repeated with the upstream deletion mutant, dl6.2 luciferase (depicted in Fig. 4.1) which was prepared by linearizing the plasmid with HindIII. Transcription from 6 hr stressed extracts directed more transcription (3-5 fold) from the deletion construct as demonstrated by increased RNA synthesis as compared to nonstressed 6 hr extracts (Fig 4.2B). These results suggest that the upstream enhancer regions are not responsible for the stress mediated increase in transcription.

The increase in transcriptional activity following physiologic stress is resistant to low concentrations of α-amanitin. RNA pol II dependent promoters are sensitive to low concentrations of α-amanitin. Recent evidence has shown that the HTLV-1 promoter
contains an overlapping transcriptional unit which is resistant to low concentrations of α-amanitin (24). Therefore, we wanted to test the effect of this compound in our in vitro transcription assay with stressed and nonstressed whole cell extracts. Transcription from the AdML promoter was used as a template control for pol II sensitivity to α-amanitin (Fig. 4.3). As reported, we found that transcription form the HTLV-1 promoter was inhibited completely only by high concentration of α-amanitin (60 μg/ml), more typical of a pol III-transcribed promoter. We found that low concentrations (6 μg/ml) of α-amanitin decreased the amount of transcription directed from the LTR. However, it did not negate the differences seen in transcriptional activity with the 6 hr stress extracts when compared to nonstressed controls (Fig. 4.3).

Physiologic stress enhances basal transcription from the HTLV-1 LTR and is Tax independent. To further confirm that physiologic stress could stimulate transcription in vivo from the HTLV-1 LTR and to map the responsive region in the viral promoter, transient transfection were performed. Hela cells were transfected with an LTR luciferase reporter plasmid, physiologic stress was induced 24 hr later and reporter gene activity measured at 3, 6, 12, and 24 hr. An increase in reporter gene activity of similar magnitude (2.0 to 2.9 mean fold difference) was observed in stressed extracts collected at 6, 12, and 24 hr as compared to nonstressed transfected controls (Fig. 4.4). To determine if the viral protein Tax could promote the stress mediated increase in reporter construct transcription, cotransfections with the LTR luciferase plasmid and an LTR Tax expressing plasmid were
performed. Cotransfections with the two constructs in nonstressed control cells had an increase in luciferase activity of 10-15 fold over LTR transfected cells alone (data not shown). However, cellular stress had a similar effect on promoter activity in cells cotransfected with the Tax expressing plasmid (2.3 fold mean increase) compared to that observed with the LTR luciferase transfected cells (2.6 fold mean increase) (Fig. 4.5). To determine if the increase in reporter gene activity was a result of posttranscriptional modifications, all transfections were repeated with the SV40 promoter (pGL2-Control plasmid, Promega), which has been show to be nonresponsive to physiologic stress (11). No difference in luciferase activity was observed between stressed and nonstressed transfected Hela cells with the SV40 luciferase plasmid. (Fig. 4.5). Therefore, these results indicate that the increase in luciferase activity from the transfected stressed cells is HTLV-1 LTR specific and transcriptionally controlled.

Physiologic stress mediates increased transcription from the LTR through sequences located between -52 and +157 of the U3/R region. To localize sequences of the LTR that are stress responsive, transient transfections were repeated with the deletion mutant, dl6.2 luciferase, depicted in Fig. 4.1. This deletion promoter lacks the upstream enhancer elements which confer Tax responsiveness. We found that physiologic stress stimulated increase transcription from dl6.2 luc as compared to nonstressed control cells and to a similar degree (2.3 mean fold difference) as the wild type promoter (2.6 mean fold difference).
Sequences downstream of the RNA initiation site have been found to be important in enhancement of basal gene expression of HTLV-1. One report demonstrated a protein homologous to YB-1 which binds to a downstream regulatory region (+195 to +240) of the LTR (9). The positive increase in transcription from this regulatory sequence was Tax independent. To determine if these downstream regulatory sequences of the LTR mediated stress enhanced transcription, transfections were repeated in the presence of a downstream deletion mutant (dl+157 luciferase) depicted in Fig. 4.1. A comparable degree of transcriptional activation was observed in the stressed transfected cells with dl+157 (2.5 mean fold increase) as compared to the wild type LTR (2.6 mean fold increase) (Fig. 4.6) suggesting that these sequences are not responsible for the stress mediated increase in transcription.

