

ORIGINAL ARTICLE

Phorbol ester-mediated re-expression of endogenous LAT adapter in J.CaM2 cells: a model for dissecting drivers and blockers of *LAT* transcription

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Linker for activation of T cells (LAT) is a raft-associated, transmembrane adapter protein critical for T-cell development and function. LAT expression is transiently upregulated upon T-cell receptor (TCR) engagement, but molecular mechanisms conveying TCR signaling to enhanced *LAT* transcription are not fully understood. Here we found that a Jurkat subline J.CaM2, initially characterized as LAT deficient, conditionally re-expressed LAT upon the treatment with a protein kinase C activator, phorbol 12-myristate 13-acetate (PMA). We took advantage of the above observation for studying *cis*-elements and *trans*-acting factors contributing to the activation-induced expression of LAT. We identified a *LAT* gene region spanning nucleotide position –14 to +357 relative to the ATG start codon as containing novel *cis*-regulatory elements that were able to promote PMA-induced reporter transcription in the absence of the core *LAT* promoter. Interestingly, a point mutation in *LAT* intron 1, identified in J.CaM2 cells, downmodulated *LAT* promoter activity by 50%. Mithramycin A, a selective Sp1 DNA-binding inhibitor, abolished LAT expression upon PMA treatment as did calcium ionophore ionomycin (Iono) and valproic acid (VPA), widely used as an anti-epileptic drug. Our data introduce J.CaM2 cells as a model for dissecting drivers and blockers of activation induced expression of LAT.

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INTRODUCTION

Linker for activation of T cells (LAT) expression is mandatory for the proper development and function of T cells.^{1,2} During ontogeny, it is first detectable within CD4–CD8–CD25+CD44+ (DN2) thymocytes and is upregulated during CD4–CD8– (DN) to CD4+CD8+ (DP) transition.^{3,4} Targeted deletion of *LAT* arrests development of Tαβ and Tγδ thymocytes at the CD4–CD8–CD25+CD44– (DN3) stage, which coincides with the insufficient pre-T-cell receptor (TCR) signaling.^{5,6} Forced expression of p56Lck kinase from its proximal promoter allows for DN-to-DP transition in LAT-deficient mice and further maturation of conventional LAT-deficient Tαβ cells. However, once in the peripheral lymphoid organs, these T cells turn into pathogenic effectors producing massive amounts of IL-4 and causing generalized Th2-like lymphoproliferative syndrome that is lethal to mice.⁷ On the other hand, transgene-mediated overexpression of LAT in the peripheral T cells causes their hyperactivation and premature acquisition of memory-like phenotype.⁸ Therefore, it seems that the maintenance of proper levels of LAT is crucial for T-cell homeostasis. TCR engagement was shown to cause a transient upregulation of LAT expression, which was further potentiated by the blockage of calcium signaling by calcineurin inhibitors cyclosporine A (CsA) and FK506.⁹ Indeed, when the calcium signaling was activated by a calcium ionophore Iono at the time of TCR engagement it blocked the upregulation of LAT expression suggesting a complex regulation of *LAT* by negative (calcium signaling) and positive (PKC–MEK–ERK) regulatory circuits. Little is known about the mechanisms by which TCR activation is integrated into the changes of *LAT* transcription. The proximal *LAT* promoter was mapped to contain multiple

GC-rich regions, which in electrophoretic mobility shift assays were shown to bind Sp1, Sp3, Elf-1 and Runx-1 transcription factors.^{10,11} Also, a heat-shock protein 90 was postulated to influence LAT expression in activated T cells.¹² Moreover, epigenetic control of *LAT* expression was suggested by a recent finding that in latently HIV-1-infected T-cell lines *LAT* locus specifically underwent histone modifications coincident with decreased transcription.¹³

In the present study, we used J.CaM2 cells as a model for dissecting signaling pathways, *cis*-elements and *trans*-acting factors influencing basal and phorbol 12-myristate 13-acetate (PMA)-induced expression of LAT. Our data suggest that the PMA-induced expression of LAT in J.CaM2 cells is facilitated by an extensive chromatin reorganization and Sp1 binding to proximal promoter, and to a novel regulatory region extending from the nucleotide position –14 to +357 relative to the transcription start site.

RESULTS

Treatment of J.CaM2 cell line with PMA induces LAT re-expression. J.CaM2 subline was produced by the radiation-mediated mutagenesis of Jurkat E6.1 cells.¹⁴ It was shown to be deficient in basal LAT expression and was used as a model for genetic *LAT* complementation assays, and to uncover LAT involvement in tonic repression of recombinase activating genes transcription.¹⁷ In Figure 1a, it is shown that when treated with a protein kinase C (PKC) activator, J.CaM2 cells unexpectedly re-expressed *LAT* at the messenger RNA and protein levels. PMA-induced LAT

