A novel Xenopus Mix-like gene milk involved in the control of the endomesodermal fates

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SUMMARY

Here we describe a novel Xenopus homeobox gene, milk, related by sequence homology and expression pattern to the vegetally expressed Mix.1. As is the case with Mix.1, milk is an immediate early response gene to the mesoderm inducer activin. milk is expressed at the early gastrula stage in the vegetal cells, fated to form endoderm, and in the marginal zone fated to form mesoderm. During gastrulation, expression of milk becomes progressively reduced in the involuting mesodermal cells but is retained in the endoderm, suggesting that it may play a key role in the definition of the endo-mesodermal boundary in the embryo. Overexpression of milk in the marginal zone blocks mesodermal cell involution, represses the expression of several mesodermal genes such as Xbra, goosecoid, Xvent-1 or Xpo and increases the expression of the endodermal gene, endodermin. In the dorsal marginal zone, overexpression of milk leads to a severe late phenotype including the absence of axial structures. Ectopic expression of milk in the animal hemisphere or in ectodermal explants induces a strong expression of endodermin. Taken together, we propose that milk plays a role in the correct patterning of the embryo by repressing mesoderm formation and promoting endoderm identity.

Key words: Xenopus, Homeobox, milk, Mix-related, Mesoderm, Endoderm, Gastrulation, Subtracted library

INTRODUCTION

In amphibians, as in all vertebrates, two fundamental mechanisms operate during early embryonic development to provoke differentiation and to pattern the three embryonic germ layers, ectoderm, mesoderm and endoderm. One, a direct consequence of the uneven spatial distribution of the egg cytoplasmic constituents, operates by the differential cellular inheritance of maternal determinants during egg cleavage which confers distinct cell-autonomous developmental potentialities. The second involves embryonic induction, whereby a group of cells receives a signal delivered by other cells, which, in conjunction with their pre-existing endowment, progressively ensures their correct differentiation and patterning.

An early developmental event that has been much studied in Xenopus, mesoderm formation, clearly reveals the operation of these two mechanisms. During the first cell cycle, the spatial reorganization of the maternal cytoplasmic constituents initiates a cascade of cell autonomous and inductive events that, by early blastula stages, sets up a vegetal dorsalizing domain, the so-called Nieuwkoop Center (Sudarwarti and Nieuwkoop, 1971; Nieuwkoop, 1977), which consists of a subset of dorsovegetal cells. During the blastula stages, the vegetal hemisphere of the egg, fated to form endoderm, sends two distinct signals to the equatorial cells to develop into two mesodermal cell types; one general mesoderm inducer (involving the TGF-β pathway) that induces mesoderm of ventral-lateral character at the marginal zone, and a second dorsal modifier (involving the wnt signaling pathway), derived from the Nieuwkoop center, that specifies the Spemann organiser (Spemann and Mangold, 1924). During gastrulation, interactions between these two mesodermal components will produce intermediate mesoderm.

At the molecular level, several secreted factors belonging to the fibroblast growth factor (FGF) and to the transforming growth factor-β (TGF-β) families have been found to simulate this vegetal signaling (reviewed by Slack, 1994; Kessler and Melton, 1994; Harland and Gerhart, 1997). Such molecules apparently activate a set of genes whose first expression is restricted to or is predominant in the mesoderm during the late blastula and early gastrula stages (reviewed by Smith, 1993; Dawid, 1994). Among the numerous mesodermal genes now identified, some encode secreted factors while others encode transcription factors.

