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Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene

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Smad proteins play a key role in the intracellular signalling of transforming growth factor β (TGFβ), which elicits a large variety of cellular responses. Upon TGFβ receptor activation, Smad2 and Smad3 become phosphorylated and form heteromeric complexes with Smad4. These complexes translocate to the nucleus where they control expression of target genes. However, the mechanism by which Smads mediate transcriptional regulation is largely unknown. Human plasminogen activator inhibitor-1 (PAI-1) is a gene that is potently induced by TGFβ. Here we report the identification of Smad3/Smad4 binding sequences, termed CAGA boxes, within the promoter of the human PAI-1 gene. The CAGA boxes confer TGFβ and activin, but not bone morphogenetic protein (BMP) stimulation to a heterologous promoter reporter construct. Importantly, mutation of the three CAGA boxes present in the PAI-1 promoter was found to abolish TGFβ responsiveness. Thus, CAGA elements are essential and sufficient for the induction by TGFβ. In addition, TGFβ induces the binding of a Smad3/Smad4-containing nuclear complex to CAGA boxes. Furthermore, bacterially expressed Smad3 and Smad4 proteins, but not Smad1 nor Smad2 protein, bind directly to this sequence *in vitro*. The presence of this box in TGFβ-responsive regions of several other genes suggests that this may be a widely used motif in TGFβ-regulated transcription.

Keywords: DNA-binding/PAI-1/Smad/TGFβ/transcription

Introduction

Members of the transforming growth factor β (TGFβ) family have pivotal roles in intercellular communication (Roberts and Sporn, 1993). Effects of these pleiotropic molecules are executed via ligand-induced heteromeric complex formation of different type I and type II serine/threonine kinase receptors (Derynck, 1994; Massagué and Weis-Garcia, 1996). The two kinases act in sequence in which the type I receptor functions as a substrate for the type II receptor (Wrana *et al.*, 1994). Activation of the type I receptor by phosphorylation with the type II receptor kinase is essential and sufficient for TGFβ signalling (Wrana *et al.*, 1994; Wieser *et al.*, 1995). Recently, our understanding of TGFβ intracellular signalling has improved dramatically through studies of TGFβ-like signalling pathways in genetically accessible species. *Drosophila Mothers against dpp* (*Mad*) and *Madea* (Sekelsky *et al.*, 1995) and structurally related *Caenorhabditis elegans Sma* genes (Savage *et al.*, 1996) were discovered in genetic screens, and found to perform essential roles in intracellular signalling downstream of serine/threonine kinase receptors (Hoodless *et al.*, 1996; Newfeld *et al.*, 1996, 1997; Wiersdorff *et al.*, 1996). In vertebrates, at least nine homologues to Mad and Sma have been identified and termed Smads (Heldin *et al.*, 1997; Kretzschmar and Massagué, 1998). Smad proteins share two regions of high similarity, termed MH1 and MH2 domains, connected with a variable proline-rich sequence.

Smad proteins can be classified according to their role in signalling of TGFβ family members. Pathway-restricted Smads interact transiently with specific activated type I receptors and thus become phosphorylated at their C-terminus (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Abdollah *et al.*, 1997; Kretzschmar *et al.*, 1997; Souchelnytskyi *et al.*, 1997). Smad2 and Smad3 are specific mediators of TGFβ and activin pathways, whereas Smad1, Smad5 and MADH6/Smad9 are involved in BMP signalling (Baker and Harland, 1996; Eppert *et al.*, 1996; Graff *et al.*, 1996; Hoodless *et al.*, 1996; Liu *et al.*, 1996; Thomsen, 1996; Zhang *et al.*, 1996; Chen,Y. *et al.*, 1997; Suzuki *et al.*, 1997). Smad4 forms hetero-oligomers with the pathway-restricted Smads and is a common mediator of TGFβ, activin and BMP signalling (Lagna *et al.*, 1996; Wu *et al.*, 1997; Zhang *et al.*, 1997). Upon heteromeric complex formation, Smads translocate to the nucleus and function as transcriptional regulators; the C-terminal MH2 domain fused to a heterologous DNA-binding domain, was found to induce a transcriptional response (Liu *et al.*, 1996; Meersseman *et al.*, 1997). In addition, Smad2 and Smad4 participate in the activin-mediated transcriptional induction of the *Xenopus Mix-2* promoter where they act as co-activators via an interaction with the FAST-1 DNA-binding factor (Chen,X. *et al.*, 1996, 1997). Furthermore, the *Drosophila* Mad member of the family, deleted in the conserved MH2 region, binds directly to GC-rich regions of various enhancers (Kim *et al.*, 1997). Recently Smad6 and Smad7, which diverge structurally from other members of the family, have been shown to act as inhibitors of these signalling pathways by interfering with activation of the pathway-restricted Smads (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997a; Hata *et al.*, 1998).

TGFβ1 activates the transcription of mammalian genes important for cell cycle regulation, including the cyclin-dependent kinase (CDK) inhibitors p21 and p15 genes (Elbendary *et al.*, 1994; Li *et al.*, 1995; Datto *et al.*, 1997),

or for extracellular matrix formation, for instance the α2(I) procollagen, fibronectin and PAI-1 genes (Keeton *et al.*, 1991; Westerhausen *et al.*, 1991; Riccio *et al.*, 1992; Inagaki *et al.*, 1994). The PAI-1 gene is highly induced by TGFβ and multiple TGFβ-responsive regions have been described in its promoter. The endogenous PAI-1 promoter, as well as the synthetic transcriptional reporter p3TP-Lux, which contains part of the PAI-1 promoter, was shown to be synergistically induced by the overexpression of combinations of Smad2, Smad3 and Smad4 (Lagna *et al.*, 1996; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997b). In the present study, we identified a short repeated DNA element in the PAI-1 promoter with which Smad3 and Smad4 interact directly, and demonstrate that these elements are essential for mediating TGFβ responsiveness.

