Abstract
Human T-cell leukemia virus type 1 Tax protein, a transcriptional activator of viral expression, promotes uncontrolled cellular proliferation. In this report, we show that Tax-expressing myoblasts do not exit the cell cycle and fail to differentiate into myotubes despite the deprivation of serum. In these cells, which displayed unchanged levels of the ubiquitous basic helix-loop-helix E2A factors and Id proteins, Tax was found to target the muscle-specific basic helix-loop-helix transcription factor MyoD. The Tax-induced increase in cyclin-dependent kinase 2 activity correlated with the phosphorylation of MyoD. However, the half-life of this hyperphosphorylated form of MyoD increased in Tax-expressing myoblasts, contrary to that in control cells. Furthermore, MyoD mRNA levels were reduced in Tax-expressing cells. Tax was found to repress MyoD expression at the transcriptional step by preventing MyoD from activating its own transcription. Interestingly, overexpression of the transcriptional coactivator p300 restored the capacity of Tax-expressing muscle cells to differentiate. These observations underscore the critical effect of the trans-repressing ability of Tax on the MyoD-controlled proliferation and differentiation processes of the myoblast lineage.

Introduction
The bHLH proteins constitute a family of transcriptional regulatory factors that play a critical role during embryonic development and cellular differentiation of various cell types, including muscle, neuron, pancreas, and T or B lymphocytes (see Ref. 1 for a review). The function of bHLH factors has been most extensively evaluated by the analysis of mechanisms that regulate skeletal muscle differentiation. This process is characterized by withdrawal of myoblasts (undifferentiated muscle cells) from the cell cycle, induction of muscle-specific gene expression, and fusion of myoblasts into multinucleated differentiated myotubes (reviewed in Refs. 2–4). These events have been found to be dependent on the activity of muscle-specific bHLH transcription factors, including MyoD, Myf-5, myogenin, and MRF4. These myogenic bHLH proteins form heterodimers with ubiquitously expressed bHLH proteins, such as E12 and E47. These heterodimers activate muscle-specific genes by binding to a DNA-specific sequence (CANNTG) known as the E-box motif (5). Furthermore, these four myogenic factors share the ability to activate the myogenic differentiation program when expressed ectopically in nonmuscle cells.

MyoD, contrary to myogenin and MRF4, is expressed in proliferating myoblasts and initiates the myogenic differentiation program. This transcription factor exerts a dual activity by initiating a transcriptional cascade that leads to the expression of the muscle-specific products and by inhibiting cell proliferation. The latter correlates with the induction of the cdk inhibitor p21 (CIP1), the up-regulation of retinoblastoma tumor suppressor protein (6–8), and possibly with the direct binding of MyoD with retinoblastoma tumor suppressor protein (9). Furthermore, MyoD represses the expression of cell cycle inducers, such as c-fos (10), and inhibits cdk4 activity by direct binding (11). Consequently, the activity of this key muscle-specific transcription factor is negatively regulated in myoblasts by the following process. HLH proteins of the Id family, which lack the basic DNA-binding domain, form heterodimers with MyoD and E proteins, preventing bHLH factors from binding to DNA (reviewed in Ref. 12). During the course of muscle differentiation, Id protein levels decrease and MyoD recovers its...
ability to dimerize with E proteins and to activate transcription of muscle-specific genes. Furthermore, cell cycle regulators also counteract MyoD activity. Thus, in myoblasts, MyoD is highly phosphorylated by cdk1 and cdk2, leading to its subsequent degradation by the proteasome (13, 14). Conversely, during differentiation, the decrease in cdk activity leads to a substantial dephosphorylation of the MyoD protein (13). Finally, the direct interaction of MyoD with cdk4 also inhibits its DNA-binding activity (11, 15).

The Tax protein is a 40-kDa nuclear phosphoprotein, encoded by the genome of HTLV-1, a complex retrovirus etiologically linked to adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (16, 17). Tax mainly functions as a transactivator of HTLV-1 proviral transcription. It also intervenes in the transcriptional activation or repression of cellular genes (Refs. 18–21; reviewed in Refs. 22–24). More importantly, Tax has also been shown to interact with and alter the expression of some cell cycle-associated genes (reviewed in Ref. 25). Hence, Tax binds p16INK4a in vitro, inactivates its activity, and also represses INK4a gene family expression (26, 27). It also forms complexes with this cdk/cyclin D inhibitor in vivo and stimulates cdk/cyclin D activity (28). Furthermore, Tax up-regulates the expression of cyclin D2, an early G1 cyclin that is associated with new partners, such as cdk2 and cdk4, in addition to cdk6 in presence of Tax (29). All of these effects lead to the increase of cdk activity shown by the hyperphosphorylation of Rb, an indicator of active progression of the cell cycle into S-phase.

