Regulation and composition of activator protein 1 (AP-1) transcription factors controlling collagenase and c-Jun promoter activities

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The activator protein 1 (AP-1) transcription factor is composed of heterodimers of the Fos/activating transcription factor (ATF) and Jun subfamilies of basic-region leucine-zipper (B-ZIP) proteins. In order to determine the identities of individual B-ZIP proteins in various AP-1 complexes we tested the effect of dominant-negative mutants to the B-ZIP proteins c-Fos, ATF2, ATF4 and CCAAT-enhancer-binding protein (C/EBP) on the activities of the collagenase and c-Jun promoters. These dominant-negative mutants inhibit DNA binding of wild-type B-ZIP proteins in a leucine-zipper-dependent fashion. Transcription of a collagenase promoter/reporter gene was induced in HepG2 hepatoma cells by expression of c-Fos and c-Jun, administration of PMA ('TPA') or by expression of a truncated form of MEK (mitogen-activated/extracellular-signal-regulated kinase kinase) kinase-1, MEKK1Δ. In all cases, the dominant-negative mutants A-Fos and A-ATF2 decreased collagenase promoter activity. However, A-ATF4 and A-C/EBP had no effect. A-Fos and A-ATF2 also reduced MEKK1Δ-induced stimulation of the c-Jun promoter. In contrast, constitutive c-Jun promoter activity was blocked solely by A-ATF2, strongly suggesting that ATF2 and/or an ATF2-dimerizing protein are of major importance for c-Jun transcription in unstimulated cells. These results demonstrate that AP-1 transcription factors of different compositions control c-*jun* gene transcription in resting or stimulated cells.

Key words: ATF2, ATF4, c-Fos, MEKK1.

INTRODUCTION

The activator protein 1 (AP-1) transcription factor was originally described as a heterodimer of c-Jun and c-Fos [1,2]. These basic-region leucine-zipper (B-ZIP) transcription factors dimerize via their leucine-zipper domains, which in turn bring together their basic domains to bind DNA in a sequence-specific manner. In recent years several homologues of c-Jun (junB and junD) and c-Fos (FosB, fra1 and fra2) have also been shown to form heterodimers. In addition, members of the activating transcription factor (ATF) family, such as ATF2, ATF3 and ATF4, can interact with members of the Fos and Jun family of proteins [3,4]. Whereas the Jun and ATF proteins each form stable homodimers as well as heterodimers, c-Fos does not dimerize and is incapable of binding DNA as a homodimer [5–8]. Thus, c-Fos exhibits no biological activity without a dimerization partner.

AP-1 plays important roles in signalling, growth control and apoptosis (reviewed in [9,10]). To understand the biological functions of AP-1, it is very important to elucidate the roles that individual B-ZIP proteins play in the cell as part of various AP-1 complexes. For example, the c-Jun–c-Fos heterodimer binds *in vitro* with high affinity to the sequence motif 5′-TGAGTCA-3′, also known as 'TPA' (PMA)-responsive element (TRE). The TRE-containing collagenase promoter has frequently been used to monitor c-Fos/c-Jun activity [11,12]. A c-Jun–ATF2 heterodimer shows low binding activity to the TRE derived from the collagenase promoter. However, this heterodimer binds *in vitro* with high affinity to the sequence 5′-TTACCTCA-3′ found in the c-Jun promoter [13]. Thus different DNA sequence elements are preferentially recognized depending on the composition of the AP-1 dimer.

One approach to studying multiple combinatorial variants of the AP-1 complex is the use of dominant-negative mutants termed A-ZIPs. These reagents inhibit DNA binding of the wild-type B-ZIP proteins in a leucine-zipper-dependent fashion. A-ZIP proteins are amphipathic molecules that contain an acidic region instead of the natural basic domain N-terminal to the leucine zipper domain. This acidic extension of the leucine zipper forms a heterodimeric coiled-coil structure with the basic region of its target that is more stable than the B-ZIP dimer bound to DNA. The A-ZIP mutants work by forming either homodimers (e.g. A-ATF2–ATF2) or heterodimer complexes (e.g. A-ATF2–c-Jun) with wild-type B-ZIPs, which are then defective for DNA binding. Moreover, given the fact that the leucine zipper of the B-ZIP protein determines its dimerization characteristics, an A-ZIP mutant can potentially form heterodimers with an entire subfamily of B-ZIP proteins. For example, A-ATF2 can bind to c-Jun and possibly to all other Jun family members to inhibit the formation of any Jun–ATF2 heterodimer. Thus compensation due to the activity of related and potentially redundant B-ZIP proteins, which occurs readily in the case of some transgenic mouse models, is reduced or eliminated by the use of A-ZIPs.

In this study we used A-ZIP mutants of c-Fos, ATF2, ATF4 and CCAAT-enhancer-binding protein (C/EBP) to analyse the occupancy of TREs found in the collagenase and c-Jun pro-

Abbreviations used: AP-1, activator protein 1; ATF, activating transcription factor; MEKK, mitogen-activated/extracellular-signal-regulated kinase kinase kinase; TRE, 12-*O*-tetradecanoylphorbol 13-acetate ('TPA'; PMA)-responsive element; C/EBP, CCAAT-enhancer-binding protein; B-ZIP, basic-region leucine-zipper; JNK, c-Jun N-terminal kinase.

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moters. The results show clear differences in the composition and the response to stimuli of the AP-1 complexes that regulate collagenase and c-Jun transcription. This study also demonstrates that the A-ZIP proteins are remarkable tools for studying the role of AP-1 in general and the role of individual B-ZIP proteins in living cells.

EXPERIMENTAL

Reporter constructs

The plasmid −517/+63CAT5, containing the human collagenase regulatory sequence, was a kind gift from Peter Angel, German Cancer Research Center, Heidelberg, Germany. A fragment containing the collagenase promoter was subcloned into plasmid pGL3-Basic (Promega), generating plasmid pColl(−517/+63)luc. The c-Jun promoter/luciferase reporter plasmids pjunwtluc−1600/+170, pjunΔ1,2luc−1600/+170 and pjunwtluc−1600/+170 have been described previously [14]. The plasmid pUAS^{5}luc containing the luciferase reporter gene, a TATA box derived from the HIV long terminal repeat, an initiator element from the adenovirus major late promoter and five binding sites for GAL4 (termed the upstream activating sequence, or UAS) has been described in [15].

