Human T-Lymphotrophic Virus Type I \textit{tax} Regulates the Expression of the Human Lymphotoxin Gene

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Human T-lymphotropic virus type-I (HTLV-I)-infected T-cell lines constitutively produce high levels of lymphotoxin (LT). To analyze the mechanisms that lead to the expression of LT in HTLV-I-infected cell lines, we studied regulatory regions of the human LT promoter involved in the activation of the human LT gene. As determined by deletional analysis, sequences between +137 and −116 (relative to the transcription initiation site) are sufficient to direct expression of a reporter gene in the HTLV-I-infected cell line MT-2. Site-directed mutation of a of the single 8-1-like motif present in the LT promoter region (positions −99 to −89) completely abrogated LT promoter activity in MT-2 cells, suggesting that this site plays a critical role in the activation of the human LT gene. Transfection of LT constructs into HTLV-I–uninfected and –unstimulated Jurkat and U937 cell lines showed little to no activity of the LT promoter. Cotransfection of the same constructs with a tax expression plasmid into Jurkat cells led to detectable promoter activity, which could be significantly increased by stimulation of the cells with phorbol myristate acetate (PMA). Similarly, cotransfection of the LT promoter constructs and the tax expression plasmid into U937 cells led to significant promoter activity upon stimulation with PMA. These data suggest that HTLV-I \textit{tax} is involved in the upregulation of LT gene expression in HTLV-I–infected cells.

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LYMHPOTOXIN (LT) is a cytokine produced by T and B lymphocytes after stimulation by antigen,1 phytohemagglutinin (PHA),2,3 or phorbol myristate acetate (PMA).4,5 Besides the initially described cytopathic effect of LT on a variety of cell lines in vitro6 and the induction of tumor necrosis of methylcholanthrene-induced sarcomas in vivo,7 a variety of other biologic effects, such as the induction of surface antigen expression in various cells,8,9 antiviral activity,10,11 the activation of osteoclasts, and the induction of bone resorption,12,13 have been assigned to this lymphokine.

The human T-lymphotropic virus type-I (HTLV-I)14 has been identified as the cause of adult T-cell leukemia (ATL), an aggressive, usually fatal T-cell malignancy.15 Recently, a severe, slowly progressive myelopathy (tropical spastic paraparesis [TSP]) that is endemic in certain Caribbean islands has also been associated with HTLV-I infection.16 An identical disease has been reported to occur in HTLV-I–infected individuals in Japan and has been termed HTLV-I–associated myelopathy (HAM).17 Autonomously growing, HTLV-I–infected T-cell lines can be established from the peripheral blood of ATL18 and from the cerebrospinal fluid (CSF) of TSP/HAM patients.19,20 Similarly, continuously growing cell lines can be established by in vitro infection of T lymphocytes with HTLV-I.21 We recently reported that HTLV-I–infected T-cell lines constitutively express high levels of LT messenger RNA (mRNA) and release biologically active LT.22 Expression of HTLV-I structural proteins is not required for the activation of the LT gene in these cells22 and we postulated that an HTLV-I regulatory gene product, most likely \textit{tax}, is involved in the activation of the LT gene. In the present report, we analyze the 5′ untranslated region of the human LT gene and demonstrate that HTLV-I \textit{tax} is indeed able to induce the expression of the human LT promoter.

MATERIALS AND METHODS

Cell lines. The human T-cell lines Jurkat (a gift of W. Greene, Howard Hughes Medical Institute, Department of Medicine, Duke University Medical Center, Durham, NC), the HTLV-I–infected T-cell line MT-2,14 and the monocytic cell line U937 were maintained in RPMI-1640 medium containing 10% fetal calf serum plus 100 U/mL penicillin and 100 \mu g/mL streptomycin at 37°C and 5% CO2.