Considering that Tax and MyoD are exerting opposite effects on cell cycle progression, the myogenic differentiation system provides a suitable model to further evaluate the effect of the Tax protein on the bHLH-controlled processes of the proliferation and differentiation paradigm. Here we show that myoblasts stably expressing Tax do not differentiate and still proliferate, as confirmed by the maintenance of cdk2 activity, when cultured under differentiation conditions. Interestingly, we found that the expression of MyoD, which normally increases during differentiation of myoblasts into myotubes, was significantly decreased in Tax-expressing cells. Conversely, the levels of E2A and Id proteins remained unchanged in the Tax-expressing muscle cells. We observed that the hyperphosphorylation of MyoD by cdk2 in these cells did not lead to its rapid degradation by the proteasome. Surprisingly, however, the MyoD half-life increased in Tax-expressing cells. We then found that Tax repressed MyoD expression at the transcriptional level by preventing MyoD from activating its own transcription. Finally, we provided evidence that the overexpression of the coactivator p300 in Tax-expressing muscle cells was followed by increased MyoD expression and by reinitiation of the differentiation pathway.

Results
C2.7 muscle cells stably expressing the HTLV-1 Tax protein (C2.7-Tax) and control G418-resistant cells (C2.7-Neo) were obtained as indicated in “Materials and Methods.” Immunoblotting analysis of C2.7-Tax and C2.7-Neo myoblasts confirmed the expression of this viral regulatory protein in the latter (see Fig. 3). When seeded in proliferation (high serum-containing) medium, both C2.7-Tax and C2.7-neo grew as mononucleated myoblasts (Fig. 1A, a and b). Their proliferation rate was similar to that of the parental cells, with a doubling time of ~20 h (Fig. 1B). When they were seeded in differentiation (low serum-containing) medium, the C2.7-Neo cells stopped proliferating (Fig. 1B) and fused into multinucleated myotubes (Fig. 1A, c). On the other hand, the C2.7-Tax cells did not differentiate into myotubes (Fig. 1A, d). Furthermore, under these conditions, they kept proliferating and were 10-fold more numerous than control cells 4 days after seeding (Fig. 1B). We also ascertained that under these conditions, Tax was still expressed in these cells (see Fig. 2). These data indicate that Tax-expressing cells are maintained in proliferation and do not differentiate, even when cultured in differentiation medium.
MyoD Expression Is Decreased in Tax-expressing Cells. The above results further suggest that Tax expressed in muscle cells affects the expression of genes involved in the differentiation of these cells. Western blot analysis revealed that the cdk inhibitor regulator p21, one of the first markers expressed in differentiating muscle cells, was overexpressed in C2.7-Neo cells placed in differentiation medium (Fig. 2A). Conversely, its expression was repressed in C2.7-Tax cells cultured in differentiation medium. The levels of myogenin, a muscle-specific bHLH factor and early marker of muscle cell differentiation, were subsequently increased in C2.7-Neo myotubes compared with C2.7-Neo myoblasts (Fig. 2A). Likewise, expression of MHC, a component of the contractile apparatus and late marker of differentiation, was absent in myoblasts and readily detected in myotubes. Conversely, neither myogenin nor MHC could be detected in C2.7-Tax cells cultured in differentiation medium. Finally, desmin, a muscle-specific intermediate filament, was readily detected at similar levels in control and Tax-expressing cells (Fig. 2A). We next assessed the expression levels of the HLH (Id) and bHLH (E2A and MyoD) proteins. The expression profiles of Id1, Id2, and Id3 proteins were found to be quite similar in both C2.7-Neo and C2.7-Tax cells cultured in proliferation medium, although a slight decrease in Id2 and Id3 expression was noticed in C2.7-Tax cells (Fig. 2B). When C2.7-Neo cells were cultured in differentiation medium for 48 h, Id1 expression decreased, whereas Id2 and Id3 could not be detected (Fig. 2B). Likewise, Id1, Id2, and Id3 were expressed in C2.7-Tax cultured in proliferation medium, but were not detected when these cells were cultured in differentiation medium. The expression of E2A was found to decrease in C2.7-Neo cells and to increase in C2.7-Tax cells when both were cultured in differentiation medium (Fig. 2B). More importantly, the MyoD protein level was found to be significantly lower in C2.7-Tax cells than in C2.7-Neo cells when both cell lines were propagated in proliferation medium (Fig. 2B). As expected, expression of MyoD protein increased in C2.7-Neo cells induced to differentiate. Conversely, in C2.7-Tax cells cultured under the same conditions, MyoD expression was still repressed. Collectively, these observations indicate that Tax expression in muscle cells correlates with decreased expression of MyoD.

