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Identification of Poly(ADP-Ribose) Polymerase as a Transcriptional Coactivator of the Human T-Cell Leukemia Virus Type 1 Tax Protein

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Human T-cell leukemia virus type 1 (HTLV-1) encodes a transcriptional activator, Tax, whose activity is believed to contribute significantly to cellular transformation. Tax stimulates transcription from the proviral promoter as well as from promoters for a variety of cellular genes. The mechanism through which Tax communicates to the general transcription factors and RNA polymerase II has not been completely determined. We investigated whether Tax could function directly through the general transcription factors and RNA polymerase II or if other intermediary factors or coactivators were required. Our results show that a system consisting of purified recombinant TFIIA, TFIIB, TFIIE, TFIIF, CREB, and Tax, along with highly purified RNA polymerase II, affinity-purified epitope-tagged TFIID, and semipurified TFIIH, supports basal transcription of the HTLV-1 promoter but is not responsive to Tax. Two additional activities were required for Tax to stimulate transcription. We demonstrate that one of these activities is poly(ADP-ribose) polymerase (PARP), a molecule that has been previously identified to be the transcriptional coactivator PC1. PARP functions as a coactivator in our assays at molar concentrations approximately equal to those of the DNA and equal to or less than those of the transcription factors in the assay. We further demonstrate that PARP stimulates Tax-activated transcription in vivo, demonstrating that this biochemical approach has functionally identified a novel target for the retroviral transcriptional activator Tax.

Human T-cell leukemia virus type 1 (HTLV-1) is etiologically associated with adult T-cell leukemia and a neurodegenerative disease called tropical spastic paraparesis/HTLV-1 associated myelopathy (87). Adult T-cell leukemia is an aggressive leukemia that develops after a long latency period in a small fraction of infected individuals (10, 39, 49, 64, 76). One HTLV-1 gene product, Tax, has been shown to activate transcription from the proviral promoter (reviewed in reference 20). In cell culture and transgenic mouse studies, Tax promotes cellular transformation (1, 30, 31, 33, 38, 42, 67). Tax is therefore believed to play a key role in promoting both HTLV-1 replication and HTLV-1-mediated transformation. One possible contribution of Tax to cellular transformation could be through loss of normal regulation of checkpoints in the cell cycle, mediated through factors such as $p16^{INK4a}$, MAD1, cdk4, and cdk6 (44, 54, 68, 83, 91). Another possible mechanism by which Tax transforms cells may derive from the ability of Tax to activate transcription of cellular genes involved in growth regulation. Examples include the genes for interleukin-2 (IL-2), IL-2 receptor alpha chain, IL-3, granulocyte-macrophage colony-stimulating factor, tumor growth factor β1, tumor necrosis factor beta, c-*fos*, c-*jun*, *junD*, *fra-1*, *egr-1*, and *egr-2* (17, 21, 22, 32, 48, 62, 70, 80, 86). Resultant T-cell hyperactivation from Tax transcriptional activation may contribute to Tax-induced neoplastic transformation of infected cells.

The HTLV-1 promoter is activated by Tax through three semiconserved, 21-bp repeats called Tax-responsive elements (TxREs) (11, 24, 74, 79, 85). Located upstream from the start site of transcription, these TxREs are nonconsensus cyclic AMP response elements (CREs), which are binding sites for the CRE-binding protein (CREB)/ATF family of transcriptional activators (36, 63). These CREs are flanked by additional G-rich and C-rich DNA sequences (28, 43). Tax does not bind independently to the TxREs (9) but instead interacts via cellular CREB (5, 19, 59, 90, 98, 99). We and others have found that Tax enhances the binding of CREB to the HTLV-1 TxRE by increasing CREB dimerization and CREB-DNA interactions (3, 8, 95). However, this activity alone cannot fully account for Tax activation in vivo or in vitro. The nuclear concentration of CREB is high enough that most CREB should already be dimerized (3). In addition, in vivo experiments with a GAL4-Tax fusion protein demonstrate that Tax can activate transcription from a GAL4-containing promoter without a TxRE or CRE in the promoter (16, 23, 25, 84). Finally, we have found that Tax can still function in vitro even when additional CREB is added to the transcription assay (4).

RNA polymerase II (RNAP II) and the general transcription factors (GTFs) have been extensively studied (reviewed in reference 69). The GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) function by directing RNAP II promoter specific transcription. Stimulatory signals from transcriptional activators have been postulated to function through a variety of mechanisms, many of which involve the recruitment of and/or stabilization of the GTFs and RNAP II on the promoter into what is called a preinitiation complex. This mechanism of stimulation has been described for the activation domain of the tumor suppressor/transcriptional activator p53, which appears

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to interact directly with TFIIH and TFIID (51, 56, 94). Similarly, the activation domain of the herpesvirus transcriptional activator VP16 interacts directly with TFIIB, TFIID, and TFIIH (52, 89). Tax has been shown to interact directly with two components of the general transcription machinery, TATA binding protein (TBP) and TAF128 (13, 14). However, these studies are in apparent conflict with a finding that Tax does not interact with native TFIID, a factor which contains TBP, TAF128, and other proteins (47). Moreover, experiments presented here show that in addition to the GTFs, at least two other coactivator proteins are required for Tax-activated transcription.

