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# CREB/ATF-Dependent Repression of Cyclin A by Human T-Cell Leukemia Virus Type 1 Tax Protein

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**Expression of the human T-cell leukemia virus type 1 (HTLV-1) oncoprotein Tax is correlated with cellular transformation contributing to the development of adult T-cell leukemia. Tax has been shown to modulate the activities of several cellular promoters. Existing evidence suggests that Tax need not directly bind to DNA to accomplish these effects but rather that it can act through binding to cellular factors, including members of the CREB/ATF family. Exact mechanisms of HTLV-1 transformation of cells have yet to be fully defined, but the process is likely to include both activation of cellular-growth-promoting factors and repression of cellular tumor-suppressing functions. While transcriptional activation has been well studied, transcriptional repression by Tax, reported recently from several studies, remains less well understood. Here, we show that Tax represses the TATA-less cyclin A promoter. Repression of the cyclin A promoter was seen in both ts13 adherent cells and Jurkat T lymphocytes. Two other TATA-less promoters, cyclin D3 and DNA polymerase α, were also found to be repressed by Tax. Interestingly, all three promoters share a common feature of at least one conserved upstream CREB/ATF binding site. In electrophoretic mobility shift assays, we observed that Tax altered the formation of a complex(es) at the cyclin A promoter-derived ATF site. Functionally, we correlated removal of the CREB/ATF site from the promoter with loss of repression by Tax. Furthermore, since a Tax mutant protein which binds CREB repressed the cyclin A promoter while another mutant protein which does not bind CREB did not, we propose that this Tax repression occurs through protein-protein contact with CREB/ATF.**

Infection with human T-cell leukemia virus type 1 (HTLV-1) has been linked to the development of several diseases: adult T-cell leukemia (ATL), tropical spastic paraparesis, and various neurological disorders termed HTLV-1-associated myelopathy (33). The HTLV-1-encoded oncoprotein Tax has been implicated in the transformation of T cells (reviewed in reference 91), as well as in tumor formation in transgenic mice (26). Although the precise mechanisms utilized by Tax to induce transformation are not known, this protein has been shown to modulate cellular genes that are involved in cellular proliferation and cell cycle control (reviewed in reference 55). Tax up-regulates expression of interleukin-2 (IL-2), IL-2 receptor, c-fos, c-Jun, erg-1, and granulocyte-macrophage colony-stimulating factor (reviewed in references 32 and 51; 52) and represses expression of the β-polymerase, c-myb, Lck, and p53 promoters (11, 39, 48, 57, 84). Tax has also been shown to affect the functions of IKKγ (10, 27, 40), c-myc (69), Bax (8), MAD1 (41), cyclin D (56), and MyoD (63).

Cyclins are critical factors in cell cycle progression (25, 71, 72). Cyclins associate with cyclin-dependent kinases and regulate the functions of cellular proteins that are required for progression through the cell cycle ($G_1$, S, $G_2$, and M) phases. The D cyclins are induced by growth factors and mediate progression through $G_1$. Cyclin A begins to accumulate after the $G_1$/S transition, and its associated kinase activity is required for both completion of S phase and entry into as well as exit from M phase (reviewed in reference 42). Aspects of cell cycle progression have been well studied in a model system utilizing a baby hamster kidney cell line, ts13, which is temperature sensitive for the $G_1$-to-S transition (82). ts13 exhibits a growth defect at the restrictive temperature (39°C), which results from a point mutation in cell cycle gene 1 (CCG1) (68). CCG1 was subsequently shown to be identical to the gene for the $TAF_{II}250$ subunit of TFIID (31). At 39°C, a subset of cell cycle-related promoters, including the cyclin A (88), cyclin D3 (81), and DNA polymerase α (44) promoters, is transcriptionally repressed. This restricted-growth phenotype of ts13 at 39°C can be complemented by overexpression of wild-type $TAF_{II}250$ (88) and the $G_1$-specific cyclin D1 (67). Interestingly, several viruses also encode functions that rescue the $G_1$-restricted phenotype of ts13. Thus, simian virus 40 (SV40) large T antigen (13) and hepatitis B virus (HBV) X oncoprotein (29) also complement the CCG1 mutation in ts13 cells.

The findings for ts13 cells suggest that many viruses might encode a CCG1/$TAF_{II}250$-like activity. In principle, this makes sense since viruses should evolve the ability to usurp the cell cycle machinery for viral replicative benefits. How the HTLV retroviruses might behave in this regard has not been extensively investigated. Because Tax's properties as a transcriptional activator and as a transforming protein resemble those of both SV40 T antigen (TAg) and HBV X protein, we reasoned that Tax might have an X- or TAg-like CCG1/$TAF_{II}250$ activity. Hence, using ts13 cells, we investigated this possibility. Unexpectedly, we found that Tax, in contrast to SV40 TAg or HBV X, failed to rescue the growth defect of ts13 cells at the

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restrictive temperature. In attempting to define the differences between Tax and TAg, we compared the transcriptional functions of the two on $TAF_{II}250$-dependent promoters. We observed that, whereas TAg activated $TAF_{II}250$-dependent expression of cyclin A in ts13 cells, Tax actually repressed the cyclin A promoter.

## MATERIALS AND METHODS

**Cell lines.** ts13 cells are temperature sensitive baby hamster kidney cells (82), which were cultured at 32°C. ts13 cells and HeLa cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone). JPX9, a derivative of Jurkat cells, contains an inducible Tax cDNA under the control of the metallothionein promoter (58). Tax expression can be induced with zinc (120 μM $ZnCl_2$) or cadmium (20 μM $CdCl_2$). TL-Su and ILT-Hod (from Mari Kannagi, Tokyo Medical and Dental University, Tokyo, Japan) cells were derived from peripheral blood lymphocytes of an HTLV-1 carrier and an ATL patient, respectively. MT4 and C8166-45 are human T-cell lines transformed by coculture with HTLV-1 producer cells. JPX9, Jurkat, C8166-45, MT4, ILT-Hod, and TL-Su cells were cultured in RPMI 1640 supplemented with 10% FBS. ILT-Hod was maintained in RPMI 1640 supplemented with 10% FBS and 20 U of IL-2 (Boehringer Mannheim) per ml.

**Plasmids.** pHpX (54), $pU_3RCAT$ (7), and TaxH52Q (70) have been described previously. LTR-luc was constructed by excising the HTLV-1 long terminal repeat (LTR) from $pU_3RCAT$ at the *Xho*I and *Hin*dIII restriction sites and reinserting the LTR into pGL3-Pr (Promega) at the *Xho*I and *Hin*dIII restriction sites. DPAΔATF was constructed by synthesis of an oligomer consisting of the fragment from −65 to +7 of the DNA polymerase α promoter (60) with *Xho*I and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively. The oligomer was then inserted into the *Xho*I and *Hin*dIII sites of the pGL3-PR luciferase reporter plasmid (Promega). pNFkB-luc was purchased from Stratagene. All other plasmids were generous gifts of R. Tjian, Howard Hughes Medical Institute, Berkeley, Calif. (CycA-luc and $pCMVhTAF_{II}250$); C. Z. Giam, U.S. Uniformed Health Services, Bethesda, Md. (TaxL90A and TaxV89A); P. Hinds, Harvard Medical School, Boston, Mass. (pCycD3-luc); T. Wang, Stanford University School of Medicine, Stanford, Calif. (pDPA LΔ5′); and K. Peden, Food and Drug Administration, Bethesda, Md. (pRSVTAg).

**Transfections.** Jurkat cells were transfected using SuperFect (Qiagen) according to manufacturer's protocol. Briefly, $5.0 \times 10^6$ cells per well (six-well plate) were transfected with 3 to 10 μg of DNA and 20 μl of SuperFect reagent. The transfection mixture was removed from cells after 4 h and replaced with complete RPMI 1640 supplemented with 10% FBS. Cells were harvested 46 to 48 h after medium replacement. ts13 cells were transfected using Lipofectamine (Life Technologies) according to the manufacturer's protocol. Six-well plates were seeded at 50 to 60% confluence and transfected the following day with 3 to 10 μg of DNA and 12 μl of Lipofectamine reagent. The transfection mixture was removed from cells after 4 h and replaced with complete DMEM supplemented with 10% FBS. Plates were incubated at 32°C for 12 h, followed by incubation at 39°C for 24 h, and then harvested. In all transfections, the total amount of DNA was equalized with pUC19. Jurkat cell transfections were normalized to β-galactosidase activity expressed from a cotransfected cytomegalovirus β-galactosidase (Invitrogen) plasmid.

