Transcriptional Coactivator CBP Facilitates Transcription Initiation and Reinitiation of HTLV-I and Cyclin D2 Promoter

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ABSTRACT

HTLV-I is the etiologic agent for adult T-cell leukemia/lymphoma (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Taxi, the major activator of this virus, is a 40- kDa (353 amino acid) phosphoprotein, predominantly localized in the nucleus of the host cell, which functions to trans-activate both viral and cellular promoters. Recently it has been shown that HTLV-I and /or Taxl expressing cells have altered gene expression of some of the cell cycle associated genes. Tax activates HTLV-I as well as number of other cellular promoters through CREB, NF-kB and SRE elements. In this study we analyzed the effect of a general coactivator, namely CBP, which may *be* involved in activation of many cellular genes. To analyze CBP transcription activation, we have utilized an *in vitro* transcription assay that allows the analysis of transcription initiation and reinitiation in the absence of chromatin effects. In this assay, which utilizes a G-free cassette downstream of the Tax-responsive 21 by repeats, polymerase II molecules responsible for the first round of transcription remain at the end of the G-free region, effectively blocking the complete elongation of reinitiated transcripts. Addition of Tax and a 682 amino acid fragment of CBP to the *in vitro* transcription reactions increased both full-length and shorter transcripts resulting from reinitiation. A CBP deletion mutant lacking the N-terminal activation domain was inactive. Preliminary data is also presented to show that, transactivation of HTLV-I and a cellular promoter, namely cyclin D2, takes place in early GO/G1 before the restriction point, "R", where Rb function has been implicated. *Iran. Biomed. 1 2: 4957, 1998*

INTRODUCTION

HTLV-I is the etiologic agent for adult T-cell leukemia/lymphoma (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/ TSP) [1, 2]. Due to the limited coding capacity of the viral genome, viral replication and transformation are largely dependent upon modification of cellular regulatory protein function. In HTLV-I, the pX region contains four ORFs, X-I, X-II, X-III and X-IV. Tax is encoded primarily by the X-IV reading frame, while Rex_i is encoded by the X-III reading frame. The AUG initiation codons for both Tax and Rex are located in the second exon of the doubly spliced mRNA. Tax_i transcriptionally activates viral mRNA synthesis, leading to an initial increase in the levels of Tax_1 and Rex [3].

HTLV-I activates and immortalizes human T-lymphocytes *in vitro,* resulting in polyclonal prolif eration of the infected cells, followed by oligo-clonal or monoclonal growth. The mechanism of HTLV-I transformation appears to be distinct from that of chronic or acute leukemia viruses. Interestingly, when the pX coding sequences were cloned into a transformation-defective herpes virus or transgenic mice containing only the Tax gene [4], it was able to transform T-lymphocytes of the same phenotype as found with HTLV-I. Of the three proteins encoded by the pX region, Tax is critical for transformation, since specific mutations of the *tax* gene AUG initiation codon eliminated the transformation potential of the herpes virus-HTLVI pX recombinant virus [5].

Tax is a 40-kDa (353 amino acid) phosphopro-

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tein, predominantly localized in the nucleus of the host cell, which functions to transactivate both viral and cellular promoters. Tax_i has not been shown to bind directly to Tax_i-responsive sequences (TREs), suggesting that Tax_i transactivation occurs through indirect effects of Tax_i on transcription factors which bind to the TREs [6]. Likely mechanisms for Tax_i trans-activation include: (1) transcriptional induction of TRE-binding transcription factors; (2) post-translational modification of TRE-binding factors; or (3) complex formation with transcription factors allowing indirect binding of Tax_i to the TRE(s).

Tax 1 has been shown to trans-activate several cellular genes (mostly by means of transfection), including IL-2 [7], IL-2R [8], GM-CSF [9], proliferating cell nuclear antigen (PCNA) [10], parathyroid hormone related protein (PTHrP) [11], *c-fos, csis* [12] and others and to negatively regulate Bpolymerase [13], a host DNA-repair enzyme. It is likely that Tax induction of cellular genes is critical for virus replication and cell transformation.

