A GENETIC TEST OF THE ROLE OF ACTIVINS IN MOUSE MESODERM FORMATION

by

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ABSTRACT

Vertebrate embryos acquire their basic body plan through a series of inductions, the first of which is mesoderm formation. In *Xenopus* embryo explants, certain polypeptide growth factors are able to induce many morphological and molecular changes that normally accompany mesoderm formation. Among the most potent inducers are the activin family of TGFβ–like factors. In exposed explants, cells normally fated to form ectoderm differentiate into mesoderm, as muscle and notochord, and undergo characteristic morphogenetic movements. Activins are expressed in many early vertebrate embryos. Conclusive demonstration of activin involvement in gastrulation however requires ablation of the molecule in the developing animal. Genetic analysis of development is not possible in *Xenopus.* In contrast, the technology of targeted mutagenesis in mice allows to generate animals lacking activins. Activins are β:β hetero or homodimers of βA and βB subunits, that also heterodimerize with an α subunit to form inhibins. Targeted disruption of the βB subunit gene leads to generation of mice lacking activins B, AB, and inhibin B. Surprisingly, they were viable, but exhibited a very specific defect in eyelid morphogenesis, and a female reproductive dysfunction. Mutant females were able to give birth to morphologically normal progeny, including homozygous βB mutants. However, many offspring died perinatally, revealing a yet incompletely defined maternal failure. Elaboration of the mouse body plan thus requires neither maternal nor embryonic activin βB. Mutant mice were found to upregulate βA expression. Because of the possible functional overlap between βA and βB, we crossed βB mutant mice with a βA deficient strain. At birth the activin βA mutant mice exhibit a number of craniofacial abnormalities, including lack of incisors and whiskers, and palate malformations. Mutants die postnatally, due to inability to suckle. Upon intercrossing βA/βB compound heterozygotes, compound homozygotes displayed at birth both eyelid defect and lack of whiskers, with palate malformations. The double mutant phenotype was thus no more severe than the sum of the two single mutant phenotypes. Therefore there is no functional overlap between βA and βB during mouse embryogenesis. The two factors mediate independent, pleiotropic, and highly specific functions in development. Although the role played by maternal βA and βB needs further investigation, we conclude that murine mesoderm and axis formation can occur in the absence of zygotic activins, in contrast to what had been speculated from amphibian studies.

Anne Vassalli
Thesis Supervisor: Rudolf Jaenisch, Professor, MIT

Whatever happens. Whatever
What is _is_ is what
I want. Only that. But that.

Galway Kinnel

"A la fin, la verite est la seule chose qui vaille d'etre possedee: elle est plus emouvante que l'amour, plus joyeuse, plus passionnee. Elle ne peut pas vous trahir. Rien ne tient, qu'elle. Moi, en tout cas, je lui donne ce qui me reste a vivre et seulement a elle."

Katherine Mansfield Journal 17 dec. 1919
(original english version out of press)

'I'm talking about this town,' F. Jasmine said in a higher voice. 'There are all these people here I don't even know by sight or name. And we pass alongside each other and don't have any connexion. And they don't know me and I don't know them. And now I'm leaving town and there are all these people I will never know.'

'But who do you want to know ?' asked Berenice.

F. Jasmine answered : ' Everybody . In the world . Everybody in the world. '

Carson Mac Cullers

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PART 1:

A GENETIC TEST OF THE ROLE OF ACTIVINS IN MOUSE MESODERM FORMATION

PART 1 : SUMMARY

Vertebrate embryos acquire their basic body plan through a series of inductive events, the first of which is mesoderm formation. The past seven years have witnessed major advances in this field. This is in great part due to work in amphibians, and the discovery that certain polypeptide growth factors were able to induce in embryonic explants many of the same morphological and molecular changes that, in intact embryos, accompany mesoderm formation. This provided an entry for the identification of gene products that participate in specification of the body plan. Candidate effectors were in turn analyzed for their presence and effects in other vertebrates. Many of the molecules suggested to be involved in these processes were found to be conserved among vertebrates in their expression pattern, and in some cases their activities. Thus, the consensus thought has been that early patterning mechanisms were shared among vertebrates. The amphibian embryo assays, such as the animal cap assay, and RNA microinjections have been extremely powerful in the identification of novel candidate genes, and have provided functional assays for the role of specific extracellular signals by the use of dominant-negative receptors. Conclusive demonstration of the involvement of a molecule in a process requires however ablation of the molecule in the developing animal. Genetical analysis of development is not possible in *Xenopus laevis*. In contrast, the existing technology of targeted mutagenesis in mice offers the means to test the involvement of any factor whose gene is available.

Among the most potent mesoderm inducers in the amphibian animal cap assay, are the activin family of TGFβ–like factors. In animal cap cells, activins induce immediate-early genes, as *Brachyury* and *goosecoid* and other homeobox and fork-head genes believed to participate in early morphogenesis. Cells differentiate into mesodermal types, such as muscle and notochord, rather than following their natural ectodermal fate. At the macroscopic level, animal caps exposed to activin 'elongate', as they undergo morphogenetic cell movements that resemble the ones that occur during gastrulation. Therefore activins appear to trigger responses that recapitulate many of the events of gastrulation. In support of their role in these processes is also the fact that activins are expressed in early fish, frog, chicken and mouse embryos.

To test the involvement of activins in early development, we have used gene targeting to generate mice lacking these factors. Activins are β:β hetero- or homodimers of two subunits, βA and βB, that also dimerize with an α subunit to form inhibins. Targeted disruption of the βB subunit gene of activin / inhibin leads to generation of mice lacking activins B, AB, as well as inhibin B. These mice were found to be viable. They exhibit a very specific defect in eyelid morphogenesis and a female reproductive dysfunction. Female mutants are able to give birth to morphologically normal progeny, including homozygous activin B mutants, but the offspring most often die

perinatally due to a yet incompletely defined maternal failure. This demonstrated that both maternal and embryonic expression of activin B is dispensable for mesoderm formation in the mouse, although maternal βB is important for survival of neonates. Because of the possible functional overlap of the related activin βA with activin βB, we have crossed the βB mutant mice with a strain of mice in which the exon encoding the activin / inhibin βA mature peptide was deleted by homologous recombination in ES cells. At birth the activin βA mutant mice exhibit a whisker-less phenotype and secondary palate malformations interfering with suckling, and they die postnatally. Upon intercrossing βA / βB compound heterozygotes, we obtained individuals displaying both eyelid defect and lack of whiskers at birth, with palate malformations. These mice were not viable, and represented the βA / βB compound homozygous mutant progeny. Thus, the double mutant phenotype was no more severe than the sum of the two single mutant phenotypes. This indicates that there is no apparent functional overlap between activins βA and βB during mouse embryogenesis. These two factors appear to mediate independent, pleiotropic, and very specific functions at various times of development. Although the role played by maternal activin A needs further investigation, we conclude from this work that murine mesoderm and axis formations can occur in the absence of zygotic activins, in contrast to what had been speculated from the earlier amphibian studies.

PART 1 : INTRODUCTION:

In this section, I attempt to introduce the concepts of embryonic inductions and of morphogens. Mesoderm induction has been thought to involve activin peptides. These peptides are the first vertebrate factors to have been shown to display the remarkable property of evoking dose-dependent cell fates in responding cells, and thus to behave as the postulated 'morphogens'. Focusing on this family of growth factors, I am next describing their wide pleiotropism of effects, their expression, particularly in the embryo, which led to the working hypothesis at the basis of this thesis.

1.1 *Embryonic inductions*

The basic body plan of a vertebrate embryo is established through a series of embryonic inductions. Underlying these events are cell to cell interactions where a cell or a group of cells send a signal to responding cells to alter their fate. The signal ('inducer') may be freely diffusible, or may be limited in its diffusion by attachment to the extracellular matrix or to the cell surface: its direct range of action is thus more or less restricted. In either instance, an inducer may organize the patterning of an entire morphogenetic field. A short range inducer may initiate a cascade of local, sequential inductions that will create a variety of cell types arranged in a specific spatial array. A more diffusible inducer may build a concentration gradient and elicit in an initially homogeneous population of cells responses that are dose-dependent; it therefore constitutes a 'morphogen' on its own. Long range inducers with concentration-dependent responses are specifically referred to as morphogens (Wolpert, 1969).

Tissues that are sources of inducers are called 'organizers' because of their ability, as assayed by ectopic transplantation, of organizing in the host tissue a new pattern that would not otherwise have arisen. Organizer regions in the embryo convey positional information to a cell (Wolpert, 1969) and modify its differentiation. Examples of organizers are:
(a) The "Spemann organizer" was the first organizer to be recognized. Hans Spemann (1938) observed that the dorsal blastoporal lip of the newt early gastrula had unique properties within the embryo. Upon transplantation to the ventral side of a host, this region was able to recruit host tissue, altering its fate and inducing the formation of a full secondary axis, comprising a neural tube, notochord, paraxial mesoderm and gut. Thus, the organizer controls long range organization of pattern and is able to specify intrinsic polarities of axes. The inducer molecule(s) responsible for this effect are still not resolved.

(b) The notochord (dorsal axial mesoderm) is essential in the induction and/or maintenance, and patterning of the neural tissue. Various newly discovered extracellular signals, as noggin, follistatin and hedgehog, have been speculated to be involved in mediating diverse aspects of neural induction and patterning (Echelard et al., 1993; Krauss et al.,1993; Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Roelink et al.,1994).
(c) The Zone of Polarizing Activity (ZPA) mediates limb patterning. A specific group of cells in the posterior margin of the limb bud specifies the pattern of digit formation across the entire limb (Tickle et al., 1975). The signalling molecule hedgehog appears to be a very good candidate mediator of the ZPA (Riddle et al., 1993). Whether hedgehog acts in this process as a morphogen (establishing a concentration gradient that specifies position to the cell) or by involving subsequent inducers is not yet known.

1.2 *Morphogens*

The first gene products to be described as morphogens were not in fact extracellular, secreted proteins but transcription factors. The *bicoid* and *hunchback* gene products control the antero-posterior (a-p) patterning of the early *Drosophila* embryo (Nusslein-Volhard et al., 1987), and nuclear dorsal protein organizes the dorso-ventral (d-v) pattern (Jiang and Levine, 1993, and ref. therein). Each of these proteins is distributed in a gradient in the early syncytial embryo, and different concentrations of nuclear protein induce the transcription of different target genes. The bicoid and hunchback gradients depend on free diffusion of the proteins from localized RNA sources within the syncytial cytoplasm of the early embryo. In contrast, morphogenetic transcription factors that act in cellularized embryos have to rely on other mechanisms to generate a gradient of their distribution. For example, the mRNA for the homeo-domain protein goosecoid was shown to be distributed in a graded fashion in the marginal zone of *Xenopus* embryos, and elicits in cells concentration-dependent responses that recapitulate a d-v axis (Niehrs et al., 1994). Establishment of the goosecoid mRNA gradient presumably reflects the graded activity of an unknown extracellular component which itself must be a morphogen.

In contrast to nuclear factors, extracellular proteins with the demonstrated property of a morphogen as defined above, are only a few and are decribed below. In *Drosophila*, the decapentaplegic and hedgehog secreted factors have both been shown by genetic means to behave as 'true' morphogens, at least in a subset of their pleiotropic activities. In vertebrates, however, the properties of morphogens have been ascribed only to the activin family of growth factors:
(a) The *decapentaplegic* (*dpp*) gene of the Transforming Growth Factor-β (TGFβ) superfamily is one of the zygotic genes essential for the specification of the d-v axis within the presumptive

ectoderm of *Drosophila* embryos. Genetic and RNA injection experiments have very elegantly demonstrated that dpp acts as a morphogen. Increasing doses of dpp specify more dorsal cell types, and different doses of the protein are sufficient to specify at least three distinct cell fates, ventral neurogenic ectoderm, dorsal epidermis, and extraembryonic amnioserosa. Thus, localized dpp RNA injections into mutant embryos that lack any inherent d-v polarity show that dpp can both define embryonic polarity and organize detailed patterning within the ectoderm (Ferguson and Anderson, 1992).

(b) The *hedgehog* (*hh*) gene product, as one of its several activities, specifies the array of cuticular cell types found across the epidermis of each *Drosophila* segment (Heemskerk and DiNardo, 1994). This was demonstrated by expressing in the epidermis increasing amounts of hh protein through the use of a heat-shock driven hh transgene, and showing that augmenting hh levels converts cell identity to a type more proximal to the endogenous hh expressing cells. Therefore, the latter cells act as a source of a long-range patterning signal.

(c) Activins A and B, members, as dpp, of the TGFβ superfamily and similarly displaying pleiotropic activities, behave in *Xenopus* embryo explants as very potent inducers of dorsal mesoderm, such as notochord and muscle (see below). When applied to disaggregated animal pole cells, they are able to elicit cell types of a progressively more antero-dorsal character as peptide concentration rises. Using a variety of histological and molecular probes (see fig.ii), Green et al. (1992) distinguished 5 distinct cell fates induced by increasing doses of activin. These were: epidermis, posterolateral mesoderm, muscle, notochord, and goosecoid-expressing organizer tissue. The responses to activin displayed a very high sensitivity to peptide dose, discriminating differences in concentration of a factor of less than 2, and were separated by sharp thresholds. Thus increasing activin concentration in vitro elicited cell fates found along an entire embryo axial dimension. This led Green et al. (1992) to postulate that an endogenous gradient of activin within the amphibian embryo plays an essential role in specifying the elements of the body plan.

Whereas the activity of dpp as a morphogen has been conclusively demonstrated in vivo in *Drosophila* , activin dose-dependent responses have still to be evidenced in vivo in *Xenopus* whole embryos [*] . However, it is extremely striking that in both *Xenopus* and *Drosophila*, a TGFβ family member evokes a series of dorsal pattern elements with small increases in concentration. The similarity of dose-response relationships in both species suggests that specification of d-v pattern by TGFβ family members may be operating in distant species, and that the biochemical mechanisms involved in establishing and interpreting these small differences in TGFβ peptide concentration within the embryo may also be conserved.

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[*] Recently, Gurdon et al. (1994) have demonstrated graded responses of cells to an activin gradient in embryonic frog tissues.

1.3 *Mesoderm induction*

The first induction to take place in the vertebrate embryo leads to mesoderm formation. Experimental embryologists have studied this process since early in this century. However an understanding of the underlying mechanisms in molecular terms has only very recently started to be possible.

The amphibian animal cap assay, pioneered by Peter Nieuwkoop (1969) and later used in a variety of experimental designs, has been an extraordinarily powerful technique to identify candidate molecules that participate in the process of mesoderm induction and patterning (Fig.i). Originally, Nieuwkoop observed that animal pole explants (presumptive ectoderm), excised from newt blastulae, would on their own differentiate into an atypical ciliated epidermis. However, when apposed to vegetal pole explants, these cells would be diverted from their natural ectodermal fate, and instead differentiate into mesodermal types, including muscle, mesothelium and notochord. This experiment localized the source of the mesoderm inducing signal within the vegetal pole (presumptive endoderm). Later experiments have searched for cell types or proteins (either supplied in soluble form, or from synthetic, injected mRNAs), that would substitute for the vegetal hemisphere in this assay, and convert presumptive ectoderm into mesoderm.

Two families of peptide growth factors have emerged as having the activity of primary inducers in the animal cap assay (for review, see Jessell and Melton, 1992; Kimelman et al., 1992; Sive, 1993). They are:

(i) Fibroblast Growth Factor (FGF) family members, such as acidic and basic FGFs, int-2 and kFGF (FGFs 1, 2, 3 and 4, respectively), tend to induce ventro-posterior types of mesoderm (mesothelium, muscle, and blood), but dorsal mesoderm such as notochord is not induced.

(ii) TGFβ family members have distinct activities. As mentioned above, activins A and B are among the most potent, and tend to induce antero-dorsal mesodermal cell types, including notochord and muscle, and, by secondary induction, neural tissue. Amphibian animal caps treated with activin can differentiate into small 'embryoids', where tissue types are arranged in a rudimentary pattern that resembles natural body axes (Sokol et al., 1990). Activins are discussed further below.

Another member of the TGFβ superfamily, Bone Morphogenetic Protein-4 (BMP-4), also has mesoderm inducing activity on the presumptive ectoderm of the animal cap (Koster et al., 1991; Dale et al., 1992; Jones et al, 1992). However, the mesoderm induced is of a ventral character (mesothelium, red blood-like cells) while dorsal mesoderm (notochord, muscle) induction is repressed. Interestingly, unlike FGFs, BMP-4 has a dominant effect on activin, being able to override the response of animal caps to activin and to ventralize it. In whole embryos, BMP-4 RNA injections inhibit formation of dorsal structures.

In addition to activins and BMP-4, yet another TGFβ superfamily member, the Vg1 peptide, was shown to have potent mesoderm inducing activity (Thomsen et al., 1993). In the *Xenopus* oocyte and unfertilized egg, the mRNA for Vg1 is localized to the vegetal pole, as expected for the natural mesoderm inducer (Weeks and Melton, 1987). Mature Vg1 peptide induces a spectrum of mesoderm which is very similar to the one induced by activins. The physiological function of Vg-1, however, remains unclear, since so far only the precursor polypeptide was detected in embryos and the mature peptide could only be seen when a chimeric BMP1-Vg1 mRNA was introduced into the embryo.

1.4 *The pleiotropism of activins*

Like many cytokines, activins and their relatives inhibins (see below) are pleiotropic factors, and their initial characterization stems from fields other than embryogenesis. They were first recognized for their regulatory role on pituitary Follicle Stimulating Hormone (FSH) release. Activin enhances FSH release from anterior pituitary cells in culture, whereas inhibin decreases it. Activins modulate secretion of other pituitary and hypothalamic peptides (ACTH, GH and oxytocin) as well. Furthermore, activins are potent inducers of erythropoietic differentiation and have been implicated in inhibition of neural differentiation (for review, see Vale et al., 1990; Hemmati-Brivanlou and Melton, 1994).

In adult vertebrate organisms, ovaries and testes are the major sites of expression of the α, βA and βB subunits of activin / inhibin (see 1.5 Structure of activins). In addition to the effects on the pituitary, several intragonadal effects of activins and inhibins have been described. These factors affect in an autocrine or paracrine manner the gonadal somatic cells, and in a paracrine manner the germ cells themselves (reviews by Vale et al., 1990; de Jong et al., 1990; Mather et al., 1992). Effects on proliferation, development of these cells, as well as on androgen biosynthesis by the testicular Leydig cells or by the ovarian thecal cells were described, often with activin and inhibin displaying opposite activities (see Table I for a summary of these effects; from de Jong et al., 1990). The most definitive demonstration of an intragonadal effect of inhibin was provided by disruption of the inhibin α gene by homologous recombination in mouse Embryonic Stem (ES) cells (Matzuk et al., 1992). This study showed that mice lacking inhibin all develop, at a few weeks of age, gonadal tumors of the sex-cord stromal type. Therefore inhibins are essential regulators of growth for gonadal somatic cells. The role played by activins in the emergence of these tumors is unknown, and is being investigated.

The variety of effects ascribed to these factors thus indicates that activins may participate in early morphogenetic events, and are used again in the adult organism to regulate the growth and differentiation of a wide variety of cell types.

1.5 *Structure of activins and their receptors*

Activins are covalently linked β:β homo- or heterodimers of βA and βB subunits (Fig. iii, after W. Vale et al., 1990). The three activin ligands, activin A (βA:βA), activin AB (βA:βB) and activin B (βB:βB) are thought to possess equivalent activities. A third subunit, inhibin α, can associate with a β subunit to form an α:β dimer termed inhibin, and which often displays opposite biological activity to activin (see below). Like other TGFβ family members, the α, βA and βB subunits are initially synthesized as precursors. The pro-regions play an essential role in intracellular assembly of the dimer (Gray and Mason, 1990), and C-terminal proteolytic cleavage yields the disulfide-linked mature peptides which constitute the biologically active ligand.

The mechanisms that underlie the antagonism of action of activins and inhibins are not known. Mesoderm induction constitutes an exception to this effect as, in the animal cap assay, inhibin (supplied as soluble factor) neither antagonizes activin nor has any effect of its own (Asashima et al., 1990; Thomsen et al., 1990). An understanding of the mechanisms of action of these peptides awaits further elucidation of their downstream effectors. Activin receptors belong to a large family of receptors that include other TGFβ superfamily member receptors (for a review, see Kingsley, 1994). Each of these receptors results from the assembly of a type I and a type II receptors, which exist in multiple isoforms. Both type I and type II receptors display a Serine-Threonine kinase motif. The patterns of ligand-receptor specificity in this family appear complex, and in some cases 'cross-talk' between various TGFβ family members and the receptors exists. In a given receptor, the type II partner is involved in specifying ligand identity, and the type I partner is involved in signal transduction (Kingsley, 1994; Wrana et al., 1994). Activins are known to bind to the products of two genes, ActRIIA and ActRIIB, and several activin type I receptors exist. Inhibin receptors are less well characterized, but are expected to belong to the same family.

1.6 *Expression of activins in embryos*

Supporting their role in early morphogenesis, activins are expressed in vertebrate embryos, starting from very early stages (Table II). In *Xenopus*, an activin bioactivity of unknown identity can already be detected in the unfertilized egg and in the blastula embryo (Asashima et al., 1991)*. Zygotic expression is detected in the late blastula for the βB subunit, and in the late gastrula for the βA subunit (Thomsen et al., 1990). Similarly, activin βB expression precedes activin βA expression in both chick and fish early embryos (Mitrani et al., 1990; Wittbrodt and Rosa, 1994).

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* Recently, activin A, AB, and B proteins were demonstrated to be present in early *Xenopus* embryos (stage 1-5) (Fukui et al., 1994).

In the mouse as well, development is initiated in the presence of activins, both maternal and embryonic in origin (Table III). Albano et al. (1993; 1994) have analyzed activin expression in mouse embryos, before and after implantation. They found that activin protein was present during all stages of preimplantation development, whereas inhibin was absent. At the RNA level, both βA and βB subunit RNAs were present as maternal messages in the oocyte, were degraded after fertilization, and reappeared as embryonic RNAs in the morula. The early postimplantation embryo, however, lacked both βA and βB subunit RNAs. At this time, in contrast, the maternal uterine decidual tissue expressed activin subunits to a high level: βA RNA was found from as early as day E5.0 in the decidua surrounding the whole embryo, and βB RNA was located more specifically around the ectoplacental cone (Manova et al., 1992; Albano et al., 1994; Feijen et al., in preparation). Intense decidual expression, in the context of a lack of expression within the embryo at the time of mesoderm formation, led Manova et al. (1992) and others to speculate that the source of activins in mammalian embryos may have shifted from embryonic to maternal.

In later mouse embryos, using RNA in situ analysis, activin subunit RNAs are first detected weakly in the heart at day E8.5, and become more abundant, each in distinct and specific locations, starting at day E10.5 (Albano et al., 1994). Feijen et al. (in preparation) have performed a detailed study of the sites of expression in postimplantation mouse embryos from day E6.5 to day E13.5. They have found that in mid-gestation embryos, βA RNA is found in mesenchyme of the face, body wall, heart, precartilage condensations of the limbs, and in mesenchyme of the digestive, respiratory and genital tracts.
βB RNA shows a distinct expression pattern. It is strongly expressed in selected regions of the brain, in particular in ependymal layers where cells have high mitotic activity, and in the spinal cord. βB RNA is also found in the epithelium of the oesophagus and stomach, and in mesenchymal condensation of the developing eye. Common sites of βA and βB expression are in blood vessels, intervertebral disc anlagen, mesenchymal condensation of the flank region, and in the gonad primordium. As the α subunit mRNA is only found in the gonad primordium, inhibin production is thought to be restricted to that tissue.

1.7 *Working hypothesis and strategy*

A genetic dissection of mesoderm formation in the mouse requires the availability of mutants in the endogenous inducer genes, and the elucidation of their interactions with other genes and mutants on this pathway.

Two genes involved specifically in mesoderm formation were identified by the virtue of the existence of a mutation in the locus:

- *Brachyury* (T) homozygous mutant embryos show abnormal mesoderm formation and migrative properties, and an absent or deficient notochord. The T gene product is a nuclear, DNA-binding protein, that presumably acts as a transcription factor (Kispert and Herrmann, 1993).
- *nodal* (413.d) mutant embryos have a defect in the establishment or maintenance of the primitive streak, do not form a mesoderm germ layer and lack a-p polarity. The gene product was identified (Zhou et al., 1993) as a TGFβ-like peptide, related to the BMP family. The *in vitro* activity of the nodal protein has not been described yet but the mutant phenotype suggests that it is a central player in mesoderm formation.

In addition, several gene targeting experiments have identified genes involved in the elaboration of mesoderm in the mouse. For instance, the *Wnt-3a* gene, which encodes a putative intercellular signaling molecule, was shown to be involved in the specification of somite fate, and essential in establishment of the tailbud or its maintenance (Takada et al., 1994). The tailbud is the site of emergence of new mesoderm after day E9.5. As a consequence, mutant embryos lack caudal somites and their body is truncated posterior to the forelimb. These mutations provide genetic and molecular tools for an analysis of gene interactions in early development.

As discussed above, activins (and especially activin B) appear to be good candidate embryonic morphogens in light of:

1) their *in vitro* activities as mesoderm inducers in amphibians, and in particular the ability to evoke dose-dependent, stepwise transitions in cell fates that mimic cell fates along a body axis,

2) their ability to induce a series of immediate-early response genes, which themselves are thought to play important roles in early patterning, as *T (Brachyury), goosecoid*, and several *fork head* genes (Fig. ii); and

(3) the observation that they are expressed in early embryos of a variety of vertebrates (fish, *Xenopus*, chick, and mouse).

I have cloned the mouse genes for the two subunits of activins (βA and βB), in the goal of generating mouse lines deficient in these factors. Because the βB subunit gene is expressed earlier in frog and chicken embryos, the βB mutant was generated initially. Following is a description of the generation and analysis of the βB deficient mice (Vassalli et al., 1994). The intercross of these mice with βA deficient mice to generate mice lacking all activins and inhibins is then described (Matzuk et al., in press).

Mesoderm induction

Amphibian animal cap assay:
presumptive ectoderm ---> mesoderm

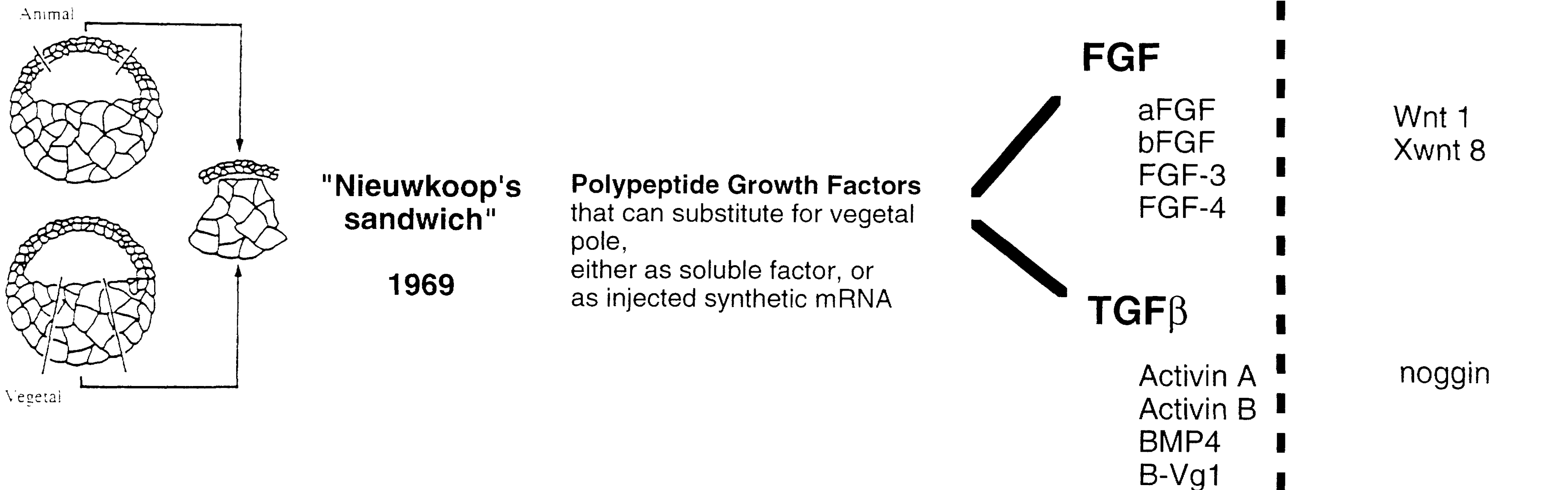

Figure i.

