JOURNAL OF VIROLOGY, July 1989, p. 3185–3189
0022-538X/89/073185-05$02.00/0

Vol. 63, No. 7

Thymic Atrophy Characteristic in Transgenic Mice That Harbor pX Genes of Human T-Cell Leukemia Virus Type I

YASUHIDE FURUTA,[1] SHINICHI AIZAWA,[1]* YOKO SUDA,[2] YOJI IKAWA,[1] HIDEHIRO KISHIMOTO,[3] YOSHIHIRO ASANO,[3] TOMIO TADA,[3] ATSUKO HIKIKOSHI,[4] MITSUAKI YOSHIDA,[4] AND MOTOHARU SEIKI[4]

Laboratory of Molecular Oncology[1] and Laboratory of Molecular Mechanisms of Aging, Frontier Research Program,[2] Tsukuba Life Science Center, Physical and Chemical Institute (RIKEN), Koyadai, Tsukuba, Ibaraki-305, and Laboratory of Immunology, School of Medicine, University of Tokyo, Hongo, Bunkyo-ku,[3] and Department of Viral Oncology, Cancer Institute, Kami-Ikebukuro, Toshima-ku,[4] Tokyo-170, Japan

Received 28 November 1988/Accepted 24 March 1989

The human T-cell leukemia viruses (HTLV) are associated with T-cell malignancies in humans. The malignant transformation occurs after a long latency in some carriers, and its mechanism appears to be distinct from that of other classes of retroviruses which induce transformation through viral or cellular oncogenes. A widely postulated explanation is that the products of novel pX genes transactivate endogenous cellular genes which lead to tumor development in T cells. To directly examine the pathological effects of pX genes in vivo, we produced transgenic mice harboring the HTLV type I pX genes under several regulatory units: HTLV type I long terminal repeat, immunoglobulin enhancer-simian virus 40 promoter, and mouse mammary tumor virus long terminal repeat. Atrophy of the thymus was characteristic in these mice no matter which regulatory unit directed the expression of the genes.

Human T-cell leukemia virus type I (HTLV-I) is an etiological agent of adult T-cell leukemia (12, 22), which is endemic to parts of Japan, the Caribbean, and Africa. The mechanism by which HTLV induces T-cell malignancies is unknown. No oncogene is encoded in the viruses (1, 15), and it is unlikely that insertional activation of the cellular proto-oncogenes accounts for the malignant transformation (5, 14). A unique pX region that contains four possible open reading frames exists in the genome of this class of viruses (15, 17). One of the products, $p40^{tax}$, has been reported to transactivate the virus long terminal repeat (LTR) (4, 18). Thus, it has been postulated that the pX product activates the expression of cellular genes, which leads to the malignant transformation of T cells. Indeed, the transactivation of cellular interleukin-2 (IL-2) receptor, IL-2 (2, 9), and c-*fos* (3, 7) genes has been reported. To examine the pathobiological effects of pX genes in vivo directly, we produced a series of transgenic mice.

Figure 1 gives a schematic representation of the HTLV-I pX genes introduced. To compare the pathobiological effects of pX genes in a variety of cell types and to examine the possibility that the effects are specific to T cells, the pX cDNA (16) was combined with the respective regulatory units of HTLV-I LTR (4), mouse mammary tumor virus (MMTV)-LTR (13), and immunoglobulin enhancer-simian virus 40 (SV40) promoter (20). Thus, the constructs could potentially produce not only $p40^{tax}$ but also $p27^{rex}$ and $p21^{X\text{-}III}$. In each construct, the production of $p40^{tax}$ was confirmed with cultured cells. In transgenic mice, MMTV LTR has been reported to direct the expression of transgenes not only in mammary glands but also to various degrees in a variety of tissues (8), and the regulatory unit composed of immunoglobulin enhancer and SV40 promoter (Ig/Tp) has been reported to direct such expression strongly in spleen or B cells and weakly in other tissues or types of cells (20; Y. Suda, S. Aizawa, Y. Furuta, Y. Ikawa, K. Saito, Y. Yamada, K. Toyoshima, and T. Yakamoto, submitted for publication).

