goosecoid expression represses Brachyury in embryonic stem cells and affects craniofacial development in chimeric mice

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ABSTRACT The homeobox gene goosecoid, originally identified in Xenopus, is expressed in the organizer or its equivalent during gastrulation in the frog, chick, zebrafish and mouse. To investigate the role of goosecoid in mouse development, we have generated embryonic stem cells that stably overexpress the murine homolog of goosecoid. These cells show a repression of the gastrulation-associated gene Brachyury. Interestingly, repression of Brachyury is conserved between Xenopus and mouse despite the lack of conservation of the Brachyury promoter. Further characterization of the goosecoid-overexpressing ES cells revealed that they maintain the expression of stage-specific embryonic antigen-1, and teratomas derived from goosecoid-overexpressing cells show the presence of cell types derived from all three germ layers. Some highly chimeric mice derived from goosecoid-overexpressing cells displayed skull defects. These observations suggest that goosecoid may play a role in specification of anterior mesendodermal fates and specifically in mouse craniofacial development.

KEY WORDS: goosecoid, Brachyury, embryonic stem cells, chimeric mice, craniofacial development

Introduction

Gastrulation is the process by which the three definitive germ layers are established during embryogenesis. In Xenopus, the dorsal lip of the blastopore functions as an organizer of mesodermal cell fate along the dorsoventral axis (Spemann and Mangold, 1924). The goosecoid gene was identified during a screen of a dorsal lip cDNA library for novel homeobox-containing genes (Blumberg et al., 1991). goosecoid is initially expressed just before the onset of gastrulation in the region of the Xenopus embryo where the dorsal lip will form. Furthermore, in the chick and the mouse, goosecoid is first expressed at the site of primitive streak formation, where gastrulation is initiated in amniote embryos (Blum et al., 1992; Izpisua-Belmonte et al., 1993). The analogous early expression patterns at the onset of gastrulation in these species suggest that the function of goosecoid may have been conserved during evolution. Further indications that mechanisms of mesodermal induction have been conserved between the frog and the mouse come from experiments in which the distal tip of the gastrulating mouse embryo, containing a region (the node) that expresses the highest levels of murine goosecoid, was transplanted into early Xenopus embryos, resulting in the induction of a partial secondary body axis (Blum et al., 1992).

In the mouse, goosecoid expression begins in the posterior epiblast, at the site where the primitive streak will form; during streak elongation, goosecoid expression becomes restricted to the node and is detected in the anterior-most mesoderm as it forms (Blum et al., 1992). goosecoid is coexpressed with HNF3β from E6.5 through E9.5 in the anterior visceral endoderm and the anterior primitive streak, the head process, forebrain and oral epithelium (Belo et al., 1997; Filosa et al., 1997). goosecoid expression at 10.5 days of gestation is restricted to portions of the facial process, branchial arches, limbs and body wall (Gaunt et al., 1993). Thus, during early embryogenesis, goosecoid expression

Abbreviations used in this paper: DMSO, dimethyl sulfoxide; ES cells, embryonic stem cells; gsc, goosecoid-like; RT-PCR, reverse transcription polymerase chain reaction; SSEA-1, stage-specific embryonic antigen 1.
begins in cells that will initiate the process of gastrulation by forming the primitive streak and then becomes restricted to a specific subpopulation of cells that will form the head process (Lawson et al., 1991); during later embryogenesis, it is expressed in head and limb organ rudiments. This expression pattern along with studies in Xenopus suggest that goosecoid may play a dual role in cell fate determination in the mouse we have derived ES cell lines that stably overexpress the gene from a heterologous promoter.

