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Transcription regulation from a TATA and INR-less promoter: spatial segregation of promoter function

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The mode of regulation of class II genes that lack the known core promoter elements is presently unclear. Here, we studied one such example, the murine *CD80* gene. An unusual mechanism was revealed wherein the pre-initiation complex (PIC) first assembled on an upstream, NF-κB enhancer element. Notably, this assembly occurred independent of contributions from the core promoter domain, and resulted in a PIC that was competent for transcription initiation. Positioning was subsequently achieved by exploiting the intrinsic architecture of the promoter, by virtue of which the tethered PIC was spatially juxtaposed with the transcription initiation site. Bridging interactions then ensued, through protein–protein contacts, which then enabled the elongation phase of *CD80* transcription.

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Introduction

The control of gene expression represents a combinatorial process involving a multiplicity of events that include chromatin recognition and reconfiguration, covalent modification of histones, recruitment of cofactors and the basal transcription components, and the assembly of an elongation-competent transcription complex (Jones and Kadonaga, 2000; Lemon and Tjian, 2000). Although transcriptional activation mechanisms for several eucaryotic genes have been deciphered to varying degrees of detail, all of these represent genes that contain well-defined core promoter elements (Smale and Kadonaga, 2003). In contrast, while genes that lack any of the conventional core promoter elements constitute a significant proportion of the eucaryotic genome, little is known about how they are regulated.

Here, we examined mechanisms regulating transcriptional activation of the gene coding for the murine CD80 protein, a member of the B7 family of costimulatory molecules. CD80 is primarily expressed on the surface of professional antigen-presenting cells of the mammalian immune system, and plays a critical role in the activation of T lymphocytes (Carreno and Collins, 2002). An intriguing aspect of the *CD80* gene is that it does not possess any of the canonical promoter elements that have been described for class II genes so far (Smale and Kadonaga, 2003; Lim *et al*, 2004). It lacks the TATA, the Initiator (Inr), the downstream promoter (DPE), the TFIIB recognition (BRE) elements, and the recently identified motif ten element (MTE). Further, the 5′-proximal sequence is also devoid of consensus sequences for Sp1 binding, a characteristic of both Inr-dependent promoters and those associated with CpG islands (Supplementary Figure 1). Finally, both the transcription start site and its proximal regions are shown to be completely histone-free, with an isolated nucleosome that was centered at −619 bp relative to the transcription start site.

A novel mechanism of gene activation was revealed wherein assembly and positioning of the pre-initiation complex (PIC) took place in two discrete and spatially segregated steps. A functional PIC competent for transcription initiation was first assembled in an NF-κB-dependent manner, at a distal site that was encapsulated by the upstream nucleosome. Activation-induced remodeling of the nucleosome regulated this assembly. The intrinsic architecture of the promoter, supported by cofactor binding, then provided for a superstructure wherein the PIC was brought to within spatial proximity of transcription start site. This juxtapositioning of PIC with the transcription start site enabled its appropriate positioning through a bridging effect, mediated by the Sp1 that was constitutively bound to the core promoter domain. Thus, cooperativity between discontiguous DNA sequence elements, operating within a three-dimensional structural context, can provide for a transcriptional regulatory mechanism that circumvents the need for a conventional core promoter element.

Results

The murine CD80 promoter and the induction of its gene product

Stimulation of the murine plasmacytoma, J558L cells with hydrogen peroxide (H_2O_2) for a brief (15 min) period led to an increase in *CD80* mRNA levels between 1 and 2 h poststimulation, and in a manner that was devoid of *de novo* protein synthesis. Transcription was initiated from the originally identified (Selvakumar *et al*, 1993; Borriello *et al*, 1994) transcription initiation site (TS1) and *in vitro* transcription assays established that the promoter sequence extending up to −957 nt from TS1 was sufficient to support expression of the CD80 gene. These cumulative results are shown in Supplementary Figure 1.

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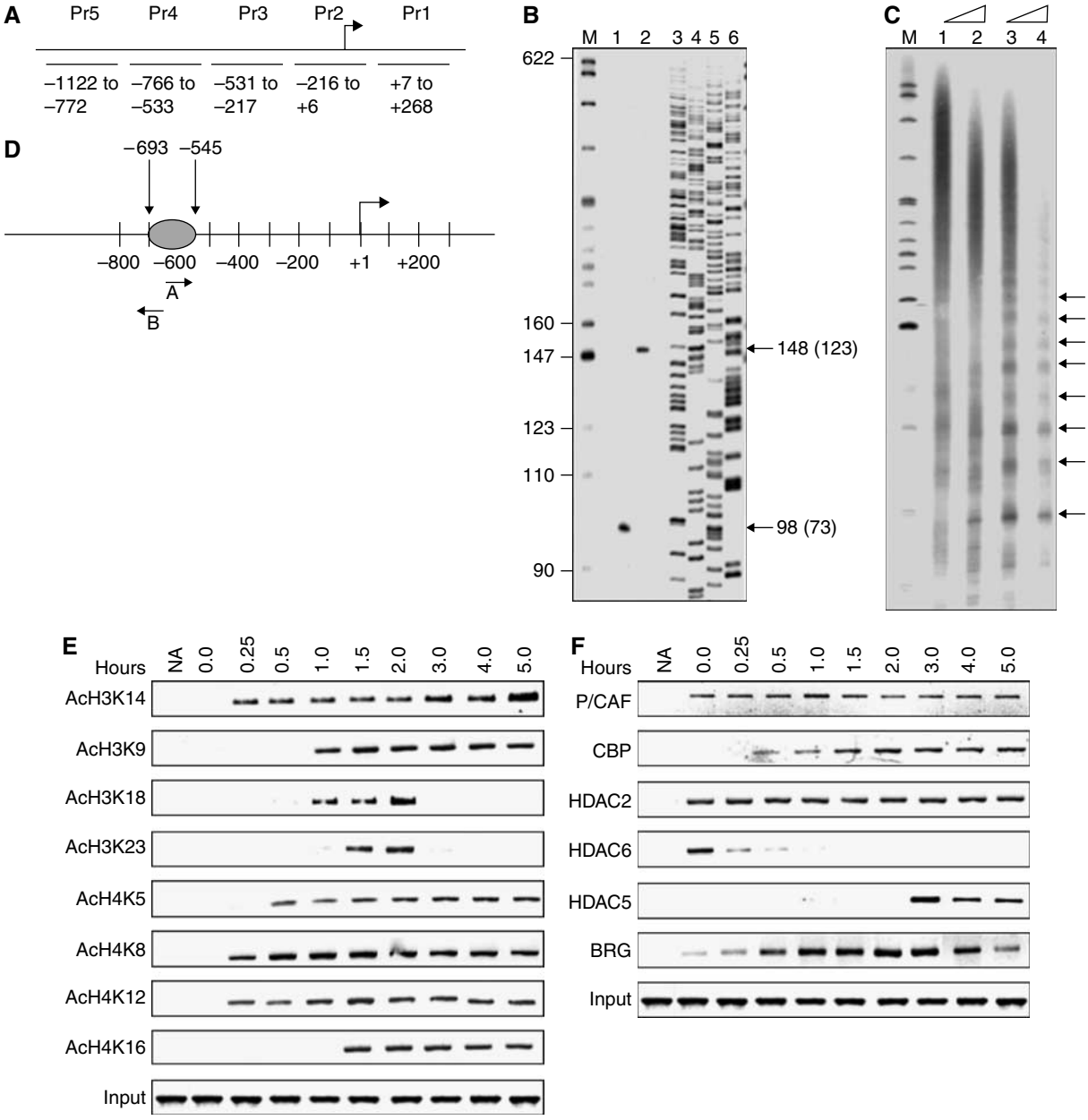

