DVR-4 (Bone Morphogenetic Protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction

C. MICHAEL JONES¹, KAREN M. LYONS¹, PETER M. LAPAN², CHRISTOPHER V.E. WRIGHT¹ and BRIGID L.M. HOGAN¹

¹Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
²Genetics Institute Inc., 87 Cambridge Park Drive, Cambridge, MA 02140, USA

Summary

Establishment of mesodermal tissues in the amphibian body involves a series of inductive interactions probably elicited by a variety of peptide growth factors. Results reported here suggest that mesodermal patterning involves an array of signalling molecules including DVR-4, a TGF-β-like molecule. We show that ectopic expression of DVR-4 causes embryos to develop with an overall posterior and/or ventral character, and that DVR-4 induces ventral types of mesoderm in animal cap explants. Moreover, DVR-4 overrides the dorsalizing effects of activin. DVR-4 is therefore the first molecule reported both to induce posteroventral mesoderm and to counteract dorsalizing signals such as activin. Possible interactions between these molecules resulting in establishment of the embryonic body plan are discussed.

Key words: mesoderm induction, posteriorization, *Xenopus*, DVR-4, activin.

Introduction

Mechanisms by which the embryonic body plan is established are being studied in species as diverse as the fruit fly, frog and mouse. *Drosophila* and murine systems emphasize genetic manipulation to study these problems, whereas the frog is amenable to dissection and biochemical studies. One of the best understood systems of embryonic patterning is the process of anterior axis specification in *Drosophila*. A gradient of bicoid protein initiates expression of genes such as *hunchback*, thereby establishing domains of anterior development. By contrast, specification of posterior regions of the fly embryo (the abdomen) requires the action of *nanos*, which seems to repress expression of *hunchback*, thereby allowing expression of posterior gap genes such as *krnps* and *giant* (reviewed by St. Johnston and Nusslein-Volhard, 1992). Therefore, it appears that in *Drosophila* an initial step in posterior development is the active repression of genes involved in anterior specification.

In contrast, molecular interactions leading to establishment of the vertebrate body plan are poorly understood. Fate mapping (Dale and Slack, 1987a) and tissue explantation experiments (Dale and Slack, 1987b) have demonstrated that mesoderm from different regions of the early *Xenopus* embryo gives rise to different tissue types. One model put forward to account for these observations is the "three signal hypothesis" of Slack and colleagues (Dale and Slack, 1987b; Smith, 1989). This theory proposes that two states of mesoderm are induced by signals originating in the vegetal pole and acting on the marginal zone of the amphibian embryo. The dorsovegetal signal induces extreme dorsal mesoderm, the future Spemann organizer, while the ventrovegetal signal induces ventral mesoderm, predominantly blood, mesenchyme and mesothelium. The third signal arises from newly induced dorsal tissue and dorsalizes the adjacent ventral mesoderm, thereby directing formation of the majority of the muscle in the amphibian embryo (Dale and Slack, 1987a). Since the formulation of this model, a great deal of effort has focused on identifying molecules that function as these signals. A major finding of these efforts is that these signalling molecules are peptide growth factors (PGF). The ventrovegetal signal is probably related to fibroblast growth factor (FGF) (Slack et al., 1987; reviewed by Smith, 1989). Moreover, recent studies involving microinjection of *wnt-1* and *wnt-8* RNAs show that a molecule of this class is capable of inducing a functional Spemann organizer (Sokol et al., 1991; Smith and Harland, 1991), and therefore is probably related to the endogenous dorsovegetal signal. The TGF-β family of growth factors is the third class of signalling molecules that has been implicated in axis specification. Isolated animal cap cells exhibit several thresholds of differential response to activin (Green and Smith, 1990), and consequently differentiate into either dorsal (high activin concentrations) or more ventral (lower concentrations) mesodermal cell types. These studies demonstrate that activin can function as a morphogen, a property that would be expected of a dorsalizing signal originating from the organizer region and acting on adjacent mesoderm. Of course, in vivo localization of these proteins and their receptors is absolutely required before firm conclusions can be reached.

