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The Interpretation of Position in a Morphogen Gradient as Revealed by Occupancy of Activin Receptors

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Summary

***Xenopus* blastula cells activate different mesodermal genes as a concentration-dependent response to activin, which behaves like a morphogen. To understand how cells recognize morphogen concentration, we have bound naturally labeled activin to cells and related this to choice of gene activation. We find that the increasing occupancy of a single receptor type can cause cells to switch gene expression. Cells sense ligand concentration by the absolute number of occupied receptors per cell (100 and 300 molecules of bound activin induce *Xbra* and *Xgsc,* respectively, i.e., 2% and 6% of the total receptors) and not by a ratio of occupied to unoccupied receptors. The long duration of occupancy explains a previously described ratchet effect. Our results suggest a new concept of morphogen gradient formation and interpretation that is particularly well suited to the needs of early development.**

Introduction

Cell differentiation during vertebrate development is determined to a large extent by a series of sequential cell interactions. In its simplest form, this process would operate as a series of binary decisions of the following kind. If the concentration of a signaling molecule is above a threshold level, a cell will adopt one particular cell fate pathway. If below this threshold, a cell will follow the pathway on which it had already embarked. However, a fundamentally different mechanism exists where an individual cell can make at least three different responses to a single signaling molecule, the "morphogen," in a concentration-dependent manner (Wolpert, 1969). In this case a cell has to be able to measure at least two concentration thresholds of a single signaling molecule to make at least three different cell fate decisions, corresponding to a nil, low, or high level of response. This mechanism is particularly important in development, because a single signaling molecule can specify the formation of different cell types spatially related to the source of the signal and hence can generate positional information. Such processes usually involve long-range signaling between inducing and responding cells located several cell diameters from each other.

There is an increasing number of examples of concentration-dependent responses to signaling in development (reviewed in Lawrence and Struhl, 1996; Neumann and Cohen, 1997). These include dorsalization of the *Drosophila* embryo by Decapentaplegic (Dpp) (Ferguson and Anderson, 1992), the specification of the expression domains of *spalt* and *omb* by Dpp in the *Drosophila* wing (Lecuit et al., 1996; Nellen et al., 1996), mesodermal signaling to the endoderm in *Drosophila* (Hoppler and Bienz, 1995), and the patterning of the vulval precursor cells in nematodes by LIN-3, an EGF-homolog (Katz et al., 1995). In vertebrate development, patterning of the ventral neural tube is believed to depend upon different concentrations of Sonic hedgehog (Shh) (Roelink et al., 1995; Ericson et al., 1997), which is also thought to play a role in patterning the limb bud (Riddle et al., 1993). Also within the vertebrates, the effect of activin on *Xenopus* blastula cells is believed to reflect the mesoderm-forming induction. Green and Smith demonstrated a concentration-dependent ability of activin to activate different genes in dissociated blastula cells (Green and Smith, 1990; Green et al., 1992). Using the same system, our previous work has demonstrated the same effect in solid tissue and has shown that activin behaves as a morphogen so that each cell recognizes activin concentration by making a nil, low, or high level gene response (Gurdon et al., 1994, 1995).

The mechanism by which cells respond to a morphogen by activating genes in a concentration-dependent way is currently obscure. The first essential step in understanding this is to determine the molecular events by which a cell perceives through its receptors different concentrations of a morphogen. Here we address several key questions regarding any situation in which a cell can activate different genes according to the concentration of an external factor. First, we ask whether increasing occupancy of one single receptor can cause the same kind of cell to express different genes. An entirely plausible and very likely alternative is that a cell has different receptors of high or low affinity for a ligand and for separate transduction pathways. Second, we ask whether a cell recognizes ligand concentration by sensing a ratio of occupied to unoccupied receptors. This would appear to be a simpler way of enabling a cell to activate (or not) a single transduction pathway than for a cell to measure in some way the absolute number of receptors that are occupied by a ligand, as cells switch from one gene expression to another. Third, we have determined the actual numbers of receptors that are occupied by ligand as cells switch from one type of gene expression to another. Last, we have determined the duration of receptor occupation by a ligand, since this can offer an explanation of the ratchet effect by which a cell can change its response to an increase, but not a decrease, in ligand concentration. These results enable us to present a novel concept of how cells respond to morphogen gradients that operate in early development.

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Results

Preparation and Characterization of Labeled Activin

In order to determine receptor binding and occupancy of activin receptors, we have generated a labeled activin ligand by mRNA injection of *Xenopus* oocytes. This has advantages over other methods of labeling proteins (for example, by iodination), since the specific activity of our preparation is very high and the activin is in an unmodified form. *Xenopus* oocytes translate injected mRNA very efficiently (Gurdon et al., 1971) and selectively release secreted proteins into the medium (Colman and Morser, 1979). We therefore injected 45 ng of mRNA encoding *Xenopus* activin βB into *Xenopus* oocytes and cultured these in medium containing [^{35}S]methionine and -cysteine (McDowell et al., 1997). Figure 1A shows the labeled proteins secreted by *Xenopus* oocytes with (Figures 1A, lanes 2 and 3) or without (Figure 1A, lane 1) prior injection of activin mRNA. The activin secreted by oocytes is predominantly of two forms: a proform (~40 kDa) and a mature dimeric activin (25 kDa) (Figure 1A, lane 2), which can be reduced into its constituent monomers (Fig. 1A lane 3). It is only the 25 kDa dimeric protein that is thought to exhibit biological activity (Husken-Hindi et al., 1994). We have determined that the [^{35}S]activin synthesized in this way has a specific activity of ~10^8 cpm/μg (McDowell et al., 1997), and this enables us to detect the binding of activin to cells at the picomolar concentrations to which they respond.

Labeled Activin Binds Specifically to Receptor Type II

To assay binding we incubate dissociated blastula cells in labeled activin for 3–30 min. Three washing steps remove unbound label, and cells are then split between samples for scintillation counting or for reaggregation and analysis of gene activation (Figure 1B). Figure 1C shows an example of a binding experiment; the vast majority of the unbound counts are removed by the three washes.

The presence of the proform of activin as seen in Figure 1A raises the question as to what form actually binds to the cells. For our results to be meaningful, we need to ensure that all the counts we are measuring represent the binding of biologically active molecules. To verify this we performed a binding assay and loaded the lysate of washed cells directly onto a protein gel under nonreducing (Figure 1D, lanes 1–3) or reducing conditions (Figure 1D, lanes 4–5). The starting activin preparation added to cells had a ratio of radioactivity of pro- to mature forms of 3 to 1 (Figure 1D, lanes 1 and 4). However, the material bound to cells consisted almost entirely of the mature form (Figure 1D, lanes 3 and 5). In addition, the ratio of pro- to mature forms had increased in the remaining supernatant (Figure 1D, lane 2). We conclude that we are able to generate naturally labeled activin protein of a very high specific activity and have developed an assay where only biologically active dimeric activin binds to embryonic cells.

It is important to appreciate that in nearly all of our experiments labeled activin is in excess in the sense that much of it remains unbound, as seen in Figures 1C and 1D (lane 2). In order to generate different levels of receptor occupancy (see below), we have carried out our binding assays with very low concentrations of ligand and over short periods of time. Under these conditions, the availability of ligand is limiting.

