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Article *in* Journal of Virology · November 1994

DOI: 10.1128/JVI.68.10.6170-6179.1994 · Source: PubMed

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JOURNAL OF VIROLOGY, Oct. 1994, p. 6170–6179
0022-538X/94/$04.00+0

Vol. 68, No. 10

Transcription of the Human T-Cell Lymphotropic Virus Type I Promoter by an α-Amanitin-Resistant Polymerase

GRAZIELLA PIRAS, FATAH KASHANCHI, MICHAEL F. RADONOVICH, JANET F. DUVALL, AND JOHN N. BRADY*

Laboratory of Molecular Virology, National Cancer Institute, Bethesda, Maryland 20892

Received 23 March 1994/Accepted 22 June 1994

The human T-lymphotropic virus type I (HTLV-I) promoter contains the structural features of a typical RNA polymerase II (pol II) template. The promoter contains a TATA box 30 bp upstream of the transcription initiation site and binding sites for several pol II transcription factors, and long poly(A)$^+$ RNA is synthesized from the integrated HTLV-I proviral DNA in vivo. Consistent with these characteristics, HTLV-I transcription activity was reconstituted in vitro by using TATA-binding protein, TFIIA, recombinant TFIIB, TFIIE, and TFIIF, TFIIH, and pol II. Transcription of the HTLV-I promoter in the reconstituted system requires RNA pol II. In HeLa whole cell extracts, however, the HTLV-I long terminal repeat also contains an overlapping transcription unit (OTU). HTLV-I OTU transcription is initiated at the same nucleotide site as the RNA isolated from the HTLV-I-infected cell line MT-2 but was not inhibited by the presence of α-amanitin at concentrations which inhibited the adenovirus major late pol II promoter (6 μg/ml). HTLV-I transcription was inhibited when higher concentrations of α-amanitin (60 μg/ml) were used, in the range of a typical pol III promoter (VA-I). Neutralization and depletion experiments with three distinct pol II antibodies demonstrate that RNA pol II is not required for HTLV-I OTU transcription. Antibodies to basal transcription factors TATA-binding protein and TFIIB, but not TFIIIC, inhibited HTLV-I OTU transcription. These observations suggest that the HTLV-I long terminal repeat contains overlapping promoters, a typical pol II promoter and a unique pol III promoter which requires a distinct set of transcription factors.

The human T-lymphotropic virus type I (HTLV-I) is the causative agent of adult T-cell leukemia and the degenerative neuromuscular disease tropical spastic paraparesis/HTLV-I-associated myelopathy (4, 6, 43, 69, 97). Replication of HTLV-I is regulated at the transcriptional and posttranscriptional levels by its own gene products, Tax_1 and Rex_1 (1, 22, 26, 27, 34, 36, 41, 82). The transactivator protein, Tax_1, activates transcription of the viral genome and expression of several cellular growth-regulatory genes and cytokines (20–22, 25, 31, 40, 44, 49, 64, 71, 82, 92). The HTLV-I promoter, which contains several *cis*-acting transcriptional regulatory sequences, is located within the 5′ long terminal repeat (LTR) (45, 70, 74). Within the LTR, several Tax_1-responsive elements have been shown to confer Tax_1 responsiveness through binding of ATF/CREB family members, Ets1, Ets2, Sp1, TIF-1, and Myb (5, 7–9, 24, 29, 58, 65, 66, 77, 88, 90, 98).

In eukaryotic cells, transcription is carried out by three different polymerases, RNA polymerases (pol) I, II, and III, distinguished primarily on the basis of their purification patterns and on α-amanitin sensitivities (13, 73, 76). Each enzyme is involved in the transcription of a different class of genes: pol I transcribes rRNAs, pol II transcribes protein-coding genes, and pol III transcribes a series of small cellular and viral RNAs (class 1 [5S RNA], class 2 [tRNA, 7SL, *Alu*, Ad2VA-I, and VA-I], and class 3 [U6, 7SK, hY1, hY3, H1, and MRP/Th RNAs] [reviewed in reference 35]). The genes transcribed by each polymerase have distinct promoter structures but include basal and modulator promoter elements.

Interestingly, transcription factors which are required for pol I, II, and III are not as distinct as once perceived. In fact, some of the basal factors or related proteins are shared by two or all three polymerases. For example, TATA-binding protein (TBP) is involved in pol I, II, and III and is associated with a different subset of TBP-associated factors, depending on the type of promoter (18, 35, 38, 46, 51, 52, 57, 79, 87, 93, 94). In yeast cells, a TFIIB-like protein (BRF-1) is involved in pol III transcription (11, 17, 53). Furthermore, TFIIA is involved in pol II and pol III transcription (61). These results suggest that RNA pol I, II, and III may differ in much more subtle ways than was originally suspected.

The experiments described in this work indicate that the HTLV-I LTR contains overlapping transcription units (OTU) which utilize the same transcription initiation site. Reconstitution experiments with purified pol II transcription factors and pol II demonstrate that pol II can transcribe this promoter. In addition, a unique α-amanitin- and tagetitoxin-resistant initiation/elongation complex is able to transcribe the HTLV-I OTU promoter in HeLa whole cell extracts (WCE) and CEM T-cell extracts. Neutralization and depletion experiments with pol II antibodies strongly suggested that classical pol II is not involved in HTLV-I OTU transcription. Interestingly, results of antibody neutralization assays indicate that two general transcription factors, TFIID and TFIIB, are required for this unique transcription complex.

MATERIALS AND METHODS

Antibodies. The RNA pol II carboxy-terminal domain (CTD) monoclonal antibody was purchased from Promega. The antibody raised against exon 5 of RNA pol II was a generous gift of R. Weinmann (Bristol Meyers Squibbs, Princeton, N.J.). The monoclonal antibody against *Drosophila* RNA pol II (ARNA-3) was obtained from International Biotechnology Laboratories, Inc. Antisera against TBP and TFIIB were purchased from Upstate Biotechnology, Inc. The antiserum against TFIIIC2 (TFIIIC2 230-kDa subunit) was a

* Corresponding author. Phone: (301) 496-6201. Fax: (301) 496-4951.