Construction of the LT/chorompenicol acetyl transferase (CAT) constructs. The 5′ flanking region of the human LT gene (−819 to +137 with respect to the CAP site) was obtained from a genomic cosmid clone (a gift of T. Spies, Department of Biochemistry and Molecular Biology, Cambridge, MA) cloned into the EcoRI and BamHI sites of the pSP72 plasmid (Promega, Madison, WI) and sequenced by the method of Sanger et al.23 Preexisting restriction enzyme sites (Fig 1C) were used to generate 5′ deletion fragments, which were blunt-end ligated either into the Bgl II site of the pCAT3M plasmid24 (Fig 1A) or into the Sal I site of a newly generated CAT plasmid that contains the Bgl II to BamHI CAT cassette of pCAT3M cloned into the BamHI site of the pSP72 plasmid (pspCAT2; Fig 1B). To generate the fragment −116/+137 of the LT gene, we used the polymerase chain reaction (PCR) technique using the original pSP72/LT plasmid as template. The 5′ primer (5′-AACACGCTGTATCTTTAAAGGACGGCCGCTCATCATC-3′) was designed from the reference site for the initiation of transcription.25 The DNA fragment resulting from amplification was digested with Pvu II and EcoRI, filled in with T4 DNA polymerase, and cloned into the blunt-ended Sal I site of the pSPCAT plasmid. For the mutation of the eB site by PCR, the 5′ primer contained in addition the desired nucleotide changes (5′-ACACACGCTGTATCTTTAAAGGACGGCCGCTCATCATC-3′). The DNA fragment containing the mutation was obtained from pSP CAT by digestion with Bgl II and would be used as template. The pSPCAT plasmid contains the Bgl II to BamHI CAT cassette of pCAT3M, cloned into the BamHI site of the pSP72 plasmid (pspCAT2; Fig 1B). To generate the fragment −116/+137 of the LT gene, we used the polymerase chain reaction (PCR) technique using the original pSP72/LT plasmid as template. The 5′ primer (5′-AACACGCTGTATCTTTAAAGGACGGCCGCTCATCATC-3′) was designed from the reference site for the initiation of transcription.25
various fragments of the 5' region of the human LT gene. (A) A circular map of pCAT3M as generated by Laimins et al. The site of transcription of the LT gene. The 5' regions of the genes that were cloned either into the Bgl II or BamHI fragment (1.6 kb) of pCAT3M was cloned into the BamHI site of the pSP72 plasmid (B) to produce a CAT plasmid (pSPCAT) containing multiple cloning sites. (C) The different fragments of the 5' regions of the LT genes that were cloned either into the Bgl II site of pCAT3M or the Sal I site of pSPCAT. The numbers in parenthesis refer to the nucleotide positions with regard to the initiation site of transcription of the LT gene. The 3' end is identical for all fragments (position +137). The Pvu II* and Pvu II mut** fragments were generated by PCR using mutagenic primers as described in Materials and Methods.

Other plasmids. The tax expression plasmid (pcTax) contains a full-length cDNA copy of the HTLV-I tax gene under the control of the human cytomegalovirus (CMV) immediate early promotor. The KS-M13+ or the pGEM4 plasmids were used as control DNA in several transfection protocols. The HTLV-I long terminal repeat (LTR)/CAT plasmid (HTLV-I LTR/CAT) consists of the LTR of the HTLV-I/CR isolate cloned 5' to the CAT gene resident in pSVOCAT.

Cell transfection and CAT assays. For DNA transfection, the diethyl aminoethyl (DEAE) dextran/cloroquine method was used. Cells (5 × 10^6) were transfected as described by Holbrook et al. MT-2 cells were transfected with 3 μg of DNA U937 and Jurkat cells were transfected with 6 μg of DNA per 5 × 10^6 cells. In the latter case, 3 μg of the different LT promoter/CAT or the HTLV-I LTR/CAT constructs was either cotransfected with 3 μg of pcTax or an irrelevant plasmid (pGEM4 or KS-M13+). All transfections were repeated at least three times with independent plasmid preparations. After transfection, cells were seeded at 10^6 cells/mL culture medium in 25 cm² culture flasks (Costar, Cambridge, MA). Cell cultures were maintained at 37°C, 5% CO₂ and harvested 48 hours after transfection. For some experiments, PMA (20 ng/mL) was added 24 hours before harvest. Cell extracts were prepared by freeze thawing and treatment at 65°C for 10 minutes. Reporter gene (CAT) activity was analyzed either according to the thin-layer chromatography (TLC) methods described by Gorman et al. or according to a liquid-phase acetylation method reported recently. In the latter case, samples were counted on a Beckman liquid scintillation counter at various time points up to 16 hours. In both assays, equal amounts of cellular protein were used as determined by the BioRad protein assay (BioRad, Richmond, CA).

RESULTS

Analysis of the human LT-promoter activity in HTLV-I-infected cells. To determine regions within the 5' regulatory part of the LT gene required for promoter activity, a series of 5' deletion fragments was inserted upstream of the CAT reporter gene (psPCAT; Fig 1). The activity of the different plasmid constructs was determined after transfection into MT-2 cells, which constitutively produce LT. Maximum promoter activity was observed with deletion constructs ranging from position -304 to -252 (Fig 2). Smaller constructs showed a decrease in activity that was most pronounced with construct pLT-116. The addition of PMA to transfected MT-2 cells 24 hours before harvest led to an increase in the promoter activity (Fig 2) of all constructs.

