A NF-κB-Dependent Dual Promoter-Enhancer Initiates the Lipopolysaccharide-Mediated Transcriptional Activation of the Chicken Lysozyme in Macrophages

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Abstract

The transcriptional activation of the chicken lysozyme gene (cLys) by lipopolysaccharide (LPS) in macrophages is dependent on transcription of a LPS-inducible Non-Coding RNA (LINoCR) triggering eviction of the CCCTC-binding factor (CTCF) from a negative regulatory element upstream of the lysozyme transcription start site. LINoCR is transcribed from a promoter originally characterized as a hormone response enhancer in the oviduct. Herein, we report the characterization of this cis-regulatory element (CRE). In activated macrophages, a 60 bp region bound by NF-κB, AP1 and C/EBPβ controls this CRE, which is strictly dependent on NF-κB binding for its activity in luciferase assays. Moreover, the serine/threonine kinase IKKβ, known to be recruited by NF-κB to NF-κB-dependent genes, is found at the CRE and within the transcribing regions of both cLys and LINoCR. Such repartition suggests a simultaneous promoter and enhancer activity of this CRE, initiating cLys transcriptional activation and driving CTCF eviction. This recruitment was transient despite persistence of both cLys transcription and NF-κB binding to the CRE. Finally, comparing cLys with other LPS-inducible genes indicates that IKKβ detection within transcribing regions can be correlated with the presence of the elongating form of RNA polymerase II or concentrated in the 3’ end of the gene.

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Introduction

Genes transcription is controlled by CREs, which are, when activated, nucleosome free regions occupied by transcription factors and identified in vivo as DNase I hypersensitive sites (DHS) [1]. A classical view separates these elements into different categories depending on their position from the transcription start site (TSS) of genes, their sequence and their chromatin signature. However, global analyses of the transcriptome suggest that the function of these CREs is not commonly restricted to a single category, genomic regions with dual promoter and enhancer activities appearing to be widespread within the genome. For example, a recent study looking at transcription sites located outside protein-coding regions in macrophages activated by endotoxins, found 70% of extragenic RNA polymerase II (RNAPII) peaks associated with genomic regions with a chromatin signature of enhancers [2], these elements generating very low abundance non-coding transcripts, suggested to be “junk” RNA. However, the idea that “enhancer-associated” extragenic transcription would represent only noise has already been challenged by several studies. Extragenic transcripts within locus control regions (LCR), these distal regions composed of several CREs able to enhance the expression of linked genes to physiological levels in a tissue-specific and copy number-dependent manner, have been identified some time ago and are believed to play a role in the chromatin remodelling observed over these regions [3–5]. More recently, the link between non-coding RNA transcription from dual promoter/enhancer elements and chromatin remodelling has been established for two chicken genes min-1 and cLys [6,7]. However these studies did not determine if these CREs are behaving simultaneously or successively as promoter and enhancer. cLys is a marker of macrophage differentiation, which rapidly responds to pro-inflammatory agents like LPS and its expression is controlled by three enhancer elements situated −6.1 kb, −3.9 kb and −2.7 kb upstream of the transcription start site, a complex promoter and a silencer element at −2.4 kb (figure 1A) [8]. We have reported that cLys expression activation was preceded by the transient transcription of LINoCR from a promoter −1.9 kb upstream of cLys TSS, this transcription being necessary for nucleosome reorganisation and eviction of the enhancer blocker protein CTCF from the silencer element [7]. Interestingly, this promoter was originally identified as a hormone response enhancer element functional in the oviduct and bound by estrogen, glucocorticoid and progesterone receptors [9,10]. However, we did not fully establish that this −1.9 kb element was driving any enhancer activity in macrophages.

