Identification and Functional Characterization of a Smad Binding Element (SBE) in the \textit{JunB} Promoter That Acts as a Transforming Growth Factor-\(\beta\), Activin, and Bone Morphogenetic Protein-inducible Enhancer*

(Received for publication, March 25, 1998)

Luigi J. C. Jonk‡§, Susumu Itoh§§, Carl-Henrik Heldin¶, Peter ten Dijke¶, and Wiebe Kruijer‡‡

From the Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, P. O. Box 14, 9700 AA Haren, The Netherlands and the Ludwig Institute for Cancer Research, Box 395, Biomedical Center, S-75, 24 Uppsala, Sweden

Smad proteins have been identified as mediators of intracellular signal transduction by members of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily, which affect cell proliferation, differentiation, as well as pattern formation during early vertebrate development. Following receptor activation, Smads are assembled into heteromeric complexes consisting of a pathway-restricted Smad and the common Smad4 that are subsequently translocated into the nucleus where they are thought to play an important role in gene transcription. Here we report the identification of Smad Binding Elements (SBEs) composed of the sequence CAGACA in the promoter of the \textit{JunB} gene, an immediate early gene that is potently induced by TGF-\(\beta\), activin, and bone morphogenetic protein (BMP) 2. Two \textit{JunB} SBEs are arranged as an inverted repeat that is transactivated in response to Smad3 and Smad4 co-overexpression and shows inducible binding of a Smad3- and Smad4-containing complex in nuclear extracts from TGF-\(\beta\)-treated cells. Bacterial-expressed Smad proteins bind directly to the SBE. Multimerization of the SBE creates a powerful TGF-\(\beta\)-inducible enhancer that is also responsive to activin and BMPs. The identification of the sequence CAGACA as a direct binding site for Smad proteins will facilitate the identification of regulatory elements in genes that are activated by members of the TGF-\(\beta\) superfamily.

The product of the \textit{JunB} gene is a member of the AP-1 family of transcription factors that activate transcription by binding to TPA response elements (TREs) within the promoter of target genes (1). AP-1 components are immediate early gene products whose expression is rapidly induced by a variety of extracellular stimuli and are encoded by the Fos and Jun families of genes that have been shown to be involved in growth control and differentiation (2). \textit{JunB} differs in biological properties from its homologs and appears to be a negative regulator of AP-1 function (3). This functional difference is because of a small number of amino acid changes in its DNA binding and dimerization motifs compared with the corresponding c-Jun sequences, as well as to differences in phosphorylation status in response to mitogenic stimulation (4).

The action of \textit{JunB} as a negative regulator of TRE response elements is consistent with its induction by negative regulators of cell growth including transforming growth factor-\(\beta\) (TGF-\(\beta\)) as well as the structurally and functionally related factors activin and bone morphogenetic protein (BMP) 2/4 (5–8). In this respect, \textit{JunB} is a member of the group of genes that are known to be induced in response to TGF-\(\beta\) stimulation, which further include the cyclin-dependent inhibitors (CDI) p15 and p21 (9, 10) and the plasminogen activator inhibitor (PAI-1) gene (11) that control, in part, cell cycle progression and extracellular matrix remodeling in response to TGF-\(\beta\), respectively. However, it is presently unclear whether the induction of these genes by TGF-\(\beta\) and related factors involves a direct mechanism.

TGF-\(\beta\) family members exert their cellular effects (12, 13) by binding to transmembrane receptors that possess serine/threonine kinase activity (14). Upon ligand binding, a heteromeric receptor complex consisting of two type II and two type I receptors is formed. Within the complex, the type I receptor is phosphorylated and activated by the type II receptor constitutively active kinase. Genetic studies in \textit{Drosophila melanogaster} and \textit{Caenorhabditis elegans} have recently led to the identification of a conserved family of proteins termed Smads that play an important role in intracellular signal transduction of serine/threonine kinase receptors (15). At present, at least nine family members have been identified in vertebrates. Smads are 40–62-kDa proteins with N- and C-terminal homology domains (MH1 and MH2) connected by a proline-rich linker. Smad1, Smad5, and presumably MADH6/Smad9 associate with and are phosphorylated after BMP-mediated BMP-RI and ActR-I activation, whereas Smad2 and Smad3 are phosphorylated after TGF-\(\beta\)-R-1 and ActR-IB activation. Following phosphorylation, which occurs at a conserved SSXS motif at the extreme C termini, these pathways-restricted Smads form heteromeric complexes with the common mediator SMAD4 and translocate to the nucleus to regulate gene tran-
Smad Binding Element in JunB Promoter