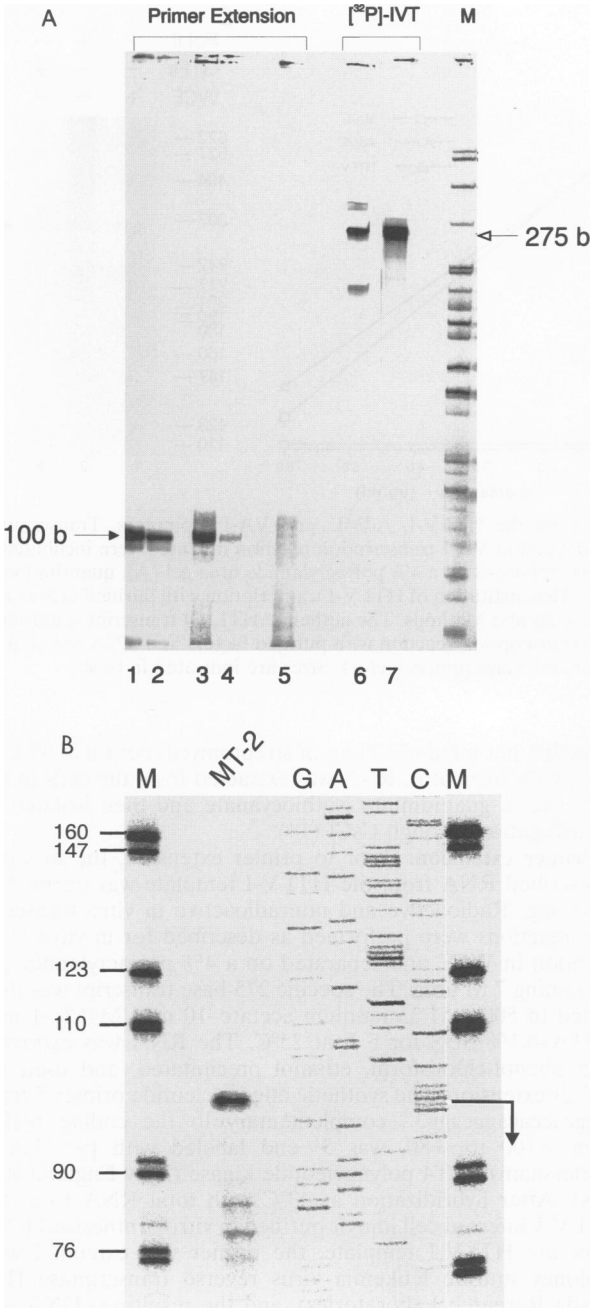

FIG. 1. Primer extension mapping of the transcription initiation site of in vivo and in vitro RNA from the HTLV-I promoter. (A) RNA from the HTLV-I template was synthesized in vitro in the presence of HeLa WCE and nonradioactive nucleotides (lanes 3 and 4) or radioactive nucleotides to monitor the purification procedure (lanes 6 and 7). The 275-base (b) specific transcript (indicated by an open arrow) was then eluted from a 4% polyacrylamide urea gel (lane 6 shows the in vitro transcription [IVT] reaction before elution, and lane 7 shows the purified transcript after elution), annealed to the 5′-end labeled primer (the primer was complementary to the region from +100 to +80 downstream of the start site), and extended as described in Materials and Methods (lanes 3 and 4). Lanes 3 and 4 show the primer extension performed on the RNA synthesized in one (lane 3) or one-half (lane 4) of an in vitro transcription reaction. Primer extension was also performed with 5 μg (lane 1) or 2.5 μg (lane 2) of RNA isolated from HTLV-I infected cell line MT-2. The principal start site of the in vitro RNA or in vivo RNA from the HTLV-I promoter is indicated by a closed arrow. (B) The primer extension cDNA from in vivo RNA was compared with the sequencing ladder obtained by dideoxynucleotide DNA sequencing reactions using the same primer on the HTLV-I template (pU3R). The 5′ end of the HTLV-I RNA corresponded to the cap site determined previously (75). Sizes are indicated in bases. Lanes M, size markers.

generous gift of A. Berk (University of California, Los Angeles).

In vitro transcription in WCE. Promoter-directed transcription in WCE was in 15-μl volumes, and nucleotide concentrations were 500 μM GTP, 500 μM ATP, 500 μM CTP, and 15 μCi of [^{32}P]UTP (Amersham). Final salt and buffer conditions were 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 3 mM $MgCl_2$, and 10% glycerol. When indicated, α-amanitin or tagetitoxin was added to the in vitro transcription reaction mixtures. Reaction conditions were as follows: for HTLV-I, 1 to 3 μl of HeLa WCE (12 mg/ml), prepared as described previously (56), and 300 ng of template (*Hin*dIII-restricted pU3R-CAT); for the adenovirus major late (AdML) promoter, 7.5 μl of HeLa WCE and 300 ng of template (*Bam*HI-restricted pf4 [63]); for VA-I, 3 μl of WCE and 40 ng of unrestricted template. Reaction mixtures were incubated for 60 min at 30°C before reactions were stopped by addition of 400 μl of 20 mM Tris-HCl (pH 8.3)–150 mM NaCl–0.2% sodium dodecyl sulfate (SDS). RNA was extracted with phenol-chloroform, ethanol precipitated, and separated by denaturing gel electrophoresis (4% polyacrylamide gel containing 7 M urea). Gels were exposed to phosphorus screens (Kodak), and bands corresponding to full-length transcripts were quantitated with a PhosphorImager (Molecular Dynamics, Inc.).

For experiments testing the effect of antibodies, HeLa WCE were preincubated for 30 min at 30°C with the antibodies (amounts of antibodies are as indicated in the figure legends) before the template and the nucleotides were added. RNasin, an RNase inhibitor (Promega), was added to all reaction mixtures containing antibodies. All experiments were repeated at least twice.

In vitro transcription with purified factors. Transcription factors (except for TBP) and RNA pol II (54) were generous gifts of D. Reinberg (Robert Wood Johnson Medical School, Piscataway, N.J.) and were purified as described previously: TFIIA (55), recombinant TFIIB (rTFIIB) (33), rTFIIE (67), and rTFIIF (3, 23, 83). All proteins were kept in buffer C containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.9), 10% glycerol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 20 mM β-mercaptoethanol. TBP was from Promega. Transcription reactions were in 35-μl volumes containing 1 μl of BC 100 (20 mM HEPES [pH 7.9], 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 mM KCl, 20% glycerol, 0.01% Triton), 2% polyethylene glycol, 17 mM HEPES, 7 mM $MgCl_2$, and 10 mM dithiothreitol. Transcription factors added to the reaction mixture were 1 μl of TFIIA, 0.12 μl of rTFIIB, 1 μl of TBP, 1.25 μl of rTFIIE, 1 μl of TFIIF, 0.5 μl of TFIIH, and 0.75 μl of RNA pol II. Nucleotide concentrations were 150 μM GTP, 150 μM ATP, 150 μM CTP, and 20 μCi of [^{32}P]UTP (Amersham); 250 ng of linearized HTLV-I template was used. All other conditions were as described above for in vitro transcription in WCE.