One class of TGF-β-related molecules, which have expression patterns consistent with a role in many inductive
interactions during vertebrate development, is the DVR family (originally known as Bone Morphogenetic Proteins; reviewed in Lyons et al., 1991). In situ hybridization studies suggest that two members of the family, DVR-2 and -4, act as signalling molecules during murine development. For example, expression patterns in the myocardium of the early embryonic heart, the apical ectodermal ridge and mesenchyme of the limb buds, and the mesenchyme and ectodermal placodes of developing hair follicles are consistent with a role in epithelial-mesenchymal interactions (Lyons et al., 1990; Jones et al., 1991). In addition, DVR-4 transcripts are localized during gastrulation, first to the allantois at 7.5 days p.c., and by 8.5 days p.c., to posterior and ventral tissues in the primitive streak region (Jones et al., 1991). This led us to hypothesize that DVR-4 specifies posterior and ventral mesodermal cell types during late gastrulation stages of mouse development. Further support for this hypothesis came from reports that DVR-4 transcripts are present throughout early Xenopus development and that DVR-4-conditioned medium induces animal cap tissue to differentiate into mesoderm, albeit in a low percentage of the cases (Koster et al., 1991).

In this report, we use the Xenopus model system to test our hypothesis that DVR-4 specifies posterior or ventral mesodermal cell types. We use a combination of RNA microinjection, tissue transplantation and animal cap induction assays to test this idea. Taken together, our results suggest that DVR-4 counteracts the dorsalizing effects of activin, and by itself induces formation of mesoderm with posteroventral characteristics.

Materials and methods

Xenopus embryo manipulation

Eggs were obtained from hormonally stimulated Xenopus laevis females and were fertilized in vitro using testis homogenate. Dejellied embryos were allowed to develop as described (Cho et al., 1988) and staged according to Nieuwkoop and Faber (1967). Animal caps were isolated at stage 8, treated with the various factors in 0.5 x MMR (1 x MMR=100 mM NaCl, 2 mM KCl, 1 mM MgSO\(_4\), 2 mM CaCl\(_2\), 5 mM HEPES, pH 7.6, 0.1 mM EDTA) for several hours until rounded, and transferred to half-strength NAM (Asashima and Grunz, 1983) for long-term culture. Tissues were fixed in Bouin’s fixative, paraffin embedded, sectioned at 7 μm, and stained with hematoxylin and eosin.

Preparation of RNA and microinjection

A human DVR-4 cDNA (kindly provided by John Wozney, Genetics Institute) was amplified through PCR so that an NcoI site was introduced over the initiation codon (5′GACACCATGGTTCCTG- GTAACCG3′) and an XbaI site introduced in the 3′ region, approximately 60 bp 3′ of the stop codon. The amplified product was then cloned into NcoI/XbaI restricted pSP64-XJM (Krieg and Melton, 1984). The β-globin 5′ leader in this construct has been shown to direct efficient production of proteins when injected into oocytes and embryos (Wright et al., 1989). XbaI-linearized template was used to produce 5′- capped RNA using SP6 RNA polymerase (Promega) and standard methods (Krieg and Melton, 1987). RNA from three separate transcription reactions was used and each produced a protein of expected size when translated in a rabbit reticulocyte lysate (Promega). Injections were carried out with a Singer micromanipulator and a gas-driven injector (Narashige). Approximately 0.5-1.0 ng RNA was injected over a 5 second period for one-celled embryo injections and approximately 1-3 pg RNA was delivered to single blastomeres for dorsal and ventral injections at cleavage stages (32-64 cell stage). Presumptive dorsal and ventral sides of embryos were identified by pigmentation (Nieuwkoop and Faber, 1967), and embryos with regularly tiered cleavage patterns were chosen. A sample of siblings was always left to develop normally to ensure that the pigmentation criterion was accurate. Control injections used only buffer (Wright et al., 1989), RNA encoding β-globin, or RNA encoding a mutant form of DVR-6, which has no effect on development. Einsteck assays were performed by transplanting half an animal cap into the blastocoel of stage 10-10.5 host embryos, and pushing the transplanted tissue to the ventral side. Surgery was performed in 1 x MBS (Gurdon, 1976) using an eyebrow hair knife, and the embryos were allowed to heal for approximately 30 minutes, transferred back to 0.199 x MBS, and allowed to develop.