Each hybrid DNA was digested with restriction enzymes to remove the sequences derived from the vector (LTR-pX was digested with *Hin*dIII and *Eco*RI; MMTV-pX was digested with *Hin*dIII and *Eco*RI; Ig/Tp-pX was digested with *Xba*I and *Bam*HI) and was injected into F_1 zygotes between C57BL/6 and CD1 mice as previously described (20). We obtained 42 transgenic founders, of which 37 harbored the transgenes in intact forms. Among these 37 founders, 3 were mosaic, 2 had double insertions, and 5 were infertile. After the founder mice were crossed with C57BL/6 mice to generate F_1 offspring, and thereafter by sibling mating, 5, 18, and 9 transgenic lines were finally established with LTR-pX, MMTV-pX, and Ig/Tp-pX DNAs, respec-

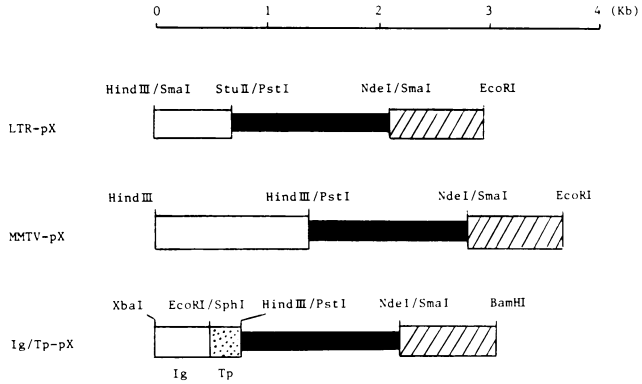

FIG. 1. HTLV-I pX transgenes. Symbols: ■, pX cDNA (16); ▨, SV40 termination unit derived from pSV2Neo (19); □ and ▭, regulatory unit. HTLV-I LTR contains the U3, R, and U5 from which 85 base pairs of the 3′ end is deleted (4). MMTV LTR was derived from pMDSG (13), and Ig/Tp contains the mouse immunoglobulin heavy chain enhancer and SV40 early gene promoter (20). The restriction sites are given as the sites in the original DNA clones for each unit. Kb, Kilobases.

* Corresponding author.

