4. Other Data Relevant to an Evaluation of Carcinogenesis and its Mechanisms

4.1 General observations on retroviral oncogenesis

Oncogenic retroviruses are naturally occurring infections of a large number of vertebrate hosts ranging from fish to humans. Retroviruses cause many types of neoplasm, including leukaemias, lymphomas, mammary and other carcinomas, and sarcomas (Weiss *et al.*, 1985; Levy, 1992–1995).

There are several distinct mechanisms by which animal retroviruses may elicit neoplasms under experimental and natural conditions. An indirect oncogenic effect occurs when neither the malignant cell nor its precursors are infected by the retrovirus. An immunodeficiency virus may permit the appearance of neoplasms as opportunistic events in the same sense as opportunistic infections occur in immunodeficient animals and humans. A directly oncogenic retrovirus inserts its provirus into a cell destined to

become malignant. Such viruses may either cause cancer after a long incubation period, or do so acutely.

The majority of nonhuman retroviruses which are directly oncogenic are C-type viruses with 'simple' genomes containing the long terminal repeats (LTR) and *gag*, *pol* and *env* genes. Such viruses do not carry transforming genes but in the tumour cells, the DNA provirus is integrated adjacent to specific cellular proto-oncogenes. These cellular genes become overexpressed through *cis*-acting promoter or enhancer functions of the LTR. Oncogenesis usually depends on a period of high virus replication. The ectopic activation of the proto-oncogene by the LTR is the crucial viral step in oncogenesis. The viruses found in the tumour cells may be either replication competent or defective variants.

Acutely transforming retroviruses carry viral oncogenes originally derived from cellular oncogenes and which are not required for viral replication. These transduced oncogenes can induce the growth of tumours with a short latency (days in contrast to months or years). Acutely transforming retroviruses are usually replication-defective, as the oncogene is substituted for viral gene sequences in the genome. Their replication relies on replication-competent 'helper' viruses which provide the missing viral proteins. Despite an excess of helper virus, acutely transforming retroviruses are seldom transmitted from one host to another.

The human T-cell leukaemia viruses, together with the related STLV of primates and bovine leukosis virus, differ from both the acutely transforming, oncogene-transducing viruses and the slowly oncogenic, replication-competent viruses in having 'complex' genomes bearing regulatory genes, such as *tax* (Section 1.1.1), required for efficient viral replication. The *tax* gene encodes a protein which also activates the expression of cellular genes (transactivation) and this effect is related to the immortalizing properties of these viruses. The transactivating effect of *tax* probably plays an important role in HTLV-I oncogenesis (Section 4.3.2). While the *tax* genes of both HTLV-I and HTLV-II exert an immortalizing effect on human T-lymphocytes *in vitro* (Section 4.3.2), only HTLV-I has been strongly linked with malignancy on the basis of epidemiological evidence (Section 2). In contrast to the *cis*-acting, slowly transforming retroviruses, the HTLV-I provirus integrates at many different chromosomal sites in ATLL cells in different patients (Yoshida *et al.*, 1984). It therefore appears that the site of integration of the viral genome is not crucial for its oncogenic effect. However, the possibility that certain sites of proviral insertion predispose to malignant transformation as a multistep process requires further investigation.

4.2 Host factors

4.2.1 *The role of the HLA system in HTLV-I infection*

HTLV-I infection can result in no disease, leukaemia or one of a range of inflammatory conditions, but particular genotypes of the virus do not appear to be associated with these different manifestations (Bangham *et al.*, 1996). It is likely that host factors strongly influence the outcome of HTLV-I infection; the HLA system is a major

candidate for such a host factor, because of its association with many diseases, including inflammatory and infectious diseases. The high degree of polymorphism of the HLA system necessitates large sample and control sizes in studies designed to test for a possible HLA association with disease. To date, large enough samples have not been tested to allow a firm conclusion to be drawn about possible association between HLA and HTLV-I infection, or to estimate the strength of an association (relative risk of disease) with confidence. However, there are strong suggestions that genetic factors, including HLA, influence the outcome of HTLV-I infection. Specifically, genetic factors might influence either the proviral load and/or the development of HTLV-I associated disease.

Most of the work examining HLA genotypes in relation to HTLV-I infection has been carried out in the island of Kyushu in southern Japan, where the seroprevalence of HTLV-I exceeds 10%. Furukawa *et al.* (1992) showed that clonal proliferation of HTLV-I-infected T cells (as shown by clonal integration of HTLV-I provirus), which is associated with a high proviral load, is commoner in TSP/HAM patients and their first-degree relatives than in unrelated healthy HTLV-I-seropositive individuals. Usuku *et al.* (1988) examined the HLA types of 27 patients with TSP/HAM, 12 patients with ATLL and healthy asymptomatic controls. They found a predominance of certain haplotypes. In ATLL patients, the haplotype A26Bw62Cw3DR5 appeared to occur in excess, but no statistical correction for multiple comparisons was applied. Sonoda *et al.* (1992) extended these observations, and again found an apparent excess of certain A25 haplotypes in ATLL patients. However, because of the way in which the study was designed, a complete statistical analysis was not made, and so these interesting observations await confirmation.

4.2.2 *Immune surveillance and escape*

(*a*) *Antibodies*

It seems unlikely that antibodies are effective in limiting viral replication in established human HTLV-I infections, because the viral load increases mainly by division of infected proviral-DNA-containing cells (Wattel *et al.*, 1995). However, it appears that the antibody titre reflects the HTLV-I proviral load (Shinzato *et al.*, 1993; Ishihara *et al.*, 1994; Miyata *et al.*, 1995).