Results

DVR-4 RNA injection into one-celled embryos

Our initial experiments involved injecting synthetic human DVR-4 RNA into one-celled Xenopus embryos. This results in the ectopic expression of DVR-4 in all cells of the developing embryo. Development of the injected embryos proceeds normally through cleavage and blastula stages, but by neurula stages it is evident that no dorsal structures form. DVR-4-injected embryos develop through the formation of a normal dorsal lip (stage 10) and yolk plug (stage 11-12), and apparent closure of the blastopore (Fig. 1). However, histological examination of the DVR-4 injected embryos reveals that gastrulation movements are inhibited (approx. 99% of injected embryos show this phenotype). No mesoderm is seen below the blastocoel roof, but a clear delineation is evident between more deeply staining tissue, tentatively identified as mesoderm (molecular evidence presented below), and yolk endoderm (Fig. 2).

To assess the mesoderm-inducing activity of the DVR-4 protein made in vivo, we explanted animal caps from injected embryos and cultured them for approximately two days (sibling stage 32) before processing them for histology. Histological examination reveals that the DVR-4 loaded caps form vesicular structures, characteristic of ventral mesodermal differentiation in this assay (Fig. 2). In addition, we treated the loaded caps with activin at concentrations (10, 20 and 50 ng/ml) known to induce dorsal types of mesoderm (striated muscle and notochord) in animal caps. DVR-4...
Fig. 1. Effect of DVR-4 RNA injections on embryonic development. Dorsal views of stage 18 control embryo (A) and DVR-4-injected embryo (D) reveal absence of dorsal structures in the injected embryo. Comparing control (B,C) and injected (E,F) embryos at earlier stages reveals formation of a normal dorsal blastopore lip (E) and yolk plug (F) in DVR-4-injected embryos. Scale bar = 300 μm.

These animal cap assays suggest that DVR-4 specifies ventral mesoderm. Evidence that DVR-4 can direct formation of posterior tissues is illustrated in Fig. 3, using the Einsteck procedure, in which tissue is transplanted into the blastocoel of host embryos. The majority of DVR-4-loaded animal caps transplanted into the blastocoel of stage 10 host embryos direct formation of a secondary posterior axis (73%, n=22). In addition to external appearance, histological exam-

Fig. 2. Histological examination of DVR-4-injected embryos and loaded animal caps. (A) Examination of injected embryo (sibling stage 18) shows that gastrulation movements are inhibited but tissue resembling mesoderm develops, as judged by cell size and staining, (delimited from yolky endoderm cells by arrowheads). The collapsed blastocoel roof emphasizes absence of supporting mesoderm. Caps isolated from stage 8 DVR-4-injected embryos and then treated with activin (B) form structures (93%, n=57) containing a large mass of tissue resembling the mesoderm-like tissue shown in A. Caps isolated from DVR-4-injected embryos and cultured without further treatment (C) form vesicles with mesothelium and blood-like cells (90%, n=31). Scale bars = 100 μm (A) or 50 μm (B,C).
ination shows that these secondary axes contain both notochord and somite arrays, indicative of true secondary axes. In control Einsteck experiments, animal caps from uninjected sibling embryos produce no secondary axes, while un.injected caps treated with 20 ng/ml activin induce extensive secondary axes containing head structures (Fig. 3). DVR-4-loaded caps treated with activin (10-50 ng/ml) never induce secondary anterior axes, consistent with the overriding of activin effects in explanted caps. In a few cases (10%), structures resembling a secondary posterior axis were formed, but in the majority of cases, the implanted tissue remained as an enlarged bump, with no external axial morphology (data not shown).

**Analysis of gene expression in DVR-4 injected embryos**

A variety of markers for different embryonic tissue types were used to assess the effects of DVR-4 at the level of gene expression. We used cardiac actin which is specific for muscle, and Brachyury (XBra; Smith et al., 1991), which is expressed initially in the marginal zone, and later around the blastopore and in the notochord, wnt-8 (expressed in ventral mesoderm), and *Xenopus DVR-4*. In addition, we chose the homeobox gene *Xhox 3* as a marker of posteroventral mesoderm. *Xhox 3* is first expressed predominantly in mesoderm, and has a graded distribution in early development, with higher levels of expression in posterior than in anterior regions (Ruiz i Altaba and Melton, 1989a).

