The critical role of human transcriptional repressor CTCF mRNA up-regulation in the induction of anti-HIV-1 responses in CD4+ T cells

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Summary

We have employed our CD4+ T cell model named HRF(+) (HIV-1 Resistance Factor) to study the inducible anti-HIV-1 responses mediated through novel soluble molecules. We found that exposure to the soluble products of HRF(+) cells activated CTCF (CCCTC binding factor) mRNA expression in HIV-1 susceptible primary and transformed CD4+ T cells and overlapped with their acquisition of transient resistance to virus. Conversely, the interference with the expression of CTCF gene in HRF(+) cells reversed the resistant phenotype and eliminated the biological potential of their cell culture supernatant to induce "HRF-like" activity in target cells. Band-shift analysis upon the nuclear fractions from HIV-1 resistant cells showed that CTCF protein bound to HIV-1 promoter and this binding prevented the formation of NF-κB/LTR complex. This evidence suggests that CTCF is an intracellular effector of HRF activity and that the acquisition of resistance to HIV-1 in CD4+ T cells is a consequence of the prior activation of CTCF gene by the soluble entity secreted by HRF(+) cells.

Keywords

CD4; T cell; HIV-1; CTCF; resistance

2. Introduction

CTCF (CCCTC binding factor) has been called a first "truly multivalent" nuclear factor performing multiple regulatory tasks in gene expression [1,2]. Through the binding of highly divergent DNA sequences and formation of various protein complexes, this multifunctional protein might suppress or activate various genes [3]. The transcription suppressive activity of CTCF protein was best described for the chicken lysozyme [4] and chicken, mouse and human c-myc genes [5]. CTCF gene is highly conserved and ubiquitously expressed throughout the Animal Kingdom, and although much has been uncovered about its structure and function, there are still some problematic issues that need to be defined. For example several mature CTCF mRNA isoforms have been detected in chicken cells [6] and CTCF gene protein products show some degree of size variability including smaller forms of unknown function [3,6,7].

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In previous studies we characterized our CD4^{+} T cell model for studies of acquired resistance to HIV-1 infection [8–12]. In this model, HIV-1 resistant cells named HRF(+), restricted transcription of viral genome through the protective activity of a novel soluble protein factor (s) named HRF (HIV-1 Resistance Factor) acting on the level of nuclear NF-κB/DNA binding [9–11]. Thorough analysis of genes involved in transcription regulation revealed that the expression of CTCF mRNA was consistently up-regulated in HRF(+) cells [8]. As CTCF is a potent suppressor of transcription [3,5,13], a characteristic that is also the "landmark" of HRF antiviral activity [10,12], we explored the significance of CTCF mRNA up-regulation and its link to the acquired resistance to HIV-1 in CD4^{+} T cells.

This investigation demonstrated that the increased transcription of CTCF gene is associated with the acquired antiviral activity in CD4^{+} T cells. We tested the CTCF mRNA expression in several HRF(+) clones by real-time PCR, and found, that elevated activity of this gene could be correlated to the extent of the antiviral protection; the higher CTCF transcriptional activity corresponded to the stronger antiviral responses. The exposure of human macrophages and transformed CD4^{+} T cells to the soluble products of HRF(+) cell line also increased the expression of their endogenous CTCF mRNA and the elevated activity of CTCF gene in these cells coincided with the induction of transient "HRF-like" resistance to virus. The antiviral activity of HRF(+) cell culture supernatant was eliminated by the RNAi - mediated depletion of CTCF. To understand the link between the HRF-mediated up-regulation of CTCF transcriptional activity and the cellular resistance to virus we analyzed the affinity of nuclear CTCF to DNA sequences on HIV-1 promoter and found that CTCF binds to LTR and this CTCF/DNA association blocks the formation of NF-κB/DNA complex. Taken altogether these observations suggest that CTCF is an intracellular effector of HRF activity regulating cellular resistance to HIV-1 through the interference with HIV-1 transcription and show a novel function for this potent transcription regulator.

3. Materials and Methods

Cell cultures and viruses

HRF(+) and HRF(-) cells distinguished by HIV-1 resistance and HIV-1 susceptibility have been described previously [8–12,14,15]. 1G5 cells stably expressing luciferase gene driven by HIV-1 long terminal promoter (LTR) [16] were obtained through NIH AIDS Research and Reagent Program Division of AIDS, NIAID. Human monocytes from healthy, HIV-1 negative volunteers were obtained by elutriation from the whole blood. Monocytes were allowed to differentiate in 12-well plates (Corning Scientific) under culture conditions as described before [17]. Briefly: cells were seeded into 12-well plates at concentration of 1.5×10^6/well and allowed to differentiate into macrophages in DMEM (Sigma) supplemented with 10% endotoxin-free, heat inactivated human serum, 10% giant cells tumor conditioned medium (Sigma), glutamate and antibiotics.

Continuous cultures of 1G5, HRF(+), and HRF(-) cell lines were maintained in RPMI 1640 (Sigma) supplemented with 5% fetal bovine serum, antibiotics and glutamate at 37°C in a 5% CO2 95% air-humidified incubator. To prepare biologically active supernatants HRF(+) and control HRF(-) cells were cultured as described before [9,10]. Briefly; 2x106/ml HRF(+) and control HRF(-) cells were cultured overnight in protein free Hybridoma medium (Sigma) supplemented with glutamate. Subsequently, cells were removed by centrifugation and supernatants were filtered through 0.45mM membranes (Millipore) and concentrated by lyophilization from 30-ml aliquots using Labconco Freeze-Dryer. Protein powder was resuspended in 10 ml distilled water and subjected to dialysis against 10mM Tris-Cl, pH 8.0 using benzoylated cellulose tubing with MW cut-off of 1.2KDa (Sigma). Dialyzed material was concentrated again by lyophilization and stored at 40C. For cell treatments lyophilized samples were resuspended in sterile distilled water to 100x concentrate followed by second

dilution in Hybridoma medium (Sigma) to 1x concentrate. This material was used to prepare conditioned culture media for macrophages.

Virus isolates used for this study was NL4-3 [18] and HIV-1 ADA [19].

Testing of HRF biological activity by Rapid Suppression Assay (RSA)

RSA was performed essentially as described before [9,10]. Briefly: 1G5 cells that are stably transfected with an inducible luciferase gene driven by HIV-1 LTR [16] were washed in PBS, resuspended in hybridoma medium to concentration of 5×10^6 cells/ml. For control titration curves, 100μl aliquots of 1G5 cells were supplemented with 0.01% to 50% volume of HRF (+), HRF(-) supernatants or medium alone, brought to a final volume of 200μl and incubated for three hours at 37^0C. Subsequently, all cells were induced with PMA at concentration of 5ng/ml. Two control tubes contained 1G5 cells were resuspended in hybridoma medium with or without PMA. Three hours later cells were collected by centrifugation and lysed in the same tubes using Reporter Lysis buffer (Promega). The expression of luciferase protein was tested based on manufacturers protocol. Cell culture supernatants for RSA testing were dialyzed for 48 hours against 10mM Tris-Cl, pH 8.0, concentrated by lyophilization and tested as 1%/vol.

