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The kinetics of HIV-1 long terminal repeat transcriptional activation resemble those of hsp70 promoter in heat-shock treated HeLa cells

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Abstract

The long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1) is activated under different conditions including heat shock. By using transient transfection assays, we have compared the thermal activation of HIV-1 LTR to that of the promoter of the gene encoding the human stress protein hsp70 which is under the control of the heat shock transcription factor HSF. In these assays, the chloramphenicol acetyl transferase (Cat) gene was used as a reporter gene. Several parameters of the heat stress were analyzed such as the temperature, the duration of heat stress and that of the recovery period. Under every condition tested, we have found that the kinetics of activation of both promoters were very similar. In addition, both showed a similar inhibition by actinomycin D. These results were compared to those obtained with a DNA construct containing the early promoter of SV-40 virus coupled to the Cat gene. In this case, no heat-mediated accumulation of CAT protein was observed, indicating that the transcriptional activation of HIV-1 LTR by heat shock is specific. HIV-1 LTR contains two NF-κB binding elements, involved in the activation of this promoter during oxidative stress, which are sequence related to the heat shock element HSE. However, under all the heat shock conditions tested, we have been unable to detect the binding of any protein to κB elements, suggesting that this site is not directly involved in the thermal activation of HIV-1 LTR. These results indicate that the thermal transcriptional activation of HIV-1 LTR and hsp70 promoters occurs through different mechanisms that are triggered by similar heat shock conditions.

Key words: Heat shock; HIV-1 LTR; NF-κB

1. Introduction

HIV-1 infection is characterized by a prolonged period of clinical latency during which the level of viral burden and virus replication is very low in blood cells but active in lymphoid organs [1,2]. Development of symptoms leading to AIDS occurs through the reactivation of viral expression [3]. As an approach to unravel the molecular mechanisms leading to this phenomenon, different agents have been studied that appear to up-regulate viral expression in infected cells. These include phorbol esters, granulocyte-macrophage colony stimulating factor, interleukin-6, inflammatory cytokines, phytohemagglutinins, protein kinase inhibitors and oxidative agents [4–11]. In addition, stresses such as UV irradiation [12] and heat shock [13,14] also activate HIV-1. Consequently, heat shock is sometime used to enhance the detection of the virus in routine identification of clinical specimens [13].

The activation of HIV-1 occurs through the stimulation of proviral transcription. This event is regulated by a complex eukaryotic promoter localized in the LTR that contains two NF-κB binding elements, three constitutive SP-1 sites and other regulatory sequences (reviewed in [15,16]). In the case of oxidative stress and tumor necrosis factor (TNFα) treatment, a common mechanism of activation involving reactive oxygen intermediates and the transcription factor NF-κB has been described [10]. In contrast, the induction by protein kinase inhibitors seems to occur independently of the binding of any transcription factor to the LTR [9]. The mechanism regulating the thermal activation of the virus is unknown. In this respect, it is interesting to note that the sequence of the NF-κB binding site (GGGACTTTC [17] is related to the consensus heat shock element (HSE) which consists of GAA and TTC blocks, arranged in alterning orientations at two nucleotides intervals [18]. Consequently, it has been hypothesized that either the heat shock transcription factor HSF regulates HIV-1 transcription through its binding to κB sites or the heat shock treatment activates NF-κB binding to DNA [14].

We report here, by using transient transfection assays and different conditions of heat shock treatment, that the kinetics of thermal transcriptional activation of HIV-1 LTR resemble those of the promoter of the gene encoding the human heat shock protein hsp70. However, in all the conditions tested, we are unable to detect a protein that specifically binds to κB elements. This suggests a complex mechanism of activation of HIV-1 LTR promoter during heat shock.

2. Materials and methods

2.1. Reagents and plasmids

Actinomycin D was from Sigma (St. Louis, MO). pLTR-Cat plasmid which contains the chloramphenicol acetyltransferase (Cat) gene under the control of the LTR of HIV-1 is a derivative from HXB2-gpt plasmid

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carrying a HIV-1 provirus genome [19,20] and from pSV2-Cat plasmid [21]. In pSV2-Cat plasmid, the chloramphenicol acetyltransferase gene was under the control of the constitutive early promoter of SV40 virus. The LTR region of the viral genome used encompasses nucleotides 8,475 to 9,197, according to the nucleotide sequence given by Ratner et al. [22]. p17-Cat neo plasmid contains the Cat gene under the control of the human hsp70 promoter. It is a derivative of pLTR-Cat and p17-hGH neo plasmids [23]; a 15 kb *Xba*I–*Bam*HI fragment from pLTR-Cat carrying the Cat gene was inserted, under the control of hsp70 promoter, in p17-hGH neo, instead of the human growth hormone gene. pCMVβ plasmid contains the gene encoding β-galactosidase under the control of the cytomegalovirus promoter (Clontech, Palo Alto).