Phosphorylation of the proline linker by growth factor-activated Erk mitogen-activated protein kinase was recently shown to have an inhibitory effect on BMP-induced nuclear accumulation of Smad1 (16). Smad6 and Smad7 are structurally and functionally distinct from other Smads in that they lack an MH1 domain and act opposite to the pathway-restricted and common mediator Smads; they block the activation of pathway-restricted signals by competition for receptor association or by preventing heteromeric complex formation between pathway-restricted and common mediator Smads (17–19).

Support for a role of Smads as transcription factors has been obtained from a number of studies. The C-terminal domains of Smad1 and Smad4 have transcriptional activity when fused to the Gal4-DNA binding domain in a Gal4-reporter transactivation assay (20, 21). Smad2 and Smad4 together with Fast-1, a winged-helix DNA binding protein, associate into activin response element (ARE) that binds to the activin response element of the Xenopus laevis Mix.2 promoter (22, 23). TGF-β as well as Smad3 and Smad4 overexpression transactivate the PAI-1 and p3TP-lux promoters (24), which has been attributed to potentiation of AP-1-dependent transcription activation (25). By contrast, Drosophila Mad domain binds a G+C-rich sequence in the Drosophila vestigial quadrant enhancer (26). However, these investigations have not been conclusive regarding the role of Smads in transcriptional activation, as well as regarding the sequences with which they interact.

In this report, we have investigated the transcriptional regulation of the immediate early gene JunB by TGF-β. Transient overexpression of Smad3 and Smad4 with various JunB promoter constructs led to the identification of a region in the upstream part of the promoter that is both Smad-transactivated and shows TGF-β-induced binding of a Smad-containing complex. Further characterization led to the identification of an inverted hexanucleotide repeated sequence binding a TGF-β-induced DNA binding activity. Multimerization of this sequence created a powerful TGF-β-inducible enhancer. Implications of these findings for Smad DNA binding and transcriptional activation will be discussed.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—An 8-kilobase EcoRI fragment was isolated from the Balb/c mouse genomic JunB clone a31 which has been described previously (27). All sequences upstream from the initiation ATG were cloned into the pGL3-basic vector (Promega). For cloning purposes, the ATG was converted to ATC by PCR (pJB1). A deletion series was constructed by restriction digestion of the pJB1 plasmid with EcoRI-PstI (pJB2), SacI (pJB3), SacI-BstBI (pJB4), or SacI-SacII (pJB5) and subsequent self-ligation. A minimal promoter construct (pGL3ti) was made from pGL3-basic by inserting oligonucleotides carrying the adenosine major late promoter TATA box (AdTu, gatcGCGGCTTATATATGGGGGGTGAGGggag, and AdTu, CCCCCTATATATATGGGGGGTGAGGgag). Nuclear extracts were preincubated with either Smad1, Smad2, Myc-Smad3, or Smad4 and pJB5 were electrophoresed on a 4% TBE. Smad3 and Smad4 antibodies (DHQ and HPP, respectively) (28) were added undiluted at 0.5 μg/ml. After 24 h, the medium was changed to DMEM supplemented with 0.3% fetal calf serum. Simultaneously, the cells were stimulated with 10 ng/ml TGF-β1 (R&D Systems), 20 ng/ml activin A, 100 ng/ml Osteogenic Protein (OP-1), 100 ng/ml BMP2, or 200 ng/ml Growth/Differentiation Factor 5 (GDF5) for 16 h. The cells were lysed for 30 min in reporter lysis buffer (Promega), followed by measurement of luciferase activity using luciferase assay system (Promega). In all transfections, β-galactosidase expression plasmids (pDM2laczZ (29) or pCH110, Pharmacia) served as internal controls to normalize the luciferase activity. β-Galactosidase activity was quantitated in 100 μl NaHPO4/NaH2PO4, 1 mm MgCl2, 100 μm 2-mercaptoethanol, and 0.67 μg/ml O-nitrophenylgalactosidase. Each transfection was carried out in triplicate and repeated at least twice.

**Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared from Mv1Lu cells using a modified Dignam protocol as described previously (30). Oligonucleotides and dephosphorylated restriction fragments were labeled with [γ-32P]ATP and T4 polynucleotide kinase. Oligonucleotides used in electrophoretic mobility shift assay experiments are shown in Scheme 1. Binding reactions contained 4 μg of nuclear extract, 100 μM KCl, 0.2 μM EDTA, 0.5 μM dithiothreitol, 20 μM glycerol, 20 μM HEPES, pH 7.9, 100 ng of poly(dI-dC) and approximately 10,000 cpm labeled probe and 500-fold molar excess of competitor oligonucleotide where appropriate. Protein binding was allowed to proceed for 30 min at room temperature. Ten percent Ficoll was added to the binding reactions in the case of pJB11 and pJB12 (Scheme 1), and samples were immediately subjected to gel electrophoresis. 4.5 μg of 5% polyacrylamide gels containing 0.5% TBE, Smad3 and Smad4 antibodies (DHQ and HPP, respectively) (28) were added undiluted at 0.5 μl, after proteins were allowed to bind the probe for 15 min. Samples were then incubated for 15 min before loading onto the gel.

**GST fusion protein expression** was induced in logarithmically growing cultures of Escherichia coli (TG1) by the addition of isopropyl-β-D-
RESULTS

Identification of a Smad-responsive Region in the JunB Promoter—Previously, we have shown that the immediate early gene JunB is a direct target for transcriptional activation in response to activation of signal transduction by TGF-β (5). To investigate the mechanism of transactivation of the JunB promoter by TGF-β, a JunB promoter-luciferase reporter construct was made containing the JunB TATA box and transcription start site, the complete 5'-untranslated region as well as approximately 6.4 kilobases of promoter upstream sequences (pJB1). Transient transfection of this construct in NIH3T3, Mv1Lu, and HepG2 cells and treatment of the cells with TGF-β did not result in a significant increase in activation of the luciferase reporter construct over uninduced levels after normalization with β-galactosidase activities from cotransfected control LacZ expression plasmid (data not shown). Recently, we and others have shown that transiently overexpressed Smad proteins transactivate target genes in a ligand-independent manner (24, 28). We therefore cotransfected pJB1 with plasmids expressing individual Smads or combinations of each pathway-restricted Smad and Smad4 into NIH3T3 cells (Fig. 1A). A 3- to 5-fold activation of the reporter construct was observed when Smads 1, 2, and 3 were co-expressed with Smad4 while individual Smads did not significantly activate the reporter. Similar results were obtained using P19 embryonal carcinoma (EC) cells and HepG2 cells. We localized the Smad-responsive region in the JunB promoter by transfecting cells with a series of deletion constructs along with Smad3 and Smad4, which is the strongest activating combination (see Fig. 1A). This analysis showed that the Smad-responsive region is located between nucleotides –3004 and –1534; whereas pJB3 has Smad indubility very similar to pJB1, this response was lost for the JB4 deletion construct (Fig. 1B). A nested set of restriction fragments was derived from this Smad-responsive region and cloned in front of a heterologous minimal promoter fused to the luciferase gene. Cotransfection of these constructs with Smad3 and Smad4 expression plasmids allowed localization of a Smad-responsive region to between nucleotides –2908 and –2611 (Fig. 1C). The position of the Smad-responsive element within the –2908/–2611 region was determined using a series of progressive 5’ and internally deleted constructs. Deletion of sequences upstream from nt –2813 did not affect Smad responsiveness while additional deletion of 26 bp (to nt –2787) abrogated inducibility. This analysis was complemented by a series of deletions of sequences located between nt –2908 and –2762. Exclusion of sequences upstream from nt –2792 abrogated transactivation by Smad3 and Smad4 (Fig. 1D). This analysis therefore defines the 22-base pair region between –2813 and –2792 as that minimally required for transactivation by Smad3 and Smad4. Interestingly, the 22-bp sequence contains a perfect 7-bp inverted repeat (CAGACAGCTGTCCTG).