RNA analyses

Total RNA was isolated using RNaeasy mini kit (Qiagen). For real-time PCR analyses total of 3μg RNA was reverse transcribed to cDNA with random hexamers using the SuperScript III First-Strand Synthesis kit (Invitrogen). The real-time detection of CTCF and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes expression was performed using TaqMan Gene Expression Assay on 7500 real-time PCR System (Applied Biosystems). Briefly; triplicates of total reaction mixture of 25μl were composed of 2μl of cDNA, 12.5μl of 2xABI TaqMan PCR Master Mix, and 0.25μl (90μM) of both sense and antisense primers and 0.5μl of the probe. After the initial denaturation at 95^0C (10min), target genes were amplified through 40 cycles of universal cycling conditions (95^0C/10s, 60^0C/1min). The GAPDH target gene was amplified using pre-designed pair of primers and a probe (Applied Biosystems) and CTCF gene was amplified by pair of primers spanning through zinc finger 4: CTCF-F 5'-TTCAAGTGTTCCATGTGCGATT-3' and CTCF-R 5'-GTATGAGAGCGAATGTG ACGTTTTA-3' (Invitrogen) and detected by probe CTCF-p 5'-6 FAMCAGTGTAGAAGTCAGCAA A^{MGBNFQ}-3' (Applied Biosystems). Standard cell dilution curves were calibrated using serial dilutions of control plasmid pTA_CTCF representing 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 10 copies of CTCF cDNA per reaction and all values are expressed as means ± standard deviation of the mean. The absolute value representing ratio target gene expression was calculated based on formula:

$$\text{Ratio target gene expression} = \frac{\text{target gene mean Qty (sample/control)}}{\text{reference gene mean Qty (sample/control)}}$$

Investigation of the induction of CTCF expression in transformed CD4+ T cell line and in human macrophages upon exposure to soluble products of HRF(+) cell line

HRF(-) and HRF(+) cells were cultured adjacent to each other in a double chamber apparatus (Cornig). Macrophages were cultured in 7.5% conditioned HRF(+) and HRF(-) medium or hybridoma medium alone. Three days later treatments were removed and cells were cultured alone in DMEM complete growth medium for up to eight days. Expression of CTCF control GAPDH genes were tested by real-time PCR using pairs of primers described above; the biological activity of their cell culture supernatants was tested by RSA and replication of HIV-1 was tested by p24 Elisa (Perkin Elmer).

siRNA silencing of CTCF mRNA in HRF(+) cells

Homo sapiens CTCF mRNA sequence (U25435) was subjected to BLAST analysis in order to exclude the common coding sequences. Based on this analysis we selected a 190bp mRNA fragment unique to CTCF as a template for dsRNA synthesis. Two primers sense 5'-GGTCTGCTATCAGAGGTTAAT GCGG-3' and antisense 5'-GTGACGATCCAAATTTGAACGCCGT-3', with appended T7 promoter sequences (5'-TAATACGACTCACTATAGG-3') were used to amplify a CTCF cDNA fragment from mRNA of HRF(+) cells. Subsequently, cDNA fragment was cloned into pCR^{TA} vector (Invitrogen) and amplified in DH5α E. coli. CTCF cDNA fragment enlarged by T7 polymerase promoter was excised from the plasmid by EcoRI digestion, separated through agarose gel electrophoresis and purified through Ultrafree-DA columns (Millipore). 3μg of this material and control Litmus 38iluc served as templates for dsRNA synthesis using ShortCut RNAi kit based on manufacturer's protocol (New England Biolabs Inc). 10μg of dsRNA from both samples was subjected to processing by shortcut RNase III for 20 min at 37^0C and purified by ethanol precipitation. The concentration of siRNA fragments was calculated based on UV absorbance and by band intensity comparison of siRNA fragments run next to serial dilutions of 21bp RNA standard (New England Biolabs Inc) on 20% TBE polyacrylamide gel (Invitrogen).

For the initial CTCF knock-out experiments $3x10^6$ HRF(+) cells were transfected with 9nM of CTCF and control Litmus 38iluc siRNA fragments by lipofectamine 2000 (Invitrogen) in Hybridoma medium (Sigma). Complete growth medium was added after 4 hours and cells were cultured in concentration of $1x10^6$cells/ml for another eight days. The CTCF mRNA degradation was tested every two days by real-time PCR as described above. The inhibition of protein synthesis was confirmed by Western blot analysis using α-CTCF and control α-tubulin antibodies (Santa Cruz biotechnology, Inc.).

Assay of the effect of CTCF siRNA silencing on susceptibility of HRF(+) cells to HIV-1 replication

To test the correlation of CTCF to HRF activity $2.1x10^7$ HRF(+) and $1.2x10^7$ HRF(-) cells were infected with HIV-1 NL4-3 [18] at concentration of 0.5pg/cell. Three days later HRF(+) culture was divided into three parts: one served as a control for restrictive infection and two others were subjected to siRNA silencing by CTCF and Litmus 38iluc fragments at 9nM concentration as described above. HIV-1 infection was monitored by Elisa (Coulter) measuring levels of extracellular antigen p24 and by RT-PCR of the single spliced *vif* transcript using pair of primers described before [9].

Assay of the effect of CTCF siRNA silencing on the biological activity of HRF(+) cell Culture supernatant

Macrophages were cultured in 7.5% conditioned HRF(+), HRF(+) $^{CTCF\ siRNA}$ and HRF(-) medium or hybridoma medium alone. Three days later treatments were removed and cells were cultured alone in DMEM complete growth medium for up to four days. The expression of CTCF and control GAPDH genes was tested by the real-time PCR using pairs of primers described above and biological activity of their cell culture supernatants was tested by RSA.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear translocation of transcription factors in HRF(+) or control HRF(-) cells was induced with 0.1pg/cell HIV-1 NL4-3 for 30 min. at 37^0C. Subsequently, the induced and un-induced cells were washed in PBS and lysed in lysis buffer (20mM Hepes, pH 7.9, 10mM NaCl, 3mM MgCl$_2$, 1mM DTT, 0.5mM EDTA, 10% glycerol, 0.1% NP-40, 1mM Na$_3$VO$_4$ and protease inhibitor cocktail) and nuclear fractions were collected by sedimentation at 500 x *g*. Nuclear

proteins were extracted with extraction buffer (20mM Hepes, 400mM NaCl, 1mM DTT, 0.5mM EDTA, 20% glycerol, 1mM Na_3VO_4 and protease inhibitors) and tested for their ability to bind labeled nucleotides representing consensus NF-κB site provided by manufacturer (Promega) or YB-1 probe 5'-361-CTGGGGACTT TCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGA-404-3' synthesized by Invitrogen Inc. All DNA-protein binding reactions were based on manufacturer's protocol (Promega) and described before [10]. Briefly; 0.5 ng of labeled probe was incubated for 20 min at 4^0C with 5μg of nuclear extracts in the presence of 1x gel shift buffer (Promega).

For the supershift band analysis 5μg of nuclear extracts were incubated for 90 min. on ice with 4μg of relevant antibody. All antibodies to p65 NF-κB, Sp1, YB-1 and CTCF were purchased from Santa Cruz, Inc. Subsequently, 1.5μl of a 10x loading buffer was added into all reactions, followed by separation by electrophoresis on 4% polyacrylamide gel until free probe was close to the bottom of the gel.

Sequencing and cloning of CTCF gene from HRF(+) cells

CTCF cDNA was amplified from HRF(+) cells by high fidelity *pfu* polymerase (Perkin Elmer) using pair of primers: sense CTCFF2 5'-ATGGAAGGTGATGCAGTCGAAGC-3' and antisense CTCFR1 5'-TCACCGGTCCATCATGCTGAGGAT-3'. Subsequently the CTCF *orf* was cloned into TA cloning vector (Invitrogen) and subjected to sequencing at DNA sequencing Core Facility at Columbia University using consecutive forward primers: CTCFF2 (as above), CTCFF5 5'-ACATGAGAACCC ATTCAGGGGA-3', CTCFF8 5'-TGAAGTGTCTAAAGAGGGCCT-3' and reverse CTCFR1 (as above) . The pTA_CTCF was used to prepare standard curves for amplification of CTCF mRNA by real-time PCR.

