Binding of host-cell factors to DNA sequences in the long terminal repeat of human T-cell leukemia virus type I: Implications for viral gene expression

(DNase I-protection assay/trans-activation/p40^{x1}/protein-DNA interactions)

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ABSTRACT Efficient expression of human T-cell leukemia virus type I (HTLV-I) genes requires both host and viral proteins and is dependent on DNA sequences in the proviral long terminal repeats (LTRs). We have used DNase I-protection assays (footprinting) to construct a map of protein-DNA interactions over a 250-nucleotide region of the LTR upstream of the start site for viral RNA synthesis. We find that a host factor (host expression factor 1, or HEF-1) binds to the imperfect 21-nucleotide repeats that have previously been implicated in HTLV-I gene expression. HEF-1 binding activity is present in preparations from both lymphoid and nonlymphoid cell lines. However, the boundaries of the protected regions and the presence of a flanking DNase-hypersensitive site vary with cell type. Several regions of binding are detected in addition to the HEF-1 sites, including a complex group of sites 40–90 nucleotides upstream of the RNA start site. A comparison of HTLV-I-transformed T lymphocytes that do and do not express the viral trans-activating protein p40^{x1} shows that none of the observed features of the DNase I footprint pattern correlate directly with the presence of this protein in the extract. These results suggest (i) that the primary recognition of promoter elements in the HTLV-I LTR involves specific interactions with host-cell proteins and (ii) that p40^{x1} influences the activity of one or more of these proteins, rather than interacting directly with the DNA.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia, an aggressive malignancy of mature T helper lymphocytes in humans (1, 2). HTLV-I is a retrovirus, and viral RNA synthesis initiates at a site in the proviral long terminal repeat (LTR). There is considerable evidence that viral gene expression is regulated in infected cells. For example, HTLV-I viral antigens generally cannot be detected in the transformed lymphocytes of adult T-cell leukemia patients, although the provirus is present (3, 4). When peripheral blood lymphocytes from these individuals are cultured, however, cells are observed that express high levels of viral RNA and protein (4–6).

High levels of viral gene expression in cultured cells require a nonstructural regulatory protein, p40^{x1}, that is encoded by the longest open reading frame in the X region at the 3' end of the genome (7–13). The p40^{x1} protein is localized primarily in the nucleus (14, 15), and because sequences upstream of the RNA start are required for trans-activation, the effect is generally believed to be at the level of transcriptional initiation. Little is known about the actual mechanism of p40^{x1} trans-activation, however, and the role of host proteins in HTLV-I gene expression has not been explored.

The sequences required for HTLV-I gene expression are contained in a region extending about 300 nucleotides upstream of the RNA start site (16–19). Recent evidence suggests that some sequences downstream of the start site may be required as well (20, 21). Because of the size of the upstream region, genetic analysis has been limited to relatively coarse-scale deletion studies and to investigation of three imperfect 21-nucleotide repeats that are conserved in both sequence and spacing between HTLV-I and the related virus HTLV-II. Deletion of sequences within the 21-nucleotide repeats lowers the level of gene expression, whereas reinsertion of the repeat sequences as a synthetic oligonucleotide, or introduction of the 21-nucleotide repeat region into a heterologous promoter, results in increased expression (16–24).

In the present study, we carried out DNase I “footprinting” (25) to identify specific protein-DNA interactions in the HTLV-I LTR, using both unfractionated nuclear extracts and fractions eluted from a heparin-agarose column. Unfractionated material allows the broadest and most unbiased survey of potential DNA-binding proteins. By contrast, heparin-agarose fractions are enriched for many DNA-binding proteins and give more consistent protection and clearer boundaries. We have used these protein preparations to construct a map of the interactions between host-cell proteins and HTLV-I LTR DNA, and have also examined cell-type-specific differences in the pattern of binding.

MATERIALS AND METHODS

Extract Preparation and Heparin-Agarose Chromatography. Nuclear extracts were prepared as described (26). For heparin-agarose chromatography (27), isolated nuclei were resuspended in 2 packed-cell volumes of extraction buffer and centrifuged, and the supernatant was diluted to 0.15 M KCl. This was applied directly to a heparin-agarose column, and bound material was eluted with TM buffer (27) containing KCl, as described in the figure legends.