(*b*) *T-cells*

The cytotoxic T-lymphocyte (CTL) is the most important antigen-specific element of the immune system for controlling most established viral infections. Kannagi *et al.* (1983, 1984) detected a CTL response specific to HTLV-I in patients with a diagnosis of ATLL. Notably, they reported that the CTL response was detectable only in those patients in whom the ATLL was in remission, and disappeared when the patients relapsed.

It is now clear that there is a very powerful, chronically activated CTL response to HTLV-I in the majority of both TSP/HAM patients (Jacobson *et al.*, 1990; Parker *et al.*, 1992, 1994; Daenke *et al.*, 1996) and healthy carriers of the virus (Parker *et al.*, 1992,

1994; Daenke *et al.*, 1996). The great majority of these CTLs are specific to the Tax protein of HTLV-I. There is evidence that the Tax-specific CTLs select antigenic variants of the Tax protein (Niewiesk *et al.*, 1995) that escape recognition by the patient's own CTLs. The selection process appears to be more efficient in healthy carriers (Niewiesk *et al.*, 1994).

The above evidence suggests that Tax-specific CTLs play a significant part in limiting viral replication, but the precise role of CTLs in protection against HTLV-I-associated diseases, or in the pathogenesis of these conditions, is not yet clear. There appears to be little difference, if any, between healthy HTLV-I carriers and TSP/HAM patients with respect to the chronic activation state, the abundance in peripheral blood, the antigen specificity or the epitope specificity of these CTL.

The observations by Kannagi *et al.* (1983, 1984) suggested that the development of ATLL might be associated with inefficient immune surveillance by CTLs. This would probably be a result of the low expression of the Tax protein by leukaemic cells.

As observed in other viral infections and malignancies, natural killer (NK) cells may also play a part in surveillance of HTLV-I infection and ATLL (see Section 4.3.3).

4.2.3 *Host genetic factors required during the transition to ATLL*

Host genetic factors are thought to be required for the transition from HTLV-I-infection of a cell to ATLL. Chromosomal abnormalities have been described in ATLL, but no specific pattern has been identified. This topic is discussed more extensively in Section 2.1.1.

At the molecular level, mutations in three tumour-suppressor genes, *p53*, *p16* and *p15*, have been identified in ATLL samples and/or HTLV-I-transformed human T-cell lines.

p53 missense mutations have been observed in 17–40% of fresh ATLL samples (Sugito *et al.*, 1991; Sakashita *et al.*, 1992; Yamato *et al.*, 1993) as well as in some HTLV-I-transformed T-cell lines. The aberrant expression of p53 protein, in the presence or absence of p53 missense mutations, has also been noted in a proportion of both fresh ATLL samples and HTLV-I-transformed T-cell lines (Sugito *et al.*, 1991; Reid *et al.*, 1993; Yamamoto *et al.*, 1993). While one report noted a correlation between Tax expression and p53 expression in HTLV-I transformed T-cell lines which lacked p53 mutations, another study did not observe any difference in p53 gene expression, methylation, and chromatin structure between HTLV-I transformed and mitogen-activated human T-cells (Lübbert *et al.*, 1989). In HTLV-I-transformed T-cells, p53 is also functionally impaired, despite an increased expression of the cell-cycle control protein $p21^{waf1/cip1}$ (Cereseto *et al.*, 1996). Thus, p53 mutation and aberrant expression may occur in less than half of ATLL cases and could conceivably play a role in tumour progression. Tax has also been demonstrated to impair $p16^{INK}$ function (Suzuki *et al.*, 1996). Homozygous deletions of the p15 (MTS2) and/or p16 (CDKN2/MTSI) tumour-suppressor genes have been reported in 10/37 (27%) ATLL patients and individual cases suggest a possible association of deletions in the genes and leukaemia progression (Hatta

et al., 1995). Deletion, mutation or aberrant expression of tumour-suppressor genes may thus play a role in the pathogenesis of ATLL.

4.3 Viral factors

4.3.1 *Proviral load and clonal integration of HTLV-I infection*

The epidemiological evidence summarized in Section 2 links HTLV-I with the emergence of ATLL in a small proportion of HTLV-I-infected individuals after a delay of several decades.

The proportion of peripheral blood mononuclear cells (PBMCs) that carry an HTLV-I provirus — the proviral load — is usually between 10 and 100 times higher in patients with HTLV-I associated inflammatory diseases such as TSP/HAM than in healthy carriers of the virus (Yoshida *et al.*, 1989; Kira *et al.*, 1991; Gessain *et al.*, 1990a,b; Kira *et al.*, 1992a,b; Kubota *et al.*, 1993; Mita *et al.*, 1993; Sugimoto *et al.*, 1993), although the ranges overlap. Typically about 10% of peripheral blood lymphocytes are provirus-positive in TSP/HAM patients, and < 1% in healthy carriers of the virus. However, the fact that TSP/HAM and ATLL appear to occur independently suggests that a high HTLV-I proviral load does not necessarily predispose to ATLL. The proviral load in ATLL is largely dependent on the number of leukaemic cells and may be very high. However, no viral genes are expressed in ATLL cells (see below). In BLV infection (Section 3.3), a high virus load early after infection is associated with an elevated risk for development of leukaemia. It will be interesting to determine whether the same is true in HTLV-I infection.