Not all activators appear to function through direct interactions with the GTFs. This was suggested by experiments where activated transcription in vitro required not only the entire set of GTFs but also additional activities referred to as cofactors or coactivators (60). Some coactivators, such as PC4, act as bridging molecules between the activators and GTFs. PC4 is a small (~15-kDa) protein that binds to the activation domains of several transcriptional activators and also to DNA-bound TBP-TFIIA complexes (26). Other coactivators that have been shown to have enzymatic activities include the CREB coactivator CBP (CREB binding protein), which has an intrinsic acetyltransferase activity (7). CBP can interact with Tax and CREB on DNA (27, 50). Acetylation of histones and/or transcription factors by CBP could play a role in Tax-activated transcription; however, a fragment of CBP lacking the catalytic domain can stimulate Tax-activated transcription in a HeLa cell extract, possibly through interactions with RNAP II (46). Recently, several large, multisubunit coactivator complexes such as SAGA, CRSP, and ARC/DRIP have been identified. Some of these complexes can function in the absence of chromatin, while others are involved with chromatin remodeling (29, 65, 77, 81). Another coactivator with enzymatic activity is poly(ADP-ribose) polymerase (PARP), or PC1. PARP catalyzes the transfer of ADP-ribose from NAD^+ to proteins and has been demonstrated to inhibit RNAP II transcription in nuclear extracts in the presence of NAD^+, most likely through ribosylation (71). However, a fragment of PARP lacking the catalytic domain can function as a coactivator for two activation domains fused to GAL4, indicating that enzymatic activity is not required for PARP coactivation (61).

We have developed an in vitro Tax-responsive transcription system in order to determine which GTFs are required for Tax activation and whether any additional activities or coactivators are required. We found that TFIIA, TFIIB, TFIID, TFIIE, TFIIF, CREB, and most likely TFIIH are required for Tax activation in vitro. Our system, however, is not activated by Tax unless it is supplemented with an additional, semipure fraction that we call the mixed coactivator. Using this assay system, we have found that the coactivator fractionates into three separable activities, LTF1 (large Tax factor 1), LTF2, and STF (small Tax factor). Our further purification and analysis has identified PARP (PC1) as the active component of LTF2. A recombinant fragment of PARP can substitute for LTF2 in this system, and we demonstrate that PARP stimulates Tax-activated transcription in transient cotransfection assays. This indicates that PARP is a coactivator of HTLV-1 Tax. Significantly, this report is unique in that it demonstrates a coactivator requirement for a natural viral transcription factor in the context of its native core promoter and binding sites.

MATERIALS AND METHODS

Plasmids. For G-less cassette constructs, oligonucleotides corresponding to nucleotides −105 to −82 of the HTLV-1 promoter and encompassing the third TxRE were synthesized as follows: 5′-CTAGCTCAGGCGTTGACGACAACC

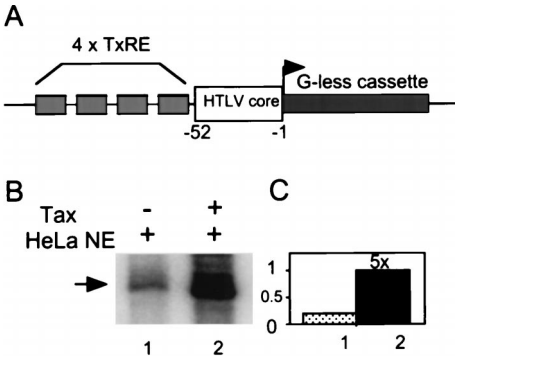

FIG. 1. HeLa cell nuclear extracts contain factors necessary for the stimulation of transcription by Tax in vitro. (A) Four copies of the HTLV-1 TxRE were placed upstream of the HTLV-1 core promoter. This region contains the natural HTLV-1 TATA box which binds TFIID and allows the assembly of the basal transcription factors. (B) Typical transcription reaction with the plasmid from panel A, HeLa nuclear extract (NE), and 100 ng of recombinant Tax where indicated. The arrow indicates the full-length, 380-base G-less transcript that is activated fivefold by the presence of Tax. For this and all figures, transcription assays were analyzed by PhosphorImager software IQMac version 1.2, and images are presented on a linear scale. (C) Quantitation of transcripts from panel B by PhosphorImager analysis. Values were normalized to full activity with Tax. Fold activation (reaction with Tax divided by reaction without Tax) is indicated (5×).

CCTCA-3′ and 5′-CTAGTGAGGGGTTGTCGTCAACGCCTGAG-3′ (the complementary HTLV-1 sequences are underlined). When the oligonucleotides are annealed, the 5′ side creates an *Nhe*I end, while the 3′ side creates an *Spe*I end. The oligonucleotides were ligated by T4 DNA ligase (Promega) and digested with *Nhe*I and *Spe*I to cleave head-to-head and tail-to-tail ligation products, leaving intact head-to-tail ligation products. These were then gel purified to obtain molecules greater than or equal to four TxREs in length. These were cloned upstream of HTLV-1 promoter positions −52 to −1 (11), followed by a 380-bp G-less region (82) as shown in Fig. 1A. For a control promoter, the −52 to −1 region was cloned into those sites upstream of the 380-bp G-less region. For the HTLV-1 luciferase construct, the region from −306 to −1, encompassing all three of the HTLV-1 TxREs, was cloned upstream of the luciferase reporter gene in the *Sma*I and *Sac*I sites of the pGL2-Basic plasmid (Promega). Constructs were verified by sequencing.

Purification of the components of the in vitro transcription system. All proteins were purified in HE buffer (25 mM HEPES [pH 7.9], 1 mM EDTA, 0.1% NP-40, 15% glycerol, 4 mM 2-mercaptoethanol [ME], 10 μg of phenylmethylsulfonyl fluoride [PMSF]) with the indicated amount of KCl (i.e., HE 0.1 is HE with 0.1 M KCl) unless otherwise indicated. Recombinant α/β and γ subunits of TFIIA were purified individually using Ni^+-nitrilotriacetic acid-agarose chromatography (72). After denaturation in 2 M urea and renaturation by dialysis, TFIIA was further purified by gel filtration on a Superdex 200 column to isolate α/β-γ complexes from uncomplexed subunits. Recombinant TFIIB (35) was purified by phosphocellulose and gel filtration chromatography. Recombinant TFIIE 34- and 56-kDa subunits were purified as described elsewhere (75). Recombinant TFIIF 30- and 74-kDa subunits (6) were purified individually by phosphocellulose and gel filtration chromatography. After denaturation in 4 M urea and renaturation by dialysis, TFIIF was further purified by gel filtration on a Superdex 200 column to isolate complexes from uncomplexed subunits (57). Recombinant $TaxH_6$ (99), a histidine-tagged recombinant Tax protein, was purified by Ni^+-nitrilotriacetic acid-agarose chromatography and gel filtration as previously described (4). The recombinant CREB protein (40) was purified by heparin-agarose chromatography and gel filtration (4).

