Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6558–6562, September 1986
Genetics

Cis-acting sequences responsible for the transcriptional activation of human T-cell leukemia virus type I constitute a conditional enhancer

(eukaryotic gene regulation/inducible enhancer/T-cell leukemia lymphoma)

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Communicated by Fotis C. Kafatos, May 19, 1986

ABSTRACT **Transcription from the long terminal repeat promoter of human T-cell leukemia virus type I is activated in the presence of a trans-activator protein, TA-I, encoded in the 3′ part of the genome. A series of long terminal repeat mutants and hybrid promoter constructs have been studied in a transient expression assay for their ability to be activated in the presence of the trans-activator protein. The sequences responsible for trans-activation have properties similar to those of transcription enhancer elements. They act relatively independent of position and orientation and activate both the homologous as well as heterologous promoters only in the presence of the trans-activator protein. Therefore, the trans-activator protein of human T-cell leukemia virus type I acts via an inducible enhancement mechanism.**

Human T-cell leukemia virus type I (HTLV-I) is an exogenous retrovirus, etiologically associated with adult T-cell leukemia lymphoma (1–3). HTLV-I is a member of a family of retroviruses that includes simian T-cell leukemia virus type I (4), human T-cell leukemia virus type II (HTLV-II) (5), and bovine leukemia virus (6). These viruses have similarly organized genomes that distinguish them from other retroviruses. In addition to the typical retroviral genomic regions *gag*, *pol*, and *env*, they contain at the 3′ part of the genome (originally called the *pX* region) additional open reading frames that encode at least two proteins (7–14). We and others have shown that the product of the longest open reading frame is a nuclear protein involved directly or indirectly in the activation of the long terminal repeat (LTR) promoter (15–17). This protein has been called variably pX, $p40^{xIV}$, X-lor, tat 1, and TA-I. We will refer to this protein as TA-I (trans-activator protein of HTLV-I) throughout this paper.

In this study, we use deletion mutagenesis and hybrid promoter constructs to identify the sequences that are required in *cis* for the trans-activation of the LTR promoter. We show that these sequences have all the characteristics of enhancer elements. Transcriptional enhancer elements have been shown to play an important role in the regulation of eukaryotic gene expression (18–23). A property of enhancers that distinguishes them from other elements is their ability to function relatively independent of position and orientation. It is thought that enhancer activation involves sequence-specific DNA-binding proteins that activate transcription from adjacent promoters by unknown mechanisms. Herein we show that the HTLV-I enhancer acts efficiently only in the presence of TA-I, indicating that TA-I is an essential element for the activation of the enhancer.

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MATERIALS AND METHODS

Plasmid Constructs. A collection of 5′ and 3′ deletions was constructed by BAL-31 treatment of plasmid pL1CAT (ref. 15 and Fig. 1). The 3′ deletions were ligated upstream from the metallothionein "TATA" box in plasmid pΔ34MTCAT that contains a fragment of the mouse metallothionein (mMT-I) promoter from nucleotide (nt) −34 to nt +68 (nt +1 is the transcriptional start site) upstream of the chloramphenicol acetyltransferase (CAT) coding sequence. The size of the deletions was determined by DNA sequencing (24). Plasmid MAXneo, a derivative of pMXL (15), produces TA-I and contains the simian virus 40 (SV40)-neo cassette of pSV2neo (25). Plasmid L1XL produces TA-I from the HTLV-I LTR promoter. Plasmid MAXneoΔL is a derivative of pMAXneo missing the entire HTLV-I LTR except for nt 1–30 of the U3 region. pMAXneoΔL produces a functional TA-I missing 16 amino acids from the carboxyl terminus (15).

Transfections. Monkey CVI cells were transfected by the calcium phosphate coprecipitation technique (26). The calcium phosphate–DNA coprecipitate at final DNA concentration of 35 μg/ml was applied to the cells for 4 hr. The coprecipitate contained the CAT producing plasmid (10 μg/ml) and the TA-I producing plasmid (10 μg/ml) as needed. The concentration was adjusted to 35 μg of DNA per ml by adding purified salmon sperm DNA. By this protocol, 10–20% of the cells scored positive for transfection as determined by an immunofluorescence assay. Human cell lines were transfected by the DEAE-dextran technique (27).