4. Results

The level of CTCF expression correlates with the level of antiviral protection in HRF(+) cells

We reported previously that HRF(+) cells have elevated levels of CTCF mRNA [8]. The over-expression of a potent transcription inhibitor [5,20–22] in cells known for secreting a novel protein factor that inhibits transcription of HIV-1 was interesting and prompted speculation that these two activities are interrelated to the acquired resistance to virus. Since the previous data were obtained for HRF(+) cell line selected for the maximum antiviral responses, we evaluated this hypothesis measuring levels of CTCF mRNA by real-time PCR in other HRF (+) clones in hand (Table 1). Although expression of CTCF was up-regulated in all HRF(+) clones; clones differed in CTCF expression. To evaluate the relation of this diversity in CTCF mRNA level to HIV-1 resistance, we challenged all tested cell lines with HIV-1, and seven days later, measured virus replication by indirect immunofluorescence for HIV-1 antigens (IF). Again, all HRF(+) clones but not the control HRF(-) cell line restricted HIV-1 infection but they could be distinguished by the extent of their anti-viral responses, and more interestingly these variations in the antiviral protection correlated to the level of CTCF mRNA up-regulation (Table 1). For example the CTCF expression in control HRF(-) cells was at 2,28 mRNA copies/ cell and at the seven-day time point 72% of the cell population was infected with HIV-1. On the other hand, the 2.4-fold increase of CTCF transcription in HRF(+) clone 5 (HRF(+)$_5$) correlated with the 58% inhibition of HIV-1 replication and greater than 3-fold increase of CTCF mRNA overlapped with the inhibition of HIV-1 replication from 66 to 70% in clones 15, 28 and 22 respectively (Table 1). These data indicate that antiviral activity in HRF(+) cells could be associated with the expression of CTCF gene and prompted subsequent question, as to whether HRF mediated protection in HIV-1 susceptible target cells could be also linked to the elevated expression of CTCF gene.

The CTCF gene is up-regulated in HIV-1 susceptible target cells upon their exposure to HRF (+) cell culture supernatant and elevated expression of CTCF coincides with the induction of "HRF-like" activity

The correlation between CTCF gene up-regulation and levels of the antiviral protection in several HRF(+) clones suggested that this protein could be the intracellular effector molecule for extracellular HRF and with this in mind we investigated whether the induction of antiviral resistance in HIV-1 susceptible cells coincided with the increased activity of CTCF transcription. Based on our previous findings that HIV-1 susceptible cells cultured in adjacent chamber with HRF(+) cells were protected from virus infection [11,12] we assayed CTCF gene expression in HRF(-) cells that were cultured for three days next to HRF$(+)_{28}$ clone in a double chamber apparatus. Exposed HRF(-) cells were harvested daily throughout the period of three days and the transcription levels of CTCF gene were analyzed by the real-time PCR (Table 2). HRF(+) treatment induced the expression of CTCF gene in HIV-1 susceptible target cells and two days after the initial exposure the CTCF mRNA was up-regulated by 1.54-fold and three-day co-culture further increased this gene expression, raising CTCF mRNA copy number to 7.4 copies/cell (Table 2).

Similar studies were subsequently performed in human macrophages – a primary target cells for HIV-1 infection. Based on our previous observations three days exposure to HRF induced endogenous "HRF-like" resistance in these cells, and the induction of antiviral activity could be achieved through soluble molecules present in HRF(+) cell culture supernatant [23]. On average, five days from the initial exposure to HRF(+) cell culture supernatant or three days after the cessation of HRF treatment, macrophages acquired an "HRF-like" activity which could be detected in their culture supernatants by Rapid Suppression Assay – (RSA) [9].

We considered the possibility, that the acquisition of "HRF-like" activity in human macrophages could be linked to the activation of CTCF mRNA transcription and to test this hypothesis macrophages from two different HIV-1 negative donors were cultured alone or in the presence of 7.5% HRF(+) or control HRF(-) conditioned medium for three days, then treatments were terminated. Expression of CTCF gene was assayed by real-time PCR, and the induction of "HRF-like" activity by RSA. In this experimental setting we investigated the "early" and "late" changes of gene expression, where the "early" time-points defined the first 48 hours of continuous treatments and the "late" time-points tested both the CTCF mRNA expression and the induction of antiviral resistance after treatments were terminated (Fig. 1).

As observed for transformed T cells, HRF(+) but not HRF(-) treatment induced the expression of CTCF gene in human macrophages, as early as 1 hour from the initial exposure the concentration of CTCF mRNA increased by 100- fold in cells from donor 1 and 5.5-fold in cells from donor 2 (Fig. 1 left *y* axis). Two days later the activation of CTCF gene still exceeded controls by 100-fold (donor 1) and by 2.4-fold (donor 2) (Fig. 1 left *y* axis). Most remarkably, the cessation of HRF(+) treatment did not stop the CTCF activation and high levels of its mRNA were still present for up to seven days later (Fig. 1 left *y* axis, 4 and 7 day-time points) which indicated that the soluble factor(s) present in HRF(+) supernatant elicited a long-term effect in target cells.

RSA evaluation of cell culture supernatants collected after treatments were terminated showed that macrophages secreted "HRF-like" activity and its induction coincided with the induction of CTCF mRNA expression (Fig. 1 right *y* axis). HRF treatment altered the composition of proteins secreted by macrophages and their culture supernatants now inhibited expression of the reporter gene by 29 to 41% 24 hours after the termination of treatment and by 30 to 50% four days later in donor 1 and 2 respectively (Fig. 1 right *y* axis; 4 and 7-day time points).

Employing the same experimental setting we assayed the efficiency of HIV-1 replication in human macrophages induced to resist virus infection. One day after the cessation of HRF treatment cells were challenged with 0.01pg/cell ADA, a well characterized macrophage tropic HIV-1 isolate [19]. The activity of CTCF mRNA was tested by Q-PCR and replication of virus was assayed by levels of the extracellular p24 core antigen. As shown in Fig. 2, replication of virus was reduced by 35 and 50% six and eight days after the initial exposure of macrophages to HRF treatment. The inhibition of HIV-1 replication correlated with the 2.2-fold increase in the activity of CTCF mRNA and provided evidence that the acquisition of antiviral activity and the induction of CTCF transcription are interrelated and suggested that CTCF protein might be the cellular partner of HRF.

Expression of CTCF is indispensable for HRF function in both intra- and extracellular compartments

To evaluate the hypothesis that CTCF protein is involved in the regulation of antiviral resistance in CD4$^+$ T cells, we tested the consequences of transient interference with CTCF expression to the antiviral activity in HRF(+) cells. We used siRNA gene targeting [24] and established experimental conditions ensuring the viability of cell culture and significant down-regulation of CTCF expression.

As shown in Table 3, CTCF but not control Litmus siRNA fragments reduced the expression of CTCF gene in these cells by 81% two days after the delivery. CTCF mRNA levels were still reduced by 65%, 50% and 34% four, six and eight days after transfection and matched the significant reduction of CTCF protein (Fig. 3).

