BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 195–201 (1998)
ARTICLE NO. RC989562

Identification of a Novel *Drosophila* SMAD on the X Chromosome

Katya D. Henderson and Deborah J. Andrew[1]
Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196

Received September 22, 1998

TGF-β signaling from the cell surface to the nucleus is mediated by the SMAD family of proteins, which have been grouped into three classes based upon sequence identity and function. Receptor-regulated, or pathway-restricted, SMADs (R-SMADs) are phosphorylated by ligand-specific serine/threonine kinase receptors. Phosphorylated R-SMADs oligomerize with the coactivating, or shared, SMAD (Co-SMAD) mediator and translocate to the nucleus where the complex directs transcription of downstream target genes. Inhibitory SMADs (I-SMADs) block receptor-mediated phosphorylation of R-SMADs. In Drosophila, one member of each class of SMAD has been reported: MAD, an R-SMAD, MEDEA, a Co-SMAD, and DAD, an I-SMAD. Here, we report the first identification of a novel *Drosophila* R-SMAD, which we have named *Smox* for *Smad on X*. We have localized the *Smox* gene to a specific interval on the X chromosome and shown that *Smox* is transcribed throughout development.

Key Words: **ACTIVIN; DPP; Drosophila; SMADs.**

The transforming growth factor-β (TGF-β) family of cytokines play important roles in development, including regulation of cell growth, and various patterning events (reviewed in [1-3]). Signaling from TGF-β family members is mediated through a family of serine/threonine kinase receptors. This receptor family consists of two classes of receptors, type I and type II. TGF-β family members bind to the type II receptor, which then recruits the type I receptor into a complex. The formation of this complex leads to phosphorylation and activation of the type I receptor by the type II receptor. In *Drosophila*, the receptors for the TGF-β famly member Decapentaplegic (DPP) are encoded by *thick veins* (*tkv*), *saxophone* (*sax*) and *punt* (*put*) [4-8]. As is seen in other systems, upon activation of these receptors, the R-SMAD encoded by the *Mothers against DPP* (*Mad*) gene is phosophorylated and forms a complex with the Co-SMAD encoded by the *Medea* (*Med*) gene [9-14]. The MH1 domain of the R-SMAD physically contacts DNA through site-specific interactions [15, 16]. In vertebrates, different R-SMADs couple with different receptors to mediate signaling from specific TGF-β family members. SMAD1, SMAD5 and SMAD8 are phosphorylated in response to BMP-2 or BMP-4 signaling, while SMAD2 and SMAD3 are phosphorylated in response to activin signaling (review in [17, 18]). Here we report on the identification of a fourth Drosophila SMAD family member, *Smad on X* (*Smox*), which encodes a protein most closely related to human SMAD3.

[1] To whom correspondence should be addressed. Fax: (410) 955-4129. E-mail: debbie_andrew@qmail.bs.jhu.edu.

MATERIALS AND METHODS

Fly stocks. The wild type flies used in all experiments were Canton S or Oregon R. The deficiencies used in this study, which include *Df(1)hl-a, Df(1)RA2, Df(1)C128, Df(1)GE202,* and *Df(1)Desi-3,* are described in Flybase.

Chromosome in situ hybridizations. Chromosome *in situ* hybridizations were done by hybridizing biotin-labelled *Smox* cDNAs to fixed larval salivary gland polytene chromosomes from either wild-type Canton S larvae or larvae heterozygous for a deficiency chromosome and a wild-type X-chromosome. We followed the procedure of [19] omitting the RNase treatment and acetylation steps, and using the Vectastain Kit (Vector Laboratories) for HRP signal detection.

Whole mount in situ hybridization. Whole mount *in situ* hybridization to embryos was carried out as described in [20] with both antisense and sense *Smox* RNA probes.

Northern analysis. Approximately 5 μg of poly-A^+-selected RNA from staged embryos, larvae, pupae, sexed adults or Schneider 2 cells was run in each track of a 1% agarose, 6 % formaldehyde gel. The size-fractionated RNA was then transferred to nitrocellulose and probed with a random-primed *Smox* cDNA probe. As a control for the amount of poly-A^+ RNA in each track, the Northern blot was also hybridized with a random-primed ribosomal protein probe, rp49 [21]

Standard molecular techniques. cDNA library screening, plasmid, phage and genomic DNA isolation, and labeling of radioactive probes were performed as described in [22]. Sequence alignments were done using the CLUSTAL X alignment program [23]. Genbank accession No. for *Smox* is AF078529.