JunB Promoter Fragments Bind TGF-β-induced Complex Containing Smad3 and Smad4—To investigate whether TGF-β induces binding of nuclear proteins to the Smad-responsive region, we successively incubated labeled probes containing nt –2908 to –2611, nt –2908 to –2788, and nt –2813 to –2792 with nuclear extracts from TGF-β-treated or -untreated Mv1Lu, HaCaT, and NIH3T3 cells (Fig. 2). Electrophoretic mobility shift assay experiments showed that extracts from TGF-β-treated cells contain an induced DNA binding activity that migrates with a lower mobility than that of a constitutively expressed binding entity. These results suggest that TGF-β activates the endogenous JunB gene by inducing binding of a nuclear factor to a JunB promoter distal element located between nucleotides –2813 and –2792. This fragment correlates with the minimal region required for transactivation by Smad3 and Smad4, suggesting that Smads may form part of nuclear complexes that bind to the 22-bp JunB promoter sequence. To analyze whether Smads are present in nuclear complexes that bind to the 22-bp JunB promoter sequence, antisera directed against Smad3 or Smad4 were added to the binding reactions of the 22-bp JunB probe with nuclear extracts (Fig. 2C). Both Smad3 and Smad4 antisera supershifted the slowest migrating TGF-β-induced complex. The Smad3 antisemur was more effective than the Smad4 antisemur, which might be due to intrinsic higher affinity of Smad3 antisemur versus Smad4 antisemur. Both antisera produced a supershifted band with extracts from uninduced cells. Possibly, these complexes were formed by stabilization of the antisemur with the interaction of contaminating cytoplasmic Smads with the probe. Neither Smad1, 2, or 5 antisemur produced supershifted bands (data not shown). These results indicate that TGF-β induces nuclear translocation of activated Smad3 and Smad4 and their subsequent binding to a defined region in the JunB promoter.

Smad Proteins Bind Directly to CAGACA Elements—Having identified Smad3 and Smad4 as components of the TGF-β-induced complexes, we next investigated whether Smad proteins directly bind the Smad-responsive fragment. We pro-
NIH3T3 cells were transfected with the pJB1 plasmid with or without Smads 1, 2, 3, and/or 4 expression plasmids. Normalized reporter activity is shown as the mean ± S.D. of triplicates. B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using 

Cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using

B, the JunB promoter is activated by Smad3 and Smad4 between nucleotides -3004 and -1534. P19EC cells were cotransfected with luciferase reporter plasmids carrying a deletion series of the mouse JunB gene with Smad3 and Smad4 expression plasmids. After 40 h, cells were lysed to measure luciferase activity. Luciferase activity was normalized using
containing probe (data not shown). When we tested the Smad binding to a probe containing four directly repeated SBEs, we found that Smad3ΔMH2, Smad4ΔMH2, and full-length Smad4 bound with much higher affinity than two repeats (Fig. 4C). The same results were obtained when the SBEs were present as two indirect repeats (data not shown). In addition, weak binding of full-length Smad3 was now observed. We could specifically compete the Smad3 or Smad4 binding with wild type oligonucleotide, but not with a mutant version in which the G3 and C5 were substituted, and no binding to mutated probe was observed (Fig. 4C). This analysis defines the central nucleotides GAC in the SBE as most important for binding Smad proteins and the TGF-β-induced complex, while the flanking nucleotides contribute less to the binding and the most 3′ nucleotide (G7) is not essential.