Results

The CAGA box is a TGFβ-inducible DNA element

We raised the possibility that a common sequence motif could be present in the TGFβ-responsive regions that have been identified along the human PAI-1 promoter (Keeton *et al.*, 1991; Westerhausen *et al.*, 1991; Riccio *et al.*, 1992). To address this question, we looked for a short DNA homology element and noticed that the sequence AG(C/A)CAGACA was found in three copies at positions –730, –580 and –280 in the human PAI-1 promoter in regions that have been shown to mediate TGFβ-transcriptional induction (Figure 1A). We named this sequence the CAGA box and cloned it in a transcriptional reporter system to determine its involvement in TGFβ-induced transcription. When cloned in multiple copies upstream of the thymidine kinase (TK) promoter, this DNA sequence confers TGFβ-mediated induction in HepG2 cells (Figure 1B), without affecting the basal activity of the vector. Similar results were observed in Mv1Lu cells (Figure 1C) or in NIH 3T3 cells (data not shown). Several hundred-fold TGFβ-mediated induction in HepG2 cells was obtained when multiple CAGA boxes were cloned upstream of a minimal promoter consisting of the TATA box and the initiator sequence of the adenovirus major late promoter (MLP) (Figure 1C). This induction was lower with the widely used TGFβ-responsive p3TP-Lux plasmid. It is noteworthy that p3TP-Lux contains the –740/–636 region of the PAI-1 promoter bearing the –730 CAGA box (Wrana *et al.*, 1992). As a control of specificity, the mutated sequence AGC-**T**A**C**A**T**A, containing three point mutations relative to the original sequence, was unable to confer TGFβ induction to the TK promoter (Figure 1B).

Mutation of the CAGA boxes in the human PAI-1 promoter abolishes TGFβ responsiveness

The wild-type human PAI-1 promoter contains three CAGA boxes. To explore the biological significance of these boxes in the TGFβ-mediated induction of this promoter, we mutated each of the three native sequences by introducing the TGFβ-non-induced mutant sequence (Figure 2). Mutation of one of the three sites led to a reduction in TGFβ induction compared with the wild-type promoter (Figure 2, Δb1, Δb2 and Δb3 mutants). With two sites, the reduction was greater (Figure 2, Δb1 + Δb2, Δb1 + Δb3 and Δb2 + Δb3 mutants) and when all three sites were mutated, the PAI-1 promoter was almost unable to respond to TGFβ (Figure 2, see Δb1 + Δb2 + Δb3 mutant). These CAGA boxes appear not to control significantly the basal activity of the promoter as, in the absence of TGFβ, the rate of transcription of the mutant promoters and the wild-type PAI-1 promoter were comparable.

The CAGA box responds to TGFβ and activin, but not to BMP

Specific serine/threonine kinase type I receptors transduce intracellular signalling of TGFβ family members; BMPR-IA (ALK-3), BMPR-IB (ALK-6) and ActR-I (ALK-2) are BMP type I receptors, whereas TGFβ and activin signal through TbR-I (ALK-5) and ActR-IB (ALK-4), respectively (Heldin *et al.*, 1997). To test the specificity of the CAGA box relative to TGFβ superfamily members, we transfected Mv1Lu cells, which are responsive to TGFβ, activin and BMP-7, with expression vectors encoding for constitutively activated versions of the type I receptors (Yamashita *et al.*, 1995). As shown in Figure 3A, expression of ALK-4/T206D and ALK-5/T204D led to transcriptional activation of the CAGA box reporter vector. In contrast, expression of ALK-2/Q207D, ALK-3/Q233D and ALK-6/Q204D did not show any effect, demonstrating that the CAGA sequence is activated by TGFβ and activin, but not by BMP-induced signalling in Mv1Lu cells. Similar results were obtained in HepG2 cells with transfection of constitutively activated versions of type I receptors (data not shown). In order to test more physiological conditions, we transfected HepG2 cells, which are responsive to activin and BMP-7 (S.Itoh and P.ten Dijke, unpublished), with a CAGA box reporter vector and incubated the cells with activin and BMP-7 (OP-1). As shown in Figure 3B, the CAGA boxes containing reporter was induced 25- and 200-fold respectively, in the presence of activin and TGFβ, whereas BMP-7 did not show any significant effect (2-fold induction). Thus, CAGA boxes respond specifically to activin and TGFβ, but not to BMP signalling.

Smad proteins participate in TGFβ-induced transcription mediated by the CAGA box

To examine whether Smad proteins were involved in the TGFβ-induced transcriptional activation observed with the CAGA box, we co-transfected HepG2 cells with a CAGA reporter construct and an expression vector encoding for the Smad7 protein, known to inhibit TGFβ/Smad-mediated transcriptional effects (Hayashi *et al.*, 1997; Nakao *et al.*, 1997a). As shown in Figure 4A, overexpression of Smad7 leads to a 50% inhibition of TGFβ-induced transcription of the CAGA box reporter construct, indicating a possible involvement of Smad proteins in CAGA box-mediated transcription.

MDA-MB468 cells, derived from a breast cancer, are human epithelial cells deficient for endogenous Smad4 expression (de Caestecker *et al.*, 1997; de Winter *et al.*, 1997). In these cells, TGFβ has no effect on a CAGA reporter construct (Figure 4B). However, co-transfection of an expression vector encoding for Smad4 restores TGFβ transcriptional induction of the CAGA boxes containing vector, demonstrating that Smad4 is necessary for the TGFβ transcriptional effect mediated by this sequence.