In these cells, activation of the −1.9 kb element and subsequent transcription of LINoCR was correlated with accumulation of the protein kinase IKKβ and histone H3 serine 10 phosphorylation (H3S10p) within LINoCR transcribed region [7]. IKKβ is part of
the IKK complex controlling the release of the NF-κB transcription factor into the nucleus to stimulate transcription of its target genes in response to pro-inflammatory stimuli. In the nucleus, NF-κB interacts with several chromatin modifiers including the histone H3K4 methyltransferase Set7/9 [11], CBP/p300 [12], TIP60 [13,14] and also IKKα for which specific chromatin modifying activity has been described [15–17]. At the promoter of NF-κB-dependent genes, IKKα phosphorylation of H3S10 is important to initiate transcription elongation [15,16,18]. Furthermore, IKKα binds to the phosphorylated RNA polymerase II C-Terminal Domain (RNAPII CTD) to target HP1α, in cooperation with AP1, C/EBPβ and NF-κB (p65) transcription factors after LPS treatment, the latter providing a rationale for IKKα detection within the coding region of LINοCR [7]. These transcription factors act cooperatively to fully activate this CRE. Additional luciferase reporter assays and in vivo chromatin immunoprecipitation analyses reveal that this 60 bp transcription factor cluster possesses concomitant promoter and enhancer activities. In addition, in contrast with what we have described for other NF-κB-dependent genes [17], IKKα is transiently recruited to both LINοCR and cLys transcribed regions. This loss of IKKα concomitant with LINοCR transcriptional inactivation is the only change identified at the -21.9 kb CRE, for which the chromatin structure and the transcription factors occupancy are still identical during or after LINοCR transcription. This result suggests an important role of IKKα to mediate the -21.9 kb CRE promoter activity and that the -1.9 kb CRE is important to initiate transcription of cLys but not to maintain this expression after CTCF eviction from the -2.4 kb silencer. This observation is

Figure 1. Rapid nucleosome loss at the -1.9 kb CRE in macrophages after LPS treatment. (a) Illustration of the chicken lysozyme locus including cis-regulatory elements and the approximate location of LINοCR transcript, grey arrow and cLys mRNA, black arrow [7]. (b) ChIP assay performed with anti-histone H3 antibody at the -1.9 kb element compared to the -6.1 kb enhancer after LPS treatment in macrophages. Horizontal axis represents time after LPS induction in minutes. Data are normalised versus input and then versus a positive control region. Data are representative of at least three independent experiments.

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linked with HP1γ poor incorporation into transcribing chromatin at cLys locus. These data may provide a paradigm for the modus operandi of CREs with dual promoter/enhancer activity and reinforce the idea that HP1γ controls IKKα associated transcriptions.

**Materials and Methods**

**Cell Culture**
The chicken cell lines monocytes HD11 [26] and erythroblasts HD37 [27] and the mouse cell line RAW264.7 were grown in Dulbecco’s modified Eagle’s medium as previously described [17,28]. Mouse primary macrophages were obtained from bone marrow by culturing in Iscove’s Modified Dulbecco’s Medium containing 10% Folical Serum and Penicillin-Streptomycin and 10% L-cell conditionned medium containing M-CSF [29] for 7 days. Where indicated, cells were treated with 1 μg/ml LPS (Sigma).

**Chromatin Immunoprecipitation Assays and Real-Time PCR Analysis**

ChIP was performed exactly as previously described [7], using dynabeads protein G (Invitrogen) with 2.4 μg per 10 μl beads of anti-p65 (Santa Cruz sc-372X), anti-Histone H3 (Abcam ab1791), anti-HP1γ (Millipore 05–690), anti-IKKα (Santa Cruz sc-7606X), anti-RNAPII S2p (Abcam ab5095) and anti-RNAPII CTD (Abcam, ab817). The primers used for the Real-Time PCR are listed in Table S1.

**Nucleosome Mapping by Indirect End Labeling**

DNase I treatment of cells and naked DNA was performed as described previously [28]. MNase digestions of HD11 and indirect end labeling were performed using isolated nuclei as described previously [30]. With 10 μg of each, different DNA preparations digested with 20 U SphII (New England Biolabs) for 3 hr at 37°C and stopped with 5 μl loading dye 20% Ficoll (Sigma), 1% SDS (Sigma), and 0.05% bromophenol blue (Sigma). The probe abutting the SphII site (~3165 to ~2865 bp) was prepared by PCR using a plasmid containing the full sequence of the lysozyme locus as a template with the following primers: fwd, TACCTAGGAGGTTGTGTTGG and rev, GCCACCTGAAGATTTGGTT. The probe was gel purified using a QIAquick Gel Extraction Kit (QIAGEN). Bands were quantified from the images generated on the pharosFX molecular imager (Biorad).