RNAP II was purified from a HeLa cell nuclear pellet by DE-52, heparin-agarose, and DEAE Hydrocell 1000 (Rainin) chromatography using the buffer system previously described (55). Native TFIID was purified to different degrees as previously described (60) with several modifications (referred to as semipurified and purified) as described in the legend to Fig. 2. Highly purified preparations of epitope-tagged affinity-purified TFIID (eTFIID) were obtained from a HeLa cell line expressing an influenza virus hemagglutinin peptide-TBP fusion protein. eTFIID was purified as previously described (100) except that the 1.0 M KCl phosphocellulose fractions that contain the eTFIID complexes were passed over a column (1 ml of beads per 60 liters starting volume of cell culture) of antiepitope antibody coupled to protein A. After washing, eTFIID was eluted with 3 ml of hemagglutinin peptide (1 mg/ml). Fractions containing eTFIID were identified by Western blots probed with anti-TBP antibody (Santa Cruz Biotechnology).

In vitro transcription assays. In vitro transcription reactions were carried out using previously described methods (4), with several modifications. For each

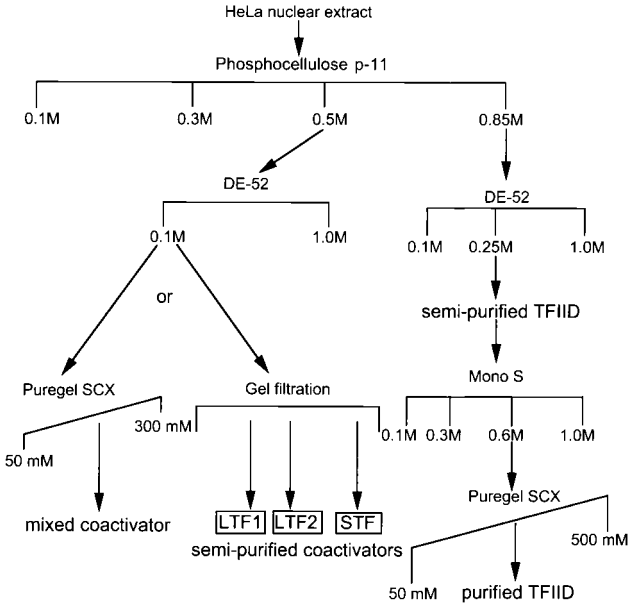

FIG. 2. Purification scheme of coactivators and TFIID. Starting material for the nuclear extract material was between 60 and 120 liters of cultured cells. Arrows indicate subsequent columns used for purification. Columns shown with horizontal bars were developed with step elutions in buffer with the salt concentrations indicated. Columns shown with slanted bars represent linear gradients with initial and final salt concentrations indicated at the left and right. Semipurified TFIID is the DE-52 0.25 M KCl peak. Purified TFIID eluted from the PureGel SCX column at approximately 150 mM KCl. The coactivator present in the DE-52 0.1 M KCl flowthrough was purified by two separate methods. Purification over a PureGel SCX column yielded the mixed coactivator peak. Purification by gel filtration yielded three activities, LTF1, LTF2, and STF.

50-μl assay, the purified factors were added in the following approximate amounts: 15 ng of TFIIA, 7 ng of TFIIB, 50 ng of 56-kDa TFIIE, 30 ng of 34-kDa TFIIE, 50 ng of the complex of 30- and 74-kDa TFIIF, 50 ng of CREB, 100 ng of Tax, 1 to 2 ng of TBP in eTFIID, and 50 to 100 ng RNAP II. Additional factors were added as indicated in the figure legends. Reactions were carried out in 0.5× HE 0.1 buffer (0.05 M KCl, 12.5 mM HEPES [pH 7.9], 0.5 mM EDTA, 0.05% NP-40, 7.5% glycerol, 2 mM 2-ME, 5 μg of PMSF/ml) supplemented with 3.4 mM $MgCl_2$. Then 250 ng of the G-less cassette promoter construct was added to the preinitiation reactions in a supercoiled form. ATP, UTP, CTP, and 3′-*O*-methyl-GTP were added after the 30-min preincubation step, permitting transcription only through the 380-bp G-less region. Extension reactions were allowed to proceed for 30 min after addition of nucleotides. Reactions containing nuclear extracts were contaminated with GTP, leading to the generation of some read-through, longer transcripts. These transcripts were cleaved after G residues by the addition of RNase T_1 for 15 min at 37°C. All reactions were terminated by the addition of NaCl (133 mM, final concentration), sodium dodecyl sulfate (SDS; 0.5% final concentration), EDTA (10.3 mM, final concentration), Tris (pH 7.9) (3.3 mM, final concentration), and carrier tRNA (100 μg/ml). Reactions were then extracted with phenol-chloroform, ethanol precipitated, and separated by urea–5% polyacrylamide gel electrophoresis (PAGE). Gels were dried and then visualized and quantitated by PhosphorImager analysis on a linear scale using IQMac v1.2.

Purification of coactivator activity. Between 60 and 120 liters of HeLa cells were grown to a density of approximately 5×10^5 per ml and used as starting material. Cells were grown in suspension-minimal essential medium (S-MEM) (Joklik modified) (GIBCO-BRL) supplemented with 3% fetal bovine serum (Sigma), 5% newborn calf serum (Atlanta Biologicals), 1 mM sodium pyruvate (Cellgro), nonessential amino acids (Cellgro), and 100 U of penicillin and streptomycin per ml. Nuclear extracts were prepared as previously described (4) and subjected to fractionation on phosphocellulose p11 (Whatman) in CB buffer (50 mM Tris [pH 7.9], 1 mM EDTA, 0.02% Tween 20, 5% glycerol, 1 mM dithiothreitol, 10 μg of PMSF/ml). Increasing KCl concentration steps were used to elute protein from the column as indicated in Fig. 2. Fractions eluting at 0.5 M KCl were dialyzed into HE 0.1 and applied to a DE-52 (Whatman) column equilibrated in HE 0.1. The complementary activity flowed through this column, was diluted with HE 0 to reduce the final KCl concentration to 50 mM, and was applied to a PureGel SCX (strong cation-exchange) column (Rainin). The column was developed with a linear gradient from 50 to 300 mM KCl. Coactivator activity eluted from the column between 100 and 140 mM KCl and is referred to as the mixed coactivator in Fig. 2. In separate preparations, the DE-52 flowthrough was applied to a Superdex 200 column and run in HE 0.1. This gel filtration step separated the activity into three peaks: one that eluted with an apparent high molecular mass of 290 to 470 kDa (LTF1), one with an apparent molecular mass of 80 to 130 (LTF2), and one with an apparent low molecular mass of about 40 kDa (STF).

