The GGTCA palindrome and cognate cellular factors in trans-regulation of human immunodeficiency virus long terminal repeat by herpes simplex virus

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Molecular interactions between herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus (HIV) were investigated in the promonocytic cell line U937. HSV-1-mediated activation was observed in transient expression assays with hybrid constructions containing the HIV long terminal repeat (LTR)-directed chloramphenicol acetyltransferase gene. Comparison of constructions that differ in the GGTCA palindrome located within the negative regulatory region of the LTR revealed four- to eightfold lower activation levels for the wild-type as compared to the mutant sequence. Three protein species, 37K, 59K/64K and 75K, that bind to the wild-type GGTCA palindrome were resolved in nuclear extracts of uninfected U937 cells by gel retardation and u.v.-crosslinking experiments. The 37K protein did not bind to the mutant palindrome sequence. However, a distinct 120K protein was detected. The 37K and 59K/64K binding proteins were not resolved in similar experiments performed with nuclear extracts from HSV-1-infected U937 cells but there was a novel p50 species that binds only to the wild-type palindrome sequence. These findings raise the possibility that interaction of these proteins at the GGTCA palindrome is involved in HSV-1-mediated regulation of the HIV LTR in U937 cells.

Introduction

Expression from the human immunodeficiency virus (HIV) long terminal repeat (LTR) is regulated by T lymphocyte activation (Bohnlein et al., 1988; Nable et al., 1987; Siekevitz et al., 1987), lymphokine action (Lowenthal et al., 1989; Osborn et al., 1989; Poli et al., 1990), macrophage differentiation (Griffin et al., 1989; Poli et al., 1990), and heterologous virus infection including infection with herpes simplex virus (HSV) (Albrecht et al., 1989; Gendelman et al., 1986; Gimble et al., 1988; Mosca et al., 1987; Ostrove et al., 1987; Pitha, 1990). In contrast to the LTR sequences that enhance the rate of RNA synthesis, other sequences present in a region called the negative regulatory element (NRE) decrease LTR-directed gene expression in established T cells and monocytes (Lu et al., 1989, 1990; Rosen et al., 1985; Siekevitz et al., 1987). A GGTCA palindrome sequence located in the NRE (at positions −353 to −327 relative to the transcription start site) was recently implicated in the negative regulation of HIV LTR in quiescent T cells (Orchard et al., 1990). However, since a positive regulatory factor, the chicken ovalbumin upstream promoter transcription factor (COUP-TF), binds to the GGTCA palindrome questions were raised about the role of the palindrome in negative regulation of the HIV LTR (Cooney et al., 1991).

Conflicting results were obtained regarding the ability of HSV to activate HIV expression. The HSV genes involved in activation depended on the cell type used in the transfection experiments and on the presence of the viral trans-activator tat (reviewed in Laurence, 1990; Pitha, 1990). Monocytes, which are a recognized reservoir for latent HIV infection (Gartner et al., 1986; Koening et al., 1986), were not studied for HSV-mediated activation, possibly because HSV does not replicate in monocytic cells (Plaeger-Marshall & Smith, 1978; Daniels et al., 1978; Albers et al., 1989; Tenney & Morahan, 1987, 1991). Efforts to identify the responsive cis-acting targets were largely unsuccessful (Laurence, 1990; Nable et al., 1988; Osborn et al., 1989; Pitha, 1990) and the possible role of the NRE remains unknown. The studies described in this report were designed to address these questions with particular emphasis on the contribution made by the GGTCA palindrome within the NRE.
Table 1. Summary of synthetic oligonucleotides for gel retardation and u.v.-crosslinking assays

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence*</th>
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<tbody>
<tr>
<td>NRE(H) (HXB2 strain)</td>
<td>GCCAGGTTAGATATCCACTGACCTTGG</td>
</tr>
<tr>
<td>NRE(P) (PV22 strain)</td>
<td>ACCAGGTACGGATATCCACTGACCTTGG</td>
</tr>
<tr>
<td>AP-1 (wild-type)</td>
<td>GATCCAAGCTATGACTCATCCGGTCTAGAA</td>
</tr>
<tr>
<td>AP-1 (mutant)</td>
<td>GATCCAAGCTATGACCCACTATCCGGTCTAGAA</td>
</tr>
</tbody>
</table>

* Base transitions and mutations are in bold. The consensus response elements are underlined. Numbers represent the positions relative to the HIV-LTR mRNA start site (+1).