Immunodepletion of HeLa WCE. HeLa WCE (40 μl at 12 mg/ml) was incubated with 10 μg (20 μl) of monoclonal anti-RNA polymerase II immunoglobulin G2a (IgG2a; Pro-

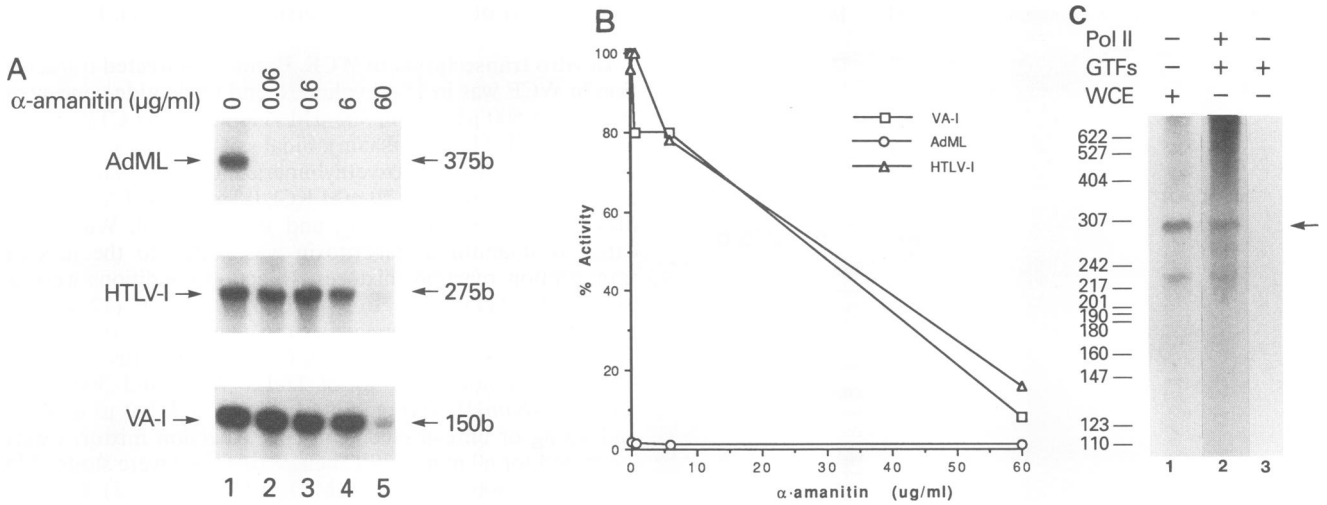

FIG. 2. (A and B) Comparison of α-amanitin sensitivity of transcription from the HTLV-I, AdML, and VA-I promoters. Transcription reactions were performed as described in Materials and Methods. HTLV-I, AdML, and VA-I transcription reaction mixtures were incubated at 30°C for 60 min in the presence of increasing amounts of α-amanitin. After electrophoresis on a 4% polyacrylamide urea gel (A), quantitation of signals was done with a PhosphorImager (Molecular Dynamics) (B). b, bases. (C) Reconstitution of HTLV-I transcription with purified factors and RNA pol II. The HTLV-I template was transcribed in vitro as described in Materials and Methods. The authentic HTLV-I transcript is indicated by an arrow. Lanes: 1, in vitro transcription reaction in HeLa WCE; 2, in vitro transcription reaction with purified factors and RNA pol II; 3, in vitro transcription reaction with purified factors and no RNA pol II. GTFs, general transcription factors. Sizes are indicated in bases.

mega) or 10 μg (10 μl) of mouse IgG2a (Sigma) for 3 h on ice and then added to a 50% slurry of protein A-Sepharose (Pharmacia) (120 μl of 50% solution in buffer D, which consisted of 20 mM HEPES [pH 7.9], 100 mM KCl, 6 mM $MgCl_2$, 0.2 mM EDTA, and 20% glycerol). Incubation with constant rotation was continued overnight at 4°C. The resin was then centrifuged in a microcentrifuge at 2,000 rpm for 2 min. The supernatant from the centrifuged resin constituted the depleted extract used in the in vitro transcription reactions and in the corresponding Western immunoblot. The resin was then washed three times in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) and eluted with SDS-polyacrylamide gel electrophoresis (PAGE) buffer.

Western blot analysis. Proteins were separated by SDS-PAGE using 4 to 20% gradient gels (Novex). Electrophoresis was performed on a Novex gel apparatus. Proteins were electrotransferred to an Immobilon-P (Millipore) membrane for 3 h in Western transfer buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 20% methanol) in a Trans-blot unit (Bio-Rad). Blots were blocked with Tris-buffered saline (TBS) (20 mM Tris-HCl [pH 7.5], 500 mM NaCl) containing 3% bovine serum albumin (BSA) for 1 h, washed in TTBS (0.05% Tween in TBS), and then reacted for 3 h with a monoclonal antibody against RNA pol II CTD (Promega) (1:3,000 dilution in TTBS–1% BSA) at room temperature. After extensive washing with TTBS, the antibody was detected with goat anti-mouse IgG conjugated to alkaline phosphatase (Promega) (1:7,500 dilution in TTBS–1% BSA) for 1 h at room temperature. After being washed twice for 5 min with TTBS and once for 5 min with TBS, the antibody-antigen complex was visualized with 40 ml of developer (176 μl of nitroblue tetrazolium and 132 μl of 5-bromo-4-chloro-3-indolylphosphate toluidinium [BCIP; Bethesda Research Laboratories] in 40 ml of 100 mM Tris-HCl [pH 9.5]–100 mM NaCl–5 mM $MgCl_2$).

Cell culture and RNA isolation. The MT-2 cell line, described previously (68), was cultivated in RPMI 1640 with 10% fetal bovine serum containing 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a 5% CO_2 incubator. RNA was extracted from the cells in the presence of guanidinium isothiocyanate and then isolated by centrifugation through CsCl (14).