0006-291X/98 $25.00

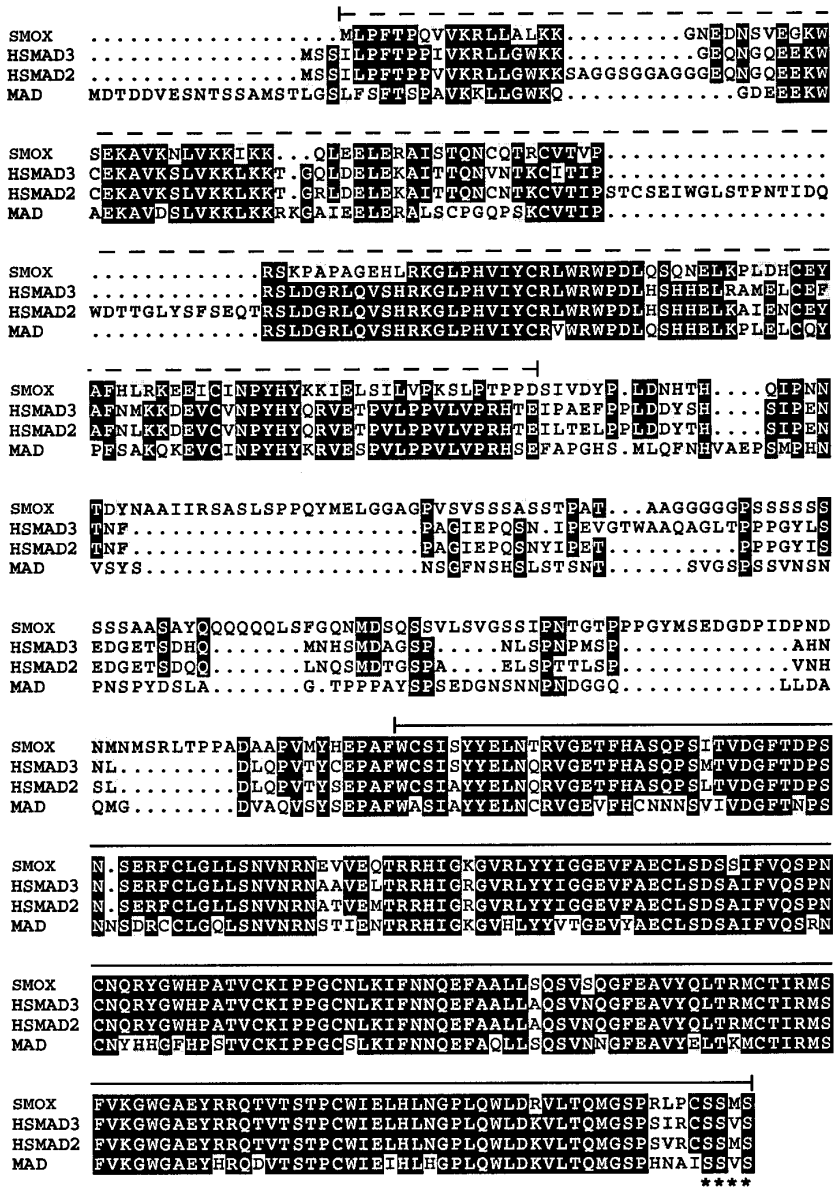

FIG. 1. Drosophila SMOX is most similar to vertebrate SMAD2 and SMAD3. The conceptual open reading frame of SMOX compared to human SMAD3, human SMAD2 and Drosophila MAD. Black indicates identical residues. Grey indicates conservative replacements. The dashed bar above the sequences indicates MAD homology domain 1 (MH1) and the solid bar above the sequences indicates MAD homology domain 2 (MH2). The asterisks indicate the C-terminal consensus phosphorylation motif SSXS found in all class I SMADs characterized to date.