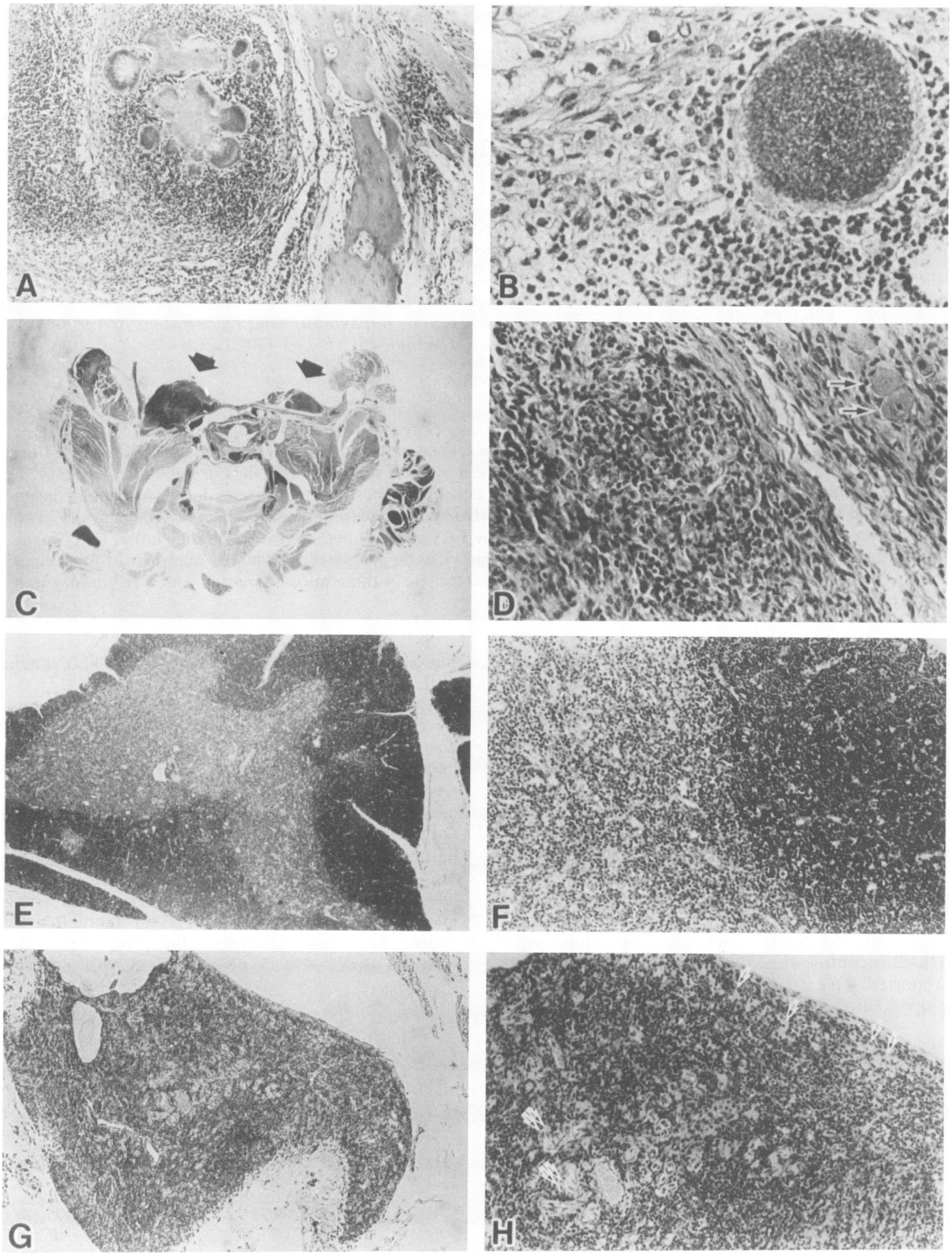

FIG. 2. Histologic features of the pX transgenic mice. (A and B) Circumscribed abscesses observed in thigh; multiple bacterial colonies were observed within bone marrow and identified bacteriologically as *S. aureus*. Neutrophils, foamy macrophages, and plasma cells were actively infiltrating the colonies. (C and D) Masses in trigeminal nerve ganglia (arrows) of a mouse with hemiparesis; these were confirmed as masses of infiltrating neutrophils and macrophages. Arrows indicate surviving glial cells among inflammatory cells. (E and F) Thymus of a control mouse; note the clear boundary between the cortex (the thick and highly cellular outer area) and the medulla (the less cellular inner area). (G and H) Atrophic thymus of a pX transgenic mouse with growth retardation; the cortex is depleted, and the corticomedullary junction is obscure. Swollen macrophages (arrows) were numerous, with "starry sky"-like structures. The medullary area was also hypocellular, partly retaining the framework structure of epithelial cells (double arrows). (I) Thymus-dependent area (arrows) in spleen of a normal mouse. (J) Involution of thymus-dependent area (arrows) in spleen correlated with thymic atrophy. (K) Atrophy of kidney glomeruli (arrows). (L) Vacuolation in cerebellum. (M) Moderate depletion of thymus observed in an apparently normal pX-transgenic mouse. The histologic observations were made on paraffin-embedded sections which were stained by hematoxylin and eosin. Magnifications: ×90 (A), ×440 (B), ×5.5 (C), ×275 (D), ×65 (E and G), ×140 (F, H, I, J, and M), ×220 (K and L).