In DVR-4 injected embryos, XBra transcripts increase transiently relative to controls after zygotic transcription begins, reaching a peak approximately 4-fold above control levels by stage 10 (Fig. 4A and data not shown). Subsequently, XBra RNA levels decline. Since XBra expression becomes restricted to the notochord after mesodermal invagination (Smith et al., 1991), and is also expressed around the blastopore during neurula stages, the absence of expression in injected embryos at these stages probably reflects the fact that morphogenesis and the progression of dorsal mesodermal differentiation is halted in the DVR-4 injected embryos. Likewise, cardiac actin RNA levels are undetectable in the injected embryos (Fig. 4A), consistent with the absence of striated muscle. In contrast, a dramatic increase in the level of *Xhox 3* is seen in the injected embryos, first evident at the midblastula transition (data not shown) and reaching high levels by stages 10 and 13 (Fig. 4B). The *Xhox 3* expression suggests that mesoderm induction occurs in the injected embryos, (Ruiz i Altaba and Melton, 1989a), and that the mesoderm is most likely a posterior or ventral type, since it does not express markers of dorsal mesoderm.

RNAse protection analysis also reveals that injection of human DVR-4 induces expression of the endogenous *Xenopus DVR-4* genes (Fig. 4A). Endogenous DVR-4 levels are approximately 2-fold more than control levels shortly after the midblastula transition, and rise to a 7-fold difference by sibling stage 18. This 7-fold difference is maintained at least until siblings reach stage 35 (data not shown). Thus, DVR-4 may upregulate expression of the endogenous *Xenopus DVR-4* genes and/or affect decay of its mRNA. Finally, wnt-8 transcripts remain at control levels until at least sibling stage 35 (Fig. 4A and data not shown).

**Localized misexpression of DVR-4 in cleavage stage embryos**

The results described above, in particular the dramatic increase in *Xhox 3* expression in injected embryos, suggest that DVR-4 inhibits the formation of anterior-dorsal mesoderm, and instead, specifies mesoderm of a posterior-ventral
Table 1. Mesodermal differentiation induced by purified, recombinant human activin and DVR-4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ciliated epidermis</th>
<th>Dorsal* mesoderm</th>
<th>Ventral* mesoderm</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39/39 (100%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Activin 10 or 20 ng/ml</td>
<td>-</td>
<td>42/58 (81%)</td>
<td>6/58 (11%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>DVR-4† 20-200 ng/ml</td>
<td>-</td>
<td>47/47 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Activin 10 or 20 ng/ml</td>
<td>-</td>
<td>9/60 (15%)</td>
<td>36/60 (60%)</td>
<td>15 (25%)</td>
</tr>
<tr>
<td>DVR-4 10 or 20 ng/ml</td>
<td>-</td>
<td>-</td>
<td>77/88 (88%)</td>
<td>11 (12%)</td>
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<tr>
<td>DVR-4 100 or 200 ng/ml</td>
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Data presented as number of caps forming particular mesodermal tissue types per number of caps treated, as determined by morphological examination. Representative caps from each experiment were sectioned and examined histologically to confirm phenotypes.

*Samples were scored as dorsal mesoderm if they contained muscle and notochord or ventral mesoderm if vesicular structures containing mesothelium and blood-like cells were present. Numbers represent a combination of three experiments.

†Similar results were observed in a total of seven separate experiments using 10, 20, 50, 100 or 200 ng/ml DVR-4.

In one other experiment, 50 ng/ml of the same DVR-4 sample induced a ventral mesodermal phenotype in a majority of animal caps. At present, we attribute this result to variability among embryo batches and stress that it does not change any conclusions of this paper.

ND = mesodermal tissue type could not be determined by morphology.