Expression constructs

Expression vectors encoding A-ATF2, A-ATF4, A-C/EBP and A-Fos have been described in [16,17]. The c-Jun and c-Fos expression vectors pMT119 and pMT35 were kindly given by Dirk Bohmann, EMBL, Heidelberg, Germany. An expression plasmid encoding MEKK1Δ, a truncated form of MEK kinase 1 (mitogen-activated/extracellular-signal-regulated kinase kinase kinase 1, MEKK1) that lacks amino acids 1–351 [18], was kindly given by Michael Karin, University of California, San Diego, CA, U.S.A. Expression vectors pRSVβ and pSV40lacZ, encoding β-galactosidase under the control of the Rous sarcoma virus long terminal repeat and the simian virus 40 promoter, respectively, have been described in [19,20].

Cell culture, transfections and reporter-gene assays

The murine neuroblastoma cell line NS20Y and the human hepatoma cell line HepG2 were cultured and transfected as described in [21]. The amounts of expression vectors transfected are indicated in the Figure legends. The luciferase reporter plasmids (1–2 μg) and the internal reference plasmids pRSVβ or pSV40lacZ (2 μg) were transfected into cells grown on 60 mm plates. Lysates were prepared 48 h post-transfection using cell-culture lysis buffer (Promega) and β-galactosidase and luciferase activities were measured [15,21]. Each experiment was repeated at least twice with consistent results. COS cells were transfected using the DEAE-dextran method as described in [22].

Western blots

Whole-cell extracts and nuclear extracts were prepared as described in [22,23]. The monoclonal antibody T7-Tag™ directed against the 12-amino-acid gene φ-10 leader peptide MASMTGGQQMGR was purchased from Novagen (catalogue no. 69522-3). Proteins from transfected COS cells were separated on a 15% Tricine/polyacrylamide gel [24] and transferred to nitrocellulose membranes (0.2 μm pore size; Schleicher & Schuell, Dassel, Germany). Blots were incubated for 30 min at room temperature with T7-Tag™ antibody (1:5000 dilution) and developed using an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma; catalogue no. A-3562) at a dilution of 1:10000. To analyse c-Jun expression in HepG2 and NS20Y cells, 40 μg of nuclear proteins was separated by SDS/PAGE and the blots were incubated with an antibody directed against c-Jun (Santa Cruz Biotechnology, Heidelberg, Germany; catalogue no. sc-1694) at a 1:1000 dilution.

RESULTS

Modular structure and expression of A-ZIP mutants

The B-ZIP family of transcription factors is characterized by the presence of a basic domain that functions as the DNA-binding domain and a leucine-zipper domain that is required for dimerization. A sequence comparison of the B-ZIP domains of c-Fos, ATF2, ATF4 and C/EBP is depicted in Figure 1(A). The A-ZIP mutant proteins preserve the leucine-zipper domain, but the basic region is replaced by an acidic region. The modular structure of the A-ZIP mutants is depicted in Figure 1(B). The A-ZIP domain is linked to two tags used for detection of the proteins by antibodies directed against either the FLAG epitope or the leader sequence of gene φ-10 derived from the T7 major capsid protein. Whole-cell extracts of COS cells transfected with expression vectors encoding A-Fos, A-ATF2, A-ATF4 and A-C/EBP were fractionated by Tricine SDS/PAGE. The proteins were identified by Western-blot analysis using antibodies targeting the gene φ-10 leader peptide. Figure 1(C) shows that all proteins were expressed as expected.

The collagenase promoter

The human collagenase promoter was chosen to test the biological activity of the A-ZIP mutants. This promoter contains an AP-1-binding site in the proximal promoter region and has been a paradigm for the study of AP-1-mediated transcription. The collagenase promoter/luciferase reporter gene transcription unit is depicted in Figure 2(A). To test the responsiveness of the promoter to increased concentrations of c-Fos and c-Jun, expression vectors encoding c-Jun and c-Fos were transfected into HepG2 hepatoma cells simultaneously with the pColl(−517/+63)luc reporter plasmid. The β-galactosidase expression vector pRSVβ was used to correct for variations in transfection efficiency. Post-transfection (48 h), cell extracts were prepared and β-galactosidase and luciferase activities were determined. Luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities. The results of these experiments are depicted in Figure 2(B). Whereas c-Fos or c-Jun alone had only slight effects upon reporter-gene transcription, co-transfection of c-Jun and c-Fos expression vectors increased transcription by approx. 20-fold, underlining the important role of a c-Jun–c-Fos dimer in regulating collagenase gene transcription.

Next, we additionally transfected expression vectors encoding A-Fos, A-ATF2, A-ATF4 or A-C/EBP. A-Fos was clearly the most effective reagent in inhibiting c-Fos/c-Jun-stimulated transcription of the reporter gene under these conditions (Figure 2C). Previous reports have shown that c-Fos does not form homodimers, but heterodimerizes with c-Jun with great stability [5–8]. A-ATF2 also showed a significant effect in blocking collagenase promoter activity. A-ATF4 and A-C/EBP dominant-negative mutants had no observable effects (Figure 2C).