Recently it has been shown that HTLV-I and/or Tax1 expressing cells have altered gene expression of some of the cell cycle associated genes. Among these changes high levels of p53, cdk inhibitor p21, Cyclin D2 and lower levels of cyclin D3, cdk inhibitor p16 have been observed [14]. *In vitro* binding assays has also indicated that Tax binds $_{pi}$ 6INK4a (cdk/cyclin D inhibitor), but not $p21^{c'P^l}$ or $p27^{k'w^l}$ and it forms complexes with $p16^{INK4a}$ *in vivo* [15, 16]. However to date, no careful analysis of Tax 1 or HTLV-I infected cells have been performed to address the functional consequence of these seemingly dramatic changes at the cell cycle level. Of particular interest and focus of this study is the notion of very early events post mitosis that Tax 1 and/or HTLV-I pose on the host cell cycle machinery. One such early event post mitosis is the activation of cyclin D family member, D2.

Cyclins are the regulatory subunits of cdc2-related protein kinase complexes in the eukaryotic cell cycle. Eight different types of cyclins have been identified in mammalian cells, and designated as cyclins A through H. Cyclins C, D (DI, D2, and D3), E, and G are believed to be G1 cyclins [17, 18]. Cyclin A is a S phase cyclin, and cyclin B (B1 and B2) is a mitotic cyclin. The initial studies of G1 cyclins were performed in budding yeast, which has three CLN-type cyclins (CLN1, CLN2, and CLN3) required for passage through START, the G1 restriction point and transition at G1/S. Three novel types of putative mammalian G1 cyclins were isolated by using human cDNA libraries to complement CLN-deficient yeast and designated as cyclins C, D, and E [19]. PRAD 1 was cloned uy a gene rearranged in an parathyroid tumor and is identical to human cyclin DI [20]. A murine homologue of cyclin D1 was independently isolated from a eDNA library prepared from murine macrophages synchronously progressing through G1 in response to colony-stimulating factor 1. The murine cyclin D1 eDNA probe was used to identify two related genes, murine cyclin D2 and D3. Unlike other types of cyclin, cyclins DE D2, and D3 have unique cell- and tissue-specific patterns of expression, suggesting that each D-type cyclin may have a distinct mechanism for transcriptional regulation. Over expression of any of D-type cyclins can accelerate the timing of START and shorten the G1 intervals [21]. With respect to general structure of cyclin D2 promoter, no TATA box was evident in the vicinity of the transcription start sites, however, putative DNA binding sites for Spl, CREB, C/EBP, PEA3, NFkB, SIF, E2F, GCF, and AP1 were identified in the cyclin D2 upstream sequence. The CAP site in the promoter was shown to be a loosely conserved sequence where number of transcription sites have been observed [22, 23].

Here we show that Tax1 associated activity with viral and, cellular cyclin D2 promoter, is at early stages of post mitosis. Preliminary data is presented to show that, *i)* Tax_i efficiently recruits CREB Binding Protein (CBP) to both promoters and that one of the functional consequence of this event is reinitiation of RNA Pol II, *ii)* The cyclin D2 over expression, as observed in HTLV-I infected cells, is related to AP2 DNA binding elements on the 5' end of the promoter, and *iii)* Trans-activation of HTLV-I and cyclin D2 promoter takes place at early GO/G1 before the restriction point, R, where Retinoblastoma (Rb) function has been implicated.

MATERIALS AND METHODS

In vitro transcription assays. The G-free DNA templates used in the *in vitro* transcription assays

were pLovTATA and pTRE-lid. *E. coli* Tax protein was purified by ammonium sulfate precipitation as described. Prior to use in the *in vitro* transcription assay, Tax (100 ng/IA1) was incubated for 30 min at 30°C with equal volume of Tax buffer (2 mM Chaps, 2 mM DTT, 10 mM-mercaptoethanol, 1 mM EDTA). For the G-free *in vitro* transcription reactions, preincubation was at 30°C for 30 mM, followed by the addition of 2 ul -^{32}P-UTP (Amersham, 400 Ci/mMol), 0.03% sarkosyl and incubation at 30°C for 60 min. Reactions con tamed Hela whole cell extract (25 pl), 1.5 µg supercoiled DNA, 75 ng Tax protein, .75 to 2.25 pg CBP(1-682) in a total volume of 50 to 65 pl. Transcription buffer (35.5 pl/reaction) contained 3 pi 20% PEG (6000), 3 41 50 mM MgC12, 3 pl 1 mM DTT, 1 pl 0.2 M Creatinphosphat (Boehringer Mannheim), 1.5 41 50 mM ATP/CTP, 5 pl 10 pM UTP, 1 pl 20 mM 3'O-Methylguanosine 5'Triphosphate (Pharmacia), 20 units RNase T1 (100 units/pl, Boehringer Mannheim) and 18 pl of Buffer D containing a final concentration 20 mM HEPES (pH 7.9), 100 mM KCI, 12.5 mM MgC12, 0.1 mM EDTA, 17% glycerol, and 1 mM DTT. For pulse-chase assays Tax and CBP were incubated in the presence of ^{32}P-UTP for 30 minutes. A 10-fold molar excess of cold UTP was added to the reaction and the polymerase complexes were allowed to elongate for 15-60 minutes. 3'-0-Methyl GTP was omitted in these assays to allow pol II elongation. Sarkosyl (0.03 %) was added to inhibit reinitiation complexes in duplicates of the 15 and 60 minute chase samples.

