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A changing morphogen gradient is interpreted by continuous transduction flow

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SUMMARY

In vertebrate development, most signalling factors behave as morphogens, eliciting divergent cell fates according to their concentration. We ask how cells interpret morphogen concentration as it changes during the establishment of a gradient. Using dissociated blastula cells of *Xenopus* exposed to activin for only 10 minutes, we have followed the phosphorylation of tagged Smad2, the principal activin transducer, from a cytoplasmic pool to the nucleus in real time. We show that a changing concentration of extracellular activin is rapidly and continuously transduced to provide a corresponding nuclear concentration of Smad2, even though gene response may be delayed for several hours. Nuclear Smad2 concentration changes up as the extracellular concentration of activin increases. We conclude that cells interpret a changing extracellular concentration by maintaining a continuous flow of activated transducer from a large cytoplasmic pool to the nucleus where it is degraded. The volume of this flow determines the steady state concentration of Smad2 in the nucleus and this is used by cells to interpret extracellular morphogen concentration.

Key words: Morphogen, TGFβ, Smad2, *Xenopus*

INTRODUCTION

Vertebrate development consists of a series of temporally and spatially controlled interactions between cells. Most of the signal factors that guide early development, including members of the TGFβ, hedgehog and Wnt families, have direct concentration-dependent and distance-related effects, and so behave as morphogens (Gurdon and Bourillot, 2001; Neumann and Cohen, 1997; Podos and Ferguson, 1999). It is almost always found that the initiation of a cell signalling event in development depends on the provision or availability of signal factors (ligands), the other components of signalling pathways being already present and in excess. This means that cells near a signal source will experience concentrations of the factor that change until a steady state is reached. Therefore a major problem in understanding signalling processes in development is to know how cells make a correct response to signal factor concentrations that change with time.

We have analysed this problem with respect to the TGFβ factor activin, which is currently believed to simulate the action of Xnrs in Nieuwkoop signalling during the first few hours of Amphibian development (Clements et al., 1999; Whitman, 2001). We concentrate here on signal transduction from the cell surface to the nucleus, thereby extending our previous work on the analysis of activin action at the cell surface.

A large amount of work has analysed the TGFβ signalling pathway, of which Smads 2, 3 and 4 are key members. Part of this work has been carried out on *Xenopus* cells (Chen et al., 1996; Chen et al., 1997; Germain et al., 2000; Graff et al., 1996; Hill, 2001; Howell and Hill, 1997; Howell et al., 1999; Howell et al., 2001; Whitman, 2001; Yeo et al., 1999), but most has been conducted on stable lines of transformed mammalian cells, which respond to TGFβ stimulation by growth arrest (for reviews, see Massague, 2000; Massague et al., 2000). The case we analyse is different for two reasons: (1) A small, threefold, change in activin concentration causes animal cap cells to choose between two different zygotic gene responses, both of which are distinct from their behaviour in the absence of activin; (2) Cells must not only choose their correct response, but they must also achieve this when the signal factor concentration changes with time as the source starts to emit its signal. These aspects of signal transduction do not take place in cultured cell lines and have not so far been investigated in embryonic cells.

Our results lead us to the following concept of how cells respond to changing signal factor concentration during development. Soon after exposure to activin, a small part of a large cytoplasmic pool of Smad2 is phosphorylated and flows rapidly to the nucleus, where it is degraded. When the extracellular concentration of signal factor rises, the volume of flow of activated Smad2 increases in proportion, and so determines the steady state concentration of nuclear Smad2. This flow is maintained by an activated receptor complex. It is by the volume of this flow and the consequent Smad2 concentration in the nucleus that cells interpret their position in a morphogen gradient.

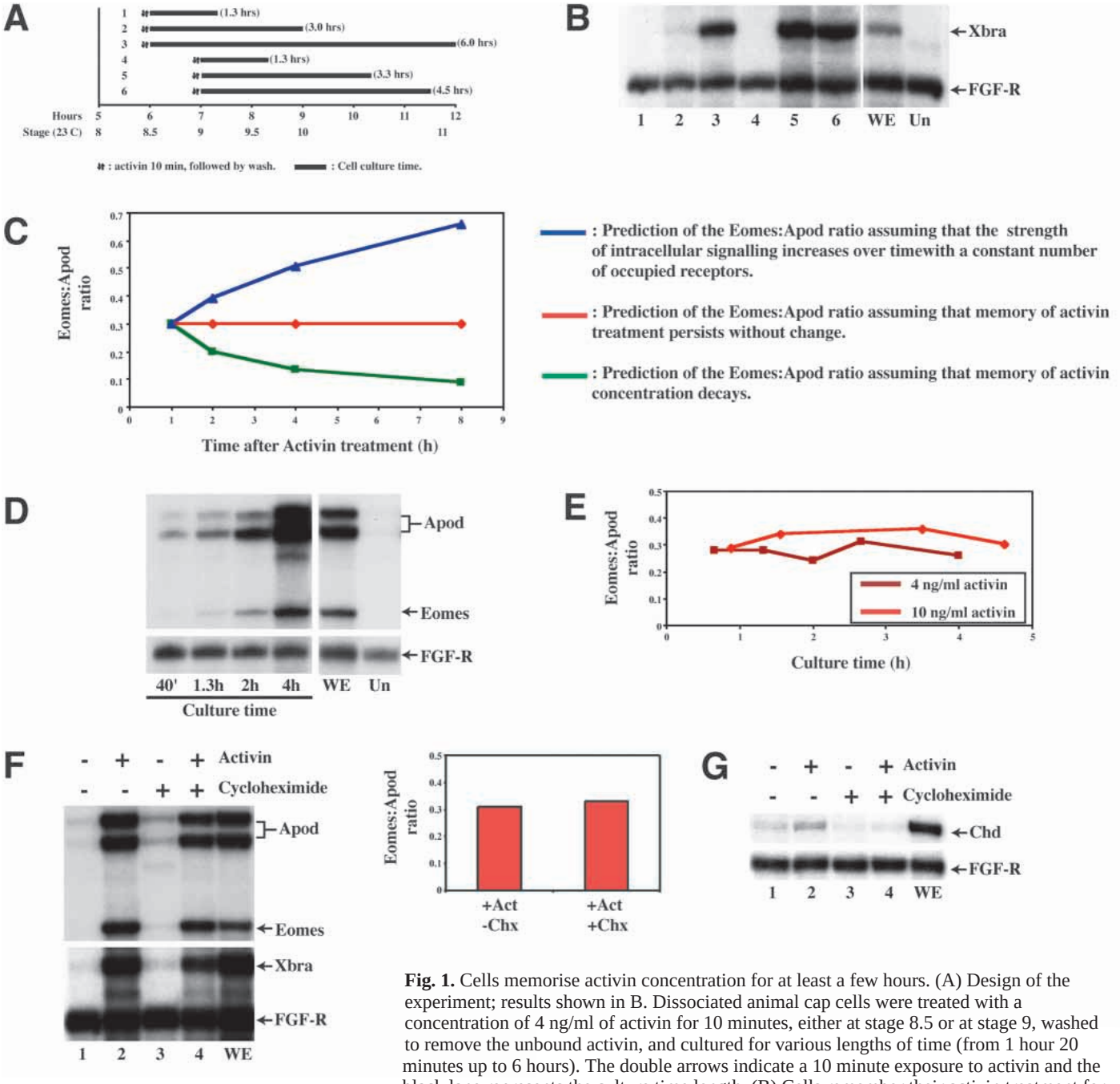

Fig. 1. Cells memorise activin concentration for at least a few hours. (A) Design of the experiment; results shown in B. Dissociated animal cap cells were treated with a concentration of 4 ng/ml of activin for 10 minutes, either at stage 8.5 or at stage 9, washed to remove the unbound activin, and cultured for various lengths of time (from 1 hour 20 minutes up to 6 hours). The double arrows indicate a 10 minute exposure to activin and the black lane represents the culture time length. (B) Cells remember their activin treatment for over 3 hours. RNAse protection analysis of Xbra expression. WE, whole embryo. Un, untreated cells. (C) Prediction of the Eomes:Apod expression ratio over time, assuming that accumulation of transcripts increases linearly with time, that degradation of transcripts is 30% per hour, that Apod and Eomes mRNAs stabilities are similar, and that Apod transcription is initiated at a quarter the activin concentration required to initiate Eomes transcription. (D) RNAse protection analysis of Apod and Eomes expression. Dissociated animal cap cells were treated with a concentration of 4 ng/ml of activin for 10 minutes, at stage 8.75, washed to remove the unbound activin and cultured for various lengths of time (from 1 hour 20 minutes up to 4 hours). (E) Summary of three separate experiments.The Eomes:Apod expression ratio does not change with time from activin treatment. Dissociated animal cap cells were treated with either of 4 ng/ml or 10 ng/ml of activin for 10 minutes at stage 8.5-9, washed to remove the unbound activin, and cultured for various lengths of time (from 40 min up to 4 hours 40 minutes). The expression of Apod and Eomes was assayed by RNase protection and quantitated. (F) Cells memorise activin concentration in the absence of protein synthesis. Dissociated animal cap cells pretreated (+) or not (-) with cycloheximide for 1 hour, were then treated with a concentration of 4 ng/ml of activin for 10 minutes at stage 9, washed to remove the unbound activin, cultured for 3 hours and gene expression was assayed by RNase protection. The Eomes:Apod expression ratio is shown on the right. (G) Chordin activation is repressed by cycloheximide. Animal caps, pretreated or not with cycloheximide for 1 hour, were then cultured with a concentration of 4 ng/ml of activin until stage 10.5 and gene expression was assayed by RNase protection.