Purification and identification of LTF2. To generate LTF2 protein for sequencing, fractions from the Superdex 200 column containing LTF2 activity were pooled, diluted with HE 0 to 50 mM KCl, and applied to a preparative 10-mm by 10-cm PureGel SCX column. The column was developed with a linear gradient from 50 to 300 mM KCl. LTF2 activity eluted from the column between 120 and 150 mM KCl, similar to the mixed coactivator. Peak fractions were pooled, $CaCl_2$ was added to complex the EDTA, and potassium phosphate (pH 6.8) was added to 20 mM (final concentration). This sample was applied to a hydroxyapatite column (CHTI-5; Bio-Rad) and washed with H buffer (5 mM HEPES [pH 7.9], 0.1 M KCl, 0.01% NP-40, 15% glycerol, 4 mM 2-ME, 10 μg of PMSF/ml) containing 20 mM potassium phosphate. The column was developed in a linear gradient from 20 to 600 mM potassium phosphate. Peak activity eluted at 260 to 290 mM, and these fractions were pooled.

Protein sequencing was performed at the Emory University Microchemical Facility by Jan Pohl. LTF2 fractions were concentrated by adsorption onto a polyvinylidene difluoride membrane by filtration and digested with trypsin. The tryptic fragments were extracted, separated by high-pressure liquid chromatography (HPLC), and rechromatographed by HPLC. Selected peptide fractions were subjected to matrix-assisted laser desorption ionization–mass spectrometry analysis to determine molecular weights. Five peptides were sequenced using Edman degradation. Matches were found by searching the SwissProt.r34 database.

Transient cotransfection assays. The mouse embryonic fibroblast cell line from the PARP knockout mouse was a generous gift from Z. Q. Wang and has been described elsewhere (96). Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, plated at a density of approximately 20% confluency, and grown for 4 to 5 h before transfection. Cells were transfected by the modified calcium phosphate method (15) with the HTLV-1–luciferase reporter construct, either alone or with an expression vector for Tax (15 μg; pHTLV-1 Tax [67]) and/or PARP (10 μg; PARP pcD-12 [2]). Cells were allowed to incubate for 24 h before the medium was changed. After an additional 24 h, cells were harvested at a density less than 70% of confluency and assayed for luciferase activity using the Promega luciferase assay system. Protein concentration of each sample was determined by the bicinchoninic acid method (Pierce) according to manufacturer's specifications, and luciferase activity was normalized to protein concentration for each sample. Background activity in this assay is less than 0.5% of the lowest values, and so it was not subtracted. Averages were determined for duplicates in experiment 1 and triplicates in experiment 2, and standard deviations were determined. Within each experiment, values were normalized to the activity observed in cells without Tax or PARP expression.

RESULTS

Development of the Tax-responsive in vitro transcription system. Previous work has demonstrated Tax responsiveness in vitro, using a linearized template containing the natural HTLV-1 promoter (4, 59). To reduce the complexity of the system and to enhance Tax dependence, a modified HTLV-1 promoter containing four tandem TxREs was placed immediately upstream of the HTLV-1 core promoter (−52 to −1) (Fig. 1A). This construct eliminates adventitious binding sites for several cellular transcription factors that are believed not to be involved in the Tax response in vivo (11, 78). To facilitate the in vitro assay, a G-less cassette (82) was fused downstream of the core promoter (Fig. 1A).

It has been demonstrated that Tax can significantly activate transcription in HeLa cells in vivo, using cotransfection experiments (88), and in vitro, using HeLa whole-cell extracts (18), indicating that HeLa cells contain the factors necessary for Tax activation. We therefore used a HeLa cell nuclear extract to test our promoter construct for transcription activation by Tax (Fig. 1B and C). Tax activated transcription fivefold, indicating that HeLa cell nuclear extracts also contain all the factors necessary for transcriptional activation of this promoter by Tax. Similar activity and Tax responsiveness were also observed when the TxREs were placed in tandem in the opposite orientation relative to the RNA start site (data not shown). In

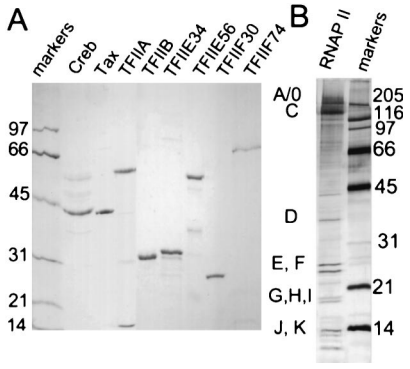

FIG. 3. Transcription factors used in in vitro assays. (A) SDS-PAGE analysis of recombinant transcription factors stained with Coomassie blue. Positions of molecular weights of markers are indicated on the left in kilodaltons. Tax and CREB are ~43 kDa. TFIIA has two polypeptides, ~55-kDa α/β subunit and ~12- to 14-kDa γ subunit. TFIIB is ~30 kDa. TFIIE contains two subunits, ~34 and ~56 kDa, and TFIIF contains two subunits, ~30 and ~74 kDa. (B) SDS-PAGE analysis of HPLC-purified RNAP II visualized by silver staining. Subunits are indicated on the left. Positions of molecular weights of markers are indicated on the right in kilodaltons.

addition, this Tax responsiveness required the presence of the TxREs (data not shown), as had been previously found (59).

