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Human T-Cell Leukemia Virus Type 1 Tax Transactivates the Human Proliferating Cell Nuclear Antigen Promoter

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The human T-cell leukemia virus type 1 (HTLV-1) transforming protein, Tax, is a potent transactivator of both viral and cellular gene expression. The ability of Tax to transform cells is believed to depend on its transactivation of cellular-growth-regulatory genes. Expression of proliferating cell nuclear antigen (PCNA) is intimately linked to cell growth and DNA replication and repair. By testing a series of PCNA promoter deletion constructs, we have demonstrated that the PCNA promoter can be transactivated by Tax. The smallest construct that was activated did not include the ATF/CRE binding site at nucleotide −50, and mutations in the ATF/CRE element in the context of a larger promoter were still activated by Tax. In addition, a Tax mutant that is defective for activation of the CRE pathway retained the ability to activate the −397 promoter construct. When a series of linker scanner mutations that span the region from nucleotide −45 to −7 were assayed, mutations in and around a repeat sequence were found to abolish Tax transactivation. Multimerized copies of either half of the repeat were Tax responsive. A single protein complex was shown to bind specifically to the Tax-responsive region, and the binding of this complex was enhanced in the presence of Tax. These results demonstrate that the PCNA promoter contains a Tax-responsive element located between nucleotides −45 and −7 whose sequence is different from those of other, previously identified Tax-responsive elements. The ability of Tax to activate the PCNA promoter may play an important role in cellular transformation by HTLV-1.

Human T-cell leukemia virus type 1 (HTLV-1) is associated with two disparate clinical syndromes: adult T-cell leukemia (41) and tropical spastic paraparesis (also known as HTLV-1-associated myelopathy) (17). In addition to the genes encoding Gag, Pol, and Env, other open reading frames exist at the 3′ end of the HTLV-1 genome. These open reading frames encode regulatory proteins, including Tax and Rex. Several studies have demonstrated that Tax is the transforming protein of HTLV-1. When Tax, under the control of the HTLV-1 long terminal repeat (LTR), was expressed in transgenic mice, the mice developed neurofibromas at 13 to 17 weeks of age (22, 39). In addition, T cells from cord blood lymphocytes infected in vitro with a recombinant herpesvirus saimiri expressing Tax and Rex became immortalized (20). Mutational analysis determined that Tax was necessary for immortalization (19). Finally, Tax cooperated with *ras* in a cotransfection transformation assay in rat embryo fibroblasts (42). The foci of transformed cells were also tumorigenic in nude mice.

Based on the transforming and immortalizing abilities of Tax, there has been much interest in determining the molecular mechanism of its function. Tax is a 40-kDa, mostly nuclear phosphoprotein which positively regulates both viral and cellular gene expression. The Tax protein transactivates the HTLV-1 LTR through *cis*-acting Tax-responsive elements (TRE-1 and TRE-2) in the 5′ U3 region (7, 40). While Tax does not bind to DNA directly, it has been shown to form a complex with the cellular TRE binding proteins cyclic AMP-responsive element binding protein (CREB) and activating transcription factor-1 (ATF-1), which bind TRE-1 (6, 59, 60), and p36, which binds TRE-2 (32).

Transactivation of cellular gene expression by Tax is thought to play an important role in HTLV-1 transformation (27, 39, 50). A number of cellular genes, including those for interleukin-2 receptor α-chain, interleukin-2, vimentin, tumor necrosis factor alpha, and mouse granulocyte-macrophage colony-stimulating factor (24, 30, 35, 46, 53), are activated by Tax through the induction of nuclear factor κB (NF-κB). Other cellular genes, including those for Krox-24 and Krox-20, are activated by Tax through CREB/ATF sites (1, 14). An additional group of cellular genes, including c-*fos*, is regulated by Tax through serum response elements (SRE), and Tax has been shown to form a physical complex with SRE binding proteins ((SRF) 15).

Although many growth factors, growth factor receptors, and transcription factors are transactivated by Tax, the precise mechanism of Tax transformation is not well understood. An intriguing target for viral oncoproteins such as Tax is the DNA replication machinery. For example, the adenovirus E1A oncoprotein has previously been shown to transactivate the proliferating cell nuclear antigen (PCNA) promoter through an ATF/CRE element (26, 36, 38). PCNA is a cofactor of DNA polymerase δ (8, 43) and functions by increasing the enzyme's processivity (52). This activity confers to PCNA an essential role in DNA replication and repair (47, 55). Following stimulation of quiescent cells, PCNA mRNA levels peak during S phase and correlate temporally and spatially with DNA synthesis (2, 8, 25, 34). PCNA has been used as a marker for cellular proliferation in a variety of tumors (10) and is expressed at higher levels in virally transformed cells than in their noninfected counterparts (16). Additionally, when the Molt-4 T-cell line was transformed with HTLV-1, PCNA accounted for over 0.6% of the total labeled protein (9, 34).

To determine whether Tax transactivates the PCNA promoter, truncated PCNA promoter constructs (38) were tested in cotransfection assays. Tax transactivated all of the truncated PCNA promoters tested, including the smallest construct, which did not contain an ATF/CRE binding site. Mutation of

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the ATF/CRE site in the context of a larger promoter did not affect Tax transactivation but did diminish transactivation by adenovirus E1A, which is known to depend on the ATF/CRE site (28, 36). In addition, mutations in Tax that affect activation of the CRE and NF-κB pathways were still able to activate the PCNA promoter. The use of linker scanner mutations localized the Tax-responsive region of the PCNA promoter to a complex repeat that spans nucleotides −45 to −7. Multimerized copies of either the distal or proximal half of the repeat confered Tax responsiveness. The nucleotide sequence of this repeat is distinct from any sequence previously described to be Tax responsive. A protein complex that specifically binds to the PCNA promoter in the absence of a functional ATF/CRE site was identified, and binding of this complex correlated with Tax responsiveness. Also, Tax enhanced the binding of this complex to the Tax-responsive repeat. The ability of Tax to activate PCNA expression may represent an important step in HTLV-1 transformation.

MATERIALS AND METHODS

Plasmids. PCNAInitpBACAT was constructed by cloning the PCNA initiator oligonucleotide (5′-AGCTTCTAGAGGCGGCATTAAACGGTTA-3′), which spans nucleotides −6 to +11, into the *Hin*dIII site in pBACAT (a gift from D. Kurtz, Medical University of South Carolina). The *Xba*I site that was used to clone all upstream sequences is underlined.

ΔATFInitCAT was constructed by generating a PCR fragment by using the −87 to +62 PCNA construct as a template and the primers PCNA-67ΔATF (5′-GCTCTAGAGGCTGGACAGCGTGG**ga**ACGTC-3′), which spans nucleotides −67 to −47, and PCNAInit (5′-CTACGCCTGCAACCG**TcTAga**GCCGC C-3′), which spans nucleotides +21 to −7. Mutations in the ATF/CRE site and in the putative initiator site are shown in lowercase in the PCNA-67ΔATF and PCNAInit primers, respectively, and the *Xba*I site in both primers is underlined. The PCR product was cut with *Xba*I and purified on an agarose gel. This fragment was then cloned into the *Xba*I site in the plasmid PCNAInitpBACAT.

