Cell Type-specific E2F Activation and Cell Cycle Progression Induced by the Oncogene Product Tax of Human T-cell Leukemia Virus Type I

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The transactivator protein Tax of human T-cell leukemia virus type I plays an important role in the development of adult T-cell leukemia probably through modulation of growth regulatory molecules including p16INK4a. The molecular mechanism of leukemogenesis induced by Tax has yet to be elucidated. We analyzed Tax function in the cell cycle using an interleukin-2 (IL-2)-dependent human T-cell line (Kit 225) that can undergo cell cycle arrest at G0/G1 phase by deprivation of IL-2. Tax activated endogenous E2F activity in IL-2-starved Kit 225 cells, resulting in activation of E2F-site-carrying promoters of genes involved in G1 to S phase transition in a cell type-dependent and p16INK4a-independent manner. The ability of Tax mutants to activate E2F coincided with that to activate nuclear factors kB and AT, sole expression of which, however, did not activate E2F, suggesting involvement of another pathway in activation of E2F. Introduction of Tax by a recombinant adenovirus induced cell cycle progression to G2/M phase in resting Kit 225 cells accompanied by endogenous cyclin D2 gene expression. Similarly, Tax-induced cell cycle progression was seen with peripheral blood lymphocytes prestimulated with phytohemagglutinin. Analyses with Tax mutants did not allow Tax-induced cell cycle progression to be differentiated from Tax-dependent activation of E2F, suggesting that Tax induces cell cycle progression presumably through activation of E2F. Nevertheless, infection with an E2F1-expressing virus, which is sufficient for induction of S phase in serum-starved fibroblasts, was not sufficient for either E2F activation or cell cycle progression in IL-2-starved Kit 225 cells, implying differential regulation of E2F activation and cell cycle progression in T-cells that is activated by Tax.

Human T-cell leukemia virus type I (HTLV-I)1 is the etiological agent of adult T-cell leukemia (1–3) and HTLV-I-associated myelopathy/tropical paraparesis (4, 5). There are several lines of evidence indicating that Tax encoded by HTLV-I plays critical roles in leukemogenesis. Introduction of the Tax gene causes persistent growth of primary T-cells in vitro, a process dependent on interleukin-2 (IL-2) (6, 7), and induction of tumors and leukemia in mice in vivo (8, 9).

Tax was initially identified as a trans-acting transcriptional activator of the HTLV-I promotor in the long terminal repeat (10–12). Subsequent studies demonstrated the ability of Tax to transactivate transcription of cellular genes involved in cell growth signaling. These include genes for growth factors/cytokines (13–16), growth factor receptors (17–19), cell adhesion molecules (20–23), cytoplasmic signal transmitters (24), and nuclear transcription factors (25–27). Modulation of expression of these genes is thought to be mainly a consequence of Tax-induced activation of enhancer elements for transcription factors, e.g. cAMP-responsive element-binding factor (CREB), serum-responsive factor (SRF), and nuclear factor (NF)-κB, which have been shown to be physically associated with Tax (28–37). In addition, Tax is known to bind molecules containing ankyrin motifs such as IκB and p16INK4a, which are inhibitors of NF-κB and cyclin D-dependent kinases, respectively (38–40). These findings indicate that Tax is a potential oncprotein that induces tumor development presumably through aberrant expression of genes related to growth signaling. However, it is not clear how Tax-induced modulation of expression of growth signal genes is involved in promotion of cell proliferation.

Progression through G1 and into S phase is controlled by G1 cyclin-dependent kinases (CDKs), including cyclin D- and cyclin E-dependent kinases. One of the roles of these kinases in G1/S progression is phosphorylation of the retinoblastoma tumor suppressor protein pRb and its family members, consequently activating the transcription factor E2F. E2F plays crucial roles in G1/S progression by regulating a variety of genes whose products are involved in cell cycle progression and DNA replication. The ability of Tax to bind and to inhibit p16INK4a activity suggested involvement of Tax in cell cycle progression. During the course of our study, we have learned of the stimulation of CDK activity and cell cycle progression by Tax in two recent reports. Schmitt et al. (41) obtained the results with an IL-2-dependent T-cell line expressing Tax in the presence of IL-2, thus providing the possibility that the CDK activation and cell cycle progression observed are mediated by stimulation with IL-2. Moreover, Neuveut et al. (42) obtained the results with an IL-2-dependent T-cell line expressing Tax in the presence of IL-2, thus providing the possibility that the CDK activation and cell cycle progression observed are mediated by stimulation with IL-2. Moreover, Neuveut et al. (42)
used the Tax-inducible T-cell line JPX-9, which we established, whose cell cycle progression is growth factor-independent and fails to be arrested by deprivation of serum with our JPX-9 cell line. Similarly, the effects of Tax on E2F-mediated transcription have been analyzed in a growth factor-independent T-cell line with exogenously introduced E2F1 and E2F4 (43). These cells are strongly resistant to arrest at G1/S phase by serum deprivation; thus, it is hard to determine the details of the effect of Tax on cell cycle progression and activation of E2F.

