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Biochemical and Biophysical Research Communications 332 (2005) 569–584
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GITR overexpression on CD4+CD25+ HTLV-1 transformed cells: Detection by massively parallel signature sequencing

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Received 25 April 2005
Available online 6 May 2005

Abstract

HTLV-I is the etiologic agent of adult T-cell leukemia (ATL), a fatal T-cell malignancy that is associated with profound immunosuppression. In this study, comprehensive gene expression profiling was performed using massively parallel signature sequencing (MPSS) to investigate virus–host interactions in acutely HTLV-1 transformed cells. The analysis revealed the modulation of numerous genes across different functional classes, many of which have not been previously implicated in HTLV-1 transformation or ATL. Differences in the transcriptomes of transformed cell lines were observed that have provided clues on how different clonal populations of cells respond to virus transformation. Quantitation of HTLV-1 transcription was possible, thus making MPSS a useful tool to study emerging pathogens and unknown microbial causes of human diseases. Importantly, overexpression of GITR, an activation marker that has not been previously reported to be upregulated by HTLV-1-infection or in transformed/leukemic cells and that is associated with the suppressor phenotype of CD4+CD25+ regulatory T-cells (Tregs), was also observed. The deep and quantitative gene expression profile generated by MPSS should provide additional leads for discovery research that can be applied to better understand the pathobiology of HTLV-1 transformation and ATL as well as to developing new therapies.

Keywords: Regulatory T-cells; Adult T-cell leukemia; Cancer; Gene expression profiling; Tax

Human T-cell lymphotropic virus type-1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL) [1–4]. ATL is characterized by a clonal malignancy of infected mature CD4+ T-cells with a CD2+, CD3+, CD4+, CD8−, CD25+, and HLA-DR1+ phenotype, resistance to chemotherapy, impairment of cell-mediated immunity, and development of opportunistic infections [5,6]. The HTLV-I genome encodes several proteins that appear to contribute to infection and oncogenesis. HTLV-1 Tax has been identified as the major oncoprotein that is believed to play a central role in the leukemogenesis of ATL [7–10]. The use of alternative splicing and internal initiation codons to produce virally encoded transcripts imparts the HTLV-1 genome with a complex organizational structure. The regulatory and accessory proteins encoded by the virus play significant roles in viral infectivity, maintenance of high viral loads, host cell activation, regulation of gene transcription, and ultimately in the regulation of virus–host cell interactions during HTLV-1 infection [11–19].

Despite extensive studies on the biology and pathology of HTLV-I-induced disease, the scale of molecular interactions that occurs between viral proteins and the host cellular transcriptional machinery, and that leads to HTLV-1 leukemogenesis, remains largely unknown. In vitro transformation of CD4+ T-cells with HTLV-1

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.162

serves as a model of the early events that lead to HTLV-1 leukemogenesis. In order to understand the complex and varied effects of the virus on the host genome, it is important to interrogate gene deregulation on a global basis—where the depth of coverage encompasses all the transcripts that are actively participating in the transformation process. In order to obtain a comprehensive and quantitative analysis of all genes expressed in HTLV-1 transformed cells, we used a new and powerful gene profiling tool called massively parallel signature sequencing (MPSS). MPSS interrogates the global transcriptome of the sample under study and the analysis is not limited to, or biased by, a pre-determined set of genes. It does this by capturing on a microbead and then sequencing every individual mRNA transcript produced from every gene in a sample and generating a quantitative measure of transcript abundance [20–22]. In addition, MPSS provides sensitivity in the range of a few molecules of mRNA per cell [23–25].

In this study, MPSS was used to compare the transcription profiles of PHA/IL-2-activated CD4+ T-cells and two acutely in vitro HTLV-1 transformed CD4+ T-cell lines. As compared to the activated CD4+ T-cells the MPSS analysis of the acutely HTLV-1 transformed cells showed significant modulation of large numbers of genes in different functional classes. The MPSS data confirmed bi-directional modulation of numerous genes that have been previously reported for HTLV-1 transformed, Tax expressing cells and ATL leukemic cells using microarray or biochemical techniques. Importantly, the MPSS data also revealed modulation of many genes that have hitherto not been implicated in acute in vitro HTLV-1 transformation and/or ATL [26–31]. In particular, overexpression of glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), an activation marker that also appears to be involved in the suppressor phenotype of CD4+ CD25+ regulatory T-cells (Treg) in vivo, was seen and confirmed by surface FACS staining of acute and chronic HTLV-1 transformed cells. MPSS was also able to capture and quantitate viral transcription. The MPSS data provide a comprehensive and quantitative transcriptional database that, together with other microarray based transcriptional studies, should aid in our understanding of HTLV-1 transformation and leukemogenesis.

Materials and methods

CD4+ T-cells and HTLV-1 transformed CD4+ T-cell lines. CD4+ T-cells were purified from peripheral blood using RosetteSep CD4+ T-Cell Enrichment Cocktail (StemCell Technologies, 15062) according to the manufacturer's instructions. Briefly, 50 μl RosetteSep cocktail was added per milliliter of blood, mixed well, and then incubated for 20 min at RT. The samples were diluted with an equal volume of PBS + 2% FBS and mixed gently. The diluted sample was layered on top of Ficoll–Paque without mixing and then centrifuged at room temperature for 20 min at 1200*g* with the brake off. The enriched cells were removed from the density medium: plasma interface and then washed with PBS + 2% FBS two times. The cells were resuspended in 3 ml ammonium chloride for 3 min to lyse RBCs followed by washing three times with 20 ml of 10% FBS/AIM-V medium, centrifugation at 1200 rpm for 10 min, and resuspension of the cells in 10% FBS/AIM-V medium (Gibco-BRL, 12055) prior to final cell counting. The two Tax expressing IL2-independent clones termed B1 and C5 used in this study were established by co-culture with HTLV-1 producing MT2 cells as previously reported. FACS confirmed that the B1 and C5 clones were CD4+, CD8−, and CD25+ [32].

Reverse transcriptase assay for analysis of virion production. B1, C5, MT2, and Jurkat E6-1 cells (5×10^7) were cultured in 50 ml RPMI 1640 plus 10% FBS for 72 h. The cells were then centrifuged, the supernatant was filtered through a 0.45 μl filter, and then 25 ml supernatant was ultracentrifuged at 27,000 rpm (Beckman, Rotor SW-28) for 2 h at 4 °C. The precipitated viruses were dissolved in 500 μl fresh media, then aliquoted, and frozen on dry ice–ethanol mixture. Two microliters of concentrated viruses was suspended in 8 μl of reverse transcriptase suspension buffer (50 mM Tris–Cl, pH 7.5, 1 mM DTT, 20% glycerol, 0.25 mM KCl, and 0.25% Triton X-100), 5× reverse transcriptase (RT) buffer (250 mM Tris–Cl, pH 7.5, 37.5 mM $MgCl_2$, and 0.25% Triton X-100) 10 μl, 0.5 M DTT 0.48 μl, poly(A) 5 μl, thymidine 5′-triphosphate (tetrasodium salt) 1 μl, and 23.52 μl distilled water. The mixture was quickly vortexed, centrifuged, and incubated at 37 °C for 1 h. The contents were then spotted on Whatman 2.3 cm filter paper (Grade DE-81). Membranes were dried on SARAN-wrap and washed three times with 2× SSC for 10 min (each wash) and 95% ethanol for 10 s. The membranes were dried on aluminum foil and placed individually in scintillation vials. Five milliliters of scintillation solution was added to each vial and counted. Samples from each cell type were analyzed in triplicate and the experiments were repeated twice.