MyoD Is Hyperphosphorylated in Tax-expressing Cells. The degradation of MyoD in proliferating muscle cells has been linked to its hyperphosphorylation by cdk2 on Ser200 (13, 14). Furthermore, Tax expression has been associated with increased cdk4 and cdk2 activity in other cellular models (28, 29). We therefore hypothesized that in Tax-expressing muscle cells, MyoD might be subjected to increased phosphorylation by cdks and, thus, rapidly degraded.
Fig. 3. Increased phosphorylation of MyoD in Tax-expressing cells does not correlate with increased MyoD degradation. A, C2.7 (Lanes 1 and 4), C2.7-Neo (Lanes 2 and 5), and C2.7-Tax (Lanes 3 and 6) cells were cultured in proliferation medium (PM; Lanes 1–3) or differentiation medium (DM; Lanes 4–6) for 2 days. cdk2 was immunoprecipitated from cell extracts by an anti-cdk2 antibody. The cdk2 kinase activity was tested against H1 histone as a substrate, using \(^{32}\)P-ATP. The kinase reaction mixture was then resolved by 15% SDS-PAGE and transferred to a PVDF membrane. Phosphorylated radioactive H1 histone was visualized after autoradiography. The same membrane was exposed either overnight (H1) or for 48 h (H1*). The presence of immunoprecipitated cdk2 was confirmed by immunoblotting of the same membrane (WB cdk2). In control reactions (Lanes 7–12), the anti-cdk2 antibody was omitted during the immunoprecipitation step. The amount of radioactive phosphorylated H1 histone was quantified by phosphorimager. The result of this quantification is shown in the graph at the bottom. B, C2.7-Neo (Lanes 1–5 and Lane 11) and C2.7-Tax (Lanes 6–10) cells were cultured in proliferation medium (PM) without or with 15 \(\mu\)g/ml cycloheximide for the indicated times and then processed for Western blot analysis. Cell extracts (100 \(\mu\)g of total proteins) were resolved by SDS-PAGE and transferred to a PVDF membrane, as described above. This membrane was probed sequentially with a polyclonal anti-Tax antiserum, a monoclonal anti-MyoD antibody (5.8A), or a monoclonal antidesmin antibody (DE-U-10), followed by the corresponding secondary HRP-coupled antibody. Antibody binding was visualized by enhanced chemiluminescence. The intensity of the MyoD protein relative to that of desmin was plotted against the time of culture in cycloheximide (bottom). The half-life was determined as indicated in “Materials and Methods.” Western blots were scanned, and the band intensities were quantified by the Image-Quant software. C, C3H10T1/2 cells were cotransfected with the MyoD and Tax expression plasmids (Lanes 3 and 4) or cotransfected with the MyoD expression vector alone (Lanes 1 and 2). Twenty-four h after transfection, the cells were further cultured in proliferation medium (PM; Lanes 1 and 3) or in differentiation medium for 2 days (DM; Lanes 2 and 4) and then harvested and lysed in antiphosphatase-containing buffer (see “Materials and Methods”). Twenty \(\mu\)g of total cell lysates were used in a Western blot analysis with a monoclonal anti-MyoD antibody. The slower migrating form of MyoD corresponds to the MyoD protein phosphorylated on Ser 200 (13). D, C3H10T1/2 cells were cotransfected with the MyoD and Tax expression plasmids (Lanes 6–10) or transfected with the MyoD expression vector alone (Lanes 1–5) and further cultured in growth medium for 2 days. Cells were then cultured in the presence of cycloheximide and analyzed as described for B. Twenty \(\mu\)g of total cell lysates were used for Western blot analysis with monoclonal anti-MyoD or antiaxin antibodies or a monoclonal anti-Tax antiserum. The intensity of the MyoD protein relative to that of actin was plotted against the time of culture in cycloheximide (bottom). The half-life was determined as indicated in “Materials and Methods.” Blots were scanned, and the band intensities were quantified by the Image-Quant software. The data presented are from one typical experiment of four performed.
To assess this hypothesis, we evaluated the activity of cdk2 in parental, control, or Tax-expressing C2.7 cells. As shown in Fig. 3A, cdk2 immunoprecipitated from C2.7 cells cultured in proliferation medium efficiently phosphorylated H1 histone. Indeed, cdk2 activity was 1.7-fold higher in C2.7-Tax cells than in C2.7-Neo cells (compare Lanes 2 and 3 in Fig. 3A). As expected, cdk2 from C2.7 parental and C2.7-Neo myotubes (cultured in differentiation medium) displayed very low to undetectable H1 kinase activity (Fig. 3A, Lanes 4 and 5). The cdk2 activity immunoprecipitated from C2.7-Tax cells in differentiation medium, although lower than that from the same cells cultured in proliferation medium, was 8-fold higher than that from C2.7-Neo myotubes (Fig. 3A, Lanes 5 and 6). These data clearly underscore the proliferative status of C2.7-Tax cells.