Gain-of-function experiments in Xenopus have implicated goosecoid in the process of axis formation. Microinjection of goosecoid mRNA into the ventral side of Xenopus embryos led to the formation of partial secondary axes (Cho et al., 1991). Cells injected with the goosecoid mRNA were able to recruit uninjected neighboring host cells into a secondary axis (Niehrs et al., 1993). Injections of goosecoid mRNA into ventral marginal zone tissue of Xenopus gastrulae led to the dose-dependent formation of mesoderm of an increasingly more dorsal nature in explant cultures (Niehrs et al., 1994). These experiments suggested a role of goosecoid in mesodermal cell fate determination. Interestingly, a goosecoid knock-out in the mouse has not resulted in an obvious gastrulation phenotype (Rivera-Pérez et al., 1995; Yamada et al., 1995; Zhu et al., 1998), but led to postnatal death due to craniofacial abnormalities related to goosecoid expression during organogenesis. This surprising result could be explained by functional complementation through a second, goosecoid-related gene. It has been proposed that the goosecoid gene family consists of at least three members in the mouse (Belo et al., 1998; Zhu et al., 1998). A full understanding of goosecoid function may therefore require the analysis of the combined action of all family members.

Brachyury, like goosecoid, is expressed in the newly forming primitive streak mesoderm from the beginning of gastrulation onwards (Wilkinson et al., 1990; Blum et al., 1992; Kispert and Herrmann, 1993). Both genes encode nuclear transcription factors, goosecoid a homeodomain protein (Blumberg et al., 1991) and Brachyury a transcription factor of the T-box type (Kispert and Herrmann, 1994, Kispert et al., 1995). Initially, when the first mesodermal cells arise (E6.4), both genes are expressed in the same population of cells (as has been shown for zebrafish embryos; Schulte-Merker et al., 1994). When the streak lengthens and reaches the tip of the egg cylinder, goosecoid expression is maintained in the anterior-most mesendodermal cells (Blum et al., 1992), which are fated to become the visible node at E7.5 (Sulik et al., 1994), while Brachyury mRNA is localized throughout the primitive streak. At E7.5 goosecoid is expressed in the prechordal plate and the neuroectoderm overlying the notochordal plate, and at E8.5 in the prechordal plate and overlying the ventral diencephalon (Belo et al., 1998). From E10.5 onwards goosecoid is found in derivatives of cranial neural crest, limb buds and external genitals (Gaunt et al., 1993; Zhu et al., 1998). By contrast, Brachyury expression persists in the streak, the notochord, and later in the tailbud for the entire period of axis formation and elongation (E6.5-E12.5; Kispert and Herrmann, 1994).

Numerous other studies in Xenopus have identified a series of genes that are able to induce or perturb the formation of mesoderm. In contrast, less is known about the specification of mesoderm identity in mammals, largely because of the difficulty of studying gastrulation, a time when the mammalian embryo has implanted and is relatively inaccessible. We address this question by using murine embryonic stem (ES) cells, pluripotent cells of the inner cell mass (Evans and Kaufman, 1981; Martin, 1981), as a model system.

In order to investigate the function of goosecoid in mesodermal cell fate determination in the mouse we have derived ES cell lines which stably overexpress the gene from a heterologous promoter. We have used these lines to form embryoid bodies in vitro, tumors in ectopic sites, and chimeras in utero. Brachyury expression in such clones displayed an inverse correlation with goosecoid transcript levels. A Brachyury reporter construct was repressed by goosecoid in ES cells, and mutating a homeobox-binding site removed this repression. Some chimeras generated with goosecoid-expressing ES cells have skull defects suggesting that goosecoid plays a role in craniofacial development. In summary, our results suggest that goosecoid was able to define discrete anterior mesendodermal cell fates.
Results

Production of ES cells that overexpress goosecoid

To derive cell lines that stably overexpress the murine goosecoid gene, the ES cell line JM-1 was electroporated with a plasmid containing the cytomegalovirus (CMV) promoter driving the expression of the entire coding region of the murine goosecoid cDNA, along with a neomycin-resistance gene driven by the Rous sarcoma virus (RSV) promoter (Fig. 1A). Colonies were selected in ES cell medium containing LIF and G418. As a control, JM-1 cells were transfected with the parent vector minus the goosecoid cDNA. RNA was prepared from individual colonies and screened by slot blot to detect clones overexpressing goosecoid. The expression of goosecoid in three representative cell lines is shown in Figure 1B. No endogenous goosecoid expression was detected in the control transfected cell lines when the cells were maintained in an undifferentiated state; however, when the cells were allowed to differentiate as embryoid bodies in the absence of LIF for 7 days, a faint signal derived from the endogenous goosecoid gene was detected in the control cells. Expression of the transfected goosecoid gene was unaffected by differentiation (Fig. 1B).