MATERIALS AND METHODS

Xenopus embryos

Eggs were in vitro fertilised then dejellied and reared in 0.1× modified Barth saline (MBS) as described previously (Gurdon et al., 1985). For protein overexpression, embryos were injected in the animal pole with an indicated dose of mRNAs at the two-cell stage into both blastomeres. Animal caps were dissected at stage 8-8.5 in 0.7× MBS and cultured to various stages, as explants, in 1× MBS or after cell dissociation and reaggregation.

Cell dissociation and activin treatment

Animal caps (five/assay) from blastulae at about stage 8-8.5 were dissociated by incubation in Ca^{2+}- and Mg^{2+}-free 1× MBS supplemented with EDTA (0.5 mM) and with 0.1% BSA, for 10-15 minutes. Cells were dispersed by gentle pipetting, incubated for 10 minutes in the desired concentration of activin (R&D Systems), washed three times in the above dissociation medium, and then washed once in 1× MBS with 0.5% BSA. Cells were then spun briefly and incubated in 1× MBS with 0.5% BSA for reaggregation. Protein synthesis was inhibited by cycloheximide treatment (10 μg/ml) for 1 hour before activin treatment. To inhibit serine-threonine kinase, after activin treatment, cells were reaggregated in 1× MBS with 0.5% BSA supplemented with the Ser-Thr kinase inhibitor H7 (Calbiochem) at 500 μM.

RNase protection

RNA was prepared and RNase protection assays were performed as previously described (Ryan et al., 1996). Quantitation was achieved by use of a Fujifilm Phosphoimager and MacBAS2.5 software.

RNA expression constructs

Capped mRNAs were synthesised in vitro using Ambion Megascript (Ambion). Myc-Smad2 was prepared from pCS_2+Myc6-Smad2 and GST-Smad2 from pT7-HA-GST-Smad2 as described before (Shimizu and Gurdon, 1999). pT7-GFP-Smad2 was constructed by replacing the HA_2 tag sequence in pT7-HA_2-Smad2 by a DNA fragment encoding the GFP, linearised by *Xba*I, and transcribed to GFP-Smad2 mRNA by T7. pT7-GR-GFP-Smad2 was constructed by inserting a DNA fragment encoding the hormone binding domain of the glucocorticoid receptor (GR) with the GFP in pT7-GFP-Smad2, linearised by *Xba*I and transcribed to GR-GFP-Smad2 mRNA by T7. pT7-HA_2-Smad2* was constructed by mutating the Ser465 and Ser467 into Glu.

GFP-Smad2 observation by confocal microscopy

Animal cap cells from embryos injected with 0.25 ng of GFP-Smad2 mRNA were cultured on fibronectin slides, as described previously, and observed by confocal microscopy (Gurdon et al., 1999). To release the GR-GFP-Smad2 protein, dexamethasone (Sigma) was added to the medium at 10 μM.

Protein analysis

Dispersed cells from GST-Smad2 injected embryos were homogenised in buffer A (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP40; 0.1 mM PMSF; 0.2 mM NaF; 2 mM Na_3VO_4) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma). Homogenates were spun three times and incubated with 40 μl glutathione sepharose slurry (pre-equilibrated in buffer A; Pharmacia Biotech) for 1 hour at 4°C. Bound proteins were resolved on SDS-PAGE and transferred to nitrocellulose. Phosphorylation of GST-Smad2 was detected by western blotting using the enhanced chemiluminescence immunoblotting-detection system (Pharmacia Biotech) with an anti-phosphoserine antibody (Zymed). Anti-GST blotting was performed according to the same protocol. Smad2 western blotting was performed as described (Faure et al., 2000).

Smad2 metabolic phosphorylation

Dissociated cells from animal caps of Myc-Smad2 injected embryos were treated with the indicated amount of activin, washed and cultured in 1× MBS with 0.5% BSA supplemented with ^{32}P-orthophosphate (0.5 mCi/ml) until control embryos reached stage 10.5. Cells were lysed in TNE buffer containing protease inhibitors (Roche) and phosphatase inhibitor (Sigma), and the lysates pre-cleared with protein-A-Sepharose beads and subjected to anti-Myc immunoprecipitation. The immunoprecipitates were subjected to SDS-PAGE and visualised by autoradiography. Anti-Myc blotting was performed according to the protocol described above.

Labelling experiment

Dissociated cells from stage 8-8.5 embryos animal caps were cultured in Ca^{2+}- and Mg^{2+}-free 1× MBS with EDTA (0.5 mM) and with 0.1% BSA supplemented with [^{35}S]methionine/cysteine (0.5 mCi/ml) for 20 minutes or with [^{32}P]orthophosphate (0.5 mCi/ml) for 1 hour. The cells were washed extensively in the dissociating medium, treated with activin as described above and cultured for the indicated lengths time in 1× MBS with 0.5% BSA supplemented with cold methionine/cysteine (2 mM) or cold PO_4 (10 mM). The cells were lysed in TNE buffer containing protease inhibitors (Roche), and the lysates pre-cleared with protein-A-Sepharose beads and subjected to anti-Smad2 (Transduction Laboratories) immunoprecipitation. The immunoprecipitates were subjected to SDS-PAGE, visualised and quantitated by use of a Fujifilm Phosphoimager.

RESULTS

The control of transduction includes a long term memory of signal factor concentration

To understand how cells respond to changing signal factor concentration, we need to know how rapidly they adjust their gene response to new factor concentrations. Do they retain a memory of a preceding concentration or do they adjust continuously to changing concentrations? In the early development of *Xenopus*, and probably of other animals, the time when zygotic genes are first expressed is determined primarily by a developmental timing mechanism, rather than by the time when cells are exposed to signalling factors that activate these genes (Cooke and Smith, 1990). Xbra expression in normal development starts 1-2 hours later than that of other early mesodermal genes (Smith et al., 1991). When dissociated animal cap cells of blastulae are exposed to activin protein for 10 minutes, washed to remove free activin and cultured (Dyson and Gurdon, 1998; Green et al., 1992), induced Xbra expression can clearly be seen within 3 hours (Fig. 1B, track 5) when cells are exposed to activin at stage 9, but is seen only at 6 hours (Fig. 1B, track 3) when exposed to activin at stage 8. Induced Xbra expression appears not to start until stage 10, and is accordingly absent in tracks 1, 2 and 4 (Fig. 1B). We conclude that cells remember their activin treatment for over 3 hours in the absence of extracellular activin, as judged by Xbra expression.

We have next asked whether cells remember activin concentration sufficiently accurately, several hours after its removal, to make the same choice of gene expression as they would have for an immediate response. As little as a threefold increase activin concentration causes cells to switch from a low dose gene response to a high dose gene response (see Fig. 6A). If cells were to lose their memory of activin concentration gradually, it would be expected that, at long times after activin

treatment, they would express a lower ratio of Eomes:Apod than at short times; this lower ratio of expression is what they would show if they were to move progressively to a lower level of activin recognition (Fig. 1C green curve). Conversely, the memory of cells might change in an upward direction if the choice of gene response were to be determined by integrating the number of occupied receptors and the time for which they had been occupied. In this case, gene response at increasing times after activin treatment would change upwards as if cells were perceiving higher concentrations of activin, i.e. they would show an increase in the ratio of Eomes:Apod expression at longer times after activin treatment (Fig. 1C, blue curve). These predictions are made assuming that the Apod and Eomes mRNA stabilities are not different. In fact, we found in several experiments, for different concentrations of activin, no change in the ratio of Eomes:Apod expression over 5 hours from the time of activin treatment (Fig. 1D,E), as predicted in Fig. 1C (red curve) if an accurate memory of activin concentration persists without change.

The delayed gene response after activin treatment could be understood if it is an indirect effect, as would happen if activin induces Xnr genes, which in turn induce early mesoderm genes. Although the activation of early genes is known to be cycloheximide insensitive (Cascio and Gurdon, 1987), we need to know whether the accurate memory of factor concentration by cells is also retained in the absence of protein synthesis. We treated animal cap cells with activin in the presence or absence of cycloheximide, and saw that it did not repress the activation of Xbra, Eomes and Apod in response to activin (Fig. 1F, tracks 2 and 4), whereas it blocks chordin activation (Fig. 1G, tracks 2 and 4), used as a control for cycloheximide treatment. We therefore conclude that cells can accurately memorise activin concentration for at least a few hours after its removal, in the absence of protein synthesis.

Modified Smad2s are valid markers of concentration-dependent activin transduction

Smad2 is a key component of the activin transduction pathway, and we need to know whether we can legitimately use modified Smad2s to follow small concentration-dependent variations in the use of this pathway. We have used Myc- or GST-Smad2 for immunoprecipitation and GFP-Smad for viewing living cells (Fig. 2A). To test whether these tagged forms correctly represent the activity of endogenous Smad2, we have compared their effectiveness in inducing Xbra and Eomes in animal cap cells. We find myc-Smad2, GST-Smad2 and GFP-Smad2 are almost equally efficient (Fig. 2B), and at most half as efficient than the wild-type Smad2 for the induction of Xbra (Fig. 2C), per amount of mRNA injected. Most importantly, these modified Smad2s induce Xbra much more strongly than Eomes at low doses, as does overexpression of unmodified Smad2 (Shimizu and Gurdon, 1999).