ΔDΔPInitCAT was constructed by cloning the oligonucleotide 5′-CTAGATG GTGACGTCG**atc**CGCGGCGCAGGGTGAGAGCGCGCGC**agt**CGA-3′, which spans nucleotides −55 to −7, into the *Xba*I site of PCNAInitpBACAT. Mutations in the distal and proximal sequences are shown in lowercase.

DistalInitCAT was constructed by cloning two copies of the distal oligonucleotide 5′-CTAGAGTCGCAACGCGGCGCAGGGT-3′, which spans nucleotides −49 to −30, into the *Xba*I site of PCNAInitpBACAT.

ProximalInitCAT was constructed by cloning three copies of the proximal oligonucleotide 5′-CTAGACGCGCTTGCGGACGCGGCGGCT-3′, which spans nucleotides −24 to +1, into the *Xba*I site of PCNAInitpBACAT.

DistalIRInitCAT was constructed by cloning two copies of the oligonucleotide 5′-CTAGACGTCGCAACGA-3′, which spans nucleotides −49 to −40, into the *Xba*I site of PCNAInitpBACAT.

ProximalIRInitCAT was constructed by cloning seven copies of the oligonucleotide 5′-CTAGAGCTTGCGGAA-3′, which spans nucleotides −17 to −9, into the *Xba*I site of PCNAInitpBACAT.

The SVTax plasmid was a gift of Zwi Berneman. The mutant SVΔTax was created by cutting the SVTax plasmid with *Hin*dIII and *Cla*I, trimming with T4 DNA polymerase to produce blunt ends, and recircularizing the plasmid with T4 DNA ligase. This deletion abolishes the translation start site for Tax while maintaining other plasmid sequences.

The adenovirus E1A expression plasmid (p1A) expresses E1A from an adenovirus early promoter (12). pU3RCAT contains the HTLV-1 LTR driving chloramphenicol acetyltransferase (CAT) expression (51). The PCNA promoter constructs −1250, −560, −397, −249, −172, −87, −46, −397ATFBam, −87ATFBam, −87InitBgl, −45, −37, −31, −27, −21, −14, and pGEM-PCNA-CAT (28, 36, 38) and CMV-E1Bp19k (57) have been previously described. The MSV-LUC plasmid was a gift of John Belmont (Baylor College of Medicine).

Antibodies. Monoclonal antibodies to ATF-1 and ATF-2 and a polyclonal antibody to Sp1 were purchased from Santa Cruz Biotechnology. The YY1 polyclonal antibody was a gift of Robert Schwartz (Baylor College of Medicine) (29). The CREB polyclonal antibody was a gift of Marc Montminy (Harvard Medical School) (18).

Transfections and CAT and luciferase assays. HeLa cells were grown in 60-mm-diameter dishes and transfected by calcium phosphate precipitation (11) with a total of 14 μg of DNA, which includes 1 μg of the MSV-LUC plasmid. The CMV-E1Bp19k plasmid was added to transfections with promoter truncations smaller than that of the −249 construct to increase the basal activities of these promoters (21). Cells were harvested at 48 h posttransfection by washing in cold phosphate-buffered saline and scraping in reporter lysis buffer (Promega). The cell pellet was disrupted by a single freeze-thaw cycle, and the cell extract was collected following centrifugation. For the luciferase assay, 5 μl of the total cellular extract was added to 20 μl of reporter lysis buffer and 50 μl of luciferase substrate (Promega). The luciferase activity was quantitated in a Turner TD-20e luminometer. CAT activity was determined by a single phase-extraction assay (45). Briefly, 50 μl of the total cellular extract was added to a mixture of 25 μl of water, 10 μl of 1 M Tris (pH 7.4), 10 μl of 2.5 mM *n*-butyryl-coenzyme A, and 5 μl of xylenes-extracted [^{3}H]chloramphenicol (NEN) at 0.2 μCi/reaction. The CAT assay mixture was incubated overnight at 37°C. The CAT reaction mixtures were processed by adding 200 μl of 2,6,10,14-tetramethyl-pentadecane and xylenes (2:1), vortexing twice for 1 min each with a 5-min rest interval, and centrifuging for 20 min. The top organic phase (140 μl) was added to 4 ml of CytoScint scintillation fluid (ICN), and radioactivity was counted in a Beckman scintillation counter. The data shown for each experiment is from at least three repetitions with different plasmid preparations and is normalized for luciferase activity. Fold activation was determined by dividing the CAT units obtained from the promoter plus Tax by the CAT units obtained from the promoter alone. CAT units were determined by dividing the ^{3}H counts by the luciferase activity for each sample.

RNA analysis. Three 60-mm-diameter dishes of HeLa cells were transfected with the −249 PCNA reporter alone, and an additional three dishes were transfected with SVTax plus the −249 PCNA reporter. Total cellular RNA was extracted by the guanidinium isothiocyanate procedure (4). RNAs from the three identically transfected dishes were pooled after being pelleted through a 5.7 M cesium chloride cushion in a Beckman SW 50.1 rotor at 35,000 rpm overnight. S1 nuclease protection assays were performed as described previously (44) with some modifications. An RNA probe was synthesized by SP6 polymerase transcription of pGEM-PCNA-CAT (38) that was linearized with *Eco*RI. The RNA probe was gel purified, and 10^5 cpm was dried with 12 μg of cytoplasmic RNA under vacuum. Following overnight hybridization at 45°C, the hybrids were digested with 200 or 400 U of S1 nuclease (Boehringer Mannheim) at 30°C for 1 h. The protected probe fragments were analyzed on a 6% polyacrylamide–7 M urea denaturing gel, dried, and exposed to X-ray film. Band intensity was quantitated by scanning the autoradiograph and using Adobe Photoshop to measure the signal intensity as a percentage of 100% black in the scanned image.

Gel shift probe preparation. The wild-type −87 to +1 probe was made by digesting the plasmid −87InitBgl with *Xho*I and *Bgl*II, which released a fragment from nucleotide −112 to +1 (36). The −87ATFBam probe was made by digesting the plasmid −87ATFBam with *Xho*I and *Mse*I, which also released a fragment from nucleotide −112 to +1. The −87 to +1 and −87ATFBam gel shift probes were gel purified, and the ends were filled in by using the Klenow fragment of DNA polymerase in the presence of dGTP, dATP, TTP, and [^{32}P]dCTP. The −46 to +1 probe was made by PCR with the −46 plasmid template, a pBACATR1 sense primer (5′-CAAGAATTCTCATGTTTGACAG C-3′) that annealed to the *Eco*RI site in the plasmid pBACAT, and a PCNA +62 to +44 antisense primer (5′-GCGACGACCGGCTGAGACC-3′). The PCR primers were labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. Following PCR, the reaction mixture was digested with *Mse*I and the labeled probe was gel purified. The competitors used for the experiments with the results shown in Fig. 6 (except −47ΔDΔP) were made by PCR with the pBACATR1 and PCNA +62 to +44 primers and the corresponding plasmid templates. The −47ΔDΔP double-stranded competitor was made by annealing the sense strand 5′-CTAGATC**cagatct**GGCGCAGGGTGAGAGCGCGCGCTT**cagatctg**GGCG GCT-3′ to its antisense strand, both of which contained 4-bp 3′ overhanging ends. The mutations, indicated by lowercase, change both the inverted and direct repeats within the distal and proximal half-sites.