These experimental conditions were used to test the effect of CTCF gene silencing on the antiviral activity in HRF(+) cells challenged with 0.5pg/cell NL4-3 HIV-1. Replication of HIV-1 was tested by RT-PCR of the single-spliced *vif* transcript and detection of the p24 core antigen (Fig. 4A & B). Consistent with our previous observations [10,11], replication of HIV-1 was inhibited in HRF(+) cells and the exposure to litmus siRNA fragments did not have any affect. However, the "knock-out" of CTCF gene expression allowed virus replication and both HIV-1 *vif* mRNA and p24 protein were synthesized at levels surpassing those observed in control HRF(-) cells. Eight days after CTCF silencing, *vif* mRNA levels in HRF(+) cells were increased by 2-fold and extracellular p24 protein by 28-fold respectively (Fig. 4A and B). Subsequent measurements of HRF activity in cell culture supernatants by RSA revealed that the silencing of CTCF gene resulted also in substantial reduction of activity in the extracellular compartment (Fig. 4C) and suggested that the elevated expression of CTCF has direct effect in the maintenance of HRF-mediated activity.

To clarify this proposition we treated human macrophages with the extracellular material from HRF(+) cells subjected to siRNA-mediated depletion of CTFC. As shown in Fig. 5, elimination of CTCF in HRF(+) cells altered the composition of their cell culture supernatant and this reagent did not induce "HRF-like" activity in macrophages; both, the expression of CTCF mRNA (Fig. 5 left *y* axis) and levels of antiviral activity (Fig. 5 right *y* axis) were comparable to cells treated with the control HRF(-) supernatant or cultured alone thus confirming our suggestion that the induction of endogenous protection in HIV-1 susceptible cells is a consequence of the prior up-regulation of CTCF mRNA expression initiated by the exposure of target cells to the soluble entity present in HRF(+) cell culture supernatant.

HIV-1 exposure induces CTCF/LTR binding in HRF(+) cells

The CTCF protein binds DNA targets with a remarkable plasticity through various combinations of its zinc fingers. It also forms complexes with other proteins leading to acquisition of novel functions by these CTCF/protein formations [25,26]. We reported

previously that the exposure to HRF affected the formation of NF-κB/LTR complex [10,27]. As CTCF is a potent inhibitor of transcription we considered the possibility that the increased concentration of this protein alters the events on HIV-1 promoter. Although so far no single consensus sequence for CTCF has been defined, data suggest that this protein binds with high affinity to GC reach motifs [3]. Sequence analysis of HIV-1 LTR revealed possible CTCF binding sites, one of them was detected within the YB-1 binding sequences. In the absence of scientific precedence of CTCF/LTR interactions we considered two possibilities: (i) that CTCF could bind LTR directly or (ii) through its interaction with YB-1 protein [25,28,29] could affect the NF-κB sequences separated from the YB-1 cognate sequence by only six nucleotides (Fig 6). To evaluate this proposition we synthesized a 43-mer probe (called here a YB-1 probe) encompassing the binding sites for NF-κB, YB-1 and Sp1 proteins and applied band–shift analyses to compare the nuclear translocation and binding activity of NF-κB, Sp1, YB-1 and CTCF proteins from HRF(+) and HRF(-) cells to this fragment of HIV-1 promoter (Fig. 6). The nuclear translocation of transcription regulators was initiated by 30 min exposure to HIV-1 and nuclear fractions were isolated based on methods described before [10]. As shown in Fig. 6A (left panel; YB-1 probe), HIV-1 exposure induced the nuclear translocation and formation of protein/DNA complex in both tested cell lines; however, nuclear extracts derived from the induced control HRF(-) cells bound to YB-1 probe with about 50% higher efficiency as compared to band intensity in sample derived from induced HRF(+) cells. The NF-κB binding site was minimally shifted by HRF(+) sample (Fig. 6A- right panel-NF-κB probe), but proteins in HRF(-) nuclei clearly shifted the probe mobility suggesting that nuclei from HRF(-) cells have more NF-κB binding activity than nuclei from HRF(+) cells. The visible YB-1 probe shifts in HRF(+) samples (Fig. 6A- left panel -YB-1 probe) could be formed by either Sp-1 or YB-1/DNA complexes as these proteins bind LTR in the exclusive manner [30].

To resolve this question the composition of protein complexes bound to YB-1 probe was analyzed by supershift EMSA using antibodies to p65 NF-κB, Sp-1 and YB-1 proteins (Fig. 6B-D). The NF-κB dimer bound to its cognate sequence on YB-1 probe only in the control HRF(-) but not in HRF(+) sample, thus confirming our conclusions of shift analysis shown in Fig. 6A. HIV-1 exposure induced also the Sp-1/LTR binding in HRF(-) cells (Fig. 6C). Binding of Sp1 protein was less efficient in HRF(+) sample, which suggested that these sequences could be occupied by YB-1 protein. This proposition was confirmed by reaction with antibody to YB-1 which detected more YB-1 protein from HRF(+) nuclei bound to the probe under both the "constitutive" and "virus-induced" culture conditions. We also tested nuclear extracts from HRF(+) and HRF(-) cells for the presence of CTCF/LTR binding and found that antibody to CTCF formed a supershift band in samples from HRF(+) but not from HRF(-) cells (Fig. 6E). This data showed for the first time that CTCF binds to HIV-1 LTR sequences and this protein/ DNA association interfered with the NF-κB/LTR complex formation. It is noteworthy that the nuclear CTCF and YB-1 proteins isolated from HRF(+) cells formed complexes with the LTR probe with similar affinity in both "constitutive" and "inducible" culture conditions thus suggesting that the cellular exposure to HRF rather than virus induction enables CTCF affinity to regulate this new target.

5. Discussion

Novel functions of CTCF protein in regulation of cellular latency of EBV and HSV-1 have been reported recently by other groups [27,31]. In one system cellular exposure to EBV activated transcription of CTCF followed by accumulation of its protein product. Consequently, CTCF protein bound to EBV sequences and inhibited transcription of virus encoded transactivator-EBNA2. In other studies a short autonomous repression motif located within N'terminal domain of CTCF was found to be important for the suppression of basal activity of SV40- and CMV promoters [13]. These newly discovered functions of CTCF protein demonstrated its remarkable regulatory diversity. We found that the exposure to active entity

secreted by HIV-1 resistant cells also induced the activity of CTCF gene. To understand this phenomenon we set our research objective to verify two hypotheses; (1) that the up-regulation of CTCF gene is linked directly to the HRF-mediated protection in HIV-1 susceptible target cells, and (2) that the CTCF gene product could be in fact an intracellular co-factor of HRF activity.

The first theory was particularly interesting in conjunction with our recent observations, suggesting that HRF treatment induced the production of endogenous "HRF-like" activity in human macrophages [23]. The acquisition of antiviral protection in HIV-1 target cells resulted from their initial exposure to the soluble products of HRF(+) cell line and persevered after treatment was terminated. Newly acquired "HRF-like" resistance was maintained through soluble molecule(s) secreted into the extracellular compartment and like "native" HRF, restricted replication of HIV-1 in virus susceptible, primary cells.

We explored the possibility that the acquisition of "HRF-like" activity in target cells could be correlated to the increased transcription of CTCF and tested this gene's activity in transformed and primary HIV-1 susceptible CD4$^+$ T cells upon their exposure to soluble products of HRF (+) cells. Probing over time the CTCF mRNA expression in target cells cultured next to HRF (+) clone confirmed our hypothesis. HRF treatment elicited rapid activation of CTCF expression coinciding with the induction of endogenous resistance. This effect was particularly strong in human macrophages thus excluding the concern that the source of CTCF observed in transformed T cell lines could be induced by a transforming virus rather than by the HRF.

Since the acquisition of "HRF-like" antiviral activity coincided with the dramatic increase of CTCF mRNA, we postulated that CTCF is in fact an intracellular effector of HRF activity and the rapid accumulation of this gene product (unpublished data) contributes directly to the "switch" from "HIV-1 susceptible" to "HIV-1 resistant" phenotype. This hypothesis was confirmed by two observations; (i) the resistant phenotype in HRF(+) cells after siRNA directed silencing of CTCF expression was reversed, and (ii) the soluble products derived from HRF (+) cells subjected to CTCF siRNA silencing did not induce the antiviral responses in human macrophages. These observations suggested that that the increased activity of CTCF gene was indispensable for the maintenance of antiviral resistance and the increased expression of CTCF protein is required to regulate the expression or export of HRF itself.