As a further test, we have asked whether GFP-Smad2 shows activin-dependent nuclear concentration, as does unmodified Smad2 when analysed by western analysis of isolated nuclei (Pierreux et al., 2000). Embryos were injected with a dose of GFP-Smad2 mRNA (0.25 ng/embryo), which is not sufficient to activate gene expression in absence of activin. We find that the nuclear accumulation of GFP-Smad2 is clearly visible in activin treated animal cap cells viewed by confocal microscopy of substrate-attached cells (Fig. 2D). We conclude that modified Smad2s can be used to follow concentration dependent responses to activin.

Signal transduction immediately follows activin treatment

We need to know how cells time their response to changing signal factor concentration. Do cells always transduce a signal as soon as receptors are activated, or do they delay transduction until they are ready to make a gene response? One mechanism would be for cells to bind and transduce signal factors only at a particular time or stage in development, defined by developmental age. Cells would be able to receive or transduce a signal only at this time. As signal factor release and subsequent build-up is probably related to developmental age, cells would be provided with the right concentration of signal at their competent time. This is not an unreasonable idea, because, as noted above, cells express genes such as Xbra in response to activin signalling mainly according to a developmental clock, and not according to when they were exposed to the signal. The alternative idea is that cells initiate intracellular transduction whenever the signal is presented, but reveal a choice of gene response only at the appropriate stage of development, defined by developmental age.

The possibility that cells are able to bind activin to their receptors only at a particular developmental age can be eliminated. ^{35}S-activin binds to the activin type II receptor at any time over the whole 5 hour period from stage 8 to stage 10.5, and this binding is competable and specific (Dyson and Gurdon, 1998). Moreover, phosphorylation of Smad2 can be detected before MBT, in embryos injected with activin mRNA at the two-cell stage (Faure et al., 2000). The first intracellular step of activin transduction, namely the phosphorylation of Smad2, is recognisable by Western analysis with an antibody that recognises phosphoserine. Dissociated animal cap cells were prepared from embryos injected with GST-Smad2 mRNA, treated with activin (3 ng/ml for 15 minutes), washed and frozen for analysis 20 to 45 minutes after activin treatment. Western blots (Fig. 3A) show that the total amount of GST-Smad2 recognised by an anti-GST antibody is the same in all the samples. An anti-phosphoserine antibody applied to the same blot shows that Smad2 phosphorylation is already detectable 20 minutes after the first activin addition, and is seen strongly by 45 minutes (Fig. 3A). This rapid phosphorylation response is observed whether cells are treated with activin at a mid (stage 8) or late (stage 9.5) blastula stage (not shown), and therefore takes place at the same rate whether or not there is a subsequent transcriptional delay.

To ask whether Smad2 phosphorylation is directly followed by nuclear migration, we have used cells from GFP-Smad2-injected embryos to follow the intracellular movement of GFP-Smad2 protein by confocal microscopy in real time. We first see that GFP-Smad2 accumulates around the nucleus (Fig. 3B, 12 minutes and 14 minutes). Nuclear GFP can then already be seen 16 minutes after activin treatment, and has become strongly concentrated by 20 minutes. This rapid nuclear accumulation is observed whether cells are treated with activin at stage 9 or stage 10. We conclude that activin treatment leads to a rapid transduction response, at least in respect of nuclear Smad2 accumulation.

We need also to know whether the nuclear Smad2 response to activin signalling is concentration related. From Fig. 3C, we

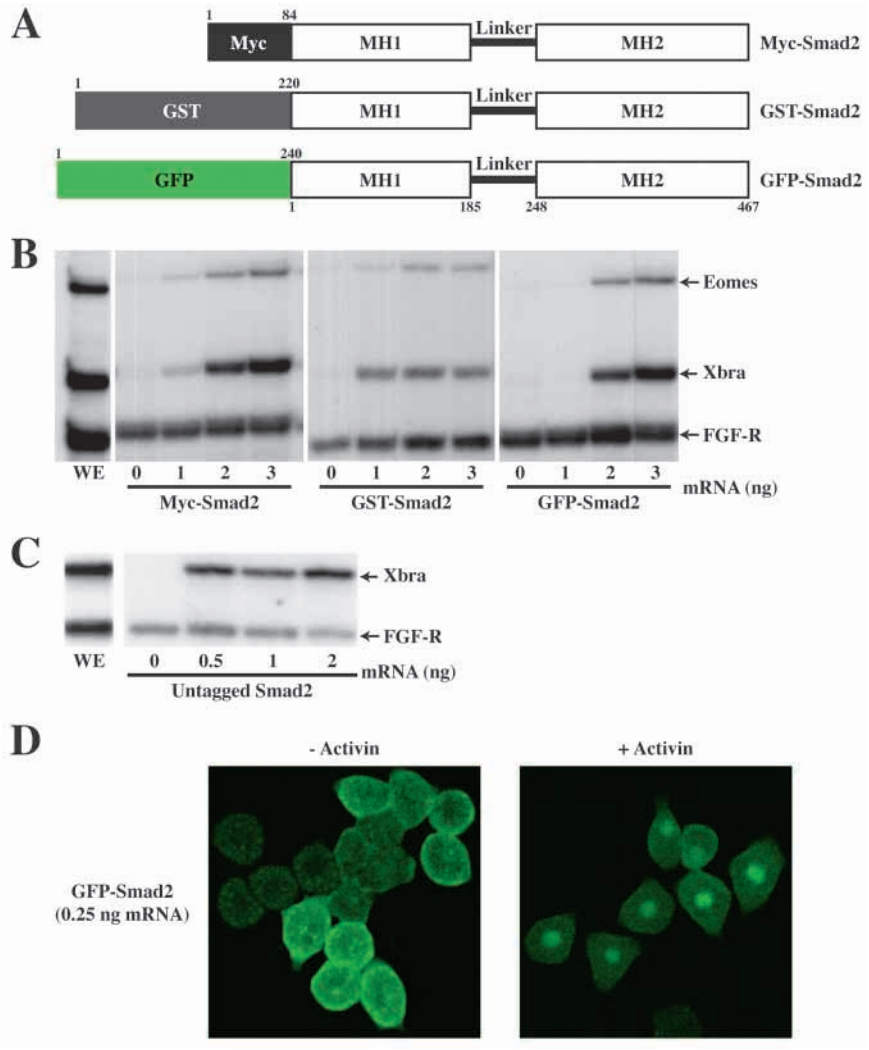

Fig. 2. Modified forms of Smad2 are valid markers of concentration-dependent activin transduction. (A) Schematic representation of the Myc-Smad2, GST-Smad2 and GFP-Smad2 fusion constructs. (B) Myc-Smad2, GST-Smad2 and GFP-Smad2 are almost equally efficient at inducing Eomes and Xbra. Each embryo was injected with the indicated amount of Myc-Smad2, GST-Smad2 or GFP-Smad2 mRNA. Animal caps were dissected at stage 8.5, cultured until stage 10.5 and gene expression was assayed by RNase protection. WE, whole embryo. (C) Induction of Xbra by injection of untagged Smad2 mRNA. Reproduced, with permission, from Shimizu and Gurdon (Shimizu and Gurdon, 1999). (D) GFP-Smad2 accumulates in the nucleus in response to activin signalling. Dissociated animal cap cells from GFP-Smad2-injected embryos were treated with activin for 15 minutes, and loaded onto a fibronectin substrate. When control embryos reached stage 10-10.5, the cells were observed by confocal microscopy.

see that, while the total amount of Myc-Smad2 remains the same, the amount of phosphorylated Smad2 increases proportionately, with threefold increments in activin concentration, in agreement with Shimizu and Gurdon (Shimizu and Gurdon, 1999). Real-time observation of nuclear GFP-Smad2 shows the same concentration related response, whenever cells are treated with activin between stage 9 and stage 10.5 (Fig. 3D).

In conclusion, we can eliminate the idea that an activin signal is transduced only at a particular developmental age. Rather we see that Smad2 phosphorylation and nuclear concentration follow directly and rapidly after activin exposure at any time during mid- and late-blastula stages, whether or not there is a transcriptional delay. We also see that the magnitude of this response is related to activin concentration.

Nuclear Smad2 concentration is inherited through mitosis

During the course of normal development between stages 8 and 10.5, each cell goes through several rounds of division. Fig. 4A shows the rate of increase in cell number during the early stages of activin response. Is the memory of activin treatment, and specifically the nuclear content of Smad2, inherited through mitosis? Low-density cultures on fibronectin slides show a uniformity of gene expression among cells after brief activin treatment (Gurdon et al., 1999). This suggests that all cells have remembered the activin concentration that they experienced, including those which have gone through a round of division. To trace nuclear Smad2 directly through mitosis, we have used cells from GFP-Smad2-injected embryos, treated with two different concentrations of activin (3 and 9 ng/ml). We have followed the intracellular movement of GFP-Smad2 protein by confocal microscopy in real time, and have looked at individual dividing cells containing GFP-Smad2 in the nucleus (Fig. 4B). Cells that received a 10 minute exposure to activin were cultured after washing to remove free activin and observed under the microscope as they underwent mitosis. For the two concentrations of activin that we used, both daughter cells deriving from a cell with nuclear GFP-Smad2 contained a similar amount of GFP-Smad2 localised in the nucleus (Fig. 4B). The quantitation of nuclear GFP-Smad2 shows no decrease in concentration after cell division (Fig. 4B). After division, daughter cells are half the volume of their parent cell, as there is no growth in these early embryo cells. Therefore the concentration of nuclear GFP-Smad2 should be the same in mother and daughter cells if receptors are working with the same efficiency. We conclude that the memory of activin concentration is inherited through, or reconstituted after, mitosis.