CAT assays and S1 mapping were performed as described (28–30). Single-stranded, uniformly labeled DNA probes were produced by primer extension from appropriate restriction fragments subcloned into M13 vectors (31). Quantitation of the CAT assays was done by liquid scintillation counting.

RESULTS

To identify the sequences necessary for the trans-activation, we constructed a series of 5′ and 3′ deletions of plasmid pL1CAT (15), and we tested their ability to act as promoters after transfection into mammalian cells in culture (Fig. 1). To study the activation of HTLV-I LTR by TA-I, pMAXneo, a plasmid producing TA-I, was cotransfected into the CV1 cell line, and the trans-activation was calculated by comparison with cells transfected only with the LTR-CAT plasmid. The results of these experiments are summarized in Fig. 2 and Table 1. The activity of the LTR promoter in the absence of the TA-I protein was detectable but low compared to other promoters such as the Rous sarcoma virus or the SV40 early

Abbreviations: HTLV-I and HTLV-II, human T-cell leukemia virus types I and II; LTR, long terminal repeat; TA-I, trans-activator protein of HTLV-I; mMT-I, mouse metallothionein; nt, nucleotide(s); SV40, simian virus-40; CAT, chloramphenicol acetyltransferase.

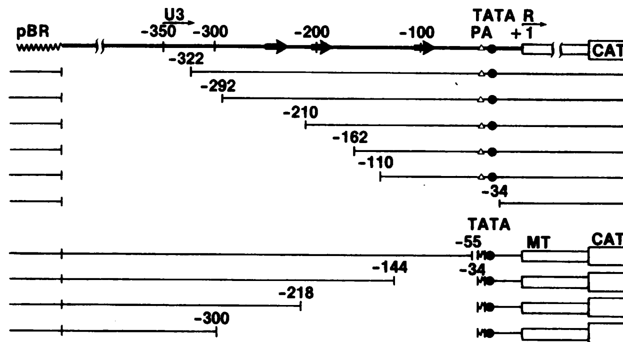

FIG. 1. 5′ and 3′ deletion mutants of the HTLV-I LTR. Plasmid pL1CAT (15) was digested with either *Sma* I or *Bgl* II and treated with BAL-31 nuclease to generate 5′ and 3′ deletions, respectively. For the 5′ deletions, the same pBR322 sequences were ligated to the deletion point. Thick arrows, three 20-base repeats that exist in the U3 region of the LTR. Solid circles, TATA box. Triangles, polyadenylylation signal (pA) AAUAAA. Thin arrows, beginning of U3 and R regions.

promoter. This basal level activity was further decreased by the deletions within the LTR (Fig. 2). Deletion to nt −322 eliminated most of the basal level expression, but the activation was not affected. Deletions to nt −210 and nt −162 affected the trans-activation marginally (Table 1), whereas deletions to nt −110 and beyond eliminated the activation completely. A gradual decrease at the level of both induced and uninduced expression of subsequent deletions was observed. Qualitatively similar results were obtained in human T-cell lines such as HUT 78.

These experiments suggested that the left border of the sequences necessary for trans-activation is after nt −162. To define the right border, deletion mutants were constructed starting from the *Bgl* II site of pL1CAT at nt +263 in the U5