Inverse correlation between goosecoid and Brachyury transcript levels in transgenic ES cell clones

In order to test if goosecoid expression in ES clones was associated with the activity of specific marker genes of the primitive streak, Brachyury and goosecoid transcript levels were analyzed in four clones: a control clone transfected with the empty expression vector (C1) and three clones stably expressing goosecoid (G185, G191, G202). Semi-quantitative analysis by radioactive RT-PCR revealed that the control clone did not express Brachyury, while a faint goosecoid signal was detected (Fig. 2), probably due to the higher sensitivity of RT-PCR compared to the Northern analysis in Figure 1. Expression levels of goosecoid and Brachyury were similar in transgenic clones G185 and G191, while the third clone, G202, expressed 57 times higher goosecoid levels and Brachyury transcripts were barely detectable (Fig. 2). An inverse correlation of the activity of the two genes was confirmed in three mass cultures each representing at least 100 independent colonies (not shown). This result indicates that goosecoid expression in ES cells induces cell fates different from primitive streak mesoderm, which is characterized by Brachyury gene expression.

To further evaluate the cell fate of transgenic goosecoid clones, the expression of lim1 and HNF3β, two anterior markers, which are both expressed in the primitive streak, prechordal plate and anterior visceral endoderm, was analyzed (Ang and Rossant, 1994; Shawlot and Behringer, 1995; Belo et al., 1997). While lim1 was not found in the goosecoid clones, HNF3β was highly expressed (not shown). This may be of significance in the light of the recent analysis of goosecoid/HNF3β double knockout mice which indicated a genetic interaction between these two genes (Filosa et al., 1997). Taken together, our analysis of markers suggests an involvement of goosecoid in cell fate determination in the anterior midline.

goosecoid acts as a repressor of Brachyury transcription in the mouse

Two recent studies have shown that in Xenopus, goosecoid acts as a repressor of Brachyury transcription (Artinger et al., 1997; Latinkic et al., 1997). The inverse correlation of transcript levels shown above argues that such a mechanism might exist in the mouse as well. However, a comparison of the promoter regions of the Brachyury genes of frog and mouse did not reveal a great degree of conservation (Fig. 3). In particular, the goosecoid-binding sequence identified by Artinger et al. (1997), through which repression of Brachyury in Xenopus was mediated, was not conserved (Fig. 3, region V).

In order to test if goosecoid repressed Brachyury gene transcription despite this lack of conservation, a mouse Brachyury reporter construct comprising the 650bp primitive streak enhancer in front of a luciferase reporter gene (Clements et al., 1996) was cloned. This construct was stably transfected into mouse embryonic stem cells (line E14.1, subclone KPA). A time course of reporter gene activity was determined in mass cultures, following the addition of 1% DMSO (v/v) in order to induce mesodermal differentiation. In agreement with published expression profiles for Brachyury and goosecoid (Johansson and Wiles, 1995), a peak of luciferase activity was found...
The effect of goosecoid on Brachyury reporter activity was investigated by transient transfection of a goosecoid expression construct into mass cultures representing at least 100 clones and subsequent DMSO treatment. As shown in Figure 4, a repression of about 50 percent was observed. This experiment was repeated several times with mass cultures and single clones. Thus, despite overall poor conservation of the promoter sequences in Xenopus and mouse, the same repression effect of goosecoid on Brachyury transcription was observed.

A careful analysis of the mouse T promoter sequence revealed the presence of one potential homeobox-binding sequence TAAT, in contrast to five such sites found in the Xenopus Brachyury promoter (Fig. 3). This element was conserved between mouse and frog (region IV; boxed and marked with asterisks in Fig. 3). This element was mutated into ACTG in the context of the 650bp promoter fragment and the effect of coexpression of goosecoid in ES cell cultures stably containing the mutated reporter gene was analyzed. As shown in Figure 4, the mutant construct was no longer repressed by goosecoid. Thus this single homeobox-binding site was necessary and sufficient for goosecoid-mediated repression of Brachyury transcription. In addition, we found that the reporter activity was markedly enhanced both in the absence and presence of goosecoid as compared to the wild-type construct (Fig. 4).