Primer extension. Prior to primer extension, the in vitro-transcribed RNA from the HTLV-I template was purified as following. Radioactive and nonradioactive in vitro transcription reactions were performed as described for in vitro transcription in WCE and separated on a 4% polyacrylamide gel containing 7 M urea. The specific 275-base transcript was then eluted in 500 mM ammonium acetate–10 mM $MgCl_2$–1 mM EDTA–0.1% SDS for 5 h at 25°C. The RNA was extracted with phenol-chloroform, ethanol precipitated, and used for primer extension. The synthetic oligonucleotide primer 5′tcaggaggcaccacaggcggg3′, complementary to the coding region from +100 to +80, was 5′ end labeled with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase (New England Biolabs). After hybridization at 43°C with total RNA from the HTLV-I-infected cell line or purified in vitro-synthesized RNA from the HTLV-I template, the primer was extended with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), and the resulting cDNA was fractionated on an 8% sequencing gel. A sequencing reaction was performed with the same +100/+80 primer. Dideoxynucleotide sequencing analysis was performed as previously described with the use of the Sequenase procedure (U.S. Biochemical Corp.) (72).

RESULTS

In vivo and in vitro RNA from the HTLV-I promoter have the same start site. HTLV-I template DNA was prepared by digesting plasmid pU3RCAT with the restriction endonuclease *Hin*dIII. Transcription from the HTLV-I promoter would generate a RNA transcript of 275 bases. The results presented in lane 6 of Fig. 1A demonstrate that the HTLV-I promoter is efficiently transcribed in HeLa WCE. The initiation site for in vitro transcription of the HTLV-I template was mapped by

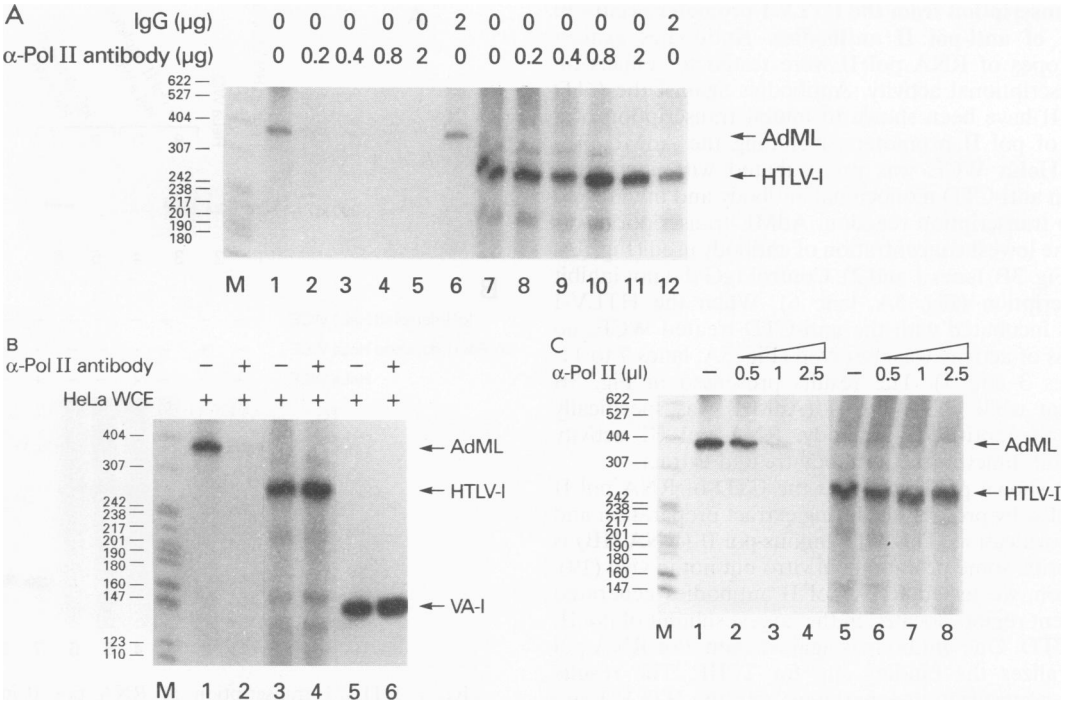

FIG. 3. In vitro transcription of the HTLV-I promoter is not inhibited by antibodies to RNA pol II. (A) HTLV-I and AdML transcription in the presence of increasing concentrations of RNA pol II CTD antibody. AdML (lanes 1 to 6) and HTLV-I (lanes 7 to 12) templates were transcribed with HeLa WCE (lanes 1 and 7) or HeLa WCE pretreated with 0.2 μg (lanes 2 and 8), 0.4 μg (lanes 3 and 9), 0.8 μg (lanes 4 and 10), or 2 μg (lanes 5 and 11) of an anti-CTD monoclonal antibody or with 2 μg of IgG (lanes 6 and 12). (B) Comparison of antibody neutralization of RNA pol II on HTLV-I, AdML, and VA-I transcription. HeLa WCE was pretreated with 2 μg of an CTD antibody before being added to the in vitro transcription reaction mixtures with the HTLV-I, AdML, or VA-I template. (C) HTLV-I and AdML transcription in the presence of increasing concentrations of an RNA pol II exon 5 antibody. AdML (lanes 1 to 4) and HTLV-I (lanes 5 to 8) templates were transcribed with control HeLa WCE (lanes 1 and 5) or HeLa WCE pretreated with 0.5 μl (lanes 2 and 6), 1 μl (lanes 3 and 7), or 2.5 μl (lanes 4 and 8) of an antibody against exon 5 of RNA pol II. Sizes in all panels are indicated in bases. Lanes M, size markers.

primer extension and compared with the start site of HTLV-I mRNA from virus-infected cells. Primer extension of the HTLV-I in vitro-synthesized RNA required purification of the specific 275-base transcript as described in Materials and Methods. The same purification was carried out with ^{32}P-labeled in vitro-synthesized RNA to monitor the procedure and is shown in Fig. 1A, lanes 6 (before purification) and 7 (after gel purification). When the initiation site of the RNA from infected cells and the in vitro-synthesized RNA from the HTLV-I template was mapped by primer extension, the predicted 100-base cDNA was obtained in both cases (Fig. 1A; lanes 1 and 2 with lanes 3 and 4). Extension of the primer in the presence of only yeast tRNA did not generate the corresponding cDNA fragment (lane 5). To confirm the authenticity of the primer extension fragment, a sequencing reaction was performed on the HTLV-I template with the primer used in the primer extension and was run together with the cDNA obtained after primer extension of in vivo-isolated RNA (Fig. 1B). These results demonstrate that HTLV-I in vitro transcription initiation site was the same as the site used in vivo.