The SBE Is a TGF-β Response Element—To investigate whether the SBE can confer inducibility to TGF-β, we inserted the SBE in different copy number in front of a minimal promoter and tested these constructs in HepG2 cells. We found that two copies were insufficient for significant induction of luciferase activity, but that with four copies a strong response was obtained (Fig. 5A). Therefore, multimerization of the SBE is sufficient to confer TGF-β inducibility to a minimal promoter. When the (SBE)4 reporter was cotransfected with Smads, we found that Smad3 alone, but not Smad2 or Smad4 alone, had a slight ligand-independent effect. The TGF-β-induced effect was enhanced by cotransfecting the Smad3 and Smad4 combination, but not with the Smad2 and Smad4 combination. Highest TGF-β-induced transcriptional response was obtained when all three Smads were cotransfected (Fig. 5A).

Smads Participate in TGF-β- induced SBE-mediated Transcription—TGF-β failed to induce the (SBE)4 reporter in MDA-MB468 cells that lack the Smad4 gene (31) (Fig. 5B). As the TGF-β response was rescued by Smad4 transfection, Smad4 appears to be required for the TGF-β-induced, SBE-mediated transcriptional response. Further transfection studies showed that Smad3 and Smad4, but not Smad2 and Smad4, cooperated in the SBE-mediated transcriptional response in these cells, which was readily observed in the absence of ligand. In fact, cotransfection of Smad3 and Smad4 resulted in a level of reporter activation over which no dramatic TGF-β-dependent response was observed. Cotransfection of the reporter plasmid into HepG2 cells along with increasing amounts of a Smad7 expressing plasmid showed a dose-dependent decrease in TGF-β-induced SBE-mediated transcriptional response (data not shown). Taken together, the results indicate that Smad3 and Smad4 are directly involved in the TGF-β-I-mediated activation of the JunB promoter-derived SBE.

SBE Is a Response Element for Other Members of the TGF-β Family—Activin and BMP2 have been shown to induce JunB mRNA expression (7, 8). To test whether the SBE can confer inducibility to activin and BMP, we transfected HepG2 cells with the TGF-β-responsive (SBE)4 construct and treated the transfected cells with either OP-1 (also termed BMP-7), activin, or TGF-β (Fig. 5C). All three ligands activate the SBE reporter albeit with different efficiencies. To corroborate these findings, we transfected Smad4-negative MDA-MB468 cells with the CATGACA reporter plasmid with or without the Smad4 expression plasmid and tested the transfected cells for responsiveness to TGF-β, activin, OP-1, BMP2, and GDF5 (Fig. 5D). In the absence of Smad4, the reporter construct was virtually unresponsive to any of the ligands. Cotransfection of Smad4 resulted in a slight activation of the reporter. However, treatment of the Smad4 cotransfected cells with the various members of the TGF-β superfamily showed that OP-1 and TGF-β strongly activated the (SBE)4 construct, whereas treatment with activin was without effect, which may indicate that our MDA-MB468 cells lack functional activin receptors. Furthermore, BMP2 and GDF5, which bind the BMP type IB receptor (BMPR-IB) (32) also strongly activated the (SBE)4 construct in agreement with these findings, constitutively active versions of activin type I receptor (ActR-I), ActR-IB, BMPR-IA, and TGF-β.

