Published online October 5, 2004

5280–5290 *Nucleic Acids Research, 2004, Vol. 32, No. 17*
doi:10.1093/nar/gkh856

Negative cross-talk between the human orphan nuclear receptor Nur77/NAK-1/TR3 and nuclear factor-κB

Hanna Harant[1,2,*] and Ivan J. D. Lindley[1]

[1]Novartis Institute for BioMedical Research, A-1235 Vienna, Austria and [2]Ludwig Boltzmann-Institute for Applied Cancer Research, Vienna, Austria

Received as resubmission July 25, 2004; Accepted September 10, 2004

ABSTRACT

The effect of orphan nuclear receptor Nur77 overexpression on activation of an interleukin-2 (IL-2) promoter–luciferase construct was analyzed in the human leukemic cell line Jurkat. Cotransfection of the IL-2 promoter construct together with the Nur77 expression plasmid resulted in a significant repression of IL-2 promoter activation compared to control cells. The repression by Nur77 requires the N-terminal activation function-1 domain. The repressive effect of Nur77 on IL-2 promoter activation is mediated through inhibition of the transcription factor complex nuclear factor-κB (NF-κB), since blocking or alteration of the IL-2 NF-κB binding sites resulted in abrogation of the repressive effect of Nur77. Moreover, further examination of a reporter gene construct containing multiple copies of the IL-2 CD28 response element (CD28RE) showed that Nur77 can inhibit transactivation mediated by the NF-κB components p65 and c-Rel. However, no effect of Nur77 was seen on p65-mediated transactivation of a construct containing multiple NF-κB binding sites of the HIV LTR. Our data suggest that Nur77 is able to block activation through NF-κB when bound to low-affinity NF-κB binding sites, such as those located in the IL-2 promoter.

INTRODUCTION

The orphan nuclear receptor Nur77 (NR4A1, NAK-1, TR3, NGFI-B) is an immediate-early response gene which is induced after stimulation with serum, growth factors and nerve growth factor (1,2). Nur77 and its closely related receptors Nurr1 (NR4A2) and Nor-1 (NR4A3) have the classical nuclear receptor structure, with an N-terminal activation function-1 (AF-1) domain, a highly homologous DNA-binding domain (DBD) and a similar ligand-binding domain (LBD), together with a C-terminal activation function-2 (AF-2) domain [reviewed in (3)]. Since no physiological ligands for these receptors have been identified, they are classed as 'orphan' nuclear receptors. Nur77, Nurr1 and Nor-1 can bind as monomers to a DNA sequence, the NBRE, or as homodimers to a palindromic sequence, the NurRE (4). Nur77 and Nurr1 can also form heterodimers with the retinoid X receptor (5).

In CD4/CD8 double-positive thymocytes and T cell hybridomas, Nur77 expression is induced through the T cell receptor (TCR) and is implicated in activation-induced apoptosis (3,6–9). Evidence exists that the DBD and a functional N-terminal AF-1 domain are required for its pro-apoptotic function, since induction of apoptosis by wild-type Nur77 can be inhibited by a dominant-negative mutant of Nur77 lacking the AF-1 domain (10,11). However, another study demonstrated that Nur77 can induce apoptosis in the prostate cancer cell line LNCaP also in the absence of the DBD, but causes cell death through translocation to the mitochondria and induction of cytochrome *c* release (12). A recent paper demonstrated that Nur77 can interact with Bcl-2, resulting in conversion of Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule (13). In thymocytes undergoing apoptosis, such mitochondrial targeting of Nur77 and cytochrome *c* release was not observed. In apoptotic thymocytes, Nur77 localizes to the nucleus, indicating that Nur77 acts by induction of genes involved in apoptosis. In fact, microarray analysis from thymocytes demonstrated that Nur77 induces known apoptotic genes, such as FasL and TRAIL, but also the novel genes Nur77 Downstream Gene 1 and 2 (NDG1 and NDG2) (14). In contrast to its role as a pro-apoptotic molecule, Nur77 has also been described as a survival factor when induced through tumor necrosis factor signaling (15). Nur77 is induced by various stimuli in different cell types, but its mere expression is not necessarily sufficient for the induction of apoptosis. Post-translational modification of the receptor may determine its pro-apoptotic activity, since phosphorylation within its DBD by Akt can inhibit its transcriptional activity and T cell apoptosis (16,17). Apart from its expression in double-positive thymocytes in mouse, Nur77 has been shown to be inducible through activation in human peripheral T lymphocytes, together with Nurr1 and Nor-1 (18,19). It is therefore likely that Nur77 plays a role in T cell activation and activation-induced apoptosis in these cells.

Of the transcription factor complexes involved in T cell activation, nuclear factor-κB (NF-κB) clearly plays an important role. Members of the NF-κB family are the proteins p50

*To whom correspondence should be addressed. Tel: +43 1 86634 330; Fax: +43 1 86634 727; Email: Hanna.Harant@novartis.com

 © Oxford University Press 2004; all rights reserved

(NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel and RelB [reviewed in (20)]. Various homo- or heterodimers can bind to NF-κB recognition sites located in regulatory regions of many genes, but the most common complex is the p50/p65 heterodimer. Activation of NF-κB is associated with phosphorylation and proteolytic degradation of the inhibitory protein IκB through activation of the IκB kinase (IKK) complex [reviewed in (21,22)].

In this study we investigate the function of Nur77 at the molecular level in activated T cells. We show that overexpression of Nur77 in the human T cell leukemia cell line Jurkat can repress activation of an IL-2 promoter–luciferase construct via negative regulation of the transcription factor NF-κB. This repression is dependent on the N-terminal sequences of Nur77, which comprise the AF-1 domain.

MATERIALS AND METHODS

Generation of Nur77 expression plasmids and deletion mutants

The Nur77 cDNA was cloned by PCR from human T lymphocytes stimulated with PMA using the sense primer 5′-CATCATGGATCCCAGGGGCAGCGGGAGCCGGCCG-GAGATGCCCTGTATCCAAG-3′ and the antisense primer 5′-CATCATGAATTCTCAGAAGGGCAGCGTGTCCAT-3′ (restriction sites for cloning are underlined). The fragment was digested with Bam HI and Eco RI and subcloned into the Bam HI and Eco RI sites of the pcDNA3.1 (+) expression vector (Invitrogen). N-terminal deletion mutants of Nur77 were created by PCR from pcDNA3.1–Nur77 by using the following primers. Nur77 amino acids 51–598 was generated using the sense primer 5′-CATCATGGATCCATGCCCAGCTT-CAGCACCTTCATG-3′. Nur77 amino acids 101–598 was generated using the primer 5′-CATCATGGATCCATGTT-CAAGTTCGAGGACTTCC-3′. Nur77 amino acids 151–598 was generated using the primer 5′-CATCATGGATCCAT-GCTCTCTCCCTGGGATGGCTCC-3′. For PCR, the antisense primer for amplification of the Nur77 cDNA was used.

C-terminal deletion mutants were created using the sense primer for amplification of Nur77 and different antisense primers. For Nur77 amino acids 1–578 the primer 5′-CATCATGAATTCTCACAGCTTGAGGTAGAAGATGCG-3′ was used. For Nur77 amino acids 1–380 the primer 5′-CATCATGAATTCTCAGGCAGTGCTGGGCCCTGAG-3′ was used. All PCR fragments were digested with Bam HI and Eco RI and cloned into the Bam HI and Eco RI sites of the pcDNA3.1 (+) vector.

The p65 and c-Rel expression plasmids were kindly provided by W. C. Greene (Gladstone Institute, San Francisco). The constructs p65 wt, p65 M1 and p65 M2 were generated by PCR from the p65 expression plasmid using the following primers (sites for cloning are underlined). p65 sense 5′-CAT-CATAAGCTTATGGACGAACTGTTC-3′; p65 antisense 5′-CATCATTCTAGATTAGGAGCTGATCTG-3′; p65 M1 antisense 5′-CATCATTCTAGATCACCCAGCCTGGGTG-GG-3′; p65 M2 antisense 5′-CATCATTCTAGATCAA-TATGTCCTTTTACG-3′. PCR products were cloned after digestion into the Xba I and Hind III sites of the pcDNA3.1 vector.

Reporter gene constructs

The 5′-flanking region of the human IL-2 gene (−471 to +49 relative to the transcription start) was cloned upstream of the firefly luciferase coding region into the pGL2-basic vector (Promega). The IL-2 promoter was cloned using the primer 5′-CATCATCCCGGGATAAAAAGGTAAAACCAT-3′ and the primer 5′-CATCATGGTACCGCAGGAGTTGAGGT-TAC-3′ (sites for cloning are underlined). The promoter sequence was amplified by PCR from an IL-2 promoter template. The digested PCR product was then subcloned into the Sma I and Kpn I sites of the pGL2-basic vector.