RESULTS

In both vertebrates and invertebrates, SMAD proteins transduce the signals from the activated TGF-β family of receptors to the nucleus. In vertebrates, three general classes of SMAD proteins have been described. R-SMADs respond to signaling by specific classes of TGF-β molecules. Examples of R-SMADs are SMADs 2 and 3 which transduce signals from the TGF-β/Activin and Veg1 receptors, and SMADs 1, 5 and 8 which transduce signals from the BMP2/BMP4 receptor (for review see [17, 24]. R-SMADs are directly phosphorylated by the activated type I receptors and form a phosphorylation-dependent complex with the only known co-SMAD, SMAD4/DPC4 [16, 25-30]. This heteromeric complex of R-SMADs and Co-SMADs translocates to the nucleus where it functions as a transcription partner to regulate gene expression. Finally, I-SMADs, such as SMAD 6 and SMAD7, function to attenuate signaling by competing with the R-SMADs

TABLE 1

Percentage Identities among the MH1 and MH2 Domains of SMOX, MAD and the Vertebrate Class 1 SMADs

Drosophila class I protein domain	SMOX		MAD		Smad2		Smad3		Smad1		Smad5	
	MH1	MH2	MH1	MH2	MH1	MH2	MH1	MH2	MH1	MH2	MH1	MH2
SMOX MH1	100	—	53.6	—	48.9	—	59.0	—	53.6	—	54.3	—
SMOX MH2	—	100	—	76.8	—	92.9	—	92.9	—	79.0	—	79.0
MAD MH1	53.6	—	100	—	54.3	—	68.1	—	92.1	—	88.9	—
MAD MH2	—	76.8	—	100	—	77.9	—	77.4	—	88.7	—	87.7

for interactions with the activated receptors [31]. I-SMADs form stable associations with the receptor complex but, unlike the R-SMADs, are not phosphorylated by the activated receptor.

There are two known R-SMAD family members in the nematode *C. elegans* [32] suggesting the existence of another R-SMAD in Drosophila in addition to the well-characterized *Mad* gene [9, 10, 33-35]. To determine if a second R-SMAD existed in *Drosophila,* we searched the Berkeley Drosophila Genome Project (BDGP) sequence databases with the MAD protein sequence. This search identified two cDNAs whose conceptual open reading frames were homologous to either MAD-homology domain 1 (MH1), cDNA clone LD07433 (Genome Systems, Inc), or MAD-homology domain 2 (MH2), cDNA clone CK01082 (C. Kopczynski, Accession #AA141008, submitted 11/16/96). To test whether either of the cDNAs corresponded to known Drosophila SMAD family members, we mapped each of the two clones to polytene chromosomes. LD07433 mapped to cytological region 100C, the known position of the *Med* gene [9], suggesting that it was a *Med* cDNA. Comparison of the LD07433 sequence with the subsequently published *Med* sequence confirmed this identity [12-14]. CK01082 mapped to cytological region 7D7-16 suggesting that the cDNA corresponded to a novel Drosophila SMAD family member since no known SMAD gene maps to this region. Since the CK01082 clone was not full length, we screened a 9-12 hr embryonic cDNA library to isolate and characterize additional cDNAs. A comparison of the conceptual open reading frame of the gene, which we have named *Smad on X* (*Smox*), to other SMAD family members reveals that it is most closely related to vertebrate SMAD2 and SMAD3 (Fig. 1; Table 1). SMOX has 60% overall identity to human SMAD3, 57.1% identity to human SMAD2, and 51.8% identity to Drosophila MAD. SMAD proteins have two highly conserved domains, known as MH1 and MH2,