Fig. 2. The 22-bp Smad response element binds Smad3 and Smad4 proteins from TGF-β-treated cells. A, the 297-bp Smad-responsive region binds a TGF-β-induced protein. An end-labeled fragment containing the 297-bp Smad-responsive region was incubated with nuclear extracts from human HaCaT keratinocyte and mink Mv1Lu lung epithelial cells treated with (+) or without (−) 10 ng/ml TGF-β1 for 1 h. Complexes were resolved on a nondenaturing 4.5% polyacrylamide gel followed by autoradiography of the dried gel. The arrow denotes the position of the TGF-β-induced complex. P indicates the lane with only the free probe. B, binding of a TGF-β-induced protein on a 120-bp Mlu NI-AfIII fragment from the Smad-responsive region. An end-labeled restriction fragment derived from the 120-bp Smad-responsive region was incubated with nuclear extracts from mouse NIH3T3 embryonic fibroblast, human HaCaT keratinocyte, and Mv1Lu lung epithelial cells treated with or without 10 ng/ml TGF-β1 for 1 h. Protein binding was visualized as in Fig. 1C. The arrow denotes the position of a TGF-β-induced complex. C, binding of endogenous Smad3 and Smad4 protein to the 22-bp Smad-responsive element (inv5, see “Experimental Procedures”) was labeled and incubated with nuclear extracts from Mv1Lu cells. Protein binding was visualized as in Fig. 1C. The nuclear extract from cells treated with TGF-β1 possessed two inducible complexes with the probe denoted as I and II. Complex I contains proteins supershifted (*) produced by the addition of Smad3 (α3) or Smad4 (α4) antibodies to the reaction mixtures.
transduction by several members of the TGF-β superfamily. We have shown that two SBEs in the JunB promoter form an inverted hexameric CAGACA repeat that readily binds a Smad3- and Smad4-containing complex from TGF-β-treated Mv1Lu cells. Bacterial-expressed full-length Smad4 and C-terminal truncated Smad3 or Smad4 bind to the JunB SBE repeat, demonstrating that the identified CAGACA sequence is a direct binding site for Smads. Binding of Smad proteins is independent of the relative orientation of the SBEs in a repeat, but multimerization of SBEs strongly increases the affinity for Smad proteins, indicating that cooperative binding is required for Smad function. This is demonstrated by the fact that activation of SBE-containing reporter constructs by TGF-β or overexpressed Smad proteins is only observed when four CAGACA elements are present.

The JunB gene is a target for BMP2, activin, and TGF-β-signaling (5–8). Ligand-mediated activation of the endogenous promoter can be mimicked by cotransfection of a reporter construct with the pathway-restricted Smad1, Smad2, and Smad3 along with Smad4. The CAGACA repeat isolated from the JunB gene, as present in the pGL3ti-(SBE)4 reporter construct, is also activated by TGF-β, activin, BMP2, OP-1/BMP7, and GDF5, implicating that these ligands activate the JunB promoter through the same response element. Members of the BMP-subfamily have been reported to signal through Smad1 and Smad5, while activin and TGF-β-signaling is mediated by Smad2 and Smad3 (15). Remarkably, the SBE repeat is only efficiently bound by bacterially produced Smad3 and Smad4. Possibly, Smads 1, 2, and 5 require additional proteins for high affinity binding to the SBE. Alternatively, in contrast to Smad3, these Smad proteins may associate with their target sequence only through Smad4. Treatment of cells with TGF-β induces the phosphorylation of both Smad2 and Smad3, which share a high degree of homology (24). However, we could not detect Smad2 protein in the TGF-β-induced complex formed with the 22-bp probe. The major difference between Smad2 and Smad3 resides in the DNA-binding MH1 domain. Possibly, the insertion in this domain of Smad2 alters its binding characteristics.