A decisive step toward deciphering the functional network
of the interactions between such genes would be accomplished by identifying other genes whose expression controls the determination and the cellular differentiation of the embryonic sheets. Here we describe a novel gene that encodes a homeodomain protein. This gene, which we named Mix like (milk), is most closely related to the Xenopus Mix.1 and Mix.2 genes (Rosa, 1989; Vize, 1996). In normal development, its expression is first detected at the late blastula (stage 9) in the entire vegetal hemisphere, including the marginal zone. During gastrulation, its expression becomes progressively excluded from the marginal zone fated to form mesoderm and persists in vegetal cells fated to form endoderm. Then it is no longer detectable from the end of gastrulation onward. We show that overexpression of milk in the marginal zone represses the mesodermal genes Xbra, goosecoid (gsc), Xpo and Xvent-1, whereas an ectopic expression of the endodermal gene, endodermin (edd) is observed. milk thus appears to be a novel type of regulatory gene in the early embryo and we suggest that it may be involved in the spatial control of endodermal specification by repressing mesoderm formation and promoting endoderm identity.

**MATERIALS AND METHODS**

**Subtraction of a blastoporal lip cDNA library**

A Xenopus blastoporal lip cDNA library constructed in λZAP II vector (Blumberg et al., 1991) was depleted of ventral sequences by subtractive hybridisation. In a first step, we prepared circular single-stranded DNA (‘Tracer’) from the library as described by Stratagene, using the XL1Blue bacterial strain and the R408 helper phage. In parallel, total RNA from 3 ventral explants extirpated at the early gastrula stage was extracted by guanidium thiocyanate procedure (Chomczynski and Sacchi, 1987), then reverse transcribed with MMLV enzyme and an oligo(dT) primer. The cDNAs were oligo(dG)-tailed using terminal transferase, and extensively purified using the XL1Blue bacterial strain. The purified cDNAs were amplified by PCR for 23 cycles with C-primer (5'-AAGGAATT(T)13-3') and T-primer (5'-GGGAGGC-CC(T)13-3') in the presence of bio-16-UTP (Boehringer). This constitutes the ‘Tracer’. Three hundred ng of Tracer were coprecipitated with a 15-fold excess of Driver and hybridized in 10 μl of buffer (0.5 M NaCl, 50 mM HEPES pH 7.6 and 2 mM EDTA) containing 50% deionized formamide at 50°C for 18 hours. Removal of the biotinylated DNA, as well as DNA hybridized to the biotinylated DNA was done by addition of streptavidin followed by phenol/chloroform extraction according to the protocol described by Sive and St John (1988). The DNA left in the aqueous phase and which represents sequences that have not found a counterpart in the ventral marginal zone cDNA, was ethanol precipitated, dissolved in water, converted to the double-stranded form and transformed into XL1 Blue strain.

**cDNA library screening**

The subtracted library was screened for dorsal-specific clones by an in situ hybridisation procedure essentially as described by Gawantka et al. (1995). Briefly, cDNA inserts from randomly selected clones were amplified from colonies using universal and reverse Bluescript primers (Stratagene). Amplified products were purified on agarose gels by GeneClean procedure (Bio 101) then used as a matrix for synthesis of digoxigenin-labeled riboprobes for whole-mount in situ hybridisation analysis. Clones showing a dorsal-specific hybridisation pattern were selected for further analysis.

One of these was a partial-length cDNA of milk. A full-length milk cDNA was isolated by screening a dorsalized Xenopus gastrula cDNA library (Lemaire et al., 1995).

**DNA sequencing**

Partial sequencing was done with a Sequenase II kit (USB) using T7 and T3 primer. The full cDNA was sequenced on both strands by Genome Express SA (France). Sequence alignment allowing analysis of homologies was generated with the Clustal V program.

**In situ hybridisation**

Whole-mount in situ hybridisation was performed according to Harland (1991), except that BM Purple was used as the alkaline phosphatase substrate. To screen the subtracted library, antisense digoxigenin probes were synthesized from PCR templates containing a cDNA insert prepared as described above. For precise localization of gene expression, probes were synthesized from rescue pBluescript plasmid containing partial milk cDNA and partial gsc cDNA linearized by EcoRI transcribed with T7 polymerase, from pSP73Xbma (Smith et al., 1991) cut by BglII transcribed with SP6 polymerase, from pBS-45edd (Sasai et al., 1996) cut by EcoRI transcribed with T7 polymerase, from pxvent-1 (Gawantka et al., 1995) cut by salI transcribed with T7 polymerase and from pxpoHK (Sato and Sargent., 1991) cut by HindIII transcribed with T7 polymerase.