**DNA In vivo Footprinting Analysis**

7.5 x 10⁷ HD11 cells were seeded in a 10 cm dish and incubated overnight before they were treated with LPS. In vivo footprinting has been performed as previously described [31] with the following primers: non-coding strand (A biotin-GGGTTAGTAATTGTTAATCTCA, B AGAAPGGCAACCTGACAGACATC, AC ACGACATCCAGCTAGGTGAAATCCTC and the respective annealing temperatures 53°C, 60°C, 66°C), coding strand (A biotin-CACCTCCCTGACCATAGCT, B GGAATTCCTGTCCCTTTGGATCAC, C TTGGATCAGGGCCCTGACCCAAAAAT, and the respective annealing temperatures 53°C, 60°C, 66°C), coding strand (A biotin-CCCTCAAGGTAACTGATGTT, B CAGAGGCAATCTGCAGTTAATCCTC, C CTTGGAATTTTTTCTCTCCGGCTGCA-CAGTT and the respective annealing temperatures 51°C, 54°C, 67°C), figure S1B (A biotin-ACCTGCTAGGATTTAATGT, B TGCAATTTCAACAAAGGACCACCTC, C AAAAGGC-GAAACCACAAGAGTGCTTTT and the respective annealing temperatures 51°C, 62°C, 67°C).

**Cloning, Mutagenesis and Transient Transfection**

DNA fragments carrying the lysozyme promoter (~376 to +17 bp) and the 1.9 kb element (~2132 to ~1877 bp) were cloned into the luciferase vector pXPG [32]. Mutants were generated by PCR amplification in the following 50 μl reaction mixtures: 1X Pfu buffer (Stratagene), pXPG-1.9AS [7] as a template, 125 ng of both forward and reverse primers, 0.25 mM dNTPs and 2.5 U Pfu Turbo (Stratagene). PCR amplification conditions were as follows: (1) denaturation at 95°C for 30 sec, (2) 16 cycles of denaturation at 95°C for 50 sec, annealing at 55°C for 50 sec and extension at 72°C for 7 min and (3) a final extension at 72°C for 7 min. Next 1 μl of 20 U/μl DpnI (NEB) was added directly to the PCR mix, incubated for 80 min at 37°C and then heat inactivated by incubation at 80°C for 20 min. Then 50 μl of stable 3 electro-competent bacteria (Invitrogen) were transformed, incubated and plated according to manufacturer recommendations. Transfection and luciferase assays were performed as previously described [7].

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay**

2 x 10⁷ HD11 cells, unstimulated or stimulated with 1 μg/ml of LPS for 1 hr and nuclear extracts prepared as previously described [33]. EMSAs were performed using end-labelled, double-stranded synthetic oligonucleotides. 2 μg of nuclear extracts was diluted in EMSA buffer [33]. The buffer-diluted samples then formed complexes with either 30 ng of unlabelled competitors or 1 μg specific antibodies, anti-α-FOS (Sc-253, Santa Cruz) and anti-C/EBPβ (Sc-61, Santa Cruz) during an incubation for 15 min at 25°C before the addition of 0.5 ng 32P γ labelled probe. After incubation with the probe for 15 min at 25°C, the samples were separated on a 5% acrylamide gel (37:5:1), 0.5 X TBE, 1/1000 TEMED and 0.1% APS. The gel was fixed, dried, exposed with a K-Screens (KODAK) for 16 hrs and analysed on pharosFX molecular imager (Biorad).

**Results**

**Identification of a 60 bp Region within the −1.9 kb CRE Occupied by Transcription Factors after LPS Treatment in Macrophages**

We have previously established that the eviction of the insulator-associated protein CTCF from its binding site and subsequent nucleosome movement over this site was dependent on transcription of LinoCR at the cLys locus in activated macrophages [7]. LinoCR is firing from a CRE located ~1.9 kb upstream of cLys TSS, this element being previously described as a hormone response enhancer in the oviduct [9,10] (figure 1a). These observations were making this CRE, one of the first characterized dual promoter/enhancer elements acting as an enhancer in macrophages. To determine, if this CRE could represent a paradigm of dual promoter/enhancer elements or if, in macrophages, it was only a promoter, we decided to undertake a more detailed characterization of this element. First, we looked at the nucleosome content in this region before and after LPS treatment in the chicken macrophage cell line HD11 compared to the −6.1 kb enhancer by chromatin immunoprecipitation (ChIP) using an antibody against total histone H3. We
NF-κB Occupies in vivo the −1.9 kb CRE and Recruits IKKα to both LINoCR and cLys Transcribing Regions Simultaneously