The "landmark" of HRF mediated antiviral activity in CD4$^+$ T cells is the inhibition of virus transcription through the interference with the formation of NF-κB/DNA complex [10,23]. We reported previously that HRF treatments did not restrict the NF-κB nuclear translocation and the active entity in HRF(+) supernatants did not compete with NF-κB dimer for its cognate site on HIV-1 promoter. We hypothesized that the intracellular HRF effector molecule could bind to LTR through its own unique DNA site, or through its association with other LTR regulatory proteins and disable the formation of NF-κB/LTR complex. The evidence of CTCF binding to HIV-1 promoter supported this proposition and enabled construction of a hypothetical model of the HRF-mediated antiviral activity involving CTCF (Fig. 6). Based on our data we suggest that HRF induces the activation of CTCF gene through the ligation of cell surface receptor or its cellular uptake. The activation of CTCF gene expression might be induced by the cellular up-take of HRF or through the activation of other cellular partners. The enhanced activity of CTCF gene is followed by the increase of the protein concentration (data not shown). Detection of CTCF/LTR complex in un-induced cells indicates that the nuclear translocation of CTCF is independent of virus stimuli; however, upon the virus invasion the cytosolic CTCF protein is relocated to the nucleus where it binds to HIV-1 LTR with a higher efficiency shown by the increase of CTCF/DNA band density. Subsequently the CTCF/LTR complex or CTCF-YB-1/LTR complex disables the capacity of NF-κB to bind its DNA target - possibly through changing the conformation of DNA as it was found for κB proteins [32].

Although we are closer to the understanding of HRF-mediated responses to virus invasion; there are still many questions waiting for an answer; for example the identity of HRF itself. This elusive molecule is yet to be defined and we believe that the identification of its intracellular co-factor will facilitate its discovery. It is also puzzling why CTCF protein bound HIV-1 promoter only in HRF(+) cells? Analysis of mRNA did not show any diversity between CTCF transcripts in HRF(+) and control HRF(-) systems (data not shown) which suggests, that HRF treatment along with the induction of transcription enables also a new function upon CTCF gene product. This new function can be achieved through the formation of CTCF/protein complexes or through the posttranslational modification. Comparable kinetics of YB-1 and CTCF binding suggested that HRF exposure enables the YB-1/CTCF binding and this protein complex targets HIV-1 LTR. Despite of these questions one fact remains solid: the CTCF is an intracellular co-factor of a novel inducible anti-HIV-1 activity in CD4^{+} T cells; This research along with reports from other research groups [13,27,31] shows that in addition to well-defined activities of CTCF, this protein might be involved in regulation of cellular responses to the viral invasion.

Acknowledgements

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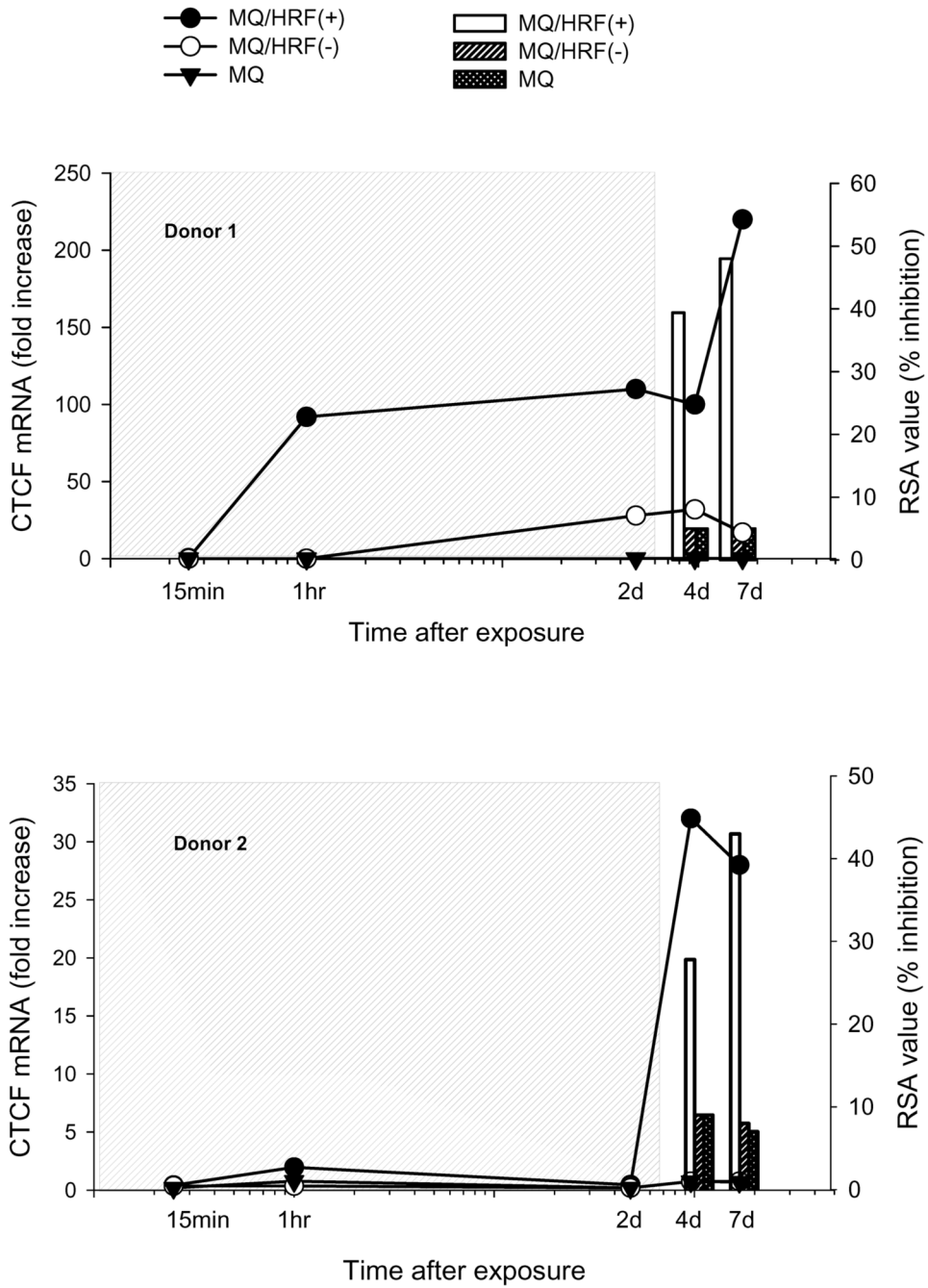
Fig. 1. Elevated expression of CTCF mRNA in primary human macrophages exposed to soluble products of HRF+ coincides with the induction of antiviral resistance
Expression of CTCF mRNA (linear plot, left *y* axis*)* was analyzed by real-time PCR; each point of the linear plot represents a mean value of three measurements. The antiviral activity of cell culture supernatants (bars right *y* axis) was tested by RSA. Grey boxes show continuous exposure of target cells to treatments; RSA values show percent inhibition of HIV-1 LTR-promoted expression of luciferase gene by 1%/Vol of the sample. MQ denotes macrophages.
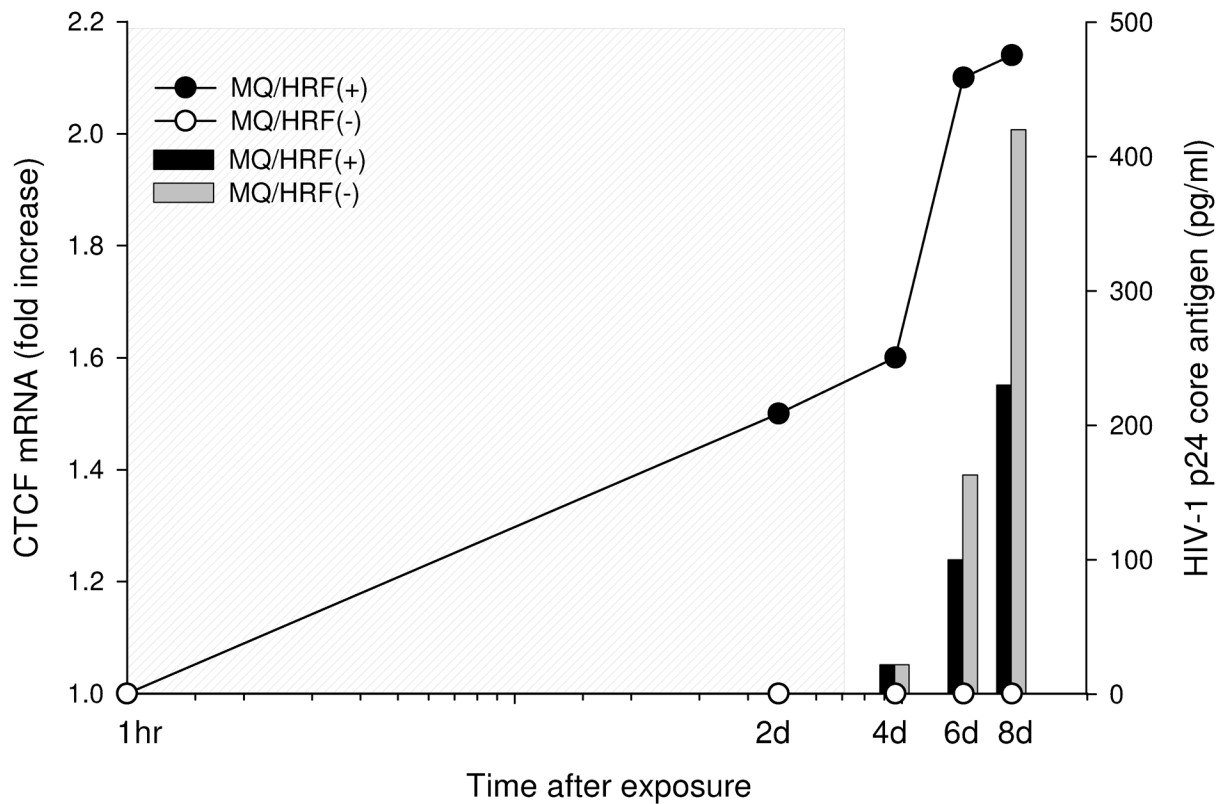
Fig. 2. Replication of R5 HIV-1 ADA is inhibited in human macrophages after their exposure to soluble products of HRF(+) cells