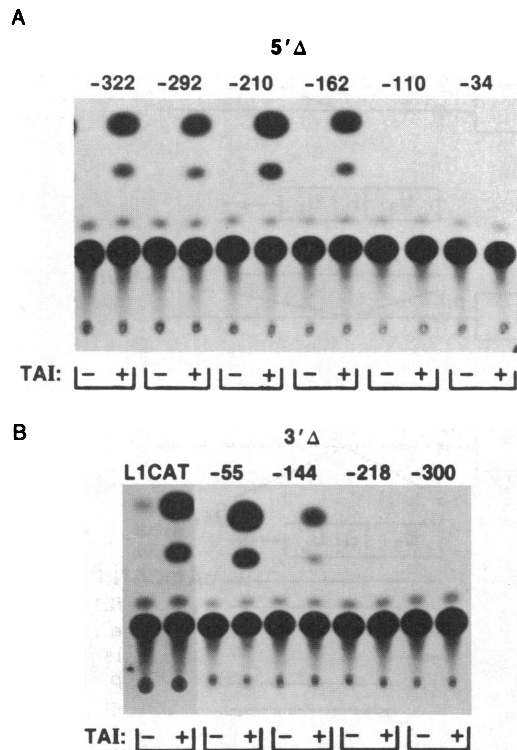

FIG. 2. CAT assays after transfections of CVI cells with the L1CAT deletion mutants in the presence (+) or in the absence (−) of the trans-activator-producing plasmid MAXneo. (*A*) 5′ deletions; (*B*) 3′ deletions.

Table 1. Activation of LTR constructs by TA-I

Plasmid	Activation*
pL1CAT	40×
p5′Δ-322	43×
p5′Δ-292	32×
p5′Δ-210	35×
p5′Δ-162	25×
p5′Δ-110	1.5×
p5′Δ-34	1.5×
p3′Δ-55	75×
p3′Δ-144	15×
p3′Δ-218	1.7×
p3′Δ-300	1×
p5′Δ-162L	36×
p5′Δ-162iL	60×
p5′Δ-110L	18×
p5′Δ-110iL	19×
pA10CATL1	7×
pA10CATiL1	6×
pA10CATe	5×
pA10CATie	6×
pA10CATp	1×
pA10CATip	1×

*CVI cells were transfected by the indicated plasmids in the presence or in the absence of the TA-I-producing plasmid pMAXneo. After autoradiography, the percent conversion of chloramphenicol was determined by liquid scintillation counting of the TLC plates. Activation was calculated by comparison of the percent conversion of chloramphenicol in the presence of TA-I to that in the absence of TA-I. Results shown are the average of three to six experiments with different plasmid DNA preparations.

region of the LTR. Deletions very close to the transcriptional start site were active in the trans-activation assay, indicating that no R region sequences are necessary for trans-activation (data not shown). To study the effect of larger 3′ deletions lacking a TATA box, a mMT-I promoter fragment from nt −34 to nt +68 ligated to the CAT gene was provided downstream from the deletions. This fragment included the TATA box and the transcriptional start site of the MT-I gene (Fig. 1). CAT enzyme measurements and S1 mapping showed that this fragment was not active as a promoter after transfections in animal cells in the absence of appropriate upstream sequences (ref. 32 and B.K.F., unpublished data). When ligated upstream from this deleted promoter, 3′ deletions to nt −55 and nt −144 were active in the trans-activation assay, while deletions to nt −218 and nt −300 were inactive (Fig. 2*B*). Deletion to nt −55 was activated equally well or better than pL1CAT, but deletion to nt −144 gave a consistently lower ratio of induced to uninduced expression (Table 1). These results indicate that the right boundary of the trans-activation-responsive sequences is approximately at nt −144. Therefore, upstream promoter elements of the LTR can induce the activation of the deleted mMT-I promoter in the absence of any HTLV-I R sequences.

To verify that levels of CAT enzyme reflect the levels of correctly initiated mRNA, the produced mRNA was examined by S1 mapping. For the 5′ deletions, a uniformly labeled single-stranded probe was used as described (15). Since pMAXneo contains one HTLV-I LTR, pMAXneoΔL was used as a TA-I producer. pMAXneoΔL produces a functional trans-activator missing 16 amino acids from the carboxyl terminus. Correctly initiated mRNA was detected only in the presence of the trans-activator. The results are consistent with those obtained by the CAT assays (data not shown). For the 3′ deletions, we used a mMT-I-specific, uniformly labeled, single-stranded probe which, upon S1 mapping, gave a 68-nt fragment indicative of the correctly initiated mMT-I mRNA. Since pMAXneo contains a mMT-I promoter, we