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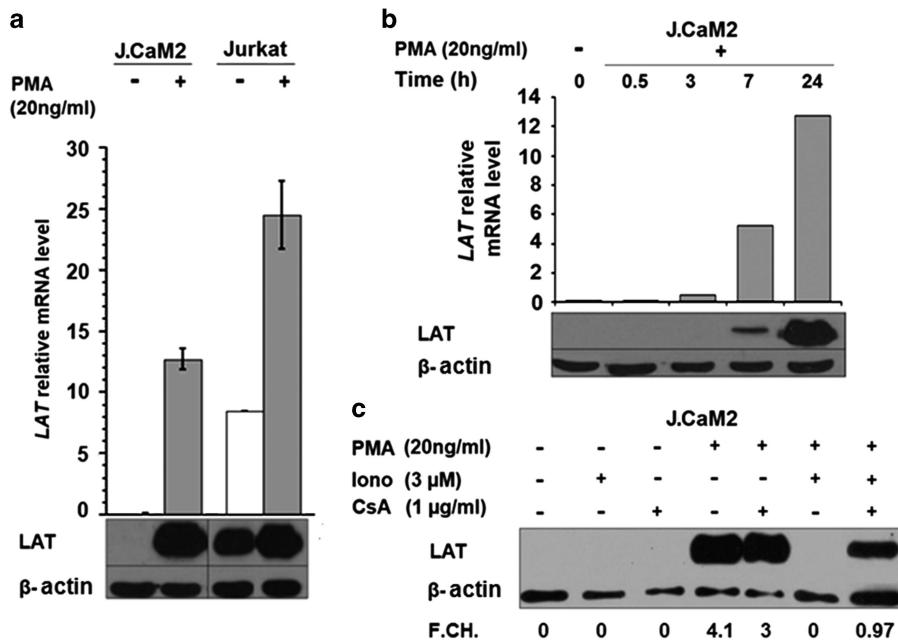


Figure 1. LAT-deficient J.CaM2 cells express LAT upon stimulation with PMA. **(a)** J.CaM2 and Jurkat leukemic T cells were either left untreated (–) or stimulated with 20 ng ml⁻¹ PMA (+). After 24 h relative LAT messenger RNA (mRNA) level was determined by quantitative PCR (qPCR) and normalized against *EEF1A1* and *HPRT1* (upper panel). Values are displayed as means ± s.d. of three independent biological replicates. LAT protein expression was assessed by western blot analysis. β-Actin expression served as a loading control (lower panel). **(b)** Kinetics of *LAT* mRNA and protein expression upon PMA stimulation were measured by qPCR and western blotting, respectively. **(c)** J.CaM2 cells were unstimulated or PMA treated in the absence or presence of ionomycin (Iono; 3 μM), cyclosporine A (CsA; 1 μg ml⁻¹) and a combination of the two drugs. Whole-cell lysates were prepared 24 h post stimulation and immunoblotted with antibodies against LAT and β-actin. Data in **b** and **c** are representative of three biological replicates. F.C.H. denotes the ratio of LAT protein expression in relation to β-actin.

re-expression in J.CaM2 cells was clearly detectable after 7 h of stimulation (Figure 1b) and as little as 2 ng ml⁻¹ of PMA was sufficient to induce LAT expression (data not shown). Calcium ionophore Iono abrogated PMA-induced LAT expression, which was restored upon the treatment with calcineurin inhibitor CsA (Figure 1c). This finding was consistent with the previous observations of a negative impact of calcium signaling on the activation-induced LAT expression in Jurkat cell line.¹⁴ Inhibition of PKC by the treatment of J.CaM2 cells with a non-specific PKC inhibitor VPA (Figure 2b) as well as inhibition of MEK/ERK, and to a lesser extent PI3K/AKT/mTOR, signaling pathways with respective inhibitors (Materials and methods) led to the abrogation of PMA-induced LAT re-expression (Figure 2a). Interestingly, VPA interfered with PMA induced but not with the basal LAT expression in Jurkat T cells (Figure 2b), suggesting that each of these mechanisms may differentially rely on the PKC activation.

A strong, dose-dependent downregulation of LAT was also observed when PMA-induced J.CaM2 cells were treated with mithramycin A, a specific inhibitor of Sp1 DNA-binding (Figure 2c). However, the effect of mithramycin A depended on the initial level of LAT expression. In Jurkat cells expressing high levels of LAT, the effect of mithramycin A treatment was barely visible, whereas in Hut-78 cells expressing moderate levels of LAT, it was clear both in untreated and PMA-treated cells (Figure 2c).

Point mutation in the *LAT* intron 1 contributes to the J.CaM2 phenotype

In a search of DNA mutations affecting basal LAT expression in J.CaM2 cells, we sequenced PCR-amplified fragments of genomic DNA, comprising a region of 2208 bp surrounding the translational start site (Materials and methods). This region was chosen because inspection of a number of chromatin immuno-

precipitation sequencing (ChIP-seq) data from LAT-expressing cells revealed that *LAT* locus (proximal promoter and part of downstream gene body) is strongly enriched for histone 3 lysine 27 acetylation (H3K27ac), that is, typical of active promoters/enhancers (Figure 5). In Figure 3, two heterozygous mutations identified in the course of sequencing are shown. Both mutations, of transition type, were absent from the Jurkat E6-1 reference line and were found to affect different alleles. One of these mutations concerned C > T transition at gene position 237 (g.237C > T) located in the first intron. The other C > T mutation found in the exon 4 at position 167 (c.167C > T) was of missense type causing the LAT threonine 56 residue to be replaced by a methionine (Figure 3a). The presence of both mutations was additionally confirmed by PCR–restriction fragment length polymorphism (RFLP) performed on genomic DNA (g.237C > T) and cDNA (c.167C > T) from J.CaM2 and Jurkat cells as a control (Figure 3a). To evaluate the effect of g.237C > T mutation on the level of *LAT* transcription, we inserted DNA fragments spanning the *LAT* proximal promoter and a region containing g.237C > T mutation, upstream of the firefly luciferase gene in the pGL4.14 [Luc2/Hygro] reporter vector (Figure 3b). When transfected to Jurkat cell line, the construct carrying the g.237C > T mutation displayed ~50% reduction in the basal and PMA-induced promoter activity as compared with its wild-type counterpart (Figure 3b). The *LAT* messenger RNA generated from the c.167C > T allele was expressed upon PMA treatment of J.CaM2 cells in a ~1:1 proportion with the other allele and was translated into a stable protein (data not shown). Of interest, the heterozygous c.167C > T *LAT* mutation was identified in five subjects (four European and one African) as a single nucleotide variant, deposited in ExAC Browser Beta database (<http://exac.broadinstitute.org/>) under the accession number rs369317401.

