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Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation

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The molecular basis for restricted cytokine expression by T helper 1 (Th1) and T helper 2 (Th2) cells is unclear. Previous studies found that P1, an element of the interleukin 4 (IL-4) promoter that binds AP-1, is important for Th2-restricted IL-4 expression. Here we show that JunB, but not the other Jun family members, was selectively induced in Th2 cells and not in Th1 cells during differentiation. JunB has previously been considered to be a negative regulator of transcription. However, we show that JunB binds directly to the P1 site and synergizes with c-Maf to activate an IL-4 luciferase reporter gene. JunB-control of IL-4 expression is mediated by the phosphorylation of JunB at Thr102 and -104 by JNK MAP kinase. The synergy between c-Maf and JunB can be attributed to cooperative DNA binding, which is facilitated by JunB phosphorylation. In transgenic mice, elevated JunB levels caused increased expression of several Th2 cytokines in developing Th1 cells. JunB also upregulated IL-4 expression in response to immunization. Thus, the early increase of JunB protein in Th2 cells can provide the specificity for c-Maf in IL-4 expression during T cell development and directs thereby Th2 differentiation.

Keywords: cytokine/JunB/phosphorylation/Th2

Introduction

In response to antigen stimulation, naïve T helper (Th) cells differentiate into different types of effector cells that are classified based on their distinct cytokine profiles and immune regulatory functions (Mosmann *et al*., 1986; Paul and Seder, 1994). Th1 cells produce interleukin 2 (IL-2) and interferon γ (IFN-γ), and play an important role in the cell-mediated immune response against intracellular pathogens. Th2 cells produce IL-4, IL-5 and IL-10, and are involved in humoral immunity and the allergic response. There is ample evidence that the dominance of either Th1 or Th2 responses directly affects the outcome of a wide array of immunologically mediated clinical conditions, including autoimmune and allergic responses, and infectious diseases (Abbas *et al*., 1996; Romagnani, 1997). It is therefore of great clinical significance to identify and manipulate factors that dictate the ratio of Th1 to Th2 cells. The course of CD4 T cell differentiation appears to be influenced by many factors. Cytokines are critical for the determination of the outcome of the differentiation to effector T cells (Paul and Seder, 1994; Constant and Bottomly, 1997). Thus, IL-4 that is present during priming promotes the differentiation of Th2 cells while preventing the differentiation of Th1 cells (Hsieh *et al*., 1992; Seder *et al*., 1992; Swain, 1993), whereas IL-12 and IFN-γ have the opposite effect (Hsieh *et al*., 1993; Manetti *et al*., 1993; Seder *et al*., 1993).

The molecular basis for T helper cell subset differentiation is still unclear. The well-established role of IL-4 in determining Th2 differentiation argues that regulating the expression of IL-4 during Th differentiation could determine the outcome. Several regulatory sites have been identified within the 5′ flanking region of the IL-4 gene which are capable of directing expression of a reporter gene in transient transfection studies (Abe *et al*., 1992; Li-Weber *et al*., 1992; Bruhn *et al*., 1993; Chuvpilo *et al*., 1993; Szabo *et al*., 1993; Todd *et al*., 1993; Rooney *et al*., 1995). These sites, designated P0, P1, P2 and P3, contain sequences that bind members of the NFAT family of transcription factors. P1, P2 and P3 also contain adjacent sites that bind activator protein 1 (AP-1) (Rooney *et al*., 1995).

Studies on *cis*-elements demonstrated that both the P0 and P1 regions are important in determining Th2-specific expression of IL-4. A region encompassing P0 was shown to confer preferential expression in long-term cultured Th2 cells but not in Th1 cells (Bruhn *et al*., 1993). This region contains an NFAT site and to its proximal side, a MARE half site which was shown to have binding affinity for both C/EBPβ and c-Maf (Davydov *et al*., 1995; Ho *et al*., 1996). NFAT plays a pivotal role in IL-4 gene activation (Chuvpilo *et al*., 1993; Szabo *et al*., 1993). However, it is present in both Th1 and Th2 cells and activates the transcription of cytokine genes characteristic for both cell types (Rooney *et al*., 1994, 1995). In contrast, c-Maf (Ho *et al*., 1996) and GATA-3 (Zheng and Flavell, 1997) were recently reported to be selectively expressed in Th2 but not in Th1 cells and both are able to drive ectopic IL-4 expression. c-Maf was shown to bind to a region proximal to P0 and activate IL-4 transcription synergistically with NFATp (Ho *et al*., 1996).

In transgenic mouse studies, a multimerized P1 region (AP-1 plus NFAT) conferred Th2-restricted expression of transcriptional activity during T cell differentiation (Wenner *et al*., 1997). In contrast, in a related study, multimerization of the NFAT binding site of the P1 element without the AP-1 site was expressed in both Th1 and Th2 subsets (Aune and Flavell, 1997). These data suggested that Th2-specific regulation of the P1 element may result from regulation of transcription factors that bind to the AP-1 portion of the element.

Our recent examination of AP-1 activity during T helper cell differentiation using AP-1 luciferase transgenic mice showed that a high level of AP-1 transcriptional activity was induced upon stimulation in Th2 cells, but not in Th1 cells (Rincón *et al*., 1997). This AP-1 activity correlated with the presence of JunB, which accumulates in Th2 cells, but not in Th1 or naïve T cells, suggesting a potential role for JunB in Th2 differentiation (Rincón *et al*., 1997). In the present study, we therefore examined whether JunB has any direct effect on IL-4 expression using both transient transfection and transgenic approaches.

Early transfection studies showed that JunB inhibits c-Jun transcriptional activity (Chiu *et al*., 1989; Schutte *et al*., 1989). This observation and later findings that the N-terminal region of JunB possesses a docking site but not phosphorylation sites for JNK (Gupta *et al*., 1996; Kallunki *et al*., 1996) both pointed to the hypothesis that JunB is a repressor of transcription mediated by AP-1. Here we show, however, that JunB is instead a strong transcriptional activator of IL-4. We demonstrate that JunB upregulates Th2 cytokines in transgenic mice and that JunB synergizes with c-Maf to activate the IL-4 promoter. JunB transcriptional activity, as well as its synergism with c-Maf for IL-4 expression, is enhanced by phosphorylation on Thr102 and Thr104 by JNK. These results provide a new molecular mechanism whereby precursor cells differentiate into effector cells secreting high levels of Th2 cytokines.

Results

Preferential expression of JunB protein in Th2 cells

Our previous study demonstrated that among the Jun family of transcription factors, AP-1 DNA-binding complexes containing JunB preferentially accumulated in Th2 cells but not in Th1 cells (Rincón *et al*., 1997). We considered it to be likely that JunB accumulation would account for the enhanced AP-1 transcriptional activity in Th2 cells. To confirm the differential expression of JunB, we performed Western blot analysis using extracts from *in vitro* differentiated Th1 and Th2 cells (Figure 1A). At day 3 of *in vitro* differentiation of purified naïve T cells, the JunB protein level was upregulated in Th2 cells relative to Th1 cells, while c-Jun and JunD protein levels were approximately the same in Th1 and Th2 cells. We further investigated the kinetics of JunB protein expression during the differentiation of Th precursors (Figure 1B). While the level of JunB protein remained relatively constant in developing Th1 cells, the JunB protein level was upregulated in developing Th2 cells at day 3 of *in vitro* priming.

Transactivation of the IL-4 promoter by JunB

The upregulation of AP-1 transcriptional activity and accumulation of JunB in Th2 cells suggested that Th2-specific AP-1 activity is contributed by JunB. The P1 site of the IL-4 promoter, previously shown to confer Th2-restricted IL-4 expression, is known to bind AP-1. These findings suggest a role for JunB in tissue-specific expression of IL-4. We therefore tested whether JunB could drive ectopic expression by the IL-4 promoter in the B lymphoma cell line M12, which does not usually express IL-4.

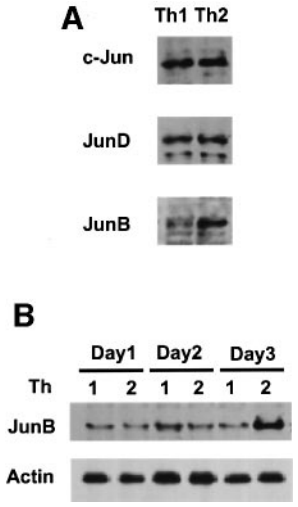


Fig. 1. Upregulation of JunB in Th2 cells. (**A**) JunB is selectively upregulated in Th2 cells. Naïve T cells were differentiated into Th1 and Th2 for 3 days and nuclear extracts were isolated. The amount of c-Jun, JunB and JunD was examined by Western blot analysis using antibodies specific for each transcription factor. (**B**) Time course of JunB protein expression during Th1/Th2 differentiation. Naïve T cells were differentiated towards the Th1 or Th2 pathways and nuclear extracts were isolated from each population at various days as indicated. JunB was detected by Western blot analysis. A polyclonal antibody against actin was used as a loading control for immunoblots.

