Molecular events involved in up-regulating human Na⁺-independent neutral amino acid transporter LAT1 during T-cell activation

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We investigated the regulation of system-L amino acid transporter (LAT1) during T-cell activation. In quiescent T-cells, L-leucine transport is mediated mainly by the system-L amino acid transport system and is increased significantly during T-cell activation by PMA and ionomycin. In quiescent T-cells, the LAT1 protein was heterocomplexed with 4F2 heavy chain (4F2hc) in the plasma membrane. During T-cell activation, the amounts of 4F2hc and LAT1 heterocomplex were significantly elevated compared with those in quiescent T-cells. In addition, by Northern-blot analysis, these increments were found to be due to elevated levels of LAT1 and 4F2hc mRNA. Transient expression of constructs comprising various *LAT1* gene promoter fragments, which contained all three of the GC boxes, was

sufficient for promoting luciferase expression in Jurkat T-cells, but the promoter of the *LAT1* gene did not respond to PMA and ionomycin. Similar observations were observed in the human *4F2hc* gene promoter. In nuclear run-on assay, the *LAT1* and *4F2hc* genes were actively transcribed even in quiescent T-cells, but the low levels of both transcripts were shown to be the result of a block to transcription elongation within the exon 1 intron 1 regions. These findings indicated that a removal of the block to mRNA elongation stimulates the induction of system-L amino acid transporter gene transcripts (LAT1 and 4F2hc) in activated T-cells.

Key words: elongation, promoter, run-on assay.

INTRODUCTION

The transport of amino acids across the plasma membranes of mammalian cells is a process of fundamental physiological importance. The system L is one of the amino acid transport systems responsible for transmembrane movement of neutral amino acids in animal cells and it exhibits high affinity for several nutritionally essential amino acids [1].

Recently, several studies have identified the important functions of the 4F2 heavy chain (4F2hc) as a component in the Na⁺-independent amino acid transport system [2–8]. This protein is associated with the system-L amino acid transporter (LAT1) [2,3]. When functionally expressed in mammalian cells together with 4F2hc, LAT1 induces the transport of neutral amino acids. When expressed independently, neither LAT1 nor 4F2hc was capable of amino acid transport to any significant extent [2,3].

LAT1 is identical to the E16/TA1 gene product, which was reported in activated T-cells and hepatocarcinoma [9–12]. The *E16/TA1* gene is present on nearly all rapidly growing tumour cells and on activated T-cells [9]. E16 transcripts are induced rapidly and degraded rapidly in human Jurkat T-cells after stimulation [12]. *E16/TA1* has a single or reiterated motif in the 3'-untranslated region of many short-lived transcripts [12]. This pattern of expression is unusual for an integral membrane protein and resembles more closely the kinetics seen for proto-oncogenes and lymphokines in the T-cell system [12]. In addition,

the function of 4F2hc was suggested to be coupled with cellular activation because of the expression pattern of 4F2hc [13,14]. 4F2hc is rapidly induced early in the G₁ phase, is expressed at high levels throughout the cell cycle and is constitutively present on the surface of activated and proliferating cells [15]. Some studies have reported the inhibitory effects of anti-4F2hc monoclonal antibodies on the growth of cell lines from lymphoid and fibroblastic origins, and on the proliferation of T- and B-lymphocytes to lectines [16]. Recent studies reported that 4F2hc is a type II transmembrane protein involved in cell-fusion events and integrin activation [13]. These observations suggest that system-L amino acid transport is very important for the activation of T-cells and neoplastic cells. However, a limited number of studies have investigated the regulation of the system-L amino acid transport [17–20]. In the present study, the mechanisms of induction of system L were investigated in activated T-cells.

EXPERIMENTAL

Materials

Restriction enzymes, reverse transcriptase and kanamycin were obtained from Takara (Kyoto, Japan) and Life Technologies; *Taq* polymerase was from New England Biolabs, Perkin-Elmer and Stratagene; total RNA from human kidney, the 5'-RACE

Abbreviations used: LAT1, system-L amino acid transporter; 4F2hc, 4F2 heavy chain; RACE, rapid amplification of cDNA ends.

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The nucleotide sequences reported in this study have been submitted to the GenBank[™] and EBI Nucleotide Sequence Data bases under accession number(s) AB023712 (exon 1), AB023713 (exon 2), AB023714 (exon 3), AB023715 (exon 4), AB023716 (exon 5), AB023717 (exon 6), AB023718 (exon 7), AB023719 (exon 8), AB023720 (exon 9), and AB023721 (exon 10).

Ready cDNA library and the human EMBL3 genomic library were from Clontech, and radioisotopes were from Amersham and DuPont Biotechnology Systems.

Transport assay for amino acids

Jurkat T-cells were cultured in minimum essential medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, penicillin (100 µg/ml), streptomycin (0.1 mg/ml), and non-essential amino acids. The cells were used 3 days after plating on 24-well plates. After T-cell activation by PMA (20 nM) and ionomycin (1 µM), the cells were cooled at 4 °C, centrifuged and resuspended for transport studies at a concentration of 2×10^7 cells/ml in Hank's balanced salt solution (HBSS). The cultured Jurkat T-cells were washed twice in HBSS and resuspended in HBSS at a concentration of 2×10^7 cells/well. In L-leucine-uptake studies, labelled [14 C]L-leucine (50 µM) was added to the incubation medium and incubated for various times. The cells were washed with cold HBSS containing 5 mM L-leucine. The methods of amino acid uptake were as described previously [21].