FACS analysis of GITR and CD25 expression. $CD4^+$ T-cells were purified from peripheral blood using RosetteSep $CD4^+$ T-Cell Enrichment Cocktail (StemCell Technologies) as described earlier [32]. Cells were separated into seven groups: group A, fresh CD4+ cells, cultured in 10% FBS/AIM-V medium without stimulation; group B, CD4+ cells, cultured for 72 h without stimulation; group C, CD4+ cells, cultured for 72 h with PHA (1 μg/ml) and IL-2 (100 U/ml); group D, CD4+ cells, cultured for 120 h with PHA (1 μg/ml) and IL-2 (100 U/ml); group E, B1 cells; group F, C5 cells; and group G, MT2 cells. For FACS analysis $5–10 \times 10^5$ cells were washed with 2% FBS/PBS in a 1.5 ml tube. Cells were blocked with 2% FBS/PBS for 30 min on ice and pelleted. Cells were resuspended in 50 μl of 2% FBS/PBS with primary antibody, incubated for 1 h on ice, and then the process was repeated with secondary antibody. Cells were washed with 2% FBS/PBS three times, fixed in 500 μl of 2% paraformaldehyde, and transferred into FACS tubes for FACS analysis. The following antibodies were used: FITC-labeled anti-human GITR MAb (R&D systems), and PE-labeled anti-human CD25 MAb (BD Pharmingen), PE-labeled isotype control mouse MAb (BD Pharmingen). The FACS analysis was repeated twice for each antibody.

RNA isolation. For isolation of total RNA, acutely HTLV-1 transformed cell lines B1 and C5 and human peripheral blood CD4+ T-cells (after 72 h of activation with 1 μg/ml PHA and 100 U/IL2) were washed three times and the cell pellets were treated with Tripure reagent (Roche Diagnostics) and the RNA was precipitated in ethanol. This procedure routinely provides yields of >200 μg of total RNA from circa 1×10^8 cells. In the MPSS experiments described below, ca. 500 ng of mRNA was used for each MPSS analysis.

Sample preparation for MPSS analysis. $Poly(A)^+$ mRNA was prepared from total RNA, then reverse transcribed and the cDNA was digested with *Dpn*II. The 21 bases adjacent to the 3′ most *Dpn*II site were cloned into a Megaclone vector as described in the MPSS standard protocol [20,21]. The resulting library was amplified and loaded

onto microbeads. About 1.6 million microbeads were loaded into each flow cell and the signature sequences were determined by a series of enzymatic reactions as outlined in Brenner et al. [20,21]. The abundance of each signature was converted to transcripts per million (TPM) for the purpose of comparison between samples. Four separate MPSS runs on each sample were performed and the TPM value(s) for a particular signature were derived from an average of two runs with the highest TPMs.

Annotation and filtering of MPSS data. To generate a complete annotated human signature database for MPSS analysis, all possible signatures (virtual signatures) from the human genome sequence (Release #13) and human UniGene sequences (Unigene build #162) based on the presence of the *Dpn*II site (GATC) were extracted. Each virtual signature was then ranked based on its position and orientation in the original sequence. The annotation for that sequence was then assigned to a signature and the resulting database was used to annotate signatures from all experiments. The results were generated as flat files containing signature sequences, TPM values, and standard deviation for each TPM (for each signature in the HTLV-1 transformed and control samples). Reliability values for all MPSS signature were based on the presence of a poly(A) tail, poly(A) signal or both, and the relative position of the signature (3′ most or 5′ most). Signatures which did not have a poly(A) signal and a poly(A) tail (designated Class 25 signatures) were considered less reliable than those with one or both features. Unless otherwise indicated, the most reliable signatures (having both a poly(A) signal and a poly(A) tail, or only a poly(A) signal or a poly(A) tail, designated as Class 1, 2, and 3, respectively) were used to make judgments on up- or downregulation of genes. The data also provided *p* values that present a measure of significance when two signatures are compared across treatments, i.e., experimental (HTLV-1 transformed CD4+ T-cells) versus control (activated CD4+ T-cells). Only signatures that had *p* values of 0.001 or lower were used for further analysis. TPM values were used to determine which signatures were upregulated (experimental TPM > control TPM) and downregulated (experimental TPM < control TPM) between samples. The reliability of the TPM values is, in part, based on the results from yeast spiking studies. For example, when 10 TPM of 11 different genes were spiked into a yeast sample 91% of the samples had observed TPM < 15 and 82% of the samples had TPM > 0. In another spike study when 25 TPM of three different genes were spiked none of the spiked samples had TPM = 0 (data not shown). From these results we assume that a TPM value of zero translates to <10 TPM. Differences in TPM values between control and transformed cells were considered significant when the change was greater than 2-fold. Wherever possible changes in individual genes were correlated with changes to other genes known to function in the same pathway to enhance reliability of deregulation. TPM values were translated into a number of transcripts per cell using the relation 3 TPM = 1 transcript [33,34]. Expression of genes in a particular sample was also considered significant if the TPM value demonstrated the presence of a large number of transcripts per cell.

Molecular characterization of HTLV-1-induced transformation. To investigate the molecular basis of HTLV-1-induced T-cell transformation, the gene expression profiles of acutely HTLV-1 transformed CD4+ B1 and C5 cells were compared with those of 72 h. PHA/IL-2-activated CD4+ T-cells. The differentially expressed genes were classified into functional categories by translating their LocusLink IDs into Gene Ontology (GO) terms (based on the GO classification available via ftp from NCBI LocusLink: ftp://ftp.ncbi.nih.gov/refseq/LocusLink/). Only a limited set of gene categories: anti-apoptosis/pro-apoptosis, NF-κB, TNFR and ligand superfamily, DNA repair, cell cycle, and mitosis were selected for further analysis based on their known or suspected involvement in HTLV-1 transformation. Since B1 and C5 cells were established as separate IL2-independent acutely HTLV-1 transformed clones, differences in gene expression between B1 and C5 cells were also analyzed simultaneously.

Results

MPSS analysis of gene signatures from control and acutely HTLV-1 transformed cells

Table 1 summarizes the results of the MPSS analysis performed on the three cell populations used in the study. Circa 2.6 million beads were successfully sequenced four times for each of the three samples tested. This resulted in the identification of 17,797–29,904 distinct signatures for each cell population. Reliable (signatures observed in more than one run across all the runs) and significant (defined as >4 TPM for a given signature) signatures varied from 9975 to 13,312. These signatures included those that could be assigned to unique genes and therefore were identifiable by UniGene Ids and those that did not match known genes. All bioinformatics analyses of MPSS datasets were performed using Perl scripts on a Unix or a Windows platform.

The results of signatures with UniGene Ids from PHA/IL-2-activated CD4+ T-cells and acutely HTLV-1 transformed B1 and C5 cells were compared. This included more than 9500 genes. We felt that this comparative analysis against PHA/IL-2-activated CD4+ T-cells from which the transformed cells were derived, as opposed to using signatures from resting CD4+ T-cells for the comparison, would be the most informative for analyzing modulations in gene transcription. Likewise, we chose for this initial analysis not to compare the acutely HTLV-1 transformed T-cell leukemia lines to non-HTLV-1 transformed T-cell leukemia lines which

Table 1
Summary of signatures sequenced from PHA/IL-2-activated CD4+ T-cells and acutely HTLV-1 transformed B1 and C5 cells using MPSS

Cell type used for mRNA isolation and MPSS analysis	Total beads successfully sequenced	Total distinct signatures	Reliable and significant signatures
Activated CD4+ T-cells	2,623,556	17,797	9,975
Acutely HTLV-1 transformed B1 Cells	2,790,701	22,100	11,130
Acutely HTLV-1 transformed C5 Cells	2,602,065	29,904	13,312

References in Tables 3–8 refer to previously reported findings from microarray-based transcription profiling analysis of HTLV-1 infected/transformed cells. In all tables, ND indicates that a transcript was not detected or was represented by a Class 25 signature which neither has a poly(A) tail nor a poly(A) signal and therefore has low reliability.

have been chronically cultured for many years and have likely accumulated multiple mutations.