The full-length cDNA of JunB or other Jun family members were co-transfected with an IL-4 promoter (–157 to +68) reporter plasmid into M12 cells. The luciferase activity generated with or without PMA/ionomycin stimulation was measured. A *LacZ* gene was also co-transfected and the β-galactosidase (β-gal) activity was used as a control to normalize the luciferase reading for transfection efficiency. Overall, c-Jun and JunB exhibited a similar ability, which was much greater than that of JunD, to activate ectopic expression of the IL-4 reporter gene (Figure 2A). Specifically, for both JunB and c-Jun, the activity of the IL-4 promoter responded in a dose-dependent manner in a range from 2–10 μg of input DNA yielding a 10-fold increase after PMA/ionomycin stimulation. In contrast, no dramatic increase in IL-4 promoter activity was observed when as much as 10 μg JunD expression vector was co-transfected.

Since the transcriptional activity of c-Jun is known to be enhanced by phosphorylation by mitogen-activated protein kinase (MAP kinases) (Whitmarsh and Davis, 1996), we tested whether the high level of transcriptional activity that was induced in response to PMA/ionomycin was mediated by MAP kinase signaling pathways. At least three distinct MAP kinase families have been identified: ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase or stress-activated protein kinase, SAPK) and p38 MAP kinase (also known as cytokine-suppressive anti-inflammatory drug-binding protein, CSBP, or reactivating kinase, RK). ERK is mainly involved in responses to signals initiated by growth factors and hormones (Robinson and Cobb, 1997), whereas JNK and p38 MAP kinase are activated by environmental stresses and pro-inflammatory cytokines (Ip and Davis, 1998). In order to investigate the possible role of MAP kinases in IL-4 expression, we first examined MAP kinase activation in M12 cells after PMA/ionomycin treatment (Figure 2B).

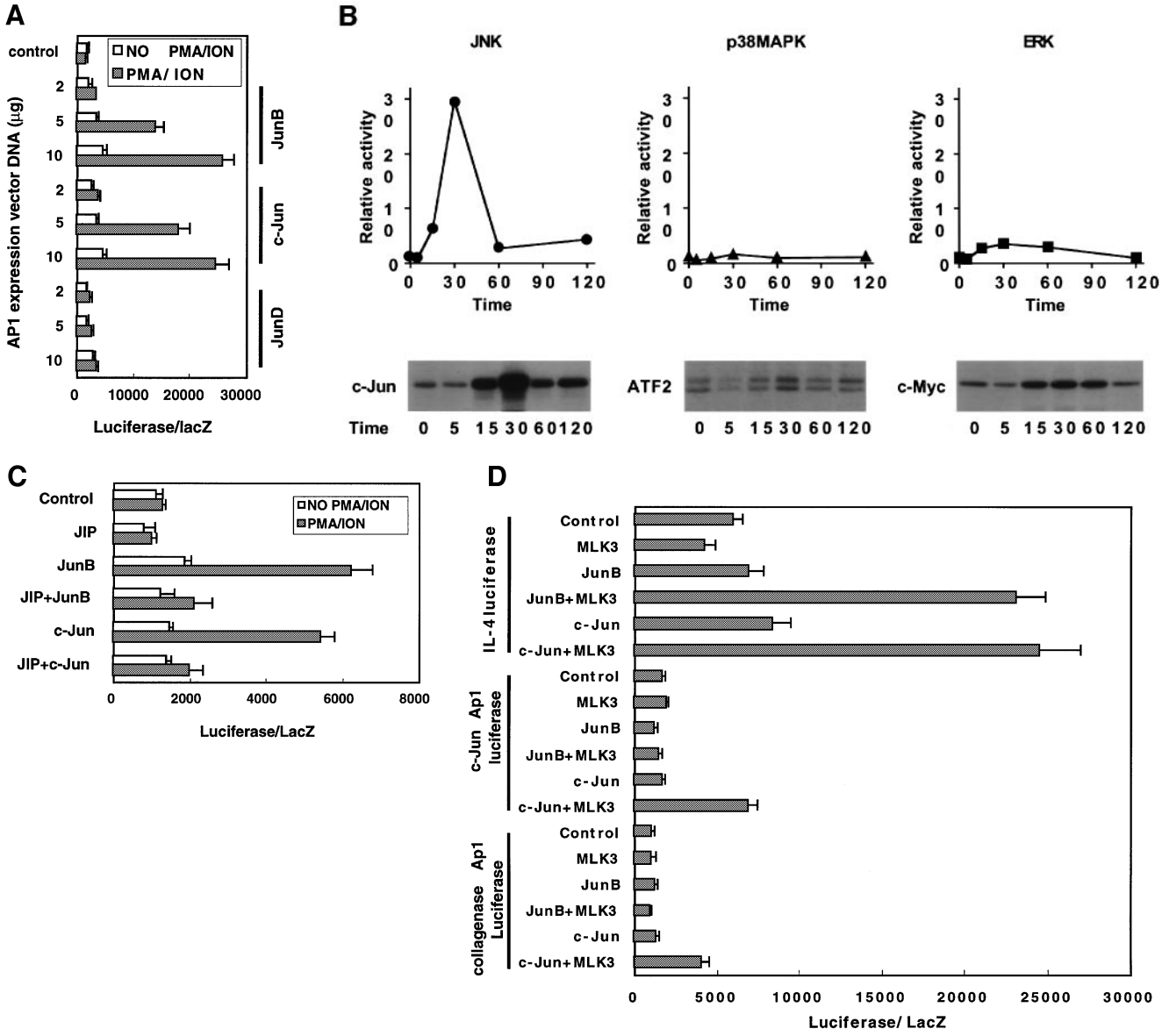


Fig. 2. Transactivation of the IL-4 promoter by JunB. (**A**) Transactivation of the IL-4 promoter by c-Jun, JunB and JunD. Various amounts of expression vector for Jun family members were co-transfected with 4 μg IL-4 promoter luciferase and 50 ng β-gal vectors into M12 cells. The cells were left overnight and then treated (black bars) or not treated (white bars) with PMA/ionomycin. Extracts were made 24 h later and relative activities of luciferase over LacZ were determined. (**B**) PMA/ionomycin stimulates JNK activity in M12 cells. The cells were treated for various times, as indicated, with PMA/ionomycin. Endogenous JNK, p38 MAP kinase and ERK were immunoprecipitated and the MAPK activity was measured in the immune complex by protein kinase assay using recombinant c-Jun, ATF2 and c-Myc as substrate, respectively. The phosphorylated c-Jun, ATF2 and c-Myc were detected after SDS–PAGE by autoradiography and quantitated by PhosphorImager analysis. MAPK activity is presented as relative protein kinase activity. (**C**) JIP-1 blocks the induction of IL-4 luciferase expression in M12 cells. M12 cells were co-transfected with c-Jun (5 μg) or JunB (5 μg), IL-4 luciferase (4 μg) and β-gal (50 ng) vectors with or without JIP-1 (4 μg). Cells were left overnight and then treated (black bars) or not treated (white bars) with PMA/ionomycin. Relative activities of luciferase over LacZ were determined. (**D**) Transcriptional activation by JunB is promoter specific. Four micrograms of IL-4 luciferase, c-Jun AP-1 luciferase or collagenase AP-1 luciferase construct and 50 ng β-gal vectors were co-transfected with 2 μg of expression vector for JunB, c-Jun or MLK3, or various combinations as indicated into M12 cells. No PMA/ionomycin was added. Extracts were made 24 h later and relative activities of luciferase over LacZ were determined.

The endogenous JNK, p38 MAP kinase and ERK activity was measured by immune complex protein kinase assay using c-Jun, ATF2 and c-Myc as substrates, respectively. Stimulation of M12 cells with PMA/ionomycin induced a potent and transient activation of JNK which reached a maximum after 30 min. However, this treatment caused only a small increase in ERK activity and only modest changes in p38 MAP kinase activity were detected. These data indicated a prominent role for JNK rather than p38 or ERK MAP kinases in the response of M12 cells to PMA/ionomycin.

We then studied the effect of the inhibition of the JNK signaling pathway on the induction of the IL-4 promoter activity. The cytoplasmic JNK binding protein JIP-1 has recently been identified as a potent inhibitor of nuclear signal transduction by the JNK pathway when it is overexpressed (Dickens *et al.*, 1997). Therefore, M12 cells were co-transfected with or without JIP-1, JunB or c-Jun, and the IL-4 promoter reporter plasmid. Figure 2C shows that JIP-1 inhibited both the c-Jun- and JunB-dependent activation of the IL-4 promoter in response to PMA/ionomycin stimulation.

We further examined the role of the JNK signaling pathway in JunB-dependent IL-4 promoter activation. Co-transfection of MLK3, a potent inducer of signal transduction by the JNK pathway (Rana *et al*., 1996; Tibbles *et al*., 1996) together with JunB or c-Jun dramatically enhanced IL-4 promoter activity (Figure 2D). Co-transfection of another JNK activator MKK7 (Tournier *et al*., 1997) together with JNK1 also enhanced JunB- and c-Jun-dependent IL-4 promoter activation (data not shown). These data demonstrated that JNK MAP kinase is involved in the regulation of JunB-dependent IL-4 promoter activity.

In one sense our results with JunB were puzzling since JunB has previously been shown to inhibit c-Jun-mediated transcription (Chiu *et al*., 1989; Schutte *et al*., 1989). However, our experiments clearly established JunB as a transcriptional activator. To examine whether JunB has similar activity in the context of other promoters, we used luciferase reporter constructs that contains either the c-Jun promoter AP-1 site or the collagenase promoter AP-1 site (Figure 2D). In all cases, c-Jun activated the promoters when co-transfected with MLK3; in contrast, JunB only showed activity at the IL-4 promoter. Hence, transcriptional activation by JunB is promoter specific.