The AP-1 motif was initially discovered as a genetic element conferring transcriptional stimulation in response to activators of protein kinase C, i.e. PMA. As shown in Figure 2(D), PMA strongly activated the transcription of a reporter gene under control of the human collagenase promoter in HepG2 cells. The

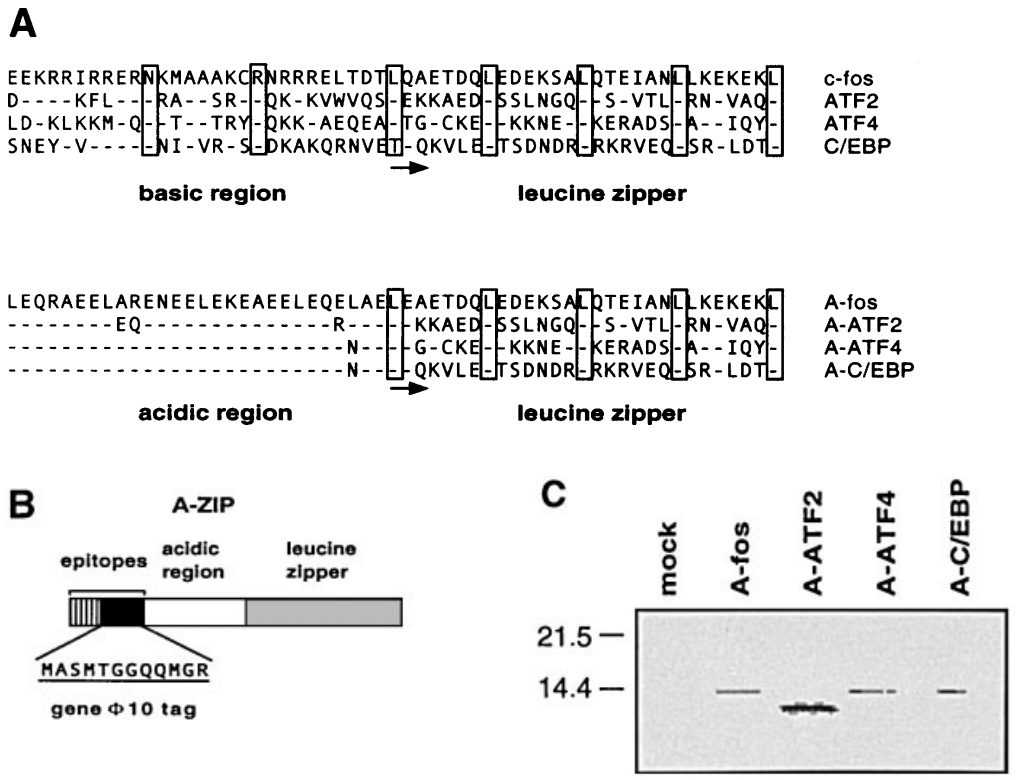

Figure 1 Modular structure of the A-ZIP proteins

(**A**) Protein sequence of the basic regions and leucine-zipper domains of the B-ZIP transcription factors c-Fos, ATF2, ATF4 and C/EBP. The leucine residues of the leucine zipper as well as the invariant arginine and asparagine residues within the basic domains are indicated. The A-ZIP proteins A-Fos, A-ATF2, A-ATF4 and A-C/EBP retain the leucine zipper. The basic domain, however, is exchanged for a domain containing acidic residues. (**B**) Schematic of the domain structure of the A-ZIP proteins. The proteins are small, consisting mainly of the acidic domain, the leucine zipper and two immunological tags (FLAG and gene ϕ-10 leader peptide). (**C**) Western-blot analysis of COS cells transfected with expression vectors encoding the A-ZIP proteins. As a control, extracts from mock-transfected COS cells were analysed. Western blots were probed with the T7-Tag™ antibody directed against the 12-amino-acid gene ϕ-10 leader peptide MASMTGGQQMGR. The numbers to the left of the gel denote molecular-mass markers (in kDa).

PMA-stimulated transcription was effectively blocked by the dominant-negative A-Fos and A-ATF2 mutants, but not by A-ATF4 and A-C/EBP (Figure 2E). The fact that similar results were seen in PMA-induced transcriptional activation as with transcription stimulated via c-Fos and c-Jun overexpression confirms that the latter results are not due to an anomalous effect of overexpression.

The activity of the AP-1 transcription factor is strongly potentiated by phosphorylation of c-Jun, ATF2 or other constituents of AP-1. c-Jun N-terminal kinase (JNK) was shown to phosphorylate Ser/Thr-Pro motifs located in the activation domains of c-Jun, JunB, JunD and ATF2 [25,26]. To stimulate JNK activity, we transfected an expression vector encoding a truncated form of MEKK1 (MEKK1Δ). MEKK1 is a strong activator of the JNK pathway. Experimentally, MEKK1Δ has been shown to activate the transcriptional activity of a fusion protein consisting of the GAL4 DNA-binding domain and the c-Jun transactivation domain [27]. We have confirmed this observation (results not shown). In HepG2 cells MEKK1Δ was additionally able to elevate the transcriptional activation potential of ATF2 (L. Steinmüller and G. Thiel, unpublished work). We used the MEKK1Δ expression vector to study the regulation of the collagenase promoter. Transfection efficiency was monitored by co-transfecting pSV40lacZ. We noticed an effect of MEKK1Δ upon Rous sarcoma virus long-terminal-repeat-controlled β-galactosidase expression. We therefore employed pSV40lacZ as the reference plasmid together with the MEKK1Δ expression vector. No effect of MEKK1Δ on simian virus 40 promoter-controlled transcription was observed. As shown in Figure 2(F), transfection of an expression vector encoding MEKK1Δ activated collagenase promoter activity strikingly. This activation was blocked by co-transfection of A-Fos or A-ATF2 expression vectors. These results indicate that MEKK1Δ activates endogenous B-ZIP transcription factors via phosphorylation, which subsequently stimulate collagenase promoter activity. The fact that A-Fos and A-ATF2 impaired MEKK1Δ-stimulated collagenase promoter/reporter gene transcription indicates that the MEKK1Δ-activated B-ZIP proteins were able to dimerize with c-Fos and ATF2.