HTLV-I-infected human T-cells show clonal expansion, even in asymptomatic individuals (Furukawa *et al.*, 1992; Wattel *et al.*, 1995). It has been suggested that after infection of human T-cells by HTLV-I and following a few rounds of reverse transcription, a clonal expansion of the infected cells predominates (Wattel *et al.*, 1996). Experiments using inverse or linker-mediated PCR have indeed indicated that HTLV-I proviral copy numbers increase predominantly via mitosis rather than via reverse transcription (Cavrois *et al.*, 1996). These findings may explain the remarkable stability of the HTLV-I genome and the high proviral load present in many HTLV-I infected individuals (Wattel *et al.*, 1995, 1996). The only viral mRNA to be reproducibly detected in PBMCs of HTLV-I-infected people is the mRNA encoding Tax/Rex/p21/Rex (Koralnik *et al.*, 1992b). This could explain the predominance of chronically activated Tax-specific CTLs in these individuals (see Section 4.2.2). In HTLV-I-infected PBMCs, the level of Tax mRNA expression per infected cell is the same in asymptomatic carriers and in TSP/HAM patients, but low or absent in uncultured ATLL samples (Franchini *et al.*, 1984; Furukawa *et al.*, 1995).

Thus, although HTLV-I-infected individuals carry HTLV-I provirus in a significant proportion of clonally expanded T-cell populations, the occurrence of ATLL is comparatively rare (Chen *et al.*, 1995).

4.3.2 *The role of Tax in cellular transformation/immortalization*

Following the discovery of the Tax protein of HTLV-I (Seiki *et al.*, 1983), intense efforts have been made to demonstrate its oncogenic properties *in vitro* and in animal models. These, as well as the possible role of other viral components in the development of ATLL, are reviewed in this section.

(*a*) *Transforming/immortalizing properties of HTLV-I Tax* in vitro

(i) *Immortalizing effects on T-cells in vitro*

In vitro, HTLV-I, as well as its close relative HTLV-II, can clearly cause human T-cells to proliferate continuously (immortalization) and, with time, acquire IL-2 independence (transformation). Co-cultivation of mitomycin-treated or lethally irradiated HTLV-I or HTLV-II producer cell lines with human peripheral blood or cord blood lymphocytes results in the immortalization of mainly $CD4^+$ and, occasionally, $CD8^+$ T-lymphocytes (Miyoshi *et al.*, 1981; Yamamoto *et al.*, 1982; Chen *et al.*, 1983; Popovic *et al.*, 1983). Several lines of evidence suggest that the viral transactivator Tax, encoded by two exons flanking the envelope gene (see Section 1.1.6), is involved in this process. Tax has immortalizing/transforming properties *in vitro:* when transduced into primary human T-cells from adult or cord blood by a retroviral vector (Akagi & Shimotohno, 1993) or a recombinant herpesvirus saimiri (Grassmann *et al.*, 1989, 1992), it is capable of altering their growth properties. Transduction of *tax* into peripheral blood T-cells by a retroviral vector leads to enhancement of the proliferation caused by IL-2 and anti-CD3 antibody (Akagi & Shimotohno, 1993). These *tax*-transduced T-cells are still dependent on IL-2, but do not require periodic restimulation with antigen and feeder cells (Akagi & Shimotohno, 1993). Transduction of cord blood cells with *tax* using a herpesvirus saimiri vector resulted in permanently growing, but still IL-2-dependent, T-cell lines (Grassmann *et al.*, 1989, 1992).

The ability of Tax to immortalize primary human T-cells may be linked to its ability to induce the expression of cellular genes which are normally involved in the early response to mitogenic and antigenic stimuli (Kelly *et al.*, 1992) as well as to a long list of cytokines and cytokine receptors (see below). However, it is possible that other viral and cellular factors contribute to the efficient immortalization of human T-cells by HTLV-I *in vitro*: whereas *tax*-transduced T-cells remain IL-2-dependent, T-cells infected in bulk culture by co-cultivation with HTLV-I producer cell lines lose their IL-2-dependence after extended passage *in vitro*. If co-cultivation is carried out with limiting numbers of HTLV-I producer cells, the resulting transformed T-cell lines can be shown to maintain their dependence on IL-2 for a longer time, and other lymphokines, such as IL-4 and IL-7, can substitute for IL-2 to some extent (Persaud *et al.*, 1995). The constitutive activation of the JAK3 and STAT kinases in HTLV-I infected T-cells could be a crucial step during the acquisition of IL-2 independence: the JAK/STAT pathway is normally required for the downstream signalling triggered by the β- and γ-chain of the IL-2 receptor and by other cytokine receptors (Migone *et al.*, 1995), suggesting that its constitutive activation would lead to IL-2 independence.

Four infectious molecular clones of HTLV-I have recently been reported (Nicot *et al.*, 1993; Kimata *et al.*, 1994; Derse *et al.*, 1995; Zhao *et al.*, 1995), some of which produce HTLV-I capable of stimulating PBMCs. However, only one of these (Zhao *et al.*, 1995) has been used successfully *in vitro* to transform (to IL-2 independence) human peripheral blood T-cells. It is also possible to achieve transformation of human PBMCs *in vitro* with a molecular clone of HTLV-II (Green *et al.*, 1995).