A continuous flow of Smad2 to the nucleus

We have seen that extracellular signal factor concentration is rapidly converted to a corresponding concentration of Smad2 in the nucleus, and this in turn is related to the choice of gene response. We now ask how the nuclear concentration of Smad2 is regulated. One way of achieving this would be for cells exposed to a certain concentration of activin, corresponding to a particular number of occupied receptors, to send a burst of phosphorylated Smad2 to their nuclei. These Smad2 molecules

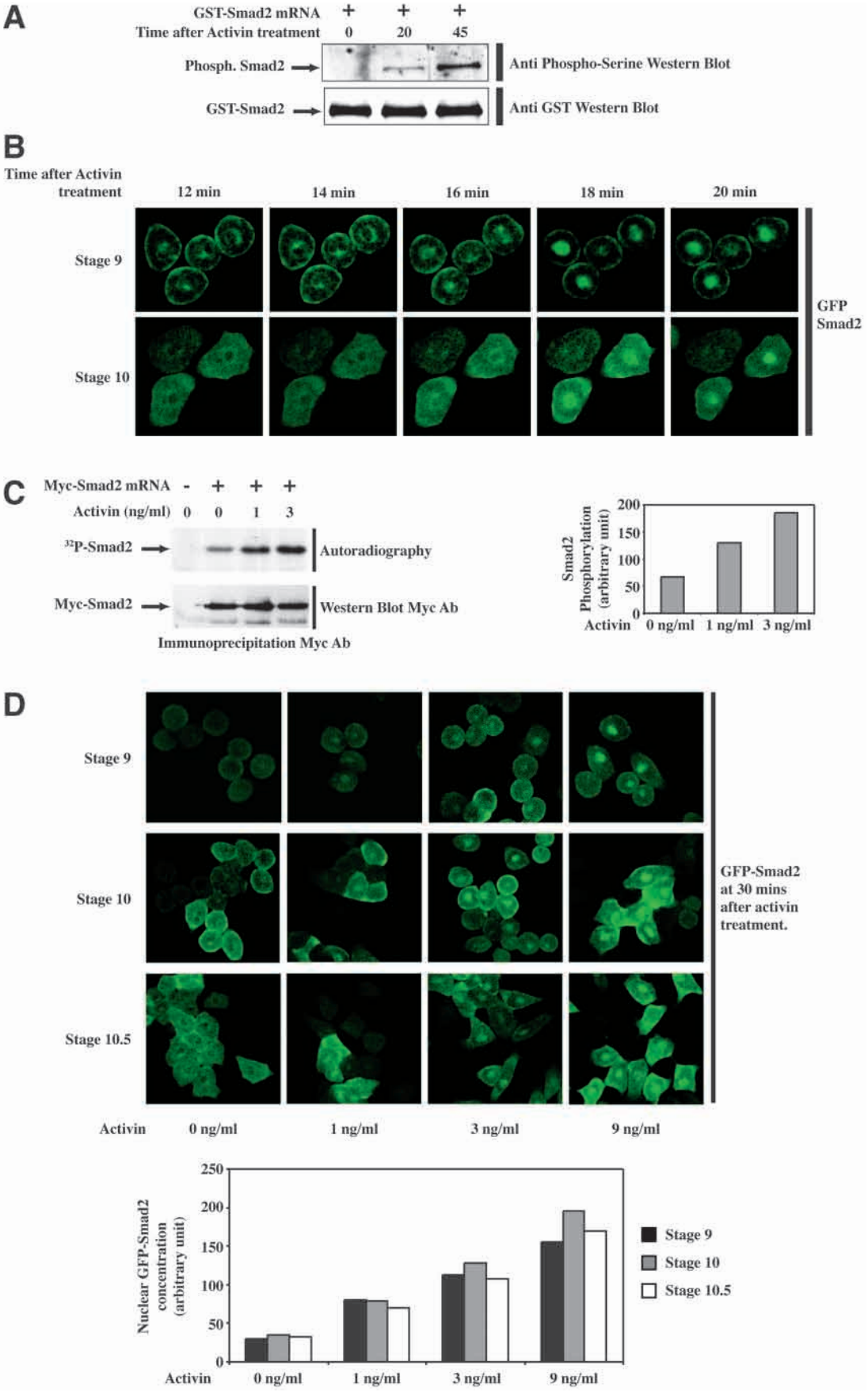
A
GST-Smad2 mRNA + + +
Time after Activin treatment 0 20 45
Phosph. Smad2
Anti Phospho-Serine Western Blot
GST-Smad2
Anti GST Western Blot
B
Time after Activin treatment
12 min
14 min
16 min
18 min
20 min
Stage 9
Stage 10
GFP Smad2
C
Myc-Smad2 mRNA - + + +
Activin (ng/ml) 0 0 1 3
32P-Smad2
Autoradiography
Myc-Smad2
Western Blot Myc Ab
Immunoprecipitation Myc Ab
Smad2 Phosphorylation (arbitrary unit)
200
150
100
50
0
Activin 0 ng/ml 1 ng/ml 3 ng/ml
D
Stage 9
Stage 10
Stage 10.5
GFP-Smad2 at 30 mins after activin treatment.
Activin 0 ng/ml 1 ng/ml 3 ng/ml 9 ng/ml
Nuclear GFP-Smad2 concentration (arbitrary unit)
250
200
150
100
50
0
Activin 0 ng/ml 1 ng/ml 3 ng/ml 9 ng/ml
Stage 9
Stage 10
Stage 10.5

Fig. 3. Phosphorylation and accumulation of Smad2 occur rapidly and in proportion to activin concentration. (A) Phosphorylation of Smad2 can be detected 20 minutes after activin treatment. Dissociated animal cap cells from GST-Smad2-injected embryos were treated with activin (3 ng/ml) for 15 minutes, reaggregated, and cultured for the indicated length of time. GST pulled down proteins from the cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose. The membrane was first blotted with an anti-phosphoserine antibody (top panel) to detect the phosphorylation of Smad2 and then blotted with an anti-GST antibody (bottom panel) to visualise total GST-Smad2. (B) GFP-Smad2 quickly enters the nucleus in cells treated with activin. Dissociated animal cap cells from GFP-Smad2-injected embryos were loaded onto a fibronectin substrate. When control embryos reached the indicated stage, the activin (5 ng/ml) was added to cells directly on the slides, and movement of GFP-Smad2 was observed by confocal microscopy in real time. (C) The phosphorylation of Smad2 is activin dose dependent. Dissociated animal cap cells from Myc-Smad2-injected embryos were treated with increasing doses of activin for 15 minutes and cultured in medium supplemented with ^{32}P-orthophosphate, until stage 10.5. Cells lysates were subjected to immunoprecipitation with anti-Myc antibody. Immunoprecipitated proteins were resolved on SDS-PAGE and transferred to nitrocellulose. The phosphorylation of Smad2 was detected by autoradiography (top panel). The membrane was then blotted with an anti-Myc antibody (bottom panel) to visualise total Myc-Smad2. The Smad2 phosphorylation was quantitated and the values plotted. (D) The nuclear accumulation of Smad2 is activin concentration related. Dissociated animal cap cells from GFP-Smad2-injected embryos were treated with increasing concentrations of activin at the indicated stages, and loaded onto a fibronectin substrate. Thirty minutes later, the localisation of GFP-Smad2 was analysed by confocal microscopy. The nuclear GFP-Smad2 concentration was quantitated using the public-domain NIH Image program (Teleman and Cohen, 2000) and the values are shown at the bottom.

would remain in the nucleus for several hours, though they could be supplemented at any time by further influxes of Smad2 if the activin concentration outside the cells is increased. The other way of achieving the same result is for Smad2 molecules that enter the nucleus to be continuously degraded as shown in cultured cell lines (Lo and Massague, 1999) and replaced by new entering molecules. The concentration of Smad2 in the nuclei of cells at any one time would reflect the balance between nuclear import and degradation.

In mammalian tissue culture experiments, phosphorylated Smad2 peaks 1 hour after a short exposure to TGFβ, and then declines (Lo and Massague, 1999). With *Xenopus* animal cap cells exposed to activin for 10 minutes, and then washed, the result is different. Phosphorylation of the Smad2 C-terminal serines, as measured by western analysis, reaches a maximum level 45 minutes after activin treatment, and then shows no decline for at least the next 4 hours (Fig. 4C). Likewise, the nuclear concentration of Smad2 that follows a 10 minute activin exposure lasts at least 4 hours in a non-dividing cell (Fig. 4D, quantitation).