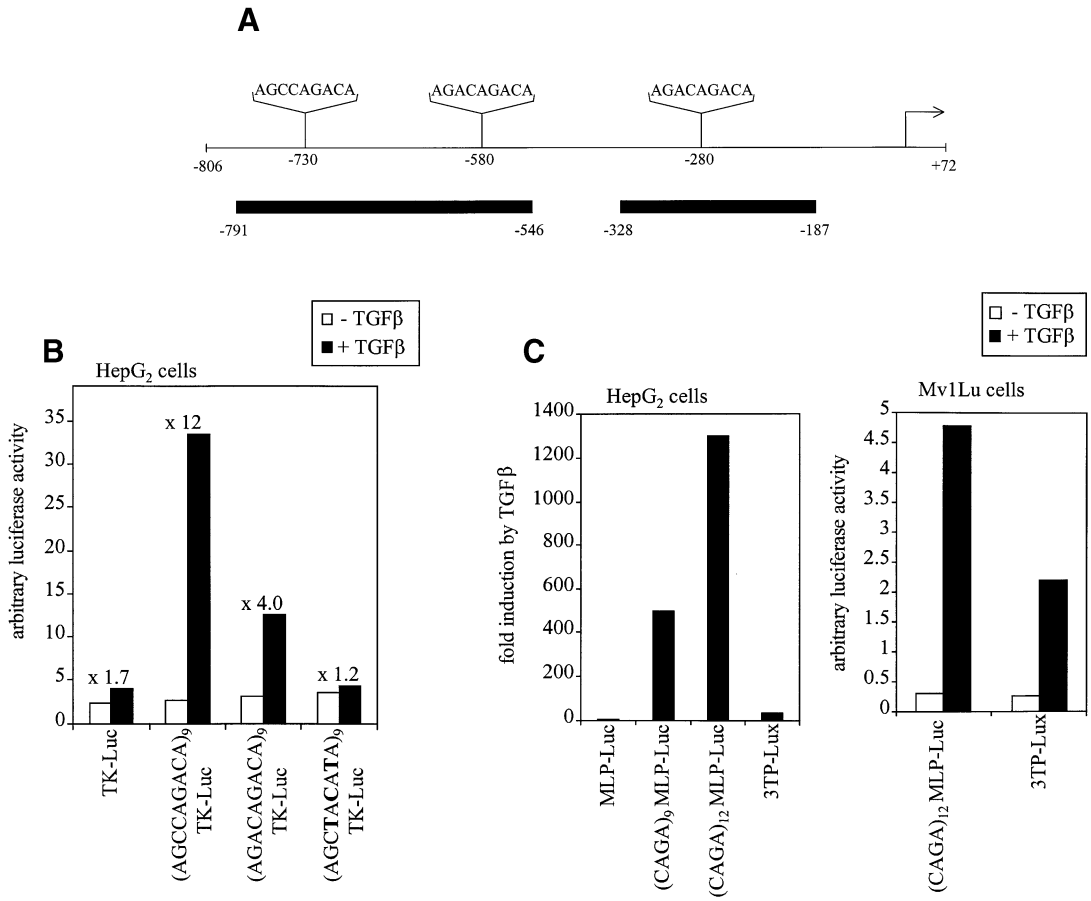

Fig. 1. The CAGA box is a TGFβ-inducible DNA element. (**A**) In the human PAI-1 promoter, two regions, depicted by black bars, have been described to respond to TGFβ. The sequences of the three CAGA boxes found in this promoter are given. (**B**) HepG2 cells were transfected with different vectors containing nine copies of the CAGA sequence cloned upstream of the HSV1-Thymidine kinase promoter (TK). AGCCAGACA is the sequence found at position –730 in the PAI-1 promoter and AGACAGACA is the sequence of the two other CAGA boxes of the PAI-1 promoter (positions –580 and –280). The last construct contains mutated CAGA boxes on 3 bp as indicated. Luciferase activities are shown and fold inductions by TGFβ are indicated. (**C**) HepG2 and Mv1Lu cells were transfected with p3TP-Lux or a vector containing nine or 12 copies of the CAGA box upstream of the minimal Adenovirus MLP. Fold inductions by TGFβ are given for HepG2 cells. Basal and TGFβ-induced luciferase levels are shown for Mv1Lu-transfected cells.

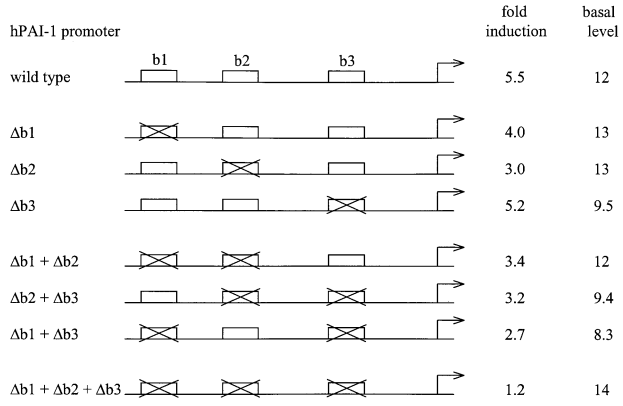

Fig. 2. The CAGA box of the human PAI-1 promoter are necessary for induction by TGFβ. Mutations of the CAGA boxes in the PAI-1 promoter were introduced by site-directed mutagenesis. The wild-type AG(C/A)CAGACA sites were replaced by the mutated AG(C/A) **TACATA** sequence. The mutated boxes are represented by a crossed rectangle. Basal levels in the absence of TGFβ and fold inductions in the presence of TGFβ in transfected HepG2 cells are given.

Previous studies have shown that overexpression of some Smad proteins activates transcription from TGFβ-responsive constructs like PAI-1-Luc or p3TP-Lux, even in the absence of TGFβ (Chen,Y. *et al.*, 1996; Lagna *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997b). As shown in Figure 4C, co-transfection in HepG2 cells of the two TGFβ-restricted pathway Smad2 and Smad3 expression vectors led to different results. Overexpression of Smad3 strongly activated a CAGA box reporter plasmid. In contrast, overexpression of Smad2 did not show any effect on the CAGA box reporter construct. This experiment indicates that Smad3 is able to increase transcription mediated by CAGA boxes and identifies a functional difference between the two closely related Smad2 and Smad3 proteins.

Smad3 and Smad4 are present in the transcription factor nuclear complexes that bind to the CAGA box

In a next step, we performed electrophoretic mobility shift assays (EMSA) using HepG2 nuclear extracts in an attempt to characterize the DNA-binding activity on the TGFβ-responsive CAGA sequence. We could identify binding complexes present only with nuclear extracts from cells induced by TGFβ (Figure 5A, compare lanes 2 and 3). Maximum binding requires a TGFβ-induction time of 30 min, but the complex can be clearly observed after a