Preparation of Probes and DNase I Footprinting. The coding-strand probe was 5' end-labeled with ³²P at a position 340 nucleotides upstream of the HTLV-I RNA start site and extended downstream to position +137. The noncoding-strand probe was 5' end-labeled at a *Bgl* II linker at position +21 and extended upstream to position –322. Binding-reaction mixtures for DNase I footprinting contained, in addition to protein preparations, 25 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 6.25 mM MgCl₂, 50 mM KCl, 10% (vol/vol) glycerol, 2 μg of poly(dI-dC) (alternating

FIG. 1. DNase I footprint of the HTLV-I proviral LTR after incubation with heparin-agarose 0.15–0.4 M KCl step-gradient fractions from HeLa cell nuclear extracts. DNA probes 5' end-labeled on the coding (a) or noncoding (b) strand were incubated with 0, 4, 8, or 18 μ g of protein, as indicated, and the DNase I cleavage products were analyzed as described (28). Maxam–Gilbert sequencing products (G and G + A) were run in adjacent lanes for reference (28). Brackets and numbers identify regions of protection.

copolymer), and 0.2 nM probe DNA in a final volume of 50 μ l (26). In binding-reaction mixtures containing unfractionated extracts, the oligonucleotide mixture p(dN)₅ (Pharmacia) was included at 40 μ g/ml. Samples were treated with DNase I, purified, and electrophoresed in denaturing 6.5% polyacrylamide gels as described (28).

RESULTS

Mapping the Protein Binding Sites in the HTLV-I LTR.

Proteins extracted from HeLa cell nuclei were subjected to

FIG. 2. Nucleotides protected within the HTLV-I proviral LTR. Sequence shown is according to Seiki *et al.* (13). Boxed nucleotides denote the sequences protected on each strand of the DNA by HeLa cell heparin-agarose fractions (from Fig. 1). Bars above and below the boxes denote the sequences protected by SLB-I cell heparin-agarose fractions (see Fig. 4). The 21-nucleotide and 11-nucleotide repeats are underlined with arrows. The "TATA" sequence and RNA start site (bent arrow; U3/R junction, proviral position 353) are indicated. Numbers above the boxed sequences correspond to regions of protection indicated in Fig. 1.

chromatography on heparin-agarose. The fraction eluted between 0.15 M and 0.4 M KCl was collected, and various amounts were incubated with singly end-labeled probes representing the coding (Fig. 1a) and noncoding (Fig. 1b) strands of the HTLV-I LTR. Protected regions were localized by alignment with DNA sequence markers and have been numbered for convenient reference.

Regions 1 and 2 correspond to two of the imperfect 21-nucleotide repeat sequences previously implicated in LTR-directed gene expression. Protection occurred with approximately the same boundaries on each strand of the DNA duplex (Fig. 2) and included essentially the entire 21-nucleotide repeat and 10–14 nucleotides of downstream sequence. Region 3 overlaps the third, most promoter-proximal 21-nucleotide repeat, but protection is shifted upstream when compared with the protection over the first two repeats (see Fig. 2).

In addition to the 21-nucleotide repeats, several other regions were protected. Region 2a lies between the second and third 21-nucleotide repeats and encompasses a portion of two imperfect tandem 11-nucleotide repeats. Region 4 begins at the downstream end of the third 21-nucleotide repeat and extends downstream to position –39.

In view of the complexity of the footprint pattern, it was desirable to compare individual fractions from a KCl-gradient elution of the heparin-agarose column. The fractions used in the experiment presented in Fig. 3 span the range 0.15–0.48 M KCl. The different DNA-binding protein species are partially resolved in such a gradient. The same fractions (Fig. 3, lanes 7–9) gave maximal protection of regions 1, 2, and 3, suggesting that the same factor may bind all three repeats. By contrast, the factor that binds region 2a was eluted earlier in the gradient and is clearly distinct.

An interesting pattern is seen in region 4, where it appears that binding activity was resolved into at least three distinct components, peaking in lanes 4 and 5, 6 and 7, and 7 and 8, respectively. Region 4 sequences lie in a position analogous to the upstream regulatory elements found in many other promoters, although the requirement for sequences in this region has not yet been examined genetically in HTLV-I.

