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Short Contribution

Artefactual gene induction during preparation of *Xenopus laevis* animal cap explants

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ABSTRACT The animal cap assay in *Xenopus laevis* was used to study the induction and regulation of the mesoderm-specific gene *Xegr-1*, a homolog of the mammalian *egr-1* genes. *Egr-1* is an immediate-early gene whose growth factor-stimulated transcriptional induction displays a transient activity profile and occurs independent of protein synthesis. The *Xegr-1* promoter contains multiple serum response elements (SREs). In this paper we show that *Xegr-1* is induced unspecifically during the process of animal cap preparation. Transcripts of *Xegr-1* appear already 30 min after cutting of animal caps. *Xfos*, another SRE-regulated immediate-early gene, is induced with the same kinetics as *Xegr-1*. In contrast, the *Xbra* gene is not induced under the experimental conditions used. *Xfos* and *Xegr-1* transcripts are not rapidly down-regulated after mechanical stimulation, but can be detected for up to 4 h later. Wounding-dependent *Xegr-1* induction is reduced by injection of either mRNA coding for the dominant inhibitory forms of both the FGF receptor and the transcription factor Elk-1. *Xegr-1* expression can be reinduced by mesoderm-inducing factors. These results led us to develop a new protocol for animal cap preparation, which circumvents the observed undesired artefactual gene activation events.

KEY WORDS: *Xenopus laevis, Ets transcription factors, Xegr-1, serum response element, animal cap*

Mesoderm formation in *Xenopus laevis* is induced and maintained by an orchestrated network of signaling pathways (for review: Heasman, 1997). The interplay of signaling cascades leads to the formation of different mesodermal identities, ranging from dorsal to ventral mesoderm. The animal cap assay represents a powerful tool to identify mesoderm-inducing factors and to characterize signaling components involved in signal transduction. Animal cap cells of blastula stage embryos are of ectodermal origin and are destined to become epidermal cells. However, they are competent to form mesoderm upon addition of inductive signals. Experimentally, mesoderm-inducing signals can either be injected into the animal hemisphere of 2-4 cell stage embryos in form of mRNA followed by dissection of animal caps by blastula stages. Alternatively, mesoderm-inducing factors are added as soluble factors to dissected animal caps of blastula stage embryos. Animal cap explants are then cultured until they reach the desired stage (e.g. gastrula stages) at which they are processed for further analysis.

During the characterization of the recently described *Xegr-1* gene (Panitz *et al.*, 1998) we noticed that untreated control animal cap explants showed strong *Xegr-1* mRNA expression when they were explanted at stage 9 (blastula, 7 h after fertilization) and lysed at stage 10 (early gastrula, 9 h after fertilization; B. Krain and A. Nordheim, unpublished). However, when caps were dissected at stage 8 or 8+ and lysed at stage 10, *Xegr-1* expression was not detectable or very low, at stage 10, respectively. This observed gene induction upon cap explantation made experiments impossible in which animal cap dissection stretched over a longer time period, e.g. starting from stage 8/8+ through stage 9/9+ until finally being processed at a fixed time point. As *Xegr-1* expression was shown to be dependent on a functional Ras/Raf/MAPK pathway (Panitz *et al.*, 1998), we wished to know whether there was a link between ectopic *Xegr-1* activation and the recently described increase in MAPK (mitogen activated protein kinase) activity, as induced by the dissection procedure of animal caps (LaBonne and Whitman, 1997).

Abbreviations used in this paper: SRE, serum response element; dnFR, dominant negative FGF-receptor; dnElk-1, dominant negative Elk-1; *Xegr-1*, Xenopus early growth-response gene; *Xbra*, Xenopus brachyury gene; NF, staging of embryos according to Nieuwkoop and Faber (1967).