(ii) *Transforming effect of Tax on fibroblast cultures* in vitro

Transfection of *tax* into RAT-1 or NIH 3T3 fibroblasts results in colony formation in soft agar and morphological changes (transformation), and the *tax*-transfected RAT-1 cells are tumorigenic in nude mice (Tanaka *et al.*, 1990). This ability to transform rat fibroblasts is dependent on the CREB/ATF pathway of Tax action (see Section 1.1.6), but does not require interaction of Tax with nuclear factor-κB (NF-κB) (see Section 4.3.3) (Smith & Greene, 1991), a pathway which is predominantly activated by Tax in T-cells. Continuous expression of *tax* is required to maintain the transformed phenotype of RAT-1 fibroblasts (Yamaoka *et al.*, 1992). Fusion of *tax*-transformed rat fibroblasts with normal human fibroblasts results in suppression of the transformed phenotype even in the presence of continued *tax* expression, suggesting the existence of a dominant inhibitory human factor acting downstream of *tax* (Inoue *et al.*, 1994b). Tax has also been shown to immortalize and, in combination with the activated Ha-ras protein, to transform primary rat embryo fibroblasts (Pozzatti *et al.*, 1990). Some naturally occurring sequence variants of Tax which are capable of activating the NF-κB or CREB/ATF pathways and of transforming rat fibroblast cell lines lack the ability to cooperate with *ras* in this manner (Matsumoto *et al.*, 1994).

(*b*) *Tumorigenic properties of* tax *in transgenic mice*

Several groups have generated transgenic mice carrying different parts of the HTLV-I genome. Transgenic mice with the *tax* gene under the control of the HTLV-I LTR (Nerenberg *et al.*, 1987) developed thymic atrophy and mesenchymal tumours (Hinrichs *et al.*, 1987; Nerenberg *et al.*, 1987), proliferation of ductal epithelial cells of the salivary glands (Green *et al.*, 1989b), muscle degeneration (Nerenberg & Wiley, 1989) and adrenal medullary tumours characterized by proliferation of undifferentiated spindle cells (Green *et al.*, 1992). Thymic atrophy was also consistently observed in other lines of mice transgenic for *tax* under the control of the SV40 promoter, the Ig enhancer and the mouse mammary tumour virus (MMTV) LTR (Furuta *et al.*, 1989).

Environmental variables may contribute to tumour formation in LTR-*tax* transgenic mice, as the development of tumours can be delayed by feeding a low-folate diet (Bills *et al.*, 1992). No lymphomas or leukaemias have been seen in LTR-*tax* transgenic mice, which may be related to the fact that the LTR-*tax* transgene was most strongly expressed in muscle, bone and cartilage, brain, pituitary, skin and salivary glands, but less so in lymphoid tissue (Bieberich *et al.*, 1993). However, the use of a Thy-1 promoter to target the *tax* expression to the thymus of transgenic mice also resulted in the formation of fibroblastic tumours accompanied by infiltration of other cell types, as in the case of the LTR-*tax* transgenic mice, but did not lead to lymphoma formation. Nor was any

expansion or phenotypic alteration of circulating lymphocytes or lymphocytes of the thymus or spleen seen in these animals (Nerenberg *et al.*, 1991).

In contrast, transgenic mice carrying the complete pX region (i.e., the genomic region containing open reading frames I, II, III and IV; see Section 1.1.6 and Figure 4) under the control of the granzyme B promoter, targeting the transgene expression to mature T-cells and NK cells, develop large granular lymphocytic leukaemia and solid tumours composed of NK-large granular lymphocytic (LGL)-like cells, which expressed FcγR, Thy 1.2, CD 44 and lacked rearranged T-cell receptor β and γ genes, and neutrophils (Grossman *et al.*, 1995). While the phenotype of these NK-LGL-like cells is clearly different from that of ATLL cells, this experiment supports the transforming potential of Tax in lymphoid cells *in vivo*. The marked neutrophil infiltration of tumours in these animals has also been noted in neurofibromas of LTR-*tax* transgenic mice and may be related to the activation of granulocyte-macrophage colony-stimulating factor expression by Tax (Green *et al.*, 1989b). LTR-*tax* transgenic mice can also exhibit marked splenomegaly and lymphadenopathy, due to a striking increase in the percentage of B-cells in these organs. This expansion of the B-lymphocyte population may also be related to cytokines secreted from *tax*-expressing fibroblastoid tumour cells, which were shown to stimulate B-cell proliferation and IgM production (Peebles *et al.*, 1995). Transforming growth factor β1 (TGF-β1) is overexpressed in several tissues from LTR-*tax* transgenic mice and stimulates the growth of cell lines derived from neurofibromas of these animals, and it has therefore been suggested that this cytokine might be involved in the development of these tumours (Kim *et al.*, 1991). Mammary carcinomas observed in *tax*-transgenic rats also expressed several cytokines, including the granulocyte chemoattractants *Gro* and *MIP-2*, but not TGF-β1 (Yamada *et al.*, 1995). Similarly, the increased bone turnover and skeletal abnormalities of LTR-*tax* transgenic mice may be related to Tax-induced local expression of cytokines (Ruddle *et al.*, 1993). Thus, in addition to a directly transforming effect of Tax are demonstrated by these in-vivo experiments, more indirect mechanisms, involving a variety of cytokines, contribute to tumour formation and may underlie other pathological effects seen in these animals.

The lymphomas described above differ from ATLL in lacking CD4 expression. Lymphomas of a $CD4^+$ phenotype were observed in 70% of bitransgenic mice carrying an LTR-c-*myc* and an Ig promoter/enhancer-*tax* construct. In addition to $CD4^+$, $CD3^+$, $CD8^-$ lymphomas, these animals develop brain tumours of neuronal lineage at very high frequency (Benvenisty *et al.*, 1992). However, the relative contributions of overexpressed c-*myc* and *tax* to tumour development in this model remain unclear.