The response element we have isolated from the JunB promoter shows no homology with Mad binding site in the Drosophila decapentaplegic-responsive vestigial quadrant enhancer has a GC-rich core and shows no homology with the Smad binding sequence as derived from the JunB promoter (23). Interestingly, like Smad3, Mad binds its recognition sequence only as a C-terminal truncated protein. These properties may be related to a proposed working mechanism for receptor-activated Smads, in which Smads become signaling-competent only when phosphorylation relieves the inhibitory action of their C-terminal MH2 domain. The sequence of the JunB SBE binding sequence shows no homology to Sp1-binding sequences that previously have been implicated in TGF-β-induced transactivation of the p15 and p21 promoters (9, 10). Smad2 and Smad4 interact with FAST-1 to form ARF, which binds through FAST-1 to the ARE in the X. laevis Mix.2 promoter (26). These Smads are required for FAST-1 binding and ARF formation. These sequences do not resemble the JunB SBE. However, one of the 6-bp sequences overlaps with a perfect SBE sequence. It will be of interest to investigate whether this SBE sequence binds either Smad2 or Smad4 present in ARF. Recently, a Smad
binding site was identified in the TGF-β-responsive promoter of the p3TP-Lux construct (25). This site contains a monomeric CAGACA sequence identical to that of the JunB SBE and overlaps with an AP-1 site. The CAGACA sequence appeared to be dispensable for TGF-β induction because mutation of this sequence did not affect TGF-β responsiveness. However, the mutated construct used in this experiment still contained a CgGACA sequence that may be a binding site for Smad proteins. By contrast, mutation of the AP-1 site completely abrogated TGF-β induction. Possibly, in the context of this promoter, Smad proteins cooperate with AP-1 to mediate TGF-β induction. In this respect, it is noteworthy that the TGF-β-inducible PAI-1 promoter contains three CAGACA elements that were able to mediate TGF-β, but not BMP-responsive-ness, of which one element is located adjacent to an AP-1 site.

Likewise, the human α2(I) collagen promoter also harbors an AP-1 site in close proximity to a monomeric CAGACA sequence.

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100.
(34). Finally, Smad proteins may regulate gene transcription through binding to AP-1 or other transcription factors without interacting with DNA in a manner analogous to AP-1/steroid hormone receptor associations. The findings may hint to a mechanism for Smad-mediated gene activation in which receptor-activated Smad proteins cooperate with ubiquitous transcription factors. Nuclear extracts from untreated and TGF-β-treated cells contain a constitutive binding activity that complexes with the 22-bp inverted CAGACA repeat-containing probe. The constitutive complex cannot be supershifted with anti-Smad antibodies. Although we cannot rule out the possibility that the constitutive activity corresponds with a Smad-anti-Smad antibodies. Therefore, they are probably not involved in the regulation of our transcription factor.

Furthermore, the interactions of Smad proteins with other Smad proteins jointly forming a transcriptionally active complex in response to members of the TGF-β superfamily can now be determined.

Acknowledgments—We are grateful to Drs. D. Melton for Smad1 and Smad2 cDNAs; R. Derynck for Smad3, GST-Smad3, and GST-Smad4 cDNAs; R. Lechleider for GST-Smad1 cDNA; M. Schutte for Smad4 cDNA; D. Eick for HaCaT cells; P. van der Saag for Mv1Lu and P19EC cells; and P. Coffer for NIH-3T3 cells. Activin-A was generously provided by Dr. T. K. Sampath (Creative Biomolecules, Inc.). We thank Loes Drenth-Diephuis and Susanne Grimsby for technical assistance and Jean-Michel Gauthier for stimulating discussions.