**Luciferase assays.** Transfected cells were harvested after two washes with PBS. Adherent cells were scraped into 250 μl, and suspension cells were resuspended into 200 μl of reporter lysis buffer (Promega). Lysates were prepared according to the protocol of the manufacturer (Promega). Luciferase activity was measured in an Optocomp II luminometer (MGM Instruments).

**Electrophoretic mobility shift assay (EMSA).** A 21-bp oligomer containing the terminal deoxynucleotidyltransferase (TdT) initiator sequence or a 28- or 61-bp oligomer containing the ATF-responsive element alone or the ATF element plus an initiator site (Inr) were labeled with [γ-$^{32}$P]ATP (Amersham Pharmacia) using T4 polynucleotide kinase (New England Biolabs). Probes were added (~30,000 cpm) to reaction mixtures (25 μl) containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 40 mM KCl, 20% glycerol, 0.5% Triton X-100, 5 mM EDTA, 5 mM dithiothreitol, 13.2 μg of salmon sperm DNA per ml, and 2 μg of nuclear extract. JPX9 and Jurkat nuclear extracts were prepared as described previously (17). MT4 and C8166-45 nuclear extracts were purchased from Geneka Biotechnology, Inc. Reaction mixtures were incubated at room temperature for 30 min. Complexes were resolved in a 4% polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer at 180 V for 2 h and visualized by autoradiography.

**Western blotting.** Approximately $10^7$ cells were harvested, washed twice in phosphate-buffered saline (PBS), and resuspended into 200 μl of 2× sample buffer (100 mM Tris [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, and 0.05% bromphenol blue). Ten microliters was loaded onto a sodium dodecyl sulfate–10% polyacrylamide gel and electrophoresed. Afterwards, the gel was electroblotted onto Immobilon-P membranes (Millipore Corp.) using a Millipore semidry blotting apparatus. Visualization of antigens on the membrane was with rabbit antiserum raised against Tax and used at a 1:1,000 dilution (38), mouse monoclonal anti-cyclin A antibody used at a 1:500 dilution (Upstate Biotechnology), or mouse monoclonal anti-β-actin antibody used at a 1:20,000 dilution (Sigma). Incubation with primary antibody was followed by incubation with goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibody. Secondary antibodies were used at a 1:10,000 dilution. Detection of secondary antibody was by chemiluminescence (Tropix). Blots of JPX9 cells and Jurkat cells (see Fig. 7) were probed for cyclin A and β-actin simultaneously. The JPX9 blot was then blocked and reprobed for Tax. The blot shown in Fig. 8 was probed for cyclin A and β-actin simultaneously.

**Cell cycle synchronization.** Cells were cultured in the presence of 2 mM thymidine (Sigma) in DMEM plus 10% FBS for 24 h, allowed to recover in complete medium with no thymidine for 12 h, and then propogated again in 2 mM thymidine for an additional 14 h.

## RESULTS

**Tax represses the cyclin A promoter in ts13 and Jurkat cells.** Because expression and replication of viruses frequently show cell phase dependence, it is reasonable that some viruses would evolve to control the cell cycle machinery of infected cells. HTLV-1 Tax, SV40 TAg, and HBV X are all transcriptional activators as well as transforming proteins. Thus, initially, we wondered whether Tax would conserve the CCG1/$TAF_{II}250$-complementing activity shared by SV40 TAg (13) and HBV X (29). To address this, we checked for functions in ts13 cells. While we could recapitulate the described activity of SV40 TAg in supporting the growth of ts13 cells at the restrictive temperature (39°C), we found that Tax provided no such function (K. V. Kibler, unpublished data).

TAg has also been shown to support temperature-sensitive $TAF_{II}250$-dependent transcription (13). Thus, to understand better the divergence between Tax and TAg in ts13 cells, we next surveyed the $TAF_{II}250$-dependent transcription of the well-characterized cyclin A promoter. We assayed for Tax effects on a cyclin A promoter-luciferase reporter (CycA-luc [88]) by transfecting ts13 cells with CycA-luc alone, CycA-luc with a Tax expression plasmid (pHpX), CycA-luc with a $TAF_{II}250$ expression plasmid (pCMV-$hTAF_{II}250$ [88]), or CycA-luc with a TAg expression plasmid (pRSV-TAg) (Fig. 1A). Compared to expression with CycA-luc alone (activity set as 100%) (Fig. 1A, lane 1), coexpression of either $TAF_{II}250$ (Fig. 1A, lane 3) or SV40 TAg (Fig. 1A, lane 4) increased luciferase expression by 50 and 180%, respectively. By contrast, in the same assay, Tax repressed CycA-luc activity by 76% (Fig. 1A, lane 2), with a dose-dependent profile (Fig. 1B). To rule out nonspecific cytotoxicity as a trivial explanation for Tax's repression of the cyclin A promoter, we also transfected ts13 cells with an HTLV-1 LTR chloramphenicol acetyltransferase reporter ($pU_3RCAT$). Figure 1C demonstrates that Tax activated $pU_3RCAT$ as it repressed CycA-luc, rendering it unlikely that observations of the latter occur from nonspecific cytotoxicity.

To verify that the effect of Tax on CycA-luc was not idiosyncratic to ts13 cells, we also tested Jurkat T cells. Because several other TATA-less promoters also show $TAF_{II}250$-dependent expression, we assayed two additional promoters, DNA polymerase α and cyclin D3, in combination with the

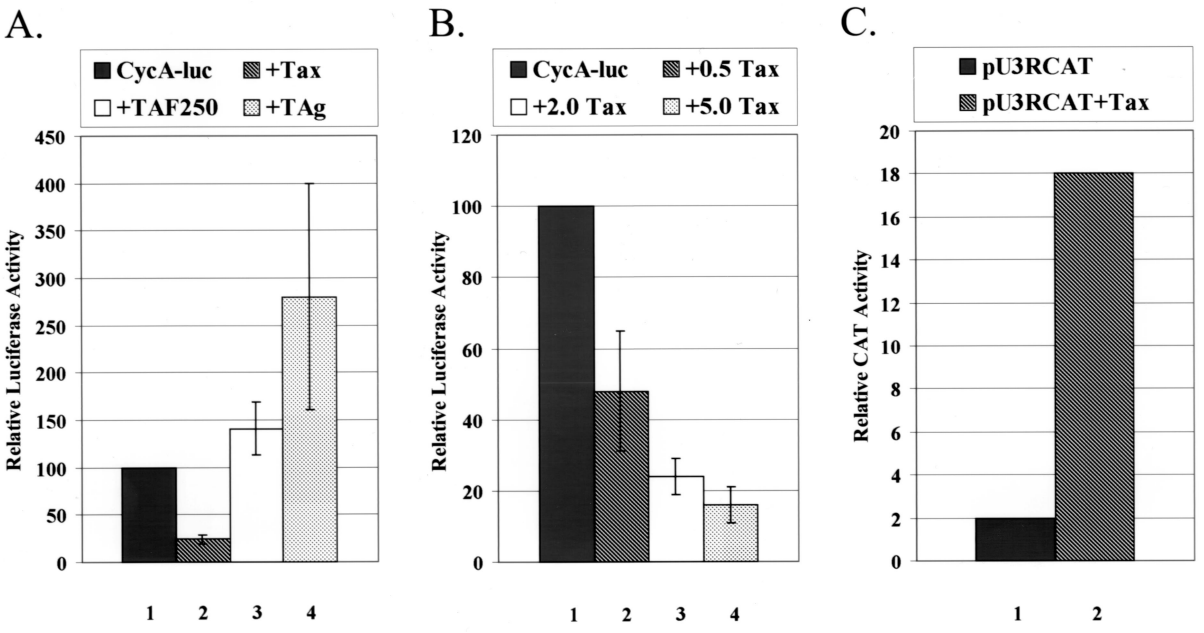



FIG. 1. Tax represses the cyclin A promoter. (A) The cyclin A promoter is activated in ts13 cells at 39°C by either human $TAF_{II}250$ or SV40 TAg but is repressed by Tax. Cells were transfected with CycA-luc alone (1 μg) (lane 1), CycA-luc plus pHpX (Tax expression vector, 2 μg) (lane 2), CycA-luc plus pCMV-h$TAF_{II}250$ (2 μg) (lane 3), or CycA-luc plus pRSV-TAg (2 μg) (lane 4). Transfected cells were incubated at the permissive temperature (32°C) for 12 h and then at the restrictive temperature (39°C) for 24 h and harvested. Results are averages from five independent experiments. Error bars show standard deviations of the means. (B) ts13 cells were transfected as described above with either CycA-luc alone (1 μg) (lane 1) or increasing amounts (as indicated) of pHpX (lanes 2 to 4). Results are averages from five independent experiments. (C) Tax activates the HTLV-1 LTR ($pU_3RCAT$) in ts13 cells at 39°C. ts13 cells were transfected with $pU_3RCAT$ (1 μg), with (lane 2) or without (lane 1) Tax (2 μg). Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (24).

luciferase reporter (DPA-luc plasmid [60] and CycD3-luc plasmid [gift of P. Hind], respectively); both of these promoters, like cyclin A, conserve a promoter-upstream CREB/ATF binding site (Fig. 2A). When these three promoter-reporter plasmids were separately assayed in Jurkat cells, we observed that Tax efficiently repressed transcription from CycA-luc (Fig. 2B, lane 2), DPA-luc (Fig. 2B, lane 6), and CycD3-luc (Fig. 2B, lane 10) to 28, 19, and 12% of baseline activities, respectively. When CREB was exogenously overexpressed by transfection, we found that Tax repression of CycA-luc was ameliorated (data not shown). These results taken together with the above findings (Fig. 1) indicate that Tax, in both ts13 and Jurkat backgrounds, exerts a consistently repressive effect on several CREB/ATF-binding-site-containing TATA-less promoters.