In situ hybridisation on sectioned embryos was performed as described by Lemaire and Gurdon (1994).

**Embryo manipulations and mRNA injections**

*Xenopus laevis* embryos were obtained by in vitro fertilisation, chemically dejellied with 2% cysteine hydrochloride (pH 7.8) at the 2- or 4-cell stage, washed with 0.1X Normal Amphibian Medium (NAM; Slack, 1984) solution and selected for reliable distinction between dorsal and ventral blastomeres based on clear pigmentation polarity. Dorsalized and ventralized embryos were obtained as previously described (Ecochard et al., 1995). For mRNA injections, embryos were transferred in 1X NAM supplemented with 3% Ficoll type 400 (Sigma). The mRNA were injected in 4.6 nl of water using a Drummond Nanoject microinjector at the 8-cell stage. For milk overexpression, mRNA was injected in two vegetal blastomeres at an equatorial region. For ectopic expression, mRNA was injected in a single animal micromere. Two hours later, the injected embryos were placed in 0.1X NAM until the control embryos reached the desired stage. Staging was performed according to Nieuwkoop and Faber (1975).

**Preparation of capped mRNA**

mRNA for microinjection was generated by in vitro transcription using the CAP Scribe kit (Boehringer) from pCS2*γFGal (Turner and Weintraub, 1994) and from pBluescript RN3milk with SP6 and T7 polymerase respectively. Templates for in vitro transcription were cut as follows. pCS2*γFGal with NotI and pBluescript RN3milk with SfiI. Staining for β-gal was performed according to Lemaire et al. (1995).

**Animal cap assay**

To study milk induction by activin, animal caps were dissected at stage 8.5 with a fine wire knife in 1X NAM then cultured in 0.75X NAM with 1 mg/ml bovine serum albumin alone or in the presence of 30 ng/ml activin A (Genentech). In some cases 10 μg/ml cycloheximide (CHX) were added to the culture 30 minutes before activin treatment. Induction was analyzed by RT-PCR at the equivalent of early gastrula stage.

To study edd induction by milk, embryos were injected in 1 or 2 blastomeres at the 4-cell stage at the animal pole with 100 pg or 1 ng milk. 800 pg of XF3 mRNA was injected (Amaya et al., 1991). Animal caps were dissected from stage 8-9 embryos and cultured until
sibling embryos reached stage 11.5 or 30 for in situ hybridisation or stage 11.5 for RT-PCR analysis.

**RT-PCR analysis**

Total RNA was extracted from 10 animal cap explants with RNA NOW kit (Biogentex) then reverse transcribed using the first-strand cDNA Synthesis kit (Amersham) in 15 μl according to the manufacturer’s instructions. PCR was performed on one-thirtieth of the cDNA in a 50 μl total volume using Expand Long Template System (Boehringer).

The thermal cycler program was 94°C for 30 seconds (denaturation), 60°C for 30 seconds (hybridisation) and 68°C for 1 minute (elongation). The primers used for amplification were Upstream: 5'-GGACTTTCCACCTACAAGACCA-3' and Downstream: 5'-CA-GTCTGACACTGACTCTGCA-3' (for milk); U: 5'-TATATCCACCCAGACTCACACC-3' and D: 5'-GATAGAGAGAGGTTGCCCCGC-3' (for Xbra) (Smith et al., 1991); U: 5'-GAGAATTACCAACAACTGG-3' and D: 5'-GGAGCATCAATGATAAGTCGAC-3' (for EF1α) (Ki et al., 1989); U: 5'-AATGCACACTGGATGGT-3' and D: 5'-GAGAATTACCATGACTCTGCA-3' (for chordin) (Sasai et al., 1991); U: 5'-TTGAAGTCTGA TGCGAGTGA-3' and D: 5'-GGGTTGT AGCAGTACTCCAT-3' (for FGFR-1) (Musci, 1990).**