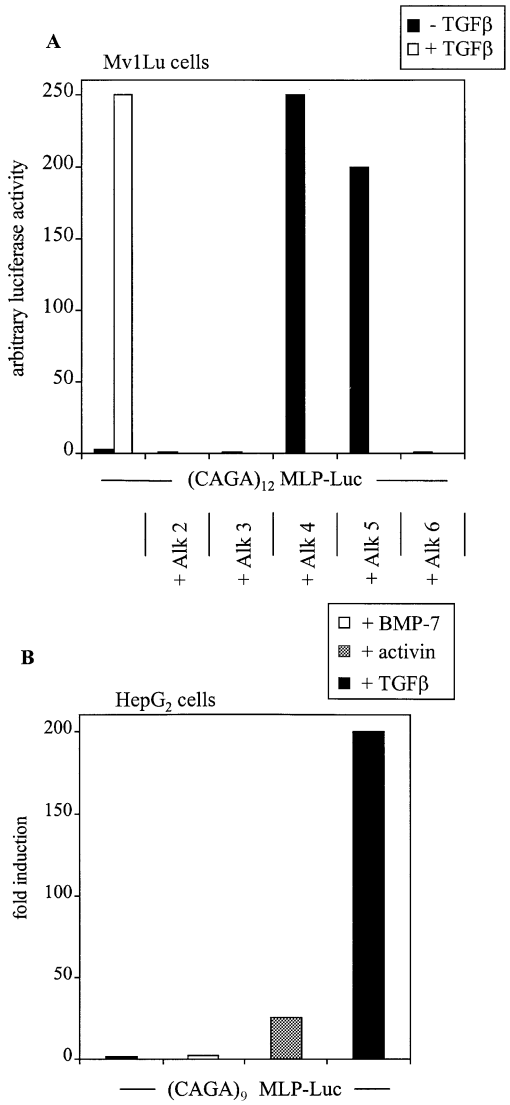

Fig. 3. The CAGA box responds to TGFβ and activin signalling but not to BMPs pathways. (**A**) Mv1Lu cells were co-transfected with a $(CAGA)_{12}$-MLP-Luc reporter construct and expression vectors encoding for constitutively activated versions of serine/threonine kinase receptors specific of TGFβ, activin or BMPs signalling. Alk-2 is the ActR-I receptor, Alk-3 the BMPR-1A receptor, Alk-4 the ActR-1B receptor, Alk-5 the TGFβR-1 receptor and Alk-6 the BMPR-1B receptor. (**B**) HepG2 cells were transfected with a $(CAGA)_9$-MLP-Luc reporter construct and induced by BMP-7, activin or TGFβ (100, 20 and 10 ng/ml, respectively).

10 min induction (data not shown). This suggests that *de novo* protein synthesis is not necessary and that a pre-existing factor is rapidly and post-translationally modified or translocated into the nucleus. This DNA-binding complex is specific since an excess of the non-radiolabelled CAGA oligonucleotide, but not of the mutated box, displaces the corresponding band (Figure 5A, lanes 4 and 5). Furthermore, this complex does not contain transcription factors proposed as potential mediators of TGFβ/activin signalling such as Sp1, AP-1, NF-1 or FAST-1 as it is not displaced by the corresponding DNA sequences to which these transcription factors bind (Figure 5A, lanes 6–10). To examine whether Smad proteins were present in the CAGA binding complex, nuclear extracts were incubated with specific antisera to Smads 1, 2, 3, 4 and 5. We could detect a supershift of the TGFβ-dependent binding complex with anti-Smad3 and anti-Smad4 antisera (Figure 5B, lanes 6 and 8). The complex was not totally supershifted with the anti-Smad4 antiserum, probably because anti-Smad4 antiserum is of lower affinity than anti-Smad3 antiserum. These supershifts were competed by addition of the immunogenic peptides used to generate the antisera, proving the specificity of the antibody recognition (Figure 5B, lanes 7 and 9). Since addition of anti-Smad1, anti-Smad2 and anti-Smad5 antisera has no effect (Figure 5B, lanes 4, 5 and 10), we conclude that the CAGA box DNA-binding nuclear complex contains the TGFβ/activin signalling Smad3 and Smad4 proteins, but not Smad2 protein or the BMP signalling Smad1 and Smad5 proteins. This is in agreement with transfection experiments showing that the CAGA reporter construct is activated by overexpression of Smad3 and the TGFβ and activin receptors which activate Smad3, but not by Smad2 overexpression or the BMP receptors which signal through Smad1 and Smad5 (Figures 3A and B and 4C).

Smad3 and Smad4 bind directly to the TGFβ-inducible CAGA box

The previous gel shift experiments that we have described demonstrate the presence of Smad3 and Smad4 in the nuclear CAGA sequence-binding complex, but cannot determine whether or not binding of Smad3 and Smad4 to DNA is direct. To address this issue, we used *Escherichia coli*-expressed GST–Smad fusion proteins in EMSA. As shown in Figure 6A, Smad3 and Smad4 deleted of the MH2 domain bound directly to a CAGA box containing probe. This binding was specific since no binding was observed even with high doses of MH2-deleted Smad3 or Smad4 proteins with an oligonucleotide containing three mutated CAGA boxes (data not shown). In line with the supershift experiments, the Smad1ΔMH2 and Smad2ΔMH2 proteins failed to bind DNA. Furthermore, and in contrast to the example of the *Drosophila* Mad protein, the full-length Smad4 protein produced in bacteria possessed a direct DNA-binding activity on the CAGA sequence (Figure 6B), whereas full-length Smad1, Smad2 and Smad3 were unable to bind DNA. Full-length Smad4 binding was specific as no complex could be detected with a probe containing mutated CAGA boxes (data not shown).

It appears from Figure 1B that the 5′-AGCCAGACA-3′ CAGA box leads to a stronger transcriptional induction than the 5′-AGACAGACA-3′ sequence. One possible explanation for this is that these two CAGA boxes present different binding affinities for Smad3 and Smad4. In order to test this hypothesis, we mixed GST–Smad4 with oligonucleotides containing both versions of the CAGA boxes. As shown in Figure 6C, Smad4 binding to the 5′-AGCCAGACA-3′ CAGA sequence was stronger than binding to the other CAGA box, thus correlating *in vitro* binding with results obtained in transfection experiments. Quantification of the bands indicates a 2-fold difference in the relative binding affinities. The same results were obtained with GST–Smad3ΔMH2 (data not shown).