Expression of CTCF mRNA (linear plot, left *y axis)* was analyzed by real-time PCR; each point of the linear plot represents a mean value of three measurements. HRF-treated macrophages were challenged with HIV-1 after termination of treatment. Replication of HIV-1 was analyzed by p24 Elisa (bars, right *y* axis) at one, three and five days after infection which corresponded to four, six and eight days from the initial exposure to HRF treatment. Grey box shows continuous exposure of target cells to treatments;
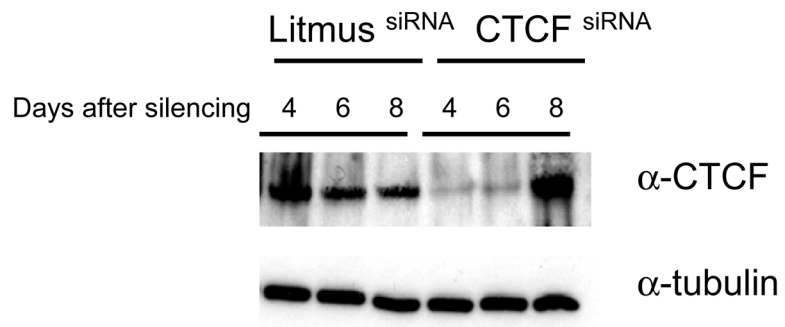
Fig. 3. The siRNA silencing of CTCF gene
HRF(+) cells were subjected to treatments by 9 nM of CTCF or control litmus siRNA fragments and effects of this interference with CTCF gene expression were assayed over time by Western blot using antibodies to CTCF and α-tubulin. Data presented in this figure show one of the three separate experiments.
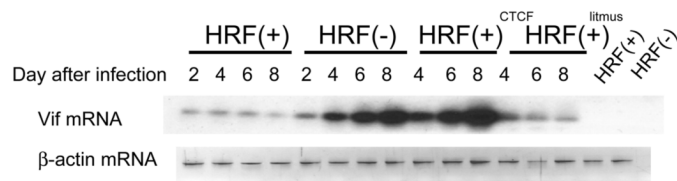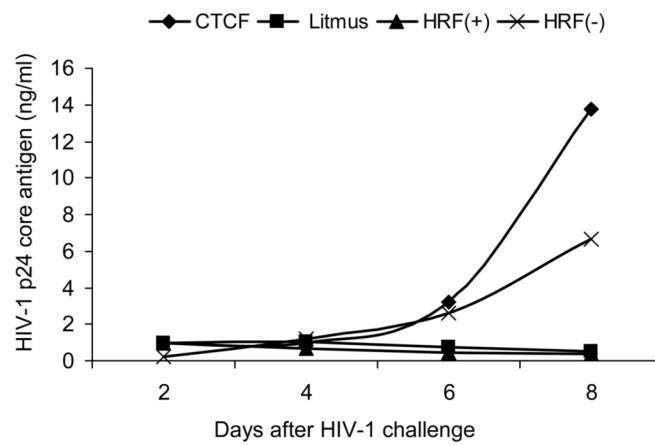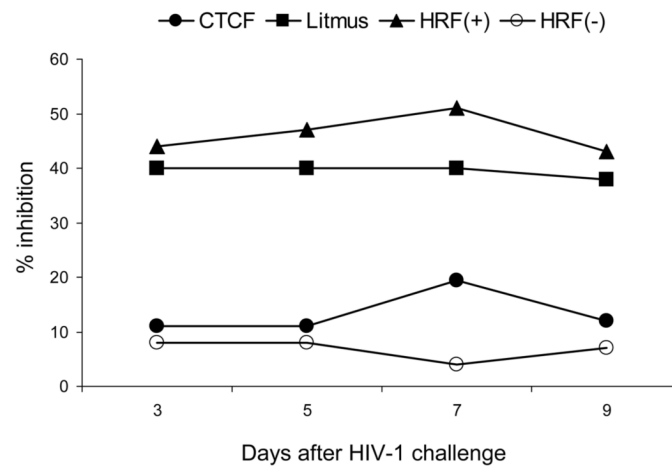
A

B

C

Fig. 4. Transient interference with the expression of CTCF mRNA reverses the HIV-1 resistant phenotype in HRF(+) cells