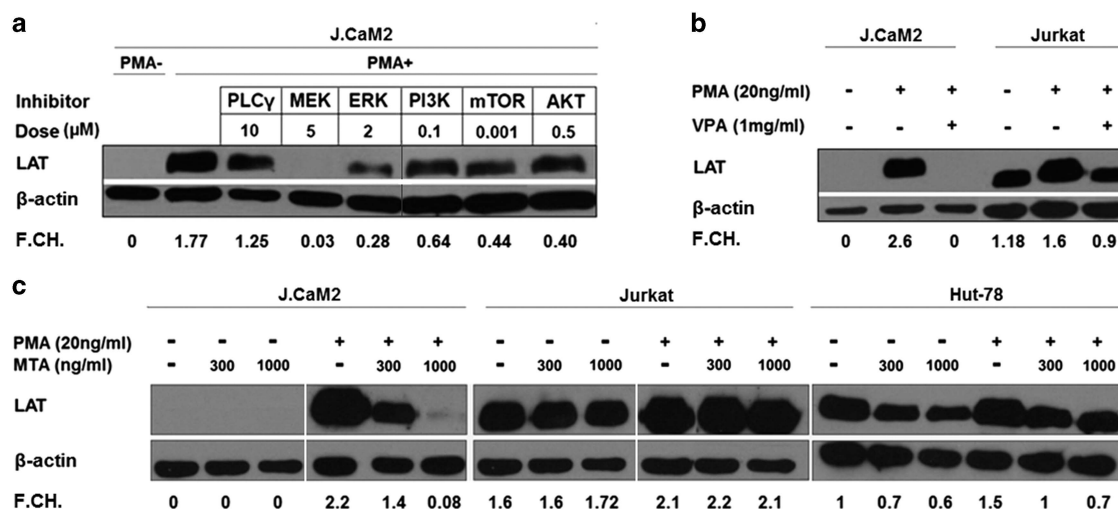


Figure 2. PMA-induced LAT expression is mediated through PKC/MEK/ERK signaling pathway and is negatively regulated by valproic acid and mithramycin A. **(a)** Assessment of the role of PLC γ /MEK/ERK and PI3K/AKT/mTOR signaling cascades in activation-induced expression of LAT: J.CaM2 cells were pretreated for 2 h with specific inhibitors targeting key components of PLC γ /MEK/ERK and PI3K/AKT/mTOR signaling pathways before addition of PMA (20 ng ml⁻¹). Twenty-four hours post activation, whole-cell lysates were processed for western blot analysis with LAT and β -actin antibodies. Results are representative of three independent experiments. **(b)** Valproic acid (VPA) exerts an inhibitory effect on PMA-induced LAT expression. VPA (1 mg ml⁻¹) was added as indicated 4.5 h before PMA supplementation. LAT expression was measured in J.CaM2 and Jurkat cells 24 h after PMA addition by western blotting. **(c)** Mithramycin A, a competitive inhibitor of Sp transcription factors DNA-binding prevents the synthesis of LAT protein: J.CaM2, Jurkat and Hut-78 cells were pretreated for 3 h with the indicated concentrations of mithramycin A (MTA) or left untreated before PMA stimulation or mock treatment. Cell lysates were prepared 24 h post activation and subjected to western blot analysis with LAT and β -actin antibody. Results are representative of three independent biological replicates. F.C.H. denotes the ratio of LAT protein expression in relation to β -actin.

Cis-regulatory elements located in the region -14/+357 of *LAT* initiation codon respond to PMA activation by promoting reporter transcription in the absence of *LAT* proximal promoter

To map *LAT* gene regions sensitive to PMA stimulation, a series of luciferase reporter constructs shown in Figure 4 were transfected to Jurkat cells treated or not with PMA (Figure 4a). In line with the previous reports, deletion of a part of proximal *LAT* promoter (Δ -916/-409) containing the binding sites of Sp1/Sp3 transcription factors, previously denoted as the ABC cluster,¹⁰ led to over 75% reduction in basal promoter activity, but strikingly had only little effect on the PMA-induced transcription. Moreover, deletion of the entire *LAT* proximal promoter (Δ -916/-14) almost entirely abrogated the basal transcription (80% reduction), whereas reducing the PMA-induced transcription only by 20%. Significant PMA-dependent promoter activity was mapped to the DNA fragment spanning a region between -14 and +126 around the *LAT* translation start site, as its deletion reduced the PMA response to < 40% of the maximal activity. Consistently, isolated mutations of all Sp1/3L-binding sites (cluster ABCDE), previously assigned to the *LAT* proximal promoter, caused the absence of basal activity and ~50% reduction of PMA-induced activity. Interestingly, when transfected to a human non-lymphoid cell line HEK293, a reporter construct spanning the region between -916 and +357 displayed comparable basal and PMA-induced transcriptional activity as in Jurkat cells (Figure 4b). This result suggested that non-lymphoid transcription factors alone are capable of supporting transcription from the *LAT* promoter. However, as the *LAT* expression was undetectable in non-stimulated or PMA-activated HEK293 cells (data not shown), this result pointed to an epigenetic mechanism involved in the control of *LAT* transcription.