Methods

Cells and virus. Two clones of the promonocytic cell line U937 that differ in CD4 phenotype were used. U937M, obtained from Dr P. Morahan (Medical College of Pennsylvania, Philadelphia, Pa., U.S.A.), contain 5% CD4+ cells. U937A, obtained from the ATCC, contain 95% CD4+ cells. They were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. HSV-1 strain F was grown and harvested 8 h post-infection (p.i.). Nuclear proteins were extracted by high-salt lysis of isolated nuclei (Wymer et al., 1989, 1992).

Plasmids. Plasmids pU3RIII and pLTRcat were established with LTR sequences from HIV strains HXB2 and PV22, respectively (Sodroski et al., 1985; Muesing et al., 1987). They have a 1 bp transversion in the 5′ half of the GGTCGA palindrome (GGTCA to GATCA) as determined by sequence analysis performed as described (Muesing et al., 1985; Starcich et al., 1985). The pal construct is mutated at position -247 of pLTRcat thereby restoring the wild-type GGTCGA palindrome. Mutagenesis was performed in a two-step PCR (Landt et al., 1990) with pLTRcat as the template. In the first PCR assay a 206 bp mutated fragment that contains HIV LTR sequences at positions -530 to -235 was generated with the 5′ primer (5′ CCAAGGTACGGATATCCACTGACCTTTGG) and the 3′ mutagenic primer (5′ CAGTCATCCGGTCTAGAAG). This fragment was isolated and purified as before, restricted with KpnI/AvaI sites of pLTRcat and inserted into the KpnI/AvaI sites of pLTRcat (at HIV LTR positions -525 to -155).

DNA transfection and chloramphenicol acetyltransferase (CAT) assays. Transfection was done by a modification of the DEAE-dextran method (Fujita et al., 1986). Cells (5 x 10⁶) were washed with warm (37°C) Tris-buffered saline (STBS; 25 mM-Tris-HCl pH 7.4, 137 mM-NaCl, 5 mM-KCl, 0.6 mM-Na₂HPO₄, 0.7 mM-CaCl₂ and 0.5 mM-MgCl₂) and resuspended in 100 μl STBS and exposed for 1 h at 37°C to 600 μl STBS containing 1.0 μg of target plasmid and 450 μg/ml of DEAE-dextran (Pharmacia). Parallel co-transfections were performed using pBR322 as a non-specific DNA to equalize nucleic acid concentration effects. HSV-1 (F) infection (5 p.f.u./cell) occurred 24 h after transfection as previously described (Mosca et al., 1987). CAT assays were performed 40 to 48 h post-transfection (Wymer et al., 1989, 1992) using 0.2 μCi of [³²P]chloramphenicol substrate incubated for 60 min. For quantitative estimates of CAT activity, the appropriate sections were cut from the thin-layer chromatography plate and placed in toluene-2,5-diphenyloxazole-12,4-bis(5-phenyloxazolyl)benzene scintillation fluid and the radioactivity was counted using a Packard liquid scintillation counter. The chloramphenicol acetate produced was measured and compared at enzyme levels on the linear part of the curve for product formation against extract concentration and time. pSV2CAT was used as a control and the parent (pCATB) was used as a negative control when comparing U937M to U937A cells for transfection efficiency.