The c-Jun promoter

The c-Jun promoter contains two TRE-like motifs termed jun1TRE and jun2TRE. Dimers of c-Jun and ATF2 bind to these DNA sequences as shown by binding assays *in vitro*. Thus the main regulators of c-*jun* gene expression are thought to be c-Jun and ATF2 [13]. Three reporter plasmids containing the luciferase reporter gene, 170 nucleotides of the 5′-untranslated region and 1600 or 290 nucleotides of the 5′-flanking region of the human c-Jun promoter, were tested (Figure 3A). In the transcription unit present in plasmid pjunΔ1,2luc−1600/170, the two TRE-like motifs were inactivated by mutation [28]. c-Jun

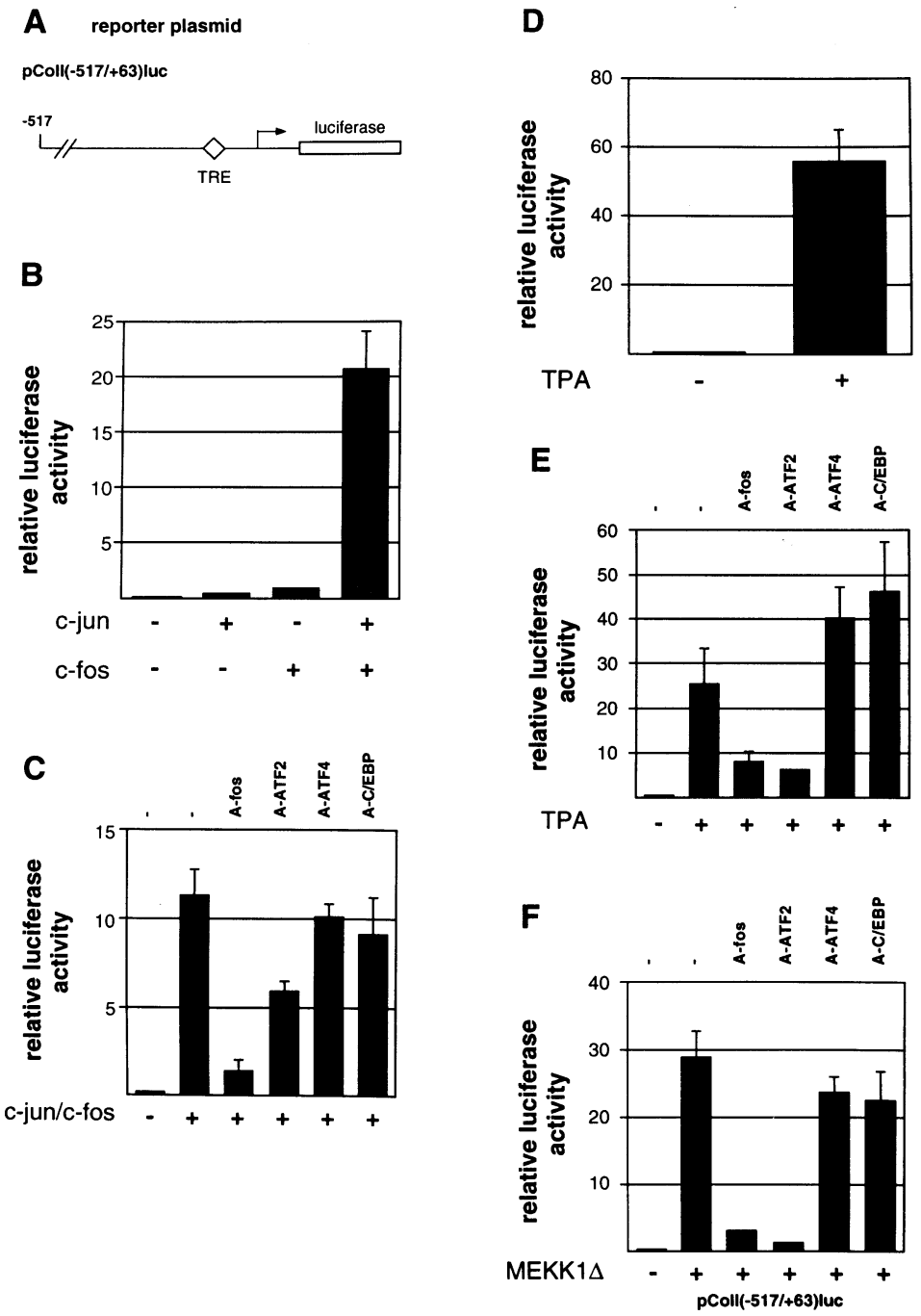

Figure 2 Biological effects of A-ZIP proteins on c-Jun/c-Fos-, PMA- or MEKK1Δ-stimulated collagenase promoter activity in HepG2 cells

(**A**) The collagenase promoter/luciferase reporter plasmid pColl(−517/+63)luc containing the luciferase reporter gene and the human collagenase promoter. (**B**) pColl(−517/+63)luc and either 750 ng/plate of the 'empty' expression vector pCMV5 (−) or expression vectors encoding c-Jun or c-Fos (+) were transfected into HepG2 hepatoma cells. The internal standard plasmid pRSVβ was included in the transfection. Then 48 h later cell extracts were prepared and the β-galactosidase and luciferase activities determined. At least four experiments were performed and the means ± S.E.M. are depicted. (**C**) HepG2 cells were transfected with pColl(−517/+63)luc, expression vectors encoding c-Jun and c-Fos, pRSVβ and 500 ng/plate of expression vector encoding A-Fos, A-ATF2, A-ATF4 or A-C/EBP. Cell extracts were prepared 48 h post-transfection. At least two experiments in quadruplicate were performed and the means ± S.E.M. are depicted. (**D**) The pColl(−517/+63)luc and pRSVβ plasmids were transfected into HepG2 cells. Cells were starved in medium containing 0.05% serum for 24 h and then incubated with PMA ('TPA') for 8 h. (**E**) HepG2 cells were transfected with pColl(−517/+63)luc, pRSVβ and 50 ng/plate of A-Fos, A-ATF2, A-ATF4 or A-C/EBP expression vector. Cells were starved in medium containing 0.05% serum for 24 h and then incubated with PMA for 8 h. At least two experiments in quadruplicate were performed and the means ± S.E.M. are depicted. (**F**) pColl(−517/+63)luc was transfected into HepG2 cells with the pSV40lacZ internal standard plasmid and an expression vector encoding MEKK1Δ (+). In addition, 150 ng/plate of A-Fos, A-ATF2, A-ATF4 or A-C/EBP expression vector was transfected. Cell extracts were prepared 48 h post-transfection. Means ± S.E.M. are shown; this experiment was perfomed twice with consistent results. For panels (**B**) to (**F**), luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities.