We also detected an increased amount of hyperphosphorylated (slow-migrating) MyoD in C2.7-Tax cells, compared with C2.7-Neo cells [Fig. 3B; compare the migration profile of MyoD in C2.7-Neo (Lanes 1 and 11) and in C2.7-Tax cells (Lane 6)]. We then determined the half-life of MyoD in C2.7-Neo and C2.7-Tax cells after cycloheximide treatment. Unexpectedly, this analysis revealed that MyoD is more stable in C2.7-Tax cells, with a half-life of 140 min compared with 30 min in C2.7-Neo cells (Fig. 3B).

In the experiments described above, the low level of MyoD in C2.7-Tax cells prevented an accurate survey of its phosphorylation and half-life. We therefore elected to transfect C3H10T1/2 fibroblasts with a MyoD-expressing plasmid either alone or together with a Tax vector. Twenty-four h later, these cells were placed in either proliferation or differentiation medium for 48 h. We found that, in the absence of Tax, hyperphosphorylated MyoD represented 70% of the amount of that protein expressed in C3H10T1/2 cells cultured in proliferation medium (Fig. 3C, Lane 1). This hyperphosphorylated form of MyoD decreased to 55% in cells cultured in differentiation medium (Fig. 3C, Lane 2). When MyoD was coexpressed with Tax, phosphorylated MyoD was found to represent 85% of the total MyoD in cells cultured in proliferation medium (Fig. 3C, Lane 3). Under differentiation conditions, phosphorylated MyoD did not decrease and still represented 80% of the total amount (Fig. 3C, Lane 4). These results indicate that Tax expression correlates with increased MyoD phosphorylation. We next transfected C3H10T1/2 fibroblasts with the MyoD vector alone or with the Tax vector and then treated them with cycloheximide for the indicated times. In the absence of Tax, the MyoD half-life was found to be ~35 min in C3H10T1/2 cells. However, in the presence of Tax, the MyoD half-life increased to 135 min (Fig. 3D). These results confirm that despite being hyperphosphorylated, MyoD acquired increased stability in the presence of Tax.

MyoD Is Repressed at the Transcriptional Level in Tax-expressing Cells. The above results indicate that Tax might interfere with transcription of the myoD gene. To verify this hypothesis, we performed a Northern blot analysis of both control and Tax-expressing muscle cells cultured in either proliferation or differentiation medium (Fig. 3A). MyoD mRNAs were readily detected during proliferation of C2.7-Neo cells and increased by 2-fold after 48 h of culture in differentiation medium (compare Lanes 1 and 2 in Fig. 4).

Conversely, MyoD mRNA levels were 3-fold lower in C2.7-Tax cells cultured in proliferation medium than in control cells cultured in that medium (compare Lanes 1 and 2 in Fig. 4). Furthermore, MyoD transcripts did not increase in C2.7-Tax cells after 48 h of culture in differentiation medium (compare Lanes 3 and 4 in Fig. 4). The results of the Northern blot analysis of E2A and Id transcripts in both C2.7-Neo and C2.7-Tax cells correlated with the results obtained in the Western blot analysis (Fig. 3B). One discrepancy was noticed between the mRNA and protein expression levels of E2A protein in C2.7-Tax cells, which may indicate that Tax modulates E2A expression at a post-translational level.