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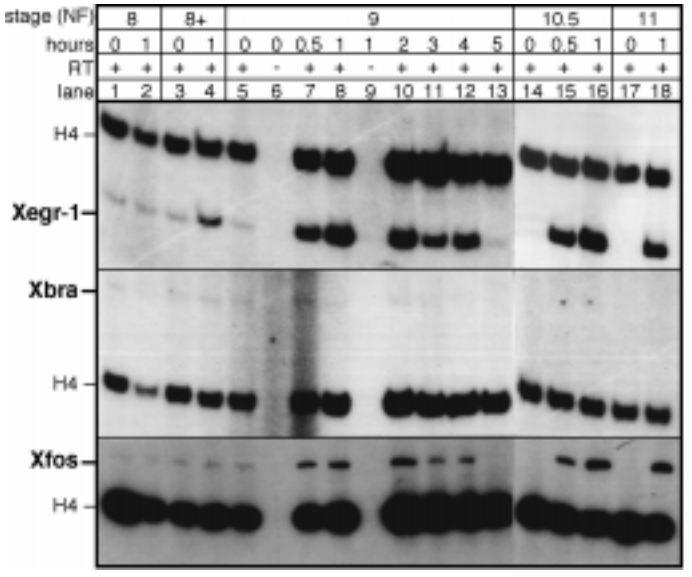

Fig. 1. Dissection of animal caps leads to gene expression of *Xegr-1* and *Xfos* but not *Xbra*. *Animal caps were explanted at different timepoints during blastula and gastrula stages (according to Nieuwkoop and Faber, 1967) and cultivated in 0.5x modified Barth´s saline for different periods of time (hours). Pools of 5 animal caps were lysed and gene expression (Xegr-1, Xfos, Xbra) was analyzed by RT-PCR. Co-amplification of histone-4 (H4) was used as an internal standard. Genomic DNA contamination was assayed in a PCR control amplification which was missing the initial reverse transcription step (RT-, lanes 6 and 9).*

Dissection of animal caps is among the most widely used preparation procedures in *Xenopus laevis* experimentation. The associated embryonic wounding, however, leads to a rapid increase in ERK (extracellular regulated kinase) activity in animal caps (LaBonne and Whitman, 1997). Increased ERK activity could be detected for over 60 min and was shown to be inhibited by the presence of the dominant inhibitory FGF-receptor. ERK activity was shown to increase by cutting dissected animal caps into quarters. This procedure could induce weak and transient expression of brachyury *(Xbra)*, a mesoderm-specific gene. Embryonic wounding apparently leads to a massive and unregulated release of FGF molecules (most likely bFGF) which bind to FGF-receptors thereby finally resulting in an increased ERK activity. Apart from *Xbra* no other downstream targets of wounding-induced ERK activity are reported to date.

We here report that in animal cap assays wounding-dependent induction of gene expression can impede studies of the FGF-signaling cascade during mesoderm induction. We find that the dissection procedure leads to a rapid activation of genes that contain serum response elements (SREs) in their promoters. Manual dissection of animal caps is shown to induce transcription of both an SRE-regulated mesoderm-specific gene (*Xegr-1*) and a gene that becomes downregulated shortly after fertilization (*Xfos*).

Transcriptional activation of Xegr-1 and Xfos by dissection

In a first step to elucidate the inducibility of *Xegr-1* mRNA expression as caused by the dissection procedure, we explanted animal caps at different timepoints (ranging from blastula stage 8 up to gastrula stage 11), cultivated them for different periods of time, lysed the caps and examined the expression of molecular marker genes by RT-PCR (Fig. 1). In addition to *Xegr-1* we examined the expression kinetics of *Xfos* and Xbra. *Xfos* was chosen, because it contains an SRE in its promoter region (Mohun *et al.,* 1989). This SRE was shown to mediate serum responsiveness to the human c-*fos* gene. *Xbra* was used because its temporal and spatial expression characteristics resemble those of *Xegr-1* (Umbhauer *et al.,* 1995; Panitz *et al.,* 1998). Animal caps that were processed within 5 min after dissection (pools of 5 animal caps; 0 h timepoint) showed only weak *Xegr-1*, *Xbra* and *Xfos* signals when animal caps were dissected at stages 8, 8+ and 9 (Fig. 1, lanes 1,3 and 5). These signals represented most likely the maternal transcripts of the three genes. Maternal transcripts were not detectable any more in animal caps from stage 10 onwards, most probably due to the (endogenous) degradation of maternal mRNA. *Xegr-1* expression could not be detected in pre-MBT (no embryonic transcription) animal caps (Fig. 1, lanes 1 and 2). In contrast, *Xegr-1* expression could be detected as early as 30 min after dissection (Fig.1, lanes 4,7,15) in all animal caps taken from stages 8+ to 11. *Xfos* expression was also induced 30 min after dissection (Fig. 1, lanes 7,15), but was not detectable until stage 9 (Fig. 1, lane 7). *Xbra* expression could not be detected beyond the maternal background signal (positive control from embryo-RNA not shown; see also Fig. 2).