To determine the true function of Tax in the cell cycle, it is crucial to examine the effects of Tax on resting human T-cells. For this, we utilized an IL-2-dependent human T-cell line that can be made quiescent by deprivation of IL-2. Our data clearly show that expression of Tax in the IL-2-deprived cells activated endogenous E2F activity, consequently activating a variety of S phase genes with E2F sites depending on the ability of Tax to activate NF-kB and NF-AT and independently of p16INK4a. In addition, using recombinant adenovirus expressing Tax, we show that expression of Tax in the resting cells indeed drove cells into S phase depending on the ability of Tax to activate E2F. More important, this effect of Tax was also observed in peripheral blood lymphocytes (PBLs), but not in fibroblasts, indicating the cell type-specific ability of Tax to induce E2F activation and cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The IL-2-dependent human T-cell line Kit225 (44) was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 0.5 nM IL-2 (Ajinomoto, Yokohama, Japan). The HTLV-I-carrying human T-cell line MT-2 (45) was maintained in RPMI 1640 medium containing 10% FCS. The rat embryonic fibroblast cell line REF52 (46) was maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS. PBLs were isolated by density gradient using Ficoll-Paque PLUS (Amersham Pharmacia Biotech) and cultured in RPMI 1640 medium containing 20% FCS with phytohemagglutinin (PHA).

**Plasmids**—The reporter plasmids pE2WTx4-Luc and pE2MTx4-Luc were generated by subcloning, into the BglII site of the pGLO promoter (Promega), of BglII fragments from pE2WTx4-CAT and pE2MTx4-CAT, which carried four tandem repeats of the adenovirus E2E enhancer with two copies of wild-type SF-2B or BglII site-replaced SF-2B (48), respectively. pE1-Luc, pW-Luc, pLTR-Luc, pNF-AT-Luc, and pCarG-Luc have been described elsewhere (38, 49–51). A series of expression vectors based on the human β-actin promoter for Tax (pMT2Tax) and its mutants (TaxSH2, TaxM22, TaxSH1, Tax703, Tadx3, and Tadx10) were described previously (52). pBAP1-1-neo was used as a control vector (53). The adenovirus major late promoter-driven expression vector pMT-56/G was used for the p56 component of NF-κB was described previously (54). pMT-2T (55) was used as a control vector. Luciferase reporter plasmids containing DNA polymerase α, thymidylate synthase, carbamoyl-phosphate synthetase/aspartate carbamoyltransferase/dihydroorotate, and c-myc promoters were generously provided by Dr. P. Fahrnam. The luciferase reporter plasmid containing the cyclin A promoter (−7300 to +245), pALUC, was a kind gift from Dr. C. Brechot (56). The luciferase reporter plasmids pHsOrcl-Luc (−1053), pHsOrcl-Luc(E2F2), pHsCdc6-Luc (−570), pHsMCM5-Luc (−1384), pHsMCM6-Luc (−754), pE2F1-Luc (−728), pE2F1-Luc(E2F2), pE2F2-Luc, pE2F2-Luc (−E2F2), and pCyCE-Luc (−207) were described previously (57–62).

For generation of a luciferase reporter plasmid with the human cyclin D2 promoter, the SacI-NcoI fragment of 5′-flanking sequences of human cyclin D2 cDNA was subcloned into the SacI-NcoI sites generated by insertion of an Neo linker into the EcoRV site of pBluescript SK- (Stratagene). To remove the ATG initiation codon that overlaps the Neo recognition site, the plasmid was religated after cutting with NeoI, followed by digestion with mung bean nuclease. The SacI-HindIII fragment was cut out and cloned into the SacI-HindIII sites of pGL2-Basic (Promega) (pCyC3-Luc−1624). Similarly, a human cyclin D3 promoter-driven luciferase plasmid was generated by cloning of the Aval fragment of 5′-flanking sequences of human cyclin D3 cDNA into the EcoRV site of pBluescript SK- after bluntning with Klenow enzyme treatment. The KpnI-XbaI fragment was isolated and inserted into the KpnI-Nhel sites of pGL2-Basic, generating pCyC3-Luc (−1319). Sequencing of these fragments identified nucleotide substitutions at nucleotides −885 (G to C), −1583 (G to C), and −1584 (C to G) in the cyclin D2 promoter and a nucleotide substitution at nucleotide −51 (T to C) and nucleotide insertions of C between nucleotides −80 and −81 and of G between nucleotides −318 and −319 in the cyclin D3 promoter, respectively, compared with sequences published previously (63).