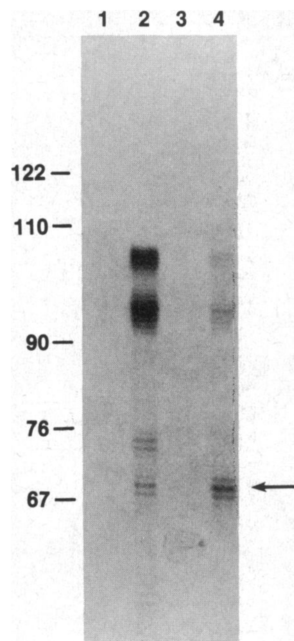

FIG. 3. S1 mapping of the 3′-deletion mutants. CV1 cells were transfected with DNA, and 2 days later total RNA was isolated and mapped. Cells were transfected with 3′Δ-55 (lane 1); 3′Δ-55 + L1XL (TA-I producer, lane 2); 3′Δ-144 (lane 3); 3′Δ-144 + L1XL (lane 4). The size of pBR322/HpaII DNA markers is indicated in bases. The arrow denotes the correct mMT-I start site. Higher bands suggest the presence of read through transcripts and aberrant initiation sites.

used pL1XL as a TA-I producer plasmid. In the presence of TA-I, correctly initiated mMT-I mRNA was observed for the deletions 3′Δ-55 and 3′Δ-144 (Fig. 3), but not for 3′Δ-218 and 3′Δ-300. In the absence of TA-I, no protected fragments are observed with any of the deletions. In addition to the correctly initiated mRNA, some higher bands are present particularly in the case of 3′Δ-55. Therefore, other start sites are used, in addition to the correct mMT-I start site. Aberrant initiation sites occur with many other constructs containing the mMT-I promoter.

We next examined whether the location and orientation of the cis-acting elements relative to the promoter were important for the trans-activation. For this, the following two deletion mutants were selected: Δ5′-162, which was the shortest active 5′ deletion, and 5′Δ-110, which was completely inactive in the trans-activation assay. An almost complete copy of the LTR was inserted in these plasmids at the 3′ part of the transcriptional unit, in the unique *Bam*HI site that exists close to the SV40 polyadenylylation site (Fig. 4). The LTR was inserted in both orientations as a *Sma* I fragment missing only the first 30 nt of the U3 region and containing human genomic DNA sequences after the U5 region. The results of the CAT assays using these constructs are shown in Fig. 5*A*. As mentioned above, 5′Δ-162 was activated to a lesser extent than pL1CAT. When the LTR fragment of HTLV-I was inserted at the *Bam*HI site in either orientation, the CAT enzyme activity was restored to the pL1CAT levels (Fig. 5*A*). Similarly, 5′Δ-110, which was inactive, could be activated very well if it contained the HTLV-I LTR fragment at the *Bam*HI site. Similar results were obtained with other deletions such as 5′Δ-55 (data not shown). We conclude that sequences within the HTLV-I LTR can act irrespective of location and orientation when ligated to these LTR deletion mutants. S1 mapping experiments after transfections with constructs p5′Δ-55e and p5′Δ-55ie, which contain the e fragment (see next paragraph) at the *Bam*HI site in either orientation, demonstrated that the correct transcription start sites were used (data not shown).