**Tax abrogates activation by $TAF_{II}250$ or TAg.** How might Tax mechanistically repress the cyclin A, DNA polymerase α, or cyclin D3 promoter? Both $TAF_{II}250$ and TAg complement the transcription of the cyclin A, DNA polymerase α, or cyclin D3 promoter at the restrictive temperature in ts13 cells (Fig. 1A and data not shown). To understand if the repressive effect of Tax directly negates the activating effects of $TAF_{II}250$ and/or TAg, we checked cotransfections of Tax with $TAF_{II}250$ or SV40 TAg. Figure 3 shows results of Tax with CycA-luc plus $TAF_{II}250$ (Fig. 3A), Tax with CycA-luc plus TAg (Fig. 3B), Tax with DPA-luc plus $TAF_{II}250$ (Fig. 3C), and Tax with DPA-luc plus TAg (Fig. 3D). In these experiments, we noted that $TAF_{II}250$ enhanced expression of CycA-luc and DPA-luc to 141% (Fig. 3A, lane 2) and 323% (Fig 3C, lane 2), respectively. However, with increasing amounts of cotransfected Tax, the activating effects of $TAF_{II}250$ were abrogated (Fig. 3A and C, lanes 3 to 5). Similar findings also documented Tax's abrogation of the activation by TAg of either CycA-luc (Fig. 3B) or DPA-luc (Fig. 3D). Collectively, these results show that Tax repression at the assayed promoters is dominant over activation by either $TAF_{II}250$ or SV40 TAg.

**Repression by Tax correlates with the CREB/ATF binding site.** In ts13 cells, it has been proposed that disruption of the $TAF_{II}250$ interaction with factors bound to a promoter-upstream CREB/ATF site upstream of the promoter (89) explains the CycA expression defect at the restrictive temperature. Because Tax additively repressed expression from the cyclin A promoter in ts13 cells at 39°C, and because Tax is known to bind CREB/ATF directly (22, 77, 83), we reasoned that physical sequestration by Tax might explain transcriptional repression. To correlate repression with Tax and CREB/ATF interaction, we transfected ts13 cells with a Tax H52Q point mutation protein (TaxH52Q) (22) which is defective in binding to CREB. Interestingly, while wild-type Tax repressed both basal (Fig. 4A, lane 2) and $TAF_{II}250$-activated (Fig. 4A, lane 5) expression of CycA-luc, TaxH52Q failed to do either (Fig. 4A, lanes 3 and 6). TaxH52Q is deficient for activation of the HTLV-1 LTR but retains the ability to activate promoters through NF-κB binding sites (70). To verify that the lack of repression of the cyclin A promoter by TaxH52Q did not result trivially from reduced protein expression, we compared levels of induction of an NF-κB-responsive reporter (pNFκB-luc) by Tax and TaxH52Q (Fig. 4B, lanes 5 and 6). Consistent with

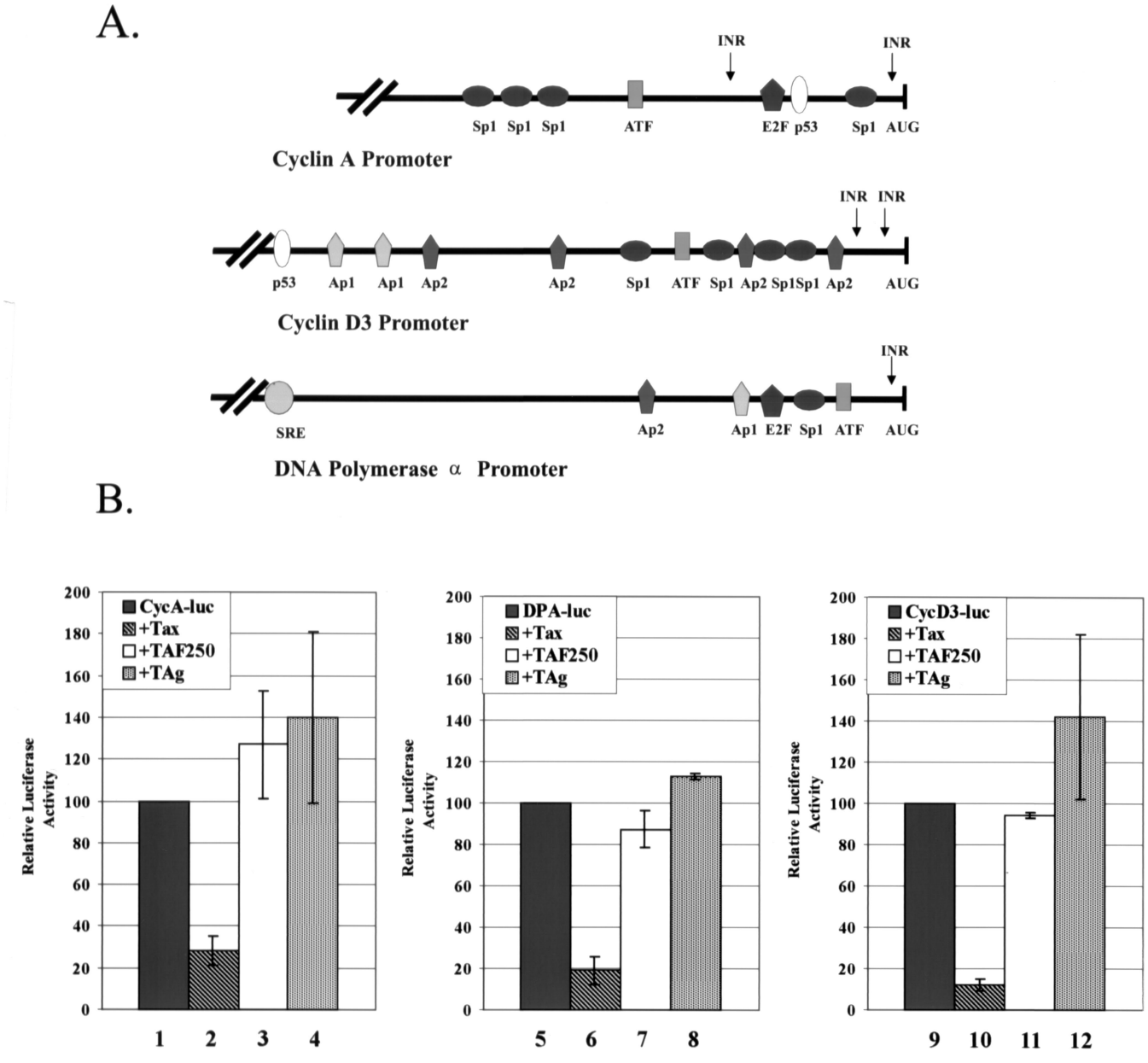



FIG. 2. Tax represses cyclinA, cyclin D3, and DNA polymerese α promoters in Jurkat T cells. (A) Schematic representations of the cyclin A (30), the cyclin D3 (9), and the DNA polymerase α (60) promoters showing approximate positions of transcription factor binding elements and the transcription start sites (Inr[INR]). SRE, serum response element. (B) Tax represses the expression of these promoters in Jurkat cells. Jurkat cells were transfected with either the promoter-reporter alone (1 μg) (lane 1), the reporter plus 2 μg of Tax (lane 2), the reporter plus 2 μg of $TAF_{II}250$ (lane 3), or the reporter plus 2 μg of TAg (lane 4). Results are averages from two independent experiments.

there being comparable levels of protein expression, TaxH52Q activated pNFκB-luc to a magnitude similar to that activated by wild-type Tax while it did not activate the HTLV-1 LTR-responsive reporter (LTR-luc) (Fig. 4B, lane 3). Similarly, repression of DPA-luc was also found to correlate with Tax proteins competent for binding CREB/ATF (data not shown). These results are consistent with Tax repression requiring physical Tax-CREB contact.