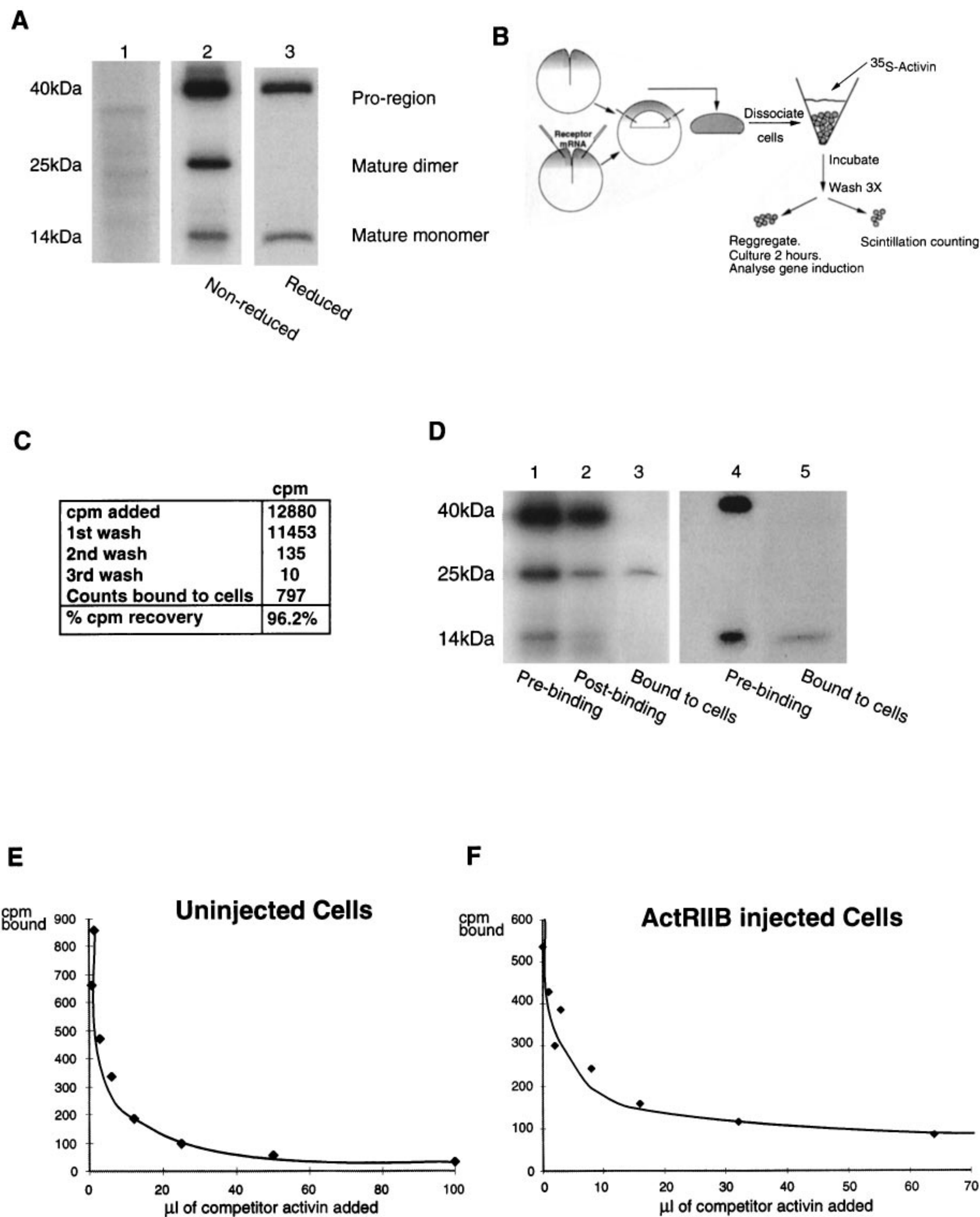
A major concern in cell binding studies is that bound counts may be due to nonspecific ligand binding to low-affinity sites. To control for this, we have used unlabeled competitor activin made in the same way as labeled activin, except that no label was added to the medium in which the oocytes were cultured. The results are shown in Figures 1E and 1F. Both uninjected cells (Figure 1E) as well as cells injected with increasing doses of mRNA encoding an activin receptor (ActRIIB) (Mathews et al., 1992) (Figure 1F) show that at least 85%–90% of the bound counts are receptor specific. It is important to note that the bound counts are reduced by half on addition of equal amounts of labeled and unlabeled competitor. We conclude that on both receptor-injected cells and on normal uninjected cells the ligand binding we measure is activin receptor specific.

To establish that nearly all labeled activin is bound to one type of receptor, we have overexpressed the *Xenopus* activin type IIB and type IIA (Nishimatsu et al., 1992) receptors by mRNA injection and have compared these results to overexpression of the activin type IB and type I receptors (ten Dijke et al., 1994), which signal but do not bind ligand (Wrana et al., 1994). As a further control, we have tested TGFβRII, which is a ligand binding receptor but which is specific for TGFβ and not activin. The results are shown in Figure 2A; overexpression of ActRIIB and ActRIIA lead to a dose-dependent increase in bound counts whereas overexpression of similar doses of ActRIB, ActRI, and TGFβRII do not. The lack of activin binding on overexpression of ActRIB and ActRI could be due to these mRNAs not being translated. We have therefore made HA-tagged receptor construct versions of these two receptors and performed a Western blot on embryos injected with 2 ng of these constructs. As shown in Figure 2B, both ActRI and ActRIB are translated effectively by *Xenopus* blastula cells.

We conclude that we can obtain increased ligand binding by overexpression of a single receptor and, moreover, that the binding characteristics determined by our assay are in agreement with results published previously for this class of receptor (Wrana et al., 1994).

Activin Binds to Type II Receptors with High Affinity and Long Occupancy

If the ligand–receptor interaction is weak and of short duration, it is clear that the receptor occupancy measured after addition of activin might not be the same as that sensed by cells 1–2 hr later. We have therefore determined the on- and off-times for activin binding to cells overexpressing the type II receptor. As shown in Figure 3A, cells bind the ligand very rapidly when exposed to a high concentration of ligand (Figure 3A, high) but relatively slowly when exposed to a lower concentration (Figure 3A, low). This dose-dependent on-rate is reflected in time-dependent gene activation in response



	cpm
cpm added	12880
1st wash	11453
2nd wash	135
3rd wash	10
Counts bound to cells	797
% cpm recovery	96.2%

Figure 1. Specificity of Binding of Labeled Activin to *Xenopus* Blastula Cells

(A) Preparation of [^{35}S]activin. *Xenopus* oocytes were injected with activin mRNA and incubated in medium containing [^{35}S]methionine and -cysteine. Each track shows labeled proteins secreted by oocytes into the culture medium. Lane 1, uninjected oocytes, nonreducing conditions. Lanes 2 and 3, mRNA injected oocytes with nonreducing or reducing conditions, respectively. The three major forms of activin are shown with their molecular weights.

