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Identification of p40x-Responsive Regulatory Sequences within the Human T-Cell Leukemia Virus Type I Long Terminal Repeat

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Distinct transcriptional regulatory sequences located within the upstream sequences required for p40x *trans*-activation of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) were chemically synthesized and cloned upstream of the basal HTLV-I LTR promoter. Plasmids containing a single 21-base-pair (bp) repeat were weakly inducible by p40x. The level of *trans*-activation by p40x was increased when two (30-fold) or three (40-fold) 21-bp repeats were present in the upstream control region. In the mutant containing two 21-bp repeats, the upstream 21-bp repeat could be positioned in either the sense (30-fold) or the antisense (16-fold) orientation. Plasmids containing a 51-bp repeat element, which included a single 21-bp repeat, were induced to levels similar to that obtained with the 21-bp repeat sequence alone. Template DNAs containing a single copy of the HTLV-I sequences between −117 and −160 were stimulated approximately 10-fold by p40x when one copy of the 21-bp element was located downstream.

Transcription of the human T-cell leukemia virus type I (HTLV-I) viral genome originates from the long terminal repeats (LTRs) located at both ends of the HTLV-I genome. Recently, it has been demonstrated that transcription from the viral LTR is increased in the presence of an HTLV-I gene product, p40x (4, 5, 7–9, 26, 29, 31, 33, 34). Analysis of the HTLV-I LTR has revealed several interesting sequences. First, there are three imperfect repeats of 21 nucleotides located at positions −84 to −104, −183 to −203, and −233 to −253 relative to the mRNA cap site. The two upstream 21-base-pair (bp) repeats are located within a larger imperfect tandem repeat sequence of 51 bp located between −158 and −269. Previous investigators have demonstrated that sequences between −159 and −350 which include the two 51-bp repeats can act as transcriptional enhancers (29). In addition, the sequences between −159 and +3 were shown to confer p40x inducibility on a heterologous promoter.

The ability of p40x to *trans*-activate the HTLV-I promoter is similar to the *trans*-acting abilities of simian virus 40 (SV40) T antigen and adenovirus E1A gene products to stimulate transcription of viral and cellular genes. We and others have shown that SV40 T antigen can stimulate transcription from the SV40 late promoter in the absence of DNA replication (1–3, 13, 20, 21). Current evidence suggests that activation of the SV40 late promoter and heterologous promoters can occur by both direct and indirect mechanisms. Similarly, the adenovirus E1A gene product is able to *trans*-activate a wide variety of eucaryotic genes, including the adenovirus E2, E3, and late genes and cellular genes such as the β-globin, β-tubulin, and heat shock genes (12, 15, 16, 19, 25, 35). Because the sequences required for E1A induction do not show any sequence homology and because specific binding of E1A to the reactive sequences has not been demonstrated (6), it is likely that E1A *trans*-activates these promoters indirectly, either through induction of synthesis of eucaryotic transcription factors or through modification of these factors (22, 23).

Sequences important for regulation of eucaryotic transcription have been identified primarily by deletion, linker scanning, or base substitution mutation. An alternative approach involves the de novo synthesis and substitution of eucaryotic transcriptional control elements. With this approach, putative control sequences are synthesized, cloned into appropriate plasmid constructs, and tested for biological activity. In this study, we have used this synthetic approach to identify sequences in the HTLV-I LTR responsive to p40x *trans*-activation. Our experiments demonstrate that two distinct regulatory sequences respond to p40x *trans*-activation at the transcriptional level.

MATERIALS AND METHODS

Cell culture and transfection. CV-1 cells were passed into 35-mm culture dishes with Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Twenty-four hours later, when cells were 60 to 80% confluent, they were transfected by the calcium phosphate precipitation technique (11). The total DNA concentration was kept constant (10 μg) with carrier plasmid DNA. Cells were incubated with transfection mixtures for 12 h, washed, and cultured with fresh medium until 48 h posttransfection.

CAT assays. Cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as described by Gorman et al. (10).

S1 nuclease analysis of RNA. RNA was extracted and analyzed by the S1 nuclease technique as described previously (24). Briefly, RNA was extracted by the hot acid-phenol method described by Queen and Baltimore (28). Purified RNA (20 μg) and labeled DNA probe were suspended in 20 μl of 0.4 M NaCl–0.04 M PIPES [piperazine-*N*,*N*′-bis(2 ethanesulfonic acid), pH 6.4]–1.25 mM EDTA–80% formamide and heat denatured at 68°C for 15 min. The samples were then transferred immediately to a 45°C water bath and incubated for 12 h. Hybridizations were terminated by the addition of 9 volumes of 0.25 M NaCl–0.03 M sodium acetate (pH 4.6)–1 mM $ZnSO_4$ which had been preequilibrated to 4°C. S1 nuclease was added to a final concentration of 1,800 U/ml, and the reaction sample was incubated for 60 min at 37°C. Protected DNA fragments were then purified and analyzed by electrophoresis in a denaturing acrylamide-urea gel.

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Synthetic oligonucleotide sequences. The following oligonucleotides were synthesized.