**Infecion Assay—**Luciferase expression plasmids and reporter plasmids were introduced into asynchronously growing Kit225 cells using the DEAE-dextran method as described previously (64). Cells were cultured in the absence of IL-2 for 48 h, and luciferase activities were measured as described previously (65). Due to transactivation of commonly used viral promoters (SV40, cytomegalovirus, and Rous sarcoma virus) by HTLV-I Tax in Kit225 cells, it was difficult to design an internal control. Accordingly, luciferase activity was adjusted by protein content. All assays were performed at least three times in duplicate, and means ± S.D. are presented.

**Northern Blotting and Reverse Transcription-PCR Assays—**Total RNA extraction and poly(A)* RNA purification were carried out using Isogen (Nippon Gene) and PolyATact (Promega), respectively, according to the protocol recommended by the manufacturers. Gel electrophoresis, transfer onto nylon membranes, and hybridization were performed as described previously (57). The probe for the cyclin D2 gene was the HindIII-XhoI fragment from pReRSV-neo containing the murine cyclin D2 cDNA (55). Glyceraldehyde-3-phosphate dehydrogenase cDNA was used as a control probe. The blot was exposed to an imaging plate and analyzed with BAS1500 and analyzer (Fuji Film).

Analyses of two distinct viral constructs from the CDKN2A locus of Kit225 and REFS2 cells were performed by reverse transcription-PCR. mRNAs from Kit225 and REFS2 cells were reverse-transcribed with primers 5′-CTCCCCGGGCAAGCCTCGGT-3′ and 5′-CCGGTACGAGGCAACAGC-3′, corresponding to sequences in human and rat p16INK4a exon 2, respectively. PCR amplification of the products was performed with appropriate primer sets: 5′-CCGGGTCGGCTAGAACGTTG-3′ and 5′-ACGGGTCGGTGAGAATGG-3′ for human p16INK4a, 5′-CCGGGCAAGTGGAGGTT-3′ and 5′-ACGGGTCGGTGAGATGGG-3′ for human p1640E, 5′-AAGCCGGGCAAGCATTCTGT-3′ and 5′-GCCGGCGCATCATACTG-3′ for rat p16INK4a, and 5′-CATGTC(G/T)G/G(T)GGCGATTGCTTCTG-3′ and 5′-GCCGTGCGCATCATACTG-3′ for rat p19INK6, respectively. The products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

**Infecion with Recombinant Adenoviruses—**The recombinant adenovirus for expression of E2F1 (Ad-E2F1) and the control virus (Ad-Con; previously Ad-CMV) have been described previously (66). The recombinant adenovirus for expression of Tax (AxCAI-Tax) and its mutant derivatives Taxd3 (67) (AxCAI-Taxd3) and Taxd17/5 (67) (AxCAI-Taxd17/5) were generated by the cosmids-terminal protein complex method (68–70) and kindly provided by Dr. M. Yoshida (University of Tokyo). For cell cycle analysis, 1.5 × 10⁷ Kit225 cells were starved of IL-2 for 48 h and infected with the recombinant adenoviruses at multiples of 100 plaque-forming units/cell in 1 ml of RPMI 1640 medium for 1 h at 37 °C. PBLs were stimulated with PHA for 72 h, washed out, cultured in the absence of IL-2 for 24 h, and infected with the recombinant adenoviruses under the same conditions. Cells were further cultured in the absence of IL-2 for 48 h or 24, 72, and 120 h, respectively, and analyzed for DNA content by flow cytometry. For Northern blot analysis, 1× 10⁷ Kit225 cells starved of IL-2 for 48 h were infected with the recombinant adenoviruses at multiples of 100 plaque-forming units/cell in 7 ml of RPMI 1640 medium for 1 h at 37 °C. Cells were further cultured under IL-2 starvation for 17 h and harvested for RNA isolation. Mock-infected cells cultured in the presence of IL-2 were used as a positive control.

**Tax Expression and DNA Content Analysis—**Cells were fixed with 1× fluorescence-activated cell sorter lysis solution (Becton Dickinson) for 10 min at room temperature and permeabilized with 1 ml of fluorescence-activated cell sorter permeabilization solution (Becton Dickinson) for 10 min. After washing with phosphate-buffered saline containing 0.5% bovine serum albumin, the cells were stained with biotin-conjugated anti-Tax monoclonal antibody (LT-4; 5 mg/ml; Pharmingen) for 30 min. For cell cycle analysis, the cells were resuspended for 30 min in the staining solution containing propidium iodide (50 μg/ml) and RNase (50 μg/ml). Cell samples were analyzed with a FACSan (Becton Dickinson).