Figure ii.

Activin Immediate-early Response Genes:

Genes	Family
Mix-1 Goosecoid Xnot Xlim-1	homeobox
Brachyury	
XFKH1 XFD-1 Pintallavis Axial	fork head

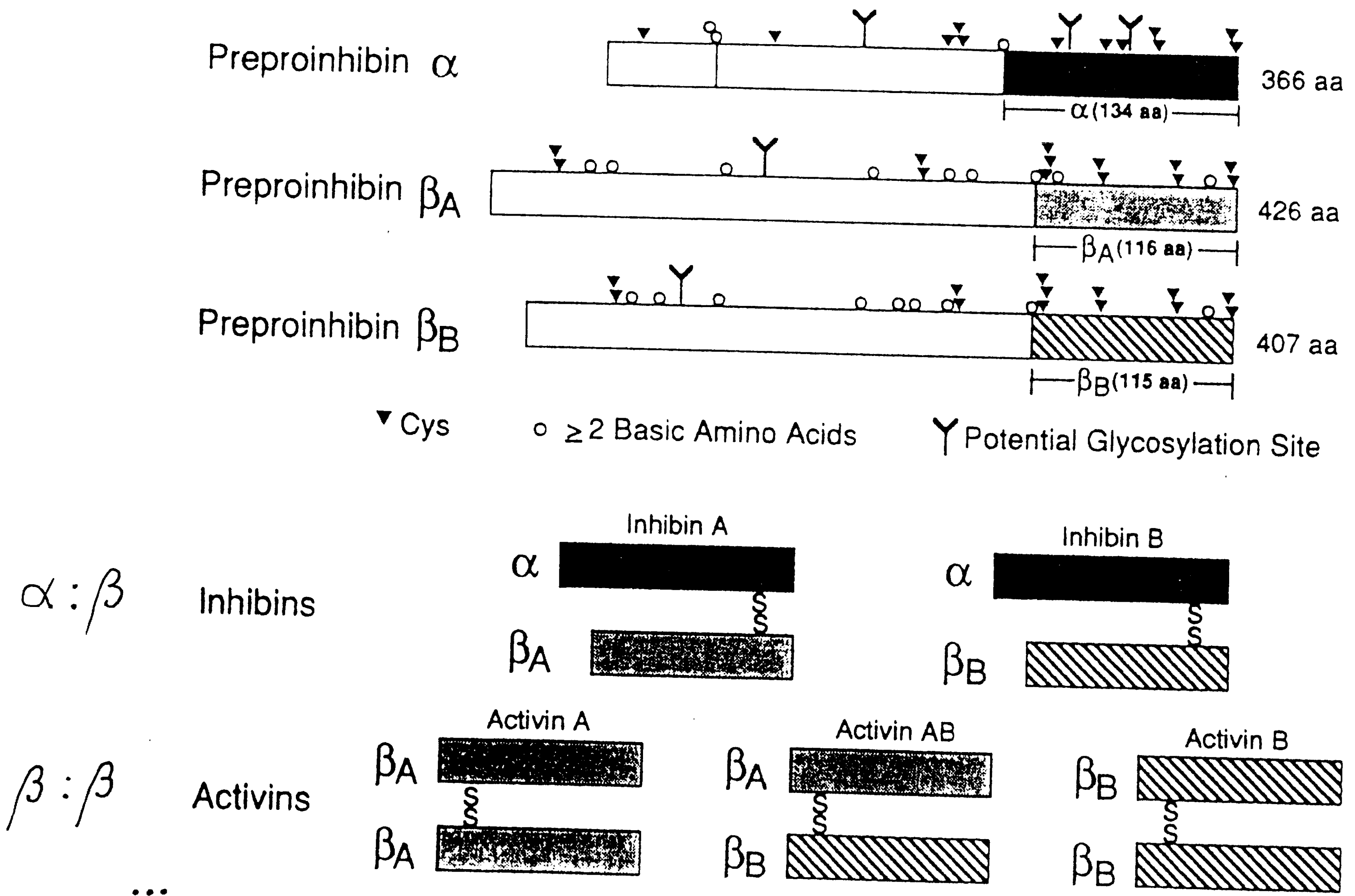

Fig. iii. Schematic of the precursors of the human inhibin α-, βA-, and βB-subunits. Mature portions of each subunit as they appear in the putative $M_r \sim 32\,000$ inhibin A (αβA) and inhibin B (αβB) are *shaded*

TABLE I. EFFECTS OF INHIBIN AND ACTIVIN ON NON-PITUITARY CELL SYSTEMS (from de Jong et al., 1990).

Organ *Parameter*	---------Effects of-------- Inhibin	 Activin
Hypothalamus		
Oxytocin secretion	n.d.	+
Testis		
Testosterone production	+	-
	=	n.d.
Spermatogonia	-	n.d.
Ovary		
Granulosa cells		
LH-receptor	n.d.	+
FSH-receptor	n.d.	+
Estradiol	-	+
	=	+
Progesterone	=	-
	n.d.	+
Inhibin	n.d.	+
Oocytes		
Meiotic division	-	=
Placenta		
GnRH; HCG	-	+
Hematopoietic cells		
Colony formation	-	+
	-	+
Cell differentiation	-	+
	n.d.	+
	n.d.	+
Cell proliferation	+	-
Fibroblasts		
Proliferation	n.d.	+
Pancreas, liver		
Insulin secretion	n.d.	+
Glycogenolysis	n.d.	+

+, stimulatory effect; -, inhibitory effect; =, no effect detected; n.d., not determined. Data are compiled by de Jong et al. (1990) from many primary sources.

TABLE IB: Pleiotropism of ACTIVINS

	Ref.
-- Induces release of FSH from cultured anterior pituitary cells	Vale et al.(1986) Nature 321:776 Ling et al.(1986) Nature 321:779 Vale et al.(1988)
-- Inhibitor of GH biosynthesis and secretion in cultured anterior pituitary cells	
-- Erythroid Differentiation Factor (EDF) (=Activin A)	Murata et al.(1988) PNAS 85:2434
-- Dorsal mesoderm induction in *Xenopus* animal cap assay	Smith (1987) Develop. 99:3 Smith et al.(1990)Nature345:729 Sokol et al(1990)Science249:561
-- Induction of partial secondary body axis upon activin mRNA injection into *Xenopus* embryos or by graft of activin-induced tissue	Cooke et al(1987)Devel. 101:893 Ruiz i Altaba & Melton (1989) Nature 341:33 Thomsen et al. (1990) Cell 63:485
-- Patterning morphogen : Stepwise transitions in cell fate in an activin concentration-dependent manner	Green, New & Smith (1992) Cell 71:731
-- Inhibition of differentiation of P19 Embryonal Carcinoma and ES cells -- Inhibition of differentiation of neural cell lines -- Mitogenic for P19 cells -- Inhibition of neural induction in *Xenopus* embryos	-Schubert et al.(1990) Nature344:868 -van den Eijnden-van Raaij et al. (1991) Mech. of Developm. 33:157 -Hashimoto et al.(1990) BBRC 173:193 -Hemmati-Brivanlou and Melton (1994) Cell 77:273
-- Increase in gap junctional permeability in *Xenopus* embryos by activin B mRNA injection -- Cell spreading and migration on fibronectin-coated substratum.	-Olson and Moon (1992). Dev. Biol. 151: 204. -Smith and Howard (1992) Develop.

TABLE II: ACTIVIN RNA EXPRESSION IN EARLY EMBRYOS

Xenopus	fertilized egg	pre-blastula	late blastula	late gastrula
βA	-	-	-	+
βB	-	-	+	+
	[Activin bioactivity present]			
Chick				
βA			-	-
βB			+ (hypoblast)	-

TABLE III: ACTIVIN EXPRESSION IN MOUSE EARLY EMBRYOS:

		PRE-IMPLANTATION				POST-IMPLANTATION	
		oocyte II	2-cell	morula	blastocyst	MATERNAL uterine decidua	embryo
RNA							
	βA	+	-	+	-	E5.0-E6.5 decidua, surrounding whole embryo	absent before E8.5
	βB	+	-	+	+	decidua, around ecto-placental cone	absent before E8.5
Protein							
	βA/βB	+	+	+	+		
	α	-	-	-	-		

ACTIVIN / INHIBIN βB SUBUNIT GENE DISRUPTION LEADS TO DEFECTS IN EYELID DEVELOPMENT AND FEMALE REPRODUCTION

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Running title: Activin B / Inhibin B deficient mice

Keywords: Mouse; Activin B / Inhibin B; gene targeting; eyelid fusion; female reproduction

Abstract

Inhibins and activins are dimeric growth factors of the transforming growth factor-β superfamily, a class of peptides that can regulate the growth and differentiation of a variety of cell types. Recently, activins have been implicated in early vertebrate development through their ability to evoke, in Xenopus embryo explants, both morphological and molecular changes characteristic of mesoderm induction. To understand these processes further, we have used homologous recombination in embryonic stem cells to create mouse strains carrying mutations in the gene encoding the activin/inhibin βB subunit. These mice are expected to be deficient in activin B (βB:βB), activin AB (βA:βB) and inhibin B (α:βB). Viable mutant animals were generated, indicating that the βB subunit is not essential for mesoderm formation in the mouse. Mutant animals suffered, however, from distinct developmental and reproductive defects. An apparent failure of eyelid fusion during late embryonic development led to eye lesions in mutant animals. Whereas βB deficient males bred normally, mutant females manifested a profoundly impaired reproductive ability, characterized by perinatal lethality of their offspring. The phenotype of mutant mice suggests that (1) activin βB plays a role in late fetal development and (2) is critical for female fecundity. In addition, we have found that expression of the related βA subunit of activin is highly upregulated in ovaries of mutant females. Altered regulation of βA activin in βB deficient mice may contribute to the mutant phenotype.

Introduction

Candidate genes and proteins that govern the formation and patterning of the mesoderm in vertebrate embryos have recently emerged. Through studies in amphibians came the realization that specific polypeptide growth factors were able to reproduce some aspects of mesoderm induction in embryo explants. These factors include members of the transforming growth factor-β (TGF-β) superfamily such as activins A and B, bone morphogenetic protein 4 (BMP-4) and Vg-1, and members of the fibroblast growth factor (FGF) family such as basic FGF (Kimelman and Kirschner, 1987; Slack et al., 1987; Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990; van den Eijnden-Van Raaij et al., 1990; Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Thomsen and Melton, 1993) . In addition, other factors that do not have the ability to induce mesoderm from presumptive ectoderm by themselves, were found to modify the pattern of mesodermal differentiation evoked by an inducer. These include members of the Wnt family such as Xwnt-8, and noggin (Smith and Harland, 1991; Sokol et al., 1991; Smith and Harland, 1992; for reviews see Kimelman et al., 1992; Sive, 1993). Among mesoderm inducing molecules, activins are unique because of their ability to induce a full range of mesodermal fates, from anterior-most mesoderm (organizer) to tail, in a concentration-dependent manner (Green et al., 1992). Genes induced by activins as an immediate-early response were identified and include the organizer-specific goosecoid gene and the Brachyury gene (Cho et al., 1991; Smith et al., 1991). In the Brachyury mouse, mesoderm formation is defective. Cloning of the mouse Brachyury (T) gene (Herrmann et al., 1990) has provided the first identification of a gene located on the pathway of mesoderm formation in vertebrates. As inducers of Brachyury, activins are thought to be part of this pathway as well. Animal caps induced by activin also undergo morphogenetic cell movements that are characteristic of gastrulation (Smith and Howard, 1992). Thus histological, molecular and cell behavior responses of animal cap explants to activin recapitulate many aspects of gastrulation. Hemmati-Brivanlou and Melton (1992) have provided the most compelling evidence of the involvement of an activin-like molecule in mesoderm formation by showing that injection of an mRNA encoding a truncated activin receptor into Xenopus embryos interferes with activin signaling and prevents mesoderm induction.

Activins are structurally related to the inhibin class of peptides. Inhibins were initially isolated from mammalian follicular fluid on the basis of their ability to inhibit the release of follicle stimulating hormone (FSH) from anterior pituitary cells (for review, see Vale et al., 1990). In the course of inhibin purification, side fractions that instead stimulated FSH release lead to the identification of activins (Ling et al., 1986; Vale et al., 1986). Inhibins and activins are dimers assembled from subunits encoded by three distinct genes: the α, βA and βB subunit genes. The precursor polypeptides encoded by these genes are proteolytically processed into mature peptides, whose dimers form the biologically active molecules. Inhibins are α:β heterodimers, whereas activins are β:β homo- or

heterodimers. The two β subunits, βA and βB, share within a species ~60% sequence identity, whereas the α subunit is more distantly related. Each β subunit is highly conserved across species and >98% identical between mammalian species (Vale et al., 1990). The βB subunit is expressed in Xenopus (Thomsen et al., 1990) and chick (Mitrani et al., 1990) late blastula embryos. In the mouse, β subunit mRNAs and β subunit immunoreactivity, in the absence of α subunit expression, were demonstrated during preimplantation stages, indicating the presence of activin in the pre-gastrulation mouse embryo (van den Eijnden-van Raaij et al., 1992; Albano et al., 1993).

In adult animals, highest expression levels of activin/inhibin subunits are found in the gonads. Recent evidence implicates inhibins and activins as intragonadal paracrine and/or autocrine regulators (reviewed by de Jong et al., 1990; Mather et al., 1992; Findlay, 1993). The major sites of inhibin/activin expression are the Sertoli cells of the testis and the granulosa cells of the ovary. Inhibins and activins were found to affect both the germ cells and the somatic components of the gonads. Activin can stimulate spermatogonial proliferation, whereas inhibin has the opposite activity. Isotypes of activin receptors were detected on specific populations of male germ cells (Kaipia et al., 1992; Kaipia et al., 1993), suggesting that these factors play a role in the regulation of spermatogenesis. Furthermore, androgen biosynthesis in testicular Leydig or ovarian thecal cells is inhibited by activin and enhanced by inhibin. In females activin and inhibin were suggested to either stimulate or inhibit, respectively, oocyte meiotic maturation, and to affect follicular development. The most conclusive evidence for a critical role of inhibin as a regulator of proliferation of somatic components of the gonad was provided by the finding that inhibin deficient mice generated by α subunit gene targeting develop gonadal stromal tumors at a very early age (Matzuk et al., 1992).

Other studies have revealed extragonadal sources of inhibin and activin synthesis and demonstrated responses in a variety of cell types (Meunier et al., 1988; Vale et al., 1990). Thus, activins have been implicated in inhibition of neural differentiation (Hashimoto et al., 1990; Schubert et al., 1990; van den Eijnden-van Raaij et al., 1991; Hemmati-Brivanlou and Melton, 1992), and induction of erythropoietic differentiation (Murata et al., 1988). In addition to their action on FSH release, their ability to regulate secretion of other hypothalamic or pituitary peptides such as oxytocin, ACTH and GH was shown (Vale et al., 1990).

To study the physiological role of these factors in mouse development and reproduction, we have used homologous recombination in embryonic stem (ES) cells to create mutant alleles where most of the first coding exon of the βB gene was deleted, and these mutations were introduced into the germline of mice. Although it remains to be seen whether embryonic development proceeds normally at a molecular level in βB deficient animals, they are born without major morphological abnormalities, with the exception of a defect in eyelid outgrowth and closure. The generation of viable mutant animals has, however, revealed a critical role for activin/inhibin βB in female reproductive function.

Results

Mice homozygous for a targeted mutation in the activin/inhibin βB subunit gene are viable

The mouse activin/inhibin βB subunit gene was isolated from a genomic DNA library derived from the 129/Sv strain using a rat partial cDNA as a probe (rinB-c2-P; Dr Kelly Mayo, unpublished). Location of the coding exons is diagrammed in Figures 1A and 1B. Two replacement type targeting vectors termed pBB1 and pBB2 (Fig.1B) were used to disrupt the βB subunit gene by homologous recombination (Thomas and Capecchi, 1987). Each vector contained 15.5 kb homology with the cognate gene. These constructs were designed to replace a 1.3 kb region of the βB subunit locus spanning the translational start codon, the signal peptide coding region as well as most of exon 1-encoded pro-region sequences with a PGK promoter driven neo selection cassette containing a PGK poly(A) signal (PGK-NEO-p(A)) (McBurney et al., 1991). The pBB1 construct contained the PGK-NEO-p(A) cassette in the same transcriptional orientation as the βB gene and the pBB2 construct contained it in reverse orientation. J1 ES cells (Li et al., 1992) were electroporated with linearized DNA and selected with G418. Homologous recombinant ES cell clones were identified by Southern blot analysis of genomic DNA from individual clones (see Materials and Methods). Both targeting constructs demonstrated similar, high frequencies of recombination at the endogenous βB subunit locus (Table 1).

Three independent ES clones heterozygous for the BB1 mutation and one clone heterozygous for the BB2 mutation were injected into host blastocysts to generate chimeric founder mice. All four clones contributed to the germline of chimeric animals, permitting the derivation of several independent mouse lines carrying the mutant alleles. Mice heterozygous for either the BB1 or BB2 mutation did not display an overt phenotype. To evaluate the possibility that the genetic background influences the mutant phenotype, animals heterozygous for the BB1 mutation were bred to homozygosity on three different genetic backgrounds. Genotype analysis of weanling offspring (Table 2) revealed that homozygous mutant animals with a 129/Sv X C57BL/6 hybrid genetic background were only marginally compromised in their viability, if at all, as they represented 21 % of the animals genotyped, instead of the expected 25 %. In the 129/Sv X BALB/c hybrid background, however, the fraction of homozygous mutants was lower (Table 2), suggesting that alleles in the BALB/c background may interact with the activin mutation and reduce viability. These results therefore indicated that mice homozygous for the BB1 or BB2 mutation are viable, although their viability may be affected by the genetic background.

Mutant mice lack wild-type mRNA and mature βB peptide

To examine how the BB1 mutation affects transcription at the βB locus, RNA was prepared from ovaries and testes of immature (3-week) or adult animals and subjected to reverse transcription followed by the Polymerase Chain Reaction (RT-PCR). When oligonucleotide primers that span the mature peptide coding region were used, a βB-specific band of the expected size was obtained both in mutants and in controls. In addition, primers that span the intron amplified the expected βB-specific band in mutant animal tissue, although with markedly reduced efficiency relative to controls (data not shown). These results indicated that transcription downstream of the neo insertion occurred in the BB1 allele. This is not unexpected as the PGK poly(A) site was previously shown to allow readthrough transcript accumulation (e.g., Lee et al., 1992).

To characterize further the nature of the transcript produced by the BB1 mutated allele, Northern blot analysis was performed (Fig. 2). The two probes used were the mACT10/11;13 probe, a PCR-generated fragment corresponding to the mouse βB mature peptide region (Fig.2A and see Materials and Methods), and a neo probe (Fig.2B). A ~4.5 kb transcript corresponding to the major wild-type mRNA (Albano et al., 1990; Manova et al., 1992) was detected with the mACT10/11;13 probe in (+/+) and (+/bb1), but not in (bb1/bb1) ovaries. In wild-type animals, a minor ovarian transcript of ~3.5 kb could be detected, but never in (bb1/bb1) animals. The 3.5 kb transcript was also seen in wild-type testes, approximately equimolar to the 4.5 kb transcript, as reported previously (Feng et al., 1989a). Neither the 4.5 nor the 3.5 kb transcripts were detected in the (bb1/bb1) mutant testes. The mACT10/11;13 probe however detected a novel transcript of ~4.0 kb in both testes and ovaries of animals containing a mutated BB1 allele, but not in (+/+) animals. The neo probe also hybridized to a band of the same size. Therefore, the 4.0 kb transcript likely corresponds to a neo-βB chimeric transcript, reading through the PGK poly(A) site and terminating at one of the βB gene endogenous poly(A) sites. Because the promoter(s) and poly(A) site(s) of the mouse βB gene have not been mapped, it is not possible to predict the exact size of such a chimeric transcript. As expected, a major ~1.0 kb band corresponding to a transcript initiated at the PGK promoter and terminated at the PGK poly(A) site was detected by the neo probe in (bb1/bb1) and (+/bb1) animals. As determined with the neo probe, the 4.0 kb neo-βB readthrough transcript was ~1/10 as abundant as the neo transcript terminated at the PGK poly(A) site. Examination of RNA from animals homozygous for the BB2 allele likewise showed the absence of the wild-type transcripts and revealed the production from this allele of a new transcript of ~3.0 kb that did not hybridize to the neo probe (Fig.2), consistent with its initiation within the PGK promoter (used in reverse orientation) and its termination at an endogenous βB poly(A) site.

In order to establish whether the aberrant transcript made by the BB1 allele could produce βB peptide, we next performed protein blot analysis. βB-specific antibodies (Vaughan et al., 1989) could readily detect a ~12 kD peptide in ovarian tissue from heterozygous or wild-type weanling females (Fig.3, upper panel, lanes 6, 7, 9, 10 and 12-14), but not in tissue from (bb1/bb1) mutant females (lanes 4, 5, 8 and 11). A polypeptide of MW ~50 kD, consistent with the βB precursor size, was also detected by the βB-specific antibodies in gonadal extracts from wild-type or heterozygous animals, but was absent in homozygous mutants (not shown).

In summary, the results of Fig.2 and 3 indicate that the BB1 mutant allele does not yield productive βB subunit mRNAs capable of generating the mature βB peptide whose dimer with another β subunit or with an α subunit forms the biologically active activin or inhibin ligand. (bb1/bb1) mutant animals are therefore expected to lack activins B and AB, as well as inhibin B , but their ability to produce activin A and inhibin A should be preserved.

The βB deficient ovary upregulates expression of the βA subunit

Expression of the activin/inhibin βA mature peptide was investigated in total ovarian tissue extract from 3-week old females. This analysis revealed a 3 to 20-fold increase in levels of βA mature peptide in animals homozygous for the BB1 mutation, relative to their heterozygous or wild-type littermates (Fig.3, middle panel, compare e.g. lanes 4,5 to lanes 6,7). In the same ovarian extracts, anti-α inhibin subunit antibodies recognized a ~45 kD polypeptide whose levels were not altered by the βB mutation (Fig. 3, lower panel).

Overexpression of the βA subunit in the βB deficient ovary may reflect altered development and cellular architecture of the mutant tissue, leading to an overrepresentation of βA expressing cells. Alternatively, regulatory mechanisms that sense the βB deficiency may exist and cause a compensatory increase in βA subunit expression.

To analyze this effect further, βA subunit RNA expression was examined by Northern analysis of ovarian extracts from immature animals. No consistent alterations in levels of the ~7.0 kb mRNA were observed in mutant tissue (not shown). Therefore, a posttranscriptional mechanism may operate to upregulate βA mature peptide production in the βB deficient ovary.

Failure of eyelid fusion at birth

Upon intercrossing animals heterozygous for the BB1 (or BB2) mutation we observed that a fraction of the neonates in the resulting litters had a distinctive phenotype. Normally, mouse pups are born with closed eyes due to fusion of the eyelids at embryonic day 16 (E16) (Rugh, 1990), and eyes open again only at ~13 days of postnatal life. In contrast, a fraction of the offspring from heterozygous

parents were born with open eyes, as illustrated in Fig. 4 A and B. All the open eyed neonates derived from four independent ES cell clones carrying either the BB1 or BB2 mutation and genotyped at birth by Southern analysis were homozygous for the mutant allele. Not all homozygotes, however, showed this phenotype, suggesting incomplete penetrance (see below). To determine whether the structure of the eye was otherwise affected in the mutant newborns, histological sections through the heads of (bb1/bb1) mutant animals and (+/bb1) control littermates were examined (Fig. 4 C, D, E). The general anatomy of the eye of the mutant newborns appeared normal, indicating a defect confined to the eyelids. Animals born with open eyes quickly developed a number of eye defects, which likely reflected traumatic damage due to the lack of eyelid protection. Hyperkeratinisation and squamous metaplasia of the corneal epithelium, accompanied by massive leukocyte infiltration of all cornea layers and eyelids, were observed as soon as the first day of life. During the subsequent days, eyelids often sealed. The timing of eye reopening appeared then delayed in mutants and revealed permanent damage that included ocular dystrophy and opacification of the cornea. This macroscopic eye pathology allowed ready identification of mutant animals during adulthood.

The open eyelid phenotype of the BB1 mutation was found to depend on the genetic background (Table 3). In the original mouse line 43, F1 animals had been backcrossed once to C57BL/6 and then intercrossed. In this line, the fraction of homozygous mutant animals exhibiting obvious eye defects during adulthood was 0.84 (N=89). A similar phenotype was observed in a 129/Sv X BALB/c hybrid genetic background, although with reduced penetrance. In contrast, when the BB1 mutation was present in a 129/Sv inbred background, the failure of eyelid fusion was never observed: none of the pups born from heterozygote intercrosses (N=137), or born from homozygous mutant males mated to heterozygous females (N=69), had open eyes at birth, and among 14 adult mutant animals, none had eye defects. This suggests that the 129/Sv inbred strain carries a gene (or genes) that suppress the open eyelid phenotype caused by the BB1 mutation. Segregation of such 129/Sv specific suppressor genes may also explain the incomplete penetrance of the phenotype in the two hybrid genetic backgrounds. Furthermore, the expressivity of the phenotype varied among affected individuals, with various degrees of completion of eyelid fusion at birth or, occasionally, a single eye being affected.

Mutant females manifest a reproductive impairment

Activins and inhibins are thought to be regulators of reproductive functions through their influence on both the pituitary and the gonads. Therefore, we next evaluated the fertility of the BB1 homozygous mutant animals. The viability of homozygous mutant embryos had also raised the possibility that maternal supply of βB peptide might rescue the potential defect in mutant embryos. If this were the

case, mutant females might be expected to be impaired in sustaining normal pregnancy and delivery of live pups.

To assess reproductive performance, we bred (bb1/bb1) mutant animals of either gender with heterozygous mates, and intercrossed homozygous mutant animals or heterozygous littermates. The average number of viable offspring per breeding pair and per month of breeding was determined for each type of cross. Fig.5 summarizes the reproductive performances observed. Homozygous mutant males bred as well as their heterozygous littermates when mated to heterozygous females. In sharp contrast, homozygous mutant females mated to either heterozygous or homozygous mutant males manifested a profoundly reduced ability to generate live offspring as compared to their heterozygous littermates. Histological examination of ovaries from βB deficient females did not show overt abnormalities (data not shown). The reproductive impairment of mutant females did not result from defective oogenesis, inability to undergo fertilization or development of embryos in utero, because they became pregnant at a frequency similar to their heterozygous littermates. Reduced fertility appeared rather to result from perinatal loss of the progeny. Offspring were born from mutant females but rarely survived beyond 24 hrs postpartum. A common observation on the day of delivery was death of an entire normal-sized litter.