Gel shift assay. The probe (50,000 cpm) was incubated with 1 μl of HeLa nuclear extract (6.2 μg/μl), 0.75 μg of poly(dI)-poly(dC), 6 μl of 5× gel shift buffer (50 mM HEPES [pH 7.9], 20% Ficoll, 250 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol), and a total of 18 μl of buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 17% glycerol, 1 mM EDTA, and 1 mM dithiothreitol) (the 18 μl includes the volume of the extract) in a 30-μl reaction mixture. For competitions, an approximately 100-fold molar excess of unlabeled DNA was added to the reaction mixture before the addition of the HeLa nuclear extract. For gel shift reactions in which the competitors were made by PCR, the amounts of the competitors were equalized by ethidium bromide staining on an agarose gel. The reaction mixtures were incubated for 20 min at room temperature. The antibody supershift experiments were performed by adding the antibody following the initial 20-min incubation and then incubating for an additional hour at room temperature. The extract was diluted 1:12 when *Escherichia coli*-expressed Tax (31) (0.36 μg/μl) was added to the gel shift reaction mixture. The samples were run on a 5% nondenaturing polyacrylamide gel in 45 mM Tris-borate–1 mM EDTA at 150 V for 3 h. The gels were dried and exposed to Kodak XAR5 X-ray film overnight at −70°C with an intensifying screen.

RESULTS

Tax transactivates the PCNA promoter. As an integral link between the cell cycle and the DNA replication and repair machinery, PCNA represents a likely target for transactivation by viral oncoproteins. To determine whether the HTLV-1 Tax protein can transactivate the PCNA promoter, a series of promoter truncations were tested for Tax transactivation. Figure

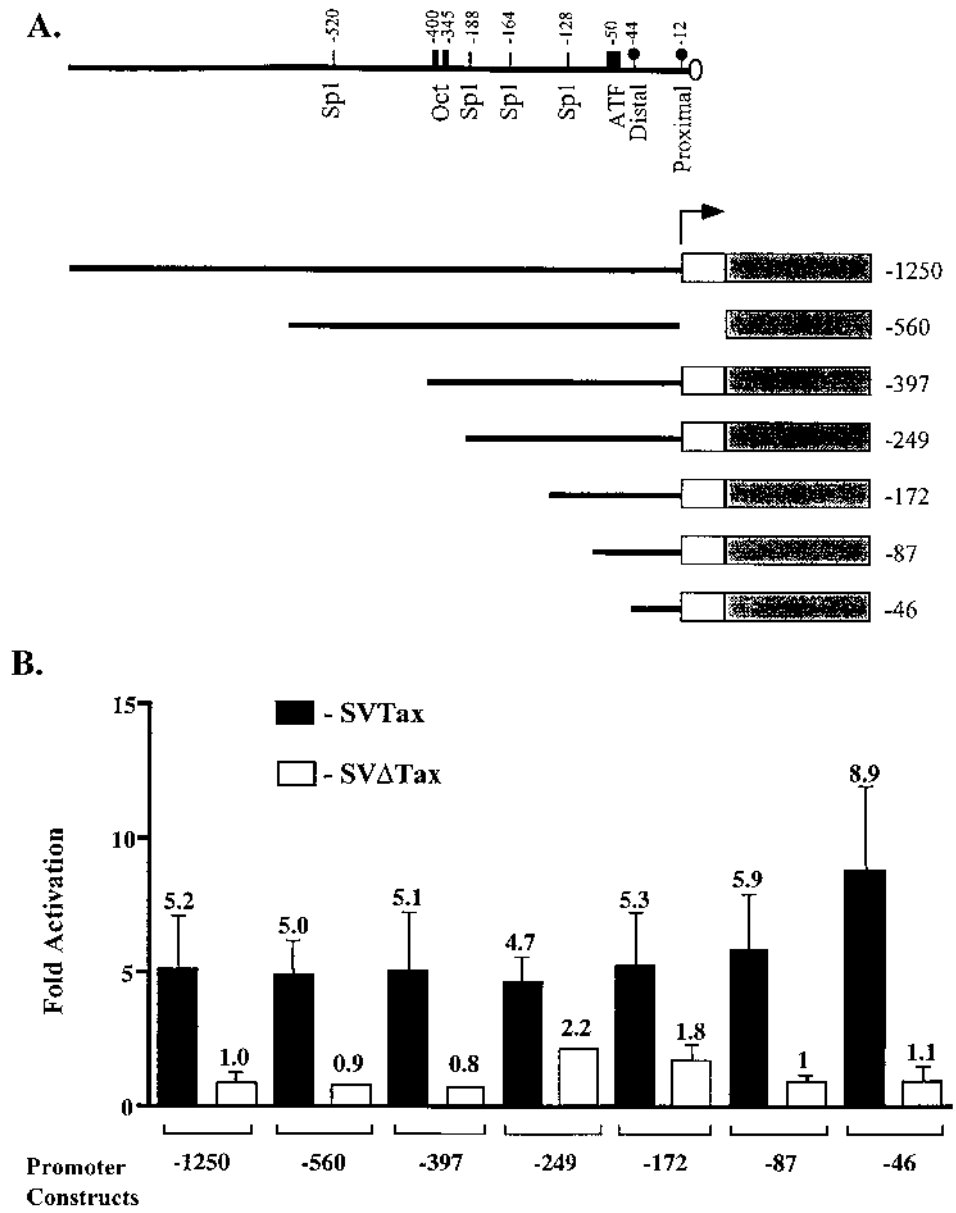

FIG. 1. Tax transactivates the PCNA promoter. (A) Schematic diagram of the PCNA promoter and promoter truncations. Putative transcription factor binding sites are shown at the top. The PCNA promoter contains a sequence with homology to an initiator element (○) which overlaps the transcription start site (┌). The open squares designate transcribed PCNA RNA from nucleotide +1 to +62, and the gray boxes designate the CAT gene in each construct. (B) Activation of the full-length and truncated PCNA promoters by Tax. PCNA promoter constructs (4 μg) were transfected with 6 μg of SVTax (■) or with 6 μg of SVΔTax (□). The resulting activities were plotted as fold activation over that with the corresponding reporter in the absence of Tax. The average fold activation is shown at the top of each bar. The result shown is the average from two experiments performed without an internal control and one with an internal MSV-LUC control plasmid. The standard deviation is shown when greater than 0.2.

