(Sigma) (7). Enzyme treatment was stopped by the addition of an equal volume of 2 M KCl in 10 mM tris-Cl. Control membranes were processed in the same way but without the addition of PI-PLC.
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Induction of Mesoderm in *Xenopus laevis* Embryos by Translation Initiation Factor 4E

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The microinjection of messenger RNA encoding the eukaryotic translation initiation factor 4E (eIF-4E) into early embryos of *Xenopus laevis* leads to the induction of mesoderm in ectodermal explants. This induction occurs without a stimulation of overall protein synthesis and is blocked by the co-expression of a dominant negative mutant of the proto-oncogene *ras* or a truncated activin type II receptor. Although other translation factors have been studied in vertebrate and invertebrate embryos, none have been shown to play a direct role in development. The results here suggest a mechanism for relaying and amplifying signals for mesoderm induction.

Unfertilized eggs contain a large store of maternal mRNAs, many of which are only translated after fertilization (*1*). A number of mechanisms have been described in vertebrates and invertebrates that regulate the translation of these mRNAs (*2*–*6*). For example, primary (such as polyadenylation) structure and secondary structure of mRNA, as well as RNA binding proteins, can affect the efficiency of translation (*4*–*6*). During early embryogenesis, the pattern of regulated mRNAs changes, so that different mRNAs may be recruited to the ribosome at different times in development, as in *Xenopus laevis* embryos (*1*, *7*). Several examples of the translational control of specific mRNAs involved in early developmental processes have been described recently in *Drosophila* and *Caenorhabditis elegans* (*8*), but translational regulation has not been shown to play a direct role in the development of vertebrate embryos.

An early step in embryogenesis is the formation of the primordial germ layers, ectoderm, mesoderm, and endoderm. In *Xenopus laevis*, mesoderm is believed to arise by an inductive signal from vegetal cells of the blastula acting on adjacent cells in the animal hemisphere (*9*). Members of the transforming growth factor (TGF)–β and fibroblast growth factor (FGF) families mimic the endogenous signal when animal pole explants (prospective ectoderm) are cultured in a solution of the respective factor [reviewed in (*10*)]. Activin, FGF, and their receptors are expressed in the embryo, and dominant negative mutants for both receptors either abolish or markedly disrupt the formation of mesoderm in embryos (*11*).

We are interested in whether mesoderm induction in *Xenopus* involves the translational regulation of specific mRNAs. Several translation factors have been studied in oocytes and embryos of *Xenopus*, sea urchins, starfish, and brine shrimp, and over-expression can lead to a modest increase in the rate of total protein synthesis in some cases (*12*). However, these factors have not been shown to regulate developmental processes.

Our initial studies have focused on the translation initiation factor eIF-4E, the mRNA cap-binding protein. This factor appears to be an important control point for the regulation of protein synthesis and cell division [reviewed in (*3*, *13*)]. The overexpression of eIF-4E in NIH 3T3 cells, rat embryonic fibroblasts, and HeLa cells can lead to aberrant growth and oncogenic transformation as determined by growth rate, morphology, focus formation in cell monolayers, and tumor formation in nude mice (*14*, *15*). Transformation by eIF-4E is blocked by a dominant negative mutant of ras (*16*). In addition, eIF-4E appears to be an early downstream target of a number of growth regulatory molecules such as platelet-derived growth factor, epidermal growth factor, tumor necrosis factor, insulin, and others (*3*), all of which induce eIF-4E phosphorylation. These findings have suggested that eIF-4E participates in the ras signal transduction pathway regulating cell division.

To address whether eIF-4E may regulate the translation of mRNAs specifically involved in mesoderm induction, eIF-4E mRNA was micro-injected into the animal pole of one to two cell embryos (*17*). The *Xenopus* translation elongation factor–1α (EF-1α) was injected into a parallel set of embryos to serve as a control (*18*). At the blastula stage [stage 8 to 9 (*19*)], animal caps were dissected and cultured in buffer until siblings had reached the late tail-bud stage. By the late neurula stage, animal caps from embryos injected with eIF-4E mRNA had elongated in a manner similar to that of animal caps treated with FGF or a low dose of activin while caps from embryos injected with EF-1α (as well as uninjected embryos) remained spherical, typical of uninduced ectodermal explants. By the tail-bud stage, the animal caps expressing eIF-4E had formed large vesicles in greater than 90% of cases (80 of 87, Fig. 1), while those expressing EF-1α, as well as uninjected caps, were uninduced (0 of 55). Histological analysis showed that eIF-4E injection had led to the induction of mesenchyme and coelomic cavities lined with mesothelium, while controls showed only atypical epidermis (Fig. 1). A mutated eIF-4E in which Ser-53 has been converted to Ala (*14*, *15*, *17*) also leads to the elongation of ectodermal explants, although in a lower percent (53%, 20 of 38) of cases. This mutant is inactive in cell transformation assays (*14*, *15*), but in some cell types the Ala-53 mutated eIF-4E appears to be functional and highly phosphorylated, suggesting that alternative sites of phosphorylation may be important for regulation in other cell types (*20*). At present, the phosphorylation sites in *Xeno-*