**goosecoid-expressing cells maintain SSEA-1 expression**

Since Brachyury was repressed by high levels of goosecoid expression, we asked if other differentiation markers were altered in our transgenic ES lines. Stage-specific embryonic antigen-1 (SSEA-1) is first expressed at the 8-cell stage of murine embryonic development (Solter and Knowles, 1978). Undifferentiated murine teratocarcinoma cells and ES cells express SSEA-1, whereas their differentiated derivatives do not (Solter and Knowles, 1978; Fox et al., 1981). In the embryonic region of 6- and 7-day egg cylinder-stage embryos, SSEA-1 is expressed by embryonic ectoderm but not by mesoderm (Fox et al., 1981). Immunofluorescence assays indicated that both control and G202 cells remained positive for SSEA-1 expression when maintained as undifferentiated cells in the presence of LIF (Fig. 5A,B). Thus, goosecoid-expressing cell lines maintained the SSEA-1 expression pattern of non-mesodermal cells.

**In vivo differentiation of goosecoid-expressing cells**

To test the in vivo developmental potential of the goosecoid-overexpressing cells, we injected control and G202 cells subcutaneously into nude mice. Both control and G202 cell lines gave rise to teratomas. RNA blot analysis of RNA isolated from the teratomas demonstrated that tumors derived from G202 cells maintained the expression of the transfected goosecoid gene (data not shown), indicating that goosecoid overexpression did not diminish tumorigenicity.
Histological analysis suggested that both control- and G202-derived tumors contained ectoderm-, endoderm-, and mesoderm-derived cell types (Fig. 5C,D).

In addition, analysis of 42 E18.5-19.5 chimeras derived from the injection of G202 cells into normal blastocysts showed that G202 cells were able to contribute to brain, heart, and liver (data not shown). Thus, goosecoid expression is compatible with differentiation into cells derived from all three germ layers. While chimeras derived from the injection of G202 cells into normal blastocysts were born, they were eaten by their mothers within 72 h of birth. Analysis of 20 G202 chimeras delivered by caesarian section revealed 3 cases of craniofacial abnormalities, including cleft palate, and shortening of the squamosal bone, whereas 66 non-chimeric littermates were normal (Fig. 6). All 3 of these chimeras had at least 50% contribution from the G202 ES line as determined by GPI analysis (data not shown).

Discussion

We have generated goosecoid-expressing ES cell lines to determine the role of goosecoid in mouse development. Three goosecoid-expressing ES lines, G185, 191, and G202, and one control ES line, c1, were identified and used in this study. Expression of downstream genes was examined in all 3 transgenic clones and the control clone. Brachyury expression was repressed in G202, the line with the highest goosecoid expression. HNF3β was induced, while there was no change in lim1 expression. Mutation of a potential homeobox-binding site in the Brachyury's leader sequence abolished the repression of Brachyury through goosecoid. Clone G202 maintains expression of SSEA-1 and these ES cells can give rise to all germ layer derivatives in teratomas formed in nude mice. Some highly chimeric mice made with the G202 line exhibited craniofacial defects.

The organizer transplantation experiments of Spemann and Mangold (1924) revealed not only that the dorsal lip of the blastopore was capable of organizing cells to form a new axis, but also suggested the existence of separate head and trunk organizers. Recent evidence has shown that the vertebrate head organizer, originally postulated to be the prechordal mesoderm, may be the anterior visceral endoderm (Thomas and Beddington, 1996; Varlet et al., 1997; reviewed in Belo et al., 1997; Bouwmeester and Leyns, 1997). The organizer genes, goosecoid, lim1 and HNF3β, are all expressed in the anterior visceral endoderm as well as in the trunk organizer suggesting that these genes may have dual organizer roles in development.