HeLa cell nuclear extracts were subjected to phosphocellulose p11 column chromatography, and proteins were eluted from the column by washing with increasing KCl salt steps as indicated in Fig. 2, recapitulating previous fractionation schemes for the GTFs (58). We expressed and purified recombinant transcription factors (Fig. 3A) from bacteria, and we purified RNAP II (Fig. 3B) from HeLa cells (see Materials and Methods). A previous report found that partially purified RNAP II and partially purified TFIID from HeLa cells (and not just the TBP subunit of TFIID), along with recombinant TFIIA, -B, and -F, were sufficient to mediate Tax activation (18). Consistent with this report, we found that Tax was able to activate transcription in a similar system consisting of recombinant TFIIA, -B, and -F, CREB, HPLC-purified RNAP II, and semipurified TFIID (Fig. 2), as shown in Fig. 4A and B, lanes 1 and 2. In addition, when recombinant TBP was substituted for semipurified TFIID, we found that Tax could not activate transcription (data not shown).

We next wanted to determine if TFIID was the only factor required from the semipurified TFIID fraction or if that fraction was supplying additional activities. We therefore focused on the purification of this fraction. Semipurified TFIID was purified over a Mono S and PureGel SCX column to make purified TFIID as indicated in Fig. 2. Most significantly, the purified TFIID required an additional activity that we call the mixed coactivator in order to obtain Tax-activated transcription (Fig. 4C and D; compare lanes 5 and 6 with lanes 7 and 8). In fact, Tax activation was somewhat higher with the mixed coactivator and the purified TFIID than with the semipurified TFIID alone or with the coactivator (5.2-fold activation versus 3.8- and 2.7-fold). The mixed coactivator had no effect on Tax-activated transcription reactions with the semipurified TFIID (Fig. 4A and B), most likely because semipurified TFIID contains some of this coactivator activity. In addition, basal transcription was slightly increased (compare lanes 1 and 3 as well as lanes 5 and 7). This mixed coactivator was derived from the 0.5 M KCl peak from the phosphocellulose column and further purified over a DE-52 and PureGel SCX column as shown on the far left of Fig. 2, with active fractions eluting as a single peak from the PureGel SCX.

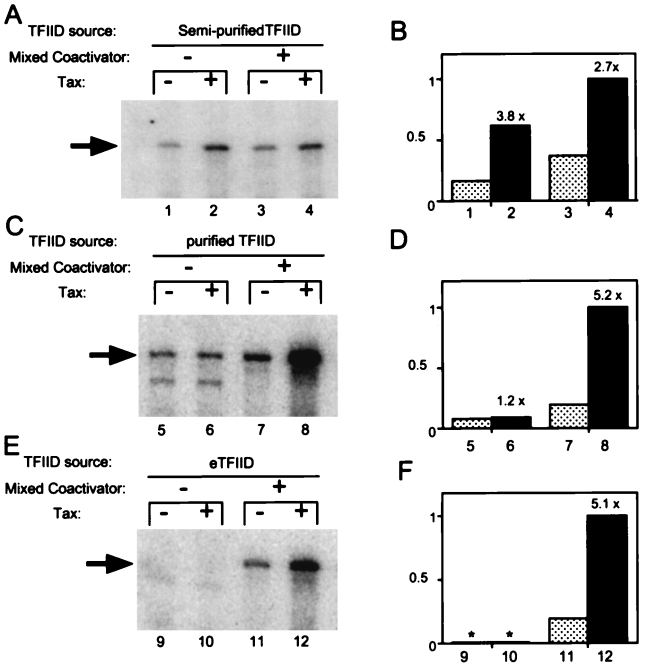

FIG. 4. Coactivator requirement depends on TFIID source. Radiolabeled RNAs from representative transcription reactions were visualized by PhosphorImager analysis. All reactions contained recombinant TFIIA, TFIIB, TFIIF, CREB, Tax (where indicated), and native, HPLC-purified RNAP II. Purification procedures for semipurified (A) and purified (C) TFIID from HeLa cell nuclear extracts are shown in Fig. 2. Highly purified TFIID (eTFIID) (E) was affinity purified from the phosphocellulose high-salt fraction from HeLa cells expressing epitope-tagged TBP. The mixed coactivator fraction was prepared as shown in Fig. 2. Arrows indicate the full-length transcript. Transcripts were quantitated as in Fig. 1 and shown in panels B, D, and F. Asterisks indicate transcripts at or below background levels.

As a more stringent test, highly purified eTFIID (100) was then substituted for the TFIID fractions. These reactions were dependent on the coactivator not only for Tax activation but also for basal transcription (Fig. 4E and F, compare lanes 9 and 10 to lanes 11 and 12). We conclude that the mixed coactivator contains not only coactivator activity but at least one activity involved with basal transcription from this promoter. Western blot analysis indicated that two GTFs, TFIIE and TFIIH, were present in mixed coactivator fractions. Since some promoters do not require TFIIE and TFIIH for in vitro transcription (41, 73), we originally had omitted these factors from the assays. Subsequent assays with more purified coactivator fractions (devoid of these factors) were dependent on recombinant TFIIE and fractions containing TFIIH (data not shown), indicating their requirement in our system.