Isolation of the membrane fraction and Western-blot analysis

The membrane fraction of Jurkat T-cells was isolated as described previously [22]. The plasma-membrane fraction was removed using a syringe and diluted with a homogenate buffer containing 5 mM PMSF. After centrifugation at 75000 *g* for 60 min, the pellet was dissolved in suspending solution (1 % Triton X-100/20 mM Tris/HCl, pH 7.5/150 mM NaCl/1 mM PMSF).

An antibody was raised against a peptide that represented the amino acid sequence CQKLMQVVPQET in the C-terminal region of human LAT1 (positions 497–507), corresponding to a putative C-terminal intracellular domain [23]. Preparation of the affinity-purified antibodies followed a method previously described [23]. The plasma-membrane fractions were prepared as described above and subjected to SDS/PAGE in the presence of protease inhibitors.

Nuclear run-on transcription assay

Nuclei were prepared from Jurkat T-cells and nuclear run-on transcription assays were performed with minor modification of techniques reported previously [24]. Nuclei were harvested from 10^8 logarithmically growing cells by hypotonic lysis in 10 mM Tris (pH 8.4)/10 mM NaCl/3 mM MgCl₂/0.25 % Nonidet P-40. Nuclei were washed once in lysis buffer, resuspended in 100 µl of glycerol storage buffer (40 % glycerol/50 mM Tris, pH 7.6/5 mM MgCl₂/0.1 mM EDTA), and used immediately, or stored at –80 °C or in liquid nitrogen. Nascent transcripts were labelled in a reaction with 0.25 mM ATP, CTP and GTP in 5 mM Tris (pH 8.0)/2.5 mM MgCl₂/25 mM KCl/2.5 mM dithiothreitol/200 µCi of [α - 32 P]UTP (3000 Ci/mmol; NEN) for 30 min at 37 °C. Reactions were stopped by the addition of 10 × stop buffer (10 % Sarkosyl or 2.5 % SDS 1 mg/ml protease K/100 mM EDTA/100 mM Tris, pH 7.6) and additional incubation at 42 °C for 30 min. RNA was purified either by extraction with 3 equal vol. of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with 0.3 M sodium acetate and 100 % ethanol using yeast tRNA as the carrier, or by purification through 3 ml Sephadex G-50 columns.

Double-stranded targets were composed of pBluescript SK⁺ (negative control), human GAPDH cDNA in pBluescript SK⁺ (positive control), various PCR amplified fragments (5 µg) for each exon of the *LAT1* and *4F2hc* genes. These were denatured

Figure 1 Characterization of L-leucine uptake in activated T-cells

(A) Changes in uptake of L-leucine with time during T-cell activation. At the indicated times after initiating activation of Jurkat T-cells by PMA and ionomycin, the Na⁺-dependent and Na⁺-independent uptake of L-leucine were tested as described in the Experimental section. The values represent means $\pm$ S.E.M. ($n = 4$). * $P < 0.01$. (B) Effects of unlabelled amino acids on the transport of radiolabelled L-leucine in activated T-cells. The cells were incubated with PMA (20 nM) and ionomycin (1 µM) for 8 h and then L-leucine uptake was determined. In the absence of Na⁺, transport of [14 C]leucine was measured for 20 s in the presence or absence of unlabelled amino acids. The concentration of radiolabelled leucine was 50 µM and that of unlabelled amino acids was 5 mM. The values represent the percentage of the transport activity of the control. Values are means $\pm$ S.E.M. ($n = 4$; ** $P < 0.01$). Control uptake in the absence of unlabelled amino acids was 40 ± 12 pmoles/mg of protein per min. BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylate; Me-AIB, *N*-(methylamino)isobutyric acid.

in a final concentration of 0.1 M NaOH/10 mM EDTA at room temperature for 30 min and applied to nylon membranes at 5 µg/slot by vacuum filtration (BioDot apparatus; Bio-Rad). Membranes were prehybridized for 1 h and then hybridized with labelled RNA (0.5×10^7 – 1.0×10^7 cpm/ml per sample) at 42 °C for 36 h. Membranes were exposed to a Phosphor Imager screen (Fuji BAS-2000) for 2–24 h.

Fluorescent automated DNA sequencing

Fluorescence-based DNA sequencing was performed using a LI-COR model 4000L sequencer. Sequencing reactions were performed using a SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies), with 0.2 pmol of template and 2 pmol of primer.

Figure 2 Induction of the LAT1 and 4F2hc heterocomplex during T-cell activation

The cell membrane from Jurkat T-cells was immunoprecipitated with anti-4F2hc (**A**) and anti-LAT1 (**B**), and electrophoresed by SDS/PAGE under either, reducing (+ DTT, dithiothreitol) or non-reducing (— DTT) conditions. The immunoreactive proteins were detected using an ABC kit (Vector Laboratories). (**A**) 4F2hc polyclonal antibodies, lower arrow, 4F2hc (85 kDa); upper arrow, heterocomplex 4F2hc–LAT1 (130 kDa). (**B**) Rat LAT1 antibodies, lower arrow, LAT1 (40 kDa); upper arrow, heterocomplex LAT1–4F2hc (130 kDa). (**C**, **D**) Densitometric analysis of the amounts of 4F2hc and LAT1 heterocomplex in the plasma membrane. Values represent means $\pm$ S.E.M. ($n = 4$), $*P < 0.01$.

