Transgenic Thymocytes Are Refractory to Transformation by the Human T-Cell Leukemia Virus Type I *tax* Gene†

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Previous transgenic work demonstrated transforming activity of the human T-cell leukemia virus type I Tax protein in fibroblasts. In the present study, a Thy-1-based vector was used to express Tax in thymocytes. These mice developed no functional or neoplastic abnormalities of T cells but developed fibroblastic tumors with a longer latency than in the previous model.

Human T-cell leukemia virus type I (HTLV-I) infection has been associated with the proliferative disease adult T-cell leukemia, with the neurodegenerative disease tropical spastic paraparesis, and with the musculodegenerative disease polymyositis (1, 2, 6, 8, 10, 15-17, 20, 21, 23, 25). An HTLV-I-encoded protein called Tax is of particular interest because it is a potent transcriptional activator of the HTLV-I viral promoter and several cellular genes.

To test the effects of the *tax* gene in vivo, we have generated Tax-expressing transgenic mice. In the first model (12), expression was directed by the HTLV-I long terminal repeat (LTR) promoter. High levels of expression were achieved by feedback of Tax on its own promoter and were seen in tail tissue, skeletal muscle, salivary gland, peripheral nerve, and bone (5, 12, 13) and resulted in induction of perineurial fibromas. No discernible effects on adult lymphoid tissue could be identified. However, expression was not detected in lymphocytes.

To assess the effects of Tax protein on the lymphoid system, we have generated a new vector derived from the murine Thy-1.2 gene capable of expressing Tax protein in the thymus of transgenic animals. This gene is highly expressed on thymocytes, with lower levels on peripheral T cells, neurons, astrocytes (18, 19, 24), and scattered fibroblasts (3). The plasmid pThy1.2 is a complete genomic clone obtained from AKR mice and contains approximately 1.6 kb of 5'- and 1 kb of 3'-flanking DNA (7) (Fig. 1a). It contains two alternate first exons, with the translation initiation codon contained in exon 2. Access to exon 2 of the native Thy-1.2 gene was obtained through a *Sma*I site at position 2694. A unidirectional deletion from positions 2490 to 2694 was created, which removed exon 2 and the 88 bp preceding it. Two synthetic oligonucleotides (Fig. 1b) were then inserted (Fig. 1c). These oligonucleotides restored authentic splice donor and acceptor sites (7) and allowed cloning of *tax* cDNA as a 1.8-kb *Bgl*II fragment from the LTR-*tax* plasmid (Fig. 2) at the unique *Bgl*II site within the vector. This *tax* fragment contained an efficient translational initiation codon and a complete copy of the HTLV-I LTR at its 3' end (Fig. 2).

Within the LTR are three copies of a *tax*-responsive

element shown to be the minimal sequence necessary for transcriptional augmentation by the Tax protein (bottom of Fig. 2). In addition, the LTR is capable of forming extensive secondary structure and contains viral polyadenylation signals. The complete transgenic vector is shown in Fig. 1d.

Northern (RNA blot) analyses demonstrated that all 10 derived transgenic lines expressed Tax-containing transcripts in the thymus. More detailed tissue surveys were performed on five of these lines. Two patterns emerged, typified by lines 20 (high-level expressor) and 11a (low expressor) in Fig. 3. Endogenous Thy transcripts are shown in Fig. 3a. For line 20, thymus was the only site of expression, whereas line 11a also expressed comparable levels in the bone marrow, intermediate levels in the brain, lung, and testes, and lower levels in other organs. Other lines showed patterns intermediate between those of lines 20 and 11a.

The Thy promoters directed two classes of Tax-containing transcripts. The first is a short spliced transcript of about 2 kb which initiates in the Thy1 promoter and terminates within the LTR in synthetic exon 2. It is slightly smaller than the transcript directed by the LTR-*tax* vector (see perineurial tumor control, Fig. 3b). The vector also directs several longer spliced transcripts of approximately 4.5 kb which terminate beyond exon 2. When thymus RNA from Thy-*tax* 20 mice was compared with perineurial tumor RNA from the LTR-*tax* mice (a highly expressing tissue), accumulation was approximately 1/5 to 1/10 of the amount in LTR-*tax* mice. Similar results were obtained by Western immunoblotting, which revealed a product indistinguishable in size from that in the infected human cell line C57-66-81. Levels of protein accumulation were also 1/5 to 1/10 of the amount of those from LTR-*tax* tumors.