We then examined whether other promoters can be acti-

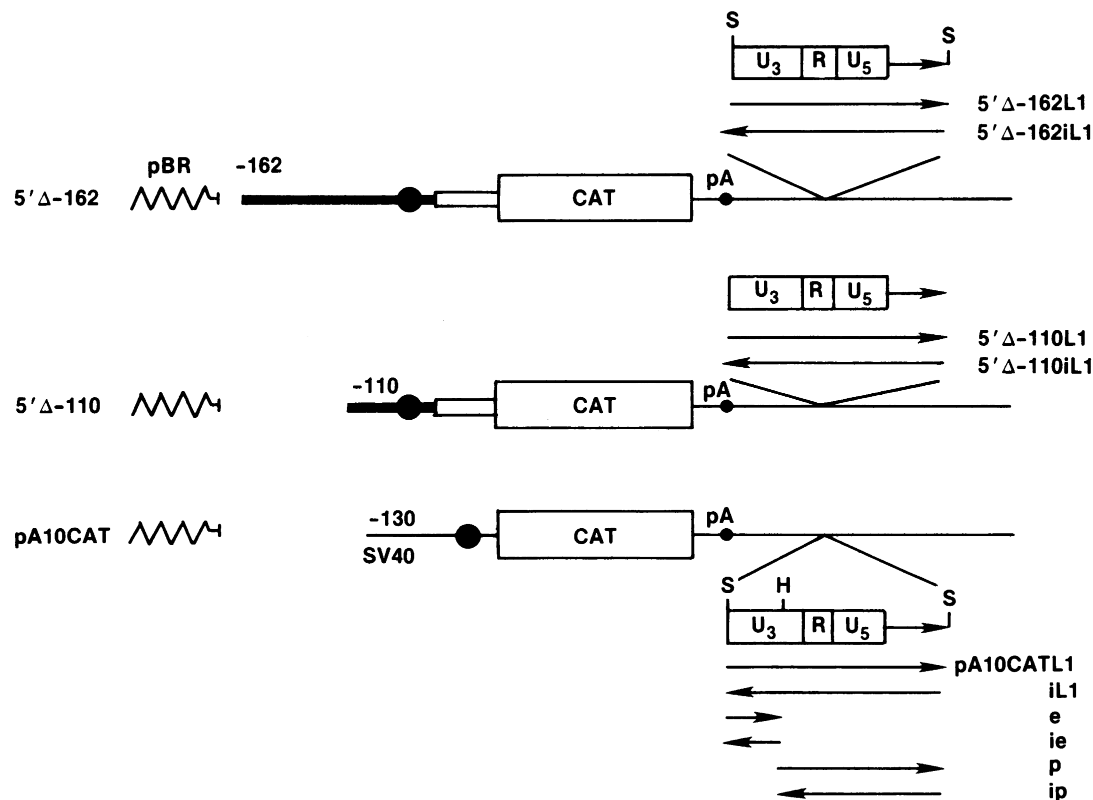

FIG. 4. Plasmids containing HTLV-I LTR fragments. A *Sma* I fragment containing an almost complete LTR except for 30 nt of the U3 region was inserted into the *Bam*HI site of 5′Δ-162 or 5′Δ-110 in both orientations. Plasmid pA10CAT contains an enhancerless SV40 early promoter ligated to the CAT gene (33). HTLV-I LTR fragments were inserted into the *Bam*HI site. Fragment e is a *Sma* I–*Hin*cII fragment containing only U3 sequences. Fragment p is a *Hin*cII–*Sma* I fragment containing 110 nt of the U3 region and the entire R and U5 regions of HTLV-I as well as human flanking genomic sequences. S, *Sma* I; H, *Hin*cII, pA, polyadenylylation signal. In all plasmids, i indicates transcriptional orientation opposite that of the CAT gene.

