Stoichiometry of Active Smad-Transcription Factor Complexes on DNA*

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Transforming growth factor-β (TGF-β)1 is the prototypical member of a large family of pleiotropic cytokines, which also includes the activins, inhibins, bone morphogenetic proteins, nodals, and others (1). TGF-β can elicit a diverse array of biological effects and has been shown to regulate cell growth, apoptosis, migration, differentiation, and specification of cell fate (2, 3).

The mechanism of signal transduction from the cell surface to the nucleus for these ligands has been intensively studied. Ligand binding results in the formation and activation of a heterotetrameric complex of serine/threonine kinase receptors. The type I receptor phosphorylates and activates the receptor-regulated Smads (R-Smads), Smad2 and Smad3, which form hetero-oligomeric complexes with the co-Smad, Smad4, and translocate to the nucleus. Smad3 and Smad4 can bind directly to consensus DNA-binding elements in the promoters of target genes, whereas Smad2/Smad4 complexes are targeted to DNA by interacting with sequence-specific DNA-binding transcription factors that contain a well-defined Smad interaction motif (SIM). The exact stoichiometry of Smad homo- and hetero-oligomers both before and after ligand stimulation is controversial. Here we determine the stoichiometry of TGF-β-induced Smad-transcription factor complexes on DNA. We show that complexes of Smad2/Smad4 with the transcription factors Fast-1 or Fast-3 contain one Fast, two Smad2s, and one Smad4. In contrast, Smad3/Smad4 complexes that bind the Smad-binding element from the c-jun promoter, are heterodimers. Furthermore, these Smad3/Smad4 complexes contain at least two additional components essential for complex formation, one of which contains a SIM. Our data suggest that the R-Smads can form heterodimers or heterotrimeric complexes with Smad4, and we propose that the exact stoichiometries of active Smad complexes on DNA may be determined by the transcription factors with which they associate.

Transforming growth factor-β (TGF-β)1 is the prototypical member of a large family of pleiotropic cytokines, which also includes the activins, inhibins, bone morphogenetic proteins, nodals, and others (1). TGF-β can elicit a diverse array of biological effects and has been shown to regulate cell growth, apoptosis, migration, differentiation, and specification of cell fate (2, 3).

The mechanism of signal transduction from the cell surface to the nucleus for these ligands has been intensively studied. Ligand binding results in the formation and activation of a heterotetrameric complex of two type II and two type I receptor serine/threonine kinases (1). The type I receptor then phosphorylates and activates receptor-regulated Smads (R-Smads), which are Smad2 and Smad3 in the case of the TGF-β receptors (1). Phosphorylation occurs on the two C-terminal serines in the SSXS motif at the extreme C termini of Smads 2 and 3. Following activation, the R-Smads form heteromeric complexes with the co-Smad, Smad4, via their C-terminal (so-called MH2) domains and translocate to the nucleus where they are directly involved in transcriptional regulation of target genes (2, 3). During active signaling, the R-Smads are continually dephosphorylated in the nucleus, resulting in the dissociation of the heteromeric complexes and the return of the Smads to the cytoplasm (4). If the receptors are still active, the R-Smads are re-phosphorylated, form complexes with Smad4, and translocate back to the nucleus. If the receptors are no longer active, the Smads are retained in the cytoplasm (4, 5).

TGF-β signal transduction results in the initiation of a new program of gene expression. It is therefore essential to determine how the Smads are regulated and how they in turn regulate transcription. Fundamental to the understanding of these processes is a detailed characterization both of the TGF-β-induced Smad heteromeric complexes, and of the complexes that assemble at promoter elements with sequence-specific transcription factors. An important issue is that of the stoichiometry of these complexes. Study of the stoichiometry of Smad complexes to date has focused solely on the Smads and remains controversial (6).

Biochemical analyses using purified full-length or C-terminal fragments of Smad2, or Smad2 from cell extracts, have demonstrated that in the unstimulated state Smad2 is monomeric (7–10). Similar analyses with Smad3 indicate that endogenous Smad3 exists in multiple oligomeric states in unstimulated cells (8), while purified C-terminal fragments of Smad3 can be either monomeric or trimeric (11, 12). Similarly, in the uninduced state, Smad4 can exist as monomers (7), dimers, or trimers (8), and crystallographic studies have shown that purified C-terminal fragments of Smad4 are trimers (13, 14). Following ligand stimulation, Smad2 has been described to form homotrimers (7), but with Smad4 it appears to form heterodimers, heterotrimeric, and also higher order complexes (7–10). Studies of purified phosphorylated full-length and C-terminal fragments of Smad2 have demonstrated that activated Smad2 preferentially forms homotrimers (9). Similarly, purified pseudo-phosphorylated C-terminal fragments of Smad3 (11, 12) and phosphorylated full-length Smad3 are also trimeric (9) and in association with Smad4, purified activated Smad3 appears to form heterotrimeric complexes with a stoichiometry of two Smad3s and one Smad4 (11, 12, 15). Activated Smad3/Smad4 complexes have not yet been fully analyzed in vivo. It is not clear whether these different results are a consequence of the methods used to determine the stoichiometry or whether Smads really can exist in several different types of complexes.