Analysis of Tax coactivators. The focus then turned to the purification of the Tax coactivator activity. To better resolve factors required for basal transcription (i.e., TFIIE) from those required for Tax activation, the coactivator was purified by gel filtration chromatography (Fig. 2 and 5). These fractions were analyzed for transcriptional activity with the recombinant factors used in the previous experiment, along with recombinant TFIIE. To achieve full basal and Tax-activated transcription, three sets of fractions were necessary (Fig. 5A and B): LTF1, LTF2, and STF. In the absence of any of these activities, transcription levels were near the limits of detection and somewhat variable (the small amount of activation seen in the absence of LTF2 in lanes 1 and 2 of Fig. 5A is not seen consistently and may be due to cross-contamination of LTF2 and

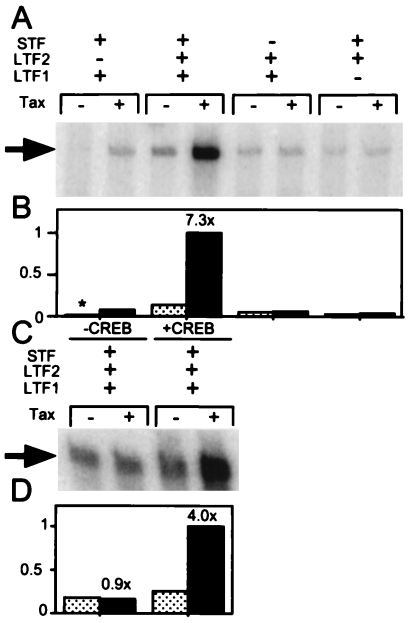

FIG. 5. Activity of LTF1, LTF2, and STF. (A) In vitro transcription reactions were carried out and analyzed as before except that recombinant TFIIE was added to all reactions. Tax (100 ng) and fractions containing LTF1, LTF2, or STF were added as indicated. The arrow indicates the full-length transcript. (B) Transcripts were quantitated as in Fig. 4. (C) In vitro transcription reactions were carried out as in panel A except that CREB was omitted from the first two lanes as indicated. (D) Transcripts from panel C were quantitated as in Fig. 4.

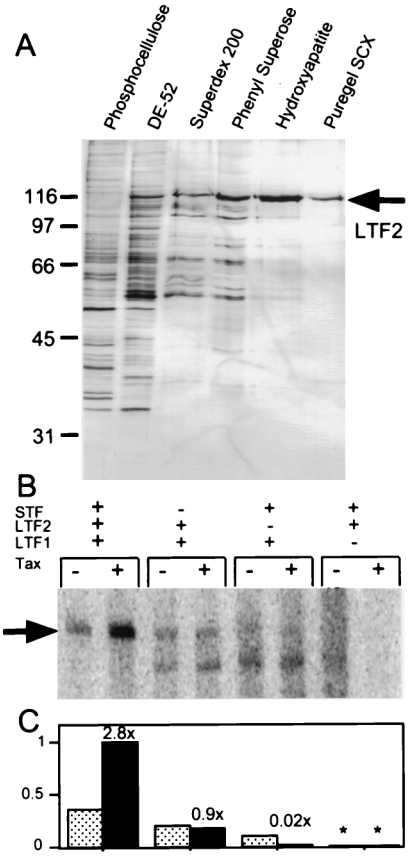

FIG. 6. Purification of LTF2. (A) The various stages of purification of LTF2 were analyzed by SDS-PAGE and visualized by silver staining. The following fractions that contained the peak of LTF2 activity are in the lanes as indicated: 0.3 to 0.5 M KCl step of the phosphocellulose column; 0.1 M KCl flowthrough of the DE-52 column; fractions corresponding to molecular weights of approximately 80 to 130 kDa of the Superdex 200 column; 0.7 to 0.8 M ammonium sulfate fraction of a linear gradient on the phenyl-Superose column; 0.25 to 0.3 M KPO_4 of a linear gradient on the hydroxyapatite column; and the 150 to 180 mM KCl fractions of a linear gradient on an analytical 4.6-mm by 10-cm PureGel SCX column. Molecular masses are indicated (in kilodaltons) on the left. (B) Highly purified PARP from the PureGel SCX column peak shown in panel A were substituted for LTF2 in transcription reactions using conditions similar to those in Fig. 5. STF, PARP, and LTF1 were added as indicated. The arrow indicates the full-length transcript. (C) Transcripts were quantitated as in Fig. 4.

LTF1). As expected, this activation of transcription by Tax is dependent on CREB (Fig. 5C and D), indicating that Tax activates transcription in this HTLV-1 promoter system through the CREB protein.

LTF2 is a single, 110-kDa polypeptide. The LTF2 fractions from gel filtration chromatography were pooled and further purified by phenyl-Superose, hydroxyapatite, and PureGel SCX column chromatography. After each column, fractions were assayed for complementation of transcription in the presence of the other necessary fractions, and active fractions were pooled and analyzed by SDS-PAGE followed by silver staining (Fig. 6A). LTF2 activity in these assays copurified with a moderately abundant, 110-kDa protein that appeared to be at least 99% of the total protein by silver staining after the last column. A transcription assay was performed using the LTF2 from the last PureGel SCX column as shown in Fig. 6A. This preparation of LTF2 was able to restore Tax-mediated activation (2.8-fold) when combined with LTF1, STF, CREB, and the basal factors TFIIA, TFIIB, eTFIID, TFIIE, and TFIIF (Fig. 6B and C).

Identification of PARP as the major component of LTF2. A similar preparation of LTF2 was obtained by chromatography as shown in Fig. 7A. Hydroxyapatite fractions were analyzed by SDS-PAGE followed by silver staining (Fig. 7B). Fractions 72 to 74 contained the peak of activity, with a three- to sixfold enhancement of transcription by Tax (data not shown). These fractions coincided with the peak of a 110-kDa protein (Fig. 7B). Since this protein was at least 99% of the protein in these fractions, total protein was concentrated on a polyvinylidene difluoride filter, digested with trypsin, and subjected to sequencing and mass spectrometry analysis as described in Materials and Methods. Of the 47 amino acids sequenced, 46 matched sequences in PARP, a 110-kDa protein (Fig. 7C). In addition, 17 peaks from the mass spectrometry analysis were also assigned to predicted tryptic fragments of PARP (Fig. 7C).