DISCUSSION

The cellular stress response is a physiologic response to adverse changes in the cellular environment. Historically, it was referred to as the heat shock response because initial studies utilized hyperthermia to induce the response. However, it is now recognized that cells can initiate a stress response when exposed to multiple other metabolic insults, including mitogens, lymphokines, viral infections, and ischemia (21). Induction of the stress response results in the production of a family of proteins referred to as cellular stress proteins. The function of these proteins are diverse and include facilitation of protein folding, oligomerization, and translocation of proteins through intracellular compartments (29). Additionally, there is evidence that cellular stress may promote
transcriptional activation of viruses. An important example is the initiation of bacteriophage lambda synthesis in *E. coli* following hyperthermia (20).

Previously, we demonstrated that the cellular response to thermal stress or Na arsenite could enhance viral protein expression in HTLV-1 infected cells (1). Induction of the stress response in these cell lines showed an increase in viral protein expression with variable increases in RNA accumulation. Results of transient transfections with an HTLV-1 LTR reporter plasmid suggested enhanced transcriptional activation following cellular stress. Our data is consistent with reports in other viral model systems which have demonstrated enhanced viral transcription following stress induction. A stable transfectant in rat cells with a CAT gene under control of the HIV-1 LTR, showed increased expression (5-fold) following either hyperthermia or chemical induction of the stress response (7). In addition, transient transfections with the HIV LTR cloned upstream of a CAT gene resulted in increased CAT activity following hyperthermia (2 to 8-fold) (27, 11) or oxidative stress (5-fold) (12). Geelen et al. (6) demonstrated transcriptional activation of a major immediate early transcription unit of the human cytomegalovirus in a stable transfection of Rat-9G cells following heat or Na arsenite treatment. An increase in viral RNA production was demonstrated following thermal stress or Na arsenite, in a stable persistent infection model of canine distemper virus (22).

Our data indicates that the cellular stress response stimulates basal transcription (2 to 3.5-fold) of the HTLV-1 promoter when an LTR luciferase plasmid was transiently transfected into Hela cells. In an initial study, we reported the results of cotransfections with an LTR reporter plasmid and a Tax expressing plasmid in HUT 78 cells (1). We
hypothesized that stress inducible transcription of the HTLV-1 promoter may be enhanced in the presence of Tax. However, both single (LTR reporter construct) and cotransfected (LTR reporter construct and Tax) HUT 78 cells demonstrated a similar increase in reporter gene activity following stress induction. In this study, with multiple trials we demonstrate similar degrees of transcription when a Tax expressing plasmid was cotransfected with an LTR luciferase plasmid compared to that with the LTR luciferase plasmid alone. We conclude that the transcriptional activation of the LTR following stress is not further enhanced in the presence of Tax. Because the degree of transcriptional activation was similar with or without Tax, we repeated the transient transfection assays with a plasmid construct which is deleted to nucleotide -52 of the U3 region of the LTR eliminating the upstream Tax-responsive regions. The increase in transcription was similar to that observed with the wild type promoter demonstrating that the Tax-responsive elements are not important in stress induced transcriptional activation of the HTLV-1 LTR. To demonstrate that the increase in luciferase activity was not related to post-transcriptional modifications such as protein or RNA stabilization events, transfections were repeated with a luciferase expressing plasmid under control of the SV40 promoter. This promoter has been shown previously to be nonstress responsive (11). There was no increase in reporter gene activity following stress induction with this promoter thus demonstrating that the increase in luciferase activity following stress induction is LTR-specific and at the transcriptional level.

Sequences downstream of the RNA initiation site in the LTR have been shown to be important for enhanced basal gene expression. Kashanchi et al. (9) reported a non Tax
responsive 45-nucleotide sequence (+195 to +240) which was found to upregulate basal expression of HTLV-I. This region was later described to bind a protein homologous to the transcriptional factor YB-1. A 136-base pair fragment (+104 to +240) was also reported to be important in basal gene expression and functioned independently of Tax (17). A recent report describes a segment of the LTR in the R region (+205 to +240) that binds a CREB factor which results in suppression of transcription (30). To determine in our study if these downstream regulatory regions were responsible for the increased rate of transcription following physiologic stress, we repeated transient transfections with a deletion construct which lack sequences +157 to +278. However, an increase in reporter gene activity was still observed following induction of the cellular stress response, indicating that these sequences do not confer stress mediated transcriptional increases.

