The Cellular Stress Response Enhances Human T-Cell Lymphotropic Virus Type 1 Basal Gene Expression through the Core Promoter Region of the Long Terminal Repeat

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Viral protein expression is postulated to play a critical role in the pathogenesis of human T-cell lymphotropic virus type 1 (HTLV-1)-associated diseases. Therefore, knowledge of the cellular events which initiate or enhance viral gene expression is important in understanding the mechanism of HTLV-1-induced disease. In this report, we examined the modulation of transcription of the HTLV-1 long terminal repeat (LTR) following induction of the cellular stress response. 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.

Human T-cell 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 (7). 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 (22). 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 (20). Recent reports have implicated the stress response in the activation of latent viruses. For example, thermal and chemical stress initiated transcription of a cytomegalovirus major immediate early transcription unit in stably transfected Rat-9G cells (4). Several reports have shown thermal stress to activate transcription of the human immunodeficiency virus type 1 long terminal repeat (LTR) (5, 8, 19). 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 (15).

We have previously reported that both thermal stress and Na arsenite increased HTLV-1 viral protein expression in persistently infected lymphocyte cell lines (1). In this study, 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 the persistently infected HTLV-1-transformed T lymphocytes MT 2.6 (11) and HUT 102 (3), as previously described (1). Whole-cell extracts prepared as described previously (15) were collected at 6, 12, and 24 h postinduction of the cellular stress response in MT 2.6 and HUT 102 cells. An HTLV-1 promoter template was prepared by digesting the LTR luciferase plasmid (14) with *Hind*III (Fig. 1). Preinitiation complexes were formed in the presence of DNA template (200 to 300 ng), whole-cell extract (3 μ g), and dialysis buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 2 mM dithiothreitol, 17% glycerol) at 30°C for 30 min. RNA synthesis was initiated with the addition of nucleotides (666 μ M each of GTP, CTP, and ATP; 10 μ Ci of [³²P]UTP [800 Ci/mmol]) and incubated for an additional 30 min at 30°C. 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 Phosphor Imager. 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. The data represent at least three independent experiments with two trials done in triplicate with the internal recovery control. Whole-cell extracts collected at 6 h following stress directed more transcription (3.1 fold-mean increase) from the HTLV-1 LTR as compared to nonstressed cells (Fig. 2A). Repeated trials demonstrated increased transcriptional activity from stressed whole-cell extracts which ranged from three to fivefold (data not shown). Whole-cell extracts collected at 12 and 24 h post-stress had an amount of transcriptional activity similar to that of the nonstressed cells (Fig. 2A). To determine if the increase in transcriptional activity from the 6-h extracts was specific for the HTLV-1 LTR, we repeated the in vitro transcription assays with the adenovirus major late promoter (AdML) (10). Transcription from the AdML directed similar amounts of transcription with the 6-h stressed extracts and nonstressed extracts (Fig. 2A). Therefore, this suggested that

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FIG. 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 (*dl6.2*) and downstream (*dl + 157*) deletion constructs.

the increased transcriptional activity in the 6-h stressed extracts was HTLV-1 LTR specific.

The *in vitro* transcription assays were repeated with the upstream deletion mutant, *dl6.2* luciferase (Fig. 1). The deletion construct was prepared from the pU3Rcat *dl6.2* plasmid (2), which is a deletion mutant of the wild-type LTR promoter (18) and lacks nucleotides -440 to -52. The *dl6.2* was digested with the restriction enzymes *Xba*I/*Bgl*II, and the promoter fragment was isolated by GENECLON II (BIO 101, Inc., La Jolla, Calif.) and cloned into the *Xba*I/*Bgl*II site of pGEM4Z (Promega, Madison, Wis.). The pGEM *dl6.2* construct was digested with *Sac*I/*Hind*II, and the promoter fragment was isolated and cloned into the *Sma*I/*Sac*I site of a luciferase reporter plasmid (pGL2-Basic; Promega, Madison, Wis.), and designated *dl6.2* luciferase.

The *dl6.2* luciferase DNA template was prepared by linearizing the plasmid with *Hind*III (Fig. 1). Transcription from 6-h stressed extracts directed more transcription (3.9-fold mean increase) from the deletion construct, as demonstrated by increased RNA synthesis compared with that in nonstressed 6-h extracts (Fig. 2B). These results suggest that the upstream enhancer regions are not responsible for the stress-mediated increase in transcription.