**RESULTS**

**Activation of Cell Cycle Regulatory Genes by HTLV-I Tax—**To examine the effects of HTLV-I Tax on cell cycle progression, we first performed transient transfection assays with...
luciferase reporter plasmids carrying promoters of genes related to cell cycle progression (the promoters of the E2F1 and E2F2 genes and the HsOrc1 gene) based on their involvement in cell cycle progression through induction of transcription of a set of genes essential for G1/S phase transition and regulation of the initiation of DNA replication in S phase (72, 73). The human T-cell line Kit 225 was used as a host cell line. Growth of these cells was IL-2-dependent, and culture in IL-2-depleted medium for 48 h caused cells to become quiescent, with ≥95% of the cell population being in G0/G1 phase (see Fig. 5A). The reporter plasmids were introduced along with pMT-2Tax or the backbone vector into Kit 225 cells, and luciferase activities were determined. WT, wild-type; Mut, mutant; DHFR, dihydrofolate reductase; DNA Polα, DNA polymerase α; TK, thymidine kinase; TS, thymidylate synthetase; CAD, carbamoyl-phosphate synthetase/aspartate carbamoyltransferase/dihydroorotase.

Involvement of E2F Sites in Tax-induced Activation—A common feature of HsOrc1, E2F1, and E2F2 promoters is that they all contain E2F-binding sites, which have been shown to predominantly function in cell cycle-dependent expression of these genes (57–59). Thus, Tax was assumed to cause activation of these promoters through E2F sites. This possibility was tested with E2F-binding site mutants with 2-base substitutions that abolished E2F binding activity (57–59). The promoter activity of these mutants (Mut) was similarly examined following transient transfection into Kit 225 cells. In contrast to the wild-type (WT) promoters, which showed significant activation in response to Tax, all mutants failed to respond to Tax (Fig. 1A). These results clearly indicate that the E2F-binding sites in the promoter regions mediate Tax function in transcriptional activation. This was further confirmed by the observation that artificially synthesized sets of four tandem repeats (E2WTx4) of the adenovirus E2 enhancer, which has two typical E2F-binding sites, could be activated by Tax, whereas its mutant (E2Mutx4) did not show any appreciable enhancement of the promoter activity in response to Tax (Fig. 1A).

We further examined whether Tax generally transactivates other promoters containing E2F-binding sites. Several genes induced in G1 and S phases have been shown to contain E2F-binding sites in their promoters, including genes for cell cycle regulators (cyclin D2, cyclin E, cyclin A, E2F1, E2F2, and c-MYC), enzymes for DNA synthesis (dihydrofolate reductase, DNA polymerase α, thymidine kinase, thymidylate synthetase, and carbamoyl-phosphate synthetase/aspartate carbamoyltransferase/dihydroorotase), and regulators of initiation of DNA replication (HsOrc1, HsCdc6, HsMCM5, and HsMCM6). Isolated
promoters of these genes were linked to the luciferase reporter plasmid and introduced into Kit 225 cells along with the Tax expression vector. As expected, all promoters exhibited 4–25-fold increases in activity in response to Tax (Fig. 1B). In contrast, Tax failed to significantly activate the cyclin D3 promoter, which does not contain recognizable E2F-binding sites. These results demonstrate that Tax activates the expression of genes governed by E2F and indicate that Kit 225 cells possess E2F molecules in an activated form in the presence of Tax, implying that Tax can generate active E2F molecules.

**Cell Line-dependent E2F Site Activation by Tax**—Tax has been demonstrated to transform rat fibroblast cell lines in vitro as well as to induce autonomous growth of normal T-cells in the presence of IL-2. Based on these findings, it was of interest to determine whether Tax induces activation of the E2F-binding site in rat fibroblast cell lines. Reporter plasmids carrying HsOrc1 and E2F1 gene promoters were transfected into rat embryonic fibroblast REF52 cells. Transfected cells were deprived of serum for 48 h to induce quiescence. Surprisingly, no activation of the two promoters was seen in the rat cell line (Fig. 2A). Furthermore, four tandem repeats (E2WTx4) of the adenovirus E2 enhancer did not result in activation by Tax transfected into rat embryonic fibroblasts. In contrast, the HTLV-I promoter containing the CREB/ATF-binding site and the NF-xB-binding site, used as controls, resulted in marked activation of the promoter activity by Tax in the same cell line, confirming the functional expression of Tax in these cells. Similar results were obtained with another rat fibroblast cell line, Rat1 (data not shown). These results suggest that Tax-induced activation of E2F-binding sites is cell line-dependent.