Mutations were introduced into transcription factor binding sites located in the IL-2 promoter sequence by overlapping PCR. The IL-2 promoter containing the disrupted NF-κB binding site within the CD28 response element (CD28RE; −159 to −156 nt relative to the transcription start) was generated using the original sense and the antisense primer 5′-TGACTCTTTGGccccTCTTTAAACCC-3′, and the primer 5′-CCAAAGAGTCATCAGAAGAGG-3′ together with the original antisense primer. The mutant, containing a disrupted NF-κB binding site (−202 to −199 nt relative to the transcription start) was generated using the original sense primer and the antisense primer 5′-CCAAAGACTGACTGAATGGATG-TAGGTGccccCCCTCTTTG-3′, and the primer 5′-CCTA-CATCCATTCAGTCAGTCTTTGG-3′ together with the original antisense primer. Conversion of the NF-κB binding site of the IL-2 promoter into the consensus NF-κB binding site of the HIV LTR was generated using the original sense primer and the antisense primer 5′-CCTACATCCATTCAGT-CAGTCTTTGG-3′ and 5′-CCAAAGACTGACTGAATG-GATGTAGGggaaagtcccCTTTG-3′ together with the original sense primer. Conversion of the NF-κB binding site of the CD28RE into the HIV LTR NF-κB binding site was created using the original antisense primer and sense primer 5′-CCAAAGAGTCATCAGAAGAGG-3′, and the primer 5′-TGACTCTTTggaaagtcccTAAACCC-3′ together with the original sense primer. All constructs were cloned into the Sma I and Kpn I sites of the pGL2-basic vector and were confirmed by sequencing.

The 4×NBREtkLUC contains four copies of the sequence 5′-GATCTTCGTGCGAAAAGGTCAAGCGCTAG-3′ fused to a 155 bp. fragment (105 to +50 relative to the transcription start) of the Herpes simplex I thymidine kinase (tk) promoter in the pGL2 basic vector. The construct 4×CD28REtkLUC contains four copies of the sequence 5′-GATCTGTTTAAA-GAAATTCCAAAGAGTCATCAG-3′ fused to the tk promoter in the pGL2 basic vector. The plasmid pRL-CMV was purchased from Promega. The plasmid pRL-tk and the truncated IL-8 promoter constructs were described previously (23). The plasmid pNF-κB-LUC was obtained from Stratagene.

Cell culture

Jurkat cells were cultivated in RPMI 1640 supplemented with 10% FCS and passaged twice a week. For transfection, 1×10^7 cells were collected by centrifugation, washed once with 2 ml phosphate-buffered saline (PBS) def., and resuspended in 100 μl PBS def. Plasmid DNA was added to a gene pulse cuvette (0.2 cm; BioRad) before addition of the cell suspension. For transfection with the IL-2 promoter constructs, 10 μg of IL-2

promoter constructs and 10 μg of the individual expression plasmids were used. Electroporation was carried out at 120 V and 960 μF. After electroporation, cells were resuspended in 2.5 ml of prewarmed RPMI 1640 + 10% FCS. 100 μl were dispensed into each well of a 96 well plate. Twenty hours post-transfection, cells were stimulated with 10 ng/ml PMA and 0.5 μg/ml ionomycin for 24 h. For stimulation with anti-CD3 and anti-CD28 antibodies, anti-CD3 antibody (Pharmingen) was coated overnight to white 96 well plates at a concentration of 10 μg/well in a volume of 100 μl. The next day, cells were added to the well and incubated with anti-CD28 (Pharmingen) antibody at a concentration of 5 μg/ml. After stimulation, cells were lysed directly in the plates and the lysates analyzed for luciferase activity using the Top count scintillation counter (HP) or the Microbeta Wallac Jet (Wallac). Individual conditions for cotransfection experiments using 600 ng of pRL-CMV or pRL-tk as internal controls are described in the figure legends. Firefly luciferase and renilla luciferase activities were measured using the Dual-luciferase assay system (Promega).

Preparation of nuclear extracts and western blot analysis

Nuclear extracts were prepared from 1×10^7 transfected Jurkat cells. Cells were centrifuged briefly and then resuspended in 400 μl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.15 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). After incubation on ice for 10 min, cells were passed three times through a 27 gauge needle. Nuclei were then sedimented by centrifugation at 14 000 r.p.m. for 30 s, followed by extraction in 25 μl of buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.42 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) for 15 min on ice with occasional vortexing. After centrifugation, the supernatant was collected and 75 μl of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) was added. For western blot analysis, 10 μg nuclear extracts were used. Western blot analysis was performed according to standard protocols. For detection, a goat anti-p50 antiserum (Santa Cruz) was used at a dilution of 1:500, a rabbit anti-p65 antiserum (Santa Cruz) was used at a 1:1000 dilution and a mouse anti-c-Rel antibody (Santa Cruz) was used at a dilution of 1:250.

Electrophoretic mobility shift assays (EMSA) were performed as described previously (23). The following double-stranded oligonucleotides were used.

NF-κB consensus (HIV LTR): 5′-GATCTTGTTACAAGG-GACTTTCCGCG-3′
NF-κB IL-2: 5′-GATCCAAAGAGGGATTTCACCTACA-3′
CD28RE: 5′-GATCTGTTTAAAGAAATTCCAAAGAGT CATCAG-3′
SP-1: 5′-GATCTATTCGATCGGGGCGGGGCGAGCG-3′.

For supershift analysis, 2 μl of rabbit anti-p65 or 2 μl of rabbit anti-p50 polyclonal serum (Santa Cruz) were used.

Western blot analysis from HEK293 cell lysates

For western blot analysis of the constructs used, 2.4×10^5 HEK293 cells were transfected with 170 ng of the individual expression plasmids and 3.4 μl lipofectamine in 6 well plates. Total cell lysates were obtained 48 h post-transfection and analyzed by western blot according to standard methods. For detection of the N-terminal truncated versions of Nur77, a rabbit anti-Nur77/Nurr1 antiserum (E-20; Santa Cruz) was used. For detection of the C-terminal deletion mutants, a mouse monoclonal antibody against mouse Nur77 (Pharmingen) was used. For detection of p65 and its C-terminal deletion mutants, a mouse anti-p65 antibody (Santa Cruz) was used.

RESULTS

Repression of IL-2 promoter activation by Nur77

We investigated the effect of Nur77 in the human T cell leukemia line Jurkat by cotransfection of a Nur77 expression plasmid together with various different promoter constructs. The activity of a Nur77 expression plasmid was confirmed by cotransfection of Jurkat cells with a luciferase reporter gene construct, containing four copies of the NBRE fused to a minimal tk promoter (4×NBREtkLUC), resulting in a 90-fold activation of reporter gene expression (Figure 1A). Reporter gene expression driven by the NBRE was also strongly enhanced by stimulation of Jurkat cells with PMA + ionomycin (Figure 1B), which induces expression of Nur77 (19).

We observed that PMA + ionomycin-mediated activation of an IL-2 promoter–luciferase construct (−471 to +49 nt relative to the transcription start) was inhibited by overexpressed Nur77, between 70 and 90% compared to pcDNA3.1-transfected cells. This repression of IL-2 promoter was observed after 6 h (Figure 1C) and 24 h (Figure 1D) with the effect being more pronounced after 24 h. Activation of the IL-2 promoter was also inhibited by transfected Nur77 when cells were stimulated with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody (Figure 1E).

Repression of IL-2 promoter activation by Nur77 is dependent on the N-terminal sequences

To analyze the mechanism responsible for the repression of IL-2 promoter activation by overexpressed Nur77, expression constructs encoding N- and C-terminal truncations of Nur77 were used for cotransfection studies (Figure 2A). The N-terminal amino acids comprising the AF-1 domain were sequentially removed, while retaining the DBD and LBD of the receptor. Cotransfection with full-length Nur77 resulted in 70% repression of IL-2 promoter activation and a similar or enhanced effect was seen after deletion of the 50 N-terminal amino acids (Nur77 amino acids 51–598). However, deletions of 100 (Nur77 amino acids 101–598) and 150 N-terminal amino acids (Nur77 amino acids 151–598) resulted in a reduced inhibition, leading to 60 and 40% repression of IL-2 promoter activation, respectively (Figure 2C). Thus, even removal of 150 N-terminal amino acids could not completely reverse the repressive effect. The extreme C-terminus of Nur77, which represents the AF-2 domain, was removed by deletion of 20 amino acids (Nur77 amino acids 1–578), and a second C-terminal Nur77 deletion mutant (Nur77 amino acids 1–380), which lacks large parts of the LBD, was also generated. A 60% repression of IL-2 promoter activation by