In order to prove that functional amounts of Tax protein were made in these cells, Thy-*tax* mice were mated with LTR-*tax* mice and doubly transgenic offspring were selected. Previous studies have shown that the LTR-*tax* construct is not expressed in the thymus of these singly transgenic mice (12, 13).

The LTR-*tax* construct in fact contains two LTR promoters, each with a complete set of *tax*-responsive elements (Fig. 2). Therefore, Tax-mediated initiation of transcription may occur from both promoters. Probing Northern blots with *tax* cDNA allows recognition of a 2-kb transcript initiating from the 5' LTR of LTR-*tax* (transcript 1 in Fig. 2). This transcript differs in size from the 4.5-kb Thy-directed *tax* transcripts but is close in size to the short Thy-directed

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FIG. 1. Construction of Thy-tax vector. (a) Map of murine genomic Thy-1.2 clone obtained from G. Evans. The clone contains four *Sma*I sites and an upstream *Bgl*II site. The two alternate first exons (E1a and E1b) are shown. (b) Sequence of oligonucleotides used to make synthetic exon 2. Sequences which match the consensus for the Thy-1.2 splice donor and acceptor regions are bracketed. Sequences flanking the consensus core were also included. The acceptor and donor sites are indicated by boxes. (c) Diagram of pThyΔE2 shows substituted synthetic exon 2. Splice donor (SD) and splice acceptor (SA) positions are indicated. The upstream *Bgl*II site has been eliminated. (d) The completed pThy-tax expression vector. *tax* cDNA has been placed into exon 2.

transcript. In contrast, probing with simian virus 40 (SV40) sequences allows identification of a 900-base transcript arising off the 3' LTR promoter of the LTR-*tax* construct (transcript 2 in Fig. 2). This is a unique marker for activity of the LTR-*tax* transgene, which is not present in the thymus of singly transgenic LTR-*tax* mice.

The results of such an analysis of doubly transgenic mice generated from the Thy-*tax* 20 and LTR-*tax* lines are shown in Fig. 4. Baseline LTR-*tax* expression was not detected in thymus tissue, as indicated by probing with *tax* (upper) or SV40 (middle) probes. However, doubly transgenic mice express more *tax* RNA in the thymus than their Thy-*tax* parent and demonstrate new activity of the 3' LTR-*tax* promoter when probed with SV40 (middle). In contrast, *tax* transcript levels were not detected in resting spleen in singly or doubly transgenic animals. The Thy-*tax* transcript may, however, be induced in spleen after concanavalin A stimulation, which also correlates with augmentation of endogenous Thy transcript. Further, expression studies on purified thymocytes showed that lymphocytes but not tissue stroma expressed Tax. These studies demonstrate that functional Tax protein was made in the thymus. To confirm this, doubly transgenic Thy-*tax* × LTR-*lacZ* mice were generated, and β-galactosidase activity in the thymus was analyzed. In agreement with RNA and protein data, these mice showed

FIG. 2. Schematic diagram of LTR-*tax* (previously called HTLV-tat-1). The position of the 5' *tax*-encoding transcript (transcript 1) and the 3' SV40 (transcript 2)-containing mRNAs are shown in the middle. The broken area on the circle indicates synthetic oligonucleotide (described in reference 12). At the bottom (hatched) are indicated the positions of the three imperfect 21-bp repeats of the *tax*-responsive elements.