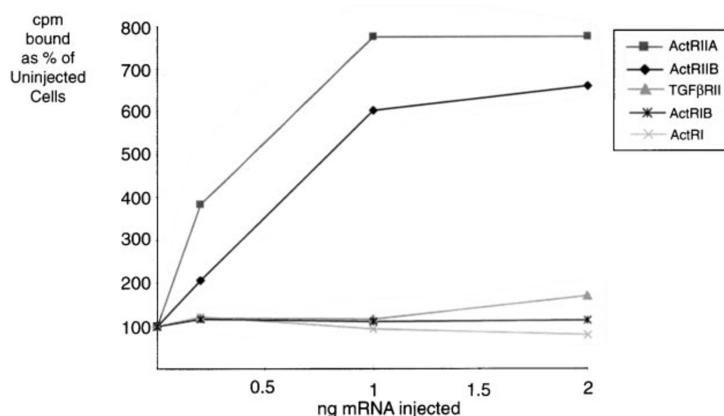
(B) Assay design for binding to cells. Cells from 5 to 15 animal caps from stage 8.5 or 9 embryos were dissected, pooled, and dissociated in 1× MBS without Ca^{2+} and Mg^{2+}. Cells were incubated in [^{35}S]activin for between 3 and 30 min. After washing, cells were either counted or reaggregated and cultured until stage 10.5 and assayed for gene transcription by RNase protection.

(C) Three washes remove all unbound counts. Dissociated cells were incubated with 30 μl of [^{35}S]activin for 10 min. The cells were washed, and after each wash the supernatants were retained and counted. The counts obtained are shown.

(D) Only the mature dimeric form of activin binds to blastula cells. [^{35}S]activin containing proregion and mature forms (lanes 1 and 4) was incubated with dissociated blastula cells for 30 min. Only the mature form of activin binds to cells (lanes 3 and 5) and is selectively depleted from the starting material (lane 2). Lanes 1–3 were analyzed under nonreducing, and lanes 4 and 5 under reducing, conditions. The ratios of mature (25 kDa) to proregion (40 kDa) forms in lanes 1–5 were 0.37:1, 0.15:1, 17:1, 0.3:1, and 15:1, respectively.

(E and F) Unlabeled activin from mRNA-injected oocytes competes out the binding of nearly all labeled activin. Samples of dissociated cells from five animal caps either uninjected (E) or previously injected with 1 ng of ActRIIB (F) were incubated in 5 μl of [^{35}S]activin and between 0.8 and 100 μl of unlabeled activin. The cells were washed and counted.

A



B

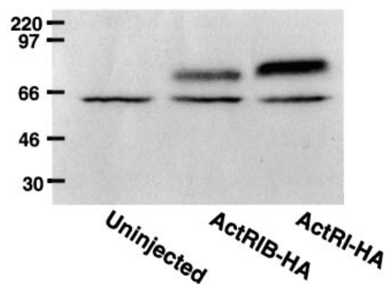


Figure 2. Characterization of Activin Binding to Its Different Receptors

(A) Activin receptors of type II but not type I bind activin directly. ActRIIA, ActRIIB, TGFβRII, ActRIB, and ActRI mRNAs (0.2, 1, or 2 ng) were injected at the 2-cell stage. The binding assay was carried out as described in Experimental Procedures. The counts per minute bound to receptor-injected cells are shown as a percentage of the bound counts to cells that did not receive injected receptor mRNA.

(B) ActRI and ActRIB are translated by *Xenopus* blastula cells. HA-tagged constructs of ActRI and ActRIB mRNA (2 ng) were injected at the 2-cell stage. The embryos were cultured until stage 8.5. Protein extracts and Western blotting were carried out as described in Experimental Procedures.

to activin (Figure 3B). As shown, at a low concentration of activin (0.5 μl), the extent of *Xbra* induction is dependent on the length of the incubation time.

Once established, the receptor–ligand interaction is extremely stable (Figure 3C). Cells were bound with [^{35}S]activin for 10 min, washed three times, and then incubated in an excess of unlabeled competitor activin. Any [^{35}S]activin that dissociates would be replaced by unlabeled activin. As shown in Figure 3C, 80% of the ligand is still bound after 2 hr and only after 5 hr has about two-thirds of the ligand dissociated. This is consistent with activin having a very high affinity for its receptor, as would be expected for a ligand that is biologically active in the picomolar range (Thomsen et al., 1990).

Interestingly, the short on-time and long off-time provide an explanation for the previously described ratchet effect (Gurdon et al., 1995). This showed that cells can rapidly alter their gene response from a low level (*Xbrachyury* [*Xbra*]; Smith et al., 1991), to a high level (*Xgoosecoid* [*Xgsc*]; Cho et al., 1991), but not in the other direction. This implies that cells respond to the highest concentration of the ligand that they experience during their window of competence. As more ligand becomes available to cells, they can fill unoccupied receptors, accounting for a switch to a higher type of gene expression. On the other hand, a reduction in activin concentration would not vacate previously occupied receptors during the next two hours, when cells sense their surrounding activin concentration, and no downward change in gene expression would take place.

Activin Receptors Are Not Significantly Internalized or Exchanged during the Late Blastula Stage

A complication affecting the interpretation of our results would exist if activin–receptor complexes are internalized. If this were the case, internalized ligand would be scored in our assay as occupied receptors whether or not the activin–receptor complex was still signaling. There are a number of other receptor systems where internalization has been observed (Koenig and Edwardson, 1997); for TGFβ, this has been reported only for a cultured mammalian cell line (Massague and Kelly, 1986). Furthermore, the rapid internalization of many growth factor receptors via clathrin-coated pits has been reported to require a Phe-Arg-X-Tyr signal sequence, which is not present in the sequence of ActRIIB (Chen et al., 1990). We sought to confirm that our results are not affected by internalization. Since it has been established that internalization of receptors is eliminated at low temperatures (Koenig and Edwardson, 1997), we bound labeled activin to dissociated cells, washed away free activin, and then cultured the cells either at 21°C or at 4°C. As shown in Figure 4A, the number of bound counts was indistinguishable in the two series when tested after 2 hr.

It is possible that the off-rate is different at 4°C and 21°C. We have therefore checked for ligand internalization in another way. Low pH can be used to release proteins bound to the cell surface, but it does not cause loss of cytoplasmic proteins (Koenig and Edwardson, 1997). This allows us to determine the extent of activin