Gel retardation assay. The oligonucleotides used in gel retardation assays are shown in Table 1. ³²P-labelled DNA probes were prepared by annealing coding strand templates (30 bases) to complementary 10 base primers 5′ CAAAAGGTCA 3′ [for NRE(H) and NRE(P)] and filling in the overhang with the Klenow fragment of DNA polymerase 1 in the presence of [³²P]dGTP, [³²P]dCTP, dATP and dTTP. Synthetic oligonucleotides AP-1 (wild-type) and AP-1 (mutant) (50 ng/ml) were used as DNA competitors for binding by annealing the appropriate chemically synthesized and complementary oligonucleotides.

The ds oligonucleotides were separated by 20% PAGE in 90 mM-Tris-borate-2.5 mM-EDTA, then isolated from the gels by overnight elution (0.5 M-ammonium acetate, 10 mM-magnesium acetate, 1 mM-EDTA, 0.1% SDS) and subsequent centrifugation to separate the DNA from the gel fragments. The supernatant was phenol- chloroform-extracted and precipitated with 2 volumes of ethanol. Nuclear extracts were prepared from U937 cells (1 x 10⁶) mock-infected with PBS pH 7.2 or infected with 5 to 10 p.f.u./cell of HSV-1 (F) and harvested 8 h post-infection (p.i.). Nuclear proteins were extracted by high-salt lysis of isolated nuclei (Wymer et al., 1989, 1992).

Typical gel retardation mixtures contained 25 mM-HEPES pH 7.9, 50 mM-KCl, 10 mM-MgCl₂, 1 mM-EDTA, 1 mM-DTT, 1 mM/ml BSA, 5% glycerol and 2.5 μg poly(dI-dC)-poly(dI-dC) (Pharmacia) used as non-specific competitor DNA. They were incubated with 5 μg of nuclear extract for 10 min at room temperature. A labelled oligonucleotide probe (10 fmol) was added (final volume 25 μl) and the mixture was incubated for an additional 10 min at room temperature. The assays were subjected to electrophoresis at 10 V/cm through a 5% polyacrylamide gel (29:1 biscrylamide) that had been pre-electrophoresed for 30 min at 10 V/cm. The gel buffer contained 45 mM-Tris-borate and 1.25 mM-EDTA. After 2.5 h the gels were dried and exposed for autoradiography as described (Wymer et al., 1989, 1992).

U.v.-crosslinking assays. The u.v.-crosslinking experiments were performed as described (Ballard et al., 1990; Moltzer et al., 1990). The photoactive ³²P-labelled probes [NRE(H) and NRE(P)] were synthesized with 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdU) instead of dTTP. They were used in DNA binding reactions with nuclear extracts and the complexes were resolved by gel electrophoresis. Crosslinking was done by u.v. irradiation in situ at 300 nm using a UVP transilluminator (Model TM-36) at 4°C for 30 min. After wet
Results

HSV-1 activation of HIV LTR in U937 cells

HIV expression is activated by HSV-1 infection in some but not all cell types and in the presence or absence of tat (reviewed in Laurence, 1990; Pitha, 1990). The ability of HSV to enhance HIV expression in monocytes, which are a recognized reservoir for latent HIV infection (Gartner et al., 1986; Koening et al., 1986), and the contribution made by the HIV strain are unknown. To address these questions, U937 cells were transfected with pU3RIII or pLTRcat which differ in the 5' half of the GGTCA palindrome, and infected with HSV-1. CAT activity was assayed 24 h after HSV-1 infection. In order to allow for the potential contribution of the clonal derivation of the U937 cells (Fenyo et al., 1988), the experiments were done in both U937M and U937A cells which differ in the proportion of CD4+ cells they contain.