We will refer to the factor that binds to the three 21-nucleotide repeats as HEF-1 (host expression factor 1). The bulk of the evidence suggests this is a single factor. As noted above, binding to all three repeats was seen with the same fractions from a heparin-agarose gradient elution. In addition, a synthetic duplex 37-mer oligonucleotide containing sequences in region 2 (–205 to –174) effectively competed for binding to regions 1, 2, and 3 (data not shown). All three regions became occupied at about the same point in the titration (Fig. 1).

Binding of HEF-1 to region 2 did not require the presence of the other repeats. Protection in region 2 was similar with the entire upstream LTR, with a subcloned *Hpa* II fragment

FIG. 3. DNase I footprint with HeLa cell heparin-agarose linear gradient fractions. A HeLa nuclear extract preparation was applied to a heparin-agarose column and eluted by a linear KCl gradient. An equal volume of each fraction was assayed by DNase I footprinting using the coding-strand probe (lanes 3–11). The corresponding KCl concentrations in the assayed fractions are as follows: lane 3, 0.15 M; lane 4, 0.19 M; lane 5, 0.23 M; lane 6, 0.27 M; lane 7, 0.31 M; lane 8, 0.35 M; lane 9, 0.39 M; lane 10, 0.44 M; lane 11, 0.48 M. Control samples without protein (lanes 1, 2, 12, and 13) and sequence markers (lanes G and G+A) are indicated.

containing only the second 21-nucleotide repeat, and with a cloned 37-mer oligonucleotide representing sequences in region 2 (data not shown).

Cell-Line-Specific Differences. One of the distinguishing features of HTLV-I and related viruses is trans-activation. This phenomenon, which requires the viral protein p40^{x1}, results in a much higher level of expression when constructs containing the LTR are transfected into infected versus uninfected cells. To test whether virus-mediated changes in the transcriptional apparatus might be reflected in the DNase I footprinting pattern, we prepared protein extracts from the T-lymphocyte line SLB-I, which is productively infected with HTLV-I (14).

As shown in Fig. 4, the overall pattern of protection with SLB-I cell heparin-agarose fractions was similar to that with HeLa. Thus, there was protection of regions 1, 2, and 3, corresponding to the 21-nucleotide repeats, as well as protection in region 2a and some protection in region 4. There were several differences, however. With SLB-I extracts the footprint in region 2 was smaller than with HeLa, with protection essentially limited to the repeat itself, rather than extending downstream (see diagram, Fig. 2). A DNase-hypersensitive site (arrow) was seen with SLB-I cell extracts just upstream of region 2a. The boundaries of region 3 were shifted. This was particularly apparent on the noncoding strand, where regions 3 and 4 were no longer separated by a DNase-sensitive area. Rather, there was partial protection over a larger, contiguous region. The protection in region 4 also showed several alterations. The differences between SLB-I and HeLa preparations are summarized in Fig. 2.

Footprinting with Unfractionated Extracts. The comparison between cell lines was extended by carrying out footprinting analysis using unfractionated nuclear extracts. However, with these preparations, protection was sometimes incomplete and it was difficult to obtain satisfactory binding in all regions in a single experiment. We chose to concentrate on regions 2, 3, and 4, where we had observed

FIG. 4. Comparison of the DNase I footprint produced using heparin-agarose 0.15–0.4 M step-gradient fractions from HeLa and SLB-I nuclear extracts. HTLV-I LTR coding (a) and noncoding (b) strand probes were used to compare the protection pattern produced by 18 μ g of protein as indicated. Sequence markers (G and G+A) and control lanes without protein (0) are indicated. Note the cell-type differences in the protection boundaries at sites 2 and 3. Regions 3 and 4 are indicated as continuous in SLB-I, as there is no obvious boundary separating them. Dashed lines within this bracket denote sequences only partially protected. Sequences protected by both HeLa and SLB-I cell extracts are summarized in Fig. 2.

significant differences between HeLa and SLB-I heparin-fractionated preparations.

In these experiments three cell lines were compared: HeLa, SLB-I, and ATL-1K. ATL-1K is a human T-cell line derived from a patient with adult T-cell leukemia (4). It is similar in phenotype to SLB-I, exhibiting antigenic determinants for CD4, interleukin-2 receptor, and HLA-DR (ref. 4 and unpublished observations). Although ATL-1K contains clonally integrated HTLV-I, it reportedly does not express the provirus (4).