2.2. Cell cultures

HeLa cells were grown at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum and in presence of 5% CO_2. Before heat shock, cells were incubated in DMEM supplemented with 5% fetal calf serum and 25 mM HEPES, pH 7.4.

2.3. Transient transfection assays

One day before transfection, exponentially growing HeLa cells were plated at a density of 1.5×10^6 cells/78 cm^2. They were then transfected with the Transfectam reagent (Promega, France). In brief, cells were gently washed with the culture medium devoid of serum; this was followed by an incubation in 2 ml of the same medium. 8 μg of pLTR-Cat, p17-Cat neo or pSV2-Cat plasmids were added to 50 μl of 150 mM NaCl and homogenized by vortexing (solution A). 24 μl of Transfectam reagent were similarly homogenized in 50 μl of a medium containing 150 mM NaCl (solution B). Solutions A and B were mixed and, 10 min later, 900 μl of serum free DMEM were added. After 10 min incubation at room temperature, the mixture was added to the 2 ml culture medium and left in contact with the cells overnight. The day after transfection, cells were trypsinized and redistributed into 60 mm culture dishes. 24 hours later, they were submitted to various heat stresses. Efficiency of transfection was estimated in parallel experiments using pCMVβ plasmid.

2.4. CAT and β-Gal assays

Twenty-four hours after the various heat stresses, transfected cells were harvested, lyzed and 50 μg of total cellular proteins were analyzed by the Boehringer CAT ELISA test, according to the manufacturer's instructions. The percentage of cells expressing β-Gal was monitored by 5-bromo-chloro-3-indolyl β-D-galactosidase staining [24].

2.5. Electrophoretic mobility shift assays

Double strand-oligonucleotides used to detect the DNA-binding activities of either NF-κB or HSF were as previously described [25,26]. Extraction of DNA-binding proteins and binding conditions were performed as described by Andrew and Faller [27]. The reaction (10 μl) contained 10 μg protein from nuclear extracts, 4 μg poly(dI-dC) (Pharmacia), 20,000 cpm (Cerenkov) ^{32}P-labeled HSE or κB DNA probe and 1 μl 10 × BB buffer (50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 5 mM EDTA, 250 mM NaCl and 10% Ficoll 400). Reaction was for 15 min at room temperature following the addition of the different ^{32}P-labeled DNA probes. Native 4% acrylamide gels were used to analyze the samples. Autoradiographs of the gels were recorded onto X-Omat AR films (Eastman Kodak Co).

3. Results

3.1. The kinetics of thermal activation of HIV-1 LTR resemble those of the human hsp70 promoter

We have compared the heat-mediated activation of HIV-1 LTR to that of the human hsp70 promoter. This was assessed by analyzing the kinetics of thermal activation of pLTR-Cat and p17-Cat-neo plasmids following their transient transfection in HeLa cells (see section 2). In these experiments, the efficiency of transfection varied between 40 and 50%. Fig. 1A shows the pattern of thermal activation of pLTR-Cat plasmid by 90 min heat shock treatments performed at different temperatures. The level of CAT produced was analyzed 24 h later. As seen in this figure, a gradual increased accumulation of CAT was observed until the temperature of 43°C. Following a 44°C heat shock treatment, the level of CAT produced was drastically reduced. When the same experiment was performed with p17-Cat-neo plasmid, we observed a rather similar pattern of activation of the hsp70 heat shock promoter (Fig. 1B). However, in this case, CAT was still produced following exposure to 44°C. Next, we have analyzed the effects of the duration of the heat shock treatment on the kinetics of activation of both promoters (Fig. 2A,B). At the temperature of 43°C, both promoters showed a maximal activation after a 90 min heat stress. Similar kinetics of activation were also observed following longer heat stresses. We also investigated the effect of the duration of the heat shock recovery period. This was assessed by exposing HeLa cells to 43°C during 90 min and allowing them to recover for