Discussion

TGFβ family members mediate their cellular actions, at least in part, by controlling the transcription of target

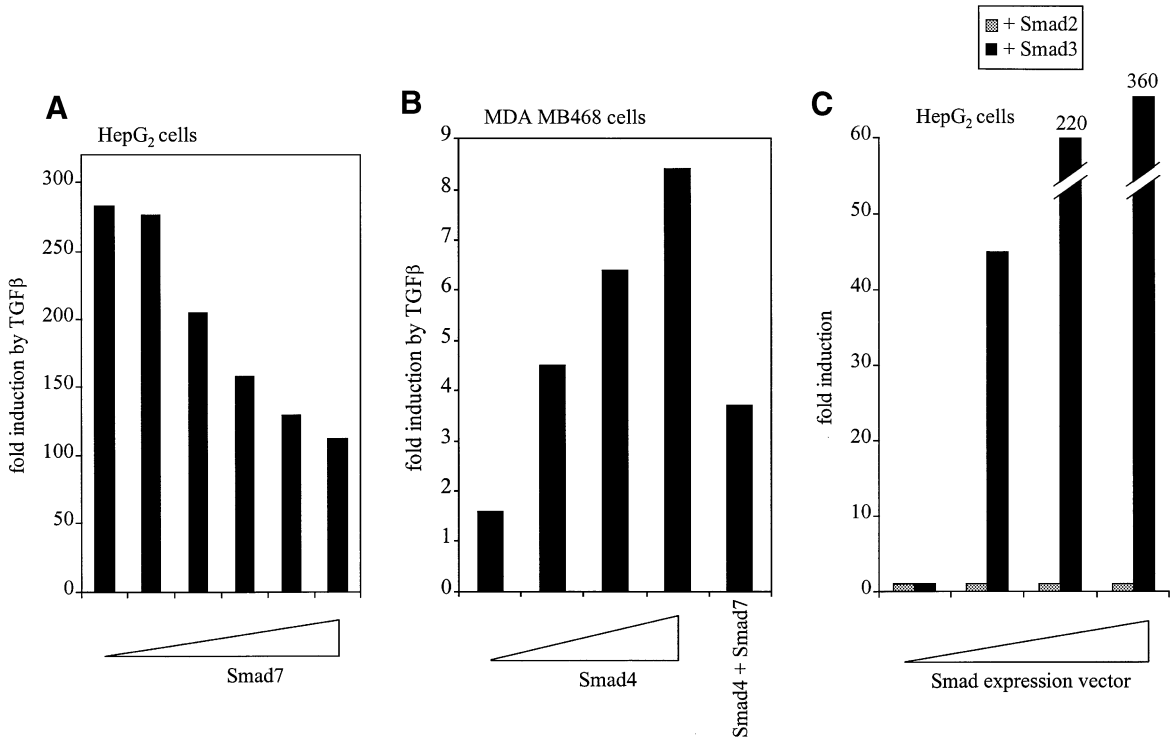

Fig. 4. Smad proteins are involved in TGFβ-induced transcription mediated by the CAGA box. (**A**) HepG2 cells were co-transfected with a $(CAGA)_9$-MLP-Luc reporter construct and increasing amounts (0, 10, 15, 20, 30 and 40 ng) of an expression vector encoding for the Smad7 inhibitory protein. (**B**) MDA-MB468 cells were transfected with a $(CAGA)_9$-MLP-Luc reporter construct and increasing amounts (0, 250, 500, 750 ng) of an expression vector encoding for the Smad4 protein. 250 ng of Smad7 expression vector with 500 ng of Smad4 expression construct were co-transfected when indicated. (**C**) HepG2 cells were transfected with a $(CAGA)_9$-MLP-Luc reporter construct and increasing amounts (0, 10, 50, 100 ng) of an expression vector encoding for the Smad2 or the Smad3 protein as indicated.

genes. In this report, we show that Smad proteins are directly involved and essential for TGFβ/activin-mediated transcriptional induction. We have identified a TGFβ/activin-inducible DNA element, termed CAGA box, which is present in three copies in the human PAI-1 promoter and specifically binds the TGFβ/activin pathway-restricted Smad3 protein and the common mediator Smad4 factor. Direct DNA-binding activity was found to be mediated by N-terminal Smad domain, similar to that of *Drosophila* Mad to the vg quadrant enhancer (Kim *et al.*, 1997). However, in contrast to GST–Smad3 and *Drosophila* Mad fused to GST, which binds to DNA only when the MH2 domain is removed, full-length GST–Smad4 protein is able to bind directly to the CAGA box. The fact that direct binding of Smad3 can be observed only with a truncation mutant in the MH2 domain, as reported for Mad, probably does not reflect a physiological situation and can be attributed to various reasons. Some studies indicate that an interaction between MH1 and MH2 domains of the Smad proteins inhibit Smad functions until receptor-mediated phosphorylation occurs (Hata *et al.*, 1997). Furthermore the material that we are using in this study are GST-fusion Smad proteins. GST is a dimer and may interfere with *E.coli*-expressed Smad protein topology, which could fold as a trimer as indicated by a recent report (Shi *et al.*, 1997). Further dedicated studies, using Smad3 expressed and purified without the GST domain and phosphorylated with activated type I receptor kinase, are required to clarify this point.

The common mediator Smad4 forms hetero-oligomers with the pathway-restricted Smads, in particular Smad3 (Heldin *et al.*, 1997). It is therefore possible to speculate, as is the case for other transcription factors, that Smad3/Smad4 complex has a better DNA-binding affinity than Smad3 and Smad4 individually. In an attempt to observe a binding cooperativity in our *in vitro* binding assays, we mixed the two Smad3 and Smad4 proteins (or MH2-deleted mutants) together with a CAGA box-containing probe. We could detect neither mutual inhibition of binding nor strong cooperativity of binding between Smad3 and Smad4 to the CAGA box (data not shown). However, these negative results were obtained with bacterially expressed GST–Smad fusion proteins and it remains possible that Smad3 and Smad4 cooperate for binding to the CAGA box *in vivo*.