Analysis of genes that may interact with goosecoid revealed that overexpressing goosecoid in ES cells results in the repression of Brachyury and induces expression of HNF3β. The repression of Brachyury that is seen in the ES line with the highest goosecoid expression is consistent with studies in Xenopus which revealed that goosecoid represses Brachyury by directly binding to consensus sites within the Brachyury promoter (Latinkic et al., 1997; Artinger et al., 1997). Both goosecoid and Brachyury are initially expressed in the same population of cells in the mouse embryo. goosecoid expression is maintained in the anterior-most part of the primitive streak (Blum et al., 1992) and is later found in the prechondal plate and in derivatives of the neural crest in the head, while Brachyury expression is maintained throughout the streak, and is found in the notochord and later in the tailbud. goosecoid may play a role in gastrulation and axis formation, as it does in Xenopus, when it is co-expressed with Brachyury in the node and primitive streak. goosecoid also has another role in craniofacial development, when goosecoid expression is found in the anterior visceral endoderm, prechordal plate and derivatives of the cranial neural crest. In these tissues, Brachyury is not expressed, perhaps as the result of repression by goosecoid. The level of goosecoid present may be a factor in the ability of goosecoid to repress Brachyury expression. Only our highest goosecoid-expressing clone was capable of repressing expression of Brachyury.

Both goosecoid and HNF3β are coexpressed in several regions of the early mouse embryo including the anterior primitive streak, prechordal plate and anterior visceral endoderm. The induction of HNF3β seen in the goosecoid-expressing clones is consistent with the observation that double mutant embryos that are goosecoid null and are heterozygous for HNF3β exhibit a loss of HNF3β expression in E8.75 embryos. goosecoid/HNF3β double mutant embryos in the most severe cases lost expression of HNF3β in axial mesoderm (Filosa et al., 1997). In these double mutants a new phenotype arises in which the forebrain was reduced in size and there were abnormal branchial arches and heart looping. The loss of HNF3β expression and the new phenotype in the goosecoid/HNF3β double mutant embryos suggests a genetic interaction between HNF3β and goosecoid (Filosa et al., 1997).

To further examine the interaction between goosecoid and Brachyury, we compared the murine and Xenopus Brachyury promoter sequences. Surprisingly, the degree of conservation was rather low and restricted to five small regions within the 500 (frog) and 650 (mouse) bp upstream of the start ATG. In particular, the binding site through which goosecoid mediates repression of Brachyury in Xenopus was not conserved (Artinger et al., 1997).
This element, located in region V (Fig. 3), is missing in the corresponding mouse sequence, although sequences upstream and downstream are conserved. Nevertheless, a Brachyury reporter construct that contained the primitive streak enhancer was down-regulated following transient cotransfection of a goosecoid expression plasmid, in agreement with the observed inverse correlation of expression of these two genes in ES cell clones (Fig. 2), and in mass cultures of ES and P19 teratocarcinoma cells stably expressing goosecoid (data not shown). Mutation of a potential homeobox-binding site in the Brachyury 5' leader sequence abolished repression of Brachyury through goosecoid. Interestingly, the promoter activity of the mutant reporter gene was markedly higher compared with the wild-type construct. This result is compatible with the proposed role of goosecoid, namely that goosecoid functions as a repressor of Brachyury in the anterior of the primitive streak resulting in distinct mesodermal characteristics. As endogenous goosecoid becomes up-regulated in ES cultures following DMSO treatment as well (Johansson and Wiles, 1995; our unpublished results), a mutant construct should not be repressed and so should display higher activity than a wild-type one.

A recent paper by Papin and Smith (2000) claimed that down-regulation of Xbra, the Brachyury homolog in Xenopus, was not directly mediated through goosecoid. In their experiments, a chimeric dominant-negative goosecoid gene construct consisting of two VP16 transactivation domains fused to the goosecoid coding region was unable to interfere with activin-induced Xbra repression. Such a chimeric protein should bind to the same target sequences as goosecoid, but instead of acting as a repressor it should activate transcription. As we have shown previously, goosecoid acts in a negative feedback loop to repress its own transcription (Danilov et al., 1998). The VP16 fusion protein thus should act as a strong inducer of the endogenous goosecoid gene, which would explain the observed unaltered repression effect. Endogenous goosecoid transcription was not analyzed in the experiments of Papin and Smith (2000).