There was no excess of phenotypically mutant animals among the pups dying perinatally. Consistent with this, we observed no distortion of genotypes among the rare pups that survived the perinatal period: of 21 weanling mice born from (bb1/bb1) mutant females mated to heterozygous males, 10 were (bb1/bb1) and 11 were (+/bb1). Thus, neonatal lethality appears unaffected by the genotype of the offspring but rather reflects a maternal defect.

Female reproductive failure was observed in lines of mice derived from three independent ES cell clones, and was manifest in the three genetic backgrounds tested: in inbred 129/Sv background, and in the two hybrid backgrounds (129/Sv X C57BL/6 and 129/Sv X BALB/c, after one backcross to C57BL/6 or BALB/c, respectively). 129/Sv X BALB/c hybrid mutant females appeared the most seriously affected: a total of 10 pregnancies carried by 4 different females failed to generate live offspring.

Mutant females show increased gestation time and decreased nursing ability

In staged matings where the day of vaginal plug (E0.5) was recorded, βB deficient females were observed to show an increase in the average duration of gestation. Among 13 control sibling females (heterozygous or wild-type), 8 had delivered the morning of day E19.5 and all but one had delivered by the afternoon of that day. In striking contrast, none of 12 mutant females delivered on day E19.5. Most mutant females delivered on day E20.5, and some in the morning of day E21.5. Occasionally,

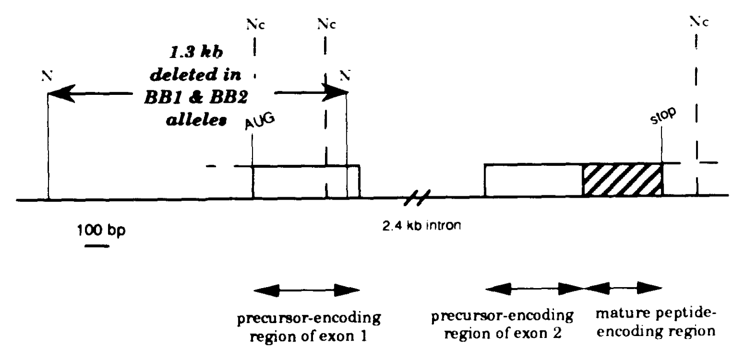
initiation of labor appeared to fail altogether, resulting in sickness of the gravid mother, and death of fetuses in utero.

Delivered babies that were about to die were morphologically unremarkable, but were cold and had empty stomachs, suggesting that their death was due to a maternal failing. Lactogenesis, however, occurred in mutant females. Histological analysis of mammary glands in a pair of control and mutant siblings at ~0.5 day postpartum is shown in Fig.6. Accumulation of milk in lobules and ducts of mutant tissue is evident. These observations suggest that either milk let-down is impaired or that the babies fail to suckle. The former possibility is supported by preliminary data showing that offspring born from mutant females and not nursed can be rescued by fostering to a normal female (not shown).

FSH measurements in mutant animals

To analyze further the cause of the reproductive defect in mutant females, and to determine whether pituitary function was altered in βB deficient animals, serum FSH levels were measured (Table 4). The interpretation of these data was complicated by the facts that (a) FSH values fluctuate widely within an experimental group, and (b) an age-dependent increase in FSH values was evident in adult animals from 11 to 24 weeks of age. Nevertheless, this study revealed an approximately 20 % increase in average serum FSH levels in mutant animals relative to heterozygotes, and the increase was consistent in all groups analyzed. It thus appears that in the context of a deficiency in both activins B, AB (which enhance FSH release *in vitro*) and inhibin B (which decreases it), the effect of inhibin deficiency is slightly dominant. The physiological significance of this modest elevation in circulating FSH is unknown.

Figure 1. Mutagenesis at the Activin/Inhibin βB Subunit Locus of Mice. 1A, Exon structure of the βB subunit gene showing the region deleted in the BB1 and BB2 mutant alleles. The transcription start and polyadenylation sites have not been mapped, and therefore the corresponding exon boundaries are indicated with dashes. The striped box indicates the coding region for the mature βB peptide. Note that the 5' splice site at the end of the first coding exon is left intact in the BB1 and BB2 recombinant alleles. Not all NarI sites within the 1.3 kb deleted fragment are represented. **1B,** Targeting scheme. A physical map of the locus is shown at the top. The structure of the targeting constructs is indicated below. Dashed lines delineate regions of homology with the chromosome. The new EcoRI site introduced by the mutation is indicated in each targeting vector. The pBB2 vector is identical to pBB1, except that the orientation of the neo cassette is reversed. Probe E is the external probe used to screen ES cell colonies and to genotype mice. rinB-c2-P represents the rat cDNA probe used to isolate genomic DNA from the βB locus. **1C,** Southern blot analysis of DNA from offspring produced by heterozygote intercrosses. DNA was digested with EcoRI and hybridized to probe E. Molecular weight markers (in kb) are shown on the right. Restriction sites : B, BglII; R, EcoRI; N, NarI; Nc, NcoI.


A.
Nc
Nc
Nc
N
N
1.3 kb
deleted in
BB1 & BB2
alleles
AUG
stop
100 bp
2.4 kb intron
precursor-encoding
region of exon 1
precursor-encoding
region of exon 2
mature peptide-
encoding region

C.
bb1/bb1
+/bb1
+/+
+/+
bb2/bb2
+/+
+/bb2
+/bb2
8.0
6.0

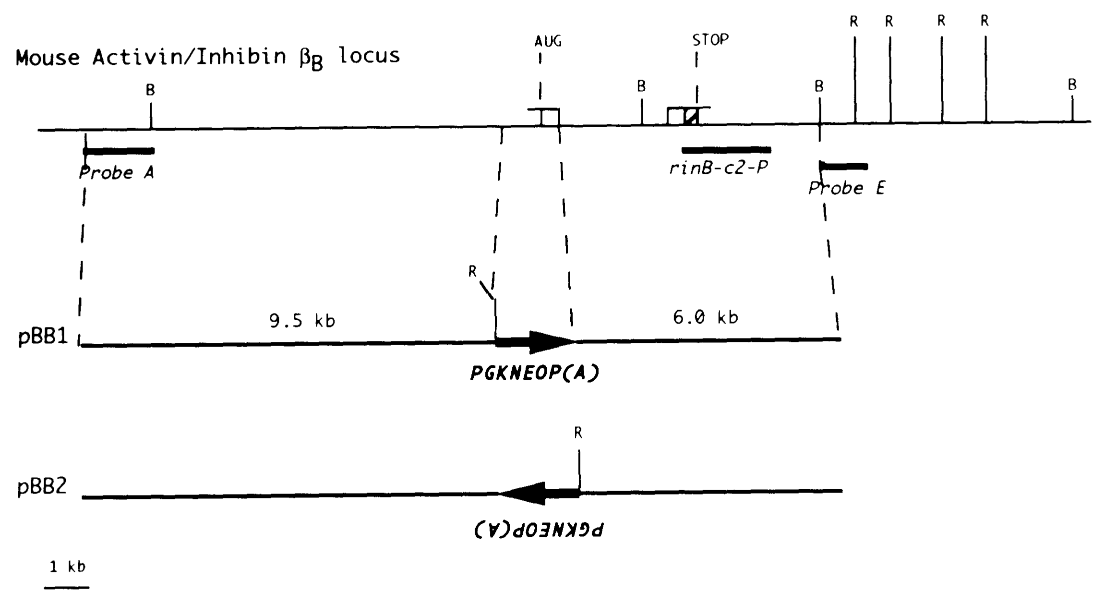
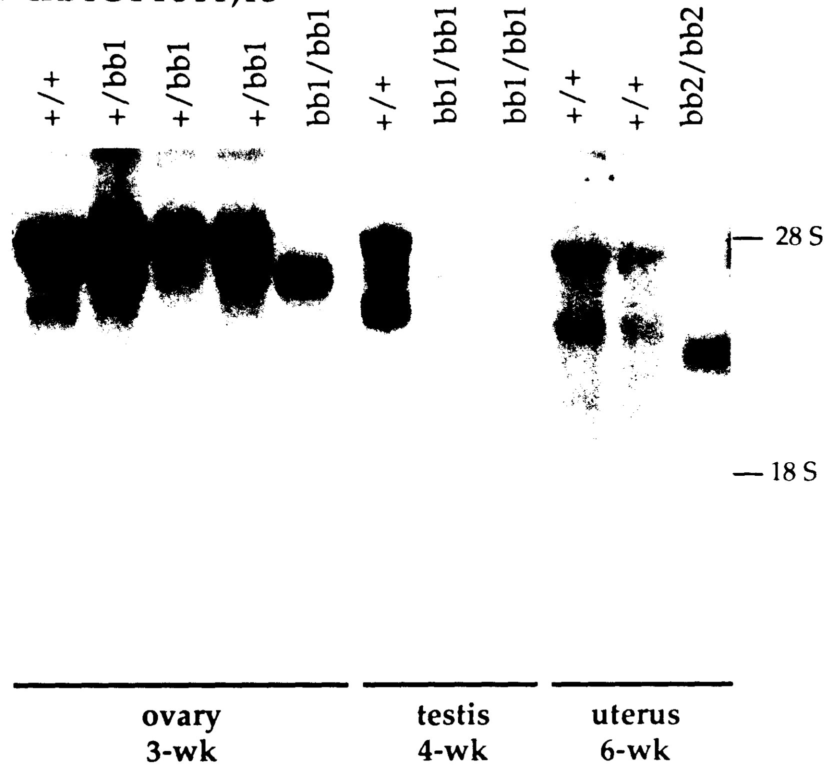

B.
Mouse Activin/Inhibin βB locus
AUG
STOP
B
R
Probe A
rinB-c2-P
Probe E
pBB1
9.5 kb
6.0 kb
PGKNEOP(A)
pBB2
1 kb

Figure 2. RNA Analysis in βB Mutant Animals. Total RNA from ovaries (~10 ug, lanes 1 to 5), testes (20 ug, lanes 6 to 8), and uterus (20 ug, lanes 9 to 11) from individual animals were examined by RNA blot analysis using a probe corresponding to the βB mature peptide encoding region (mACT1011;13, panel A) or a neo probe (panel B). Genotypes of animals are indicated at the top and positions of ribosomal RNAs are shown on the right.


A. mACT1011;13
+/+
+/bb1
+/bb1
+/bb1
bb1/bb1
+/+
bb1/bb1
bb1/bb1
+/+
+/+
bb2/bb2
28 S
18 S
ovary
3-wk
testis
4-wk
uterus
6-wk

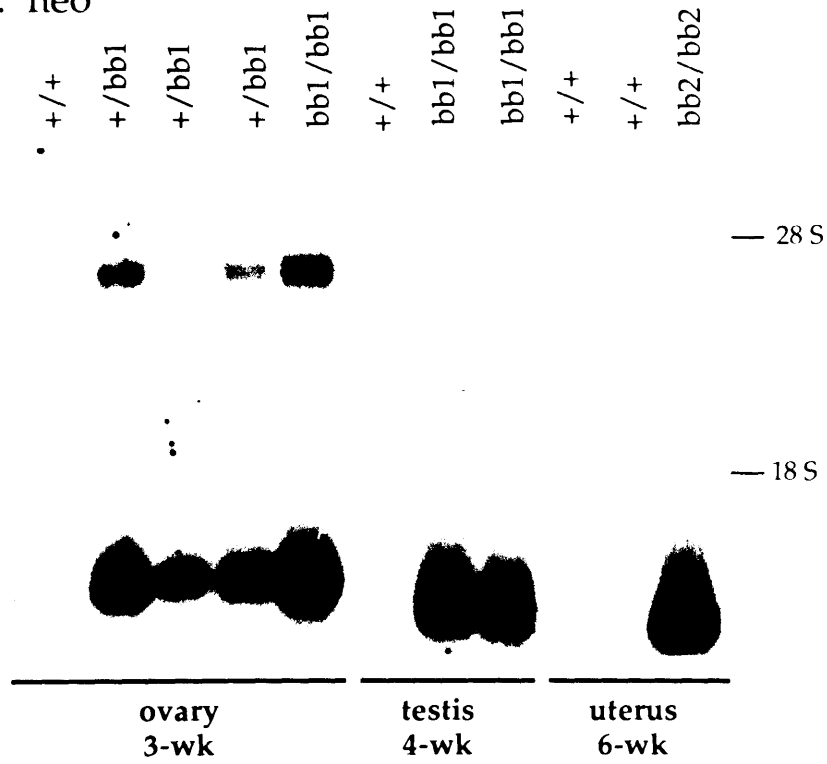
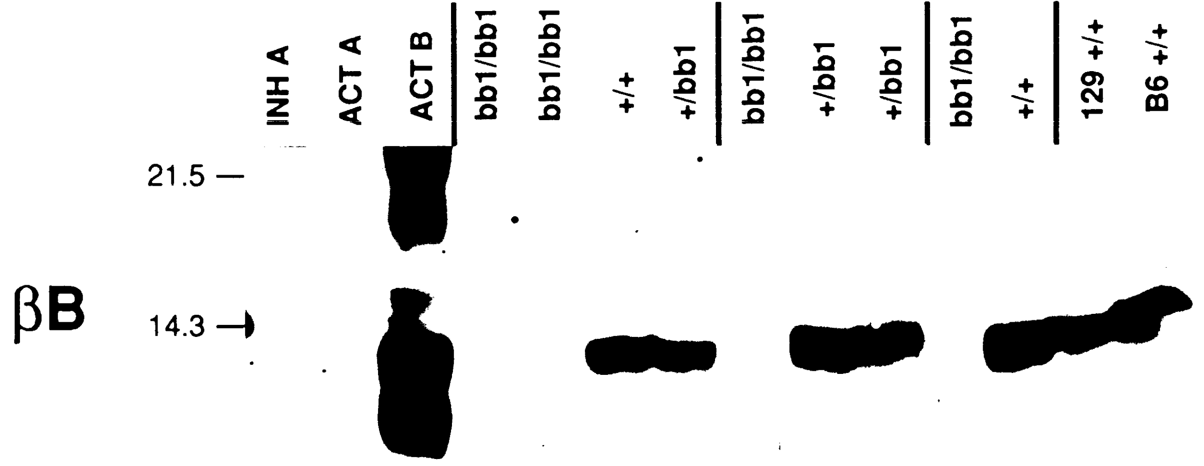
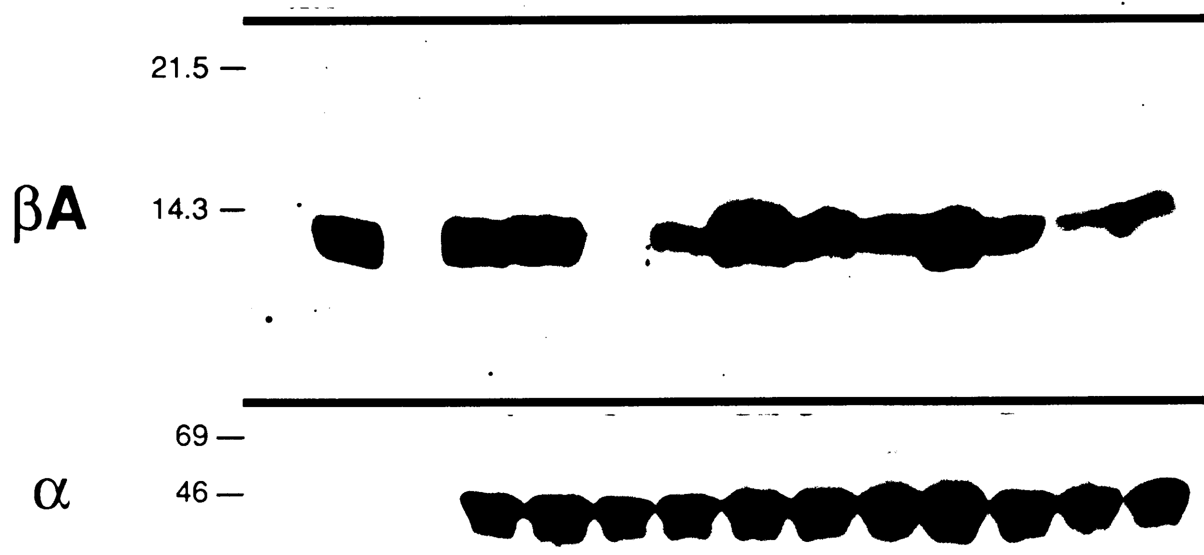

B. neo
+/+
+/bb1
+/bb1
+/bb1
bb1/bb1
+/+
bb1/bb1
bb1/bb1
+/+
+/+
bb2/bb2
28 S
18 S
ovary
3-wk
testis
4-wk
uterus
6-wk

Figure 3. Animals homozygous for the BB1 mutation lack mature βB peptide and upregulate βA peptide expression. Ovarian extracts from individual immature (21-day old) females were run on a SDS-PAGE gel under reducing conditions and examined by immunoblot analysis using anti-βB antibodies (upper panel). These antibodies recognize a peptide in the C-terminal region of the βB subunit. The same blot was stripped and re-analyzed using anti-βA antibodies (middle panel) or anti-α antibodies (lower panel). Lanes 1 to 3 contain 1 ng each of human recombinant inhibin A (lane 1), activin A (lane 2), activin B (lane 3). Lanes 4 to 14 contain 100 ug each of total protein from ovary. Genotypes are indicated at the top. Lanes 13 and 14 represent a control wild-type 129/Sv, and a control wild-type C57BL/6 female, respectively. Extracts in lanes 4 to 7 originate from siblings with a 129/Sv X BALB/c hybrid genetic background (BALB-N2 backcross), lanes 8 to 10 are from siblings with a 129/Sv X C57BL/6 background (B6-N1), and lanes 11 and 12 are from siblings from a (129/Sv X C57BL/6) N4 backcrossed generation. Migrations of molecular weight markers (in kD) are shown on the left.



INH A
ACT A
ACT B
bb1/bb1
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+/+
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129 +/+
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1 2 3 4 5 6 7 8 9 10 11 12 13 14

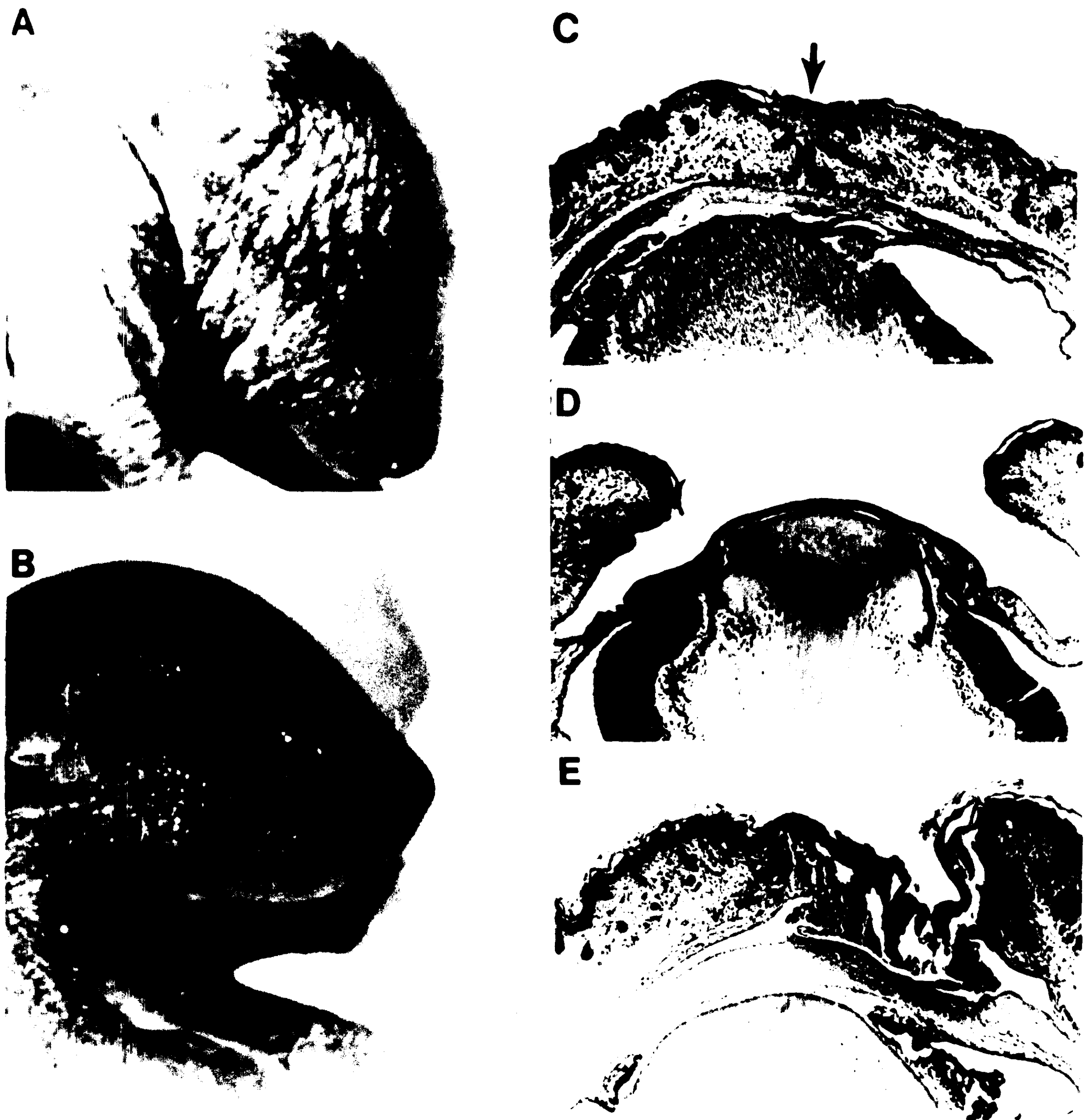

Figure 4. Failure of Eyelid Closure at Birth. A, a heterozygous control pup showing fused eyelids covering the eye, **B**, homozygous mutant littermate with open eyelids. Photographs in A and B were taken within a few hours of birth. **C, D, E**, Transverse sections through the eyes of (C) a heterozygous control neonate; an arrow indicates the site of eyelid fusion, (D) a mutant neonate a few hours after birth, before inflammation is evident, and (E) a mutant neonate at ~0.5-1 day of age, displaying acute leukocyte infiltration in the cornea and eyelids, corneal edema and corneal hyperkeratinization. Objective magnification was 10 X.

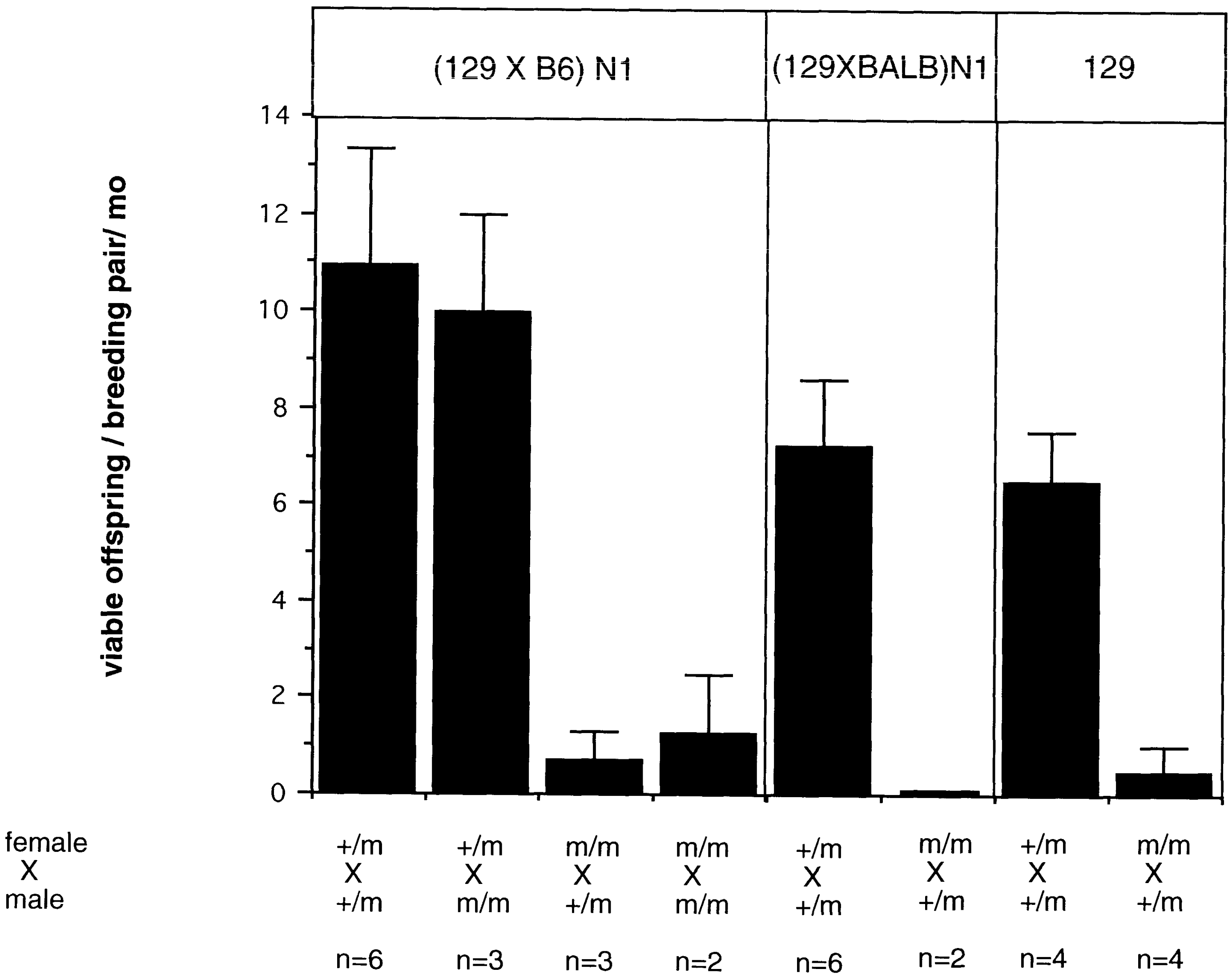

Figure 5 . Reproductive Performance of Animals Heterozygous or Homozygous for the BB1 Mutation.
The number of viable progeny per breeding pair and per month of breeding was averaged for each type of cross in each genetic background. Genetic background is indicated at the top and the number of breeding pairs (n) is indicated at the bottom. Several m/m (129 X BALB) females died during their first pregnancy from failure to deliver, and were not included in the figure.