Smad2 and Smad4 were found to participate with FAST-1 in an active DNA-binding complex termed activin response factor (ARF), to regulate *Xenopus mix.2* gene expression in an activin-dependent fashion (Chen,X. *et al.*, 1996, 1997). FAST-1 is thought to be the principal DNA-binding component of ARF (Chen,X. *et al.*, 1996, 1997). However, Smad4 promotes binding of the ARF complex (Liu *et al.*, 1997). There are two sequences with similarity to CAGA boxes flanking the 6 bp repeats of ARF to which FAST-1 binds. It is thus a possibility, which remains to be determined, that Smad2 and/or Smad4 contribute with FAST-1 to DNA-binding activity of ARF through these CAGA-like sequences. In HepG2 cells, FAST-1 appears not to be present and an ARE reporter construct is induced by TGFβ only when a FAST-1 expression

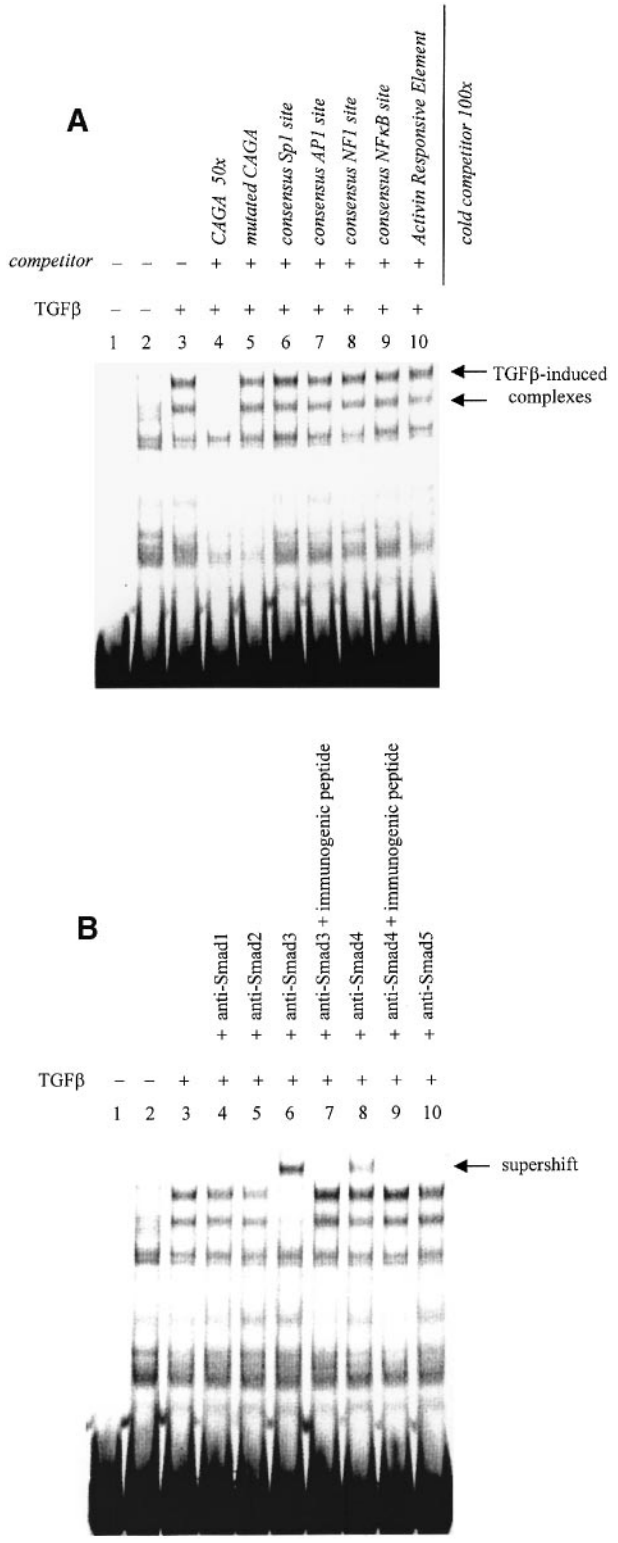

Fig. 5. Smad3 and Smad4 bind to the TGFβ-inducible CAGA box. (**A**) An EMSA was performed using a ^{33}P-labelled probe containing the CAGA sequence and nuclear extract from HepG2 cells induced for 30 min by TGFβ or not induced. Bands corresponding to specific TGFβ-induced complexes are indicated. Fifty or 100 molar excesses of various non-radiolabelled oligonucleotides were added as competitors, including the wild-type and mutated CAGA sequences. (**B**) Specific anti-Smad antisera were incubated with TGFβ-induced HepG2 nuclear extracts before mixing with the CAGA probe. The supershifted complexes are indicated. The antigenic peptides used to generate the reactive antisera were added in lanes 7 and 9 to show the specificity of the anti-Smad3 and anti-Smad4 antisera.

vector is co-transfected (Hayashi *et al.*, 1997; our unpublished data). However, co-transfection of a FAST-1 expression vector in HepG2 cells has no effect on basal or TGFβ-induced transcription of a CAGA reporter (data not shown), suggesting that FAST-1 is not involved in the TGFβ-induced binding of the Smad3/Smad4-containing complex to the CAGA box.

Two lines of evidence show that the CAGA box is specific for TGFβ/activin signalling. First, this sequence, when fused to a luciferase reporter gene, does not mediate transcriptional induction upon BMP stimulation. Second, the BMP pathway-restricted Smad1 and Smad5 proteins did not show any DNA-binding activity to CAGA boxes. In contrast with Smad3, the TGFβ/activin-restricted Smad2 protein was able neither to *trans*-activate nor to bind to this sequence, indicating a functional difference between the closely related Smad2 and Smad3 proteins. Smad2 differs from Smad3 and Smad4 mainly in the MH1 domain; Smad2 contains two stretches of amino acid residues that are lacking in Smad3 and Smad4. These additional extra sequences may change the structure, altering the DNA-binding characteristics of Smad2. Therefore, Smad2 and Smad3 may have a different subset of target genes and regulate distinct cellular processes.

TGFβ is known to mediate transcriptional effects on various promoters. In addition to the human PAI-1 promoter where the three CAGA boxes are necessary for TGFβ induction, we have found CAGA boxes in several other TGFβ-inducible regions of promoters regulated by TGFβ (see Table I). The box B of the α2(I) procollagen contains the related ATGCAGACA sequence that mediates TGFβ stimulation (Inagaki *et al.*, 1994; our unpublished results). Similarly, the CAGA sequence is present in the described TGFβ-responsive elements of the germline Igα constant region (Lin *et al.*, 1992) and TGFβ1 promoters (Kim *et al.*, 1989a,b) and may represent a more widely used motif to confer TGFβ induction to promoters and enhancers.