By observing animal cap cells dissociated from GFP-Smad2-injected embryos and treated with activin, we have distinguished between the burst and the continuous turnover explanations for the maintenance of phosphorylated nuclear Smad2 (Fig. 4E). These cells contain different amounts of GFP, as some cells received, by chance, more mRNA, and hence more GFP-Smad2 protein (Fig. 4E, red arrows) than others (Fig. 4E, yellow arrows). Because there is such a low amount of GFP-Smad2 in some cells (Fig. 4E, yellow arrows), a small decrease of GFP-Smad2 renders them invisible quicker than cells with a bigger pool of GFP-Smad2 (Fig. 4E, red arrows). After activin treatment, nuclear GFP-Smad2 can be easily seen to disappear in cells with a lower amount of GFP (Fig. 4E, yellow arrows). This favours the idea of continuous nuclear Smad2 turnover and replacement.

To determine whether the long lasting high level of nuclear Smad2 is maintained by turnover, we labelled activin-treated cells with ^{35}S-methionine for 20 minutes. Two and a half hours after the end of the labelling period, immunoprecipitation showed that half of the labelled Smad2 pool had been degraded (Fig. 5A, black line), whereas in non-activin treated cells the amount of labelled Smad2 remained constant (Fig. 5A, grey line). To determine the turnover of activated (as opposed to total) Smad2, we labelled cells with ^{32}P-orthophosphate which marks only activated Smad. After immunoprecipitation, the Smad2 signal was quantitated (Fig. 5B). Phosphorylation of Smad2 can not be detected more than two hours after the removal of activin. The Smad2 turnover must have a half-life of 1 hour or less, because the ^{32}P-orthophosphate pool is not effectively chased in our experiments. These results are inconsistent with the burst hypothesis and support the idea of a continuous turnover and replacement.

If there is a continuous activation of Smad2, the phosphorylation of Smad2 should continue to be detectable long after removal of activin. Animal cap cells were treated with activin for 15 minutes and ^{32}P-orthophosphate was added either immediately or 1.5 hours after activin washing. After immunoprecipitation, we observed the ^{32}P labelling of Smad2 (Fig. 5C). We saw that Smad2 activation could be detected when ^{32}P-orthophosphate was added to the cells long after activin removal. We conclude that Smad2 is continuously phosphorylated in the absence of activin.

To demonstrate more directly the continuing activation and nuclear entry of Smad2 in response to activin, we fused GFP-Smad2 to the hormone-binding domain of the glucocorticoid receptor (GR-GFP-Smad2) (Fig. 5D) to provide a conditional release of Smad2. The glucocorticoid domain causes sequestration of the fusion protein in the heat-shock apparatus; this sequestration is relieved and the protein released by addition of dexamethasone (DEX) (Kolm and Sive, 1995; Mattioni et al., 1994). Nuclear accumulation of GR-GFP-Smad2 can be observed in cells treated with dexamethasone more than 3 hours after the end of activin treatment (Fig. 5E). We conclude that the concentration of Smad2 is maintained in the nucleus of activin treated cells by continuous entry and degradation.

The Smad2 flow changes up but not down

In normal development, Nieuwkoop centre signalling is thought to start at the 250-cell stage (Jones and Woodland, 1987) and to become increasingly strong during stages 8 and 9 (Wylie et al., 1996). Animal cap cells near the Nieuwkoop signalling source must therefore experience a continuous increase in the concentration of the factor(s) during this time. As already shown by bead-exchange experiments (Gurdon et al., 1995) and confirmed in Fig. 6A, cells activated genes according to the highest concentration of activin they received.

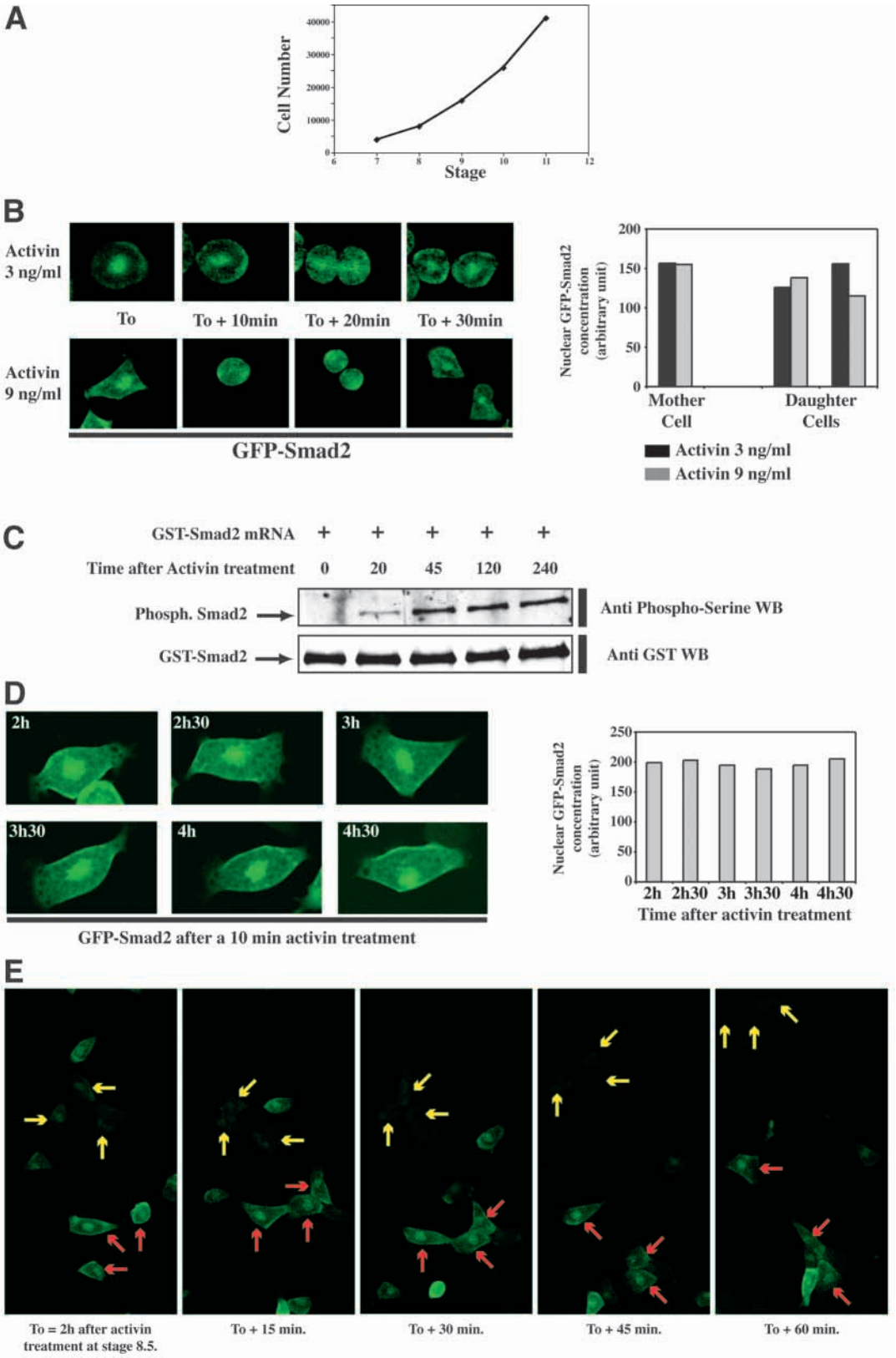

Fig. 4. An activated nuclear Smad2 pool is inherited through mitosis and persists for at least 4 hours. (A) The absolute increase in cell number, during the activin competence period, as a function of stage is depicted. (B) Nuclear Smad2 is relocalised to the nucleus after mitosis. Confocal microscopy observation, in real time, of GFP-Smad2 localisation in activin treated cells undergoing mitosis. The nuclear GFP-Smad2 concentration was quantitated and the values are shown on the right. (C) Transient activin treatment leads to the formation of a stable pool of phosphorylated Smad2. The experiment was designed as described in Fig. 3A. (D) GFP-Smad2 is localised in the nucleus for several hours after activin treatment. Dissociated animal cap cells from GFP-Smad2-injected embryos were treated with activin and loaded onto fibronectin substrates. The localisation of GFP-Smad2 was observed in the same cell, over a period of 4.5 hours, by confocal microscopy in real time. The quantitation of the nuclear GFP-Smad2 concentration is shown on the right. (E) Confocal microscopy observation, in real time, of GFP-Smad2 distribution in activin treated cells cultured on a fibronectin substrate. Yellow arrows indicate cells containing a small pool of GFP-Smad2, and red arrows cells with a bigger pool of GFP-Smad2.