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Smad3 and Smad4 can bind directly to the Smad-binding DNA element (SBE), which contains only four base pairs (5'-GCTT-3' or its reverse complement, 5'-AGAC-3'), via their N-terminal (so-called MH1) domains (16, 17). These interactions are weak and insufficient to convey promoter selectivity (17), and hence Smad3/Smad4 complexes synergize with other transcription factors such as members of the AP-1 family and TPEs (18, 19) to regulate gene expression. Smad2/Smad4 complexes do not bind DNA alone but require other transcription factors to target them to specific sequences (19-21). For example, in early Xenopus embryos, complexes of Smad2 with Smad4 (a novel Xenopus Smad4 homologue) (22, 23) are recruited to the activin responsive element (ARE) of the Mix.2 gene by the forkhead/winged helix proteins, Fast-1 (22, 24, 25) or Fast-3 (26) and to the distal element (DE) of the goosecoid promoter by the paired-like homeodomain transcription factors Mixer, Milk, and Bix.3 (27, 28). Smad2/Smad4 transcription-factor interaction is mediated by a defined proline-rich Smad interaction motif (SIM) present in the transcription factor (27, 28), which binds to a hydrophobic pocket in the MH2 domain of Smad2 (28).

Due to the hydrophobic surrounding the stoichiometry of active Smad-transcription factor complexes on DNA. We demonstrate that Fast-1/Smad2/Smad4 (ARF1) and Fast-3/Smad2/Smad4 (ARF2) complexes contain one Fast, two Smad2s, and one Smad4. In contrast, we show that Smad3/Smad4 complexes interacting with the c-jun Smad binding region (29), which we refer to as the SBR, are heterodimers.

Further analysis reveals that these Smad3/Smad4 complexes also contain an essential SIM-containing subunit and a further stabilizing protein that interacts with a repeated CCAG motif in the 3' region of this element. Our data suggest that R-Smads may form heterodimers or heterotrimers with Smad4, and we propose that the exact stoichiometries of active Smad complexes on DNA may be determined by the transcription factors with which they associate.

**EXPERIMENTAL PROCEDURES**

*Plasmids*—The following plasmids have been described previously: XSmad2 and hSmad4 in FLAG-, HA-, and Myc-containing EF expression vectors (26, 27). T7-tagged Fast-1 and Fast-3 were generated by subcloning the Fast sequences into an EF-T7 expression vector, which was generated from EF-plink (30). Myc- and FLAG-tagged hSmad3 in EF expression vectors were subcloned from pcDNA3-hSmad3 (a gift from Peter ten Dijke).

*Cell Culture, Transfections, and Inductions*—NIH3T3, HaCaT, and Smad2-null mouse embryo fibroblasts were all maintained in Dulbecco’s modified Eagle medium containing 10% fetal calf serum. NIH3T3 cells were transfected with LipofectAMINE (Invitrogen). TGF-$\beta_1$ (Peprotech) was dissolved in 4 mM HCl containing 1 mg/ml bovine serum albumin and used at a final concentration of 2 ng/ml. Inductions were for 1 h throughout.

*Nuclear Extracts and Bandshift Assays*—Nuclear extracts were prepared from HaCaT and NIH3T3 cells as previously described (29). The following $^{32}$P-labeled oligonucleotide probes were generated by annealing and filling in the overhanging oligonucleotides (previously described or shown below) by PCR in the presence of $\alpha^{32}$PdCTP and $\alpha^{32}$PdATP. Xenopus Mix.2 ARE (22); c-jun SBR (31); c-jun mutant 1, GGAGGTCGGACTGCGAGC-ACATAGACAGCAG (sense) and TGCCGACCTGCTGCTGCTGC (antisense); c-jun mutant 2, GGAGGTCGGACTGCGAGC-ACATAGACAGCAG (sense) and TGCCGACCTGCTGCTGCTGC (antisense); c-jun mutant 3, GGAGGTCGGACTGCGAGC-ACATAGACAGCAG (sense) and TGCCGACCTGCTGCTGCTGC (antisense); c-jun mutant 4, GGAGGTCGGACTGCGAGC-ACATAGACAGCAG (sense) and TGCCGACCTGCTGCTGCTGC (antisense); c-jun mutant 5, GGAGGTCGGACTGCGAGC-ACATAGACAGCAG (sense) and TGCCGACCTGCTGCTGCTGC (antisense); c-jun mutant 6, GGAGGTCGGACTGCGAGC-ACATAGACAGCAG (sense) and TGCCGACCTGCTGCTGCTGC (antisense).
complex was entirely supershifted by the T7 and Smad4 antibodies indicating that it contains both Fast-1 and Smad4. Supershifting with either the FLAG or Myc antibodies resulted in a partial supershift of the complex indicating incorporation of both the FLAG- and Myc-tagged Smad2s in the complex. Inclusion of both antibodies in the bandshift reaction resulted in a stronger supershift and the appearance of a super-supershifted band (Fig. 2A). This result indicates that the ARF1 complex contains at least two Smad2s. The residual non-supershifted complex (Fig. 2A) is due to the presence of endogenous Smad2, as the whole complex is supershifted with the Smad2/3 antibody (data not shown and Fig. 2C). In order to determine if this complex contains more than two Smad2s we performed analogous experiments with FLAG-, Myc-, and HA-tagged Smad2. Inclusion of each pair of antibodies in the bandshift reaction resulted in a super-supershifted complex with identical mobility (data not shown). Inclusion of all three antibodies in the bandshift reaction resulted in the appearance of a super-supershifted complex, but not a triply-supershifted complex (Fig. 2B), even after prolonged exposure of the film (data not shown). Note that it is possible to detect triply supershifted complexes, for instance upon inclusion in the bandshift reaction of two antibodies that recognize the tags on Smad2 together with an antibody that recognizes Smad4 (see right lane, Fig. 2A). Thus from these experiments we conclude that ARF1 contains two Smad2s.