Figure 1 MNase and DNase analysis-based positioning of a solitary nucleosome. (**A**) The probes used for Southern blot analysis and the start site (arrow) are depicted in a line diagram (not to scale). (**B**) LM-PCR-based positioning of the nucleosome. Lane 1 represents the amplified product of Primer A, and lane 2 of Primer B as depicted in (D). The lengths of LM-PCR products are indicated on right. The number in parentheses corresponds to the lengths minus that of the 25 bp linker. M is pBR322*Msp*I-digested marker. Lanes 3–6 are products of sequencing for accurate positioning. (**C**) *In vivo* DNase I footprinting of the positioned nucleosome. Lanes 1, 2 represent naked DNA and lanes 3, 4 represent nuclei digested with DNase I. M is pBR322*Msp*I-digested marker. Arrows indicate laddering in lanes 3 and 4. (**D**) Schematic representation of mouse CD80 proximal promoter region. A and B represent primer positions used for LM-PCR mapping. The shaded oval represents the positioned nucleosome. (**E**) Time kinetics of histone acetylation in response to H_2O_2 stimulation, as determined by chromatin immunoprecipitation (ChIP). Antibodies specific for the various acetylated derivatives of the lysine residues are indicated and the specificity of immunoprecipitation was ensured in parallel experiments where the primary antibody was omitted (NA). (**F**) Time-dependent recruitment of the indicated nucleosome modifiers, following H_2O_2 stimulation of the cells. Results are representative of four separate experiments.

We next examined the *CD80* promoter sequence (extending up to −955 nt) for the presence of nucleosomes. Initial Southern blot hybridization analysis on MNase-digested DNA, using the probes depicted in Figure 1A, yielded a positive signal only with the probe Pr4 (Supplementary Figure 2), although all of the remaining probes were equally capable of hybridizing with the *CD80* promoter. We then performed ligation-mediated PCR (LM-PCR) experiments on MNase-digested, mononucleosomal DNA preparations. Using nested primer sets internal to each of the probe sequences shown in Figure 1A, we again obtained amplification in both 3′- and 5′-directions only when primer sets from within the Pr4 regions were employed (Figure 1B). These results indicated the presence of a solitary nucleosome that was positioned between −693 and −545 nt with reference to TS1. This was further confirmed by mononucleosome chromatin

immunoprecipitation (ChIP) assays using antibodies specific for H3 histone (Supplementary Figure 2). A similar analysis on the B lymphoma cell line, A20, and on splenocytes derived from BALB/c mice gave identical results, confirming that the nucleosomal positioning described here was not artefactual to J558L cells (Supplementary Figure 2). Finally, *in vivo* DNase I footprinting analysis of this nucleosome yielded the typical 10 bp ladder (Figure 1C, lanes 3 and 4), establishing that this single nucleosome was both translationally and rotationally positioned (illustrated in Figure 1D).

Stimulation of cells with H_2O_2 induces histone acetylation and nucleosome remodeling

Stimulation also led to histone acetylation, which was first detectable by 15 min. Acetylation occurred at both H3 and H4 histones, with a temporal increase in the spectrum of lysine residues that were acetylated (Figure 1E). Both Lys14 of H3 and Lys18 of H4 are known substrates for PCAF/GCN5, implying that these early acetylation events are probably PCAF dependent (Grant *et al*, 1999; Roth *et al*, 2001). This is consistent with our findings that, in addition to HDAC2 and HDAC6, PCAF was also constitutively associated with the promoter region (Figure 1F). Whereas the association of HDAC2 remained stable, stimulation of cells led to an immediate dissociation of HDAC6 (Figure 1F). Thus, we postulate that the H_2O_2-induced dissociation of HDAC6 leads to a shift in steady-state equilibrium from HDAC to PCAF-associated HAT activity, and the consequent activation of histone acetylation events. That treatment of cells with the HDAC inhibitor, TSA, yields a similar profile of histone acetylation (Supplementary Figure 3) supports such a possibility. The subsequent expansion of the spectrum of lysine residues acetylated is probably due to the recruitment of CBP that is first detected at 30 min of poststimulation, to then saturate by 1.5 h (Figure 1F). On the other hand, the transience of the acetylation at Lys 8 and 23 of H3 could be a consequence of the HDAC5 engagement observed at 3 h (Figure 1F).

Acetylation also led to recruitment of BRG1, the ATPase subunit component of SWI/SNF remodeling complexes. This was initiated by 15 min poststimulation, with saturation being obtained by 30 min (Figure 1F). Since ChIP assays on mononucleosome preparations revealed that BRG1 was recruited on the nucleosome (Supplementary Figure 3), we examined if there was any reconfiguration of the nucleosome as a consequence. While nucleosome sliding was not detected (not shown), increased accessibility of the *Acc*I restriction site indicated in Figure 2A was, however, observed. Stimulation of cells with H_2O_2 led to a substantial increase in the accessibility of this site by 30 min poststimulation, which then remained constant up to 4 h (Figure 2B). Thus, stimulation of J558L cells with H_2O_2 results in rotational, but not translational, repositioning of the nucleosome.

Increased accessibility of the *Acc*I site at 30 min poststimulation was also observed for BALB/c mouse splenic B cells stimulated with H_2O_2. In contrast, this site was already accessible in unstimulated A20 cells—a cell line that constitutively expresses high levels of CD80—with no further modification upon stimulation (Supplementary Figure 3). Collectively, these findings point to the relevance of nucleosome remodeling in defining the transcriptional status of the *CD80* gene.

Nucleosome remodeling facilitates formation of the PIC

Components of the PIC, RNA polymerase II (Pol II), TFIIB, and TBP were also all recruited by 30 min of stimulation of cells with H_2O_2 (Figure 2C). This time point correlates with that of the nucleosome remodeling event (see Figure 2B), suggesting a causal relationship between these two events. Significantly, soon after its recruitment, Pol II was phosphorylated in the C-terminal domain (CTD) of its largest subunit at Ser5 (Figure 2C), indicative of its acquisition of an initiation-competent state. Phosphorylation at Ser2 of the CTD, a marker for entry of Pol II into the elongation phase (Prelich, 2002), was detected by 2 h (Figure 2C)—consistent with the time course of appearance of the CD80 transcript.

We also detected the constitutive association of HMGI(Y), Sp1, and C/EBPβ with the promoter (Figure 2C), and three distinct HMG-binding sites located between −87 ando −72 nt, −380 and −363 nt, and −542 and −536 nt could be identified (Supplementary Figure 4). While HMGI(Y) and Sp1 associations were stable, C/EBPβ dissociated by 1.5 h after stimulation. Significantly, C/EBPβ was found to be coassociated with Cdk8, implying its presence in a nonactivated form wherein it functions as a repressor of gene expression (Figure 2C) (Mo *et al*, 2004). Finally, our ChIP experiments revealed that NF-κB complexes were also recruited at 30 min of stimulation, and this association then remained stable for the remainder of the experiment (Figure 2C). Importantly, inhibition of NF-κB recruitment with specific inhibitors (described below) also prevented the dissociation of C/EBP and Cdk8 (not shown).

