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Characterization of Cellular Factors That Interact with the Human T-Cell Leukemia Virus Type I p40^x-Responsive 21-Base-Pair Sequence†

KUAN-TEH JEANG,‡* IMRE BOROS, JOHN BRADY, MICHAEL RADONOVICH, AND GEORGE KHOURY
Laboratory of Molecular Virology, National Cancer Institute, Bethesda, Maryland 20892

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Transcriptional activation of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) by viral protein p40^x requires a 21-base-pair (bp) sequence which is repeated three times within the LTR. This sequence contains a core octanucleotide (TGACGTCT) which has been attributed to be a cyclic-AMP (cAMP)-responsive element. We demonstrate here that the HTLV-I LTR can be specifically stimulated by cAMP regulators and have identified four proteins in HeLa cells that bind to the HTLV-I 21-bp sequence. We correlated the in vitro binding and transcriptional activity of one of these cellular factors (M_r, 180,000) to the *trans*-activation of the HTLV-I LTR by p40^x. Point mutations were generated within the cAMP octanucleotide of the HTLV-I 21-bp sequence that simultaneously abolished biological responsiveness to *trans*-activation by p40^x and to stimulation by cAMP. We found that these mutations also eliminated the binding of the 180-kilodalton HeLa factor to the HTLV-I 21-bp element. In the absence of a demonstrable DNA-binding property for p40^x, we hypothesize that cellular proteins are involved, possibly through signal transduction pathways, in its *trans*-activation of responsive promoters.

Human T-cell leukemia virus type I (HTLV-I) has been established as the etiological agent for one particular form of adult T-cell leukemia (for a review, see reference 19). At the 3′ end of the HTLV-I genome is an open reading frame that encodes, through a double-splicing event, a single 2.1-kilobase (kb) mRNA which can be alternately translated into three distinct proteins (p40^x, p27^x, and p21^x) (33, 47). A similar open reading frame has also been described for HTLV-II (27) and bovine leukemia virus (9, 42). In HTLV-I, the p40^x protein, a 40-kilodalton (kDa) polypeptide, has been directly implicated as the viral gene product responsible for positive transcriptional *trans*-regulation of the promoter element found within the HTLV-I long terminal repeat (LTR) (14, 17, 53, 58).

The HTLV-I LTR contains three copies of an imperfectly conserved 21-base-pair (bp) sequence (32, 51). Deletion analysis of the HTLV-I LTR and chimeric constructions utilizing LTR sequences linked to reporter genes illustrated that two or more copies of this 21-bp sequence, regardless of orientation, can serve as a sufficient target for p40^x *trans*-activation (4, 50, 54). It has also been shown that some cellular genes are transcriptionally stimulated by p40^x but do not contain this 21-bp element (8, 29). These findings suggest an absence of one specific sequence exclusively required for p40^x-mediated *trans*-activation.

This theme of a lack of exclusive target sequence requirement has been similarly described for other viral *trans*-activators (for a review, see reference 34). The adenovirus E1a, the herpesviruses immediate-early proteins, and the SV40 large-T antigen all *trans*-activate many different genes. E1a, for example, can transcriptionally induce genes as diverse as rat preproinsulin (21), human β-globin (60), simian virus 40 (SV40) early promoter (61), and other adenovirus promoters (3, 31). An equally large recitation can be compiled for the herpes and SV40 *trans*-activators. Since the stimulated genes do not share common target sequences for induction, it is unlikely that these viral proteins mediate their transcriptional effects through a direct sequence-specific binding mechanism. Thus generalized cellular factors and pathways are probably involved in many viral *trans*-activation events.

It is possible, however, that p40^x acts through a direct interaction with the upstream sequences of responsive genes (19). We tested this possibility and failed to detect significant binding of p40^x to the HTLV-I LTR or even generalized binding to neutral DNAs. Hence, we undertook to identify cellular proteins that may interact with the HTLV-I LTR as a step toward a better understanding of the regulatory properties of p40^x. The present study utilized a UV-cross-linking procedure to probe for proteins in HeLa cells that bind to the p40^x-responsive 21-bp element of HTLV-I. We found four groups of proteins with molecular weights of 76,000, 120,000, 180,000, and >200,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), that preferentially bound to the HTLV-I 21-bp sequence.

Results from point mutations generated within the HTLV-I 21-bp sequence suggest that the binding of one of these cellular proteins, the 180-kDa species, correlated with the *trans*-activation function of p40^x. These mutations also revealed the importance of a highly conserved octameric sequence (5′-TGACGTCT-3′), shown in other systems to be a cyclic-AMP (cAMP)-responsive element, for p40^x-mediated regulation of the HTLV-I LTR. We present evidence suggesting that similar target sequences may be shared within the HTLV-I LTR for activation by both p40^x and cAMP.

* Corresponding author.
† Dedicated to the memory of George Khoury.
‡ Present address: Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.

MATERIALS AND METHODS

UV cross-linking of proteins to DNA probes. Synthetic oligonucleotide probes were generated using an Applied Biosystems model 380A synthesizer. The DNA was purified according to the manufacturer's instructions. A 1-μg portion of oligonucleotide was nick translated at 17°C for 90 min in 10 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–100 mM NaCl–1 mM dithiothreitol–250 μM dGTP–250 μM dATP–1.5 μM [^{32}P]dCTP (specific activity, 400 Ci/mmol; Amersham Corp.)–250 μM bromodeoxyuridine (BUdR)-triphosphate (Sigma Chemical Co.) by using 5 U of Kornberg polymerase (Boehringer Mannheim Biochemicals). Where necessary, DNA was preincubated with 10^{-8} g (final concentration) of DNase I at room temperature for 1 min to generate nicks. After radiolabeling, the oligonucleotides were analyzed by gel electrophoresis and visualized by autoradiography to verify that they remained intact.

In the UV-cross-linking reactions, 50 μg of HeLa nuclear extract (11) was first incubated on ice in a 10-μl reaction volume in buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.9; 1.5 mM $MgCl_2$; 40 mM KCl; 1 mM spermidine) containing 2 to 4 μg of poly(dI-dC) (Pharmacia Fine Chemicals) for 15 min. Radiolabeled DNA probe (10 ng) was added, and incubation was continued for an additional 15 min. The entire reaction mixture was then transferred onto a piece of parafilm and irradiated with a handheld UV lamp (Mineral Light model R-51) at a distance of 2 cm for 90 s. After irradiation, 10 μl of SDS sample buffer (50 mM Tris hydrochloride, pH 7.0; 2% SDS; 1.5 M β-mercaptoethanol; 5% glycerol; 0.01% bromophenol blue) was added. The mixture was heated at 68°C for 5 min and then electrophoresed in a 10% SDS-polyacrylamide gel.

Gel mobility shift assay. Gel mobility shift assays were performed as previously described (16, 20). In the competition experiments, 1 μg of unlabeled competitor oligonucleotide or 2 to 4 μg of poly(dI-dC) was added to the HeLa protein extract and incubated on ice for 15 min before the addition of labeled probe. Electrophoresis of protein-DNA complexes was conducted in 4, 6, or 8% acrylamide gel in 0.25× TBE (1× TBE is 90 mM Tris hydrochloride plus 90 mM boric acid plus 1 mM EDTA [pH 8.3]) at a constant current of 11 mA. In some experiments, the protein-DNA mixture was subjected to UV irradiation before electrophoresis. Relevant bands from these experiments were visualized by autoradiography of wet gel, followed by razor blade excision of the gel slices. After saturation at 68°C for 15 to 30 min in SDS sample buffer, the gel slices were "stuffed" into the stacking wells of an SDS-polyacrylamide gel. This second gel electrophoretically resolved the covalently linked protein-DNA complexes, as determined from the migration properties of the protein component.

Oligonucleotide sequences and plasmid constructions. The oligonucleotides used in the experimental protocols included:

(i) wild-type (WT) HTLV-I 21-bp oligonucleotide (32)

5′-GATCAAGGCTCTGACGTCTCCCCCC
TTCCGAGACTGCAGAGGGGGGCTAG

(The seven mutated versions of this 21-bp sequence are listed in Fig. 2.)