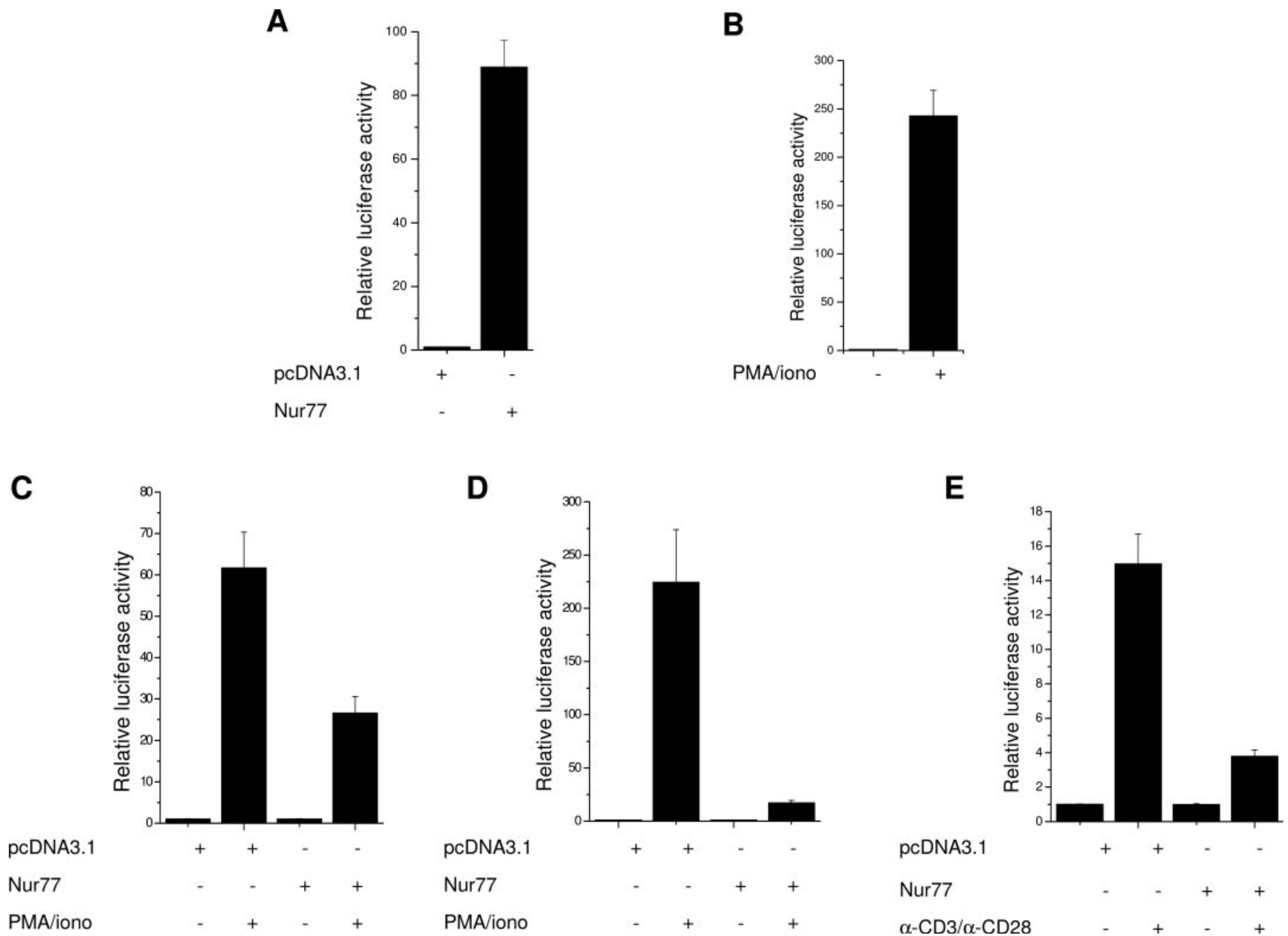

Figure 1. Effect of Nur77 overexpression on activation of the IL-2 promoter. (**A**) Jurkat cells were cotransfected with pcDNA3.1 alone or pcDNA3.1–Nur77 with the 4×NBREtkLUC and pRL-CMV plasmids. Firefly luciferase activities were normalized to Renilla luciferase activities. Luciferase activity relative to the pcDNA3.1-transfected control is shown. (**B**) Activation of the 4×NBREtkLUC through endogenous Nur77 was analyzed by transfection of 4×NBREtkLUC and stimulation with PMA + ionomycin (iono) for 24 h. Luciferase activities relative to untreated controls are shown. Data are means ± SD from three independent transfections. Jurkat cells were cotransfected with the IL-2 promoter together with either pcDNA3.1 alone or pcDNA3.1–Nur77. Twenty hours post-transfection, cells were stimulated with PMA + ionomycin (iono) for 6 h (**C**), for 24 h (**D**) or with plate-bound anti-CD3 and soluble anti-CD28 antibody for 24 h (**E**). Luciferase activities relative to untreated controls are shown. Data are means ± SD from six independent transfections.

Nur77 amino acids 1–578 and approximately 70% repression of IL-2 promoter activation by Nur77 amino acids 1–380 was observed. These data indicate that the N-terminal amino acids of Nur77 participate in the repressive effect on IL-2 promoter activation, while the C-terminal putative LBD appears to be dispensable (Figure 2C). The functionality of the constructs was further confirmed by cotransfection with the 4×NBREtkLUC reporter gene plasmid. While full-length Nur77 caused 90-fold activation of NBRE-driven luciferase expression, removal of the 50 N-terminal amino acids, as in construct Nur77 amino acids 51–598, reduced its transactivation ability to yield only 50-fold activation. Removal of 100 (Nur77 amino acids 101–598) and 150 (Nur77 amino acids 151–598) N-terminal amino acids resulted in a severe decrease in transactivation, down to a residual 15- to 20-fold activation of luciferase expression. Removal of C-terminal amino acids caused approximately 40-fold activation of NBRE-driven reporter gene expression (Figure 2D).

All constructs used in the experiments described above were analyzed in HEK293 cells transfected with the individual Nur77 mutants. Since expression of Nur77 and its mutants was relatively low in Jurkat cells, HEK293 cells were chosen as an alternative, because of the high transfection efficiencies obtained with this line. Western blot analysis demonstrated that each of the Nur77 mutants was expressed at levels comparable to that of the full-length Nur77 (Figure 2B).

IL-2 promoter repression by Nur77 is mediated via NF-κB

We wished to determine the mechanism that underlies the repressive activity and asked whether overexpressed Nur77