(i) p40x region I: 21-bp repeat (−84 to −103, −183 to −202, and −233 to −253);

5′-CTAGACTCGAGAAGGCTCTGACGTCTCCCCCCGGA-3′
3′-TGAGCTCTTCCGAGACTGCAGAGGGGGGCCTGATC-5′

(ii) p40x region II: 51-bp repeat (−156 to −212, −214 to −269);

5′-CTAGACTCGAGGGTCAGGGCCCAGACTAAGGCTCTGACGTCTCCCCCCGGAGGGCAGCTCAGCACCA-3′
3′-TGAGCTCCCAGTCCCGGGTCTGATTCCGAGACTGCAGAGGGGGGCCTCCCGTCGAGTCGTGGTGATC-5′

(iii) p40x region III: (−117 to −160);

5′-CTAGACTCGAGCCTCCGGGAAGCCACCAAGAACCACCCATTTCCTCCCCATGTTTGA-3′
3′-TGAGCTCGGAGGCCCTTCGGTGGTTCTTGGTGGGTAAAGGAGGGGTACAAACTGATC-5′

(iv) p40x region III (mutant 1);

5′-CTAGACTCGAGCCTCCGGGAAGAAATTAAGAAAAATTCATTTCCTCCCCATGTTTGA-3′
3′-TGAGCTCGGAGGCCCTTCTTTAATTCTTTTTAAGTAAAGGAGGGGTACAAACTGATC-5′

(v) p40x region III (mutant 2):

5′-CTAGACTCGAGCCTCCGGGAAGAAATTAAGAAAAATTCATTTAAATTCCATGTTTGA-3′
3′-TGAGCTCGGAGGCCCTTCTTTAATTCTTTTTAAGTAAATTTAAGGTACAAACTGATC-5′

RESULTS

Analysis of promoter deletion mutants. A series of BAL-31 deletion mutants was prepared by first restricting the plasmid pU3Rcat (34) at the unique *Xho*I site (HTLV-I map position [mp] 8213). The linearized DNA was digested with BAL-31 exonuclease for various lengths of time to generate a series of progressive 5′ deletions and restricted with *Hin*dIII (HTLV-I mp 8916) to create a defined 3′ end. DNA fragments 250 to 700 bp in length were isolated from an agarose gel. These fragments were ligated into the pCAT3M vector at the unique *Bgl*II site, immediately upstream of the CAT coding sequence (10). A schematic of the deletion mutants is presented in Fig. 1.

Deletion mutant DNAs were either transfected into CV-1 cells alone or cotransfected with a recombinant clone encoding the HTLV-I p40x protein (9). At 48 h posttransfection, cell extracts were prepared and gene expression was assayed for CAT activity (Fig. 2, Table 1). In the absence of p40x protein, the pU3Rcat (−440) extract gave approximately 10% conversion of the [^{14}C]chloramphenicol substrate to the acetylated form (Fig. 2A, lane 3; Table 1). Deletion to nucleotide −306 (pU3Rcat *dl*10-1) decreased the basal level of gene expression 50 to 70% (Fig. 2A, lane 4). Deletion to nucleotide −242 (pU3Rcat *dl*6-3) led to a further drop in the level of basal gene expression (Fig. 2A, lane 5). In the representative experiment presented here, approximately 0.4% CAT activity was detected. Further deletion to −101 (pU3Rcat *dl*11-2) or −52 (pU3Rcat *dl*6-2) abolished detectable basal promoter activity (Fig. 2A, lanes 6 to 8).

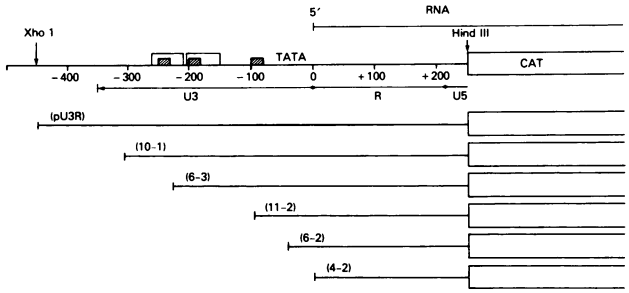

FIG. 1. Schematic of HTLV-I LTR and promoter deletion mutants. pU3Rcat (34) was digested with *Xho*I, digested with BAL 31 exonuclease, and digested with *Hin*dIII, and DNA fragments 200 to 700 bp in length were isolated. These DNA fragments were then ligated into the pCAT3M vector at the unique *Bgl*II site. The endpoints of each deletion mutant were determined by DNA sequence analysis.

In parallel experiments performed in the presence of the p40x protein, the level of expression for pU3Rcat, pU3Rcat *dl*10-1, and *dl*6-3 was increased to 98, 63, and 38% conversion, respectively (Fig. 2B, lanes 3 to 5). Since the value of 98% is beyond the linear range of the enzyme reaction, assays were repeated with 1/10 the amount of the pU3Rcat extract from cotransfection with HTLV-I p40x. The p40x induction ratios for plasmids pU3R, 10-1, and 6-3 were 67-, 21-, and 100-fold above their respective basal levels. Weak p40x induction was observed with deletion mutants 11-2, 6-2, and 4-2 (Fig. 2B, lanes 6 to 8), indicating that sequences critical for efficient *trans*-activation were located between −242 and −101.