Recently, Yingling and collaborators have shown that Smad3 and Smad4 form a complex that recognizes a bipartite binding site within the AP-1 sites of the artificial TGFβ-responsive p3TP-Lux vector (Yingling *et al.*, 1997). This site overlaps the AP-1 binding site and contains the CAGA box-similar sequence 5′-AGTCAGACA-3′. Furthermore, this bipartite binding site can compete for Smad binding to a CAGA boxes-containing probe in our *in vitro* gel shift experiments (data not shown). However, in contrast to our conclusions, they report that these Smad-binding CAGA-like boxes are dispensable for TGFβ-inducibility. Instead, they conclude that AP-1 sites are required and are in part sufficient for TGFβ induction. Our conclusions differ, as our results clearly indicate that CAGA boxes are necessary and sufficient to mediate TGFβ-transcriptional effects. Furthermore, we observed in HepG2 cells that TGFβ has no inductive effect on a reporter containing several copies of the sequence 5′-TGAGTCATCCC-3′ containing a consensus AP-1 binding site (underlined) with no CAGA-related sequence (data not shown). In Yingling *et al*. (1997) the fragment of the collagenase promoter used as an AP-1 site contains the **CAGA**-related sequence 5′-TG**AGTCAGAC**-3′ which is destroyed when the AP-1 site is mutated. The partial overlap of the AP-1 and CAGA sites complicates

the analysis and could explain the discrepancy between the results obtained by these authors and our data. However, our observations do not exclude the possibility that the Smad-containing complex that binds to the CAGA box interacts and cooperates with other transcription factors in natural promoters to increase the rate of transcription. It is noteworthy that the –730 CAGA box of the PAI-1 promoter and the box B CAGA sequence of the α2(I) procollagen promoter are respectively located very close to an AP-1 and Sp1 sites, suggesting a potential transcriptional synergy between these different transcription factors.

Very recently, Zawel and collaborators, by using a random pool of oligonucleotides in a PCR/selection strategy, have isolated a palindromic sequence that binds Smad3 and Smad4 (Zawel *et al.*, 1998). Strikingly, this site contains the 5′-AGACA-3′ sequence which is also present in the 5′-AGCCAGACA-3′ and 5′-AGACAGACA-3′ CAGA boxes of the PAI-1 promoter and which may represent therefore a core binding site for Smad3/Smad4. These findings open several lines of future research. Determining the optimal and consensus binding sites for pathway-restricted Smads and common mediator Smad4 would require further studies and allow the screening of gene banks for putative Smad binding sites in promoters or enhancers of genes that are known to be regulated by TGFβ family members, as well as the discovery of possible novel TGFβ family responsive genes. These studies should also help in understanding the

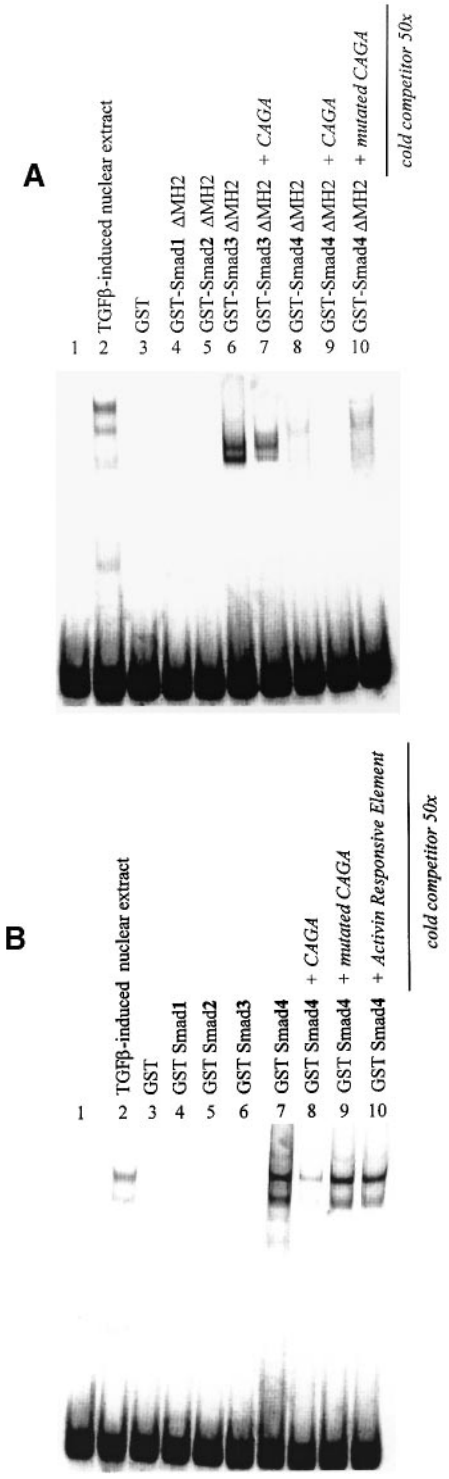

Table I. Various TGFβ-inducible promoters contain CAGA sequences

	CAGA sequence	Location
Human PAI-1 promoter	AGCCAGACA	–730
	AGACAGACA	–580
	AGACAGACA	–280
Human $TGF\beta_1$ gene	AGCCAGACA	+22
Human α2(I) collagen promoter	ATGCAGACA	–264
Human germline Igα constant region	AGCCAGACC	–120
	GGCCAGACA	–35

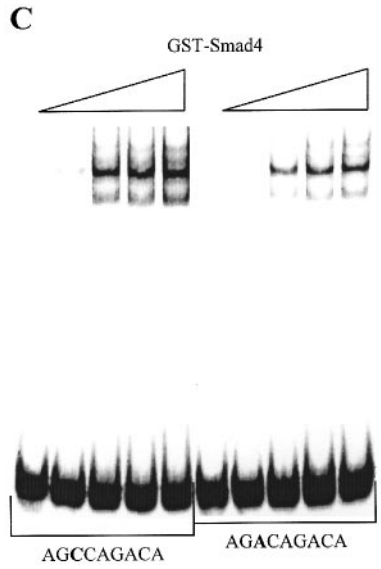

Fig. 6. Direct binding of Smad3 and Smad4 to the CAGA sequence. (**A**) *E.coli*-expressed GST–Smad1, 2, 3 and 4 proteins, deleted of the conserved carboxy-terminal MH2 region, were incubated with a ^{33}P-labelled CAGA probe. A 50 molar excess of non-radiolabelled oligonucleotide competitors was added when indicated. Nuclear extracts of TGFβ-treated HepG2 cells have been added to the probe in lane 2 to locate the nuclear DNA-binding complex. (**B**) A similar experiment where full-length Smad proteins, fused to the GST domain, produced in bacteria were used. (**C**) Increasing amounts of GST–Smad4 protein (0, 200, 300, 400, 500 ng) were incubated with oligonucleotides containing the two CAGA box sequences found in the hPAI-1 promoter: 5′-AGCCAGACA-3′ and 5′-AGACAGACA-3′.

mechanisms of potential DNA-binding specificities between the Smads. Finally, as the CAGA box binds a Smad3/Smad4 complex and drives TGFβ-dependent transcription, the domains and critical residues in these Smads involved in DNA-binding and transactivation can be identified through structure–function studies.