**RESULTS**

**Isolation of milk, a novel Xenopus homeogene**

In a previous study (Ecochard et al., 1995) a differential screen for zygotic dorsal-specific cDNAs of a dorsal lip cDNA library derived from stage 10.25 Xenopus gastrula (Blumberg et al., 1991) enabled us to isolate, in addition to previously identified genes such as goosecoid (Blumberg et al., 1991) or chordin (Sasai et al., 1994), a novel nodal-related gene, fugacin, identical to Xnr-3 reported by Smith et al. (1995). In an extension of this search, we performed a subtractive hybridisation of the dorsal lip cDNA library with amplified cDNAs obtained from mRNAs isolated from ventral mesodermal explants (see Material and Methods).

A differential screen of this subtracted library performed by colony filter hybridisation, with probes enriched in either dorsal- or ventral-specific cDNA, prepared as previously described (Ecochard et al., 1995), gave no clear signal with most of the clones. To identify clones preferentially expressed in the dorsal territories of early gastrulae, we carried out a screen by whole-mount in situ hybridisation with digoxigenin-labeled riboprobes made from individual randomly selected clones from the subtracted library. Of 17 clones, two were preferentially expressed in the dorsal territories of stage 10.25 embryos. The first one codes for a novel potentially secreted protein which will be described elsewhere. The second clone contained a 1.2 kb insert and it codes for a novel homeodomain protein whose expression and functional properties are described in this study.

As this clone contained only a partial reading frame, longer cDNAs were isolated by screening a dorsalized gastrula cDNA library (Lemaire et al., 1995). The largest of these, 2.0 kb in length, was sequenced and found to contain a single open reading frame of 400 amino acids (Fig. 1A). The homeodomain of milk is most closely related to those of Xenopus Mix.1 and **Fig. 1. Sequence analysis of milk.**

(A) The deduced amino acids sequence of the longest milk cDNA is compared to those of Mix genes. Conserved amino acids are printed on a black background. Residues homologous to those of Mix are printed on a grey background. The homeodomain (HD) is indicated by the arrows. Helix 2 and helix 3 of the homeodomain as well as the acidic region at the C terminus are underlined. (B) Schematic structure of milk protein. Percentage amino acid homology with Mix are indicated for the different regions. The accession number for milk is AF005999.
Expression of the milk gene in development

*milk* transcripts were first detected at stage 9 in the dorsal marginal zone and to a lesser extent in ventral marginal zone (Fig. 2A,C). At the early gastrula stage, the distribution of *milk* transcripts was nearly identical (Fig. 2B,D). As gastrulation progressed, the *milk* hybridisation signal decreased to become undetectable by the late-gastrula stage and through later development as judged by whole-mount in situ hybridisation (not shown).

For a more precise definition of the expression domains of *milk* mRNA, the expression of the early mesodermal gene *Xbra* and *milk* during gastrulation were compared by in situ hybridisation on sectioned embryos. As described in Materials and methods, hybridisations were performed on alternate sagittal or parasagittal sections under identical experimental conditions. At stage 10.25, *Xbra* staining was restricted to the presumptive mesoderm (Fig. 2E), while *milk* staining, like that of *Mix.1*, appeared more widespread encompassing most of the presumptive mesoderm and endoderm (Fig. 2F,G). Consistent with whole-mount in situ data, a more intense *milk* hybridisation signal was observed in dorsal cells. At stage 11.5, a comparative analysis of the expression patterns of *Xbra* and *milk* revealed that *milk* was no longer expressed around the blastopore in involuting mesodermal cells (presumptive chordomesoderm and caudal mesoderm) (Keller, 1976) which constitute the major site of expression of *Xbra*. Therefore, during gastrulation the expression domains of *milk* and *Xbra* become mutually exclusive, *milk* expression being transiently maintained in the yolky vegetal mass.