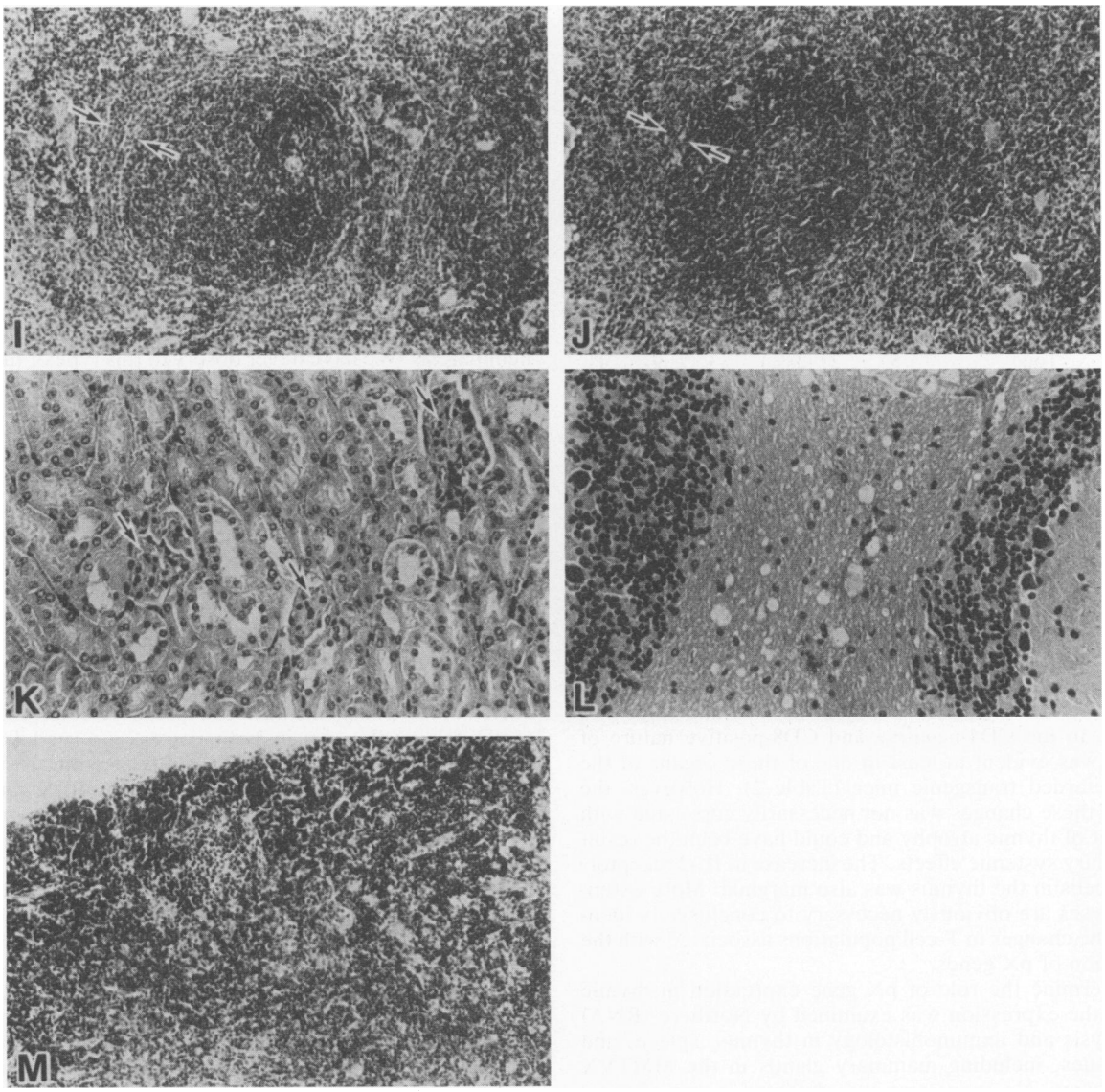

FIG. 2—*Continued.*

tively. In these mice, 1 to 60 copies of the transgenes were incorporated tandemly in either head-to-tail or head-to-head and tail-to-tail fashion (data not shown).

Slender mice and mice bearing abscesses or hemiparesis of thoracic or pelvic extremities or both were observed among these transgenic mice. Abscesses were due to infection by *Staphylococcus aureus* (Fig. 2A and B), which is known to occur frequently in immunosuppressed mice. Neutrophils, foamy macrophages, and plasma cells actively infiltrated the colonies, suggesting no defects in myelogenesis or lymphogenesis of these cell types. In dissected hemiparesis-bearing mice, masses were observed in the trigeminal nerves of brain tissue (Fig. 2C), and these were histologically confirmed to be composed of neutrophils and macrophages (Fig. 2D). Atrophy of the thymus was commonly observed in all these unhealthy mice (Fig. 2G and H). The cortex was depleted, dendritic macrophage was prominent, and there was remarkable loss of normal small lymphoid cells in both cortex and medulla. Occasionally concomitant with thymic atrophy was the involution of a thymus-dependent area in the spleen (Fig. 2J). Other lesions histologically observed in these mice included atrophy of kidney glomeruli (Fig. 2K) and vacuolation in the cerebellum associated with the degeneration of myelinated fibers (Fig. 2L).