The results of four independent experiments, summarized in Table 2, indicate that expression from pLTRcat is significantly (80- to 94-fold) increased in HSV-1-infected as compared to uninfected U937 cells. The increase is not an artefact due to the clonal selection of U937 cells since similar results were obtained in both U937M and U937A cells. HSV-1 infection also increased expression from pU3RIII. However, the levels of CAT activity in cells transfected with pU3RIII (10 to 14% conversion) were significantly lower than those seen with pLTRcat (78 to 94% conversion). This presumably reflects HIV strain variation since the experiments were done in duplicate with the same HSV-1 strain, and transfection efficiencies were similar for both LTRcat sequences and in both U937 cell lines (Table 2). It was significant that activation does not require HSV-1 replication. Indeed, consistent with previous findings (Albers et al., 1989; Daniels et al., 1978; Plaeger-Marshall & Smith, 1978; Linnauvori & Hovi, 1981; Tenney & Morahan, 1987, 1991) HSV-1 titres in duplicate samples of the infected U937 cells were 5 and 0.09 p.f.u./cell at 0 and 24 h p.i., respectively.

The LTR sequences in pLTRcat and pU3RIII have a 1 bp difference in the GGTCA palindrome that was previously implicated in negative regulation of HIV LTR (Orchard et al., 1990). To determine whether this may explain the different levels of HSV-1-mediated activation seen with the two constructs, we used a mutant of pLTRcat (pal) in which the GGTCA palindrome was restored to the wild-type sequence. As shown in Table 2, HSV-1-mediated activation of the pal construct in U937 cells (22.8% conversion) was similar to that seen for pU3RIII. We interpret these findings to indicate that the GGTCA palindrome is involved in HSV-1-mediated activation of HIV LTR.

The GGTCA palindrome forms complexes with nuclear extracts from uninfected U937 cells

To determine whether U937 proteins bind to the GGTCA palindrome and whether similar species bind to both the wild-type (pU3RIII) and mutant (pLTRcat) sequences, we used gel retardation analysis with 30 bp oligonucleotides that contained the wild-type GGTCA palindrome [NRE(H)] or its mutant [NRE(P)] respectively (Table 1). In this assay the labelled oligonucleotides were incubated with the protein fractions and subjected to gel electrophoresis. Specific protein-DNA complexes were detected by changes in the electrophoretic mobility of the oligonucleotides. The experiments were performed in the presence of competitor poly(dI-dC)-poly(dI-dC) to reduce non-specific binding, suggesting that complexes are formed by proteins that have a greater affinity for the respective oligonucleotides than for poly(dI-dC)-poly(dI-dC).

Three complexes, designated H1, H2, and H3 in order of increasing electrophoretic mobility, were seen in gel retardation assays with NRE(H) and nuclear extracts from uninfected U937 cells (Fig. 1a, lane 2). Complexes designated P1, P2 and P3 were seen in similar assays with NRE(P) and nuclear extracts from uninfected U937 cells (Fig. 1a, lane 11). Since the GGTCA palindrome overlaps the AP-1 binding site in HIV LTR (Orchard et al., 1990), we sought to determine the relative contribution of the GGTCA and the AP-1 binding sites. NRE(H) or NRE(P) were mixed with U937 nuclear extracts in the presence (or absence) of various concentrations (20 to 2000 fmol, representing a two- to 200-fold excess) of the homologous oligonucleotides or oligonucleotides containing the AP-1 binding site or its

Table 2. HIV LTR activation in HSV-1-infected U937 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>pLTRcat (PV22)</th>
<th>pU3RIII (HXB2)</th>
<th>pal</th>
<th>pSV2cat†</th>
<th>pCATB</th>
</tr>
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<tbody>
<tr>
<td>U937M</td>
<td>1±1±0±2</td>
<td>13±0±1</td>
<td>0±8</td>
<td>18±0±2</td>
<td>0±09±0±01</td>
</tr>
<tr>
<td>Mock</td>
<td>14±2±4</td>
<td>104±2±8</td>
<td>22±8+2</td>
<td>NA†</td>
<td>NA</td>
</tr>
<tr>
<td>HSV-1</td>
<td>94±3±2</td>
<td>104±2±8</td>
<td>22±8+2</td>
<td>NA†</td>
<td>NA</td>
</tr>
<tr>
<td>U937A</td>
<td>80±0±4</td>
<td>12±0±2</td>
<td>0±9±0±01</td>
<td>2±0±4</td>
<td>0±08±0±02</td>
</tr>
<tr>
<td>Mock</td>
<td>78±3±6±8</td>
<td>143±1±3</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>HSV-1</td>
<td>78±3±6±8</td>
<td>143±1±3</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M. for four independent experiments.
† Conversion rates with pSV2cat are 1±6±0±8, 1±4±0±1 and 5±9 for C6, Vero and 293 cells, respectively.
‡ NA, Not applicable.
§ ND, Not determined.
a) 