(ii) *lac* operator oligonucleotide (57)

5′-GATCAATTATGAGCAGATAATAATT
TTAATACTCGTCTATTATTAACTAG

(iii) *ori*-P oligonucleotide (66)

5′-GATCCAGATTAGGATAGCATATGCTACCCA
GTCTAATCCTATCGTATACGATGGGTCTAG

(iv) H23 oligonucleotide

5′-GATCAGAGCCTCCCAGTGAAAAACA
TCTCGGAGGGTCACTTTTTGTAAAG

(v) rat somatostatin oligonucleotide (46)

5′-GATCCCTTGGCTGACGTCAGAGAGA
GGAACCGACTGCAGTCTCTCTCTAG

For in vivo chloramphenicol acetyltransferase (CAT) assays, tandem synthetic copies of WT (pWT$A_{10}CAT_2$), mutant HTLV-I 21-bp (e.g., pH19$A_{10}CAT_2$), or rat somatostatin (pRS$A_{10}CAT_2$) oligonucleotides were inserted in the sense orientation into the unique *Bgl*II site of plasmid $A_{10}CAT_2$ (35). This *Bgl*II restriction site is located approximately 102 bp upstream of the SV40 AT-rich promoter element.

Column chromatography. A HeLa cell nuclear extract (11, 64) was made from 10 liters of suspension culture. The extract equilibrated in buffer (10 mM Tris hydrochloride, pH 7.9; 100 mM KCl; 10% [wt/vol] glycerol) was fractionated with a fast protein liquid chromatography (FPLC) Mono-S column (Pharmacia). A 60-ml linear salt gradient (starting buffer: 10 mM Tris hydrochloride, pH 7.9; 100 mM KCl; 10% [wt/vol] glycerol; end buffer: 10 mM Tris hydrochloride, pH 7.9; 1.0 M KCl; 10% [wt/vol] glycerol) was used for protein elution.

DNase I footprinting. DNase I footprinting (18) was performed with end-labeled DNA fragments. Single or multiple copies of the WT or H19 synthetic HTLV-I 21-bp was placed in the sense orientation into the unique *Bgl*II site of CAT vector p$A_{10}CAT_2$ (35). The resulting plasmids were restricted with *Acc*I and labeled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham) and Klenow enzyme (New England BioLabs, Inc.). Redigestion with *Sfi*I generated uniquely end-labeled fragments which were then gel purified.

In a typical experiment, 5,000 cpm of probe and 2 μg of poly(dI-dC) were incubated on ice with various amounts of protein in buffer (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 100 mM KCl) for 15 min. The reaction was then brought to room temperature and digested with DNase I (50 μg/ml, final concentration) for 60 s, followed by phenol-chloroform extraction, ethanol precipitation, and analysis in a urea-polyacrylamide gel.

In vitro transcription and RNA analysis. Cell-free transcription was performed with whole-cell extract (44). A typical assay contained 0.5 μg of supercoiled test DNA, 0.5 μg of an internal control β-globin (πSVHPBΔ128) (58) DNA, and 1.5 μg of HeLa whole-cell extract in 15 μl of buffer (20 mM HEPES, pH 7.9; 100 mM KCl; 12.5 mM $MgCl_2$; 0.1 mM EDTA; 2 mM DTT; 17% glycerol; 1 mM each of UTP, ATP, CTP, and GTP). Incubations were for 1 h at 30°C. Equal volumes of buffer or 100 ng of protein column fractions were added to some reactions.

RNA transcribed in vitro was isolated by phenol-chloroform extraction, followed by ethanol precipitation, and then analyzed by using a primer extension assay. A 5′-TTGGGA TATATCAACGGTGG-3′ oligonucleotide primer was synthesized that was complementary to the 5′ end of the CAT gene. Details of this analysis have been described previously (51). The M13 probe and conditions for S1 nuclease analysis

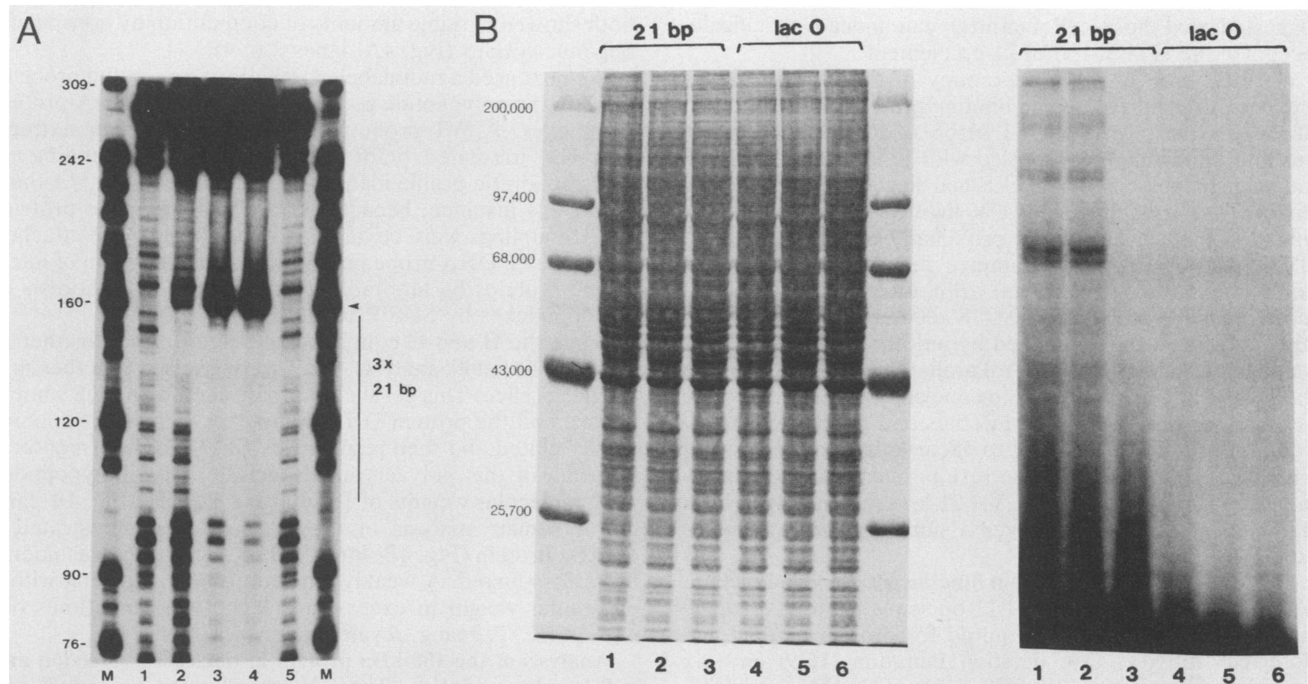

FIG. 1. Identification of HeLa cell factors that bind to the 21-bp repeated sequence of the HTLV-I LTR. (A) Protection of the HTLV-I 21-bp sequence from nuclease digestion. A 10-ng portion of a ^{32}P-labeled DNA fragment containing three tandem copies of the HTLV-I 21-bp sequence was incubated on ice for 15 min with (i) buffer alone (lanes 1 and 5), (ii) buffer with 10 μg of HeLa nuclear extract (lane 2), (iii) buffer with 50 μg of HeLa nuclear extract (lane 3), or (iv) buffer with 70 μg of HeLa nuclear extract (lane 4). After incubation, each reaction mixture was digested with 50 μg of DNase I per ml and electrophoresed in a 6% urea-acrylamide gel. The region of protection over the HTLV-I 21-bp sequences is labeled 3 × 21 bp. The arrowhead points to a generated DNase I-hypersensitive region. Molecular size markers are end-labeled pBR322 *Hpa*II fragments (lane M). (B) A WT 21-bp HTLV-I probe or a *lac*-O probe (see Materials and Methods) was used in UV-cross-linking experiments with 50 μg of HeLa cell nuclear protein extract. Reaction conditions were as follows: UV irradiation for 90 s (lanes 1 and 4); UV irradiation for 90 s followed by DNase I "trimming" at 1 μg/ml (final concentration) for 5 min at room temperature (lanes 2 and 5); and no UV-irradiation (lanes 3 and 6). (Left) Coomassie blue-stained gel. (Right) Autoradiographic exposure of the same gel. The four groups of bands identified in lanes 1 and 2 of the autoradiogram migrated with approximate molecular weights of >200,000, 180,000, 120,000, and 76,000 (top to bottom).