Conserved sequence elements between the mouse and frog Brachyury promoter contain potential binding sites for two other transcription factors that are important regulators of early vertebrate development. LEF-1 binding sites are present in both the mouse and frog promoter (Love et al., 1995; Giese et al., 1997). One of these is located in conserved region I (Fig. 3). The presence of these sites indicates that Brachyury may be a direct target of the Wnt signaling pathway active in early vertebrate embryogenesis. This pathway results in the activation of the architectural transcription complex LEF-1/β-catenin and leads to epithelial-mesenchymal transitions concomitant with primitive streak formation (Huber et al., 1996; Schneider et al., 1996). Conserved region III comprises a perfect binding site for the homeobox transcription factor caudal (Margalit et al., 1993). As the expression of caudal genes overlaps with that of Brachyury in mouse, chick and frog (Meyer and Gruss, 1993; Epstein et al., 1997; Pillemer et al., 1998), they may be positive modulators of Brachyury expression in the forming mesoderm.

The appearance of craniofacial defects in some goosecoid chimeras is consistent with the defects seen in goosecoid null mutants (Rivera-Pérez et al., 1995; Yamada et al., 1995). The goosecoid null mutants have a reduction in the palatine bone, whereas one chimera, generated from the G202 goosecoid-overexpressing cell line, had a severe cleft palate. Two goosecoid chimeras have a shortened squamosal bone, while this bone appears to be normal in goosecoid null mutants. None of the G202 goosecoid-overexpressing chimeras had the tympanic ring bone defect characteristic of the null mutants. The similarities in phenotypes between the goosecoid null and chimeric embryos has a precedent in the similar phenotype resulting from ectopic expression of Hox-3.1 and the targeted disruption of Hox-3.1, which display similar vertebral transformations (Le Mouellic et al., 1992; Pollock et al., 1992). It has been proposed that the Hox-3.1 gene is regulated antipodally; that is, overexpression of the gene and the loss of expression both result in similar phenotypes (Pollock et al., 1992).
Thus, the level of goosecoid overexpression may affect the severity of the phenotype seen. It is therefore possible that higher levels of ectopic goosecoid expression would result in more of the severe phenotypes seen in the null mutant. The degree of chimerism and the regions of the embryo to which the ES cells contribute will also affect the phenotype that is seen.

The observation that mice homozygous for a null mutation of goosecoid undergo apparently normal gastrulation (Rivera-Pérez et al., 1995; Yamada et al., 1995) suggests the existence of other goosecoid-related genes (De Robertis, 1995). A second goosecoid gene, gscl, has been identified in the mouse and is expressed at the time of gastrulation (Galili et al., 1997; Wakamiya et al., 1997). It has been argued that the goosecoid gene family consists of at least three genes (Belo et al., 1998; Zhu et al., 1998), and a full understanding of goosecoid function will require the analysis of the whole family.

Our observation that goosecoid overexpression leads to the repression of Brachyury and induces HNF3β expression demonstrates that these cell lines are a potentially valuable resource for the identification of other genes, such as chordin (Sasai et al., 1994), whose expression is regulated by goosecoid. Studies are under way to determine targets of goosecoid regulation in the mouse.

Materials and Methods

Cloning of p205

mRNA was isolated from 500 E6.5 mouse gastrulae, and a cDNA library was prepared in lambda MOSSlox (Amersham) (Danilov et al., 1998). Two full-length goosecoid cDNA clones were isolated by using the genomic probe P2 (Blum et al., 1992). The goosecoid cDNA was inserted into the expression vector pRc/CMV (Invitrogen) and designated p205.

Production of goosecoid-expressing cell lines

The ES cell line JM-1 (Miettinen et al., 1995; Meneses, J.J. and Pedersen, R.A., unpublished observations) was electroporated with 10 µg of PvuI-digested p205. Colonies were selected in medium containing 150 µg/ml G418 and transferred to duplicate 24-well plates containing mitomycin-treated G418-resistant STO feeder cells in high-glucose Dulbecco’s modified Eagle’s medium. The medium was supplemented as described by Robertson (1987) and by the addition of 5-10% conditioned medium from recombinant leukemia inhibitory factor (LIF)-producing CHO cells (a gift of Genetics Institute, Cambridge, MA). One plate was used to freeze cells; colonies in the duplicate plate were grown until confluent. Individual colonies were characterized by slot-blotting RNA isolated from each well and probing the filters sequentially for goosecoid and cytoplasmic β-actin.