JunB is phosphorylated by the JNK MAP kinase

c-Jun transcriptional activity is known to be regulated by phosphorylation on two serines (residues 63 and 73) by the JNK MAP kinase (Whitmarsh and Davis, 1996). In contrast, these phosphorylation sites are not conserved in JunB, although the JNK binding site is conserved in both c-Jun and JunB. The presence of the JNK binding site in JunB suggests that JunB may be a substrate for JNK. However, previous studies using fragments of JunB did not support this hypothesis (Gupta *et al*., 1996; Kallunki *et al*., 1996). We therefore examined potential sites of JunB phosphorylation by JNK using full-length recombinant JunB. These studies demonstrated that JunB is indeed an excellent substrate for JNK (Figure 3A). In contrast, JunB was a weak substrate for the p38 MAP kinase and was not significantly phosphorylated by ERK MAP kinase. Positive control experiments were performed by investigating the phosphorylation of c-Jun by JNK, ATF2 by p38 MAP kinase, and c-Myc by ERK2 to ensure that all kinases were active.

Examination of the sequence of JunB revealed the motif Thr-Pro-Thr-Pro (residues 102–105) which is in a primary sequence context that is similar to the sites of phosphorylation of the transcription factor ATF2 by JNK (Gupta *et al*., 1995). To examine whether Thr102 and Thr104 are sites of JunB phosphorylation by JNK, we examined the effect of replacement of these residues with Ala (Figure 3B). To determine the sites on JunB phosphorylated by JNK MAP kinase, phosphoamino-acid analysis was performed using wild-type and mutant glutathione *S*-transferase (GST)–JunB. The mutant JunBala protein was constructed by replacing Thr102 and Thr104 of JunB with Ala residues. We immunopurified JNK from control (–) and UV-stimulated (+) cells, and recombinant wild-type or mutant JunB proteins were used as substrates in an immune complex protein kinase assay. A marked increase in the level of [^{32}P]phosphothreonine and a small increase in the level of [^{32}P]phosphoserine was observed with wild-

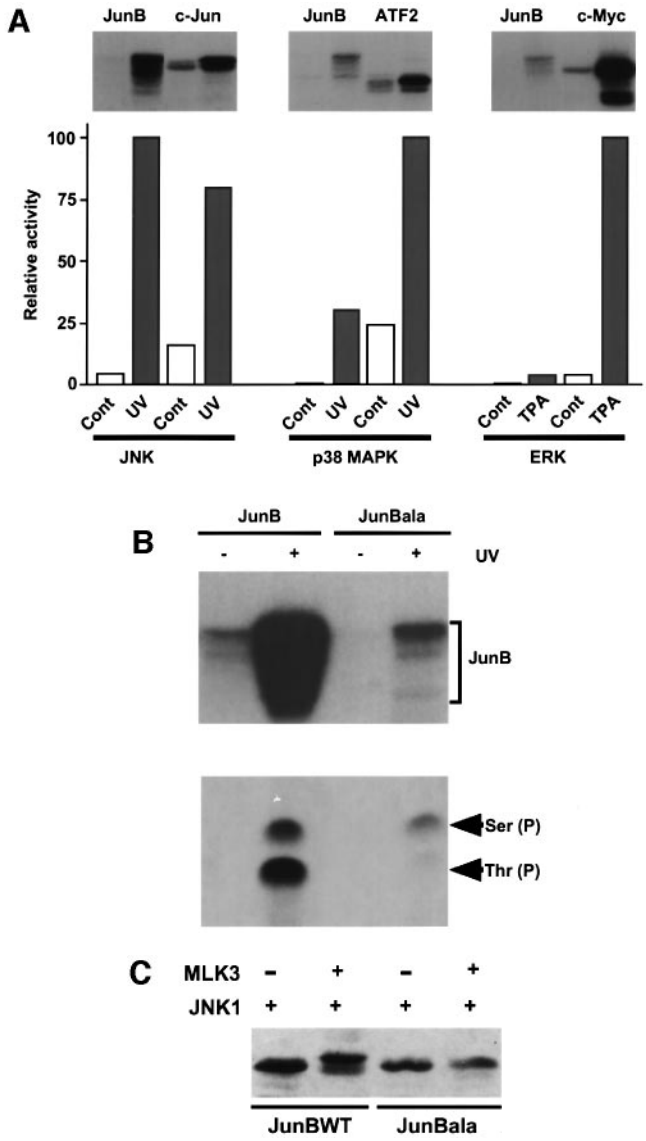


Fig. 3. JunB is phosphorylated by JNK. (**A**) JunB is a better substrate for JNK than for p38 MAP kinase or ERK. COS-7 cells were transiently transfected with expression vectors (pCDNA3) encoding either epitope-tagged-JNK1, -p38a MAP kinase or -ERK2. Thirty-six hours after transfection, the cells were untreated (–) or treated (+) with UV-C (80 J/m^2) or TPA (100 ng/ml). The cells were harvested after incubation at 37°C (60 and 15 min, respectively). Epitope-tagged MAP kinases were immunoprecipitated and the MAP kinase activity was measured in the immune complex by protein kinase assay using recombinant JunB, c-Jun, ATF2 or c-Myc as indicated. The phosphorylated recombinant proteins were detected after SDS–PAGE by autoradiography and quantitated by PhosphorImager analysis. MAP kinase activity is presented as relative protein kinase activity. (**B**) JNK phosphorylates JunB on Thr102 and Thr104. Epitope-tagged-JNK1 was isolated by immunoprecipitation from extracts of cells stimulated (+) or not (–) with UV-C (80 J/m^2) for 60 min and used for protein kinase assays with recombinant wild-type or mutant JunB, as substrates. The mutant JunBala protein was constructed by replacing Thr102 and Thr104 with Ala residues. The phosphorylated recombinant proteins were detected after SDS–PAGE by autoradiography and were analyzed by [^{32}P]phosphoamino-acid analysis. (**C**) JunB is phosphorylated on Thr102 and Thr104 in M12 cells. A Flag-tagged JunB expression vector was co-transfected with a JNK1 expression vector with or without MLK3 expression vector into M12 cells. Cell extracts were isolated 24 h later and Western blot analysis was performed using a monoclonal antibody to Flag.