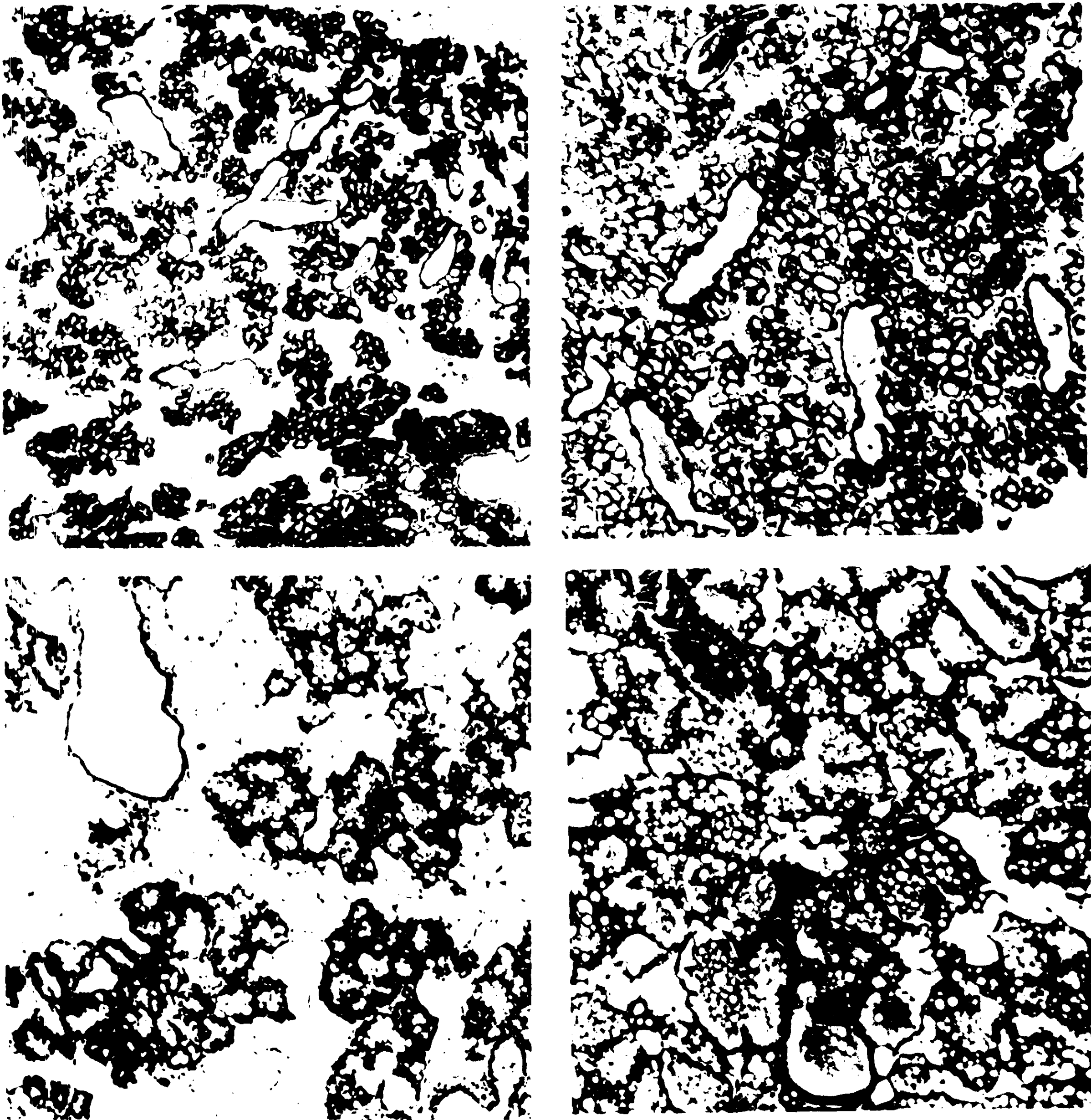

Figure 6. Postpartum Histology of Mammary Glands in Mutant and Control Heterozygote Siblings. Panels A and C show control tissue from a heterozygous female and panels B and D show tissue from a mutant βB deficient sibling. Tissue was taken ~0.5 day after delivery. Note the increased glandular:adipose tissue ratio, the accumulation of milk and the vacuolation of lobules in mutant mammary tissue. The mutant animal had delivered a litter that was not nursed. Both animals were from a BALB-N1F2 generation. Objective magnification was 4X (A and B), and 20X (C and D).

Table 1. Homologous recombination at the activin/inhibin βB locus

Targeting construct	Cells electroporated	$G418^r$ colonies	$G418^r$ clones screened	Positive clones[1]	Targeting frequency targeted clones / $G418^r$ clones	Targeting frequency targeted clones / transfected cells
pBB1	5×10^7	5100	172	41	1/4.2	2.4×10^{-5}
pBB2	5×10^7	2900	58	10	1/5.8	1×10^{-5}

(1) as determined by Southern blot analysis using genomic probe E (see fig.1).

Table 3. The eyelid closure defect: phenotypic variation with genetic background.

Cross (female X male)	Mouse line	Genetic background	oeb/total[a] (neonates)	Penetrance[b] (adults)
(+/m) X (+/m)	129-134	129	0.00 (N=137)	0.00 (N=14)
	BALB-210	(129 X BALB) F1	0.04 (N=90)	0.21 (N=24)
	BALB-15	(129 X BALB) N1	0.12 (N=102)	ND
	B6-134	(129 X B6) F1	0.11 (N=299)	0.33 (N=21)
	B6-43	(129 X B6) N1	0.20 (N=367)	0.84 (N=89)
(+/m) X (m/m)	B6-43	(129 X B6) N1	0.46 (N=225)	
(m/m) X (+/m)	B6-43	(129 X B6) N1	0.53 (N=36)	
(m/m) X (m/m)	B6-43	(129 X B6) N1	0.86 (N=78)	

[a] oeb/total, fraction of total offspring with one or both eye(s) open at birth or displaying resulting eye defects as observed within one week of life. The total number of offspring examined for each cross is indicated in parentheses. All pups whose eyes could be examined are entered irrespective of their viability.

[b] For each mouse line, the penetrance is calculated as the fraction of adult homozygous mutant animals displaying obvious eye defects (ocular dystrophy and/or corneal opacification) of one or both eye(s), as determined by simple visual inspection. The total number of homozygous mutant animals examined figures in parentheses.

ND, not determined.

Table 4. Serum FSH values in βB deficient mice.

Mouse line	Genetic background	Gender	Age (wk)	FSH (ng/ml)		
				+/+	+/m	m/m
129-134	129	Male	11-16	ND	90.4±14.6 (N=9)	105.7±18.5 (N=6)
B6-43	(129 X B6) N1	Male	11-25[a]	141.6±33.2 (N=8)	172.0±42.8 (N=14)	206.0±57.7 (N=14)
			6.5-14.5[a]	124.1±62.6 (N=6)	110.6±33.8 (N=11)	171.0±111.4 (N=11)
B6-43	(129 X B6) N1	Female	12-24	78.9±22.4 (N=6)	73.2±18.1 (N=9)	86.4±27.4 (N=11)

[a]Two experimental groups of males of the B6-43 line had FSH values determined in separate experiments.

Discussion

We have created mouse lines carrying two different mutant alleles for the gene that encodes the activin/inhibin βB subunit. In both alleles the coding capacity (in exon 1) for approximately a third of the βB precursor polypeptide was deleted at its N-terminal. Gray and Mason (1990) have shown that the pro-region of the activin A precursor is essential for the intracellular assembly of the homodimer and secretion of biological activity. It is not known where the dimerization activity resides within the pro-region and whether the exon 2-encoded pro-region would suffice in this function. It is likely, however, that the alteration generated loss-of-function alleles because the signal sequence and approximately half of the propeptide sequence were deleted. Protein analysis confirmed the lack of mature βB peptide in mutant tissue. The mutation, therefore, should prevent the synthesis of activins B, AB and of inhibin B. Nevertheless, mutant mice completed embryonic development and were viable. Their birth with open eyes indicated, however, that some aspects of development were altered in the absence of a wild-type βB subunit gene. The generation of viable mice has furthermore uncovered a maternal effect by which maternal βB chain expression is required for efficient perinatal survival of newborns. As a result, βB deficient females had an extremely low fecundity, despite their ability to sustain gestation up to the time of birth.

Recent evidence implicates an activin-like molecule as a patterning morphogen during early vertebrate development (reviewed by Kimelman et al., 1992; Sive, 1993). Remarkably, interference with the activin pathway by injection of an mRNA encoding a truncated activin receptor into Xenopus embryos prevents mesoderm induction and formation of axial structures (Hemmati-Brivanlou and Melton, 1992). Of the two activin genes, the βB subunit gene is the first expressed in Xenopus (Thomsen et al., 1990; Dohrmann et al., 1993) and chick embryos (Mitrani et al., 1990) which has led to the hypothesis that activin B may be involved in mesoderm induction. Activin activity is already detectable in the unfertilized Xenopus egg (Asashima et al., 1991), but zygotic expression occurs only at the late blastula stage (Thomsen et al., 1990). Thus, it is not clear whether activins are involved in the induction of mesoderm itself or rather in patterning and regionalization of already induced mesoderm. In the mouse embryo, activin βB specific RNA as well as activin β subunit protein have been detected in blastocysts and at cleavage stages, respectively (van den Eijnden-van Raaij et al., 1992; Albano et al., 1993). Survival of βB mutant mice to adulthood, therefore, indicates that activins B, AB and inhibin B are dispensable for embryonic development in the mouse. Matzuk et al. (1992) have previously shown that α subunit deficient mice, lacking inhibins A and B, develop to term. These molecules may not play a function in early mouse development, or there may exist overlapping pathways allowing compensation for their deficit at these stages. The βA subunit gene in particular may 'cover' the βB deficiency in our mutants. Our finding of upregulated expression of the βA subunit in the βB deficient ovary possibly reflects a regulatory mechanism that also operates in the mutant

embryo and provides a means for normal morphogenesis in the absence of activin B. Generation of an activin-less mutant by targeting the βA gene and intercrossing βA and βB mutant mice should provide definitive answers to some of these questions. In addition, examination of the expression of activin immediate-early response genes, as Brachyury and goosecoid, in the βB deficient embryos should help to determine whether development proceeds normally, both temporally and spatially.

Although βB subunit mutant embryos develop to term, the deficiency in either activin B, activin AB or inhibin B affects prenatal development, as revealed by a defect in eyelid fusion at birth. Inhibin deficient mice are born with closed eyes, suggesting that the open eye phenotype specifically results from activin deficiency. Unfused eyelids at birth could result from either premature (prenatal) eye opening or, perhaps more likely, from failure of fusion at E16. The event of eyelid closure depends on the proliferation of mesenchymal cells to effect apposition and eventual fusion of the eyelids at E16. These cells are neural crest derivatives that populate the first branchial arch (see Juriloff and Harris, 1993). The significance of the open eye phenotype is unclear. A variety of mutations are known to cause birth with open eyes in mice (Lyon and Searle, 1989). Some reside at unknown loci and others result from targeted disruption of specific genes. The latter category includes mutants in the c-abl and TGF-α genes (Schwartzberg et al., 1991; Luetteke et al., 1993; Mann et al., 1993). The variety of primary defects causing open eyes at birth suggests an unspecific nature to this phenotype. The process of eyelid outgrowth and fusion may be especially sensitive to sublethal developmental perturbations. For example, it may rely upon events that can only occur in a narrow window of time. Alternatively, as suggested by the fact that the genes for both the βB subunit and an activin receptor are expressed in the eye anlagen of Xenopus embryos (Dohrmann et al., 1993), a more specific effect of βB on the eye may be operating. A different mutation in the βB subunit gene, that results in deletion of part of the mature peptide encoding region, was also found to affect eyelid fusion in mouse embryos (H. Schrewe and T. Gridley, personal communication).

Expression of the eye phenotype was affected by the genetic background. The defect was never observed in 129/Sv inbred mice but was seen when the mutation was introduced into either of two hybrid genetic backgrounds (129/Sv X C57BL/6 or 129/Sv X BALB/c). The incomplete penetrance observed in hybrid mice may therefore be due to the existence of suppressor allele(s) contributed by the 129/Sv genome. Further backcrosses of the mutant animals to C57BL/6 or BALB/c mice will clarify this question. In addition, the occasional individuals displaying a single affected eye suggest that not all variation is due to genetic factors. In contrast to the eye phenotype, the reproductive failure of mutant females (see below), appears fully penetrant in the 129/Sv inbred genetic background, suggesting that the suppressor(s) carried by the 129/Sv genome act(s) specifically during embryonic development.

A large body of evidence exists for the relevance of activins and inhibins in the biology of reproduction of both males and females. Initially discovered as antagonistic regulators of FSH secretion from anterior pituitary cells, activins and inhibins were also shown to exert a variety of local effects in the gonads, their primary site of expression in adults (see reviews by Vale et al., 1990; Mather et al., 1992). The decidual tissue, forming in the uterine wall in response to nidation of an embryo, has also been found to express activin/inhibin β subunits. Expression of the βA subunit mRNA has been demonstrated in the mouse uterine decidua shortly after implantation (Manova et al., 1992) and βB subunit expression in human decidua was reported to increase in the course of pregnancy (Petraglia et al., 1990). It seemed possible, therefore, that maternal peptide, secreted by the decidua, functions in the developing embryo to promote its normal development to birth. We have thus tested the reproductive capacity of mutant animals. Histological examination of testes and ovaries from βB deficient animals had failed to detect overt abnormalities. However, when the fertility of mutant animals mated to heterozygous partners was assayed, it became apparent that mutant males bred indistinguishably from heterozygous controls, but mutant females suffered an impaired reproductive performance. While mutant females were able to become pregnant and to carry pregnancies to the end of gestation, they failed to raise their offspring normally. Perinatal lethality appeared to affect equally homozygous mutant and heterozygous neonates. This demonstrated that embryonic development can be completed in the absence of both maternal and fetal βB peptide. The viability of βB deficient embryos is therefore not dependent upon maternal supply of βB peptide. Our experiments have, however, revealed a critical role for maternal βB expression in perinatal survival of the newborns.

The causes that underlie the reproductive failure in mutant females are not resolved. The newborn pups appeared morphologically normal, suggesting that their death was due to a defect in the delivery process or in maternal nursing behavior. βB deficient mothers may manifest a defect in parturition, in postpartum events such as uterine remodeling, lactation, or of the hormonal milieu affecting maternal behavior. Examination of mammary tissue indicated that lactogenesis was not impaired in mutant females. The abnormal accumulation of milk in mutant mammary glands suggests, however, that milk let-down might be impaired. Oxytocin is a hypothalamic nonapeptide which is very potent in inducing uterine contractions and controlling milk ejection. It is significant that Sawchenko et al. (1988) have demonstrated β subunit immunoreactivity in the central neural pathways involved in oxytocin secretion. Furthermore, it was reported that local administration of activin in the hypothalamus of rats leads to a rapid increase in plasma oxytocin level and infusion of anti-β subunit serum was reported to inhibit milk ejection (Vale et al., 1990). A local source of oxytocin expression by the epithelium of the uterine endometrium peaks at parturition (Lefebvre et al., 1992), and is a potential target of the βB mutation as well. Preliminary analysis of postpartum uteri suggests morphological alterations in mutant females (A. Vassalli, H. Gardner, unpubl.). It is interesting to note

that the mutation of another pleiotropic cytokine, leukemia inhibitory factor (LIF), was shown to affect specifically the reproductive function of females by interfering with the ability of the mutant uterus to allow implantation of blastocyst embryos (Stewart et al., 1992). Various cytokines may be involved in the timely regulation of the critical physiological changes which occur in the uterus during pregnancy.

Whether the βB subunit gene is active in pathways of early development that display redundancy can best be addressed by studying the interaction of the mutated gene with other, early-acting mutations. For example the presence of the βB mutation may alter the phenotype of embryos heterozygous or homozygous for mutations that affect mesoderm formation, such as the Brachyury mutation at the T locus (Lyon and Searle, 1989) or the 413.d mutation in the nodal gene (Zhou et al., 1993). When mutated in mice, the T gene causes axial deficiencies whose severity are dependent on the dose of the residual wild-type T function (Mac Murray and Shin, 1988) . Because activin is an inducer of T, and levels of T expression affect development in a concentration-dependent manner, it is possible to envisage that activin B deficiency would cause an altered, perhaps enhanced, T mutant phenotype.

βB deficient mice may also have relevance to the study of tumorigenesis. α subunit mutant mice (inhibin deficient) develop gonadal tumors early in life with complete penetrance (Matzuk et al., 1992). In contrast the βB subunit mutant mice do not develop such tumors. Inhibin deficient mice would be expected to have increased levels of effective activin activity due to the lack of antagonist activity. The involvement of the βB gene in the mechanisms of tumorigenesis can be studied in α/βB double mutants.

In summary, the mutations in the βB subunit gene indicate that activins B, AB and inhibin B are dispensable for embryonic development in the mouse. The gene is, however, active during prenatal development, as revealed by the failure of eyelid closure in mutant neonates. Secondly activins B, AB and/or inhibin B function(s) later in adult female life to regulate events that surround parturition. A deeper understanding of the causes underlying these phenotypes may allow future study of processes relevant to both development and reproduction.

Methods

Cloning of the mouse activin/inhibin βB subunit gene and construction of targeting vectors

To clone the mouse βB subunit gene, a 129/Sv mouse strain genomic DNA library derived from the D3 ES cell line (Doetschman et al., 1985; gift of Drs. En Li and Doug Gray) was screened with a partial rat βB cDNA fragment (rinB-c2-P, gift of Dr Kelly Mayo, Northwestern University, Evanston, Illinois). A total of 23 kb of genomic DNA, centered on the βB subunit coding regions, were isolated in overlapping phages and restriction-mapped. Exons were mapped by Southern blot hybridization with exon-specific probes (see PCR cloning of mouse βB subunit exon probes). To construct the targeting vectors, the 17 kb λ1 phage insert was cloned into pBluescriptKS$^-$ (Stratagene), digested with Nar1 enzyme and religated, yielding a construct designated pλ1ΔNar1. This results in the deletion of a 1.3 kb Nar1 fragment that comprises the mouse coding regions homologous to the coding regions for amino acids 1 to 137 of the rat precursor chain (Feng et al., 1989b). For positive selection, a PGK-NEO-p(A) cassette derived from p(KJ1) (gift of Dr Michael Rudnicki; Tybulewicz et al., 1991) and ligated to Cla1 linkers, was inserted into the Nar1 site of pλ1ΔNar1. The resulting targeting vector containing the neo gene in the same transcriptional orientation as the βB subunit gene is termed pBB1, and pBB2 contains the neo cassette in reverse orientation. Both vectors have the same arms of homology, of 9.5 kb (5') and 6.0 kb (3').

The 3' breakpoint of the ΔNar1 deletion was confirmed by PCR amplification on tail DNA of a BB1 allele-specific fragment, "010"/ACT15b, that flanks the breakpoint of the deletion, using a PGK p(A) primer ("010", 5' AA-CGA-GAT-CAG-CAG-CCT-CTG 3') and an exon 1 βB primer (ACT15b) (see PCR cloning of mouse βB subunit exon probes). This yielded the expected fragment of 127 bp in animals carrying a BB1 mutant allele but not in (+/+) littermates. Moreover, the ACT16 primer (5' ATG-GTC-ACG-GCC-CTG-CGC-AA 3'), which lies within the ΔNar1 deletion, was used in conjunction with the ACT15b primer to amplify a 138 bp wild-type allele-specific fragment in (+/+) and (+/bb1) but not in (bb1/bb1) animals, further confirming the 3' breakpoint of the deletion.

PCR cloning of mouse βB subunit exon probes

A mouse exon 1 probe, mACT14/15, and a mouse exon 2 probe, mACT10/11;13, were cloned from D3 genomic DNA with the use of the Polymerase Chain Reaction. Published rat (Esch et al., 1987; Feng et al., 1989b) and human (Mason et al., 1989) sequences were used in the choice of primers. Limited degeneracy was introduced in the primer sequences. For mACT14/15, 700 ng of D3 genomic DNA was amplified using ACT14 (5' CGA-ATT-CCA-GGA-CAC-CTG-TAC-GTC-GTG 3') and ACT15

(5' GCG-GAT-CCC-TCT-GCA-AAG-CTG-ATG-AT(CT)-TC 3') primers (0.2 uM each) in a 50 ul PCR reaction comprised of 10 mM Tris-HCl, pH 9.0 at 25 C, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 100 ug/ml gelatin, 200 uM dNTPs and 1.5 U Taq polymerase (Cetus). Cycling parameters were 95 C for 1 min., 56 C for 30 sec., and 72 C for 1 min. (5 cycles) followed by 40 cycles of 95 C for 1 min. and 72 C for 2 min. The 318 bp PCR product was gel purified, digested with EcoRI and BamH1, and cloned into pKS. All 12 clones analyzed were identical and contained the Nar1 site at the homologous position to codons for amino acids 137-138 in the rat (Feng et al, 1989b). For mACT10/11;13, ACT10 (5' CGG-ATC-CGG-C(CT)T-GGA-GTG-(CT)GA-(CT)GG 3') and ACT11 primers (5' CGA-ATT-CCC-ACA-CTC-CTC-CAC-(AGT)AT-CAT 3') (0.5 uM each) were used with the same reaction components as above, and amplification was carried out by 40 cycles of 94 C for 1 min., 55 C for 1 min., and 72 C for 1 min. The 350 bp PCR products were purified and cloned into pKS as above. The ACT10 and ACT11 primers amplify both βA and βB gene fragments (see Mitrani et al., 1990; Thomsen et al., 1990) which are distinguishable by their restriction maps.

Transfection and selection of ES cells

The J1 ES cell line (Li et al., 1992) was established from a male 129/Sv embryo and grown essentially as described by Robertson (1987). ES cells were cultured on a feeder layer of γ-irradiated embryonic fibroblasts (EF) in Dulbecco's Modified Eagle's Medium supplemented with 15% heat inactivated fetal bovine serum (Hyclone), non-essential amino acids (Gibco), 10^{-4} M β-mercaptoethanol and antibiotics. Leukemia inhibitory factor (LIF) was added to the medium at 200-500 U/ml. G418^r EFs were prepared from E13.5 embryos carrying a targeted mutation in the β2-microglobulin gene (Zijlstra et al., 1989).

Two T75 flasks of subconfluent J1 cells at passage 9-10 were trypsinized and resuspended at 2.5 x 10^7 ml^{-1} in PBS. Linearized pBB1 plasmid DNA was added to the cell suspension at a concentration of 30 ug/ml, and cells were electroporated at 250 V and 250 uF using a BTX 300 electroporator. Electroporated cells were plated on feeder layers of G418^r EF cells at a density of 5 x 10^6 cells per 9 cm plate. Selection with G418 at 350 ug/ml (dry powder, Gibco) was initiated 20 to 30 hrs later, and carried out for 7-10 days. At that time individual G418^r colonies were picked, dissociated in trypsin, and plated on wells of feeder-covered 24-well plates. After a 3 to 4-day expansion, 2/3 of the cells in each well were frozen, and the rest further expanded in the absence of feeders for DNA preparation.

The pBB2 targeting plasmid was electroporated in similar conditions, but the cells were resuspended at 5 x 10^7 ml^{-1} in the presence of 25 ug linearized DNA and electroporated in one cuvette of a Bio-Rad Gene Pulser set at 800 V and 3 uF.

Screening of recombinant ES clones and animal genotyping

ES cell and tail genomic DNAs were prepared according to Laird et al. (1991), digested with EcoRI and submitted to Southern blot analysis, using a 850 bp BglII-BamHI genomic fragment as a probe (E in Fig. 1), which lies adjacent 3' to the right arm of the targeting constructs. Of 172 G418^r ES clones transfected with the pBB1 vector, 41 were homologous recombinants, as evidenced by the presence of a 8.2 kb recombinant EcoRI restriction fragment in addition to the ~20 kb wt fragment. Further characterization of the mutated alleles in 22 targeted clones was done using probe E on BamH1 or HindIII genomic digests, and using an internal probe corresponding to the other end of the targeting vectors (probe A, Fig. 1), as well as a neo probe. Probe A is a 1.5 kb SalI-BglII fragment corresponding to the 5' extremity of the left arm of the vectors. These analyses confirmed that 20 of the 22 clones had undergone the predicted homologous recombination event with no additional event. The same screening strategy was employed in the pBB2 transfection experiment. Ten ES clones among 58 that were screened contained the predicted 6.4 kb recombinant EcoRI fragment, indicative of the reverse orientation of the PGK-NEO-p(A) cassette in this allele. Final washes with both probes A and E were in 0.2 X SSC, 0.5 % SDS at 65 C.

Blastocyst injections and breeding of chimeras

Embryo manipulations were carried out as described by Bradley (1987). Subconfluent ES cells were trypsinized, resuspended in injection medium (45% DMEM, 45% Hepes-buffered saline, supplemented with 10% fetal bovine serum, 10^{-4} M β-mercaptoethanol and non-essential amino acids), and injected into either C57BL/6 or BALB/c blastocyst embryos. Resulting chimeric animals of both genders were bred to C57BL/6 or BALB/c mates. Both female and male chimeras transmitted the mutated allele to some of their progeny. To obtain the BB1 mutation in an inbred genetic background, a chimeric male which had demonstrated high chimerism of the germline was bred to 129/Sv females.

RNA analysis

RNA was isolated from tissue by the Acid Guanidinium Thiocyanate-Phenol-Chloroform extraction method (Chomczynski and Sacchi, 1987), electrophoresed on a 2.2 M formaldehyde, 1% agarose gel and transferred onto Zetabind membrane. Blots were hybridized to the mACT10/11;13 probe (see above) or a neo probe, consisting of a 600 bp PstI fragment of p(KJ1).

Protein analysis

Ovaries from individual 3-week old females were homogenized in 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, in the presence of 2 mM PMSF, 5 ug/ml Chymostatin, 5 ug/ml Pepstatin A, 5 ug/ml Leupeptin, and 25 ug/ml Aprotinin. Protease inhibitors were from Sigma. Extracts were spun and total protein in supernatants was quantitated by BCA assay (Pierce). Extracts were run in reducing conditions on 15% polyacrylamide-SDS gels and transferred to nitrocellulose. Equal loading of protein was verified by Ponceau S staining of the membrane after transfer. All subsequent incubations and washes were done at room temperature. Blots were blocked with 5% Carnation nonfat dry milk, 0.2% NP-40 in Tris-buffered saline (TBS; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl) for 30 min., and then incubated overnight in primary antibodies diluted in blocking buffer. After being washed once in 0.2% NP-40 in TBS and twice in 0.1% Tween-20 in TBS, blots were blocked again, incubated for an hour in anti-rabbit HRP-conjugate antibodies (Sigma), that had been diluted (1:10,000) in blocking buffer. Blots were then washed as above, rinsed in TBS, and detected by Enhanced Chemiluminescence (Amersham).

Anti-peptide affinity purified rabbit antibodies were a generous gift of Dr Wylie Vale (Vaughan et al., 1989). The βB-specific antibodies were directed against peptide (80-112)-NH_2 of the mature βB subunit and the βA-specific antibodies recognize peptide (81-113)-NH_2 of the mature βA subunit. Both antibody samples were used at a dilution (1:300). The anti-α subunit antibodies were directed against a human inhibin α(1-25)-Gly-Tyr peptide and were used at a (1:150) dilution. Specificity was confirmed using human recombinant activins A and B, and inhibin A as standards run in parallel in Western blotting assays. Human recombinant proteins were kindly provided by Drs Jennie Mather and Lynne Krummen (Genentech).

Histological analysis

Neonate mice were fixed in 10 % buffered formalin. Heads were dehydrated, paraffin embedded, and serially sectioned in the transverse plane. Sections were stained with hematoxylin and eosin. Abdominal mammary glands were processed similarly and sectioned longitudinally.

FSH measurements

Blood (0.5 ml) was taken by retroocular bleeding of animals that had been ether anesthetized. All mice used in the study shown in Table 4 were non-breeding animals. Serum was prepared and FSH values were determined by radioimmunoassay, as described by Matzuk et al. (1992).

Acknowledgments

We thank Kelly Mayo, Wylie Vale, Joan Vaughan, Jennie Mather and Lynne Krummen for the kind gift of reagents. A.V. wishes to thank En Li for his advice on ES cell culture; Jessie Dausman for her critical, dedicated help in blastocyst injections; Ruth Curry for expert animal care and Wu-teh Tu for histology; Gerry Thomsen for initial advice on PCR of the mature peptide coding region; Mike Frohman for initial encouragement and Peter Mombaerts for advice on targeting; Arlene Sharpe and George Mutter for interpretation of histology. We thank Jordan Kreidberg and Paul Soloway for their useful editorial comments. This research was supported by a grant from Amgen Inc. to KFL and National Institutes of Health grant R35 CA 44339-06 to RJ.