Identification and characterization of the human *LAT1* gene

The 5' ends of *LAT1* gene transcripts were determined using 5'-rapid amplification of cDNA ends (RACE) (Clontech) [25]. The first PCR amplification (reaction volume, 100 μ l) was performed with 5'-RACE Ready human Jurkat cDNA, anchor primer A (5'-CCATCCTAATACGACTCACTATAGGGC-3'), and primer RACE1 (5'-CGTGGGCGCCATCATCATCGGC-TCGGGC-3', nt +177 to +201 relative to the translation start site based on human *LAT1* cDNA) or primer 3b (5'-GAGTG-TGTCTGGAGTTGGCCTGGAAG-3', nt +499 to +516). A portion (2 μ l) of the first PCR product was subjected to 35 cycles of a second PCR amplification with anchor primer AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') and primer RACE2 (5'-GGCGCCGCGCAGGCGAGGCGAGGGCGT-3', nt +104 to +131 of *LAT1* cDNA). Amplified DNA products were subcloned into pBluescript II SK and

sequenced using a SequiTherm Long-Read Cycle Sequencing Kit-LC.

To isolate exons 1 and 2, a human EMBL3 genomic library was screened with 32 P-labelled DNA probes, which were amplified using 5'-RACE and PCR. Several overlapping clones containing the human *LAT1* gene were identified by high-stringency screening as described previously [25]. The distances between exons 1 and 2 were estimated by Southern-blot analysis, restriction-enzyme mapping and long-and-accurate PCR (LA-PCR) in a yeast artificial chromosome (YAC) clone (Tahoka, Tokyo, Japan) [25].

Primer-extension analysis

The oligonucleotide LAT1-1 (5'-GGCGCCGCGCAGGCGAGG-GCGAGGGCGT-3', nt +104 to +131 relative to the translation start site) was labelled at a concentration of 20 pM using a 5'-

terminal DNA-labelling system (Amersham) [25]. Free nucleotides were removed by two consecutive rounds of ethanol precipitation in the presence of ammonium acetate, and the labelled oligonucleotides were collected in 20 μ l of nuclease-free water. Labelled primer (2.5 pM) was co-precipitated with 20 μ g of total RNA from Jurkat T-cells using ethanol and sodium acetate. The dried pellets were dissolved in 30 μ l of hybridization buffer containing formamide. Following hybridization, the mixture was diluted with 130 μ l of a solution containing 50 mM Tris/HCl, pH 7.5, 40 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 75 μ g/ml actinomycin D, 0.5 mM of each deoxyribonucleotide and 2 units of avian myeloblastosis virus reverse transcriptase, and incubated at 37 °C for 1 h [25]. The products were precipitated using ethanol, size-fractionated on a 5% acrylamide/7 M urea gel, and visualized using autoradiography with an intensifying screen.

RNase-protection assay

RNase protection was performed with ³²P-labelled transcripts of the 640 bp (nt -462 to +178, including exon 1 and intron 1) *Sma*I/*Hind*III fragment of the human *LAT1* gene [26]. Total RNA was isolated from Jurkat T-cells following the method of Chomczynski and Sacchi [27].

Construction of luciferase plasmids for transfection

The plasmid pLAT1-3.2 was constructed by subcloning a *Sac*I/*Hind*III fragment of the human *LAT1* gene, including the 5' flanking sequence and exon 1, into the *Hind*III site of the pGL-3 basic vector (Promega, Madison, WI, U.S.A.). Plasmids LAT1-2100, LAT1-1120, LAT1-900, pLAT1-480, pLAT1-350, LAT1-300 and LAT1-100 were similarly constructed from progressively smaller fragments of the *LAT1* gene ligated into the *Hind*III site of the pGL-3 basic vector [26]. The orientation and precise limits of the constructs were verified by sequencing.

To isolate exons 1 and 2 of the human *4F2hc* gene, a human EMBL3 genomic library was screened with ³²P-labelled DNA probes amplified using 5'-RACE and PCR. Several overlapping clones containing the human *4F2hc* gene were identified by high-stringency screening as described previously [26]. p4F2hc-1, p4F2hc-2, p4F2hc-3, p4F2hc-4, p4F2hc-5 and p4F2hc-6 (containing the human *4F2hc* promoter [14] or enhance-cloned into pGL3 vector) were constructed from progressively smaller fragments of the *4F2hc* gene ligated into the *Eco*RI site of the pGL-3 basic vector [26].

DNA transfection and luciferase assay

All plasmids used in the luciferase assay were purified using Qiagen ion-exchange columns. Jurkat T-cells (A.T.C.C.) in 5 cm dishes were transfected with 5 μ g of luciferase construct and 1 μ g of pCMV β -galactosidase with the use of Transfast (Life Technologies, Gaithersburg, MD, U.S.A.) [26]. Typically, 10⁷ Jurkat cells were transfected with the reporter and constructed vectors and treated 24 h post-transfection with PMA plus ionomycin for 12–24 h. Cell lysates were prepared and assayed for β -galactosidase activity to determine transfection efficiency; the amount of cell lysate used for assaying luciferase activity was then adjusted based on the galactosidase activity.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. The differences between the means of two groups and three or more groups were estimated

Figure 3 Induction of LAT1 and 4F2hc mRNAs during T-cell activation

The cells were stimulated with PMA and ionomycin. At various times the cells were harvested using centrifugation and total RNA was isolated. Total RNA (20 μ g) was loaded on a 1.2% denaturing gel using a ³²P-labelled 4F2hc cDNA probe or LAT1 cDNA probe. Northern-blot analysis was performed as described in the Experimental section. Densitometric analysis of the amounts of 4F2hc and LAT1 mRNA was determined as described previously [7]. Values represent means $\pm$ S.E.M. ($n = 4$), * $P < 0.01$.

using Student's *t* test and one-way ANOVA, respectively. A *P* value of < 0.05 was considered significant.