The involvement of CREB/ATF in repression was further analyzed using CREB/ATF binding site-intact or CREB/ATF binding site-deleted (DPAΔATF-luc) forms of DPA-luc. In these assays, we tested both Tax (Fig. 4C) and a Tax point mutation protein, TaxV89A, which binds CREB with wild-type affinity (Fig. 4D). Figure 4D shows that in contrast to TaxH52Q, TaxV89A effectively repressed DPA-luc (Fig. 4D, lanes 2 to 4). On the other hand, CREB/ATF-independent expression from DPAΔATF-luc (Fig. 4C and D, lanes 6 to 8) was insignificantly affected by either Tax or TaxV89A. Collectively, the results in Fig. 4A, C, and D verify that Tax interferes with CREB/ATF-dependent activity at the cyclin A and DNA polymerase α promoters and that this interference correlates with the ability of Tax to bind CREB.

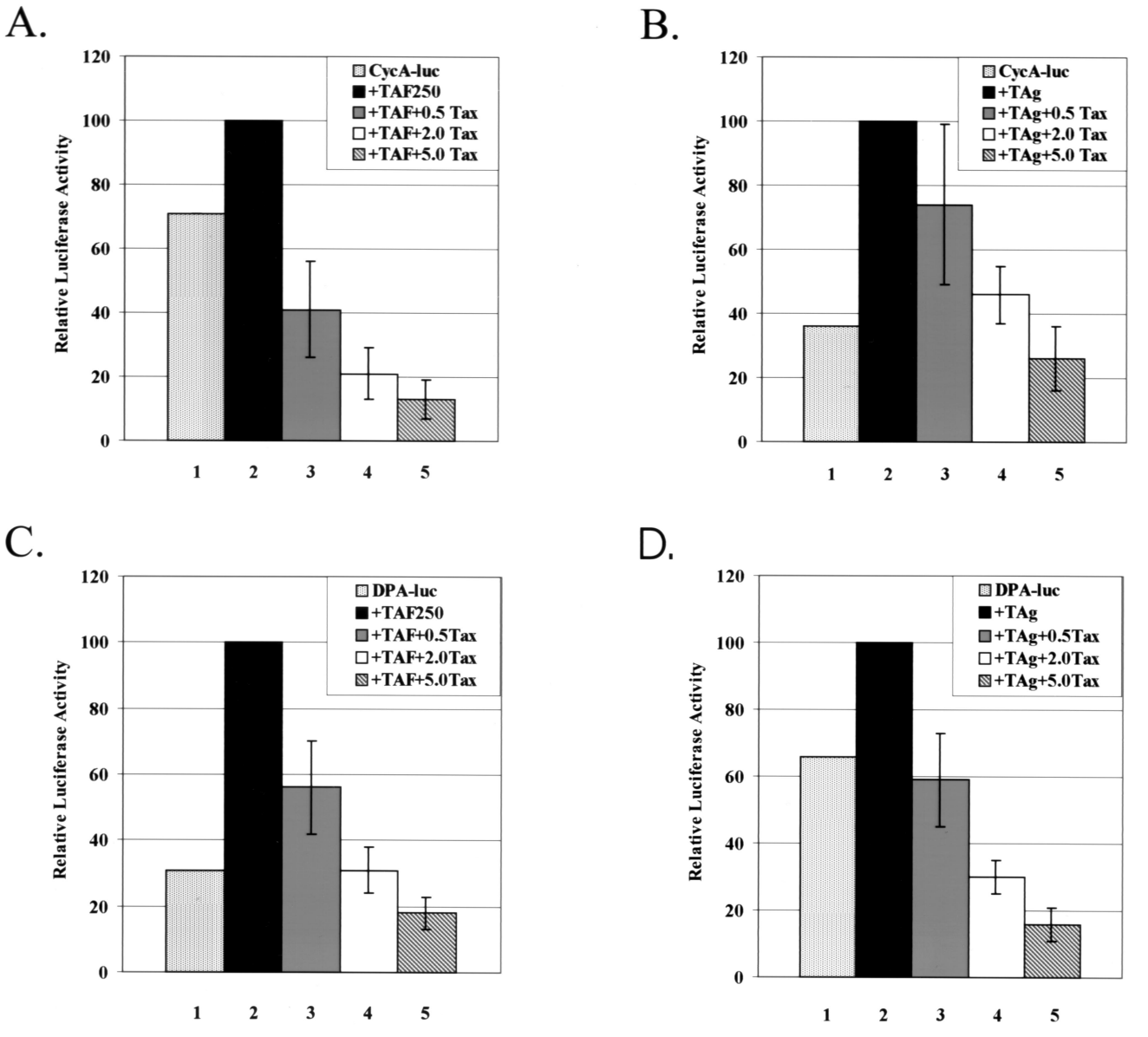



FIG. 3. Activities of $TAF_{II}250$ and TAg are repressed by Tax in ts13 cells. ts13 cells were transfected with 1 μg of CycA-luc (A and B) or DPA-luc (C and D). (A) CycA-luc was cotransfected with $TAF_{II}250$ (2 μg) and increasing amounts of Tax. (B) CycA-luc was cotranfected with TAg (2 μg) and increasing amounts of Tax. (C and D) Transfections of DPA-luc with either $TAF_{II}250$ (C) or TAg (D) and increasing amounts of Tax. Results are averages from a minimum of five independent experiments.

**Tax repression does not require CBP binding.** It has been shown that optimal Tax function requires binding not only to CREB but also to CREB-binding protein (CBP) (20). Interestingly, Tax sequestration of CBP has also been proposed as a mechanism which explains the repression of MyoD-dependent (63) and p53-dependent (85) transcription. In view of these findings, we wished to clarify whether repression of the cyclin A, DNA polymerase α, and cyclin D3 promoters was also a consequence of CBP binding by Tax. To address this question, we interrogated the activities of two Tax mutant proteins, TaxL90A and TaxV89A, in ts13 cells. TaxL90A and TaxV89A have been characterized for binding to CBP (28); the former binds CBP with wild-type affinity, while the latter (although intact for CREB binding) binds CBP negligibly. When these two mutant proteins were tested, both were found to repress indistinguishably the cyclin A (Fig. 5A) and the cyclin D3 (Fig. 5B) promoters. Similar repression was also observed for both TaxL90A and TaxV89A on the DNA polymerase α promoter (data not shown). These findings clarify that Tax repression of the cyclin A and cyclin D3 promoters does not require CBP binding.

**Tax affects protein complex formation at the CREB/ATF binding site.** The HTLV-1 LTR contains three CREB/ATF binding sites (37). Highly efficient activation of this viral LTR by Tax is, in part, explained by Tax-CREB complex formation at cognate sites in the LTR (23, 46, 59). This ability of Tax to