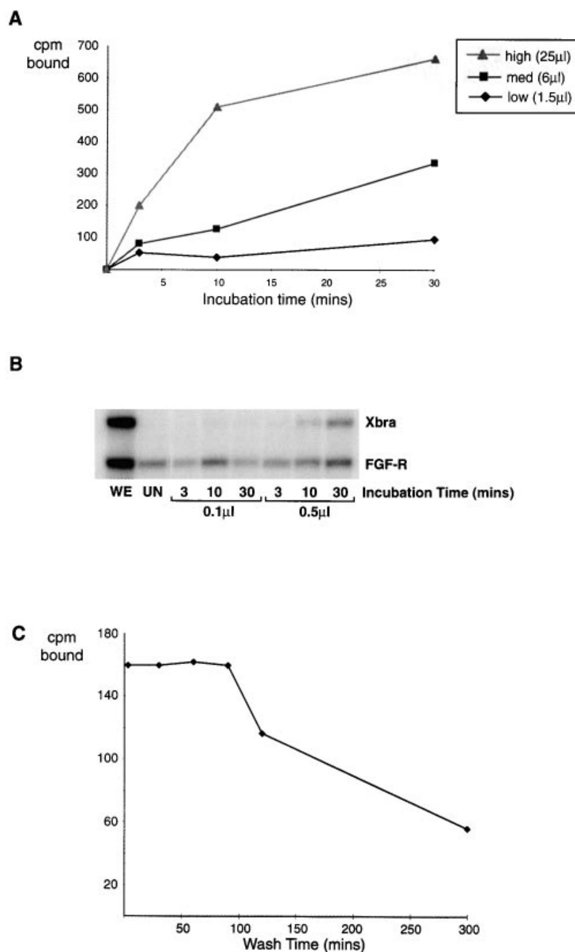


Figure 3. Determination of On- and Off-Rates for Activin Binding to Its Receptor

(A) Activin binds to its receptor with a rapid on-time. Dissociated animal cap cells injected with 1 ng ActRIIB receptor mRNA per embryo were incubated for 3, 10, or 30 min in 1.5, 6, or 25 µl of [^{35}S]activin. Cells were washed and counted. The counts per minute bound to the cells are shown.

(B) Gene induction is dependent on incubation time. Dissociated cells were incubated in either 0.1 or 0.5 µl of [^{35}S]activin for between 3 and 30 min. The cells were washed, reaggregated, and cultured until stage 10.25. *FGF-R* and *Xbra* gene induction was assayed by RNase protection. This activin preparation was older and less active than that used in Figure 5C.

(C) Activin remains bound to its receptor for at least 2 hr. Dissociated animal cap cells injected with 2 ng ActRIIB receptor mRNA were incubated for 10 min in [^{35}S]activin. Cells were washed three times and then incubated for increasing lengths of time from 0 to 5 hr in a 3-fold excess of unlabeled activin. After washing, cells were counted. The cpm bound to the cells are shown. The counts shown are an average of three independent experiments.

internalization. We therefore bound [^{35}S]activin to cells for 10 min, washed the cells, and then washed further in 1× MBS (pH 7.5, 5, or 2.5) either immediately or after 40 min. If extensive internalization had occurred, fewer counts would be removed by washing in pH 2.5 after 40 min than after washing immediately. As shown in Figure 4B, this is not the case. The same number of counts are removed by low pH washing after 3 and 40 min. Obviously, if the low pH washes lysed the cells, internalized activins might be scored as being on the cell surface. We have therefore determined the extent of cell lysis by using cells injected with the lineage marker GFP (Zernicka-Goetz et al., 1996). Figure 4C shows that leakage of GFP from within cells does not take place in cells washed at pH 7.5 or pH 2.5 but is readily detectable in cells permeabilized by lysolecithin (Gurdon, 1976). We therefore conclude that there is no appreciable ligand dissociation during the course of our experiments and that the cell-associated activin is a true measure of occupied receptors.

We also wanted to know if there was appreciable synthesis of new receptors during the course of our experiments. If a cell continually increases the number of receptors on its surface and if cells measure a ratio of filled to unfilled receptors, then our measurements of total receptor number at early time points might not apply at the time when cells establish their response. To determine whether cells transfer new receptors to the cell surface, we prebound cells with unlabeled activin for 10 min, washed the cells, and then challenged with [^{35}S]activin after 3, 10, and 30 min incubations (Figure 4D). We compared the result with cells which had been treated in the same way but had not been pretreated with competitor activin. As shown in Figure 4D, there is no increase with time in the binding of labeled activin to cells prebound with competitor as would have been expected if new receptors were being transported to the cell surface. We conclude that during the course of our experiments there is little or no net increase of receptors at the cell surface.

Increasing Occupancy of the Same Receptor Activates Different Genes

We are now in a position to ask the major question of whether increasing occupancy of a single receptor species can lead to the activation of different genes. An alternative and entirely plausible hypothesis is that each cell has receptors of different affinity. According to this idea, high-affinity receptors would bind ligand at a low concentration, whereas low-affinity receptors would require a high concentration of ligand to become occupied. If low- and high-affinity receptors were linked to different transduction processes, this could readily account for the low and high types of gene expression.

To distinguish these two explanations of cell response to morphogen concentration, we have used cells in which the mRNA encoding a single species of activin receptor (ActRIIB) has been overexpressed. This ensures that the vast majority of the receptors on the cell are of a single type. We ask the question whether increasing occupancy of this single receptor is able to cause a switch in gene activation. mRNA (1 ng) encoding ActRIIB was injected into 2-cell stage embryos. These were cultured until stage 9 when animal cap tissue was dissected and the cells dissociated by placing them in a Ca^{2+}/Mg^{2+}-free 1× MBS. The cell binding assay with [^{35}S]activin was then carried out as described in Experimental Procedures. Half of the cells for each concentration point were counted for radioactivity, while the other half were reaggregated and cultured until stage 10.25, when the cells were assayed for gene induction by an RNase protection assay. Figure 5A shows that increasing the concentration of radioactive activin leads to increasing occupancy of the ActRIIB receptor. This figure

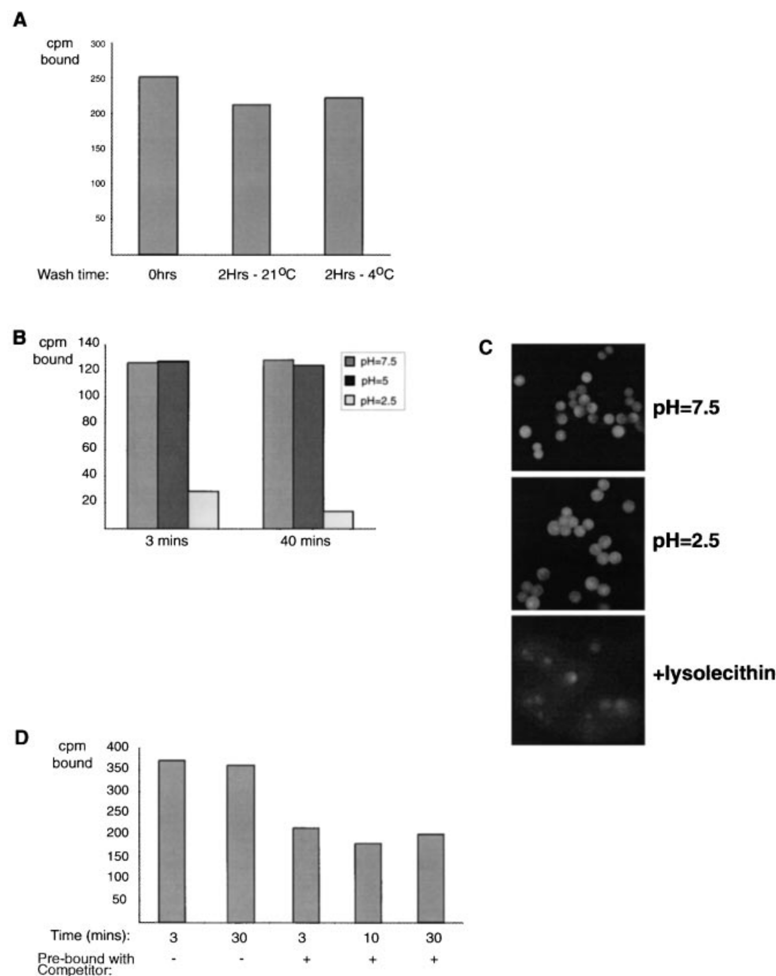


Figure 4. Activin Receptors Are Not Significantly Internalized or Exchanged during the Late Blastula Stage

(A) Significant internalization of labeled ligand does not occur. Dissociated cells were incubated in 5 μl of labeled activin for 10 min. The cells were washed and either counted immediately (0 hr), or cultured for 2 hr at 21°C or 4°C before counting.