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pus eIF-4E have not been identified, and it is not clear which (if any) sites are required for mesoderm induction.

The induction of mesoderm was also assessed by the detection of molecular markers specific to mesoderm. The *Xenopus* homolog of brachyury, *Xbra*, is expressed throughout the presumptive mesoderm of early gastrulas (*21*) and serves as a useful early marker of mesoderm induction by peptide growth factors such as FGF and activin. Embryos were injected into the animal pole at the one-cell stage with eIF-4E or EF-1α mRNAs. Animal caps were dissected during the blastula stage (stage 8 to 9) and cultured until sibling embryos had entered gastrulation (stage 10). RNA was isolated and analyzed by Northern (RNA) analysis and by reverse transcription polymerase chain reaction [RT-PCR (*22*)]. Figure 2A (lane 1) shows that *Xbra* is expressed in animal caps from embryos injected with eIF-4E but not with EF-1α (lane 2, Fig. 2A), nor is it expressed in animal caps from uninjected embryos.

Late markers for differentiated mesoderm, including α-hemoglobin and muscle-specific actin (*23, 24*) are also induced by eIF-4E, as judged by Northern blotting (Fig. 2B) and by RT-PCR (Fig. 2C). Muscle actin is undetectable in uninjected controls (lane 3, Fig. 2B; lane 1, Fig. 2C) or in animal caps injected with EF-1α. In uninjected animal caps, α-T1 hemoglobin is detectable at low levels (lane 1, Fig. 2C) but is markedly induced in animal caps injected with eIF-4E (lane 2). Thus, the increased expression of eIF-4E in presumptive ectoderm leads to mesoderm induction as determined morphologically, histologically, and by the expression of early and late markers of mesoderm. The induction of blood distinguishes this response from mesoderm induction by FGF and activin, which do not induce globin, but is similar to the response to injection of bone morphogenetic protein–4 (BMP-4) mRNA (*25*).

The induction of mesoderm by eIF-4E could be caused by the increased translation of endogenous mesoderm-inducing factors such as FGF, Vg-1, activin, or BMP-4. If these factors in turn activate eIF-4E, as observed for other growth factors (*3*), this cycle could amplify an inductive signal and provide a means of relaying that signal from one cell to the next. Cells responding to mesoderm-inducing factors can induce mesoderm from adjacent ectodermal cells, a process termed homoiogenetic induction (*26*). A positive feedback loop involving eIF-4E could be a molecular mechanism for homoiogenetic induction. Such an autocrine loop has been proposed to explain the transformation of tissue culture cells by eIF-4E, which appears to function both upstream and downstream of ras. This is suggested by the observations that phosphorylation and activation of eIF-4E are stimulated by ras-dependent signals and inhibited by the dominant negative form of ras, Asn-17 ras^H, while ras in turn is activated by the over-expression of eIF-4E (*16, 27, 28*). Because ras has been implicated in the response to mesoderm-inducing factors