In vitro transcription from HTLV-I promoter in WCE is not sensitive to α-amanitin RNA pol II-specific inhibitor. At low concentrations, α-amanitin, a bicyclic octapeptide from the mushroom *Amanita phalloides*, selectively inhibits RNA pol II (42, 48, 50). α-Amanitin inhibits transcription through binding to the largest subunit of RNA pol II and blocking transcription elongation (16, 91). We tested the effect of the drug in an in vitro transcription reaction with HeLa WCE containing the HTLV-I promoter and compared it with classical pol II or pol III promoter (Fig. 2). As expected, transcription from the AdML promoter (RNA-pol II) was inhibited at very low concentrations of α-amanitin (0.06 μg/ml; Fig. 2A, lane 1 versus 2). Transcription from the VA-I promoter (RNA-pol III) was resistant to low concentrations of α-amanitin (0.06 to 6 μg/ml) but was inhibited at higher concentrations of 60 μg/ml of α-amanitin (Fig. 2). When α-amanitin was titrated on the HTLV-I template, transcription was not inhibited at low concentrations of α-amanitin (Fig. 2). The titration curve on HTLV-I resembled more the RNA pol III-transcribed promoter VA-I titration curve. This result is not peculiar to transcription in HeLa cell extracts, since similar results were obtained with CEM T-lymphocyte cell extracts (data not shown).

The HTLV-I promoter contains most features of a classical pol II promoter. In view of the results presented above, we were interested in determining if the HTLV-I promoter could be transcribed by pol II in vitro. Consistently, specific HTLV-I transcription could be reconstituted with purified factors and purified RNA pol II. Human TFIIA (hTFIIA), recombinant human TFIIB (rhTFIIB), rhTFIIE, rhTFIIF, and hTFIIH were added to in vitro transcription assays in the presence or absence of RNA pol II purified from HeLa cells. As shown in Fig. 2C, the HTLV-I promoter was accurately transcribed in the reconstituted reaction (lane 2). Purified pol II was required for HTLV-I transcription in the reconstituted system (lane 3).

In vitro transcription from the HTLV-I promoter occurs in the presence of anti-pol II antibodies. Antibodies against different epitopes of RNA pol II were tested for effects on HTLV-I transcriptional activity. Antibodies against the CTD of RNA pol II have been shown to inhibit transcription of a wide variety of pol II promoters, including the AdML promoter (89). HeLa WCE was preincubated with increasing amounts of an anti-CTD monoclonal antibody and then tested in an in vitro transcription reaction. AdML transcription was inhibited at the lowest concentration of antibody used (Fig. 3A, lanes 2 to 5; Fig. 3B, lanes 1 and 2). Control IgG did not inhibit AdML transcription (Fig. 3A, lane 6). When the HTLV-I template was incubated with the anti-CTD-treated WCE, no significant loss of activity was observed (Fig. 3A, lanes 7 to 11; Fig. 3B, lanes 3 and 4). The results presented in Fig. 3B confirmed that while RNA pol II (AdML) was specifically inhibited by the anti-CTD antibody, RNA pol III activity (VA-I) was still intact in the antibody-treated extract.

It has been shown previously that the CTD of RNA pol II can be degraded by proteolysis during extract preparation and polymerase purification. This CTD-minus pol II (RNA IIB) is able to transcribe some promoters in vitro but not in vivo (19). For this reason, we tested RNA pol II antibodies generated against different regions located in the largest subunit of pol II, outside the CTD. One antibody is against exon 5 of RNA pol II and neutralizes the binding site for TFIIF. The results obtained in a neutralization experiment with the HTLV-I and AdML promoters with this antibody are presented in Fig. 3C. Consistent with the results presented above, transcription from the AdML promoter, but not the HTLV-I promoter, was inhibited by the pol II antibody. The third antibody was raised against *Drosophila* RNA pol II. This antibody inhibited AdML transcription but not HTLV-I transcription (data not shown). These results argue against the possibility that HTLV-I was transcribed by a CTD-minus RNA pol II and confirmed that the transcript is synthesized by a polymerase distinct from the classical pol II.

A HeLa WCE depleted from RNA polymerase II is able to transcribe the HTLV-I promoter. Neither a pol II-specific drug (α-amanitin) nor three different pol II-specific antibodies were able to inhibit transcription from HTLV-I promoter in vitro. In both types of experiments, pol II is inhibited, as suggested by the lack of synthesis of AdML transcription. Nevertheless, pol II was still physically present in the extract, allowing the possibility that a modified pol II transcription complex transcribes the HTLV-I promoter. We therefore depleted RNA pol II from HeLa WCE by immunodepletion in order to analyze transcription of the HTLV-I promoter in the absence of pol II. Anti-CTD antibody or control IgG was incubated with HeLa WCE before addition of protein A-Sepharose. After centrifugation, the supernatant (Fig. 4A, lanes 4 and 6) and pellet fractions (lanes 3 and 5) were analyzed by Western blotting with anti-CTD antibodies. While no RNA pol II could be detected in the control IgG-bound fraction (lane 5), most of the pol II present originally in the WCE was depleted when anti-CTD was used (lane 3).

The control and pol II-depleted supernatant fractions were then incubated with an HTLV-I, AdML, or VA-I template, and in vitro transcription activity was analyzed. As shown in Fig. 4B, while the depleted extract could not support transcription from the AdML promoter (lane 2), the pol III VA-I promoter was efficiently transcribed (lane 8). Interestingly, the pol II-depleted extract was also able to transcribe the HTLV-I promoter (lane 5), giving the same level of activity as the control IgG-depleted extract (lane 6). Similar depletion experiments were performed with antibody against exon 5 of the large subunit of RNA pol II (binding site for TFIIF). Similar to the results presented above, the depleted extract was inactive on the AdML promoter, but the HTLV-I promoter was transcribed efficiently (data not shown).