These abnormalities were observed in two transgenic lines with LTR-pX (LTRX-3 and LTRX-9), three with MMTV-pX (MMTVX-2, MMTVX-5, and MMTVX-12), and three with Ig/Tp-pX (IgX-3, IgX-4, and IgX-9). No other abnormalities, including tumors, were noted in any lines during the observation period of 1 year. These abnormalities have not previously been observed in control or other transgenic mice with the strains and conditions we used; *Pseudomonas aeruginosa* was detected, but in other colonies. Table 1 gives the incidence of abnormal mice in the transgenic lines described above that could be phenotypically detected and histologically confirmed to have an atrophic thymus. The incidence was somewhat greater in the IgX series of mice and lower in the MMTVX series, with about four-fifths of the animals apparently remaining healthy during the year-long

TABLE 1. Incidence of thymic atrophy in pX-transgenic mice

Transgene	Transgenic line	Incidence		Onset (mo)		
		Total no.[a]	No. atrophic[b]	1–2	3–6	7–12
LTR-pX	LTRX-3	12	3	1	1	1
	LTRX-9	18	4	0	2	2
	Total	30	7 (23%)	1	3	3
MMTV-pX	MMTVX-2	25	4	0	2	2
	MMTVX-5	42	7	1	3	3
	MMTVX-12	36	2	0	1	1
	Total	103	13 (13%)	1	6	6
Ig/Tp-pX	IgX-3	32	10	2	4	4
	IgX-4	28	7	1	3	3
	IgX-9	22	6	0	2	4
	Total	82	23 (28%)	3	9	11

[a] Number of mice observed for 1 year after birth from F_0 to F_3 generations.
[b] Number of mice exhibiting growth retardation, abscesses, and/or hemiparesis and in which the thymus was atrophic.

observation period. These seemingly healthy mice of MMTVX-2, IgX-3, and IgX-4 lineages were sacrificed 4 months after birth at either the F_4 or F_5 generation to examine possible thymus abnormality histologically. Of 32 mice examined, 4 showed atrophy, though not extensive (Fig. 2M), while the others were normal.

Since histologic analyses suggested an abnormality in T-cell lymphogenesis, a group of each subset of T cells was analyzed by flow cytofluorometry in thymus and spleen. A reduction in the CD4-negative and CD8-positive nature of the cells was evident at least in one of these organs of the growth-retarded transgenic mice (Table 2). However, the extent of these changes was not necessarily correlated with the extent of thymic atrophy and could have been the result of secondary systemic effects. The increase in IL-2 receptor-positive cells in the thymus was also marginal. More extensive analyses are obviously necessary to conclusively identify specific changes in T-cell populations associated with the introduction of pX genes.

To determine the role of pX gene expression in thymic atrophy, the expression was examined by Northern (RNA) blot analysis and immunohistology in thymus, spleen, and other tissues, including mammary glands in the MMTVX series of mice. No expression was detected in any of these tissues. Certain genes, such as the SV40 T gene and those of procaryote and viral origin, are likely to be suppressed in transgenic mice (11, 20, 21). Derepression and expression may occur infrequently in cells, depending on the regulatory unit used. One possible explanation of the expression and thymic atrophy with pX genes is that these genes are also suppressed in normal cells during the course of development. In contrast to the expression of the SV40 T gene (20), which leads to tumor development, the expression of pX genes might be lethal to cells relating to the thymus and therefore cause thymic atrophy.