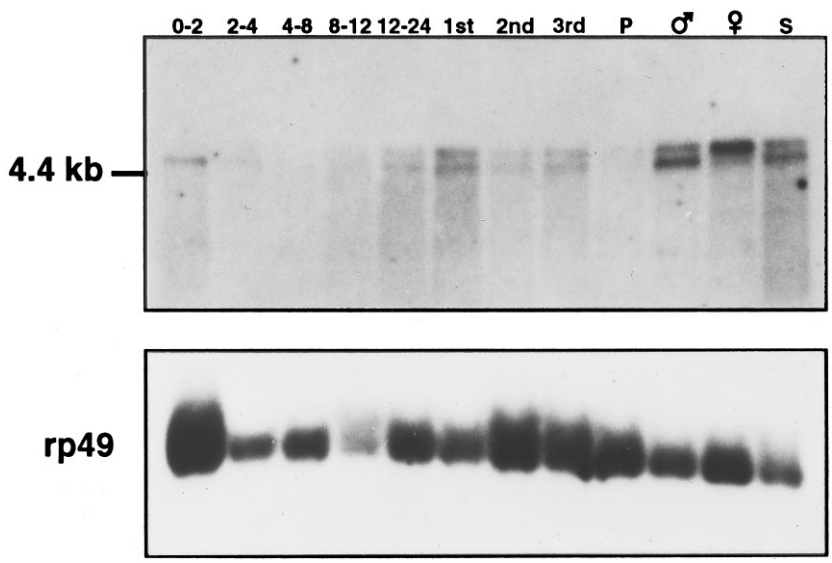

FIG. 2. Drosophila SMOX encodes two transcripts expressed throughout development. 0-2, 2-4, 4-8, 8-12, 12-24 refer to hours of embryogenesis, 1st, 2nd and 3rd refer to the three larval stages, P refers to pupae, S refers to Drosophila Schneider 2 cells. The slower migrating form is the most abundant form in adult females and 0-2 hr embryos, suggesting that this transcript is provided maternally. The same Northern is shown hybridized to *rp49* probes as a control for loading.