The same experiment was performed with FLAG- and Myc-tagged Smad4 and untagged Smad2 (Fig. 2C). Equivalent supershifts were readily detected with the FLAG and Myc antibodies, and inclusion of both antibodies increased the intensity of the single supershift, but did not result in the detection of a super-supershifted complex (Fig. 2C). Thus ARF1 contains one Smad4.

Finally we determined the number of Fast-1 molecules present in the ARF1 complex employing the same methodology, and using FLAG- and HA-tagged Fast-1 with endogenous Smads. Inclusion of the Flag or HA antibodies in the bandshift reactions resulted in a partial supershift of the ARF1 complex, whereas inclusion of both antibodies resulted in a complete supershift, but did not reveal a super-supershifted complex. Thus ARF1 contains one Fast-1 (Fig. 2D) (33).

ARF2 Contains Two Smad2s, One Smad4, and One Fast-3 Molecule—We have recently identified a second complex (ARF2), which forms on the Mix.2 ARE in Xenopus embryos in response to endogenous TGF-β family members (22) and have identified its transcription factor component as Fast-3 (26). We therefore performed an identical analysis to that described above to determine the stoichiometry of ARF2. NIH3T3 cells were transiently transfected with T7-tagged Fast-3, untagged Smad4, and FLAG-, Myc-, and HA-tagged Smad2, and ARF2 was analyzed by bandshift assay. Supershifting with the anti-FLAG-, -Myc, or -HA antibodies resulted in a partial supershift of the complex (Fig. 3A). Inclusion of each pair of antibodies in the bandshift reaction resulted in a super-supershifted complex

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**Fig. 1.** Schematic representation of the methodology employed to determine Smad-transcription factor complex stoichiometry on DNA. NIH3T3 cells are transiently transfected with Smads (Smad2 shown in green and Smad4 shown in red), and the transcription factor Fast-1 (shown in blue). The Smad2s in this example are N-terminally tagged with different epitopes. Cells are treated with TGF-β, and nuclear extracts are made and analyzed by bandshift assay using the activin responsive element (ARE) from the Xenopus Mix.2 gene as a probe. The ARE with a Fast binding site and an SBE is shown at the bottom of the figure. Supershifting with combinations of antibodies recognizing the different tags enables determination of the complex stoichiometry. The presence of a single supershifted complex in extracts incubated with both antibodies indicates that only one Smad2 is present in the complex (upper panel). In contrast, the presence of a super-supershifted complex in extracts incubated with both antibodies would indicate that the complexes contained both tagged versions of Smad2, and would demonstrate that at least two Smad2s are present in the complex (lower panel).

**Fig. 2.** ARF1 contains two Smad2s, one Smad4, and one Fast-1. NIH3T3 cells were transiently transfected with different combinations of tagged Smad2s and Fast-1 followed by treatment with TGF-β for 1 h. Nuclear extracts from these cells were analyzed by bandshift assay using the ARE probe. Supershifting with the indicated antibodies; S4, Smad4; S2/3, Smad2/3. The ARF1, supershifted, super-supershifted, and triply supershifted complexes are shown. A, cells were transfected with FLAG- and Myc-tagged Smad2, untagged Smad4, and T7-tagged Fast-1. B, cells were transfected with FLAG-, Myc-, and HA-tagged Smad2, untagged Smad4, and T7-tagged Fast-1. C, cells were transfected with FLAG- and Myc-tagged Smad4, untagged Smad2, and T7-tagged Fast-1. D, cells were transfected with FLAG- and HA-tagged Fast-1.

The same experiment was performed with FLAG- and Myc-tagged Smad4 and untagged Smad2 (Fig. 2C). Equivalent supershifts were readily detected with the FLAG and Myc antibodies, and inclusion of both antibodies increased the intensity of the single supershift, but did not result in the detection of a super-supershifted complex (Fig. 2C). Thus ARF1 contains one Smad4.
Inclusion of all three antibodies in the band-shift reaction resulted in the appearance of a super-super-shifted complex, but not the appearance of a triply supershifted complex, indicating that ARF2 contains two Smad2s (Fig. 3A).

To determine how many Smad4s were present in ARF2, we performed the same experiment with FLAG- and Myc-tagged Smad4 and untagged Smad2. Supershifts of similar intensity were detected with the FLAG and Myc antibodies, and inclusion of both antibodies increased the intensity of the single supershift, but did not result in the detection of a super-super-shifted complex (Fig. 3B). Thus ARF2 contains one Smad4.

Finally bandshift analysis with FLAG- and HA-tagged Fast-3 revealed that ARF2 contains one Fast-3 (Fig. 3C).