Induction of H3K27ac and enhanced Sp1/Sp3 binding to the *LAT* proximal promoter and downstream gene sequence coincide with PMA treatment of J.CaM2 cells

To confirm that the basal *LAT* transcription is driven by a single promoter and to assess the pattern of epigenetic modifications

across *LAT* gene, we first analyzed public ChIP-seq data available for Jurkat T cells. Figure 5a depicts profiles of RNA polymerase II (Pol II), H3K4me3 and H3K27ac binding for Jurkat cells. This analysis suggested that (1) no alternative promoter is involved in the steady-state transcription of *LAT* and (2) H3K4me3 and H3K27ac histone marks of active promoters/enhancers are enriched across both the *LAT* proximal promoter and downstream intragenic region encoding exons/introns 1-4. To find out whether the lack of *LAT* transcription in J.CaM2 cells was coincident with a loss of H3K27ac mark and altered Sp1/Sp3 binding, we performed chromatin immunoprecipitation assay focused on three genomic DNA fragments encompassing the *LAT* proximal promoter (-404/-220) and downstream regions (fragments -14/+186 and +186/+357) that promoted PMA-induced transcription in luciferase reporter assays (Figure 4a). In Figure 5b, we showed that in untreated J.CaM2 cells, the level of H3K27ac as well as Sp1/Sp3 binding was low for all the fragments analyzed, whereas upon PMA stimulation, an enrichment of H3K27ac and an increase in Sp1 binding were observed. Interestingly, a weak Sp3 binding was detected for the region -404/-220, but was virtually absent from the intragenic regions (-14/+186 and +186/+357), both in quiescent and activated cells, suggesting a prevalent role of Sp1 in the regulation of *LAT* expression.

DISCUSSION

In the present study, we documented an unexpected observation of PMA-induced expression of *LAT* in J.CaM2 cells that till present were regarded as permanently *LAT* deficient. Phorbol esters are known to bypass the TCR requirement by mimicking diacyl glycerol-mediated activation of PKC and suppressing the calcium-dependent signaling cascade.¹⁸ Indeed, when the calcium ionophore Iono was used along with PMA for J.CaM2 cell treatment, no *LAT* re-expression was observed (Figure 1c), suggesting fine balanced and dynamic control mechanisms over *LAT* transcription emanating from the TCR. We used PMA-induced *LAT* re-expression in J.CaM2 cells as a read-out for testing inhibitors of PKC-dependent signaling pathways in