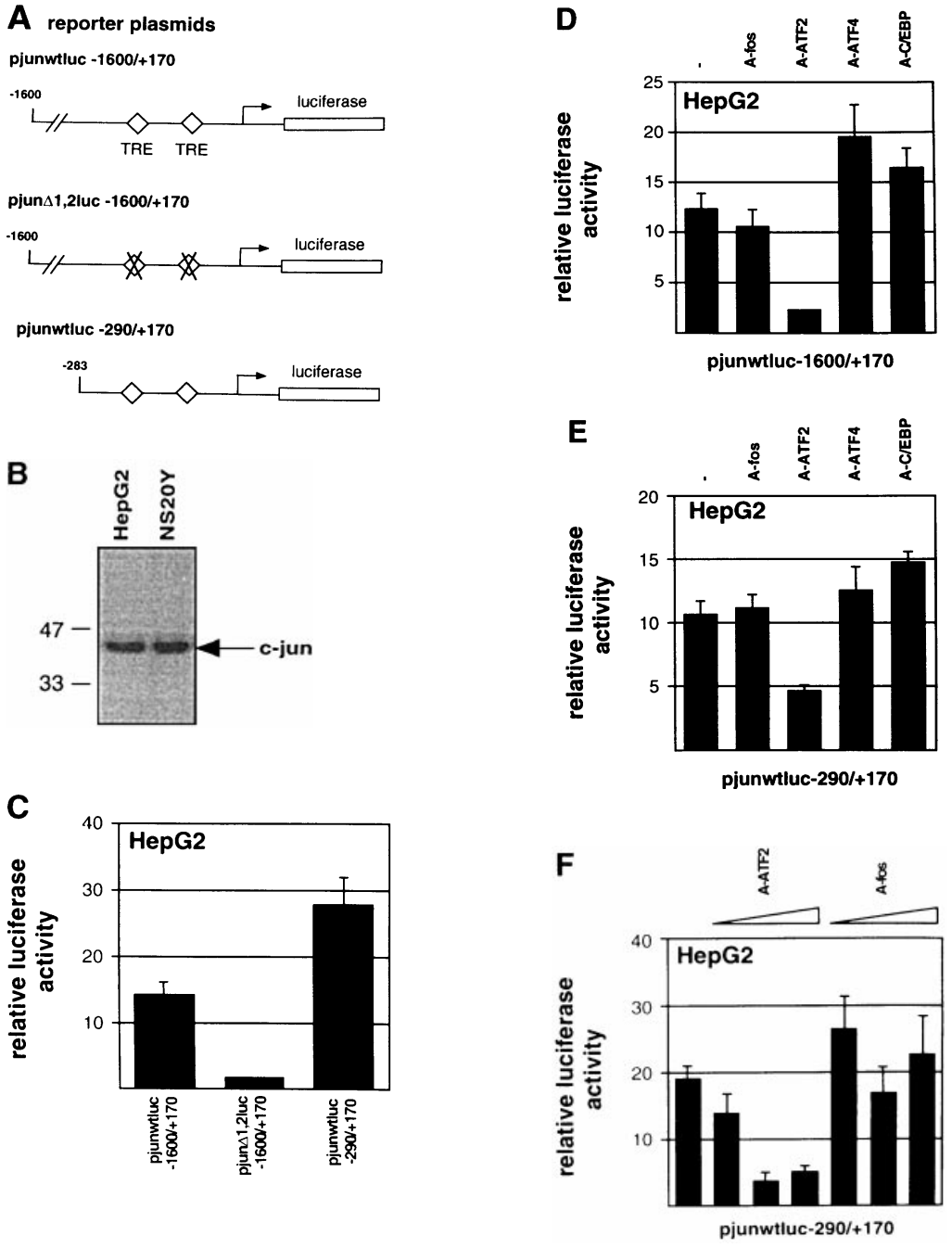

Figure 3 Biological effects of A-ZIP mutants upon constitutive c-Jun promoter activity in HepG2 hepatoma cells

(**A**) The reporter plasmids pjunwtluc −1600/+170, pjunwtluc −290/+170 and pjunΔ1,2luc −1600/+170 contain the luciferase reporter gene and the regulatory region derived from the human c-*jun* gene. The c-*jun* TRE motifs jun1TRE and jun2TRE were inactivated in the transcription unit present in pjunΔ1,2luc −1600/+170. (**B**) Nuclear extracts were prepared from the HepG2 and NS20Y cells. Proteins (40 μg) were separated by SDS/PAGE (10% gel) and blotted to nitrocellulose membranes. The membranes were probed with antiserum directed against c-Jun. The Western blot was developed using a peroxidase-coupled goat anti-rabbit antibody and ECL. The numbers to the left of the gel denote molecular-mass makers (in kDa). (**C**) Plasmids pjunwtluc −1600/170, pjunwtluc −290/170 and pjunΔ1,2luc −1600/170 were transfected into HepG2 cells together with the pRSVβ internal standard plasmid. (**D**, **E**) HepG2 cells were transfected with (**D**) pjunwtluc −1600/+170 or (**E**) pjunwtluc −290/+170, pRSVβ and 150 ng/plate of A-Fos, A-ATF2, A-ATF4 or A-C/EBP expression vector. (**F**) HepG2 cells were transfected with pjunwtluc −290/+170, pRSVβ and 50, 100 or 150 ng/plate of expression vector encoding A-Fos or A-ATF2. For panels (**C**) to (**F**), after cell extracts had been prepared, luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities.