The Smad3/Smad4 Complex on the c-jun SBR Is a Heterodimer—Having established that the Smad components of the ARF1 and ARF2 complexes are trimeric and consist of two Smad2s and one Smad4, we investigated the stoichiometry of Smad3/Smad4 complexes. We chose to analyze the Smad3/Smad4 complex that binds the SBR in the 5' untranslated region of the c-jun gene (29). Binding of this complex to the SBR is absolutely required for TGF-β-mediated induction of the c-jun gene (29). NIH3T3 cells were transiently transfected with empty vector or FLAG-Smad4, and HA- and Myc-tagged Smad3. Cells were then induced for 1 h with TGF-β and nuclear extracts prepared, which were analyzed by bandshift assays using the c-jun SBR as a probe (illustrated in Fig. 4D) (29, 31). A weak TGF-β-inducible complex was detected using extracts from empty vector-transfected NIH3T3 cells (Fig. 4A). Confirmation that this contained endogenous Smad3 and Smad4 was obtained by supershift analysis with appropriate antibodies (data not shown). In extracts from the epitope tagged Smad-transfected cells, a more intense lower mobility complex was detected comprising the transfected Smads, which are slightly bigger than the endogenous Smads due to their tags (Fig. 4A, white arrow). Inclusion of the HA or Myc anti-

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**Fig. 3.** ARF2 contains two Smad2s, one Smad4, and one Fast-3. NIH3T3 cells were transiently transfected with different combinations of tagged Smads and Fast-3 followed by treatment with TGF-β for 1 h. Nuclear extracts from these cells were analyzed by bandshift assay using the ARE probe. Supershifts were performed with the indicated antibodies; S4, Smad4; S2/3, Smad2/3. ARF2, supershifted and super-supershifted complexes are indicated. A, cells were transfected with FLAG-, HA-, and Myc-tagged Smad2, untagged Smad4, and T7-tagged Fast-3. B, cells were transfected with FLAG- and Myc-tagged Smad4, untagged Smad2, and T7-tagged Fast-3. C, cells were transfected with FLAG- and HA-tagged Fast-3.

**Fig. 4.** The Smad3/Smad4 complex on the c-jun SBR contains one Smad3 and one Smad4 and requires only two SBEs. A, NIH3T3 cells were transiently transfected with FLAG-tagged Smad4 and HA- and Myc-tagged Smad3 followed by treatment with TGF-β for 1 h. Nuclear extracts from these cells were analyzed by bandshift assay using the c-jun SBR as a probe (illustrated below panel D). Supershifts were performed with the indicated antibodies. The endogenous Smad3/Smad4 complexes (filled arrow), the complexes containing transfected tagged Smad3 and Smad4 (open arrows) and supershifted complexes are shown. B, cells were transfected with Myc-tagged Smad3 and FLAG- and HA-tagged Smad4 and analyzed as in A. C and D, nuclear extracts were prepared from untreated or TGF-β-treated HaCaT cells and analyzed for the formation of endogenous Smad3/Smad4 complexes using the c-jun SBR or mutant (Mut) probes illustrated below. SBEs in the probes are indicated by black arrows, and dashes indicate identical residues. Supershift analysis was performed with the antibodies shown, and the supershifted and Smad3/Smad4 complexes are indicated.
bodies in the bandshift reactions resulted in the appearance of a supershifted complex. Inclusion of both antibodies further increased the intensity of this supershifted complex, but did not generate a super-supershifted complex (Fig. 4A) even after prolonged exposure of the film (data not shown). These findings indicate that there is only one Smad3 in this Smad3/Smad4 complex. Analogous transfections were performed with Myc-tagged Smad3 and FLAG- and HA-tagged Smad4. Similarly, supershift analysis revealed that this Smad3/Smad4 complex contained only one Smad4 molecule (Fig. 4B).

Crystallographic analysis has demonstrated that the MH1 domain of Smad3 contacts the 5′-AGAC-3′ SBE (17). Our findings were therefore surprising, as the c-jun SBR used in this analysis contains three such SBEs (Fig. 4D) and might therefore be capable of binding three Smad MH1 domains. We therefore generated three mutant probes containing only two SBEs elements in three possible arrangements (illustrated in Fig. 4D). Nuclear extracts were prepared from uninduced and TGF-β-induced HaCaT cells. An endogenous Smad3/Smad4 complex is readily detected in extracts from induced cells when assayed by bandshift using the c-jun SBR probe (Fig. 4C) (29). Bandshift analysis with the wild type and all three mutant probes (normalized for specific activity) demonstrated the appearance of a TGF-β-inducible endogenous Smad3/Smad4 complex on these probes that could be supershifted with antibodies directed against Smad2/3 and Smad4 (Fig. 4C). Importantly the mobility of this complex was identical for all the probes tested. Thus the Smad3/Smad4 complex, which binds to the c-jun SBR appears to contact only two SBEs regardless of their position relative to each other (Fig. 4C). This finding is entirely consistent with our observation that the Smad3/Smad4 complex is a heterodimer. Further bandshift analysis with mutant probes containing only one or no SBEs (mutants 4 and 5, respectively) revealed that the presence of two SBEs is required for efficient complex formation on the c-jun SBR (Fig. 4D).

Recent biochemical analysis using purified C-terminal domains of Smad3 and Smad4 indicates that mixtures of these molecules are capable of forming heterotrimers containing two Smad3s and one Smad4 (11, 12). It has also been shown that endogenous Smad2 and Smad3 can form hetero-oligomers following TGF-β stimulation (32). Smad2 however is unable to bind to DNA directly, as it contains a 30-residue insertion immediately upstream of the DNA-binding β-hairpin present in the conserved MH1 domain (21). We therefore investigated whether the Smad3/Smad4 complex that associates with the c-jun SBR might also contain Smad2. Bandshift analysis using nuclear extracts prepared from uninduced and TGF-β-induced Smad2-null fibroblasts demonstrated that the Smad3/Smad4 complex does not contain Smad2 (data not shown). Similarly, we can rule out the presence of any other known Smads in the Smad3/Smad4 complex, as we have not detected either transfected or endogenous Smad1, Smad5, or Smad8 using NIH3T3 or HaCaT extracts and appropriate antibodies (data not shown). We therefore conclude that the Smad3/Smad4 complex does not contain other Smads and contains a heterodimer of Smad3 and Smad4.