1A depicts the structures of the PCNA promoter constructs and the positions of potential transcription factor binding sites (28, 38). The Sp1, Oct, and ATF elements were identified by sequence similarity to other known elements. Binding of ATF to the site at nucleotide −50 was shown to be responsible for a DNase footprint in that region (36); however, binding of Sp1 and Oct to their predicted sites has not been demonstrated. The PCNA promoter does not contain a TATAA box and presumably utilizes an initiator element for accurate initiation of RNA transcription (37, 48). Transfection of Tax with the various promoter deletion constructs resulted in Tax activation ranging from 4.7- to 8.9-fold (Fig. 1B). Tax transactivated the PCNA promoter to similar levels in Jurkat cells, a human T-lymphocyte line (data not shown). Although the degree of Tax transactivation of the PCNA promoter appears to be modest, it is equivalent to that previously reported for E1A activation of this promoter (38) and is greater than the reported fluctuation in PCNA expression throughout the cell cycle (37). A Tax translation mutant (SVΔTax) failed to activate the PCNA promoter deletions (Fig. 1B), confirming that activation is dependent on the Tax protein and is not due to spurious effects of either the simian virus 40 promoter or the backbone of the Tax expression plasmid. Since both the −560 reporter, which contains promoter sequences from nucleotide −560 to

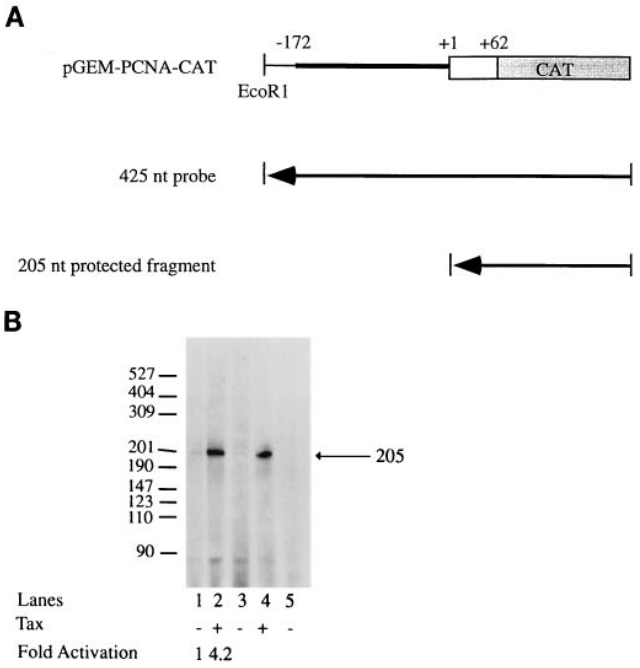

FIG. 2. Tax activation of the PCNA promoter occurs at the level of transcription. (A) A 425-nucleotide (nt) RNA probe was generated from the plasmid pGEM-PCNA-CAT. The expected 205-nucleotide protected fragment is illustrated at the bottom. (B) S1 nuclease protection analysis of RNA prepared from untransfected cells (lane 5), cells transfected with the −249 promoter alone (lanes 1 and 3), or cells transfected with both the −249 promoter and SVTax (lanes 2 and 4). Lanes 1 and 2 were digested with 200 U of S1 nuclease, and lanes 3, 4, and 5 were digested with 400 U of S1 nuclease. The position of the protected RNA fragment is shown by the arrow on the right, and the locations of molecular size markers (in nucleotides) are shown on the left. Duplicate plates of cells transfected with the −249 promoter alone or in combination with Tax were analyzed for CAT activity. The fold activation from the CAT assay is shown at the bottom.

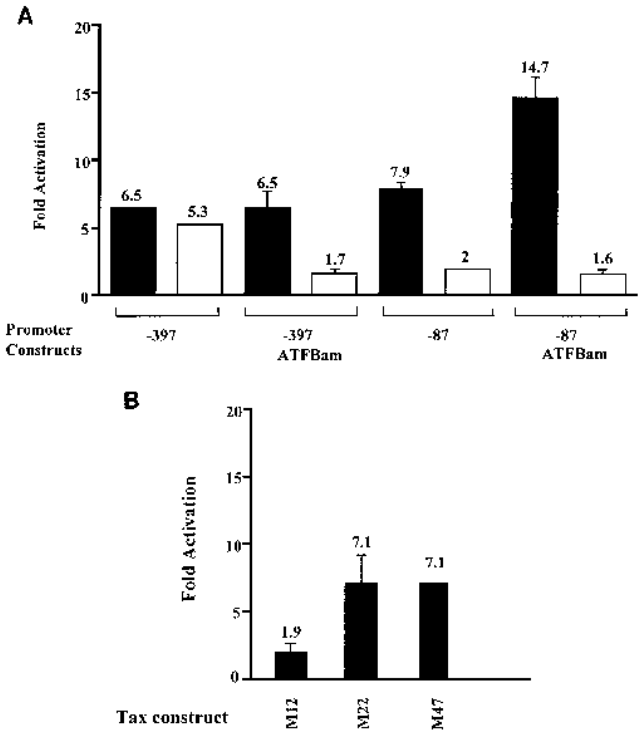

FIG. 3. Tax transactivation of the PCNA promoter is independent of the ATF element. (A) Cells were transfected with 4 μg of −397, −397 bearing a 4-bp mutation in the ATF site (−397ATFBam), −87, or −87 bearing a 4-bp mutation in the ATF site (−87ATFBam). The cells were cotransfected with 8 μg of SVTax (■) or 2 μg of p1A-expressing adenovirus E1A (□). (B) Cells were transfected with 4 μg of the −397 reporter alone or together with 6 μg of Tax mutant M12, M22, or M47. The fold activation was calculated as described in the legend to Fig. 1 and is shown above each bar. The error bars depict the standard deviations from two independent experiments.

−2, and the −46 reporter, which contains promoter sequences from nucleotide −46 to +62, are activated by Tax, the minimal Tax-responsive element must reside between nucleotides −46 and −2.

Tax activates the PCNA promoter at the level of RNA. An S1 nuclease protection assay was used to ascertain whether observed increases in CAT enzyme activity in the presence of Tax reflect elevated mRNA synthesis. RNA prepared from cells transfected with the −249 PCNA promoter either alone or in combination with Tax was analyzed by an S1 nuclease protection assay. A diagram of the RNA probe and the expected size of the protected fragment is shown in Fig. 2A. A 4.1-fold increase in PCNA-CAT mRNA derived from the −249 promoter was observed in Tax-transfected cells (Fig. 2B, lanes 2 and 4) compared with that seen in cells transfected with the promoter alone (Fig. 2B, lanes 1 and 3). It was difficult to visualize bands in lanes 1 and 3 due to low expression of the PCNA promoter in the absence of Tax. No specific band was seen in untransfected cells (Fig. 2B, lane 5). Tax activated the −249 promoter 4.2-fold as determined by measuring CAT activity in similarly transfected cells. Thus, the level of RNA expression corresponded well with CAT activity, demonstrating that Tax transactivation of the PCNA promoter results primarily from increased levels of correctly initiated mRNA.