Collectively, these data indicate that Tax mainly represses MyoD expression at a transcriptional level and suggest that Tax expression is linked to decreased activity of the myoD gene promoter. To verify this hypothesis, we used the pEnh-myoD-Lac-Z plasmid, in which the reporter Lac-Z gene, which codes for β-galactosidase, is under the control of 258-bp fragment of the myoD gene enhancer (30, 31). This 258-bp element directs the expression of the Lac-Z gene in a spatiotemporal pattern indistinguishable from the expression of the normal myoD gene during development (30). This
construct was transfected in C2.7 myoblasts, either alone or with a Tax expression plasmid (pLTR-Tax). In cells cultured in proliferation medium, the activity of this myoD gene enhancer measured in the absence of Tax was found to decrease by 50% in its presence (Fig. 5A). In cells cultured in differentiation medium, a 5-fold increase in the activity of this enhancer was observed compared with that in cells cultured in proliferation medium. However, in the presence of Tax, such an increase was not observed, and the activity of the myoD gene enhancer remained at a level comparable to that observed in cells expressing Tax and cultured in proliferation medium. Because MyoD increases its own transcription via the E boxes present in its gene enhancer, the effect of Tax on MyoD expression was then assessed by transfecting C3H 10T1/2 cells with the pEnh-myoD-Lac-Z construct together with a MyoD vector. An increase of the myoD expression was then assessed by transfecting C3H 10T1/2 cells with the pLTR-Tax vector re-

tact by segregated the p300/CBP coactivators from bHLH myoblasts, Tax, a retroviral regulatory protein, disturbs the myogenic bHLH transcription factors (21, 32). Consequently, it might be expected that overexpression of these coactivators in Tax-expressing muscle cells will lead to increased MyoD expression and allow these cells to resume the differentiation process. To verify this hypothesis, C2.7-Tax cells were transfected with a CMV-p300 vector and then cultured in differentiation medium for 3 days. Immunofluorescence analysis showed that the percentage of cells expressing MHC, a late differentiation marker, correlated with the amount of the transfect vector (Fig. 6A). Western blot analysis confirmed the increased expression of MHC in CMV-p300-transfected C2.7-Tax cells compared with C2.7-Neo cells, as indicated in “Materials and Methods.” The enzyme activities are expressed as fold activation of the β-galactosidase activity in cells transfected with the pEnh-myoD-Lac-Z plasmid cultured in proliferation medium. B, C3H10T1/2 cells were transfected with 0.5 μg of the pEnh-myoD-LacZ plasmid, alone or together with 1 μg of pLTR-Tax and/or 1 μg of pCMV-MycD. Forty-eight h after transfection, cells were harvested and assayed for β-galactosidase activity, as indicated in “Materials and Methods.” The enzyme activities are the means of three experiments (bars; SD). Results are expressed as fold activation of the β-galactosidase activity in cells transfected with the pEnh-myoD-Lac-Z plasmid.

Discussion

During developmental events, cell proliferation and terminal differentiation appear to be mutually exclusive processes, controlled by the balance between opposing cellular signals. Skeletal muscle differentiation provides a model suitable for the analysis of the mechanisms governing this balance. In this field, most studies have demonstrated the critical role of the myogenic bHLH transcription factors of the MyoD family in the initiation and maintenance of the myogenic program. Indeed, after receiving differentiation signals, MyoD induces cell cycle arrest and initiates the coordinated expression of muscle-specific genes (4).

In this report, we provide evidence that when expressed in myoblasts, Tax, a retroviral regulatory protein, disturbs the coordinated events accompanying muscle cell differentiation. We first confirmed previous observations by showing that, contrary to control (parental and C2.7-Neo) cells, C2.7 myoblasts stably expressing Tax failed to form multinucleated myotubes when placed in differentiation-inducing medium (28). We then extended these observations by showing that they failed to express differentiation markers, such as myogenin and MHC. Likewise, under these culture conditions, C2.7-Tax cells continued to proliferate, contrary to
control cells. Finally, we observed that the inhibition of muscle cell differentiation by Tax was associated with decreased expression of MyoD.