It was important to confirm that our ATL-1K cells had not spontaneously begun to express p40^{x1} at the time of our experiments. This was done using antisera specific for the COOH-terminal portion of the protein. Fig. 5b shows that p40^{x1} was readily detected by immunoblotting with SLB-I whole-cell and nuclear preparations but was not detected in ATL-1K, HeLa, or the uninfected T-cell line HUT-78. This conclusion was supported by hybridization/nuclease S1 analysis of total cell RNA (Fig. 5a). These assays detect RNA spliced to the second acceptor site present in the doubly spliced p40^{x1} mRNA (29). RNA spliced to this acceptor was detected in SLB-I but not in ATL-1K cells or in either of the uninfected T-cell lines Jurkat and HUT-78. The absence of functional p40^{x1} in ATL-1K was further confirmed by transfection experiments, where trans-activation of HTLV-I LTR constructs was not observed (data

FIG. 5. Analysis of *X* mRNA and $p40^{x1}$. (a) Hybridization/S1 nuclease analysis was performed as described (29), using total cell RNA from cell lines SLB-I, ATL-1K, Jurkat (30), and HUT-78 (31). The probe was a 396-nucleotide DNA fragment labeled at a *Cla*I site (proviral position 7473) downstream of the second *X*-region mRNA splice acceptor. The 174-nucleotide band marked SA represents *X*-specific spliced mRNA. The band marked probe reflects full-length probe protected by hybridization to HTLV-I genomic or envelope mRNA. Lane M: *Sau*3A1-digested pBR322 DNA. (b) Immunoblotting, performed essentially as described (32), using PrA rabbit antiserum specific for the carboxyl terminus of $p40^{x1}$. The blot was developed with a goat anti-rabbit antibody coupled to horseradish peroxidase. Each lane contained 100 μ g of protein from the cell lines SLB-I, ATL-1K, HeLa, and HUT-78. Whole-cell extracts (W) were prepared by freeze-thawing. Nuclear extracts (N) were prepared as described in *Materials and Methods*. The band at M_r 40,000 ($p40$) is $p40^{x1}$.

not shown). The inclusion of both ATL-1K and SLB-I cell preparations in the footprinting experiments helps one to distinguish the possible effects of $p40^{x1}$ itself from other features that may be specific to transformed lymphocytes.

In footprinting experiments using the unfractionated extract, there were prominent differences between cell lines in regions 3 and 4 (Fig. 6a). No protection in region 3 was seen with HeLa cell extract and only a portion of region 4 was protected. By contrast, SLB-I and ATL-1K lymphocyte extracts clearly protected a contiguous area encompassing regions 3 and 4. The enhanced protection seen with these extracts in the area between regions 3 and 4 parallels that with the SLB-I heparin-agarose fractions, although the effect here is somewhat more pronounced.

The footprint pattern in region 2 also varied with cell type (Fig. 6b). A prominent hypersensitive site was seen at the downstream boundary of region 2 with SLB-I and ATL-1K cell extracts, but not with HeLa cell extract. Cleavage at this site did not occur in control experiments where DNase I was omitted, ruling out an endogenous nuclease in the lymphocyte extracts. Moreover, poly(dA-dT) was found to inhibit formation of the hypersensitive site, whereas this polynucleotide did not interfere with protection in region 2 (data not shown). This differential inhibition, together with the cell-line specificity, suggests that the hypersensitivity is attributable to a biochemical activity separate from the HEF-1 binding activity.

In each of the regions shown here, the footprint patterns produced by ATL-1K and SLB-I cell extracts were essentially identical. This result, together with the absence of detectable $p40^{x1}$ in ATL-1K cell extracts, suggests it is unlikely that any features of the footprint patterns seen here are attributable to direct interaction with $p40^{x1}$. The footprints with ATL-1K and SLB-I cell extracts differ from those obtained with HeLa, however, suggesting that several features are specific to the differentiation state of the cells.

FIG. 6. Comparison of the footprint patterns produced by unfractionated nuclear extracts from HeLa, SLB-I, and ATL-1K cells. The coding-strand probe was used. Regions 3 and 4 are shown in *a* and region 2 is shown in *b*. Reaction mixtures contained 0 (–) or 200 (+) μ g of protein, as indicated.