To establish that the level of CAT expression accurately reflected a change in the steady-state levels of CAT mRNA, total cell RNA was isolated 48 h after transfection and assayed by quantitative S1 nuclease protection (Fig. 2C). The probe used in these studies was synthesized from an M13 vector containing a portion of the CAT coding sequences (24). The intact probe was 458 nucleotides in length. A protected DNA band of 256 nucleotides corresponded to complete protection of the CAT sequences within the probe. The intensity of the 256-nucleotide DNA fragment observed following hybridization of the single-stranded probe to RNA extracted from pU3Rcat-transfected cells (Fig. 2C, lane 1) was significantly increased in the presence of p40x (Fig. 2C, lane 2). While similar levels of the CAT-specific protected

TABLE 1. Activity of HTLV-I deletion mutants in the presence and absence of p40x[a]

Plasmid	Deletion endpoint	CAT activity[b] (U)	
		Without p40x	With p40x
pU3Rcat	−440	10	670
*dl*10-1	−306	3	63
*dl*6-3	−242	0.4	38
*dl*11-2	−101	0.08	1.1
*dl*6-2	−52	0.11	0.78
*dl*4-2	−21	0.12	1.3

[a] The transfection protocol used in this experiment is described in the legend to Fig. 2.

[b] The values presented are based on the percentage of [^{14}C]chloramphenicol substrate converted to the acetylated form. Each unit represents 1% conversion.

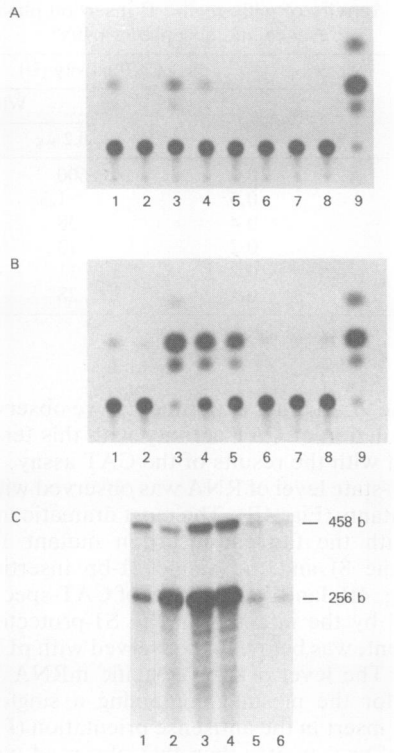

FIG. 2. Analysis of transcriptional activity of HTLV-I promoter deletion mutants. The HTLV-I promoter deletion mutants (2 μg) depicted in Fig. 1 were transfected into CV-1 cells in the absence (A) or presence (B) of p40x (0.2 μg). At 48 h, extracts were prepared and CAT enzyme activity was determined. Lane 1, pSV2CAT; lane 2, pA10CAT2; lane 3, pU3Rcat; lane 4, *dl*10-1; lane 5, *dl*6-3; lane 6, *dl*11-2; lane 7, *dl*6-2; lane 8, *dl*4-2; lane 9, enzyme control. (C) Samples (20 μg) of whole-cell RNA were probed by S1 nuclease protection analysis for CAT mRNA as described in Materials and Methods. The position of the intact S1 probe (458 bases) and the DNA fragment protected by CAT mRNA (256 bases) are indicated. Lane 1, pU3Rcat; lane 2, pU3Rcat plus p40x; lane 3, *dl*10-1 plus p40x; lane 4, *dl*6-3 plus p40x; lane 5, *dl*11-2 plus p40x; lane 6, *dl*6-2 plus p40x.

DNA fragment were observed in cells transfected with deletion mutants 10-1 and 6-3 in the presence of p40x (Fig. 2C, lanes 3 and 4, respectively), little CAT-specific RNA was detected in samples obtained from cells transfected with mutant 11-2 or 6-2 plus p40x (Fig. 2C, lanes 5 and 6). These data confirm that p40x *trans*-activation of the HTLV-I LTR occurs at the transcriptional level and requires regulatory sequences between −242 and −101.

Synthesis and cloning of synthetic oligonucleotides corresponding to suspected transcriptional control sequences. In view of the DNA sequence and the deletion mutant analysis results, three potentially important nucleotide sequences were synthesized and analyzed for transcriptional activity. The first sequence was the HTLV-I 21-bp repeat, which we will refer to as p40x region I; the second sequence was the 51-bp repeat, which we will refer to as p40x region II; and the third oligonucleotide, which we will refer to as p40x region III, encompassed DNA sequences which are located between the 51-bp repeats and the downstream 21-bp repeat. The oligonucleotides were cloned into HTLV-I deletion mutant vectors 11-2 and 6-2, which exhibited no basal promoter activity. The series of plasmids derived from this procedure are outlined in Fig. 3. The 11-2 deletion mutant

TABLE 2. Activity of 21-bp insertion plasmids in the presence of p40x[a]

Plasmid	CAT activity (U)
*dl*11-2	0.8
*dl*11-2-35S	42
*dl*11-2-35AS	26
*dl*11-2-35S/AS	59

[a] See Table 1, footnotes *a* and *b*.

contained one 21-bp sequence at the extreme end of the deletion (Fig. 3A). Mutant 11-2-35S contained an additional 21-bp repeat cloned in the sense orientation (Fig. 3B), while mutant 11-2-35AS contained one additional 21-bp repeat in the antisense orientation (Fig. 3C). Mutant 11-2-35S/AS contained two additional 21-bp repeats (Fig. 3D). Plasmids containing a single copy of the 21-bp repeat in the *dl*6-2 mutant were cloned in both the sense (Fig. 3E) and antisense (Fig. 3F) orientation. Two clones were isolated which contained p40 region II in the sense (11-2-67S) and antisense (11-2-67AS) orientation upstream of the single 21-bp repeat in mutant *dl*11-2 (Fig. 3G and H).