The HTLV-1 LTR 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 (16). This overlapping transcriptional unit is resistant to low concentrations of α -amanitin not typical of polymerase II but sensitive to high concentrations more characteristic of polymerase III (16). To determine if the stress-mediated increase in transcriptional activity was directed by polymerase II predominantly or the overlapping transcriptional unit, *in vitro* transcription assays were repeated in the presence of low (6 μ g/ml) and high (60 μ g/ml) concentrations of α -amanitin. In the presence of low concentrations of α -amanitin, stressed extracts still directed increased transcription from the LTR compared to that in nonstressed extracts and to the same degree as that observed in the absence of α -amanitin (Fig. 3). Therefore, suggesting that the stress-mediated effect is through the overlapping transcriptional unit of the viral promoter. The AdML promoter was used as a prototypic promoter transcribed by polymerase II, and as expected was sensitive to low concentrations of α -amanitin (Fig. 3).

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 transfections were performed. HeLa cells were transfected with plasmid

FIG. 2. Whole-cell extracts collected from MT-2.6 cells at 6 h following physiologic stress direct more transcription from the HTLV-1 LTR than nonstressed extracts. (A) *In vitro* transcription assays were performed with whole-cell extracts collected at 6, 12, and 24 h 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 (616 bp) was used as an internal recovery control. One of three reactions is demonstrated. The mean relative ratio (triplicate assays) of RNA:DNA loading control is shown graphically. 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-h extracts collected from stressed MT 2.6 cells continued to direct more transcription from this truncated promoter than did those from nonstressed control extracts. The data represents at least three independent experiments with two trials done in triplicate with the internal recovery control. NS, nonstressed; S, stressed.

constructs by using a commercially available lipid-mediated delivery system (Transfectam; Promega, Madison, Wis.). Cells were transfected for 3 h following the recommended assay protocol for media without serum by the manufacturer (Promega). HeLa cells were transfected with the luciferase reporter

FIG. 3. 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 h following induction of the cellular stress response in HTLV-1-infected cells. The reactions were also performed in the presence of 6 or 60 μ g of α -amanitin per ml. Transcription from the AdML promoter was also performed in the presence of 6 μ g of α -amanitin per ml. NS, nonstressed; S, stressed.

FIG. 4. The cellular stress enhances basal transcription from the HTLV-1 LTR as early as 6 h following stress induction. HeLa cells were transfected with an HTLV-1 LTR reporter plasmid, and luciferase activity was measured at 3, 6, 12, and 24 h following induction of the cellular stress response with Na arsenite. All transfections were performed in triplicate in 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 are presented as the mean fold increase in luciferase activity. NS, nonstressed; S, stressed.

plasmids, physiologic stress was induced 24 h later, and luciferase activity was measured in cell lysates by liquid scintillation counting following manufacturer's recommendations (Promega). All transfections were performed in triplicate in at least three independent trials. A significant ($P < 0.05$) increase in reporter gene activity of similar magnitude (2.0- to 2.9-fold mean difference) was observed in stressed extracts collected at 6, 12, and 24 h compared to that in nonstressed transfected controls (Fig. 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 (pHTLV-1 Tax) (13) were performed. Cotransfections with the two constructs in non-stressed control cells had an increase in luciferase activity of 8- to 12-fold over that of LTR-transfected cells alone (Fig. 5B). 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. 5A and 5B). To determine if the increase in reporter gene activity was a result of posttranscriptional modifications, all transfections were repeated with the simian virus 40 promoter (pGL2-Control plasmid; Promega), which has been shown to be nonresponsive to physiologic stress (8). No difference in luciferase activity was observed between stressed and non-stressed transfected HeLa cells with the simian virus 40 luciferase plasmid (Fig. 5A and 5B). Therefore, these results indicate that the increase in luciferase activity from the transfected stressed cells is HTLV-1 LTR specific and transcriptionally controlled.