Increased transcription of the HTLV-1 LTR following stress induction was also addressed by performing in vitro transcription assays. Whole cell extracts collected after stress induction, enhanced promoter directed transcription from the HTLV-1 wild type LTR (3 to 5 fold) and the deletion mutant dl6.2 (3 to 5 fold) compared to nonstressed extracts. Parallel in vitro transcription assays were performed with the AdML to assay the specificity of the extracts for the HTLV-1 LTR. A similar degree of activity was seen with the 6 hr stressed extracts as compared to nonstressed control extracts with this promoter.

We determined the kinetics of the stress mediated response and found that whole cell extracts collected at 6 hr post stress increased transcription while extracts collected at 12 and 24 hr transcribed a similar amount of RNA as nonstressed controls. Transcriptional
activation of the LTR as evaluated by transient transfections showed no increase in stress mediated transcription at 3 hr but an increase at 6 hr as well as 12 and 24 hr post stress. This continued increase in luciferase activity from stressed extracts at 12 and 24 hr is likely a consequence of accumulation of luciferase (25). The immediate phase following physiologic stress is the recovery phase, in which there is a decrease in protein synthesis secondary to both decreased transcription and translation (14). However, during this recovery phase transcription and translation of cellular stress proteins increase with the peak in stress protein synthesis at 3-6 hr following stress induction (14). This preferential gene expression is not completely understood, but includes phosphorylation of preexisting proteins (28, 3, 12) and changes in chromatin structure (23). If the stress mediated increase in HTLV-1 transcription followed similar kinetics as an hsp gene, one would expect transcriptional activation during this recovery phase. However, our data demonstrates no increase in transcription until 6 hr following stress induction. Therefore, this data suggests that the stress-mediated increase in transcription of the LTR does not occur during the recovery phase following physiologic stress, and stress protein synthesis may be important in this event.

The HTLV-1 promoter is unique in that it contains a typical eukaryotic RNA polymerase II promoter and an overlapping promoter in the LTR which uses the same transcriptional initiation site. This overlapping transcriptional unit is resistant to low concentrations of α-amanitin not typical of pol II but sensitive to high concentrations more characteristic of pol III (24). A recent report demonstrates that Tax can stimulate
transcription by RNA pol III, mediated through the transcriptional initiation factor, TFIIIB (8). We performed in vitro transcription assays with whole cell extracts from HTLV-1 infected cells in the presence of low and high concentrations of α-amanitin. The AdML promoter was used as a prototypic promoter transcribed by pol II, and as expected was sensitive to low concentrations (6 μg/ml) of α-amanitin. Transcription from the LTR promoter was resistant to low concentration of α-amanitin (6 μg/ml), but inhibited at high concentrations (60 μg/ml). The in vitro transcription assays were repeated with α-amanitin added to stressed extracts and compared to nonstressed control extracts. If the increase in stress mediated transcription was through protein components of this overlapping transcriptional unit, we expected no change in the difference in transcription would be seen with α-amanitin. In the presence of low concentrations of α-amanitin, stressed extracts still directed increase transcription from the LTR as compared to nonstressed extracts and to the same degree as observed in the absence of α-amanitin.