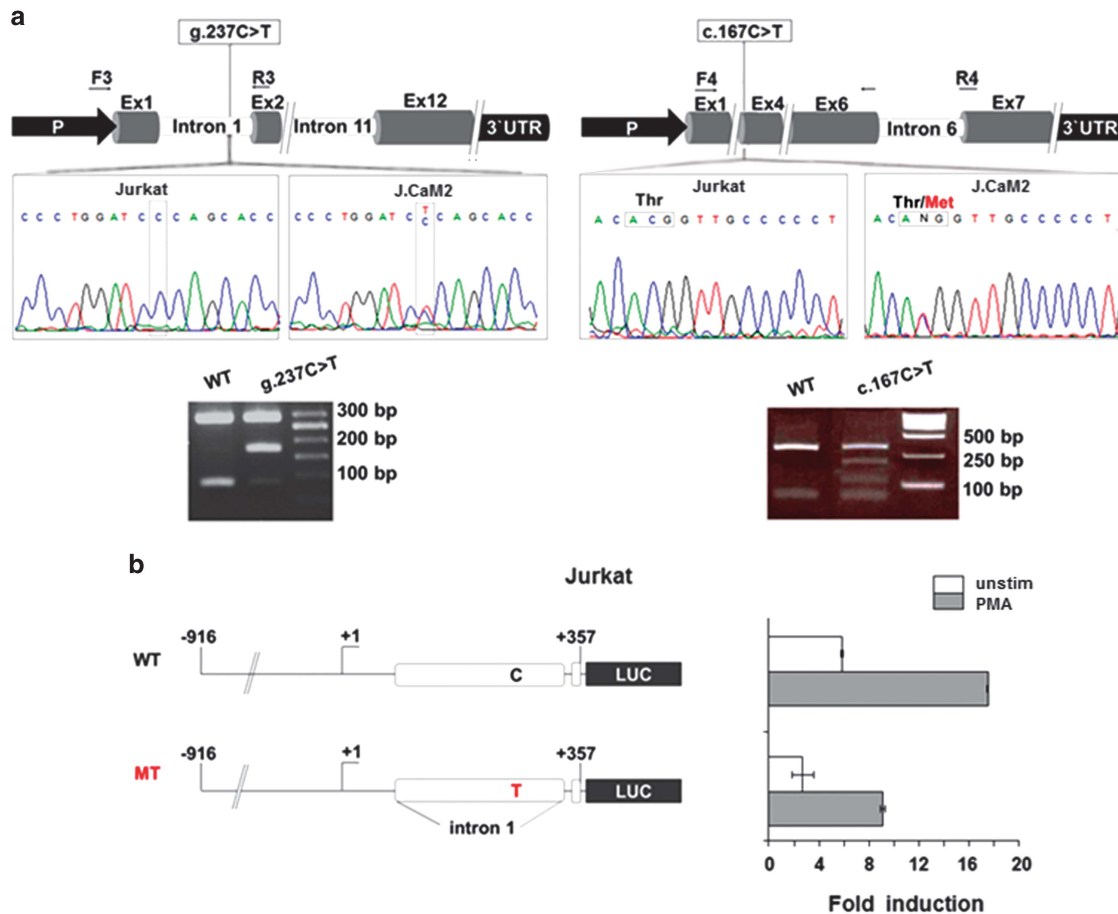


Figure 3. Identification of two point mutations in *LAT* gene of J.CaM2 cells by DNA sequencing and PCR-RFLP. (a) Left panel: histograms showing homozygous wild type 'C' nucleotide in intron 1 at the position g.237 in Jurkat and heterozygous variant C>T found in J.CaM2. The mutation was further confirmed by the digestion of PCR product (amplified from genomic DNA) with *Bam*HI endonuclease. Right panel: histogram of homozygous, wild type 'C' base in exon 4 at position c.167 in Jurkat and heterozygous c.167C>T missense mutation in J.CaM2 changing threonine to methionine at position 56. Presence of the variant was verified by the digestion of PCR product (generated using cDNA) with *Nla*III. (b) Scheme of the pGL4.14-*LAT* (-916/+357) plasmids used to test the effect of g.237C>T mutation on *LAT* promoter activity in Jurkat cells under resting and activation conditions. The results of dual-luciferase assay are representative of four technical replicates that represent two independent experiments ($P < 0.05$). Data are reported as fold change with respect to the pGL4.14 [luc2/Hygro] basic vector. Error bars represent s.d.

T cells. Apart from MEK/ERK and to a lesser extent PI3K/Akt/mTOR inhibitors (Figure 2a), one of such potential inhibitors, the anti-epileptic drug VPA, turned out to efficiently counteract the PMA-induced upregulation of *LAT* in J.CaM2 and Jurkat cells, but had only little effect on the basal *LAT* expression (Figure 2b). Reportedly, an immunosuppressive effect of VPA in the course of experimental autoimmune encephalomyelitis was linked with its direct pro-apoptotic effect exerted on activated but not naive T cells.¹⁹ Our data suggest that VPA could also block the unfolding of immune response at its early stage by blocking TCR-mediated signals leading to the activation of T cells. As the action of VPA involves number of cellular targets including PKC²⁰ and histone deacetylases,²¹ we sought to delineate which of these activities was responsible for the observed effect on *LAT*. For this purpose, we treated PMA-activated J.CaM2 and Jurkat cells with a histone deacetylase inhibitor, *N*-butyrate, but found no evidence for its involvement in *LAT* expression (data not shown). Therefore, it is likely that VPA exerts its *LAT*-modulating effect through the inhibition of PKC.

We also took advantage of J.CaM2 model for studying *cis*-elements and *trans*-acting factors involved in basal and activation-induced expression of *LAT*. First, to find out whether the deficiency of *LAT* in J.CaM2 cells was due to mutations in the core *LAT* promoter, we sequenced 2208 bp long DNA fragment that included the entire *LAT* promoter on both alleles. Sequence

comparison revealed that the only mutations that differentiated J.CaM2 from parental Jurkat cells were located outside of the core promoter, in the gene body (Figure 3a). One of them, present in intron 1 (g.237C>T), drew our attention because it reduced *LAT* promoter activity by 50% (Figure 3b). It is known that first introns are often enriched in *cis*-regulatory motifs enhancing transcription. Although human *LAT* intron 1 is relatively short (195 bp), nevertheless it fulfills number of criteria associated with such regulatory functions,²² that is, elevated percentage of G+C nucleotides (64% for human *LAT*, data not shown) and their CC, GG and CG dinucleotide enrichment often associated with the presence of Sp1-binding sites. Moreover, *LAT* intron 1 spanning DNA fragment promoted PMA-induced reporter transcription in the absence of upstream core *LAT* promoter (Figure 4). This result, although obtained with non-chromatinized reporter constructs, supported *cis*-regulatory activity of this region. In addition, ChIP assay revealed that, indeed, both *LAT* core promoter and downstream gene sequence (-14/+357) underwent chromatin remodeling, probed by an increase in H3K27ac in response to PMA treatment (Figure 5b). The above observations are consistent with an explanation that not only *cis*-regulatory regions present in the core promoter, but also those located downstream from transcription start site of *LAT* cooperatively regulate its basal and activation-induced expression. It is however very likely that other