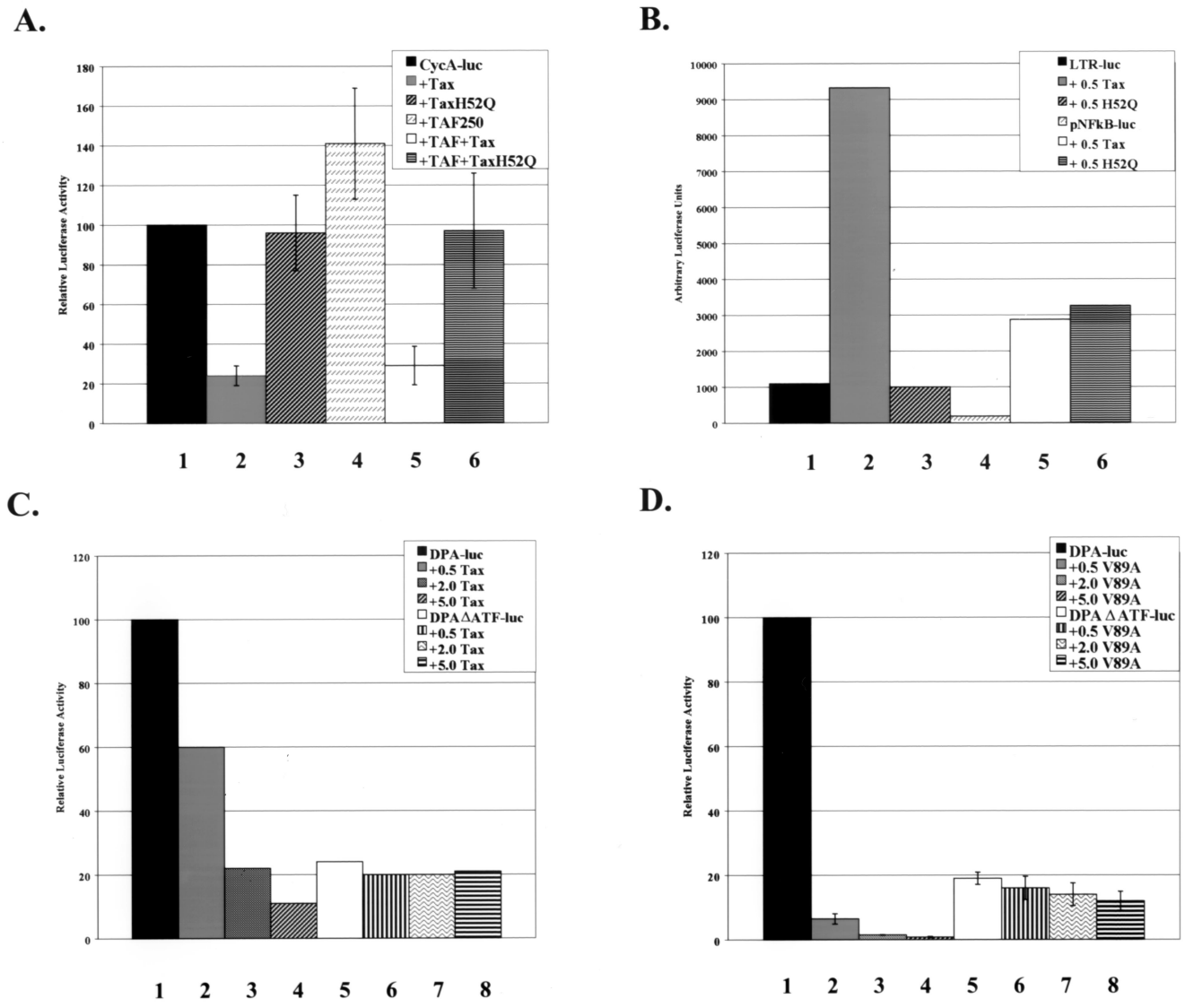



FIG. 4. Repression of the cyclin A and DNA polymerase α promoters by Tax involves interaction with CREB/ATF. (A) ts13 cells were transfected with CycA-luc alone (1 μg) (lane 1); CycA-luc plus Tax (2 μg) (lane 2); CycA-luc plus a Tax mutant protein which cannot bind CREB, TaxH52Q (2 μg) (lane 2) (22); CycA-luc plus $TAF_{II}250$ (2 μg) (lane 4); CycA-luc plus $TAF_{II}250$ and Tax (2 μg) (lane 5); or CycA-luc plus $TAF_{II}250$ and TaxH52Q (2 μg) (lane 6). (B) ts13 cells were transfected with a luciferase reporter containing either an HTLV-1 LTR promoter (LTR-luc) (lanes 1 to 3) or an NF-κB-responsive promoter (pNFκB-luc) (lanes 4 to 6). Transfections were with the reporter alone (0.5 μg) (lanes 1 and 4), the reporter plus Tax (0.5 μg) (lanes 2 and 5), or the reporter plus TaxH52Q (0.5 μg) (lanes 3 and 6). (C) ts13 cells were transfected with either DPA-luc (wild-type promoter; lanes 1 to 4) or DPAΔATF-luc (promoter with the ATF site deleted; lanes 5 to 8). Transfections were with DPA-luc alone (1 μg) (lane 1), DPA-luc plus increasing amounts of Tax (lanes 2 to 4), DPAΔATF-luc alone (1 μg) (lane 5), or DPAΔATF-luc plus increasing amounts of Tax (lanes 6 to 8). (D) ts13 cells were transfected with DPA-luc (lanes 1 to 4) or DPAΔATF-luc (lanes 5 to 8). Transfections were with DPA-luc alone (1 μg) (lane 1), DPA-luc plus increasing amounts of TaxV89A (lanes 2 to 4), a Tax point mutant protein with wild-type CREB-binding activity (28), DPAΔATF-luc alone (5 μg) (lane 5), or DPAΔATF-luc plus increasing amounts of TaxV89A (lanes 6 to 8). Results are averages from three independent experiments (A and D).

activate transcription via CREB/ATF sites is context specific since at other CREB binding sites (i.e., those found in cellular promoters), Tax-CREB complex formation may occur (46, 59, 77), but no activation is seen. The above CycA-luc, DPA-luc, and CycD3-luc results are compatible with an alternative functional interpretation: Tax-CREB interaction at some TATA-less promoters manifests as repression.

To check that functional repression by Tax correlates with "altered" protein complex formation at CREB/ATF sites, we performed EMSAs using nuclear extracts from several T-cell lines (Jurkat, C8166-45, MT4, uninduced JPX9, and metal-induced JPX9 cells). Jurkat is a well-established T-cell line whose transformation is unrelated to HTLV-1. C8166-45 (64) and MT4 (53) are HTLV-1-transformed T cells which express Tax constitutively. JPX9 cells are derived from Jurkat cells and contain an integrated Tax gene under the control of a metal-inducible metallothionein promoter (58) (Fig. 6D). Using these extracts, we examined complex formation with either a