Tax has been demonstrated to bind and to inhibit p16INK4a, an inhibitor of cyclin D-dependent kinases (39, 40). We examined whether Kit 225 and REF52 cells express p16INK4a. Northern blot analysis with p16INK4a cDNA as a probe showed a single 2.3-kb band in Kit 225 cells in the presence or absence of IL-2 (data not shown). Recently, it was reported that the CDKN2A locus generates two distinct transcripts for p16INK4a and ARF derived from alternative first exons E1α and E1β, respectively (74–77). To discriminate between these transcripts, reverse transcription-PCR with sets of primers specific for p16INK4a and ARF was performed. As shown in Fig. 2B, a PCR-amplified band from the transcript for ARF, but not for p16INK4a, was detected in Kit 225 cells during both growth and quiescent stages, indicating that Kit 225 cells express only ARF. The same PCR examination revealed that REF52 cells expressed both transcripts for p16INK4a and ARF. These results suggest that Tax-dependent activation of the E2F-binding site is independent of p16INK4a.

**Tax Mutants and Activation of E2F-binding Sites**—Tax mutants are categorized into at least three groups based on their ability to activate target elements: NF-xB-, SRF-, and CREB/ATF-binding sites. In addition, Tax was recently shown to inhibit p16INK4a, an inhibitor of cyclin D-dependent kinases (39, 40). We examined whether Kit 225 and REF52 cells express p16INK4a. Northern blot analysis with p16INK4a cDNA as a probe showed a single 2.3-kb band in Kit 225 cells in the presence or absence of IL-2 (data not shown). Recently, it was reported that the CDKN2A locus generates two distinct transcripts for p16INK4a and ARF derived from alternative first exons E1α and E1β, respectively (74–77). To discriminate between these transcripts, reverse transcription-PCR with sets of primers specific for p16INK4a and ARF was performed. As shown in Fig. 2B, a PCR-amplified band from the transcript for ARF, but not for p16INK4a, was detected in Kit 225 cells during both growth and quiescent stages, indicating that Kit 225 cells express only ARF. The same PCR examination revealed that REF52 cells expressed both transcripts for p16INK4a and ARF. These results suggest that Tax-dependent activation of the E2F-binding site is independent of p16INK4a.

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of the SRF-binding site (CArG box), and Taxd3 did not activate CREB/ATF-binding sites. The mutant TaxM22, which activated CREB/ATF- and SRF-binding sites, could not induce activation of E2F-, NF-κB-, or NF-AT-binding sites. Under our experimental conditions, Tax mutants capable of activating the E2F site could not be distinguished from those that activated the NF-κB- and NF-AT-binding sites, suggesting that NF-κB and NF-AT transcription pathways are involved in activation of the E2F-binding site.

In this context, it was intriguing to examine whether exogenous expression of subunits of NF-κB activates the E2F-binding site. The E2F-binding site reporter plasmid (E2WTx4) was introduced into IL-2-depleted Kit 225 cells along with an expression vector for the p65 subunit of NF-κB. Introduction of the p65 subunit activated the authentic NF-κB-binding site in a p65 dose-dependent manner, whereas dose-dependent reduction rather than enhancement of the activity of the E2F-binding sites was observed in the same cells (Fig. 4). It should be noted that Kit 225 cells have active NF-κB irrespective of the presence of IL-2. This could explain weak activation of the typical NF-κB site by introduction of the p65 expression vector. It should also be noted that Tax could not activate the E2F-
binding site in rat fibroblast cell lines in which it could activate the NF-κB-binding site (Fig. 2A). Similarly, introduction of the NF-ATp or NF-AT4a expression vector did not activate the E2F-binding site (data not shown). These results suggest that activation of E2F by Tax is mediated by another pathway that coincides with the pathway that activates NF-κB and NF-AT.

Cell Cycle Progression Induced by HTLV-I Tax—Tax-dependent activation of E2F molecules resulted in induction of expression of genes involved in cell cycle progression, including genes required for the initiation of DNA replication and DNA synthesis. Therefore, Tax itself may induce cell cycle progression far from G1 phase. To assess this possibility, the G1 to S phase transition was examined by measuring the DNA contents of Kit 225 cells following expression of Tax. Tax was expressed in Kit 225 cells by infection with the recombinant adenovirus AxCAIY-Tax, which has a Tax expression cassette in place of the E1A and E1B genes. Approximately 65% of the Kit 225 cells treated with the recombinant adenovirus were infected as determined by flow cytometric analysis of Tax expression (Fig. 5A). Kit 225 cells positive for Tax showed significant increases in populations of cells in S and G2/M phases (Fig. 5A). In addition, reduction of cell numbers in G1 phase was also associated with cells positive for Tax. In contrast, Tax-negative cells showed no apparent increases in the number of cells in S and G2/M phases compared with control virus-infected cells (Ad-Con). The same infection experiments were carried out with normal PBLs prestimulated with PHA to examine whether the cell cycle progression induced by Tax is specific to Kit 225, which is an immortalized cell line. After stimulation with PHA for 72 h, PBLs were cultured without PHA for 24 h and infected with the recombinant adenoviruses.