Tax transactivation of the PCNA promoter is not dependent on the ATF/CRE element. Since ATF/CRE elements are known to be important targets for Tax transactivation of the HTLV-1 promoter as well as certain cellular genes, we wanted to confirm the results of the deletion analysis which suggested that the ATF/CRE site was not involved in Tax transactivation of the PCNA promoter. Specifically, the smallest PCNA promoter truncation tested, the −46 construct was transactivated nearly ninefold by Tax (Fig. 1B). This deletion construct does not contain the core ATF/CRE site, TGACGT, which is centered at nucleotide −50 and is important both for basal activity of the PCNA promoter and for transactivation by adenovirus E1A (38). To further determine whether the ATF/CRE binding site was necessary for Tax transactivation, it was mutated in the context of the larger −397 PCNA promoter construct. When the wild-type −397 construct was compared to the mutant −397ATFBam construct (36), the levels of Tax transactivation were similar (Fig. 3A) (6.5-fold). In contrast, E1A transactivation of the wild-type −397 construct was greater than that of the mutant −397ATFBam construct (5.3- and 1.7-fold, respectively), in agreement with previously published results (36). Tax and E1A transactivation was also tested on the shorter −87 promoter and its corresponding ATF/CRE mutant (−87ATFBam). Tax activation of the mutant −87ATFBam construct was not reduced when compared to that of the wild-type −87 construct (14.7- and 7.9-fold, respectively), again demonstrating that Tax transactivation of the PCNA promoter does not depend on the ATF/CRE binding site. E1A activation of the −87 and −87ATFBam constructs was not detected, in agreement with the results of others, who reported that E1A activation of these reporters is difficult to detect due

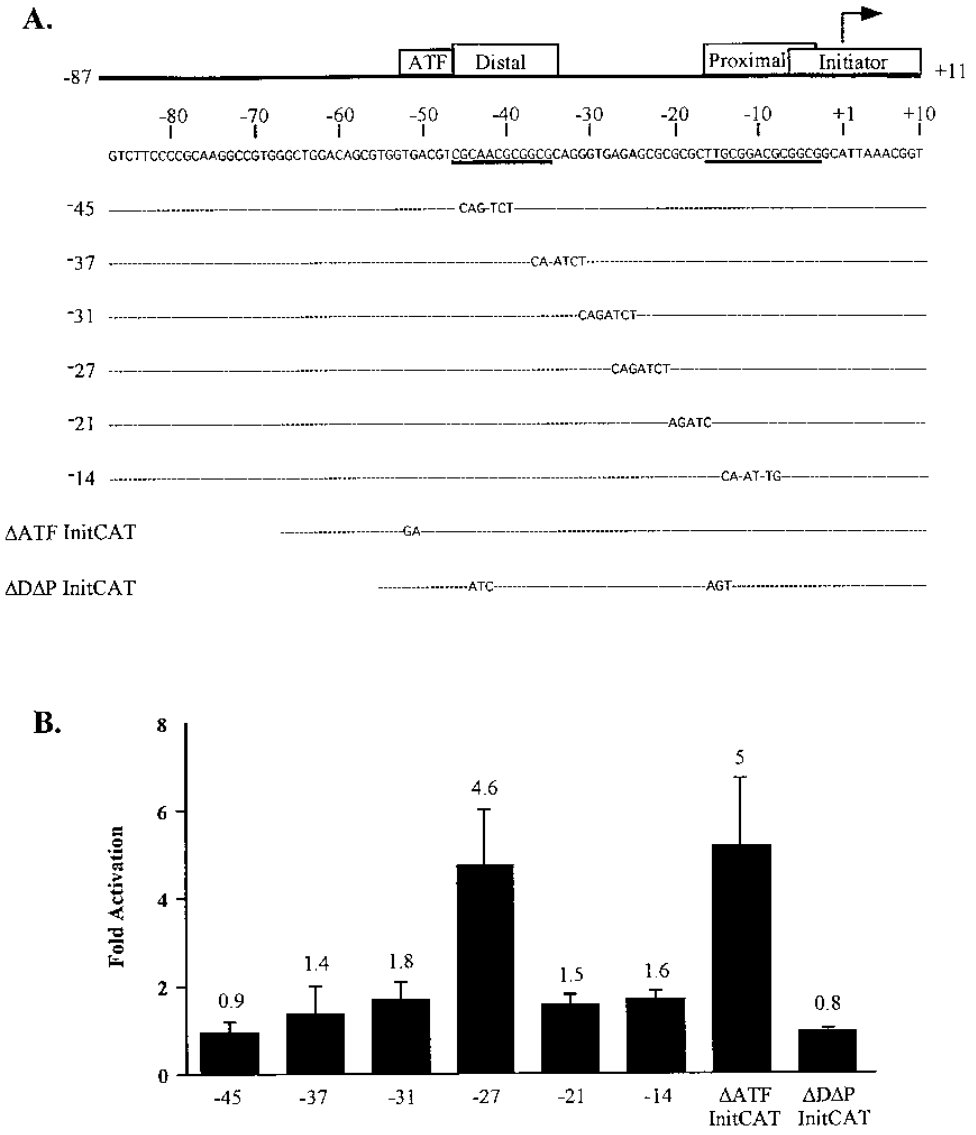

FIG. 4. Tax activation of the PCNA promoter is dependent on a repeat region. (A) The PCNA promoter from nucleotide −87 to +11 with putative transcription factor binding sites is diagrammed at the top. The distal and proximal repeats are underlined. The specific mutations in each linker scanner mutant are shown below. (B) Cells were transfected with 4 μg of a linker scanner mutant alone or in combination with 6 μg of SVTax. Fold activation was calculated as described in the legend to Fig. 1 and is shown above each bar. The error bars depict the standard deviations from three independent experiments.

to low basal activity (38). The observation that Tax transactivation actually increased on the −87ATFBam mutant reporter suggests that the binding of ATF may compete for the binding of an adjacent factor required for Tax transactivation of the PCNA promoter.

Tax mutants that are defective for activation of the CRE and NF-κB pathways activate the PCNA promoter. Tax has previously been shown to activate promoters containing ATF/CRE, SRE, or NF-κB elements. To confirm that the ATF/CRE binding site within the PCNA promoter was not required for Tax activation, Tax mutants that fail to activate ATF/CRE (M47)- or NF-κB (M22)-dependent promoters (49) were analyzed for their ability to activate the −397 PCNA promoter construct (Fig. 3B). Both M47 and M22 activated the −397 PCNA promoter (7.1-fold) to levels similar to that seen with wild-type Tax (6.5-fold) (Fig. 3A), while another mutant, M12, which is defective for activation of both ATF/CRE- and NF-κB-dependent promoters, did not activate the PCNA promoter (1.9-fold). Together with Tax activation of a PCNA promoter containing a mutation in the ATF site, these results demonstrate that Tax activation of the PCNA promoter does not depend on the ATF/CRE pathway. Thus, Tax activation of the PCNA promoter likely involves a previously uncharacterized Tax-responsive element.