Efficient Formation of the Smad3/Smad4 Complex on the c-jun SBR Requires an Additional Factor(s) Containing a SIM—We recently observed that different sequence-specific DNA-binding transcription factors are capable of recruiting Smad2/Smad4 molecules to DNA via their conserved SIMs (27, 28), and we have demonstrated that peptides derived from the Mixer, Fast-1, and Fast-3 SIMs are capable of disrupting the interaction of these transcription factors with Smads on their DNA-binding sites (26–28). We therefore investigated whether the Smad3/Smad4 complex also contained an additional component bearing a SIM and tested this directly using a SIM peptide competition assay. A peptide corresponding to the Mixer SIM was able to efficiently disrupt the formation of the Smad3/Smad4 complex, whereas a mutant form of this peptide (which is no longer able to bind to the hydrophobic pocket in the Smad2 MH2 domain, Ref. 28) had no effect on complex formation (Fig. 5A). To interpret this finding, it was obviously essential to prove that the SIM peptide did not disrupt the interaction between Smad3 and Smad4. We did this using a peptide pull-down assay (28). The SIM peptide was immobilized on beads and incubated with whole cell extracts from uninduced and TGF-β-induced Smad2-null mouse embryonic fibroblasts. The wild-type peptide was able to efficiently pull down Smad3 from extracts from both uninduced and TGF-β-induced cells (Fig. 5B). This peptide pulled down Smad4 only from the extracts from TGF-β-induced cells through its interaction with Smad3 (Fig. 5B). These data thus prove that the SIM peptide does not disrupt Smad3/Smad4 complex formation. Thus we conclude that the Smad3/Smad4 complex on the c-jun SBR includes an additional factor(s) that contains a SIM and that binding of this factor is essential for complex formation on DNA.

Efficient Formation of the Smad3/Smad4 Complex on the c-jun SBR Requires the SBEs and the Repeated CCAG Motifs in the 3′-Region of the SBR—Having established that the Smad3/
were detected by bandshift assay using the c-SBR probe or mutant
produced and TGF-
repeated CCAG motifs.

A Smad4, and at least one other factor, which binds to the re-
the c-SBR requires the coordinated assembly of Smad3,
-Jun
Smad3/Smad4 complex with faster mobility on the 5
-H11032
derivatives of this probe as illustrated. Note the formation of a weaker
-Jun
SBR probe and a 3
CaT nuclear extracts on the c
open arrows
FIG.6.
Efficient formation of the Smad3/Smad4 complex on
induced HaCaT cells, and Smad3/Smad4 complexes
,
bandshift assays were performed with HaCaT nuclear extracts on the c
-H11032
probes with the flank-
non-homologous flanking sequences (middle; Fig. 6A, Smad3/Smad4
, white arrow), indicating that additional sequences in the 3
-region of the c
-Jun
SBR probe are required for formation of the wild-type complex. We were unable to detect a
-Jun
inducible complexes that bound three these probes with that which bind the wild-type probe.

Only an extremely weak TGF-
-Jun
inducible Smad3/Smad4 complex was detected on the 5
 probe, that had a slightly higher mobility relative to the wild-type Smad3/Smad4 complex (Fig. 6A, Smad3/Smad4
, white arrow), indicating that additional sequences in the 3
-region of the c
-Jun
SBR probe are also required for the formation of the Smad3/Smad4 complex, or that the presence of an additional stretch of DNA upstream of the SBEs is necessary. We could prove that the latter was the case since we analyzed complexes that bound a probe identical to the 3
 probe, but which also contained an additional non-homologous sequence (TTGGTGTGG) 5
 to the SBEs, and demonstrated that a wild-type Smad3/Smad4 complex efficiently bound this probe (data not shown). This finding probably reflects the topological requirement for additional DNA upstream of the first SBE contacted by a Smad. The middle region probe, which contains the SBEs flanked by se-
sequences unrelated to the c
-Jun
SBR, strongly and constitutively bound factors that obscured the normal location of the Smad3/
-Jun
SBR (Fig. 6A). (We deduce that these factors bind the GT-rich sequences at the ends of this probe; data not shown). However, we were able to conclude that this probe, which does not contain the 5
 or 3
-flanking sequences of the c
-Jun
SBR, was unable to bind Smad3/Smad4 complexes, be-
cause a supershifted complex (which would not be obscured) was not detected in the presence of the Smad2/3 antibody (Fig. 6A).

From these data we conclude that the SBEs themselves are not sufficient for formation of a TGF-
-Jun
inducible Smad3/Smad4 complex on the c
-Jun
SBR. Specific sequences are required 3
 to the SBEs and additional DNA is required 5
 to the SBEs, although its precise sequence is not important. Examination of the sequence of the 3
-region of the c
-Jun
SBR revealed a direct triple repeat of the 4-bp motif, CCAG. We therefore generated a mutant bandshift probe by mutating each of these sites to CATG (Fig. 6B). Bandshift analysis using this probe revealed that these sites were absolutely required for the efficient for-
mation of the wild-type Smad3/Smad4 complex (Fig. 6B).