of β-globin-specific transcripts were as described by Laimins et al. (36).

cAMP regulator experiments. K562 erythroleukemia cells (10^7 cells per transfection) were treated with 5 μg of either a human immunodeficiency virus (HIV) LTR-CAT (pBENN CAT) (22) or an HTLV-I LTR-CAT plasmid (pU3RCAT) (58) by the DEAE-dextran procedure (8). At 24 h after treatment the cells were either mock induced or induced with cAMP regulators (25 μM forskolin, 1 mM 8-bromo-cAMP, and 0.5 mM 3-isobutyl-1-methylxanthine; Sigma). After an additional 24 h (i.e., 48 h after DNA introduction), cellular CAT activities were assayed. The same experiments were also performed with plasmids $pWTA_{10}CAT_2$ and $pH19A_{10}CAT_2$.

Other techniques. Standard approaches for recombinant DNA manipulations, gel electrophoresis (43), and CAT assays (24) were used. CAT assay quantitations were based upon the average percentage of incorporation from three separate experiments, as assessed by scintillation counts.

RESULTS

Identification of cellular factors that bind to the HTLV-I LTR. The LTRs of several retroviruses are important in determining the pathogenicity and tissue specificity of these viruses (5, 10, 39; for a review, see reference 13). The HTLV-I LTR contains a promoter element that is strongly and efficiently *trans*-activated by a virally encoded gene product, $p40^x$, which is also known as *tat*-I (14, 17, 53, 58). Because biologically active HTLV-I $p40^x$ produced in large amounts by either procaryotic (22) or eucaryotic (29) expression systems shows no detectable DNA-binding property (unpublished observations), we believe that cellular factor(s) probably play an important role in this *trans*-activation process.

Three repeated 21-bp sequences are found in the HTLV-I LTR and are conserved at the sequence level in the HTLV-II LTR (55). This 21-bp sequence, when present in more than one copy, is a sufficient target for $p40^x$-mediated transcriptional *trans*-activation (4, 50, 54). We reasoned that the 21-bp repeated sequence would be a likely binding site for putative cellular factors. This reasoning was tested by DNase I footprinting and a UV-cross-linking procedure to identify factors within eucaryotic cells that specifically recognize this sequence.

We inserted three synthetic copies of the HTLV-I 21-bp repeat in tandem (see Materials and Methods) upstream of an enhancerless SV40 promoter. This construction permitted a biochemical analysis of the 21-bp repeats that were independent of other HTLV-I LTR sequences. A uniquely end-labeled DNA fragment containing the three copies of the HTLV-I 21-bp repeat was gel isolated and incubated with HeLa cell nuclear proteins. Subsequent DNase I treatment revealed a strong level of protection of the three copies of HTLV-I 21-bp sequence from nuclease digestion by HeLa cellular protein(s) (Fig. 1A, lanes 2 to 4). This in vitro finding

suggested that host cell factor(s) can indeed specifically recognize the HTLV-I viral 21-bp element.

We next used one synthetic copy of the HTLV-I 21-bp sequence to identify specific binding protein(s) in HeLa cell nuclear extract. An HTLV-I 21-bp oligonucleotide probe was first uniformly incorporated with BUdR and [^{32}P]dCTP and then incubated with HeLa cell nuclear proteins. This mixture was irradiated with UV light to generate covalent linkages between DNA and proximally attached proteins. SDS-PAGE of the treated samples delineated four proteins (some migrating as doublets) with molecular weights of approximately 76,000, 120,000, 180,000, and >200,000 (Fig. 1B, right) that were visualized by autoradiography by their coupling to radiolabeled DNA. Parallel experiments using an oligonucleotide probe containing one copy of the procaryotic *lac* operator sequence (see Materials and Methods) demonstrated no significant binding to eucaryotic nuclear proteins (Fig. 1B, lanes 4 to 6). These results illustrated the preferential recognition of the HTLV-I 21-bp sequence by at least four nuclear polypeptides over a similarly sized but unrelated DNA fragment.

Correlation of in vitro protein binding with in vivo function. We mutagenized the HTLV-I 21-bp sequence to investigate the nucleotide sequences required for protein binding and biological activity. One deletion mutation (H19) and six transition mutations (H21, H29, H31, H33, H45, and H47) spanning the 21 bases were independently generated (Fig. 2, top). Two copies of each were inserted, in a sense orientation, upstream of an SV40 early promoter-CAT hybrid gene ($pA_{10}CAT_2$ [36]; Fig. 2, middle). In the resulting plasmids the HTLV-I 21-bp sequences were positioned roughly 100 bases from the SV40 early TATA element. Each plasmid was cotransfected into CV-1 cells with a $p40^x$-producing plasmid (48). CAT activities were assayed to determine their relative responsiveness to $p40^x$. We found that only the H19 and H21 mutations, when compared with WT, were inactive to *trans*-activation by $p40^x$ (Fig. 2, bottom). The other five mutations (H29, H31, H33, H45, and H47) did not differ significantly from WT in biological activity (Fig. 2, bottom).

WT oligonucleotide and oligonucleotides of each of the seven mutations were radiolabeled and used in gel mobility shift experiments (Fig. 3). The WT probe, when incubated with HeLa nuclear extract, produced four migration-retarded DNA-protein complexes (Fig. 3, lane 1, bands A, B, C, and D). All of the mutant probes shared the D complex (Fig. 3, lanes 2 to 8); however, the two mutations that inactivated biological activity (H19 and H21) did not form either the B or the C complexes (Fig. 3, lanes 2 and 3). In contrast, both the B and C bands were seen when the five biologically intact mutants (H29, H31, H33, H45, and H47; Fig. 3, lanes 4 to 8) were used as probes. The A band was inconsistently present (Fig. 3, lanes 1 and 6 to 8) and often variable in intensity. These results correlated the formation of the B and C protein-DNA complexes in vitro with their responsiveness to $p40^x$ *trans*-activation in vivo.

Identification of polypeptides contained within the protein-DNA complexes. The gel shift experiment described above correlated the in vitro binding by the cellular factors responsible for the formation of the B and C complexes with the in vivo biological activity of the HTLV-I 21-bp sequence. We performed an additional set of experiments using unrelated oligonucleotides as competitors to further verify the specificities of these two complexes (Fig. 4). We found that when four different oligonucleotides were used in competition studies, the B and C complexes were erased only by the WT sequence (Fig. 4A, lane 5), whereas the A and D complexes both showed variable amounts of competition by nonspecific oligonucleotides (Fig. 4A, lanes 2 to 4).

We next used a radiolabeled, BUdR-incorporated probe to identify the polypeptide component of the four DNA-protein complexes. A WT probe, incubated with nuclear extract, was UV irradiated before electrophoresis, generating an electrophoretic profile identical to that seen in Fig. 4A (lane 1). In this instance, because of UV induction, the protein-DNA couplings were covalent in nature. Thus, the attached radiolabeled DNA probe permitted the visualization of interacting proteins by autoradiography after electrophoresis of the complexes by SDS-PAGE.

Since the B and C complexes migrated close together in the mobility shift analysis, they were excised together in a single gel slice. This gel slice was saturated with SDS sample buffer, and the protein-DNA complexes were electrophoretically eluted and then resolved by SDS-PAGE. An autoradiogram of this polyacrylamide gel identified polypeptides with molecular weights of 180,000 and 120,000 (Fig. 4B, lane 1). A similar analysis of the D complex demonstrated a 76-kDa protein (Fig. 4B, lane 2). The A complex was poorly UV cross-linked. A weakly labeled protein migrating with a molecular weight in excess of 200,000 was sometimes observed (K.-T. Jeang, results not shown).