Fig. 4. (A) RNAse protection analysis of gene expression in control (−) and DVR-4-injected (+) embryos. Expression profiles of the molecular markers used suggest that development proceeds normally in the DVR-4-injected embryos until gastrulation stages, when expression of the dorsal mesodermal markers, Brachyury (XBra) and cardiac actin, is dramatically reduced, while wnt-8 and DVR-4 continue to be expressed. Human DVR-4 autoinduces expression of endogenous Xenopus DVR-4 (2-fold) shortly after the midblastula transition (Stage 9). The protection pattern for the DVR-4 probe results from nucleotide variations in DVR-4 transcripts from the tetraploid Xenopus genome. Densitometric quantitation shows that XBra expression also increases 2-fold over control levels shortly after stage 9. (B) Xhox 3 expression is also seen to dramatically increase in the DVR-4-injected embryos, reaching peak levels in late gastrula embryos, remaining elevated through stage 17 (data not shown), but returning to levels comparable to control by late neurula stages. Absence of a Xhox 3 band in the stage 10 control embryo lane is due to exposure time and low yield of RNA. EF-1α is used to standardize amounts of RNA in each assay.

Character. Since injection of DVR-4 into one-celled embryos causes an inhibition of gastrulation, only limited information can be gained as to the role DVR-4 plays in overall patterning of the embryonic mesoderm. We therefore attempted to find an assay allowing more localized misexpression of the protein. In particular, we wanted to test the effects of overexpressing the protein in presumptive dorsoanterior regions of the embryo. DVR-4 RNA injected into two prospective dorsal or ventral blastomeres of 4- and 8-cell embryos consistently arrests gastrulation in injected embryos. However, if relatively small amounts of RNA (approximately 1-3 pg) are injected into 1 or 2 blastomeres of 32- to 64-cell embryos, the injected embryos can proceed past gastrulation stages. Fig. 3 shows that misexpression of DVR-4 on the presumptive dorsal side of the embryo results in an inhibition of anterior structures (82% of dorsally injected embryos). Embryos injected in this manner have a much more defined antero-posterior axis than one-cell injected embryos, but lack anterior structures (heads). This is strikingly similar to the phenotype resulting from injection of Xhox 3 RNA into a similar location (Ruiz i Altaba and Melton, 1989b). This phenotype is consistent with the hypothesis that DVR-4 specifies more posterior structures or inhibits dorsoanterior differentiation. Furthermore, if the same amount of DVR-4 RNA is injected into presumptive ventral blastomeres, the embryos are morphologically indistinguishable from un.injected sibling controls at least until swimming tadpole stages.

An reasonable hypothesis from the experiments described
above is that DVR-4 and activin could interact to establish an ordered anterior-posterior pattern in the *Xenopus* embryo. We therefore used purified factors in animal cap induction assays in an attempt to quantitate interactions between the two molecules. From the above experiments, one prediction is that purified DVR-4 should induce a ventral type of mesoderm, and 10-50 ng/ml activin more dorsal mesoderm, while a combination of the two factors should induce ventral and/or intermediate types of mesoderm. When we treated animal caps with DVR-4 protein alone (10-200 ng/ml), in seven separate experiments, less than 5% of treated caps form recognizable mesodermal tissues. The majority differentiate, like controls, into ciliated atypical epidermis, when characterized histologically (Table 1, Fig. 5, and data not shown). Consistent with other reports, caps treated with activin alone develop into elongated structures containing notochord, muscle and neural tissue. However, when the two factors are added in combination, the majority of caps develop into vesicular structures, representative of ventral mesodermal differentiation (Fig. 5, panel D). Moreover, the caps treated with a combination of DVR-4 and activin express higher levels of Xhox 3 than do those treated with activin alone (Fig. 6A), again suggesting that the mesoderm induced in these caps has a posterior and/or ventral character.

To further assess the actions of purified DVR-4 in animal cap mesoderm induction assays, and as a control for toxic effects of our purified protein, we treated isolated explants with either bFGF alone, or bFGF in combination with DVR-4. Animal caps treated under both conditions are histologically indistinguishable (data not shown), and Xhox 3 expression levels are comparable in both groups (Fig. 6B). Xhox 3 levels are however, substantially higher in caps treated with bFGF in combination with DVR-4 than in caps treated with DVR-4 alone, suggesting that DVR-4 does not non-specifically inhibit the response to all mesoderm inducers. These results argue that the effects seen in combination with activin are not due to toxic contaminants in the DVR-4 protein preparation.