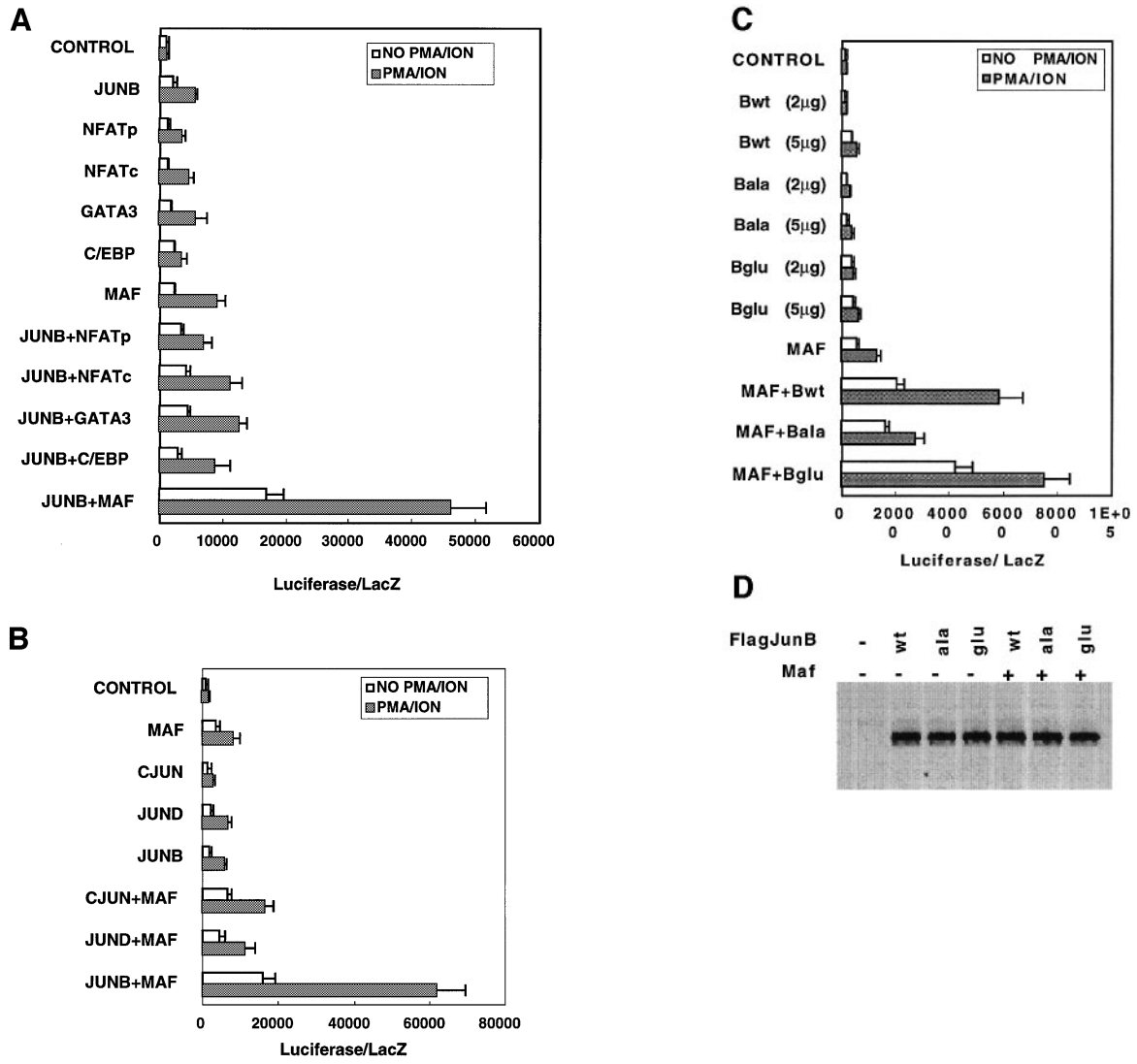


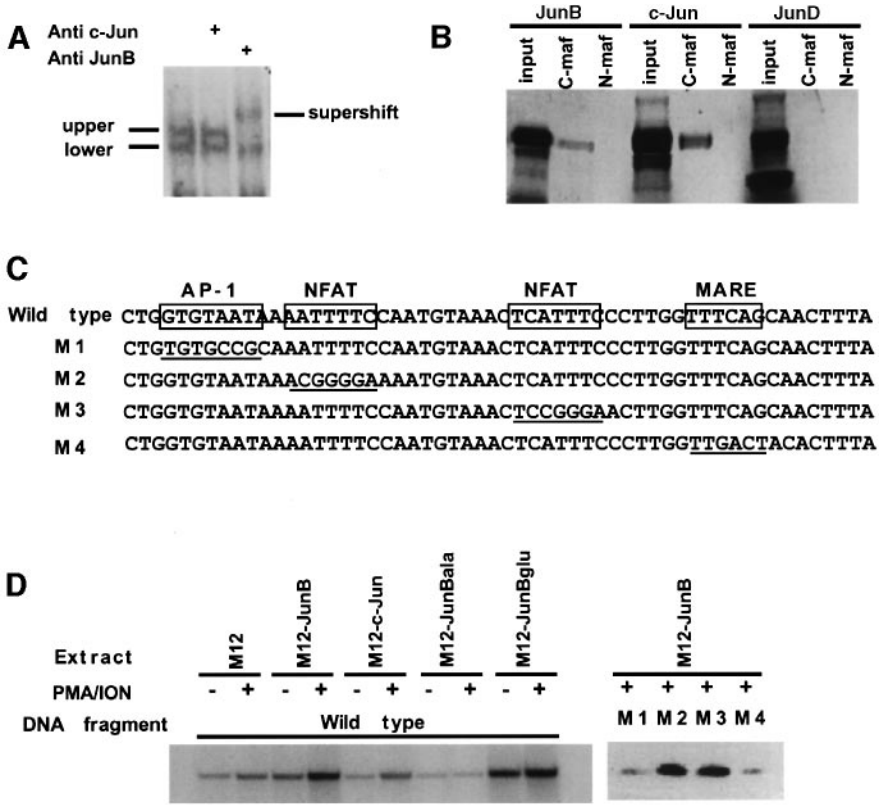
Fig. 4. JunB activates the IL-4 promoter synergistically with c-Maf. (**A**) JunB and c-Maf synergistically activate the IL-4 promoter. Various combinations of expressions vectors as indicated were co-transfected with IL-4 luciferase and β-gal vectors into M12 cells. The amount of DNAs used are IL-4 promoter luciferase 4 μg, β-gal 50 ng, c-Maf 0.8 μg, and all other DNAs 2 μg. Luciferase and LacZ values were determined with or without stimulation. (**B**) Among Jun family members only JunB synergizes with c-Maf to activate the IL-4 promoter. A JunB expression vector was co-transfected with expression vectors for different transcription factors, as indicated. Luciferase assays were then performed. The amount of DNA used was the same as above. (**C**) The synergy between JunB and c-Maf was enhanced by JunB phosphorylation. JunB wild type (JunB), JunB Thr102/104→Ala mutant (JunBala) and JunB Thr102/104→Glu mutant (JunBglu) together with other DNAs were used in transient transfection assays. The amount of JunB vectors used without c-Maf is indicated. JunB vectors (2 μg) were used in the transfection when both JunB and c-Maf were co-expressed. (**D**) Phosphorylation does not change JunB protein expression in the transfection experiment. Figure 4C was repeated with Flag-tagged JunB, JunBala and JunBglu constructs. The amount of JunB protein in the last six samples of Figure 4C was determined by Western blot analysis using a monoclonal antibody against the Flag epitope.

type JunB. In contrast, when JunBala was used as a substrate for JNK, only a small increase in the level of [^{32}P]phosphoserine was observed. Control studies were performed using wild-type c-Jun and mutated c-Jun in which the JNK phosphorylation sites Ser63 and Ser73 were replaced with Ala residues (c-Junala). As expected, the marked serine phosphorylation of c-Jun by JNK was abrogated in the c-Junala mutant (data not shown). These data demonstrated that JunB is phosphorylated by JNK MAP kinase on Thr102 and Thr104.

To further confirm the involvement of JunB phosphorylation in the transfection assays described in this report, we examined whether JunB is phosphorylated in M12 cells. A Flag-tagged JunB expression vector was co-transfected with JNK1 expression vector with or without a MLK3 expression vector into M12 cells. Cell extracts were isolated 24 h later and Western blot analysis was performed using a monoclonal antibody against Flag. Co-transfection of MLK3 induced phosphorylation of JunB, which was manifested as a slower-migrating band. In contrast, the phosphorylated, slower-migrating band was not observed in co-transfection assay using MLK3 and JunBala. Thus, in M12 cells, JunB is phosphorylated on Thr102 and Thr104 residues.

JunB and c-Maf activate the IL-4 promoter synergistically

Next we co-transfected with JunB other transcription factors that are known to activate the IL-4 promoter, including NFATp, NFATc, GATA-3, C/EBPβ and c-Maf.



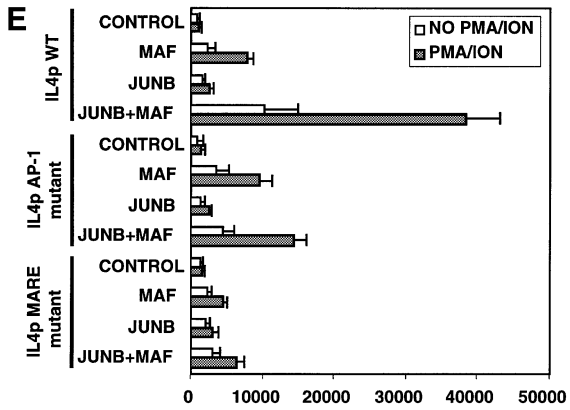


Fig. 5. JunB binds the IL-4 promoter synergistically with c-Maf. (**A**) Electrophoretic mobility shift analysis (EMSA) of the P1 element. Radiolabeled oligonucleotides containing the AP-1 and NFAT site of the P1 element was used in the gel shift assay. Extracts are made from activated Th2 cells (see Materials and methods). C-Jun and JunB antibodies were used for supershift analysis. (**B**) Interaction between JunB and c-Maf. The C-terminal (b-Zip domain) and N-terminal halves of c-Maf with *S*-tag were immobilized on agarose. *In vitro* translated c-Jun, JunB and JunD labeled with [^{35}S]methionine were incubated with the beads. Bound proteins were resolved on SDS–PAGE. C-Maf and N-Maf refer to proteins bound to beads coupled with C- and N-terminal halves of c-Maf. (**C**) The wild-type and mutant DNA fragments of the IL-4 promoter. Sequences of the DNA fragment spanning –87 to –31 of the IL-4 promoter and various mutants. M1 represents the DNA fragment that has mutated the P1 AP-1 site; in similar fashion: M2, P1 NFAT; M3, P0 NFAT; M4, MARE. (**D**) C-Maf binding to IL-4 promoter DNA is facilitated by phosphorylated JunB. Wild-type or mutant DNA fragments were incubated with c-Maf beads and with or without various protein preparations as indicated. Bound DNA fragments were resolved on a non-denaturing polyacrylamide gel. (**E**) The AP-1 and MARE sites are required for synergistic activation of IL-4 promoter by JunB and c-Maf. IL-4 promoter luciferase constructs with either the AP-1 site or the MARE site mutated were compared with the wild-type IL-4 promoter construct with a luciferase assay.

A synergistic effect on IL-4 activation was observed between JunB and c-Maf, but not with any other factors (Figure 4A). While c-Maf alone or JunB alone stimulated the IL-4 promoter activity by ~6- and ~4-fold, respectively, c-Maf and JunB together generated an ~50-fold stimulation. Remarkably, the synergy between c-Maf and JunB was specific, as this effect was not observed when JunB was substituted with the other Jun family members c-Jun and JunD (Figure 4B).