The in vivo DMS footprinting revealed the specific sites within the −1.9 kb element at which transcription factors were binding in response to LPS. The subsequent analysis of the sequence encompassing the footprints implied that NF-κB, C/EBP and AP1 were binding. This was confirmed by electrophoretic mobility shift assays (EMSA) (figures S2 and S3a–d). In these experiments, NF-κB appeared to bind only weakly to the −1.9 kb element. However, additional EMSA experiments with a 40 bp oligonucleotides encompassing both the proposed C/EBP and NF-κB sites showed a cooperative binding of both proteins to this CRE (figure S3e).

We have previously shown that the −1.9 kb CRE cloned immediately upstream of δ35 promoter in sense orientation was increasing this promoter LPS-dependent inducibility suggesting that this CRE was an enhancer in macrophages [7].

Having determined the location, identity and binding ability of transcription factors present at the −1.9 kb element in the activated HD11 cells, their individual contribution to promoter activity was assessed in transient transfection. An extensive set of constructs containing the individual or combination of binding inactivation mutations, revealed by EMSAs, were cloned into the pXPG luciferase reporter plasmid and transfected into HD11 cells (figure 4b). The mutation preventing NF-κB binding to the −1.9 kb element completely abolishes both basal and LPS inducible activity of this promoter. In addition, mutations of C/EBP, X or AP1 show similar impact on this promoter’s basal and LPS-inducible expression, expression being further reduced by double mutants C/EBP and AP1 or X and AP1. Taken together, these results show that NF-κB is essential for the promoter activity of the −1.9 kb element but does not act alone as each individual mutation has a significant impact on the promoter activity in the transient transfection assays.

We have shown previously that C/EBPβ and Fos were binding to the −1.9 kb element after LPS treatment in HD11 [7]. In this work, C/EBPβ and Fos were found enriched at the −1.9 kb element from 20 min post LPS treatment in agreement with experiments above describing a quick activation of this element in response to LPS. If this element is activated early after stimulation, we did not detect any change in δ35 basal mRNA level before 45 min post LPS. Because detectable changes in total mRNA level are delayed compared to transcriptional activation, we could not determine if the −1.9 kb element was first acting as a promoter and then as an enhancer after LINoCR expression was stopped or...
if this CRE could act simultaneously as promoter and enhancer. Using a transgenic mouse line harbouring the 21 kb cLys domain inserted into the HPRT locus [35], we first performed additional chromatin immunoprecipitation experiments looking at the NF-κB protein family member p65 in primary macrophages. As expected we detected enrichment for p65 in both cLys promoter and the 21.9 kb element after 30 min and 120 min of LPS treatment (figures 5a and S4c). Interestingly if p65 binding was stable after short-term and long-term LPS treatment, total RNAPII or elongating RNAPII (RNAPII S2p) occupancies were higher in cLys coding region (0.2 kb to 3.6 kb) after 30 min than after 120 min of LPS treatment (figures 5b, S4a and S4b). As expected, RNAPII was only detectable within LINoCR transcrib-
IKKα Recruitment to NF-κB-dependent Genes can Follow Different Kinetics

IKKα enrichment profile was not temporally and spatially comparable with our previous observations made for TNF, Ccl3 or Il1b other NF-κB-dependent genes [17]. At these genes, IKKα accumulates in the 3′ end, with 10 times more IKKα detected in 3′ compared to the promoter region, binds to chromatin in an HP1c-dependent manner and is still detectable after 2 h of LPS stimulation. We hypothesised that IKKα was not interacting with chromatin because of the poor incorporation of HP1c to transcribing chromatin within cLys locus. Indeed, we detected only 3 to 4 times HP1c enrichment in the coding region of cLys and no enrichment at all in LINoCR-transcribing region compared to a CTCF-binding site located upstream of the murine Il6 TSS where HP1γ was undetectable in macrophages (figures 5d and S4e). TNF, Ccl3 or Il1b respond to LPS with similar kinetics.