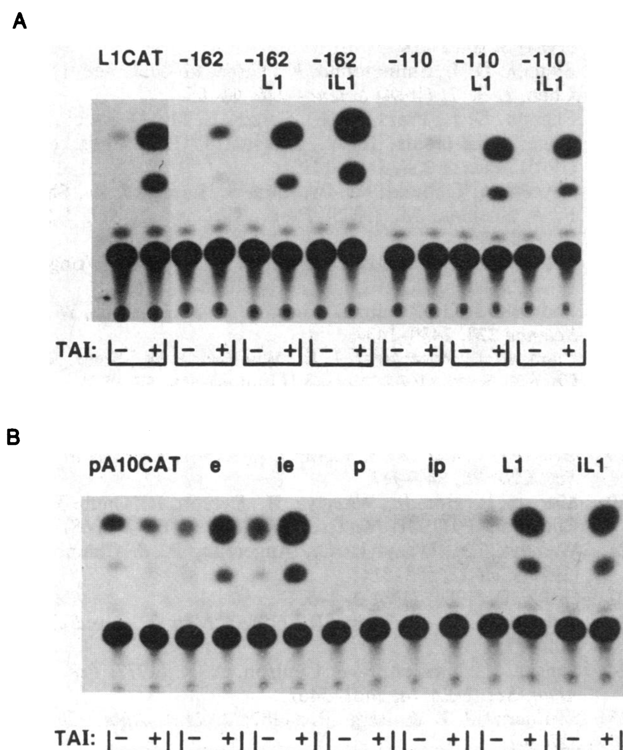

FIG. 5. CAT assays after cotransfections with the constructs shown in Fig. 3. CV1 cells were transfected with various plasmids in the presence (+) or absence (−) of a trans-activator-producing plasmid, MAXneo. (*A*) pL1CAT deletion mutants containing an LTR on the 3′ side of the promoter at two different orientations. (*B*) pA10CAT constructs containing either an almost complete LTR (pA10CATL1, pA10CATiL1) or fragments e and p at two different orientations.

vated in the presence of TA-I if they contain the HTLV-I LTR sequences in *cis*. We chose to insert the LTR, or fragments derived from it, in the *Bam*HI site of plasmid pA10CAT (33), a plasmid that contains an enhancerless SV40 promoter (Fig. 4). The same *Sma* I LTR fragment described above was inserted in pA10CAT as well as two smaller fragments designated e and p in Fig. 4. Fragment e contained exclusively U3 sequences (nt −322 to −96). Fragment p contained the remaining U3 region and the complete R and U5 regions of the LTR and human flanking DNA. Fig. 5*B* shows that the plasmids that contain either the *Sma* I fragment or the e fragment are activated in the presence of TA-I. Since both orientations are active, we conclude that within the U3 region of HTLV-I LTR, there exist sequences that act as a conditional enhancer, i.e., they act independently of position or orientation and only in the presence of TA-I. On the contrary, fragment p is completely inactive in the trans-activation assay. Further analysis has shown that subfragments of e as well as synthetic oligonucleotides can also act as conditional enhancers (unpublished results).

DISCUSSION

One major conclusion of this work is that the region responsible in cis for the trans-activation of HTLV-I LTR by the virally encoded trans-activator TA-I lies entirely within the U3 region of the LTR. Furthermore, this region can function when inverted or positioned further away from the LTR promoter or from heterologous promoters such as the SV40 early promoter and the mMT-I promoter. These characteristics indicate that the cis-acting element in the LTR promoter is a conditional enhancer, active only in the presence of the trans-activator TA-I. In addition they indicate that TA-I acts entirely at the transcriptional level to increase the rate of transcription of the homologous or even a heterologous promoter. It is unlikely that mRNA stabilization contributes to the activation, since we have shown that the mMT-I and SV40 early promoters are not affected by TA-I (15), while constructs of these promoters containing HTLV-I U3 sequences are. In addition, nuclear runoff experiments (unpublished results) indicate that there is an increase in the rate of transcription in the presence of TA-I.