Nerenberg et al. reported two types of in vivo effects of the HTLV-I pX gene which had its own LTR (6, 10). One group of their mice which expressed $p40^{tax}$ in muscle developed mesenchymal tumors of perineural cell origin, and these tumors also developed in trigeminal nerve tissue with hemiparesis. We have never observed this type of tumor; the lesions which we found in the trigeminal nerve may have been caused by the inflammatory response of neutrophils and macrophages. Another group of their mice also had $p40^{tax}$ expression in the thymus and exhibited thymic depletion and growth retardation. All these mice died neonatally (between 3 to 6 weeks) before reaching the age at which mesenchymal tumors develop, while in our transgenic lines the onset of the disease extended over a wide period and about four-fifths of the mice remained apparently healthy for more than 1 year (Table 1). We limited our analyses of F_0 mice to those which survived beyond weaning, and thus a late-onset type of mouse would have been selected in our study. Histologically, the thymic depletion was limited to the cortex in the transgenic mice of Nerenberg et al., in contrast to the hypocellularity in both cortex and medulla in our mice. The greatest discrepancy between our results and theirs is that we detected no expressions of pX gene products. One possible explanation may be the lower expressions of pX genes in our transgenic lines, consistent with later onset and lower incidence of disease. This might also account for our mice not having developed neurofibromas. Differences exist in the transgene construction used by these researchers, which include introduction of Kozak consensus sequence and duplicate termination with 3′ LTR and SV40 sequences. Their transgene possibly produced only $p40^{tax}$, but in our constructs, $p27^{rex}$ and $p20^{X\text{-}III}$ might also be produced. The genetic background of mice used might also explain some of the differences. It is always possible that growth retardation and thymic atrophy were the result of an unclarified primary event brought on by pX genes. However, no spontaneous thymic atrophy was observed in our non-

TABLE 2. Flow cytofluorometric analyses of T-cell populations[a]

Cell type and mouse no.	% of total cells											
	Thymocytes						Splenocytes					
	4 8⁻	4⁺8⁻	4 8⁺	4⁺8⁺	+−/−+[b]	IL-2R⁺	Thy-1⁺	4⁺8	4 8⁺	4⁺8⁺	+−/−+[b]	IL-2R⁺
Control												
1	8.1	7.1	10.1	73.7	0.70	0.6	28.6	61.2	39.2	9.4	1.56	2.4
2	9.8	6.6	8.8	74.8	0.75	1.2	27.6	63.7	37.8	8.6	1.68	3.5
3	8.8	9.2	11.1	70.9	0.82	0.9	26.5	65.8	35.3	7.8	1.86	2.0
Transgenic												
1	10.0	8.6	3.6	76.6	2.39		17.9	73.7	37.4	6.1	1.97	2.0
2	10.7	8.2	5.4	74.7	1.52	3.1	20.0	78.0	32.5	6.5	2.40	3.2
3	5.0	11.4	13.3	70.3	0.86	1.6	27.2	65.1	22.4	2.6	2.91	2.8
4	6.1	6.5	8.7	75.6	0.75	2.8	18.9	65.6	19.0	7.4	3.45	2.6
5	7.3	8.4	13.9	68.4	0.60	2.3	21.7	67.7	21.2	6.0	3.19	4.0
6	10.0	11.8	2.3	75.8	5.13	3.0	8.1	75.3	18.5	8.6	4.07	3.0

[a] Abbreviations: 4, CD4; 8, CD8; IL-2R, IL-2 receptor. The values for CD4-CD8 poulations in spleen are given as ratios to Thy-1-positive cells.
[b] Ratio of CD4-positive and CD8-negative cells to CD4-negative and CD8-positive cells.

transgenic or other series of transgenic mice; this condition was commonly induced by pX genes regardless of the regulatory unit used. Thus, it seems likely that pX genes are responsible, directly or indirectly, for this phenotype.