Cells were further cultured for 24, 72, and 120 h and examined for DNA content. Cells did not show appreciable changes in population in S and G2/M phases until 24 h after infection with Ad-Tax compared with cells infected with control virus (Fig. 5B). However, Ad-Tax infection increased populations of cells in S and G2/M phases up to 30% at 72 h and 38% at 120 h. In contrast, populations of cells in S and G2/M phases decreased to 16% after infection with the control virus at 120 h. These results indicate that expression of Tax induces cell cycle progression in PHA-stimulated PBLs.

To examine which pathway of Tax-mediated activation is important for cell cycle progression, we examined the ability of recombinant adenoviruses carrying Tax mutants to promote the cell cycle in Kit 225 cells. Infection with a recombinant adenovirus carrying a mutant Tax gene (AxCAIY-Taxd3) capable of activating NF-κB, SRF, and NF-AT pathways, but not the CREB/ATF pathway, induced an increase in the cell populations in S and G2/M phases similar to cells infected with wild-type Tax recombinant adenovirus (AxCAIY-Tax) (Fig. 5C). Comparable levels of expression of the Tax proteins were confirmed by Western blot analysis (data not shown). No significant progression of the cell cycle was, however, seen in Kit 225 cells infected with another Tax mutant-carrying recombinant adenovirus (AxCAIY-Taxd17/5) incapable of activating any of these pathways as confirmed by reporter assay.

These results demonstrate that Tax can induce progression of the cell cycle from G1 to S phase and probably to G2/M phase. The cell cycle progression was seen only with Tax that was able to activate the NF-κB, NF-AT, and SRF-binding sites, consistent with the ability to activate the E2F-binding site. It should be noted that no progression of the cell cycle was induced by infection with Ad-E2F1 (Fig. 5B). This was in contrast to the results observed in fibroblast cells (81).

Tax-induced Expression of the Endogenous Cyclin D2 Gene—In G1 phase of normal T-cell growth, cyclin D2 is predominantly compared with cyclin D1 in fibroblasts. Based on the observation that the cyclin D2 promoter was transactivated by Tax in the transient transfection assay (Fig. 1), we examined Tax-dependent activation of the endogenous cyclin D2 gene. Kit 225 cells were cultured in the absence of IL-2 for 48 h and then infected with recombinant adenoviruses expressing wild-type or mutant Tax. RNA was extracted from cells 17 h post-infection, and expression of the cyclin D2 transcript was monitored by Northern blot hybridization. In the absence of IL-2, Kit 225 cells infected with a recombinant adenovirus (AxCAIY-Taxd3) incapable of activating any of the known pathways expressed a very low level of the 1.0-kb transcript for cyclin D2. Similar to the 1.0-kb transcript, Tax increased the level of the 1.0-kb transcript by 2-fold. The level of the known to generate at least two transcripts of 1.0 and 7.0 kb. Gene—

Fig. 4. Effect of overexpression of p65 on E2F activation. Kit 225 cells were transfected with expression plasmid for the p65 component of NF-κB along with the luciferase reporter plasmid pE2WTx4-Luc or pκB-Luc. The total amount of expression vector DNA was adjusted to 10 μg with the backbone vector. Cells were further cultured for 48 h without IL-2 and harvested for luciferase activity measurement. RLU, relative light units.
The major finding of this study is that HTLV-I Tax induces cell cycle progression to G2/M phase in human T-cells arrested at G0/G1, and that this effect is, at least in part, mediated through the activation of E2F. In this study, we used a human T-cell line, Kit 225. Growth of these cells is absolutely dependent on IL-2; and thus, they could be easily induced to the resting or G0/G1 state by depletion of IL-2. Approximately 95% of Kit 225 cells were at G0/G1 phase 48 h after IL-2 starvation, and little or no apoptosis was observed, unlike the mouse IL-2-dependent T-cell line CTLL-2, which showed apoptosis as early as 6 h after IL-2 depletion (82). In a series of preliminary experiments, we used the IL-2-independent human T-cell line JPX-9, which we have established previously to be a derivative of the human T-cell line Jurkat, which could express Tax upon induction (26). However, it was difficult to make JPX-9 or Jurkat cells pass into a quiescent stage. Under our experimental conditions, withdrawal of serum even for 36 h did not cause cell cycle arrest at G0/G1 phase (data not shown), which is inconsistent with recently reported observations (42). PHA-stimulated PBLs are the same as JPX-9 cells in that growth factor deprivation is not sufficient to make cells arrest at G0/G1 phase. Nevertheless, an increase in the population in S and G2/M phases of PHA-stimulated PBLs by Tax is significant. Based on these results together with the observations obtained with Kit 225 cells, we conclude that Tax induces cell cycle progression, although further studies to elucidate the molecular mechanisms associated with Tax-induced cell cycle progression in PHA-stimulated PBLs are needed.