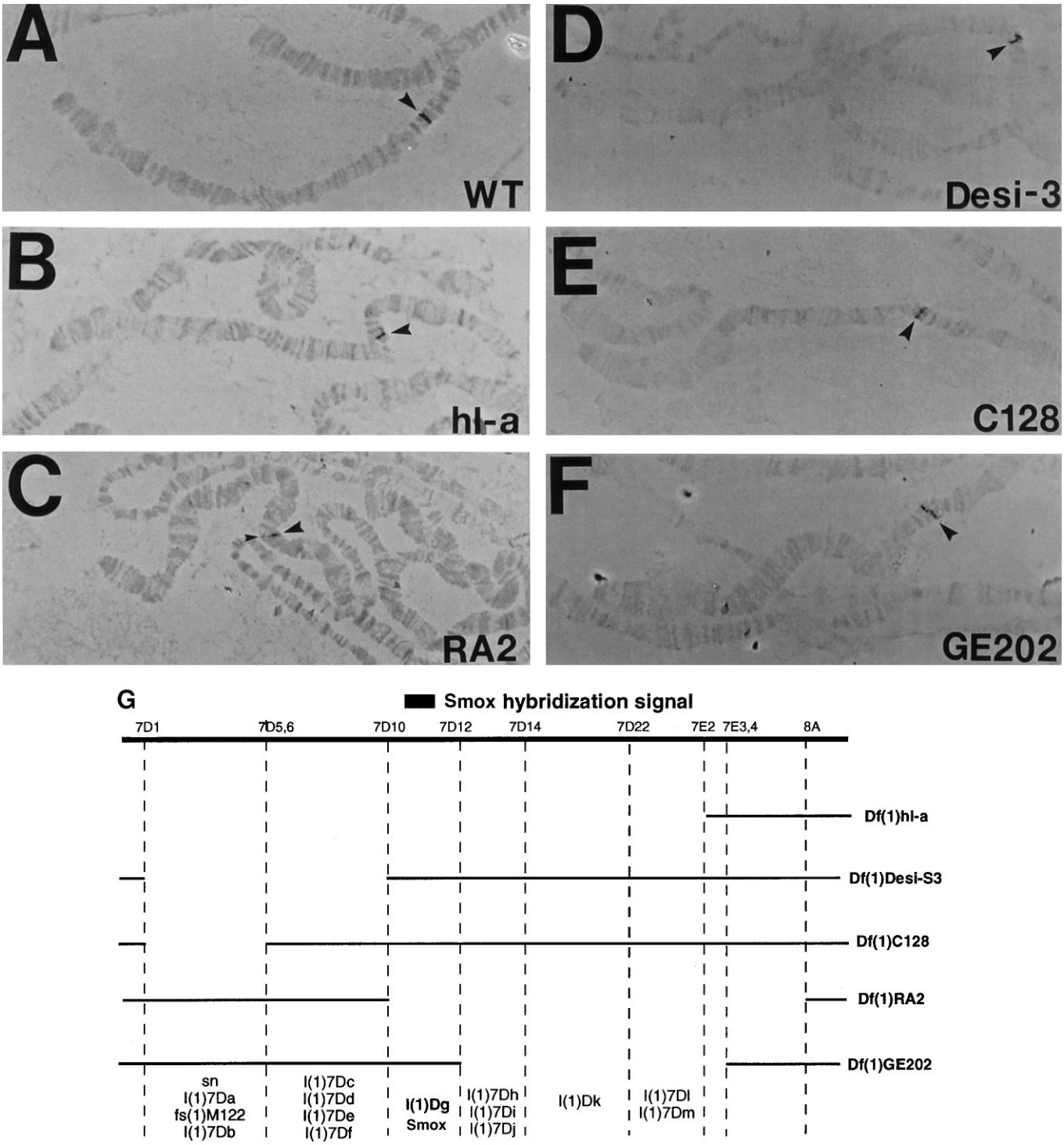

FIG. 3. *Smox* maps to cytological region 7D10, a region containing only a single known complementation group, *l(1)7Dg*. Hybridization of a *Smox* cDNA clone to polytene chromosomes of either wild-type larvae or larvae heterozygous for deficiencies in the 7D interval of the X chromosome: wild-type chromosomes (A); *Df(1)hl-a/+* chromosomes (B); *Df(1)RA2/+* (C); *Df(1)Desi-S3/+* (D); *Df(1)C128/+* (E); *Df(1)GE202/+* (F). The arrowheads indicate the hybridization signals. The position of *Smox* in the genome relative to genes mapping to the 7D-E region (G). The chromosomal region deleted in the deficiency is indicated by a break in the dark horizontal lines. The only extant allele *l(1)7Dg* complemented Df(1)RA2 but not Df(1)C128, suggesting that the original mutation in the *l(1)7Dg* stock has been lost.

TABLE 2

Components of the ACTIVIN and BMP Signaling Pathways in Vertebrate and Drosophila Development

	ACTIVIN pathway		BMP pathway	
	Vertebrate protein	Drosophila protein	Vertebrate protein	Drosophila protein
Ligand	ACTIVIN	ACTIVINβ_B	BMP-2/BMP-4	DPP
			BMP-5/BMP-6/BMP-7	60A/SCREW
Type II receptor	ACTR-II		ACTRII	
	ACTR-IIB	PUNT?	ACTRIIB	PUNT
			BMPR2	
Type I receptor	ACTR-I	ATR-I	BMPR-IA	TKV
	ACTR-IB		BMPR-IB	
			ACT-RI	SAX
Class I Smad	SMAD2	SMOX?	SMAD1	MAD
	SMAD3		SMAD5	
			SMAD8	
Class II Smad	SMAD4/Dpc4	MED?	SMAD4/Dpc4	MED
Class III Smad	SMAD6		SMAD6	
	SMAD7	DAD?	SMAD7	DAD
Inductive event(s)	Dorsal mesoderm		Ventral mesoderm	Early dorsal patterning
	Erythroid differentiation	?	Cartilage and bone	Midgut morphogenesis
	FSH release		Apoptosis	Imaginal disc patterning
				Mesoderm patterning
				Oogenesis
				Salivary gland limits

separated by a linker region which is less conserved. SMOX MH1 has 59.0 % identity to the MH1 domain of SMAD3 and 48.9 % identity to the MH1 domain of SMAD2 (sequence below dashed line in Fig. 1 and Table 1). SMOX MH2 has 92.9 % identity to the MH2 domains of both SMADs2 and 3 (sequence below solid line in Fig. 1 and Table 1). Like SMAD3, SMOX does not have the two insertions of nine and thirty residues found in the MH1 domain of SMAD2. SMOX has an SSXS motif at the C-terminus which has been found in all receptor-regulated SMADs characterized thus far (indicated by asterisks in Fig. 1). The two most C-terminal Ser residues of this motif in other class I SMADs are phosphorylated by activated type I receptors [36-39].

To determine where *Smox* may be functioning, we determined the expression of *Smox* both by Northerns and by *in situ* hybridization to whole mount embryos. The Northern analysis revealed two *Smox* transcripts, both approximately 4.5 kb (Fig. 2). The larger of the two transcripts is abundant in adult females and in 0-2 hr embryos suggesting that the larger *Smox* transcript is provided maternally. To determine the spatial expression pattern of *Smox,* we hybridized embryos with both sense and antisense *Smox* RNA probes. We detected global expression throughout embryogenesis with the antisense probe but not with the negative control sense probe, suggesting that *Smox* is expressed to equivalent levels in all cells of the embryo (data not shown).