To determine whether the state of phosphorylation of JunB has any effect on the synergy between JunB and c-Maf, we co-transfected c-Maf with JunBala or JunBglu mutants. JunBglu was constructed by replacing Thr102 and Thr104 with glutamic acid residues. JunBglu would therefore be expected to mimic a constitutively active form of JunB. Both mutants and the wild-type JunB vector when co-transfected alone with the IL-4 reporter gene generated modest activation of the IL-4 promoter (Figure 4C). Although there was still some synergy with c-Maf when JunBala was co-transfected, the activity was lower than when wild-type JunB was used. However, the JunBglu mutant generated a very potent synergy with c-Maf in activating the IL-4 promoter. These data indicated that phosphorylation of JunB enhanced its ability to

synergize with c-Maf in transcriptional activation. Since the protein stability of c-Jun is known to be influenced by phosphorylation (Ip and Davis, 1998), we examined the JunB protein level in the above transfection experiments by Western blot analysis. Similar amounts of JunB and JunB-mutant proteins were detected in each sample regardless of the status of PMA/ionomycin treatment or c-Maf co-transfection (Figure 4D). Thus, the different levels of luciferase activity measured using the different JunB proteins was not caused by changes in the amount of JunB protein.

Cooperative binding of JunB and c-Maf to IL-4 promoter DNA

Previous gel-shift experiments using the Th2 cell-specific P1 element showed that JunB is able to bind to the AP-1 site (Rooney *et al*., 1995). Extracts from Th2 cells cultured from primary T cells form a similar pattern of shifted bands on the P1 element (Wenner *et al*., 1997), and competition experiments using various oligonucleotides showed that the AP-1 site was bound. To examine whether JunB is the AP-1 factor that binds to P1 in primary T cells, we performed gel-shift experiments using P1 oligonucleotides and nuclear extracts from day 3 Th2 cells differentiated *in vitro*. The double-shifted bands characteristic of the P1 element were formed, and the upper band was supershifted by JunB antibody but not by c-Jun antibody (Figure 5A).

The synergy between JunB and c-Maf raised the possibility that JunB interacts directly with c-Maf, since c-Jun was known to bind c-Maf directly (Kerppola and Curran, 1994). Alternatively, indirect interaction through a common binding partner, a transcriptional co-activator for example, was also possible. We expressed both the C-terminal half b-Zip domain and the N-terminal half of the recombinant c-Maf protein in *Escherichia coli*, and immobilized the purified proteins on agarose beads taking advantage of the *S*-tag in the expression vector construct. We incubated the beads with radiolabeled JunB, c-Jun or JunD protein obtained from *in vitro* transcription–translation reactions. Both JunB and c-Jun bound to the C-terminal half of c-Maf which contains the leucine-zipper motif (Figure 5B). In contrast, JunD did not bind to either moiety of c-Maf. c-Maf has been reported to bind the MARE half site around –40 bp of the IL-4 promoter (Ho *et al*., 1996). We therefore sought to determine whether JunB also bound this site, potentially in cooperation with c-Maf. However, an EMSA experiment using a probe of –62 to –25 bp of the IL-4 promoter did not form any bands that could be supershifted by JunB antibody (data not shown).

To examine whether JunB potentiated the binding of c-Maf to DNA, we took beads carrying immobilized c-Maf and added *in vitro* translated JunB and radiolabeled DNA fragments spanning bps –87 to –31 of the IL-4 promoter and various mutants of this DNA element (sequences listed in Figure 5C). We did not observe any substantial increase in bound DNA when *in vitro*-translated JunB was used (data not shown). However, we did see a large increase in DNA bound to c-Maf when we substituted the *in vitro*-translated JunB with extracts from M12 cells transfected with JunB and treated with PMA/ionomycin (Figure 5D). We did not see the increase when using extracts from c-Jun transfected cells, which argues that JunB specifically increased c-Maf binding to DNA. Significantly, PMA/ionomycin treatment was required to increase DNA binding, which suggests that JunB phosphorylation may be required since PMA/ionomycin dramatically induces JNK activity. We tested this hypothesis by using extracts from JunBala or JunBglu transfected cells. Extracts from JunBala transfected cells did not

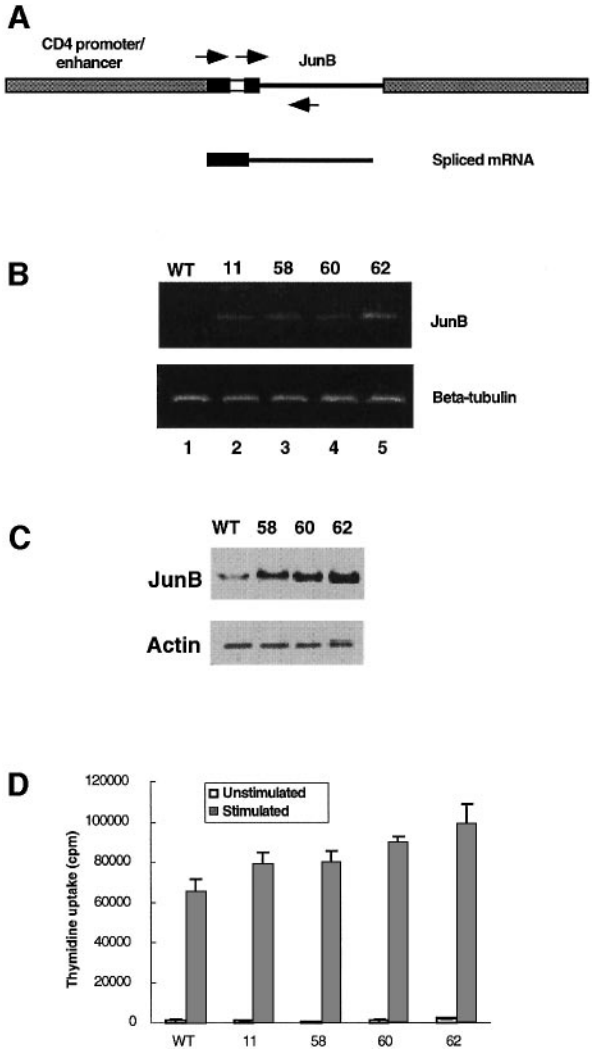


Fig. 6. Expression of JunB in CD4-JunB transgenic mice. (**A**) Schematic diagram of the CD4-JunB transgene. The JunB cDNA was fused with the second exon of the CD4 gene. The arrow at the bottom indicates the downstream primer used for screening both the transgene and the expression of transgene mRNA. The first arrow from the left above the construct indicates the primer used for screening the transgene mRNA in RT–PCR. The second arrow indicates the primer used for screening the transgene incorporation. (**B**) RT–PCR showing expression of transgene in mice. Total RNA was isolated from T cells in peripheral blood of transgene positive mice and wild-type littermates. RT–PCR was done using primers described in Figure 1A and Materials and Methods. β-tubulin was used as a control. (**C**) Increase of JunB protein in transgenic mice. JunB Western blot analysis was done with T cells isolated from lymph nodes of transgenic mice and wild-type littermates. Western blot analysis using a polyclonal antibody against actin was used as a loading control. (**D**) Normal proliferation of transgenic T cells. Naïve T cells were isolated from transgenic mice or wild-type littermates and cultured with ConA, IL-2 and APC (see Materials and methods) for 2 days and [^{3}H]thymidine was added. Radioactivity incorporation was measured 18 h later in triplicate.