It has previously been shown that the turnover of MyoD in proliferative myoblasts is negatively regulated by direct phosphorylation of this myogenic transcription factor (on Ser200) by cdk1 and/or cdk2. Indeed, phosphorylation of Ser200 appears to be required for targeting MyoD to the ubiquitin-proteasome pathway (13, 14). These observations, together with the finding that Tax stimulates cdk2 activity in T cells (29), led to the hypothesis that MyoD could be hyperphosphorylated and thus rapidly degraded in Tax-expressing muscle cells. We indeed demonstrated increased cdk2 activity in C2.7-Tax muscle cells, confirming a similar observation in Tax-expressing T cells and extending previous studies demonstrating increased cdk4 activity in Tax-expressing myoblasts (28, 29). As expected, MyoD was hyperphosphorylated in C2.7-Tax cells as well as in fibroblasts coexpressing Tax and MyoD, when compared with the relative control cells. Surprisingly, however, when hyperphosphorylated in Tax-expressing cells, MyoD escaped rapid degradation by the ubiquitin-proteasome pathway. The mechanism responsible for the inhibition of MyoD degradation in Tax-expressing cells remained to be elucidated. Likewise, the antioncogene p53 was stabilized and functionally repressed in either HTLV-1-infected or Tax-expressing cells (33–35). Here again, the mechanism of p53 stabilization by Tax has not been established. Conversely, Tax was shown to facilitate degradation of the inhibitor of nuclear factor-κB, thus allowing activation of the nuclear factor-κB pathway (36–42). These data rule out the possibility that Tax represses MyoD degradation by shutting off the proteasome and ubiquitin pathways. Likewise, other viral oncoproteins, such as adenoviral E1A, were found to specifically inhibit the proteasome-mediated degradation of specific cellular proteins. For example, E1A stabilizes p53 by binding to and inhibiting the 19S subunit of the proteasome (43). Because Tax also directly interacts with the two subunits, HsN3 and HC9, of the 20S proteasome (44, 45), it cannot be excluded that Tax stabilizes MyoD by a similar mechanism.

Consequently, the decreased MyoD protein expression in Tax-expressing muscle cells could not be explained by increased degradation of the MyoD protein. We rather demonstrated that Tax is acting at the transcriptional level. Indeed, when compared with control cells, MyoD mRNA levels were reduced by 3-fold in C2.7-Tax cells cultured in proliferation medium. Likewise, no increase was observed in these cells shifted to differentiation medium, contrary to results obtained in control cells. MyoD is known to directly activate its own transcription through an autoregulatory loop mediated by E boxes present in the enhancer of this gene (46–48). We have previously provided evidence that Tax is able to down-regulate the transcriptional activity of MyoD (21). We demonstrated that this trans-inhibiting property was linked to the inhibition by Tax of the interaction between MyoD and the KIX domain of the p300 coactivator. Therefore, the repression of MyoD gene expression in Tax-expressing cells could be a consequence of Tax-mediated inhibition of MyoD transcriptional activity that blocks the MyoD autoregulatory loop. Using the pEnh-myoD-Lac-Z reporter plasmid (30), we demonstrated that Tax was found to inhibit the activity of the myoD gene enhancer in muscle cells. Indeed, we observed that the MyoD-dependent β-galactosidase expression was repressed in muscle cells and in fibroblasts cotransfected with Tax and myoD vectors. Furthermore, overexpression of the p300 coactivator in cells stably expressing Tax was found to increase the expression of MyoD in these cells, which are then able to enter the differentiation pathway. Taken together, these data indicate that by inhibiting MyoD transcriptional activity, the HTLV-1 Tax protein represses the differentiation of muscle cells and also down-regulates MyoD expression by blocking the MyoD autoregulatory loop.

As indicated in the “Introduction,” the Tax protein has been shown to function mainly by trans-activating or trans-repressing the transcription of specific cellular genes and by promoting cell cycle progression. The results reported in this study underline that Tax affects cellular proliferation and differentiation of muscle cells by trans-repressing the activity of myogenic bHLH transcription factors. Interestingly, recent findings support that bHLH factors behave as tumor suppressors: when overexpressed in leukemic cells, they repress proliferation and induce the death of these cells (48, 50). Furthermore, the tight association between the transcriptional repression of bHLH factors and the development...
of T-cell leukemia (51–55) might further implicate Tax in T-cell leukemogenesis associated with HTLV-1 infection.

Materials and Methods

Cells. The C3H10T1/2 fibroblasts (CCL 226; American Type Culture Collection) and the C2.7 myoblasts (56) were propagated in DMEM (Life Technologies, Inc., Cergy Pontoise, France) supplemented with 10% FCS, 5 mM L-glutamine, 20 IU/ml penicillin, and 20 μg/ml streptomycin. Their differentiation into myotubes was achieved by culturing C2.7 myoblasts in growth medium until they reached 80% confluence and then placing them in differentiation medium (DMEM containing 2% horse serum). Maximum formation of myotubes was usually observed 48–72 h later.