To complete our analysis, we chose two additional NF-κB-dependent genes with different temporal patterns of expression, BTG2 and IL10, for which expression peaks before or after 2 h of LPS treatment respectively (figure S5). ChIP experiments performed 30 min, 2 h and 4 h post LPS stimulation showed p65 binding to the promoter of both genes (figure 7a). Moreover, enrichment for HP1γ correlated with RNAPII S2p at these genes in agreement with what we observed for TNF and cLys (figures 7c, 7d and S6a), indicating that the amount of HP1γ detected within the coding regions of these genes shadows the rate of transcription. The analysis of IKKα recruitment to these loci unveiled a more heterogeneous association of this kinase with the different NF-κB-dependent genes studied. At the BTG2 promoter, the dynamics of p65 and IKKα occupancy mimicked our observation for the cLys promoter (figures 7b and 7c). At the 3′ end of the gene, IKKα enrichment was 4 fold higher than at the promoter whereas HP1γ was also 4 fold more associated with BTG2 than with cLys but 4 fold less than with TNF (figures 7b and 7c). In contrast, the

Figure 3. in vivo DMS footprinting of the −2000 to −2150 bp region, part of the −1.9 kb CRE, reveals transcription factor occupancy in response to LPS stimulation. HD11 cells were, in order from left to right, either unstimulated or LPS (1 μg/ml) stimulated for 30 min, 60 min or 240 min. Cells were then treated with DMS before the isolation of genomic DNA for hot piperidine cleavage and LM-PCR analysis. The HD37 erythroid cell line which do not express clys and the naked HD11 genomic DNA, G reaction, reference sequence are also shown. (a) non-coding strand and (b) coding strand. The filled circles represent DMS hyper-methylation and the open circles base protection from DMS. The positions of the selected G bases are indicated relative to the clys transcription start site. The potential transcription factors are indicated adjacent to a single line encompassing their proposed binding site. LINoCR transcription start site is indicated with an arrow. These images are representative of the result of two independent experiments.
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promoter of *IL10*, for which LPS-mediated transcription is delayed compared to *BTG2*, was not bound by p65 before 2 h post LPS stimulation (figure 7a). In addition, if p65, HP1γ and RNAPII S2p enrichments within this locus were comparable at 4 h with the one observed at *BTG2* locus after 30 min of LPS treatment, IKKα was 2.5 to 3 fold less recruited to *IL10* locus compared to the latter (figures 7a–d). These data would suggest that IKKα is mainly playing a role during the earliest stage of LPS-mediated transcription. However, we have shown previously that the presence of IKKα at transcribing NF-κB-dependent genes could be maintained after 2 h of transcription [17]. At these genes, the accumulation of IKKα downstream of the transcription end site (TES) is independent of RNAPII S2P (figure S6b). Taken together, these data highlight the fact that IKKα recruitment dynamics can obey different rules during transcription and suggest that HP1γ controls this dynamics.

**Discussion**

Enhancers with a “cryptic” promoter activity are widespread along the genome, but the functions of the produced ncRNAs, if functional at all, are still unknown [2]. Concomitantly, two dual

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**Figure 4. The −1.9 kb element is a NF-κB-dependent LPS inducible promoter and enhancer in transient transfection.** HD11 macrophages were transfected for 18 hrs with Jetpeii 2 µl and 1 µg DNA prior to 7 hrs 1 µg/ml LPS stimulation, black bars, or remained untreated for 7 hrs, grey bars. The constructs are illustrated adjacent to the y-axis. (a) Clys promoter is a black arrow, the −1.9 kb CRE is a dark grey arrow, the firefly luciferase coding region is grey. (b) The −1.9 kb element is cloned in antisense orientation, Position of the NF-κB, C/EBP, X unknown protein and AP1 are indicated next to the Y axis. (a and b) The data are plotted as the mean value of two independent experiments, individual experiments had triplicate samples for each condition. Inter sample variation has been corrected by Renilla normalisation. Positive error bars indicate standard deviations.