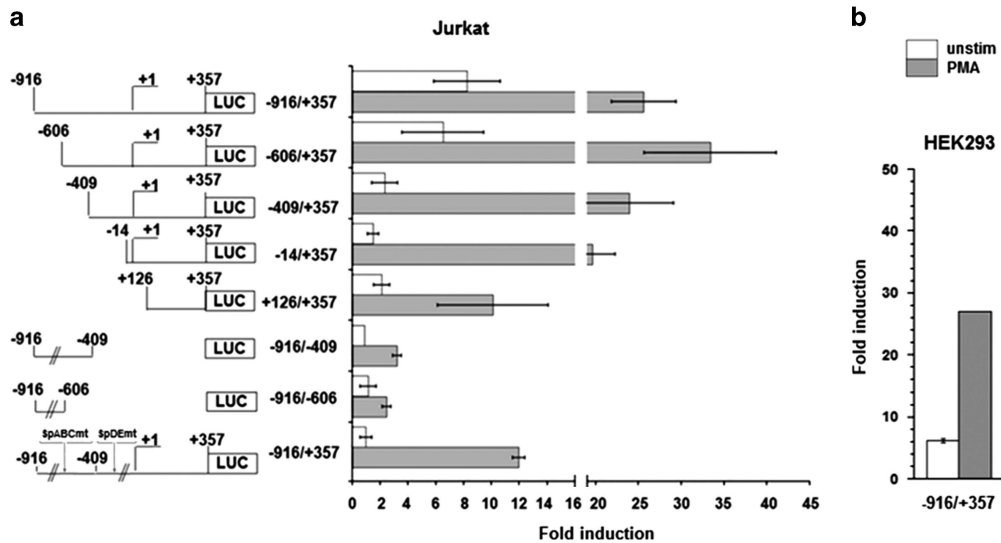


Figure 4. Mapping PMA-responsive sequences in *LAT* proximal promoter and downstream gene body. **(a)** Relative luciferase activities for pGL4.14-*LAT* deletion constructs assessed in Jurkat cells cultured in the presence or absence of PMA. Cells were activated with PMA 24 h post transfection. To measure reporter activity under basal and stimulated conditions, cells were assayed 24 h after transfection and 24 h post PMA addition, respectively. The average of three independent biological replicates is shown, and error bars indicate s.d. **(b)** Luciferase activity for pGL4.14-*LAT* (-916/+357) construct assayed in non-lymphoid HEK293 cell line unstimulated or stimulated with PMA. Luciferase activity in basal and activation state was determined 24 h after transfection and 24 h post addition of PMA, respectively. The average of three independent biological replicates is shown, and error bars represent s.d.

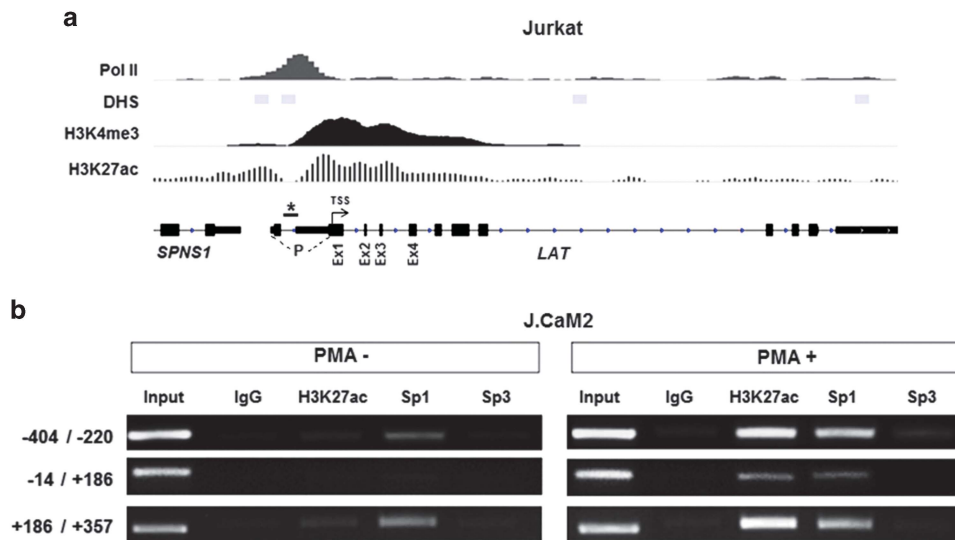


Figure 5. Histone modifications and recruitment of Sp transcription factors associated with the steady-state and PMA-induced expression of *LAT*. **(a)** ChIP-seq profiles derived from publicly available ChIP-seq data sets for RNA Polymerase II (Pol II; GSE50622), DNase I hypersensitive sites (DHS; GSE29692), H3K4me3 (GSE35583) and H3K27ac (GSE59257)—visualized for *LAT* gene in resting Jurkat cells. The region defined earlier using dual-reporter assay¹⁰ as *LAT* proximal promoter (P) is bound by Pol II and displays DNase I hypersensitivity. The promoter is also occupied by H3K4me3 and H3K27ac that are indicative of active promoters. **(b)** Representative ChIP-PCR results revealing binding signals of H3K27ac, Sp1 and Sp3 on chosen *LAT* gene regions in untreated and PMA stimulated J.CaM2 cells. The experiment was repeated four times giving reproducible results.

essential but as yet unidentified mutations in distant *cis*-elements and/or *trans*-acting factors contribute to the *LAT* deficiency in J.CaM2 cells. In a study by Roose *et al.*,¹⁷ 193 genes were identified as differentially expressed between Jurkat and J.CaM2 cells. We speculate that the depletion of H3K27ac mark across the *LAT* locus in quiescent J.CaM2 cells (Figure 5) suggests that some of these mutations may affect chromatin remodeling factors necessary for the acquisition of permissive transcriptional conformation. Indeed, epigenetic silencing of *LAT* transcription was recently reported in latently HIV-1-infected cell lines, in which H3K4me3 and other

transcription activation mark associated with *LAT* gene, H3K9ac, were specifically suppressed by the action of HIV-1 gene products.¹³