Having demonstrated the requirement for specific sequences 3
 to the SBEs for formation of the Smad3/Smad4 complex, we investigated, using competition experiments, whether a dou-
ble-stranded oligonucleotide corresponding to this region could compete out the potential cooperating binding factors. Cold competitor oligonucleotide corresponding to the 5
 or 3
-flanking sequences (top strand illustrated in Fig. 6C) were unable to compete out the Smad3/Smad4 complex. The middle region competitor oligonucleotide, which contains just the SBEs, how-
C, bandshift assays were performed with HaCaT nuclear extracts on the c
-Jun
SBR probe as in A, except that increasing molar excesses of cold double-stranded competitor oligonucleotides illustrated at the bot-
tom of the figure were also included in the bandshift reactions as indicated. In all cases supershifts with anti-Smad2/3 and also anti-
Smad4 antibodies (B and C) were performed to establish that the TGF-
-Jun
induced complexes contained Smad3 and Smad4. SBEs are indi-
cated by black arrows, and dashes indicate identical residues.
whether these factors are the same or distinct, we determined sequences binding of active Smad3/Smad4 complexes to DNA requires the factor oligonucleotide), but, as demonstrated above, efficient binding of active Smad3/Smad4 complexes to DNA requires the additional nonspecific sequences 3' to the SBEs and specific sequences 3' to the SBEs.

The Smad3/Smad4 Complex SIM-containing Factor Is Distinct from the Repeated CCAG Motif-binding Factor—We have demonstrated that the Smad3/Smad4 complex that binds the c-jun SBR contains an additional component bearing a SIM and a factor that interacts with the CCAG sequences. To resolve whether these factors are the same or distinct, we determined whether the (Smad3/Smad4)* complex, which forms on the c-jun 5' SBR probe (that does not contain the CCAG repeats), contained the SIM-containing factor. We performed peptide competition analysis using the wild-type and mutant Mixer SIM peptides. Titration of the wild-type, but not the mutant, SIM peptide was able to efficiently disrupt the formation of the (Smad3/Smad4)* complex on the 5' probe (Fig. 7A). This finding demonstrates that the (Smad3/Smad4)* complex does include the SIM-containing factor and hence this factor is distinct from the repeated CCAG motif-binding factor.

Recent data from several laboratories has demonstrated that a Smad3/Smad4 complex forms on the palindromic dimeric SBEs from the promoters of both the mouse and human Smad7 genes (34–39) (illustrated in Fig. 7B). We performed bandshift analysis with the Smad7 SBE probe, and using peptide competition analysis demonstrated that the Smad3/Smad4 complexes that form on this probe also contain a SIM-containing factor (Fig. 7B). This finding further supports our notion that this factor is distinct from the repeated CCAG motif-binding factor, as the CCAG sequences are not present in this probe.

**DISCUSSION**

Smad-Transcription Factor Complex Stoichiometry—The stoichiometry of Smad homo- and hetero-oligomers both before and after ligand stimulation has previously been studied using a variety of biochemical techniques. The outcome of these studies is controversial, with some models concluding that Smad2/Smad4 complexes are heterodimers and others concluding they are heterotrimmers or higher order complexes (Ref. 6 and references therein). Here we have analyzed active Smad-transcription factor complexes on DNA. We demonstrate that the ARF1 and ARF2 complexes contain a heterotrimer of Smad2 and Smad4 in the stoichiometric ratio of 2:1. These complexes also contain one Fast-1 and one Fast-3 molecule, respectively (Fig. 8). These findings are consistent with the observation that the ARE in the Xenopus Mix.2 promoter contains one Fast binding site in close proximity to one SBE, to which the Smad4 binds (33). These findings also strengthen our previous hypothesis that the increased mobility of the ARF2 complex relative to the ARF1 complex is due to the fact that Fast-3 is smaller than Fast-1 and not due to a difference in the numbers of Smads in these complexes (26). We have previously demonstrated that the Fast molecules present in these complexes contact a hydrophobic binding pocket in the Smad2 MH2 domain via their conserved proline-rich SIMs (26, 28). Interestingly we have recently identified another region of homology between Fast-1 and Fast-3 adjacent to the SIM motif (26), which is also capable of contacting Smad2.

We have demonstrated that the Smad3/Smad4 complexes that form on the c-jun SBR in contrast are not trimeric, but rather contain a heterodimer of Smad3 and Smad4. Consistent with this we have shown that only two of the three SBEs present in this region are required to form a wild-type Smad3/Smad4 complex, suggesting that one Smad3 and one Smad4 contact these sites via their MH1 domains. This Smad3/Smad4 complex contains at least two other factors, one containing a SIM, and the other binding to the CCAG repeats 3' to the SBEs in the c-jun SBR (Fig. 8 and see below).

Thus we have demonstrated that in the context of certain DNA-bound Smad-transcription factor complexes, Smad2/Smad4 complexes are trimeric and Smad3/Smad4 complexes are dimeric. This result suggests two possible models. First, in **vivo**, activated Smad2 may always form trimers with Smad4, and activated Smad3 may always form dimers with Smad4. Alternatively, both R-Smads may exist in both heterodimeric and heterotrimeric complexes with Smad4 and the precise stoichiometries may be dependent on the precise binding elements, as well as the associated transcription factors. Given

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*2 R. A. Randall, M. Howell, C. Page, P. Bates, and C. S. Hill, manuscript in preparation.*
components of many Smad complexes on DNA. The factor(s) that have been suggested to act as degenerate Smad binding motifs.