RESULTS

Induction of the amino acid transport system L in Jurkat T-cells

L-Leucine was employed as the test substrate for system L in Jurkat T-cells. We studied the effect of Na⁺ on L-leucine uptake in this cell line. Uptake of L-leucine (50 μ M) was measured from two different uptake media, one containing NaCl and the other containing choline chloride in place of NaCl. The Na⁺-independent component of L-leucine uptake was greater than 80% of the total uptake (Figure 1A). After treatment with PMA plus ionomycin, L-leucine uptake was increased by about 2.5-fold of the control at 8 h. The increase in L-leucine uptake was mainly due to the Na⁺-independent component, but the Na⁺-dependent component.

The specific inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylate (BCH) for system L markedly blocked the leucine uptake in Jurkat T-cells (Figure 1B). In addition, uptake of radiolabelled leucine was found to be strongly inhibited by aromatic, as well as branched-chain, amino acids (Figure 1B).

(B)

Exon-intron structure of the human LAT1 gene

Intron number	Exon	Intron (size)	Exon
1	CAC TGC GTG C H C V L	gt gattacggggc. (17K) . . . cctctgtctccac ag	TG CTG CTC ACG L L T
2	ATC GGG AAG G I G K G	gt tgagtacgtcc. (11K) . . . ctttggccctgcc ag	GT GAT GTG TCC D V S
3	TAT GGA GGA TG Y G G K	gt aggttcttgaa. (576) . . . ttctctaaatttt ag	G AAT TAC TTG N Y L
4	AAC CCC TAC AG N P Y R	gt acgtgcaagaa. (604) . . . ccctgttctttcc ag	A AAC CTG CCC N L P
5	GAT GCC GTG V A V	gt gagccgctacc. (883) . . . gttgtgcctgcct ag	GAC TTC GGG D F G
6	ACA TCC TCC AG T S S R	gt gagcacagccc. (766) . . . cttgtctgtccac ag	G CTC TTC TTC L F F
7	GTG TTC ACG V F T	gt aagcctgctgc. (1.2K) . . . ccgcttctcttcc ag	TGT GTG ATG C V M
8	CCC ATC AAG P I K	gt gagcccggggc. (1.9K) . . . tgtcctctgcccc ag	GTG AAC CTG V N L
9	CAG GGC ATC T Q G I F	gt gagtatctcgc. (1.3K) . . . cttctctcctgac ag	TC TCC ACG ACC S T T
gt ag			

Figure 4 Structural organization of the human LAT1 gene

(A) The structural organization of the human LAT1 gene locus, comprising 10 exons spanning 30 kb of DNA, is depicted. A 1 kb scale is indicated to the right. The location of exons relative to the mRNA transcript is illustrated. The relative distances between exons (filled boxes) are shown. Values were obtained from analysis of the four genomic clones: LAT-1, λLAT1-2, λLAT1-3 and λLAT1-4. The distance between exons 1 and 2 was determined using restriction mapping and Southern-blot analysis with region-specific probes and PCR sequencing products. **(B)** Sequences of exon-intron junctions for the human LAT1 gene. Exon sequences (upper case) are indicated together with 50 bases of intronic sequence (lower case) at their 5' and 3' junctions. Consensus intronic splice junctions are shown as bold letters.

Expression of 4F2hc and LAT1 proteins

Recently [2], we identified the important functions of 4F2hc as a component in the Na⁺-independent amino acid transport system. This protein is associated with LAT1 and can act as a functional transport system. We therefore tested whether 4F2hc proteins could be detected in Jurkat T-cells. As shown in Figure 2, using 4F2hc-specific antibodies, two 4F2hc-specific protein bands of 85 and 130 kDa were detected in quiescent Jurkat T-cells under non-reducing conditions (Figure 2A). The 130 kDa band was not visible under reducing conditions, suggesting that this band might represent LAT1 heterodimers linked by a disulphide bridge. In activated T-cells, the intensity of the 130 kDa band was increased about 1.8-fold compared with that of quiescent Jurkat T-cells. Under reducing conditions, this band was reduced markedly and the 85 kDa band was prominent.

In addition, using LAT1-specific antibodies, two LAT1-specific protein bands of 40 and 130 kDa were detected in non-activated Jurkat T-cells under non-reducing conditions (Figure 2B). On treatment with PMA plus ionomycin, the intensity of the 130 kDa band was increased about 2.0-fold. Under reducing conditions, the 40 kDa protein band was prominent. These findings indicated that the 130 kDa band corresponded to a heterodimer of 4F2hc and LAT1, and that the increase in system-L transport activity was due to the concomitant induction of 4F2hc and LAT1 proteins in the plasma membrane. Thus LAT1 and 4F2hc proteins form a heterodimeric structure via a disulphide bond in the Jurkat T-cell membrane.