Although both nucleosome remodeling and recruitment of NF-κB, along with components of the PIC, occurred simultaneously (Figure 2B and C), we established that the recruitment events were in fact dependent upon the former process. First, inhibition of NF-κB activation with either capsaicin (Singh *et al*, 1996) or the NEMO peptide (May *et al*, 2000) yielded no significant effect on H_2O_2-induced nucleosome remodeling (not shown), suggesting that NF-κB does not mediate nucleosome remodeling. We then attempted to inhibit nucleosome remodeling by using the approach of siRNA. To avoid possible complications due to redundancy, we employed a combination of siRNAs that targeted both the *BRG1* and *BRM* genes. Silencing of *BRG1/BRM* expression led to a marked suppression of H_2O_2-induced remodeling at the nucleosome (Figure 2D). Further, association of both NF-κB and the PIC components was also inhibited in *BRG1/BRM*-silenced cells, but not in those transfected with nonsilencing siRNA (Figure 2E). Thus, nucleosome remodeling constitutes an obligatory prerequisite for the recruitment of NF-κB, in addition to assembly of the PIC.

A role for NF-κB in CD80 regulation

An analysis of the promoter sequence for the presence of NF-κB target sites revealed only a cognate half-site for p50 binding (5′-GGGAA-3′), located between −545 and −541 nt relative to TS1. Interestingly, this sequence was present towards the 3′ end of the nucleosome; and 60 nt downstream of the *Acc*I restriction site that displayed increased accessibility upon stimulation of cells with H_2O_2 (Figure 2B). Electrophoretic mobility shift assays (EMSA) using a double-stranded probe spanning the positions from −551 to −530 yielded retarded mobility for this probe when incubated with nuclear extracts from peroxide-stimulated, but not unstimu-

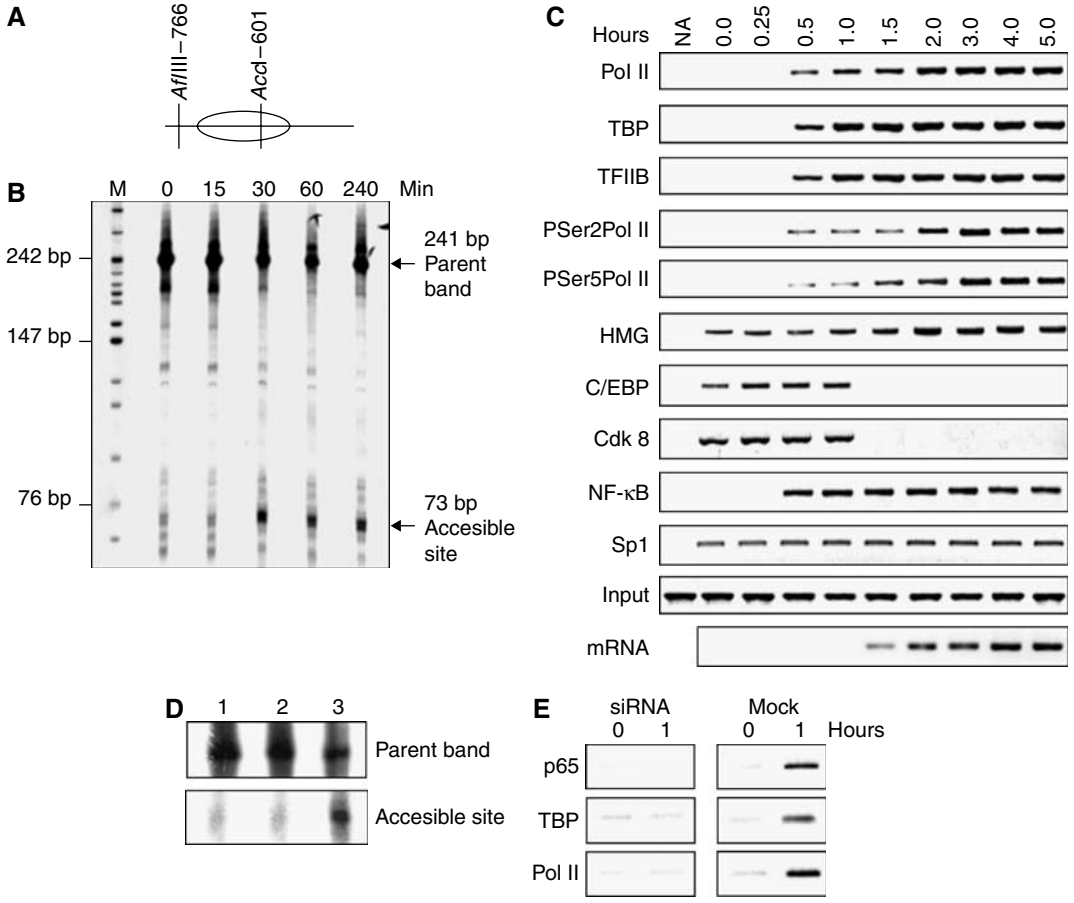

Figure 2 Stimulation with H_2O_2 induces remodeling of the nucleosome. (**A**) Schematic representation of the restriction enzyme sites analyzed in (B) corresponding to the positioned nucleosome. (**B**) Restriction enzyme accessibility was performed on unstimulated and H_2O_2-stimulated J558L cells for the indicated time points. Arrows to the right denote the fragments resulting from *in vitro* and *in vivo* digestion, respectively. M is pBR322*Msp*I-digested marker. (**C**) Time course for the recruitment of indicated factors as analyzed by ChIP in unstimulated and H_2O_2-stimulated J558L cells. The specificity of immunoprecipitation was ensured in parallel experiments where the primary antibody was omitted (NA). The corresponding time kinetics of CD80 mRNA in unstimulated (0.0) and H_2O_2-stimulated cells is represented in the lowest panel. The results are representative of four separate experiments. (**D**) Restriction enzyme accessibility was performed on J558L cells transfected with BRG1 and BRM targeting-siRNA that were either unstimulated (lane1), or stimulated with H_2O_2 for 1 h(lane2). A parallel set of cells was transfected with nonsilencing siRNA (GFP-specific), prior to stimulation with H_2O_2 (lane 3). The corresponding products are indicated. Results are from one of three experiments. (**E**) J558L cells were either transfected with siRNA targeted to BRG1 and BRM or to GFP (Mock). After 24 h, they were stimulated with H_2O_2 for 1 h and ChIP was performed with the indicated antibodies.

lated, cells (Figure 3A). Further, binding of the native probe with the extract from stimulated cells could be competitively inhibited with an excess of an unlabeled probe bearing the consensus sequence for NF-κB recognition (Figure 3A). Finally, binding was also substantially inhibited in the presence of antibodies specific for either p50 or p65, and completely inhibited with a combination of both (Figure 3A). Collectively, the results in Figure 3A identify the sequence between −545 and −541 nt as the locus for recruitment of the p50-p65 NF-κB heterodimer, in stimulated cells.

We next cloned the *CD80* promoter fragment between −955 and +372 nt into the EGFP vector such that EGFP expression was now under its control. An additional construct was also generated with a mutation within the cognate NF-κB half-site. These constructs were separately transfected into J558L cells, and *EGFP* expression analyzed by flow cytometry. Low levels of *EGFP* expression were detected in unstimulated cells transfected with either construct (Figure 3B, panel a). Stimulation with H_2O_2 led to a significant increase in *EGFP* expression in cells transfected with the plasmid encoding the native (Figure 3B, panel b), but not the mutant (Figure 3B, panel c), promoter. These results, therefore, identify a central role for the nucleosome-localized NF-κB binding half-site in regulating H_2O_2-dependent *CD80* expression. Importantly, cotransfection of J558L cells with an NF-κB p65-expressing plasmid along with the above native *CD80* promoter-containing EGFP-1 vector resulted in constitutive expression of high levels of EGFP (Figure 3B, panel d). Thus in addition to it being obligatory, the recruitment of NF-κB is alone sufficient to activate the *CD80* promoter.