In previous studies, an important potential contribution of ubiquitously expressed Sp1 and Sp3 transcription factors to the *LAT* promoter activity were reported. We confirmed and extended these findings by showing, using the ChIP assay, that in addition to the *LAT* proximal promoter also a downstream regulatory region bound Sp1 but virtually no Sp3 (Figure 5). An interesting parallel between the *LAT* and the *IL-10* genes in respect to their common regulation by the Sp1/Sp3 could be drawn. Although

the expression patterns of these genes were shown to be restricted to hematopoietic cells, both genes strongly depended on ubiquitously expressed Sp1/Sp3 factors recruited upon cellular activation that could be mimicked by the PMA treatment. Consistently with the above, the Sp1 DNA-binding inhibitor mithramycin A downmodulated both basal and PMA-induced expression of LAT (Figure 2c). It was also interesting to observe that in non-lymphoid human cell line HEK293, LAT promoter was able to drive basal and PMA-induced reporter transcription in the absence of lymphoid transcription factors (Figure 4b). This finding suggested a predominant role of non-lymphoid transcription factors in enhancing LAT transcription.

In conclusion, these data introduce J.CaM2 line as an useful cellular model for uncoupling *cis*-elements and *trans*-acting factors controlling basal and activation-induced expression of LAT. Both, Sp1/Sp3 recruitment to the LAT promoter and the adjacent intragenic region contribute to controlling the transcription pattern of this gene. J.CaM2 cells can also serve as a sensor for assessing biological activity of potential immunosuppressive therapeutics impairing LAT expression through the PKC-dependent signaling pathways.

MATERIALS AND METHODS

Reagents

PMA, Iono, CsA, mithramycin A, VPA were purchased from Sigma-Aldrich (St Louis, MO, USA) and were dissolved in dimethyl sulfoxide. Inhibitors of PLC γ (Et-18-OCH $_3$), MEK1/2 (PD 98059), ERK1/2 (FR 180204), PI3K (LY294002), AKT (AKT inhibitor VIII, Isozyme-selective, AKT 1,2) and mTOR (rapamycin) were from Merck Millipore (Watford, UK) and were dissolved in dimethyl sulfoxide. Rabbit, polyclonal anti-LAT antibody was obtained from Merck Millipore (#06-807). Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Sigma-Aldrich (#AP156P). β -Actin (C4) mouse monoclonal IgG1 antibody (sc-47778) was supplied by Santa Cruz Biotechnology (Heidelberg, Germany). ChIP-grade antibodies used in ChIP assays were as follows: normal rabbit IgG (#12-370), H3K27ac (#07-360), Sp1 (#07-645) and Sp3 (#07-107) purchased from Merck Millipore and normal rabbit IgG (#2729) from Cell Signaling Technologies (Danvers, MA, USA). FastDigest restriction enzymes *Bam*HI and *Nla*III were from Fermentas (Hanover, MD, Germany). DreamTaq polymerase PCR kit was purchased from Fermentas and KapaTaq PCR set was from Kapa Biosystems (Wilmington, MA, USA).

T-acute lymphocytic leukemia cell lines

Human acute lymphocytic leukemia cell lines: Hut-78, J.CaM2 and Jurkat (clone E6.1) were maintained in Roswell Park Memorial Institute 1640 medium, and HEK293 cell line was maintained in Dulbecco's modified Eagle's medium. The basal media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM L-glutamine (Sigma-Aldrich), 1% streptomycin/penicillin (Sigma-Aldrich) and the cells were cultured in a 5% CO $_2$ humidified incubator at 37 °C.

RNA isolation, cDNA synthesis and conventional PCR

Cytoplasmic RNA was purified using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Qiagen) according to the manufacturer's protocol. RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and used in quantitative PCR and conventional PCRs with DreamTaq DNA Polymerase (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative RT-PCR

The qRT-PCR assay was performed in three biological replicates on an ABI 7300 (Applied Biosystems, Foster City, CA, USA) instrument. Amplifications were carried out with 5 \times HOT FIREPol EvaGreen Quantitative PCR Mix Plus with ROX (Solis BioDyne, Tartu, Estonia). Each qRT-PCR run included 5 ng of cDNA and the reaction conditions were as follows: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 61 °C and 30 s at 72 °C. To control the formation of primer dimers and unspecific amplification products, the melting curve analysis was employed. The relative expression of LAT (amplified with F1/R1 primer pair) was normalized to the expression of *EEF1A1* and *HPRT1*. The nucleotide sequences of primers used for the qRT-PCR are listed in Supplementary Table 1.

Sequencing

A 2208-bp LAT gene fragment ranging from -916 to +1292 was amplified from J.CaM2 and Jurkat genomic DNA with F2/R2 primer pair and cloned into pJET1.2/blunt cloning vector (Fermentas, Thermo Fisher Scientific). The obtained clones and PCR products were sequenced with panel of primers listed in Supplementary Table 1 by the Laboratory of DNA Sequencing and Oligonucleotides Synthesis, IBB PAS, Warsaw, Poland.

PCR-RFLP

PCR fragments amplified with DreamTaq polymerase using F3/R3 primer pair (Supplementary Table 1) from J.CaM2 and Jurkat genomic DNA was digested with *Bam*HI endonuclease to confirm the presence of heterozygous g.237C>T mutation in J.CaM2. Digestion of the wild-type 443-bp PCR product generated 273- 86- and 84-bp fragments, whereas mutant allele was digested into 273- and 170-bp fragments. F4/R4 primers were used to amplify 348-bp PCR product from J.CaM2 and Jurkat cDNA, and detect heterozygous c.167C>T change in J.CaM2. *Nla*III digestion produced 288- and 60-bp fragments from wild-type DNA, whereas mutant sequence was cut into 183-, 105- and 60-bp products.