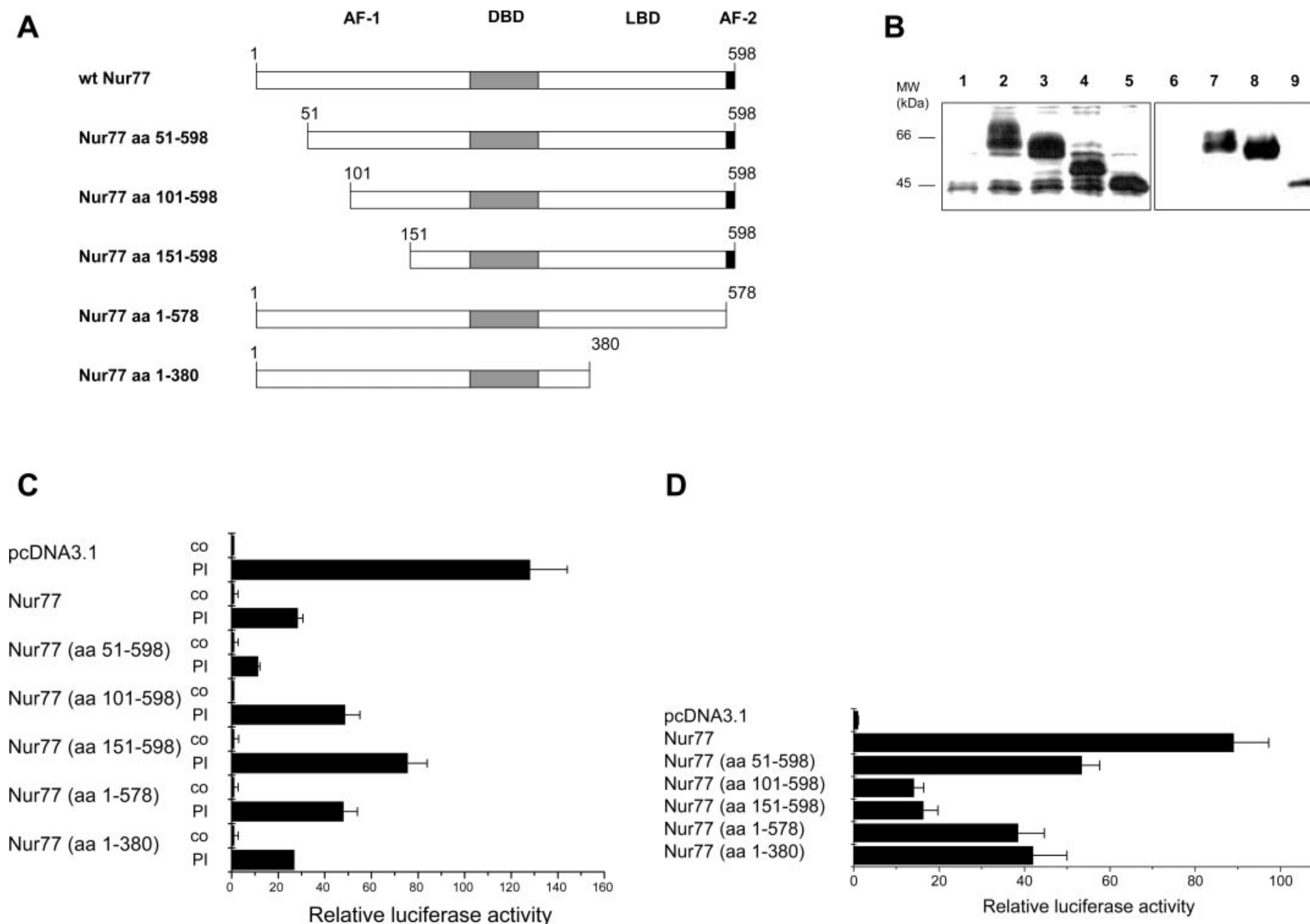

Figure 2. Effect of various deletion mutants of Nur77 on activation of the IL-2 promoter. (**A**) A schematic representation of the individual constructs is shown. (**B**) HEK293 cells were transfected with the individual Nur77 deletion mutants. Expression levels were analyzed by western blot analysis. Lane (1) pcDNA3.1, (2) Nur77, (3) Nur77 (amino acids 51–598), (4) Nur77 (amino acids 101–598), (5) Nur77 (amino acids 151–598), detected with the anti-Nurr1 antiserum; (6) pcDNA3.1, (7) pcDNA3.1–Nur77, (8) Nur77 (amino acids 1–578), (9) Nur77 (amino acids 1–380), detected with the anti-Nur77 antibody. (**C**) Jurkat cells were cotransfected with the IL-2 promoter construct together with expression plasmids encoding Nur77 or Nur77 deletion mutants and stimulated with PMA + ionomycin (iono) for 24 h. Luciferase activities relative to untreated controls are shown. (**D**) Jurkat cells were cotransfected with the 4×NBREtkLUC plasmid, pRL-CMV and the individual Nur77 deletion mutants. Firefly luciferase activities were normalized to Renilla luciferase activities. Luciferase activities relative to pcDNA3.1-transfected controls are shown. Data are means ± SD from four independent transfections.

interferes with transcription factors involved in the regulation of IL-2 promoter activation. We analyzed the effect of transfected Nur77 on activation of reporter gene constructs, representing multiple binding sites for NFAT and AP-1 transcription factors, which are essential for the regulation of IL-2 promoter activity. However, Nur77 overexpression had no effect on constructs monitoring activation of NFAT or AP-1 (data not shown). We also analyzed the effect of Nur77 overexpression on activation of a reporter gene construct driven by four copies of the CD28RE of the IL-2 promoter (4×CD28REtkLUC), which represents a low-affinity binding site for NF-κB upstream of a non-consensus AP-1 binding site (24). Cotransfection with the Nur77 expression plasmid resulted in a 40% repression of PMA + ionomycin-induced CD28RE-driven reporter gene expression (Figure 3A). The NF-κB binding site of the CD28RE and the NF-κB binding site of the interleukin-8 (IL-8) promoter are very similar and have been shown to be preferentially bound by p65- or c-Rel homodimers or p65-c-Rel heterodimers (25). Although the effect of Nur77 overexpression on activation of the IL-8 promoter was less pronounced than its effect on the IL-2 promoter, a 55% repression of PMA + ionomycin-induced expression of luciferase was observed (Figure 3B). However, cotransfection of Nur77 had no effect on expression of luciferase driven by five copies of a different NF-κB binding site, that of the HIV LTR (pNF-κB-LUC) (Figure 3C).

We next asked whether activation of these promoter constructs by overexpression of the NF-κB subunit p65 can be repressed by cotransfected Nur77. Strong activation of luciferase expression was seen when the construct 4×CD28REtkLUC was cotransfected with the p65 expression plasmid. When Nur77 was coexpressed, a significant, reproducible 80% reduction of this p65-mediated transactivation was observed (Figure 3D). However, Nur77 expression had no effect on p65-mediated activation of the construct with five copies of the HIV LTR NF-κB site (pNF-κB-LUC), analogous to the PMA + ionomycin stimulation of this construct (Figure 3E). The transfections were performed under identical

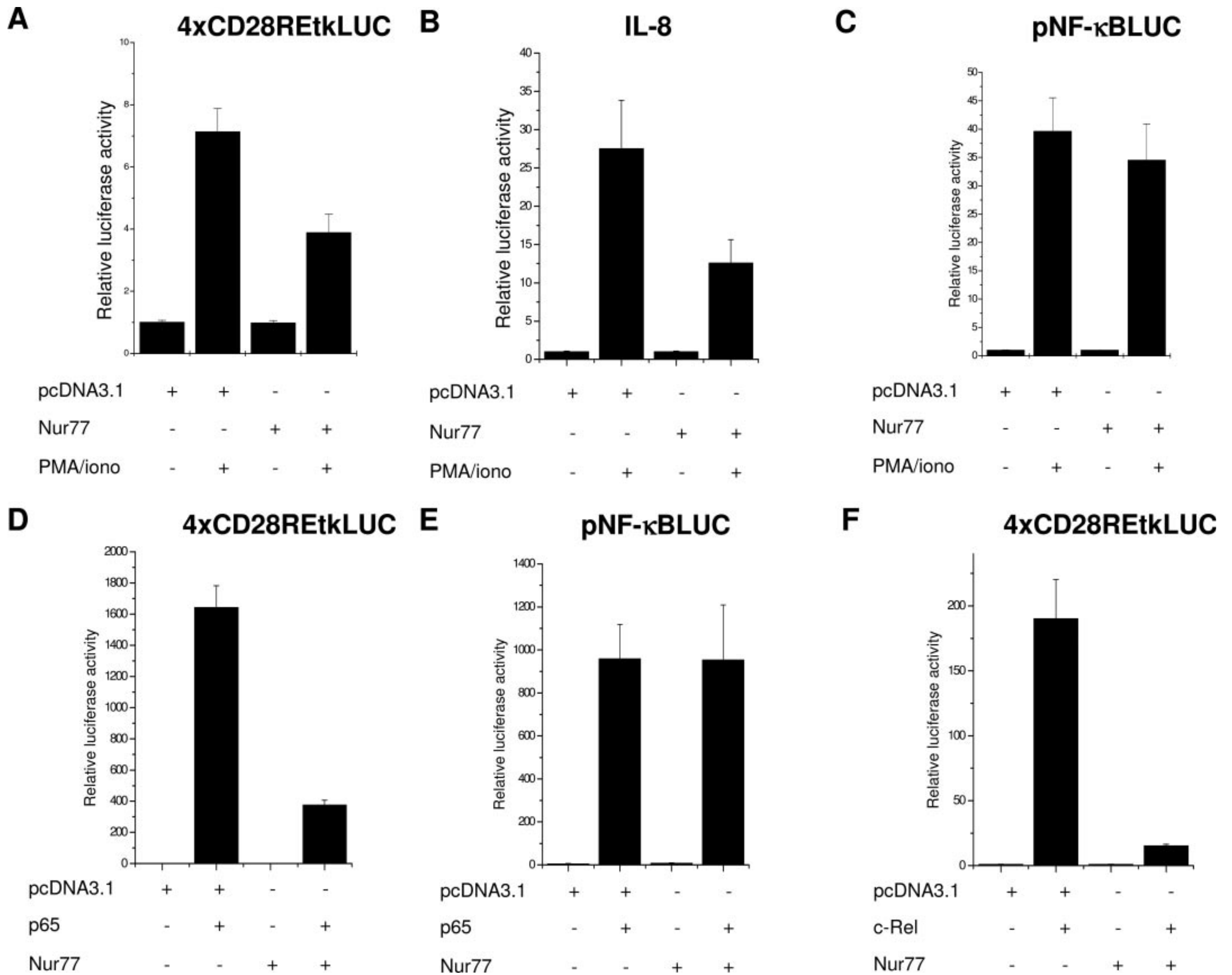

Figure 3. Effect of Nur77 overexpression on activation of constructs containing different binding sites for NF-κB. Jurkat cells were cotransfected with various reporter gene constructs representing (**A**) four copies of the CD28RE of the IL-2 promoter (4×CD28REtkLUC), (**B**) a truncated IL-8 promoter, (**C**) a construct containing five binding sites for NF-κB (pNF-κB-LUC) and stimulated with PMA + ionomycin (iono) for 24 h. Jurkat cells were cotransfected with (**D**) 4×CD28REtkLUC or (**E**) pNF-κB together with a p65 expression plasmid and pRL-tk, in presence and absence of Nur77. (**F**) Jurkat cells were cotransfected with 4×CD28REtkLUC together with a c-Rel expression plasmid and pRL-tk in the presence and absence of Nur77. In transfections containing pRL-tk, firefly luciferase activities were normalized to Renilla luciferase activities. Relative firefly luciferase activities compared to vector-transfected controls are shown. Data represented are means ± SD from four individual transfections.

conditions and in parallel. We also analyzed the effect of c-Rel on activation of CD28RE-dependent reporter gene expression in the presence and absence of Nur77. While c-Rel overexpression strongly activated luciferase expression, overexpression of Nur77 caused a significant repression of this effect (Figure 3F), suggesting that both p65 and c-Rel can interfere with Nur77.