Analysis of the 180-kDa protein in nuclease protection and cell-free transcription. The UV-cross-linking procedure can be used to visualize relevant polypeptides during purification. This approach is sensitive enough to detect relatively small amounts of DNA-binding activity against a large background of total cellular proteins. This is how we monitored the chromatographic separation of the four HTLV-I 21-bp binding proteins.

A preparation of HeLa nuclear extract was applied to a FPLC Mono-S resin column (see Materials and Methods) and eluted with a linear (0.1 to 1.0 M KCl) salt gradient. Sequential column fractions were collected, and a small portion of every second fraction was tested by UV cross-linking to a radiolabeled BUdR-incorporated WT oligonucleotide probe. These samples, after treatment, were resolved by SDS-PAGE, stained with silver, and exposed for autoradiography. Although the silver-stained profiles of the column fractions were unremarkable for any particular polypeptide (Fig. 5A, left), relatively clear patterns identifying the 76-, 120-, 180-, and >200-kDa proteins were seen in the autoradiogram of the same gel (Fig. 5A, right). This autoradiogram also delineated a partial separation of the four groups of DNA-binding activities that could not otherwise be appreciated in the stained gel.

This separation of the four DNA-binding proteins allowed us to assess each protein for its individual ability to protect the HTLV-I 21-bp sequence from DNase I digestion. A DNA probe containing one copy of the HTLV-I 21-bp sequence was uniquely radiolabeled at an *Acc*I restriction site (Fig. 5B). This 249-bp fragment, following incubation with whole HeLa nuclear extract (Fig. 5C, lane w) or with selective elution fractions from the FPLC Mono-S column (Fig. 5C, lanes 1, 5, and 7), was treated with DNase I. As expected, we observed a protecting activity for the HTLV-I 21-bp sequence in the whole unfractionated nuclear extract (Fig. 5C, lane w). This activity in the unfractionated nuclear extract was specific for binding to the WT HTLV-I 21-bp element, since it showed poor DNase I protection for a biologically inactive mutated 21-bp sequence (Fig. 5D).

In serial assays of column fractions, we found that fraction 1, which contained the 76-kDa protein (Fig. 5A, lane 1), showed no protection over the HTLV-I 21-bp sequence (Fig.

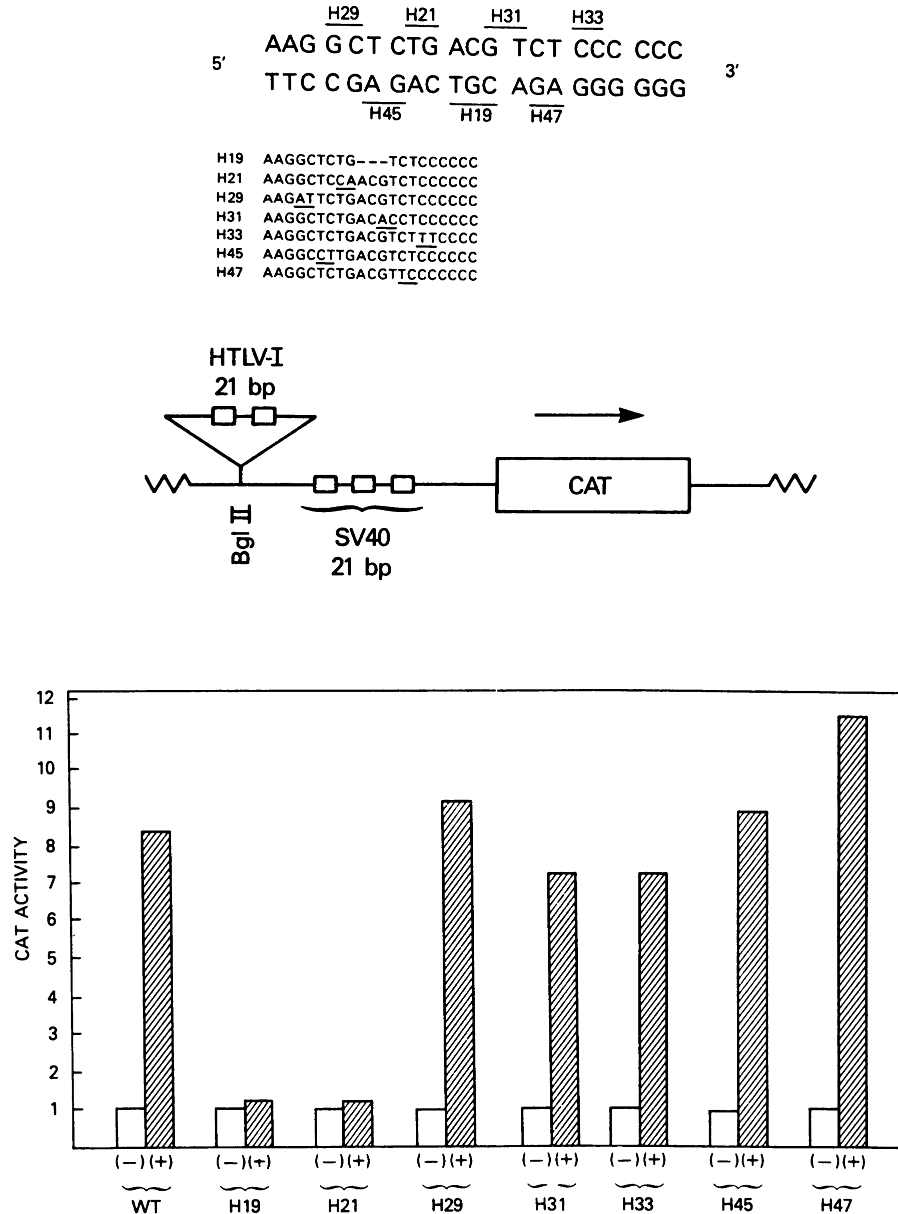

FIG. 2. Mutational analysis of the HTLV-I 21-bp sequence. (Top) The WT HTLV-I 21-bp sequence is presented in duplex form. The relative positions of seven individual mutations are indicated by over- or underlining. The actual sequences of the mutated oligonucleotides are presented in smaller letters showing the sense strand only. (Middle) Schematic representation of a SV40-CAT vector ($pA_{10}CAT_2$) (35) that contains two tandem copies of HTLV-I 21-bp repeat. (Bottom) Relative CAT activities in arbitrary units for the various HTLV-I 21-bp $A_{10}CAT_2$ constructions transfected into CV1 cells with (+) or without (−) a $p40^x$-producing plasmid (48). The graph represents the average values from three separate experiments.

5C, lane 1). Column fraction 5, which contained primarily the 76- and 120-kDa proteins, along with small contaminating amounts of the 180- and >200-kDa proteins (Fig. 5A, lane 5), also did not protect the HTLV-I 21-bp sequence (Fig. 5C, lane 5). Fraction 7, however, which was enriched with the 180-kDa protein (Fig. 5A, lane 7), did protect the 21-bp sequence from nuclease digestion (Fig. 5C, lane 7). Since the DNA probe used for footprinting also contained three copies of the SV40 GC box, a prominent SP1 activity over the SV40 21-bp repeats (12) could also be seen in the unfractionated HeLa nuclear extract (Fig. 5C, lane w), as well as in column fraction 7 (Fig. 5C, lane 7).