Plasmid 11-2-57S contained p40x region III in the sense orientation immediately upstream of the 21-bp repeat in mutant 11-2 (Fig. 3I). The final construct contained one copy of the p40x region III in the sense orientation (6-2-67S) (Fig. 3K) in mutant 6-2.

Transcriptional activity of 21-bp insertion templates in the presence and absence of p40x. The recombinant plasmids containing insertions of the 21-bp oligonucleotide in various orientations and duplications (Fig. 3A to F) were transfected into CV-1 cells in the presence or absence of p40x (Fig. 4, Table 2). Consistent with the data presented above, in the absence of p40x, deletion mutant 11-2, which contained one

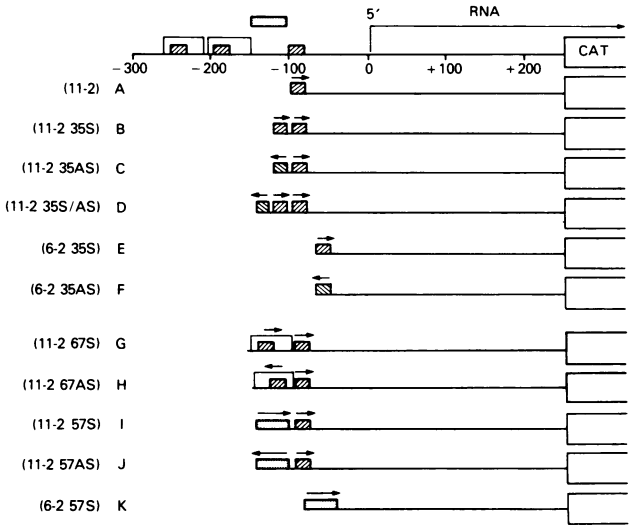

FIG. 3. Schematic of HTLV-I promoter construct containing inserts of p40x regions I, II, and III. Synthetic oligonucleotides corresponding to the HTLV-I 21-bp repeat (p40x region I), 51-bp repeat (p40x region II), or the DNA sequences located between the 51-bp repeats and the downstream 21-bp repeat (p40x region III) were inserted into HTLV-I promoter deletion mutant 11-2 or 6-2. The number and orientation of inserts were determined by restriction enzyme analysis. Symbols: Arrows indicate orientation of 21-bp repeat; ▨, location of 21-bp repeat units (p40x region I); ▭, position of 51-bp repeats (p40x region II); ▩, position of p40x region III.

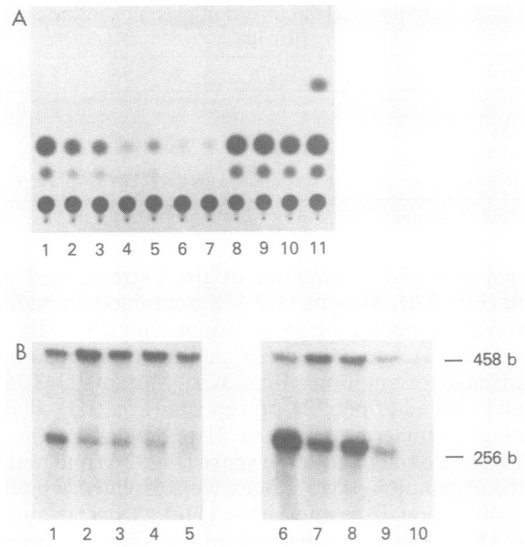

FIG. 4. Analysis of p40x region I insertion mutants. The HTLV-I promoter mutants (2 μg) containing an insert(s) of p40x region I were transfected into CV-1 cells in the absence or presence of p40x (0.2 μg). (A) At 48 h posttransfection, extracts were prepared and CAT enzyme activity was determined. Lane 1, pU3Rcat; lane 2, mutant 10-1; lane 3, 6-3; lane 4, 11-2; lane 5, 11-2-35S; lane 6, 11-2-35S/AS; lane 7, 11-2-35AS; lane 8, 11-2-35S plus p40x; lane 9, 11-2-35S/AS plus p40x; lane 10, 11-2-35AS plus p40x; lane 11, enzyme control. (B) Samples (20 μg) of whole-cell RNA were probed by S1 nuclease protection analysis for CAT mRNA as described in Materials and Methods. The position of the intact S1 probe (458 bases) and the DNA fragment protected by CAT mRNA (256 bases) are indicated. Lane 1, pU3Rcat; lane 2, 11-2-35S; lane 3, 11-2-35S/AS; lane 4, 11-2-35AS; lane 5, 11-2; lane 6, pU3Rcat plus p40x; lane 7, 11-2-35S plus p40x; lane 8, 11-2-35S/AS plus p40x; lane 9, 11-2-35S plus p40x; lane 10, 11-2 plus p40x.

copy of the 21-bp repeat sequence, was inactive (less than 1% conversion of [^{14}C]chloramphenicol to the acetylated form) (Fig. 4A, lane 4). The addition of either one 21-bp repeat in the sense (Fig. 4A, lane 5) or antisense (Fig. 4A, lane 7) orientation or the insertion of the two 21-bp repeats (Fig. 4A, lane 6) had little effect on the basal CAT activity.