*milk* is activated by the inducer activin in animal caps

The similarity of the expression patterns of *Mix.1* and *milk* led us to examine whether, like *Mix.1*, *milk* is a target of the TGF-β-like mesoderm inducing factor activin. As shown in Fig. 3, when blastula animal caps were exposed for 3 hours to 30 ng/ml of activin, a clear accumulation of *milk* mRNA was observed, as was the expression of *Xbra* by the early gastrula equivalent stage. To test whether *milk*, like *Mix.1*, is a direct target of the activin pathway, the protein synthesis inhibitor cycloheximide was added 30 minutes before activin addition. This treatment did not prevent the activation of *milk* in response to activin. In contrast, *chordin*, whose activation by activin is not direct but requires de novo protein synthesis (Sasai et al., 1994), was not activated in the presence of CHX, thus demonstrating the effectiveness of our treatment. These results suggest that *milk* is a direct target of the signal transduction pathway triggered by activin-like growth factors.

Overexpression of milk in the marginal zone blocks mesodermal cell involution

To investigate the role that *milk* might play during early development and to better understand the importance of its exclusion from the involuting marginal zone during gastrulation, we injected synthetic *milk* mRNA into the equatorial region of either the two dorsal or the two ventral blastomeres of a 4-cell stage embryo.

When ventral blastomeres were injected, embryos appeared externally normal until the early to mid-gastrula stages. Developmental defects first became apparent by stage 11.5, when injected embryos failed to form a ventral blastopore lip.
Endoderm induction by milk

whereas controls had formed a circular blastopore (not shown). As development proceeded, blastopore closure did not occur completely (Fig. 4Aa). These gastrulation disturbances were observed when as little as 100 pg were injected but became more severe with large amounts (1 ng) of milk (not shown). By the tailbud stage these gastrulation defects led to a reduction in the posterior-most structures, without affecting anterior axial structures (Fig. 4Ab).

When dorsal blastomeres were injected, embryos developed normally until the late blastula stage. Thereafter, when controls began to gastrulate, embryos injected with either 100 pg or 1 ng failed to show any involution of dorsal cells and did not form a dorsal lip (not shown). By stage 12.5 (Fig. 4Ba), the dorsally injected embryos formed only a normal ventral lip (Fig. 4Bb). Most of the injected embryos did not neurulate (Fig. 4Bd,f) while control embryos injected with lacZ mRNA developed normally (Fig. 4Bc,e). From this stage onward, two main phenotypes developed that depended essentially on the amount of injected RNA. Embryos injected with a low dose (100 pg) of milk mRNA developed slightly reduced trunk and tail structures and did not differentiate discernible head structures (Fig. 4Ca,b). Embryos injected with higher doses (1 ng) lacked both head and trunk/tail structures and resembled UV-ventralized embryos having no apparent polarity (Fig. 4Cd).

Overexpression of milk represses the mesodermal genes Xbra, goosecoid, Xpo and Xvent-1 and induces endodermin

The inhibition of mesodermal cell involution provoked by overexpression of milk suggested that injected cells had lost their mesodermal identity. To test this hypothesis, we examined the effect of the overexpression of milk on mesodermal markers Xbra, gsc, Xpo and Xvent-1.

The comparison of the spatial distribution of Xbra transcripts, analysed by whole-mount in situ hybridisation at stage 11.5, between normal embryos and embryos coinjected with milk and the lineage tracer lacZ mRNA in the dorsal marginal zone reveals a clear repression of Xbra. This repression was restricted to injected cells, which are positive for X-gal staining (Fig. 5A,B).

Overexpression of milk in the dorsal marginal zone also repressed gsc. In the control embryo at stage 10.5, gsc staining

**Fig. 3.** milk is an immediate-early target of activin.
Animal caps were removed at stage 8.5 and cultured until stage 10 in the presence or absence of activin (30 ng/ml) and with or without addition of 10 μg/ml cycloheximide. All markers were analysed by RT-PCR.