The in vitro footprinting results are consistent with the 180-kDa factor (complex B, Fig. 3 and 4) playing a role in the biology of $p40^x$-mediated *trans*-activation. To verify the function of this factor in cell-free transcription, fractions from the FPLC Mono-S column containing partially purified 120-kDa (Fig. 6A, lane 5) or 180-kDa (Fig. 6A, lane 7) factors were separately added to Manley transcription lysates prepared from HeLa cells. Two templates were compared in the in vitro transcription assay. The first was plasmid pH19 $A_{10}CAT_2$, which contained two synthetic copies of the inactive H19 mutations placed upstream of an SV40 early-promoter-driven CAT gene (Fig. 6B). The second plasmid, $pWTA_{10}CAT_2$, contained tandem copies of the WT HTLV-I

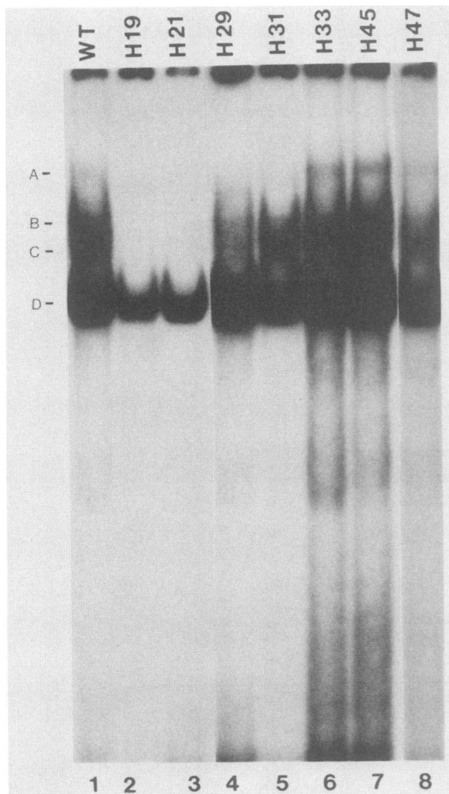

FIG. 3. Binding of cellular factors to WT and mutated HTLV-I 21-bp oligonucleotides. ^{32}P-labeled WT or mutant oligonucleotides (10 ng) and 4 μg of poly(dI-dC) were incubated with 50 μg of HeLa cell nuclear protein. The protein-DNA complexes were resolved in a 6% gel. The free probe has migrated off the gel. The individual probes used in the different incubations are labeled at the top. The four protein-DNA complexes described in the text are designated A, B, C, and D.

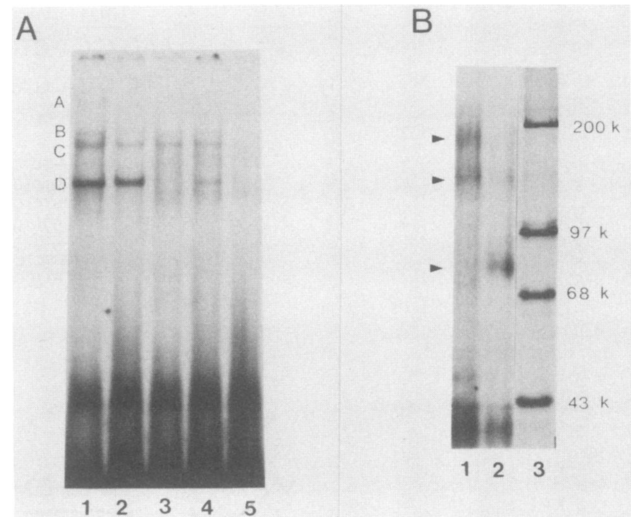

FIG. 4. Characterization of HTLV-I 21-bp-sequence-binding proteins by substrate competition. (A) A 10-ng sample of WT 21-bp probe incorporated with [^{32}P]dCTP and BUdR was incubated with 50 μg of HeLa nuclear extract in the presence of 2 μg of poly(dI-dC) (lane 1), 1 μg of H23 oligonucleotide (see Materials and Methods; lane 2), 1 μg of *ori*-P oligonucleotide (lane 3), 1 μg of *lac*-O oligonucleotide (lane 4), or 1 μg of unlabeled WT oligonucleotide (lane 5). Samples were electrophoresed in a 4% polyacrylamide gel followed by autoradiography. Complexes A, B, C, and D (described previously) are labeled. (B) A 10-ng sample of WT 21-bp probe was incubated with HeLa nuclear extract as in lane 1 (panel A) except that the reaction was UV irradiated before gel electrophoresis and autoradiography. Regions of the gel containing complexes B and C together and complex D alone were separately excised and analyzed by SDS-PAGE. Lanes: 1, proteins eluted from the B and C complexes; 2, protein from the D complex; 3, ^{14}C-labeled molecular weight markers. The three arrowheads point to proteins of molecular weights 180,000, 120,000, and 76,000 (top to bottom, respectively).

21-bp repeat placed upstream of the SV40 early promoter (Fig. 6B).

The templates were separately added with a β-globin control plasmid (61) to Manley HeLa cell extract. Incubation was conducted at 30°C for 60 min in the presence or absence of supplementation with FPLC column fractions enriched for either the 120- or 180-kDa protein. Half of the in vitro-generated RNA from each reaction was used for assay by primer extension with a CAT oligonucleotide (Fig. 6C, top). The other half was quantified for β-globin-specific RNA by S1 nuclease protection by using a single-stranded M13 probe (Fig. 6C, bottom). These results showed an approximately 10-fold stimulation by the 180-kDa-protein-containing fraction of $pWTA_{10}CAT_2$ when compared with the mutant $pH19CAT_2$ template (Fig. 6C; compare lane 4 with lane 8). In contrast, addition of the 120-kDa-protein-containing fraction to the incubation mixtures failed to cause significant differences in the relative transcriptional rates between the two templates (Fig. 6C; compare lane 3 with lane 7). Transcription from the internal control β-globin promoter remained relatively constant in each of the different reactions (Fig. 6C, bottom). Although we cannot exclude either positive or negative contributions from contaminating cofractionated proteins, one interpretation of these findings is that specific binding by the 180-kDa protein to the HTLV-I 21-bp repeats in plasmid $pWTA_{10}CAT_2$ positively augments transcription.

Control of the HTLV-I LTR through a signal transduction pathway. The identification of cellular proteins that can interact with the HTLV-I 21-bp sequence agrees with a molecular model whereby $p40^x$-mediated *trans*-activation occurs via a cellular pathway. One possible route for the actions of $p40^x$ was suggested by Goodman and co-workers (46). These investigators found, by comparing nucleotide sequences, the presence of an octameric sequence within the HTLV 21-bp element that is highly conserved in genes that are transcriptionally regulated by cAMP (Fig. 7A). This finding, if significant, argues that HTLV-I could have incorporated an element of cellular metabolism in its transcriptional repertoire. We therefore experimentally determined whether the HTLV-I LTR promoter could be regulated by changes in intracellular cAMP levels.

Initial screening experiments showed that the HTLV-I LTR was inducible by cAMP in a variety of different cell types. Erythroleukemia K562 cells, however, consistently gave the highest level of induction. We transfected K562 cells with either an HIV LTR-CAT (pBENNCAT) (22) (Fig. 7B, samples 1 to 3) or an HTLV-I LTR-CAT (pU3RCAT) (58) (Fig. 7B, samples 4 to 6) plasmid. After 24 h, cells were mock treated (Fig. 7B, samples 1 and 4), treated with adenyl-cyclase stimulator (forskolin; Fig. 7B, samples 2 and 5), or treated with cAMP analog (8 bromo-cAMP; Fig. 7B, samples 3 and 6). cAMP regulators significantly induced the HTLV-I LTR-CAT plasmid but not the control HIV LTR-