Plasmids, Transfections, and β-Galactosidase Assay. The pLTR-Tax plasmid containing the HTLV-1 Tax cDNA under the control of the HTLV-1 LTR has been described previously (57). The pLTR-Neo and the pSV-Neo plasmids contain the neomycin resistance gene under the control of HTLV-1 and the SV40 virus promoter, respectively. The pEnh-myOD-LacZ plasmid (a generous gift of S. Tajbakhsh, Institut Pasteur, Paris) contains the 258-bp core sequence of the myoD gene enhancer, which has been found to direct the expression of the lac-Z gene in a spatiotemporal pattern indistinguishable from the expression of the myoD gene (30). The pCMV-Tax, pCMV-MyoD, and pCMV-p300 vectors contain the Tax, MyoD, and p300 coding sequences, respectively, under the control of the CMV promoter (21). The pCMV-EGFP plasmid contains the coding sequence of the fluorescent EGFP protein under the control of the CMV promoter (pEGFP-C1; Clonetech, Ozyme, France). The C3H10T1/2 and C2.7 cells were transfected using FuGene 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany) as described previously (21). The amount of DNA in each transfection experiment was normalized using the same empty vectors or identical vectors expressing irrelevant proteins to maintain an identical amount of each promoter.

Forty-eight h after transfection, the cells were harvested by scraping and lysed in Mammalian Protein Extraction Reagent (Pierce). After centrifugation, protein concentrations of cell lysates were determined by the Bradford assay (Bio-Rad). Identical amounts of proteins were assayed for lysates were determined by the Bradford assay (Bio-Rad). Identical amounts of proteins were assayed for lysates were determined by the Bradford assay (Bio-Rad). Identical amounts of proteins were assayed for lysates were determined by the Bradford assay (Bio-Rad). Identical amounts of proteins were assayed for lysates were determined by the Bradford assay (Bio-Rad). Identical amounts of proteins were assayed for lysates were determined by the Bradford assay (Bio-Rad).

Stable Transfections. C2.7 myoblasts stably expressing Tax were generated by cotransfection of C2.7 cells with the pLTR-Tax and pLTR-neo plasmids at a molar ratio of 10:1, using the FuGene transfection reagent. The use of the pLTR-Neo plasmid allowed the selection of Tax-expressing cells because Tax is a potent trans-activator of HTLV-1 LTR transcription. Transfected cells were cultured in growth medium containing genetin (Life Technologies, Inc.) at a final concentration of 1 mg/ml. Cells were subcultured at a low density for 2–3 weeks, at which time a polyclonal population of resistant cells, referred to as C2.7-Tax cells, was obtained. In parallel, control cells, referred to as C2.7-Neo cells, were engineered by cotransfecting C2.7 myoblasts with the pSV-Neo vector and culturing the transfected cells in geneticin at 1 mg/ml.

Western Blotting, Immunofluorescence, and Antibodies. For Western blot analysis, cells washed in PBS were then harvested by scraping and resuspended in lysis buffer. This lysis buffer contained 100 μg/ml Tris (pH 7.8), 1% NP40, 1% SDS, 20 μg/ml protease inhibitor for mammalian cell extract (Sigma Chemical Co.), 1 mM dithiothreitol (DTT), 362 units/ml endonuclease, and phosphatase inhibitors (10 μg NaF and 250 μg β-glycerophosphate). Identical amounts of proteins (determined by Bradford assay) were diluted with 2× Laemmli buffer [125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.4% bromphenol blue, 200 mM DTT]. After being boiled for 3 min, the cell lysates were resolved by 10% SDS-PAGE. The proteins were then transferred to PVDF membranes (Hybond-P; Amersham, Les Ulis, France) with a semidry electric transfer device. The membranes were blocked in PBS containing 10% nonfat milk and 0.1% Tween 20. Membranes were washed once and incubated in saturation solution containing the appropriate primary antibody for at least 2 h at room temperature, rinsed, and then incubated with antimouse (Dako) or antirabbit (Immunotech) immunoglobulin conjugated with HRP. Proteins recognized by antibodies were visualized by the enhanced chemiluminescence detection system (Renaissance; NEN).

For immunofluorescence analysis, C2.7-Tax cells were fixed for 35 min in 3% paraformaldehyde in PBS, washed in PBS containing 10 μg/ml glycine, permeabilized in PBS containing 1% Triton for 5 min, and washed twice with PBS containing 10 μg/ml glycine and once with in PBS containing 25 μg/ml glycine. The cells were blocked in the antibody buffer (PBS containing 1% BSA, 0.5 mM NaCl, and 0.5% Tween 20) containing 3% NBCS for 1 h at room temperature. The cells were then incubated in the presence of the primary antibody diluted in the antibody buffer containing 1% NBCS for 2 h. After three washes in PBS containing 10 μg/ml glycine, the cells were incubated with the secondary antibody (FITC-conjugated goat antimouse IgG; Sigma Chemical Co.) diluted in the antibody buffer containing 1% NBCS for 45 min. After three washes in PBS containing 10 μg/ml glycine, fluorescent cells mounted in 50% glycerol were enumerated with a fluorescent microscope.