In these CCAG motifs after very long exposures (data not shown), one Smad3, at least one SIM-containing Smad4, and one Fast-1; ARF2 contains two Smad2s, one Smad4, and one Fast-3; the Smad2/Smad4 complex on the c-jun SBR contains one Smad3 (yellow), one Smad4, at least one SIM-containing factor (X, purple) and at least one CCAG motif-binding factor (Y, pink). For discussion, see text.

The evidence from other studies indicating that in vitro phosphorylated Smad2 can form heterodimers with Smad4 in the absence of transcription factors (10), and that pseudo-phosphorylated Smad3 appears to form heterotrimers with Smad4, we favor the latter model. However, it will only be possible to resolve this issue when further endogenous TGF-β-induced Smad-transcription factor complexes are fully characterized.

Complexities of Smad Complexes—Our analysis of the Smad3/Smad4 complex reveals that formation of a complex of wild-type strength and mobility requires the interaction of another, as yet unidentified, component with the region of the c-jun SBR 3' to the third SBE. This region is characterized by the triple repeat of a 4-bp motif CCAG. Mutation or deletion of these motifs abolished formation of the wild-type Smad3/Smad4 complex. In bandshifts performed with the probe deleted of this region, we were able to detect an extremely weak Smad4 complex.

The Smad-interacting factors c-Ski and SnoN are also candidates for the additional factors present in the Smad3/Smad4 complexes. Both proteins bind to the MH2 domain hydrophobic pocket precludes formation of active TGF-β-inducible Smad3/Smad4 complexes on DNA, which strongly suggests that another distinct SIM-containing transcription factor is required to stabilize Smad3/Smad4 complexes on DNA. This factor is unlikely to contact DNA itself (Fig. 8) as it is still present in the faster mobility (Smad3/Smad4)* complex and in the Smad3/Smad4 complexes that bind the Smad7 SBE, which shares no obvious sequence homology to the c-jun SBR outside the SBEs. The presence of a factor(s) that presumably binds to the hydrophobic pocket of the Smad3 MH2 domain in both the c-jun SBR Smad3/Smad4 complexes and the Smad7 SBE Smad3/Smad4 complexes raises the interesting possibility that other Smad3/Smad4 complexes may also contain this additional factor(s).

Smad3/Smad4 Complex Components—Antisense-mediated inactivation of menin expression (the product of the multiple endocrine neoplasia gene) was recently shown to disrupt Smad3/Smad4 complex formation on an element from the plasminogen activator inhibitor 1 promoter (44). Menin can interact with the MH2 domain of Smad3 and is therefore a potential candidate for one of the components of the Smad3/Smad4 complex described here. We have, however, been unable to supershift the endogenous HaCat Smad3/Smad4 complex with antibodies specific for menin, and similarly overexpression of FLAG-tagged menin in NIH3T3 cells did not result in any appreciable change in the intensity or mobility of the Smad3/Smad4 complex and neither was the complex supershifted with anti-FLAG antibodies.3 The Smad-interacting factors c-Ski and SnoN are also candidates for the additional factors present in the Smad3/Smad4 complexes. Both proteins bind to the MH2 domains of Smads, and site selection studies with c-Ski resulted in the identification of the SBE as a consensus DNA-binding motif (reviewed in Ref. 45). We have been unable to supershift the Smad3/Smad4 complex with anti-c-Ski or SnoN antisera and hence have no evidence to suggest that these proteins are present in this complex.3 We are currently employing biochemical techniques to identify the additional factors we have demonstrated to be present in these Smad3/Smad4 complexes.

Determinants of Smad Stoichiometry—Here we have shown that TGF-β-inducible Smad-transcription factor complexes can contain either heterotrimeric or heterodimeric Smad oligomers. Our data also suggest that not only do Smad2/Smad4 complexes interact with SIM-containing transcription factors; this is also a feature of Smad3/Smad4 complexes. It is attractive to speculate that the exact stoichiometry of the Smad complexes

3 G. J. Inman and C. S. Hill, unpublished observations.
bound to different promoter elements may be determined by their associated transcription factors and adjacent binding sites. The number of SIM or SIM-like motifs present in these interacting transcription factors may play a role in this. These transcription factors could select the correct Smad hetero-oligomer from a pool of ligand-induced hetero-oligomers present in the cell, or alternatively induce the formation of the correct hetero-oligomer.