Induction of 4F2hc and LAT1 transcripts

Next, we investigated whether this elevation was mediated by changes in LAT and 4F2hc mRNA levels (Figure 3). The expression of LAT1 mRNA first occurred 2 h after PMA and ionomycin stimulation and peaked at 4 h, after which the levels of LAT1 mRNA gradually decreased. The levels of LAT1 mRNA were 6-fold increased in activated T-cells at 4 h. In comparison, the 4F2hc mRNA level was 5-fold increased at 4 h and then gradually decreased by 24 h. When we examined the effect of inhibitors on the induction of LAT1 and 4F2hc mRNA, actinomycin D suppressed the enhancement of LAT1 and 4F2hc mRNA in Jurkat T-cells immediately and almost completely; in contrast, cycloheximide showed no significant inhibition until 12 h after its addition (results not shown).

Structure of the 5'-untranslated region of human LAT1 mRNA

To identify exon 1 of the human *LAT1* gene, we used 5'-RACE to characterize the 5' end of a previously unidentified human LAT1 mRNA detected in a Jurkat T-cell 5'-RACE cDNA library and an oligo Cap cDNA library of human testis. We confirmed that the RACE products were derived from human LAT1 mRNA by showing that their uncharacterized sequences were identical to those of products obtained by reverse transcriptase-PCR (results not shown). As shown in Figure 4, the *LAT1* gene is organized into 10 exons spanning approximately 30 kb. The translation initiation codon exists in exon 1. Sequences around the exon-intron junctions are shown in Figure 4. All the exon-intron boundary sequences conform to the GT/AG rule. All the introns also contained the consensus sequence for RNA splicing, including an adenosine residue as a potential lariat acceptor site 8–36 bp upstream of the 3'-splice junctions [26].

Characterization of the LAT1 promoter

To identify the transcription start site for human LAT1 mRNA, primer-extension and RNase-protection analyses was performed.

Figure 5 Identification of transcription-initiation sites for exon 1 from primer-extension analysis and the nucleotide sequence of the human *LAT1* gene promoter

Primer-extension analysis was performed with a primer specific for transcripts containing exon 1. The ³²P-labelled LAT1 oligonucleotide was hybridized with total RNA from Jurkat T-cells and the resulting extension products were separated using electrophoresis adjacent to a DNA sequencing ladder generated from a human LAT1 genomic subclone with the same primer. Identical findings were obtained in two separate experiments. Two closed triangles indicate the cap site.

Primer extension was performed with ³²P-labelled LAT1 primer and total RNA from quiescent and activated Jurkat T-cells. The extended products were separated by electrophoresis together with a sequencing reaction mixture as a source of size markers. Two extended products of 189 and 191 bp were observed (Figure 5).

For RNase-protection analysis, ³²P-labelled antisense RNA probe was hybridized to total RNA from Jurkat T-cells and subjected to RNase digestion. The expected sizes of the protected fragments, on the basis of the location of the start site identified above, were 170 and 171 bp for the sequence primer of intron 1 (results not shown). The RNase-protection data are thus consistent with those obtained by primer-extension analysis. The findings of these analyses thus indicated that the cap site was either 111 or 115 bp upstream of the exon 1–intron 1 junction.

As shown in Figure 6, the putative promoter lacks consensus TATA or CATT boxes, but contains three GC-rich elements that include the consensus Sp1-binding motif (GGGGCGG), characteristic of the promoters of housekeeping genes. The 5'-flanking region contains one AP-2-binding site at nt –282 to –270. In addition, three heat-shock-factor-binding-like sequences are presented (accession number AB023712).

Functional analysis of the LAT1 and 4F2hc gene promoters

Reporter constructs were made in which the 5' upstream sequences of exon 1 were fused to a luciferase reporter gene (Figure 7, closed bars). The site of fusion was within the untranslated leader sequence of the *LAT1* gene at position +13 relative to the transcription initiation site. When transfected into Jurkat T-cells, a construct containing sequences –2100 to +13 from LAT1 (pLAT1-2100) was able to direct expression of luciferase activity (37 ± 5.2-fold increase). Progressive deletion to positions –1110,