As a first step toward identifying mutations in *Madr-7D,* we obtained deficiencies that spanned cytological region 7D. These deficiencies were used to map *Smox* relative to complementation groups in the region by hybridizing a *Smox* cDNA to polytene chromosomes from salivary glands of larvae heterozygous for each deficiency (Fig. 3A-F). *Df(1)hl-a* deleted *Smox* (Fig. 3B,C), whereas *Df(1)Desi-3, Df(1)C128,* and *Df(1)GE202* did not (Fig. 3D-F). We consistently detected a diminished signal on the *Df(1)RA2* chromosome relative to the signal on the wild-type chromosome *in trans* (Fig. 3C). The mapping of *Smox* relative to these deficiencies places the *Smox* gene at the proximal breakpoint of *Df(1)RA2* in a genomic interval containing only a single known gene, *l(1)7Dg,* of which there is one reported allele, VA334 (Fig. 3G) [40]. However, VA334 complemented *Df(1)RA2,* suggesting that the existing VA334 chromosome contains a functional allele of *l(1)7Dg.*

DISCUSSION

In this work, we have discovered a second R-SMAD in *Drosophila, Smox.* SMOX is most similar to SMADs 2 and 3 whereas MAD is most similar to SMADs 1 and 5 (Table 1). In vertebrates, SMADs 2 and 3 function in signaling pathways distinct from the pathways in which SMADs 1 and 5 function, although several components from each pathway are shared. Based on its homology to the vertebrate proteins, SMOX may func-

tion in a related TGF-β signaling pathway, termed the "activin" pathway. A Drosophila activin β_B-like ligand has been described [41] and two Drosophila activin-like type I receptors have been identified, ATR-I and SAX [4, 7, 33, 42]. However, many of the phenotypes described for mutations in SAX suggest a role for this receptor in the DPP (BMP) signaling pathway, so it is not always obvious from sequence comparisons which components function in which pathway. Table 2 compares the proposed activin- and BMP- signaling pathways in vertebrates and Drosophila (modified from [17]). "?"s indicate that the protein has been proposed to act within a particular biochemical pathway but the function of that pathway and the specific involvement of a given protein has not been established genetically. Mutations in neither the Drosophila activin β_B-like ligand nor ATR-I receptor have been described. Thus, the function of the Drosophila activin pathway, in which SMOX may participate, remains largely unknown.

ACKNOWLEDGMENTS

We thank D. Barrick and P. Bradley for their critical comments on this manuscript. We are grateful for the fly stocks that were provided by the Indiana and Umea Stock Centers, and A. Christensen. We thank the Berkeley Drosophila Genome Project for their progress and for the ease with which their databases are accessed and desired clones provided. Sequencing was done by the Johns Hopkins University School of Medicine Genetics Core DNA Analysis Facility. We thank C. Machado for providing a developmental Northern and all the members of the Andrew laboratory for their help throughout this work. This work was supported by NIH grant # R01 GM51311.

