erty or determinant of myeloid cells or of the neutrophil lineage. Moreover, whereas the bHLH proteins active in muscle cells and B cells are thought to both stimulate differentiation and to transcriptionally define a cellular phenotype, the bHLH proteins of myeloid cells may serve only to promote a specific, transient phase of differentiation.

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20. We thank F. Rauscher III, R. Wilson, and H. Weintraub for comments on the manuscript. Supported by grants CA 10815, CA 21124, and CA 25875 from the National Cancer Institute (NCI) (to G.R.), by a grant from the National Institutes of Health (to H. Weintraub), and by funds from the Howard Hughes Medical Institute (to T.K.). B.L.K. was supported by training grant CA 09171 from the NCI.

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Cloning of a Second Type of Activin Receptor and Functional Characterization in *Xenopus* Embryos

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A complementary DNA coding for a second type of activin receptor (ActRIIB) has been cloned from *Xenopus laevis* that fulfills the structural criteria of a transmembrane protein serine kinase. Ectodermal explants from embryos injected with activin receptor RNA show increased sensitivity to activin, as measured by the induction of muscle actin RNA. In addition, injected embryos display developmental defects characterized by inappropriate formation of dorsal mesodermal tissue. These results demonstrate that this receptor is involved in signal transduction and are consistent with the proposed role of activin in the induction and patterning of mesoderm in *Xenopus* embryos.

POLYPEPTIDE GROWTH FACTORS AND their receptors are believed to mediate the induction and patterning of mesodermal tissues in early *Xenopus* embryos. The inducing activities of these factors have been characterized in part by their effects on animal cap tissue from blastula embryos. Untreated caps form ectodermal tissues such as skin, whereas caps treated with different members of the fibroblast growth factor (FGF) and transforming growth factor (TGF)-β families form a variety of mesodermal tissues (*1*). Among the members of the TGF-β family, the activins are particularly potent in this assay, inducing a full complement of mesodermal tissues in a concentration-dependent manner (*2, 3*). The inducing activity of activin has also been measured by injecting activin RNA into embryos that have been rendered axis-deficient by ultraviolet (UV) irradiation. Activin RNA induces only partial, posterior structures in these embryos, whereas RNA for *Xwnt-8*, a member of the *wnt* oncogene family, induces a complete dorsal axis (*4*). Although the precise role of activin in embryos is not known, these results suggest that activin acts downstream from the creation of Spemann's organizer to induce and pattern specific mesodermal derivatives. One way to determine the role of activin in embryos is to study the receptors for these molecules, as has been done for FGF (*5*). Several high-affinity activin-binding proteins have been identified in mammalian cells, and we have recently cloned an activin receptor (ActR) from a mouse corticotroph cell line, AtT20, which is predicted to be a transmembrane protein serine kinase (*6*). To determine if a similar receptor mediates mesodermal induction in early embryos, we cloned a *Xenopus laevis* activin receptor. Introduction of this second type of ActR into early embryos causes

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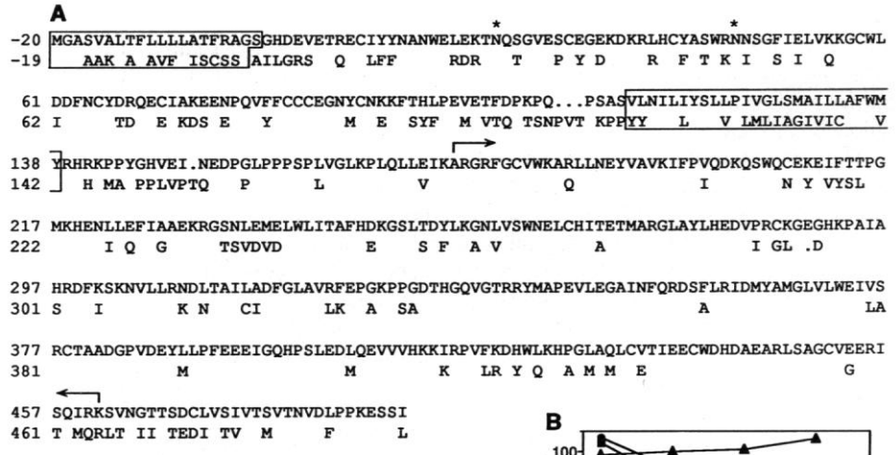
anomalous mesodermal development in a manner consistent with a function for activin in mesodermal induction and patterning.