Two groups have used normal T-cells as hosts for Tax expression (6, 7, 41). In these studies, infection with recombinant herpesvirus or retrovirus was performed to overcome the low efficiency of Tax delivery to primary human T-cells. In both cases, growth of T-cells became IL-2-dependent presumably due to the constitutive expression of functional IL-2 receptor, which is induced by Tax-mediated transactivation of genes for receptor subunits (17–19). Nevertheless, no IL-2-independent growth was observed with T-cells infected with Tax-expressing viruses. Considering our observation that Tax was able to induce cell cycle progression in IL-2-dependent T-cells in the absence of IL-2, Tax may be involved in two different steps in different ways, finally resulting in IL-2-independent growth of primary T-cells through the IL-2-dependent state. Tax-induced escape from IL-2-dependent growth may reflect the final step of development of adult T-cell leukemia. Thus, elucidation of the molecular mechanism of this step is important for a full understanding of HTLV-I-infected T-cell transformation.

CTLL-2 cells failed to activate NF-κB in the absence of IL-2, resulting in apoptosis. We previously reported that constitutive expression of Tax induced IL-2-independent growth of CTLL-2 cells (82). Tax in CTLL-2 cells induced activation of NF-κB in the absence of IL-2, which is involved in the prevention of apoptosis necessary for IL-2-independent growth of CTLL-2 cells. In contrast, the active form of NF-κB is present in Kit 225 cells irrespective of the presence of IL-2 and/or Tax; and thus, apoptosis is prevented for at least 36 h after IL-2 depletion. Tax expression is thus thought to be necessary for growth stimulation in addition to prevention of apoptosis through activation of NF-κB. Kit 225 cells therefore seem to be adequate for studying the cell cycle progression associated with Tax expression without interference by autonomous cell cycle progression and apoptosis.

We demonstrated Tax-dependent activation of the E2F-binding sites in Kit 225 cells depleted of IL-2. There was no appreciable activation of the E2F-binding site when the reporter plasmid and Tax expression plasmid were separately transfected, followed by mixing the cells together, indicating that activation of the E2F-binding site is not mediated by activation of growth factor genes such as IL-2 by Tax (data not shown). Furthermore, cell cycle progression in IL-2-starved Kit 225 cells was observed only in Tax-expressing cells (Fig. 5A). We thus concluded that the E2F-binding site activation and cell cycle progression induced by Tax are mediated by intracellular events intrinsic to T-cells. In contrast to the activation in Kit 225 cells, Tax could not activate E2F-binding sites in rat fibroblast cell lines (Fig. 2). Tax may show species-specific activation. However, this seems unlikely because Tax activated the CREB and NF-κB transcription pathways in these cell lines. Activation of E2F-binding sites by Tax may be cell line-dependent.

In agreement with the previous observation that HTLV-I-infected cells and adult T-cell leukemia cells contain high levels of E2F that bind to E2F-binding sites (83), our results indicate that Tax activates endogenous E2F activity in Kit 225 cells.
During the course of our study, enhancement of E2F-mediated transcription by Tax was independently reported with a growth factor-independent human T-cell line, CEM (43). It was shown that Tax-dependent augmentation of exogenously introduced E2F1 activity and Tax-mediated trans-activation of the E2F1 promoter required the ability of Tax to transactivate the CREB transcription pathway, but did not require its ability to transactivate the NF-κB transcription pathway. This is in marked contrast to our observation that the NF-κB-activating ability of Tax is associated with activation of endogenous E2F activity and that trans-activation of the E2F1 promoter by Tax is largely mediated through E2F. Differences between the two studies might be due to differences in the cell lines and assay conditions used; they assayed the effects of Tax on exogenously introduced E2F1 in a growth factor-independent T-cell line in the growing state, whereas we examined the effects of Tax on endogenous E2F activity in an IL-2-dependent T-cell line at G0/G1 arrest. We observed the same results with another IL-2-dependent human T-cell line, TY8-3 (data not shown).