Tax transactivation of the PCNA promoter is dependent on a novel Tax-responsive sequence. After narrowing of the Tax-responsive region of the PCNA promoter to nucleotides −46 to −2, visual inspection of this sequence revealed a complex element containing an inverted repeat (TTGCG) adjacent to a direct repeat (ACGCGGCG). The combined inverted and direct repeats are found in both the distal and proximal elements, which are underlined in Fig. 4A. These GC-rich repeats have no direct homology to known transcription factor binding sites or to previously characterized Tax-responsive elements. A

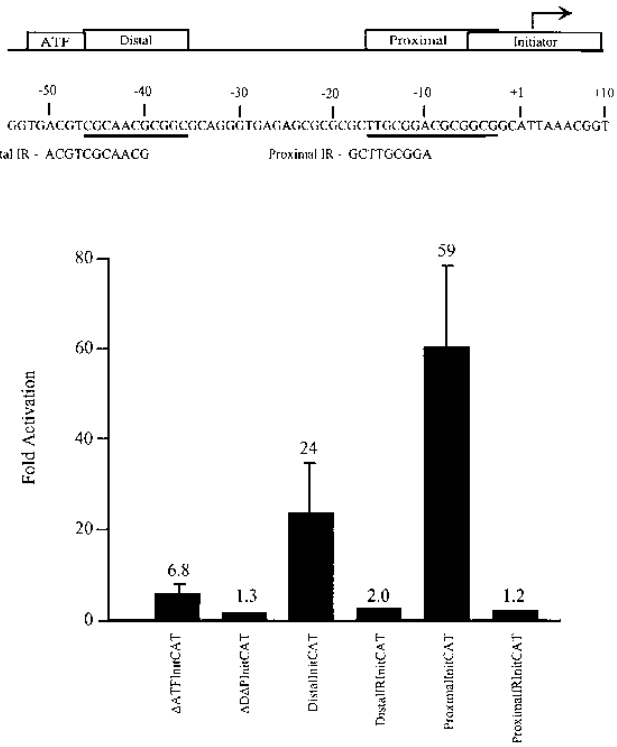

FIG. 5. Multiple copies of the distal or proximal halves of the Tax-responsive repeat are Tax responsive. Four micrograms of the indicated reporter plasmids was transfected alone or with 6 μg of SVTax into HeLa cells. The fold activation was calculated as described in the legend to Fig. 1 and is shown above each bar. The error bars depict the standard deviations from two independent experiments.

set of six linker scanner mutants spanning nucleotides −45 to −7 (28) was used to further define the Tax-responsive element. The two linker scanner mutations that affected the distal half of the repeat (−45 and −37) abolished Tax activation (0.9- and 1.4-fold, respectively). An additional linker scanner mutation, −31, did not directly change any nucleotides of the direct or indirect repeats yet showed reduced Tax transactivation (1.8-fold). The proximity of this mutation to the direct repeat may affect protein binding and thus Tax transactivation of this construct. Two mutations that affected the proximal half of the repeat region (−21 and −14) also abrogated Tax activation (1.5- and 1.6-fold, respectively). Only the −27 mutant, which contains wild-type distal and proximal repeats, was activated by Tax (4.6-fold), and this activation was similar to Tax activation of the ΔATFInitCAT reporter (5-fold), which also contains wild-type repeats. This result suggests that the Tax-responsive element in the PCNA promoter resides within a complex repeat sequence (−46 to −36 [distal] and −16 to −3 [proximal]) (Fig. 4). Confirming this result, a construct containing a more subtle 3-bp mutation in both the distal and proximal halves of the repeat, ΔDΔPInitCAT, was not Tax responsive.

Multimerized copies of the distal or proximal half-site confer Tax responsiveness. To further define the Tax-responsive element within the PCNA promoter, multiple copies of the distal or proximal PCNA promoter sequences were cloned upstream of the PCNA initiator driving CAT gene expression. These constructs were transfected into HeLa cells either alone or with SVTax, and their activities were compared (Fig. 5). Relative to Tax activation of a construct containing a wild-type PCNA promoter sequence (ΔATFInitCAT) (6.8-fold), Tax activated multiple copies of either the distal element (DistalInitCAT) (24-fold) or the proximal element (ProximalInitCAT) (59-fold). When multiple copies of truncated versions of the distal and proximal elements, Distal IR (nucleotides −49 to −40) and Proximal IR (nucleotides −17 to −8), were tested, Tax activation was reduced (2- and 1.2-fold, respectively). Finally, the ΔDΔPInitCAT reporter, containing a wild-type copy of the ATF/CRE site but mutations in both the distal and proximal sites, displayed reduced Tax activation (1.3-fold). Thus, Tax transactivation of the PCNA promoter correlates with the presence of an imperfect, GC-rich, bipartite element. At least two copies of the distal (CGCAACGCGGCG) or proximal (TTGCGGACGCGGCG) half of this repeat are required for Tax transactivation.

Formation of a protein complex on the PCNA promoter independent of the ATF/CRE site. Since Tax does not bind DNA directly, its transactivation depends on interactions with cellular DNA binding proteins. Thus, the binding of cellular proteins to the Tax-responsive repeat region of the PCNA promoter was analyzed. It has previously been suggested that the inverted repeat may provide binding sites for the cellular transcription factor YY1 (38). However, further inspection of the repeat region of the PCNA promoter revealed little homology to a consensus YY1 binding site. To characterize cellular binding proteins and to directly determine whether YY1 binds the Tax-responsive repeat within the PCNA promoter, a probe containing sequences spanning nucleotides −87 to +1 was used in a gel shift assay. When a nuclear extract was incubated with the −87 to +1 probe, four specific complexes were resolved (Fig. 6A, lane 2). These complexes were all competed in the presence of unlabeled −87 to +1 DNA (Fig. 6A, lane 3).

To establish the identities of the complexes, a series of antibodies were incubated with the probe and nuclear extract. When a polyclonal antibody to YY1 was added, no supershift was seen (Fig. 6A, lane 4). In a concurrent experiment the YY1 antibody supershifted YY1 protein when bound to the skeletal α-actin SRE 1 (29) (data not shown), suggesting that YY1 does not bind to the Tax-responsive region in the PCNA promoter. In the presence of a monoclonal antibody to ATF-1, complexes 1, 2, and 3 were either obliterated or shifted to a position above complex 4, while complex 4 remained unaffected (Fig. 6A, lane 5). Complexes 1, 2, and 3 were also either obliterated or shifted in the presence of a polyclonal antibody to CREB, while complex 4 again remained unaffected (Fig. 6A, lane 8). Monoclonal antibodies to either ATF-2 or Sp1 did not shift any of the bands (Fig. 6A, lanes 6 and 7, respectively), demonstrating the specificity of the ATF-1 and CREB antibody shifts.

To confirm that complexes 1, 2, and 3 were dependent on the ATF/CRE site at nucleotide −50, a −87 to +1 sequence containing a 4-base mutation in the ATF/CRE site (−87ATF-Bam) was used as a probe. Complexes 1, 2, and 3 did not form on this probe, while a complex comigrating with complex 4 was observed (Fig. 6A, lane 9). In addition, complexes 1, 2, and 3 were competed by an unlabeled ATF/CRE element, while complex 4 was unaffected (data not shown). Thus, the human PCNA promoter, as previously observed for the mouse PCNA promoter (23), can bind members of the ATF/CREB family of transcription factors. Complexes 1, 2, and 3 contain CREB/ATF family members, and formation of these complexes is dependent on the presence of the ATF/CRE site. Complex 4 results from the binding of a factor(s) other than CREB/ATF family members, and the formation of this complex is dependent on interactions with sequences other than the ATF/CRE element, making it a candidate for binding to the Tax-responsive repeat within the −87 to +1 PCNA promoter sequence.