The left border of the cis-acting sequences was identified at nt −162 by the 5′ deletions. The right border defined by the 3′ deletions was nt −144. However, in both series of mutants, sequences upstream or downstream apparently contribute to the overall strength of the promoter. Moreover, the sequences between nt −162 and nt −144 by themselves cannot activate a heterologous promoter (unpublished results). Therefore, additional sites within the U3 region are necessary for the activation of the HTLV-I LTR. One hypothesis that would explain the results of the deletion mutagenesis is that repeated binding sites exist within the U3 region for the binding of TA-I and/or other factors affected by the presence of TA-I. The most striking repeated elements in the U3 region are the three 20-base repeats at positions −251 to −233, −203 to −185, and −103 to −85 indicated by arrows in Fig. 1. These repeats exist also in the U3 regions of simian T-cell leukemia virus type I (4) and HTLV-II (34, 35) and constitute the only conserved elements in the U3 region between HTLV-I and HTLV-II other than the TATA box, the polyadenylylation site, and a region around the cap site. Similar repeats exist also in the U3 region of bovine leukemia virus (refs. 9 and 10 and our unpublished results). Since the activators of HTLV-I and HTLV-II are interchangeable, and since the activator of HTLV-II interacts with the HTLV-I LTR deletions in a similar way (unpublished results), it is attractive to postulate that the 20-base repeat is the place of interaction between trans-activators and LTR for both HTLV-I and HTLV-II and that more than one repeat is necessary for trans-activation. Repeated elements appear to be the rule for many enhancers (for review see ref. 36). In this model, deletion mutants with two or more repeats would be active in the presence of TA-I, whereas those with one repeat would be inactive. With the exception of the 5′Δ-162, all the deletion mutants obey this rule. To explain the inducibility of the 5′Δ-162, we hypothesize that binding sites exist in this deletion for factor(s) that act in cooperation with the factor(s) binding to the remaining 20-base repeat and facilitate the activation. We noticed an imperfectly repeated sequence at positions −155 and −144 (GGAAGCCACC and GGAA−C-CACC). Although such sequences may contribute to the final strength of the LTR promoter independent of the presence of TA-I, our data indicate that the most important element for the transcriptional activation of the HTLV-I LTR is an enhancer that is induced in the presence of TA-I in the same cell. We have now demonstrated that this enhancer is contained within the 20-base-pair repeats (unpublished results).

The mechanism by which HTLV-I causes leukemia is not understood. HTLV-I has some properties of chronic leukemia viruses—i.e., it is replication competent, causes a monoclonal malignancy after a long latency period, and does not contain any transduced cellular oncogenes (3). However, the sites of integration in the leukemic cells are apparently random (37), which rules out the activation of cellular genes by insertion of viral promoters/enhancers. Also, HTLV-I can efficiently immortalize human lymphocytes *in vitro* (38, 39), a property associated with acute transforming viruses containing oncogenes. It has already been proposed by several investigators that TA-I is the transforming protein of HTLV-I analogous to other viral proteins that are modulators

of viral gene expression [SV40 and polyoma tumor (T) antigens and E1A protein of adenovirus]. Although this hypothesis has not been proven up to date, it is reasonable to propose that TA-I may activate some cellular genes by interacting with certain cellular enhancers.

The results presented above are in disagreement with data presented by Rosen *et al.* (40). These authors divided the LTR into an "enhancer" fragment (nt −350 to nt −55) and a "promoter" fragment (−55 to +315). They concluded that sequences responsive to the trans-acting factors are not present in their entirety within the region −350 to −55 that contains the HTLV-I enhancer and that the HTLV-I enhancer element is not responsive to the virus-associated trans-acting regulatory factors. At the present time, we do not understand the reason for this disagreement.

It has been shown that the acquired immune deficiency syndrome (AIDS) retrovirus human T-cell leukemia virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) is also activated by a viral factor (40, 41). The interaction of this factor with the LTR is also specific, but it does not appear to be similar to HTLV-I in that the HTLV-III/LAV trans-activator requires sequences within the R region for appropriate function (refs. 41 and 42 and our unpublished results). Therefore, the possibility exists that mechanisms other than transcriptional regulation are involved in the activation of HTLV-III/LAV expression.

Note Added in Proof. Similar conclusions were published by Fujisawa *et al.* (43). These authors reported also that deletions with one copy of the 20-base-pair repeat such as 3′Δ-216 are transactivated, which is in disagreement with our results.

We thank Gillian Kaplin for excellent technical assistance; F. Wong-Staal and R. C. Gallo for materials and discussions; S. Hughes for helpful comments; and H. Marusiodis and K. Bivens for expert editorial assistance. This research was sponsored by the National Cancer Institute, Department of Health and Human Services, under Contract N01-CO-23909 with Bionetics Research, Inc. H.P. was supported by the Leukemia Society of America. B.K.F. was supported by a Swiss National Science Foundation grant.

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