Northern blot. Total cellular RNA was extracted using the Trizol reagent (Gibco/BRL). Total RNA (5 pg) was spotted onto 0.2 micron nitrocellulose (Millipore Inc.), UV cross-linked and hybridized overnight at 42°C with various 40 mer end-labelled, ^{32}P-labelled, Cyclin D2, D3, E, HTLV-I LTR (R region) and Actin probes [19, 22]. Next day, they were washed 2 times (10 mls), 15 minutes each, with 0.2% SDS, 2x SSC at 37°C, exposed and counted on a PhosphorImager cassette (Molecular Dynamics). *Cell cycle analysis.* Various infected or control cells were either blocked with hydroxyurea for 18 hours, or blocked with hydroxyurea, washed and released for 1 hour followed by addition of nocodazole for 14 hours (50 ng/ml). Following the block, cells were washed with PBS (2x) and

released using complete media. Whole cell extracts were made from 5 x 10^7 cells/time point. Samples were collected every three hours and the cells were used to make whole cell extracts for *in vitro* transcription, isolated total cellular RNA for Northern analysis, or processed for cell sorting. Single color flow cytometric analysis of DNA content was performed on all cell tested. Cells were washed with PBS and approximately 2 x 10^6 cells were fixed by addition of 500 pl of 70% ethanol. Cell pellets were washed with PBS (3x, 10 ml each time), incu bated in 1 ml PBS with 150 pg/m1RNaseA (Sigma) and 20 pg/ml propidium iodide (Sigma) at 37°C for 30 minutes. The stained cells were analyzed for red (FL2) fluorescence on a FACScan (Becton Dickenson) and the distribution of cells in the G1, S and G2/M phases of the cell cycle was calculated from resulting DNA histogram using Cell FIT software, based on a rectangular S-phase model (Fast Systems, Inc., Gaithersburg, MD).

Transfections and CAT Assays. The wild type and various mutant cyclin D2 constructs were electroporated along with Tax$_i$, into Jurkat cells using 230 volts, and 800 uF (Cell porator, Life Technologies). Extracts were prepared 18 hours later for CAT assay. Cells were harvested, washed once with PBS without Ca^{++} and Mg^{++}, pelleted, and resuspended in 150 41 of 0.25 M Tris (pH 7.8). Cells were freeze/thawed 3X, with vortexing after each thawing. Tubes were then incubated for 5 mM at 68°C followed by centrifugation. The supernatants were transferred to 1.5 ml Eppendorf tubes. After one final spin, the supernatant was again transferred to 1.5 ml Eppendorf tubes and the protein concentration was determined. CAT assays were performed with 10 pg protein according to the method of Gorman and Howard.