Transgenic mice carrying the *env* and pX region of HTLV-I [i.e., with the potential to encode the envelope proteins, Tax, Rex, $p21^{rexIII}$, $p12^{I}$, $p30^{II}$, $p13^{II}$; see Section 1.1] under the control of the HTLV-I LTR develop an inflammatory arthropathy resembling human rheumatoid arthritis, in addition to the thymic atrophy, mesenchymal tumours and adenocarcinomas reported in LTR-*tax* transgenic mice (Iwakura *et al.*, 1991, 1994). In addition to increased c-*fos* and c-*jun* expression in the tumours and normal skin and muscle of these animals (Iwakura *et al.*, 1994), a variety of inflammatory cytokines, including IL-1α, IL-1β, IL-6, TNF-α, TGF-β1, interferon-γ and IL-2, as well as MHC genes, are over-

expressed in transgenic joints (Iwakura *et al.*, 1995). This widespread activation of components of the immune system is probably related to the expression of the transgenic construct in many organs, including brain, salivary gland, spleen, thymus, skin, muscle and mammary gland (Iwakura *et al.*, 1994, 1995).

Taken together, the evidence emerging from studies with these different lines of HTLV-I transgenic mice suggests that *tax* has a relatively weak oncogenic effect *in vivo* which is apparent only in transgenic animals with high levels of *tax* expression. Lymphoid cells are not particularly sensitive to the transforming effect of Tax and T-cell malignancies have been found only when a strong, non-HTLV-I-derived, promoter was used, or after simultaneous expression of *tax* and c-*myc*. Some aspects of the pathology induced by *tax in vivo* may be related to the aberrant expression of a variety of lymphokines, resulting in marked neutrophil infiltration of mesenchymal tumours and increased bone turnover.

4.3.3 *Pathways of Tax-mediated transactivation of cellular genes*

The transforming properties of the Tax protein, demonstrated in transgenic mice, transduced T-cells and transfected fibroblasts (see above) are the result of its ability to induce the expression of a wide variety of cellular genes in addition to the HTLV-I LTR (see Table 8). Tax negatively regulates the expression of β-polymerase, a cellular DNA repair enzyme (Jeang *et al.*, 1990), and the tumour-suppressor genes *p53* (Uittenbogaard *et al.*, 1995) and $p16^{INK4A}$ and represses the tumour-suppressor role (Suzuki *et al.*, 1996).

Transcriptional activation by Tax of these various cellular and viral genes requires the presence of specific target sequences in the promoter DNA. Three different such target sequences, the cyclic AMP-responsive element (CRE), the NF-κB binding site and the serum response element (SRE), are known to mediate the Tax-induced transactivation of most of the cellular genes listed above. Additional, so far unidentified, pathways probably account for the activation of a few other Tax-responsive genes.

Modified CREs are present in the HTLV-I LTR within three 21-bp repeat elements, at least two of which are required for Tax-induced transactivation (Fujisawa *et al.*, 1986; Shimotohno *et al.*, 1986). CRE binds members of the bZIP family of cellular transcription factors which include CRE binding protein (CREB) (Zhao & Giam, 1992; Suzuki *et al.*, 1993), CRE modulator (CREM) (Foulkes *et al.*, 1991), activating transcription factor (ATF) (Hai *et al.*, 1989), Tax-responsive element binding protein (TREB) (Yoshimura *et al.*, 1989) and HEB (21-bp binding proteins) (Béraud *et al.*, 1991). Tax activates transcription from the HTLV-I LTR as well as the CRE-containing promoters of the *c-fos*, *c-egr* and nerve growth factor genes by binding to one or several of these transcription factors and enhancing their interaction with the target DNA (Zhao & Giam, 1992) or altering their DNA-binding specificity (Paca-Uccaralertkun *et al.*, 1994). Tax achieves this by binding to the basic domain of bZIP transcription factors (Baranger *et al.*, 1995; Perini *et al.*, 1995), thus enhancing their dimerization (Wagner & Green, 1993) and stabilizing a complex consisting of DNA, bZIP and Tax (Wagner & Green, 1993; Baranger *et al.*, 1995). As part of this process, Tax also alters the relative affinity

Table 8. Tax-activated cellular genes[a]

Interleukin (IL)-related genes	
IL-2Rα	Inoue *et al.* (1986)
IL-1	Sawada *et al.* (1992)
IL-2	Siekevitz *et al.* (1987)
IL-3	Wolin *et al.* (1993)
IL-6	Yamashita *et al.* (1994)
IL-8	Mori *et al.* (1995)
'Housekeeping' genes	
Vimentin	Lilienbaum *et al.* (1990)
MHC class I	Sawada *et al.* (1990)
Growth-factors/hormone genes	
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Miyatake *et al.* (1988)
Nerve growth factor (NGF)	Green (1991)
Transforming growth factor β1 (TGF-β1)	Kim *et al.* (1990)
Tumour necrosis factor α (TNF-α)	Dhib-Jalbut *et al.* (1994)
Tumour necrosis factor β (TNF- β)	Paul *et al.* (1993)
Parathyroid-hormone-related protein	Watanabe *et al.* (1990)
Proenkephalin	Joshi & Dave (1992)
Early response cellular genes	Alexandre *et al.* (1991); Kelly *et al.* (1992)
Cellular oncogenes	
c-*egr*	Fujii *et al.* (1991)
c-*fos*	Fujii *et al.* (1988)
c-*jun*	Fujii *et al.* (1991)
c-*myc*	Duyao *et al.* (1992)
c-*rel*	Li *et al.* (1993)
c-*sis*	Pantazis *et al.* (1987)
Viral promoters	
HTLV long terminal repeat	Sodroski *et al.* (1984); Felber *et al.* (1985)
HIV long terminal repeat	Jeang *et al.* (1988b)
Cytomegalovirus IE enhancer	Moch *et al.* (1992)
SV40 promoter	Fujisawa *et al.* (1988)