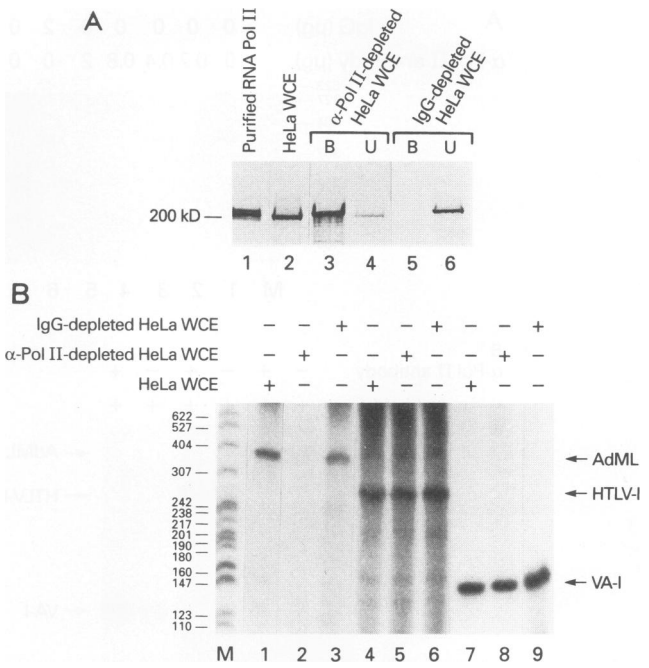

FIG. 4. HTLV-I transcription in RNA pol II-immunodepleted HeLa WCE. (A) HeLa WCE was depleted with antibodies specific for RNA pol II (α-pol II) or with IgG as described in Materials and Methods. Pellet (bound [B]) and supernatant (unbound [U]) fractions were subjected to SDS-PAGE (4 to 20% gel) and Western blotted with an anti-pol II CTD antibody. Purified RNA pol II was used as a positive control (lane 1). (B) The supernatant fractions were used in transcription assays with the AdML, HTLV-I, or VA-I template. Sizes are indicated in bases. Lane M, size markers.

In vitro transcription from the HTLV-I promoter is not affected by the pol III-specific inhibitor tagetitoxin or antibodies to TFIIIC. We were interested in determining if the HTLV-I promoter was transcribed by pol III in the WCE. Tagetitoxin, a bacterial phytotoxin, preferentially inhibits RNA pol III. In HeLa WCE, accumulation of 5S rRNA, U6 small nuclear RNA, tRNA, and VA-I was inhibited at low to intermediate concentrations (0.03 to 0.3 U/15 μl) of tagetitoxin (84, 85). In contrast, transcription from the AdML promoter (pol II) was not significantly affected at 3.5 U/15 μl (84, 85). The effect of tagetitoxin was tested on HTLV-I transcription in comparison with RNA pol II- and pol III-directed transcription (Fig. 5). While VA-I transcription was inhibited at low concentrations of tagetitoxin (0.3 U/15 μl), AdML transcription was not inhibited significantly at 1.2 U/15 μl (Fig. 5A). Interestingly, HTLV-I transcription was not affected at low concentrations of tagetitoxin (Fig. 5A). The titration curve of this inhibitor on HTLV-I promoter was similar to the curve of a classical pol II promoter (AdML) (Fig. 5B). In a separate set of experiments, we assayed the activity of the HTLV-I promoter in the presence of both α-amanitin and tagetitoxin. No significant inhibition was observed (data not shown).

Previous studies have shown that class 1 and 2 RNA pol III promoters require two basal transcription factors, TFIIIB and TFIIIC, in addition to pol III (reviewed in reference 35). Class

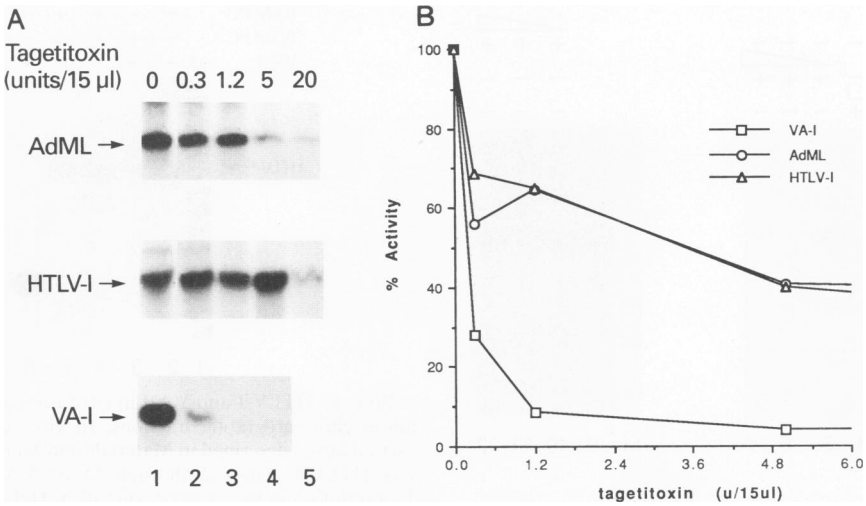

FIG. 5. Determination of tagetitoxin sensitivity of HTLV-I, AdML, and VA-I transcripts. Tagetitoxin, at the concentration indicated above each lane, was added to the in vitro transcription reaction mixture. The in vitro [^{32}P]RNA was purified and electrophoresed on a 4% polyacrylamide–urea gel (A). RNA transcripts were quantitated with a PhosphorImager (Molecular Dynamics) and plotted (B).

3 promoters require TFIIIB but not TFIIIC. WCE were preincubated with a TFIIIC2 antibody before being added to reaction mixtures containing the HTLV-I, AdML, or VA-I template promoter. As shown in Fig. 6, HTLV-I and AdML transcription was observed at the same level in the presence or absence of the TFIIIC2 antibody. In contrast, VA-I transcription was inhibited with the lowest amount of antibody tested, consistent with the known requirement of TFIIIC for transcription of this promoter.

Transcription of the HTLV-I promoter requires TBP and TFIIB. We were next interested in determining if any known general transcription factor was required for transcription of the HTLV-I promoter in HeLa WCE. Extracts were preincubated with antibodies against two general transcription factors, TBP and TFIIB, before being added to an in vitro transcription reaction mixture with the HTLV-I, AdML, or VA-I template. A significant effect was observed when an antibody against either TFIIB or TBP was tested on HTLV-I transcription (Fig. 7A). An unrelated serum did not affect HTLV-I transcription when used at the same concentrations (Fig. 7A, lanes 9 to 12). Antibodies to TBP and TFIIB were also effective on AdML transcription, which has been shown to require both factors for initiation (Fig. 7B). TBP antibodies and, to a lesser extent, TFIIB antibodies inhibited VA-I transcription (Fig. 7B).