RESULTS

To analyze transcriptional activation by Tax and CBP, purified proteins were added to *in vitro* transcription reactions with a G-free template containing the Tax-responsive 21 by repeats upstream of the TATA box. Our initial studies utilized a fragment of CBP containing amino acids 1-682, CBP (1-682), which can activate transcription when fused to a Gal-4 DNA binding
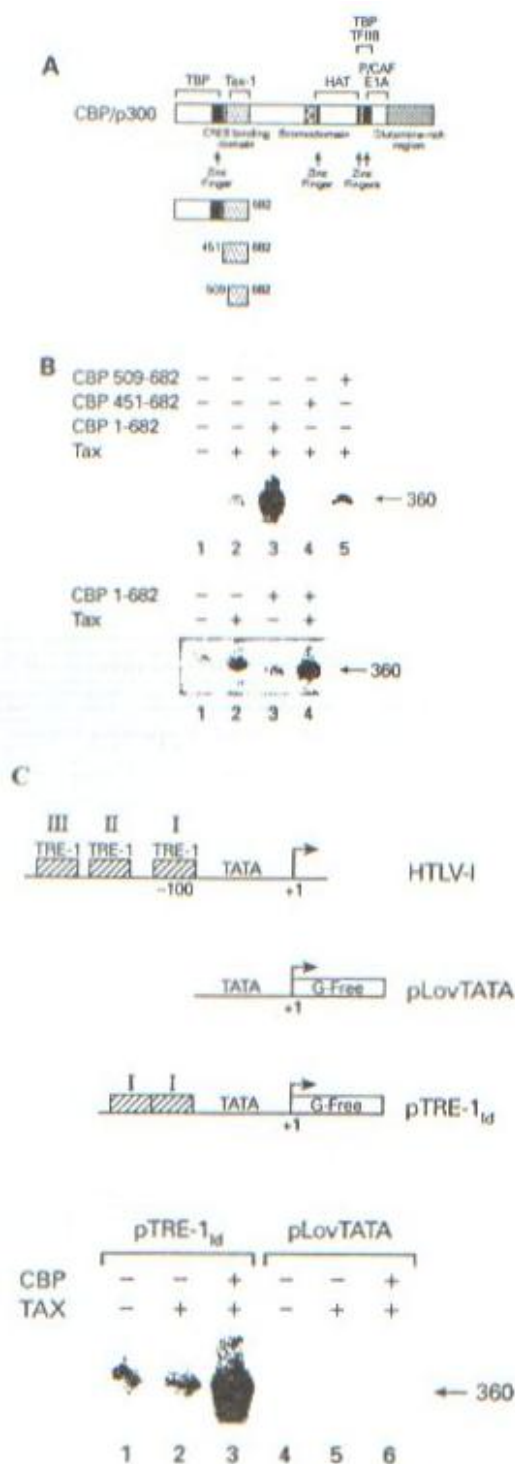
Fig. 1. *In vitro* transactivation of CRE response element by Tax and CBP. (A) Schematic of CBP protein. Binding sites for TATA binding protein (TBP), CREB, Tax, well as acetyltransferase- (HAT), zinc finger-, and glutamine rich-domains are indicated. (B) and (C) *In vitro* transcription assay. Tax and CBP were added at 0 time, followed by the addition of 0.03% sarkosyl and ^{32}P-UTP at 30 minutes. Samples were then incuiy,ted for 1 hour at 30°C and further processed for RNA product analysis [38].

domain and has protein interaction domains for TBP, CREB and Tax (Figure 1A). This region of CBP is sufficient for transcription activation and formation of the Tax-CREB-CBP complex. We subsequently tested two deletion mutants of this CBP domain, CBP (451-682) and CBP (509-682). In the absence of exogenous CBP and limiting Tax protein, Tax only modestly activated the HTLV-I CRE promoter (Figure 1B, lanes 1 and 2). The addition of Tax and CBP (1-682) stimulated the appearance of the 360 base (360 b) transcript ten-fold (Figure 1B, lane 3). The difficulty in obtaining an efficient *in vitro* Tax transactivation system in the past may be due to the fact that CBP is a limiting component within the extracts. CBP (451-682) or (509-682) failed to activate transcription (Figure 1B, lanes 4 and 5), suggesting that the CBP amino terminus is essential for Tax activity. Swope et al. have recently reported that CBP(1-400) interacts with the basal transcription factor TBP [24[. Thus, our results suggest that the interaction of CBP with TBP (TFIID) may be important for Tax-dependent CBP activation. As controls for the *in vitro* transcription assays, addition of either CBP- or Tax-, but not control-, antibodies inhibited the *in vitro* transcription (data not shown). In addition, a template which lacked the 21 by repeats was not transactivated by the addition of Tax and CBP (Figure 1C).

To determine whether Tax and CBP affect both the initiation and reinitiation of transcription, we used 3'-0-Methyl GTP in the *in vitro* transcription assay. As described by Szentirmay and Sawadogo [25], in the presence of 3'-0-Methyl GTP, the first round of poi II elongation complexes remain at the end of a G-free region, blocking the elongation of pol II complexes that result from reinitiation (Figure 2C). As a result, successive polymerases stack up from the end of the cassette, producing shorter transcripts representing rounds of reinitiation. Figure 2A shows that addition of both Tax and CBP stimulate the appearance of the full length 360 b transcript as well as discrete shorter transcripts of approximately 280, 240, 210, 180, 165, and 144 bases. Hybridization analysis of the full length and shorter RNAs confirmed that they originated from the G-free cassette (data not shown). These data suggest that Tax and CBP stimulate both transcription initiation and reinitation.