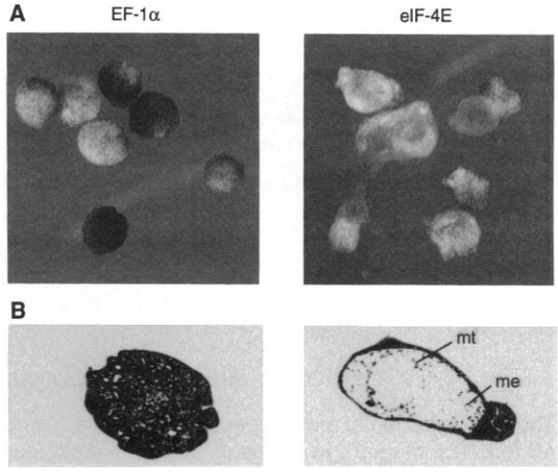

Fig. 1. Induction of mesoderm in *Xenopus* blastula animal caps by eIF-4E. (**A**) Embryos at the one-cell stage were injected in the animal pole with mRNA encoding mouse eIF-4E. Animal caps were removed from late blastulas [stage 9 (*19*)] and cultured in buffer [×0.5 Marc's modified Ringer's (MMR) (*29*)] until siblings had reached the tadpole stage. The right panel shows representative animal caps from eIF-4E injected embryos. The left panel shows animal caps from control embryos injected with mRNA encoding EF-1α. This experiment has been repeated four times in over 80 embryos with similar results. (**B**) Animal caps fixed, sectioned, and stained with Giemsa (Sigma). The right panel shows a representative animal cap from an embryo injected with eIF-4E mRNA. There is a central coelomic cavity lined with mesothelium (mt) and surrounded by mesenchyme (me) (×160 magnification). The left panel shows the histology of an uninduced animal cap with atypical epidermis throughout. The dose of injected eIF-4E mRNA was 2 to 4 ng per embryo (higher doses were also effective and apparently not toxic to explants). At these doses, eIF-4E was just detectable on electrophoresis, and fluorography of ^{35}S-methionine labeled proteins and did not appear to be a major cytosolic protein, suggesting that exogenous eIF-4E mRNA may not be efficiently translated, as observed by others (*20*).

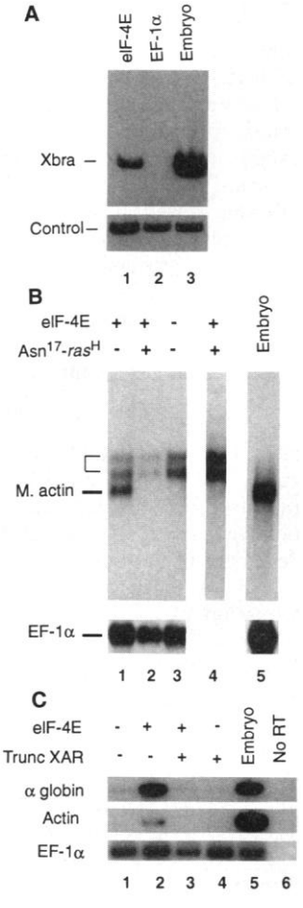

Fig. 2. Molecular markers of mesoderm induction induced by eIF-4E. Embryos were injected as described in Fig. 1; animal caps were removed at stage 9 and cultured in ×0.5 MMR (*29*). (**A**) Brachyury expression: When sibling embryos had entered the gastrula stage (stage 10), RNA was isolated from animal caps and embryos and analyzed by RT-PCR to identify the *Xenopus* homolog of brachyury (Xbra). Lane 1, animal caps from eIF-4E injected embryos; lane 2, animal caps from EF-1α injected embryos; lane 3, whole embryos. Similar results were seen by Northern blotting. As a control, primers for the XAR 1 were also used (lower panel), because the levels of this mRNA are not affected by mesoderm induction by activin or FGF in animal caps (*38*). (**B**) Inhibition of eIF-4E induction of the late mesodermal marker muscle actin by dominant negative ras. Embryos were injected with eIF-4E alone or with asn^{17} – ras^H mRNA (3 ng), as indicated in the figure with plus and minus signs. Isolated animal caps were cultured until the early tadpole stage, when RNA was isolated and analyzed by Northern blotting with a fragment of the muscle actin gene (*23*) as a probe. The abbreviation M. actin indicates muscle-specific actin; the bracket indicates cytoskeletal actin bands, which are not specific to mesoderm. Lanes 1 to 3 were exposed for 4 hours. Lane 4 is the same as lane 2, but exposed for 24 hours. Lane 5 (whole embryo) was exposed for 0.5 hour. The filter was reprobed with EF-1α as a control for equal loading. (**C**) Both α-globin and muscle actin induction mediated by eIF-4E are blocked by co-expression of the truncated activin receptor. Embryos were injected with mRNAs for eIF-4E (as in Fig. 1), the truncated activin receptor (Trunc XAR, 1 ng), or both, as indicated. Isolated animal caps were cultured until stage 30. RNA was isolated and analyzed by RT-PCR with primers directed against muscle actin or αT1 hemoglobin (*22, 23, 24*). Primers for EF-1α were used as a control for equal input of cDNA (lower panel). Lane 5 shows the signal from whole embryos, and lane 6 is a mock reverse transcription.

including FGF and activin (*29*), it may function in the same pathway as does eIF-4E in *Xenopus*.