REFERENCES

1. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Junat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729–739.
2. Angel, P., and Karin, M. (1991) Biochem. Biophys. Acta 1072, 128–157.
3. Chiu, R., Angel, P., and Karin, M. (1989) Cell 59, 979–986.
4. Nikolakaki, E., Coffer, P., Hemelsoet, R., Woodgett, J. R., and Defize, L. H. K. (1993) Oncogene 8, 833–840.
5. de Groot, R. P., and Kruijver, W. (1991) Biochem. Biophys. Res. Commun. 185, 1074–1081.
6. Li, J., Hu, J.-S., and Olsen, E. N. (1990) J. Biol. Chem. 265, 1556–1562.
7. Hashimoto, M., Gaddy-Kurten, D., and Vale, W. (1993) Endocrinology 133, 1934–1940.
8. Chalaux, E., Lopez-Rovira, T., Rosa, J. L., Bartrons, R., and Ventura, F. (1998) J. Biol. Chem. 273, 5571–5578.
9. Li, J.-M., Nichols, M. A., Chandrasekharan, S., Xiong, Y., and Wang, X.-F. (1995) J. Biol. Chem. 270, 26750–26753.
10. Datto, M. B., Yu, Y., and Wang, X.-F. (1985) J. Biol. Chem. 270, 28623–28628.
11. Westerhausen, D. R., Jr., Hopkins, W. E., and Billadello, J. J. (1991) J. Biol. Chem. 266, 1092–1100.
12. Roberts, A. B., and Sporn, M. B. (1996) in Peptide Growth Factors and Their Receptors, Part 1 (Sporn, M. B., and Roberts, A. B., eds) Vol. 95, pp. 419–472, Springer-Verlag New York Inc., New York.
13. Reddi, A. H. (1997) Cytokine Growth Factor Rev. 8, 11–20.
14. Wran a, J., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Nature 370, 341–347.
15. Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1998) Nature 390, 465–471.
16. Kretschmar, M., Doody, J., and Massagué, J. (1997) J. Biol. Chem. 272, 618–622.
17. Imamura, T., Fukase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) Nature 389, 622–626.
18. Nakao, A., Arafah, M., Mor 74 n, Nakayama, T., Christian, J. L., Heuchel, R., Isho, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., and ten Dijke, P. (1997) Nature 389, 631–635.
19. Hayashi, H., Abdollah, S., Qi, Y., Cai, J., Xu, Y.-Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Wran a, J., and Falb, D. (1997) Cell 89, 1165–1173.
20. Liu, F., Hata, A., Baker, J. C., Doody, J., C cramo, Harland, R. M., and Massague, J. (1996) Nature 381, 620–623.
21. Meersman, G., Verschueren, K., Nelkes, K., Blumentock, C., Kraft, H., Wuytens, G., Remacle, J., Kozak, C. A., Tylzanowski, P., Niehrs, C., and Huebler, D. (1997) Proc. Natl. Acad. Sci. USA 94, 127–130.
22. Chen, X., Rubock, M. J., and Whitman, M. (1996) Nature 383, 691–696.
23. Chen, X., Weissberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997) Nature 389, 85–89.
24. Zhang, Y., Feng, X.-H., Xu, R.-Y., and Derynck, R. (1996) Nature 383, 168–172.
25. Yingling, J. M., Datto, M. B., Wong, C., Frederick, J. P., Liberati, N. T., and Wang, X.-F. (1997) Mol. Cell. Biol. 17, 7019–7028.
26. Kim, J., Johnson, R., Chen, H. J., Carroll, S., and Laughon, A. (1997) Nature 388, 304–308.
27. de Groot, R. P., Aubert, J., Karpen, M., Staels, B., and Kruijver, W. (1991) Nuclear Acids Res. 19, 775–781.
28. Nakao, A., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Junat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729–739.
29. Angel, P., and Karin, M. (1991) Biochem. Biophys. Acta 1072, 128–157.
30. Chiu, R., Angel, P., and Karin, M. (1989) Cell 59, 979–986.
31. Nikolakaki, E., Coffer, P., Hemelsoet, R., Woodgett, J. R., and Defize, L. H. K. (1993) Oncogene 8, 833–840.
32. Nakao, A., Arafah, M., Mor 74 n, Nakayama, T., Christian, J. L., Heuchel, R., Isho, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., and ten Dijke, P. (1997) EMBO J. 16, 5353–5362.
33. Boer, P. H., Potten, H., Adra, C. N., Jardine, K., Mulhoffer, G., and McBurney, M. W. (1990) Biochem. Genet. 28, 299–308.
34. Jonk, L. C. J., de Jonge, M. E. J., Pals, C. G. E. M., Wissink, S., Verraart, J. M. A., Scholemmer, J., and Kruijver, W. (1994) Mech. Dev. 47, 81–89.
35. Schutte, M., Hruban, R. H., Hedrick, L. E., Cho, R. K., Nudasy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Nawroz, H., Sidransky, D., Casero, C. A., Melzer, P. S., Hahn, S. A., and Kern, S. E. (1996) Cancer Res. 56, 2527–2530.
36. Alevizopoulos, A., and Mermod, N. (1997) Bioessays 19, 581–591.
37. Greenwel, P., Inagaki, Y., Hu, W., Walsh, M., and Ramirez, F. (1997) J. Biol. Chem. 272, 19738–19745.