Previous results from Szentirmay and Sawadogo have demonstrated that reinitiated pol II complexes are more sensitive to low concentrations of sarkosyl
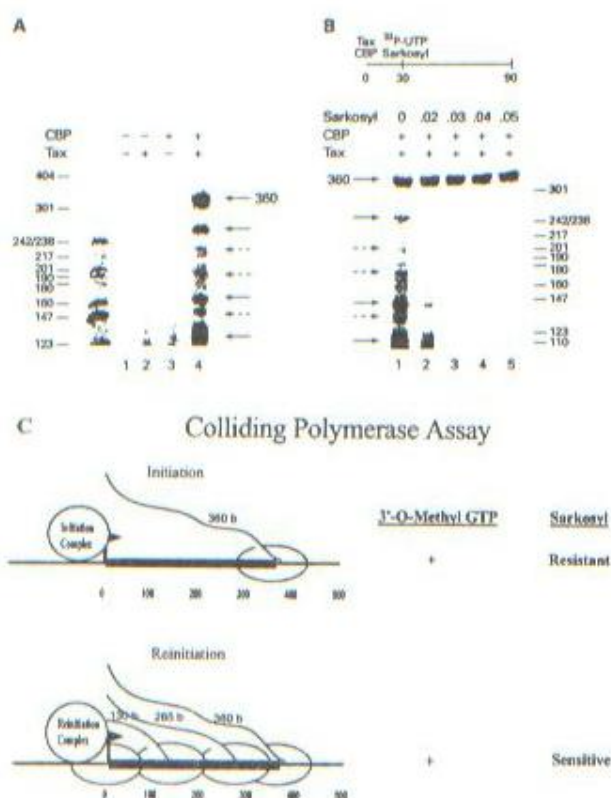
Fig. 2. CBP facilitates transcription initiation and reinitiation *in vitro.* (A) *In vitro* transcription products in the presence of Tax and CBP using the CoPRA assays (25). (B) Reinitiation complexes are sensitive to addition of sarkosyl. Tax and CBP were added at 0 time, followed by the addition of sarkosyl and ^{32}P-UTP at 30 minutes. Samples were then incubated for 1 hour at 30°C. (C) Schematic for colliding polymerase reinitiation assay.

[25]. To confirm that the shorter RNAs represented reinitiation transcription complexes, template DNA, extract, CBP and Tax were preincubated for 30 minutes, followed by the addition of sarkosyl and ^{32}P-UTP. Consistent with Szentirmay and Sawadogo's results, our studies demonstrate that the reinitiated transcription complexes were prefer-entially sensitive to the addition of sarkosyl after the preincubation period (Figure 2B). Following the assembly of the transcription complexes during a preincubation period, addition of sarkosyl to 0.03% inhibited the reinitiated pol II complexes, but failed to inhibit primary initiation complexes.

Promoter effect of number of genes post-mitosis including HTLV-I, cyclin D2, D3 and E, were next examined using slot blot RNA hybridization analysis. HTI_V-I-infected cells (MT-2), and uninfected CD4 lymphocytes (CEM), were blocked at M phase with nocodazole, washed and released with complete media in the presence of sodium butyrate. We have previously shown that sodium butyrate can rapidly induce viral gene expression in ACH2, U I [26], and in MT-2 and MT-4 cells (data not shown). FACS analysis of blocked and released cells are shown in Figure 3, Panel B. Most of the MT-2 and CEM cells had traversed into early G 1 following nocodazole release. Cells at time zero and 2 hours post-release were also processed for RNA analysis. Results of such an experiment are shown in Figure 4, Panel C, where both HTLV-I and cyclin D2 promoter showed an increase in gene expression in MT-2 cells 2 hours post-mitosis. Cyclin D3 and E, however, were not activated under these conditions.
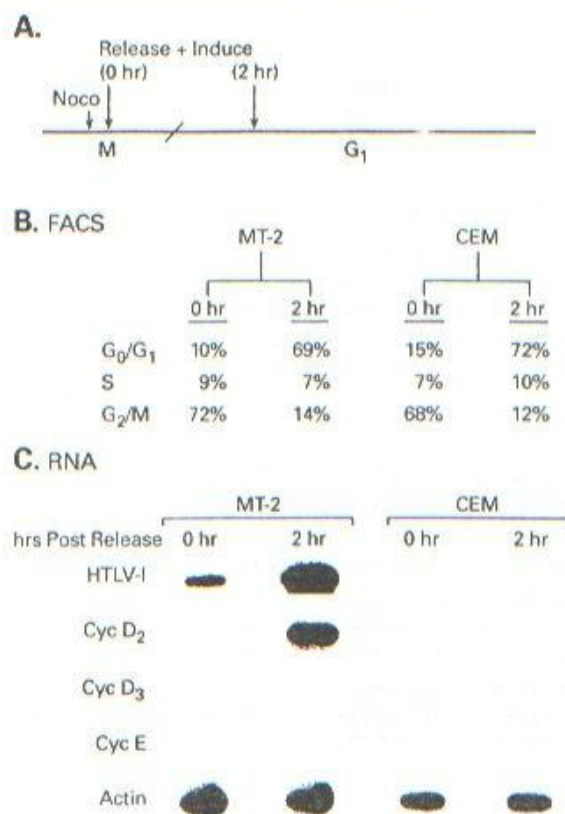