RNA isolation and RNA blot analysis

RNA was prepared by using Ultraspec (Biotecx) according to the manufacturer’s instructions. For RNA blot analysis, 20 µg of total RNA was dissolved in 3 µl of water. To each 3.3 µl of sample buffer containing 65% deionized formamide, 8.1% formaldehyde, 1.3xMOPS buffer (10xMOPS buffer=0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) were added. Each sample was heated at 65°C for 5 min, then cooled on ice. The RNA was fractionated by electrophoresis on 1% agarose gels containing 6.2% formaldehyde and 1xMOPS buffer. RNA was transferred to nitrocellulose filters (Schleicher and Schuell) by capillary action overnight in 20xSSC (1xSSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Filters were baked at 80°C under vacuum for 2 h, wet with 2xSSC, and prehybridized overnight at 42°C in 50% formamide, 6.6xSSC, 5xDenhardt’s solution, 0.1% sodium dodecyl sulfate (SDS). 0.1 µg/ml denatured salmon sperm DNA, and 10% dextran sulfate. Filters were hybridized with 32P-labeled DNA probes (Random Priming Kit, Boehringer Mannheim) in the same solution at 42°C overnight. Filters were washed for 30 min in a solution of 2xSSC, 0.1% SDS at ambient temperature, followed by 2 washes at 65°C in a pre-heated solution of 0.2xSSC, 0.1% SDS. Filters were exposed to Kodak X-Omat film at -70°C.

Semiquantitative RT-PCR

Total RNA was isolated from cultured cells by the guanidinium thiocyanate/phenol method using the QGENE TriPure™ kit according to the manufacturer’s instructions (peqlab Biotechnologie GmbH, Erlangen, Germany). Total RNA was subjected to DNaseI treatment prior to RT reactions. Ten µg of RNA, 10xDNase-buffer (500 mM Tris HCl, pH 7.5, 50 mM MgCl2), 40 U RNAsin (Pharmacia, Freiburg Germany), 10 U DNaseI (RNase free, Boehringer Mannheim, Germany) and 10 µg yeast t-RNA were incubated in a total volume of 50 µl for 10 min at 37°C. Following the addition of 40 µl 4M LiCl and 325 µl H2O, RNA was extracted and resuspended in 25 µl DEPC-H2O.

For cDNA synthesis, 800 ng DNasel-treated total RNA was incubated in a mixture of 5xRT buffer, 13 µM dT20, 400 µM dNTPs (Pharmacia, Freiburg Germany), 10 µM DTT, 10 U RNAsin, 60 U Superscript II reverse transcriptase, at 42°C for 45 min. Amplifications were performed with Taq-Polymerase (Pharmacia) and radioactively-labeled α[32P]dCTP (mCi; Amersham-Buchler GmbH, Braunschweig) with gene specific primers. GAPDH, 5’: 5’TGTCC-A GTATGATTCTCACC-3’, 3’: 5’CCATCCAC-AGTCTTCTGAG-3’, amplification of 440bp; Brachyury, 5’: 5’TGCTGCCTGTGAGTCATAAC-3’, amplification of 285bp.
3', 3': 5'-TGCTGCGGTGATCATAAC-3', amplification of 343 bp; goosecoid, 5': 5'-GGTCTGGATC-TGGTGTCTCCGG-3', 3': 5'-TCAGCTGTCGCGATTCGCAAAAT-3', amplification of 280 bp.

GAPDH and Brachury primers were incubated in the same reaction (10°/94°C, 10°/55°C, 10°/72°C, 27 cycles, followed by a 2° extension at 72°C). To detect GAPDH in combination with goosecoid; GAPDH reactions were run for 8 cycles, then fresh PCR mix was added which contained goosecoid primers and PCR was performed for 22 additional cycles (30°/94°C, 30°/55°C, 30°/72°C).

RT-PCR reactions were analyzed on a 6% polyacrylamide gel. The gel was dried and autoradiographed on X-ray film (Hyperfilm). Bands were quantified on a phosphorimager (Fuji Co. Phosphorimager, Mac BAS software package).