Reporter plasmids

All reporter constructs used in dual-luciferase reporter assay were based on the pGL4.14[luc2/Hygro] basic, promoterless vector (Promega, Madison, WI, USA). The cloned inserts were created by PCR amplification performed on genomic DNA from J.CaM2 or Jurkat using F2/R2 primer pair (Supplementary Table 1). Individual inserts were prepared by restriction enzyme digestion of the PCR product. Where needed insert ends were blunted using Pol II Klenow fragment to generate ends compatible with pGL4.14[luc2/Hygro] MCS. Site-directed mutagenesis of SP_{ABCDE} consensus sequences was performed using SP_{ABCmut} F/R and SP_{DEmut} F/R primer pairs. Incorporated mutations were the same as previously described. The constructs pGL4.14-LAT (-916/+357 wild type) and (-916/+357_{SPABCmut}) were sequenced to confirm the presence of mutations (Primer sequences included in Supplementary Table 1).

Transient transfection

Jurkat cells (clone E6-1) were seeded in a 24-well plate at the density of 0.4 \times 10⁶ cell per well in 0.5 ml of Roswell Park Memorial Institute 1640 without antibiotics. Two hours before DNA transfection, 10 μ l of K2 Multiplier (Biontex, Munich, Germany) was added to each well. Jurkat cells were co-transfected with 60 ng of pRL-TK Renilla luciferase plasmid control vector and 600 ng of target pGL4.14-LAT [luc2/Hygro] construct using K2 transfection reagent (Biontex). To study promoter activity in basal state, the cells were lysed and subjected to dual-luciferase reporter assay (Promega) 24 h post transfection. To examine PMA-induced promoter activity, the cells were treated with PMA 24 h post transfection and the lysates were obtained 24 h post stimulation. The results are expressed as the ratio of firefly to Renilla luciferase activity. The value obtained for pGL4.14[luc2/Hygro] basic vector was set as onefold induction. Each bar on the chart represents the average fold induction and error bars denote s.d.

ChIP-PCR and analysis of public ChIP-seq data sets

The ChIP assays were performed on unstimulated and PMA-treated J.CaM2 cells using the EZ-Magna ChIP Chromatin Immunoprecipitation kit (17-408, Millipore, Watford, UK) according to the manufacturer's instruction. Small modifications were incorporated into chromatin preparation step. After cells were cross-linked and lysed in cell lysis buffer, the nuclei were pelleted and resuspended in the MNase Lysis buffer (50 mM Tris-HCl (pH 7.6), 0.2% NP-40, 50 mM CaCl $_2$) and left for 20 min on ice. Chromatin was digested with 4 μ l of Micrococcal Nuclease (New England Biolabs, Hitchin, Hertfordshire, UK) for 4 min at 37 °C, and the reaction was stopped by adding 20 mM EDTA and 20 mM EGTA. Nuclei were pelleted and resuspended in the Nuclear Lysis buffer. Remaining integral, nuclear membranes were disrupted by five cycles of sonication (HD 2070, Bandelin Sonopuls, Germany) using the following settings: duty cycle 30% and power 60%. Cell debris were pelleted by centrifugation and remaining supernatant containing chromatin was further processed according to the manufacturer's instruction. The protocol was repeated four times and each time the reproducibility of DNA fragmentation pattern was verified on agarose gel. A volume of 2 μ l of purified ChIP-DNA was subjected to PCR with KapaTaq polymerase (Kapa Biosystems) and three primer pairs targeting the following LAT regions: -404/-220, -14/+186 and +186/+357. The region upstream of

the –400 bp was difficult to amplify probably because it was strongly fragmented by MNase. ChIP assay was repeated three times and the results were reproducible.

Public ChIP-seq data sets sources: For the purpose of the study, four publicly available ChIP-seq data sets were utilized that were extracted from GEO database:²⁶ GSE50622,²⁷ GSE35583/ENCODE Consortium,²⁸ GSE59257,²⁹ GSE29692, GSE35583 and GSE17312/NIH Roadmap Epigenomics Mapping Consortium (<http://nihroadmap.nih.gov/epigenomics/>).³⁰ Visualization of the data was performed using the Integrative Genomics Viewer—IGV (Broad Institute).^{31,32}

Western blot

Cells were lysed in the radioimmunoprecipitation assay buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 20 mM methyl- β cyclodextrin) that was freshly supplemented with protease inhibitor cocktail (Sigma-Aldrich). Whole-cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were then incubated with anti-LAT at a concentration of 1:1000 and horseradish peroxidase-conjugated anti- β -actin at a 1:10000 dilution. The chemiluminescence assay was carried out as described previously.^{33,34}

Bioinformatics

Densitometric analysis was performed using ImageJ software, downloaded from NIH website (<http://imagej.nih.gov>).

Statistics

All bar charts demonstrate the mean \pm s.d. The graphed data are representative of at least three biological replicates. Messenger RNA relative quantification and statistical analysis were carried out using Relative Expression Software Tool (REST) 2009.³⁵ Student's *t*-test was used to analyze the data from dual-luciferase assay. $P \leq 0.05$ was considered to indicate a statistically significant difference. The asterisk denotes statistically significant differences between the indicated samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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