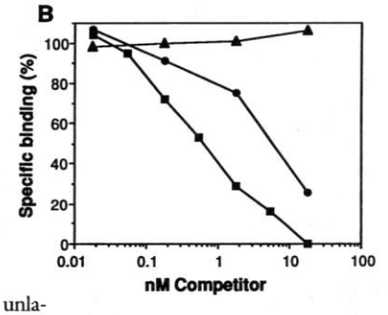
To clone a cDNA encoding a *Xenopus* ActR, a cDNA library from stage 17 (early neurulae) embryos (*7*) was screened by low stringency hybridization with the coding sequence of the mouse activin receptor as a probe (*6, 8*). The 4.5-kb insert of one positive clone was sequenced and found to code for a protein of 510 amino acids (Fig. 1A). This protein, like the mouse ActR, appears to be a transmembrane protein serine-threonine kinase. Nucleotide sequences of the two receptors are 60% identical; amino acid sequences are 69% identical. The intracellular kinase domains were significantly more conserved (77%) than the extracellular ligand-binding domains (58%). The positions of all the cysteine residues and potential sites of N-linked glycosylation in the extracellular domain are conserved between the two receptors, as are all the kinase subdomains and the unique kinase domain inserts in the intracellular domain. To be consistent with the nomenclature for TGF-β receptors (*9*), we now designate this class of activin receptor ActRII, because the receptors represent the type II, higher molecular weight, affinity-labeled complex. Because the sequence of this *Xenopus* (x)ActRII is more similar to a second ActRII that has been cloned from chicken and mouse cells (*10*), this receptor is designated xActRIIB.

To verify that xActRIIB codes for an activin receptor, we measured binding of iodinated activin A on COS cells transfected (*6*) with xActRIIB. Competition binding experiments (*6*) revealed that xActRIIB bound activin A with a dissociation constant (K_d) of 640 pM; this binding could be competed at lower affinity with inhibin A but could not be competed with TGF-β1 (Fig. 1B). Chemical cross-linking of iodinated activin A to transfected COS cells generated a single affinity-labeled complex of 88 kD. Formation of this complex could be completely inhibited by activin A and partially inhibited by inhibin A (Fig. 1C), consistent with the results of competition binding. These binding characteristics are similar to those of mActRII, although it is possible that the type II and type IIB receptors may have different binding specificities for other related ligands.

Previous studies have shown that activins are likely to be expressed in early embryos during mesodermal induction; RNA encoding the *Xenopus* activins B and A can be first detected in stage 9 and stage 13 embryos, respectively (*3*), and activin-like activity has been found in unfertilized eggs and blastulae (*11*). RNA encoding xActRIIB was detected by ribonuclease protection in fertilized eggs and in all embryonic stages through tadpole stages; furthermore, measurement of RNA amounts from different regions of the embryo suggested that xActRIIB expression is uniform throughout the embryo (*12*). Thus xActRIIB is likely to be expressed during stages when mesodermal induction occurs.

To determine whether xActRIIB can transmit a signal in response to activin, we synthesized xActRIIB RNA in vitro and injected *Xenopus* embryos at two different concentrations. Injected embryos were allowed to develop to stage 9, at which time animal caps were dissected and treated overnight with different concentrations of activin (*13*). The response of the caps to activin was assessed by quantifying muscle-specific actin RNA with a ribonuclease protection assay (*7*). Embryos injected with 0.4