The primary antibodies were as follows: a rabbit polyclonal anti-Tax, rabbit polyclonal antinmyogenin (M-225; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-MyoD (clone 5.8A; PharMingen), mouse monoclonal antiantigen (clone AC-40; Sigma, St. Quentin Fallavier, France), mouse monoclonal anti-MHC (clone MY32; Sigma), mouse monoclonal antidesmin (clone DE-U-10; Sigma), mouse monoclonal anti-GAPDH (MAB 374; Chemicon, International Interchim), mouse monoclonal anti-E2A (clone Yae; Santa Cruz Biotechnology), and rabbit polyclonal anti-Id1, -Id2, or -Id3 antibodies (Santa Cruz Biotechnology).
Determination of cdk2 Activity. Cells (C2.7, C2.7-Neo, or C2.7-Tax cells) were washed twice in 1× PBS, scraped in 1 ml of PBS, and finally harvested. After centrifugation, the cell pellet was resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.4% NP-40, 2 mM EDTA, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM ATP, 2 μg/ml each of leupeptin and aprotonin, 2 mM sodium vanadate, 2 mM DTT). After 10 passages through a 21-gauge needle, cell lysates were cleared by centrifugation at 13,000 rpm. Protein concentrations in the supernatant were then determined by the Bradford assay. Two hundred μg of protein were incubated for 2 h at 4°C with a 1:50 dilution of a polyclonal anti-cdk2 antibody (M2; Santa Cruz Biotechnology). Protein G-Sepharose beads were then added and incubated for 1 h at 4°C. Immune complexes bound to beads were then centrifuged at 12,000 rpm for 5 min at 4°C. The beads were washed three times with the lysis buffer, twice with the same buffer containing 400 mM NaCl, and finally twice in a kinase buffer (25 mM HEPES (pH 7.4), 25 mM MgCl2, 25 mM β-glycerophosphate, 2 mM DTT, 0.1 mM NaVO3). Beads were finally incubated in 20 μl of kinase buffer containing 50 μM ATP, 5 μCi of [γ-32P]ATP, and 1 μg of purified H1 histone. The reaction mixtures were then subjected to Western blot analysis. The membranes were autoradiographed and immunoblotted with the anti-cdk2 antibody. The amount of radioactive phosphorylated H1 histone was quantified using a phosphorimager and the anti-cdk2 antibody. The amount of radioactive phosphorylation of nuclear MyoD is required for its rapid degradation. Mol. Cell. Biol., 25: 119–126, 2005.

Half-Life Determination. Cells (C2.7-Neo, C2.7-Tax, or transfected C3H10T1/2) were incubated in DMEM supplemented with 10% FCS and 15 μg/ml cycloheximide for 0.5, 1, 2, and 3 h and harvested by scraping. Whole-cell lysates were subjected to Western blot analysis as described above. The PVDF membranes were successively probed with the mouse monoclonal anti-MyoD (clone 5.8A; PharMingen), mouse monoclonal antisera (clone DE-U-10; Sigma), and rabbit polyclonal anti-Tax antibodies. The amount of immunoblotted protein was determined by scanning of the autoradiogram and analysis using the Image-Quant software (Molecular Dynamics).

Northern Blot Analysis. RNAs were prepared using the Total RNA Isolation system (Promega, Charbonnieres, France) as described by the manufacturer. Twenty μg of each RNA sample were electrophoresed on a 1% agarose-30% formaldehyde gel and transferred by capillary action in 20× SSC to nylon membranes (Biotrans; ICN). RNA transfer was verified by ethidium bromide fluorescence under UV illumination. The nylon membrane was saturated in a hybridization solution (50 mM sodium phosphate (pH 7), 0.1% SDS, 1 M NaCl, 2.5 mg/ml denatured herring sperm DNA). DNA fragments corresponding to the specific probes were purified by low-melting agarose gels, labeled with [α-32P]dCTP in the Prime-a-Gene labeling system (Promega) according to the manufacturer’s instructions, purified on Sephadex G-50 fine columns (Sigma), denatured, and added to hybridization solution at 106 cpm/ml. The same membrane was stripped by boiling in a 1% SDS solution and reprobed several times.

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References