To investigate the regulatory role of NF-κB, J558L cells were stimulated with peroxide either in the presence or absence of the inhibitors of NF-κB, and then subjected to ChIP assays using antibodies against p65, CBP, Pol II, TBP, or TFIIB. In addition to inhibition of NF-κB recruitment, the inclusion of NF-κB inhibitors also led to a marked reduction in recruitment of CBP, Pol II, TFIIB, and TBP; with the NEMO peptide showing the more pronounced inhibitory effect (Figure 3C). Thus, NF-κB recruitment appears to be critical for assembly of the PIC.

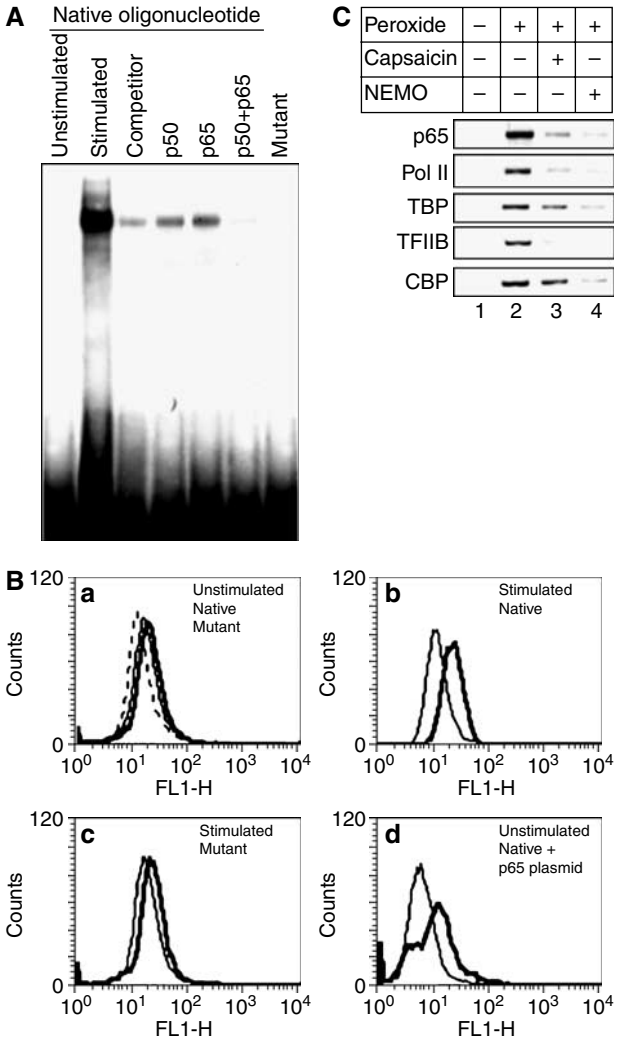

Figure 3 NF-κB is critical for recruitment of the pre-initiation complex. (**A**) EMSA was performed with an oligonucleotide spanning the NF-κB half-site and nuclear lysate obtained from unstimulated J558L cells (lane 1) and H_2O_2-stimulated cells for 2 h (lanes 2–7). Specificity of shifted protein–DNA complex (lane 2) was assessed through competition with a 100-fold excess of unlabeled oligonucleotide representing the NF-κB consensus sequence (lane 3), and by the absence of binding to a mutant of the oligonucleotide used in lane 2 containing the GG→CC mutation (lane 7). Also shown are the effects of inclusion of antibodies specific for p50 (lane 4), p65 (lane 5), and a mixture of both anti-p50 and anti-p65 antibodies (lane 6). (**B**) Plasmids containing the native CD80 promoter, or its mutated derivatives, cloned upstream of EGFP (see text) were transfected into J558L cells and examined for EGFP expression by flow cytometry. Profiles obtained in unstimulated cells transfected with either the native (thin line), the NF-κB mutant promoter (thick line), and mock-transfected cells (dotted line) are given in panel 'a'. Panels 'b' and 'c' show the extent of EGFP induction (thick line) obtained in cells transfected with native (b) or mutant (c) promoter at 4 h after stimulation with H_2O_2, along with the corresponding profile in unstimulated cells (thin line). Panel 'd' compares EGFP induction in unstimulated cells transfected with the native promoter alone (thin line), along with the p65 expression plasmid (thick line). The subpopulation of cells lacking EGFP expression in the latter group likely represents an untransfected subset. (**C**) J558L cells were pretreated with capsaicin or NEMO prior to stimulation with H_2O_2. After 1.5 h, ChIP analysis was performed with the indicated antibodies, using CD80 promoter-specific primers. Parallel experiments established that both capsaicin and the NEMO peptide also inhibit H_2O_2-dependent CD80 mRNA induction by >85% (data not shown).

NF-κB-dependent PIC assembly does not require the core promoter domain

Since such interactions have been previously described (Schmitz *et al*, 1995; Vanden Berghe *et al*, 1999), we next ascertained whether CBP, Pol II, TFIIB, and TBP also associate with p65 in J558L cells. As expected, stimulation of cells with H_2O_2 led to an increase in nuclear accumulation of phosphorylated p65 (Figure 4A). Phosphorylation of p65 has been reported to enhance DNA binding, and the transactivation activity of p65 containing NF-κB dimers (Naumann and Scheidereit, 1994). Significantly, immunoprecipitation of p65 from peroxide-stimulated cells also resulted in the co-precipitation of CBP, Pol II, TFIIB, and TBP (Figure 4A),

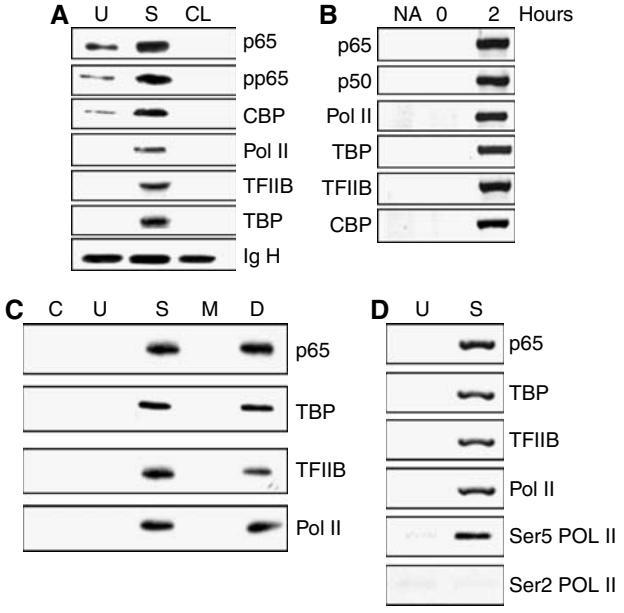

Figure 4 NF-κB nucleates formation of a functional PIC. (**A**) Nuclear lysates from unstimulated cells (U), cells stimulated with H_2O_2 for 1.5 h (S) were immunoprecipitated with antibodies specific for p65. Co-immunoprecipitated proteins were detected by Western blot using the antibodies indicated on the right. IgH was used as the loading control, and rabbit anti-mouse IgG was used as the mock-immunoprecipitation control (CL). (**B**) Nuclei were isolated from formaldehyde-fixed unstimulated and H_2O_2-stimulated (2 h) J558L cells. Mononucleosome-sized chromatin was immunoprecipitated with antibodies indicated on the left. PCR was performed with primers within the region protected by the nucleosome. The efficiency of MNase digestion was verified by using primers immediately outside the nucleosome (data not shown). (**C**) The results of a pull-down experiment wherein nuclear extracts from unstimulated (U) and H_2O_2-stimulated (S) cells were incubated with a biotinylated-ds-DNA fragment representing the native promoter sequence (−753 to +154) are shown. In parallel, nuclear extracts from stimulated cells were also incubated with biotinylated probes that contained a mutation within the nucleosomal NF-κB half-site (M) and a deletion of the region between −120 and +154 nt (D). These probes were then precipitated with streptavidin–agarose, and the bound proteins identified by Western blot using indicated antibodies. Lane C indicates the control group where streptavidin–agarose beads alone were used. Results are from one of four experiments. (**D**) The deletion mutant (D) described in (C) above was cloned into an EGFP-1 vector (see text), and then transfected into J558L cells. After 36 h, transfected cells were then either left unstimulated (U), or stimulated with H_2O_2 for 1 h (S), followed by ChIP analysis using the indicated antibodies. Immunoprecipitated DNA was analyzed by PCR using promoter and EGFP-specific primers. Results are a representative of three separate experiments.