FIG. 3. Northern blot analysis of tissues from *Thy-tax* transgenic mice. (a) Analysis of RNA from a nontransgenic C57BL/6 mouse probed with nucleotides 2694 to 5322 (*Clal-KpnI*) of pThy1.2. (b and c) RNA from organs of transgenic lines *Thy-tax* 20 and *Thy-tax* 11a. Ten milligrams of total cellular RNA was used per lane. Probed with *tax* cDNA. Perineurial (PN) tumor RNA was obtained from a *tax*-induced fibroma which occurred in *LTR-tax* mice (12, 15).

levels of transactivation which were about one-fourth of the maximal levels in *LTR-tax* × *LTR-lacZ* mice. Complete data on *LTR-lacZ* mice will be discussed in detail elsewhere (22).

Thy-tax mice appeared phenotypically normal throughout the first year of life. However, between 16 and 24 months, mice from *Thy-tax* lines 1, 20, 21, and 22 developed characteristic tumors on the tail, ears, and hind limbs (Table 1). The

TABLE 1. Tumor development^a

Mouse line	No. observed	No. with fibroblastic tumor	% with tumor
Control	67	0	0
<i>Thy-tax</i> 1	21	5	24
<i>Thy-tax</i> 20	18	7	39
<i>Thy-tax</i> 21	5	1	20
<i>Thy-tax</i> 22	3	2	67

^a Mice from each of the lines were observed for more than 18 months. Mice with macroscopic tumors were killed, and histologic confirmation was performed.

distribution and appearance of these tumors were similar to those seen for the *LTR-tax* mice. Histologic analyses showed these tumors to be formed of fibroblastic elements similar in appearance and pleiomorphism to those described for the *LTR-tax* mice. However, less than half of the *Thy-tax* mice developed tumors, with a median latency of greater than 18 months (Table 1). This contrasts with tumor development in *LTR-tax* mice, in which nearly 100% had tumors by 8 months.

In order to examine the consequences of Tax protein on lymphocytes, thymocytes and splenocytes from singly or doubly transgenic mice were examined by fluorescent analysis. Thymocytes were doubly labeled with CD4 plus CD8 to quantitate subpopulations. No differences were seen between normal and transgenic mice in the fraction of singly positive, doubly negative, or doubly positive cells. In order to measure the effect of Tax on activation of these cells, cells were stained for levels of surface interleukin-2 receptor (IL-2R). When thymocytes or splenocytes were examined for both IL-2R plus either CD8, CD4, or Thy1.2 expression, no significant difference was seen in the proportion of cells which were IL-2R positive in transgenic or normal mice. Further studies revealed no differences in proliferative response to concanavalin A between normal and transgenic splenocytes. Singly transgenic mice have been observed for up to 24 months and doubly transgenic mice for up to 12 months. These mice exhibited no obvious immune system abnormalities or obvious increased incidence of spontaneous leukemia.

The above experiments suggest that *tax* expression alone confers minimal direct phenotypic or functional effects on thymocytes. However, it is possible that *tax* has an indirect effect, serving as an oncogenic cofactor. To test this, we subjected *tax*-expressing mice to sublethal doses of irradiation. Early gamma-irradiation studies by Kaplan et al. (9) demonstrated that varying the schedule of doses delivered as well as the age at exposure could greatly influence the frequency with which C57BL/6 mice develop lymphoma. However, the latency period between radiation and disease was minimally affected. Radiation resulted in no significant change in latency for three *Thy-tax* lines, for the *LTR-tax* line, or for double transgenic offspring compared with the median 159 days of latency seen for nontransgenic C57BL/6 mice. In addition, histologic analysis revealed no characteristic differences between the thymic lymphomas of the transgenic mice and their normal littermates.

In previous studies of the *LTR-tax* transgenic mice, we have noted an augmentation of expression in transformed fibroblasts or salivary gland (5, 12, 13; unpublished observations). Figure 5 shows comparisons of *tax* RNA levels from unmanipulated mice and from those in which radiation was used to induce lymphomas. Radiation-induced transforma-

FIG. 4. Northern blot analysis of *Thy-tax* × *LTR-tax* doubly transgenic mice. *Thy-tax* 20 mice were mated with *LTR-tax* mice, and doubly transgenic offspring were selected. Previous studies (Fig. 3) showed that *tax* expression was limited to the thymus in line 20 mice. Expression in thymus and spleen of singly or doubly transgenic mice is compared in adjacent lanes. NI, Normal mouse control. Blots were probed with cDNA for *tax*, SV40, or actin.