is constitutively expressed in HepG2 and NS20Y neuroblastoma cells, as shown by immunoblot analysis (Figure 3B). The c-Jun promoter/luciferase reporter plasmids pjunwtluc −1600/+170, pjunwtluc −290/+170 and pjunΔ1,2luc −1600/+170 were transfected individually into HepG2 cells together with the internal reference plasmid pRSVβ. Figure 3(C) shows constitutive reporter gene activity generated by transfection of plasmids pjunwtluc −1600/+170 and pjunwtluc −290/+170. Interest-

ingly, the transcription unit containing the truncated form of the c-Jun promoter (plasmid pjunwtluc−290/+170) activated reporter gene activity approx. 2-fold more than the longer version (plasmid pjunwtluc−1600/+170). In contrast, the reporter gene under control of the mutated c-Jun promoter showed little basic activity in HepG2 cells, supporting the view that the TRE-like motifs jun1TRE and jun2TRE are of major importance for constitutive c-Jun expression. Next, we tested the effect of the dominant-negative A-ZIP mutants on constitutive c-Jun promoter activity. The reporter plasmid pjunwtluc−1600/+170 and the internal reference plasmid pRSVβ were transfected into HepG2 hepatoma cells together with expression vectors encoding A-Fos, A-ATF2, A-ATF4 and A-C/EBP. Post-transfection (48 h), cell extracts were prepared and β-galactosidase and luciferase activities were determined. Only A-ATF2 impaired c-Jun promoter activity, whereas A-Fos, A-ATF4 and A-C/EBP had no effect (Figure 3D). Similar results were obtained using the pjunwtluc−290/+170 reporter plasmid. Only A-ATF2 impaired constitutive c-Jun promoter activity (Figure 3E). The A-ATF2 protein was shown to be expressed at higher levels than the other mutants (Figure 1C). To exclude the possibility that the level of expression of the A-ZIP mutants could be responsible for the observed effects, we performed a titration analysis. The results show that lower amounts of expression vector encoding A-ATF2 were already biologically active in repressing constitutive c-Jun promoter activity, whereas no effects were observed using an A-Fos-encoding vector (Figure 3F). Taken together, these results suggest that ATF2 and/or an ATF2-dimerizing protein other than c-Jun are the major transcription factors responsible for constitutive c-Jun promoter activity in HepG2 cells.

To exclude the possibility that this is a peculiarity of HepG2 hepatoma cells, we studied constitutive c-Jun promoter activity in NS20Y neuroblastoma cells. Again, we observed an impairment of constitutive c-Jun promoter activity by A-ATF2, but not by A-Fos, A-ATF4 or A-C/EBP, using the reporter plasmids pjunwtluc−1600/+170 (Figure 4A) and pjunwtluc−290/+170 (Figure 4B). Titration experiments revealed that even the lower amounts of A-ATF2 were biologically active in repressing constitutive c-Jun promoter activity (Figure 4C).

Under the experimental conditions described, c-Jun played no role in regulating its own gene transcription. However, phosphorylation of c-Jun by JNK increases its transcriptional activity ([27], and L. Steinmüller and G. Thiel, unpublished work), suggesting that phosphorylated c-Jun may play a role in c-*jun* gene transcription under 'stimulating' conditions. Therefore, we transfected the c-Jun promoter/luciferase reporter constructs with intact or inactivated TRE-like motifs into HepG2 cells together with a MEKK1Δ expression vector. MEKK1Δ strongly activated transcription mediated by the c-Jun promoter (plasmid pjunΔ1,2luc−1600/+170, Figure 5A). However, an inactivation of the TRE-like sequences resulted only in a marginal stimulation of reporter-gene transcription, indicating that the TRE motifs are targets for the MEKK1Δ-induced signalling pathway (plasmid pjunΔ1,2luc−1600/170, Figure 5A). Similar results were obtained with NS20Y neuroblastoma cells (Figure 5C). In HepG2 cells, MEKK1Δ-stimulated transcription of the c-Jun promoter was effectively blocked by the dominant-negative A-Fos and A-ATF2 mutants, but not by A-ATF4 and A-C/EBP (Figure 5B). Likewise, A-Fos and A-ATF2 repressed MEKK1Δ-stimulated c-Jun promoter activity in NS20Y cells (Figure 5D). The A-ZIP mutants A-ATF4 and A-C/EBP did not function as dominant-negative mutants. Rather, an activation of c-Jun promoter activity was observed in NS20Y cells following transfection of A-ATF4- or A-C/EBP-encoding expression vectors (Figure 5D). In summary, the fact that A-Fos impaired MEKK1Δ-stimulated

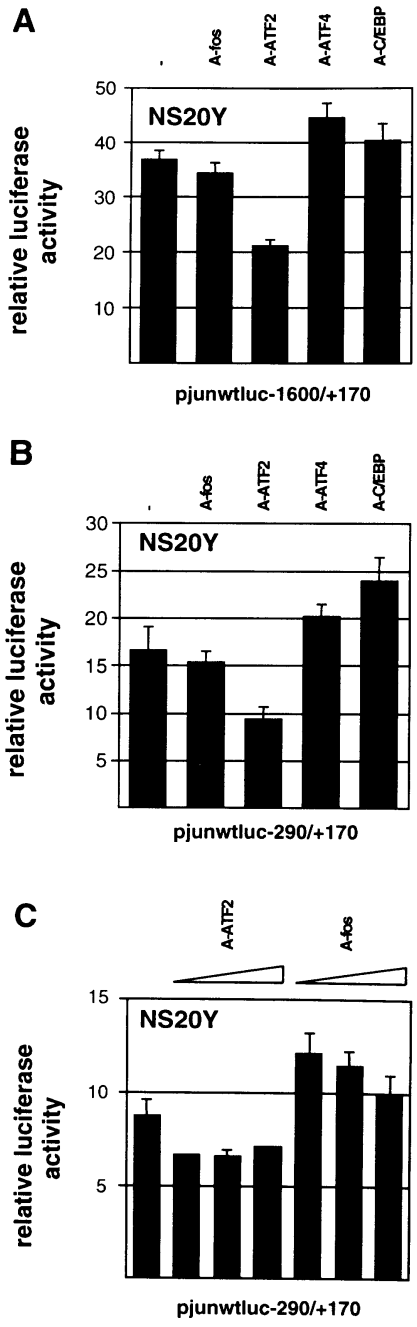

Figure 4 Biological effects of A-ZIP mutants upon constitutive c-Jun promoter activity in NS20Y neuroblastoma cells

(**A**, **B**) NS20Y neuroblastoma cells were transfected with the (**A**) pjunwtluc−1600/+170 or (**B**) pjunwtluc−290/+170 reporter plasmids, the pRSVβ reference plasmid and 500 ng/plate of expression vector encoding A-Fos, A-ATF2, A-ATF4 or A-C/EBP. Cell extracts were prepared and the β-galactosidase and luciferase activities determined. (**C**) NS20Y cells were transfected with pjunwtluc−290/+170, pRSVβ and 150, 300 or 500 ng/plate of A-Fos or A-ATF2 expression vector. Cell extracts were prepared and the β-galactosidase and luciferase activities determined.

transcription in both HepG2 and NS20Y cells indicates that, under these conditions, the AP-1 complex is composed of proteins that can form dimers with c-Jun and ATF2. These results demonstrate that AP-1 transcription factors of different compositions control c-*jun* gene transcription in resting or stimulated cells.