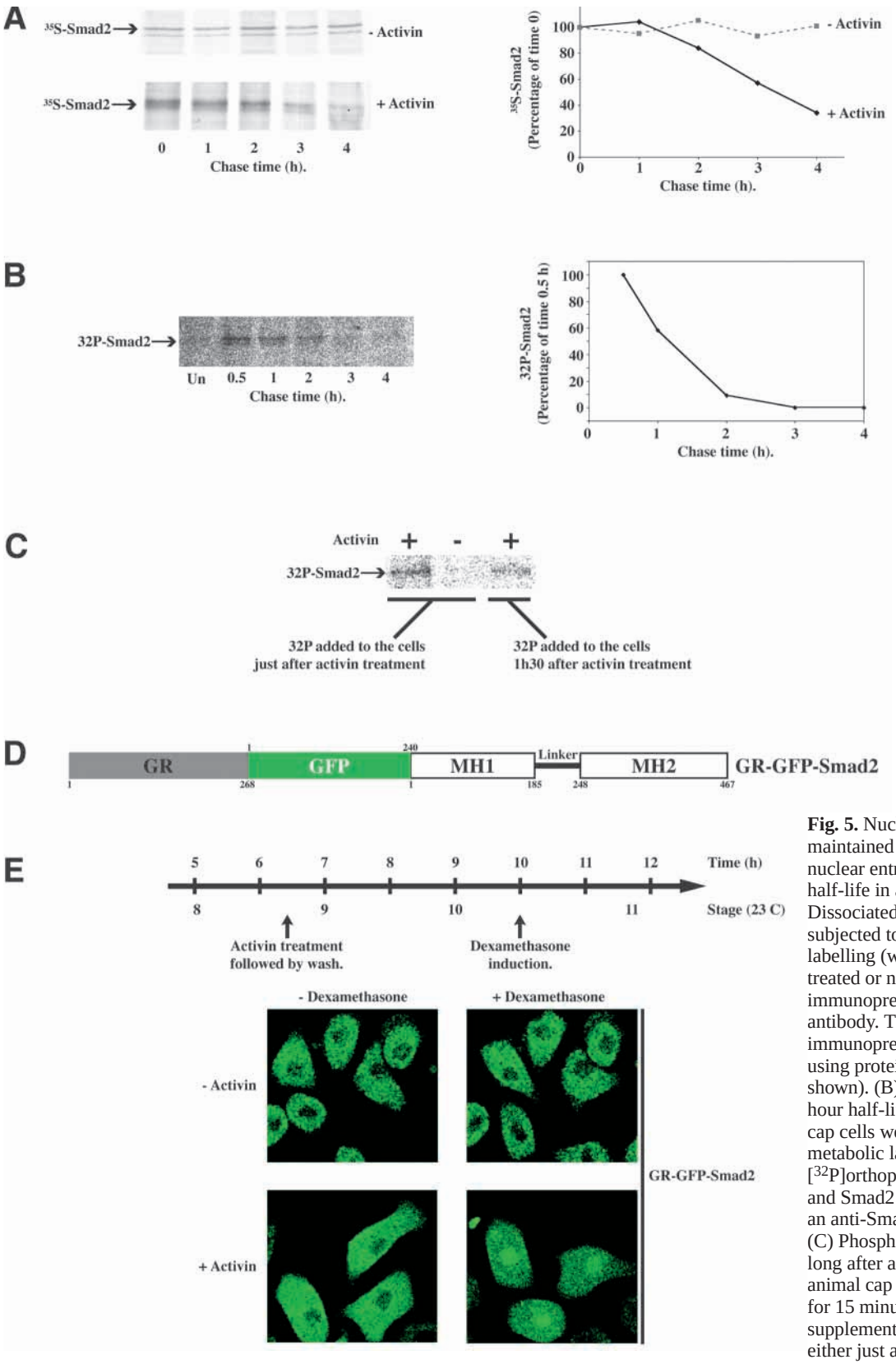

Fig. 5. Nuclear Smad2 concentration is maintained by continuous degradation and nuclear entry. (A) Smad2 has a 2-3 hour half-life in activin-treated cells (graph). Dissociated animal cap cells were subjected to a pulse-chase metabolic labelling (with [^{35}S]methionine/cysteine), treated or not with activin, and Smad2 was immunoprecipitated with an anti-Smad2 antibody. The specificity of immunoprecipitation was confirmed by using protein-A-sepharose alone (data not shown). (B) Activated Smad2 has a 1-1.5 hour half-life (graph). Dissociated animal cap cells were subjected to a pulse-chase metabolic labelling (with [^{32}P]orthophosphate), treated with activin, and Smad2 was immunoprecipitated with an anti-Smad2 antibody. Un, untreated. (C) Phosphorylation of Smad2 is detected long after activin treatment. Dissociated animal cap cells were treated with activin for 15 minutes and cultured in medium supplemented with [^{32}P]orthophosphate either just after or 1.5 hours after activin treatment. Smad2 was immunoprecipitated with an anti-Smad2 antibody. (D) GR-GFP-Smad2 fusion construct. (E) Dexamethasone treatment can induce nuclear Smad2 localisation, several hours after activin has been removed. Dissociated animal cap cells from GR-GFP-Smad2-injected embryos were treated with activin for 15 minutes, extensively washed, loaded onto a fibronectin substrate before control embryos reached stage 9. Three hours after stage 9, GR-GFP-Smad2 release was induced by addition of dexamethasone and GR-GFP-Smad2 localisation observed by confocal microscopy in real time.

Cells exposed to 1 ng/ml activin for 15 minutes expressed Xbra but not Eomes at stage 10.5 (Fig. 6A, track 1). Cells given a 15 minute treatment of activin at 4 ng/ml expressed Eomes more strongly than Xbra (Fig. 6A, track 2). Exactly the same ratio of Eomes:Xbra expression was induced by 1 ng/ml followed by 4 ng/ml, as well as by 4 ng/ml followed by 1 ng/ml (Fig. 6A, tracks 3 and 4, respectively). Because an extracellular concentration of activin is transduced to the nucleus in 20 minutes, and as cells remember an activin concentration for several hours, we need to understand how cells react at the level of transduction to external activin concentrations that go up with time.

Cells exposed to a weak concentration of 1 ng/ml activin for only 15 minutes show a visible but weak GFP accumulation in the nucleus, and this does not increase with time (Fig. 6B,C). The same 1 ng/ml activin for 15 minutes followed 30 minutes later by a concentration of 4 ng/ml activin results in a clear increase of the GPF nuclear accumulation (Fig. 6B,C). Not surprisingly, cells that received an initial concentration of 4 ng/ml, followed 30 minutes later by a 1 ng/ml concentration, do not show a decrease in nuclear GFP; the level of nuclear GFP remains at a high concentration (Fig. 6B,C).

We have shown above that a particular dose of activin, which equates to a constant number of activated receptor complexes and to continuous flow of Smad2 to the nucleus, elicits the same choice of gene response over several hours, and that there is no integration of number of occupied receptors with time. We now conclude that cells quite rapidly change their perception of activin concentration in an upwards direction, but not downwards, over the course of a few hours, by adapting the flow of Smad2 to the highest concentration of activin. We therefore suppose that, in the absence of anti-factors, when the concentration of signal factor has reached its