DISCUSSION

In the present experiments, we have identified specific host-cell protein–DNA interactions in the HTLV-I LTR. We have shown that the imperfect 21-nucleotide repeats appear to be occupied by a single factor, for which we propose the name HEF-1. In view of prior genetic evidence showing a role for these sequences, it is likely that HEF-1 is a host factor that mediates HTLV-I gene expression. Because the HEF-1 recognition sites lie upstream of the RNA start site, HEF-1 may act at the level of transcriptional initiation, although this has not been shown directly.

The HEF-1 binding activity is present in all cell lines tested, a finding consistent with observations that the HTLV-I LTR is active and can be trans-activated by $p40^{x1}$ in cells from a wide variety of lineages (3, 6). The boundaries of HEF-1 protection in regions 2 and 3 differed for partially purified material from HeLa and SLB-I cell lines, however, suggesting that a modified or different form of the protein is present. This finding of an apparently cell-line-specific form of a DNA-binding factor is analogous to the situation with the immunoglobulin octamer-binding factor, which has different chromatographic behavior (33) and yields complexes with altered mobilities (33, 34) when prepared from lymphoid cell lines.

Preparations from both SLB-I and ATL-1K cells consistently showed protection over the entire third 21-nucleotide repeat, in contrast to HeLa cell preparations, which showed either no protection (Fig. 6) or protection centered somewhat upstream (Fig. 1; also see Fig. 2). The enhanced protection seen with SLB-I and ATL-1K cell extracts may

reflect the ability of a modified HEF-1 to better position itself on the third 21-nucleotide repeat.

In addition to the altered boundaries of protection seen with heparin-agarose fractions, we observed a hypersensitive site flanking region 2 with unfractionated extracts of SLB-I and ATL-1K cells, but not HeLa cells. In additional experiments, we have seen this hypersensitive site with extracts from the HTLV-II-transformed cell line Mo-T and the gibbon leukemia virus-transformed cell line MLA-144, but not with the immature transformed T-cell line Jurkat. Thus, although the functional significance of the hypersensitive site remains obscure, we have seen it so far only in mature, transformed T lymphocytes, suggesting it may correlate with the differentiation state of the cell.

Throughout this study we were aware of the possibility that one or more of the binding sites described here might be recognized by factors previously defined with other promoters. For example, sequences within the 21-nucleotide repeats (CTGAC) and region 4 (CTcAaTAA) are somewhat similar to the consensus sequence for the transcription factor AP-1, $\text{C}_{\text{GTGACT}}\text{A}_{\text{A}}$ (35). We have performed footprinting experiments using affinity-purified AP-1 and found that, under conditions that gave saturating binding to an authentic recognition site in simian virus 40 DNA, there was little specific protection of HTLV-I LTR sequences (data not shown). Another sequence within the 21-nucleotide repeats [GGG-GAC(C)] is similar to the conserved sequence (GGG-GACTTTCC) found in the NF- κ B binding site in the immunoglobulin enhancer (36), the simian virus 40 enhancer, and the human immunodeficiency virus LTR (37). NF- κ B binding activity is reportedly absent from extracts of unstimulated HeLa cells, however (38), whereas HEF-1 activity is present in these preparations. The 21-nucleotide repeats also contain a sequence (TGACGTC) that is very similar to a consensus sequence found in several cAMP-responsive genes (TGACGTCA) (39). Purification of a protein that binds this sequence in the promoter of the somatostatin gene was described recently (40); we do not know whether it is related to HEF-1.

What are the implications of this work for HTLV-I transcriptional regulation? We have detected a number of host-factor binding sites in the HTLV-I LTR, several of which correspond to known control elements. By contrast, we have seen no region of protection in the footprints that correlates with the presence of the viral gene product p40^{x1}. These findings suggest that the primary recognition of promoter elements in the HTLV-I LTR involves specific interactions with host-cell proteins. It is possible that p40^{x1} also recognizes DNA sequences in this region, but that the complexes were not detected because they form inefficiently or are unstable under footprinting conditions. We consider it more plausible, however, in view of the footprinting result, that p40^{x1} interacts with one or more of the host-cell factors described here to accelerate a rate-limiting step in mRNA synthesis. Such an interaction might involve protein modification or protein-protein contact, neither of which would necessarily affect the footprinting pattern.

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