At the RNA level, a modest increase in the level of steady-state CAT mRNA was observed with the 21-bp repeat insertions. The level of the 256-base CAT mRNA-specific DNA fragment was increased with each of the mutants (Fig. 4B, compare lanes 2 to 4 and lane 5). The level of the CAT mRNA S1-protected DNA fragment was significantly below that observed with the control plasmid pU3Rcat (Fig. 4B, lane 1).

In the presence of p40x, each of the 21-bp repeat insertion mutants demonstrated a significant increase in both CAT activity (Fig. 4A, Table 2) and CAT mRNA (Fig. 4B). The single 21-bp insertion mutant in the sense orientation, 11-2-35S (Fig. 3B), generated 42% conversion of [^{14}C]chloramphenicol to the acetylated form (Fig. 4A, lane 8). The plasmid containing a single 21-bp insertion in the antisense orientation, 11-2-35AS, resulted in approximately 26% conversion (Fig. 4A, lane 10), suggesting that the orientation of the two 21-bp repeats contributes to the induction by p40x. The plasmid containing three copies of the 21-bp sequence, 11-2-35S/AS (Fig. 3D), gave approximately 59% conversion of the [^{14}C]chloramphenicol substrate in this experiment (Fig. 4A, lane 9). In other experiments, we observed an even greater stimulation of CAT activity with this template.

TABLE 3. Activity of p40x region II insertion plasmids in the presence and absence of p40x[a]

Plasmid	CAT activity (U)		
	Without p40x	With p40x	
		0.2 μg	1.0 μg
pU3Rcat	10.7	600	750
*dl*11-2	0.5	1.3	5
*dl*11-2-35S	0.4	38	92
*dl*11-2-35AS	0.2	13	93
*dl*11-2-67S	0.2	11	88
*dl*11-2-67AS	0.4	28	91

[a] See Table 1, footnotes *a* and *b*.

Consistent with the results of the CAT assay, an increase in the steady-state level of RNA was observed with the 21-bp insertion mutants (Fig. 4B). The most dramatic increase was observed with the tandem insertion mutant 11-2-35S/AS (Fig. 4B, lane 8) and the single 21-bp insertion plasmid 11-2-35S (Fig. 4B, lane 7). The level of CAT-specific mRNA, as measured by the intensity of the S1-protected 256-base DNA fragment, was below that observed with pU3Rcat (Fig. 4B, lane 6). The level of CAT-specific mRNA was significantly less for the plasmid containing a single additional 21-bp repeat insert in the antisense orientation (Fig. 4B, lane 9). This level was greater than that observed with the 11-2 mutant containing only a single copy of the 21-bp repeat (Fig. 4B, lane 10).

We have also assayed the mutants containing 21-bp inserts in the sense and antisense orientation upstream of the sequences retained in mutant 6-2 (Fig. 3E and F). No CAT activity or RNA could be detected with these plasmids in either the absence or presence of p40x (data not shown).

Transcriptional activity of 51-bp insertion templates in the presence and absence of p40x. The recombinant plasmids containing insertions of the 51-bp repeat (p40x region II) in the sense and antisense orientation were transfected into CV-1 cells in the presence or absence of p40x. The addition of one 51-bp repeat in the sense (Fig. 3G) or antisense (Fig. 3H) orientation had little effect on the basal CAT activity (Table 3).

In the presence of p40x, the level of gene expression was significantly increased in plasmids containing the 51-bp repeat (Table 3). The plasmid containing a single copy of the 51-bp repeat in the sense orientation (11-2-67S) (Fig. 3G) generated 11% conversion of [^{14}C]chloramphenicol to the acetylated form, while the plasmid containing a single copy of the 51-bp repeat in the antisense orientation (11-2-67AS) (Fig. 3H) generated approximately 28% conversion. These conversion levels were approximately 10- and 28-fold, respectively, above the activity observed with the basal 11-2 mutant. Higher levels of induction were observed when the plasmids were cotransfected with 1.0 μg of p40x. For comparison, insertion mutants containing a single copy of the 21-bp repeat were included in the assay. The results of this experiment demonstrate that the 51-bp repeat insertion mutants were not significantly more inducible by p40x than the 21-bp repeat insertion mutants. This result suggests that the nucleotides which flank the 21-bp sequence within the 51-bp repeat are not functionally important in p40x *trans*-activation in CV-1 cells.

Transcriptional activity of p40x region III insertion templates in the presence and absence of p40x. We next tested the

level of gene expression observed when a single copy of the p40x region III was cloned upstream of a single copy of the 21-bp repeat sequence (Fig. 5, Table 4). Similar to the results presented in Fig. 2, pU3Rcat (96%), 10-1 (84%), and 6-3 (53%) were quite active in the presence of p40x (Fig. 5A, lanes 1 to 3). In addition, mutants 11-2 (0.9%), 6-2 (0.5%), and 4-2 (0.4%) showed almost no transcriptional activity in the CAT assay (Fig. 5A, lanes 6 to 8). The addition of the p40x region III to mutant 11-2 (Fig. 3I) partially restored its inducibility to the p40x *trans*-acting protein. Two independent isolates (Fig. 5A, lanes 4 and 5) of this plasmid induced CAT enzyme activity to 11 and 9% conversion of [^{14}C]chloramphenicol. This activity was approximately 10-fold more than that observed with deletion mutant 11-2 (Fig. 5A, lane 6). S1 nuclease analysis demonstrated that activation of gene expression by p40x region II occurred at the transcriptional level (Fig. 5B, lanes 5 and 6). In the absence of a single 21-bp repeat in the basal HTLV-I promoter element, mutant *dl*6-2-57S (Fig. 3K) gave no detectable induction by p40x.