supporting that NF-κB can actively provide an interacting surface that contributes towards at least stabilizing PIC assembly/recruitment in the context of *CD80* expression. Furthermore, ChIP assays using mononucleosome preparations verified that it was the nucleosome-localized p50-p65 NF-κB heterodimer that was involved in engagement of components of the PIC (Figure 4B).

To rule out the possibility that additional noncanonical-NF-κB-binding sites may also exist, we generated a biotinylated-ds DNA probe representing the segment between −753 and +154 nt relative to TS1. This probe was incubated with the nuclear extract from either stimulated or unstimulated cells and then precipitated with streptavidin–agarose prior to examination by Western blot analysis. Binding of the p65 subunit of NF-κB could clearly be detected when the probe was incubated with extracts from stimulated, but not from unstimulated cells (Figure 4C). Further, coassociation with TBP, TFIIB, and Pol II was also detected in this case (Figure 4C). Importantly, a mutation within the NF-κB cognate half-site completely abolished the ability of the probe to interact with activated NF-κB, as also with TBP, TFIIB, and Pol II (Figure 4C). Thus, the nucleosome-encapsulated domain contains the sole NF-κB-binding site that facilitates PIC assembly.

Notably, a 3′ truncation of the above probe from −120 to +154 nt had no effect on the binding of activated p65-containing NF-κB. Further, this 3′-deletion also did not affect the coassociation with Pol II, TBP, and TFIIB (Figure 4C). These findings were additionally verified by *in vivo* ChIP experiments where the truncated promoter was cloned upstream of *EGFP* into an EGFP-1 vector, and then transfected into J558L cells. Stimulation of these cells with H_2O_2 led to assembly of the PIC on the transfected template even in the absence of the core promoter region (Figure 4D).

Thus, the above results together suggest that NF-κB recruitment at its cognate half-site is alone sufficient to ensure formation of a stable PIC, and that contributions from the TS1-core promoter domain are not necessary for this process. A significant aspect of the data in Figure 4D is the fact that phopshorylation of CTD on Ser5, but not on Ser2, could also be detected. This suggests that nucleosomal recruitment of the p65-p50 NF-κB heterodimer is alone sufficient to nucleate formation of a mature PIC that is functionally capable of promoter clearance.

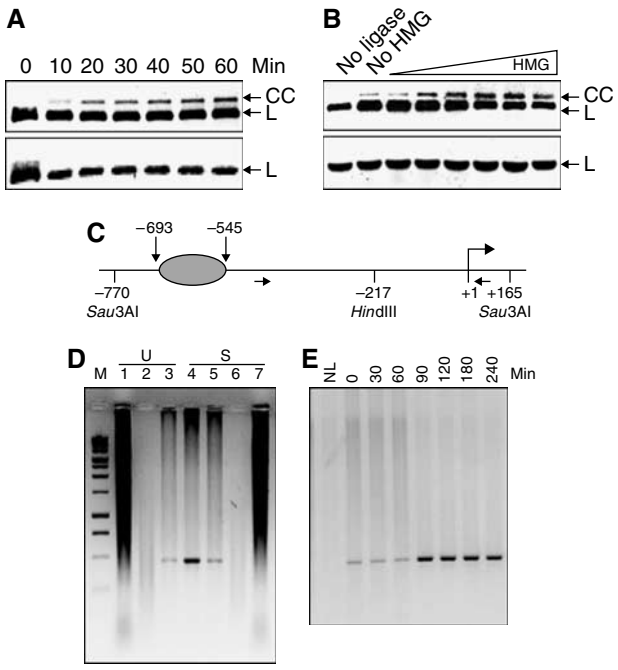

Figure 5 Stabilization of the intrinsic bending ability of the CD80 promoter. (**A**) Time-dependent ligation of the region from −667 to +36 of CD80 promoter in the absence of HMGI(Y). Upper panel shows ligation of the CD80-derived segment, whereas the lower panel shows ligation of a control DNA taken from the cDNA region of the murine LAB gene. Reaction products were resolved on an agarose gel (1.2%) followed by staining with ethidium bromide (0.5 μg/ml). Linear DNA (L) molecules run faster than the closed circular products (CC). (**B**) The results of the ligation reaction that was performed in the presence of increasing concentrations of HMGI(Y) with a constant reaction time of 20 min. (**C**) Schematic representation depicting *Sau*3AI and *Hin*dIII cut sites. *Sau*3AI has multiple cut sites within the CD80 promoter but one of the fragments spanning −770 to +165 relative to the start site defines the region of interest. *Hin*dIII cuts once within the promoter at −217 bp. PCR primers spanning −427 to +53 bp are represented by arrows. (**D**) 3C assay-based ligation products detected by PCR. Lane 1–3 are unstimulated, lanes 4–7 are stimulated with H_2O_2 (2 h), and lane 5 is pretreated with the NEMO peptide prior to stimulation. Lanes 1 and 7 are not subjected to either restriction digestion or ligation. Lanes 2 and 6 are digested with *Sau*3AI and *Hin*dIII only. Lanes 3–5 were first restriction digested followed by ligation. M is the 1 kb Promega ladder. (**E**) The time kinetics for loop configuration as identified by 3C assay on J558L cells stimulated with H_2O_2 for the indicated time points. Control sample with no ligase is marked as NL.

DNA-encoded structural elements facilitate communication between the nucleosome and TS1

While a functional PIC was assembled at a distal, 5′-upstream, site, transcriptional activation would—nonetheless—require it to interact with TS1 and/or its proximal sequences. To probe whether bending of the intervening DNA sequence could potentially support this long-range effect, we performed T4 ligase-mediated DNA cyclization assays (Kahn and Crothers, 1992) with a 704 bp probe, representing the sequence between −667 and +36 nt. A significant amount of the monomeric circular DNA product was indeed generated in a time-dependent fashion. (Figure 5A). Notably, the presence of HMGI(Y) during the ligation reaction resulted in a concentration-dependent increase in the amount of the closed circular DNA product formed (Figure 5B). Thus, the intervening sequence between the nucleosome and TS1 possess an intrinsic bending ability, which is further stabilized through HMGI(Y) binding. This bending, in turn, is likely to bring the nucleosome to within spatial proximity of the transcription start site (TS1).

To further confirm the spatial colocalization of the nucleosome and TS1 *in situ*, we adapted the Chromosome Conformation Capture assay, a technique that permits the identification of DNA segments that are spatially juxtaposed (Dekker *et al*, 2002). Using the combination of restriction enzymes and the primer pair depicted in Figure 5C, a moderate degree of interaction could be detected between the nucleosomal and the TS1-proximal segments in unstimulated cells, confirming the intrinsic bending propensity of the intervening DNA *in situ*. Importantly, stimulation of cells led to a marked increase in the extent of this interaction, and this increase was inhibited upon inclusion of the NF-κB inhibiting NEMO peptide (Figure 5D). Thus, activation-dependent

recruitment of the p65-p50 NF-κB at its nucleosomal target site, presumably along with components of the PIC, further stabilizes this interaction between the nucleosomal and the core promoter DNA segments. This stabilization occurred by 90 min poststimulation (Figure 5E), suggesting that it is consequent to PIC assembly, and its timing correlates well with that for the induction of *CD80* transcription.