(B) Significant internalization of labeled ligand does not occur. Dissociated cells were incubated in 5 μl of labeled activin for 10 min. The cells were washed to remove unbound label and then further washed twice in 1× MBS (pH 7.5, 5, or 2.5) after 3 or 40 min and then counted.

(C) pH 2.5 washing of cells does not cause permeabilization. Dissociated cells previously injected with 1 ng of GFP mRNA were washed as in (B) with 1× MBS (either pH 7.5 or pH 2.5). They were then photographed. Cells were made permeable by addition of lysolecithin (Gurdon, 1976).

(D) There is no net increase in activin receptors on the cell surface. Dissociated cells were cultured in 5 μl of unlabeled activin for 10 min. They were washed and challenged with 5 μl of labeled activin for 5 min after 3, 10, or 30 min of incubation. The bound counts were compared to cells that had not been prebound with competitor.

also shows that at low concentrations of activin, binding is linearly dependent on its concentration, but that at higher concentrations saturation of binding is seen. From this data a Scatchard plot can be generated (Figure 5B) as has been done for other ligands with *Xenopus* cells (Gillespie et al., 1989; Marigo et al., 1996).

To confirm that these binding data are consistent with the activin ligand binding only to the overexpressed ActRIIB receptor, we analyzed the data (Figures 5A and 5B) using the program Ligand (Munson and Rodbard, 1980). This program is used to determine how many classes of ligand binding affinity (receptor types) are present. It analyzes the data first by assuming ligand binding to receptors of one affinity and then by assuming binding to two types of receptor sites of different affinity. These results are then compared statistically using an F-test to see which model best fits the data. The data in Figure 5A can only be fitted to a model involving one class of receptor. The gel analysis in Figure 5C shows that increasing occupancy of ActRIIB also leads to the activation of different genes. As shown graphically in Figure 5D, it is clear that at low occupancies *Xbra* is induced, whereas at higher occupancies *Xgsc* and *Xeomes* (Ryan et al., 1996) are induced and *Xbra* is much reduced. We conclude that increasing occupancy of a single receptor can lead to a switch in gene expression.

We have asked what proportion of a cell's receptors must be occupied for *Xbra* or *Xgsc* to be induced. To determine this we needed to know the maximal or saturation level of ligand binding. From the Scatchard plot we can determine the saturation binding (intercept of the x-axis) to be 4750 cpm (Figure 5B). Assuming that the saturation of ligand binding occurs at full occupancy of the receptors, we are then able to calculate the percentage of total occupancy at which genes are activated. We calculate the occupancy at which *Xbra* and *Xgsc* are induced by expressing the bound counts per minute (cpm) (from Figure 5A) at the concentration when these genes are first expressed (Figure 5C) as a percentage of saturation binding (in this case, 4750 cpm). Results from different experiments were averaged, and as shown in Figure 5E, we see that *Xbra* and *Xgsc* are activated at extraordinarily low receptor occupancies of 0.3% and 0.8%, respectively, the switch in gene expression taking place between these two values.

Receptor Occupancy in Normal Cells

We now ask whether the conclusions drawn from cells overexpressing receptor IIB are also applicable to normal embryonic cells. To determine the number of receptor classes involved in binding activin to uninjected cells, we have used the same approach as for receptor-injected

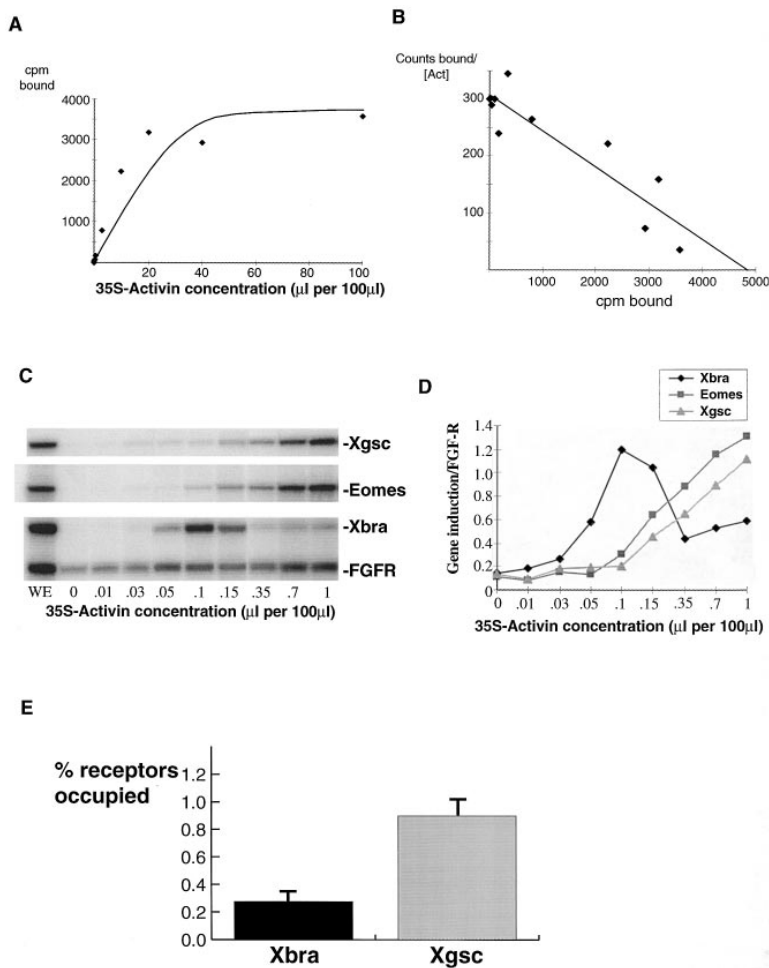


Figure 5. Occupancy of Overexpressed Receptor ActRIIB Required to Induce *Xbra* and *Xgsc*

(A) Activin binds to ActRIIB receptors in a concentration-dependent manner. Blastula cells previously injected with 1 ng of ActRIIB mRNA were incubated with increasing concentrations of [^{35}S]activin for <5 min. After washing, the cells were counted. The graph shows that increasing cpm bind to the cells as the activin concentration is increased, but at high concentrations saturation is reached.

(B) Activin binds to one receptor species. Scatchard analysis of data in (A).

(C) Induction of *Xbra* and *Xgsc* by activin is concentration dependent. Cells from the experiment shown in (A) were reaggregated and cultured until stage 10.5 and then frozen for RNase protection analysis of gene expression. The gel analysis shows that at low concentrations *Xbra* is induced; at higher concentrations, *Xgsc* is induced and *Xbra* repressed. WE, whole embryo.