FIG. 5. Northern comparison of RNA from thymus, lymphoma, and fibroma tissues from Thy-*tax* transgenic mice. For thymus and lymphoma tissue, 5 mg of polyadenylated RNA was used per lane. For fibroma samples, 10 mg of total cellular RNA was used. Probes are indicated on the right-hand side.

tion is in fact associated with a decrease, not an increase, in *tax* expression. This contrasts sharply with the high levels of expression seen in spontaneously developing fibroblastic tumors (far right lanes in Fig. 5).

The method of exon substitution and Tax-mediated feedback amplification described in this article provides a general technique for targeting cDNAs in a variety of transgenic mouse tissues. By generating doubly transgenic Thy $\times$ LTR-*tax* mice, high levels of *tax*-encoding transcripts were achieved. These were only twofold lower than those seen in the most highly expressing tissues of LTR-*tax* line 6-2 mice and were comparable to those in other LTR-*tax* lines (e.g., 8-4), which efficiently developed perineurial fibromas.

In the LTR-*tax* model, disease induction was ultimately associated with high levels of Tax expression in a subpopulation of parenchymal cells. This latency period varied with tissue type. This event also occurred in fibroblasts of the Thy-*tax* mice but was greatly delayed. Both constructs contained a complete copy of the HTLV-I LTR which included the *tax*-responsive elements. In the LTR-*tax* construct, this was adjacent to the promoter, whereas this is approximately 4 kb downstream of the Thy promoter. Feedback amplification through this element may be responsible for discontinuous levels of expression, which appear to be either on or off. In contrast, transgenic lymphocytes underwent no further upmodulation of *tax*, nor did they change phenotype. Tax expression alone in these cells failed to augment genes, such as those for IL-2R and cytokines (data not shown), which are known to be associated with HTLV-I transformation. Tax failed to hasten or augment radiation-induced leukemogenesis, nor was radiation-induced proliferation associated with upmodulation of *tax*. Radiation also was not associated with a shift in latency for development of fibroblastic tumors. Previous studies of radiation-induced leukemogenesis show a strong correlation with *ras* mutations (14). This suggests that *ras* cooperation may not be an important event in Tax-mediated tumorigenesis in vivo. Ongoing studies will test this.

Together, the data suggest that Tax-mediated disease induction is a multistep process to which different tissues have various susceptibilities. Steps may occur independently or be linked. An early step may be upmodulation of *tax*. In fibroblasts, skeletal muscle, and salivary gland, upmodulation occurs spontaneously and ultimately leads to disease induction. In the present study, simple upmodulation

in lymphocytes may occur without disease. In fact, our data and that from epidemiologic studies suggest that HTLV-I products are very inefficient at transforming T cells (14). In these cells, additional steps may be required (taking up to 40 years and occurring in less than 4% of patients) to develop the fully transformed phenotype. It is likely that these secondary events occur more frequently in fibroblasts. An important distinction between fibroblasts and lymphocytes is in their growth response to stimulation. Fibroblasts characteristically remain activated and proliferate for a longer period of time than lymphocytes, which rapidly return to a resting state. This may affect the differences in the ability of these two cells to proliferate in response to Tax.

It is unclear how commonly HTLV-I infects fibroblasts in humans. The partially defective character of the HTLV-I envelope proteins may limit infection of this cell type in patients. To date, only a single case of HTLV-I-associated fibroblastic malignancy has been reported (9a). However, the availability of a model in which fibroblasts are reproducibly transformed by *tax* should facilitate elucidation of the biochemical pathways of transformation in this cell. Subsequent comparisons between fibroblasts, Thy-*tax* thymocytes, and virally transformed human cells will be important. In addition, the ability to generate tissue culture lines from our mice will allow detailed investigation of early events in pathogenesis, such as breakage of latency.

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