We next wished to know the time period for which explantation-induced gene expression persisted. In all timepoints examined we could detect *Xegr-1* transcripts at 1 h after dissection (for *Xfos* it is identical in stages 9, 10.5 and 11). Animal caps dissected at stage 9 were cultivated for up to 5 h after dissection. In these cultivated caps *Xegr-1* and *Xfos* expression persisted at a comparable level for 4 h (Fig. 1, lanes 5-13) after dissection. Five hours after dissection expression levels of *Xegr-1* and *Xfos* dropped to control levels (Fig. 1, lane 13). The time-span of *Xegr-1* and *Xfos* expression subsequent to dissection was found to vary between experiments (data not shown). We observed this artefactual gene expression throughout blastula and gastrula stages.

These data show that expression of *Xegr-1* and *Xfos* is most likely induced by the dissection process itself, although formally it can not be ruled out that incubation of animal caps in the saline is the cause of *Xegr-1* and *Xfos* expression.

Effects of dominant-interfering mutants of signaling components on dissection-induced gene expression

In a further attempt to characterize the nature of wounding-induced gene expression we overexpressed dominant-negative constructs that interfered with the FGF/Ras/ERK signaling pathway. mRNAs coding for dominant-negative mutants of the FGF-receptor (dnFR) and the transcription factor Elk-1 (dnElk-1) were injected into the future animal cap region of 4 cell-stage embryos. Animal caps were cut at blastula stages (NF8-9+) and incubated for different time periods (0 h and 1 h), lysed, and gene expression was analyzed by RT-PCR (Fig. 2). *Xegr-1* expression was found to be severely inhibited by dnElk-1 (Fig. 2, lanes 8,12,16). Overexpression of dnFR also inhibited *Xegr-1* expression, albeit less efficiently than dnElk-1 (Fig. 2, lanes 7,11,15). Overexpression of dnFR and dnElk-1 showed relatively little effect on wounding-induced *Xfos* expression, but dnElk-1 was more effective in inhibiting *Xfos* expression than was dnFR. Again, *Xbra* expression was not induced by dissection of animal caps.

It was surprising that dnFR could only weakly inhibit dissection-induced *Xegr-1* expression. We have previously shown that dnFR and dnElk-1 are equally effective in inhibiting *Xegr-1* expression upon mesoderm induction (Panitz *et al.*, 1998). These data therefore indicate that the dissection process induces not only the FGF/Ras/Raf/ERK pathway but other signal transduction pathways as well. Alternatively, the dissection process leads to the activation of the Ras/Raf/ERK pathway by unknown means and is independent of FGF and FGF-receptors.

Wounding-induced gene activation: qualitative differences to XeFGF-induced mesoderm-specific gene expression

We have shown that manual dissection of animal caps leads to unspecific, wounding-induced activation of SRE-regulated genes. This effect requires zygotic transcription as it does not occur in caps dissected in pre-MBT stages (Fig. 1). Although effects of dissection procedures on cell fate have been reported (Dale and Slack, 1987), a detailed analysis has only recently been provided (LaBonne and Whitman, 1997; Christen and Slack, 1999). LaBonne and Whitman were able to show that manual dissection leads to long-lived (over 60 min) activation of ERK-type MAP kinases that is completely inhibited by overexpression of dnFR (as assessed by kinase assays). Christen and Slack (1999) found that wounding of embryos by injecting a needle leads to phosphorylation of ERK. This process could not be inhibited by previous injection of mRNA coding for the dominant-negative FGF-receptor (as assessed by immunostaining).