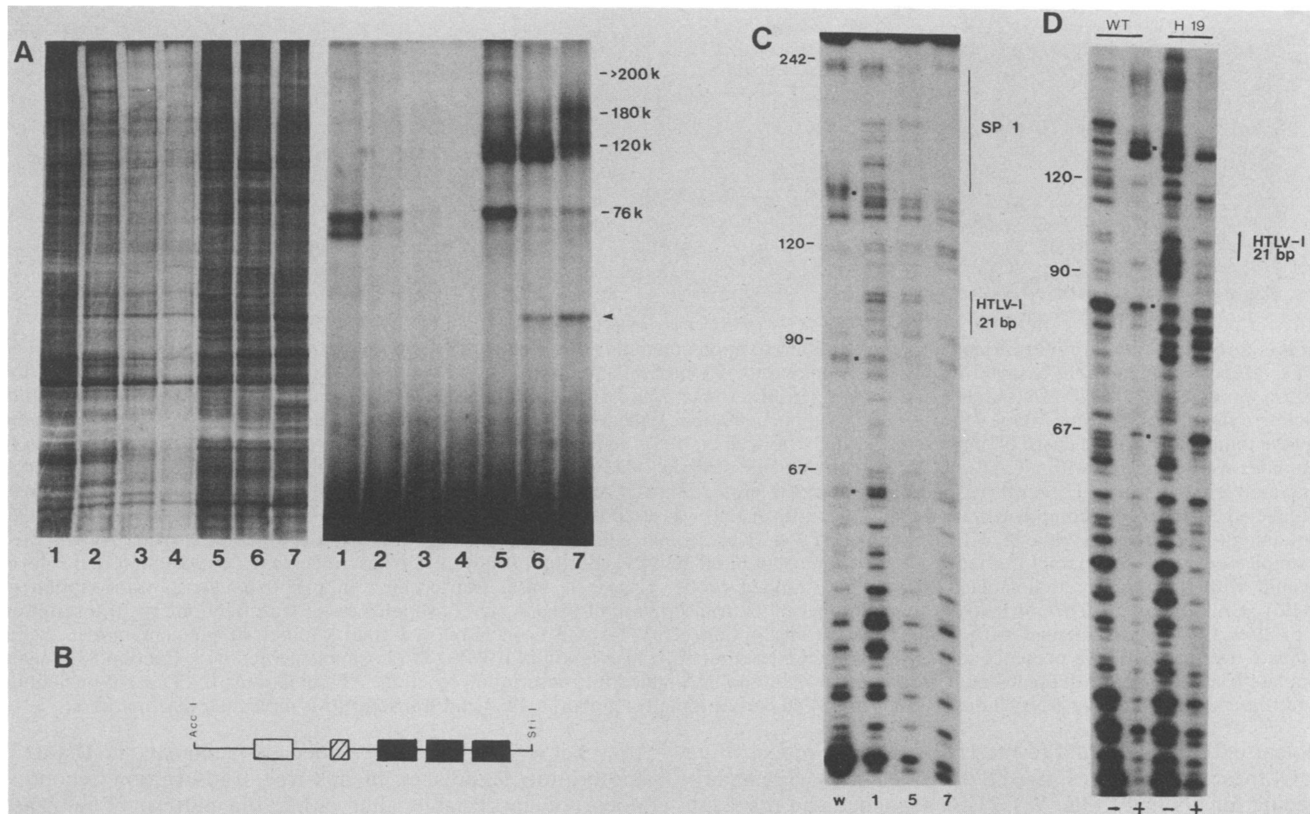

FIG. 5. Partial separation of HTLV-I 21-bp-sequence-binding proteins and analysis by nuclease protection. (A) Sequential column fractions (lane 1, low salt; lane 7, high salt) of HeLa nuclear extract from an FPLC Mono-S column were assayed by UV cross-linking to WT probe. (Left) Silver-stained SDS-PAGE of the assayed fractions. (Right) Autoradiogram of the same gel showing the presence of the 76-, 120-, 180-, and >200-kDa proteins. The arrowhead points to a 46-kDa protein that is observed in different HeLa nuclear preparations (lanes 6 and 7) and may represent a proteolytic product. (B) Schematic representation of the DNA fragment used for DNase I footprinting. The relative positions of a single copy of the HTLV-I 21-bp sequence in the sense orientation (□), a one-third copy of the SV40 72-bp repeat (▨), and three copies of the SV40 21-bp repeat (■) are shown. The latter two elements are as described for the plasmid $A_{10}CAT_2$ (35). (C) DNase I protection of HTLV-I 21-bp sequence with whole HeLa nuclear extract or selective column fractions. Lanes: w, unfractionated whole HeLa extract; 1, fraction 1 from panel A; 5, fraction 5 from panel A; 7, fraction 7 from panel A. Nucleotides are numbered by their distance from the *Acc*I site. Protected regions over the SV40 21-bp repeat (SP 1) and the HTLV-I 21-bp sequence are labeled. (D) DNase I assay of WT or H19 sequences with whole HeLa nuclear extract. WT probe fragment is as described for panel B. The H19 probe is identical to WT except that a single copy of the H19 oligonucleotide in the sense orientation is substituted in place of the WT sequence. DNase I digestion patterns in the absence (−) or presence (+) of HeLa protein extract are shown. Equivalent positions in panels C and D are indicated by filled squares.

CAT plasmid. When these cells were treated simultaneously with two separate cAMP modulators (e.g., 8 bromo-cAMP and isobutyl-methylxanthine), a positive induction of an up to 25-fold increase over the basal strength of the LTR promoter was observed (Fig. 7B, compare lane 7 with lane 11). This increase is comparable to that seen for $p40^x$-mediated *trans*-activation of the HTLV-I LTR in K562 cells (results not shown).

In other experiments, we used $pA_{10}CAT_2$ plasmid constructions which contained tandem copies of either mutated ($pH19A_{10}CAT_2$; Fig. 7C) or WT ($pWTA_{10}CAT_2$; Fig. 7C) HTLV-I 21-bp oligonucleotides. We introduced $pH19A_{10}$ CAT_2, $pWTA_{10}CAT_2$, or pU3RCAT (HTLV-I LTR; Fig. 7C) into K562 cells and tested the inducibility of each by cAMP regulators. CAT assays showed that the H19 plasmid, which was not *trans*-activated by $p40^x$ (Fig. 2), was also not stimulated by increased levels of intracellular cAMP. In contrast, cells transfected with either $pWTA_{10}CAT_2$ or pU3RCAT showed elevated acetylase activities after treatment with 8 bromo-cAMP and isobutyl-methylxanthine).

The results described above indicated that the HTLV-I 21-bp sequence, under the appropriate conditions, can serve as a cAMP-responsive element. This finding is of interest since it suggested the cross-utilization of target sites by both $p40^x$ and cAMP. We tested this possibility with the construction of $pRSA_{10}CAT_2$, a plasmid that contained two synthetic copies of the rat somatostatin cAMP-responsive element (see Materials and Methods) upstream of an SV40 promoter-driven CAT gene. We found that while $pA_{10}CAT_2$ (control; Fig. 7D) did not respond to $p40^x$ induction, $pRSA_{10}CAT_2$ (rat somatostatin; Fig. 7D) in the presence of $p40^x$ protein did show an increase in CAT activity comparable to the increase seen for $pWTA_{10}CAT_2$ (HTLV-I 21 bp; Fig. 7D).

DISCUSSION

In this study we have used a UV-induced indirect labeling technique to identify cellular factors that bind to the HTLV-I 21-bp $p40^x$-responsive sequence. This approach is similar to that reported by Sharp and co-workers (6). We and others (65) have utilized this method in conjunction with mobility shift assays to further characterize protein factors responsible for gel-retarded complexes (Fig. 4).