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promoter/enhancer CREs have been described, for which remodelling of the surrounding chromatin domains depends on their promoter activity and associated transcription of ncRNAs [6,7]. However in these examples, the exact dynamic of promoter and/or enhancer activity during transcription of the associated protein-coding gene is still unclear. For example, cLys −1.9 kb CRE promoter and enhancer activities have been documented but in different cell types, enhancer in the oviduct and promoter in macrophages [7,9,10]. In this study, we determined that the 2 −1.9 kb CRE is a LPS-responsive element in macrophages controlled by a unique 60 bp transcription factors cluster occupied by AP1, C/EBP, NF-κB and a still unidentified factor, additional ChIP experiments confirming that Fos, C/EBPβ [7] and NF-κB (p65) were binding in vivo to this CRE. The key inflammatory factor NF-κB is the main regulator of this CRE, which does not show any activity in absence of NF-κB binding in luciferase assays. If NF-κB is necessary, AP1, C/EBP and an undetermined factor act synergistically with NF-κB to provide full activity of this element. Such cooperative function between AP1, C/EBPβ and NF-κB has been described for multiple LPS-inducible genes including IL-6, CXCL8 or IL-8 [36–38]. In vitro, NF-κB binding is stronger in presence of C/EBP bound next to its binding site, like observed for IL-6 and IL-8 promoters, where C/EBPβ and NF-κB directly associate with each other [39].

In transient transfection, C/EBP mutant alters only slightly the −1.9 kb promoter activity suggesting that C/EBP-independent binding of NF-κB is stronger in vivo compared to EMSA or that the alteration of the −1.9 kb promoter activity in C/EBP mutant is mainly caused by the reduction of NF-κB binding. The fact that cLys contains three functional C/EBP binding sites bound by C/EBPβ [7,40] and shows only a slight increase in activity after LPS treatment reinforce the second hypothesis.

This cluster of LPS-inducible transcription factors is distinct from the steroid receptor binding sites involved in regulating this CRE in the oviduct [9] and from the mapped progesterone transcription factor binding sites, which are at a greater distance from LINoCR TSS [41], but still within the 200 bp nucleosome free region. These differences would argue that this CRE accomplishes different functions in the oviduct and in macrophages. However, additional transient transfections with the −1.9 kb CRE cloned at the 3′ end of a luciferase gene driven by cLys promoter confirms that the same 60 bp transcription factor cluster also drives a LPS-inducible enhancer activity in macrophages. Because our previous data suggest that cLys transcriptional activation was a strict consequence of CTCF eviction induced by LINoGR, the −1.9 kb CRE was expected to act successively as

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**Figure 5.** LPS induces recruitment of NF-κB, IKKα, RNAPII and HP1γ with different kinetics. (a–d) ChIP performed with primary macrophages treated with LPS for the indicated time points in minutes and the following antibodies (a) anti-p65 (NF-κB), (b) anti-RNAPII CTD, (c) anti-IKKα and (d) anti-HP1γ. Horizontal axis indicates primers used for the Real time PCR (distance in kb from the transcription start site of cLys). Data are normalized versus input. Error bars represent SD from three independent qPCR replicates. These data are representative of at least three independent experiments.

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a promoter and then as an enhancer consequently to LINoCR expression being turned off. Surprisingly, this is not the model this study is revealing. ChIP experiments show p65 binding to d1.9ys promoter and the −1.9 kb CRE after both 30 min and 120 min post LPS treatments. In contrast, IKKα is only recruited to the −1.9 kb CRE and not to d1.9ys promoter and this recruitment is seen only after the shortest period of LPS treatment. The absence of IKKα at d1.9ys promoter could be explained by the fact that the NF-kB heterodimer p65:c-Rel and not p65:p50 occupies d1.9ys promoter [42]. If p65:p50 dimer recruits IKKα to the promoter of NF-kB-dependent genes [15,16], p65:c-Rel has been shown to recruit IkBβ to a selected group of genes in response to LPS including TNF and IL1β [43,44]. Furthermore, IKKα is detected in both transcribing regions probably bound to the phosphorylated RNAPII CTD as reported previously [17]. This observation suggests a direct connection between d1.9ys promoter and the −1.9 kb CRE and simultaneous promoter and enhancer activities of this element. This contact could be mediated by C/EBPβ, which has been shown to form long range interaction and DNA looping [45]. If the short distance between these two elements does not allow chromosome conformation capture (3C) analysis, the hypothesis of a direct interaction is reinforced by recent views regarding the chromatin organization in euchromatin. These two nucleosome free regions should be indeed physically in close proximity within the 30 nm chromatin fiber structure, which has been shown to be conserved at transcribing regions [46].