We have previously found that mesoderm-specific *Xegr-1* expression is dependent on a functional Ras/Raf/ERK pathway (Panitz *et al.*, 1998). Overexpression of either dnFR or dnElk-1 inhibited *Xegr-1* expression to control levels. Injection of the same amounts of mRNAs coding for either dnFR and dnElk-1 showed, however, different effects on wounding-induced *Xegr-1* expression. While overexpressed dnElk-1 severely reduced *Xegr-1* expression, dnFR was less efficient in inhibiting *Xegr-1* expression. This result may indicate that the dissection process leads to the activation of the Ras/Raf/ERK pathway by unknown means downstream of FGF and the FGF-receptor. Such an explanation is supported by the recent results of Christen and Slack (see above). Another explanation implies that in addition to the Ras/Raf/ERK pathway other signaling pathways are activated upon cap dissection. While dnFR inhibited signal transduction by the Ras/Raf/ERK pathway, dnElk-1 might have also inhibited *Xegr-1* expression induced by the JNK- and p38 pathway, as well as that induced by the Ras/Raf/ERK pathway (Treisman, 1996). Although the JNK-pathway and p38-pathway remain to be identified in *Xenopus* (a JNK kinase homolog has been identified in *Xenopus*, Yashar *et al.*, 1993), it has been shown in cell culture studies that Elk-1 can be a target of all three MAP kinase pathways (Whitmarsh *et al.*, 1995; Price *et al.*, 1996; reviewed in Treisman, 1996). It is conceivable, that signaling pathways other than the ERK-, JNK- and p38-pathways are activated upon wounding. This assumption could explain the finding that both dnFR and dnElk-1 have only little effect on wounding-induced *Xfos* expression compared to the effect on *Xegr-1* expression. While *Xegr-1* contains several putative SREs in its promoter (Panitz *et al.*, 1998), *Xfos* contains only one SRE (Mohun *et al.*, 1989). It is known that elements other than the SRE play a role in transcriptional regulation of the human *c-fos* gene (Hill and Treisman, 1995).

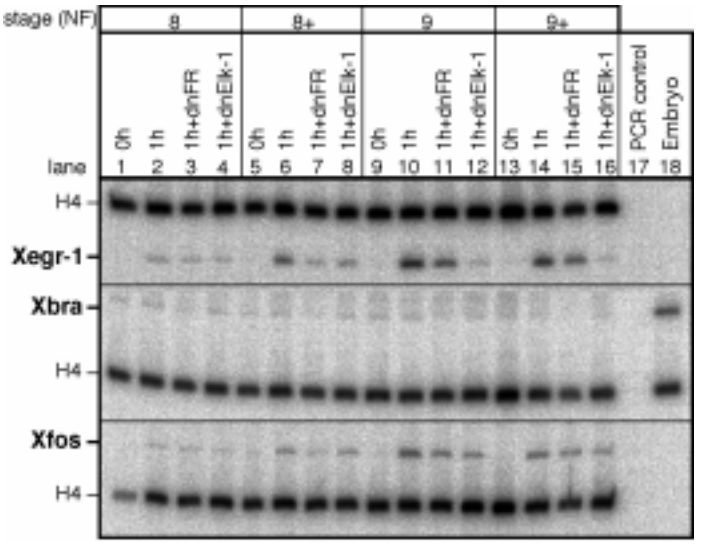

Fig. 2. Dominant-negative mutants of the FGF-receptor (dnFR) and Elk-1 (dnElk-1) affect dissection-induced gene expression. *Animal caps from uninjected or mRNA-injected embryos were explanted at different blastula stages (stage 8-9+), incubated for 1 h; 0 h controls were lysed immediately after dissection. Expression of Xegr-1, Xbra and Xfos was analyzed by RT-PCR. Co-amplification of histone-4 (H4) was used as an internal standard. A template-free RT-PCR is given in lane 17. In lane 18, a gastrula stage embryo was used as positive control for RT-PCR using Xbra primers. dnFR mRNA was injected at 2 ng and dnElk-1 mRNA at 0.3 ng per embryo.*

We find that *Xfos* expression is not induced in animal caps upon embryonic injection of XeFGF mRNA in animal caps (data not shown). This finding supports our conclusion that wounding-induced gene expression differs qualitatively from FGF-induced mesodermal gene expression.