PARP has recently been identified as PC1 and shown to act as a coactivator of fusion proteins containing GAL4 DNA binding domains and activation domains from either GAL4 or NF-κB (61). In this report, full-length PARP as well as PARP(1-450), a deletion mutant containing the DNA binding region (positions 1 to 450), were expressed in *Escherichia coli*, and both were found to function in this system when added in approximately 80-fold molar excess over the DNA template (61). Titration experiments of LTF2 (PARP) determined that a much lower concentration of PARP (approximately equimolar to the DNA and transcription factors) was required for optimum activation in our system (data not shown). We expressed recombinant PARP(1-450) (61) in *E. coli* and purified it by Ni^{+}-agarose and gel filtration chromatography. We next tested it as a substitute for LTF2 in our assay system. Again, a low concentration of PARP was found to functionally replace LTF2 in Tax-mediated transcriptional activation (Fig. 8). Tax

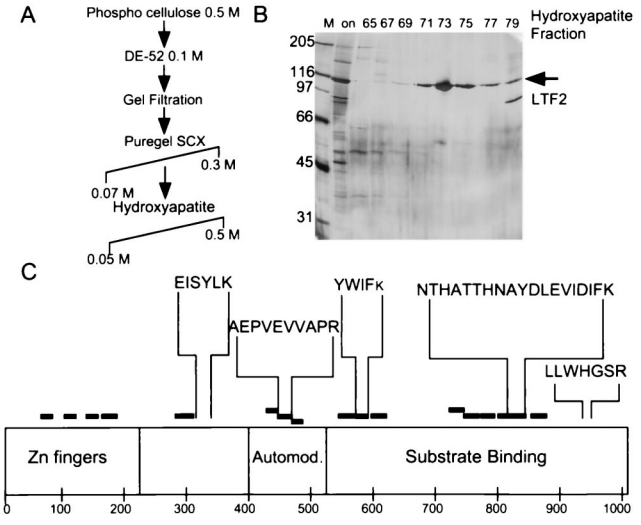

FIG. 7. Identification of the 110-kDa protein in LTF2. (A) LTF2 fractions were obtained as shown in Fig. 2 and further purified over phenyl, Superose, and hydroxyapatite. (B) LTF2 fractions from the hydroxyapatite column were analyzed by SDS-PAGE followed by silver staining. M, markers (molecular masses are indicated at the left); on, the onput to the column. Individual fraction numbers are indicated. The 110-kDa protein that copurifies with LTF2 is indicated by the arrow. Fractions 72 to 74 were pooled and sequenced, and the results are shown in panel C. The 1,014 amino acids of PARP are represented by boxes with the amino acid number indicated below. Using the SwissProt.r34 database, 46 of the 47 amino acids in our 110-kDa protein sequence corresponded to sequences within the protein PARP (entry P09874). The actual sequences of the peptides are displayed in the single-letter code and are positioned over the region to which they mapped onto PARP. The lysine indicated by a smaller K is the one mismatch (arginine in PARP). The position of mass spectrometry peaks that matched the predicted tryptic fragments of PARP are shown by bold black lines. Functional domains within PARP are labeled. Putative Zn finger domains are in the amino-terminal region. The automodification domain (Automod.) is the region that is extensively autoribosylated. The catalytic domain that binds substrate is in the carboxy-terminal region.

was found to activate transcription 3.8-fold, close to the levels seen in parallel reactions with native purified LTF2 (3- to 6-fold). In this and similar experiments, optimal activation was seen at approximately equimolar concentrations of DNA template and PARP. The ability of PARP to function at lower concentrations may be indicative of a higher specificity for PARP in the HTLV-1 Tax system versus the GAL4-activation domain system.

Enhancement of Tax-specific transcription in vivo by PARP. PARP is a fairly abundant, ubiquitous protein. To determine

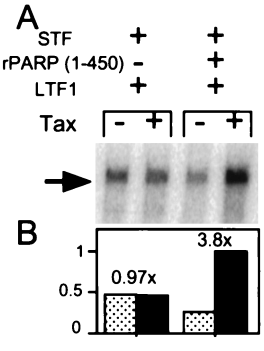

FIG. 8. PARP(1-450) can substitute for LTF2 in transcription. (A) Transcription reactions were carried out as before, in the absence of LTF2 or with 40 ng of recombinant PARP(1-450) where indicated. The arrow indicates the full-length transcript. (B) Transcripts were quantitated as in Fig. 4.

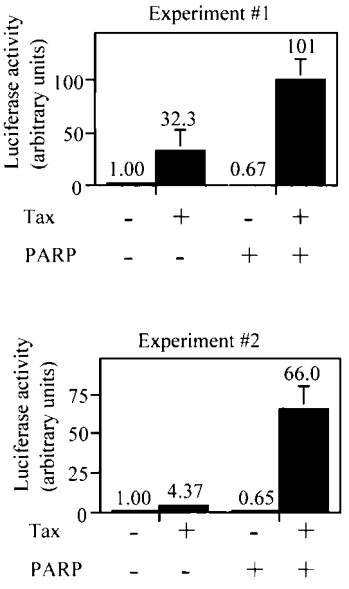

FIG. 9. PARP enhances Tax-activated transcription in vivo. A mouse embryonic cell line deficient for PARP was transfected with 10 μg of a luciferase reporter plasmid driven by the HTLV-1 promoter (positions −306 to −1) alone or with 15 μg of an HTLV-1 Tax expression vector; 10 μg of PARP expression vector (pcD-12) was added as indicated. Each reaction received additional pUC DNA to a total of 35 μg of DNA. Luciferase activity was normalized to protein concentration and then divided by the activity in the absence of Tax and PARP; the numerical value is indicated above each bar. Experiment 1 is the average of duplicates, while experiment 2 is the average of triplicates. The positive error bar indicates the standard deviation for each average.

the effect of PARP on Tax-activated transcription in vivo, we therefore chose an embryonic fibroblast cell line derived from a mouse that had PARP expression eliminated by targeted gene disruption (96). These cells were negative for PARP by Western blotting; however, transient transfection of a PARP cDNA plasmid (2) generated levels of PARP expression that could be detected by Western blotting (data not shown). An HTLV-1 luciferase reporter construct (see Materials and Methods) was transfected in the presence or absence of an HTLV-1 Tax expression vector (Fig. 9). As expected (88), Tax activated transcription in these cells. Parallel cultures of cells were also transfected under the same conditions with the PARP expression vector. Transcription from the HTLV-1 promoter was slightly decreased by the expression of PARP (0.67 and 0.65 times that seen without PARP in experiments 1 and 2, respectively). This finding parallels the slight decrease in basal transcription seen in the in vitro system and recombinant PARP (Fig. 8). Most significantly, the amount of Tax-specific transcription was enhanced due to the expression of PARP in these cells (3.1 and 15.1 times more in experiments 1 and 2, respectively). This led to a PARP-induced increase in Tax activation of 4.6- and 23.2-fold, indicating that in vivo PARP functions as a coactivator of Tax-activated transcription.