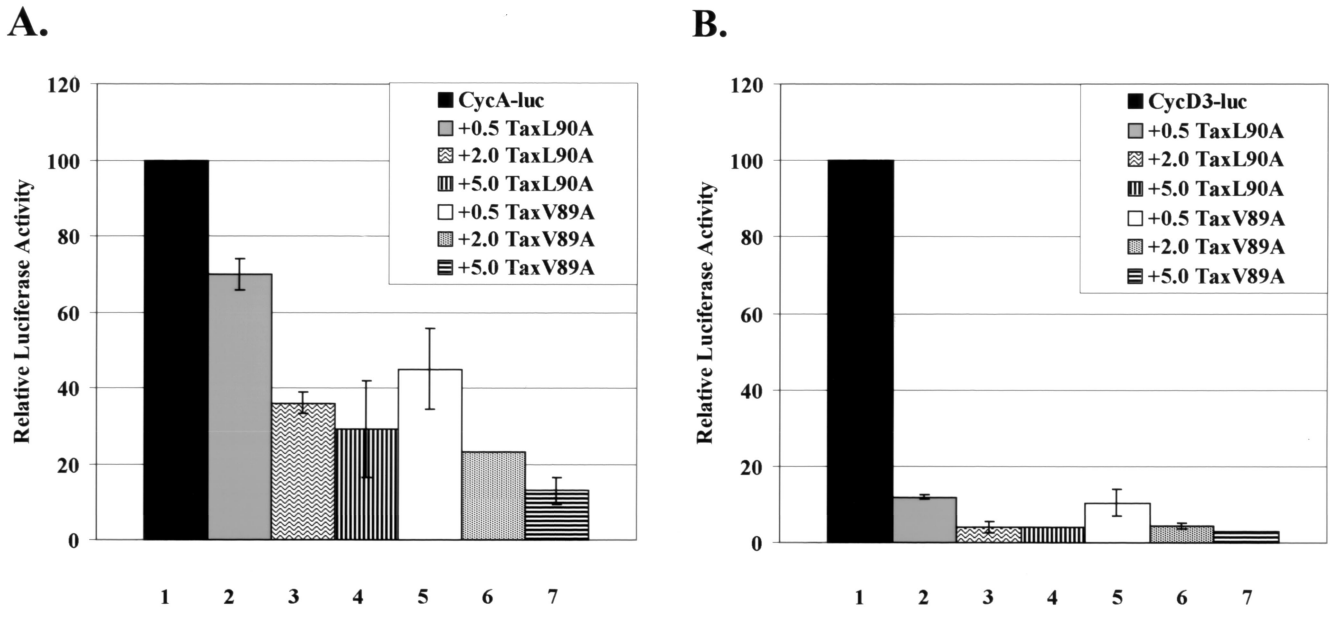


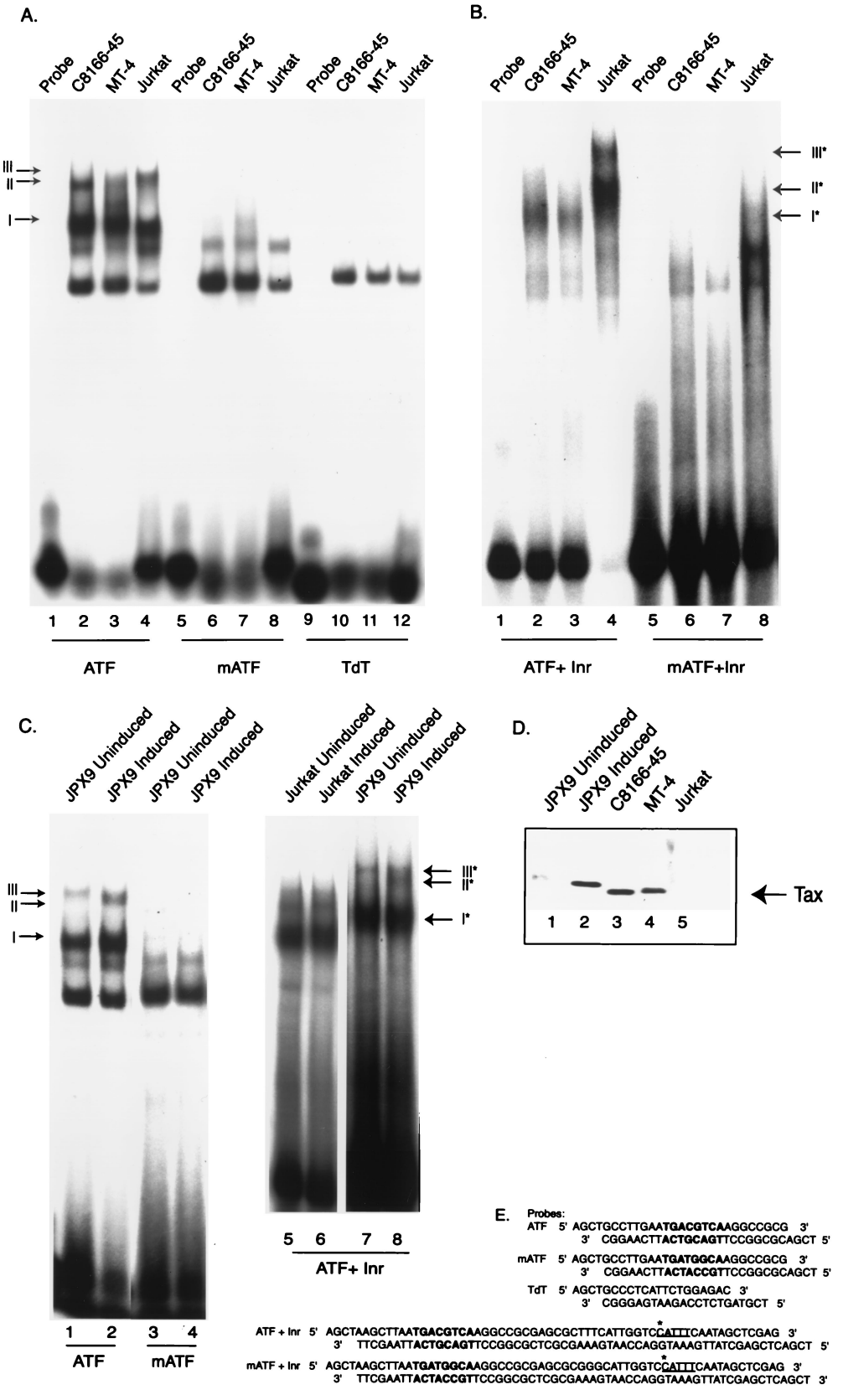

FIG. 5. Tax represses expression of cyclin A and cyclin D3 promoters through a CBP-independent mechanism. (A) ts13 cells were transfected with CycA-luc alone (1 μg) (lane 1) or CycA-luc plus increasing amounts (as indicated) of either TaxL90A (lanes 2 to 4) or TaxV89A (lanes 5 to 7). (B) The same transfections were repeated using CycD3-luc. Note that TaxL90A is functionally and physically intact for interaction with CBP but that TaxV89A is deficient in both respects. Results are averages from two independent experiments.

probe which contains the sequence of the ATF element from the cyclin A promoter (Fig. 6A, lanes 1 to 4, and C, lanes 1 to 2) or a second probe which contains a mutated ATF sequence (mATF) (Fig. 6A, lanes 5 to 8, and C, lanes 3 to 4). Comparing ATF to mATF (Fig. 6A and B), we could resolve three sequence-specific moieties (I, II, and III,) together with several nonspecific bands. Among the three sequence-specific complexes, the profiles of bands II and III changed when Tax-expressing C8166-45 cells or MT4 cells were compared to Jurkat cells. When JPX9 cells were induced with zinc to express Tax (Fig. 6C, lane 2), corresponding changes in the moiety II and III complexes were also noted (Fig. 6C, lanes 1 and 2). In both instances, Tax expression led to an enhanced band II and a reduced prominence in band III. A complex formed on a probe containing the TdT Inr sequence was used as a parallel control to indicate that equivalent concentrations of nuclear factors were used for the Jurkat, C8166-45, and MT4 extracts (Fig. 6A, lanes 9 to 12). Addition of anti-Tax antibody to the C81 nuclear extract prior to addition of the labeled probe resulted in a shift of band II (data not shown), consistent with the presence of Tax protein in this complex. These results are compatible with an interpretation that Tax affects the composition of a complex(es) formed at the cyclin A-derived CREB/ATF site.

We next used an EMSA probe which included both the ATF binding site and the Inr sequence from the cyclin A promoter (30). With the longer probes, resolution of protein-DNA complexes was less distinct. Nevertheless, the protein-DNA complexes formed on the ATF-Inr probe using nuclear extracts from two Tax-expressing cell lines (C8166-45 and MT4 cells) (Fig. 6B, lanes 2 and 3) were clearly different from those formed using a non-Tax-expressing extract (Jurkat) (Fig. 6B, lane 4). Changes in complex formation on this probe were also apparent when we compared uninduced JPX9 cells to induced JPX9 cells (Fig. 6C, lanes 7 to 8). This change was not a consequence of zinc induction, as no change was detected in nuclear extracts of Jurkat cells induced with $ZnCl_2$ (Fig. 6C, lanes 5 to 6). While we do not fully understand why complexes form differently in the various extracts, the results collectively support an interpretation that these are Tax-mediated changes.

**Reduced cyclin A expression in Tax-expressing and in HTLV-1-transformed cells.** From several perspectives, the above findings would be fully compatible with perturbed cyclin A expression in Tax-expressing and in HTLV-1-transformed cells. Levels of cyclin A protein normally oscillate during the cell cycle, with rapid accumulation at the beginning of S phase (reviewed in reference 42). To examine at the intracellular level Tax effects on cyclin A in early S phase of the cell cycle, we synchronized JPX9 and Jurkat cells using a double thymidine block protocol which enriches for nascent S cells (reviewed in reference 75). Cells released from the double thymidine block commence to progress from $G_1$ into S. Cyclin A expression in thusly processed cells was monitored for JPX9 (Fig. 7A, lanes 1 to 5), as well as JPX9 cells treated with zinc to express Tax (Fig. 7A, lanes 1 and 6 to 9). As controls, Jurkat cells, untreated (Fig. 7B, lanes 1 to 5) or treated with zinc (Fig. 7B, lanes 1 and 6 to 9), were also assessed to determine any effects which might occur solely from zinc treatment.