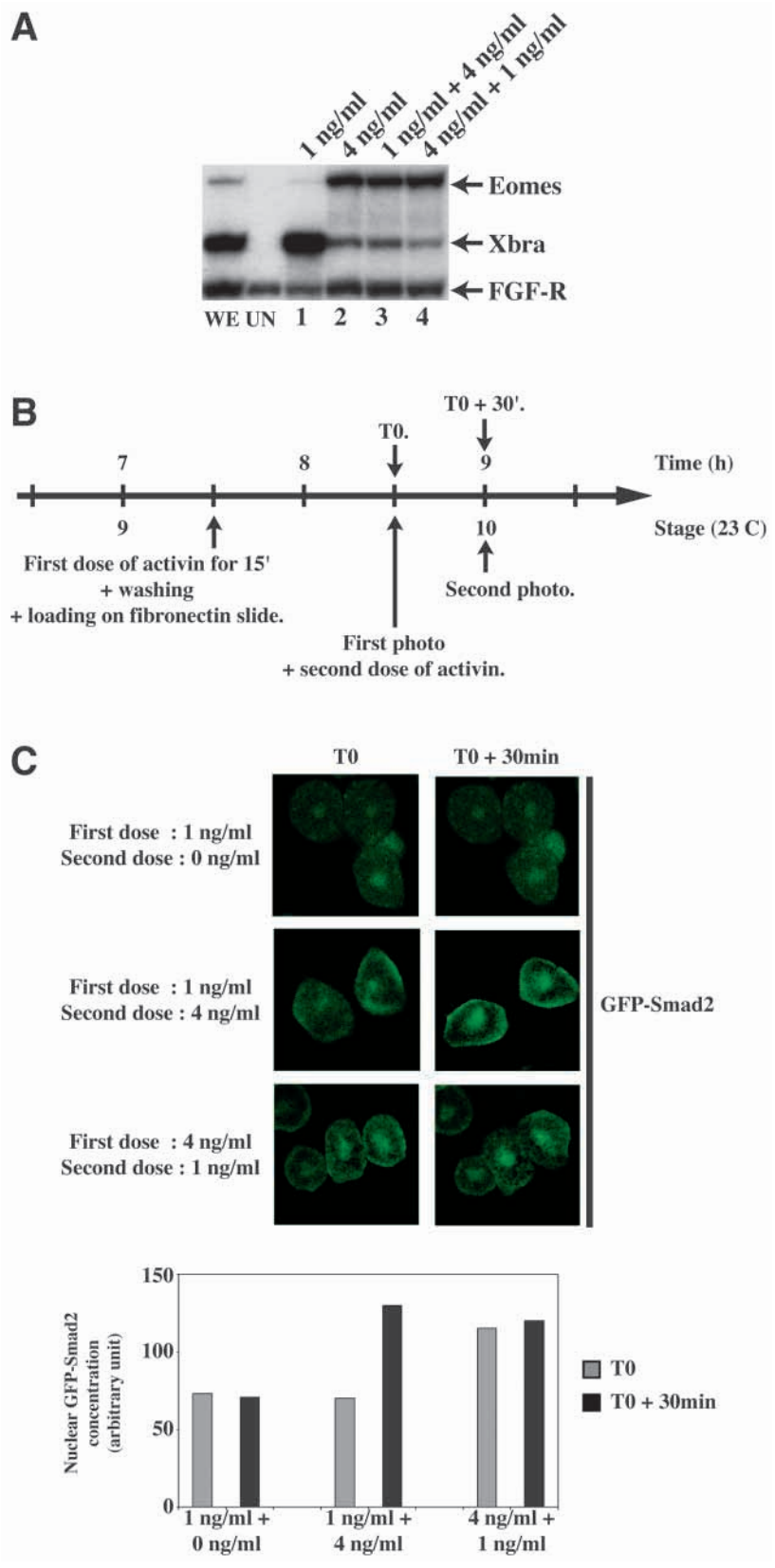

Fig. 6. The Smad2 flow volume is used by the cells to interpret changing morphogen concentration. (A) Cells can switch from a low dose to a high dose gene response, but not from a high to a low dose gene response. RNase protection analysis of Apod and Eomes expression. Dissociated animal cap cells were treated either with 1 ng/ml of activin for 15 minutes (track 1), or with 4 ng/ml for 15 minutes (track 2), or with 1 ng/ml for 15 minutes and with 4 ng/ml for 15 minutes (track 3), or with 4 ng/ml for 15 minutes and with 1 ng/ml for 15 minutes (track 4). Cells were extensively washed after each dose of activin and cultured until control embryos reached stage 10.5. WE, whole embryo. UN, untreated cells. (B) Design of the experiment; results shown in C. Dissociated animal cap cells from GFP-Smad2-injected embryos were treated with a first dose of activin (1 or 4 ng/ml) for 15 minutes and loaded onto fibronectin substrates. At time T0, a first photo of the cells is taken and the cells receive a second dose of activin (4 or 1 ng/ml) or only buffer. At T0 + 30 minutes, a second photo of the same cells is taken. (C) The amount of nuclear Smad2 increases in response to higher concentrations of activin, but does not decrease with lower concentrations. GFP-Smad2 localisation is observed by confocal microscopy in real time. The nuclear GFP-Smad2 concentration was quantitated using the public-domain NIH Image program and the values are plotted on the bottom graph.

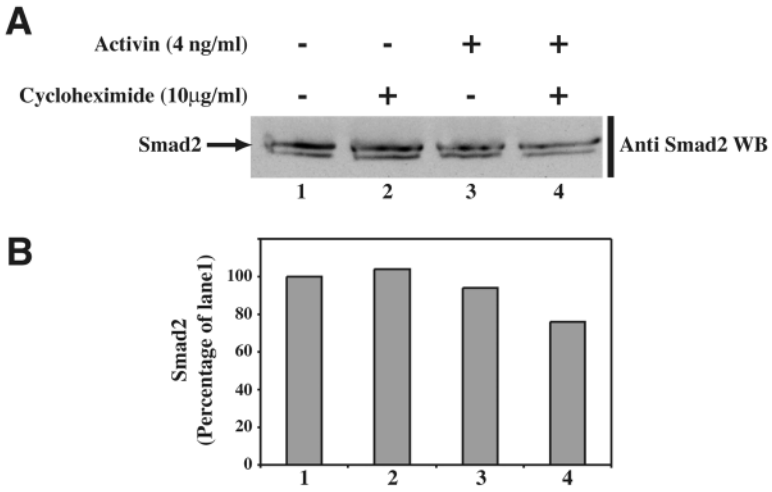

Fig. 7. (A) The cytoplasmic pool of Smad2 is big enough to supply the Smad2 flow for several hours. Dissociated animal cap cells, pretreated or not with cycloheximide for 1 hour, were then treated with a concentration of 4 ng/ml of activin for 15 minutes at stage 9, washed to remove the unbound activin, cultured for 3 hours and the total amount of Smad2 was determined by western blot, using an anti-Smad2 antibody. (B) Quantitation of the total amount of Smad2 from the experiment shown in A, using the public-domain NIH Image program.

highest level in part of an embryo, cells in that region will continue to express the appropriate genes, even if the concentration of factor subsequently decreases.

The Smad2 pool

We concluded that (1) cells remember the activin concentration for several hours, (2) the basis of this memory is reflected in the nuclear concentration of Smad2, and (3) this nuclear concentration of Smad2 is maintained in steady state by a continuous turnover and replacement. This model raises the following question: is the cytoplasmic pool of Smad2 big enough to refresh the nuclear pool of Smad2 for several hours, even in the presence of cycloheximide?

To answer this question, we analysed the Smad2 protein level in animal cap cells treated with activin in the presence or absence of cycloheximide (Fig. 7A). Cells were cultured for 3 hours after activin treatment and the total amount of Smad2 determined by western blot with an anti-Smad2 antibody. The quantitation of two separate experiments (Fig. 7B) shows that 3 hours after activin exposure and cycloheximide treatment, the amount of Smad2 decreases only by 25% from the initial level. We conclude that there is enough Smad2 in the cytoplasm to supply the Smad2 flow and to maintain the nuclear concentration for several hours, even in absence of protein synthesis.

Transduction flow is maintained by an activated receptor complex

The flow of Smad2 continues for several hours after the complete removal of activin from around cells, and is not therefore maintained by the continuing extracellular presence of the factor that induced the flow. The flow could be maintained at any step in the transduction pathway from occupied receptors to a chromatin configuration responsible for selective transcription. It is hard to imagine how a chromatin state could be responsible because the two hours (Fig. 5B) or less for activated Smad2 turnover time requires its supply to be replenished to account for its persistence for at least 4 hours in the absence of ligand supply; a chromatin configuration is unlikely to be able to maintain transduction flow from cytoplasm to nucleus.

To test whether persistent receptor signalling is responsible for continuing Smad2 transduction, we have used the specific serine-threonine kinase inhibitor H7 to cause discontinuation of receptor signalling at various times after activin treatment. Animal cap cells were exposed to activin for 10 minutes and washed, as usual, and then treated with serial dilutions of the inhibitor H7. High concentrations of H7 damage cells, as judged by the inhibition of the FGF-R transcripts (Fig. 8A, tracks 3 and 4). A low concentration of inhibitor has no effect (Fig. 8A tracks 7 and 8). Middle concentrations (tracks 5 and 6) reduce or inhibit the transcription of Xbra, Eomes and Apod, but do not reduce the level of FGF-R.

Is H7 really inhibiting the phosphorylation of Smad2 by the type I receptor? It could be that H7 represses a downstream step of the activin-signalling pathway. Animal cap cells from GST-Smad2 injected embryos were exposed to activin for 10 minutes, washed and then cultured minus or plus H7 inhibitor. The phosphorylation of Smad2 is monitored by western analysis with an anti-phosphoserine antibody, after immunoprecipitation (Fig. 8B). We see that H7 inhibits the phosphorylation on the C-terminal serine of Smad2, in response to activin.

To confirm that H7 represses specifically the phosphorylation of Smad2, we used a constitutively active type I receptor (Alk4*) (Armes and Smith, 1997) or a constitutively activated Smad2 (Smad2*), where the C-terminal serines have been mutated to glutamine, to mimic the phosphorylation. The constitutively activated Smad2* activates gene transcription at a lower concentration of injected mRNA than Smad2 (data not shown). H7 should repress gene activation by Alk4* but not activation by Smad2*. Alk4*- or Smad2*-injected animal cap cells were treated, or not, with two concentrations of H7 and gene activation was analysed by RNase protection assay (Fig. 8C). The two effective concentrations of H7 repress the activation of Apod, Eomes and Xbra by Alk4* (lanes 6 and 7), but not their activation by Smad2* (lanes 3 and 4). We conclude that H7 specifically inhibits receptor signalling, but not downstream transduction.

A prediction from this result is that the inhibitor H7 should have no effect on gene expression the nearer it is administered to the time when gene transcription starts. This is because activin induces a continuing flow of Smad2 from the cytoplasm to the nucleus. The inhibitor arrests this flow, and the nuclear content of Smad2 will decrease in accord with its turnover, remaining at up to half of its normal level for 1-1.5 hours. To