This proposed cycle offers specific predictions that can be tested directly. For example, the inhibition of steps putatively upstream of eIF-4E should block mesoderm induction by eIF-4E. Co-expression of eIF-4E with Asn-17 ras^H (*30*) completely blocks the induction of mesoderm, as judged by the elongation of explanted animal caps, histology, and the induction of mesodermal markers including muscle actin (lanes 2 and 4, Fig. 2B) or αT1-hemoglobin. Similarly, the dominant negative form of the *Xenopus* activin type II receptor (*11*) completely blocks the induction of muscle actin and αT1-hemoglobin by eIF-4E (lane 3, Fig. 2C). We conclude from these results that a TGF-β related receptor is essential for mesoderm induction by eIF-4E, but it is not clear at present whether the truncated activin receptor inhibits only activin signaling or other TGF-β related signaling pathways in *Xenopus*.

A second prediction is that eIF-4E should stimulate the translation of a mesoderm-inducing factor, either specifically or by stimulating total protein synthesis. However, eIF-4E did not stimulate total protein synthesis in embryos, as determined by ^{35}S-methionine incorporation, nor did it affect the pattern of newly synthesized embryonic proteins as determined by protein electrophoresis and fluorography. More likely, increased eIF-4E levels lead to preferential translation of a limited set of low-abundance mRNAs that includes mesoderm-inducing factors. A similar argument, proposed to explain eIF-4E mediated oncogenic transformation, suggests that an increased level of eIF-4E preferentially facilitates the translation of growth factor mRNAs with inhibitory secondary structure in the 5′ end, a feature commonly found in mRNAs encoding growth regulatory molecules (*14, 15, 31*). To test whether eIF-4E can stimulate the translation of a mesoderm-inducing factor in *Xenopus* without stimulating total protein synthesis, we examined the effect of increased concentrations of eIF-4E on the translation of *Xenopus* activin βB or a reporter mRNA [chloramphenicol acetyltransferase (CAT) in pSP64T] in *Xenopus* oocytes (Fig. 3). Coexpression of eIF-4E led to an approximately fourfold stimulation of the translation of activin but had no effect on the translation of CAT or on total protein synthesis. This result shows that activin βB mRNA can be a target of eIF-4E, although activin is not necessarily the primary or only agent of eIF-4E mediated mesoderm induction.

In tissue culture cells, eIF-4E can abolish the repression of translation due to inhibitory sequences (a stem-loop structure) in the 5′ region of mRNA (*31*). In *Xenopus*, the presence of a stem-loop structure in the 5′ end of mRNA inhibits translation in oocytes and late blastulas, but the same mRNA is translated efficiently during the early cleavages following fertilization (*5*), when mesoderm induction is believed to begin (*10, 32*). A developmentally regulated RNA unwinding activity is present in early embryos in a parallel pattern, with the highest levels during early cleavages (*33*), and may account for the translation of this mRNA (*5*). The translation initiation factor eIF-4E is a subunit of the initiation complex eIF-4F, which also has RNA helicase activity. It is interesting to speculate that, in response to an inductive signal, eIF-4E may activate this unwinding activity and lead to increased translation of maternal mesoderm-inducing signals during early cleavage stages.

Thus, we have shown that expression of eIF-4E mRNA in early *Xenopus* embryos leads to mesoderm induction in ectodermal explants and that this induction is dependent on ras- and TGF-β related signaling. We have also shown that eIF-4E can preferentially stimulate the translation of an mRNA encoding a mesoderm-inducing factor without stimulating total protein synthesis [as proposed for growth factor mRNAs in other systems (*16, 31*)]. A positive feedback loop wherein the eIF-4E stimulated translation of a mesoderm-inducing factor mRNA leads to the increased activation of eIF-4E may be a mechanism for the amplification and relay of inductive signals in vertebrate embryos.