Transcriptional activation of CD80 involves protein-mediated bridging between the nucleosomal and the core promoter regions

To determine how the PIC was positioned in the vicinity of the transcription start site, we first performed *in vivo* DNase I footprinting experiments for the DNA sequence between −120 and +48 nt. Four broad regions of protection were observed in the DNA obtained from unstimulated cells, the most notable of which were a strong footprint between +6 to +16, and another between −41 and −14 nt. Thus, the DNA sequences flanking TS1 appear to be constitutively bound with protein factors. Notably however, these footprints remained largely unaltered in peroxide-stimulated cells, suggesting that transcriptional activation of *CD80* did not involve the formation of any new protein–DNA contacts at least in the immediate vicinity of TS1 (Figure 6A and B). The alternate possibility that remained was if PIC positioning could be mediated through contact with one or more of the protein factors that were stably associated with the TS1 core promoter region.

To first determine which of the protein-bound TS1-flanking sequences were critical for transcriptional activation, we again took the EGFP-CD80 promoter construct described in Figure 3B and performed site-directed mutations that separately targeted the above footprint regions described by the nucleotide sequences +6 to +16, −21 to −14, and −41 to −31, respectively. While the mutation within the −21 to −14 sequence (Mut2) completely abolished the H_2O_2-inducible EGFP expression, no such inhibitory effect was obtained for the other mutations (Figure 6C). Further, the resistance of Mut2 to induction could not be overcome by cotransfection with the NF-κB p65-bearing plasmid described in Figure 3B (Figure 6C). Thus, at least the critical determinant of the core promoter activity of the *CD80* gene resides within the sequence between −21 to −14 nt, relative to TS1. Consequently, PIC positioning could involve the protein(s) associated with this segment of DNA. That the region defined by Mut2 constituted the only *cis*-regulatory element in the TS1-proximal region was also confirmed by performing additional, and extensive, mutations within the segment extending from −36 to +13 nt. None of these additional mutations, however, had any significant effect on inducible EGFP expression in transfected cells (Supplementary Figure 5).

Although a bioinformatics analysis of the −21 to −14 DNA sequence did not reveal a consensus binding sequence for any of the known transcription factors, a probe representing the −30 to −10 nt sequence, however, displayed retarded mobility, in an EMSA assay, when incubated with the nuclear extract from unstimulated J558L cells (Supplementary Figure 5). Further, this interaction was abolished upon mutation of the nucleotides present in the sequence between −19 and −15 nt. In competitive inhibition experiments, we observed significant inhibition of the nuclear extract-induced retarded mobility of the native −30 to −10 probe, when incubated in

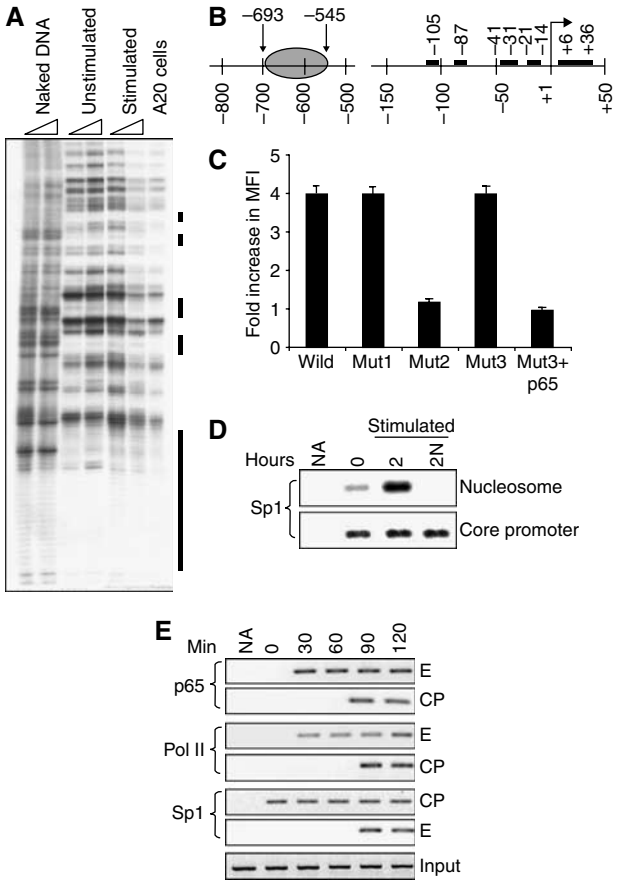

Figure 6 PIC positioning on the core promoter involves protein-mediated contacts. (**A**) *In vivo* footprinting of either unstimulated (lanes 3, 4) or H_2O_2 stimulated (for 2 h, lanes 5 and 6) J558L cells with increasing DNase I concentrations. Lane 7 shows footprints in A20 cells, which constitutively express CD80. Lanes 1 and 2 show naked DNA controls. The vertical solid black lines indicate the significant regions of protection. (**B**) Schematic representation of significant *in vivo* footprints. Probes positions are indicated and the footprint regions are shown as solid black lines. (**C**) Bar graph representing fold increase in MFIs corresponding to EGFP expression as measured by FACS analysis (see Figure 3B). J588L cells transfected with wild-type promoter fused to EGFP (see text), or various mutants Mut1 (mutated in region spanning −41 to −31), Mut2 (−21 to −14), Mut3 (+6 to +16). The final group is cotransfected with Mut2 and p65. (**D**) Nuclei were isolated from formaldehyde-fixed J558L cells that were either left unstimulated (0), or stimulated with H_2O_2 for 2 h either in the absence (2) or presence (2 N) of the NEMO peptide. Mononucleosome ChIP was performed as described in Figure 4B with Sp1 antibody (top panel). Parallel Sp1-specific ChIP experiments, using sonicated preparations, were also performed as described for Figure 1E to monitor for changes in the overall level of Sp1 binding as a results of cell stimulation (lower panel). The specificity of immunoprecipitation was ensured in parallel experiments where the primary antibody was omitted (NA). (**E**) ChIP analysis was performed as described earlier with the variation that sonication was replaced by restriction enzyme digestion to ensure precise fragmentation of DNA. PCR analysis was carried out simultaneously in the nucleosomal-enhancer (E) and core promoter (CP) region and the efficiency of digestion was verified with various primer combinations. Results are representative of three separate experiments.

the presence of an excess of a probe bearing the Sp1-binding sequence (Supplementary Figure 5). These findings could subsequently be confirmed by the supershift obtained upon incubating the complex between nuclear protein and the CD80 promoter-derived probe with anti-Sp1 antibody

(Supplementary Figure 5). Thus, the functional determinant within the core promoter domain of TS1 is constitutively bound with Sp1. Importantly, no additional Sp1-binding site could be detected at least in the TS1-upstream region extending up to −120 nt (not shown).