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-1740 TAAGTAAGTG GGGCGGAGCC TCCCTGGTGG CCGGTCGTCA GCCCAGTGCC TGAAATGCAT
                                     CREB
-1680 GCAAAGTCTA TTTTATTTTT GCGTAGTTGA ACCTAAGCAA AAAATCTGGC CCTACAATCT
-1620 CTAACCTTGC TCCTAACTGG CACCTACCCT ATTGCACAAG AAGCTATCTA GGGGAGGACT
                                     C/EBPα
-1560 CAGTGAGCAC TCGGTGAGTT TGCCAGCCA TGGTGGTACT GTCTCCTTCC CTCTGCAGGC
                                     AARE
-1500 ACAGCGGGAA GAATAGGCGG GATGGAGGGC TCTCTGCACC TTCCACAGCC AGCAGCGTGG
                                     GATA-1
-1440 ACGGCAGATT ACAAGTTAGT ATCTGTGTGA CCTCCGCACT CCCCTGCCGG TGGTGCCTTG
-1380 GGCTGCTGAG ATTTGCTTGG GTTTTATCAC TGCAGAGGAA TGCCCGCCAT ACCGGGGGGC
                                     GATA-X
-1320 GAGCAAGGTC CAGAATGACA AGCCGTGGCA TTCCTCAGCC GCCCCCTCCC CGGGGTGGGG
-1260 CCTGGACATT CCTGGCTGCA GCTAGAGGTC CCAGCATGAC AGGGATTCTG ACCCCCCCTC
-1200 CACCACGGCT ATTGTTCAGG ATTCTGTGGA TTCCAGAGGC ATCTGCCAGC CAGACTGTCT
-1140 TGCTGTGAAC ACCCAGCTCT GCAACCGGTG TTTGCGTGTG TGCCCTGGGA GGCTGAACGC
-1080 TCACCCAGAA CAAAGGCCTC CCCACATGCC TGCTGTGAGC TTCCAAGGAC TCGTTAGGAC
                                     HSF
-1020 ACACGCCAAG GAACCCCGGG TGGGGGTGAT ACCGAGGAGC TTCTCCCTG ACCCACACAC
                                     GATA-1,2
-960 ACCCTTTGAA GAGGTCTTTG GCTGCTCACC AGTCTGGTCA ATCTCGGGC AGTATTTATA
                                     cdx-A
-900 TTCTTCCCG TTTCCAGGCC TAGGAGCCTT GGCATCCTGC TCTCGCCTCT AACTGAAATT
-840 ACCTCTCAAC CCTTTTTCAG TAAGATGCCT CTCGCATTCC CCAGGAATAA CAAGCTTTAA
-780 AGCCCTTCA CACTGAAGCC TGCTTTTGAA TTCCCAGCT AGATTGGGG GTGGGGGTCT
-720 TATGGCTCCC GGTGAACCCC CGGTTCTGTT GATTAAATTG AGCTCAGGCA GATTGCTGGC
                                     HSF
-660 TTCCCAAAGC CTGTGTATT ACTGCTTCTC AAAGCTACGG GCACCGGATC GCCATCTTTG
-600 GCAAGATTCT TTTGGGATAC AATGGGAATT ACTGGGTGGC CTCGGTTCGG TTCTTCCCTC
                                     HSF
-540 GTCTCAGTTC GCGGCTCACC AGCCACATTG ATGCAGCCCC CAGGCTGGAA GGAGGCTGCA
-480 GGAGCTTCCC CTCAGGTCAT CCTCTCATCC CTCCCCGTG CCCCAGGAGC TGGTTGTGGG
-420 GCGGTTTCCA TCCCCTAGGC CCATCCGGGA CAGGAGCCTA GGTTCCTTTC GGGGGGTACC
                                     GATA-1
-360 CCAAACCCCA TCCTTGGCCT CAGGCCAGCC CTGGTGCAC TCCCGCTCCC AGGCTTGACG
-300 AGAGGCTGCG GGCCAGTGGG TGAAGGGGCT CGCCCTGACT GCCAGGCCCC GCCCAGGCGC
-240 ACCCGGGAGG ACGGGGTGGG ATGACGCGGG CGCCGGGGGG GTAGGTCGCG AGGCCGGGGT
                                     AP-2
-180 CTCCATGGCG CAGGAGGACG GGGGCCTTCG AGGACCACGC GGGCCTGGGA ATCGCCTGCC
-120 AGGCTAGGCC GGAGACGCA CGTGCTCCGA GCTGGGTGGA GGGGCGGGG CTGAGAGCCG
                                     Sp-1
-60 CGGCCGGGCC ACGGGGCGGG GAGGAGCCGC GGACGGTGGG CGGGGCCAGC GGGCCGGGGC
                                     Sp-1
+1 CTAAAAGGCG GCGCGGGCGG GGTCTTGAC GCAGCTGCGG GCGGCGAGCG GCGCGCACAA
    L→START
+61 TCCTCGCTCG GCTGCGGCTC TCGGGTGTCC CAGGCCCGGC CGGTAAGCAG AGCATGGCGG

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Figure 6 The nucleotide sequence of the region surrounding the first exon of the human *LAT1* gene

The sequenced region was from the *Bam*HI and *Hind*III restriction sites. Location of the transcription-initiation site is indicated by an arrow. HSF, heat-shock transcription factor; GATA, GATA-binding protein; AARE, amino acid-responsive element; CREB, CAMP-response-element-binding protein; C/EBP, CCAAT-enhancer-binding protein.

–893, –784, –469, –342 and –281 resulted in an increase in activity, reaching a maximum of 240 % of the pLAT1-2100 construct. In contrast, lower activity was apparent with the pLAT1-100. Deletion of nt –281 to –100, including the three

GC boxes, resulted in a 10-fold drop in promoter activity, indicating that this region contains strong positive elements. Transfection with the control vector resulted in no measurable luciferase activity. We investigated the stimulation of *LAT1* gene

Figure 7 Functional analysis of the human *LAT1* gene promoter

Jurkat T cells were transfected with the pGL-3 basic vector containing the indicated fragments of the *LAT1* (A) and *4F2hc* (B) gene promoters upstream of the luciferase gene. Construction of luciferase plasmids for transfection was described in the Experimental section. Luciferase activity, expressed as light units/s per mg of protein, was subsequently assayed in cell lysates as described in the Experimental section. The effect of PMA (20 nM) and ionomycin (1 μ M) on the promoter function (closed bars) was determined 24 h after stimulation. Values are the means $\pm$ S.E.M. from four independent experiments.

transcription in Jurkat T-cells by PMA plus ionomycin. As shown in Figure 7(A) (closed bars), the activities for the reporter vectors containing various deletion fragments did not respond to PMA plus ionomycin.