These experiments provided evidence that Nur77 targets the NF-κB binding sites of the IL-2 promoter, located within the CD28RE (−161 to −154 nt relative to the transcription start) and further upstream (−205 to −198 nt relative to the transcription start). These sites were analyzed in more detail, by introducing mutations into the IL-2 promoter (Figure 4A). Mutation destroying either of the NF-κB binding sites attenuated IL-2 promoter activation, but did not reverse the repressive effect caused by cotransfection of Nur77 (Figure 4B). Mutations introduced into both NF-κB binding sites resulted in an IL-2 promoter construct, which was not inducible, thus preventing analysis of the effect of Nur77. Other binding sites for the transcription factors NFAT, AP-1, SP-1 and octamer-binding factors were also mutated, but had no effect on the repressive action of Nur77 (data not shown). To analyze whether the sequence of the NF-κB binding sites of the IL-2 promoter plays a role in the repressive effect of Nur77, these sites were changed into the consensus NF-κB binding site of the HIV LTR. This sequence was chosen since it is present in the construct containing multiple NF-κB binding sites, where activation was unaffected by overexpressed Nur77. When both IL-2 NF-κB binding sites were altered to the HIV LTR NF-κB binding site, no Nur77-mediated repression was observed

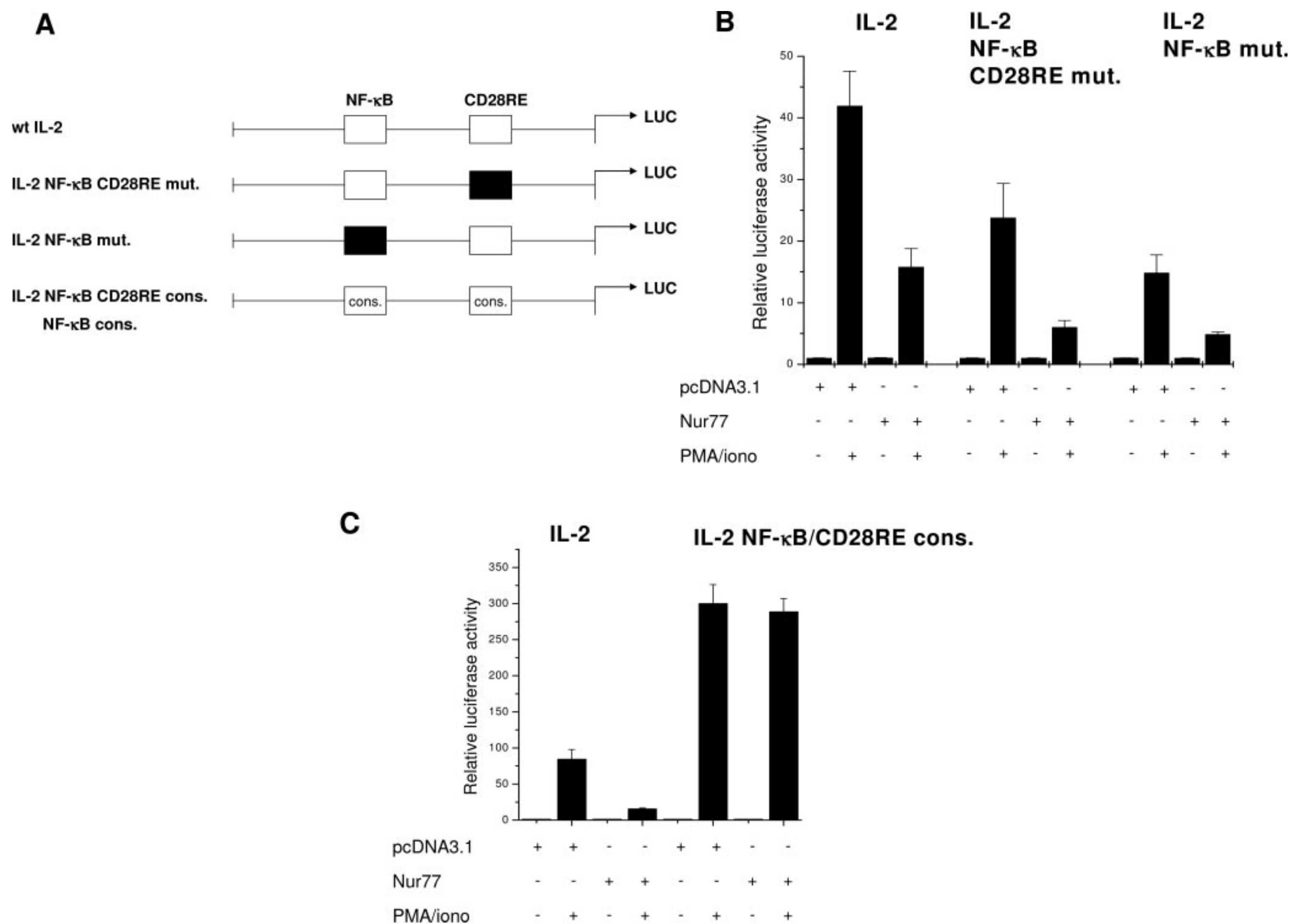

Figure 4. Effect of Nur77 overexpression on activation of mutated IL-2 promoter constructs. (**A**) A schematic representation of the individual IL-2 promoter constructs is shown. Jurkat cells were cotransfected with various mutated IL-2 promoter constructs and either pcDNA3.1 or pcDNA3.1–Nur77. (**B**) Jurkat cells were transfected with either the IL-2 promoter, IL-2 promoter containing a mutated NF-κB binding site within the CD28RE (IL-2 NF-κB CD28RE mutant) or an IL-2 promoter construct containing a mutated NF-κB binding site (IL-2 NF-κB mutant); (**C**) cells were transfected with either the IL-2 promoter construct or an IL-2 promoter construct with both NF-κB binding sites changed to the consensus NF-κB binding site (IL-2 NF-κB CD28RE consensus NF-κB consensus). Cells were stimulated with PMA + ionomycin (iono) for 24 h. Data represented are means ± SD from three (B) and four (C) independent transfection experiments.

(Figure 4C). These results indicate that the NF-κB binding sites of the IL-2 promoter are indeed involved in the repressive effect of Nur77.

Next, cotransfections with C-terminal truncations of NF-κB p65 together with the IL-2 promoter were performed, with the aim of blocking binding of endogenous NF-κB to its binding sites in the IL-2 promoter. The p65 C-terminal mutation 1 (p65 M1 amino acids 1–431) lacks C-terminal amino acids representing the transactivation domains, while the p65 C-terminal mutation 2 (p65 M2 amino acids 1–305) lacks larger parts of the C-terminal domains (26). When the constructs were cotransfected with the NF-κB-dependent reporter gene construct pNF-κB-LUC, stimulation of luciferase expression by PMA + ionomycin was significantly inhibited (Figure 5A). This result indicates that the mutants are able to compete for binding of endogenous NF-κB to its binding site. The expression of the mutants was further confirmed by western blot analysis using lysates obtained from transfected HEK293 cells (Figure 5D). When the p65 mutants were cotransfected with the IL-2 promoter, a decrease in inducibility by PMA + ionomycin was observed. While p65 M1 inhibited activation of the IL-2 promoter by only 35%, some further decrease was still observed in the presence of coexpressed Nur77. However, cotransfection with p65 M2 inhibited IL-2 promoter activation by 65%, and overexpressed Nur77 did not cause further repression (Figure 5B). The p65 mutant M1 was able to slightly transactivate the CD28RE. Cotransfection with Nur77, however, did not inhibit transactivation by p65 M1 (Figure 5C). These data indicate that the C-terminal domain of p65 is required for repression by Nur77.

We also analyzed the effect of p65 on activation of the NBRE by Nur77. Cotransfection of 4×NBREtkLUC and Nur77 in the presence of increasing amounts of p65 expression plasmid decreased activation of the NBRE-dependent luciferase expression (Figure 6A). However, cotransfection with the p65 mutants, p65 M1 or p65 M2, did not reduce NBRE activation by Nur77 (Figure 6B).