To probe for an interaction between the Sp1-occupied domain and the nucleosome, we next performed mononucleosome ChIP assays using antibodies specific for Sp1. Stimulation of cells with H_2O_2 led to a marked increase in the interaction between Sp1 and the nucleosomal region, and this increase was inhibited in the presence of the NEMO peptide, (Figure 6D). These effects were independent of the fact that the overall level of Sp1 association with the CD80 promoter remained unaffected under these various conditions (Figure 6D). Nuclear preparations from stimulated cells were also digested with the combination of restriction enzymes shown in Figure 5C. This was followed by ChIP using antibodies against p65, Pol II, or Sp1, and an analysis of the precipitates for the presence of either the core promoter or the nucleosome-encapsulated DNA segments. As shown in Figure 6E, co-precipitation of both DNA segments was clearly detectable by 90 min poststimulation, which correlates well with the time point at which *CD80* transcription is activated. A role for Sp1 in positioning PIC in the context of TS1 is, therefore, supported by the collective results from these experiments.

PIC positioning and entry into the elongation phase is facilitated through 'bridging' between NF-κB and Sp1

Next, we performed *in vitro* experiments wherein a biotinylated probe representing the core promoter sequence of *CD80* was incubated with nuclear extracts from activated cells either in the presence or absence of an additional probe derived from the upstream nucleosome-encapsulated DNA sequence. The rationale here (see Figure 7A) was to determine whether a macromolecular complex of Sp1, NF-κB, and components of the PIC indeed assembles on the core promoter in a manner that is dependent upon both NF-κB and Sp1. The biotinylated probe was precipitated with streptavidin–agarose, and the associated protein constituents analyzed by Western blot. In addition, we also performed PCR to determine whether the probe representing the upstream enhancer sequencer was co-precipitated. Incubation of the core promoter probe alone with the nuclear extract yielded only Sp1 reactivity in the Western blot, whereas addition of the enhancer probe to this mixture resulted in the co-precipitation—in addition to Sp1—of p65, TFIIB, Pol II, and TBP (Figure 7B, lanes 2 and 3). Importantly, the PIC components were not co-precipitated either when the core promoter probe contained a mutation in the Sp1-binding site, or when the NF-κB site was mutated in the enhancer probe (Figure 7B, lanes 4 and 5). These collective results, therefore confirm that positioning of the PIC on the *CD80* core promoter requires the upstream, nucleosomal DNA sequence, and that this positioning is dependent upon the integrity of the respective Sp1 and NF-κB target sites. Importantly, depletion of Sp1 from the nuclear extract prior to incubation with the native core promoter and enhancer probes also led to an inhibition of PIC association with the core promoter (Figure 7B, lane 6). These latter findings provide direct evidence for involvement of the Sp1 protein in PIC positioning in the vicinity of TS1.

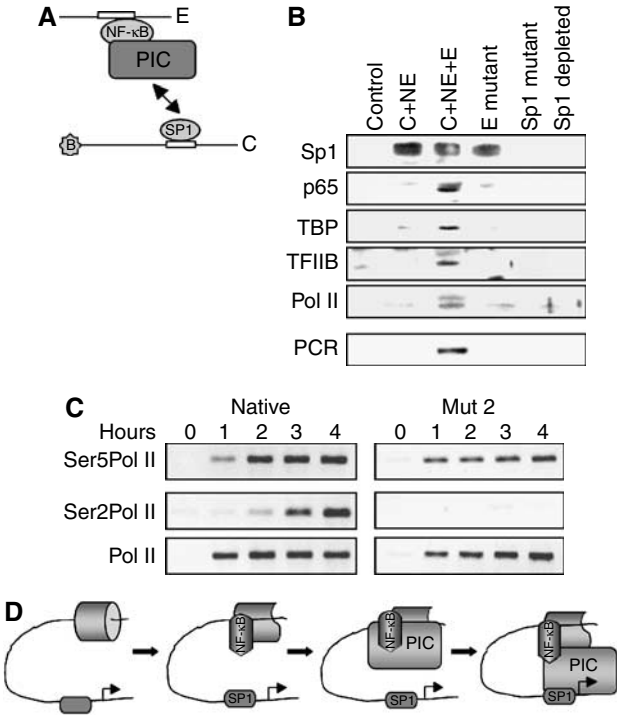

Figure 7 NF-κB recruits PIC at a distal location and Sp1 positions it at the start site. (**A**) Diagrammatic representation of experimental design in (B). E and C represent the enhancer and the core probes, respectively, while B indicates the biotinylated end. (**B**) Western blot with the indicated antibodies showing *in vitro* pull down with the biotinylated core probe. Lane 1 represents beads alone control; lane 2, biotinylated core probe (C) incubated with nuclear extract (NE); lane 3, C + NE + enhancer probe (E); lane 4, C + NE + mutant E (mutated at NF-κB site); lane 5, mutant C (mutated at Sp1 site) + NE + E; lane 6, C + E + NE depleted of Sp1. The bottom panel shows the result of PCR performed on the pulled down template with enhancer probe-specific primers. (**C**) ChIP was performed with the indicated antibodies on J558L cells transfected with native and mutant 2-EGFP constructs (described in text). At 36 h post-transfection, cells were stimulated with H_2O_2 for the time points shown. PCR was performed with promoter and EGFP-specific primers. (**D**) Illustration of the sequence of events leading to CD80 transcription. A single distal nucleosome is positioned 600 bp upstream of start site encapsulating the critcal NF-κB site. Sp1 is constitutively bound to the core promoter. Nucleosme remodeling is a prerequiste for NF-κB recruitement. NF-κB recruits a functional PIC that is then positioned by Sp1 at the start site resulting in transcription.

Essentially similar results were also obtained in a 'reverse' experiment where it was the enhancer probe that was biotinylated instead of the core promoter probe (Supplementary Figure 6). Finally, consistent with our interpretation that the PIC pull-downs represent bridged complexes involving both the core promoter and nucleosomal enhancer sequences, the presence of the enhancer probe could be detected by PCR in precipitates obtained only when the native probe sequences were employed. This was not the case when probes bore mutations either in the Sp1- or the NF-κB-binding sites. Similarly, co-precipitation of the enhancer probe could not also be detected when probes with the native sequences were incubated with an Sp1-depleted nuclear extract (Figure 7B, bottom panel).

We next transfected cells with the EGFP vector containing either the native or the Mut2 versions of the *CD80* promoter described in Figure 6C. Following stimulation with H_2O_2,

phosphorylation of the CTD of Pol II was then determined by ChIP. In cells transfected with the construct containing the native promoter sequence, stimulation led to the expected recruitment of Pol II (Figure 7C). Soon thereafter, the CTD underwent phosphorylation at Ser5 to be followed by Ser2 phosphorylation by 3 h poststimulation (Figure 7C). As expected from Figure 4D, stimulus induced Pol II recruitment and phosphorylation of CTD on Ser5 was also observed in cells transfected with Mut2 (Figure 7C). However, no phosphorylation at Ser2 could be detected even at the later times (Figure 7C). These latter results identify that the loss of CD80 promoter activity of Mut2 observed in Figure 6C is due to the inability of Pol II to enter into the elongation phase of transcription which, in turn, is critically dependent upon the integrity of the Sp1-binding site. Thus, while the assembly of a transcription initiation-competent PIC occurs on the upstream NF-κB target site, it is the Sp1-dependent positioning on the core promoter domain that permits its entry into the elongation phase.

Discussion

Our cumulative findings suggest that the obligatory nature of NF-κB recruitment, during the regulation of CD80 expression, arises from its ability to mediate two separate—but complementary—events. At one level, NF-κB engagement induces the displacement of C/EBPβ and Cdk8 although the mechanism by which this is achieved is presently unknown. The observed co-occurrence of C/EBPβ with Cdk8—a constituent of the repressive CRSP130/Sur2-based Mediator complex and its dissociation by 1 h poststimulation—suggests that it is this complex that maintains the *CD80* gene in its transcriptionally inactive form, in the absence of the appropriate exogenous stimulus.