Similarly, we isolated the human *4F2hc* gene and characterized its promoter. The human *4F2hc* gene promoter contained a G+C-rich 5'-flanking housekeeping promoter which lacked TATA and CAAT sequences, but contained binding sites for the ubiquitous Sp1 transcription factor [14]. In a previous study,

Gottesdiener et al. indicated that nuclear proteins bind the NF-4A and NF-4B sequences of intron 1 of the mouse *4F2hc* gene [14]. The enhancer element of intron 1 of the mouse *4F2hc* gene is also necessary, at least in part, to respond to PMA and ionomycin stimulation. We tested whether the first intron of the human *4F2hc* gene contains a transcriptional enhancer element that binds multiple nuclear proteins and responded to PMA. In the present study, such positive enhancer regions were detected in the intron 1 of the human *4F2hc* gene. As shown in Figure 7

Figure 8 Half-life of LAT1 and 4F2hc mRNA in activated T-cells

The cells were unstimulated or stimulated as indicated with ionomycin and PMA. After added actinomycin D ($5 \mu\text{g/ml}$), total RNA was isolated at various times, and the amount of LAT1 or 4F2hc mRNA was measured using Northern-blot analysis and plotted on semi-logarithm scale against the time of actinomycin D addition. Each point represents the mean $\pm$ S.E.M. ($n = 4$) of three experiments (A), 4 h after activation and (B) 8 h after activation.

(closed bars), the activities for various deletion fragments of intron 1 of the human 4F2hc gene did not respond to PMA and ionomycin.

Half-life of LAT1 and 4F2hc mRNAs

To clarify the mechanisms involved in the elevation of LAT1 and 4F2hc transcripts in activated T-cells, we investigated (i) alterations in mRNA stability and (ii) modulation of a block to transcription elongation.

First, the effect of PMA and ionomycin on the stability of 4F2hc and LAT1 mRNAs was investigated (Figure 8), because LAT1 transcript has a single or reiterated motif in the 3'-untranslated region of many short-lived transcripts. After treatment of PMA plus ionomycin for 4 h and 8 h, the RNA-synthesis inhibitor actinomycin D was added to the cells, and incubation was continued for 1, 2, 3 and 6 h. As shown in Figure 8, the half-life of LAT1 mRNA was 3 h in the activated T-cells and 6 h in the quiescent T-cells. The half-life of 4F2hc mRNA was about 10 h in both the activated and quiescent T-cells. PMA and ionomycin did not significantly alter the stability of 4F2hc

mRNA. In contrast, the half-life of LAT1 mRNA was shortened by PMA and ionomycin.

Elimination of the block to transcription elongation by PMA and ionomycin

Run-on transcription assays were performed to determine whether the increases in LAT1 and 4F2hc mRNA levels observed following PMA and ionomycin stimulation were due to increased transcription (Figure 9A). ^{32}P -Labelled run-on transcription products were hybridized to Southern blots containing fragments derived from different regions of the LAT1 gene. In the first experiment, each PCR fragment, encoding exons 1 (probe A), 2 (probe B), 8 (probe C) and 9 (probe D), was used. The intensity of hybridization to probe A reflected the level of transcription of exon 1, whereas hybridization to probe B reflected the level of transcription of exon 2. In quiescent Jurkat T-cells, the intensity of hybridization to probe A was about 10-fold higher than those of probes B and C. This indicated that only a portion of the initiated transcripts continued through the first intron.

After 4 h of PMA/ionomycin stimulation, the intensity of hybridization to probes B and C was approximately equal to that

Figure 9 Run-on transcription assay in the nuclei isolated from activated T-cells

Nuclei were prepared from Jurkat T-cells that were treated with PMA and ionomycin from 4 h. Transcriptions in the isolated nuclei were analysed by hybridization of the ^{32}P transcripts to 5 μg of linearized plasmids containing various fragments for each exon (probes A–D) of the *LAT1* or *4F2hc* genes, GAPDH cDNA (probe E), or no insert (pBluescript II) (Probe F). In the *LAT1* gene (A), probe A is the partial sequence of exon 1 (179 bp, +325 to +504). Probes B–D are the complete sequences of each exon (E1, E8, E9): exon 2, probe B, 126 bp; exon 8, probe C, 150 bp; exon 9, probe D, 178 bp. In the *4F2hc* gene (B), probes A–D are the partial sequences of exon 1 (E1), 2 (E2), 6 (E6) and 9 (E9) (203, 170, 181 and 520 bp respectively), respectively. Probes A–D were amplified using PCR and subcloned into the pBluescript II vector. After purification of the amplified fragment, these were denatured in a final concentration of 0.1 M NaOH/10 mM EDTA at room temperature for 30 min and applied to nylon membranes at 5 $\mu\text{g}/\text{slot}$ by vacuum filtration. Quantification of transcription rate was performed using a densitometer and the rates were normalized with respect to the GAPDH signal. Run-on transcription assays of quiescent (–) and PMA/ionomycin-activated (+) T-cells are shown. Values are means $\pm$ S.E.M. from four experiments.

of probe A. PMA-plus-ionomycin treatment stimulated a 7-fold increase in exon 2 transcription (intensity of probe B/GAPDH) and also a 10-fold increase in that of exon 7. In contrast, the elevation of the exon 1 transcript was minimal.