Data from LaBonne and Whitman (1997) do not contradict our results. The authors identified ERK activity to be increased after dissection. This, however, does not rule out that other signaling pathways are induced in addition to the ERK pathway. As dnElk-1 inhibits *Xegr-1* expression completely, all wounding-induced signaling pathways that lead to *Xegr-1* expression must have an Ets protein component in common whose activity is blocked by dnElk-1. It is conceivable that other genes apart from *Xegr-1* and *Xfos* are activated by animal cap dissection procedures. The dissection procedure, however, does not lead to elongation of animal caps, a feature characteristic of induced animal caps treated with mesoderm-inducing factors. Mesoderm-specific expression of *Xegr-1* can, however, be reinduced after the initial unspecific gene expression has ceased (data not shown).

Several publications have noted and examined artefacts linked to embryonic manipulations. Tiedemann (1986) showed that neural differentiation of gastrula ectoderm of *Triturus alpestris* is dependent on different saline conditions. Animal caps of *Xenopus* transiently express the cement gland marker XAG1 and the early neural marker Sox2 (Streit and Stern, 1999, and references therein). Our short communication describes artefactual gene expression under the experimental conditions we are routinely using in our laboratory. We have neither examined the role of different salines, nor the impact of other tools for excision of animal caps on artefactual gene expression. We did not check, whether

excision of animal caps by fine glass needles, instead of using forceps, leads to less cell wounding and therefore to less artefactual gene expression. Recent results by Christen and Slack (1999) indicate that even minimal wounding of an embryo, as was caused by injection of a fine glass needle, leads to an increase in activated ERK after 15 min (and presumably to gene expression of genes that are activated by the Ras/Raf/ERK pathway). We have not examined the effect of wounding on the expression of neural-specific markers. Animal caps were incubated in 0.5xMBS. This buffer promotes rounding-up of animal caps and prevents their disintegration. Hence we can exclude artefactual gene expression to be caused by disintegration of animal cap cells during incubation.

Whatever the nature of wounding-induced signaling pathways might be, the fact that unspecific gene expression of SRE-regulated genes takes place should be taken into consideration in any gene expression study directed at SRE-regulated genes.

As a consequence of our findings, we established a modified protocol for dissection of animal caps. In order to minimize an unspecific *Xegr-1* signal in animal caps, we omitted the incubation of animal caps before lysis. After injection of mRNA in the animal cap region of 4 cell embryos we cut animal caps at stage 10 and lysed them immediately (within 5 min) for further analysis by RT-PCR. This procedure prevented accumulation of artefactually induced transcripts and led to undetectable *Xegr-1* levels in uninjected animal caps (negative controls). This modified protocol can, however, not be applied to experiments in which cutting is followed by further incubation procedures (e.g. external inducers).

Experimental Procedures

Embryo manipulation

Handling of *Xenopus laevis* embryos, injection of mRNAs, primer sequences and RT-PCR conditions have been described in detail previously (Panitz *et al.*, 1998). Briefly: *Xenopus laevis* embryos were incubated in 0.1x modified Barth´s saline (MBS). For cutting of animal caps, embryos were transferred in agarose-coated petri-dishes filled with 0.5x MBS. Animal caps were cut using watchmaker forceps and cultured for different time-periods in agarose-coated 96-well microtiter plates filled with 0.5x MBS.

Xfos primers: sense. 5´- CTCTGTACACATCAGAATGG-3´; antisense 5´-AATGTCCTTCAGCATTACAG-3´. *Xfos* primers amplify a 251bp fragment (nucleotide positions 1791-2041; Mohun *et al.*,1989). *Xfos* fragments were amplified for 28 cycles at conditions described in Panitz *et al.*, 1998. Staging of embryos was done according to Nieuwkoop and Faber, 1967.

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