APPENDIX 1:

Functional Analysis of Activins during Mammalian Development

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ABSTRACT

Activins are hetero-dimeric (βA:βB) and homo-dimeric (βA:βA; βB:βB) members of the TGF-β superfamily.[1] The βA and βB subunits are expressed in multiple embryonic and extraembryonic tissues during murine development.[1-7] Gain of function and dominant negative loss of function experiments in *Xenopus laevis* and *Oryzias latipes* have suggested a role of activins in mesoderm induction and neurulation.[8-15] To define the *in vivo* functions of activins in mammals, mice with mutations in activin-βA or both activin-βA and activin-βB were generated. Activin-βA-deficient mice develop to term, but die within 24 hours of birth. They lack whiskers and lower incisors and have defects in the secondary palate including cleft palate, demonstrating a role of activin-βA in craniofacial development. Mice lacking both activin-βA and βB display the defects of both the individual mutants and no additional defects, indicating that there is no functional redundancy between these proteins in embryogenesis. In contrast to the suggestion from experiments in lower vertebrates,[8-15] zygotic expression of activins is not essential for mesoderm formation in mice.

Activins (Figure 1A) demonstrate high conservation during vertebrate evolution.[1, 8-10, 16-18] Components of the activin signal transduction cascade are expressed during embryonic development in fish, amphibians, birds, and mammals.[1-10, 14, 15, 17, 19-21] In the mouse, activin-βA and βB subunits are expressed zygotically prior to implantation,[23] but only in the maternal deciduum[3, 6, 7] until E10.5 when activin-βA begins to be expressed in mesenchymal cells of the developing face, whiskers, hair follicles, heart, and digestive tract.[7, 20, 21] This broad spectrum of expression and the extreme evolutionary conservation suggest that activins play a key role in mammalian development.

Mice deficient in activin-βB have been reported previously.[22] They are viable but exhibit a defect in eyelid development and impaired female reproduction but no apparent mesoderm defects.[22] To further address the functional roles of activins in mammalian development, mice with a disrupted activin-βA allele were generated using embryonic stem cell technology. Heterozygous ($act\beta A^{m1}/+$) mice were fertile and viable and were intercrossed to obtain $act\beta A^{m1}/act\beta A^{m1}$ mice (activin-βA-deficient). Genotype analysis of the progeny from these intercrosses (Figure 1C) revealed normal Mendelian ratios with 25.1% wild-type (68/271), 49.4% heterozygotes (134/271) and 25.5% homozygotes (69/271) indicating that $act\beta A^{m1}/act\beta A^{m1}$ mice survived to birth. The activin-βA-deficient mice (Figure 2A) appeared healthy at birth, they breathed normally, and were similar in weight to controls [$act\beta A^{m1}/act\beta A^{m1}$, 1.19_0.12g (n=22); littermate controls, 1.24_0.11g (n=23)]. Although $act\beta A^{m1}/act\beta A^{m1}$ mice progressed to term at the expected frequency, they died within 24 hours of birth. The $act\beta A^{m1}/act\beta A^{m1}$ mice lacked whiskers and incisors (Figure 2B-D), consistent with the expression of activin-βA mRNA in the mesenchyme of the whisker follicle and teeth primordia.[7, 20, 21] Analysis of skeletal preparations demonstrate the absence of lower incisors (complete absence in 22/25 mice) compared to controls (Figure 2B). Immature tooth buds could be detected in histological sections (Figure 3). Histologic analysis of the whisker-pads demonstrate an apparent delay in growth and/or differentiation of the whisker follicles (Figure 2E,F). Unlike control littermate mice, the root sheath, if present, appears immature, the cells at the base of the hair bulb often form an irregular pattern, and the hair papilla always lies closer to the surface (Figure 2F). Thus, activin A is required for the normal development of whiskers and teeth (incisors).

$act\beta A^{m1}/act\beta A^{m1}$ newborn mice, identified by the lack of whiskers, failed to suckle. Gross anatomical observations revealed that these mice had a cleft palate which is a major cause of the feeding problems. 12 of 42 (29%) hybrid background (C57Bl/6/129SvEv) and 3 of 9 (33%) 129SvEv inbred $act\beta A^{m1}/act\beta A^{m1}$ newborn mice demonstrated cleft secondary palate (Figure 3). Skeletal preparations of 22 $act\beta A^{m1}/act\beta A^{m1}$ newborn mice (hybrid background) which did not have clefts of the secondary palate revealed that 7 of 22 (32%) lacked a hard palate whereas the majority of the remaining mice exhibited incomplete development of the hard palate (Figure 3).

To generate mice deficient in all activins and therefore lacking both activin- βA and activin-βB subunits (Figure 1A), $act\beta A^{m1}/+$ mice were interbred with $act\beta B^{m1}/+$ mice[22] to generate compound heterozygous mutant mice ($act\beta A^{m1}$, $act\beta B^{m1}/+, +$). Compound heterozygous mutant mice were

interbred and compound homozygous mutant mice ($act\beta A^{m1}$, $act\beta B^{m1}/act\beta A^{m1}$, $act\beta B^{m1}$) deficient in all activins and inhibins were viable at birth (Figure 3F) and seen at the expected frequency (Table 1). $act\beta A^{m1}$, $act\beta B^{m1}/act\beta A^{m1}$, $act\beta B^{m1}$ mice were readily identified since they exhibit phenotypic characteristics of the single mutant lines. These mice lacked whiskers and had eyelid defects (*i.e.*, open eyes; Figure 3F), and appear to have died of palate defects with 3 out of 9 (33%) having cleft secondary palate. Furthermore, the $act\beta A^{m1}$, $act\beta B^{m1}/act\beta A^{m1}$, $act\beta B^{m1}$ mice were comparable in weight (1.08 _ 0.11 g; n = 9) to their littermates (1.08 _ 0.13 g; n = 35) and did not appear to have any additional defects that were not observed in the individual activin-βA or βB mutant mice.

The absence of whiskers and incisors and the defects in the formation of the secondary palate in the activin-βA-deficient mice are consistent with the expression of activin-βA mRNA,[7, 19, 20] and the *in vitro* effects of activin on chondrogenesis and bone formation.[23, 24] The cleft palate and mandible and incisor defects are similar to the defects seen in *Msx1*-deficient mice.[25] The incomplete penetrance of the "cleft" is reminiscent of human cleft palate and the multifactorial nature of its development.[26] Interestingly, mice with the recessive mutation, *oel*, had cleft palate, open eyelids, and died perinatally[27] similar to the compound homozygous activin-βA/βB mutant mice. The phenotype in the *oel* mice is consistent with a defect in a common downstream component of both the activin A and B signal transduction cascades. The observation that activin βA/βB double mutant mice have a phenotype which is an additive combination of the single mutant phenotypes indicates that the individual activin homodimers are not functionally compensating for one another during development and that the activin-βA:βB heterodimer does not appear to have a unique function during embryogenesis.

Although we have shown that zygotic activins are not essential for mesoderm induction in mice, a recent study with TGF-β1 mutant mice suggests that maternal TGF-β1 may cross the placenta to partially rescue the mutant mice.[28] Since activin-βB-deficient mice demonstrate normal mesoderm-derived tissue when delivered from activin-βB-deficient mothers,[21] clearly the maternal or decidual activin B is not essential. Similar studies with activin-βA-deficient females cannot be performed. The overexpression of activin subunits, receptors, and truncated receptors in *Xenopus laevis* and fish might influence mesoderm induction[8-15] because of interactions with other TGF-β family members[29-31] and their receptors. For example, a truncated *Xenopus* activin receptor type II has been shown to block the activity of Vg1, a related TGF-β family member.[29] Identification of the serine/threonine kinase receptors expressed around the time of mesoderm induction will give us a better understanding of the TGF-β-related proteins involved in this process. Lastly, mutant mice deficient in one of the activin receptors, activin receptor type II (ActRcII), (accompanying manuscript) do not overlap phenotypically with the activin-βA/βB compound mutants. This raises the question about the relevance of ActRcII, as a receptor for activin A and/or activin B during embryogenesis. Alternatively, the other type II activin receptor, activin receptor type IIB, might be the major receptor during embryogenesis. The final conclusion on specificity of the ligand/receptor interactions must come from analysis of ActRcIIB mutant mice and the demonstration of epistasis in compound mutant ligand/receptor mice.

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Figure 1. Structures of activins and inhibins, targeting of the activin-βA gene in ES cells, and generation of activin-βA-deficient mice. a, The dimeric forms of activins and inhibins. Absence of βA and βB subunits abolishes all activin and inhibin activity. b, The targeting vector to delete exon 2 (1.8 kb of sequence) of the activin-βA gene is shown. The mouse activin-βA gene is a two exon gene with an 8.8 kb intron. Exon 1 encodes the signal peptide sequence and 101 amino acids of the propeptide, and exon 2 encodes 181 amino acids of the propeptide and the 116 amino acid mature activin-βA peptide. Homologous recombination between this targeting vector and the endogenous mouse activin-βA locus deletes exon 2. The presence of a 4.0 kb fragment using a 5' probe or a 12.3 kb fragment using a 3' probe versus a 14.5 kb wild-type fragment upon restriction with BamHI is diagnostic of the mutant allele. c, Southern blot analysis of DNA from offspring derived from heterozygous matings. Genomic DNA (~5 ug), isolated from the tails of offspring from one litter, was digested with BamH1 and analyzed as described using a 5' probe. The presence of a single 4.0 kb fragment in the four lanes at the left indicates a homozygous mutant (-/-) genotype. The four homozygotes at the left did not have milk in their stomachs and lacked whiskers.

METHODS. 21.6 kb of isogenic DNA sequence encompassing the 2 exon mouse activin-βA gene was isolated from a mouse 129SvEv library. Linearized vector (25 ug) was electroporated into the hprt-negative AB2.1 ES cell line, selected in HAT and FIAU, analyzed by Southern blot analysis, and injected into chimeras as described.[32, 33] Enrichment in HAT and FIAU was 14.4-fold compared to HAT alone. Southern blot analysis of ES cell DNA identified 14 targeted clones out of 115 clones screened. The mutant allele was transmitted from chimeras derived from independent clones βA5-F12 and βA6-D5. To confirm the nature of the mutant allele, a cDNA probe encoding the activin-βA C-terminal (mature) sequence was hybridized to DNA from the $act\beta A^{m1}/act\beta A^{m1}$ mice. The lack of hybridization to a wild-type activin-βA allele confirmed that the mutant $act\beta A^{m1}$ allele was null (data not shown).

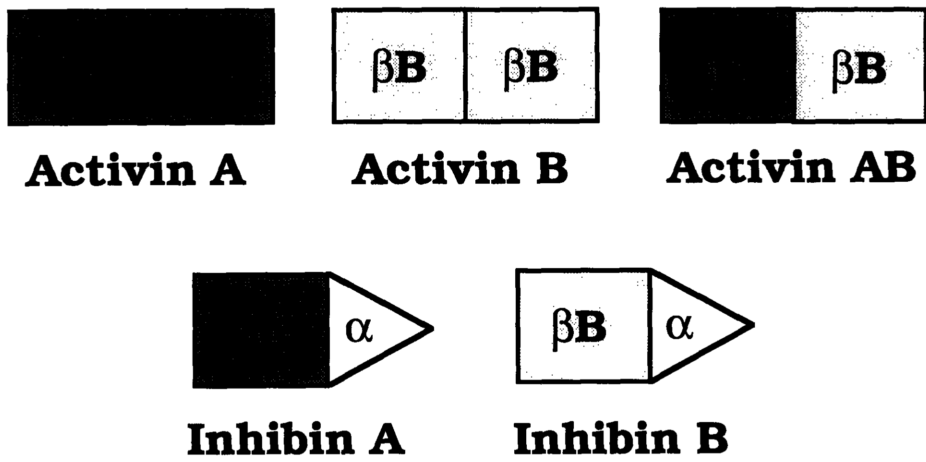


Figure 1A

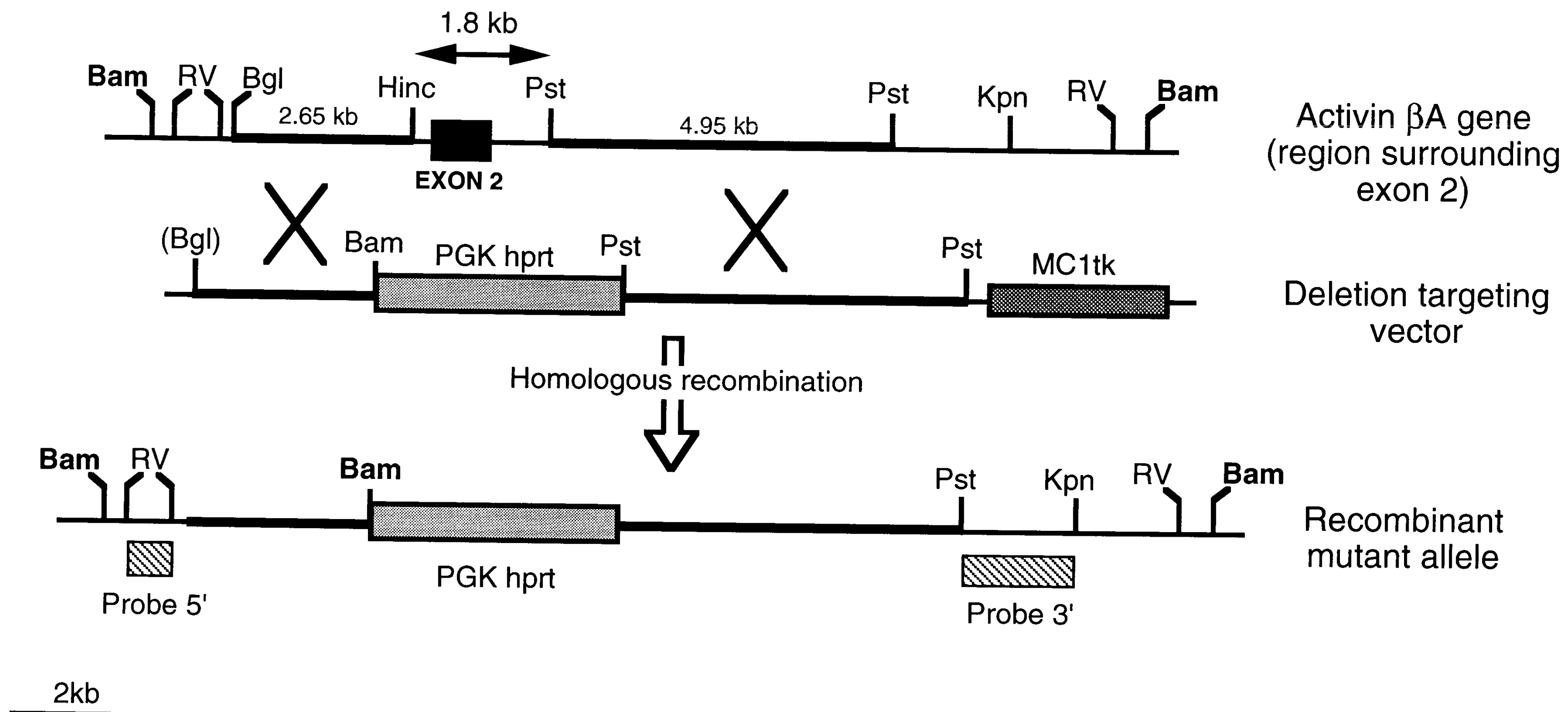
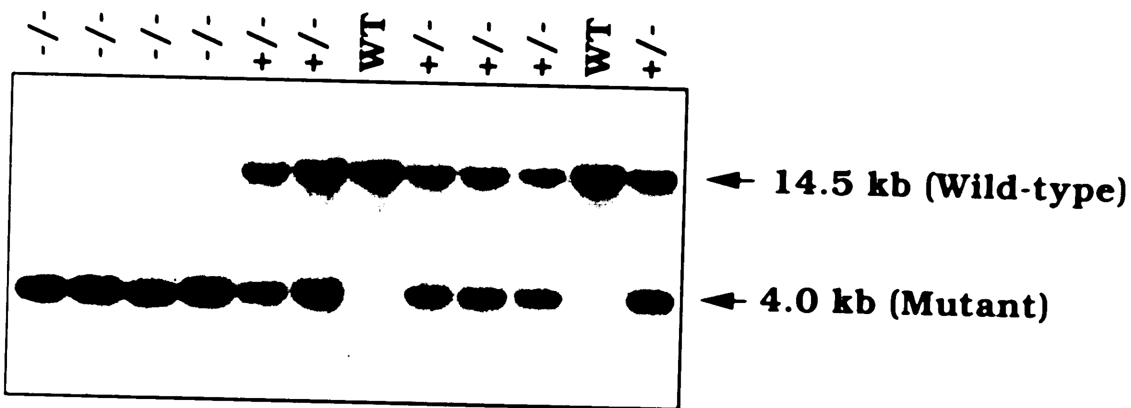


Figure 1B

SOUTHERN BLOT OF TAIL DNA

βA +/- X βA +/-



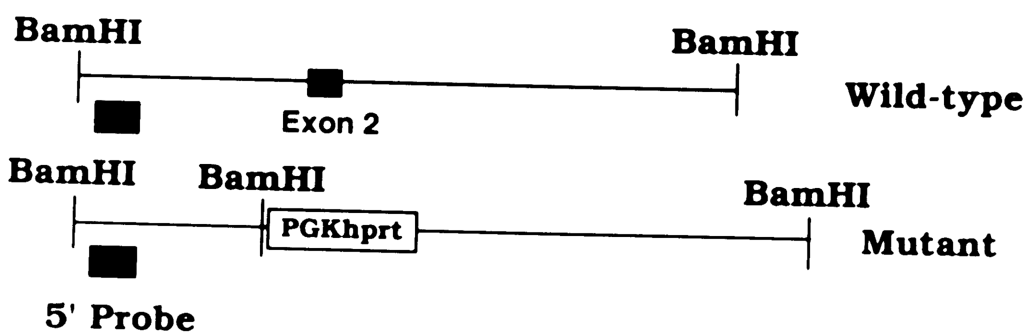
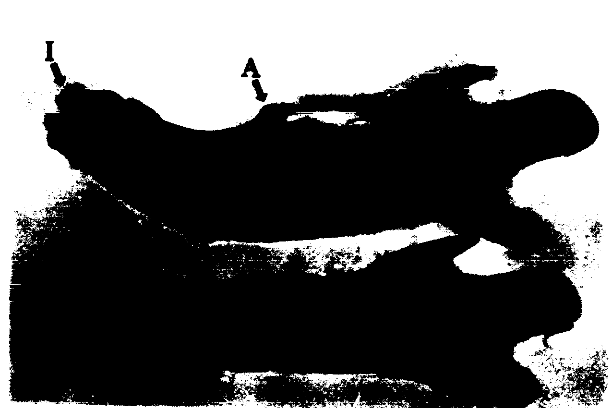


Figure 2. Morphologic and histologic analysis of activin-βA mutant and control mice. a, wild-type (left) and activin-βA-deficient homozygote (right) newborn littermate mice. Note the absence of milk in the stomach of the activin-βA-deficient mouse compared to the wild-type control (arrow). b, medial view of wild-type (top) and $act\beta A^{m1}/act\beta A^{m1}$ (bottom) dissected mandibles stained with alizarin red and alcian blue. The alveolar ridge (A) is less prominent and the incisor (I) is missing in the mutant. c,d, gross analysis of the whisker pads and face of a wild-type control (c) and $act\beta A^{m1}/act\beta A^{m1}$ (d) newborn mice demonstrating absence of whiskers in the mutant (d) mouse. e,f, histologic analysis (hematoxylin and eosin stain as described)[33] of the whisker follicles of control (e) and $act\beta A^{m1}/act\beta A^{m1}$ (f) mice. Photographs e,f, were taken at the same magnification. Transverse sections through the whisker pads were performed. Note that the distances of the hair papillae (P) from the surface in the mutant (f) are shortened compared to the control (e). In addition, the hair bulbs (B) in the mutants (f) are less well-formed than in the control (e). The root sheath (S) is obvious as it approaches the epidermis in the control (e), but is rarely visible in multiple sections examined for mutants.

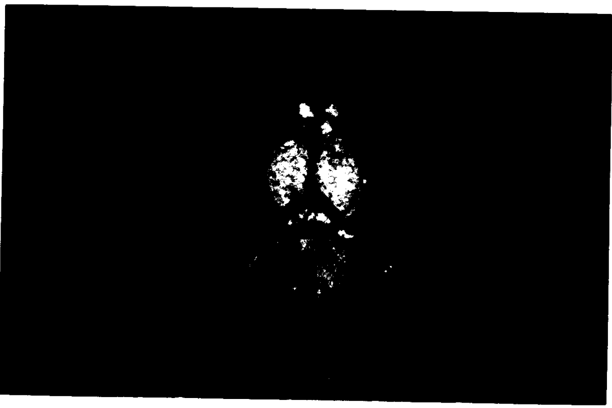
A

B

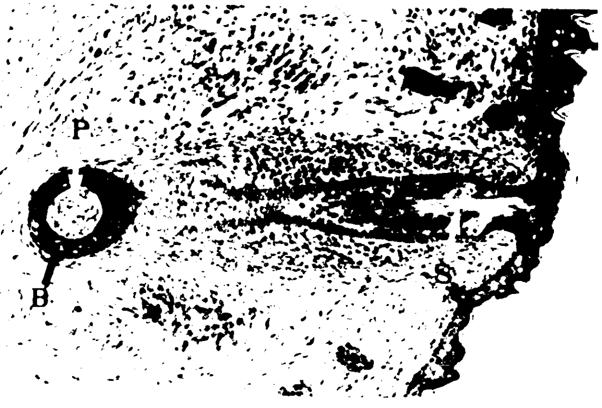


C

D



E



F

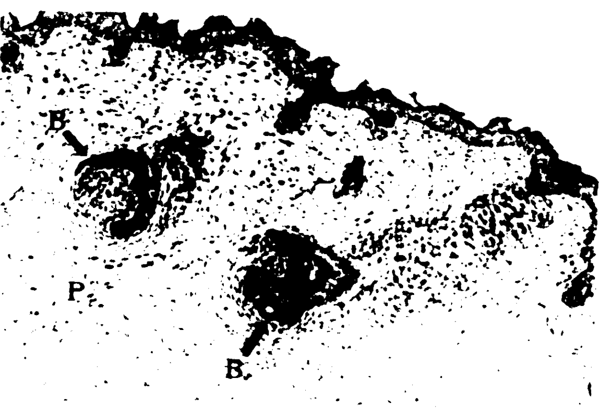
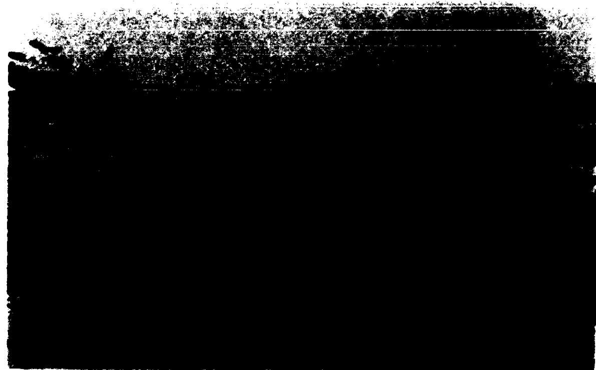


Figure 3: Histologic analysis of the palate and surrounding tissues (a-e) and morphologic analysis of compound homozygote mice (f). Coronal sections at the level of the eye of wild-type (a, b) and $act\beta A^{m1}/act\beta A^{m1}$ (c-e) mice. Note the normal joining of the lateral palatine processes to form the hard and soft palate in the wild-type mouse (arrow) photographed at low (a) and high (b) magnification. The hard palate (arrow) has not formed in the mutant photographed at low (c) and high (d) magnification which is evident by a lack of bone matrix and a "sagging" of the palate. There is a cleft palate (CP; absence of both hard and soft palates) in another mutant (e). f, newborn control (left) and compound homozygote $act\beta A^{m1}, act\beta B^{m1}/act\beta A^{m1}, act\beta B^{m1}$ (right) mice. Both mice were living at the time of the photograph. The mutant mouse (right) had open eyes and lacked whiskers and was approximately the same size and weight as the control.

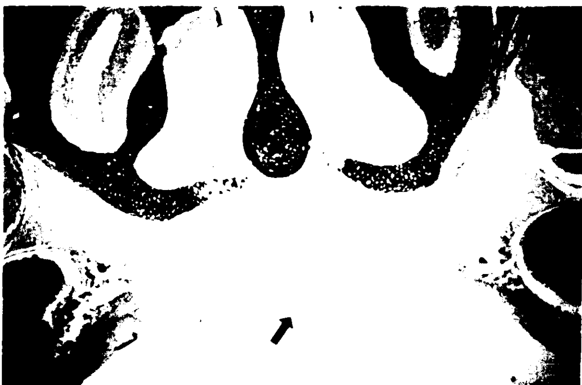
METHODS: Coronal sections through the palates were processed as described[32] and stained with alcian blue and neutral red. Southern blot analysis of DNA from newborn offspring born from intercrossing of compound heterozygous activin-βA/βB mutant mice was as described.[22, 23] Southern blot analysis to determine the activin-βA genotype utilized the 5' probe described above and analysis of the activin-βB genotype utilized a 3' probe as described.[22] Tail DNA was digested with BamH1.

A

B



C



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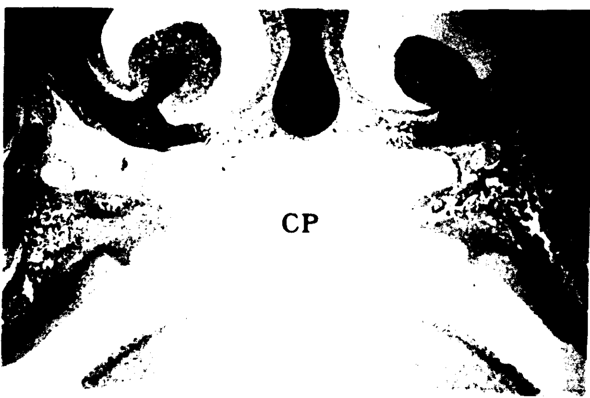
E



F

Table 1. Genotype analysis of offspring generated from compound heterozygous mice.

_A	_B	Number	Obs. %	Exp. %
WT*	WT	10	8.0	6.25
WT	+/-	13	10.4	12.5
WT	-/-	10	8.0	6.25
+/-	WT	11	8.8	12.5
+/-	+/-	34	27.2	25
+/-	-/-	16	12.8	12.5
-/-	WT	4	3.2	6.25
-/-	+/-	13	10.4	12.5
-/-	-/-	14	11.2	6.25
TOTAL		125	100.0	100.0

*WT, wild-type; +/-, heterozygote; -/-, homozygote.

PART 1 : DISCUSSION:

I will now briefly summarize the phenotype of the activin / inhibin βB deficient mice and the conclusions we can draw from it. Surprisingly, these mice did not show an overt mesodermal defect, or other early developmental failure. They are viable and suffer from eye defects and female reproductive dysfunction.

The limitations of single gene knockouts when they do not yield the expected phenotype will be discussed. Lack of a scorable phenotype may stem from the existence of overlapping, "redundant" pathways in development, to the activation of compensatory by-pass events that do not normally occur, or altogether from irrelevancy of the particular gene in the function studied. Further studies aimed at distinguishing these possibilities will be proposed.