Fig. 3. Endogenous promoter activities of HTLV-I and some cyclin genes. Both MT-2 and CEM cells were blocked with Nocodazole (see Fig. 5 legend), washed next day and released in presence of 5 inM sodium Butyrate. Samples were collected at zero, or 2 hrs post release for RNA analysis. Panel A, represents the diagram for this experiment. Panel B, indicates the FACS analysis of both cell types using propidium Iodide DNA staining (FAST systems, Gaithersburg, MD), and panel C, represents hybridization of 10 1.1g of total RNA using HTLV-I (Nick translated sequence of HTLV-1 LTR R region, +1 to +260, and cyclins D2, D3, E and actin probes [15].
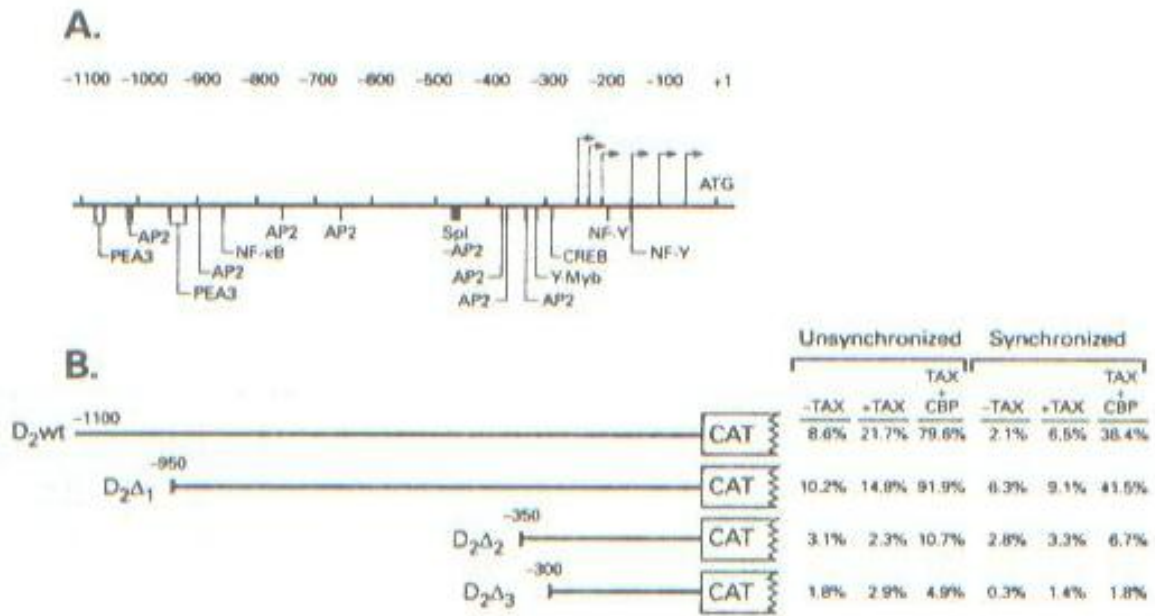
A.

B.