HRF(+) and HRF(-) cells were transfected by CTCF or control Litmus siRNA fragments then challenged with NL4-3 HIV-1 at concentration of 0.5pg/cell. Replication of HIV-1 was tested by RT-PCR of the single-spliced *vif* transcript (A); detection of the p24 core antigen (B); biological activity of cell culture supernatants was tested by RSA (C). Data presented in this figure show one of the three separate experiments.
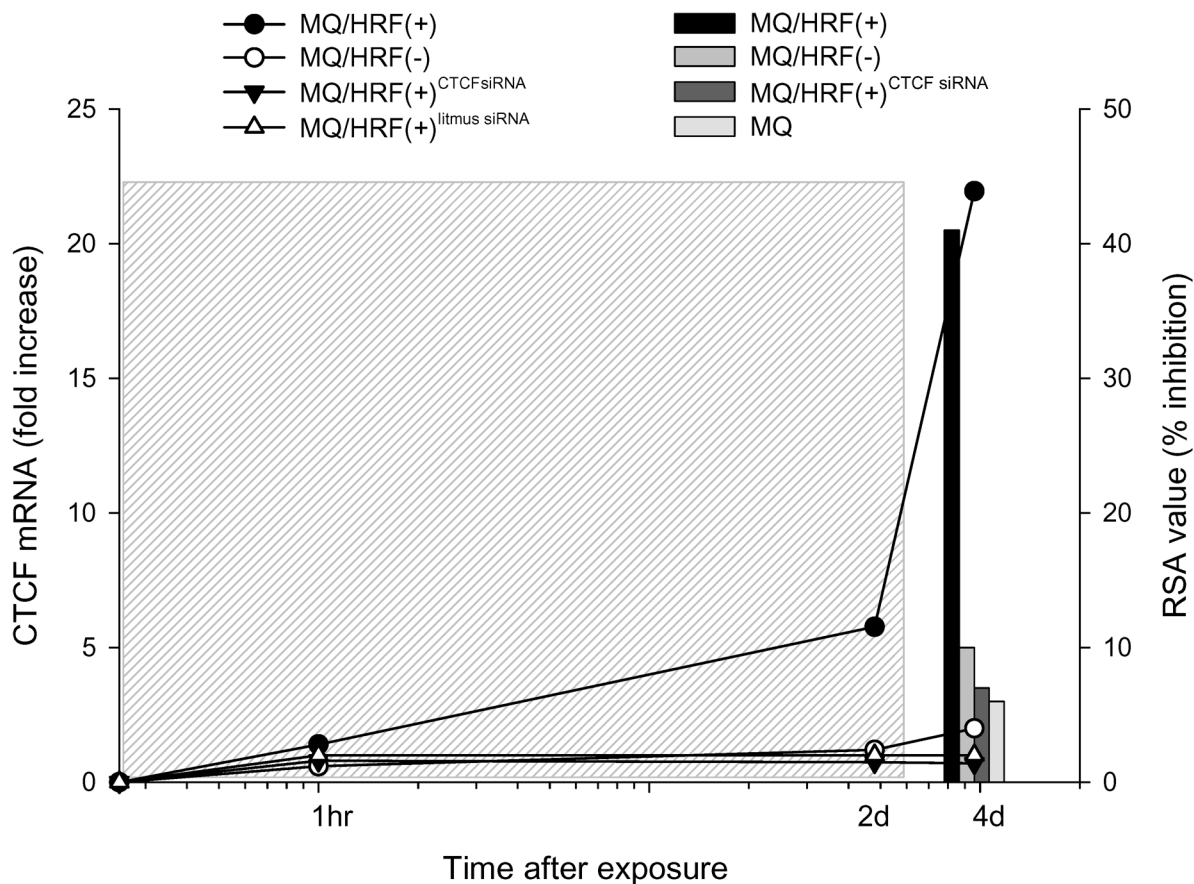
Fig. 5. Transient interference with the expression of CTCF mRNA in HRF(+) cells inactivates the biological potential of their cell culture supernatant to induce the antiviral activity in target cells Expression of CTCF mRNA (linear plot, left *y* axis*)* was analyzed by real-time PCR, and the antiviral activity of cell culture supernatants (bars, right *y* axis) was tested by RSA. Grey box shows continuous exposure of target cells to treatments; RSA values show percent inhibition of HIV-1 LTR-promoted expression of luciferase gene by 1%/Vol of the sample and the each point of the linear plot represents a mean value of three measurements.
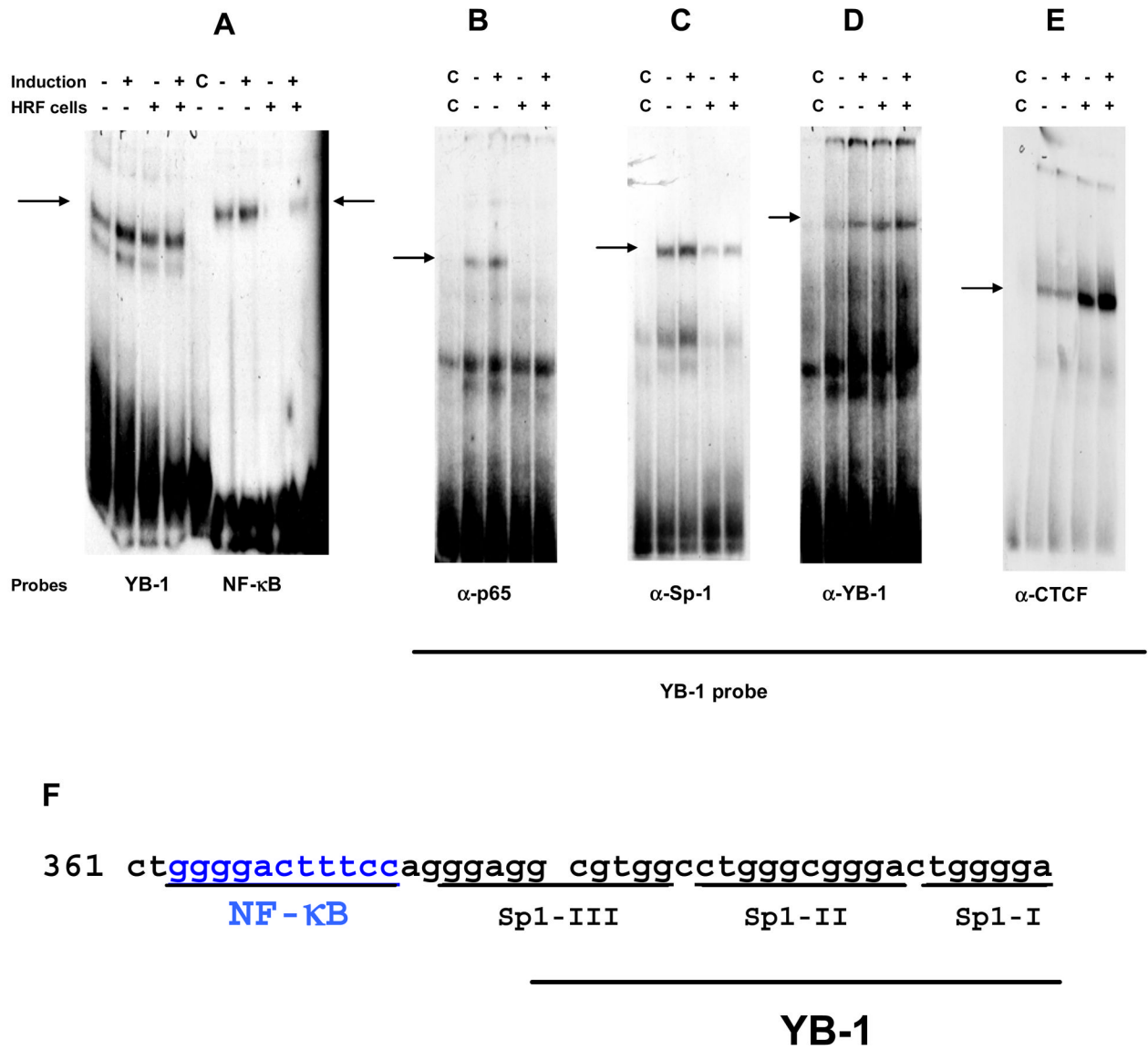
A