Analysis of HTLV-1 transcription by MPSS and virus production from B1 and C5 cells

One unique capability of MPSS, as compared to microarray analysis, is to quantitatively analyze HTLV-1 transcription simultaneously with cellular gene transcription. An analysis of the HTLV-1 genome based on the *Dpn*II digest revealed 46 signatures, 23 each from the plus and minus strands. Out of the 46, 13 and 24 signatures were detected, respectively, in B1 and C5 cells, and none in the PHA/IL-2-treated CD4+ T-cells by MPSS. Table 2 shows signatures that were detected from the plus strand of the HTLV-1 genome. The most abundant signature (GATCCGTCTGAAAACAG, 5746 TPM in B1, 25,086 TPM in C5, Table 2) was the 3′ most signature on HTLV-1 genome which maps to transcripts in the pX region of the HTLV-1 genome (Fig. 1A) and is identified by the *Dpn*II site at 7105 bp on the HTLV-1 genome (Fig. 1B). This may arise by several mechanisms including, for example, a bicistronic Tax/Rex mRNA which uses the pX splice acceptor at position 6950 bp, a transcript containing exon 1 (nucleotide 1–119) joined to the splice acceptor site at 6950 bp to form p21 Rex mRNA, and others [35,36] as shown in Fig. 1C. The signatures originating at *Dpn*II sites 3886 and 4587 bp (which lie 3′ to exon 1) may arise by premature termination of transcription beyond exon 1. In contrast, the *Dpn*II site at position 4801 bp lies between the alternative splice acceptors 4641/4658 bp and the splice donor site at position 4831. These splice junctions define exon 2 and suggest that these signatures may arise again by transcription termination after splicing of exon 1 and exon 2. The relative low abundance of the signatures located near the more 5′ *Dpn*II sites, as well as those located in the *env* gene compared to the most abundant signature located near the most 3′ *Dpn*II site, suggests that premature termination of transcription in B1 and C5 cells is an infrequent event. This is not surprising since Tax, which is required for viral transcriptional elongation and transactivation, is expressed in these cells [32]. Abundance of transcripts arising from the 3′ *Dpn*II site also suggests that virus particles were being produced by these cells. Indeed, as shown in Fig. 2, there was increased reverse transcriptase activity in both B1 and C5 cells compared to the control cell line Jurkat E-6. The RT activity was greater for C5 cells as compared to B1 cells (in agreement with the higher TPM values of HTLV-1 transcripts for C5 cells compared to B1 cells shown in Table 2). In addition, the cell free RT activity for C5 cells was at the same level as for MT-2 cells. However, the RT activities in the B1, C5 as well as MT2 supernatants are all weak. These results indicate that the HTLV-1 transformed cell lines do not produce large amounts of cell-free HTLV-1 virions. This result is in agreement with the known observation that HTLV-1 infection of CD4+ T-cells with virus containing supernatants is inefficient unless there is direct cell-to-cell contact [37].

Upregulation of anti-apoptotic/pro-apoptotic genes

Previous studies have documented the role of anti-apoptosis genes in HTLV-1 transformation. Therefore, this was one of several functional gene categories that was analyzed in this investigation. As shown in Table 3, several genes in this functional category showed significant upregulation in the HTLV-1 transformed cells compared to PHA/IL-2-activated CD4+ T-cells. This included the anti-apoptotic gene BIRC3 that was upregulated 18-fold in both B1 and C5 cells. BCL2L1 (bcl-xL) was also observed to be highly expressed in B1 and C5 cells with TPM values that were 25- and 10-fold upregulated. Other genes which showed a smaller and/or variable changes in expression levels included Akt2, a member of the Akt family with cell cycle progression and transforming activity, that was differentially upregulated in B1 and C5 cells, and BCL2 which was upregulated 2-fold in B1 cells but unchanged in C5 cells. The anti-apoptotic gene

Table 2
Detection of plus strand HTLV-1 transcripts in B1 and C5 cells

Signature	HuB1 (TPM)	HuC5 (TPM)	Location of *Dpn*II site (bp)	Genes
GATCCCCGAGACCAAAT	ND	83	3028	gag-pro-pol, pol
GATCATTCCCCCTTCCG	ND	14	3623	gag-pro-pol, pol
GATCCCATCTCCAGGCT	18	82	3886	gag-pro-pol, pol
GATCCCAGAGACACATT	6	9	4587	gag-pro-pol, pol
GATCCCAAAGAAAAAGA	10	32	4801	rex, pol
GATCAGGCCCTACAGCC	ND	11	5006	env
GATCGTGCCAGCCTATC	291	1336	5516	env
GATCCCGCTCCCGCCGA	25	187	5748	env
GATCTCCTGTTCTGGGA	ND	13	5975	env
GATCACCTGGGACCCCA	5746	25,086	7105	tax, rex

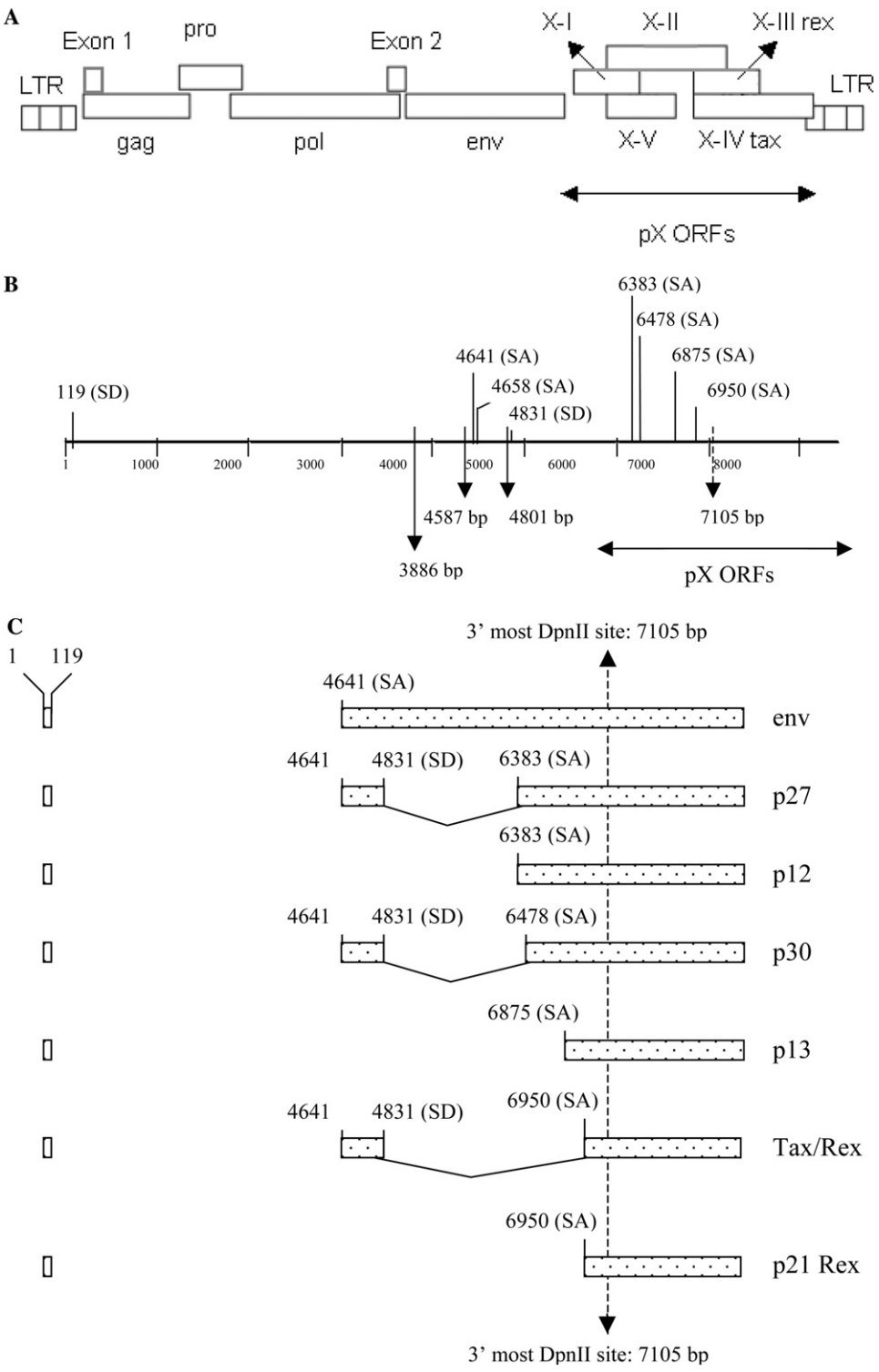

Fig. 1. MPSS analysis of the HTLV-1 genome. (A) The structural gag, pol, env, and regulatory tax (X-IV) and rex (X-III) genes along with the regions comprising exons 1, 2 and the remaining open reading frames (ORFs X-I, X-II, and X-V) spanning the pX region are shown. (B) Distribution of splice acceptor (SA) and donor (SD) sites and the location of 3′ most *Dpn*II site (dotted arrow at 7105 bp) along the HTLV-1 genome (represented by solid line marked at intervals of 1000 bp). Only the locations of the more proximal *Dpn*II sites (positions 3886, 4587, and 4801 bp, solid arrows) and the pX region are shown. (C) Analysis of 3′ most signature derived from HTLV-1 genome by MPSS. Figures on top indicate positions along the HTLV-1 genome in base pairs. Numbered boxes represent exons beginning from the first exon on the left shown by coordinates 1–199 bp. Shown are the predicted spliced transcripts that contain the 3′ most *Dpn*II site (dotted arrow) and contribute to the most abundant signature. The corresponding HTLV-1 transcripts are shown on the right of the boxes. Location of splice acceptor (SA) and donor (SD) sites is shown as in (A) above (figure drawn to scale with reference to B).