p40x region III contains a pentanucleotide sequence, CC(T/A)CC, which is repeated four times within the 43-base HTLV-I regulatory sequence (see Materials and Methods). To test the importance of this sequence, we constructed mutations within the pentanucleotide. Mutant *dl*11-2-57S-1 base substitutions changed the sequence of the second and third repeat to AAATT, while mutant *dl*11-2-57S-2 changed repeats two, three, and four to AAATT. The base substitution mutants and parent plasmid *dl*11-2-57S were transfected into CV-1 cells in the presence of p40x. Both mutants were approximately seven- to eightfold less active than the wild-type plasmid (Table 4), suggesting that the CC(T/A)CC pentanucleotide repeat may be an important sequence within p40x region III.

TABLE 4. Activity of p40x region III insertion plasmids in the presence of p40x[a]

Expt	Plasmid	CAT activity[b] (U)
1	pU3Rcat	96
	*dl*10-1	84
	*dl*6-3	53
	*dl*11-2-57S	11
	*dl*11-2-57S	9
	*dl*11-2	0.9
	*dl*6-2	0.5
	*dl*4-2	0.4
2	*dl*11-2-57S	22
	*dl*11-2-57S-1	3
	*dl*11-2-57S-2	3

[a] The transfection protocol used in these experiments is described in the legend to Fig. 5.
[b] See Table 1, footnote *b*.

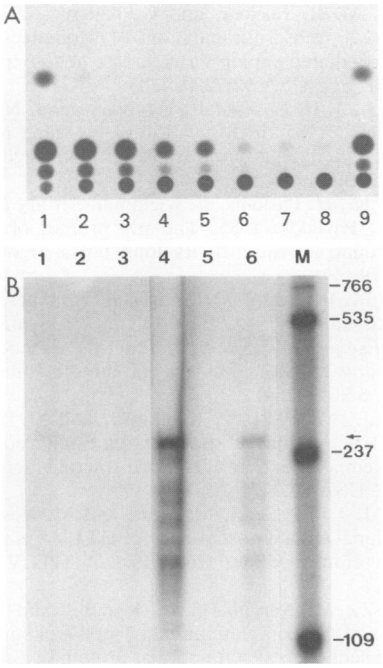

FIG. 5. Analysis of p40x region III insertion mutants. The HTLV-I promoter mutants (2 μg) containing an insertion of p40x region III were transfected into CV-1 cells in the presence of p40x (0.2 μg). (A) At 48 h posttransfection, extracts were prepared and CAT enzyme activity was determined. Lane 1, pU3Rcat; lane 2, 10-1; lane 3, 6-3; lane 4, 11-2-57S; lane 5, 11-2-57S; lane 6, 11-2; lane 7, 6-2; lane 8, 4-2; lane 9, enzyme control. (B) Samples (20 μg) of whole-cell RNA were probed by S1 nuclease protection analysis for CAT mRNA as described in Materials and Methods. The intact S1 probe was 458 bases. The DNA fragment protected by CAT mRNA (256 bases) is indicated by the arrow. Lanes 1 and 4, pU3Rcat; lanes 2 and 5, *dl*11-2; lanes 3 and 6, *dl*11-2-57S. Transfections for lanes 4 through 6 were performed in the presence of p40x. Lane M, Size markers.

DISCUSSION

The experiments presented in this manuscript provide information about the sequences required for basal and p40x *trans*-activation of the HTLV-I LTR. Deletion of sequences between −440 and −306 decreased HTLV-I basal gene expression approximately 50 to 70%. Further deletion to approximately −242 decreased the level of HTLV-I gene expression to essentially background levels (<1%). Thus, the DNA sequences between −306 and −242 are apparently important for basal activity. Our data are consistent with those of Rosen et al. (29), who previously demonstrated that basal HTLV-I promoter activity decreased to background levels following deletion of sequences between −294 and −240. More recently, Paskalis et al. (26) presented evidence that sequences upstream of −322 were required for basal HTLV-I promoter activity. While we observed a decrease in gene expression after deletion of similar sequences, our −300 deletion mutant retained some basal activity. With a HeLa cell nuclear extract, a DNase I footprint region extending from −262 to −314 overlaps the basal-level upstream control region (K.-T. Jeang, personal communication). Further experiments will determine whether the DNase I-resistant sequence and the transcriptional control element are identical.

We have shown that two specific sequences within the HTLV-I LTR can be activated by the *trans*-acting protein p40x. The first sequence constitutes the 21-bp repeat located in the HTLV-I upstream control region (p40x region I). Activation of this sequence by p40x was dependent on the presence of at least two tandem 21-bp repeats. In the presence of p40x, we observed up to a 30-fold induction of CAT activity. Further oligomerization increased the extent of p40x inducibility to approximately 40-fold. Quantitative S1 nuclease analysis demonstrated that the activation occurred at the level of transcription. It is interesting that a simple duplication of the 21-bp repeat sequence allowed

p40x induction. A direct duplication of the 21-bp repeat is not observed in the wild-type LTR sequence; the closest 21-bp repeats are separated by approximately 50 bp of DNA.