HTLV-I in vitro transcription requirements for basal factors. Transcription factors required for RNA pol I, II, or III transcription can be functionally separated from each other by chromatography on phosphocellulose. This scheme separates a cellular extract into four fractions termed A, B, C, and D (reviewed in references 35 and 59). RNA pol II transcription requires fractions A, C, and D; RNA pol III requires fractions B and C. To further characterize the properties of the HTLV-I promoter, we tested phosphocellulose fractions in an in vitro transcription assay with HTLV-I and VA-I templates. The experiment presented in Fig. 8 demonstrates, as previously

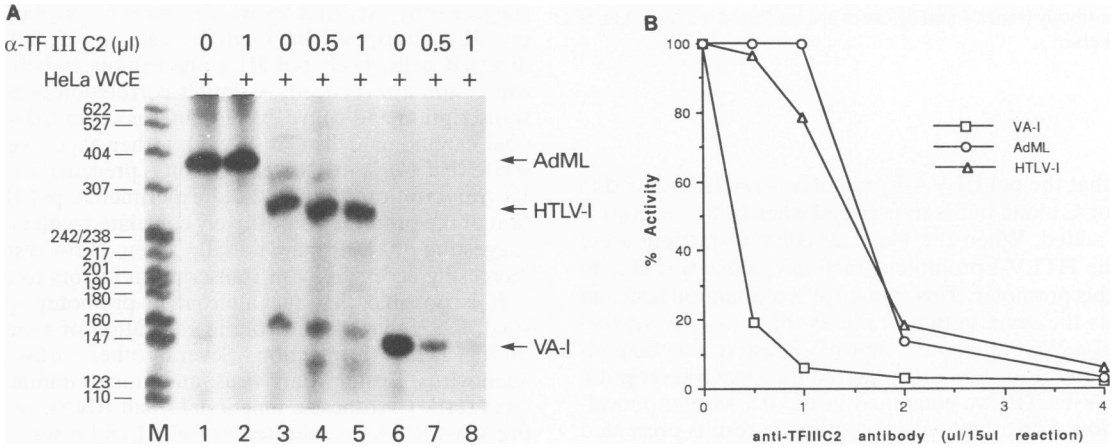

FIG. 6. In vitro transcription of the HTLV-I LTR does not require TFIIIC. HeLa WCE was preincubated with an anti-TFIIIC2 antibody (α-TF III C2) for 30 min at 30°C before the HTLV-I, AdML, or VA-I template was added. In vitro [^{32}P]RNA was purified and electrophoresed on a 4% polyacrylamide–urea gel (A). Specific transcripts were quantitated with a PhosphorImager (Molecular Dynamics) and plotted (B). Sizes are indicated in bases. Lane M, size markers.

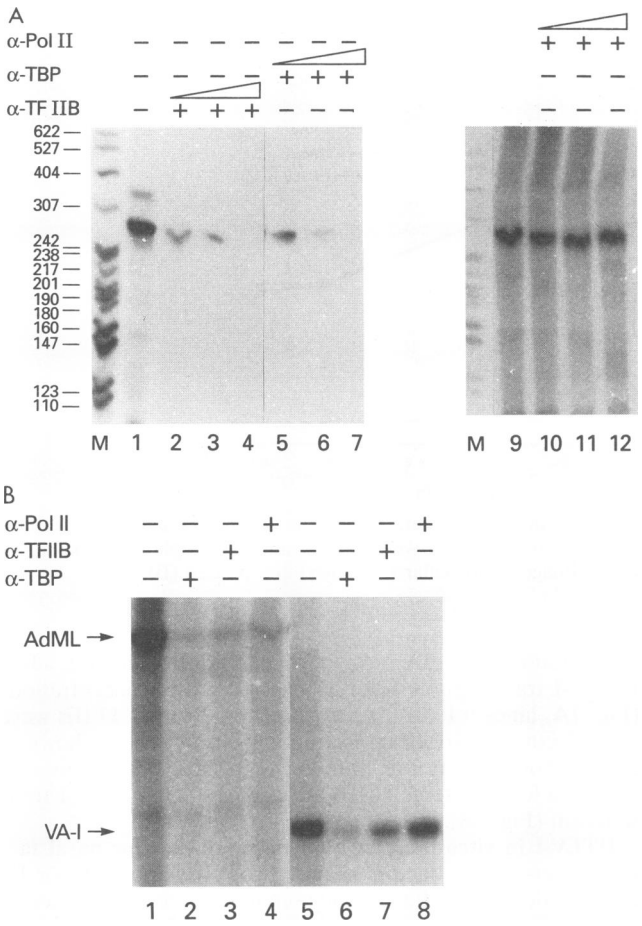

FIG. 7. HTLV-I transcription in the presence of increasing concentrations of TFIIB and TBP antibodies. (A) HTLV-I template DNA was transcribed in the presence of HeLa WCE (lanes 1 and 9) or HeLa WCE pretreated with 1 μl of TFIIB, TBP, and pol II antibodies (α-TF IIB, α-TBP, and α-Pol II; lanes 2, 5, and 10), 2 μl of TFIIB, TBP, and pol II antibodies (lanes 3, 6, and 11), or 4 μl of TFIIB, TBP, and pol II antibodies (lanes 4, 7, and 12). (B) AdML and VA-I transcription in the presence of increasing concentrations of TFIIB and TBP antibodies. AdML and VA-I templates were transcribed in the presence of HeLa WCE (lanes 1 and 5) or HeLa WCE pretreated with 2 μl of TBP antibody (lanes 2 and 6), 2 μl of TFIIB antibody (lanes 3 and 7), or 2 μl of pol II antibody (lanes 4 and 8). Sizes are indicated in bases. Lanes M, size markers.

described, that the pol III VA-I promoter is not transcribed in fraction B or C alone but is transcribed when both fractions B and C are added. When the identical column fractions were tested on the HTLV-I promoter, fraction C alone was able to transcribe this promoter. This transcript is α-amanitin resistant and presents the same initiation site as the transcript synthesized in HeLa WCE (data not shown). These results demonstrate that transcription factors required for basal transcription of the HTLV-I LTR are contained in the 0.5 M phosphocellulose fraction. Consistent with the antibody results presented above, this column fraction contains both TFIIB and a fraction of the cellular TBP. This fraction is currently being fractionated by DEAE, Mono S, and single-stranded DNA chromatography.