**Fig. 4.** Effect of overexpression of milk on Xenopus development. (A) milk RNA injection in the ventral marginal zone results in no marked phenotypic effect. (a) Neurula-stage embryos injected with 100 pg of milk RNA in the ventral marginal zone. Note that ventral cells have failed to involute. (b) The same embryos as in a at the tailbud stage. The two embryos have developed apparently normally. (B) milk overexpression in the dorsal marginal zone causes abnormal migration of cells. (a,c,e) Control embryos injected with 500 pg of lacZ RNA. (b,d,f) Embryos injected with 100 pg of milk RNA. (a,b) Late stage gastrula, vegetal view, dorsal is up. (c,d) Neurula stage, dorsal view. (e,f) Late stage neurula, frontal view, dorsal is up. Note that the dorsal cells have failed to involute resulting in an absence of gastrulation and neurulation. (C) Effect of the injection of different doses of milk mRNA in the dorsal marginal zone. (a) The top embryo was injected with 500 pg of lacZ RNA. Below, two embryos injected with 100 pg of milk RNA at the tailbud stage show microcephaly. (b) The same embryos as in a at a later stage. (c) Different phenotypes observed in embryos injected with 1 ng of milk mRNA. (d) Dorsal view of an embryo in c; no axial structures have developed.
appeared clearly in the marginal zone directly above the dorsal blastoporal lip (Fig. 5C), as previously reported (Cho et al., 1991). In embryos injected with milk mRNA and lacZ mRNA (Fig. 5D), no gsc transcripts were detected in the injected cells that were X-gal-positive. Repression of the ventrolateral mesodermal marker Xvent-1 and the posterior mesodermal marker Xpo (Sato and Sargent, 1991) was observed in embryos injected with milk mRNA in the ventral marginal zone (as shown for Xvent-1 in Fig. 5E,F). The effect of milk is not a general property of all homeobox genes as overexpression of the BMP4-target homeogene msx1 (Suzuki et al., 1997) does not have the same effect (Lemaire et al., 1998).

The repression of key mesodermal genes suggests that overexpression of milk diverts marginal cells from their normal mesodermal differentiation pathway. As the expression pattern of milk is reminiscent of the endodermal gene endodermin (edd), we tested if ectopic expression of milk led to an activation of edd in injected cells. As reported earlier (Sasai et al., 1996) edd expression, analysed by in situ hybridisation at stage 11.5, was found in the entire ring of the blastopore (Fig. 5J). In the same stage embryos, overexpressing milk and β-gal in the dorsal marginal zone, the edd staining extended more dorsally than in control embryos and colocalised with the lineage tracer (Fig. 5H,G).

Although these results are suggestive of a cell autonomous activation of edd in response to milk, it is difficult to rule out that ectopic edd expression is due to an alteration of the migration of cells that normally express edd during development. To determine whether ectodermal expression of milk could likewise induces edd, embryos were injected at the animal pole with milk mRNA (1 ng) in one micromere at the 4-cell stage. The spatial distribution of edd mRNA as well as of Xbra and Xvent-1 mRNAs was then analysed by in situ hybridisation at stage 11.5. All the injected embryos displayed a large patch of ectopic edd transcription as shown in Fig. 5I. In contrast, no activation of the mesodermal markers was detected (not shown). To explore this effect further, we performed experiments on isolated animal caps. milk-injected animal caps were explanted at stage 8.5, cultured until stage 11.5 and assayed for the expression of edd, Xbra and Xvent-1. As shown in Fig. 6A clear patches of edd expression were observed by in situ hybridisation at the mid-gastrula stage; in contrast, Xbra and Xvent-1 induction was never detected (not shown). Activation of edd in milk-injected animal explants at gastrulation stages was also confirmed by the RT-PCR assays (Fig. 6H). These data demonstrate that milk activates edd expression both in the marginal zone of the embryo and ectodermal explants. Although edd is expressed in both presumptive mesoderm and endoderm at this stage, lack of detectable expression of Xbra in milk-injected caps suggest that injected cells have acquired an endodermal identity. To test this idea, we analysed the expression of edd in stage 30 injected caps. At this stage, strong expression of edd is restricted to the endodermal layer (Sasai et al., 1996). As in experiments carried out at the gastrula stage, patches of cells strongly expressing edd were found in stage 30 animal caps injected with 1 ng milk mRNA (Fig. 6B). Finally, it has been reported that FGF signaling can negatively interfere with the induction of edd by
the secreted factor chordin in animal caps (Sasai et al., 1996). To test the influence of FGF signaling on the regulation of \( \text{edd} \) by milk, embryos were coinjected with milk mRNA and mRNA of a dominant negative form of the FGF receptor (XFD; Amaya et al., 1991). At the two doses of milk mRNA tested (100 pg and 1 ng), the expression of \( \text{edd} \) in caps harvested at stage 8.5 from injected embryos then cultured until stage 11.5 was not increased in the presence of 800 pg of XFD, and in fact appears to be weaker as revealed by in situ hybridisation (Fig. 6D-G) or by RT-PCR (Fig. 6H).