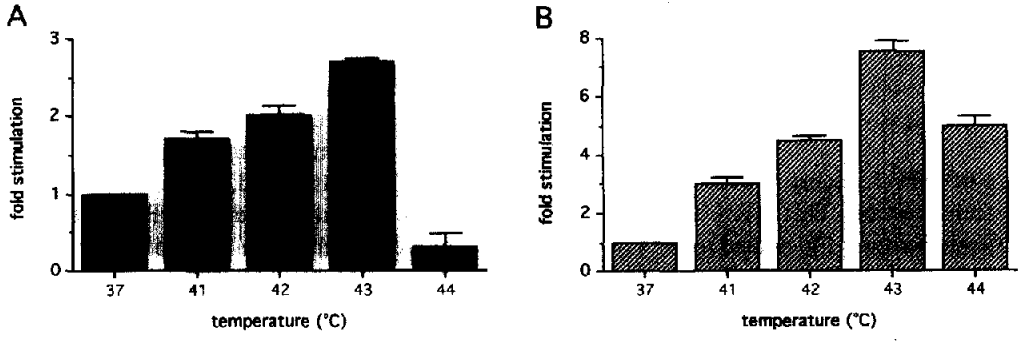

Fig. 1. Kinetics of activation of HIV-1 LTR and hsp70 promoter by heat shock. HeLa cells transiently transfected with either pLTR-Cat or p17-Cat neo plasmids were submitted to various heat stresses. Transfected HeLa cells were heat shock treated for 90 min at either 41, 42, 43 or 44°C and allowed to recover for 24 h before being analyzed. Control cells were left untreated. The level of cytoplasmic CAT enzyme was quantified by ELISA test as described in section 2. The degree of activation was calculated by dividing the CAT concentration of the different samples by the CAT concentration of the standard non stressed cells. The histograms shown are representative of five identical experiments, standard deviations are presented (n = 3). (A) HIV-1 LTR, black plots and (B) hsp70 promoter, hatched plots.

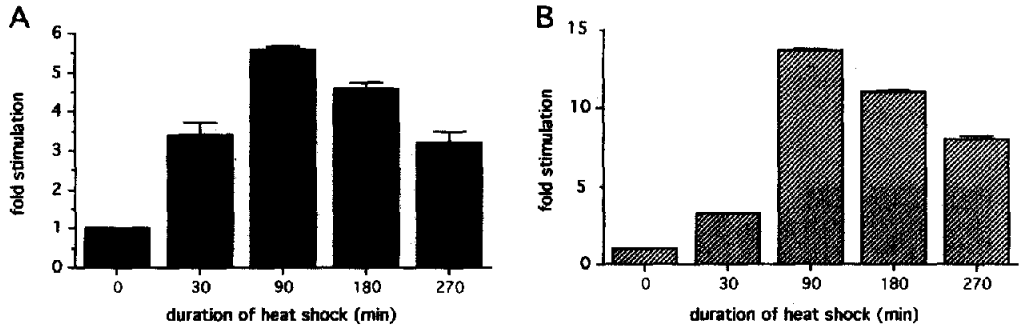

Fig. 2. Effect of the duration of the heat shock treatment on HIV-1 LTR and hsp70 promoter activation. HeLa cells transfected with LTR-Cat or p17-Cat-neo plasmids were incubated at 43°C during either 30, 90, 180 or 270 min and allowed to recover for 24 h before being analyzed. Control cells were left untreated. Determination of CAT concentration and presentation of the results are as in Fig. 1. (A) HIV-1 LTR, black plots and (B) hsp70 promoter, hatched plots.

1, 3, 24 and 48 h at 37°C before being analyzed. In both cases, we observed that the overall patterns of CAT accumulation were similar and that the maximal accumulation of this reporter protein occurred after 24 h of heat shock recovery (Fig. 3A,B). Taken together, these results indicate that the kinetics of thermal activation of HIV LTR, though not identical, resemble those of the hsp70 promoter.