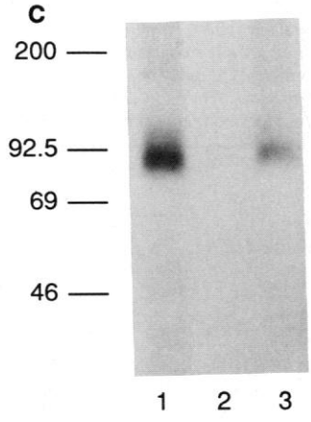


Fig. 1. Sequence and binding characteristics of *Xenopus* activin receptor. (**A**) *Xenopus* ActRIIB (top) and mouse ActRII (bottom) (*6*) protein sequences; only those residues that differ are shown in the mouse sequence. The predicted signal sequence and transmembrane domain are boxed, and potential sites of N-linked glycosylation are marked with asterisks. The kinase domain is indicated by arrows. Dots indicate gaps introduced to align the sequences. (**B**) Competition binding of iodinated activin A to COS cells transfected with xActRIIB. Binding was performed on COS cell monolayers and competed with unlabeled activin A (■), inhibin A (●), or TGF-β1 (▲). Data are expressed as percent specific binding, where binding was normalized to the specific binding in the absence of competitor (5.4% of input cpm). Nonspecific binding was 0.9% of input cpm. Binding parameters were determined with the use of the program LIGAND (*20*). (**C**) Chemical cross-linking of iodinated activin A to COS cells transfected with xActRIIB. Cells were incubated with iodinated activin in the absence of competitor (lane 1), or in the presence of 36 nM activin A (lane 2) or 36 nM inhibin A (lane 3). After binding, the cells were cross-linked with disuccinimidyl suberate, and the fraction soluble in Triton X-100 was analyzed by denaturing polyacrylamide gel electrophoresis in the presence of 2 mM dithiothreitol (DTT). The sizes of molecular weight markers (kilodaltons) are indicated. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

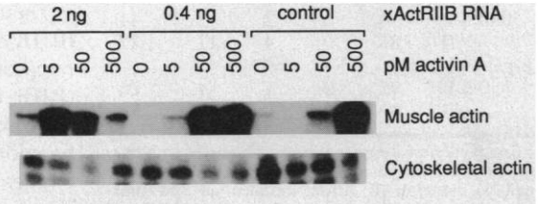


Fig. 2. Activin A induction in animal cap tissue injected with xActRIIB RNA. Stage 9 animal caps from uninjected embryos (control), or from embryos injected with different concentrations of xActRIIB RNA, were treated with different concentrations of activin A (0, 5, 50, and 500 pM) and assayed for muscle-specific actin RNA (top panel) and for cytoskeletal actin (bottom panel), which hybridizes to the same probe and serves as a control for RNA recovery. Variation in the amount of cytoskeletal actin RNA is likely because the probe used in this assay was not in excess.

and 2.0 ng of xActRIIB RNA were approximately 10- and 100-fold more sensitive, respectively, to activin than control embryos (Fig. 2). The low amount of muscle actin found in animal caps in the absence of added activin A is probably a consequence of contamination of the animal cap with a small amount of marginal zone tissue.

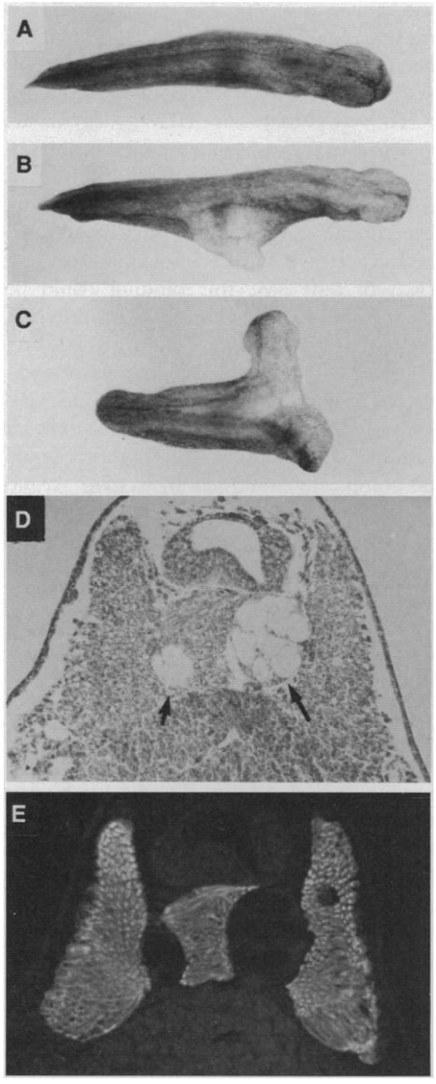


Fig. 3. Developmental defects in embryos injected with xActRIIB RNA. Embryos injected with a control RNA (**A**) or with xActRIIB RNA (**B** and **C**) were photographed at stage 29/30 from the dorsal side. Anterior is to the right. Embryo in (B) was injected with 1.0 ng of xActRIIB RNA and has a secondary trunk-like protrusion; embryo in (C) was injected with 0.2 ng of xActRIIB RNA and is double-headed. (**D** and **E**) Adjacent transverse sections through an embryo (stage 30) injected with 1 ng of xActRIIB RNA were either stained with hemotoxylin-eosin and photographed under bright field (D) or stained by immunofluorescence with a muscle-specific antibody, 12/101 (*21*), and photographed under epifluorescent illumination (E). This embryo has a split dorsal axis as marked by two notochords (arrows) surrounded by somitic mesoderm.