DISCUSSION

The major goal of this work was to determine whether or not a coactivator is required for Tax-mediated activation of transcription and, if so, to identify it. We wanted to use a system in which Tax was recruited to the promoter via its natural protein and DNA interactions (Tax-CREB-TxRE complexes). To this end, we have developed an in vitro system based on recombi-

nant and highly purified factors. This system includes recombinant TFIIA, TFIIB, TFIIE, TFIIF, CREB, and Tax, along with highly purified RNAP II, affinity-purified eTFIID, and semipure TFIIH (in our LTF1 fractions). In addition to these factors, additional fractions, LTF2 and STF, were also required. We have identified PARP as a coactivator (LTF2) of Tax in this well-defined in vitro system containing full-length CREB and Tax.

PARP is a ubiquitous protein found in most cell types, including T cells (reviewed in reference 53). It binds to DNA strand breaks and catalyzes the transfer of ADP-ribose from NAD^+ to proteins. PARP has been proposed to contribute to genome stability, but the exact role that it plays is unclear. Targeted disruption of the PARP gene in mice was not lethal; however, cells from these mice had higher sister chromatid exchange and increased micronucleus formation after DNA damage (97). There are several other examples of factors that are believed to be involved in both DNA repair and transcription. TFIIH is a GTF that contains subunits that are also involved in nucleotide excision and repair (92). Similarly, the Cockayne's syndrome proteins CSA and CSB interact with RNAP II complexes and are believed to link transcription and DNA repair (37, 93). Therefore, there are precedents for DNA repair factors to also function in transcription. PARP was recently identified to be the coactivator PC1, although its mechanism of activation is less well understood (61). The authors reported that PARP was required for activated transcription from two model activators: the activation domain from NF-κB fused to the GAL4 DNA binding domain as well as a similar fusion protein containing the acidic activation domain from GAL4. In addition, it was found that in this GAL4 fusion system, PARP could function as a coactivator on preformed TFIID-TFIIA-DNA complexes, but PARP had to be added before the complete preinitiation complex was formed. PARP has also been shown to be present in a complex with the muscle-specific transcription element MCAT1 and the TEF-1 transcriptional activator (12). In vivo, PARP can relieve repression of transcription by overexpressed AP-2, indicating that PARP may also be a coactivator for AP-2 (45).

Our report is the first to demonstrate PARP function as a coactivator of a natural transcription factor in vitro. We were able to demonstrate that purified recombinant PARP(1-450) could substitute for PARP/LTF2 purified from HeLa cells and function as a required coactivator. With recombinant PARP, the likelihood of contaminants having coactivator activity is extremely low, confirming that the active component of LTF2 is indeed PARP. In an earlier report, Meisterernst et al. (61) found that a molar ratio of PARP to DNA template and transcription factors of approximately 80 to 1 was necessary for PARP coactivator function. In contrast, we found that PARP functioned optimally at near stoichiometric ratios of PARP to DNA and transcription factors. This may imply a more direct mechanism of coactivation in the Tax system versus the GAL4 or NF-κB fusion system. One possible model is a specific mechanism of recruitment in the HTLV-1 Tax system. We are currently examining complexes formed at the promoter. While these analyses are difficult due to the ability of PARP to bind DNA nicks and ends, we have developed techniques with end-blocked oligonucleotides that indicate that PARP binds specifically to the HTLV-1 promoter (Z. Zhang and M. G. Anderson, unpublished observations).

It appears unlikely that direct communication of Tax to the GTFs is the sole mechanism of activation since two of the required coactivators, STF and PARP, do not contain GTFs. It is still possible that Tax may function to make the cellular transcription factors to which it binds (such as CREB) activate more effectively. Although the bulk of the CREB coactivator CBP fractionates elsewhere, it is also detected in the LTF1 fraction by Western blotting (data not shown). Tax has been shown to help CREB recruit CBP (27, 50). CBP then could activate transcription through recruitment of an acetyltransferase or through its intrinsic acetyltransferase. Acetylation of histone proteins has been associated with more transcriptionally active chromatin (34). The histone acetyltransferase activity may be important in vivo but is probably not involved in the in vitro activation that we detect, since our template is naked plasmid DNA without histones. An additional contribution of CBP could be the stabilization of Tax-CREB complexes (27), but our system uses saturating levels of Tax and CREB. It is still possible that in our system, CBP functions as a coactivator through interactions with RNAP II that are mediated by RNA helicase A (46, 66). While contributions by CBP to the Tax activation that we observe in vitro cannot be ruled out, two (STF and PARP) of the three required coactivator activities that we have identified do not contain CBP. This implies that both CBP and PARP may be required for complete Tax-activated transcription. Finally, since the presence of PARP stimulates Tax-activated transcription in vivo, we conclude that PARP therefore plays a role in the HTLV-1 life cycle.

We are currently in the process of further purifying STF in order to determine its identity and examine its function. Since the precise role of STF and PARP in coactivation remains unclear, future experiments will need to address the mechanism of coactivation with Tax. Is there a direct protein-protein interaction between PARP, STF, and Tax? Our initial experiments have not been able to detect protein-protein interactions between PARP and Tax. It is possible that PARP may form DNA-bound complexes with Tax and the GTFs. Our Tax-stimulated in vitro transcription system provides a tool for dissecting the mechanism of transcriptional activation in the context of a natural transcription factor.

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