In conclusion, we report that induction of the cellular stress response increases basal transcription of the HTLV-1 LTR mediated through the core promoter region. Additionally, our data suggests that the effect on the promoter may be mediated through an intermediate pol II/pol III complex. The stress mediated increase in transcription does not follow the kinetics of a stress protein gene, but suggests that stress protein synthesis may be required, however further investigation is needed. Future studies will be performed to further map the stress responsive sequences in the LTR and to determine the role that stress proteins may have in transcriptional activation of this viral promoter.
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Figure 4.1: HTLV-1 LTR promoter constructs including the wild type LTR and deletion mutants. The stippled line represents the sequences deleted in both the upstream (d6.2) and downstream (d1+157) deletion constructs.
Figure 4.2: In vitro transcription assays of whole cell extracts collected from MT 2.6 cells post induction of the cellular stress response. (A) In vitro transcription assays were performed with whole cell extracts collected at 6, 12, and 24 hr following induction of the stress response in MT-2.6 cells. The HTLV-1 LTR specific transcript is 278 bases. A $^{32}$P-end-labeled segment of DNA (616bp) was used as an internal recovery control. The data represents at least three independent experiments with two trials done in triplicate with the internal recovery control. The mean relative ratio of RNA:DNA loading control is shown. The in vitro transcription reactions were repeated with the AdML promoter. The AdML specific transcript is 375 bases in length. (B) In vitro transcription reactions were repeated with a truncated LTR promoter (dL6.2) which lacks the Tax-responsive enhancer elements. The 6 hr extracts collected from stressed MT 2.6 cells direct more transcription from the HTLV-1 wild type and truncated promoter as compared to nonstressed control extracts. (NS, nonstressed; S, stressed)
Figure 4.3: The transcriptional sensitivity of stressed and nonstressed whole cell extracts of MT 2.6 cells to α-amanitin from the HTLV-1 LTR. The stress mediated increase in transcriptional activity of whole cell extracts from the HTLV-1 LTR is resistant to low concentrations of α-amanitin. In vitro transcription assays were performed with whole cell extracts collected at 6 hr following induction of the cellular stress response in HTLV-1 infected cells. The reactions were also performed in the presence of 6μg/ml or 60μg/ml of α-amanitin. Transcription from the AdML promoter was also performed in the presence of 6μg/ml of α-amanitin. (NS, nonstressed; S, stressed)
Figure 4.4: Transient transfection assays with an HTLV-1 LTR reporter construct in Hela cells. Hela cells were transfected with an HTLV-1 LTR reporter plasmid and luciferase activity measured at 3, 6, 12, and 24 hr following induction of the cellular stress response with Na arsenite. The stress response enhances basal transcription from the LTR as early as 6 hr post stress induction. All transfections were performed in triplicate in at least three independent trials. Luciferase activity of extracts collected at the same time points from nonstressed transfected cells was assigned a value of 1.0. The data is presented at the mean fold increase in luciferase activity. (NS, nonstressed; S, stressed)
Figure 4.5: Transient cotransfection assays with an HTLV-1 LTR reporter construct and Tax-expressing construct in Hela cells. Hela cells were transfected with an HTLV-1 LTR luciferase expression plasmid alone and cotransfected with an LTR-Tax expressing plasmid. The stress response was induced at 24 hr following transfections. Twenty-four hours following physiologic stress, luciferase activity was determined and compared to nonstressed cells. The SV40-luciferase plasmid was transfected as above and used as a nonstress responsive control. Stress induction stimulates basal transcription of the LTR and is Tax independent. All transfections were performed in triplicate in at least three independent trials. Luciferase activity of extracts from nonstressed transfected cells was assigned a value of 1.0. The data is presented at the mean fold increase in luciferase activity. (NS, nonstressed; S, stressed)
Figure 4.6: Transient transfection assays demonstrating the cellular stress response stimulates transcription of the HTLV-1 LTR through sequences localized between -52 and +157 of the U3/R region. Hela cells were transfected with HTLV-1 LTR deletions mutants and the cellular stress response was induced with Na arsenite. Luciferase activity was determined in cell extracts collected 24 hr following stress and compared to nonstressed cell extracts. All transfections were performed in triplicate in at least three independent trials. Luciferase activity of extracts from nonstressed transfected cells was assigned a value of 1.0. The data is presented at the mean fold increase in luciferase activity. (NS, nonstressed; S, stressed)
CHAPTER 5
THE CELLULAR STRESS RESPONSE INCREASES TRANSLATION OF THE
VIRAL ENVELOPE PROTEIN IN HTLV-1 TRANSFORMED CELL LINES

INTRODUCTION

Surface expression of human T-lymphotropic virus type 1 (HTLV-1) envelope (env) protein is essential for the syncytia forming ability of infected cells (9). Consequently, syncytia formation assays are frequently used to measure changes in viral env biological activity. HTLV-1 viral env proteins are synthesized as a precursor protein, which is subsequently cleaved to a surface protein (gp46) and transmembrane protein (gp21) (4, 16). The precursor protein is translated from a singly spliced mRNA species on membrane (endoplasmic reticulum) bound polyribosomes. The protein is then modified by glycosylation events, proteolytically cleaved and inserted into the host cell membrane during virion assembly and release (14, 10).

Previously we reported that induction of the cellular stress response by Na arsenite or thermal treatment increased syncytia formation in HTLV-1-infected cells (1). The stress mediated increase in syncytia formation was inhibited by incubating the cells in the presence of a monoclonal antibody against gp46 (1). Western blot analysis revealed increased intracellular viral env protein (gp68) expression following induction of the stress
response. However, northern blot analysis revealed mild differences in env mRNA in response to cellular stress and the differences did not compare to the magnitude of intracellular env protein expression and syncytia formation. Therefore, suggesting that in HTLV-1 transformed cell lines the accumulation of viral env protein in response to cellular stress is a result of post-transcriptional events.