Cyclin A expression was assessed by immunoblotting using specific antiserum. On the same blots, we also checked for expression of Tax (Fig. 7A) and the cellular β-actin protein (Fig. 7A and B). Signals were quantitated by densitometry, and values were normalized to those for β-actin (Fig. 7C). Based on quantitations from the Western blots, we deduced that cyclin A levels increased, as expected, in Jurkat and JPX9 cells


A.
Probe
C8166-45
MT-4
Jurkat
III
II
I
1 2 3 4 5 6 7 8 9 10 11 12
ATF
mATF
TdT
B.
III*
II*
I*
1 2 3 4 5 6 7 8
ATF+ Inr
mATF+Inr
C.
JPX9 Uninduced
JPX9 Induced
Jurkat Uninduced
Jurkat Induced
1 2 3 4
ATF
mATF
5 6 7 8
ATF+ Inr
D.
JPX9 Uninduced
JPX9 Induced
C8166-45
MT-4
Jurkat
Tax
1 2 3 4 5
E.
Probes:
ATF 5' AGCTGCCTTGAATGACGTCAAGGCCGCG 3'
3' CGGAACTTACTGCAGTTCCGGCGCAGCT 5'
mATF 5' AGCTGCCTTGAATGATGGCAAGGCCGCG 3'
3' CGGAACTTACTACCGTTCCGGCGCAGCT 5'
TdT 5' AGCTGCCCTCATTCTGGAGAC 3'
3' CGGGAGTAAGACCTCTGATGCT 5'
ATF + Inr 5' AGCTAAGCTTAATGACGTCAAGGCCGCGAGCGCTTTCATTGGTCCATTTCAATAGCTCGAG 3'
3' TTCGAATTACTGCAGTTCCGGCGCTCGCGAAAGTAACCAGGTAAAGTTATCGAGCTCAGCT 5'
mATF + Inr 5' AGCTAAGCTTAATGATGGCAAGGCCGCGAGCGCGGGCATTGGTCCATTTCAATAGCTCGAG 3'
3' TTCGAATTACTACCGTTCCGGCGCTCGCGAAAGTAACCAGGTAAAGTTATCGAGCTCAGCT 3'

as the cells entered into S phase (Fig. 7C). Zinc treatment of Jurkat cells had an unexpected effect of enhancing cyclin A expression. However, zinc treatment of JPX9 cells (which clearly induced Tax expression [Fig. 7A, lanes 6 to 9]) had a markedly suppressed cyclin A expression (Fig. 7C). Thus, whereas zinc treatment nonspecifically enhanced cyclin A in Jurkat cells, the same treatment in JPX9 cells distinctly established Tax expression with cyclin A suppression.

The correlation between Tax expression and cyclin A repression in JPX9 cells prompted us to investigate authentically HTLV-1-transformed cell lines. We compared cyclin A expression in Jurkat, HeLa, and four HTLV-1 cell lines: C8166-45, MT4, ILT-Hod, and TL-Su. MT4 and C8166-45 were derived from coculture of human cord leukocytes and HTLV-1-infected cells (53, 64). TL-Hod was derived from an ATL patient (3), while TL-Su was derived from an HTLV-1 carrier (3). Immunoblotting with cyclin A-specific serum showed that amounts of cyclin A were greatly reduced in all four HTLV-1-positive cells (Fig. 8A, lanes 3 to 6) when compared to the amount in Jurkat (Fig. 8A, lane 2) or HeLa (Fig. 8A, lane 1) cells. In Fig. 8B, cyclin A expression values are graphed after normalization to β-actin values. These results, together with a previous report of a reduced level of cyclin A mRNA in HTLV-1-infected T-cell lines (2), are consistent with reduced cyclin A as a characteristic of HTLV-1 infection and transformation.

## DISCUSSION

Viruses are obligatory host cell parasites. Consequently, it is not surprising that the life cycles of viruses importantly depend on the cell cycle of the host. Parvoviruses, for example, rely on the host cell S phase to replicate viral-DNA genomes (14). Herpesviruses interact with several cyclins and cyclin-dependent kinases, implicating critical participation by these cell cycle proteins in virus expression and replication (66, 86). The human immunodeficiency virus utilizes the host $G_1$ phase to complete reverse transcription and to prepare for integration of its proviral genome (76, 94), and emerging evidence indicates that the HTLV-1-encoded Tax protein plays important roles in modulating cell cycle progression (reviewed in reference 55). Here, we unexpectedly found that the levels of an S- and M-phase cyclin, cyclin A, is repressed by HTLV-1 Tax.

Expression of Tax by HTLV-1 has been correlated with cellular transformation (62; reviewed in reference 91). Arguably, effects of Tax on cell cycle progression are important to the transforming biology of HTLV-1. Historically, Tax was first characterized as a potent activator of gene expression (reviewed in reference 91). Hence, the ability by Tax to activate mitogenic factors such as IL-2 (34, 73), IL-2 receptor α (5), Jun (19), and Fos (18) was predictable and is fully compatible with its expected cell growth-promoting phenotype. Recently, it has, however, become apparent that several prototypic transcriptional gene activators such as p53 (21, 35, 50), E2F (96), and E1a (6, 47) are also potent transcriptional repressors of other genes. Thus, it is suggested that the ambient outcomes of transactivator proteins reflect the collective balance of up- and down-regulatory effects on different subsets of genes. Indeed, for HTLV-1 Tax, the initial suggestion of its potential as a *trans*-repressor (39) has been rapidly extended by a flurry of studies describing its repressive activity on factors such as p53 (84), p16INK4a (49, 79), lck (48), p18INK4c (80), c-Myc (69), MAD1 (41), and c-Myb (57), among others.

In considering transcriptional repression by Tax, there are currently two proposed mechanisms. First, a series of examples indicate that Tax works repressively through its interaction with an E-box-binding basic-helix-loop-helix protein (39, 48, 63, 69, 78, 80, 84). Second, other studies support mechanistic repression by Tax through its sequestration of p300/CBP coactivator proteins (4, 78, 85). Here, our descriptions of the cyclin A, cyclin D3, and DNA polymerase α promoters suggest a third route through which Tax manifests transcriptional repression: context-specific binding to CREB/ATF.

Several findings helped us define the mechanism utilized by Tax to repress the promoter activities of cyclin A, cyclin D3, and DNA polymerase α. Initially, we observed that Tax proteins competent for CREB binding (e.g., wild-type Tax and TaxV89A) exhibited repression but that a Tax mutant protein (TaxH52Q) which cannot bind CREB failed to exert this repression (Fig. 4A). Next, that DPA-luc, but not DPAΔATF-luc, was repressed by Tax delineated a requirement for CREB/ATF in this repressive process (Fig. 4C and D). Last, similarly to another example of the down-regulation of the cyclin A promoter through its upstream CREB/ATF site (92), we found distinct changes in protein-DNA complex formation when using CREB/ATF-motif-containing probes to compare nuclear extracts with or without Tax (Fig. 6). These observations, coupled with the demonstration that cyclin A, cyclin D3, and DNA polymerase α repression is CBP independent (Fig. 5), provided a first illustration of Tax-mediated repression through context-specific sequestration of CREB/ATF. We note that in other systems, context-specific activation and repression is not without precedent. For instance, at many promoters the YY1 protein activates transcriptional initiation by stimulating recruitment of RNA polymerase II while at other promoters YY1 represses transcription by sequestering CREB/ATF (95). Similarly, depending on context, the cyclin A gene has also been

FIG. 6. Tax affects protein-DNA complexes formed at the cyclin A promoter. (A) EMSA using an ATF binding site probe. Nuclear extracts, as indicated, were incubated with labeled probes consisting of either the wild-type ATF binding site (lanes 1 to 4), mATF (lanes 5 to 8), or a TdT promoter sequence (as a parallel control to indicate factor concentration of extracts; lanes 9 to 12). Probe alone is shown in lanes 1, 5, and 9. (B) EMSA using an ATF plus Inr probe derived from the cyclin A promoter. Probe alone is shown in lanes 1 and 5. (C) EMSA using the ATF probe (lanes 1 to 2), the mATF probe (lanes 3 to 4), and the ATF plus Inr probe (lanes 5 to 8) in nuclear extracts of JPX9 cells (lanes 1 to 4 and 7 to 8), which were either uninduced or induced with $ZnCl_2$ or Jurkat cells (lanes 5 to 6) that had or had not been induced with $ZnCl_2$. (D) Western blot of cells using anti-Tax serum. C8166-45 and MT4 cells express Tax constitutively, JPX9 cells induced with 120 μM $ZnCl_2$ express Tax, and Jurkat cells and uninduced JPX9 cells do not express Tax. Note that we have consistently observed a difference in the migration size of the Tax protein from JPX9 cells. An explanation for this is currently unknown. (E) Sequences of probes are shown with the ATF site in bold (wild type or mATF) and the Inr underlined; the transcription start site is denoted by asterisk.