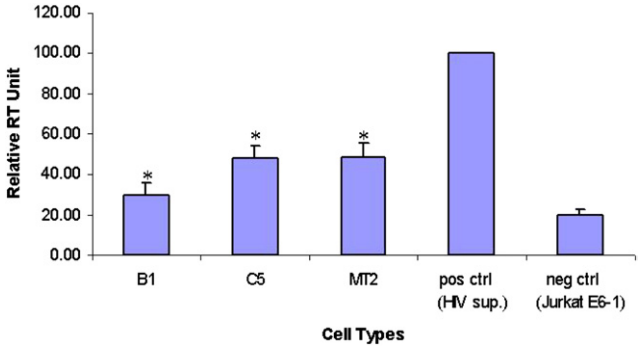

Fig. 2. Reverse transcriptase activity in cell culture supernatants of acutely HTLV-1 transformed (B1 and C5) and chronically transformed (MT-2) cell lines. The RT units were normalized against the positive control (supernatant from 293 T cells transfected with HIV_{NL4-3} provirus). Each error bar represents the mean ± SD of two independent experiments. *t* test shows that RT values in B1, C5, and MT2 group are statistically different to the negative control ($*p < 0.05$).

c-FLIP, a caspase 8 homolog that acts as a dominant negative inhibitor of TNF receptor-induced apoptosis [38], was upregulated 3- and 8-fold in C5 cells and B1 cells, respectively. Caspase 8 itself was not detected in B1 cells and was only expressed at very low levels in C5 cells. The expression of the anti-apoptotic gene DAD1 was upregulated ca. 2-fold in both B1 and C5 cells. Intriguingly, members of the pro-apoptotic BCL2/adenovirus E1B 19 kDa interacting protein (BNIP) family—BNIP3 and BNIP3L (BNIP3-like)—were both upregulated in C5 cells, although BNIP3 was not detected in B1 cells. Both DAD1 and BNIP3 have been previously reported to be upregulated in Tax expressing cells [29].

Activation of the NF-κB pathway

HTLV-1 Tax is known to activate NF-κB and activation of NF-κB plays a central role in HTLV-1 transformation. As shown in Table 4, NFKB2 was upregulated more than 40-fold in both B1 and C5 cells whereas NFKB1 was either unchanged (B1 cells) or downregulated (C5 cells). RELB was also upregulated ca. 4- and 10-fold in B1 and C5 cells, respectively. Interestingly, IKBKB was upregulated 2-fold in B1 as compared to activated CD4+ T-cells and upregulated more than 6-fold in C5 cells. NIK, an upstream kinase that causes IKKα and IKKβ activation, was upregulated more than 2-fold in both B1 and C5 cells. COT, a member of the MAP3K family, was upregulated 10-fold in C5 cells and detected at basal levels in B1 cells. NAK was detected only in activated CD4+ T-cells.

Neutralization of TNFR-mediated apoptotic pathways

The largest number of genes that were analyzed are members of the TNF-related superfamily of membrane-anchored and secreted ligands that are known to play important roles in regulating T-cell growth and survival. As shown in Table 5, of the eight TNFR family members that were detected, four were downregulated and four were upregulated in the HTLV-1 transformed

Table 3
Expression of apoptotic and anti-apoptotic genes in PHA/IL-2 activated CD4+ T-cells and acutely HTLV-1 transformed CD4+ T-cells

HUGO name/LocusLink ID	Gene name	PHA/IL-2-activated CD4+ T-cells (TPM)	Acutely HTLV-1 transformed cells (TPM)		References
			B1 cells	C5 cells	
Akt2, LL.208	v-akt murine thymoma viral oncogene homolog 1, protein kinase B	14	25	68	—
BCL2, LL.596	B-cell CLL/lymphoma 2	9	20	9	Harhaj et al. [26], de La Fuente [28]
BCL2L1 (bcl-xL), LL.598	B-cell CLL/lymphoma 2-like 1	5	125	52	Harhaj et al. [26], de La Fuente [28,29], Pise-Masison et al. [27]
BIRC2, LL.330	Baculovirus inhibitor of apoptosis protein (IAP) repeat containing (BIRC) 2	ND	10	ND	Harhaj et al. [26]
BIRC3, LL.330	Baculovirus inhibitor of apoptosis protein (IAP) repeat containing (BIRC) 3	17	311	320	Harhaj et al. [26], Ng et al. [31], Pise-Masison et al. [27]
BNIP3, LL.664	BCL2/adenovirus E1B 19 kDa interacting protein 3	ND	ND	171	Harhaj et al. [26], Ng et al. [31], de La Fuente [29]
BNIP3L, LL.665	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	ND	64	48	—
c-FLIP, LL.8837	Cellular FLICE inhibitory protein	41	248	135	—
CASP8 (caspase 8), LL.841	Caspase 8, apoptosis-related cysteine protease, FLICE	ND	ND	5	Harhaj et al. [26], Pise-Masison et al. [27]
DAD1, LL.1603	Defender against cell death 1	85	197	162	Harhaj et al. [26], de La Fuente [28,29]

Table 4
Expression NF-κB subunits and activating factors in acutely HTLV-1 transformed cells

HUGO name/ LocusLink ID	Gene name	PHA/IL-2-activated CD4+ T-cells (TPM)	Acutely HTLV-1 transformed cells (TPM)		References
			B1 cells	C5 cells	
NFKB1, LL.4790	Nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (p105)	31	33	6	Harhaj et al. [26]
NFKB2, LL.4791	LYT10, p49/p100	5	202	252	—
IKBKB, LL.3551	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase β	12	24	80	—
RELB, LL.5971	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of κ light polypeptide gene enhancer in B-cells 3	5	18	48	Ng et al. [31], Pise-Masison et al. [27]
NIK, LL.9448	NF-κB inducing kinase, mitogen-activated protein kinase kinase kinase kinase 4	40	103	83	—
COT, LL.1326	Mitogen-activated protein kinase kinase kinase 8	4	4	41	—
TBK1/NAK, LL.29110	TANK-binding kinase 1, NF-κB-activating kinase	35	ND	ND	—

Table 5
Deregulation of TNFR-mediated apoptotic pathways in acutely HTLV-1 transformed cells