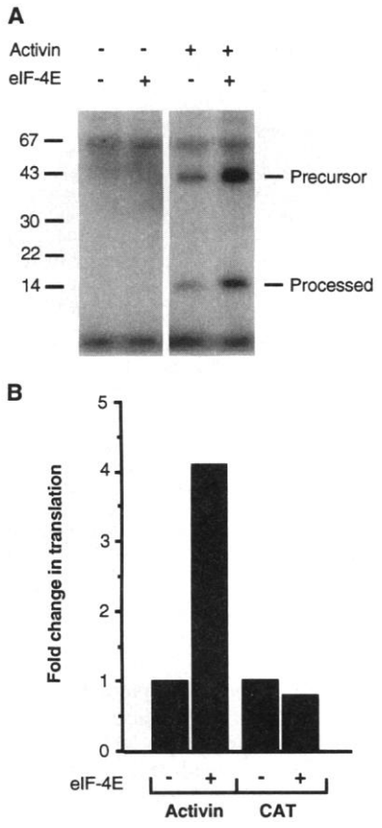

Fig. 3. Translation of activin stimulated by eIF-4E without affecting total protein synthesis or translation of a reporter mRNA. Defolliculated *Xenopus* oocytes (*40*) were injected with mRNAs for activin βB, eIF-4E, or CAT as indicated in the figure. All three mRNAs were cloned into pSP64T (*35*) and had identical 5′ and 3′ untranslated regions. (**A**) Injected oocytes were cultured for 3 days in the presence of ^{35}S-methionine. Supernatants were subjected to SDS-polyacrylamide gel (15%) electrophoresis (in the presence of β-mercaptoethanol) and fluorography. Precursor (40 kd) and processed (14 kD) forms of activin are both secreted from the oocyte. On longer exposures, other secreted proteins were noted specifically from oocytes injected with eIF-4E; the identity of these endogenous proteins is currently unknown. The broad band (68 kD) pres- ent in all four lanes is bovine serum albumin, which was added to the culture medium. (**B**) Translation of activin (given in arbitrary units) quantitated from autoradiographs with laser densitometry (including precursor and processed forms in quantitation). The translation of CAT was inferred from CAT activity (*41*), which was quantitated with a Fuji (Stanford, Connecticut) bio-imager. Values were normalized with the level of activin or CAT translation in the absence of eIF-4E defined as 1.0. The change in the level of activin protein or CAT activity when co-expressed with eIF-4E is plotted on the *y* axis.

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An Osmosensing Signal Transduction Pathway in Mammalian Cells

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The osmotic balance between the cytoplasmic and extracellular compartments of cells is critical for the control of cell volume. A mammalian protein kinase, Jnk, which is a distant relative of the mitogen-activated protein kinase group, was activated by phosphorylation on threonine and tyrosine in osmotically shocked cells. The activation of Jnk may be relevant to the biological response to osmotic shock because the expression of human Jnk in the yeast *Saccharomyces cerevisiae* rescued a defect in growth on hyper-osmolar media. These data indicate that related protein kinases may mediate osmosensing signal transduction in yeast and mammalian cells.

The maintenance of homeostasis is a fundamental property of the cells that form the tissues of the body. Exposure to anisotonic media initiates a response that maintains cellular volume. This response is thought to be mediated by changes in plasma-membrane ion transport (*1*) and the accumulation of nonpolar solutes (*2, 3*). The signal transduction pathway that controls this response to osmotic shock in mammalian cells is not understood. However, detailed studies of osmoregulation in enteric bacteria have established the importance of a two-component pathway involving a histidine kinase sensor (*EnvZ*) and a transcriptional regulator (*OmpR*) that is activated by phosphorylation on Asp (*4*). In the yeast *Saccharomyces cerevisiae*, both a histidine kinase sensor and a member of the MAP kinase group [high osmolarity glycerol response 1 (Hog1)] have been implicated in osmosensing (*5, 6*).

The exposure of cultured mammalian cells to anisotonic media causes an increase in the phosphorylation of several proteins (*7*). This reaction suggests that osmotic shock may cause the activation of one or more protein kinases. Consistent with this hypothesis, the exposure of Chinese hamster ovary (CHO) cells to hyper-osmolar media caused a marked increase in Jnk activity, which was detected in an immune complex protein kinase assay by the use of a polyclonal anti-Jnk1 antibody. Activation of Jnk in response to osmotic shock was also observed in CHO cells transfected with epitope-tagged Jnk1 (Fig. 1A). The increase in Jnk1 activity was observed within 5 min of exposure of the cells to hyper-osmolar media (Fig. 1B). Maximal activity was observed 15 to 30 min after osmotic shock, with a progressive decline in Jnk1 activity at later times. The activation of Jnk1 caused by ultraviolet radiation requires the phosphorylation of Thr^{183} and Tyr^{185} (*8*). Therefore, we investigated whether these phosphorylation sites were required for the activation of Jnk1 by osmotic shock. The replacement of Thr^{183} (with Ala) and Tyr^{185} (with Phe) blocked Jnk1 activation (Fig. 1C). Together, these data demonstrate that the Jnk signal transduction pathway (*8, 9*) is activated by the exposure of cultured mammalian cells to hyper-osmolar media.

Comparison of the sequence of human Jnk1 with the GenBank database revealed that this enzyme is similar to the *Saccharomyces cerevisiae* protein kinase Hog1 (Fig. 2). Yeast that are defective in Hog1 expression cannot grow after osmotic shock (*5*). This

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