The present study was designed to determine the effects of physiologic stress on env protein metabolism in HTLV-1 transformed cell lines. We show that the increase in HTLV-1-mediated syncytia formation following stress response induction is a result of increase cell surface expression of gp46. Cellular stress in MT 2.6 cells did not alter the turnover of intracellular gp68 as no changes in viral protein half-life were demonstrated as compared to nonstressed cells. However, env expression in stressed cells treated with a protein synthesis inhibitor (cycloheximide) indicate the effect is mediated through increase protein synthesis. Our results indicate that induction of the stress response in virally infected cells modulates viral env protein metabolism to enhance HTLV-1-mediated syncytia formation.

MATERIALS AND METHODS

Cell lines and stress response induction. MT-2 (8) cells are persistently infected HTLV-1 transformed T-lymphocytes derived from patients with ATLL. MT-2, clone 6 (MT 2.6) was derived by limited dilution cloning of the parent strain MT-2. The cells were maintained at 37°C in RPMI 1640 containing 10% fetal calf serum, 1.2 mM glutamine, 60
units/ml penicillin, and 60 µg/ml streptomycin (cRPMI).

The cellular stress response was induced using Na arsenite as previously described (1).

Immunofluorescence analysis. HTLV-1 surface envelope expression was determined following stress induction by indirect immunofluorescence staining of 1 x 10^6 cells for gp46. The primary antibody was a 1:200 dilution of a murine MAb against gp46 (IC11, 11). The secondary antibody was a 1:20 dilution of a rabbit anti-mouse IgG FITC-conjugate (Sigma, St. Louis, MO). All staining was performed on ice and antibodies were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.1% sodium azide to prevent internalization of antigen-antibody complex. Following staining the cells were fixed in 2% paraformaldehyde. Specific staining was measured by flow cytometric analysis using a Coulter Elite cytometer (Coulter Corp., Hialeah, FL). Isotypic FITC labeled MAb controls (mouse IgG, Sigma) were included in each trial. The immunofluorescent flow cytometric data was analyzed using the Coulter Immuno-4 analysis program (Coulter). Intracellular expression of hsp 72, the major inducible member of the hsp 70 family, was determined as previously described (1). Dual expression of surface gp46 expression and intracellular hsp72 expression was determined in MT 2 6 cells following stress induction. Cells were first labeled for gp46 as described above and fixed in 2 ml of Permeafix (Ortho Diagnostic Systems, Raritan, NJ) for 30 min. Fixed cells were washed in PBS with 1% BSA and 0.1% sodium azide, and stained for intracellular expression of hsp72 as described (1).
Radioimmunoprecipitation (RIPA). RIPA was performed as previously described with some modifications (3). MT 2.6 cells were metabolically labeled with $[^{35}S]$cysteine and $[^{35}S]$methionine (Trans$^{35}$S-label, ICN, Irvine, CA) after a 20 min incubation in methionine/cysteine-free RPMI. After a 30 min pulse-labeling, cells were washed and resuspended in cRPMI with 5 fold excess methionine/cysteine. The cellular stress response was induced by exposing the cells to Na arsenite for 1.5 hr and lysates collected at 3, 6, 12, and 24 hr following stress induction. Cells were disrupted with RIPA lysing buffer (1X PBS, 0.1% SDS, 0.5% deoxycholate 1% Triton X-100) containing protease inhibitors and clarified for 1 hr at 45,000 rpm. Supernatants were collected and precleared with a 50% suspension of protein A-Sepharose beads (CL-4B, Sigma) for 60 min. The lysates were incubated with a MAb (1:20 dilution) against HTLV-1 gp46 (IC11) for 12-18 hr at 4°C. Immune complexes were precipitated with protein A-Sepharose, washed with RIPA lysing buffer and eluted by boiling in 2X Laemmli sample buffer. Samples were electrophoretically resolved in an SDS/10% polyacrylamide gel and relative amounts of radiolabeled proteins determined with a Molecular Dynamics Phosphor Imager. The t$_{1/2}$ was calculated as $(.693 \times t) \ln(N_0/N)$, where $N_0$ represents the relative amount of pulse sample and $N$ represents the relative amount of chase sample (7).