[a]Referencing is not exhaustive

of a bZIP protein for different DNA binding sites, thus modifying DNA binding site selection (Perini *et al.*, 1995). This explains the ability of Tax to transactivate a wide range of promoters containing recognition sites for members of the bZIP family. There are, however, subtle differences between the activation of the CRE-related sequences in the HTLV-I LTR and that of cellular CREs: in the case of the viral LTR, Tax binds to unphosphorylated CREB, increases its association with the 21 bp repeats and thus promotes interaction with CREB, an essential component of the transcription factor complex (Chrivia *et al.*, 1993; Kwok *et al.*, 1996). In contrast, Tax does not bind directly to CREB associated with a cellular CRE (Kwok *et al.*, 1996), and phosphorylation of CREB, which is required for its normal activation of cellular CRE-containing promoters

and interaction with CREB (Chrivia *et al.*, 1993), is also necessary for the activation of these cellular promoters by Tax. Rather, by also binding to CREB, Tax promotes its interaction with phosphorylated CREB associated with a cellular CRE (Kwok *et al.*, 1996). Thus, for activation of the viral LTR, Tax is able to bypass a normal control mechanism of this pathway, whereas this control step is still operative in the case of Tax-activated cellular genes.

In a related manner, Tax binds to all members of the NF-κB family of transcription factors to activate transcription (Suzuki *et al.*, 1994). The NF-κB binding site is present in several genes known to be activated by Tax, such as those encoding IL-2Rα, GM-CSF, TNF-β and the HIV LTR. In addition to binding to NF-κB in the nucleus, Tax also interacts with the NF-κB pathway at an earlier, cytoplasmic stage, in a completely different manner. Tax complexes with two proteins, I-κBα and I-κBγ, that are inhibitors of NF-κB. I-κB proteins normally bind to members of the NF-κB family in the cytoplasm and prevent their entry into the nucleus. Following stimulation of cells, I-κB proteins are phosphorylated and the NF-κB/I-κB complex dissociates, allowing the free NF-κB to enter the nucleus. Tax has been shown to bind to I-κBα and I-κBγ and it causes dissociation of NF-κB/I-κB complexes, thus increasing the turnover of NF-κB and its import into the nucleus (Hirai *et al.*, 1994; Suzuki *et al.*, 1995). As in the case of members of the CREB/ATF family, binding of Tax thus mimics the effect of phosphorylation and thus interferes with physiological control mechanisms of these different pathways.

The third target sequence known to be involved in Tax-mediated activation of cellular genes is the SRE. An SRE is found in the promoters of early response genes known to be activated by Tax (Kelly *et al.*, 1992) and interacts with the transcription factor SRF. Tax binds to SRF, including unphosphorylated SRF, and the SRF/Tax complex activates transcription (Fujii *et al.*, 1992).

An additional Tax-responsive sequence, TRE-2, is present in the HTLV-I LTR. TRE-2 alone is not sufficient to mediate a Tax response, but can do so in the presence of one single 21-bp element (Marriott *et al.*, 1990). A 36-kDa zinc finger protein, termed TIF-1 or THP and related to the GLI family of proteins, interacts with TRE-2 (Marriott *et al.*, 1990; Tanimura *et al.*, 1993). Thus, cooperative binding of a CREB/ATF protein (binding to CRE in the 21-bp element) and THP may be required for Tax-mediated activation of this target sequence.

Thus, the pleiotropic effect of Tax on at least three different enhancer sequences is explained by its ability to interact with a variety of different transcription factors or inhibitors. Binding of Tax to these proteins may substitute for modifications such as phosphorylation or dimerization which normally occur in these proteins as a result of intracellular signalling. Different regions in Tax are required for the activation of individual pathways and mutants with specificity for individual pathways have been designed (Smith & Greene, 1990, 1991). There is also evidence that extracellular Tax, released from infected cells, may induce NF-κB-site-containing promoters, such as the IL-2Rα or TNF-β promoter, and thus induce the activation of uninfected cells (Lindholm

et al., 1992; Marriott *et al.*, 1992). However, a role of extracellular Tax in the pathogenesis of ATLL, over and above that of Tax produced within infected cells, is uncertain.

In addition to its role in cellular activation, Tax may be involved in increasing the likelihood of DNA damage in infected cells, possibly by increasing DNA instability (Saggioro *et al.*, 1994) and/or through its inhibitory effect on the expression of the repair enzyme, β-polymerase (Jeang *et al.*, 1990).

4.3.4 *Differences between HTLV-I-transformed T-cells and ATLL cells*

Although the experiments summarized above indicate that *tax* has some transforming potential *in vitro,* as well as *in vivo*, it is clear that HTLV-I-transformed T-cell lines, or T-cells transduced with *tax*, are not representative of ATLL.