![Gel retardation assays of NRE(H) and NRE(P) with nuclear extracts of U937 cells.](image)

(b) 

![Gel retardation assays of NRE(H) and NRE(P) with nuclear extracts of U937 cells.](image)

Fig. 1. Gel retardation assays of NRE(H) and NRE(P) with nuclear extracts of U937 cells. (a) 32P-labelled NRE(H) (lanes 1 to 9) or NRE(P) (lanes 10 to 18) oligonucleotides (10 fmol; sequences shown in Table 1) were incubated with nuclear extracts from mock- (lanes 2, 4 to 9, 11, 13 to 18) or HSV-l-infected (lanes 3, 12) U937M cells. Bands H1 to H3 and P1 to P3 represent complexes formed by the NRE(H) and NRE(P) probe, respectively. Unlabelled NRE(H) (lanes 4 to 6, 16 to 18) or NRE(P) (lanes 7 to 9, 13 to 15) oligonucleotides were included in the competition assays. Concentrations of competitors (20 to 2000 fmol) are shown above each lane.

(b) 32P-labelled NRE(H) (lanes 4 to 6) or NRE(P) (lanes 1 to 3) probes were incubated with nuclear extracts from mock-infected U937 cells in the presence of 2000 fmol of unlabelled AP-1 wild-type (lanes 2, 4) or a mutant AP-1 (lanes 3, 5) oligonucleotide. Nucleoprotein complexes were resolved by gel retardation assays. Similar results were obtained in U937A cells and in PBMCs obtained as described (Bernstein et al., 1991).

mutant (Table 1). Formation of the H1 complex was eliminated by incubation with the unlabelled homologous [NRE(H)] oligonucleotide even at equimolar (20 fmol) concentrations (Fig. 1a, lane 4). The H2 and H3 complexes were eliminated by competition with 2000 fmol of the NRE(H) oligonucleotide (Fig. 1a, lane 6). Formation of the P1 and P3 complexes was eliminated by incubation in the presence of 2000 fmol of the unlabelled homologous probe (Fig. 1a, lane 15); P2 was affected by 20 fmol of competing NRE(P) (Fig. 1a, lane 13). The formation of the complexes was not prevented by competition with an oligonucleotide containing the consensus API binding site (Fig. 1b, lanes 2, 4) or its mutant (Fig. 1b, lanes 3, 5).

To determine whether similar proteins are involved in complexes formed by the wild-type [NRE(H)] and mutant [NRE(P)] palindrome sequences we did cross-competition experiments. The labelled oligonucleotides [NRE(H) or NRE(P)] were incubated with nuclear extracts from uninfected U937 cells in the presence of 20 to 2000 fmol of the heterologous unlabelled oligonucleotide used as competitor. It was significant that formation of the P1 complex was not eliminated by competition with the unlabelled NRE(H) oligonucleotide even at 200-fold excess concentrations (Fig. 1a, lanes 16 to 18), whereas all three NRE(H) complexes (H1 to H3) were affected by competition with the unlabelled NRE(P) oligonucleotide (Fig. 1a, lane 9). We interpret these findings to suggest that proteins in the P1 complex are distinct from those binding to NRE(H). Similar results
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Effect of HSV-1 infection on cellular factors that bind the GGTCA palindrome