To localize sequences of the LTR that are stress responsive, transient transfections were repeated with the deletion mutant, *dl6.2* luciferase, depicted in Fig. 1. This deletion promoter lacks the upstream enhancer elements which confer Tax responsiveness. We found that physiologic stress stimulated a significant ($P < 0.05$) increase in transcription from *dl6.2* lu-

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LTR	LTR	LTR + Tax	LTR + Tax
ns	s	ns	s
1.52±.12*	3.47±.56	12.83±3.79	39.44±7.44
.94±.15	2.91±.37	11.62±3.04	22.72±4.01
.55±.10	1.33±.16	4.36±.70	8.92±2.06

SV40	SV40
NS	S
1.47±.22	1.48±.13
3.07±.63	3.41±.54
nt	nt

*luciferase activity (cpm × 10⁵); mean±standard deviation

FIG. 5. The cellular stress response stimulates basal transcription of the HTLV-1 LTR and is Tax independent. 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 h following transfections. Twenty-four hours following physiologic stress, luciferase activity was determined and compared to nonstressed cells. The simian virus 40-luciferase plasmid was transfected as described above and used as a non-stress-responsive control. All transfections were performed in triplicate in three independent trials. (A) Luciferase activity of extracts from nonstressed transfected cells was assigned a value of 1.0. The data are presented as the mean fold increase in luciferase activity. NS, nonstressed; S, stressed. (B) Absolute values of luciferase activity from the 3 trials performed in triplicate expressed in counts per minute (cpm) × 10⁵.

ciferase (Fig. 6A and 6B) compared to that in nonstressed control cells and to a similar degree (2.3-fold mean difference) as the wild-type promoter (2.6-fold mean difference).

Sequences downstream of the RNA initiation site in the LTR have been shown to be important for enhanced basal gene expression. Kashanchi et al. (6) reported a non-Tax-responsive 45-nucleotide sequence (+195 to +240) which was found to upregulate basal expression of HTLV-1. This region was later described to bind a protein homologous to the transcriptional factor YB-1. A 136-bp fragment (+104 to +240) was also reported to be important in basal gene expression and functioned independently of Tax (12). 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 tran-

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LTR ns	LTR s	dl+157 ns	dl+157 s
1.51±.15*	3.04±.55	.52±.02	1.04±.13
.70±.13	2.01±.63	.17±.03	.43±.03
4.03±.24	12.5±1.88	.79±.03	2.33±.21

dl6.2 ns	dl6.2 s
.29±.02	.58±.11
.07±.02	.18±.03
.44±.06	1.11±.23

*luciferase activity (cpm × 10⁵); mean±standard deviation

FIG. 6. 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 h following stress and compared to nonstressed cell extracts. All transfections were performed in triplicate in three independent trials. (A) Luciferase activity of extracts from nonstressed transfected cells was assigned a value of 1.0. The data are presented as the mean fold increase in luciferase activity. NS, nonstressed; S, stressed. (B) Absolute values of luciferase activity from the three trials performed in triplicate expressed in counts per minute (cpm) × 10⁵.

scription (21). 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 the deletion construct, *dl + 157* luciferase, which lacks sequences +157 to +278 (Fig. 1). The pU3Rcat *dl + 157* plasmid (6) was digested with *Bgl*II, and the promoter fragment was purified, cloned into the luciferase reporter plasmid pGL2-Basic, and designated *dl + 157* luciferase. However, a significant increase ($P < 0.05$) in reporter gene activity was still observed (Fig. 6A and 6B) following induction of the cellular stress response, indicating that these sequences do not confer stress-mediated transcriptional increases.

We determined the kinetics of the stress-mediated response and found that whole-cell extracts collected at 6 h post-stress increased transcription while extracts collected at 12 and 24 h

transcribed a similar amount of RNA as nonstressed controls (Fig. 2). Transcriptional activation of the LTR as evaluated by transient transfections showed no increase in stress-mediated transcription at 3 h but an increase at 6 h as well as 12 and 24 h post-stress (Fig. 4). This continued increase in luciferase activity from stressed extracts at 12 and 24 h is likely a consequence of accumulation of luciferase (17). 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 (9). However, during this recovery phase transcription and translation of cellular stress proteins increase with the peak in stress protein synthesis at 3 to 6 h following stress induction (9). Our data demonstrate no increase in transcription until 6 h following stress induction. Therefore, these data suggest that the stress-mediated increase in transcription of the LTR does not occur during the recovery phase following physiologic stress and therefore stress protein synthesis may be important in this event.

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 suggest that the effect on the promoter may be mediated through the overlapping transcriptional unit. The stress-mediated increase in transcription does not follow the kinetics of a stress protein gene, but it 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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