The amount of muscle actin decreased with increasing concentrations of activin in the embryos injected with 2 ng of xActRIIB RNA. This is consistent with the observation that isolated animal cap cells uniformly exposed to different concentrations of activin only form muscle cells in response to a narrow range of activin concentrations (*14*). Our results (Fig. 2) indicate that the concentration of ligand and the amount of receptor are both important in determining the signal transmitted. Thus, the range of activin concentrations that leads to muscle differentiation is lower in animal cap cells from injected embryos, which are expressing more receptor than normal, than from uninjected embryos.

The precise role of activin signaling in the formation of the embryonic body axis has not been established (*4, 14, 15*). Increasing the amount of receptor in early embryos should change the activin response in regions of the embryo where activin signaling normally occurs. Because the activin receptor is most likely to play a role in the formation of mesoderm, xActRIIB RNA was injected bilaterally into two-cell embryos at the boundary between the animal and vegetal pole, to get greater than normal amounts of receptor in the marginal zone. Injected embryos were left to develop to tadpole stages (stage 28), fixed, and examined externally and histologically for developmental defects.

Embryos injected with xActRIIB RNA that developed abnormally were divided into several categories based on characteristic defects (Table 1). Both the percentage of embryos displaying these defects and the type of defect were dependent on the amount of xActRIIB RNA injected. In embryos injected with the highest levels of RNA, the most common defect was a failure to close the blastopore during gastrulation, which resulted in spina bifida. Although spina bifida occurs in response to some toxic treatments, and in varying frequency when embryos are injected with a variety of RNAs (*16*), embryos injected with large amounts of activin RNA also display a high frequency of spina bifida (*4*). Furthermore, closure of the blastopore is mediated by the movements of a specific population of dorsal mesodermal cells, and those movements can be induced in animal cap cells by activin (*17*). Thus, spina bifida observed in embryos injected with large amounts of xActRIIB RNA may reflect an alteration in the induction or patterning of this population of dorsal mesodermal cells.

Table 1. Defects produced in embryos injected with xActRIIB RNA. The xActRIIB and β-galactosidase (β-gal) RNA were injected twice into the marginal zone of two-cell stage embryos, and the embryos were allowed to develop to stage 28. The results from two independent experiments are shown.

RNA injected (ng)		Embryos with phenotype (%)				Total (*n*)
		Normal*	Morphological defect†		Spina bifida‡	
			A	P		
xActRIIB						
1.0	I	19	0	35	46	43
	II	21	0	13	65	97
0.02	I	65	5	21	8	37
	II	60	4	22	14	93
0.004	I	96	0	2	2	44
	II	85	0	4	11	27
β-gal						
0.5	I	92	0	4	4	26
	II	92	0	0	8	50

*Based on external appearance. †Embryos with extra dorsal structures on the lateral or ventral sides of the embryo. Posterior (P) defects included tail- and trunk-like protrusions. Anterior (A) defects had duplicated head structures. The presence of extra dorsal tissue was confirmed by histology. ‡Embryos in which the posterior end of the embryo was bifurcated.

In embryos injected with intermediate amounts of xActRIIB RNA, the frequency of spina bifida dropped and abnormal embryos most frequently showed a defect consisting of inappropriate dorsal tissues in trunk regions of the embryos (Fig. 3, A and B). These defects were similar to those observed in embryos injected with RNA encoding activin A or B (*3, 4*). Histological analysis revealed a variety of defects in the formation of dorsal mesodermal tissues, including splitting of the notochord, fusion of somitic mesoderm across the midline, and an extension of somitic mesoderm into ventral regions of the embryo (Fig. 3, D and E). In a few cases, there was sufficient additional dorsal tissue to be classified as a duplication of the dorsal axis, although it should be emphasized that these secondary axes were relatively disorganized. Because activin alone cannot generate an axis in UV-irradiated embryos (*4*), the defects observed in embryos injected with xActRIIB RNA are likely the result of inappropriate development of mesodermal tissue along a predetermined axis. Finally, a rare group of embryos formed duplicated anterior structures, consisting in most cases of two heads (Table 1 and Fig. 3C).