To determine the effect of HSV-1 infection (albeit non-permissive) on cellular proteins that bind the GGTCA palindrome, we performed gel retardation assays with nuclear extracts from HSV-1-infected U937 cells. Complexes co-migrating with H2 and H3 or P1 and P3 were formed by NRE(H) and NRE(P) respectively (Fig. 1a, lanes 3, 12; Fig. 2, lanes 2, 3, 8). The H1 and P2 complexes were not formed suggesting that HSV-1 infection inhibits the synthesis of cellular protein(s) involved in the formation of these complexes, or modifies their binding potential. Complex formation was eliminated by competition with the homologous probes (Fig. 2, lanes 4, 5, 9, 10) but not by the AP-1 or irrelevant oligonucleotides (data not shown). As was the case with uninfected U937 cell extracts, incubation in the presence of unlabelled NRE(P) competitor eliminated the formation of the complexes formed by NRE(H) (Fig. 2, lane 6) while incubation in the presence of unlabelled NRE(H) competitor did not eliminate formation of the P1 complex (Fig. 2, lane 11).

GGTCA-binding proteins in U937 cells

In order to begin to identify the cellular proteins that complex with the GGTCA palindrome, we did cross-linking experiments with radiolabelled BrdU-containing oligonucleotides. Following gel mobility shift assays using NRE(H) or NRE(P) oligonucleotides and nuclear extracts from uninfected U937 cells and subsequent u.v.-crosslinking within the polyacrylamide matrix, the DNA–protein adducts were excised from the irradiated gels and subjected to SDS–PAGE analysis (Molitor et al., 1990).

Four proteins of approximate Mr of 75K, 64K, 59K and 37K were resolved in the H3 complex (Fig. 3, lane 5). The 75K species was also present in the H1 and H2 complexes and the 64K and 59K species were resolved in...
the H1 and H2 complexes, respectively (Fig. 3, lanes 1, 3). The 37K protein was seen only in the H3 complex (Fig. 3, lane 5). The finding of all four protein species in the H3 complex which migrates with the highest mobility is difficult to interpret and may reflect a contribution of the secondary and/or tertiary structure of the complex towards its mobility. The p37 is present only in the H3 complex and may be responsible for these secondary structural effects. The 59K, 64K and 75K species were also seen in the P2 complex (Fig. 3, lane 9) and p75 was seen in the P3 complex (Fig. 3, lane 10). However, the P1 complex contained a distinct protein of Mr, 120K (Fig. 3, lane 7) and this finding is consistent with the results of the competition experiments. The p37 species was not seen in any of the complexes formed with the NRE(P) oligonucleotide (Fig. 3, lanes 7 to 11). These findings suggest that the A residues in NRE(P) (Table 1) are the critical contact bases for p120, whereas the G residues at these same positions in NRE(H) are the critical contact bases for p37.

It should be pointed out that the levels of p75 appeared somewhat higher in the complexes formed with NRE(P) as compared with NRE(H). However, the extent of crosslinking of each protein species may vary due to the positioning of the photoreactive BrdU in the oligonucleotides, and the critical contact bases for the various protein species. Therefore, quantitative interpretations of the levels of individual protein species in each complex are not possible. Similar results were obtained in both U937M and U937A cells.

**GGTCA-binding proteins in HSV-1-infected U937 cells**

To identify the effect of HSV-1 infection on GGTCA-binding proteins, the crosslinking experiments were done with nuclear extracts from HSV-1-infected U937 cells. The p59, p64 and p37 species were not seen in complexes formed by either oligonucleotide [NRE(H) or NRE(P)] with HSV-1-infected cell extracts (Fig. 3, lanes 2, 4, 11). A novel 50K species was resolved in the H2 complex (Fig. 3, lanes 2, 4), but HSV-1 infection had no effect on the complexity of the p75 or p120 species to their respective oligonucleotides (Fig. 3, lanes 6, 8, 11). Similar results were obtained in U937M and U937A cells.