HUGO name/LocusLink ID	Gene name	PHA/IL-2-activated CD4+ T-cells (TPM)	Acutely HTLV-1 transformed cells (TPM)		References
			B1 cells	C5 cells	
TNF receptor superfamily (SF) members					
TNFRSF1A, LL.7132	TNF receptor SF, member 1A, TNF-α receptor	24	ND	4	Harhaj et al. [26], Pise-Masison et al. [27]
TNFRSF1B, LL.7133	TNF receptor SF, member 1B, TNF-β receptor	466	6	3	Harhaj et al. [26]
TNFRSF5, LL.958	TNF receptor SF, member 5, CD40	ND	24	176	—
TNFRSF7, LL.939	TNF receptor SF, member 7, CD27	95	ND	ND	Harhaj et al. [26], Pise-Masison et al. [27]
TNFRSF8, LL.943	TNF receptor SF, member 8, CD30	ND	40	52	Harhaj et al. [26]
TNFRSF10B, LL.8795	TNF receptor SF, member 10B, TRAIL-R2, DR5	ND	35	37	—
TNFRSF18, LL.8784	TNF receptor SF, member 18, GITR	76	1256	1490	
TNFRSF25, LL.8718	TNF receptor SF, member 25, DR3, APO-3	221	ND	ND	—
TNF ligand superfamily (SF) members					
TNFSF1, LL.4049	TNF ligand SF, member 1, TNFβ, lymphotoxin-α	ND	867	181	—
TNFSF2, LL.7124	TNF ligand SF, member 2, TNFα	144	8	34	Harhaj et al. [26], Pise-Masison et al. [27]
TNFSF3, LL.4050	TNF ligand SF, member 3, lymphotoxin-β	668	ND	ND	Harhaj et al. [26]
TNFSF4, LL.7292	TNF ligand SF member 4, Tax-transcriptionally activated glycoprotein 1, OX40L	ND	140	130	Harhaj et al. [26], de La Fuente [28], Pise-Masison et al. [27]
TNFSF5, LL.959	TNF ligand SF, member 5, CD40L	2	ND	ND	Harhaj et al. [26]
TNFSF7, LL.970	TNF ligand SF, member 7, CD27L, CD70	ND	1564	2990	Harhaj et al. [26], de La Fuente [28], Pise-Masison et al. [27]
TNFSF10, LL.8743	TNF ligand SF, member 10, TNF-related apoptosis inducing ligand (TRAIL), APO2L	21	6	9	—
TNFSF12, LL.8742	TNF ligand SF, member 12, TNF-related weak inducer of apoptosis (TWEAK), APO3L, DR3LG	64	3	3	—
TNF pathway-related pro- and anti-apoptotic and associated genes					
c-FLIP, LL.8837	Cellular FLICE inhibitory protein	41	248	135	—
CASP3, LL.836	Caspase 3, apoptosis-related cysteine protease	3	39	ND	de La Fuente [29]
CASP8, LL.841	Caspase 8, apoptosis-related cysteine protease, FLICE	ND	ND	5	Pise-Masison et al. [27]
CRADD, LL.8738	CASP2 and RIPK1 domain containing adapter with death domain	2	282	216	
RIPK1, LL.8737	receptor-interacting serine–threonine kinase 1	ND	13	ND	—
SIVA, LL.10572	CD27-binding protein	42	215	136	Ng et al. [31]
TTP, LL.7538	Tristetraprolin	141	28	97	—
TRADD, LL.8717	TNFRSF1A-associated via death domain	10	10	45	Ng et al. [31]

B1 and C5 cells. TNFRSF1A (TNFR1) and TNFRSF1B (TNFR2), the receptors for TNFα and TNFβ, respectively, were downregulated in both B1 and C5 cells. The expression of TNFRSF25 (DR3), a member of the TNF receptor family of death receptors, which binds to downstream signal transducers TRAF2, RIP, FADD, and caspase 8 through association with TRADD [39] was also downregulated. However, there was also upregulation of TNFRSF5 (CD40), TNFRSF8 (CD30), TNFRSF10B (DR5), and TNFRSF18 (GITR). Of the latter, TNFRSF18 was upregulated ca. 20-fold to very high levels in both B1 and C5 cells.

Simultaneously, a number of TNF ligand superfamily members TNFSF2 (TNFα), TNFSF3 (lymphotoxin β), TNFSF10 (APO2L, TRAIL), and TNFSF12 (APO3L, TWEAK) were downregulated. TNFSF7 (CD70, CD27L) was highly upregulated and SIVA, a CD27-binding protein, was upregulated 3- to 5-fold in both B1 and C5 cells, whereas its receptor TNFRSF7 (CD27) was downregulated to undetectable levels in both B1 and C5 cells. Also upregulated were TNFSF1 (TNFβ, lymphotoxin α), TNFSF4 (OX40L), and TNFRSF10B (DR5). These observations are in agreement with the transcription profile for HTLV-1 trans-

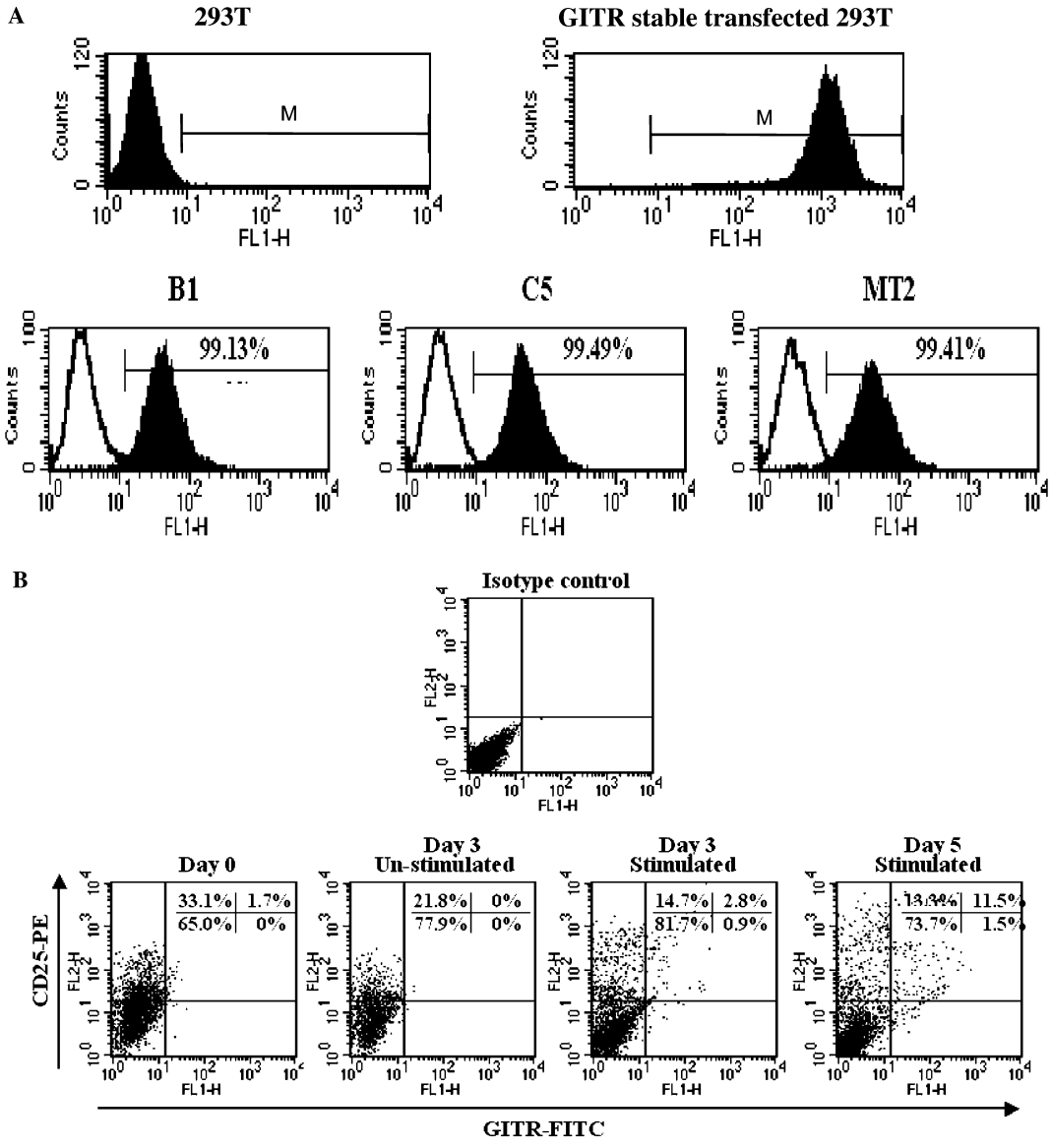

Fig. 3. FACS analysis of GITR expression. (A) CD4+, CD25+ acute (B1 and C5 cells), and chronic (MT-2) HTLV-1 transformed cell lines were stained for GITR expression with FITC-labeled anti-human GITR (solid) or FITC-labeled isotype control IgG (open). The percentage figures indicate the proportion of cells that were positive for GITR expression. Stably transfected GITR expressing 293T and parental 293T cells served as positive and negative control cells. (B) Freshly harvested and purified CD4+ T-cells were stained immediately after purification (day 0), were incubated in culture for three days without stimulation (day 3), or were stimulated with 1 μg/ml PHA plus 100 U/ml IL-2 for 3 days (day 3, stimulated) or 5 days (day 5, stimulated) before double staining with FITC-anti-human GITR and PE anti-CD25 Mabs.

formed cells reported by Harhaj et al. [26]. Among the TNF pathway-related apoptosis adapters, CRADD was upregulated more than 100-fold in B1 and C5 cells, and TRADD was upregulated 4-fold in C5 cells. RIPK1, however, was undetectable in C5 cells and present at very low levels in B1 cells.