LITERATURE CITED

1. **Chen, I. S. Y., J. McLaughlin, J. C. Gasson, S. C. Clark, and D. W. Golde.** 1983. Molecular characterization of genome of a novel human T-cell leukaemia virus. Nature (London) **305:** 502–505.
2. **Cross, S. L., M. B. Feinberg, J. B. Wolf, N. J. Holbrook, F. Wong-Staal, and W. J. Leonard.** 1987. Regulation of the human interleukin-2 receptor a chain promoter: activation of a nonfunctional promoter by the transactivator gene of HTLV-I. Cell **49:**47–56.
3. **Fujii, M., P. Sassone-Corsi, and I. M. Verma.** 1988. c-*fos* promoter trans-activation by the tax_1 protein of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **85:**8526–8530.
4. **Fujisawa, J., M. Seiki, T. Kiyokawa, and M. Yoshida.** 1985. Functional activation of the long terminal repeat of human T-cell leukemia virus type I by a trans-acting factor. Proc. Natl. Acad. Sci. USA **82:**2277–2281.
5. **Hahn, B., V. Manzari, S. Colombini, G. Franchini, R. C. Gallo, and F. Wong-Staal.** 1983. Common site of integration of HTLV in cells of three patients with mature T-cell leukaemia-lymphoma: a retraction. Nature (London) **305:**340.
6. **Hinrichs, S. H., M. Nerenberg, R. K. Reynolds, G. Khoury, and G. Jay.** 1987. A transgenic mouse model for human neurofibromatosis. Science **237:**1340–1343.
7. **Katoh, I., Y. Yoshinaka, and Y. Ikawa.** 1989. Bovine leukemia virus trans-activator $p38^{tax}$ activates heterologous promoters with a common sequence known as a cAMP-responsive element or the binding site of a cellular transcription factor ATF. EMBO J. **8:**497–503.
8. **Leder, A., P. K. Pattengale, A. Kuo, T. A. Stewart, and P. Leder.** 1986. Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: multiple neoplasms and normal development. Cell **45:**485–495.
9. **Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J. Inoue, M. Yoshida, and T. Taniguchi.** 1987. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-1-encoded $p40^{X}$ and T3/Ti complex triggering. Cell **48:**343–350.
10. **Nerenberg, M., S. H. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay.** 1987. The tat gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice. Science **237:**1324–1329.
11. **Palmiter, R. D., and R. L. Brinster.** 1986. Germ-line transformation of mice. Annu. Rev. Genet. **20:**465–499.
12. **Popovic, M., M. S. Reitz, Jr., M. G. Sarngadharan, M. Robert-Guroff, V. S. Kalyanaraman, Y. Nakao, I. Miyoshi, J. Minowada, M. Yoshida, Y. Ito, and R. C. Gallo.** 1982. The virus of Japanese adult T-cell leukaemia is a member of the human T-cell leukaemia virus group. Nature (London) **300:**63–66.
13. **Ringold, G., B. Dieckmann, and F. Lee.** 1981. Co-expression and amplification of dihydrofolate reductase cDNA and the *Escherichia coli* XGPRT gene in Chinese hamster ovary cells. J. Mol. Appl. Genet. **1:**165–175.
14. **Seiki, M., R. Eddy, T. B. Shows, and M. Yoshida.** 1984. Nonspecific integration of the HTLV provirus genome into adult T-cell leukaemia cells. Nature (London) **309:**640–642.
15. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA **80:**3618–3622.
16. **Seiki, M., A. Hikikoshi, T. Taniguchi, and M. Yoshida.** 1985. Expression of the pX gene of HTLV-I: general splicing mechanism in the HTLV family. Science **228:**1532–1534.
17. **Slamon, D. J., K. Shimotohno, M. J. Cline, D. W. Golde, and I. S. Y. Chen.** 1984. Identification of the putative transforming protein of the human T-cell leukemia viruses HTLV-I and HTLV-II. Science **226:**61–65.
18. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. *Trans*-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225:**381–385.
19. **Southern, P. J., and P. Berg.** 1981. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1:**327–341.
20. **Suda, Y., S. Aizawa, S. Hirai, T. Inoue, Y. Furuta, M. Suzuki, S. Hirohashi, and Y. Ikawa.** 1987. Driven by the same Ig enhancer and SV40 T promoter *ras* induced lung adenomatous tumors, *myc* induced pre-B cell lymphomas and SV40 large T gene a variety of tumors in transgenic mice. EMBO J. **6:**4055–4065.
21. **Suda, Y., S. Hirai, M. Suzuki, Y. Ikawa, and S. Aizawa.** 1988. Active *ras* and *myc* oncogenes can be compatible, but SV40 large T antigen is specifically suppressed with normal differentiation of mouse embryonic stem cells. Exp. Cell Res. **178:** 98–113.
22. **Yoshida, M., I. Miyoshi, and Y. Hinuma.** 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc. Natl. Acad. Sci. USA **79:**2031–2035.