C1.9ys expression is maintained several hours after that LINoCR has been shut down [7], but this expression is IKKα independent. The reasons, for IKKα disappearance from d1.9ys locus, are unclear since the transcription factors and especially NF-kB (p65) still occupy their binding sites at the −1.9 kb CRE several hours post LPS treatment. It could be explained by the inactivation of the transcription factors, bound to this regulatory element, by post-translational modifications. For example, p65 can be activated by Msk1-dependent phosphorylation of its serine 276 and deactivated by PP2A phosphatase without affecting p65 DNA binding [47,48]. Post-translational modifications also regulate C/EBPβ activity [49–51], C/EBPβ preventing NF-kB phosphorylation and thus its activation in TNF tolerant cells [52]. Finally, the composition of the AP1 dimer alters the transcriptional activation capability of this transcription factor [53]. Together, these results suggest that the transcription factors bound cluster within the −1.9 kb CRE can be inactivated without observable changes in DNA binding. In this model, the −1.9 kb CRE would initiate transcription of d1.9ys and would concomitantly abrogate CTCF enhancer blocker activity. The maintained expression of d1.9ys would thereafter be controlled by the three-enhancer elements upstream of CTCF, the −1.9 kb element playing a minor role or no role at all after CTCF eviction. Such a transient role of IKKα is observed for BTG2, for which p65 binding to the promoter is maintained after loss of IKKα and in absence of transcription. However IL10 expression, which is delayed compared to the other analysed genes, is concomitant with p65 binding to its promoter arguing for a role of this protein in transcription of late LPS responsive genes. NF-kB and IKKα binding to the IL10 promoter is induced by the HIV-1 TAT protein but observed 30 min after stimulation [54]. In contrast, when compared to BTG2, IKKα recruitment to IL10 locus after LPS stimulation is poor suggesting that NF-kB (p65) can activate genes independently of IKKα or that p65 is playing a minor role in IL10 expression, as suggested by other studies [55,56]. Nevertheless, the presence of IKKα can be measurable after 15 min of LPS stimulation and maintained beyond 2 h within transcribing regions in correlation with NF-kB (p65) promoter occupancy as observed for TNF, Ccl3 or IL1β [17]. This “extended” detection of IKKα correlates with a dense distribution of HP1γ throughout the transcribing regions of these genes [17]. As described for IKKα, HP1γ directly interacts with the elongating polymerase [24,25] and closely mimics the distribution of the latter during transcription. In addition, chromatin accumulation of IKKα downstream of the TNF transcription end site in activated macrophages is HP1γ-dependent [17]. This chromatin association of IKKα is not observed for d1.9ys and BTG2 for which the distribution of this kinase with both HP1γ and RNAPII S2p are correlated.

In macrophages, the d1.9ys −1.9 kb CRE is driven by a 60 bp transcription factors cluster and is a NF-kB/IKKα-bound dual promoter and enhancer with both activities being simultaneous. Its promoter activity is associated with chromatin remodelling of the
transcribed region and CTCF eviction and its enhancer activity is characterised by initiation of cLys transcription. In addition, we have determined that IKKα association with coding regions can be restricted to the earliest stage of LPS-mediated macrophages activation, in contrast with genes, like TNF, which display chromatin associated IKKα in the 3' end of the gene [17]. These two scenarios suggest a NF-κB-dependent recruitment of IKKα during the initiation step of transcription, followed for some genes.