**DISCUSSION**

*milk*, a novel Mix.1-related gene, which progressively demarcates the boundary between mesoderm and endoderm

*milk* is a member of the paired-like homeogene family and displays striking similarities in sequence, embryonic expression and transcriptional response to activin with the previously isolated *Xenopus* genes Mix.1 and Mix.2 (Rosa, 1989; Vize, 1996). At the sequence level, these genes bear two highly conserved regions: the homeodomain, which shows 86% of conserved amino acids and a carboxy-terminal acid region (65% similar in a stretch of 17 amino acids). Outside these regions, the degree of homology is lower (36-46%). These features suggest that Mix and milk genes could be derived from a duplication of an ancestral gene that took place before the occurrence of the *Xenopus* tetraploidization event.

Both genes are expressed transiently following the mid-blastula transition in closely overlapping domains including the presumptive mesoderm and endoderm. As gastrulation proceeds, milk transcripts are predominantly localized in the presumptive endoderm, becoming progressively excluded from the mesodermal domain of Xbra expression. Such a segregation strongly suggests that negative interactions between these two genes may operate to further delimit the meso-endodermal boundary.

*milk* behaves as a mesodermal repressor and an endodermal activator

Genetic or epigenetic inactivation of Brachyury in mouse, zebrafish and *Xenopus* has revealed a requirement for this gene in mesoderm formation (reviewed in Yamada, 1994; Conlon et al., 1996; Smith, 1997). Hence suppression of Xbra expression by milk suggested that overexpression of milk may prevent mesoderm formation. Consistent with this idea, we find that expression of all early mesodermal markers tested (Xbra, gsc, Xpo and Xvent-1) is suppressed in cells overexpressing milk. These data indicate that milk diverts marginal cells from their normal mesodermal differentiation pathway. In this respect, milk appears different from the Mix.1 gene, which has been recently demonstrated to exhibit a ventralising activity towards the mesoderm, acting as a downstream component of the BMP-4 signaling pathway (Mead et al., 1996). More recently, Latinkic et al. (1997) demonstrated a clear ventral and dorsal suppression of Xbra at gastrula stages by expressing high levels of activin mRNA. This suppressive effect appears to be mediated by the activity of a number of paired-like homeobox genes, which is consistent with our result. It is worth noting though that the presence of a C-terminal acidic domain in milk suggests that milk might act indirectly to regulate Xbra by activating the transcription of a direct repressor of this gene. It is also possible that milk act synergistically with other factors to repress mesoderm, since it is a member of the family of