Similar findings were obtained in the run-on assay of the human 4F2hc transcript. PMA-and-ionomycin treatment resulted in an approx. 10-fold increase in the exon 2 transcript (intensity of probe B/GAPDH) of the 4F2hc gene compared with that in the non-treated cells. Despite the increase in the intensity of hybridization to probes B, C and D in PMA-plus-ionomycin-treated cells, no elevation of intensity of probe A was detected (Figure 9B).

DISCUSSION

System L is predominantly responsible for supplying the cells with a majority of essential amino acids, and so studies on the physiological regulation of system L are of interest and importance [1]. In the present study, we characterized system-L amino acid transport in activated T-cells. The transport properties found in activated T-cells resembled those of LAT1. The immunoprecipitation assay using LAT1- and 4F2hc-specific antibodies suggested that system-L amino acid transport was composed of a LAT1–4F2hc complex in the cell membrane. The increase in the amount of 4F2hc–LAT1 complex paralleled the elevation of L-leucine uptake in activated T-cells.

Since the regulation of 4F2hc gene expression has been studied extensively [17–20], we focused on the mechanisms of the induction of LAT1. The 5'-RACE analysis suggested that utilization of human *LAT1* gene promoters do not change. Run-on assay indicated that the elevation of LAT1 was due to increases in transcriptional activity, but not the elongation of mRNA half-life. PMA and ionomycin did not stimulate the promoter activity of the *LAT1* gene. The first intron of the mouse 4F2hc gene contained a transcriptional enhance-element that binds multiple nuclear proteins and responds to PMA [17]. The nuclear protein binds the NF-4A and NF-4B sequences of intron 1 [17]. The enhancer element is necessary, at least in part, to respond to PMA and ionomycin stimulation. In contrast, such positive enhancer regions were not detected in intron 1 of the human 4F2hc gene. In addition, the changes in the promoter utilization did not contribute to the expression of the LAT1 transcript in the activated T-cells.

Interruption of transcription in the first intron of 4F2hc has already been worked out by Leiden's group [14,15], who showed that PMA releases the block, resulting in full-length transcripts. Indeed, the steady-state levels of mature 4F2hc mRNA were very low in resting T-cells. This was due to blocked transcription elongation within the exon 1/intron 1 region of the gene. In the present study, phorbol ester activation removed this block to transcription elongation, resulting in increased levels of full-length 4F2hc mRNA. Similar observations were obtained in the study of the *LAT1* gene.

Control at the level of transcriptional elongation has also been observed in several eukaryotic genes themselves. Blocks to transcription elongation regulate the abundance of proto-oncogenes such as *c-myc* [28,29], *c-myb* [30,31] or *c-fos* [32–34] and other genes such as adenosine deaminase [35–39], ornithine decarboxylase [40], histone 3.3 [41], *Drosophila* heat-shock genes [42] or apolipoprotein A-1 [43]. Eukaryotic DNA sequences representing transcript blocks have been identified, but these sequences are not the sole determinants of regulating read-through, as termination can be modulated by initiation from different promoters. Hence, modification of the transcription complex by accessory factors may alter the process of RNA

polymerase. In addition, the RNA secondary structure may play a critical role in attenuation events. The regulation of proto-oncogenes *c-myc* and *c-myb* includes blocks to elongation within intron 1, and phorbol ester removed this block in cellular activation [28,29]. Both the increase in transcription initiation and the loss of normal attenuation mechanisms contributed to the *c-myc* overexpression that occurs in human Burkitt's lymphoma [30].

The patterns of expression in LAT1 and 4F2hc in the activated T-cells are unusual for an integral membrane protein and resemble more closely the kinetics observed in proto-oncogenes (*c-myc*, *c-myb*) and lymphokines in the T-cell system [30]. Indeed, several previous studies indicated that the expression of 4F2hc and LAT1 mRNA is very rapid and essential to the activation of T-cells and neoplastic cells. Regulation of system-L amino acid transport appears to play an important role in decisions about cellular growth versus differentiation, in addition to c-Myc [28–31]. The attenuation mechanisms are important to the rapid and concomitant induction of 4F2hc and LAT1 in activated T-cells.

In addition, Nakamura et al. [44] demonstrated that 4F2hc alone is expressed efficiently on the cell surface as a monomer. In contrast, LAT1 is expressed minimally at the plasma membrane in mammalian cells, remaining mostly in the Golgi area, and requires 4F2hc to be sorted to the cell surface. These findings indicate that one of the functions of 4F2hc is to guide LAT1 to the plasma membrane. Therefore, the induction of LAT1 may be necessary to accompany the expression of the guidance molecule 4F2hc. Thus a removal of the block to mRNA elongation of the *LAT1* and 4F2hc genes appears to be an adequate mechanism for the rapid induction of system-L amino acid transport in activated T-cells.

Finally, Jurkat T-cells possess an inducible system L, which can be activated by PMA and ionomycin. The concomitant increase in LAT1 and 4F2hc is essential for the expression of the L-system amino acid transport, and the rapid induction of system L in activated T-cells is, at least in part, due to modulation of a block to transcriptional elongation in the 4F2hc and *LAT1* genes. Further studies are needed to map the location of the elongation block more precisely using a cell-free transcript of the LAT1 template.

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