Insertion of the 51-bp repeat sequence (p40x region II) upstream of the basal HTLV-I promoter increased gene expression to approximately the same level as did an insertion of the 21-bp repeat sequence. This result suggests that the nucleotides which flank the 21-bp sequence within the 51-bp repeat are not functionally important in p40x *trans*-activation in CV-1 cells. In view of the sequence homology between the two 51-bp repeats, this result may not be unexpected. While the two 21-bp repeats are 85 to 90% homologous, the flanking sequences are less than 50% homologous. Due to the somewhat artificial nature of our promoter constructs, we cannot rule out the possibility that the context of the 21-bp sequence within the 51-bp repeat is important for HTLV-I transcription in an intact LTR. In addition, the 51-bp repeats might play an important role in HTLV-I transcription in a T cell. Experiments to address these questions are under way.

The second sequence which conferred inducibility in the presence of p40x was p40x region III, which consisted of the upstream HTLV-I sequences between −160 and −117. When this sequence was positioned upstream of a single 21-bp repeat, we reproducibly observed an approximately 10-fold induction in p40x *trans*-activation. This level of induction is significant, but less than that observed with the 21-bp repeats. This result at first suggests that p40x region III is a weaker transcriptional control element. However, it should be kept in mind that our selection of sequences for the p40x region III oligonucleotide was somewhat arbitrary. Thus, it is possible that the synthetic oligonucleotide does not contain critical regulatory sequences of this domain. Within the p40x region sequence, the pentanucleotide sequence CC(T/A)CC was repeated four times. Our results demonstrate that specific mutations within the second and third repeat decrease inducibility by p40x (Table 4), suggesting a functional role for these sequences.

It is not clear at present how p40x stimulates transcription from a promoter containing p40x region I or III. One possibility is that p40x stimulates the expression of or modifies preexisting transcription factors which interact with these DNA sequences. This model is similar to that recently proposed for the adenovirus E1A protein *trans*-activation of the E2 promoter (22). An increase in the binding activity of transcription factors which interact with upstream adenovirus E2 promoter sequences has been observed. In still another set of experiments, it has been suggested that the adenovirus E1A protein specifically induces the level of the active polymerase III transcription factor, TFIIIC (14, 36). Alternatively, p40x may bind to region I and III either directly, by sequence recognition, or indirectly, through an intermediate transcription factor. Further studies are required to distinguish among these various possibilities.

The position of the p40x region III, between the two 21-bp repeat sequences, raises an interesting possibility concerning the function of p40x in the *trans*-activation mechanism of the intact HTLV-I LTR. In one model, transcription factors binding to p40x region III might act to stabilize the binding of transcription factors to the 21-bp repeats. Interaction between individual transcription factors binding to separate domains of the DNA has been reported for other eucaryotic promoter elements (3, 17, 30). In the case of SV40 late gene *trans*-activation by the early gene product T antigen, efficient and stable binding of transcription factors to T-antigen-binding site II and sequences within the SV40 72-bp repeats apparently occurs only when the two transcriptional domains are correctly spaced (3). Similarly, Sawadogo and Roeder have demonstrated that the binding of transcription factor TFIID is stabilized by the binding of transcription factor USF to the adenovirus major late upstream control region (30). Jones and Tjian have also demonstrated that efficient transcription of the herpesvirus thymidine kinase gene requires the presence of two transcription factors, SP1 and CTF (17). The CTF-binding sequence is located directly between the two SP1-binding sites and perhaps acts to stabilize SP1 binding. To test this hypothesis in HTLV-I, we increased the spacing between the tandem 21-bp repeats to the wild-type spacing interval, but without the intervening p40x region III sequences. Analysis of these mutant templates should help to determine the importance of the p40x target sequences.

During final editing and preparation of this manuscript, Shimotohno et al. (32) published experiments demonstrating that the HTLV-I 21-bp repeats conferred p40x inducibility on a basal HTLV-I promoter.

LITERATURE CITED

1. **Alwine, J. C.** 1985. Transient gene expression control: effects of transfected DNA stability and *trans* activation by viral early proteins. Mol. Cell. Biol. **5**:1034–1042.
2. **Brady, J. N., J. B. Bolen, M. Radonovich, N. P. Salzman, and G. Khoury.** 1984. Stimulation of simian virus 40 late expression by simian virus 40 tumor antigen. Proc. Natl. Acad. Sci. USA **81**:2040–2044.
3. **Brady, J., M. R. Loeken, and G. Khoury.** 1985. Interaction between two transcriptional control sequences required for T-antigen-mediated simian virus 40 late gene expression. Proc. Natl. Acad. Sci. USA **92**:7299–7303.
4. **Cann, A. J., J. D. Rosenblatt, W. Wachsman, N. P. Shah, and I. S. Y. Chen.** 1985. Identification of the gene responsible for human T-cell leukemia virus transcriptional regulation. Nature (London) **318**:571–574.
5. **Felber, B. K., H. Paskalis, D. Kleinman-Ewing, F. Wong-Staal, and G. N. Pavlakis.** 1985. The pX protein of HTLV-1 is a transcriptional activator of its long terminal repeat. Science **229**:675–679.
6. **Ferguson, B., B. Krippl, O. Andrisani, N. Jones, H. Westphal, and M. Rosenberg.** 1985. E1A 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind DNA. Mol. Cell. Biol. **5**:2653–2661.
7. **Fujisawa, J., M. Seiki, T. Kiyokawa, and M. Yoshida.** 1985. Functional activation of the long terminal repeat of human T-cell leukemia virus type 1 by a *trans*-acting factor. Proc. Natl. Acad. Sci. USA **82**:2277–2281.
8. **Fujisawa, J. I., M. Seiki, M. Sato, and M. Yoshida.** 1986. A transcriptional enhancer sequence of HTLV-I is responsible for *trans*-activation mediated by p40x of HTLV-I. EMBO J. **5**:713–718.
9. **Giam, C.-Z., M. Nerenberg, G. Khoury, and G. Jay.** 1986. Expression of the complete human T-cell leukemia virus type 1 pX coding sequence as a functional protein in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:7192–7196.
10. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
11. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
12. **Green, M. R., R. Tresiman, and T. Maniatis.** 1984. Transcriptional activation of cloned human β-globin genes by viral immediate early gene products. Cell **35**:137–148.
13. **Hartzell, S. W., B. J. Byrne, and K. N. Subramanian.** 1984. The simian virus 40 minimal origin and the 72-base-pair repeat are required simultaneously for efficient induction of late gene expression. Proc. Natl. Acad. Sci. USA **81**:6335–6339.