Materials and methods

Plasmids constructs

CAGA reporter vectors were generated using pGL3 basic plasmid (Promega). TK or MLP promoters were PCR-amplified and inserted between the *Bgl*II and *Hin*dIII sites. The CAGA boxes containing oligonucleotides were cloned into the *Xho*I site. The sequences of the oligonucleotides cloned are: CAGA boxes containing oligonucleotides: 5′-TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC-3′ and its complementary strand; 5′-TCGAGAGACAGACAAAAAGA-CAGACATTTAGACAGACAC-3′ and its complementary strand. CAGA mutant oligonucleotide: 5′-TCGAGAGCTACATAAAAAGCTACAT-ATTTAGCTACATAC-3′; 3′-CTCGATGTATTTTTCGATGTATAAAT-CGATGTATGAGCT-5′.

All the constructs were sequence-checked. Expression vectors for Smad7, Smad4 and constitutively active forms for ALK-2 to ALK-6 have been described previously (Nakao *et al.*, 1997a,b). The site-directed mutagenesis in the human PAI-1 promoter was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

Cell culture

The human hepatoma cell line HepG2 (HB 8065), the human breast adenocarcinoma cell line MDA-MB468 (HTB 132) and the Mv1Lu mink lung epithelial cell line (CCL 64) were purchased from the American Type Culture Collection. HepG2 and Mv1Lu cells were grown in a 5% CO_2:95% air atmosphere in BME or MEM medium respectively (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 10 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (complete medium). MDA-MB468 cells were grown in a 7.5% CO_2:92.5% air atmosphere in DMEM/F12 (1:1) medium (Life Technologies, Inc.) with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (complete medium).

Transfection and luciferase assays

HepG2 and MDA-MB468 cells were transiently transfected, with the indicated constructs and the internal control pRL-TK vector, using the calcium phosphate co-precipitation method. When increasing amounts of expression vectors were transfected, total DNA was kept constant by addition of pCMV5. Cells were serum starved for 8 h before stimulation with 7 ng/ml of human recombinant TGFβ1 (R&D) and luciferase activities were quantified 14 h later using the Dual Luciferase Assay (Promega). For activin and BMP-7 (Creative Biomolecules) induction, 20 and 100 ng/ml respectively, were used. Values were normalized with the renilla luciferase activity expressed from pRL-TK. Mv1Lu cells were transfected using the DEAE–dextran method. Luciferase values shown in the figures are representative of transfection experiments performed in duplicate in at least three independent experiments.

Nuclear extracts

Nuclear extracts were prepared from control and TGFβ-treated HepG2 cells. Cells were harvested 30 min after treatment and processed according to the protocol of Sadowski and Gilman (1993). Briefly, confluent cells from eight 100 mm dishes were washed with phosphate-buffered saline (PBS) and scraped. After another washing, cells were suspended in 2 ml of cold buffer A (20 mM HEPES pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 0.13 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.4 mM ammonium molybdate, 1 mM DTT, 0.5 mM PMSF and 1 μg/ml each leupeptin, aprotinin and pepstatin). The cells were allowed to swell on ice for 15 min and then lysed by 30 strokes of a Dounce all-glass homogenizer. Nuclei were pelleted by centrifugation and resuspended in 600 μl of cold buffer C (buffer A, 420 mM NaCl and 20% glycerol). The nucleus membrane was lysed by 15 strokes of a Dounce all-glass homogenizer. The resulting suspension was stirred for 30 min at 4°C. The clear supernatant was aliquoted and frozen at –80°C.

Electrophoretic mobility shift assays (EMSA)

Oligonucleotides were end-labelled with [α-^{33}P]dCTP and [α-^{33}P]dATP using the Klenow fragment of DNA polymerase. Binding reactions containing 10 μg of nuclear extracts (or 400 ng of GST–Smad fusion proteins) and 2 ng of labelled oligonucleotides were performed for 20 min at 37°C in 18 μl of binding buffer (20 mM HEPES pH 7.9, 30 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.8 mM NaPi, 20% glycerol, 4 mM spermidine, 3 μg polydI–dC). Protein–DNA complexes were resolved in 5% polyacrylamide gels containing 0.5× TBE. The sequence of the double stranded oligonucleotide used as a probe was: 5′-TCG-AGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACAC-3′ and its complementary strand. The CAGA oligonucleotides used for Figure 6C were: 5′-TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCA-GACAC-3′ and 5′-TCGAGAGACAGACAAAAAGACAGACATTTA-GACAGACAC-3′ and their complementary strands. The sequence of the competitor CAGA mutant oligonucleotide was: 5′-TCGAGAGCTA-CATAAAAAGCTACATATTTAGCTACATAC-3′ and its complementary strand. Competitor oligonucleotides containing other transcription binding sites are: Fast-1 site: 5′-TCGAGGCTGCCCTAAAATGTGTAT-TCCATGGAAATGTCTGCCCTTCTCTC-3′ and its complementary strand, AP-1 site: 5′-CCGGGGATGACTCAGCC-3′ and its complementary strand. NF-1 site: 5′-CCGGTTTGGATTGAAGCCAATATG-3′ and its complementary strand, Sp1 site: 5′-TCGAGGACAGGGGG-CGGAGCCTC-3′and its complementary strand.

Production and purification of Smad fusion proteins

The full-length Smad proteins and the MH2-deletion mutants fused to GST were expressed in *E.coli* and partially purified by column chromatography using Pharmacia's protocol. Briefly, bacteria were grown in 2× YTA medium and induced with 0.1 mM IPTG. After sonication, the GST fusions were isolated using glutathione–Sepharose 4B, washed three times, eluted, then dialysed against PBS supplemented with 2 mM DTT and 0.5 mM PMSF.

Preparation of polyclonal antisera

Antisera raised against Smad peptides have been described previously (Nakao *et al.*, 1997b).

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