**Discussion**

**RNA microinjection suggests a role for DVR-4 in mesodermal patterning**

In order to ectopically express DVR-4 during early stages of development, we injected RNA encoding human DVR-4 into one-celled *Xenopus* embryos. The 100% amino acid sequence identity in the presumed mature region (last 100
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Fig. 6. RNAse protection analysis of Xhox 3 expression in animal caps treated with activin A, DVR-4, bFGF, or combinations. (A) Xhox 3 expression in caps treated with activin and DVR-4 compared with caps isolated from injected embryos. Xhox 3 is induced to highest levels in DVR-4-loaded caps, while activin in combination with DVR-4 protein induces higher levels than either activin or DVR-4 alone (a faint band, not photographically reproducible, is present in the DVR-4 lane; see Fig. 6B). (B) Xhox 3 induction in explants treated with bFGF and DVR-4. When standardized to EF-1α, Xhox 3 levels are comparable in bFGF and bFGF + DVR-4-treated caps, and both treatments induce the same type of (ventral) mesodermal morphology (data not shown). Additionally, purified DVR-4, while not inducing mesodermal tissue recognizable by histology, induces a very low level of Xhox 3 expression, and control caps are negative. In other experiments (e.g. Fig. 6A), where RNA yields were higher, a protected Xhox 3 fragment in control caps is never detected, and is consistent with published reports (Ruiz i Altaba and Melton, 1989c). Numbers in parentheses are concentrations in ng/ml.
posterior character. DVR-4 is therefore the first molecule reported both to induce ventral mesoderm, and to act dominantly over the dorsalizing effects of activin.

Further support for our hypothesis that DVR-4 actively specifies posteroventral mesoderm is obtained when DVR-4-loaded animal caps are used in Einstech assays. The caps from injected embryos instruct the formation of secondary posterior axes when transplanted into the host blastocoel (Fig. 3). This is similar to the results reported for bFGF-treated (Ruiz i Altaba and Melton, 1989c) and XlHbox 6-loaded caps (Cho et al., 1991). Moreover, DVR-4-induced secondary axes contain notochord and aligned somites extending throughout the secondary structures (data not shown), and are therefore more complete than those reported for XlHbox 6 (Cho et al., 1991). While an organized neural tube has not been observed in our secondary axes, they do contain melanocytes, a neural crest derivative, but the origin of these cells in the secondary axes is unknown, and they could represent cells that have migrated in from the primary axis. Nevertheless, our results suggest that DVR-4 alone initiates a cascade of signals that establishes an extensive posterior axis in the Xenopus embryo. Furthermore, localized overexpression of DVR-4 on the presumptive dorsal side of the embryo inhibits formation of dorsoanterior structures, while the same amount of RNA injected into future ventral blastomeres has no apparent effect on development (Fig. 3). These results are consistent with the hypothesis that DVR-4 induces posteroventral mesoderm and counteracts the effects of dorsalizing signals such as activin (see below).

DVR-4 loaded animal caps used in Einstech assays interact with naive ventral tissues to induce secondary tails, but DVR-4 mRNA injected into prospective ventral blastomeres has no apparent effect on development. These data suggest that the loaded caps act as an organizing center to direct production of secondary posterior structures, a situation different from injection of mRNA into similar ventral regions.

Exogenous DVR-4 counteracts the effects of activin, and acts in establishing ventral mesoderm

To better characterize interactions between DVR-4, activin and bFGF, the purified factors were used in animal cap assays for mesoderm induction. Surprisingly, purified DVR-4 is inefficient at inducing mesoderm when applied to animal cap explants at concentrations between 10 and 200 ng/ml (0%, Table 1; no more than 5%, unpublished observations). The fact that animal caps respond to DVR-4 produced in vivo from injected RNA by forming ventral mesoderm, but not to purified exogenous protein suggests that the caps must be exposed to high local concentrations of the factor for prolonged periods of time in order to differentiate into ventral mesodermal tissues. This is a condition achieved by RNA injection but not by incubation in the purified factor. Alternatively, the DVR-4 produced from injected mRNA by embryonic cells could be qualitatively different from the purified protein (e.g. post-translational modifications). Nevertheless, the purified protein preparation does counteract the effects of activin when the two factors are applied in combination.