Western blot analysis. Western blot analysis was performed for detection of HTLV-1 envelope glycoproteins and hsp 72. MT 2.6 cells were lysed and solubilized in lysis buffer [6.25 mM Tris-HCL (pH=6.8), 2% SDS, 10% glycerol, 50 mM DTT], separated on a SDS/10% polyacrylamide gel and transferred to nitrocellulose as described (1). HTLV-1
gp68 or hsp 72 was visualized by enhanced chemiluminescence following manufacturers recommendations (ECL™, Amersham Life Science). Cycloheximide (Sigma) was added to selected cultures at a final concentration of 100 ug/ml to inhibit protein synthesis.

RESULTS

Induction of the cellular stress response in MT 2.6 cells increases the surface expression of HTLV-1 viral envelope protein (gp46). Previously we reported that induction of the stress response in two HTLV-1 infected cell lines (MT 2.6 and HUT 102) significantly increased the formation of syncytia as compared to nonstressed controls (1). To determine if the increase in syncytia formation in stressed cells was a result of enhanced surface expression of the HTLV-1 envelope protein (gp46), we determined the expression of gp46 by flow cytometric analysis following stress induction at 24 and 48 hr. We demonstrate that induction of the stress response increased surface expression of gp46 at both 24 and 48 hr (Fig. 5.1) as compared to nonstressed controls. We then performed a kinetic study to follow the expression of gp46 post physiologic stress. Cells were labeled for gp46 at 1, 3, 6, 24 hr post stress and expression of hsp72 was determined simultaneously (Fig. 5.2). Hsp72 is the highly inducible member of the hsp70 family, and a very sensitive indicator of an active stress response (7). Increased surface expression of gp46 was observed as early as 3 hr with peak expression at 6 hr following stress induction. The expression of hsp72 followed a similar pattern as gp46, however peak expression occurred prior to gp46 in MT 2.6 cells. (Fig. 5.2).
By using hsp72 as a marker for cells undergoing an active stress response, we wanted to determine if the cells expressing the greatest amount of gp46 were also the cells expressing the highest degree of hsp72. Therefore, dual labeling was performed for both surface expression of gp46 and intracellular hsp72 and analyzed by flow cytometric analysis. Similar to our kinetic study we observed increases in both gp46 and hsp72 expression, however the expression did not appear to directly correlate (data not shown).

Cellular stress had no effect on the protein turnover of HTLV-1 gp68. To address the effect cellular stress had on the turnover of gp68, we determined the $t_{1/2}$ of gp68 following induction of the cellular stress response in MT 2.6 cells. MT 2.6 cells were pulse-labeled for 30 min with $[^{35}S]$cysteine and $[^{35}S]$methionine (Fig. 5.3A, lane 1 and 5), the stress response induced and chased in excess cold methionine/cysteine for the indicated times (Fig. 3a, lanes 2-4 and 6-8) before immunoprecipitation with anti-gp46 MAb. Pulse-chase experiments determined that the turnover of gp68 in nonstressed cells had a $t_{1/2}$ of 9-14 hr. A previous study reported a $t_{1/2}$ of 7-12 hr for gp68 in MT-2 cells (10). Pulse-chase experiments in stressed and nonstressed cells revealed a similar $t_{1/2}$ of 13 and 14 hr respectively (Fig. 5.3A and 3B) therefore indicating minimal change in gp68 turnover in stressed MT 2.6 cells.

Inhibition of protein synthesis in MT 2.6 cells blocked the stress-mediated increase in gp68 expression. To determine the stress-mediated effect on viral env synthesis at the translational level, stressed and nonstressed cells were incubated in the presence of
cycloheximide, a protein translation inhibitor, to evaluate decay of steady-state gp68 in the absence of new protein synthesis. In stressed, cycloheximide treated cells the rate of protein synthesis was decreased to 35% of control (stressed, non-treated) cells (Fig. 5.4). In addition, increased expression of gp68 was observed following stress induction (Fig. 5.4). Non-stressed cycloheximide treated cells decreased the rate of protein synthesis to 49% of control (non-stressed, non-treated) cells (Fig. 5.4). Therefore, concluding that the increase expression of gp68 following stress induction is primarily a result of increase protein synthesis and not due to changes in protein turnover (t₁/₂).