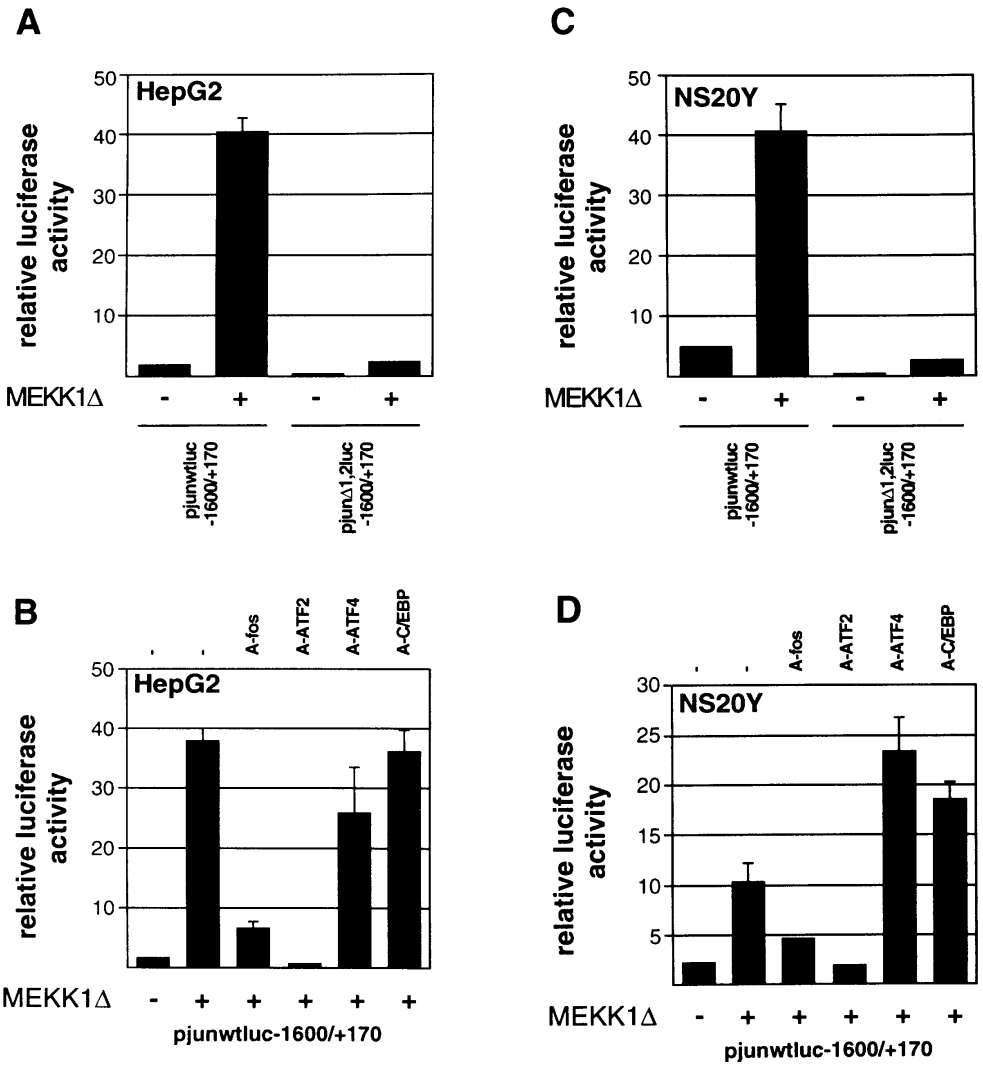

Figure 5 Expression of MEKK1Δ enhances c-Jun promoter activity in HepG2 cells

(**A**, **C**) The c-Jun promoter/luciferase reporter plasmids pjunwtluc −1600/+170 and pjunΔ1,2luc −1600/+170 were transfected into (**A**) HepG2 or (**C**) NS20Y cells together with the pSV40lacZ internal standard plasmid and 100 ng/plate of MEKK1Δ expression vector. Luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities. (**B**) HepG2 or (**D**) NS20Y cells were transfected with pjunwtluc −1600/+170 reporter plasmid, pSV40lacZ, MEKK1Δ expression vector (100 ng/plate) and (**B**) 150 or (**D**) 500 ng/plate of A-Fos, A-ATF2, A-ATF4 or A-C/EBP expression vector. Cell extracts were prepared and the β-galactosidase and luciferase activities determined.

DISCUSSION

The complexity of AP-1 activation of gene transcription has become appreciated in recent years as a result of the identification of an increasing number of B-ZIP proteins that are able to bind to the TRE or related sequences. The objective of this study was to characterize the regulation and composition of the AP-1 transcription-factor complex in two AP-1-regulated transcription units. Initially described as a heterodimer of c-Jun and c-Fos that binds to the 5′-TGAGTCA-3′ TRE motif, the current view is that AP-1 actually consists of several distinct homodimers or heterodimers composed of various members of the Fos, Jun and ATF B-ZIP subfamilies. Although the core sequence of the AP-1-like binding sites found in the collagenase and c-Jun promoters shows a high degree of homology, the variation in these binding sites suggests that different AP-1 complexes may bind with varying affinities and differentially affect transcriptional activity. Results from this paper support this view. First, overexpression of c-Fos and c-Jun was able to increase selectively the activity of the collagenase promoter. The c-Jun promoter did not respond to increases in c-Jun and/or c-Fos concentrations (L. Steinmüller and G. Thiel, unpublished work). Second, stimulation by PMA increased collagenase promoter activity, but not c-Jun promoter activity (L. Steinmüller and G. Thiel, unpublished work). Further differences between the collagenase and the c-Jun AP-1 sites have been reported using UV light, phorbol ester and expression of E1A as stimuli [29,30]. Expression of MEKK1Δ, however, stimulated both collagenase and c-Jun promoters very efficiently. The promiscuous effect of MEKK1Δ is most probably due to the ability of this kinase to activate different B-ZIP proteins (e.g. c-Jun and ATF2).