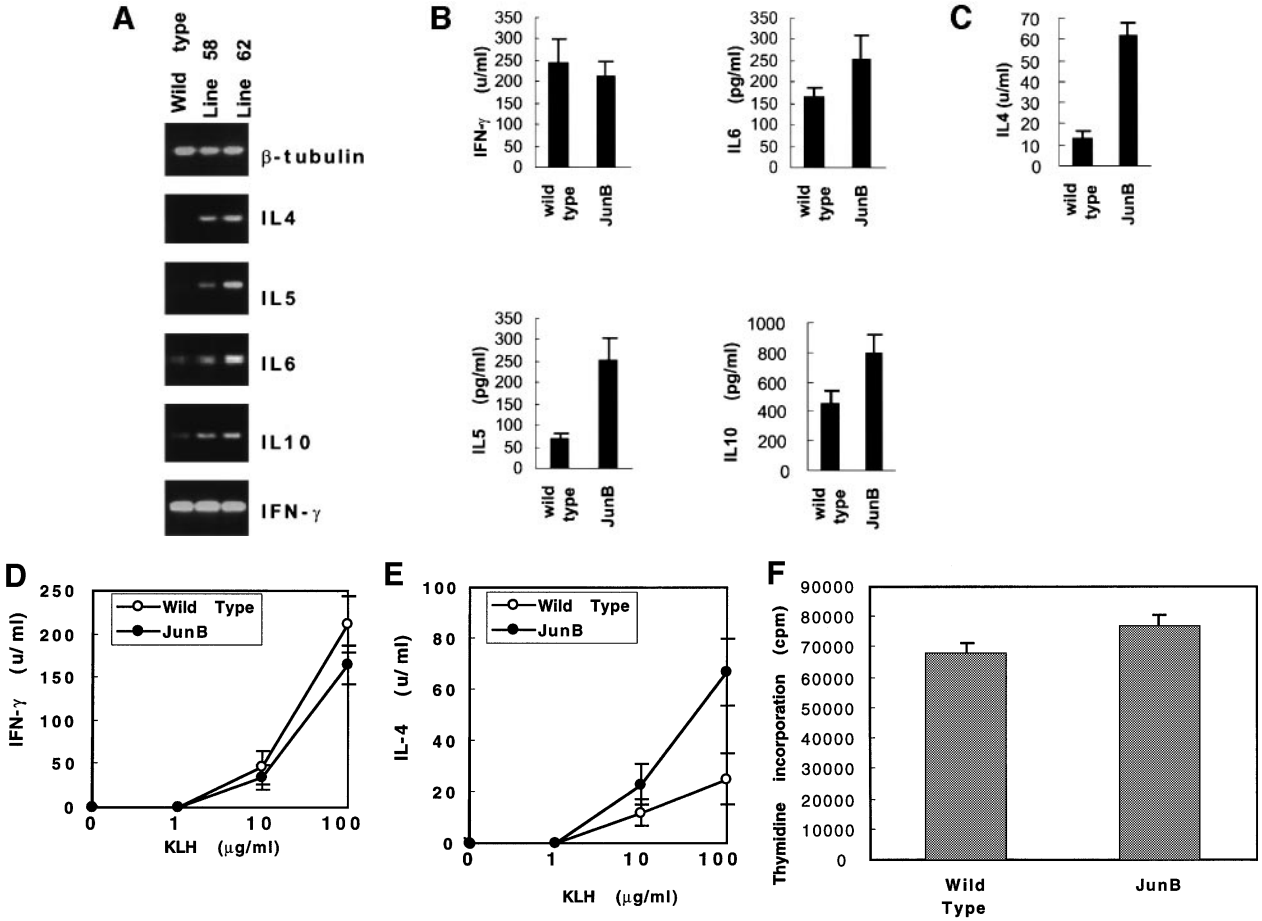


Fig. 7. Upregulation of Th2 cytokines in JunB transgenic mice. (**A**) RT–PCR. Naïve T cells were isolated from transgenic or wild-type littermates and differentiated *in vitro* toward Th1 using ConA, IL-12 and anti-IL-4 antibody. Three days later, total RNA was isolated and cytokine RT–PCR was done. β-tubulin was used as a control. (**B**) ELISA. Cytokine ELISA was performed with the supernatant of the day 3 culture used in Figure 7A. Average values from line 58 and line 62 were used for the graph. (**C**) *In vitro* T cell differentiation was performed as described in Figure 7A except that no anti-IL-4 antibody was added. The amount of IL-4 secreted was examined by cytokine ELISA. (**D** and **E**) Cytokine production in KLH-immunized mice. T cells from draining lymph node of KLH immunized mice were cultured with various amount of KLH for 4 days. IL-4 and IFN-γ was measured using ELISA. The wild-type mice used were the transgene negative littermates. (**F**) [^{3}H]thymidine was added to the culture at day 3 and incorporation of radioactivity was measured 18 h later in triplicate.

facilitate DNA binding even with PMA/ionomycin treatment, while extracts from JunBglu transfected cells were sufficient to mediate binding even without PMA/ionomycin treatment. These experiments argue strongly that the cooperative DNA binding of JunB and c-Maf is facilitated by JunB phosphorylation.

To map the DNA sequence that is required for this cooperative binding, we used the –87/–31 bp fragment of the IL-4 promoter with mutations at various sites. Mutations at the P1 AP-1 site and the MARE half site greatly reduced DNA binding while mutations at either NFAT sites had no effect (Figure 5D). These experiments indicated that JunB and c-Maf bind distant elements (centered on –80 and –40, respectively) of the IL-4 promoter and that cooperativity of binding is facilitated through JunB phosphorylation. To test further whether the AP-1 and MARE sites are required for JunB and c-Maf mediated synergistic activation of IL-4 promoter, we generated IL-4 promoter luciferase constructs with either the AP-1 site or the MARE site mutated. Figure 5E shows that both constructs lost the ability to support synergistic promoter activation by JunB and c-Maf in luciferase assays. Thus, synergy between JunB and c-Maf requires functional AP-1 and MARE sites.

Upregulation of cytokines in differentiating Th1 cells from JunB transgenic mice

To examine whether JunB plays a causal role in Th2 cytokine expression *in vivo*, we generated transgenic mice that express JunB in T cells (Figure 6A). Since the CD4 promoter construct we employed lacks the CD4 silencer, JunB is expressed in both CD4 and CD8 cells (Killeen *et al.*, 1993). The expression of the JunB transgene in peripheral T cells was verified by RT–PCR using one primer derived from the first exon of CD4 gene and the other primer from the 5′ region of JunB cDNA (Figure 6A). Of the nine transgenic lines generated, four lines tested positive for transgene expression. Among them, line 62 has a higher copy number and higher expression level than other lines (Figure 6B). We then examined JunB protein levels in T cells by Western blot analysis (Figure 6C). JunB protein was significantly increased in transgenic mice compared with wild-type littermates. It should be noted that this increase is roughly equivalent to the

increase of the endogenous JunB protein in Th2 cells (Figure 1). Expression of the JunB transgene did not have any overt effect on lymphocyte development since the transgenic lines had normal profiles of thymocyte subpopulations staining for CD4 and CD8 and spleen cells (CD4 and CD8 cells, and B cells) (data not shown). The proliferation of naïve CD4 T cells in response to Con A, IL-2 and APC was slightly enhanced in the transgenic lines compared with the wild-type littermates (Figure 6D).

To test whether JunB is sufficient to turn on Th2 cytokine expression, we examined the effect of the JunB transgene in Th1 cells, a condition where Th2 cytokine expression is not usually observed. We reasoned that elevation of the JunB level in Th1 cells by transgenesis to an equivalent level as found in Th2 cells would test whether JunB plays a similar role in Th2 differentiation. Naïve CD4 T cells from transgene-positive animals and negative littermates were differentiated *in vitro* to the Th1 subset by culture with ConA plus IL-2, IL-12 and anti-IL-4 mAb for 3 days, by which time T helper cells have already differentiated to produce the corresponding cytokines. The expression levels of several cytokines were examined by RT–PCR (Figure 7A). In both transgenic lines examined, the level of IFN-γ was essentially the same as that in the wild-type littermate controls. In contrast, a much higher level of IL-4 and IL-5 mRNA was observed in both transgenic lines than in the negative littermate controls. The levels of IL-6 and IL-10 were also increased. The slightly stronger signal seen in line 62 corresponded with its higher level of JunB expression than in other lines.

To quantitate the amount of cytokines produced in the culture, we performed ELISA with the supernatant of the day 3 culture used above (Figure 7B). In the transgenic T cells, IFN-γ remained unchanged at high level. The amount of IL-5 was increased by 3- to 4-fold; the amount of IL-6 and IL-10 were both increased, although to a lesser extent. IL-4 secretion by both wild-type and transgenic cells was virtually undetectable due to the fact that anti-IL-4 mAb was added to the culture to skew the response toward Th1 differentiation (data not shown). To quantitate the difference in IL-4 secretion, we repeated the above experiment without anti-IL-4 mAb. ELISA measurements showed that T cells from JunB transgenic mice produced 4- to 5-fold more IL-4 than negative littermates (Figure 7C). These data indicated that overexpression of JunB was sufficient to turn on Th2 cytokine expression, even in a Th1 population.

Upregulation of IL-4 in antigen re-stimulated T cells from KLH-immunized mice

To test whether JunB transgenic T cells were predisposed to develop into IL-4 producing cells *in vivo*, we immunized mice using Keyhole Limpet Hemocyanin (KLH) with complete Freund's Adjuvant (CFA) and harvested T cells from draining lymph nodes after 9 days. Cytokine production by these cells in response to KLH was measured by ELISA (Figure 7D and E). KLH in CFA elicits an immune reaction skewed toward Th1. As expected, we saw a relatively high level of IFN-γ production using wild-type T cells. The amount of IFN-γ produced in transgenic T cells, however, was slightly lower (Figure 7D). Interestingly, although the amount of IL-4 produced in both wild-type and transgenic cells was quite low, there was a consistently higher amount of IL-4 produced by the transgenic T cells (Figure 7E). At the same time, the proliferative ability of the transgenic T cells was essentially the same as the negative littermate T cells (Figure 7F). Thus, elevation of JunB levels to that of Th2 cells is sufficient to direct the synthesis of Th2 cytokines in Th1 cell populations.

Discussion

The molecular basis for T-helper-restricted expression of cytokines is poorly understood. IL-4 is the key cytokine that determines the Th2-differentiation pathway. Recently, c-Maf (Ho *et al*., 1996) and GATA-3 (Zheng and Flavell, 1997) have been shown to be selectively expressed in Th2 but not in Th1 cells. Both factors are able to induce IL-4 gene expression in populations that normally do not express this gene. In addition, GATA-3 has a profound global effect on the expression of most Th2 cytokines. While it is at present unclear how GATA-3 acts, either locally at cytokine promoters or globally at the Th2 locus or both, c-Maf directly binds and activates the IL-4 gene. However, it is not yet clear how c-Maf plays its role in Th2 differentiation since by the time c-Maf upregulation is manifested at day 8 after T cell activation (Ho *et al*., 1996), Th2 differentiation has been completed for several days. Our studies (Rincón *et al*., 1997; this report) show that a third transcription factor, JunB, is selectively expressed in developing Th2 cells but not in Th1 cells. Moreover, we now demonstrate that JunB plays a causal role in this process since JunB overexpression in transgenic mice upregulated all Th2 cytokines assayed. We further demonstrate that JunB and c-Maf activate IL-4 expression synergistically. The early upregulation of JunB in Th2 cells could provide the tissue specificity for c-Maf during Th2 differentiation while the accumulation of large amounts of c-Maf later might serve to maintain a high level of IL-4.