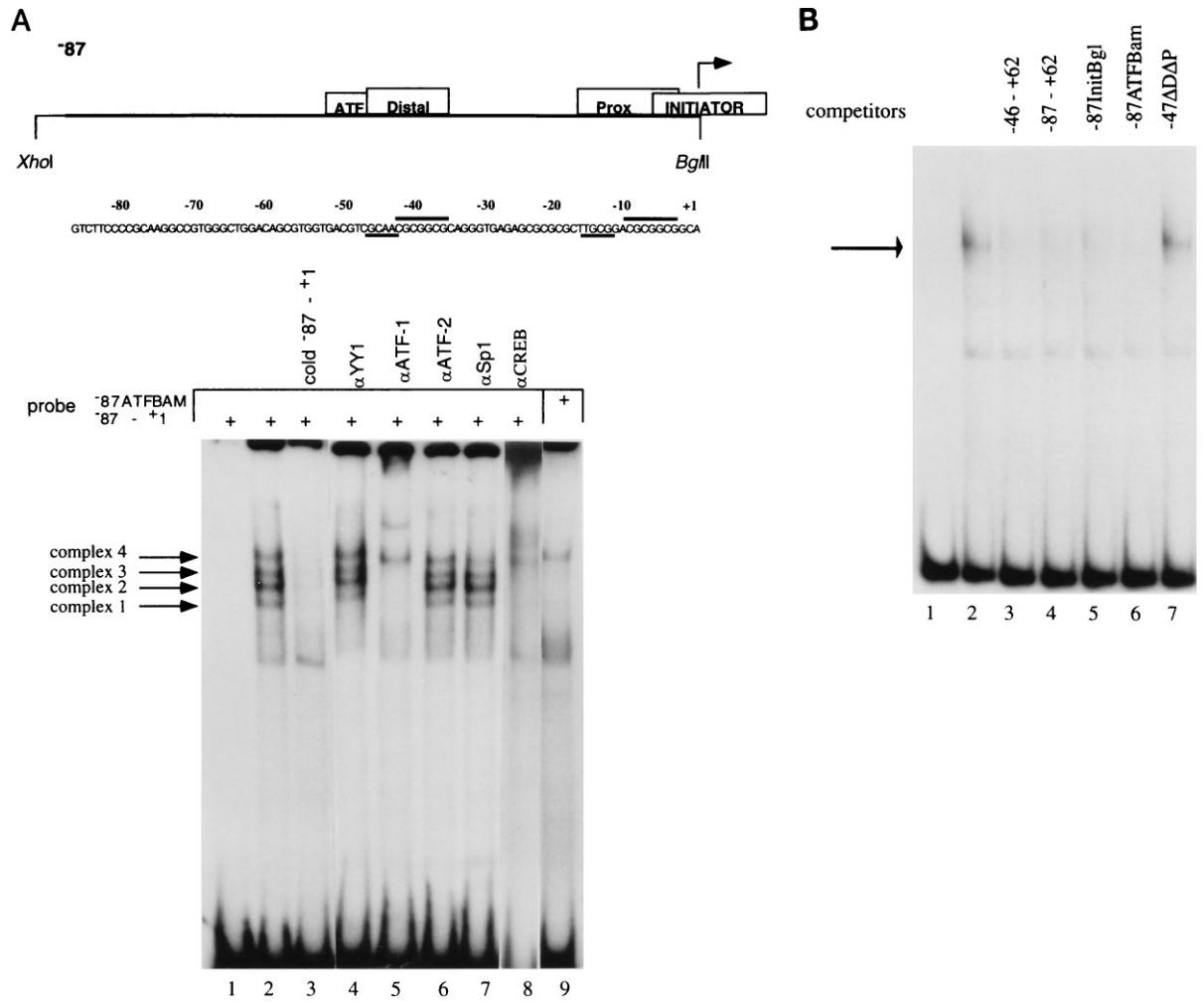

FIG. 6. A specific protein complex binds to the PCNA promoter independent of the ATF site. (A) The sequence of the −87 to +1 probe is shown at the top. The positions of distal and proximal sequences are shown relative to the ATF site, and the inverted and direct repeats are underlined and overlined, respectively, in the nucleotide sequence. The −87 to +1 probe was incubated with nuclear extract (lanes 2 to 8). A specific competitor was added in lane 3, and antibodies were added as indicated in lanes 4 to 8. The complex formed on the −87ATFBam probe is shown in lane 9. (B) The probe (−46 to +1) was mixed with HeLa nuclear extract. The competitors indicated above each lane were in approximately 100-fold excess over the probe concentration. The arrow indicates the position of the specific complex.

Specific binding of a protein complex to the Tax-responsive element. To determine whether protein binding was directly dependent on the Tax-responsive region of the PCNA promoter, the smallest promoter element that was activated by Tax in vivo was used in a competition gel shift experiment. A single complex formed on the −46 to +1 probe, which does not contain the ATF/CRE element (Fig. 6B, lane 2). Promoter elements that contained the wild-type Tax-responsive region, i.e., −46 to +62, −87 to +62, −87InitBgl, and −87ATFBam, each competed for complex formation (Fig. 6B, lanes 3, 4, 5, and 6, respectively). All of these competitors, except −87InitBgl, which has not been tested, were responsive to Tax in vivo. Importantly, the −47ΔDΔP competitor, which contains mutations in the distal and proximal repeats, did not affect the formation of the complex on the −46 to +1 probe (Fig. 6B, lane 7), and when −47ΔDΔP was used as a probe, the specific complex was not formed (data not shown). In addition, a construct containing similar mutations, ΔDΔPInitCAT, was not responsive to Tax in vivo (Fig. 4 and 5). These experiments demonstrate a correlation between Tax activation of the PCNA promoter in vivo and the formation of a specific DNA-protein complex in vitro.

Tax enhances the binding of a complex to the Tax-responsive repeat of the PCNA promoter. Tax has the ability to increase the DNA binding affinity of bZIP transcription factors (56). Therefore, the effect of Tax on the binding affinity of the specific complex formed on the −46 to +1 Tax-responsive repeat was examined (Fig. 7, lane 1). When the extract was diluted 1:12, the specific complex was not visible (Fig. 7, lane 2), but addition of increasing amounts of purified Tax protein in combination with the diluted extract increased the affinity of the specific complex (lanes 3 and 4). A bacterial extract that does not express Tax did not augment the binding of the specific complex to the −46 to +1 probe (Fig. 7, lanes 5 and 6). This result suggests that Tax has a direct affect on the protein(s) that binds the Tax-responsive repeat and suggests a mechanism for Tax transactivation of the PCNA promoter.