To analyze whether Nur77 can influence translocation or DNA binding of NF-κB proteins, Jurkat cells were transfected with the Nur77 expression plasmid and nuclear extracts were

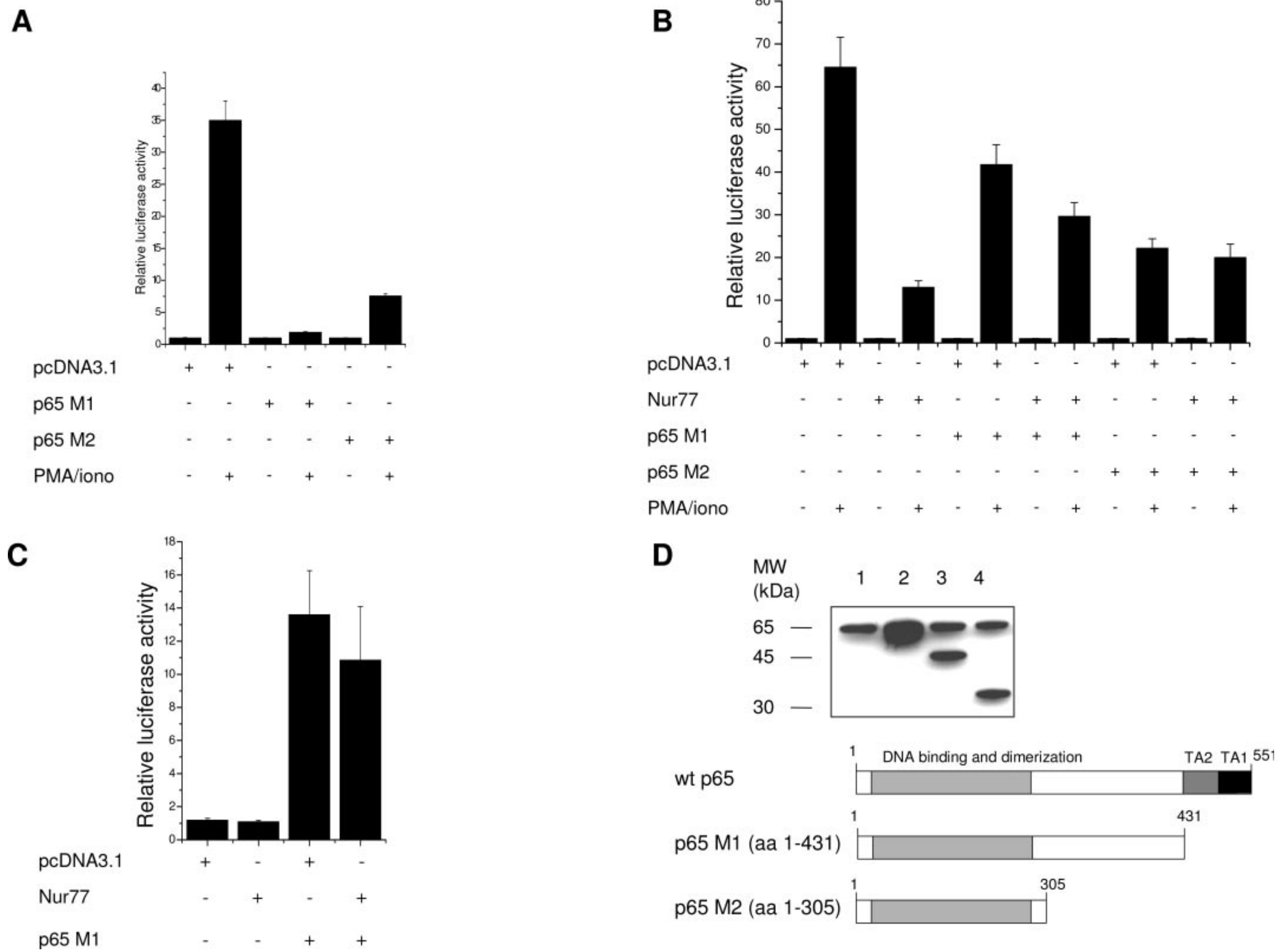

Figure 5. The repressive effect of Nur77 on IL-2 promoter activation can be overridden by cotransfection of NF-κB p65 deletion mutants. (**A**) Jurkat cells were cotransfected with pNF-κB and p65 M1 or p65 M2. Cells were stimulated with PMA + ionomycin (iono) for 24 h. Means ± SD from two independent experiments are shown. (**B**) Jurkat cells were cotransfected with the IL-2 promoter, pcDNA3.1-p65 M1 or pcDNA3.1-p65 M2 with or without the Nur77 expression plasmid. Cells were stimulated with PMA + ionomycin (iono) for 24 h. Luciferase activities relative to untreated controls are shown. Means ± SD from three independent experiments are shown. (**C**) Jurkat cells were cotransfected with 4×CD28REtkLUC and either pcDNA3.1 or pcDNA3.1-p65 M1 with or without Nur77 expression plasmid. Cells were stimulated with PMA + ionomycin (iono) for 24 h. Luciferase activities relative to untreated controls are shown. Means ± SD from three independent experiments are shown. (**D**) HEK293 cells were transiently transfected with the individual p65 expression constructs. Total lysates were subjected to western blot analysis. Lane 1, pcDNA3.1; lane 2, pcDNA3.1-p65; lane 3, pcDNA3.1-p65 M1; lane 4, pcDNA3.1-p65 M2. A schematic representation of the individual p65 constructs is shown.

prepared from unstimulated cells and cells stimulated with PMA + ionomycin for 4 h to induce activation of NF-κB. Nuclear extracts were then analyzed in EMSA experiments for binding of NF-κB to the three types of binding sites used in this study. A reduction of the complex binding to the IL-2 NF-κB binding site and NF-κB binding site of the CD28RE was seen in Nur77-transfected cells, stimulated with PMA + ionomycin (Figure 7A). In addition, the presence of the NF-κB proteins p50, p65 and c-Rel in the nucleus was analyzed by western blot analysis, and no difference in nuclear localization between pcDNA3.1- or Nur77-transfected cells after stimulation with PMA + ionomycin was observed (Figure 7A).

Finally, the strength of NF-κB binding to the individual binding sites from nuclear extracts of PMA + ionomycin stimulated cells was examined. While there is very strong specific binding of NF-κB to the consensus NF-κB binding site of the HIV LTR, there is only a weak complex seen when the IL-2 NF-κB binding site or the CD28RE NF-κB binding site are used in EMSA analysis. Competition with increasing amounts of unlabeled oligonucleotide representing the HIV LTR NF-κB binding site showed that binding to both the IL-2 NF-κB and the CD28RE NF-κB binding sites is readily competed by the unlabeled oligonucleotide (Figure 7B). In summary, our data indicate that the inhibitory effect of Nur77 is mediated via the IL-2 NF-κB sites, and that the conversion of the two low-affinity NF-κB binding sites of the IL-2 promoter into two high-affinity binding sites of the HIV LTR overrides this inhibitory effect. It can therefore be speculated that Nur77 is an inhibitor of NF-κB, when bound to low-affinity binding sites.

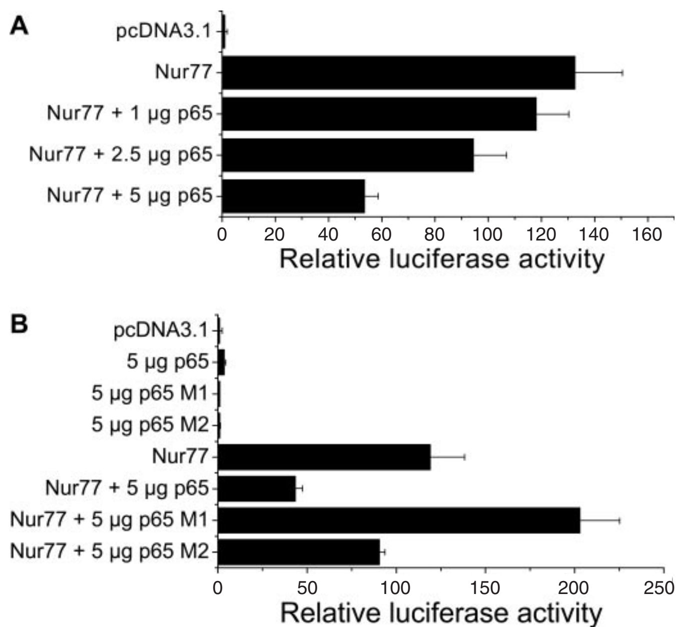

Figure 6. Repression of NBRE activation by p65. (**A**) Jurkat cells were cotransfected with 4×NBREtkLUC and either pcDNA3.1 or pcDNA3.1–Nur77 and increasing amounts of p65, and with pRL-tk as internal control. (**B**) Jurkat cells were cotransfected with 4×NBREtkLUC and either pcDNA3.1 or pcDNA3.1–Nur77 and the different p65 mutants, and pRL-tk. Firefly luciferase activities were normalized to Renilla luciferase activities. Relative firefly luciferase activities compared to vector-transfected controls are shown. Data represented are means ± SD from three individual transfections.

DISCUSSION

The human orphan nuclear receptor Nur77 is crucially involved in activation-induced apoptosis in immature double-positive thymocytes and T cell hybridomas (6,7,9). Since Nur77 has been shown to be expressed in human peripheral T lymphocytes after activation (18,19), it is likely to be involved in the regulation of proliferation and activation-induced cell death also in mature T cells. In this investigation, we analyzed the effects of Nur77 at the molecular level in an activated T cell, using the human T cell leukemia cell line Jurkat. These cells express Nur77, Nurr1 and Nor-1 after stimulation with anti-CD3 or chemical stimuli, such as PMA + ionomycin [(19) and *own observations*]. We observed that overexpression of Nur77 resulted in a clear, reproducible repression of IL-2 promoter activation by PMA + ionomycin or anti-CD3 and anti-CD28 antibodies compared to vector-transfected cells.