DISCUSSION

In this study, we determined the influence of the cellular stress response in mediating changes in HTLV-1 viral env protein metabolism. We demonstrate that the increase in stress-mediated syncytia formation in HTLV-1 transformed cells is a result of increase surface expression of the viral gp46. By following the kinetics of gp46 and hsp72 expression following stress induction with Na arsenite, we found that the peak increase in the intracellular expression of hsp72 was a few hours prior to peak gp46 expression. However, both followed similar patterns with increase expression early (3 hr) and gradual decline by 48 hr. While their expression was parallel we were unable to detect a correlation between gp46 and hsp72.

Our data indicates that the increase expression of gp46 is a result of viral protein synthesis at the translational level. Previously, we observed there was a difference between the magnitude of stress-induced change of viral gene expression in HTLV-1 transformed
cell lines in that the amount of intracellular envelope protein generally was 2-3 fold greater than the increase in viral RNA transcripts suggesting a posttranscriptional event (1). In this study, we determined the half-life of gp68 by metabolically labeling MT 2.6 cells following stress induction. We demonstrate that there is no difference in gp68 turnover in stressed cells as compared to nonstressed controls. However, when protein synthesis was inhibited by cycloheximide and the amount of gp68 determined following stress induction, we found there was no increase in expression of gp68 in the stressed cells as compared to controls. Thus, indicating that the stress-mediated increase in envelope expression in HTLV-1 transformed cell lines is likely a result of increased synthesis due to increase translation.

Induction of the cellular stress response induces the expression of a set of proteins referred to as hsp or cellular stress proteins (5). Following physiologic stress, there is increased transcription and translation of hsp with decrease synthesis of most other cellular proteins (15, 12). This preferential pattern of protein synthesis is to promote survival of the cell following stress induction (12). The mechanism of translational control is not completely understood however it appears there is suppression of translation of non-hsp mRNA rather than degradation (17). One major role of regulation is inhibition of polypeptide chain initiation (13, 6). There is also evidence that elongation is another mechanism of translational regulation of hsp messages (2, 18). It has been shown that ribosomes elongate faster on hsp mRNA following stress compared to nonstressed cells (12). One study evaluating the turnover of c-myc and c-myb following thermal stress demonstrated not only an increase in the half-life of c-myc (five- to eightfold) and c-myb
(twofold) but also increase protein synthesis (two-fold) of c-myc at the translation level (7). This resulted in an increase of c-myc protein expression following thermal stress (7). The reason for the selective synthesis and increased turnover of c-myc is unknown, but speculated that there is either a need for the protein during stress recovery or for the reinitiation of proliferation (7).

Although the mechanism is unknown, we speculate that the increase in synthesis of HTLV-1 env protein may be translationally controlled similar to hsp following stress induction (e.g. preferential mRNA translation). Biologically, this may provide an escape mechanism for virion release from a damaged cell or perhaps a protective cellular mechanism to allow enhanced recognition of the highly immunogenic env protein.

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Figure 5.1: Surface expression of gp46 following induction of the cellular stress response in MT 2.6 cells. Immunofluorescent staining for surface gp46 on MT 2.6 cells was determined by flow cytometric analysis at 24 and 48 hr post cellular stress. The data is expressed as the number of cells (%) which are more positive for gp46 as compared to nonstressed controls. ±SEM of 5 trials for 24 hr and 3 trials for 48 hr.
Figure 5.2: Kinetic expression of gp46 and hsp72 in MT 2.6 cells following induction of the cellular stress response. MT 2.6 cells were labeled for gp46 or hsp72 at 1, 3, 6, and 24 hr following stress induction and compared to nonstressed labeled controls.
Figure 5.3: Effect of cellular stress on HTLV-1 gp68 turnover. A) MT 2.6 cells were pulsed labeled with $[^{35}\text{S}]$-methionine and $[^{35}\text{S}]$-cysteine, and chased after Na arsenite treatment. Cell lysates from nonstressed and stressed cells were immunoprecipitated with anti-gp46 MAb. B) Graphic representation of the degradation of gp68 shown in panel A.
Figure 5.4: Effect of cellular stress on translation of HTLV-1 gp68. A) MT 2.6 cells were stressed with Na arsenite, then cycloheximide (100ug/ml) was added. The expression of gp68 was determined 24 hr later by western blot analysis. B) Graphic representation, the data points represent the mean of two trials. s=stressed cells, ns=nonstressed cells, cont.=non-treated, cyhx=cycloheximide treated
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