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REFERENCES

1. Massagué, J. (1998) Annu. Rev. Biochem. 67, 753–791
2. Massagué, J., Blain, S. W., and Lo, R. S. (2000) Cell 103, 295–309
3. Wakefield, L. M., and Roberts, A. B. (2002) Curr. Opin. Genet. Dev. 12, 22–29
4. Inman, G. J., Nicolas, F. J., and Hill, C. S. (2002) Mol. Cell. 10, 283–284
5. Pierreux, C. E., Nicolas, F. J., and Hill, C. S. (2000) Mol. Cell. Biol. 20, 9041–9054
6. Kawabata, M., Inoue, H., Hanyu, A., Imamura, T., and Miyazono, K. (1998) EMBO J. 17, 4056–4065
7. Jayaraman, L., and Massagué, J. (2000) J. Biol. Chem. 275, 40719–407217
8. Wu, J. W., Hu, M., Chai, J., Seoane, J., Huse, M., Li, C., Rigotti, D. J., Yin, S., Muir, T. W., Fairman, R., Massagué, J., and Shi, Y. (2001) Mol. Cell 8, 1277–1289
9. Wu, J. W., Fairman, R., Penry, J., and Shi, Y. (2001) J. Biol. Chem. 276, 20688–20694
10. Chacko, B. M., Qin, B., Correia, J. J., Lam, S. S., de Caestecker, M. P., and Lin, K. (2000) Nat. Struct. Biol. 7, 248–253
11. Correia, J. J., Chacko, B. M., Lam, S. S., and Lin, K. (2001) Biochemistry 40, 1473–1482
12. Shi, Y., Hata, A., Lo, R. S., Massagué, J., and Pavletich, N. P. (1997) Nature 388, 87–93
13. Qiu, B., Lam, S. S., and Lin, K. (1999) Structure Fold. Des. 7, 1493–1503
14. Qin, B. Y., Lam, S. S., Correia, J. J., and Lin, K. (2002) Genes Dev. 16, 1959–1963
15. Zawel, L., Dai, L., Backhaus, P., Zhou, S., Kimler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617
16. Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massagué, J., and Pavletich, N. P. (1998) Cell 94, 585–594
17. Zhang, Y., and Derynck, R. (1999) Trends Cell Biol. 9, 274–279
18. Massagué, J., and Wotton, D. (2000) EMBO J. 19, 1745–1754
19. ten Dijke, P., Miyazono, K., and Heldin, C. H. (2000) Trends Biochem. Sci. 25, 64–70
20. Shi, Y. (2001) Bioessays 23, 223–232
21. Howell, M., Itoh, F., Pierreux, C. E, Valporredor, S., Ioh, S., ten Dijke, P., and Hill, C. S. (1999) Dev. Biol. 214, 354–369
22. Massagué, N., Hanafusa, H., Kusakabe, M., Shibuha, Y., and Nishida, E. (1999) J. Biol. Chem. 274, 12163–12170
23. Chen, X., Rubock, M. J., and Whitman, M. (1996) Nature 383, 691–696
24. Chen, X., Weisberg, E., Frimdrncher, W., Watanabe, M., Naco, G., and Whitman, M. (1997) Nature 385, 85–89
25. Howell, M., Inman, G. J., and Hill, C. S. (2002) Development 129, 2823–2834
26. Germain, S., Howell, M., Esslelmont, G. M., and Hill, C. S. (2000) Genes Dev. 14, 435–451
27. Randall, A. A, Germain, S., Inman, G. J., Bates, P. A., and Hill, C. S. (2002) EMBO J. 21, 145–156
28. Wang, C., Rougier-Chapman, E. M., Frederich, J. P., Datto, M. B., Liberati, N. T., Li, J. M., and Wang, X. F. (1999) Mol. Cell. Biol. 19, 1821–1830
29. Marais, R., Wyne, J., and Tresiman, R. (1993) Cell 73, 381–388
30. Lehmann, K., Janda, E., Pierreux, C. E., Rytoama, M. Schulze, A. McMahan, M., Hill, C. S., Beug, H., and Downward, J. (2000) Genes Dev. 14, 2610–2622
31. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaka, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) EMBO J. 16, 5353–5362
32. Yoo, C. Y., Chen, X., and Whitman, M. (1999) J. Biol. Chem. 274, 26584–26590
33. Eresh, S., Riese, J., Jackson, D. B., Bohmann, D., and Bienz, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2354–2357
34. Eresh, S., Riese, J., Jackson, D. B., Bohmann, D., and Bienz, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2354–2357
35. Brodin, G., Ahgren, A., ten Dijke, P., Heldin, C. H., and Heuchel, R. (2000) J. Biol. Chem. 275, 29023–29030
36. van Gersdorff, G., Susztak, K., Rezvani, F., Bitzer, M., Liang, D., and bottiger, E. P. (2000) J. Biol. Chem. 275, 11320–11326
37. Stopa, M., Anhuf, D., Terstegen, L., Gatsios, P., Gressner, A. M., and Dooley, S. (2000) J. Biol. Chem. 275, 29308–29317
38. Xu, X., Miller, Z. A., Benchabane, H., Wrana, J. L., and Lodish, H. F. (2000) J. Biol. Chem. 275, 33205–33208
39. Nagarajan, R. P., Chen, F., Li, W., Vig, E., Harrington, M. A., Nakshatri, H., and Chen, Y. (2000) Biochem. J. 348, 591–596
40. Demisscova, N. G., Pouponnot, C., Long, J., He, D., and Liu, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6397–6402
41. Kuczeny, A., and ten Dijke, P. (2002) J. Biol. Chem. 277, 4883–4891
42. Xu, X., Yin, Z., Huse, M., Ponnapan, C., Long, J., He, D., and Liu, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6397–6402
43. Eresh, S., Riese, J., Jackson, D. B., Bohmann, D., and Biezn, M. (1997) EMBO J. 16, 2014–2022
44. Hata, A., Seoane, J., Lagna, G., Montalvo, J. B., Kern, S. E., and Massagué, J. (2000) Cell 100, 229–240
45. Kaji, H., Canaff, L., Lebrun, J. J., Goltzman, D., and Hendy, G. N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3837–3842
46. Liu, X., Sun, Y., Weinberg, R. A., and Lodish, H. F. (2001) Cytokine Growth Factor Rev. 12, 1–8
Additions and Corrections

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