1.8 *The phenotype of activin / inhibin B deficiency: i. Viability*

Upon intercrossing animals heterozygous for the βB subunit mutation, we obtained a frequency of 21% (n=223) (in the 129/Sv X C57BL/6 background), or 17% (n=158) (in the 129/Sv X BALB/c background) of weanling mice homozygous for the mutation, instead of the Mendelian expectation of 25%. Thus activin / inhibin B deficiency is not a lethal condition and may only be marginally deleterious to embryonic development. The generation of viable mice provided the definitive evidence that the single mutation in the βB subunit gene does not prevent mesoderm formation in mouse embryos. We cannot however conclude on the normality of early inductive events in βB deficient embryos, as embryos themselves have not been analyzed yet. Such an analysis may reveal alterations in morphogenetic pathways (see below). Our data, however, establish the dispensability in mice of activins AB, B, as well as inhibin B, for the generation of a normal body plan.

It is very difficult to speculate on the significance of this result with regard to other vertebrate species. A vast amount of literature exists on the roles of activins on mesoderm formation in frog and chick embryos. In addition to the data demonstrating expression in embryos and responsiveness of embryonic explants to activin A or B, there also is functional evidence for such a role. Interference with the activin pathway in *Xenopus* embryos by injection of a synthetic mRNA encoding a truncated activin receptor prevents mesoderm induction and axial structure formation (Hemmati-Brivanlou and Melton, 1992). As a control of the specificity of this effect, responsiveness to FGF is preserved in injected embryos. It nevertheless remains possible that the truncated activin receptor interferes in this experiment with signaling by TGFβ-like molecules other than activin. This caveat aside, the data point to the existence of an essential activin signaling pathway in *Xenopus* embryos. At this point of analysis, considering the lack of

mesodermal phenotype in activin B deficient mice, it would appear that either frog and mouse development differ in the roles played by activin B, or that a different factor (possibly activin A, see below) is able to participate in mesoderm formation in both species.

Despite the lack of an overt early defect, the βB mutant mice were found to suffer from distinct later developmental and reproductive defects and alterations which are described below.

ii Failure of eyelid fusion

βB mutant mice manifested a distinctive phenotype at birth. During late embryogenesis, mesenchymal cells of the eyelids normally proliferate to undergo apposition and eventual fusion of the eyelids at embryonic day 16 (E16). Mice are thus normally born with closed eyelids, and eyes re-open at ~13 days of postnatal life. In contrast, many βB deficient mice were born with open eyes, and quickly developed a series of secondary eye lesions.

It is intriguing to note that many apparently unrelated mutations similarly cause birth with open eyes in mice. This had suggested that the open eyelid phenotype may not be specific, but indicative of subtle morphogenetic alterations occurring in the context of a variety of genetic defects. For example, the process of eyelid outgrowth and fusion may be especially sensitive to sublethal developmental perturbations, such as found in c-abl and TGFα deficient mice, as well as in a variety of lines carrying mutations that have not yet been identified, all of which get born with open eyes (Lyon and Searle, 1989; Schwartzberg et al., 1991; Luetteke et al., 1993; Mann et al., 1993).

However, expression of the activin βB mRNA was recently demonstrated in a variety of specific locations in the postimplantation mouse embryo (Feinjen et al., in preparation), and this suggested a more specific role of activin B in the developing eye. Condensations of mesenchymal cells in the tarsus of the developing eye, which is precursor of the eyelid, were found to highly express βB mRNA at E12.5 of development. Activin B may thus act as an important, surprisingly specific, growth factor for these cells.

The eyelid fusion phenotype was found to depend on the genetic background of the mice. It was observed in the two hybrid genetic backgrounds tested (129/Sv X C57BL/6 and 129/Sv X BALB/c) (with incomplete penetrance), but was never observed in the 129/Sv inbred background. After four generations of backcross to C57BL/6, the eyelid fusion defect displays complete penetrance in mutant newborns. This suggests that the 129/Sv genome contains an allele(s) that acts as a suppressor of this phenotype.

In contrast to the eyelid defect, the other overt phenotype of the βB mutation (see next section) was fully penetrant in all three genetic backgrounds, suggesting that the 129/Sv suppressor acts specifically during embryonic development.

iii Female reproductive dysfunction:

As secreted factors, activins and inhibins potentially gain access to the developing embryo from maternal sources during gestation. Decidual or placental activins, for example, may participate in normal development of the embryo, and may rescue the zygotic null genotype. Additionally, as was mentioned earlier, the mouse oocyte contains both RNA and protein for activin. Thus initiation of development occurs in the presence of maternal activin. For these reasons, to fully address the question of the roles of activins during mouse embryogenesis, development must be assessed in the context of a maternal deficiency as well. This was made posssible by the fact that βB deficient females are viable, and they were thus tested for their ability to produce live offspring.

Furthermore, among the pleiotropic functions attributed to activins and inhibins are various activities in reproduction itself (for reviews, see Vale et al., 1990; Mather et al., 1992), thus prompting us to assess reproductive performances in both sexes. In adult animals, activins and inhibins are mainly produced by the gonads, and are thought to be important regulators of reproductive functions through their influence on both the pituitary and the gonads. In addition to their regulatory effects on FSH release from the anterior pituitary, intragonadal autocrine and paracrine activities on both somatic and germ cells have been described. For example, activins and inhibins were reported to affect spermatogonial proliferation in the male and follicular development in the female (Vale et al., 1990; Mather et al., 1992). When reproductive performances of mutant animals were assessed, mutant males were found to breed normally. On the other hand, mutant females showed a profoundly impaired reproductive ability.

Most βB deficient females generated few, if any, viable progeny in their lifetime. The impairment was found not to be caused by a defective oogenesis, or an unability to sustain gestation. Mutant females were found to be able to give birth to live, morphologically normal neonates including homozygous mutants, thus disproving the hypothesis that maternal activins rescue the βB mutant animals in utero. Rather, the poor fecundity of mutant females resulted from a maternal defect leading to perinatal lethality of the offspring. The nature of this defect is incompletely resolved. It may lie at multiple levels of pre- and postpartum female physiology, as suggested by the three following observations:

(1) Even though mutant females often failed to nurse their offspring, lactogenic lobular development was seen in mutant mammary tissue. Excess milk accumulation was observed in mutant ducts and lobules. This suggested a defect in milk let-down. In this context, it is significant that activins have been implicated in regulation of hypothalamic oxytocin secretion (Vale et al., 1990). Oxytocin is a nonapeptide implicated in milk ejection and uterine contractions.

(2) Postpartum histological analysis of mammary glandular and uterine endometrial tissue suggested hyperplasia of both epithelia (unpublished observations).
(3) Timed mating experiments have shown that mutant females have a prolonged gestation. Normal delivery occurred on day E19.5 of gestation (in 12/13 control females). In contrast, very few βB deficient females (1/13) had delivered on that day (Table IV, page 82). Mutant females delivered on day E20.5 or E21.5. Furthermore, they were occasionally found to experience a failure in the delivery process, resulting in death of the offspring in utero.

In summary, maternal βB expression was found to be dispensable for embryonic development of the offspring, as mutant females were able to give birth to live babies, but was found to be critical for their perinatal survival.

1.9 *Limitations of single gene knockouts and strategies to overcome them*

As the activin B mice illustrate, knowledge of the expression pattern of a gene and of the *in vitro* activity of its product are not sufficient for a prediction of its loss-of-function phenotype. Disruptions in, for example, the c-src, MyoD, or TGFα genes have also produced mice with a considerably milder phenotype than expected (Soriano et al., 1991; Rudnicki et al., 1992; Luetteke et al., 1993; Mann et al., 1993). c-src deficient mice develop apparently normally, but suffer osteopetrosis later in life. TGFα mutant mice are viable, but with wavy hair and whiskers, and mice lacking MyoD are viable and fertile.

When inactivation of a candidate effector in a particular process fails to reveal abnormality in this process, it is desirable to know whether this results from compensation by other genes (redundancy or use of a 'by-pass', alternate pathway), or rather because the gene in question simply does not participate in the process. Here I will describe three ways to address this question, and how they apply to the activin βB subunit mutant mice.

i . Spatial and temporal analysis of development

The disruption of a gene that acts during embryogenesis may not arrest development, but nevertheless alter its course. This may not be readily apparent in the adult mutant animal.

βB mutant embryos have not been analyzed. Morphological analysis and expression studies of the known activin response genes, and of other genes thought to participate in early inductive events, may indicate whether development is spatially or temporally altered. A delay in onset of expression of mesodermal genes, such as Brachyury, Evx1, goosecoid or Sna would indicate that activin B is involved in elaboration of mesoderm, but that compensatory pathways exist or are activated in βB deficient embryos. Such an analysis was very informative in the case of the Myf-5 gene (Braun et al., 1992). Myf-5 is temporally the first of a series of four myogenic Helix-Loop-Helix (HLH) transcription factor genes to be expressed in mouse embryos and transfection of a Myf-5 expression vector into a variety of cell lines converts them into muscle types. Most surprisingly, Myf-5 deficient mice develop normal muscle. When embryos were analyzed at stages of myotomal differentiation however, activation of expression of several muscle markers was delayed by several days. Therefore the Myf-5 gene belongs to a pathway of muscle differentiation that displays redundancy.

ii . Analysis of expression of related genes

Genes of the same multigene family as the mutated gene may be altered in their expression in the mutant animal.

Inactivation in mice of the gene that encodes the myogenic HLH transcription factor MyoD did not result in an overt phenotype. Remarkably, apparently normal muscle development occurs in mice lacking MyoD (Rudnicki et al., 1992). Molecular analysis revealed that mutant animals up-regulate the expression of the related HLH gene Myf-5. The latter gene itself is not essential for muscle development , as Myf-5 deficient mice, although lacking normal ribs, develop normal muscle (Braun et al., 1992). The role played by Myf-5 is however essential in the MyoD mutant to achieve normal muscle development, as the MyoD / Myf-5 compound homozygous mutant lacks skeletal muscle altogether (Rudnicki et al., 1993) (see below).

Expression of the two other activin / inhibin subunits (α and βA) in βB mutant immature ovary (3-week) was examined both at the protein and RNA levels. It was found that whereas inhibin α subunit levels were unaltered in βB mutant tissue, the related βA subunit was highly up-regulated (3- to 20-fold). This up-regulation is not accompanied by similar alterations at the RNA

level. It is therefore achieved by a post-transcriptional mechanism. Regulation may be translational, or may be at the level of peptide stability.

The finding of up-regulation of βA subunit in the βB deficient ovary opens the possibility that similar regulatory mechanisms operate in the embryo, and provide a means for normal morphogenesis in absence of activin B. Addressing this question requires the generation of a true activin-less embryo by inactivation of the βA subunit gene and intercross of both mutants (see below).

iii . 'Double KOs'

The full potential of the ES cell gene targeting technology becomes realized when individual single mutants are intercrossed, and multiple genetic interactions are studied in the context of the developing animal.

In one of the first genetic analyses of redundancy in the mouse, the individual roles of non-receptor src-like Tyrosine protein kinases, and the level of their mutual functional overlap, are being studied in single and compound targeted mutant lines (Soriano et al., 1991; Stein et al., 1992; Grant et al., 1992; Lowell et al., 1994). As an example, hck-/- ; fgr -/- mutant mice displayed an increased susceptibility to infection with *Listeria monocytogenes*, an intracellular bacterium, a phenotype not observed in either single mutant (Lowell et al., 1994). Therefore, either hck or fgr is required to maintain natural immunity in mice.

A genetic analysis of the family of insulin-like growth factors (IGFs) and their receptors, using targeted mutants in the IGF1, IGF2 and IGF1R genes, has revealed interactions between these genes, showing increasingly severe deficiency in body growth and viability in some of the compound mutants (Baker et al., 1993; Liu et al., 1993). In addition, a spontaneous deletion exists that encompasses the IGF2R gene, T-hairpin (T^{hp}). This mutation shows a mid-gestation dominant lethal effect when transmitted by the mother, due to imprinting of the IGF2R gene, expressed solely from the maternal allele. Interestingly, a rescue of the T^{hp} lethal maternal effect was achieved by crossing T^{hp}/+ females with IGF2-/- males and generating mice lacking both IGF2 and IGF2R expression (Filson et al., 1993). This suggested that the maternal lethal effect of the T^{hp} mutation is in fact due to defective clearance, and consequent excess of IGF2 in embryos that lack the IGF2R.

One of the most striking demonstrations of functional overlap within a gene family was provided by the MyoD / Myf-5 mutant mice (Rudnicki et al., 1993). As mentioned earlier, MyoD mutant mice have no overt phenotype, whereas Myf-5 mutants die perinatally because of defective thoracic cage formation. In the double mutant, however, there is a complete block in

skeletal muscle formation. Therefore, these genes can substitute for one another in skeletal muscle formation.

More subtle effects of gene dosage can be observed. Thus, whereas the lack of MyoD has no apparent muscle phenotype, combined with heterozygosity at the Myf-5 locus (in MyoD -/-; Myf-5 +/- animals), it leads to a lethal muscle insufficiency (Rudnicki et al., 1993). Therefore a recessive mutation (Myf-5) can show co-dominance if associated with a second mutation. As (MyoD +/-; Myf-5 -/-) animals have normal muscle, this also shows that, although the two genes possess overlapping functions in myogenesis, they differ in their dose-effect relations: one allele of MyoD appears sufficient to complete myogenesis, but one allele of Myf-5 does not.

In an attempt to determine whether activin B participates in early development, we have intercrossed the βB mutant with *Brachyury* heterozygous mice (T/+). The T mutation leads to a semidominant phenotype of tail-shortedness in heterozygotes, whereas homozygosity is lethal in mid-gestation. T/T mutants manifest a defect in migration of nascent mesoderm and a deficient notochord (see Beddington et al., 1992). It has been shown that the phenotype of various mouse T mutants is T dose-dependent, in that a graded severity in deficiency of posterior body development is observed as wild-type T function is diminished (Mac Murray and Shin, 1988). As inducers of T expression (Smith et al., 1991), activins may contribute to normal T levels. Thus an activin B homozygous deficient condition may further reduce T dose in T/+ heterozygotes, and potentially aggravate their phenotype, leading to further truncations in the posterior body, such as spina bifida. In T/T homozygotes, activin B deficiency might also increase the severity of the phenotype.

Upon intercrossing compound heterozygous animals, we have obtained a normal frequency of βB -/-; T +/- animals. These mice displayed eye defects and short tail, with no overt additional defect, indicating that at the heterozygous level, the T mutant phenotype was not aggravated by the lack of activin B. Although βB -/-; T -/- embryos were not analyzed, these preliminary data do not suggest a genetic interaction between the two mutations.

Activins A, AB, and B are thought to have identical activities, although different expression patterns. Matzuk et al. (in press) have generated mice carrying a deletion in exon 2 of the gene encoding activin / inhibin βA subunit. Homozygotes, deficient in activins A, AB and inhibin A, can be recognized at birth by their lack of whiskers. They also suffer defects in secondary palate formation, including sometimes cleft palate, and die shortly after birth from inability to feed. In order to create a mouse deficient in all activins, we have intercrossed the βB and the βA mutants. In compound heterozygote matings (βA -/+; βB -/+) X (βA -/+; βB -/+), all classes of progeny were recovered at birth in Mendelian frequencies. A fraction of the offspring displayed both defective eyelid fusion and absence of whiskers and died shortly after birth. These animals represented the compound homozygotes (βA -/-; βB -/-), deficient in all activins and all

inhibins. Thus, the compound mutant phenotype appeared no more severe than the sum of the individual phenotypes, indicating that no apparent functional overlap exists between the βA and βB genes. It can therefore be concluded that embryonic activins are dispensable for early mouse development. This is in contrast to the evidence in amphibians which points to an essential role of activins in mesoderm induction and axial structure formation (Hemmati-Brivanlou and Melton, 1992).

One caveat of the mouse experiments is that we cannot assess the importance for embryonic development of the expression of the βA subunit gene in the uterine decidua of the mother, since βA deficient females invariably die shortly after birth. The importance of maternal effects is described in the next section.

1.10 The importance of maternal effects

In *Drosophila*, genes that affect early development are of two fundamental classes, maternal or zygotic. Similarly in *Xenopus*, early development is governed by maternal determinants, stored in the egg in form of mRNAs or proteins. At the midblastula transition, zygotic gene expression takes over control of further development. In contrast, mouse embryos are already active in transcription at the late 1-cell stage, and are thus largely controlled by their own genes. Therefore, the genetics of early embryogenesis in mice are usually adequately addressed by performing intercrosses between parents heterozygous for the mutation of interest. However, in two sets of cases, heterozygote intercrosses are not adequate to determine the phenotype of a true 'protein knockout' in the embryo. These are the cases when the heterozygous mother contributes the wild-type gene product during development:

1) The female gamete itself can contribute wild-type RNA and/or protein to the early developing embryo. This is plausible in the case of activins, because mouse oocytes contain RNA and protein for both βA and βB subunits (Albano et al., 1993). In the fish *Oryzias latipes*, maternally provided activin, in contrast to zygotically expressed activin, was recently shown to be essential for mesoderm and axis formation (Wittbrodt and Rosa, 1994).

2) In all mutations that affect extracellular, secreted gene products, transfer of the wild-type protein potentially occurs from maternal sources during development and rescues the embryo. The maternal protein may be expressed by the uterine decidua, the placenta, or gain access to the embryo via the blood circulation.

For example, TGFβ1 mutant mice undergo apparently normal development in utero, but die shortly after weaning from a multi-focal inflammatory syndrome (Shull et al., 1992; Kulkarni et al., 1993). Considering TGFβ1 expression and postulated functions, it was a surprise that these mice lack an embryonic phenotype. Recently, it was shown that iodinated TGFβ1 administered

intracardially to the mother transfers efficiently to the fetus (Letterio et al., 1994). In addition, several tissues of TGFβ1 -/- neonates born from heterozygous mothers were found to immunostain for TGFβ1. In this study a single TGFβ1 -/- female was rescued to breeding age by daily injections of dexamethasone. Her four homozygous pups displayed severe cardiac abnormalities and died within one day, whereas three heterozygous littermates appeared normal. These data suggested that maternal transfer of TGFβ1 rescues the TGFβ1 -/- progeny to weaning age.

As mentioned above, these limitations in phenotype analysis apply to the activin β mutants as well. Initially, the oocyte contributes both activins to the early embryo (Albano et al., 1993). After implantation, both βA and βB subunits are strongly expressed in the maternal uterine decidua (Manova et al., 1992; Albano et al., 1994). In the case of the βB subunit, the viability of mutant females allowed to demonstrate that lack of both maternal and fetal βB subunit was compatible with normal development. In contrast, because βA mutant animals die at birth, the fertility of mutant females cannot be tested. It remains possible that an essential determinant of early development is supplied maternally in mammalian embryos, as activin A.

1.11 Constitutive KO vs inducible KO

It is important to reflect on the notion that inactivation of a gene throughout development ("constitutive knock-out") may give a distorted picture of the process we seek to understand. In the context of a constitutive gene inactivation, the developing organism may develop alternate, compensatory pathways, that do not prevail during normal development. These changes in developmental pathways may not be apparent in the final phenotype (e.g. a healthy mouse). On the other hand, a gene ablation that occurs only at the develomental stage of interest ("inducible or conditional knock-out") may give different answers. An inducible gene disruption is a better strategy to investigate the participation of the gene in a specific process. This is especially important for genes that are active, as activins, at several times of ontogeny. In the case of genes that are active both during development and adulthood, this strategy is necessary to investigate the adult function of the gene. For example, contrary to what in vitro evidence could have predicted, activin / inhibin βB deficient female mice undergo normal oogenesis. In the immature βB deficient ovary, however, expression of the other activin / inhibin βA subunit is highly upregulated. It is possible that this upregulation is critical to the development of a functional ovary, and that a gene inactivation specific to adult stages would lead to female sterility.

A variety of techniques are being tested to generate conditionally inactivated alleles, using conditionally-expressed site-directed recombinases to catalyze the gene inactivation reaction in a cell- or developmental stage-specific manner.

1.12 The real mesoderm inducer?

If not activins, what are the real inducers ? The gene mutated by the 413.d retroviral integration encodes an essential, non cell-autonomous function in mesoderm formation (Conlon et al., 1991), which when cloned proved to be a TGFβ family member (nodal; Zhou et al., 1993). Its expression in the mouse node is consistent with it being a direct player in the events that surround gastrulation. It will be very interesting to see whether nodal protein has mesoderm inducing activity and whether it is capable, as activins are, of inducing early-acting genes such as *Brachyury* and *goosecoid*. Another mutation has been shown to affect specifically mesoderm induction in mouse embryos, the *msd* mutation (mesoderm defective; Holdener et al., 1994). This mutation, contrary to the 413.d mutation, is cell-autonomous. Although primitive streak formation is blocked in both mutants, their phenotype differ markedly. The *msd* mutation has the features of a mutation that would affect the receptor or other downstream effector of a mesoderm inducing signal. The molecular cloning of the genes affected in such mutants, in combination with the targeting of known genes, will initiate a genetic understanding of mesoderm induction, and allow tests of epistasis. By comparison with the genetics of the zebrafish, for instance, it will be possible to appreciate to what extent these genetic pathways have been conserved throughout vertebrate species.

Table IV: βB deficient females show delayed delivery

Delivery

	E19.5		E20.5		E21.5
	AM	PM	AM	PM	AM
Control:	8/13	12/13	13/13		
Mutant:	0/13	1/13	7/13	8/13(1)	10/13(1)

(1) The remaining 3 females were sacrificed, and 2 had embryos dead *in utero*.

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APPENDIX 2 :

RANDOM INSERTIONAL MUTAGENESIS: THE *MOV34* EMBRYONIC LETHAL

ABSTRACT

Retroviruses can be used to create novel, random insertional mouse mutants. The retrovirus serves both as the mutagen and as a molecular tag to allow efficient cloning of the affected locus. A total of 66 mouse strains carrying single retroviral insertions were previously generated. When bred to homozygosity, 4 of them displayed an overt mutant phenotype. The *Mov34* strain carries an early embryonic lethal mutation. While *Mov34/Mov34* mutant embryos develop apparently normally up to the blastocyst stage, they arrest shortly after implantation, at day ~ E4.5-E5.5. Molecular cloning and analysis of the disrupted locus had identified a novel gene, termed *Mov34*, highly conserved among species including Drosophila, but with no known function. An unusual feature of the protein sequence was the presence of a highly charged C-terminal tail consisting of alternating positive and negative charges. Expression of the *Mov34* 1.7 kb transcript appeared almost ubiquitous in mouse tissues, suggesting that this gene may serve a general, "house-keeping" function. In this context, the question of the survival of the homozygous mutant embryos until implantation is of particular interest. In contrast to other vertebrates, early development in mammals is controlled primarily by embryonic gene expression, rather than by maternal determinants. We have been interested in assessing the causes that underlie the lethality of this mutant at the time of implantation . Here we show that the *Mov34* transcript is maternally encoded in the primary oocyte, remains present throughout continuation of meiosis, and is degraded in the one-cell embryo. During later preimplantation development, the transcript reappears at the 4-cell stage and becomes prominent by the blastocyst stage, behaving as a constitutively expressed mRNA. Therefore, the expression pattern of the 1.7 kb transcript is consistent with its deficiency causing the *Mov34* phenotype. This expression pattern may further indicate that maternal transcript provides sufficient Mov34 protein for survival until implantation.

We have generated anti-peptide antibodies directed against Mov34 sequences. These antibodies recognize a 39 kD protein in mouse cell lines. Preliminary evidence suggests that they stain an intracellular membrane compartment.

INTRODUCTION

The development of techniques in the early1980s that allow the introduction of cloned DNA into the germline of mice has transformed mammalian molecular genetics (Jaenisch, 1988). It became a major tool in the analysis of gene expression and function, but also permits to apply methods of insertional mutagenesis for the discovery of novel mouse genes, in a manner similar to the way transposable elements were used in the molecular genetic studies of yeast, maize or the fly (for a review, see Rudnicki and Jaenisch, 1991). The process of generating transgenic mice involves the integration of foreign DNA into host chromosomal sequences, and thus potentially disrupts or insertionally mutates endogenous genes at a certain frequency. Indeed most insertional mutations were initially recovered serendipitously when generating transgenic mice for a different purpose. The generation of mice carrying foreign DNA for the purpose of studying the expression and function of the transgenic sequences involves DNA microinjection into the male pronucleus of a one-cell embryo. A distinct way to efficiently introduce exogenous DNA into the germline of mice (which however does not, in most cases, leads to expression of the introduced sequences) is by retroviral infection of preimplantation embryos. Both methods of DNA microinjection and retroviral infection induce insertional mutations at similar frequencies of ~5-10 %. However, they highly differ in the way the exogenous DNA integrates into host sequences, and therefore in the resulting structure of the mutation and the clonability of the affected locus. Pronuclear microinjections lead most of the time to a profound alteration of the integration site, frequently with tandem arrays of copies of the transgene and complex rearrangements of the flanking sequences, including deletions, inversions, duplications and translocations. Cloning of the disrupted locus is in most cases extremely difficult. Retroviral infection instead produces integration of a single provirus, with only a few bp duplication on either side, and therefore is a superior method for mutagenesis, allowing efficient cloning of the flanking regions.

More recently, the advent of ES cell technology has contributed a major advance in the field of insertional mutagenesis, permitting a more rational and directed approach to the generation of mutants. The availability of cells in culture with a germ line potential allows to apply screening and selection procedures to enrich for insertional mutations before the production of an animal. This involves the design of new vectors that function as enhancer traps or promoter traps allowing to select for the insertions that occurred in the vicinity of, or within a gene. The use of vectors that express β-galactosidase only if integrated within a host gene from which they acquire regulatory control sequences allows to screen for expression patterns of interest upon introduction of the cells within a host embryo. Although in its simpler form, the ES cell approach selects for genes that are expressed in ES cells, it has proven to allow identification of genes with

a variety of expression patterns, including developmentally regulated ones. Improved strategies are being developed for generation of mutations affecting specific biological processes.

Initial experiments that used the retroviral infection approach employed the wild-type, replication-competent Moloney-Murine Leukemia Virus (Mo-MLV) (for a review, see Rudnicki and Jaenisch, 1991). However, replication of the virus often occurs in the animal, leading to viremia and leukemia. More recently, defective or replication-incompetent recombinant retroviruses can be produced from packaging cell lines in the absence of formation of wild-type virus. In a total of 66 mouse strains carrying individual proviral integrations, four displayed a mutant phenotype when bred to homozygosity. The *Mov13* strain was the first insertional mutant described. The phenotype of embryonic lethality at E13 was found to be associated with integration of the Mo-MLV provirus in the first intron of the $\alpha 1$(I) collagen gene. The recombinant retrovirus MPSVneo produced two other insertional mutants. The *Mpv17* mouse strain manifested a lethal nephrotic syndrome in homozygous adults (Weiher et al., 1990), and retroviral integration at the *Mpv20* locus resulted in preimplantation lethality (Gray et al.).