(D) Induction of *Xbra* and *Xgsc* by activin is concentration-dependent. Quantitation of gel analysis shown in (C).

(E) *Xbra* and *Xgsc* are induced, respectively, at an occupancy of 0.3% and 0.8% of the total receptors, determined as follows. Saturation binding corresponding to the maximum number of receptors was determined from (B) to be 4750 cpm. Induction of *Xbra* and *Xgsc* was deemed to have occured when each of these was clearly above background (0.05 μl/100 μl for *Xbra* and 0.15 μl/100 μl for *Xgsc* [C and D]). The actual numbers of cpm bound at these concentrations was determined from a tabulated form of the data shown in (A). These numbers are expressed as a percentage of the saturation binding. (E) shows the values and standard deviations of the average of several such experiments.

cells. Increasing ligand concentration leads to increased binding, again linearly at low concentration and with saturation at high concentrations (Figure 6A). The Scatchard plot (Figure 6B) indicates that activin binds to one receptor class. To confirm this we have again analyzed the data using the program Ligand. As with receptor-injected cells, the data can only be fitted to a model involving one receptor binding class, which is of similar affinity to the binding class seen for receptor-overexpressing cells (ActRIIB). We conclude that normal (uninjected) cells bind activin by one class of receptor that has an affinity similar to that of ActRIIB.

We are now able to ask at what occupancy of functional receptors normal cells respond to activin and switch gene response. The experiment was performed in the same way as for receptor-injected cells (Figure 5) and the results are shown in Figures 6C–6E. As with receptor-injected cells, activin binds to cells in a concentration-dependent manner (Figure 6A) and saturation binding occurs at 1300 cpm (Figure 6B). The analysis of gene transcription is shown in Figure 6C. As was shown before (Green and Smith, 1990; Green et al., 1992), low-response genes such as *Xbra* and *Xwnt8* (Smith and Harland, 1991; Sokol et al., 1991) are induced at a lower activin concentration than high-response genes such as *Xgsc* and *Xeomes* (Figure 6D). By determining a maximal or saturation binding, we show first that the occupancy at which low response genes such as *Xbra* are first induced is 2% and second that the occupancy at which cells switch gene response and start to induce high-response genes such as *Xgsc* is 6% (Figure 6E). We conclude that normal cells using their endogenous receptors respond to a morphogen at a very low receptor occupancy (2%) and also that they can switch gene response with only a 3-fold increase in occupancy and at a very low level (6%).

Type I Receptors Do Not Affect Response to Activin

Our results have shown that the abundance of Type II receptors is intimately involved in gene response to activin concentration. Since type I receptors are required for activin signal transduction, and since constitutively active type I receptors (ActRI and ActRIB) have different effects on gene induction (Armes and Smith, 1997), we have tested whether overexpression of type I receptors influences the pattern of gene response to activin concentration. We therefore treated *Xenopus* animal caps previously injected with 2 ng of ActRI or ActRIB mRNA with increasing doses of activin. These were cultured

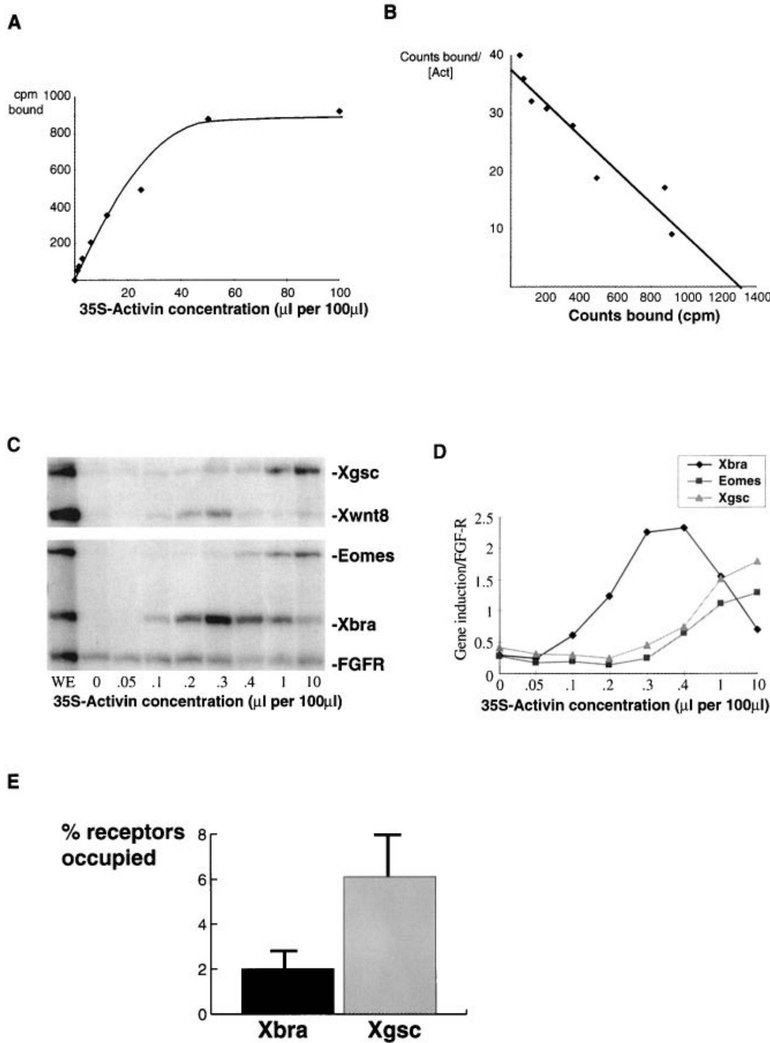


Figure 6. Occupancy of Activin Receptors in Normal Cells as Required to Induce *Xbra* and *Xgsc*

(A) Activin binds to endogenous receptors in a concentration-dependent manner. Blastula cells from uninjected embryos were dissociated and incubated with increasing concentrations of [^{35}S]activin. After washing, the cells were counted. The graph shows increasing cpm bound to the cells on increasing activin concentration, but at high concentrations saturation is observed.

(B) Activin binds to one receptor species. Scatchard analysis of data in (A).

(C) Induction of *Xbra* and *Xgsc* by activin is concentration-dependent. Cells from the experiment shown in (A) were reaggregated and cultured until stage 10.5 and then frozen for RNase protection analysis of gene expression. The gel analysis shows that low concentrations of activin induce *Xbra* and *Xwnt8* and higher concentrations induce *Xgsc* and *Xeomes*. WE, whole embryo.

(D) Induction of *Xbra* and *Xgsc* by activin is concentration-dependent. Quantitation of gel analysis shown in (C).

(E) *Xbra* and *Xgsc* are induced at an occupancy of 2% and 6% of the total receptors. This was determined as for Figure 5E.

until stage 10.25 and the induction of marker genes determined. As shown in Figure 7, there is no effect of overexpressing of either wild-type type I or type IB receptors on the response to activin compared to uninjected cells. We conclude that type I receptors, although mediating the downstream effects of activin signaling, do not affect the response to activin concentration.