Nur77 has the classical nuclear receptor structure of a DBD and LBD, and a long N-terminal domain, designated the AF-1 domain. This domain has been implicated in the constitutive, 'ligand-independent' activation by Nur77, since mutational analysis has demonstrated that it is essentially involved in transactivation by the receptor (6). The AF-1 domain has been shown to recruit several cofactors, which influence cell-specific transactivation by this nuclear receptor (27). By the use of N- and C-terminal truncated versions of Nur77 we were able to link its ability to transactivate via the NBRE and to repress IL-2 promoter activation, suggesting that the same domains are involved in both activities. These data indicate that Nur77 can repress IL-2 promoter activation only when it is transcriptionally competent, as has also been proposed for the induction of apoptosis (10).

Using mutations in the IL-2 promoter, we could identify the two NF-κB binding sites as the targets of the repressive action of Nur77. One binding site for NF-κB is located at −205 to −198 nt relative to the transcription start (28). The second NF-κB binding site is located within the CD28RE, which contains a low-affinity binding site for NF-κB adjacent to an AP-1 site, but which has also been shown to be bound by NFATp (29–31). The NF-κB family members p50, p65 and c-Rel can bind to the CD28RE NF-κB site, but it is preferentially bound by either p65- or c-Rel-homodimers, or p65- and c-Rel-heterodimers (31). The CD28RE NF-κB binding site is almost identical in sequence to the IL-8 NF-κB binding site (25). We saw a repression of both the CD28RE and IL-8 promoter activation by coexpressed Nur77. However, activation of a construct containing multiple binding sites of the HIV LTR NF-κB element was not influenced by Nur77. The HIV LTR NF-κB binding site has been shown to be preferentially bound by the p50/p65 heterodimer or a p50/p50 homodimer (32). In addition, conversion of the two IL-2 promoter NF-κB binding sites into the sequence of the NF-κB element in the HIV LTR resulted in a construct that was not repressed by Nur77. While the two NF-κB sites of the IL-2 promoter are both low-affinity binding sites, the HIV LTR NF-κB element has been demonstrated to be bound by NF-κB with high-affinity, a finding which we confirmed by EMSA. One reason for the repressive effect of Nur77 on the IL-2 promoter NF-κB element may be that it is not able to repress NF-κB when tightly bound to an element, such as that in the HIV LTR.

When we activated the CD28RE reporter construct by overexpression of p65, a clear repression of luciferase expression was seen when Nur77 was cotransfected. Also cotransfection with selected Nur77 deletion mutants, which caused differential repression of IL-2 promoter activation, showed a similar effect on p65-mediated transactivation (data not shown). Very similar effects of Nur77 were seen when the CD28RE–luciferase construct was cotransfected with a c-Rel expression plasmid. Our data indicate that Nur77 can interfere with p65 and c-Rel, both NF-κB members relevant for activation of the IL-2 promoter through the CD28RE. Furthermore, cotransfection experiments with the p65 deletion mutants showed that the C-terminal sequences of p65 are required for the repressive action of Nur77. While this manuscript was in preparation, Hong *et al*. demonstrated a direct interaction between p65 and Nur77. They showed that p65 can inhibit Nur77-mediated transactivation of steroidogenic genes, such as the P450c17 gene, and that transactivation of a synthetic NBRE construct by Nur77 was inhibited through p65 (33). In this study we also observed p65-mediated inhibition of Nur77-driven transactivation, but we can further show that this inhibition is reciprocal. However, we observed that Nur77-mediated inhibition of NF-κB-dependent transactivation requires the N-terminal domains of Nur77, while Hong *et al*. show that the C-terminal region of Nur77 is targeted by p65 (33). Thus, an interaction between the N- and C-terminal domains of Nur77 is probably required for an optimal interaction.

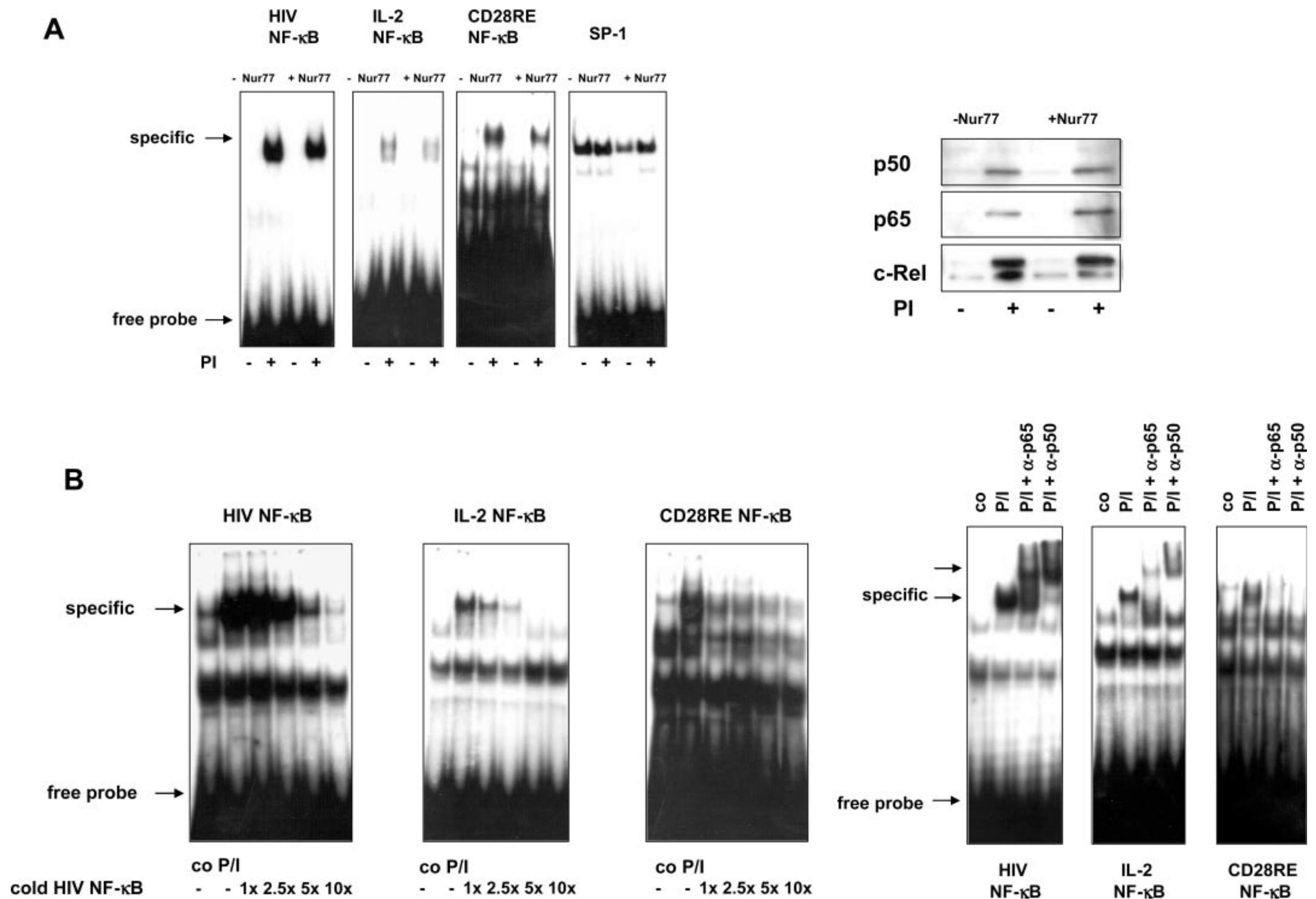

Figure 7. Analysis of NF-κB proteins in the nucleus of vector- or Nur77-transfected Jurkat cells. (**A**) Jurkat cells were transfected either with the pcDNA3.1-vector or pcDNA3.1–Nur77. Nuclear extracts were prepared 40 h post-transfection of unstimulated cells or cells stimulated with PMA + ionomycin (iono) for 4 h. Left panel: EMSA analysis of the binding of NF-κB to the NF-κB binding site of the HIV LTR, IL-2 NF-κB and NF-κB of the CD28RE in pcDNA3.1 or pcDNA3.1–Nur77-transfected cells with and without stimulation with PMA + ionomycin (iono) for 4 h. As control, binding to a SP-1 site was also analyzed. Right panel: western blot analysis was performed for the detection of the NF–κB protein p50, p65 and c-Rel. (**B**) Binding of NF-κB from nuclear extracts from Jurkat cells, stimulated for 4 h with PMA + ionomycin (iono), to the different NF-κB binding sites. Binding of NF-κB to the radiolabeled oligonucleotides was competed with increasing amounts of unlabelled HIV NF-κB oligonucleotide (left panel). The presence of the NF-κB components p50 and p65 was confirmed by supershift analysis (right panel).