Whereas HTLV-I-transformed T-cell lines (either cell lines obtained by co-cultivation of irradiated HTLV-I producer cell lines with fresh human primary T-cells, or non-ATLL cells grown from HTLV-I infected individuals) express viral mRNAs *in vitro*, cell lines originating from ATLL do not (Maeda *et al.*, 1985; Imada *et al.*, 1995). Early experiments suggested that ATLL cells *in vivo* also do not express HTLV-I mRNAs (Franchini *et al.*, 1984). While some expression of viral mRNA in fresh ATLL samples has been seen using RT-PCR (Berneman *et al.*, 1992a; Koralnik *et al.*, 1992b), it is unclear whether this occurred in ATLL cells or other HTLV-I infected T-cells. In-situ hybridization suggested some expression of *tax* mRNA in ATLL cells (Setoyama *et al.*, 1994). However, ATLL-derived cell lines which engraft in SCID mice show no, or reduced, expression of viral mRNAs (Imada *et al.*, 1995).

Leukaemic cells from ATLL patients do not usually grow in the presence of IL-2 (Maeda *et al.*, 1985), but occasionally ATLL cells have been found to respond to IL-2, and grow as permanently IL-2-dependent cell lines, suggesting that at some stage during their development ATLL cells require IL-2 to proliferate (Maeda *et al.*, 1985, 1987).

Primary cultures of ATLL cells, as well as ATLL-derived cell lines, can grow in SCID mice to form tumours with the same phenotypic profile and HTLV-I integration patterns as the ATLL samples from which they were established (Feuer *et al.*, 1993; Kondo *et al.*, 1993; Imada *et al.*, 1995). While uncultured HTLV-I-infected T-cells from a few asymptomatic individuals and those from about one third of TSP/HAM patients will persist in SCID mice, they do not form tumours (Feuer *et al.*, 1993). In contrast to ATLL cells or cell lines, HTLV-I-transformed T-cell lines not derived from ATLL cells will only grow in SCID mice which have been pretreated with antibodies to asialo GM1 (Ishihara *et al.*, 1992; Feuer *et al.*, 1995) to reduce NK cell activity. However, this distinction may not be absolute: untreated animals can be successfully engrafted using increased numbers of an HTLV-I-transformed, non-leukaemic cell line (Ohsugi *et al.*, 1994), while blocking of NK function with monoclonal antibody TM-β1 or the β-chain of the murine IL-2 receptor may enhance the rate of engraftment of fresh ATLL cells. However, taken together, these reports suggest that ATLL cells have a higher tumorigenic potential *in vivo* than HTLV-I-transformed T-cell lines because of their ability to evade NK-mediated cell lysis (Feuer *et al.*, 1995).

Thus, infection of T-cells with HTLV-I may provide some proliferative advantage and oligoclonal expansion, probably related to the pleiotropic activating properties of *tax*. NK-cell activity as well as CTL activity (see Section 4.2.3) may play an important role in limiting the expansion of HTLV-I-infected T-cells at this stage, and progression to ATLL requires a number of additional events. Whereas HTLV-I producer T-cell lines express high levels of the adhesion molecules LFA-1, LFA-3 and ICAM-1, ATLL-derived cell lines show reduced expression of these surface markers (Fukudome *et al.*, 1992). As these molecules play an important role in the recognition of tumour cells by the immune system, it is conceivable that their reduced expression on ATLL cells may facilitate their escape from immunosurveillance. At present, there is no convincing evidence that variation in viral sequences (see Section 4.2.3) will allow the emergence of more 'leukaemogenic' clones of HTLV-I-infected cells.

4.3.5 *The role of other viral and host cell proteins in lymphocyte stimulation and leukaemogenesis*

Apart from Tax, the HTLV-I envelope protein and the recently described $p12^{I}$ protein have been investigated with regard to their potential roles in T-cell stimulation and/or leukaemogenesis. Purified HTLV-I viruses have been reported to stimulate human T-cells via the HTLV-I envelope protein and a CD2/LFA-3-dependent pathway (Gazzolo & Duc Dodon, 1987; Duc Dodon *et al.*, 1989), but the interpretation of this phenomenon remains controversial. Recombinant HTLV-I envelope protein, expressed in a vaccinia virus vector (Cassé *et al.*, 1994) does not induce T-cell proliferation. Whereas a $(YXXL/I)_2$ signalling motif in the cytoplasmic domain of the BLV envelope protein mediates activation of B-lymphocytes *in vitro* (Beaufils *et al.*, 1993), and is required for efficient replication *in vivo* (Willems *et al.*, 1995), the cytoplasmic domain of the HTLV-I envelope has only a truncated (YXXL) motif which appears functionally inactive (Beaufils *et al.*, 1993).

Several adhesion molecules, such as LFA-3, ICAM-1, LFA-1, and the cell surface markers CD28, CD69 and CD5 show increased expression on the surface of HTLV-I-infected (Fukudome *et al.*, 1992; Imai *et al.* 1993) or *tax*-transfected (Chlichlia *et al.*, 1995; Tanaka *et al.*, 1995) cells. Antibodies to CD2 and LFA-3 inhibit the mitogenic activity of HTLV-I-infected T-cell lines (Kimata *et al.*, 1993) and the spontaneous proliferation of PBMCs from HTLV-I-infected asymptomatic carriers or TSP/HAM patients (Höllsberg *et al.*, 1992; Wucherpfennig *et al.*, 1992), suggesting that these molecules contribute to the process of proliferation.