As seen in Fig. 4A, the heat-mediated production of CAT driven by the LTR-Cat plasmid was abolished when cells were incubated with 0.5 μg/ml actinomycine D added 10 min prior to heat stresses performed at different temperatures. A similar observation was made when the p17-Cat-neo plasmid was used (Fig. 4B). In these experiments, the analysis of the level of CAT produced was performed 3 h after the heat stress to minimize the cytotoxic effect induced by actinomycin D in heat-treated cells. Control experiments were also performed by using a plasmid containing the Cat gene under the control of a constitutive promoter, such as the early promoter of SV40 virus, which is devoid of HSE or kB sites. This was assessed by performing transient transfection of HeLa cells with pSV2-Cat plasmid. Transfected HeLa cells were then exposed to 90 min heat stresses performed at different temperatures and the level of CAT produced was analyzed 24 h later. As seen in Fig. 5, in this case, a decreased level of CAT correlated with the increased temperature of the heat stress. A similar observation was made when the duration of the heat stress or that of the recovery period were investigated (not shown). Thus, these observations suggest that the production of CAT driven by HIV-1 LTR in heat shock-treated HeLa cells is specific and transcriptionally regulated.

3.2. *The thermal activation of HIV-1 LTR does not occur through the binding of a protein factor to LTR κB elements*

We have analyzed whether the activation of HIV-1 LTR observed in different conditions of heat shock treatment correlated with the binding of a protein factor to κB elements. This was analyzed in HeLa cells either left untreated or exposed to 43°C for 90, 180, 270 and 360 min. Nuclear extracts were prepared and electrophoretic mobility shift assays were performed using DNA probes encompassing either HSE or κB motifs (see section 2). As seen in Fig. 6A, a 90 min heat shock treatment performed at 43°C efficiently induced the binding of HSF

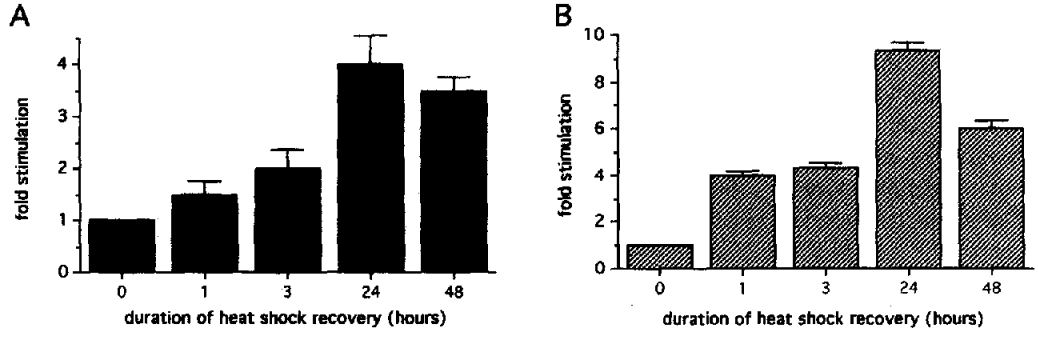

Fig. 3. Analysis of HIV-1 LTR and hsp70 promoter activation during heat shock recovery. HeLa cells transfected with pLTR-Cat or p17-Cat-neo plasmids were exposed to 43°C during 90 min and allowed to recover for either 0, 1, 3, 24 or 48 h at normal temperature before being analyzed. Control cells were left untreated. Determination of CAT concentration and presentation of the results are as in Fig. 1. (A) HIV-1 LTR, black plots and (B) hsp70 promoter, hatched plots.