**Discussion**

Conflicting results were obtained regarding HSV-mediated activation of HIV LTR reflecting, at least in part, the use of different cell types and experimental conditions (for reviews, see Laurence, 1990; Pitha, 1990). Monocytes, which are a recognized reservoir for latent HIV infection (Gartner et al., 1986; Koening et al., 1986), were not studied, and the contribution of NREs is unknown. The salient feature of the data presented in this report is the observation that the GGTCA palindrome within the NRE is involved in HSV-1-mediated regulation of HIV LTR in the promonocytic cells U937. The following comments seem pertinent with respect to these findings.

Our conclusion that the palindrome is a cis-response element potentially involved in HSV-1-mediated regulation of HIV LTR rests on the observations that (i) the levels of HSV-1-mediated activation are significantly higher (sixfold) for the construct (pLTRcat) that has the mutant as compared to that which has the wild-type (pU3RIII) GGTCA palindrome although the basal expression is similar for both constructs, and (ii) the levels of HSV-1-mediated activation of pLTRcat were reduced to those seen for pU3RIII by a 1 bp mutation that restored the wild-type palindrome sequence (pal construct). Our findings are not an artefact due to clonal selection of U937 cells (Fenyo et al., 1988) since similar results were obtained in both U937M and U937A cells that differ in the proportion of CD4+ cells and could therefore be considered as functionally distinct. Furthermore, at least as pertains to gel retardation assays, similar results were also obtained in peripheral blood monocytes (C.-P. Feng et al., unpublished).

Gel retardation and u.v.-crosslinking reproducibly (at least three independent experiments) resolved four to six proteins that bound to the GGTCA palindrome. Binding was specific for the palindrome in that complex formation was not affected by competition from oligonucleotides that contain the overlapping AP-1 binding site. Two uninfected cell proteins (p37 and p120) were specific for the wild-type and mutant palindromes, respectively. All other cellular proteins (p75, p59 and p64) bound to both palindrome sequences. The respective contribution of the various proteins toward complex formation is unclear. In HSV-1-infected cells, the p59, p64 and p37 species were not resolved but a novel 50K protein was detected and it bound only to the wild-type sequence. The failure to detect the p59, p64 and p37 species may reflect inhibition of their synthesis or modification of their binding potential. The use of HSV host shutoff mutants, not presently available to our laboratory, could help differentiate between these two interpretations.

The exact identity of the protein species that bind the GGTCA palindrome sequences is unknown. Based on Mr, p75 may correspond to the high Mr (68K) T cell COUP-TF previously shown to bind to the GGTCA palindrome (Cooney et al., 1991). The slightly increased mobility presumably reflects the presence of the covalently bound oligonucleotide. Supershift mobility assays with specific COUP-TF antibody, not presently available to our laboratory, should help answer this question.
Since p59 and p64 sometimes appeared as a doublet and they were similarly down-regulated by HSV-1 infection they may represent different levels of post-translational modification of the same protein. The p37 may be analogous to the negative-regulatory factor Rpt-1 that down-regulates HIV LTR in resting T cells and the target sequence of which is unknown (Patarca et al., 1988). We believe that the 120K protein is a distinct species rather than a multimer consisting of the lower M<sub>r</sub> proteins, since (i) it did not complex with NRE(H) which binds the lower M<sub>r</sub> proteins, and (ii) it was detected in NRE(P) complexes with HSV-1-infected cell extracts in which p59 and p64 are down-regulated. The HSV-1-induced p50 protein may be a member of the heat-shock protein family that is induced by HSV-1 infection even in the absence of DNA synthesis (LaThangue et al., 1984). We assume that down-regulation of the p37 and p59/p64 species is mediated by HSV virion host shutoff proteins (Read & Frenkel, 1983) whereas induction of the p50 species involves the trans-activating immediate early (IE) genes, directly or through interaction with other cellular factors (Feldman et al., 1982; LaThangue & Latchman, 1987).