Fig. 4. Effect of Tax and CBP on cyclin D2 promoter. CEM (12D7) cells were transfected with 51.tg of the reporter genes, and either one .tg of *E. coli* His-tag recombinant Tax protein, or 1 tg of CMV-CBP using the electroporation method. Cells were incubated overnight for CAT assay. Panel A represents a diagram of the human cyclin D2 promoter with its putative DNA binding sites for various transcription factors (22,23). Panel B represents PCR amplified fragments of cyclin D2 promoter elements inserted upstream of a promoter less (pCAT-basic) plasmid at Hind III site. Right panel (synchronized) represents cells blocked overnight with Nocodazole (M phase blocker, 50 ng/ml), washed next day with PBS (without Ca^{++} or Mg^{++}), transfected with reporter DNA and Tax protein, released with complete medium for 2 hrs, and subsequently blocked with Hydroxy-urea (Gl/S blocker, 2 mM) for 24 hrs. Cells were collected and processed for CAT assay after 24 hrs. This procedure scores Transcription events related to GI phase of cell cycle, since majority of cells (70-80%) remain at end of G1 phase.
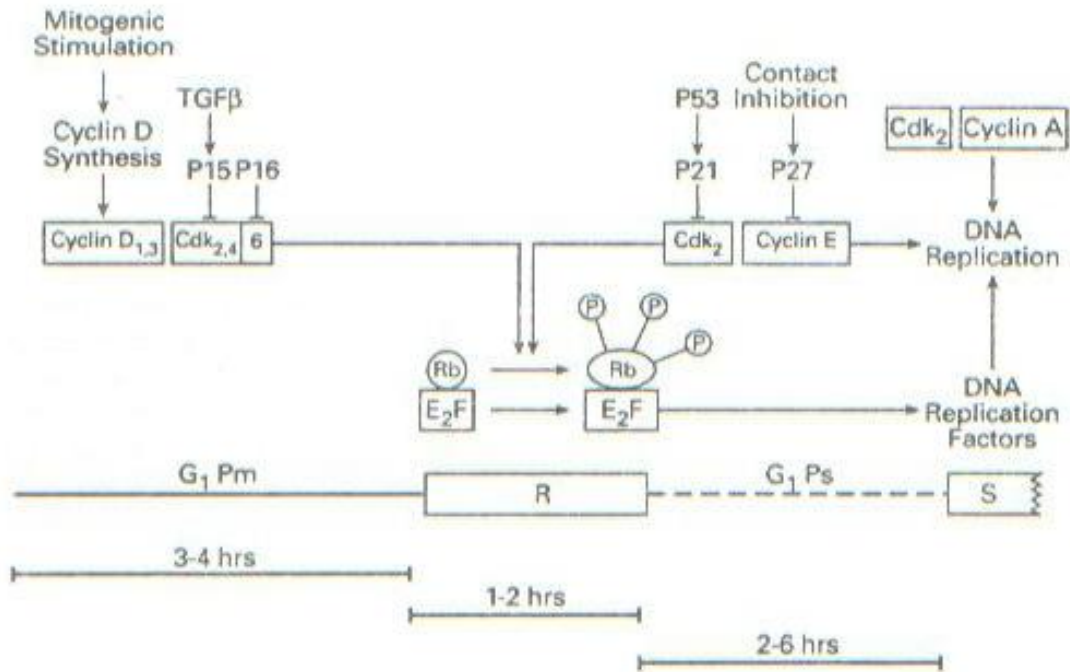
Fig. 5. A model of events in GI phase of cell cycle. G1 -pm represent the time post mitosis where cyclin D2 and HTLV-I gene expression takes place. The "R" represents the time where Rb and possibly CBP/p300 (data from this manuscript) are phosphorylated by cyclin D2 associated complex, and G1 -ps represents events before S phase, including E2F transactivation of genes such as cyclin E and PCNA synthesis. Diagram modified from Zetterberg et al [39].

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Subsequent experiments have shown that cyclin D3 is repressed in MT-2, but not CEM, cells (data not shown), consistent with a previously published report on cyclin D3 down regulation in all HTLV-I-infected cells [15, 16]. Cyclin D2 and D3 levels were slightly higher in CEM control cells 2 hours post-release (Figure 3, right panel), however no dramatic induction of these promoters were observed as compared to HTLV-I infected cell.