Induction - + - + C - + - +
HRF cells - - + + - - - + +

B C - + - +
       C - - + +

C C - + - +
       C - - + +

D C - + - +
       C - - + +

E C - + - +
       C - - + +

Probes   YB-1   NF-κB

α-p65   α-Sp-1   α-YB-1   α-CTCF

YB-1 probe

F

```
361 ctggggactttccagggagg cgtggcctgggcgggactggggga
         NF-κB            Sp1-III      Sp1-II      Sp1-I
```

YB-1

Fig. 6. EMSA analysis of the nuclear fractions from HRF(+) and HRF(-) cells upon HIV-1 NL4-3 induction

The nuclear translocation of transcription factors was initiated by 30 min exposure to HIV-1 at concentration of 0.1pg p24/cell. (A) The nuclear translocation of proteins was tested by formation of protein/DNA complexes to YB-1 or to consensus NF-κB probes; (B through E) Protein complexes bound to the YB-1 probe were detected by antibodies raised to p65-NF-κB, Sp-1, YB-1 and CTCF proteins; [C] annotation in the gels indicates that competing unlabeled nucleotide was added into the reaction]; (F) The YB-1 probe enclosing cognate sequences for NF-κB (363–373); Sp1-III (376–385); Sp1-II (387–396); Sp1-I (398–402); YB-1 (381–402) was designed based on the HIV-1 HXB2 promoter sequence; Data presented in this figure show one of the three separate experiments.
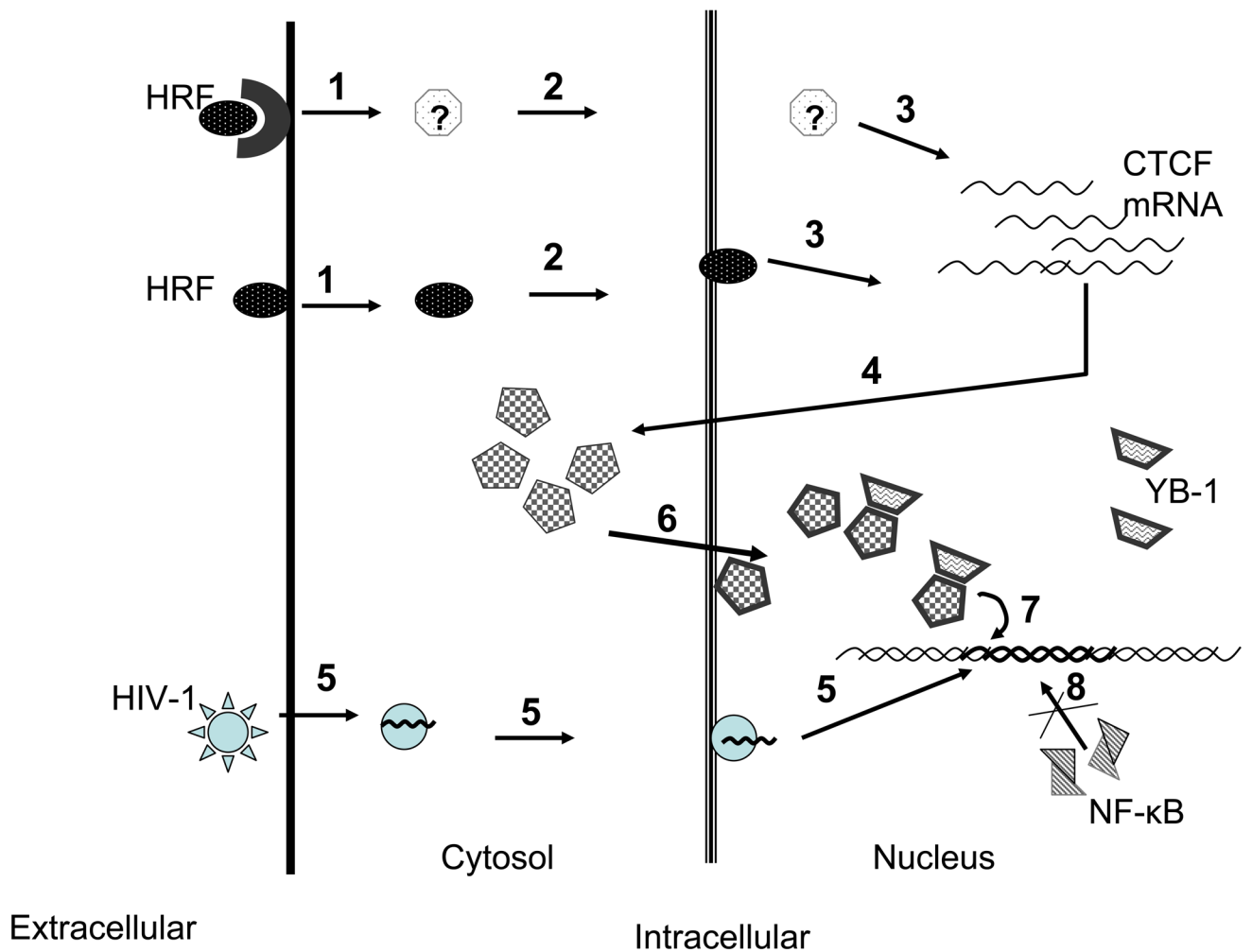
Fig. 7. The hypothetical scenario for HRF-mediated antiviral activity in HIV-1 susceptible CD4$^+$ T cells

Through the ligation of cell surface receptor and activation of the intracellular effector or through its intracellular uptake HRF induces transcription of CTCF mRNA followed by the increased protein levels (1–4). Upon virus invasion (5) CTCF protein is relocated to the nucleus where it binds to HIV-1 LTR possibly through its interaction with YB-1 protein (6–7) and inhibits transcription of HIV-1 through direct interaction with LTR; this CTCF/LTR complex blocks binding of NF-κB to its cognate sequence on HIV-1 promoter (8).

Table 1

Correlation between the levels of CTCF mRNA expression and the levels of antiviral protection in several HRF(+) clones.

Analysis	HRF(-)	HRF(+)$_5$	HRF(+)$_{15}$	HRF(+)$_{28}$	HRF(+)$_{22}$
CTCF mRNA (copy/cell)	2.28	5.56	7.4	8.1	12.8
CTCF mRNA (fold increase)	1	2.4	3.25	3.55	5.6
HIV-1 replication (% IF+)	72	14.3	5.8	2.9	1.6

Table 2

Exposure to soluble products of HRF+ cell line induces expression of CTCF mRNA in target cells.

Analysis	HRF(-)/HRF(+) day 1	HRF(-)/HRF(+) day2	HRF(-)/HRF(+) day 3	HRF(+)28	HRF(-)
CTCF mRNA (copy/cell)	2.1	3.4	7.4	9.8	2.2
CTCF mRNA (fold increase)	0.95	1.54	3.36	4.45	1

Table 3

Comparison of CTCF mRNA levels in cells subjected to treatment with CTCF or control litmus siRNA fragments.

Analysis Day 2	CTCF mRNA copy/cell		
	Day 4	Day 6	Day 8
HRF(+) CTCF siRNA	2.4	4.5	6.2	8.6
HRF(+) litmus siRNA	8.3	13	12	12
HRF(+)	13	12.8	12.5	13