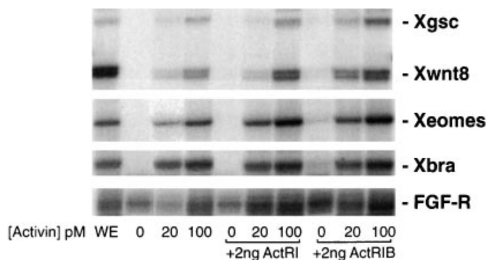


Figure 7. Type I Receptors Do Not Affect Response to Activin

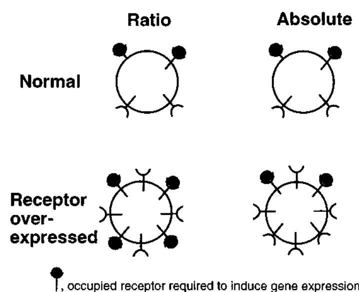
Overexpression of type I receptors does not affect response to activin. Cells were injected with either 2 ng of ActRI or ActRIB mRNA at the 2-cell stage. They were then cultured to stage 8.5, when animal caps were cut and incubated in either nil, 20, or 100 pM of activin. The animal caps were frozen at stage 10.25 for RNase protection analysis of gene expression.

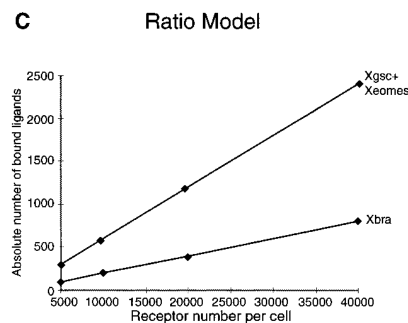
The Absolute Numbers of Receptors and Ligands Required to Induce *Xbra* and *Xgsc*

It is known that the concentration of activin required to induce a suspension of *Xenopus* blastula cells to express *Xbra* and *Xgsc* is in the range of 20–50 pM (Green et al., 1992). However, this does not tell us the number of molecules of activin that need to be bound to a cell for these responses. Nor is it at all clear that a 2-fold difference in activin concentration in the medium corresponds to only a 2-fold difference in number of occupied receptors. We can now determine from our experimental results the actual number of molecules bound to cell surface receptors when different gene responses are elicited. To do this, we need to know the specific activity of our activin preparations. By comparing the biological activity of oocyte-labeled and -purified activin (Genentech), we find that three different preparations of [^{35}S]activin have a specific activity within the range of 0.3–3 $\times$ 10^8 cpm/μg in agreement with a value of 10^8 cpm/μg cited by McDowell et al. (1997). From the molecular

A

		Uninjected	0.2ng ActRIIB	0.5ng ActRIIB	1ng ActRIIB	Average
Activin :	Molecular weight	25000 kDa				
	Specific Activity	10^8cpm/µg				
	cpm per molecule	$4.2x10^{-6}$				
Blastula cells:	No. per animal cap	6,000				
Activin bound to cells:						
	No. of an. caps	10	5	5	5	
	Max cpm per sample	1309	1272	2667	4750	
	Max cpm per cell	0.02	0.04	0.09	0.16	
	No. of receptors per cell	5236	10172	21336	38000	
Activin required to induce Xbra:						
	Receptor occupancy	2.0%	0.9%	0.5%	0.3%	
	Molecules per cell	105	96	111	114	106+/-8
Activin required to induce Xgsc:						
	Receptor occupancy	5.7%	2.8%	1.3%	0.8%	
	Molecules per cell	298	288	277	304	291+/-11





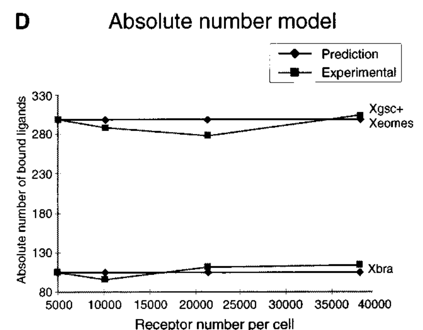


Figure 8. Cells Detect Absolute, Not Relative, Occupancy of Receptors

(A) Summary of numbers of ligand and receptor molecules involved in response to activin concentration.

(B) Design of experiments to distinguish between the absolute and ratio models of cell response to a morphogen. Receptor overexpression generates different predictions for absolute and ratio models. If cells sense a ratio of bound/unbound receptors, the number of bound ligands required to generate the same gene response would increase on receptor overexpression. If cells sense absolute numbers of bound ligands, the number of bound ligands required to generate the same response would remain the same in cells with normal or overexpressed receptors.

(C) Ratio model predictions. As receptor number per cell increases, increasing numbers of bound ligand are required to maintain a fixed ratio of occupied to unoccupied receptors.

(D) Experimental data supports absolute occupancy model.

weight of dimeric activin, we can calculate the cpm/molecule (Figure 8A). We have determined the number of cells in a stage 9 *Xenopus* animal cap to be 6,000 by counting cells and from published DNA values (Dawid, 1965). From our values for saturation binding (Figures 5B and 6B), we calculate the number of receptors per cell to be 5,000 (Table I), a value within the normal range for other receptors of other cells (e.g., Koenig and Edwardson, 1996). This value is increased by 7-fold to 38,000 on receptor-injected cells. Using the occupancies for gene inductions calculated in Figures 5 and 6, we show that the actual number of molecules needed for induction is 100 for *Xbra* and 300 for *Xgsc* (Figure 8A). To confirm this result, we have repeated our analysis for cells overexpressing both 0.2 and 0.5 ng of ActRIIB. As would be expected, these cells have 10,000 and 21,000 receptors, respectively, and induce *Xbra* at 0.9% and 0.5% and *Xgsc* at 2.8% and 1.3% receptor occupancies, respectively (Figure 8A). We conclude that only 100 molecules of activin need to be bound to a single normal cell for that cell to begin to induce *Xbra*. When 300 molecules of activin are bound, the cell switches response and begins to transcribe *Xgsc*, *Xeomes*, and other genes.

Cells Detect Absolute, Not Relative, Occupancy of Receptors

Figure 8B explains two ideas of how cells could perceive a change in morphogen concentration. One is that cells sense a ratio of occupied to unoccupied receptors. This mechanism could be achieved if, for example, unoccupied receptors have a phosphatase activity and occupied receptors a kinase activity. A switch in intracellular activity could be readily envisaged as change from below to above 50% receptor occupancy. Furthermore, a ratio mechanism of detection could also operate at much lower levels than 50% if the kinase activity of an occupied receptor exceeded the opposite activity of an unoccupied receptor; consider, for example, inhibitory versus activatory SMADs (Hayashi et al., 1997; Nakao et al., 1997). An alternative idea is that cells can count the absolute number of occupied receptors, sensing as little as a 2- to 3-fold increase.

These two ideas can be distinguished experimentally by comparing the receptor occupancy at which genes are activated in normal and receptor-injected cells (Figure 8B). In the latter, the large excess of receptors generated by mRNA injection will increase the ratio of unoccupied to occupied receptors when the ligand is limiting.