We have identified, using this UV-labeling approach, four cellular polypeptides of 76, 120, 180, and >200 kDa in HeLa

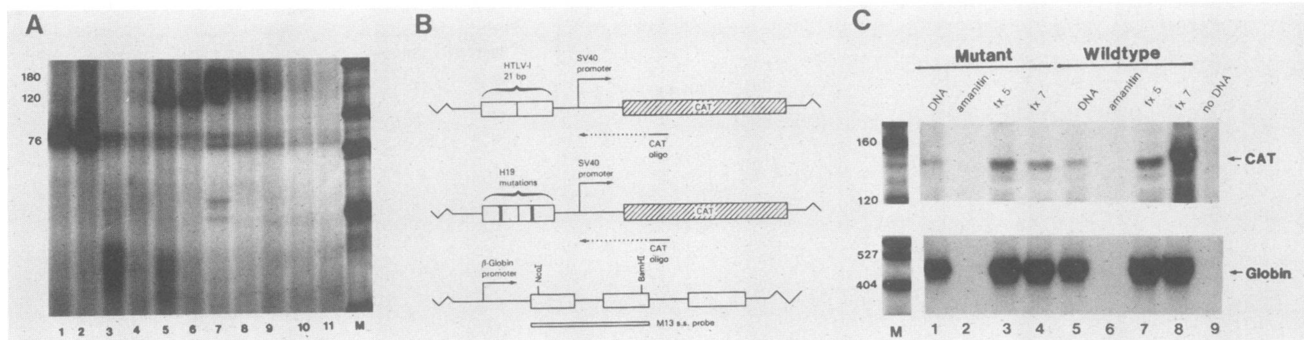

FIG. 6. Stimulation of in vitro transcription by column fraction containing the 180-kDa protein. (A) Sequential column fractions from an FPLC Mono-S column were assayed by UV cross-linking to WT probe (lanes 1 to 11). An autoradiograph showing the separation of the 76-kDa (lanes 1 and 2), 120-kDa (lanes 5 and 6), and 180-kDa (lanes 7 and 8) proteins is presented. For in vitro transcription assays either fraction 5 (lane 5) or fraction 7 (lane 7) was used for supplementation. Lane M, ^{14}C-labeled protein molecular weight markers. (B) Schematic representations of the analysis of in vitro-generated transcripts. RNA synthesized from the HTLV-I $pWTA_{10}CAT_2$ or $pH19A_{10}CAT_2$ templates were analyzed with a CAT-specific primer. A single-stranded M13 probe (426 bp) was used to analyze β-globin-specific transcript generated from an internal control plasmid by S1 nuclease protection. (C) Analyses of RNA from in vitro transcription reactions. (Top) A ^{32}P-labeled 20-bp primer complementary to the CAT mRNA (59) was used to quantitate RNA transcribed from a $pA_{10}CAT_2$ template (35) containing two copies of synthetic WT or mutant HTLV-I 21-bp sequence (see panel B). Either fraction 5 or fraction 7 (panel A) was used to supplement a Manley extract titrated to give a low level of basal activity. (Bottom) Analysis of transcripts from a β-globin internal control plasmid with a uniformly radiolabeled M13 single-stranded probe. Lanes: 1, basal transcription of $pH19A_{10}AT_2$; 2, transcription of $pH19A_{10}CAT_2$ in the presence of 1 μg of α-amanitin per ml; 3, transcription of $pH19A_{10}CAT_2$ supplemented with fraction 5; 4, transcription of $pH19A_{10}CAT_2$ supplemented with fraction 7; 5, transcription of $pWTA_{10}CAT_2$ in Manley extract without supplementation; 6, same template transcribed in the presence of 1 μg of α-amanitin per ml; 7, transcription of $pWTA_{10}CAT_2$ supplemented with fraction 5; 8, same template transcriptionally supplemented with fraction 7; 9, no DNA added to transcription reaction. Abbreviations: CAT, a 140-nucleotide band, the expected size for RNA initiated from the SV40 early promoter; globin, a 426-band transcript; M, molecular size markers.

nuclear extracts that bind to the HTLV-I 21-bp repeat (Fig. 1). Of these four proteins, two (120 and 180 kDa) were highly specific for binding to the WT 21-bp sequence and resistant to competition with heterologous DNAs (Fig. 4). In mobility shift assays, seven oligonucleotides that mutated at different positions within the HTLV-I 21-bp element demonstrated that the in vitro binding of the 120- and 180-kDa proteins (but not the 76- or >200-kDa proteins) correlated well with the in vivo responsiveness of these mutants to *trans*-activation by $p40^x$ (Fig. 2). Comparing the 120- and 180-kDa factors, we found that column fractions enriched with the latter contained an HTLV-I 21-bp DNase I protecting activity and were active in the transcriptional stimulation of HTLV-I 21-bp-sequence-containing templates in vitro (Fig. 5 and 6). Preliminary experiments suggested that the 120- and 180-kDa proteins shared some common partial proteolytic fragments (K.-T. Jeang, unpublished data). Given the possibility of degradation during the protein extract preparation, the 120-kDa molecule may represent a proteolytic product of the larger, biologically active 180-kDa protein.

We considered that the 180-kDa protein may play a role in the *trans*-activation of the HTLV-I LTR by $p40^x$. While the exact mechanism of this regulation remains to be elucidated, one suggestion has been that $p40^x$ may interact directly with sequences upstream of responsive promoters (19). We have used biologically active protein from overproducing procaryotic cells (23), from overproducing eucaryotic cells (30), and from an HTLV-I-transformed T-cell line (37) and have been unable to attribute any in vitro DNA-binding properties to $p40^x$ (unpublished observations). Hence, it appears probable that $p40^x$ utilizes cellular protein intermediaries in interactions with target promoter elements. Since the completion of our study, other investigators (1, 49) have also demonstrated binding of host cell factor(s) to the HTLV-I 21-bp element and have suggested an indirect pathway in $p40^x$-mediated activation of the HTLV-I LTR.

The underlying mechanistic interplay between $p40^x$ and cellular factors is not obvious. We have noted that the presence of $p40^x$ protein, added exogenously, in DNase I footprinting assays or in cell-free transcription reactions does not appreciably alter either the pattern of nuclease protection or the level of template-specific transcriptional stimulation (unpublished observations). Thus the effects of $p40^x$ may not be easily reproducible in vitro by simple protein-protein interactions. These observations suggest a complex schema of $p40^x$-mediated regulation which is compatible with published reports of the *trans*-activation by $p40^x$ of heterologous promoters that do not share overt sequence homology with the HTLV-I LTR or with each other (8, 29).

How might $p40^x$ then activate a number of unrelated genes? One possibility is that this protein utilizes a ubiquitous signal transduction pathway such as a cAMP-dependent cascade. The cAMP kinase cascade is operative in virtually all eucaryotic cells (for a review, see reference 25) and probably represents an extension of a procaryotic regulatory mechanism (63). Hence, it is perhaps more than coincidence that the HTLV-I, HTLV-II (Fig. 7A), and bovine leukemia virus (9) 21-bp repeated sequences all conserve, with slight variations, the consensus octanucleotide commonly found in cAMP-responsive genes (46). In the present study, we have demonstrated that intracellular cAMP levels can specifically coordinate the expression of a reporter gene that is linked to the HTLV-I LTR (Fig. 7B). Three pieces of evidence suggest that this cAMP-mediated regulation is relevant to $p40^x$ *trans*-activation. First, the HTLV-I 21-bp element, in multimers, can serve as a sufficient target for both $p40^x$ (Fig. 2) and cAMP (Fig. 7C). Second, a mutation (H19) within the cAMP 5′-TGACGTCA-3′ consensus (46) of the 21-bp sequence inactivated both $p40^x$ (Fig. 2) and cAMP (Fig. 7C) responsiveness. Third, an unrelated cAMP-responsive element from the rat somatostatin gene can apparently substitute for the HTLV-I 21-bp sequence in $p40^x$-mediated *trans*-activation (Fig. 7D). These findings suggest that $p40^x$ and cAMP may share a convergent, but not necessarily identical, pathway in the regulation of some genes. Similar observations correlating an interaction between the adenovirus E1a