HSV-1 does not replicate in U937 cells and expression is limited to the IE110 and IE175 genes (Tenney & Morahan, 1987, 1991). Vmw65 is a component of the virion and is therefore present in HSV-1-infected cells. However, at least in cotransfection experiments, the LTR is activated only by IE110 DNA in U937 cells. It is not activated by Vmw65 or IE175 DNA (C.-P. Feng et al., unpublished).

It is tempting to interpret our findings within the context of multiprotein interactions that convert COUP-TF from a positive to a negative regulator thereby addressing its ability to bind to a negative cis-response element (Cooney et al., 1991). According to this interpretation p59/p64 and p37 are a family of proteins [the thyroid/steroid receptor superfamily (Wang et al., 1989; Cooney et al., 1991)] that converts the positive regulator COUP-TF (p75) into a repressor protein by masking its activating domain or by interfering with its ability to interact with a modulatory protein (S300-II) (Tsai et al., 1987). Presumably HSV-1 infection counteracts the negative effect of this family of proteins by inhibiting their synthesis and/or interfering with their binding potential/function. Implicit in such an interpretation is the assumption that the higher levels of HSV-1-mediated activation seen for the mutant palindrome reflects the ability of the HSV-1-induced p50 species (which binds only to the wild-type palindrome) similarly to convert COUP-TF into a negative regulator. An alternative interpretation is that p120, which binds only to the mutant palindrome, is a positive regulator that functions together with (or independent of) COUP-TF. Its activity in uninfected cells is similarly counteracted by the presence of the p59/p64 species that are down-regulated by HSV-1 infection. Final conclusions pertaining to the validity of these interpretations must await the results of ongoing studies designed to characterize the GGTCB-binding proteins further.

Notwithstanding, the findings provide a potential interpretation for the present discrepancies relating to HSV activation of HIV LTR (reviewed in Laurence, 1990; Pitha, 1990). Within the limitation imposed by extrapolation from the in vitro to in vivo situations, they have interesting implications for HIV production from the latent proviral genome i.e. before tat expression (similar to the tat<sup>-</sup> conditions used in our experiments). Indeed, it has been estimated that in asymptomatic HIV-seropositive individuals there is one HIV provirus molecule per 6000 to 80000 peripheral blood monocytes (PBMCs) (Simmonds et al., 1990). On the average, 2 x 10<sup>4</sup> PBMCs circulate through 1 cm<sup>3</sup> of skin tissue per min (Golde & Groopman, 1990; Schlant & Sonnenblick, 1990). Therefore, during a recurrent HSV episode, approximately three or four HIV provirus-carrying PBMCs will become infected with HSV each minute. The normal HSV-infected patient experiences on average five episodes of recurrent herpes, from which infectious virus can be isolated for at least 3 to 5 days (Aurelian & Kessler, 1985). Furthermore, the frequency of HSV infection is higher in HIV seropositive individuals (Quinn, 1990). Accordingly, there is a high probability that the monocytes carrying HIV provirus will become infected with HSV. Although HSV infection of PBMCs is non-permissive (Albers et al., 1989; Tenney & Morahan, 1987, 1991), our findings indicate that it can modify the balance of HIV regulation by cellular proteins, ultimately breaking latency.

We thank Dr Roberta Kamin-Lewis for CD4 analysis of U937 cells. Rita Fishelevich for technical assistance, Denise Garnett for help with the figures and Irene Gervis for help with the manuscript. Chupei Feng is the recipient of a GRA fellowship from the University of Maryland Graduate School. This study was supported by Public Health Service grant NS 26665 from the National Institute of Neurological and Communicative Disorders and Stroke.

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(Received 28 April 1992; Accepted 10 November 1992)