We have considered the possibility that DVR-4 could attenuate the actions of activin by blocking access to the activin receptor. While it is theoretically possible that this mechanism could in fact be used by the embryo, recent reports show only very low levels of competition between activin and DVR-4 (BMP-4) for several different splice variants of the activin receptor in vitro (Artisano et al., 1992), thereby arguing against this being the sole mechanism for DVR-4 inhibiting the effects of activin. If DVR-4 does not block the activin receptor, it must be eliciting some molecular change in the animal cap cells that alters their response to activin. While the majority of animal caps treated with purified DVR-4 were histologically indistinguishable from control caps (forming atypical epidermis), we found that XhoX 3 was induced, albeit to very low levels, in the DVR-4-treated caps (Fig. 6B). It therefore seems that the purified factor (at least as applied here, for a limited time to whole animal caps) induces a program of gene expression that is not sufficient by itself to induce mesoderm differentiation, but is still able to direct mesoderm induced by "high concentrations" of activin toward more posteroventral development. Clearly, more information is needed concerning receptors, protein-binding specificities, and the intracellular signalling molecules they generate, before precise mechanisms can be established.

Another PGF widely studied in amphibian mesoderm induction is bFGF. Since bFGF is thought to induce mesoderm of a ventral or intermediate type (Slack et al., 1987), its interactions with DVR-4 were also studied. When caps are cultured in bFGF at concentrations that normally result in the formation of intermediate types of mesoderm (50-150 ng/ml), no histological differences are detectable between caps treated with bFGF or bFGF in combination with DVR-4 (data not shown), and XhoX 3 expression is comparable in both groups (Fig. 6B). The fact that bFGF and DVR-4 seem to induce similar tissue types does not make this result surprising. However, it is possible that the two factors induce tissue types that have different molecular characteristics. In support of this hypothesis is the one important difference between the effects of adding bFGF and DVR-4. Experiments show that tissue induced by bFGF can be dorsalized when exposed to PGFs such as activin (i.e. activin is dominant over bFGF; Cooke, 1989). However, our experiments using DVR-4-loaded caps treated with activin, and purified activin in combination with exogenous DVR-4, suggest that tissue exposed to DVR-4 cannot express a dorsalized phenotype (i.e. that the effect of DVR-4 is dominant over that of activin).

A revised model for mesoderm patterning

The data reported here regarding inductive properties of DVR-4 could warrant a revision of the "three signal model" for mesoderm patterning (Dale and Slack, 1987b). DVR-4 is capable of inducing mesoderm with ventral and posterior characteristics, as assayed by animal cap induction and Einstech assays, respectively. Furthermore, DVR-4 produced in vivo through RNA injection, and purified DVR-4 added to isolated animal caps, are both capable of overriding the dorsalizing effects of activin. We therefore propose that the ventrovegetal signal of induction may involve not only FGFl-like molecules, but also DVR-4.

Our hypothesis would place DVR-4 as an inducer and/or specifier of extreme ventroposterior mesoderm, while FGF, or FGF in combination with DVR-4, would induce...
mesoderm with more intermediate or lateral characteristics. Moreover, mesoderm induced by FGF is capable of responding to dorsalizing signals from the organizer. DVR-4 and low concentrations of activin or bFGF induce indistinguishable phenotypes in animal cap induction assays. However, at high concentrations, both activin and FGF can induce dorsal and intermediate mesodermal tissues, respectively, while this is not the case for DVR-4. In addition, DVR-4 actions are dominant over those of activin while FGF effects are not.

In addition to DVR-4 actively inducing ventroposterior mesoderm in vivo, we propose that the protein also maintains tissue in state of ventroposterior differentiation. In support of this idea, we note that injection of DVR-4 autoinduces expression of the endogenous genes and that this induction is maintained for an extended period of time (Fig. 4A and data not shown). In light of these results, we suggest that endogenous DVR-4 might actively oppose the dorsalizing signal (possibly activin), thereby inhibiting expression leading to differentiation of dorsoanterior phenotypes, a situation analogous to the posterior determination system in the Drosophila embryo (see Introduction). In addition, DVR-4 induces genes such as Xhox 3, which may set up a cascade of gene expression that directs posteroventral development, whereby dorsalizing signals are disregarded. In this way, establishing the posterior and ventral regions of the amphibian embryo is probably an active process and not simply a default program due to absence of dorsalizing signals.

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