![Fig. 6. milk induces edd expression in isolated animal caps. Animal cap explants from milk-injected embryos (1 ng) harvested at stage 9 then cultured until the equivalent of stage 11.5 (A) or 30 (B) express high level of edd mRNA, as revealed by in situ hybridisation. Control caps at the equivalent of stage 11.5 do not express edd (C). (D-H) Inhibition of the FGF signaling decreases edd activation by milk. Animal caps injected with 100 pg (D,E) or 1 ng (F,G) of milk alone (D,F) or with 800 pg of XFD RNA (E,G) were hybridized with the edd antisens probe at a stage equivalent to stage 11.5. The clear decrease of edd expression in XFD injected caps is confirmed by RT-PCR analysis (H).](image-url)
paired-like genes (such as Mix.1, gsc, siamois or XANF-1 (Zaraisky et al. 1993, 1995, reviewed by Gehring et al., 1994) which encode transcription factors that can interact with each other by cooperative homo- or heterodimerization on DNA (Wilson et al., 1993, 1995). It is obviously of importance therefore to look for milk-responsive regions in the promoter of Xbra.

The repression of mesoderm formation raises the question as to the novel fate of the milk injected cells. The sustained vegetal expression of milk, suggest that, in addition to its role as a mesodermal suppressor, milk may contribute to the definition of the endodermal layer. Indeed, we find that overexpression of milk in the marginal zone or in animal caps leads to the activation of edd. In animal caps, this activation is not transient and persists until tailbud stages at a time when in vivo edd behaves as a pan-endodermal marker (Sasai et al., 1996).

Taken together, our data indicate that the milk-injected cells have adopted an endodermal state. In ectodermal explants, this endodermal shift cannot be considered as a consequence of a mesodermal repression. This strongly suggests that milk can actively induce endodermal fate and the presence of a C-terminal acid motif which has been observed in several transcriptional activators (Ptashne, 1988) raises the possibility that this gene may function as an activator of endoderm specific genes. Whether the transcriptional activation of the edd gene by milk is direct or achieved by some indirect means, for example by regulating two recently identified AMG-box genes (Xsox17α and -β) necessary and sufficient for endoderm formation, (Hudson et al., 1997) remains to be elucidated.

The role of milk in early Xenopus development

Previous work has postulated the existence of a mesendodermalizing activity that operates in the very early embryo to induce or ‘vegetalize’ the entire vegetal hemisphere (Takada and Yamada, 1960; Kocher-Becker and Tiedemann, 1971; Grunz, 1983; Asashima et al., 1991; Jones et al., 1993; Henry et al., 1996). In vitro experiments have shown that activin or the related TGF-β growth factor Vg1 display such an activity, inducing mesoderm and endoderm in ectodermal explants in a concentration-dependent manner (Green et al., 1992). Moreover, recent evidence suggests that activin can function in the embryo as a long-range morphogen (Gurdon et al., 1994, 1995; Jones et al., 1996). This raises the question as to the mechanism that operates in vivo to exclude the vegetal pole cells from the mesodermal pathway, as they themselves are exposed to mesoderm inducing factors. Cornell and colleagues (1995) proposed that lack of FGF signaling in endodermal cells may account for the failure of these cells to adopt a mesodermal fate in response to the presence of activin. Consistent with a negative influence of FGF signaling on endoderm formation, Sasai and colleagues (1996) recently reported that inhibition of FGF signaling in animal caps could synergise with chordin to activate an endodermal pathway. However, more recently La Bonne and Whitman (1997) showed that there are comparable levels of FGF signaling in marginal zones and vegetal halves of blastula explants. In this report, we demonstrate that inhibition of FGF signaling had no positive effect on edd induction by milk and may even have weakened the activation of edd, suggesting that induction of endoderm by milk or by chd probably proceeds via different mechanisms. Thus, in contrast to the inhibitory effects on an endoderm response to chordin signaling, it seems that endogenous FGF signaling promotes endodermal induction by milk.

In conclusion, milk appears to be able to ‘lock’ the program of mesodermal differentiation and to activate the endodermal one. While it is very likely that milk acts in concert with other transcription factors, its identification will contribute significantly to deciphering the molecular mechanisms that underlie endoderm formation in Xenopus.

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