DISCUSSION

In an effort to understand the transforming and immortalizing properties of the HTLV-1 Tax protein, we have investigated the ability of Tax to transactivate the PCNA promoter. This investigation demonstrated that (i) Tax can activate the

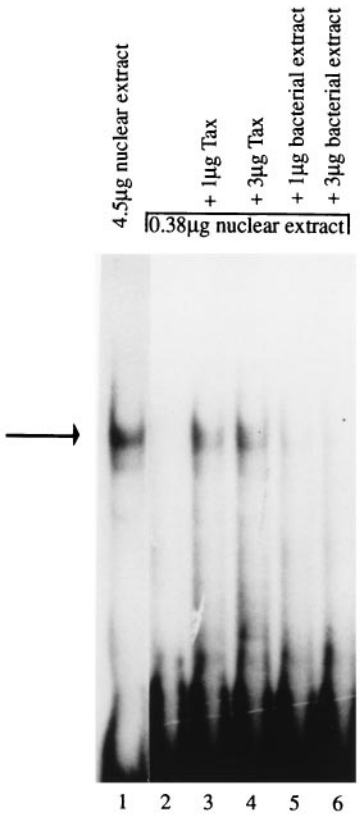

FIG. 7. Tax can enhance the formation of the Tax-responsive repeat binding complex. The −46 to +1 probe was mixed with HeLa nuclear extract (4.5 μg) for gel shift analysis (lane 1). The extract was diluted 1:12 (lanes 2 to 6). Either 1 μg (lane 3) or 3 μg (lane 4) of Tax protein was added to the gel shift reaction mixture. Equivalent concentrations of an identically prepared *E. coli* extract that did not express Tax were added to lanes 5 and 6. The specific complex that forms on the −46 to +1 probe is indicated by the arrow. Lanes 2 to 6 were exposed to film five times longer than lane 1.

PCNA promoter, (ii) the Tax-responsive element in this promoter resides within a complex repeat element that is different from known cellular transcription factor binding sites and from previously characterized Tax-responsive elements, and (iii) the Tax-responsive region binds a specific cellular protein complex whose binding is enhanced by Tax. The Tax-responsive region of the PCNA promoter has been narrowed to a 44-bp sequence located between nucleotides −46 and −2 which contains a direct and an indirect repeat. Although the distal and proximal halves of the repeat are both required for Tax activation, we have localized Tax responsiveness specifically to either the direct or indirect components of the repeats. The −45 and −14 linker scanner mutations alter both the direct and indirect repeats within the distal or proximal half-sites, respectively, and abolish Tax activation. Linker scanner mutations that affect only the distal direct repeat (−37) and a mutation that is near the distal direct repeat (−31) abrogate Tax activation. A linker scanner mutation within the proximal indirect repeat (−21) also had defective Tax transactivation. A mutation between the distal and proximal halves of the repeat (−27) does not affect Tax activation, illustrating that the distal and proximal halves are discrete units. Truncation of the distal and proximal elements affected the direct repeat within each half-site and abrogated Tax responsiveness. These results demonstrate that sequences within the direct repeat are necessary for Tax transactivation. From these studies, we cannot discount a possible role of sequences within the indirect repeat in Tax transactivation. The direct and indirect repeats are sufficient for Tax transactivation, as demonstrated by the ability of multimerized copies of the distal or proximal sequences to support Tax transactivation. It is possible that the Tax-responsive element in the PCNA promoter does not directly require the repeats but rather may encompass this region while utilizing an extended set of nucleotides.

Since Tax has previously been shown to function through ATF elements, it was important to confirm that the ATF element around nucleotide −50 is not involved in Tax activation. Toward this end, we have demonstrated that Tax can transactivate promoters containing a mutation in the ATF site (−397ATFBAM and −87ATFBAM) and the truncated −46 promoter that does not contain the ATF site. In corroboration, a Tax mutant that is defective for activation of ATF/CRE-dependent promoters was capable of activating the PCNA promoter. We have also shown that ATF-1 and CREB form complexes on probes which contain the ATF site, indicating that this site is functional. Promoter sequences which lacked a functional ATF site failed to bind CREB/ATF family members but formed a unique DNA-protein complex (complex 4) in gel shift experiments. A mutant affecting both the direct and indirect repeats was not transactivated by Tax in vivo and did not compete for the specific complex in a gel shift. Therefore, the ability to respond to Tax in vivo correlates with the ability to form a specific gel shift complex in vitro. At present we do not know the identity of the factor(s) bound to the Tax-responsive element.

A number of possible mechanisms for Tax regulation of the PCNA promoter can be envisioned. Tax could physically bind to the protein(s) in this complex, bringing it into the proximity of the PCNA promoter where it could influence transcriptional activity. Tax has previously been shown to form a physical complex with the cellular transcription factors CREB, SRF, and p36, and this association appears to affect both viral and cellular gene expression (15, 32, 60). Alternatively, Tax may affect the subcellular localization of cellular transcription factors that regulate PCNA expression. For example, Tax has previously been shown to increase the nuclear concentration of NF-κB/Rel family members, resulting in activated expression of genes containing a κB element (5). Finally, Tax could affect the binding affinity of the protein(s) which binds to the Tax-responsive element in the PCNA promoter, resulting in increased activity of the promoter. Tax has been shown to increase the affinity of a diverse group of transcription factors for their binding sites (3). Based on the ability of Tax to increase formation of the specific complex assembled on the Tax-responsive repeat in the PCNA promoter, it is likely that Tax affects the binding affinity of the protein(s) that forms this complex and that this increase in binding affinity plays a role in Tax activation of the PCNA promoter. The Tax-induced increase in affinity of bZIP transcription factors such as CREB and ATF has been attributed to an increase in dimerization of these factors (56). By inference, the cellular factor(s) that binds the Tax-responsive repeat in the PCNA promoter may function as a dimer. It remains possible that additional mechanisms such as direct binding or alteration of subcellular localization may also contribute to Tax regulation of the PCNA promoter.

The activation of PCNA gene expression by Tax may play an important role in HTLV-1-mediated transformation. Because transcriptional regulation of PCNA during the cell cycle appears to be nominal (two- to threefold during the G_1-to-S transition) (37), even relatively small increases in transcription induced by Tax could manifest profound changes in cell-cycle-

controlled DNA replication. Although it is not well understood how PCNA expression is controlled transcriptionally during the cell cycle, the function of PCNA at the protein level is regulated by the cdk inhibitor p21 (58). p21 can bind to PCNA and inhibit PCNA-dependent DNA replication in vitro (13, 54). Overexpression of PCNA by Tax may increase the pool of uncomplexed PCNA protein. This free PCNA protein could then induce DNA replication to proceed without pausing in G_1 to repair DNA damage, resulting in continuous cell proliferation. The inability to survey DNA for deleterious mutations prior to replication may lead to an accumulation of harmful mutations, resulting in transformation. Indeed, cells taken from HTLV-1-infected adult T-cell leukemia and tropical spastic paraparesis/HTLV-1-associated myelopathy patients exhibit a variety of DNA damage (33). This type of mechanism could account for the low percentage of infected individuals who develop disease and for the long latency period between infection by HTLV-1 and the onset of disease.

HTLV-1 Tax joins adenovirus E1A in constituting a group of viral oncoproteins that activate PCNA gene expression. Identification of this common target of viral oncoproteins strengthens the likelihood that PCNA plays an important role in viral transformation and immortalization. Further analysis of Tax transactivation of the PCNA promoter is likely to yield important insights into the mechanism of HTLV-1 transformation.

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