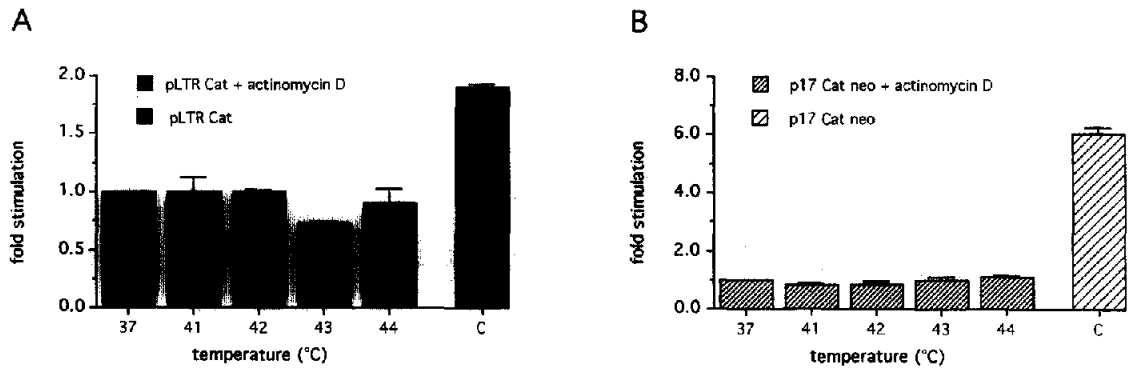

Fig. 4. Effect of Actinomycin D on HIV-1 LTR and hsp70 promoter activation by heat shock. HeLa cells transiently transfected with either pLTR-Cat (A) or p17-Cat-neo (B) plasmids were incubated for 10 min with 0.5 μg/ml actinomycin D before being exposed 90 min at either 41, 42, 43 or 44°C. After 3 h recovery at 37°C, the level of CAT accumulation was determined as described in section 2. C: controls, activation of either LTR-Cat or p17-Cat-neo by 43°C heat shock treatment performed during 90 min in the absence of actinomycin D and analyzed 3 h later. The histograms shown are representative of five identical experiments, standard deviations are presented ($n = 3$). In this experiment, the level of CAT was analyzed already as 3 h (instead of 24 h) after the heat stress. This was done to minimize the inhibitory effect of actinomycin D on the recovery of normal cellular functions after the heat stress.

to HSE. In contrast, following heat shock, no protein interacted with the κB element (Fig. 6B). A similar result was observed even after longer exposure of the cells to 43°C (Fig. 6B) or following treatments performed at other temperatures (not shown). In contrast, a 2 h treatment of HeLa cells with 250 μM hydrogen peroxide induced the binding of a protein factor, probably NF-κB, to the κB motif (Fig. 6C, see also [10]). These results suggest that the activation of HIV-1 by heat shock does not occur through the binding of a specific factor to the κB motifs of the LTR.

4. Discussion

Toward a better understanding of the molecular mechanisms that regulate the heat-mediated activation of HIV-1 LTR, we have performed kinetic analysis of this phenomenon in HeLa cells. In addition, we have compared the LTR activation to that of the promoter of the major human heat shock protein hsp70. Different parameters of the heat shock response were analyzed, such as the temperature, the duration of the heat stress or that of the recovery period. Under every condition tested, the overall kinetics of heat-induced CAT accumulation in LTR-Cat transfected cells resembled those observed in cells transfected with a plasmid containing the Cat gene under the control of the hsp70 promoter. In particular, the conditions of maximal activation (90 min at 43°C) were similar. The slight differences detected in the pattern of expression of the two promoters were not greater than those observed between different heat shock genes [29]. In cells transfected with the same Cat gene under the control of the heterologous SV40 early promoter, which contains no HSE or kB sites, no heat-mediated accumulation of CAT was observed. Thus, the heat-mediated production of Cat mRNA is specific of the presence of the LTR or hsp70 promoter 5′ of the Cat gene. This excludes the activation or synthesis of a heat-inducible factor which could affect the steady-state levels of Cat mRNA by altering its half-life. Moreover, experiments performed with actinomycin D confirmed that the heat-mediated production of CAT driven by HIV-1 LTR was transcriptionally regulated. Hence, these observations suggest closely related mechanisms leading to the thermal activation of heat shock and LTR promoters that depend on the physiology of the heat shock treated cells.

Except for HIV-1 LTR, no other eukaryotic promoter, devoid of HSE elements, has been shown to be strongly activated by heat shock [28,29]. Most cellular and viral genes usually display a reduced or unaltered rate of tran-

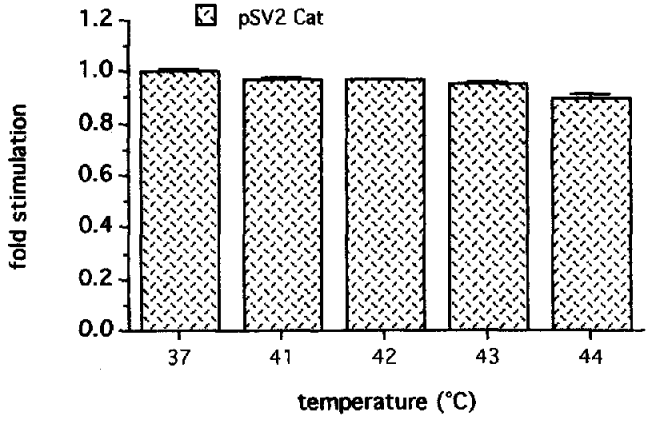

Fig. 5. Analysis of the expression of a SV-40 early promoter-Cat construct in heat shock-treated HeLa cells. HeLa cells transiently transfected with pSV2-Cat plasmid were exposed 90 min at either 41, 42, 43 or 44°C. After 24 h recovery at 37°C, the level of CAT was determined as described in section 2. Determination of CAT concentration and presentation of the results are as in Fig. 1. The histograms shown are representative of five identical experiments, standard deviations are presented ($n = 3$). Note the lack of activation of pSV2-Cat plasmid by heat shock.