Consistent with transactivation of the cyclin D2 promoter by Tax in the transient transfection assay, endogenous cyclin D2 gene expression was induced in Kit 225 cells infected with recombinant Tax-expressing adenoviruses (Fig. 6). The endogenous cyclin D2 gene expression by Tax may contribute to Tax-mediated activation of CDK4 and CDK6 in addition to physical interaction between Tax and cyclin D3 molecules, as recently reported (42). Our results did not demonstrate any appreciable increase in transcription of cyclin D3 in the transient transfection assay. These results may explain the previous observations that HTLV-I-transformed T-cell lines expressed high levels of cyclin D2 and very low levels of cyclin D3 (84).

In addition to these scenarios, Tax-dependent E2F activation may be mediated by suppression of cyclin D/CDK4 and CDK6 inhibitors belonging to the INK4 family. Earlier studies demonstrated that Tax binds to and inhibits p16INK4a (39, 40). This suppressive effect of Tax on the inhibitor may mediate activation of E2F through the relief of suppression of cyclin D/CDK4 and CDK6 activities. In Kit 225 cells, however, we confirmed no expression of p16INK4a as also reported recently by Stott et al. (85). It is of interest to note that Tax did not activate E2F in REF52 cells, in which p16INK4a is expressed. These results indicate that Tax can activate endogenous E2F activity independently of p16INK4a. This is consistent with a recent report that Tax can augment exogenously introduced E2F1 activity in a growth factor-independent human T-cell line negative for p16INK4a (43).

Our investigation using Tax mutants demonstrated significant correlations between activation of the cell cycle transcription factor E2F and signaling pathways associated with transcription factors NF-κB and NF-AT, i.e. Tax mutants that could activate the NF-κB and NF-AT transcription pathways could induce E2F activation. No such correlations were observed between activation of the CREB transcription pathway and E2F activation or between activation of the SRF transcription pathway and E2F activation. We thus expected that exogenous expression of NF-κB or NF-AT could activate E2F independently of cell cycle progression. The results of introducing the p65 subunit of NF-κB, which enhanced a typical NF-κB-binding site in Kit 225 cells, were opposite to our expectations. The same was true with c-Rel and a combination of the p65 and p50 subunits of NF-κB (data not shown). Moreover, introduction of an NF-ATp or NF-AT4a expression vector did not activate E2F activity either (data not shown). Based on these observations, we speculate that activation of the NF-κB and/or NF-AT transcription pathway may be necessary but not sufficient for E2F activation. Alternatively, Tax utilizes another pathway that coincides with the NF-κB and NF-AT pathways to activate E2F and to drive cell cycle progression.

There is sufficient evidence to indicate that E2F is critical for cell cycle progression through induction of expression of a series of genes mainly required for G1 and S phases. These genes are categorized into three groups according to the functional properties of their products: regulators of the cell cycle itself such as members of the cyclin, E2F, and myc families; genes encoding enzymes essential for DNA synthesis; and molecules that regulate the initiation of DNA replication, including Hs-Or1 and members of the MCM family. Promoters of these genes have been shown to contain typical E2F-binding sites or related sequences. Our results clearly demonstrated that these promoters with E2F-binding sites were activated by Tax. As expected from these results, Tax promoted cell cycle progression in the human T-cell line Kit 225. This is similar to the observation that introduction of E2F1 into rat fibroblast cell lines activates a set of these promoters and induces cell cycle progression (86). However, Kit 225 cells displayed different characteristics when transfected with E2F; exogenous E2F1–5 did not activate E2F-binding site-containing promoters (data not shown), and E2F1 did not induce cell cycle progression (Fig. 5B). In contrast, Tax activated all promoters tested in the transient transfection assay and induced cell cycle progression. These results suggest that Kit 225 cells have a cell type-specific regulatory mechanism in E2F activation and cell cycle progression distinct from that in fibroblasts and that Tax has the ability to stimulate the cell type-specific regulatory pathway(s) to activate E2F and to induce cell cycle progression.

As Tax induced cell cycle progression, we expected to observe an increase in the number of Kit 225 cells following introduction of Tax. No increase was, however, associated with infection with the Tax recombinant adenovirus (data not shown). Instead, we noted a time-dependent increase in the number of dead cells after infection. Overexpression of E2F, typically E2F1, has been shown to induce not only cell cycle progression, but also apoptosis (87). It is possible that cell cycle progression in Kit 225 cells induced by Tax resulted in apoptosis similar to that in rat fibroblasts cells forced to undergo cell cycle progression by overexpression of E2F.

Taken together, our findings indicate different regulatory mechanisms involved in E2F activation and cell cycle progression in rat fibroblast cells compared with human T-cells, further suggesting the involvement of Tax in a cell growth mechanism specific for T-cells. Elucidation of an as yet unknown Tax-mediated pathway implicated in cell cycle progression, which is presumably activated by IL-2 (88), may allow the design of new methods to study tumorigenesis induced by infection with HTLV-I.

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