To further analyze the effect of cyclin D2 promoter, we constructed a series of wild type and 5' deletion promoter plasmids using PCR and inserted them upstream of the CAT reporter gene. The reporter CAT gene was inserted 10 bases upstream of the authentic cyclin D2 AUG codon, where the only translational start site is donated from the CAT open reading frame. Following transfection of wild type cyclin D2 promoter into unsynchronized CEM cells, we have observed a 2.5-fold activation with Tax protein [46] and a 9.5-fold increase with a full length CBP construct (Figure 4). The same amount of Tax protein alone (1 ug) is capable of activating HTLV-I promoter by 40-fold (data not shown). Wild type full length CBP plasmid was not capable of activating HTLV-I, or any of the cyclin D2 promoters, in the absence of Tax (data not shown). Similar data, albeit less overall activity, was obtained with transfection of G1 cells where the Tax$_i$ and CBP effect is more pronounced than Tax$_i$ alone (Figure 4, synchronized panel). G1 cells represent cells blocked with nocodazole at M (for 18 hrs), washed with PBS, transfected with various plasmids, released for 2 hours with complete media and blocked for the second time with hydroxyurea at Gl/S border. Cells were harvested for CAT assay 24 hours post-hydroxyurea block. Interestingly, transfection in unsynchronized and synchronized cells show the possible effect of AP2 DNA binding sites to be most crucial in cyclin D2 promoter. High levels of AP2 activity have been reported in HTLV-I-infected cells [27].

DISCUSSION

The human T-lymphotropic virus type I (HTLV-I) is associated with an aggressive malignancy of mature CD4+ T-cells, adult T-cell leukemia (ATL) and the degenerative neuromuscular disease tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM).

It seems likely that the HTLV-I virus, through expression of the viral regulatory proteins Tax$_i$ and Rex , provides some initial alteration in cell metabo'ism predisposing the development of ATL. Subsequently, the rearrangement or altered expression of a cellular oncogene(s) may provide the "second hit", leading to development of ATL. In fact, there have been reports that Tax$_i$ triggers DNA damage. Diverse cytogenetie abnormalities have been observed in ATL patient peripheral blood lymphocytes. Although several karyotypic abnormalities have been found, including trisomies 3 and 7, and rearrangements in the long arm of chromosome 6, no single chromosomal defect is pathognomonic for ATL [28].

Tax, which is critical for viral replication, transformation and gene regulation. Tax activates HTLV-I gene expression through a sequence resembling the cyclic AMP-responsive element (CRE) in the HTLV-I LTR. The transcription coactivator CBP was first identified as a critical component of the CREB activation pathway [29, 30]. Subsequent to DNA binding, CREB is phosphorylated at serine-133, facilitating its interaction with CBP (31). Interestingly, in the presence of Tax, the requirement for CREB phosphorylation is bypassed for transactivation of the viral 21 by repeats [32]. Similar to transcription activators, CBP has been reported to interact with basal transcription factors such as TFIIB and TBP. The N-terminal half of CBP, which contains the TBP binding site, is important for CREB-mediated transcription and contains a strong activation domain. It has also been reported that CBP interacts with holo pol II [33], and contains a histone acetyltransferase domain. Thus, CBP may contain several domains which are important for transcription. Given the importance of CBP/p300 in CREB, NF- B, AP1, MAPK, PKA, and nuclear hormone receptor signaling, elucidating the mechanism of action of the CBP/p300 co-activators has important implications for gene regulation [34, 35]. These observations, collectively, suggest that Tax and CBP interactions have broad transcriptional implications and possibly are involved in more than just activating the HTLV-I promoter. To that end, result from Figure 3 is an indication to that effect, where cyclin D2 is also upregulated by Tax.

Genetically, the human cyclin D2 gene (CCND2) has been mapped to chromosome 12p13 and trisomy 12 which is the commonest chromosomal change in B-CLL and immunocytomas. Cyclin D2 mRNA was found to be over expressed in 29 of 34 B-CLL cases and in all cases of LPL. The level of cyclin D2 expression in these disorders was, on

average, 5- to 10-fold higher than in normal resting B lymphocytes [36]. Cyclin D3 was not detected in any sample from B-CLL or LPL patients, whereas cyclin D1 was expressed only in the 3 cases (LPL, 1; MCL, 2) associated with a t(11;14) translocation. Other interesting observations on the Cyclin D2 gene have also been noted where retroviral sequences found adjacent to the open reading frame. The *vin-1* gene first identified as the common site of provirus integration in retrovirus-induced rodent T-cell leukemia was shown to be identical to the cyclin D2 gene [37]. The possible role of *vin1/cyclin* D2 gene in oncogenesis is suggested by the over expression of cyclin D2 that results from provirus integration. It will be of interest to determine whether HTLV-I, much like the rodent family member, is also using the cyclin D2 loci for its integration site. Finally, a model of where tax and CBP may have their effect in a cell cycle dependent manner is depicted in Figure 5. Perhaps, future fine tuned experiments will determine if the Tax/CBP involvement at early G1 (G1Pm) touches any other cyclin promoters, rising to a more active cdkicyclin complex.

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