Negative cross-talk between NF-κB and members of the nuclear receptor family has been described for the glucocorticoid receptor (GR), members of the peroxisome proliferator activated receptors (PPARs), retinoid-related orphan receptors (ROR), and steroid hormone receptors amongst others, taking place at various regulatory levels. The cross-talk can be mediated via direct protein-protein interaction, common coregulators, or regulation of IκBα expression (34–38). The mechanism responsible for the repression of NF-κB activity by Nur77 appears to be very similar to that described for GR. The highest degree of homology between p65 and c-Rel exists within the N-terminal Rel homology domain (RHD), which is targeted by GR and, presumably, also Nur77. Moreover, in the case of GR, the C-terminal transactivation domains of p65 are necessary for repression (26), and this is also evident from our own data for Nur77.

In summary, we have demonstrated negative cross-talk between Nur77 and NF-κB. The potential role of this interaction in the induction of apoptosis by Nur77 will be the focus of further studies.

ACKNOWLEDGEMENTS

We wish to thank R. Reuschel and W. Mayer-Granitzer for DNA sequencing. We also thank B. Wolff for critical reading of the manuscript.

REFERENCES

1. Milbrandt,J. (1988) Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron*, **1**, 183–188.
2. Hazel,T.G., Nathans,D. and Lau,L.F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl Acad. Sci. USA*, **85**, 8444–8448.
3. Winoto,A. and Littman,D.R. (2002) Nuclear hormone receptors in T lymphocytes. *Cell*, **109 (suppl.)**, S57–S66.
4. Philips,A., Maira,M., Mullick,A., Chamberland,M., Lesage,S., Hugo,P. and Drouin,J. (1997) Antagonism between Nur77 and glucocorticoid receptor for control of transcription. *Mol. Cell Biol.*, **17**, 5952–5959.

5. Perlmann,T. and Jansson,L. (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes Dev.*, **9**, 769–782.
6. Woronicz,J.D., Calnan,B., Ngo,V. and Winoto,A. (1994) Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature*, **367**, 277–281.
7. Liu,Z.G., Smith,S.W., McLaughlin,K.A., Schwartz,L.M. and Osborne,B.A. (1994) Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene Nur77. *Nature*, **367**, 281–284.
8. He,Y.W. (2002) Orphan nuclear receptors in T lymphocyte development. *J. Leukoc. Biol.*, **72**, 440–446.
9. Calnan,B.J., Szychowski,S., Chan,F.K., Cado,D. and Winoto,A. (1995) A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity*, **3**, 273–282.
10. Cheng,L.E., Chan,F.K., Cado,D. and Winoto,A. (1997) Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J.*, **16**, 1865–1875.
11. Kuang,A.A., Cado,D. and Winoto,A. (1999) Nur77 transcription activity correlates with its apoptotic function *in vivo*. *Eur. J. Immunol.*, **29**, 3722–3728.
12. Li,H., Kolluri,S.K., Gu,J., Dawson,M.I., Cao,X., Hobbs,P.D., Lin,B., Chen,G., Lu,J., Lin,F. *et al.* (2000) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science*, **289**, 1159–1164.
13. Lin,B., Kolluri,S.K., Lin,F., Liu,W., Han,Y.H., Cao,X., Dawson,M.I., Reed,J.C. and Zhang,X.K. (2004) Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. *Cell*, **116**, 527–540.
14. Rajpal,A., Cho,Y.A., Yelent,B., Koza-Taylor,P.H., Li,D., Chen,E., Whang,M., Kang,C., Turi,T.G. and Winoto,A. (2003) Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor. *EMBO J.*, **22**, 6526–6536.
15. Suzuki,S., Suzuki,N., Mirtsos,C., Horacek,T., Lye,E., Noh,S.K., Ho,A., Bouchard,D., Mak,T.W. and Yeh,W.C. (2003) Nur77 as a survival factor in tumor necrosis factor signaling. *Proc. Natl Acad. Sci. USA*, **100**, 8276–8280.
16. Pekarsky,Y., Hallas,C., Palamarchuk,A., Koval,A., Bullrich,F., Hirata,Y., Bichi,R., Letofsky,J. and Croce,C.M. (2001) Akt phosphorylates and regulates the orphan nuclear receptor Nur77. *Proc. Natl Acad. Sci. USA*, **98**, 3690–3694.
17. Masuyama,N., Oishi,K., Mori,Y., Ueno,T., Takahama,Y. and Gotoh,Y. (2001) Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis. *J. Biol. Chem.*, **276**, 32799–32805.
18. Feske,S., Giltnane,J., Dolmetsch,R., Staudt,L.M. and Rao,A. (2001) Gene regulation mediated by calcium signals in T lymphocytes. *Nature Immunol.*, **2**, 316–324.
19. Riley,J.L., Mao,M., Kobayashi,S., Biery,M., Burchard,J., Cavet,G., Gregson,B.P., June,C.H. and Linsley,P.S. (2002) Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. *Proc. Natl Acad. Sci. USA*, **99**, 11790–11795.
20. Baeuerle,P.A. and Baltimore,D. (1996) NF-kappa B: ten years after. *Cell*, **87**, 13–20.
21. Karin,M. and Delhase,M. (2000) The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin. Immunol.*, **12**, 85–98.
22. Karin,M. and Ben Neriah,Y. (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol.*, **18**, 621–663.
23. Harant,H., Eldershaw,S.A. and Lindley,I.J. (2001) Human macrophage inflammatory protein-3alpha/CCL20/LARC/Exodus/SCYA20 is transcriptionally upregulated by tumor necrosis factor-alpha via a non-standard NF-kappaB site. *FEBS Lett.*, **509**, 439–445.
24. Serfling,E., Avots,A. and Neumann,M. (1995) The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. *Biochim. Biophys. Acta*, **1263**, 181–200.
25. Huang,D.B., Chen,Y.Q., Ruetsche,M., Phelps,C.B. and Ghosh,G. (2001) X-ray crystal structure of proto-oncogene product c-Rel bound to the CD28 response element of IL-2. *Structure (Camb.)*, **9**, 669–678.
26. Wissink,S., van Heerde,E.C., Schmitz,M.L., Kalkhoven,E., van der Burg,B., Baeuerle,P.A. and van der Saag,P.T. (1997) Distinct domains of the RelA NF-kappaB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J. Biol. Chem.*, **272**, 22278–22284.
27. Wansa,K.D., Harris,J.M. and Muscat,G.E. (2002) The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment. *J. Biol. Chem.*, **277**, 33001–33011.
28. Kang,S.M., Tran,A.C., Grilli,M. and Lenardo,M.J. (1992) NF-kappa B subunit regulation in nontransformed CD4+ T lymphocytes. *Science*, **256**, 1452–1456.
29. McGuire,K.L. and Iacobelli,M. (1997) Involvement of Rel, Fos, and Jun proteins in binding activity to the IL-2 promoter CD28 response element/AP-1 sequence in human T cells. *J. Immunol.*, **159**, 1319–1327.
30. Ghosh,P., Tan,T.H., Rice,N.R., Sica,A. and Young,H.A. (1993) The interleukin 2 CD28-responsive complex contains at least three members of the NF kappa B family: c-Rel, p50, and p65. *Proc. Natl Acad. Sci. USA*, **90**, 1696–1700.
31. Maggirwar,S.B., Harhaj,E.W. and Sun,S.C. (1997) Regulation of the interleukin-2 CD28-responsive element by NF-ATp and various NF-kappaB/Rel transcription factors. *Mol. Cell Biol.*, **17**, 2605–2614.
32. Kunsch,C., Ruben,S.M. and Rosen,C.A. (1992) Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. *Mol. Cell Biol.*, **12**, 4412–4421.
33. Hong,C.Y., Park,J.H., Ahn,R.S., Im,S.Y., Choi,H.S., Soh,J., Mellon,S.H. and Lee,K. (2004) Molecular mechanism of suppression of testicular steroidogenesis by proinflammatory cytokine tumor necrosis factor alpha. *Mol. Cell Biol.*, **24**, 2593–2604.
34. Delerive,P., Fruchart,J.C. and Staels,B. (2001) Peroxisome proliferator-activated receptors in inflammation control. *J. Endocrinol.*, **169**, 453–459.
35. De Bosscher,K., Vanden Berghe,W. and Haegeman,G. (2000) Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *J. Neuroimmunol.*, **109**, 16–22.
36. McKay,L.I. and Cidlowski,J.A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.*, **20**, 435–459.
37. Delerive,P., Monte,D., Dubois,G., Trottein,F., Fruchart-Najib,J., Mariani,J., Fruchart,J.C. and Staels,B. (2001) The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. *EMBO Rep.*, **2**, 42–48.
38. He,Y.W., Beers,C., Deftos,M.L., Ojala,E.W., Forbush,K.A. and Bevan,M.J. (2000) Down-regulation of the orphan nuclear receptor ROR gamma t is essential for T lymphocyte maturation. *J. Immunol.*, **164**, 5668–5674.