Fig 2. Activity of LT-promoter deletion constructs in MT-2 cells. The different pLT constructs were transfected into MT-2 cells by the DEAE dextran method. Cytoplasmic extracts from unstimulated cells (left side) or cells stimulated for 24 hours with PMA (20 ng/mL, right side) were prepared after 48 hours. CAT activity of extracts was assessed by a 16-hour liquid scintillation assay. CAT conversion as percentage of the total added 3H-coenzyme A is indicated on the y-axis. The bars represent the mean percentage of acetylation calculated from five individual experiments. The designation of the different constructs is given at the bottom of the figure. The pSPCAT plasmid served as negative control. Lysates of MT-2 cells transfected with an HTLV-I LTR/CAT construct were used as control and yielded greater than 90% conversion within several hours (data not shown).
promoter, we wanted to ascertain the relevance of this NF-κB binding site for the activation of the human LT promoter. For this purpose, we introduced a mutation into the critical region by PCR amplification (Fig 3A). Transfection of the mutated LT/CAT plasmid (pLT-116mut) into MT-2 cells showed that the activity of the LT promoter (pLT-116) was completely lost after the substitution of three nucleotides within the κB sequence (Fig 3B). Stimulation of MT-2 cells with PMA could not restore wild-type promoter activity of the mutated plasmid (Fig 3B).

Effect of tax and/or PMA on the LT-promoter activity in Jurkat and U937 cells. Transfection of the pspCAT control plasmid into U937 and Jurkat cells yielded high background activity after stimulation with PMA. We therefore established a series of LT/CAT constructs using the pCAT3M plasmid as the basic vector (Fig 1A and C). Transfection experiments showed that these plasmids were active in MT-2 cells, albeit to a lower extent than the LT/CAT constructs described above (data not shown). To test the effect of HTLV-I tax on the LT promoter, the LT/CAT deletion constructs were cotransfected into Jurkat and U937 cells either together with a plasmid containing the HTLV-I tax gene under the control of a CMV promoter (pcTax) or an irrelevant plasmid.

As shown in Fig 4A, cotransfection of LT/CAT constructs and pcTax resulted in the detection of promoter activity. The activity could be significantly increased by stimulation of the cells with PMA (Fig 4B). In contrast, LT/CAT constructs cotransfected with irrelevant plasmid DNA were inactive in unstimulated (Fig 4A) Jurkat cells and stimulation with PMA led to a modest increase in CAT activity only with the construct LT-149 (Fig 4B). Using a liquid-phase CAT assay, we could confirm the data obtained with the TLC assay. As shown in Fig 4A, cotransfection of LT/CAT constructs with an HTLV-I tax expression plasmid led to a pronounced increase in LT-promoter activity in both U937 and Jurkat cells. These data indicate that cellular factors induced by PMA are important for the trans-activation by tax of the human LT gene. The relative inducibility of the different LT/CAT constructs by tax was comparable in U937 and Jurkat cells and maximal promoter activity was obtained after deletion of the 5′ untranslated region up to nucleotide position −149. In contrast, maximal promoter activity in MT-2 cells was achieved with constructs extending to position −304. A similar phenomenon, i.e., the loss of murine LT-promoter activity in constructs extending further 5′ than position −293 of the murine LT gene, has been observed by Fashena et al. A possible explanation for this finding might be the presence of negative

Fig 3. Analysis of LT-promoter function after site-directed mutation of the NF-κB binding site. (A) The sequence of the NF-κB binding site obtained from the pLT-116 (wt) or the pLT-116mut (mut) plasmid after site-directed mutagenesis by PCR is as described in Materials and Methods. Wild-type (pLT-116) and mutant (pLT-116mut) plasmids were transfected into Jurkat and U937 cells either together with a plasmid containing the HTLV-I tax gene under the control of a CMV promoter (pcTax) or an irrelevant plasmid.

DISCUSSION

We have analyzed the human LT promoter using deletion constructs of the 5′ untranslated region of the LT gene joined to a CAT reporter gene. These LT-promoter constructs were highly active when transfected into HTLV-I-infected MT-2 cells. Maximal activity was observed with constructs extending to positions −304 and −252 of the LT gene, but even deletions progressing as far as position −116 did not completely abrogate LT-promoter activity.