Previous studies of the role of AP-1 in T helper cell development have generated somewhat inconsistent results. First, different observations were made as to whether AP-1 complexes are differentially accumulated in Th1 versus Th2 cells. In gel-shift experiments using extracts from long-term Th1 and Th2 clones AE7 and D10, Rooney *et al*. (1995) found that AP-1 complexes from both AE7 and D10 cells were able to bind the P1 element of the IL-4 promoter, and that JunB was present in both of the complexes that were detected by EMSA. However, in another study using primary T cells (Wenner *et al*., 1997), the AP-1-containing complex which shows a lower mobility in the gel-shift assay was only observed using activated Th2 extracts. A parallel study (Rincón and Flavell, 1997) also found accumulated AP-1 complexes with some c-Fos, and high levels of JunB-containing complexes in Th2 cells but not in Th1 cells. Furthermore, AP-1 transcriptional activity was also enhanced in Th2 cells relative to Th1 cells. Secondly, different conclusions have been made as to whether AP-1 contributes to Th2-specific IL-4 expression. The observation that the NFAT/AP-1 sites of the IL-2 and IL-4 promoters were functionally interchangeable in AE7 and D10 cells (Rooney *et al*., 1995) led to the proposal that AP-1 does not determine

Th1/Th2 specificity. However, in transgenic mice, multimerization of the same NFAT/AP-1 site was found to direct luciferase reporter gene expression only in Th2 cells (Wenner *et al*., 1997) as was the expression of similar AP-1 and NFAT/AP-1 sites from the IL-2 promoter (Rincón and Flavell, 1997; Rincón *et al*., 1997). These discrepancies could simply represent the different behaviors of long-term T cell clones and primary cells, or alternatively, differences in experimental systems. The analysis of promoter activity during the process of Th1/Th2 differentiation not only provides a normal cellular context, but also addresses developmental requirements and we have therefore used this approach (Rincón and Flavell, 1997; Rincón *et al*., 1997; this study).

Our finding that JunB is specifically induced in developing Th2 cells provides a molecular explanation for the observation that P1 NFAT/AP-1 elements drive Th2-restricted expression. Several mechanisms assure the specificity of IL-4 gene activation by JunB. First, the differential induction of JunB was not observed for either c-Jun or JunD. Thus, JunB is the only Jun family member that is differentially regulated and has the potential to contribute to Th-specific gene expression. Secondly, among the Jun family members, only JunB is capable of synergizing with c-Maf to activate the IL-4 gene. As shown by Western blot analysis, c-Jun is present in both Th1 and Th2 cells, and c-Jun alone possesses an ability to activate the IL-4 promoter that is similar to JunB. However, the synergy between JunB and c-Maf generates much higher IL-4 promoter activity, while c-Jun can only generate at most low level activity in this context. Finally, the fact that JunB was found to inhibit c-Jun transcriptional activity could provide another mechanism for this specificity (Chiu *et al*., 1989; Schutte *et al*., 1989). Although it was proposed that JunB might form heterodimers with c-Jun and thus attenuate the formation of c-Jun homodimers, the mechanism for JunB inhibition of c-Jun transcriptional activity is not clear. Our discovery that JunB is an excellent substrate for JNK raised the possibility that in those experiments overexpressed JunB might have competed with c-Jun for JNK binding and thus led to reduced c-Jun phosphorylation.

The fact that JunB has a much lower activity than c-Jun in driving transcription of a TRE-luciferase reporter and its apparent inhibitory effect on c-Jun transcriptional activity (Chiu *et al*., 1989; Schutte *et al*., 1989) has loosely classified JunB as a transcriptional repressor. However, our study has clearly shown that JunB directly binds the IL-4 promoter and activates its transcription. Interestingly, JunB does not activate two other constructs containing AP-1 binding sites, while c-Jun does. Thus, JunB differs in its promoter selectivity from c-Jun and activates or inhibits transcription within a specific promoter context.

A striking finding of our current study is the role of phosphorylation on JunB function in T helper cells. Previously JunB had not been thought to be a phosphorylation substrate for JNK, despite the fact that this molecule has a conserved JNK binding site (Gupta *et al*., 1996; Kallunki *et al*., 1996). We found, however, that the synergy of JunB with c-Maf was strongly potentiated by stimulation of M12 cells with PMA plus ionomycin. Furthermore, direct studies of JunB phosphorylation showed that two threonine residues flanked by prolines (Thr102 and Thr104) were phosphorylated equally efficiently as the prototypic JNK phosphorylation sites at Ser63 and Ser73 of c-Jun (Figure 3A). The JunB phosphorylation sites are similar to the phosphorylation sites on ATF-2, also recognized by JNK (Gupta *et al*., 1995). Consistent with these findings, removal of these phosphorylation sites by replacement of both Thr residues with Ala, abolished phosphorylation and reduced the transcriptional synergy with c-Maf. Conversely, replacement of these Thr residues with Glu, mimicking constitutive phosphorylation at these sites, gave stronger synergy in the absence of stimulation with PMA plus ionomycin than was observed with wild-type or Ala mutants (Figure 4C).

The initial observation of transcriptional synergy between JunB and c-Maf and the fact that they are both b-Zip proteins raised the possibility that they might form heterodimers (Kerppola and Curran, 1994). Although we found that JunB directly interacts with c-Maf *in vitro*, we could not identify a DNA element that binds JunB within the vicinity of the c-Maf binding site. The identification of the P1 element as the binding site for JunB indicated that JunB and c-Maf probably bind to independent sites, possibly as homodimers. The DNA-binding experiment using c-Maf beads suggests that JunB and c-Maf interact with each other, directly or indirectly (Figure 5). This interaction is enhanced by JunB phosphorylation, which also contributed significantly to the transcriptional synergy between JunB and c-Maf suggesting that this interaction mediates this synergy. The potentiation of c-Maf DNA binding by JunB appears to be mediated, at least in part, by the phosphorylation of JunB by JNK at Thr102 and Thr104. This may explain why c-Jun only poorly mediates this interaction and therefore does not synergize with c-Maf to drive transcription mediated by the IL-4 promoter. In contrast, c-Jun is strongly phosphorylated at Ser63 and Ser73, and is only poorly phosphorylated by JNK on other sites.

Similar to our previous findings with GATA-3, JunB stimulates expression of multiple Th2 cytokine genes. JunB strongly actives IL-4 and IL-5, and modestly activates IL-6 and IL-10 expression (Figure 7) whereas GATA-3 strongly increased IL-4, IL-6 and IL-10, and to a lesser extent IL-5. JunB could induce IL-4 expression directly and increase the expression of other cytokines indirectly through the effect on IL-4. However, the fact that little IL-4 was present in the Th1 culture due to the addition of IL-4 antibody argues against the latter scenario. Alternatively, JunB could directly activate each cytokine gene.

JunB emerges as a new Th2-specific transcription factor that regulates Th2-restricted cytokine expression. It would be interesting to further dissect in future experiments the immediate early events that lead to upregulation of JunB in Th2 cells but not in Th1 cells, which ultimately influences the fate of Th1/Th2 differentiation.

Materials and methods

Plasmid constructs and site-directed mutagenesis

The IL-4-luciferase reporter plasmid pIL4-157luc has been described previously (Zheng and Flavell, 1997). The expression vectors c-Jun/pMexNeo, JunD/pMexNeo and JunB/pMexNeo II were kind gifts of Dr R.Bravo. pREP4/NFATc was a kind gift from Dr L.Glimcher. pSH210 (expression vector for human NFATc2/NFATp) was kindly provided by Dr G.Crabtree. pSKMSV/EBPβ (expression vector for C/EBPβ) was

kindly provided by Dr S.McKnight. Bacterial expression of GST–Jun (Dérijard *et al*., 1994), GST–ATF2 (Gupta *et al*., 1995) and GST–Myc (Alvarez *et al*., 1991) have been described previously. The mammalian and the bacterial expression vectors for wild-type and mutants JunB were constructed by subcloning the JunB full-length DNA into pCDNA3 (Invitrogen) and a PCR fragment corresponding to residues 1–248 of JunB into pGEX-5X1 (Pharmacia-LKB Biotechnology, Inc.). The Flag epitope (-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-; Immunex Corp.) was inserted between codons 1 and 2 by insertional overlapping PCR (Ho *et al*., 1989). To replace Thr102 and Thr104 with Ala or Glu, site-directed mutagenesis was performed with overlap extension by PCR as described previously (Ho *et al*., 1989). The structure of the expression vectors was confirmed by automated sequencing using a model 373A machine (Applied Biosystems Inc.). The GST fusion proteins were purified by glutathione affinity chromatography (Smith and Johnson, 1988). The IL-4 luciferase constructs with AP-1 or MARE sites mutated were generated using Quick Change Kit (Stratagene). The primers used were: CCAGAATAACTGACAATCTGGCTGCAGAAAATTTTCCAA-TGTAAACTC and GAGTTTACATTGGAAAATTTTCTGCAGCCA-GATTGTCAGTTATTCTGG for the AP-1 site mutation; CTCATTTT-CCCTTGGTTTCGAATTCTTTAACTCTATATATAGAGAG and CTCT-CTATATATAGAGTTAAAGAATTCGAAACCAAGGGAAAATGAG for the MARE site mutation.