According to the ratio model, cells will require 2% of their receptors to be occupied to induce *Xbra* and 6% to induce *Xgsc*, and this will be true in normal cells as well as in cells overexpressing receptor. Therefore, the actual number of bound ligands needed to induce these genes will have to increase in proportion to the increase in receptor number to ensure that the appropriate ratios of occupied to unoccupied receptors are maintained (Figure 8C). Conversely, the absolute occupancy model makes an entirely different prediction. The prediction is that the number of receptors that need to be occupied to activate a particular gene will remain constant, however many more receptors are present on cells (Figure 8D). It is clear that our results are incompatible with the ratio model (Figure 8C) and are entirely in agreement with the absolute occupancy model (Figure 8D).

Discussion

How cells interpret their position in a morphogen gradient is not understood at the level of cell surface receptors. Most of what we know has come from two types of analysis. A large number of studies has measured the binding and affinities of signaling molecules for their receptors. For example, using transfected or overexpressed receptors and labeled ligand, it has been shown that Patched binds Hedgehog (Marigo et al., 1996), and biacore measurements have determined ligand–receptor affinities in vitro (e.g., for BMP-4 [Natsume et al., 1997]). The other type of investigation has analyzed the effects of receptor mutations (Furriols et al., 1996) or constitutively active receptors (Nellen et al., 1996; Armes and Smith, 1997). In the present study, we have directly related receptor occupancy to gene activation as a response to the concentration-dependent effects of a morphogen. We use the results to present a model of how cells interpret their position in a morphogen gradient.

Gradient Interpretation

An unexpected result is that cells sense morphogen concentration and switch gene response when a remarkably small proportion of their receptors (2%–6%) is occupied by ligand. However, this seems to us very understandable if we envisage cells' interpretation of a morphogen gradient in the following way. We suppose that cells in one region of an embryo actively secrete morphogen for a few hours, during which time the concentration increases. After this, cells discontinue emitting morphogen, and its concentration will decrease. We believe that responsive cells monitor morphogen concentration continuously and respond by a ratchet mechanism to the highest concentration that they experience within their competent life.

This proposed mechanism has several advantages for early development. First, we have shown that cells can bind ligand and respond rapidly to the morphogen and do not therefore need to wait for it to reach equilibrium. Through the ratchet effect, cells would always respond to the highest concentration that they experience within the few hours of their competent life even when the number of occupied receptors per cell might temporarily decrease, as during cell division. The ratchet effect, as we have pointed out, would operate by the very high affinity of ligand for its receptors. Second, if cells were to respond at high occupancy but still measure the absolute numbers of occupied receptors, there could be some inconsistency of response due to titration of type I receptors. Under these conditions, the ligand type II receptor complexes first formed would see a much higher concentration of type I receptors than subsequent complexes. We would then predict that overexpression of Type I receptors would change response to activin concentration. Our results (Figure 7B) show that this is not the case. We conclude that at the very low occupancies seen both type I and type II receptors are in such excess that the few receptors actually used for signaling do not significantly reduce the overall pool of available receptors, and so increased occupancy can be directly reflected in increased signaling.

The mechanism we propose can explain an apparent paradox. On the one hand, the ligand must be limiting in order to account for the concentration-dependent responses that are observed. On the other hand, the ligand must be in excess to be able to create a concentration gradient in distant cells. If this were not the case, most if not all the ligand would be sequestered by cells nearest the source, as may happen in the case of Hedgehog (Chen and Struhl, 1996). We explain this paradox in the following way. We have shown that cells can respond when very low levels of ligand are bound (100–300 molecules). This means that only a small proportion of the ligand in the intercellular space needs to be bound by receptors within the time available. In this way, cells are able to generate a concentration-dependent response without significantly reducing the concentration of ligand around them and therefore without disturbing the gradient.

These special characteristics of high-affinity receptors, low absolute ligand concentration, low receptor occupancy, and a ratchet mechanism of response may be especially suitable for embryos that need to elicit concentration-dependent responses to changing ligand concentration over a short time scale. By comparison, T cells respond to about 8000 bound ligands at up to 100% receptor occupancy (Rothenberg, 1996; Viola and Lanzavecchia, 1996).

Postreceptor Signaling

Future work will analyze the regulation of events that follow different levels of receptor occupancy and different strengths of type I activin receptor signaling. At present there are many uncertainties. Different kinds of type I receptor may be involved in several different signaling pathways (Chang et al., 1997). Activin signaling is thought to be transduced by Smad2 and Smad4 (Graff et al., 1996) but inhibited by Smad7 (Hayashi et al., 1997; Nakao et al., 1997). Overexpression of Smad2 seems to lead to activation of *Xgsc* at a higher Smad2 concentration than *Xbra* but permits coexpression of both genes (Graff et al., 1996). Since this is not the case with activin, it appears that the transduction of the activin signal is more complex than merely a concentration-dependent activation of Smad2, a matter under current investigation. This suggests that other intracellular molecules

may be involved. Eventually, the strength of receptor signaling must be related to the occupation of activin response elements in the promoters of activin-responsive genes. A number of different elements of this kind have so far been identified (Watabe et al., 1995; Chen et al., 1996). A knowledge of these elements together with the results presented here should provide a full understanding of how cells respond to their position in a morphogen gradient, from the binding of ligand on the cell surface to gene activation in the nucleus.

Experimental Procedures

Oocyte Synthesis of Activin

As described elsewhere (McDowell et al., 1997).

Egg Injection and Detection of Receptor mRNA

Embryos were injected with 0.2, 0.5, 1, or 2 ng of *Xenopus* ActRIIB mRNA (original plasmid gift from Dr. C. Kintner); ActRIB, ActRI, and TGFβRII mRNA (original plasmid gift from Dr. A. Bhushan); or ActRIIA mRNA (original plasmid gift from Dr. N. Ueno) at the 2-cell stage. For tagged receptors, the receptor open reading frames were cloned into pT7TSHA (Zorn and Krieg, 1997). Synthetic mRNA was produced using Ambion Megascript T7 kit. Expression was detected on a 10% SDS-PAGE gel by Western blot with anti-HA (Boeringer) by chemiluminescense (Amersham).

Activin Binding to Cells

Animal caps from uninjected or previously receptor-injected embryos were dissected at stage 8.5. The cells were dissociated in Ca^{2+}/Mg^{2+} free 1× MBS containing 0.5 mM EDTA and 0.1% BSA. This medium was used for all subsequent incubation and washing steps. The dissociated cells were centrifuged in polyhema-treated 1.5 ml Eppendorf tubes in a horizontal bench-top microfuge and resuspended in the assay volume of 100 μl. Labeled activin was added and incubations were carried out at 23°C with cell dispersal every few minutes for a total period ranging from 3 to 30 min. After incubation, the cells were washed three times by dispersal and centrifugation in 1 ml MBS-BSA medium, a procedure that removed more than 99% of the unbound radioactivity. Each sample of washed cells was divided into parts for direct scintillation counting and reaggregation by addition of $CaCl_2$ (to final 2 mM) and further culture at 23°C.

SDS-PAGE Analysis

SDS loading buffer (± β-mercaptoethanol) was added to samples that were heated at 100°C for 5 min. The samples were run on a 15% polyacrylamide gel, fixed, and analyzed by fluorography (using Amersham Amplify reagent).

RNase Protection Analysis

RNA was prepared and RNase protection assays were performed as previously described (Ryan et al., 1996) using antisense probes prepared for *Xbra*, *Xgsc*, *Xwnt8*, *Xeomes*, and *FGF-Receptor*.

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