By mutation studies we could demonstrate that a single κB site present at positions −89 to −99 is crucial for the function of the human LT promoter because elimination of this site abolished promoter activity in unstimulated as well as PMA-stimulated MT-2 cells. This has been previously shown only for the murine LT gene. For transfection experiments involving HTLV-I-uninfected cell lines, a different set of LT-promoter constructs was used, as indicated in Results. These LT-promoter/CAT constructs showed no significant activity in unstimulated Jurkat and U937 cells and induction with PMA was minimal. Cotransfection of LT/CAT constructs with an HTLV-I tax expression plasmid led to the detection of significant promoter activity in Jurkat cells, but not in U937 cells. Stimulation of the cells with PMA after cotransfection of LT/CAT constructs and the tax expression plasmid led to a pronounced increase in LT-promoter activity in both U937 and Jurkat cells. These data indicate that cellular factors induced by PMA are important for the trans-activation by tax of the human LT gene. The relative inducibility of the different LT/CAT constructs by tax was comparable in U937 and Jurkat cells and maximal promoter activity was obtained after deletion of the 5′ untranslated region up to nucleotide position −149. In contrast, maximal promoter activity in MT-2 cells was achieved with constructs extending to position −304. A similar phenomenon, i.e., the loss of murine LT-promoter activity in constructs extending further 5′ than position −293 of the murine LT gene, has been observed by Fashena et al. A possible explanation for this finding might be the presence of negative
Fig 4. HTLV-I tax–induced LT-promoter activity in Jurkat cells. LT-CAT constructs as indicated at the bottom of the figure were cotransfected into Jurkat cells with pcTax (+tax) or an irrelevant plasmid (–tax). Cytoplasmic extracts were obtained after 48 hours from unstimulated transfected cells (left panel) or cells stimulated for 24 hours with PMA (20 ng/mL) as described in Materials and Methods. Percentage of CAT conversion after 16 hours of reaction: (A): LT-572, (+tax) = 3.2%, (–tax) = 0.3%; LT-304, (+tax) = 5%, (–tax) = 0.1%; LT-149, (+tax) = 7.3%, (–tax) = 0.9%; pCAT3M, (+tax) = 1.35%, (–tax) = 0.8%; HTLV-I LTR, (+tax) = out of range/89%, (–tax) = 3.1%; (B): LT-572, (+tax) = 6.2%, (–tax) = 0.75%; LT-304, (+tax) = 11.9%, (–tax) = 2.3%; LT-149, (+tax) = 41%, (–tax) = 4%; pCAT3M, (+tax) = 2%, (–tax) = 1%; HTLV-I LTR, (+tax) = out of range/94%, (–tax) = 31%.

regulatory elements upstream of position -149 of the human LT gene. Unlike MT-2 cells, both Jurkat and U937 cells do not produce high levels of LT even after stimulation with PMA (data not shown) and factors responsible for the suppression of LT gene expression might be operant in these cells.

The human LT gene is located within 8 kb in a tandem configuration with the tumor necrosis factor-α (TNF) gene. Enhancer elements have been identified within the 5′ region of the TNF gene located within a distance of less than 3.5 kb from the LT promoter, and a restriction enzyme fragment length polymorphism within the 5′ region of the LT gene has been reported to be associated with its inducibility. This complex distribution of possible control elements makes it very likely that regulatory elements other than those addressed in this report also participate in the regulation of LT gene expression.

Among the various biologic effects of LT, its antitumor and antiviral activities are the most relevant clinically. The demonstration that HTLV-I tax is able to induce the LT promoter suggests that the activation of the LT gene in HTLV-I–infected cells is not due to cellular defense mechanisms in response to viral infection, but rather is a direct or indirect effect of the HTLV-I regulatory gene product tax on the LT-promoter region. The activation of LT in HTLV-I–infected cells suggests the participation of this lymphokine in the pathogenesis of HTLV-I–induced diseases. Hypercalcemia (with and without osteolytic bone lesions) is frequently observed in ATL patients. Several cytokines produced by HTLV-I–infected cells have been suggested in the past to contribute to these symptoms. LT activates osteoclasts, induces hypercalcemia, and acts synergistically with other osteoclast-activating factors in bone resorption. It is therefore conceivable that induction of LT by HTLV-I in vivo contributes to the development of the osteolytic bone lesions and hypercalcemia frequently observed in ATL patients. This assumption is supported by data from a study by Ishibashi et al, who reported that Japanese ATL patients with hypercalcemia exhibit elevated serum levels of LT.

The association of TSP/HAM, a demyelinating disease of the central nervous system, with HTLV-I infection has only recently been established. HTLV-I–infected cell lines can be established from the CSF of such patients, and polyclonal integration of HTLV-I has been demonstrated in up to 10% of their peripheral T lymphocytes. It is of interest that the ability of myelin basic protein (MBP)-reactive T-cell clones to induce experimental autoimmune encephalitis (EAE) in mice is positively correlated with the amount of LT and TNF production by these cells. In addition, the development of EAE can be prevented by the administration of anti-LT/TNF antibodies to the mice after transfer of the MBP-specific T-cell clones. Because EAE represents a reliable model for demyelinating neurologic disorders, it is conceivable that induction of the LT gene by HTLV-I tax in cells infiltrating TSP/HAM lesions plays a role in the pathogenesis of this disease. Extensive clinical studies will be necessary in the future to define the in vivo significance of LT activation by HTLV-I tax and its participation in disease processes during HTLV-I infection.
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