In vitro differentiation of CD4 T cells

Naïve CD4 T cells were isolated from transgenic mice or wild-type littermates using methods described previously (Kamogawa *et al*., 1993). Preparation of APC has also been described previously (Kamogawa *et al*., 1993). To differentiate CD4 T cells *in vitro*, naïve T cells (1×10^6/ml) were cultured with an equal number of APC, 2.5 mg/ml Con A and 30 U/ml IL-2 in Bruff's medium containing 5% fetal calf serum (FCS). For Th1 differentiation, IL-12 (3.5 ng/ml) and the anti-IL-4 monoclonal antibody 11B11 were added; for Th2 differentiation, IL-4 (1000 U/ml) and the IFN-γ monoclonal antibody XMG1.2 were added. For re-stimulation, cells were harvested 4 days after stimulation, washed and cultured for 24 h in Bruff's medium supplemented with 2.5 μg/ml Con A.

Transgenic mice

The JunB cDNA was subcloned into the *Sal*I site of the CD4 promoter/enhancer plasmid p37.1 (a gift from Dr D.Littman), which expresses in both CD4 and CD8 cells. A *Not*I fragment of this plasmid was isolated and injected into fertilized eggs of $(B6\times C3H)F_2$ and transgenic mice were generated as previously described (Hogan *et al*., 1986). Founders were screened with primer CD4seq2 derived from the CD4 second exon and primer JunBr1 derived from the 5′ end of JunB cDNA. Positive founders were confirmed by Southern blotting. The expression of the transgene was checked with RT–PCR on peripheral lymphocytes using primer CD4seq1 derived from the first exon of CD4 gene and the JunBr1. Founders were bred with B6 mice and the F_1 mice were used for experiments. The sequences of the primers are as follows: JunBr1, 5′-GTGGGTTTCAGGAGTTTGTAGTCG-3′; CD4seq2, 5′-GCTCA-GATTCCCAACCAACAAGAG-3′; CD4seq1, 5′-GTGAAGGAAGG-ACTGGCCAGAG-3′.

RT–PCR

Total RNA was prepared using Trizol from Gibco-BRL according to the manufacturer's instructions. Reverse transcription was done using Superscript II (Gibco-BRL). The primers for cytokines and PCR conditions were described previously (Reiner *et al*., 1994; Zheng and Flavell, 1997). The primers for β-tubulin are 5′-TGTCCATGAAGGAGGTGG-ATGAG-3′ and 5′-ATGTTGCTCTCAGCCTCGGTGAAC-3′.

Immunization

Mice were immunized with 50 μg KLH in complete Freund's adjuvant (CFA) in each hind footpad. CD4 T cells in draining lymph nodes were isolated 9 days later as described previously (Kamogawa *et al*., 1993) and 2×10^5 cells were cultured with APC in the presence of the indicated amount of KLH. After 4 days, supernatant IL-4 and IFN-γ was assayed using an ELISA kit as recommended by the manufacturer (PharMingen). For proliferation assays, 1 μCi [^{3}H]thymidine was added to the culture. Cells were harvested after 18 h and the radioactivity was measured with a β-counter (Beckman). Triplicates of each sample were measured and average values determined.

EMSA

Naïve T cells were differentiated to Th2 *in vitro* for 3 days. The cells were activated with PMA (50 ng/ml) and ionomycin (1 μM) for 2 h and nuclear extracts were isolated as previously described (Schreiber *et al*., 1989; Tugores *et al*., 1992). Complementary oligo nucleotides containing P1 element (5′-GACAATCTGGTGTAATAAAATTTTCCAATG-3′ and its complementary strand) were labeled with [^{32}P]ATP (ICN) using T4 polynucleotide kinase (NEB), annealed, and purified using a G50 Sepharose column. Radiolabeled probe (2×10^4 c.p.m.) was incubated in the binding reaction buffer 10 mM HEPES pH 7.4, 1 mM EDTA, 50 mM NaCl, 8% glycerol, 1 mM DTT, 0.5 mM PMSF together with 4 μg of nuclear extract, 4 μg of poly(dI–dC) for 30 min on ice. In the supershift experiments, JunB and c-Jun polyclonal antibodies (Santa Cruz) were pre-incubated with extract for 20 min before adding probe. The total volume for the binding reaction is 20 μl, of which, 15 μl was loaded onto a 4% polyacrylamide gel with 0.5× TBE and run at 16 mA at 4°C for 2.5 h.

Western blot analysis

Nuclear extract (20 μg) was run on a precast 10% SDS–PAGE gel (Novex) and transferred to Immobilon-p transfer membrane (Millipore) with a semi-dry transfer unit (Hoefer). The membrane was blotted with polyclonal JunB, c-Jun, JunD antibodies (Santa Cruz) or a monoclonal antibody against the Flag epitope (Kodak) and processed using the ECL Western blot reagents (Amersham). A polyclonal antibody against actin was used as a control for loading.

Tissue culture and transfection assays

COS-7 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified environment with 5% CO_2. Transient transfections were performed with the LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. After 36 h, the cells were serum-starved overnight and treated with UV-C (80 J/m^2), or TPA (100 ng/ml) for 60 and 15 min, respectively, prior to lysis. Cell extracts were prepared in lysis buffer (20 mM Tris pH 7.4, 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride] and centrifuged at 915 000 *g* (15 min at 4°C). The concentration of total soluble protein in the supernatant was quantitated using the Bradford method (Bio-Rad). The B cell lymphoma M12 was cultured in Bruff's medium supplemented with 5% FBS. The transfection of M12 cells was done essentially as previously described (Ho *et al*., 1996).

Protein kinase assays

Epitope-tagged MAPKs were immunoprecipitated from cell extracts by incubation for 3 h at 4°C with the M2 monoclonal antibody to Flag (IBI-Kodak) or 12CA5 monoclonal antibody to HA (Boehringer Mannheim) bound to protein G–Sepharose beads (Pharmacia-LKB Biotechnology, Inc.). Endogenous JNK, p38 MAPK and ERK were immunoprecipitated with polyclonal antibodies pre-bound to protein A–Sepharose beads (Pharmacia-LKB Biotechnology, Inc.). The immune complexes were washed twice with lysis buffer and three times with the kinase buffer (25 mM HEPES pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). The MAPK activity was measured at 30°C for 20 min in 30 μl of kinase buffer containing 1 μg of recombinant GST–Jun, GST–JunB, GST–ATF2 or GST–Myc and 50 μM [γ-^{32}P]ATP (10 Ci/mmol, 1 Ci = 37.6 Gbq). The reactions were terminated by addition of Laemmli sample buffer. Proteins were resolved by 10% SDS–PAGE and identified by autoradiography. The incorporation of [^{32}P]phosphate into the GST fusion protein was quantitated by PhosphorImager analysis.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed by partial acid hydrolysis (1 h at 110°C in 6 M HCl) and thin layer electrophoresis as described (Davis and Czech, 1985).

Protein immobilization and DNA binding

The expression vectors pET29 containing N-terminal (amino acid residues 1–170) and C-terminal (amino acid residues 171–370) fragments of c-Maf were kind gifts from Dr L.Glimcher. The truncated c-Maf proteins were expressed using T7 polymerase in the BL21 (DE3) strain. The proteins were induced as described (Ho *et al*., 1996) and purified using the *S*-tag purification kit (Novagen). Proteins were immobilized on the beads without the final elution step. *In vitro* transcription–translation was done using the Promega TNT coupled rabbit reticulocyte lysate system with [^{35}S]methionine incorporated. Proteins from a 12.5 μl

reaction were used as input to assay for c-Maf binding. The reaction also contains 20 µl c-Maf beads in a total volume of 50 µl. The binding buffer was the same as the EMSA binding buffer. The samples were mixed on a rotator at room temperature for 2 h and washed with binding buffer three times. Proteins immobilized on the beads were separated on a 10% SDS–PAGE gel and exposed to film. The transcription–translation vectors JunDpKS$^+$, JunBpKS$^+$ and c-JunpTZ18R were kind gifts of Dr R.Bravo. For the c-Maf-beads DNA binding experiments either 12.5 µl TNT product or 100 µg extract from M12 cells (2×10^6 M12 cells transfected with 20 µg JunB, JunBala, JunBglu or c-Jun expression vector; PMA/ionomycin was added 1 h before making extracts), 40 µl c-Maf Beads, 30 µg poly(dI–dC) with or without radiolabeled DNA fragment (1×10^5 c.p.m.) in 100 µl total volume were incubated at room temperature for 2 h with constant mixing. After binding, beads were washed three times and extracted with phenol. DNA was ethanol precipitated and resolved on an 8% polyacrylamide gel.

Luciferase assay

For luciferase assays, 4 µg IL-4 promoter luciferase reporter plasmid and 0.8 µg c-Maf expression plasmids were used. For all other expression plasmids, 2 µg of was used unless otherwise noted in the figure legends. Protein concentration of the M12 extracts were measured using the Bio-Rad protein assay reagent. Two-hundered micrograms of extract was used to assay luciferase activity with the Promega luciferase assay substrate. Fifty nanograms of a LacZ reporter plasmid was co-transfected in all the experiments. Fifty micrograms of extract was incubated with 8 mM chlorophenolred-β-D-galactopyranoside (CPRG), 0.2 M Na_2HPO_4 pH 7.3, 1 mM $MgCl_2$, and 45 mM β-mercaptoethanol for 20 min at 37°C and OD_{570} readings were measured.

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