Infection of preimplantation embryos with a recombinant replication-competent Mo-MLV, containing a bacterial *supF* gene within each LTR to facilitate cloning of the targeted locus (Reik et al., 1985; Soriano and Jaenisch, 1986), was used to generate 31 transgenic mouse strains (*Mov15* to *45*). One of these, *Mov34*, exhibited a recessive lethal phenotype. Embryos homozygous for the *Mov34* proviral integration develop to blastocysts but die soon after implantation, before reaching the egg cylinder stage. The chromosomal locus containing the provirus was cloned and analyzed. Integration occurred in the first intron of a 7-exon gene. Unlike *Mov13*, proviral insertion occurred in the same transcriptional orientation as the host gene. The *Mov34* locus gene was found to express an abundant and widely expressed 1.7 kb transcript, whose sequence is widely conserved among species. The *Mov34* locus maps to mouse chromosome 8, human chromosomal region 16q23-q24, and the *Drosophila* homologue was mapped to 60B,C on chromosome 2 (Gridley et al., 1990). The murine and *Drosophila* proteins share 62 % amino acid identity, and have no significant homology with proteins of known function. The murine amino acid sequence predicts a protein of ~ 35 kD, displaying an unusual highly hydrophilic C-terminal domain, that consists of alternating positive and negative charged residues (Gridley et al., 1990).

The timing of lethality of the mutant shortly after implantation may indicate that, in the mouse, maternal components of the oocyte operate to rescue the homozygous mutant for 5 days of preimplantation development. Together, the conservation of *Mov34* sequences among species, the wide tissue distribution of the associated transcript, and the mutant early lethality suggest that, rather than a function specific to implantation events, the *Mov34* gene carries a function general to the biology of the cell or to the biology of tissues.

RESULTS

Expression of the *Mov34* mRNA in oocytes, preimplantation embryos and adult tissues

Mov34 homozygous mutant embryos die shortly after implantation. It has not been possible to recover any live conceptuses with a *Mov34/Mov34* genotype at any postimplantation stage. In intercrosses between animals heterozygous for the *Mov34* mutation, the frequency of dead or resorbed embryos is 25% at the egg-cylinder stage (E6.5), indicating that mutant embryos do undergo implantation, but fail very soon any further developement (Soriano et al., 1987).

Because the *Mov34* gene is an abundantly and nearly ubiquitously transcribed gene, it has been speculated to encode a general function, common to all cells. One hypothesis is that survival of homozygous mutant embryos during the 5 days preceding implantation is attributable to maternal rescue by the *Mov34* mRNA in the oocyte. To assess this possibility, we have examined mRNA expression in oocytes and later preimplantation stages. Northern blot analysis with a riboprobe was performed, as this method was previously shown to allow detection of specific maternal RNAs in single oocytes (Huarte et al., 1987).

Primary oocytes were isolated manually from the ovary. Care was taken not to include attached follicular cells, by repeated pipetting and visual inspection. Secondary oocytes were isolated from the oviducts of superovulated females. A single 1.7 kb transcript was previously identified at the *Mov34* locus (Soriano et al., 1987). Northern analysis of the oocyte samples revealed that the transcript was abundant as a maternal message in the primary oocyte (Fig.1, lane 1), and that in the course of meiotic maturation, the transcript decreased in size (lane 2), likely due to deadenylation of transcripts occurring at this time (see Discussion). A profile of expression of the *Mov34* transcript throughout meiosis and preimplantation stages is shown in Fig.2. Primary oocytes in lane 1 were separated from their zona pellucida, in order to rule out the possibility that the signal obtained in oocytes is contributed by the follicular cell extensions existing through the zona. The same signal intensity was obtained as in intact primary oocytes (compare lanes 1 and 2), confirming that the transcript is associated with the oocyte only. After reduction in size and amount in secondary oocytes (lane 4), the *Mov34* transcript was degraded after fertilization (1-cell embryos, lanes 5,6). Reappearance of the transcript by embryonic gene expression occurred at the 4-cell stage (lane 9), followed by accumulation throughout the morula and blastocyst stages (lanes 10 and 11). Thus the early expression pattern of the *Mov34* gene is the one of a constitutively active gene. Activation of the gene is concomitant with activation of the embryonic genome.

We next examined expression of the 1.7 kb *Mov34* transcript in adult mouse tissues (Fig.3), and confirmed its presence in similar amounts in all solid tissues tested (Soriano et al., 1987). In whole blood, however, the transcript was absent (not shown), as previously found (Soriano et al., 1987). In mouse cell lines, mRNA expression was found to be ubiquitous as well. Embryo Carcinoma (F9, before and after differentiation), Embryonic Stem cells, a variety of hematopoietic cells (EL4, MEL), and fibroblasts (3T3) were found to express the *Mov34* gene highly (not shown).

Raising of antisera to the predicted Mov34 protein sequence

In order to localize the *Mov34* gene product within the cell, and begin a functional analysis, we raised antisera in rabbits. The longest open reading frame found in the full-length *Mov34* cDNA (Gridley et al., 1991) consists of 321 amino acids. Two peptide sequences were chosen in this predicted amino acid sequence (see the boxed regions in Fig. 4) and synthesized chemically. Peptide 1 represents the 15-amino acid C-terminal of the protein. This encompasses about one half of the highly charged region, consisting of alternated positive and negative residues, which is found at the C-terminal of the Mov34 protein sequence. Peptide 2 is a 12-residue stretch consisting of amino acids 272 to 283 (Gridley et al, 1991).

Each peptide was coupled to thyroglobulin using SMCC as crosslinker. These peptide-thyroglobulin conjugates were used to immunize rabbits (2 rabbits per conjugate). We next synthesized 2 other conjugates using the same peptides, with BSA as carrier instead of thyroglobulin, and MBS crosslinker instead of SMCC. These conjugates share structurally only the peptide with the ones used as immunogens, and were used to monitor the peptide-specific response in the antisera. This analysis was done by Western blotting, where various amounts of the BSA-peptide conjugates were run on a SDS-PAGE gel. Each rabbit was found to have raised a strong response, exclusively of the expected specificity (not shown).

To verify that the antisera recognized also the peptides in the context of the whole protein, we next synthesized in vitro the Mov34 protein. The p28 cDNA was transcribed using SP6 RNA polymerase, the transcripts were isolated and used as templates in an in vitro translation reaction catalyzed by rabbit reticulocyte lysates. Whole antisera, or affinity-purified antibodies (see below), were then used to immunoprecipitate the translation products. Figure 5 shows that both rabbits immunized with peptide 1-conjugate (animals #1 and #2) produced antibodies that efficiently immunoprecipitated the *Mov34* gene product (lanes 5 and 8). This reaction was inhibited by an excess of peptide 1 (lanes 6 and 9), but not of peptide 2 (lanes 7 and 10). Rabbits immunized with peptide 2-conjugate (animals #3 and #4), however, manifested a much weaker response in this assay. One animal antiserum weakly immunoprecipitated the Mov34 protein (lane

11, clearly visible upon longer exposure), and the other not detectably. As the antibody response against the peptide 2-BSA conjugate as assayed by Western analysis was strong in both these animals (see above), this may indicate that peptide 2 is poorly accessible in the context of the whole polypeptide structure.

Identification of the Mov34 protein in mouse cells

We next sought to identify the product of the *Mov34* gene in mouse cells. #1 antiserum was titered on cytoplasmic + membrane (C) and nuclear (N) extracts of NIH-3T3 fibroblasts by Western analysis, and found to recognize in both cellular fractions, and at dilutions from (1:1,000) to (1:16,000), a protein of approximate 39 kD MW as major antigen (Fig. 6, lanes 4 and 5). The recognition was specifically abolished by co-incubation with competitor peptide 1 (lanes 6 and 7). At a dilution of (1:16,000), the 39 kD protein was the only band recognized. #2 antiserum at (1:16,000) also recognized a 39 kD band as only protein in the cytoplasmic fraction, but in addition strongly recognized a ~ 65 kD protein in the nuclear fraction (not shown). In this Western assay, antisera #3 and #4, raised against peptide 2, did not recognize a 39 kD protein (not shown).

Affinity purification of the antisera on columns coupled with the relevant peptides greatly enriched the titer of antibodies recognizing the 39 kD protein (Fig. 7). In this experiment, whole antisera #1, #2 and #3 (lanes labelled 'W') faintly recognized the 39 kD protein in cytoplasmic and nuclear extracts of 3T3 fibroblasts ('3') or EL4 lymphoma cells ('E'). However, the 39 kD protein was strongly recognized by all three corresponding fractions that were eluted from the affinity columns (lanes labelled 'A'). In cytoplasmic extracts from fibroblasts, antibodies from antisera #1 and #2 were specific for the 39 kD protein. However in nuclear fractions of fibroblasts and in EL4 cells, one to three additional protein species were also recognized. The relatedness of these other antigens to the *Mov34* gene product is unknown. In summary, antibodies raised against two different regions of the Mov34 protein both recognized a 39 kD protein antigen that partitions in cytoplasmic as well as nuclear cellular fractions. In the cytoplasm of fibroblasts, this is the only protein recognized by the anti C-terminal (peptide 1) antibodies.

Localization of the Mov34 protein in mouse cells

We used these antibodies in immunostaining experiments. 3T3 fibroblasts were allowed to attach on coverslips, and indirect cell staining was performed using FITC-labeled goat-anti-rabbit secondary antibodies. All 4 sources of antibodies stained a cytoplasmic, vesicular compartment, displaying sometimes a reticulate organization (Fig. 8). Using antibodies from #2 antiserum, some cells displayed in addition a punctuate nuclear pattern, that could be the only pattern present in

the cell, or that could be (even in an adjacent cell) associated with the punctuate cytoplasmic pattern (not shown).

This staining pattern, observed both with the anti-C-terminal and the anti-internal peptide antibodies, suggests that the Mov34 protein is associated with a membrane-bound intracellular compartment. The specificity of the staining, however, remains to be confirmed by competition with the cognate peptide. If this specificity is confirmed, the precise localization of the Mov34 antigen in the cell should be determined by performing co-staining in presence of markers of the various subcellular compartments in conditions of fixation that preserve the ER tubular network (Munro and Pelham, 1987). Subcellular fractionation experiments should complement these data. As the secretory apparatus of the cell can be morphologically and functionally manipulated with a variety of drugs and treatments, these should be used as well, and may provide informative clues to the function of this protein.

Figure 1. The *Mov34* mRNA is maternally encoded in the oocyte. Northern blot analysis of (OI) 205 primary oocytes isolated from ovaries, and (OII) 319 secondary oocytes isolated from oviducts of superovulated females. The probe is a T7 riboprobe transcribed using p28 linearized with ScaI as a template.

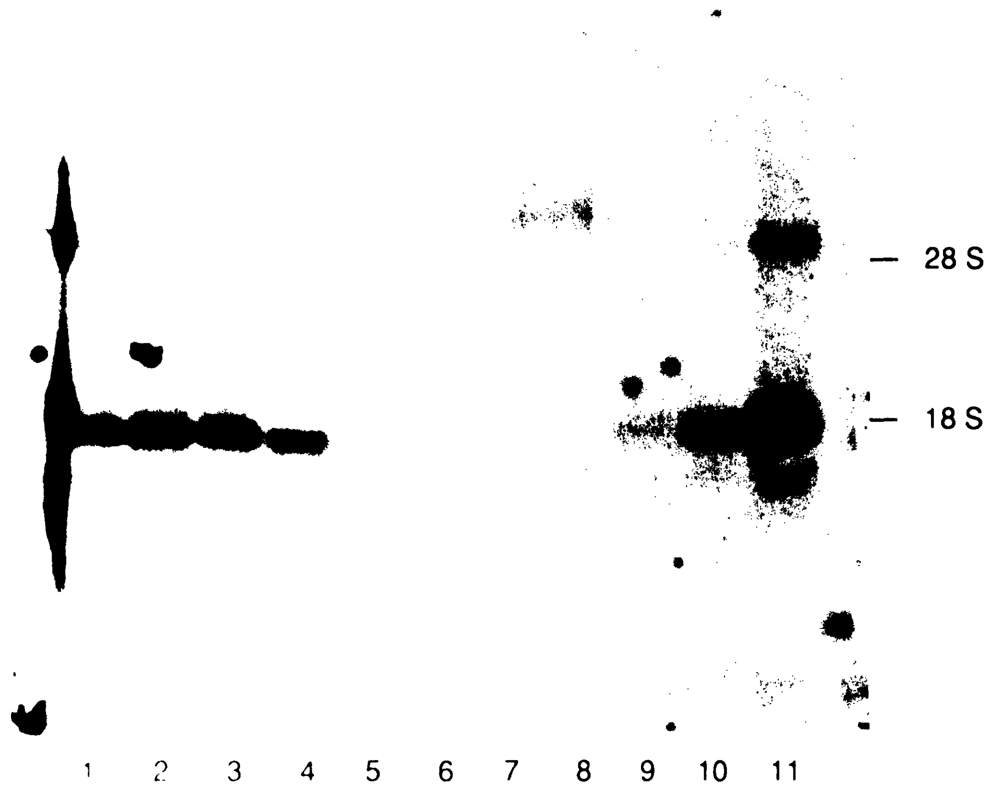

Figure 2. Expression of the *Mov34* mRNA during preimplantation development. Northern blot analysis of total RNA from (lane 1) 50 zona pellucida-denuded primary oocytes, (lane 2) 50 primary oocytes (strain FVB), (lane 3) 50 primary oocytes (strain 129), (lane 4) 50 secondary oocytes, (lane 5) 50 1-cell embryos,(lane 6) 47 1-cell embryos, (lane 7) 30 2-cell embryos, (lane 8) 31 2-cell embryos, (lane 9) 27 4-cell embryos, (lane 10) 28 morulae, (lane 11) 29 blastocysts; hybridized with p28 riboprobe as in Fig.1 This experiment was repeated two other times with similar results.

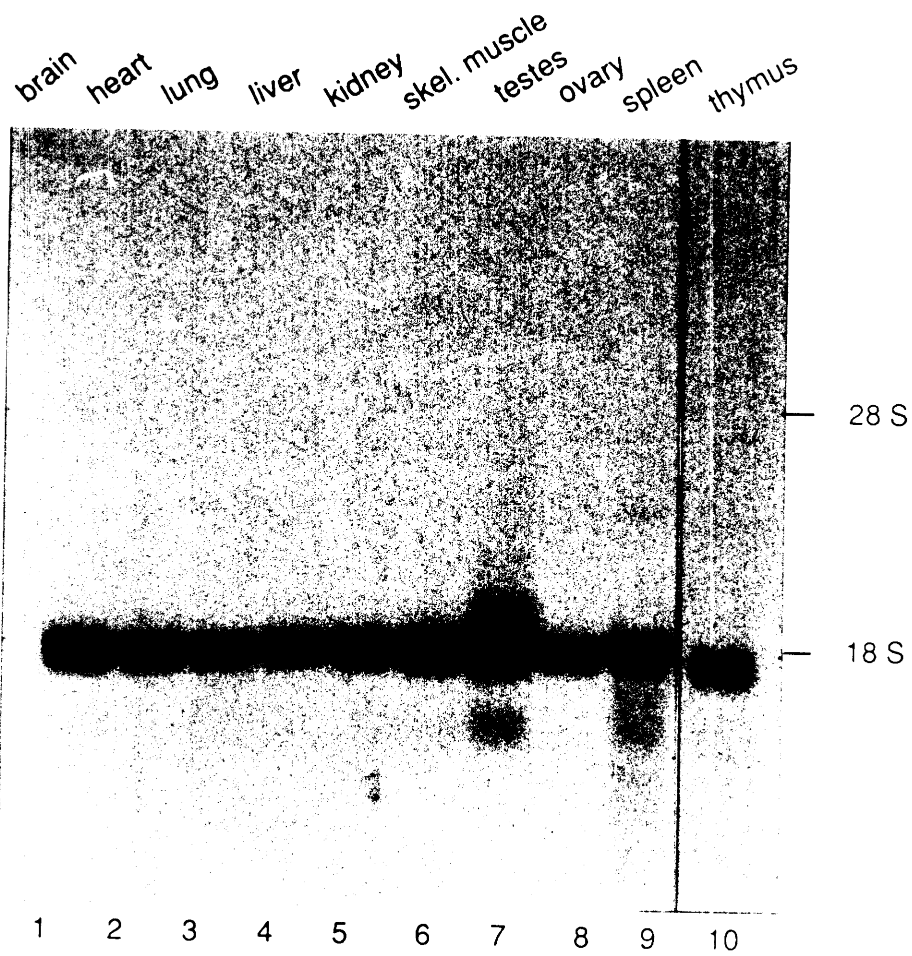

Figure 3. Expression of the Mov34 mRNA in adult tissues. Northern blot analysis of RNA from the organs indicated. Each lane contains 10 ug of total RNA, except 8 (ovaries) which contains half this amount, and 9 (spleen) which contains twice this amount.

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 -78  GAGGAGCGGCCTGTGTCGCTGCGGTTGCTGGTGTTTGCGGGTTGCAGGGAGCCGGGCCGG
 -18  TGGTCGGGCTGTGTCGCGATGCCGGAGCTGGCGGTGCAGAAGGTGGTGGTTCACCCCCTG
                          MetProGluLeuAlaValGlnLysValValValHisProLeu   14

  43  GTGCTGCTCAGTGTGGTGGATCATTTCAACCGAATTGGCAAGGTTGGAAACCAGAAGCGG
      ValLeuLeuSerValValAspHisPheAsnArgIleGlyLysValGlyAsnGlnLysArg   34

 103  GTAGTTGGTGTGCTTTTGGGATCATGGCAAAAGAAAGTACTTGATGTATCCAACAGTTTT
      ValValGlyValLeuLeuGlySerTrpGlnLysLysValLeuAspValSerAsnSerPhe   54

 163  GCAGTACCTTTTGATGAAGATGACAAAGATGATTCTGTCTGGTTTTTAGACCATGATTAT
      AlaValProPheAspGluAspAspLysAspAspSerValTrpPheLeuAspHisAspTyr   74

 223  TTGGAAAACATGTATGGGATGTTCAAGAAGGTCAATGCCAGAGAAAGGATAGTTGGGTGG
      LeuGluAsnMetTyrGlyMetPheLysLysValAsnAlaArgGluArgIleValGlyTrp   94

 283  TACCACACAGGCCCCAAACTGCACAAGAATGATATCGCCATCAATGAACTCATGAAGAGA
      TyrHisThrGlyProLysLeuHisLysAsnAspIleAlaIleAsnGluLeuMetLysArg  114

 343  TACTGCCCCAACTCAGTATTGGTCATTATCGACGTGAAGCCAAAGGACCTAGGACTTCCC
      TyrCysProAsnSerValLeuValIleIleAspValLysProLysAspLeuGlyLeuPro  134

 403  ACCGAAGCCTACATCTCAGTGGAGGAAGTTCATGACGATGGGACGCCAACGTCAAAAACT
      ThrGluAlaTyrIleSerValGluGluValHisAspAspGlyThrProThrSerLysThr  154

 463  TTTGAGCATGTGACTAGCGAGATTGGAGCAGAGGAGGCGGAGGAAGTCGGAGTGGAGCAC
      PheGluHisValThrSerGluIleGlyAlaGluGluAlaGluGluValGlyValGluHis  174

 523  TTACTAAGAGACATCAAGGACACTACAGTGGGGACTCTCTCCCAGCGGATCACAAACCAG
      LeuLeuArgAspIleLysAspThrThrValGlyThrLeuSerGlnArgIleThrAsnGln  194

 583  GTCCATGGCTTGAAGGGACTGAACTCCAAGCTCCTGGATATCAGGAGCTACCTGGAGAAG
      ValHisGlyLeuLysGlyLeuAsnSerLysLeuLeuAspIleArgSerTyrLeuGluLys  214

 643  GTAGCCAGCGGCAAGCTGCCCATCAACCACCAGATCATATACCAGCTGCAGGACGTCTTC
      ValAlaSerGlyLysLeuProIleAsnHisGlnIleIleTyrGlnLeuGlnAspValPhe  234

 703  AACCTGCTGCCGGACGCCAGCCTGCAGGAGTTTGTCAAGGCCTTCTACCTGAAGACCAAT
      AsnLeuLeuProAspAlaSerLeuGlnGluPheValLysAlaPheTyrLeuLysThrAsn  254

 763  GACCAGATGGTGGTGGTGTACCTGGCCTCGCTGATCCGCTCTGTGGTCGCCTTGCATAAC   pep2
      AspGlnMetValValValTyrLeuAlaSerLeuIleArgSerValValAlaLeuHisAsn  274

 823  CTCATCAACAACAAGATTGCCAACCGGGATGCCGAGAAGAAGGAGGGACAGGAAAAGGAG
      LeuIleAsnAsnLysIleAlaAsnArgAspAlaGluLysLysGluGlyGlnGluLysGlu  294

 883  GAGAGCAAGAAGGAGAGAAAAGACGACAAAGAGAAGGAGAAGAGCGACGCAGCGAAGAAA   pep1
      GluSerLysLysGluArgLysAspAspLysGluLysGluLysSerAspAlaAlaLysLys  314

 943  GAAGAGAAAAAGGAGAAAAAGTAAATGGTGTAGCTTTTTTAATTAGTAAATTAAAATCTT
      GluGluLysLysGluLysLysEnd                                      334

1003  ATAACCATAACTCCGTGTGCCACTAGGAGGGTCCTTTGTCACATTCAGTGCTTGTGGACA
1063  AGCGCTCTGCCCTGGTCACCCTTGCTGTGGCATTGTGGAGATGAGTGGACAAAAGGACGG
1123  ATCTCAGCCCTACGATACAGCTTCAGTTGCATGAGTTGGGGGTGTGGTGGCTCAGGTGTC
1183  CGACTTCATGAGGAGAACAACCTAGCCTTGGGCACTCTTCCTTTTATCCTTGAAAGGGAT
1243  TTTTTTTCCTATCTTACAAGATCCATGGGATCTGTTGGTTGTAATTTTCATAATTGCTAA
1303  ATGGAATTCATGGATCCCATACAATGCCGCCCAACACTTGAGCTTGGACCCTTGTGAACT
1363  TTTGAGACCACACCACCTTTTGGACCTGTAACTAGCATTCTTAGGGCTTGGGGTCCAAGG
1423  CCATGTATTGTTTACCTTCAAAGATACAATTAAATGGCTTGGTTTTTAAAAAAAAAAAAA
1482
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Figure 4. Sequence of the *Mov34* cDNA. Shown are the combined nucleotide sequence of the overlapping cDNA clones and the predicted amino acid sequence (Gridley et al., 1991). The adenylate residue of the predicted translation initiation codon is numbered nucleotide +1. Nucleotide numbering is shown to the left, and predicted amino acid numbering is shown to the right. The amino acid sequence of the two peptides used to raise antibodies are boxed, pep1 (C-terminal 15 amino acids) and pep2 (an internal 12-amino acid sequence). Wavy lines indicate the position of the hypothetical 21-residue membrane-spanning region. The central Arginine residue is circled.

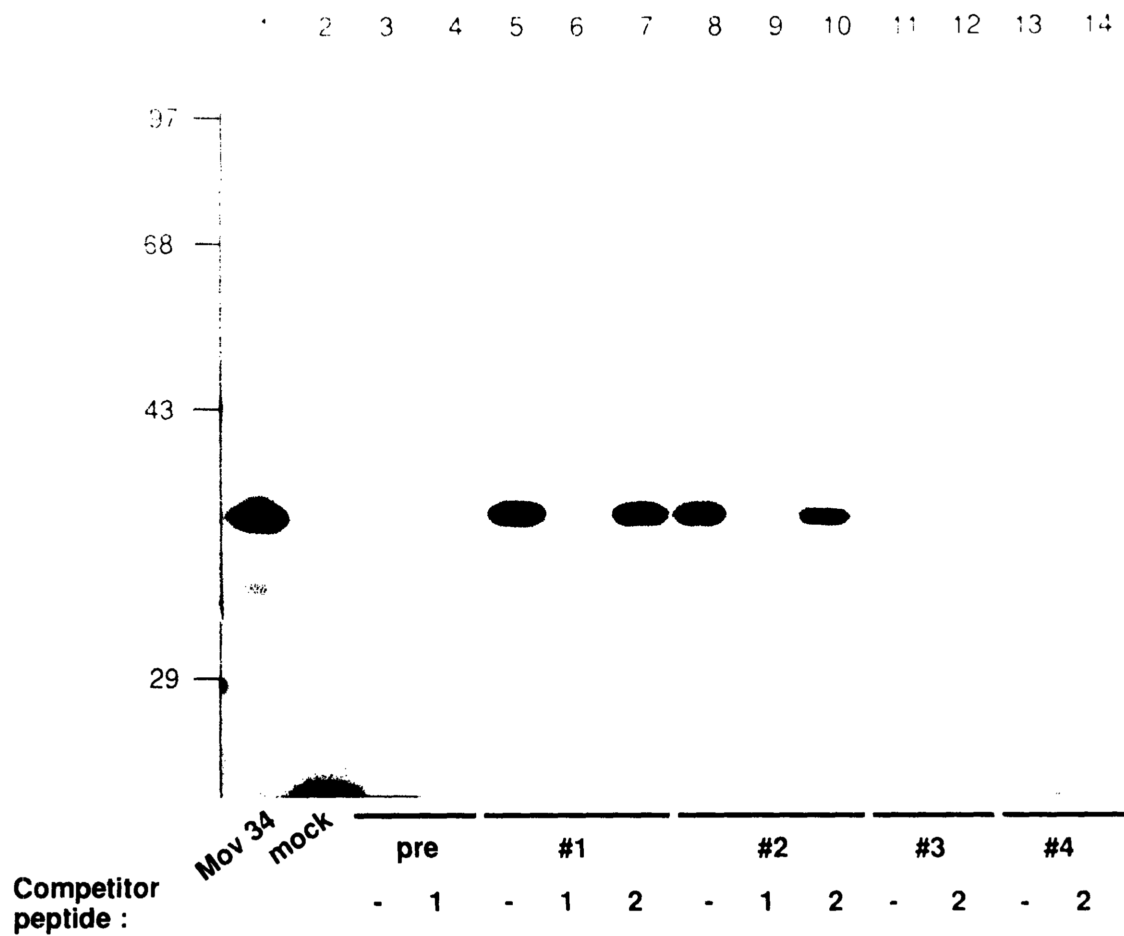

Figure 5. Immunoprecipitation of the *Mov34* cDNA product. The p28 cDNA was linearized with XbaI, and in vitro transcribed. Transcription products were used as templates in a rabbit reticulocyte lysate, and immunoprecipitated with anti-C terminal (#1 and #2), or anti-internal peptide (#3 and #4) affinity-purified antibodies, in presence or absence of competitor peptide 1 or 2. Peptide 1 corresponds to the C-terminal 15 amino acids of the *Mov34* open reading frame, and peptide 2 corresponds to a 12-residue internal sequence. Lane 1, 1 ul *Mov34* in vitro translation, lane 2, 4ul mock in vitro translation. Indicated on the left, Molecular Weight standards (kD).