The finding that GITR is overexpressed in HTLV-1 transformed cells has not been previously reported using transcriptional profiling microassays. This is of particular interest because CD4+, CD25+, and GITR+ cells represent a phenotype for regulatory T-cells that have been implicated in the suppression of host immune responses including anti-tumor immunity [40–43]. Therefore, this observation provided an important test both to validate the MPSS data and to determine if the high TPM values for GITR mRNA expression correlated with high protein expression levels on B1 and C5 cells. As can be seen in Fig. 3A, FACS analysis of the acutely transformed B1 and C5, as well as chronically HTLV-1 transformed MT2 cells, showed uniformly high levels of surface GITR expression on the entire population of cells. Thus, both acute and chronic HTLV-1 transformed cell lines are of CD4+, CD25+, and GITR+ phenotype. Table 5 also shows that the TPM values for GITR mRNA expression in fresh CD4+ T-cells that were treated for three days with PHA/IL-2 were comparatively lower than the HTLV-1 transformed cell lines. This finding was also confirmed by FACS analysis in which fresh CD4+ T-cells (Fig. 3B, left panel), cells that were either left unstimulated for three days, or cells that were stimulated with PHA/IL-2 for 3 and 5 days and then dually stained for CD25 and GITR were examined. As can be seen in Fig. 3B, there was a time-dependent increase in the percentage of CD4+, CD25+, and GITR+ cells from 0% (day 3 unstimulated) to 2.8% (day 3 stimulation) to 11.5% (day 5 stimulation).

Deregulation of DNA repair genes

The MPSS analysis of B1 and C5 cells also revealed variable downregulation of several genes from different repair pathways: ADPRT (base excision repair (BER) pathway), ERCC5, pol δ (pol δ subunit 2), XAB2 and XAP1 (nucleotide excision repair (NER) pathway), and XRCC3 (recombination repair (RR) pathway) in the HTLV-1 transformed cells as compared to PHA/IL-2-activated CD4+ T-cells (Table 6). For example, ADPRT is downregulated 6-fold in C5 cells but was minimally changed in B1 cells. Expression of DNA polymerase β (pol β), a central participant of the BER pathway [44,45], that was not detected by MPSS has been reported to be suppressed by Tax [46]. Subunit two of DNA polymerase δ (pol δ, POLD2), a principal DNA replicase in eukaryotes which participates in the NER, mismatch repair, and long patch BER pathways [47,48], was downregulated 3- to 4-fold in B1 and C5 cells, whereas pol δ subunits 1 and 4 were expressed at similar levels as PHA/IL-2-activated CD4+ T-cells. HTLV-1 has been reported to enhance pol δ activity via Tax-mediated transactivation of the PCNA pro-

Table 6
Deregulation of DNA repair proteins in acutely HTLV-1 transformed cells

HUGO name/LocusLink ID	Gene name	PHA/IL-2-activated CD4+ T-cells (TPM)	Acutely HTLV-1 transformed cells (TPM)	
			B1 cells	C5 cells
ADPRT[a], LL.142,	ADP-ribosyltransferase (NAD+; poly(ADP-ribose) polymerase), PARP	95	69	16
ERCC3, LL.2071	Excision repair cross-complementing rodent repair deficiency, complementation group 3, XPB, RAD25	ND	27	ND
ERCC5, LL.2073	Excision repair cross-complementing rodent repair deficiency, complementation group 5, XPG	87	10	13
ERCC6, LL.2074	Excision repair cross-complementing rodent repair deficiency, complementation group 6, RAD26	ND	ND	17
HMGN1, LL.3150	High-mobility group nucleosome-binding domain 1	803	472	82
PCNA[b], LL.5111	Proliferating cell nuclear antigen	210	690	170
POLD1, LL.5424	Polymerase (DNA directed), δ1, catalytic subunit 125 kDa	11	1	31
POLD2, LL.5425	Polymerase (DNA directed), δ2, regulatory subunit 50 kDa	143	34	41
POLD4, LL.57804	Polymerase (DNA-directed), δ4	14	31	9
POLS, LL.11044	Polymerase (DNA directed) σ	89	ND	7
RUVBL1, LL.8607	RuvB-like 1 (*E. coli*)	5	8	20
RUVBL2, LL.10856	RuvB-like 2 (*E. coli*)	ND	40	119
XAP1, LL.1642	Damage-specific DNA-binding protein 1, 127 kDa, DDB1	74	ND	23
XAB2, LL.56949	XPA-binding protein 2	203	47	54
XRCC3, LL.7517	X-ray repair complementing defective repair in Chinese hamster cells 3	29	1	ND

[a] Also reported by de La Fuente et al. [29].
[b] Also reported by Pise-Masison et al. [27].

moter [49]. PCNA was upregulated 3-fold in B1 cells and detected at essentially unchanged levels in C5 cells. PCNA has been shown to increase the processivity of pol δ and pol δ-directed synthesis past lesions in DNA resulting in increased nucleotide misincorporation rates [50] and therefore may compensate for the low level expression of pol δ. This is in agreement with the data reported by Pise-Masison et al. [27].

Other proteins that are involved in DNA repair were also deregulated in the HTLV-1 transformed cells. For

Table 7
Deregulation of cell cycle machinery in acutely HTLV-1 transformed cells

HUGO name/LocusLink ID	Gene name	PHA/IL-2-activated CD4+ T-cells (TPM)	Acutely HTLV-1 transformed cells (TPM)		References
			B1 cells	C5 cells	
BUB3, LL.9184	Budding uninhibited by benzimidazoles 3 homolog	28	191	89	—
CCNB1, LL.891	Cyclin B1	46	71	66	Harhaj et al. [26], de La Fuente [28], Pise-Masison et al. [27]
CCNB2, LL.9133	Cyclin B2	115	32	70	—
CCNC, LL.892	Cyclin C	3	ND	2	—
CCND2, LL.894	Cyclin D2	76	294	654	Mori et al. [30], Harhaj et al. [26], de La Fuente [29], Ng et al. [31]
CCND3, LL.896	Cyclin D3	320	ND	14	Mori et al. [30], Harhaj et al. [26]
CCNE1, LL.898	Cyclin E1	22	ND	2	Harhaj et al. [26]
CCNG1, LL.900	Cyclin G1	ND	72	169	Harhaj et al. [26], de La Fuente [28]
CDC10, LL.989	Cell division cycle 10	69	10	4	—
CDC20, LL.991	Cell division cycle 20	46	ND	ND	—
CDC45L, LL.8318	Cell division cycle 45-like (*S. cerevisiae*)	8	14	45	—
CDC25B, LL.994	Cell division cycle 25	68	4	20	de La Fuente [28]
CDK1, LL.983	Cell division cycle 2, G1 to S and G2 to M, CDC2	82	ND	ND	Pise-Masison et al. [27]
CDK2, LL.1017	Cyclin-dependent kinase 2	78	142	380	Pise-Masison et al. [27])
CDK3, LL.1018	Cyclin-dependent kinase 3	ND	ND	6	Pise-Masison et al. [27]
CDK4, LL.1019	Cyclin-dependent kinase 4	65	123	78	Pise-Masison et al. [27]
CDK5, LL.1020	Cyclin-dependent kinase 5	159	4	ND	—
CDKN1A, LL.1026	Cyclin-dependent kinase inhibitor 1A (p21a, Cip1)	ND	3	19	de La Fuente [28], Ng et al. [31], Pise-Masison et al. [27]
CDKN1B, LL.1027	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	3	21	ND	—
CDKN1C, LL.1028	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	ND	2	19	—
CDKN2A, LL.1029	Cyclin-dependent kinase inhibitor 2A (p16INK4)	ND	21	ND	de La Fuente [28]
CSK, LL.1445	c-src tyrosine kinase	263	8	22	Harhaj et al. [26]
CEN3, LL.1070	Centrin, EF-hand protein, 3	ND	37	41	—
DNCH1, LL.1778	Dynein	2	45	6	—
DCTN1, LL.1639	Dynactin p150	ND	12	34	—
DCTN2, LL.10540	Dynactin p50	10	110	83	—
GADD34, LL.23645	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	47	81	150	—
GADD45β, LL.4616	Growth arrest and DNA-damage-inducible, β	37	184	207	de La Fuente [28]
GADD153, LL.1649	DNA-damage-inducible transcript 3	4	156	93	—
MAD1L1, LL.8379	MAD1 mitotic arrest deficient-like 1 (yeast)	7	1	ND	—
MAD2L2, LL.10459	MAD2 mitotic arrest deficient-like 2 (yeast)	ND	3	18	Pise-Masison et al. [27]
NUDC, LL.10726	Nuclear distribution gene C homolog (*A. nidulans*)	223	7	32	—
PRC1, LL.9055	Protein regulator of cytokinesis 1	125	125	215	—
PLK1, LL.5347	Polo-like kinase	133	238	492	Ng et al. [31]
RAN, LL.5901	RAN, member RAS oncogene family	1363	2357	1913	—
PTTG1, LL.9232	Pituitary tumor-transforming 1 (Pds1p, securin)	63	90	58	—