These results demonstrate that the xActRIIB cDNA encodes a new type of activin receptor. Because overexpression of xActRIIB in *Xenopus* embryos renders them more sensitive to activin, this receptor can functionally mediate at least one of the biological activities associated with activin signaling. Moreover, increasing the concentration of receptor in embryos results in developmental defects primarily in the for-

mation of dorsal mesodermal derivatives in posterior regions of the embryo. The defects observed in these receptor studies are remarkably similar to the defects that have been observed when activin is expressed in embryos by RNA injection. Activin is thus further implicated in the later steps of the multistep process by which specific mesodermal tissues are induced and patterned in the vertebrate embryo.

Note added in proof: Since the submission of this work, the xActRII (*18*) and mActRIIB (*19*) sequences have been reported; injection of xActRII RNA into *Xenopus* embryos was also found to affect development.

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An Amino Acid Mutation in a Potassium Channel That Prevents Inhibition by Protein Kinase C

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A slowly activating, voltage-dependent potassium channel protein cloned from rat kidney was expressed in *Xenopus* oocytes. Two activators of protein kinase C, 1-oleoyl-2-acetyl-*rac*-glycerol and phorbol 12,13-didecanoate, inhibited the current. This inhibition was blocked by the kinase inhibitor staurosporine. Inhibition of the current was not seen in channels in which Ser^{103} was replaced by Ala, although other properties of the current were unchanged. These results indicate that inhibition of the potassium current results from direct phosphorylation of the channel subunit protein at Ser^{103}.

MODULATION OF ION CONDUCTANCE by transmitters and second messengers is important in the regulation of cell excitability (*1*). K^+ currents in several types of cells are affected by agents that change the activity of protein kinase A (PKA) and protein kinase C (PKC) (*2*). In the case of a delayed-rectifier K^+ channel (*3*) and a Ca^{2+}-activated, voltage-dependent K^+ channel (*4*), phosphorylation by PKA results in increased activity of the channel when the channel is reconstituted into lipid bilayers.

A cDNA was cloned from rat kidney, and RNA transcripts from this cDNA injected into *Xenopus* oocytes directed expression of a K^+ current, I_{sK} (*5*). This current activates slowly when the membrane is depolarized and does not reach a steady state even after several minutes (*5*). Nucleotide sequences encoding this same protein have been isolated from heart (*6*) and uterus (*6, 7*) cDNAs, and from human genomic DNA (*8*). The predicted protein has 130 amino acids (129 in human) and, apparently, a single transmembrane domain (*5, 9*) (Fig. 1). Phorbol esters that activate PKC also inhibit I_{sK} in *Xenopus* oocytes (*10*). We now show by site-directed mutagenesis that this inhibition of current probably results from phosphorylation of a single serine residue.

Depolarization of *Xenopus* oocytes injected with in vitro–transcribed RNA encoding the putative K^+ channel (*11*) evoked a slowly developing outward current that reached steady state only after 50 s (Fig. 2). Phorbol 12,13-didecanoate (PDD) (50 nM, applied for 5 min) inhibited this current (Fig. 2) maximally within 10 min. The effect continued for at least 45 min. This inhibition was much more marked at negative potentials; the current measured at the end of a 20-s depolarization was inhibited by 88.8 ± 5.1% at −40 mV but only by 18.1 ± 3.7% at +40 mV (n = 6) (Fig. 2). The rate at which the current developed during the depolarizing pulse was not changed by treatment with PDD (Fig. 2).

The partially activated current could be evoked repeatedly with 5-s depolarizing pulses to −10 mV (Fig. 2). Under this condition, inhibition by PDD reached a maximum after 10 min (Table 1). In contrast, a similar application of the enantiomer 4α-phorbol 12,13-didecanoate, which does not activate PKC, had no effect on I_{sK} (n = 3). Staurosporine (3 μM) prevented the action of PDD (control current = 370 ± 41 nA; in PDD, current = 395 ± 66 nA, n = 4; staurosporine itself inhibited the current by about 20%). Similar results were obtained with another PKC activator, 1-oleoyl-2-acetyl-*rac*-glycerol (OAG) (Fig. 3A). OAG inhibited the current by 19.6 ± 3.1% (n = 4) and 31.8 ± 3% (n = 10) at concentrations of 1 μM and 10 μM, respectively; in some batches of oocytes the action of OAG reversed quickly (within 10 min) when the application was discontinued (n = 11), but in others it did not reverse even after the oocytes were washed for 30 to 45 min (n = 14). In

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