Cloning of wild-type and mutant Brachury promoter luciferase constructs

Point mutations in Brachury reporter constructs were introduced by site-directed mutagenesis using the PCR-overlap extension protocol (Dieffenbach et al., 1995). At position +35 the putative homeobox binding site TAAT was changed to ACTG. Primers were designed to add BamH I and Hind III restriction sites at the 5'- and 3'-ends of the promoter, respectively. The following primer pairs were used:

sense: MS-1: 5'- GAGAAGATCCGCGGGCCAAAGT -3',
anti-sense: MS-2: 5'- AAAGCGAGCTACCGGTCTGGCACA -3',

sense: MS-3: 5'- TGTCAGGGACCCAGGTAGCTGTTTT -3',
anti-sense: MS-4: 5'- GAGAAGCTCCAGGAGTCCTGATCCC-3'

The final PCR product was digested with BamH I and Hind III, gel purified, and inserted into pT81-LUC-vector (Nordeen, 1988), digested with BamH I/Hind III. The wild-type promoter fragment was obtained from a PCR reaction containing primers MS-1 and MS-4. The promoter constructs were verified by sequencing.

Transfections and luciferase reporter gene assay

ES cells (E14.1 subclone KPA) were cultured on gelatinized dishes in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum in the presence of LIF (500 U/ml). ES cells (6x10^4) were cotransfected with 25 µg pT81-LUC plasmid (empty vector, wild-type or mutant Brachury promoter) and 4 µg of a plasmid containing the neomycin phosphotransferase gene (PGK-neo) by electroporation in PBS (Mg 2+/Ca 2+-free), 10 mM sodium chromate, 140 mM potassium chloride, 10 mM sodium phosphate, pH 7.4. Gels were stained with 1 ml of staining solution (0.1 mM methylthiazolium tetrazolium, 0.2 mg phenazine methylsulfate, 1 mg bromothymol blue) mixed with 1 ml 2 M glycine, pH 8.5). Gels were done, followed by one wash in PBS. Samples were mounted with 70% glycerol in PBS with p-phenylenediamine added to decrease quenching of fluorescence.

ES cell differentiation in vivo

Control and G202 cells (10^6 cells in 0.1 ml PBS) were injected subcutaneously into male nude mice (BALB/c nu/nu). Mice were killed when tumors were 1-2 cm in size (3.5 weeks after injection) and fixed for 6 h in Bouin's fixative and dehydrated through ethanol. Tumors were embedded in plastic, sectioned, and stained with hematoxylin-and-eosin for histological analysis.

Chimeras were generated by injecting G202 or C1 cells into C57BL/6/J or (C57BL/6/J x CBA)F1 blastocysts. Blastocysts were transferred to CD1 surrogates.

Generation of goosecoid chimeras

C1, G202, G185 or G191 ES cell lines were injected into C57Bl/6/J or CD-1 blastocysts and transferred into the uteri of pseudopregnant CD1-female mice. Embryos were harvested at E14.5 and E18.5.

Glucose phosphate isomerase assay

Tail, brain, heart and liver tissue was removed from embryos delivered by caesarean section (Hogan et al., 1994) at E18.5-19.5, placed in 100 µl of water and freeze-thawed 4 times (dry ice/ethanol bath to warm water). The lysate (1 µl) was loaded onto an equilibrated cellulose acetate plate (Helena) and run for 30-40 min at 180 V in running buffer (50 mM Tris, 375 mM glycine, pH 8.5). Gels were stained with 1 ml of staining solution (0.1 M Tris pH 8, 20 mM MgCl2, 15 mg fructose 6-phosphate, 1 mg methylthioimidazole tetrazolium, 0.2 mg phenazine methosulfate, 1 mg NADP and 10 U glucose-6-phosphate dehydrogenase) mixed with 1 ml warm 1% low melt agarose. The color was developed for 15-30 min in the dark.

Skeletal preparations of chimeras and non-chimeric littersmates

Embryos were delivered by caesarean section at E18.5-19.5 (Hogan et al., 1994). Fetuses were fixed in 95% ethanol and stained with alcian red and alizarin red and alizarin blue BSG according to the method of McLeod (1980).

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