Contrary to this conclusion, results obtained with both the SCID model (see above) and the rabbit model suggest that the potential of HTLV-I-infected cell lines to stimulate lymphocyte proliferation *in vitro* does not necessarily correlate with their leukaemogenic potential *in vivo*. An experiment reported by Leno *et al.* (1995) suggests that the leukaemogenic potential of an HTLV-I-infected T-cell line could be linked to its ability to induce apoptosis, which was demonstrated in thymic cells *in vivo* and peripheral blood T-cells *in vitro,* rather than its ability to induce T-cell stimulation, and that this phenotype is due to a cellular, rather than viral, factor. However, in another report (Seto

& Kumagai, 1993), the leukaemogenic potential of individual cell lines in (B/J × Chbb:HM) F1 rabbits did correlate with their ability to induce leukocytosis *in vivo* in the parental Chbb:HM rabbit strain and this phenotype seemed to be linked to the surface expression of a cellular 65 kDa glycoprotein, the precise role of which remains to be established. While these experiments may help to identify cellular factors promoting the growth of HTLV-I-transformed cell lines in rabbits, they do not necessarily reflect events occurring in human ATLL.

The Tax protein itself has been shown to induce apoptosis in Jurkat cells (Chlichlia *et al.*, 1995), in particular when Tax-expressing Jurkat cells were stimulated via the T-cell receptor. *Tax*-transfected RAT-1 cells are also prone to apoptosis via a BCL-2-dependent pathway when cultured in the absence of serum (Yamada *et al.*, 1994). In RAT-1 cells, *tax* induces apoptosis less efficiently than the cellular oncogenes c-*myc* and c-*fos* and through a different pathway (Fujita & Shiku, 1995). The ability of Tax to induce apoptosis is probably related to its pleiotropic effect on cellular promoters (see above), and reflects an increased susceptibility of activated cells to undergo apoptosis in the absence of essential stimuli. These experiments may not explain the observations reported by Leno *et al.* (1995).

The $p12^{I}$ protein of HTLV-I (Koralnik *et al.* 1992b, 1993) has been shown to cooperate with the E5 protein of bovine papilloma virus in the transformation of C127 mouse cells (Franchini *et al.* 1993) and may thus have oncogenic properties. $p12^{I}$ and E5 share some structural similarity: both proteins localize to the cellular endomembranes and interact with another very hydrophobic protein, the 16 kDa subunit of H+ vacuolar ATPase (Schlegel *et al.*, 1986; Goldstein *et al.*, 1991; Franchini *et al.*, 1993). Both E5 and $p12^{I}$ interact with distinct growth factor receptors. E5 activates the platelet-derived growth factor receptor (Petti *et al.*, 1991; Goldstein *et al.*, 1994) and $p12^{I}$ specifically interacts with the β and γ, but not the α chains of the IL-2R (Mulloy *et al.*, 1994). Possibly, binding of $p12^{I}$ to the IL-2R chains could alter the receptor signalling by inducing their cytoplasmic juxtaposition, an event thought to be crucial in kinase activation and IL-2 signalling (Nelson *et al.*, 1994). In this regard, it is noteworthy that constitutive activation of STAT and JAK3 kinases has been demonstrated in HTLV-I-transformed T-cells (see Section 4.3.2). In fact, constitutive activation of the IL-2R signalling pathway is correlated with IL-2 independence (Migone *et al.*, 1995). The DNA sequence of the HTLV-I $p12^{I}$ gene from 21 HTLV-I positive individuals (7 healthy carriers, 8 TSP/HAM and 6 ATLL) has been found to be highly conserved (Franchini, 1995).

However, the precise role of $p12^{I}$ in T-cells is not yet understood. $p12^{I}$ is not required for the transformation of cord blood lymphocytes *in vitro* (Ratner *et al.*, 1985) and is dispensable for *tax*, *rex* or envelope expression *in vitro* (Roithmann *et al.*, 1994).

In proviral DNA extracted from ATLL, the genes encoding $p13^{II}$ and $p30^{II}$ appear to be subject to frequent mutations leading to premature translational termination codons, suggesting that these proteins might not be essential in maintaining disease (Berneman *et al.*, 1992b; Chou *et al.*, 1995).

4.3.6 *Differences between HTLV-I and HTLV-II*

As discussed in section 2.1, HTLV-I is associated with human leukaemia whereas HTLV-II is not. Section 1.1.7 summarized the differences in genomic structure between HTLV-I and HTLV-II.

It is unlikely that there is a functional homologue of HTLV-I $p12^{I}$ in HTLV-II, but deletions in the region between the HTLV-II *env* gene and the second *tax* exon, which would eliminate expression of any potential homologue of HTLV-I $p12^{I}$, $p13^{II}$ or $p30^{II}$, have no effect on virus production, envelope function or transforming potential *in vitro* (Green *et al.*, 1995). The effect of disrupting the G4 and R3 reading frames of BLV, located in a similar region of the BLV genome but of only limited similarity to HTLV-I $p12^{I}$, $p13^{II}$ or $p30^{II}$ (Alexandersen *et al.*, 1993), is discussed in Sections 1.1.7 and 3.3. In conclusion, it is not clear whether any of the small accessory proteins found only in HTLV-I, e.g., $p21^{I}$, is responsible for the leukaemogenic properties of HTLV-I in humans, and the precise roles of HTLV-I $p12^{I}$, $p13^{II}$ and $p30^{II}$ during in-vivo leukaemogenesis remain to be established.

It is also conceivable that as yet unidentified minor differences in the in-vitro transforming potential between HTLV-I and HTLV-II might translate into a weak oncogenic effect *in vivo* (for example, a long latency period for leukaemia development) for HTLV-I but not for HTLV-II.