Figure 7. NF-κB-dependent genes show specific temporal patterns of IKKα recruitment (a–d) ChIP performed with RAW264.7 cell lines treated with LPS for the indicated time points in minutes and the following antibodies (a) anti-p65 (NF-κB), (b) anti-IKKα, (c) anti-HP1γ and (d) anti-RNAPII S2p. Horizontal axis indicates primers used for the Real time PCR. Data are normalized versus input and then versus the Myc – 20 Kb control region. Error bars represent SD from three independent qPCR replicates. These data are representative of at least three independent experiments. doi:10.1371/journal.pone.0059389.g007
by a conservation of the kinase within the transcribing locus, this conservation being possibly NF-κB independent.

Supporting Information

Figure S1 In vivo DMS footprinting of the distal part of the −1.9 kb promoter. HD11 cells were, in order from left to right, either unstimulated or LPS (1 μg/ml) stimulated for 30 min, 60 min or 240 min. Cells were then treated with DMS before the isolation of genomic DNA for hot piperidine cleavage and LM-PCR analysis. The HD37 erythroid cell line which do not express AP1, C/EBP and NF-κB (right lane) is blue and DMS footprints for X (unidentified) are purple. LINoCR transcription start site (TSS) and proposed TATA box are shaded grey. Numbers located above the sequence are base pair positions relative to the clyS transcription start site. The filled circles represent DMS hyper-methylation and the open circles base protection from DMS. The positions of the selected G bases are indicated relative to the clyS transcription start site. The potential transcription factors are indicated adjacent to a single line encompassing their proposed binding site. These images are representative of at least three independent experiments. (TIFF)

Figure S2 Sequence of the −1.9 Kb promoter/enhancer element and detailed EMSA’s probes. (a) The proposed transcription factor binding sites are double lined and colour coded; the AP1 site is gold, C/EBP site is green, NF-κB site is blue and DMS footprints for X (unidentified) are purple. LINoCR transcription start site (TSS) and proposed TATA box are shaded grey. Numbers located above the sequence are base pair positions relative to the clyS transcription start site. (b) Probes designed for EMSA experiments. Base pair exchanges in designed mutants (Mt) are indicated at the top of the specific sequence. (TIFF)

Figure S3 AP1, C/EBP and NF-κB transcription factors bind to the proposed site in the −1.9 kb CRE in vitro. Electromobility Shift Assay demonstrating specific binding of (a) AP1, (b) X (unidentified), (c) C/EBP, (d) NF-κB and (e) C/EBP and NF-κB to the −1.9 Kb element. Supershift, when the reaction mixture was incubated with 1 μg of anti-c-Fos antibody (a, Lane 5), is indicated with an asterisk (*). Probes and cold competitors (100x) or antibodies (anti-) are indicated at the top of each lane. Sequences of the probes are detailed in figure S2. These figures are representative of at least three independent experiments. (TIFF)

Figure S4 IKKα recruitment to the clyS locus is transient. (a–e) ChIP performed with primary macrophages treated with LPS for the indicated time points in minutes and the following antibodies (a) anti-RNAII CTD, (b) anti-RNAII S2p, (c) anti-p65 (NF-κB), (d) anti-IKKα and (e) anti-H1pγ. Horizontal axis indicates primers used for the Real time PCR (distance in Kb from the transcription start site of clyS). Data are normalized versus input and then versus a background control region designed within a CTCF binding site at the IL6 locus. Error bars represent SD from three independent qPCR replicates. These data are representative of at least three independent experiments. (TIFF)

Figure S5 Changes in BTG2 and IL10 expression in response to LPS treatment. Time course of BTG2 (blue squares) and IL10 (red squares) mRNA levels in RAW264.7 cells in response to LPS treatment. Results are expressed relative to GAPDH expression. Error bars represent SD from three independent experiments. (TIFF)

Figure S6 IKKα accumulates downstream of TNF TES independently of the elongating polymerase. (a and b) scatter plots showing the degree of correlation between the elongating polymerase (Y axis) and (a) H1pγ (x axis) or (b) IKKα (x axis). Blue rectangles display values from data presented in figure 6. Red lozenges are the same values without ‘TNF TES 30’, 120” and 240”. Trend lines and R-squared values are display on the figures. (TIFF)

Table S1 List of primers used in this study. (TIFF)

Author Contributions
Conceived and designed the experiments: PFL. Performed the experiments: JW LO PFL. Analyzed the data: JW PFL. Wrote the paper: PFL.

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