14. **Hoeffler, W. K., and R. G. Roeder.** 1985. Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. Cell **41**:955–963.
15. **Imperiale, M. J., L. T. Feldman, and J. R. Nevins.** 1983. Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell **35**:127–136.
16. **Imperiale, M. J., R. P. Hart, and J. R. Nevins.** 1985. An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and E1A-induced transcription. Proc. Natl. Acad. Sci. USA **82**:381–385.
17. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter *in vitro*. Cell **42**:559–572.
18. **Kalyanaraman, V. S., M. Sarngadharan, P. Bunn, J. Minna, and R. C. Gallo.** 1981. Antibodies in human sera reactive against an internal structural protein of human T-cell lymphoma virus. Nature (London) **294**:271–273.
19. **Kao, H.-T., and J. R. Nevins.** 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. Mol. Cell. Biol. **3**:2058–2065.
20. **Keller, J. M., and J. C. Alwine.** 1984. Activation of the SV40 late promoter by T-antigen: direct effects in the absence of DNA replication. Cell **36**:381–389.
21. **Keller, J. M., and J. C. Alwine.** 1985. Analysis of an activatable promoter: sequences in the simian virus late promoter required for T-antigen-mediated *trans* activation. Mol. Cell. Biol. **5**:1859–1869.
22. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. E1A transcription induction: enhanced binding of a factor to upstream promoter sequences. Science **231**:719–722.
23. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A *trans*-activation. Cell **45**:219–228.
24. **Loeken, M. R., G. Khoury, and J. Brady.** 1986. Stimulation of the adenovirus E2 promoter by simian virus 40 T-antigen and E1A occurs by different mechanisms. Mol. Cell. Biol. **6**:2020–2026.
25. **Murthy, S. C. S., G. P. Bhat, and B. Thimmappaya.** 1985. Adenovirus EIIA early promoter: transcriptional control elements and induction by the viral pre-early EIA gene, which appears to be sequence independent. Proc. Natl. Acad. Sci. USA **82**:2230–2234.
26. **Paskalis, H., B. K. Felber, and G. N. Pavlakis.** 1986. Cis-acting sequences responsible for the transcriptional activation of human T-cell leukemia virus type I constitute a conditional enhancer. Proc. Natl. Acad. Sci. USA **83**:6558–6562.
27. **Poiesz, B. J., R. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with T-cell lymphoma. Proc. Natl. Acad. Sci. USA **77**:7415–7419.
28. **Queen, C., and D. Baltimore.** 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell **33**:741–748.
29. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. Location of cis-acting regulatory sequences in the human T-cell leukemia virus type I long terminal repeat. Proc. Natl. Acad. Sci. USA **82**:6502–6506.
30. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:167–175.
31. **Seiki, M., J.-I. Inoue, T. Takeda, and M. Yoshida.** 1986. Direct evidence that p40x of human T-cell leukemia virus type I is a *trans*-acting transcriptional activator. EMBO J. **5**:561–565.
32. **Shimotohno, K., M. Takano, T. Teruuchi, and M. Miwa.** 1986. Requirement of multiple copies of a 21-nucleotide sequence in the *U3* regions of human T-cell leukemia virus type 1 and type 2 long terminal repeats for *trans*-acting activation of transcription. Proc. Natl. Acad. Sci. USA **83**:8112–8116.
33. **Sodroski, J. G., C. A. Rosen, W. C. Goh, and W. A. Haseltine.** 1985. A transcriptional activator protein encoded by the x-lor region of the human T-cell leukemia virus. Science **228**:1430–1434.
34. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. *trans*-Acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225**:381–384.
35. **Stein, R., and E. Ziff.** 1984. HeLa cell β-tubulin gene transcription is stimulated by adenovirus type 5 in parallel with viral early genes by an E1a-dependent mechanism. Mol. Cell. Biol. **4**:2792–2801.
36. **Yoshinaga, S., N. Dean, M. Han, and A. J. Berk.** 1986. Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A-dependent increase in transcription factor IIIC concentration. EMBO J. **5**:343–354.