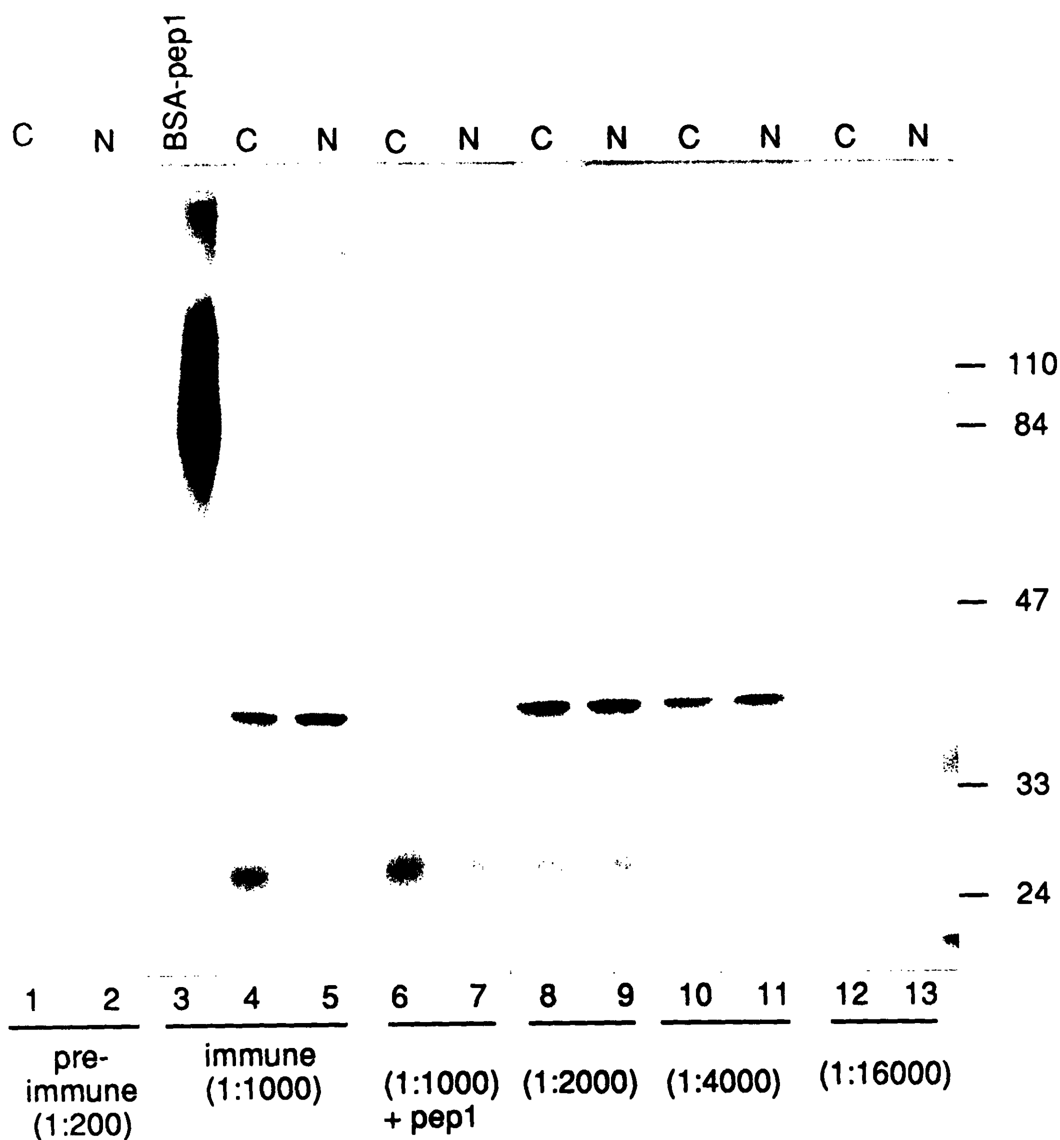

Figure 6. Identification of the 39 kD *Mov34* gene product in cell lines.
Western blot analysis of 3T3 fibroblast cytoplasmic and cell membrane fraction (C), or high salt nuclear extract (N), run in reducing conditions. The immunoblots were allowed to bind to antiserum #1 at the indicated dilution, in the presence (lanes 6 and 7) or absence of competitor peptide. Lane 3 contains BSA conjugated with peptide 1 (50 ng), as a positive control. On the right, approximate Molecular Weights (kD).

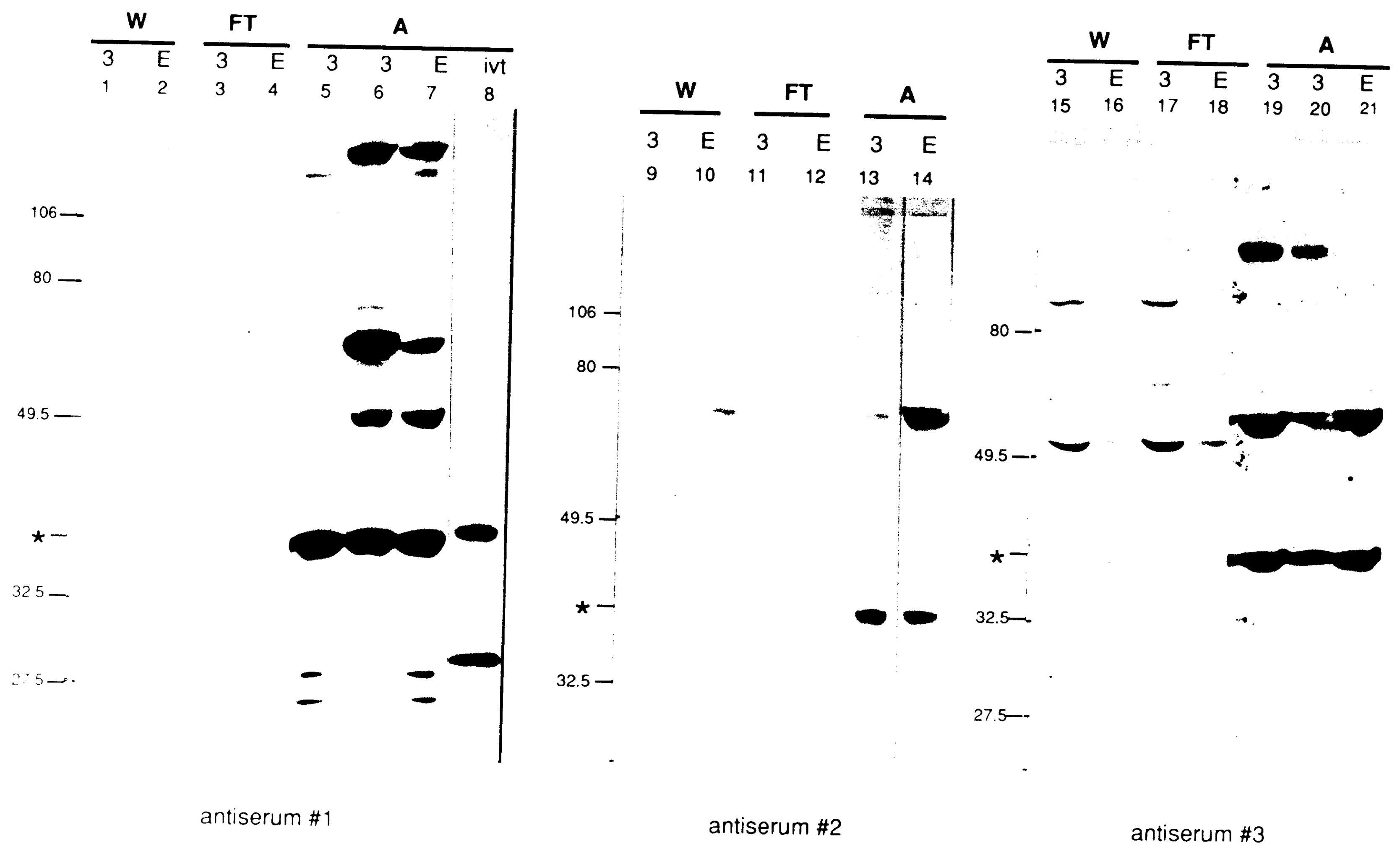

Figure 7. **Affinity-purification of antibodies.** Column fractions were tested by Western analysis of cell extracts from 3T3 fibroblasts(3) [illegible] EL4 lymphoma (E). All lanes contain 100 ug of total protein from cytoplasmic extracts, except lanes 6 (100 ug nuclear extract), lane [illegible] ug nuclear extract), and lane 8 (in vitro translation). Blots were incubated in dilutions of W, whole serum; FT, affinity column flow [illegible] A, affinity column eluate. The asterisk indicates the position of the 39 kD putative Mov34 gene product.

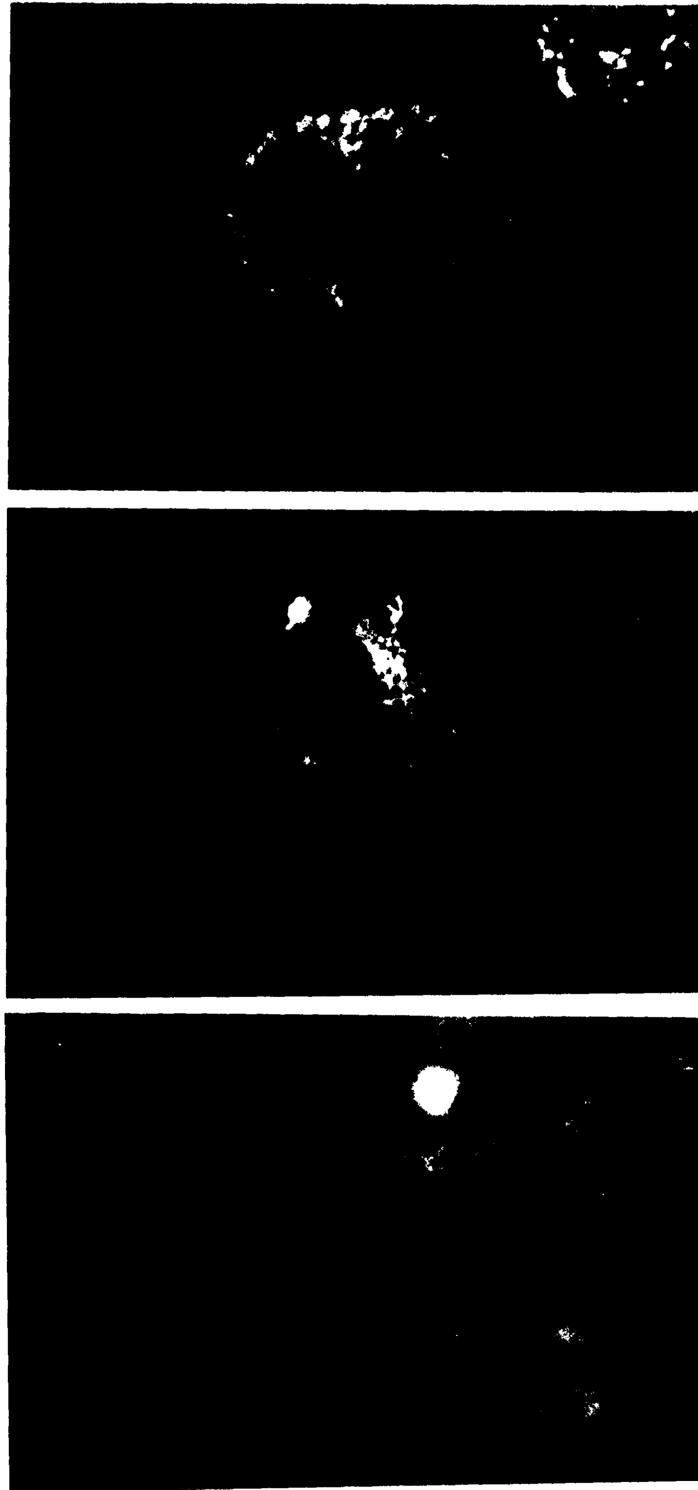

Figure 8. Indirect immunofluorescence of NIH-3T3 fibroblasts using affinity-purified anti-Mov34 peptide antibodies. Upper and middle panels. Anti C-terminal primary antibodies [antiserum #[illegible]]. Lower panel: Anti-internal sequence antibodies [antiserum #4]. The second antibody used [illegible] conjugated GARIg

DISCUSSION

The *Mov34* retroviral insertion is one of few mutations in the mouse known to arrest development after uterine implantation but prior to formation of the egg cylinder, and thus before the onset of gastrulation. Recently, targeted disruption of the *even-skipped* gene *evx1* was shown to cause early postimplantation lethality of the mouse conceptus (Spyropoulos and Capecchi, 1994). The *evx1*⁻ mutant phenotype manifests itself the earliest when compared to the other previously described, periimplantation, pregastrula mutants (Spyropoulos and Capecchi, 1994; and references therein). This mutant fails to differentiate extraembryonic tissues or to form a yolk sac cavity. *evx1* encodes a homeobox transcription factor that has been implicated in the response to growth factors through the TRE/AP-1 promoter element. The inadequate function of critical growth factors may provide an explanation for the mutant phenotype (Spyropoulos and Capecchi, 1994). Other mutants are known to form abnormal egg cylinders, with varying degrees of tissue disorganization (t^0, *fug1*, t^{w73}, lethal yellow (A^y), *velvet coat* (Ve), *blind* (Bld), and c^{6H}). Among these mutants, only *fug1* and lethal yellow were molecularly characterized and both shown to disrupt expression of a protein involved in RNA binding or metabolism (Michaud et al., 1993; DeGregori et al., 1994).

The *Mov34* mutant is similar to the *evx1*⁻ mutant in terms of timing, but the morphological basis of the lethality has not been fully described. Molecular characterization of the proviral integration site led to the identification of a 7-exon gene, in the first intron of which the recombinant Moloney-Leukemia Virus had integrated, apparently causing the lack of expression of the wild-type 1.7 kb transcript (Gridley et al., 1991). This transcript was found to encode a novel protein of predicted MW ~35 kD and to be widely expressed in adult mouse tissues (Soriano et al., 1987; Gridley et al., 1991).

In this study, we have analyzed the onset of expression of the *Mov34* gene in wild-type early embryos. We have shown that the 1.7 kb transcript is present as a maternal message in the primary oocyte. Resumption of meiosis preceding ovulation leads to a shortening of the *Mov34* transcript, as is observed for actin messenger RNA (Bachvarova et al., 1985). Shortening of the actin mRNA during meiotic maturation was shown to be the result of a deadenylation process that affects some mRNAs, while others are subject to further polyadenylation instead (Huarte et al., 1987). Similar to actin mRNA, the *Mov34* transcript was found to be degraded after fertilization. During preimplantation development, we first detected the *Mov34* transcript at the 4-cell stage, and observed its accumulation at the morula and blastocyst stages, preceding the appearance of the mutant phenotype in *Mov34/Mov34* embryos. Thus the early expression pattern of the 1.7 kb transcript is consistent with its deficiency causing the lethality in mutant embryos, and follows the

pattern of a constitutively expressed mRNA. We have raised antibodies directed against two distinct peptide sequences in the open reading frame of the cDNA corresponding to the 1.7 kb transcript, and found that both of them recognized a 39 kD protein antigen in mouse cell lines, consistent with the size of the *Mov34* gene product. Immunostaining of fibroblast cells using these antibodies revealed a punctuate/reticulate pattern, resembling an intracellular membrane compartment, such as the endoplasmic reticulum (ER). The observation that the 39 kD protein recognized in Western blots by the antibodies partitioned both in the cytoplasmic and nuclear fractions was consistent with an ER localization. These data, although very preliminary, therefore suggested that the *Mov34* gene product is an ER resident protein. Signals that confer ER localization to a protein have been known for both soluble and transmembrane proteins. A variety of soluble proteins that reside in the lumen of the ER contain the extreme C-terminal sequence KDEL, and this sequence was shown to constitute a signal sufficient to retain a reporter soluble protein in the ER (Munro and Pelham, 1987). Retention signals for transmembrane proteins have also been studied, and found to consist of less stringently defined short cytoplasmic sequences that occupy also the extreme C-terminal position in the protein, and that were present in proteins possessing a putative transmembrane domain (Nilsson et al., 1989; Jackson et al., 1990; Shin et al., 1991). Mutational analysis demonstrated that two lysines positioned three and four or five residues from the C-terminal, and a variety of related motifs, conferred ER retention to transmembrane proteins (Jackson et al., 1990). The Mov34 protein N-terminal displays a hydrophobic character and potentially acts as a signal peptide. In addition, the protein contains a ~21 amino acid stretch at positions 257 to 277 that displays a high hydrophobicity score (Eisenberg, 1984), with the exception that it contains an arginine residue exactly in its middle, at position 267. This region may potentially assume a transmembrane configuration. It is closely followed in sequence by the extremely hydrophilic C-terminal ~36 amino acid domain, which displays a characteristic alternation of positively and negatively charged residues, with 30 out of 36 residues being charged. This domain does not contain a KDEL sequence. In contrast, comparison between the motifs that confer ER retention to transmembrane proteins and the C-terminal of the *Mov34* gene product shows close similarities, although the exact sequence present in the *Mov34* gene product was not tested. The presence of these sequence motifs, together with the cell staining data, led us to hypothesize the following topology for this novel protein. The N-terminal and the bulk of the polypeptide (256 out of 321 amino acids) would be located within the ER lumen, the polypeptide would traverse the ER membrane, and the C-terminal tail would face the cytoplasmic compartment. Although no functional domain has been identified in the protein, such a topology would predict an intra-luminal function.

Future studies of the *Mov34* gene function and of the causes that underly the mutant phenotype should include a confirmation of the protein intracellular localization, in particular by

cell fractionation and co-staining of cells with markers of specific cellular compartments. Morphological analysis, using both light and electron microscopy, of the mutant embryos surrounding the time of lethality, appears essential for an understanding of the basis of the developmental arrest. A complementary approach to elucidate the function of the *Mov34* gene will be to derive Embryonic Stem cells from blastocysts produced by heterozygote intercross. This would allow a determination of whether the *Mov34* mutation is a cell-lethal, and if not, what competence *Mov34* deficient cells have for participating in normal development upon reintroduction into a host embryo. The present genetic background of the *Mov34* mutation (a BALB/c derivative) may however not be permissive for the generation of ES cells. Backcrossing animals carrying the mutation to C57BL/6, for example, may thus be necessary.

The prospect of the Mov34 protein being a novel ER resident membrane protein, if confirmed, is exciting. In view of the wide expression of the *Mov34* associated transcript in embryonic and adult tissues, and of the early lethal phenotype, it can be envisaged that the protein carries out an essential function in the cell secretory machinery, such as transport within the lumen, protein modification or targeting.

METHODS

Mouse oocyte and preimplantation embryo collection

Fully-grown primary oocytes were isolated manually from ovaries, and secondary oocytes were isolated from the oviducts of superovulated females, as described by Huarte et al. (1985).

RNA extraction from oocytes and preimplantation embryos

RNA from a small number of oocytes or embryos was prepared as described in Huarte et al. (1987). The riboprobe used in Northern blot analysis was synthesized using T7 RNA polymerase (Promega) on a template of p28 (Gridley et al., 1990), linearized with ScaI.

Raising of antisera

Two sequences of the predicted Mov34 protein (Gridley et al., 1990) were chosen and synthesized with an Applied Systems solid-phase peptide synthesizer. 'pep1' is the C-terminal 15 amino acid peptide: EKSDAAKKEEKKEKK. 'pep2' is an internal 12 amino acid peptide: LHNLINNKIANR (see Fig.4).

Each peptide was coupled to Thyroglobulin using SMCC cross-linker (from Pierce, see below), and Thyroglobulin conjugates were used as immunogens in New Zealand white rabbits (2 rabbits per immunogen). The primary immunization was a subcutaneous injection of 1 mg Thyroglobulin conjugate in complete Freund's adjuvent. Subsequent boosts were with 0.5 mg conjugate in incomplete Freund's adjuvent, and rabbits were bled 10-15 days after immunization.

To verify the reactivity of the antisera, each peptide was coupled to Bovine Serum Albumin (BSA) using MBS cross-linker (Pierce), and a titration of BSA conjugates were run on SDS-PAGE gels, transferred to nitrocellulose, and tested for reactivity with each antiserum. All 4 rabbits were found to have mounted an antibody response with the expected specificity.

Peptide-carrier coupling

10 mg of carrier protein (Thyroglobulin, BSA or Keyhole Limpet Hemocyanin (KLH)) was dissolved in 0.75 ml of PBS and stirred on a magnetic platform. 1.7 mg of cross-linker (SMCC or MBS), dissolved in 200 ul dimethylformamide, was added dropwise to the carrier protein, and the reaction allowed to proceed for 30' at r.t., with continued stirring. After addition of 1.55 ml of 0.1 M Sodium Phosphate pH 6, the unreacted cross-linker was removed on a Sephadex G-25 gel

filtration column (PD-10, Pharmacia) equilibrated in 0.1 M Sodium Phosphate pH 6.0. Peptide (5 mg) was then added to the purified reacted carrier, and allowed to couple overnight at r.t. with stirring. The conjugates were dialyzed against 0.1 M Hepes pH 7.4 at 4 C, and the absorbancy at 280 nm was measured.

Immunoaffinity purification of antibodies

To prepare the peptide antigen column, slurry of Affigel-10 (or -15, BioRad) was transferred to a glass fritted funnel and washed with 20 volumes of ice-cold deionized water. The moist gel cake (to create a ~2-ml bed volume) was transferred to a test tube, and peptide-carrier conjugate diluted in 5 ml of 0.1 M Hepes pH 7.4 (see section Peptide carrier coupling) was immediately added. The gel slurry was gently agitated on a rocker for 2 hrs at 4 C. To block the remaining active esters at the end of the reaction, 0.1 ml of 1 M ethanolamine-HCl (pH 8) was added and allowed to react for 1 hr at 4 C with agitation. The gel slurry was then poured into a column (BioRad Econocolumn). The supernatant draining off the column was collected and the coupling efficiency checked (see Harlow and Lane, 1988). The gel was washed with 0.1 M Hepes pH 7.4, until the OD280 of the eluent was zero. The gel was then submitted to a wash with 0.58% acetic acid (used later to elute specific antibodies), followed by a wash with PBS to restore the pH. A basic wash with 0.1 M triethylamine (pH 11.5) (also used to elute specific antibodies), followed by neutralization with PBS, was next performed. The column was then ready to use, or otherwise stored at 4 C, in PBS with 0.02% NaN3.

To affinity purify antibodies from serum, 4 ml of antiserum were added to 4 ml of PBS, and added to the antigen-coupled gel. The mixture was agitated by rotating overnight at 4 C. The column was then washed with PBS (20 bed volumes) until the effluent read zero at 280 nm. A high salt wash with 5 ml of PBS / 2 M NaCl was performed, followed by a wash with 20 bed volumes of10 mM Sodium Phosphate pH 6.8. Antibodies bound by acid-sensitive interactions were eluted from the column by passing 10 bed volumes of 0.58% acetic acid through the column, collecting 1 ml fractions (in tubes containing 110 ul 1 M Tris base, for quick neutralization of the antibodies), and monitoring absorbancy at 280 nm. Optionally, after a 10 mM Sodium Phosphate pH 8 wash, a basic elution step was carried out by passing 10 bed volumes of 0.1 M triethylamine (pH 11.5) through the column, and collecting 1 ml fractions in tubes that contained 110 ul 1M monobasic Sodium Phosphate. The column was restored to neutral pH with a PBS wash, and stored at 4 C for later use. The fractions which absorbed at 280 nm were pooled and dialyzed overnight against PBS, 0.02% NaN3. Antibodies were stored frozen in small aliquots.

In vitro translation and immunoprecipitation of the Mov34 gene product

The p28 plasmid was linearized with BamHI and used as template in a transcription reaction by SP6 RNA polymerase (Promega) in presence of $m^7G(5')ppp(5')G$ cap (Pharmacia). The RNA was translated in rabbit reticulocyte lysate (Promega). Methods were as suggested by the manufacturer.

The in vitro translation reaction (50 ul) was made to 1.2 ml volume with NET-BSA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 2.5 mg/ml BSA, 0.02% Na N3), and precleared by addition of 36 ul preimmune serum, incubation for an hr on ice, and adsorbtion on Protein A Sepharose (400 ul) for an hr at 4 C. After centrifugation in a microfuge for 30", the supernatant was split in 12 tubes, which were made to 500 ul each with NET-BSA. Each tube was incubated overnight on ice with 5 ul antiserum or 2 ug of affinity-purified antibodies, in the presence or absence of BSA-peptide conjugate (15 ug) as competitor. Protein A Sepharose (100 ul) was added, adsorbtion allowed for an hr at 4 C with agitation, and the tubes were centrifuged for 30". The beads were washed 2 X with NET-BSA buffer, 1 X with NET-BSA-0.5 M NaCl, and 1 X with 10 mM Tris-HCl (pH 7.5), 0.1% NP-40. Immune complexes were dissociated by addition of 40 ul of 2 X Laemmli sample buffer in presence of 0.1 M DTT. The tubes were heated at 90 C for 10', and loaded on a 10% acrylamide SDS-PAGE gel. The gel was fixed and fluorography was performed using Amplify (Amersham).

Cell protein extracts

Cells were harvested and the pellet washed 3 times in cold PBS and resuspended in 5-10 volumes of lysis buffer A (0.3 M sucrose, 20 mM Tris-HCl pH 8.0, 20 mM Hepes pH 7.3, 50 mM NaCl, 0.4% NP-40, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0) in the presence of 2 mM PMSF and 1 U/ml aprotinin. The lysed cells were immediately centrifuged for 5' at 1,500 rpm, and resulting supernatant was saved as the cytoplasm and membrane fraction (C). The pellet was washed once in buffer A, and resuspended in 5 volumes of lysis buffer B (25% glycerol, 20 mM Tris-HCl pH 8.0, 20 mM Hepes pH 7.3, 530 mM NaCl, 0.1% NP-40, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0) in presence of protease inhibitors as above. Nuclei were then incubated on ice for 20' and spun for 5' at 1,500 rpm. The supernatant was used as nuclear extract (N). Protein quantitation was performed using the BCA assay (Pierce).

Western analysis

Western analysis was as described in Vassalli et al. (1994). In some experiments (Fig.6), the alkaline phosphatase method (Lane and Harlow, 1988) was used for detection, instead of Enhanced Chemiluminescence (Amersham).

Immunofluorescence

NIH-3T3 fibroblasts were trypsinized, resuspended at 30,000 cells/ml, seeded onto sterile glass coverslips in 24-well dishes, and allowed to attach overnight. After 2 washes in Hepes-Buffered Saline (HBS) (37 C), cells were fixed in Methanol : Acetone (1:1) for 5'. Organic solvents were prechilled at -20 C and mixed just before use. Coverslips were allowed to air dry for 5', and washed 3 X 10' in PBS on a rotating platform. All subsequent washes were performed at r.t. with agitation on a rotating platform. Preincubation of the fixed cells was done in PBS / 1% BSA / 3% Normal Goat Serum for 1 hr at r.t. The primary antibodies were diluted in PBS / 1% BSA, with or without competitor peptide (as peptide-BSA conjugate), incubated on ice for an hour, spun in microfuge for 10' at 4 C, and applied onto the fixed cells in a volume of 100 ul or more. Binding was allowed overnight at r.t. Cells were washed 1 X 10' in PBS / 1% BSA, and 2 X 10' in PBS alone. Fixed cells were then preincubated as before, and incubated for 45' at r.t. in FITC or RITC conjugated Goat-Anti-Rabbit secondary antibodies that had been freshly diluted (1:100) in PBS / 1% BSA / 3% normal goat serum. Fixed cells were washed 3 X 10' in PBS, before mounting.

Immunofluorescence alternate protocol

Cells were seeded at 30,000 cells / ml / well onto sterilized glass coverslips in 24-well plates, and allowed to attach overnight. After 2 washes with HBS or PBS (37 C), cells were fixed with 3% paraformaldehyde in PBS for 10' at 25 C, washed 3 X 5' with PBS on a rotating platform, and permeabilized with PBS / 0.1% BSA with saponin at 0.15%, and rinsed 2 X 5' in PBS. After a preincubation step for an hr in PBS / 1% BSA / 0.01% saponin, the fixed cells were incubated overnight at 4 C in primary antibodies diluted in the same buffer. Cells were washed 3 X 10' in PBS / 0.01% saponin, preincubated as before, and incubated in secondary antibodies diluted (1:100) in PBS / 1% BSA / 0.01% saponin for 45' at r.t. Three washes of 10' each in PBS / 0.01% saponin were done, and the coverslips mounted.

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