REFERENCES

1. Kingsley, D. (1994) *Genes and Development* **8,** 133–146.
2. Hogan, B. L. M. (1996) *Genes and Development* **10,** 1580–1594.
3. Derynck, R., and Feng, X.-H. (1997) *Biochim. Biophys. Acta* **1333,** F105–F150.
4. Brummel, T. J., Twombly, V., Marqués, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massagué, J., O'Connor, M. B., and Gelbart, W. M. (1994) *Cell* **78,** 251–261.
5. Nellen, D., Affolter, M., and Basler, K. (1994) *Cell* **78,** 225–237.
6. Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J. L., Attisano, L., Szidonya, J., Cassil, J. A., Massagué, J., and Hoffman, F. M. (1994) *Cell* **78,** 239–250.
7. Xie, T., Finelli, A. L., and Padgett, R. W. (1994) *Science* **263,** 1756–1759.
8. Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffman, F. M., Gelbart, W. M., Massagué, J., and O'Connor, M. B. (1995) *Cell* **80,** 899–908.
9. Raftery, L. A., Twombly, V., Wharton, K., and Gelbart, W. M. (1995) *Genetics* **139,** 241–254.
10. Newfeld, S. J., Chartoff, E. H., Graff, J. M., Melton, D. A., and Gelbart, W. M. (1996) *Development.*
11. Raftery, L. A., and Wisotzkey, R. G. (1997) *Annals of the New York Academy of Sciences,* 318–320.
12. Das, P., Maduzia, L. L., Wang, H., Finelli, A. L., Cho, S.-H., Smith, M. M., and Padgett, R. W. (1998) *Development* **125,** 1519–1528.
13. Hudson, J. B., Podos, S. D., Keith, K., Simpson, S. L., and Ferguson, E. L. (1998) *Development* **125,** 1407–1420.
14. Wisotzkey, R. G., Mehra, A., Sutherland, D. J., Dobens, L. L., Liu, X., Dohrmann, C., Attisano, L., and Raftery, L. A. (1998) *Development* **125,** 1433–1445.
15. Kim, J., Johnson, K., Chen, H. J., Carroll, S., and Laughon, A. (1997) *Nature* **388,** 304–308.
16. Shi, Y., Hata, A., Lo, R. S., Massagué, J., and Pavletich, N. (1997) *Nature* **388,** 87–93.
17. Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) *Nature* **390,** 465–471.
18. Whitman, M. (1998) *Genes and Development* **12,** 2445–2462.
19. Pardue, M.-L. (1994) *in* Drosophila melanogaster: Practical Uses in Cell and Molecular Biology (Goldstein, L. S. B., and Fyrberg, E. A., Eds.), Vol. 44, pp. 755, Academic Press, San Diego.
20. Lehmann, R., and Tautz, D. (1994) *in* Drosophila melanogaster: Practical Uses in Cell and Molecular Biology (Goldstein, L. S. B., and Fyrberg, E. A., Eds.), Vol. 44, pp. 755, Academic Press, San Diego.
21. O'Connell, P., and Roshbash, M. (1984) *Nucleic Acids Research* **12,** 5495–5513.
22. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning A Laboratory Manual, 2nd ed., Vol. 1,2,3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Higgins, D. G. (1993) ClustalV: Multiple Alignment of DNA and Protein Sequence, Human Press, Totowa, N. J.
24. Massague, J., Hata, A., and Liu, F. (1997) *Trends in Cell Biology* **7,** 187–192.
25. Hahn, S. A., Schutte, M., Hoque, A. T. M. S., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996) *Science* **271,** 350–353.
26. Lagna, G., Hata, A., Hemmati-Brivnlou, A., and Massagué, J. (1996) *Nature* **383,** 832–836.
27. De Caestecker, M. P., Hemmati, P., Larischbloch, S., Ajmera, R., Roberts, A. B., and Lechleider, R. J. (1997) *J. Biol. Chem.* **272,** 13690–13696.
28. Hata, A., Lo, R. S., Wotton, D., Lagna, G., and Massagué, J. (1997) *Nature* **388,** 82–87.
29. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J.-i., Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) *EMBO J* **16,** 5353–5362.
30. Zhang, Y., Musci, T., and Derynck, R. (1997) *Current Biology* **7,** 270–276.
31. Nakao, A., Afrakhte, M., Morén, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., and ten Dijke, P. (1997) *Nature* **389,** 631–635.
32. Savage, C., Das, P., Finelli, A. L., Townsend, S. R., Sun, C.-Y., Baird, S. E., and Padgett, R. W. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 790–794.
33. Childs, S. R., Wrana, J. L., Arora, K., Attisano, L., O'Connor, M. B., and Massagué, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9475–9479.
34. Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H., and Gelbart, W. M. (1995) *Genetics* **139,** 1347–1358.
35. Newfeld, S. J., Mehra, A., Singer, M. A., Wrana, J. L., Attisano, L., and Gelbart, W. M. (1997) *Development* **124,** 3167–3176.
36. Macias-Silva, M., Abdollah, S., Hoodless, P., Pirone, R., Attisano, L., and Wrana, J. L. (1996) *Cell* **87,** 1215–1224.

37. Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) *J. Biol. Chem.* **272,** 27678–27685.

38. Kretzschmar, M., Liu, F., Hata, A., Doody, J., and Massague, J. (1997) *Genes and Development* **11,** 984–995.

39. Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin, C. H. (1997) *J. Biol. Chem.* **272,** 28107–28115.

40. Lindsley, D. L., and Zimm, G. G. (1992) The Genome of *Drosophila melanogaster,* Academic Press, San Diego.

41. Kutty, G., Kutty, R. K., Samuel, W., Duncan, T., Jaworski, C., and Wiggert, B. (1998) *Biochem. Biophys. Res. Comm.* **246,** 644–649.

42. Wrana, J. L., Tran, H., Attisano, L., Arora, K., Childs, S. R., Massagué, J., and O'Connor, M. B. (1994) *Molec. Cell. Biology* **14,** 944–950.