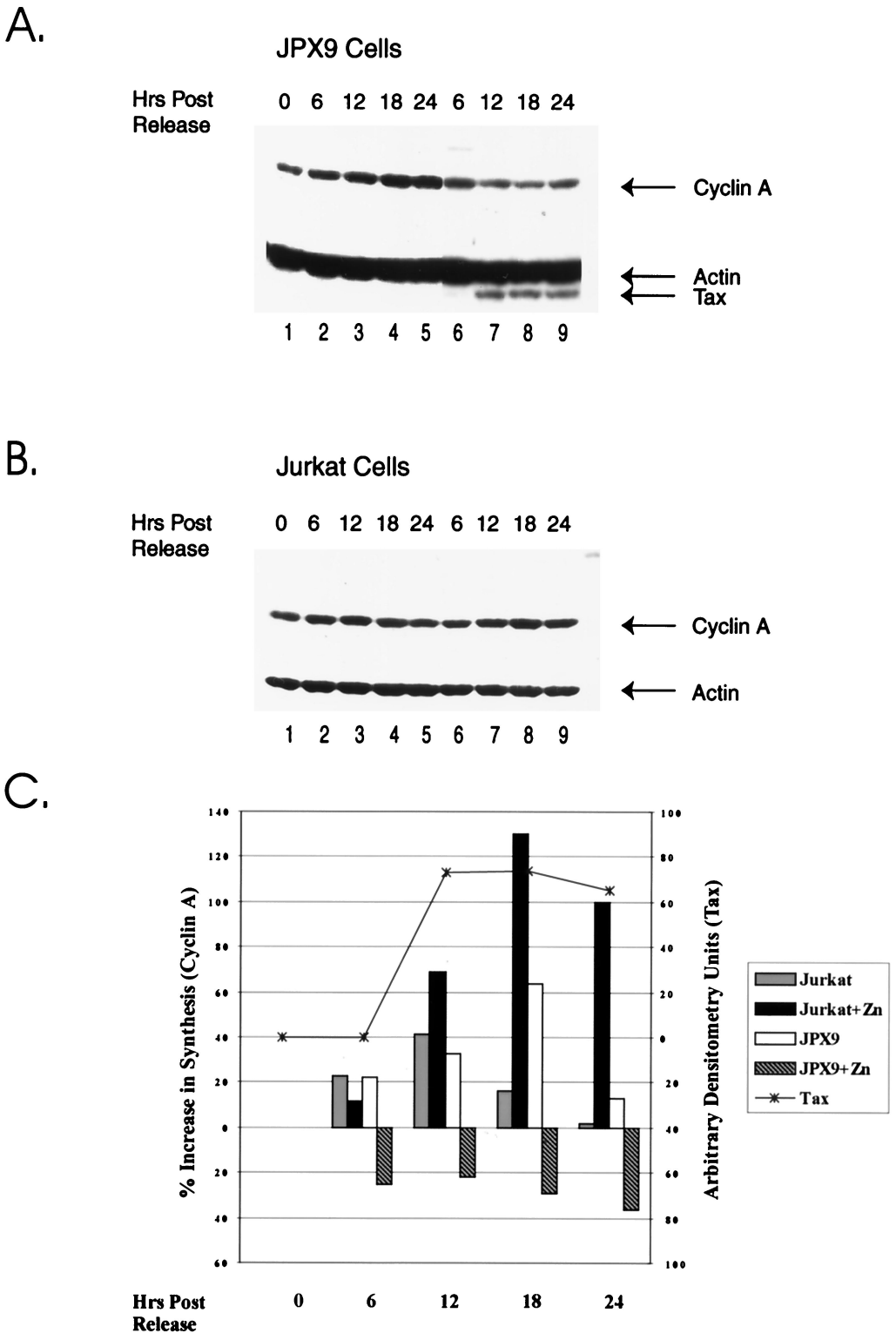



FIG. 7. Expression of Tax is correlated with reduced expression of cyclin A in synchronized T cells. (A) JPX9 cells were synchronized with a double thymidine block. One set of uninduced cells was harvested at 0, 6, 12, 18, or 24 h after release from the block (lanes 1 to 5). A second set was induced with 120 μM $ZnCl_2$ at time zero after release and harvested at the same time points (lanes 6 to 9). (B) Jurkat cells were treated as described for panel A for JPX9. Samples in panels A and B were probed with specific antisera to cyclin A, β-actin, and Tax. (C) Graphic quantitation of relative levels of cyclin A and Tax synthesis in cells. β-Actin bands were measured by densitometery (a lighter exposure was used for the JPX9 cells), and the values were used to normalize for densitometric quantitations of cyclin A and Tax. Results for cyclin A (bars; left *y* axis) are shown quantitatively as percentages of increase over the amount at time zero. Tax (line; right *y* axis) expression in induced JPX9 cells is shown in arbitrary densitometric units.

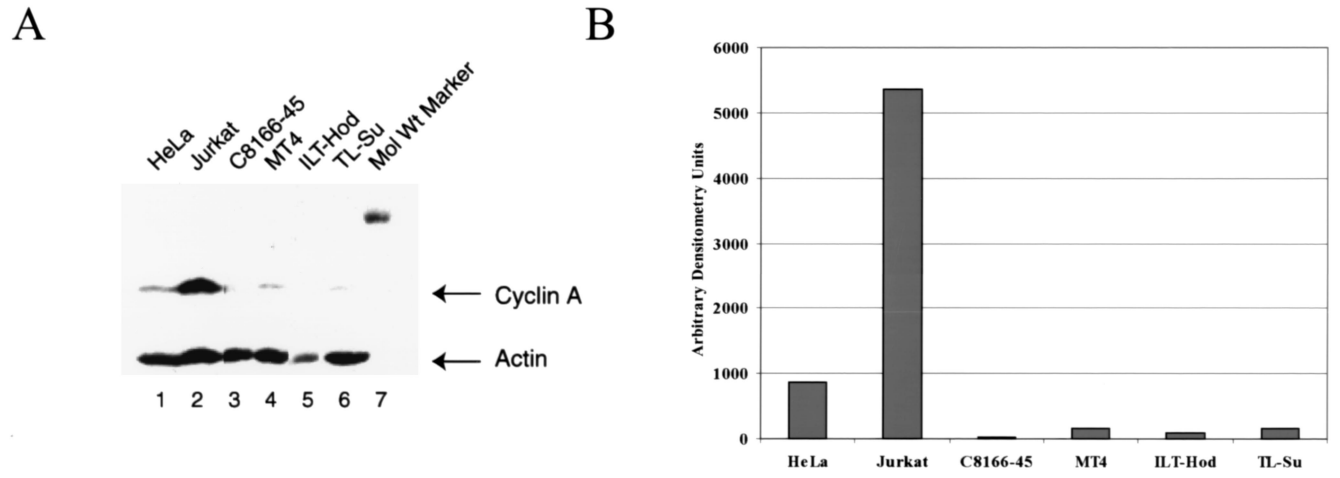



FIG. 8. HTLV-1-transformed cells express reduced amounts of cyclin A compared to levels expressed in HeLa and Jurkat cells. ILT-Hod and TL-Su are cell lines established from ATL patients; C8166-45 and MT4 cells are derived from in vitro cocultivation of cord blood with HTLV-1 producer cells. (A) Representative Western blot of the indicated cells; (B) quantitation of amounts of cyclin A after normalization to β-actin amounts. Mol Wt, molecular weight.

shown to be either up- or down-regulated through its upstream CREB/ATF site (15, 16, 92, 93).

How might HTLV-1 benefit from repressed expression of cyclin A? In relevant T-cell lines, we clearly observed that amounts of cyclin A are significantly reduced both by HTLV-1 transformation (Fig. 8) and by the singular expression of Tax (Fig. 7). These findings agree with a previous report of reduced cyclin A mRNA in HTLV-1-infected T-cells (2). Interestingly, in other virological settings, cyclin A is similarly repressed by cytomegalovirus (36, 65) and herpes simplex virus (1) infection of cells. While we do not fully understand why viruses should repress cyclin A, a few thoughts come to mind. First, we note that cyclin A negatively regulates E2F-1 activity (45) during the S phase of the cell cycle. One speculation is that reduced amounts of cyclin A result in a prolonged S phase, which thereby benefits the replication of viral genomes. Second, a role in preventing aberrant reassembly of DNA initiation complexes in the S phase of the cell cycle was recently further attributed to cyclin A-cdk2 (12). In this perspective, normal cyclin A-cdk2 activity ensures that only one round of DNA replication occurs within a single S phase. Considered thusly, Tax repression of cyclin A may engender aberrant DNA reduplication, providing another explanation for how this oncoprotein induces aneuploidogenic abnormalities in cells (reviewed in reference 43). Finally, in its role as a mitotic cyclin, cyclin A also regulates egress of cells from mitosis (74, 87). Suppression of cyclin A may result in accelerated progression through mitosis, further accounting for the failure in HTLV-1-transformed cells to faithfully execute the mitotic spindle assembly checkpoint (41).

The unexpected observation that Tax represses cyclin A provides a further illustration of the intimate relationship between viruses and host factors. It additionally highlights the delicate balance between positive and negative events in maintaining cellular homeostasis. Our Tax-cyclin A results add to the growing literature stating that this cyclin is commonly targeted by viruses. Thus, HTLV-1 joins adenovirus (61), HBV (90), and herpesviruses (1) in subverting the function(s) of cyclin A. Future studies on virus-cyclin interplays are likely to advance our understanding of the symbiosis between viruses and cells.

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