example, HMGN1 is part of the cellular mechanism that regulates the rate of DNA repair induced by UV through its interaction with chromatin and enhancement of the rate of removal of UV damage from DNA [51]. As compared to PHA/IL-2-activated CD4+ T-cells, HMG1 expression was downregulated ca. 2- and 10-fold in B1 and C5 cells, respectively. RUVBL2 is a DNA helicase that is essential for homologous recombination and ds DNA break repair, it was detected at elevated levels in both B1 and C5 cells. The related gene RUVBL1, that maps to a region with frequent rearrangements in different types of leukemia and solid tumors [52,53], was upregulated in C5 cells.

Deregulation of cell cycle and mitotic genes

A characteristic of HTLV-1 transformed cells is deregulation of cell cycle machinery leading to enhanced cell survival and genomic alterations. As Table 7 indicates, cyclin-dependent kinases, CDK1 and CDK5, were markedly downregulated to nearly undetectable levels in both B1 and C5 cells. In contrast, CDK2 and CDK4 were moderately elevated in both cells, which is in agreement with the report of Pise-Masison et al. [27]. High levels of cyclin D2 were found in both cell lines while cyclin D3 was detected at very low levels in B1 and C5 cells. These data are in agreement with the results described by Mori et al. [30]. However, Mori et al. [30] also observed high levels of cyclin D1 which was not detected in the MPSS data. CDC25B, a member of the CDC25 family of phosphatases, was detected at only low levels in B1 and C5 cells. The CDK inhibitor of the Cip/Kip family p21Cip1, which acts as a potent inhibitor of cell growth in lymphoid cells, inhibits the late G1 and S phase kinase CDK2, and has been previously reported to be strongly activated by HTLV-1 Tax [28,54,55], was variably upregulated in C5 cells. Additionally, p57 Kip2 was variably upregulated, whereas the levels of p27 Kip1 and p16 INK4 were only upregulated in B1 cells.

Several growth arrest and DNA damage inducible (GADD) family genes were upregulated in B1 and C5 cells. These include GADD34, GADD45β, and GADD153. Several genes involved in cytokinesis and mitotic spindle assembly were detected by MPSS. Many of these genes were consistently (BUB3, CEN3, DNCH1, DCTN1, and DCTN2) or variably (PTTG1 (Pds1p, securin), PLK1, PRC1, and RAN) upregulated. The PLK1 substrate and binding protein NUDC were markedly downregulated in B1 and C5 cells. RAN, a member of the RAS oncogene family, was also expressed at high levels in these cells.

Comparison of transcriptomes of acutely HTLV-1 transformed B1 and C5 cells

Peripheral blood CD4+ T-cells from one individual were used to derive the IL2-independent HTLV-1 transformed CD4+ T-cell lines B1 and C5 by single-cell cloning. Therefore, these cell lines represent two independent populations of transformed cells with possibly unique responses to HTLV-1 infection and unique molecular signatures. Of the several thousand reliable and significant signatures that were sequenced in B1 and C5 cells and which had UniGene Ids, there were 4599 signatures (transcripts) that were common to both B1 and C5 cells. However, there were 1867 and 2322 signatures (with unique UniGene Ids) that were uniquely present in B1 and in C5 cells, respectively. This observation can likely lead to differences between the cells at the biochemical level. For example, there were genes such as MALT1, RECQL, and BRRN1 that were not detected in B1 cells (Table 8). These genes are involved in chromosomal translocation and TCR-induced NF-κB activation (MALT1), DNA repair and genomic stability (RECQL), and mitosis (BRRN1) [56–58]. Conversely, CDKN2A,

Table 8
Genes uniquely expressed in acutely HTLV-1 transformed B1 and C5 cells

HUGO name/LocusLink ID	Gene name	PHA/IL-2-activated CD4+ T-cells (TPM)	B1 cells (TPM)	C5 cells (TPM)
ADPRT, LL. 142	ADP-ribosyltransferase (NAD+; poly(ADP-ribose) polymerase), PARP	95	65	16
BCL2, LL.596	B-cell CLL/lymphoma 2	9	20	9
BCL2L1, LL.598	B-cell CLL/lymphoma 2-like 1, bcl-xL	5	125	52
BIRC2, LL.330	Baculovirus inhibitor of apoptosis protein (IAP) repeat containing (BIRC) 2	ND	10	ND
BNIP3, LL. 664	BCL2/adenovirus E1B 19 kDa interacting protein 3	3	ND	171
BRRN1, LL.23397	Barren homolog (*Drosophila*)	20	ND	83
c-FLIP, LL.8837	Cellular FLICE inhibitory protein	41	248	135
CDKN2A, LL.1029	Cyclin-dependent kinase inhibitor 2A	ND	21	ND
CPR2, LL.9238	Cell cycle progression 2 protein	19	33	ND
MALT1, LL. 10892	Mucosa-associated lymphoid tissue lymphoma translocation gene 1	2	ND	37
RECQL, LL.5965	RecQ protein-like (DNA helicase Q1-like)	17	ND	84
STK6, LL.6790	Aurora/IPL1-like kinase	ND	62	ND

an inhibitor of CDK4 and an important tumor suppressor gene [59], CPR2, a gene involved in overcoming G1 arrest signals [60], and STK6, a protein kinase implicated chromosome segregation or spindle formation [61], were detected in B1 but not C5 cells. B1 cells also had higher levels of expression of genes involved in cell survival, anti-apoptosis, and DNA repair including: BCL2, BCL2L1, c-FLIP, and ADPRT (Table 8).

Discussion

Systems biology is a new paradigm for conducting research on complex biological systems where the organism or biological system under investigation is analyzed in its entirety rather than examining individual transcriptional or biochemical pathways. In this study, systems biology was applied to the study of HTLV-1 transformation and the MPSS data were compared to published reports of gene profiling on HTLV-1 transformed, Tax-expressing, and ATL cells. In particular, the depth of the MPSS, defined as the detection and discovery of genes that might be missed using a microarray system, was evaluated. The results demonstrate that MPSS can identify and quantitate a large number of gene transcripts that are found to be modulated by HTLV-1 in published reports using microarrays [26,27,29–31]. However, MPSS was also able to capture and identify a number of novel transcripts that not been previously implicated in HTLV-1 transformation and/or ATL.

Because MPSS interrogates the global transcriptome of the sample and is not biased by a pre-determined set of genes, it could capture and quantitate expression of endogenous viral transcripts in the HTLV-1 transformed cell lines. The majority of viral transcripts were derived from the 3′ most *Dpn*II site (Fig. 1C) which could encode a number of different pX region genes including the doubly spliced Tax/Rex transcript (Table 1). Western blot studies have confirmed the expression of Tax protein in these cells [32]. However, the expression of other pX region encoded proteins has not been evaluated. The results shown in Fig. 2 provide evidence that the transformed cells released cell-free RT activity suggesting the production of virus particles. Further studies to evaluate the production of infectious virus by these HTLV-1 transformed cells will be required to investigate this observation in more detail. There are likely to be important interplays between the viral and cellular genes that result in the modulation of cellular transcription. This property of MPSS may also provide a powerful new tool for the detection of emerging pathogens or unknown infectious agents of cancer and other human diseases.