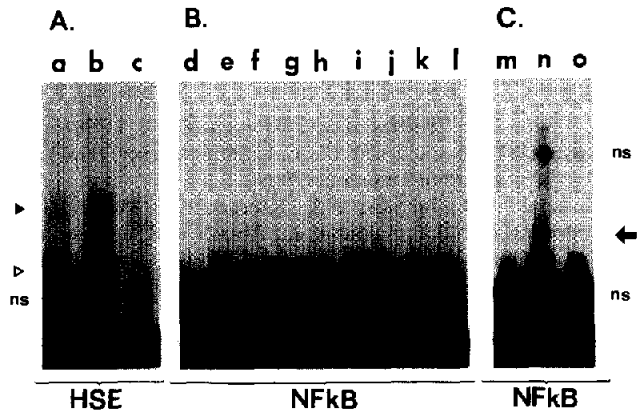

Fig. 6. Activation of HIV-1 LTR by heat shock does not implicate the binding of a protein factor to κB element. (A) HeLa cells were either left untreated (lane a) or exposed to 43°C for 90 min (lanes b and c). Nuclear extracts were prepared and equal proportions (10 μg of proteins) were incubated with a ^{32}P-labeled DNA probe encompassing the HSE motif. Samples were analyzed on native 4% polyacrylamide gel. A fluorogram of the gel is presented. Heat shock was sufficient to induce the binding of HSF to HSE motif (lane b) and the specificity of the complex is shown by competition reaction performed with 200 ng of the unlabeled HSE motif (lane c). ns, non specific DNA-binding complex; open arrow-head, constitutive HSE binding activity; black arrow-head, induced HSF binding. (B) HeLa cells were exposed to 43°C during either 90 (lane e), 180 (lane f), 270 (lane g), or 360 min (lane h), or left untreated (lane d). The nuclear extracts were incubated with a ^{32}P-labeled DNA probe encompassing κB motif and analyzed as above. The hypothetic binding of a transcription factor (lane e) was competed with respectively 5, 100, 200 and 300 ng of the same unlabeled probe (lanes i, j, k and l, respectively). Note that the lack of protein binding to the κB motif after heat shock. (C) HeLa cells were left untreated (lane m) or treated with 250 μM of hydrogen peroxide for 2 h (lanes n and o). Nuclear extracts were incubated with the κB motif as described previously. Lane n is a positive control showing the binding of a protein factor (probably NF-κB) to the κB motif (black arrow); the specificity of the binding is shown by competition reaction with 200 ng of the unlabeled κB motif (lane o). Non-specific (ns) and specific (black arrow) DNA-binding complexes are shown.

scription during heat shock [28,29]. Hence, as an approach to the molecular mechanisms regulating the thermal activation of HIV-1 LTR and also because of the sequence similarity between κB and HSE elements, we have investigated whether heat shock could induce the binding of a protein factor to the κB motif. Electrophoretic mobility shift assays demonstrated that no protein interacted with κB in extracts of heat shock-treated HeLa cells. This absence of binding was observed in all the heat shock conditions tested, including those that induced maximal LTR activation. This confirms a preceding report showing no specific protein binding to κB motifs in Jurkat cells treated for one hour at 42°C [10]. However, this contrasts with a deletion analysis of HIV-1 LTR κB sites that resulted in a reduced activation of this promoter in heat shock-treated U937 promonocytic cells [14]. An explanation for this descrepancy could be that other functional domains in HIV-1 LTR, perhaps in cooperation with NF-κB, are involved in the thermal activation of this promoter. In this respect, it is interesting to note that the LTR contains three binding sites for the constitutive SP-1 transcription factor that are proximal to κB sites, since a cooperation between NF-κB and SP-1 seems to be required for HIV-1 enhancer activation in response to mitogens [30]. Extensive mutational analysis of the LTR will help to define if the thermal activation of this promoter depends on specific sites or appears devoid of *cis*-acting sequences as previously shown in the case of the LTR activation by protein kinase inhibitors [9].

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