Additionally, NF-κB recruitment was also central to formation of the PIC. Our data support that this function was achieved by providing the interacting surface necessary for this process. Importantly, incorporation of the NF-κB site within a positioned nucleosome also provided for another level of regulation during *CD80* expression. Thus in addition to NF-κB activation, induction of *CD80* was also dependent upon the activation of events leading to remodeling of this nucleosome.

Nucleosomes are known to regulate gene expression either through regulating access to the transcription initiation site (Agalioti *et al*, 2000), regulating access to promoter proximal enhancer elements (Weinmann *et al*, 1999), or by acting as a barrier to control the escape of Pol II from the promoter region (Soutoglou and Talianidis, 2002). In all of these examples, however, the regulatory nucleosomes were always positioned either at or in proximity to the start site of transcription. In contrast, the regulatory nucleosome on *CD80* promoter was positioned at several hundred nucleotides upstream from the transcription initiation site. Importantly, our recent results reveal that by controlling access of the NF-κB site, the nucleosome provides for a filtering mechanism that maintains signal specificity of *CD80* regulation. Thus, while a variety of cellular stimuli are known to activate NF-κB, only those that could simultaneously induce histone acetylation at the nucleosome were found to be capable of inducing *CD80* expression (Supplementary Figure 7).

It was particularly intriguing that a functional, initiation-competent, PIC could assemble on the NF-κB template in the absence of any assistance from the core promoter domain. This was confirmed in experiments wherein a deletion of the core promoter domain was found to have no significant effect either on PIC formation or its acquisition of an initiation-competent state. In this connection, previous studies have shown that the mammalian Pol II can naturally exist in a holoenzyme, and transcription factor-dependent recruitment of the Pol II holoenzyme in a single step has been demonstrated (Ossipow *et al*, 1999). Thus a similar single-step, NF-κB-mediated, recruitment of the Pol II holoenzyme complex may well explain the independent assembly of the PIC at the distal upstream site.

Functionally, the core promoter has been defined as the minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription by the Pol II machinery. Typically it is localized within a stretch that extends up to ~35 nt either upstream or downstream of the transcription start site, and contains one or more of sequence motifs that are capable of binding to either components of TFIID, or to TFIIB (Smale and Kadonaga, 2003). In the present case, however, PIC nucleation occurred at a distal site, with the conformation of promoter DNA being responsible for bringing it in proximity with the transcription start site. It was this NF-κB-mediated, and DNA conformation-dependent tethering of the PIC then, that collectively provided the cooperativity necessary to facilitate interactions between molecular components of the PIC and the core promoter-associated Sp1. While Sp1 is known to be capable of directly interacting with TFIID, the tandem presence of multiple Sp1-binding sites on a DNA sequence appears to be normally required for this (Smale and Kadonaga, 2003). In the present case, therefore, the inability of Sp1 to directly recruit TFIID may probably be due to the fact that a single Sp1 target site exists within the core promoter domain of TS1. In any event, it was the Sp1-mediated positioning that then ensured entry of Pol II into the elongation phase.

The expression of a given eucaryotic gene is generally considered to represent an involved process that is mediated through the coordinated assembly of a unique enhancer–transcription factor complex termed as the enhanceosome (Merika and Thanos, 2001). Activation of the *CD80* gene, however, presents a simplified case where NF-κB recruitment constitutes the sole controlling parameter. The remaining components of the enhanceosome, which include HMGI(Y) and at least some of the proteins bound to the TS1-flanking segments, were constitutively associated with the promoter prior to the activation of cells. This is somewhat similar, although not identical, to the situation observed with the TCR-α enhancer (Spicuglia *et al*, 2000). Unique about the present instance, however, is the three-dimensional context of this pre-assembly, which circumvents the need for a conventional core promoter element. Thus, while the upstream NF-κB site was responsible for recruiting a functional PIC, the role of the core-promoter bound Sp1 was restricted to positioning of this nucleosome-tethered PIC. This is schematically illustrated in Figure 7D.

In summary, this report delineates the mechanism of transcriptional activation of a gene that is devoid of all of

the known core promoter elements. Importantly, this mechanism presents several novel aspects, the most notable of which is that PIC assembly and its positioning occur in discrete steps, and involves spatially segregated sites. Indeed, at least from an operational perspective, the core promoter function of the murine *CD80* gene can be said to reside within two widely separated DNA sequence elements, with their cooperative action being dependent upon the promoter architecture. It will be of interest to determine whether such a discontiguous mode of transcriptional control also exists for other genes that lack canonical core promoter elements.

Materials and methods

Cell culture and reagents

J558L and A20 cell lines (ATCC) were maintained in RPMI 1640 (Invitrogen Corp.) supplemented with 10% fetal bovine serum and 1 × penicillin/streptomycin. Cells were stimulated with 1 mM H_2O_2 for 15 min, resuspended in fresh medium and further cultured for the indicated periods of time. In experiments evaluating importance of p65, J558L cells were pretreated for 2 h prior to stimulation with 250 μM NEMO and 300 μM Capsaicin. Cycloheximide (10 μg/ml) and TSA (100 nM) were purchased from (Sigma).

Total RNA was isolated with TRIZOL® and the manufacturer's instructions were followed for Real-time PCR (QuantiTect SYBR Green RT–PCR Kit, Qiagen), RT–PCR (QIAGEN One-Step RT–PCR), and 5′ RACE (Invitrogen Corp.). The source of the various antibodies used in this manuscript, as well as the sequences of primers used for the various experiments are listed in Supplementary Table 1.

General protocols

For site-directed mutagenesis, the QuikChange XL kit (Stratagene) was used in accordance with the protocol supplied by manufacturer, and *in vivo* DNase I footprinting was performed as described earlier (Thanos and Maniatis, 1995). The ChIP procedure was performed as per the manufacturer's instructions (Upstate Biotechnology) and precipitated DNA samples were analyzed by PCR (30–35 cycles) on a 1% agarose gel.

Nuclease digestions, LM-PCR, and nucleosome mapping

MNase and restriction enzyme digestion followed by LM-PCR was performed as described in Weinmann *et al* (1999) with slight modifications. The amplification PCR cycles were 20 (restriction enzyme), 18 (DNase I), and 22 (MNase).

Mononucleosomes were isolated from formaldehyde-fixed cells as described in Soutoglou and Talianidis (2002) with the modification that nuclei were digested with 150 U MNase at 37°C for 30 min. For Southern hybridization, 20 μg of purified MNase-digested DNA was analyzed.

siRNA

Target-specific siRNA for *Brm* and *Brg-1* (mouse) was purchased from Santa Cruz Biotechnology, Inc. and transfected using RNAiFect kit (Qiagen) at $10\,\mu g/5 \times 10^6$ cells as recommended by the manufacturer. The negative control siRNA was targeted against GFP. After 24 h in culture, ChIP analysis was performed. The inhibition in expression obtained for both cases was at least 75% as determined by Western blot analysis (Supplementary Figure 6).

Chromosome conformation capture assay

Chromosome conformation capture was performed as described in Dekker *et al* (2002) only with a change in enzyme, specific to this study. The reaction was diluted 15 times and ligation was preformed with T4 DNA ligase and detected by PCR.

Cyclization kinetics measurements

Ligation reactions were carried out with 0.5 μg/ml of PCR amplified DNA using Pfu (Stratagene), in the presence of 16 U/ml of T4 DNA ligase in standard buffer (Roche Inc.). Ligation products were analyzed by TBE–agarose gel electrophoresis and then stained with 0.5 μg/ml ethidium bromide.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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