A hallmark of HTLV-1 transformed cells and ATL cells is the expression of CD4+CD25+ T-cell phenotype. An important new finding of the present study includes upregulation of GITR transcription and its' surface overexpression on acute and chronic HTLV-1 transformed cell lines (Table 5 and Fig. 3A). Regulation of GITR expression on human CD4+ T-cells during the course of T-cell activation or infection is not well understood and the molecular basis for GITR upregulation is only beginning to be elucidated. Certain conditions of cell activation that mimic TCR stimulation can result in upregulation of GITR expression [62] however, the role of viral infections in this process has not been reported. Previous studies have demonstrated that Tax is able to transactivate the IL-2 receptor α-chain (CD25) expression via activation of NF-κB and binding to the κB enhancer in the IL-2Rα promoter [62–64]. Whether Tax or other HTLV-1 viral proteins play a role in the upregulation of GITR transcription and its overexpression will be important to determine.

Recently, Karube [65] demonstrated by RT-PCR and immunostaining expression of forkhead/winged helix transcription factor (FoxP3), a specific marker that is important for the function of CD4+CD25+ regulatory T-cells, in peripheral blood and lymph node samples of 10 of 17 adult T-cell leukemia/lymphoma cells. Immunostaining for Fox3P varied in intensity in these samples from no staining to >50% of peripheral blood cells and leukemic cells in lymph node biopsy samples from patients with lymphomatous ATL. Chronic HTLV-1 transformed MT-2 cells were also examined and were reported to be positive for RT-PCR but negative for immunostaining of FoxP3. Matsubara [66] reported that FoxP3 mRNA expression was variably upregulated in ca. 50% of ATL cases and TGF-β secretion, which has been implicated in the immunoregulatory function of Tregs, was detected in one of the four samples. Ishida [67] also detected the expression of FoxP3 mRNA and protein in CD4+CD25+ T-cells from ATLL patients but established HTLV-1 transformed cell lines, including MT-2 cells, had undetectable to very low levels of FoxP3 mRNA. We attempted intracellular staining for forkhead/winged helix transcription factor (FoxP3) protein expression by FACS analysis of MT-2, B1, and C5 cells. Using this assay only circa 1% of cells were positive (data not shown). Whether this represents a technical limitation of our study is uncertain at this time however, in vitro HTLV-1 transformed cells differ from leukemic cells of ATL in several aspects. For example, virus production is almost completely absent in ATL cells. It will be important to investigate if FoxP3 expression can be induced by activation of CD4+CD25+ GITR+ HTLV-1 transformed cells. It will also be important to determine if CD4+CD25+FoxP3+ ATL cells are also GITR+ since in vitro studies have demonstrated that ectopic FoxP3 expression can result in upregulation of GITR expression [68]. The report by Matsubara [66] that

only a small population of each ATL sample expressed low levels of GITR provides initial observations in this regard. In addition, anti-GITR Mabs can abrogate the suppression mediated by Tregs [69] and removal of CD4+CD25+ Tregs can evoke tumor immunity in vitro and in vivo [70,71]. Moreover, tumor antigen specific CD4+ Treg cells expressing CD25 and GITR have been recovered from tumor-infiltrating lymphocytes (TILs) of cancer patients and in vitro studies have provided evidence that these Tregs could have a profound effect on the inhibition of T-cell responses against cancer [69]. Despite these intriguing possibilities, the in vivo role of GITR expression and Treg function in ATL disease can only be speculated at the present time until additional phenotypic and functional studies are reported [72,73].

Other interesting trends in the transcriptional profiling data of several functional classes of proteins were observed in the MPSS studies although additional biochemical studies will be required to verify these findings. For example, upregulation of the NF-κB, Akt2-mediated survival pathways and anti-apoptotic BCL2 and BIRC family genes and downregulation or neutralization of TNF receptor mediated pro-apoptotic pathways were observed in the HTLV-1 transformed cells and may represent defining biochemical events that lead to enhanced cell survival in acutely HTLV-1 transformed cells (Tables 3–5). The MPSS analysis also demonstrated marked upregulation of CD70 (CD27L) and downregulation of CD27 (Table 5). A similar inverted profile (high CD70 and low CD27) has been observed in lymphoproliferative diseases such as chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma [74]. TNFSF7/TNFRSF7 (CD70/CD27) interactions are believed to play an important role in the maturation and activation of B- and T-cells [75–77]. It has been reported that CD70-expressing glioma cells induce the release of soluble CD27, decrease CD27 expression, and induce apoptosis of peripheral blood mononuclear cells (PBMC) and the investigators have proposed a role for CD70 in mediating immune escape of human malignant gliomas [78]. The MPSS data revealed a general defect in DNA repair mechanisms in the HTLV-1 transformed cells. Many of the DNA repair proteins detected at low levels in these transformed cells are involved in important steps in the repair process. For example, XAB2, a component of the NER transcription-coupled repair and transcription pathway, interacts with XPA (a factor central to NER pathway) and with the transcription-coupled repair-specific proteins ERCC5 and ERCC6 [79]. ADPRT (PARP) is a nuclear enzyme that binds with both the single-stranded (ss) and double-stranded (ds) DNA breaks and, along with β-polymerase, is associated with base excision repair of nuclear DNA [80–82]. With the exception of RUVBL2, the fact that none of the other DNA repair genes are consistently upregulated beyond the levels found in activated CD4+ T-cells and, in many cases, are downregulated suggests an apparent inability of the transformed cells to mount a vigorous DNA repair response. This study also revealed the modulation of several genes associated with mitosis that have not been previously reported in the context of HTLV-1-induced transformation. For example, a relation between HTLV-1 and RAN, which has a role in regulating microtubule-associated processes and spindle assembly in mitosis [83–88] and is expressed at high levels in B1 and C5 cells, has not been established. Similarly, the role of NUDC, a PLK1 substrate and binding protein that has been reported to cause multiple mitotic defects including multinucleation in COS-7 cells [89], is also not known.

Finally, although the two acutely HTLV-1 transformed B1 and C5 cells were derived from a single individual, there were significant differences in their gene expression profiles. These in turn may produce distinct phenotypic effects—for example, increased or decreased susceptibility to apoptosis or DNA damage—and may affect the outcome of survival pathways by altering the balance of factors that predispose the cell to succumb to or resist HTLV-1-induced deregulation. Indeed, oligoclonality of leukemic cells is observed in ATL patients [90–92] and may contribute to the heterogeneity of cell killing and survival after chemotherapy [93–98]. An additional factor that may be responsible for the observed variation is the probable difference in the level of HTLV-1 genes expressed in the two cell lines. For example, the most 3′ *Dpn*II signature of the HTLV-1 genome was more than 4-fold more abundant in C5 cells (25086 TPM) as compared to B1 cells (5746 TPM) and the relative levels of these genes may therefore fundamentally alter the behavior of transformed cells.

In summary, the MPSS analysis has provided a new set of transcriptional profiling data that complements and extends similar analyses that have been performed using microarray and traditional biochemical techniques. The particular value of the MPSS platform lies in its ability to provide a quantitative, comprehensive, and unbiased picture of gene modulation in the system being analyzed. Additional studies will be required to validate these gene profiling results through biochemical and functional assays on HTLV-1 transformed and ATL cells. Our study demonstrates that GITR is overexpressed in acute and chronic HTLV-1 transformed cells lines. This observation provides an important entry point for investigations that examine the functional role of GITR in HTLV-1 transformation and ATL, and the role of viral genes such as Tax on GITR overexpression. It is our goal to utilize the MPSS data to develop a better understanding of the biochemical changes that lead to this fatal disease so that new targeted chemo- and immunotherapies can be developed.

Acknowledgment

This work was supported by R21 AI053822 from the National Institutes of Health.

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