In transfection assays using the collagenase promoter, A-Fos and A-ATF2 exhibited negative effects upon c-Fos/c-Jun-, PMA- or MEKK1Δ-induced collagenase promoter activity. A-Fos, like

c-Fos itself, is unable to homodimerize and acts as a dominant-negative to c-Jun or other Jun-family members, preventing transcriptional activation [16]. Likewise, ATF2 has been shown to dimerize with c-Jun *in vitro* [13]. The fact that A-ATF2 inhibits collagenase promoter activity indicates that a dimerization partner of ATF2 is part of the AP-1 complex regulating collagenase promoter activity. We observed frequently that A-ATF2 was as active as A-Fos in inhibiting PMA-induced collagenase promoter activity. In contrast, A-ATF2 was not as active in repressing c-Fos/c-Jun-stimulated transcription. This may indicate that the B-ZIP proteins forming these particular AP-1 complexes are different under different conditions. For example, ATF2–ATF2 and ATF2–jun dimers may be a more prominent component of the collagenase promoter complex under PMA-induced conditions, whereas Fos–Jun heterodimers are probably dominant in c-Fos/c-Jun-stimulated transcription. However, the full details of this scenario remain to be elucidated further. Nevertheless, these results point to a complicated pathway with redundant members.

Previous studies of binding *in vitro* suggested that a heterodimer of c-Jun and ATF2 binds to the c-Jun promoter and functions as the main regulator of c-*jun* gene transcription [13]. This heterodimer interacts with the TRE-like sequences jun1TRE and jun2TRE within the c-Jun promoter. The importance of these genetic elements was underlined by the fact that mutations within them blocked constitutive c-Jun promoter activity, as shown in the Results section (Figure 3C). Using transient-transfection experiments, we were unable to stimulate c-Jun promoter activity, using combinations of expression vectors encoding c-Jun, c-Fos or ATF2, or chemical inducers, such as PMA or the calcium ionophore A23187 (L. Steinmüller and G. Thiel, unpublished work). Similar observations have been reported by others [31]. The fact that PMA induces c-*jun* gene transcription in Jurkat cells [32] indicates that there are cell-type-specific variations in the regulation of the c-*jun* gene. We observed that the c-Jun promoter was constitutively active in HepG2 or NS20Y cells, giving us the opportunity to test the biological effects of the dominant-negative A-ZIP mutants upon constitutive c-Jun promoter activity. The results of these experiments show that ATF2 and/or an ATF2-dimerization partner are the major transcription factors responsible for constitutive c-Jun promoter activity in hepatoma and neuroblastoma cells. c-Jun is unlikely to be involved in regulating constitutive c-Jun promoter activity, as indicated by the fact that A-Fos did not show any impairment in reporter-gene transcription. Thus, ATF2 or an ATF2-dimerization partner distinct from c-Jun or other Jun-family members regulates c-*jun* gene expression under resting conditions. These data are in agreement with findings that c-Jun transcription can be initiated in cells that lack basal levels of c-Jun, such as F9 embryonal carcinoma cells [28,33]. Consistent with our results is the finding that ATF2 homodimers have been reported to bind very efficiently to the jun1TRE and jun2TRE motifs [28].

MEKK1Δ has been described to enhance the transcriptional activation potential of c-Jun [27], which we have confirmed (results not shown). In addition, we found that the biological activity of ATF2 is strikingly increased by MEKK1Δ (L. Steinmüller and G. Thiel, unpublished work). The fact that two major AP-1-constituting B-ZIP proteins are activated by MEKK1Δ made it very likely that the c-Jun promoter is sensitive to MEKK1Δ expression. As expected, the c-Jun promoter activity increased following transfection of a MEKK1Δ expression plasmid. Interestingly, stimulation of c-Jun promoter activity by expression of MEKK1Δ was impaired by A-Fos and A-ATF2, indicating that, under these conditions, an AP-1 complex that contains a c-Fos-dimerizing B-ZIP protein regulates c-Jun promoter activity. Thus the composition of AP-1 bound to the c-Jun promoter depends on the environment of the cells, e.g. resting or stimulating conditions.

No effects were observed using the dominant-negative mutants A-ATF4 and A-C/EBP. Whereas A-C/EBP was used as a negative control, we expected biological activity of A-ATF4 upon c-Jun-mediated transcription. ATF4, also known as CREB2, has been described in several studies to heterodimerize with c-Jun [4,34]. Moreover, recombinant A-ATF4 binds to recombinant c-Jun in binding assays *in vitro* (C. Vinson, unpublished work). Surprisingly, we observed that A-ATF4 and A-C/EBP occasionally stimulated transcription, e.g. in MEKK1Δ-expressing NS20Y cells, but not in MEKK1Δ-expressing HepG2 cells. Similar observations have been reported for keratinocytes [35]. The fact that A-ZIP mutants activate transcription in certain cell types indicates that these mutants may function as de-repressors. In this context, it is of interest that ATF4 was initially described as a transcriptional repressor [36]. Naturally, impairment of the activity of a repressor protein would result in the de-repression of transcription. The precise mechanism by which A-ATF4 and A-C/EBP enhance transcription awaits further investigation.

Taken together, we have shown that AP-1 transcription factors of different compositions are involved in the regulation of collagenase and c-Jun transcription. Clear differences were observed using over-expression of c-Jun and c-Fos, expression of a truncated MEKK1 (MEKK1Δ) or stimulation with PMA. Interestingly, MEKK1Δ activated the collagenase and the c-Jun promoter, most probably by the activation of several signalling pathways. This study shows furthermore that the A-ZIP proteins are remarkable tools for the study of AP-1 composition and activity in living cells. The A-ZIPs can be used, as shown here, to elucidate the dimerization preferences of B-ZIP proteins in constituting different AP-1 complexes. This information is required to understand the role of B-ZIP proteins in signalling, growth control and apoptosis.

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