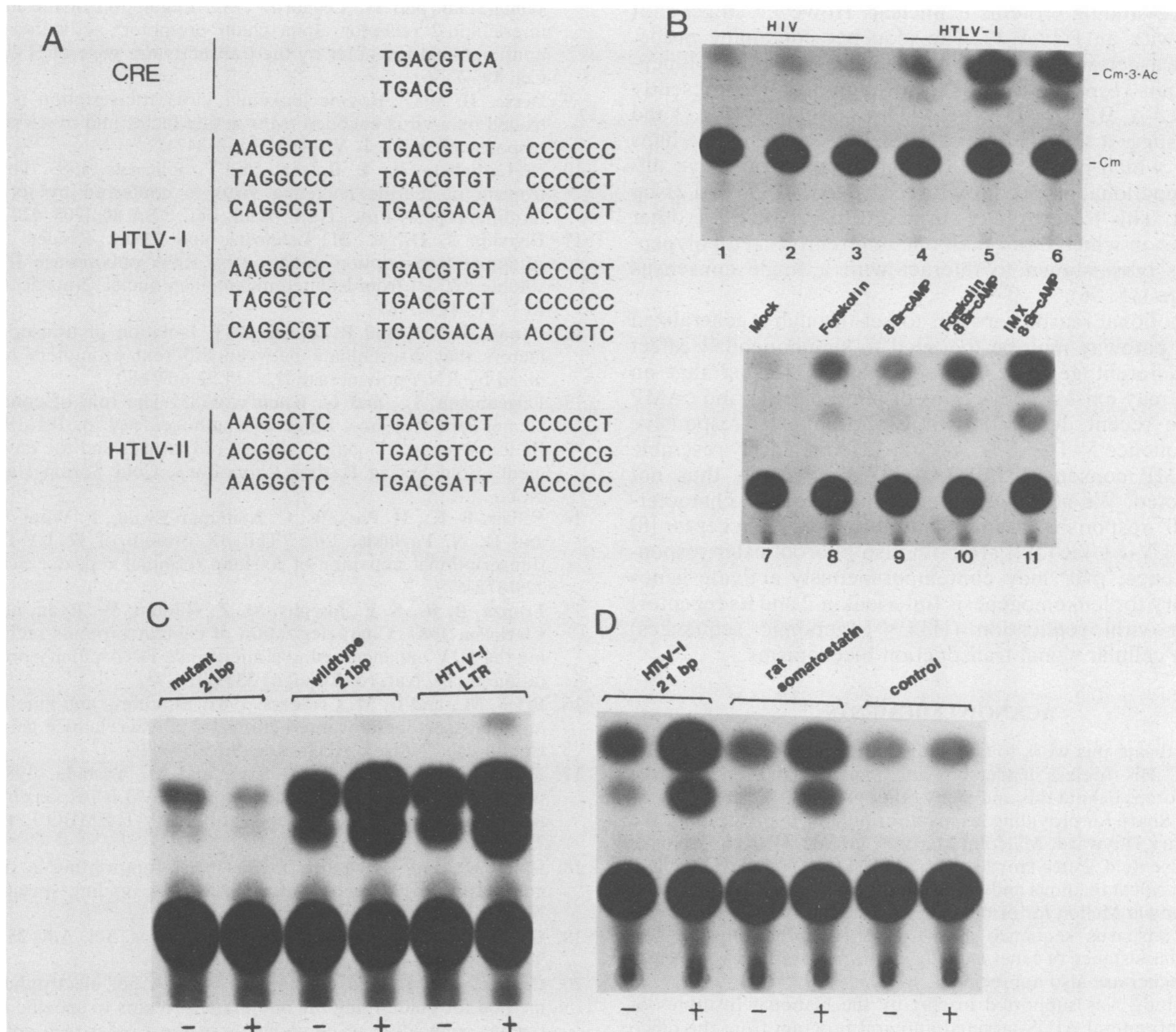

FIG. 7. The HTLV-I LTR contains a cAMP-responsive element. (A) The octameric (46) and pentameric (62) cAMP-responsive element (CRE) are compared against the three 21-bp sequences found in an American HTLV-I isolate (upper rows [32]), a Japanese HTLV-I isolate (lower rows [52]), and a Japanese HTLV-II isolate (55). (B) An HIV LTR-CAT plasmid (pBENNCAT) (22) or an HTLV-I LTR-CAT plasmid (pU3RCAT) (58) was separately introduced into K562 cells. CAT activities were assayed 48 h after transfection and 24 h after regulator treatment from mock-induced cells (samples 1 and 4), cells induced with 25 μM forskolin (samples 2 and 5), and cells induced with 1 mM 8 bromo-cAMP (samples 3 and 6). Samples 7 through 11 compare the relative induction values of the HTLV-I LTR by single (forskolin, sample 8; 8 bromo-cAMP, sample 9) or double (forskolin plus 8 bromo-cAMP, sample 10; isobutyl-methylxanthine plus 8 bromo-cAMP, sample 11) cAMP regulators. Abbreviations: Cm, chloramphenicol; Cm-3-Ac, acetylated chloramphenicol. (C) CAT activities from K562 cells transfected with plasmids pH19$A_{10}CAT_2$ (mutant 21 bp), pWT$A_{10}CAT_2$ (wildtype 21 bp), or pU3RCAT (HTLV-I LTR) that were mock induced (−) or induced (+) simultaneously with 1 mM 8 bromo-cAMP and 0.5 mM isobutyl-methylxanthine. (D) K562 cells were transfected separately with plasmid DNAs pWT$A_{10}CAT_2$ (HTLV-I 21 bp), pRS$A_{10}CAT_2$ (rat somatostatin), and p$A_{10}CAT_2$ (control). −, Samples that are plasmid DNAs transfected alone; +, samples that are DNAs cotransfected with an HTLV-I $p40^x$-producing plasmid (48).

and cAMP-responsive elements have also been reported (26, 40, 41).

Should $p40^x$ and cAMP utilize a similar target sequence, the role of cAMP-dependent kinases in transcriptional activation by $p40^x$ should not be overlooked. Since mutations (H19 and H21) within the highly conserved pentameric subset (62) of the cAMP octamer (46) not only resulted in the loss of biological activity but also in the inability of the HTLV-I 21-bp sequence to bind the 120- and 180-kDa proteins (Fig. 3), we suggest that these polypeptides could be participating factors in a $p40^x$-interactive cellular cascade. Recently, Montminy and Bilezikjian (45) purified a 43-kDa protein from PC12 cells that bound specifically to the cAMP-responsive element of the somatostatin gene. Lin and Green (41) have also independently identified a 46-kDa protein from HeLa cells that bind to cAMP-inducible promoters by a UV-cross-linking approach. We have also noted in our analyses with HeLa cell nuclear proteins the variable presence of a 46-kDa protein that can bind to the HTLV-I 21-bp element (Fig. 5A, lanes 6 and 7, arrow; Fig. 6A, lane 7). Using an oligonucleotide affinity column containing the HTLV-I 21-bp sequence, we have preparatively purified this 46-kDa species to homogeneity, as assayed by silver staining on SDS-PAGE (unpublished results). The exact relationship between the 43-kDa PC12 protein, the 46-kDa HeLa protein, and the higher-molecular-weight HeLa cell HTLV-I 21-bp-

sequence-binding proteins is unclear. However, in gel shift assays with an HTLV-I 21-bp-sequence-containing probe, we have ascertained that the purified HeLa 46-kDa polypeptide is not responsible for the formation of the presently described A, B, C, or D protein-DNA complexes (Fig. 3 and 4). We suggest that there may be multiple proteins, perhaps related, which have similar binding affinities but have different functional properties with regard to the HTLV-I 21-bp element. This is not totally surprising since there are other examples in which more than one differently sized polypeptide has been shown to interact with a single consensus sequence (15, 56).

A functional reason for p40^x to act through a generalized cellular pathway may be the need to simultaneously affect many different genes. One report has suggested that an overlap may exist in the actions of phorbol ester and cAMP (7). The recent description of a phorbol-ester-responsive core sequence 5′-TGACTCA-3′ (2, 38) that closely resemble the cAMP consensus 5′-TGACGTCA-3′ (46) is thus not unexpected. We note with interest that two well-characterized p40^x-responsive promoters (interleukin-2 α receptor [8] and HTLV-I LTR [28]) are both also phorbol ester responsive. Hence, p40^x may contemporaneously activate genes necessary for leukemogenesis (interleukin-2 and its receptor) and for viral replication (HTLV-I genomic sequences) through cellular signal transduction mechanisms.

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