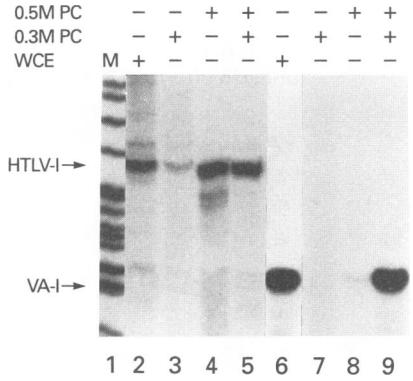

FIG. 8. HTLV-I and VA-I in vitro transcription using phosphocellulose chromatography fractions. In vitro transcription assays were carried out as described in Materials and Methods. The DNA template was HTLV-I (lanes 2 through 5) or VA-I (lanes 6 through 9). Transcription in the presence of 1 μl of HeLa WCE (lanes 2 and 6), 5 μl of 0.3 M phosphocellulose (PC) fraction (lanes 3 and 7), 5 μl of 0.5 M phosphocellulose fraction (lanes 4 and 8) and 5 μl of 0.3 M and 5 μl of 0.5 M phosphocellulose fractions (lanes 5 and 9) was assayed.

DISCUSSION

The HTLV-I LTR contains all of the features of a typical pol II transcription template. The promoter contains a TATA box 30 bp upstream of the transcription initiation site and binding sites for several pol II transcription factors, and long poly(A)$^+$ RNA is synthesized from the integrated HTLV-I proviral DNA in vivo. In addition, HTLV-I transcription can be reconstituted in vitro with pol II and recombinant pol II transcription factors. The data presented in this report strongly suggest that the LTR also contains an overlapping promoter that is not transcribed by pol II. The HTLV-I OTU might represent a novel class of promoters that have transcription factor requirements and properties which are intermediate between those of typical pol II and III promoters. As discussed below, the HTLV-I promoter has similarity to the U6 promoter.

The c-*myc* and the adenovirus E2E promoters have the unusual property of being transcribed by both pol II and pol III. Interestingly, the possibility that pol III transcription of c-*myc* plays a role in the regulation of transcription by pol II is suggested by experiments which report a correlation between pol III transcription and c-*myc* expression (15, 86). In growth-arrested cells, both pol III transcription activity and c-*myc* expression are reduced. A similar correlation between pol III transcription and c-*myc* expression was observed in F9 embryonal carcinoma cells (12, 15, 95). Other experiments demonstrate that c-*myc* mRNA expression is preceded by a rise in pol III transcription (28). In these experiments, pol III transcription of the promoter is positively correlated with pol II activity, suggesting that it may initially open chromatin structure, increasing accessibility of transcription factors to the DNA.

It is possible that the alternative promoter synthesizes a small RNA that plays a role in regulation of gene expression during HTLV-I infection. Several other viruses, including adenovirus, Epstein-Barr virus, and human immunodeficiency virus (HIV), synthesize functional small RNAs. In adenovirus, the VA-I RNA is generated by pol III and plays a critical role in regulation of protein synthesis (reviewed in reference 30). Similarly, the HIV TAR RNA has been reported by Gunnery et al. to regulate translation in HIV-infected cells (32). In view of these observations in other viral systems, it will be important

to determine if a small transcript is generated from the HTLV-I promoter in vivo.

Transcriptions by pol I, II, and III all require the common transcription factor TBP. For the TBP complex utilized in pol II, D-TFIID, and pol III, TFIIIB, transcription can be separated by chromatography on phosphocellulose. The OTU of HTLV-I apparently requires a fraction of TBP that is distinct from either D-TFIID or TFIIIB. In vitro transcription of the HTLV-I promoter is supported by the 0.5 M phosphocellulose fraction. Interestingly, Hernandez recently demonstrated that this 0.5 M fraction is required for transcription of the U6 promoter (35). U6 transcription does not require the TFIIIC complex present in the 0.5 M fraction but does require a large multiprotein complex, SNAPc, which binds to an upstream regulatory sequence (proximal sequence element) in the U6 promoter. The SNAPc complex is a TBP-containing complex. In fact, the overlapping HTLV-I promoter resembles, in several ways, the U6 class 3 pol III promoter. Class 3 pol III promoters are regulated by an upstream control region, contain a TATA box, do not require TFIIIC, and have intermediate sensitivity to tagetitoxin (reviewed in reference 35). Interestingly, when the U6 TATA box is mutated, transcription activity is changed from pol II to pol III. We have changed the HTLV-I TATA box to the HIV TATA box and found that the level of transcription decreases both in vitro and in vivo, suggesting a level of regulation at the HTLV-I TATA box (unpublished data). Studies are under way to determine the α-amanitin sensitivity of the TATA mutant.

In vitro transcription of the HTLV-I promoter has been reported by Matthews et al., using nuclear extracts from either HeLa or CEM lymphocyte cells. In the latest report, the authors present evidence that in the presence of Tax_1 and CREB, HTLV-I transcription is sensitive to low concentrations of α-amanitin (60). The α-amanitin sensitivity of HTLV-I basal transcription was not quantitatively addressed in these reports. In experiments in this laboratory, we have utilized CEM extracts and found that, similar to our results for HeLa cell extracts, HTLV-I basal transcription is resistant to low levels of α-amanitin (data not shown). It will be of interest to determine if the addition of both Tax_1 and CREB converts the HTLV-I transcription initiation/elongation complex to a typical pol II α-amanitin sensitivity.

At this point, we cannot rule out the possibility that the overlapping promoter of HTLV-I is transcribed by a polymerase that is intermediate between pol II and pol III. RNA polymerases are multisubunit enzymes of about 500 to 600 kDa and composed of 10 to 15 subunits (76). In HeLa cells, RNA pol II is composed of 10 subunits ranging from 240 to 10 kDa (99). The two largest subunits share homology with the b and b′ subunits of the RNA polymerase from *Escherichia coli* (96). The largest subunits of all three polymerases are also closely related to each other (10, 37, 39). In addition, some of the smaller subunits are shared among the three polymerases (47, 81, 96). In eukaryotes, the large subunits of pol II and pol III are more closely related to each other than to that of pol I (62). The large subunit of pol II uniquely contains an unusual CTD consisting of tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (19). Given the conserved amino acid structure of the large subunit, it is not inconceivable that polymerase-associated proteins, which may regulate enzymatic activity, could form intermediate polymerase complexes. Future experiments will be performed to further characterize the in vitro properties of this interesting bimodal promoter and to address the in vivo relevance of this observation.

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