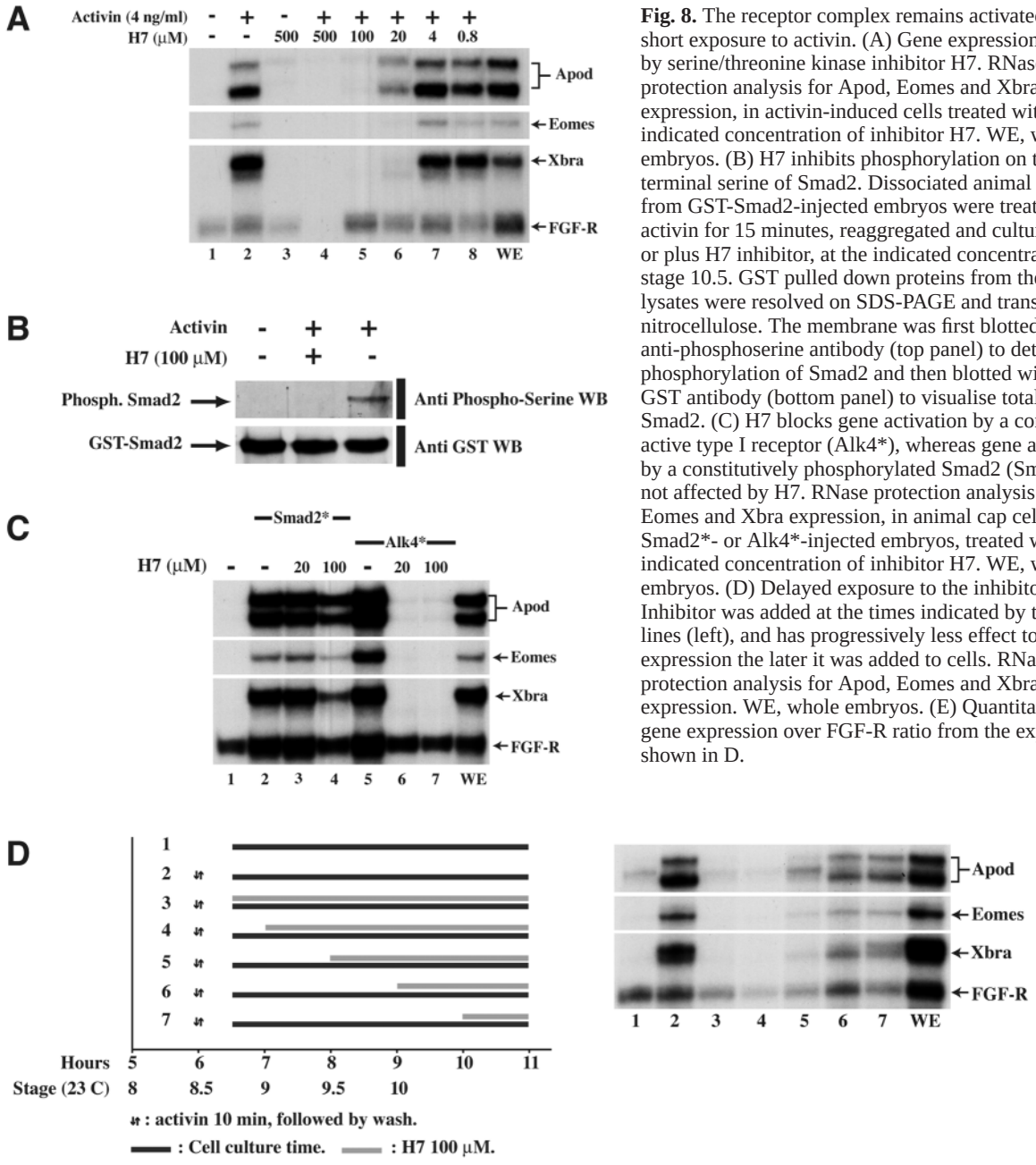

Fig. 8. The receptor complex remains activated after a short exposure to activin. (A) Gene expression is blocked by serine/threonine kinase inhibitor H7. RNase protection analysis for Apod, Eomes and Xbra expression, in activin-induced cells treated with the indicated concentration of inhibitor H7. WE, whole embryos. (B) H7 inhibits phosphorylation on the C-terminal serine of Smad2. Dissociated animal cap cells from GST-Smad2-injected embryos were treated with activin for 15 minutes, reaggregated and cultured minus or plus H7 inhibitor, at the indicated concentration, until stage 10.5. GST pulled down proteins from the cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose. The membrane was first blotted with an anti-phosphoserine antibody (top panel) to detect the phosphorylation of Smad2 and then blotted with an anti-GST antibody (bottom panel) to visualise total GST-Smad2. (C) H7 blocks gene activation by a constitutively active type I receptor (Alk4*), whereas gene activation by a constitutively phosphorylated Smad2 (Smad2*) is not affected by H7. RNase protection analysis for Apod, Eomes and Xbra expression, in animal cap cells from Smad2*- or Alk4*-injected embryos, treated with the indicated concentration of inhibitor H7. WE, whole embryos. (D) Delayed exposure to the inhibitor H7. Inhibitor was added at the times indicated by the red lines (left), and has progressively less effect to gene expression the later it was added to cells. RNase protection analysis for Apod, Eomes and Xbra expression. WE, whole embryos. (E) Quantitation of the gene expression over FGF-R ratio from the experiment shown in D.

test this prediction, animal cap cells were treated with activin for 10 minutes, washed and the inhibitor added at different times. In Fig. 8D,E, we see that gene expression is progressively restored to the nil-inhibition level, the shorter the time between inhibitor addition and the assay of gene expression. We conclude that the continuing flow of Smad2 to the nucleus is maintained by a receptor, or ligand-receptor complex, that remains active in the absence of any extracellular supply of activin. An activated receptor complex is the most likely basis of the memory described above.

DISCUSSION

The timing of transduction

In many examples of extracellular signalling, cells make an almost immediate response, so that the expression of induced genes can be seen within minutes. An example is the activation of Fos which can be seen within 5 minutes at 37°C of transferring cells into growth-promoting medium (Greenberg and Ziff, 1984). In this and others examples of rapid effects, cells are making an all-or-none metabolic or growth response to a signal. In the case of metabolic or growth responses, cells are primed to make the particular activity for which they are prepared. For differentiation responses, cells must choose one response out of several possibilities. In spite of this significant difference, the activin response analysed here starts to be transduced on a similar time scale to Fos, that is within 15 minutes of signal factor presentation at 23°C, as seen by the nuclear entry of activated Smad2. This is true even if the eventual outcome of the signalling, that is gene expression indicative of the differentiation pathway, does not emerge for up to 4 hours.

The timing of gene response, reviewed by Cooke and Smith (Cooke and Smith, 1990) is a peculiar characteristic of animal development. The mechanism of this developmental timing is not understood. However, our results described here have eliminated one possible explanation for it. This is that it depends on the delayed transduction of a signal until a certain developmental age has been reached. We find that an activin signal is rapidly transduced to the level of nuclear Smad2 whenever the signal is presented. Evidently the timing mechanism resides at a level beyond that of nuclear Smad2 accumulation.

Response to changing morphogen concentration

The reason we initiated the work described here was to determine how cells make a correct response to changing signal factor concentration. Although there are only a few cases where this has been directly measured, nearly all signalling molecules believed to guide early vertebrate development have concentration-related effects, whether directly like members of the TGFβ class that contribute to Nieuwkoop signalling, or indirectly like the anti-factors noggin and chordin in Spemann signalling. In the case of activin and probably in other early developmental signalling molecules, cells are competent to respond to signals over a wide time span of several hours. Therefore cells must experience factor concentrations that change with time, but must nevertheless select the correct concentration to which to make their major response.

Our results suggest the following process by which cells respond to changing signal factor concentration during development. As soon as signalling starts, cells bind the extracellular factor to their receptors, and quickly transduce the signal through activated Smad2 to create a nuclear concentration of Smad2. A steady-state nuclear Smad2 concentration is maintained by a continuous flow from the cytoplasm to the nucleus, where Smad2 is degraded. In transformed cell lines, phosphorylated Smad2 is degraded by ubiquitination (Lo and Massague, 1999). But in these cells, the significance of ubiquitin-dependent degradation of Smad2 remains to be defined. It may ensure a swift elimination of the TGFβ signalling that regulates very dynamic physiological processes, or it may remove the surplus activated Smad from the nucleus (Massague and Chen, 2000). In the case we have analysed here, it ensures a constant nuclear concentration of Smad2, which reflects the extracellular concentration of activin. A gene response, and hence cell-fate decision, is made at a particular developmental stage unrelated to when signalling commenced. Even in the absence of a continuing signal source, cells remember the highest concentration of signal that they have received by maintaining the same nuclear concentration of activated Smad through the continuing activity of an activated receptor complex. As the extracellular concentration of signal factor goes up with the continuing emission of a signal from its source in the embryo, the nuclear Smad2 concentration increases in proportion by an increase in the volume of intracellular Smad2 flow. In this way, cells always select their choice of gene response according to the highest signal factor concentration that they experience in their competent life.

Relationship to transduction response in normal development

All our experiments described here have treated blastula animal cells with activin. It is currently believed that activin reflects the activity of related TGFβ signal molecules such as Xnr1-Xnr6, derriere, etc (Hill, 2001; Whitman, 2001). Most animal cap cells do not normally receive TGFβ signals, but nevertheless respond to experimentally supplied activin by following the same gene responses and cell fate pathways as equatorial blastula cells, thought to be the normal recipients of TGFβ (Nieuwkoop) signalling. As activin protein is much easier to acquire than Xnr protein, and as animal cap cells have received no TGFβ signals at the time of isolation, we use this protein and these cells to understand the principles of cell response to changing signal factor concentration in development.

We suggest that our model is likely to apply to signalling in normal development, except for the absence of anti-factors, such as Cerberus, that are secreted from the dorsal lip of gastrulae and are absent from animal cap cells. Such molecules may be responsible for the loss of phosphorylated Smad2 in the dorsal region of mid-gastrulae (Lee et al., 2001), and would presumably prevent an indefinite increase in extracellular concentration of TGFβ factors. The decrease of phosphorylated Smad2 in the dorsal region compared with the ventral region occurs around stage 11 (Lee et al., 2001). The loss of competence to respond to TGFβ factors, which happens at stage 11 (Gurdon et al., 1985; Jones and Woodland, 1987), can also explain this loss of phosphorylated Smad2 in the dorsal region. In all other respects, we believe our conclusions are consistent with what is known about TGFβ signalling in normal embryos, where the timing of signal factor exposure is not as accurately known as it is in our protein addition experiments.

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