Expression of the *Brachyury* gene during mesoderm development in differentiating embryonal carcinoma cell cultures

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SUMMARY

When aggregated and treated with dimethyl sulfoxide (DMSO), P19 embryonal carcinoma cells differentiate into cell types normally derived from the mesoderm and endoderm including epithelium and cardiac and skeletal muscle. The *Brachyury* gene is expressed transiently in these differentiating cultures several days before the appearance of markers of the differentiated cell types. The expression of *Brachyury* is not affected by DMSO but is induced by cell aggregation, which requires extracellular calcium. Expression of *Brachyury* is also induced by various members of the TGFβ family such as activin and bone morphogenetic proteins. D3 is a mutant clone of P19 cells selected for its failure to differentiate when aggregated in DMSO. Aggregated D3 cells express *Brachyury* mRNA suggesting that the mutation(s) responsible for the phenotype of D3 cells is downstream of the chain of events initiated by *Brachyury* expression.

Key words: mesoderm, *Brachyury*, P19 cells, embryonal carcinoma cell, mouse

INTRODUCTION

In *Xenopus* embryos, mesoderm is generated when cells from the vegetal pole instruct cells from the overlying animal area to differentiate into mesoderm. The signals involved in mesoderm induction are called mesoderm-inducing factors. Various molecules have been shown to possess potent mesoderm-inducing or competence-modifying activities (Smith and Howard, 1992) in vitro when added to isolated *Xenopus* animal cap explants or in vivo when ectopically expressed in *Xenopus* embryos. These include the products of the *Wnt* (McMahon and Moon, 1989) and *noggin* (Smith and Harland, 1992; Smith et al., 1993) genes, peptide growth factors such as fibroblast growth factor and members of the TGFβ family including activin and bone-morphogenic proteins (BMP)-2 and -4 (Thomsen et al., 1990; Smith et al., 1991; Dale et al., 1992; Green et al., 1992). Which if any of these molecules function in mesoderm induction in the *Xenopus* embryo is not yet clear.

In mammals very little is known about signals triggering mesoderm induction. The *Brachyury* (T) gene is essential for the development of posterior mesoderm in the mouse. Homozygous embryos lacking a functional *Brachyury* gene die at 10 days of gestation due to insufficient mesoderm formation and the absence of a notochord and allantois (Beddington et al., 1992). *Brachyury* transcripts (Herrmann et al., 1990) are first detected in early murine gastrulae next to the primitive streak, in posterior mesoderm and in ectoderm destined to form mesoderm (Wilkinson et al., 1990). The expression becomes restricted to the notochord and the tail bud (Wilson et al., 1993). No transcripts are detected in adult tissues. Thus, *Brachyury* mRNA is found in tissues affected by the mutation, suggesting that *Brachyury* protein is essential for mesoderm formation or maturation (Beddington et al., 1992). In *Xenopus* embryos, mesoderm-inducing factors have been shown to induce *Brachyury* expression (Smith et al., 1991; Green et al., 1992) and injection of *Brachyury* mRNA results in ectopic mesoderm development (Cunliffe and Smith, 1992).

Embryonal carcinoma (EC) cells are the multipotential stem cells of teratocarcinomas whose development potential resembles that of early embryonic cells. EC cells can be cultured and induced to differentiate into many specialized cell types. The P19 line of EC cells (McBurney and Rogers, 1982; McBurney, 1993) differentiates into ectodermal-like cells including neurons and glia cells when aggregated and exposed to retinoic acid (RA) at concentrations greater than 0.1 μM (Jones-Villeneuve et al., 1982, 1983; Aizawa et al., 1991). In contrast, when aggregated and treated with 0.5-1.0% DMSO or 10-100 nM RA, P19 cells differentiate into mesoderm-like cells including cardiac and skeletal muscle (McBurney et al., 1982; Edwards et al., 1983; Edwards and McBurney, 1983). We investigated the expression of *Brachyury* during P19 cell differentiation and found its expression restricted to cultures containing cells destined to differentiate into mesoderm.

MATERIAL AND METHODS

Cell culture and differentiation

The cell lines P19, RAC65 and D3 were maintained as described (Rudnicki and McBurney, 1987). They were kept in exponential growth phase by routinely sub-culturing the cells at intervals of 48
hours in minimum essential medium alpha (MEMα) (Gibco, Burlington, Ontario) supplemented with 2.5% fetal calf serum and 7.5% calf serum (Cansera International Inc., Toronto, Ontario).

To induce differentiation, cells were allowed to aggregate in Petri-
grade dishes in the presence of 0.8% dimethyl sulfoxide (DMSO) for 4 days. The treated aggregates were then transferred to tissue culture-
grade dishes or gelatin-coated coverslips and cultured without DMSO
(Rudnicki and McBurney, 1987). Differentiated cells were harvested for RNA analysis at daily intervals throughout the differentiation
regime or fixed for immunofluorescence 7 days after initiation of
differentiation. Days of differentiation were numbered consecutively
after the first day of aggregation (day 0).

To prepare calcium-free culture medium, calf serum was dialysed for 36 hours at 4°C against phosphate-buffered saline (PBS) using a
dialysis membrane having a molecular weight cutoff of 6000-8000
and then filter-sterilized. Cells were plated into bacterial grade Petri
dishes in calcium-free Joklik’s medium (Gibco, Burlington, Ontario)
supplemented with 10% dialysed calf serum and 0-4.5 mM calcium
chloride.

Activin and BMP-2, -3, -4 and -5 were obtained from the Genetic
Institute (Cambridge, Mass.). Activin was prepared from conditioned
media of a CHO cell line (Chinese hamster cell line overexpressing
activin A). The activin preparation was found to be active in the
Xenopus animal cap assay at 1:200 dilution and was used at this
concentration in culture medium. BMP-2, -3 and -5 were 90% pure and
BMP-4 was 75% pure. In our assays, BMP-2, -4 and -5 were used at
1.0 nM and BMP-3 was used at 0.7 nM. They were all handled using
siliconized plasticware. Leukemia inhibitory factor (LIF) was
obtained at 10^6 units/ml with a specific activity of 10^3 units/µg from
GIBCO BRL (Burlington, Ontario). 3-isobutyl-1-methylxanthine
(IBMX) and forskolin were purchased from Sigma (St Louis,
Missouri).

Northern blot analysis
Total RNA was prepared from cells by the lithium/urea method
(Auffray and Rougeon, 1980). 15 µg aliquots of RNA were elec-
trophoresed through 0.9% agarose gels containing 20 mM 3-[N-Mor-
pholino]propane-sulfonic acid, 1 mM EDTA, 5 mM sodium acetate,
pH 7.0 and 10% formaldehyde. The electrophoretically separated
RNAs were transferred to Hybond-N membranes (Amerham,
Oakville, Ontario) that were then treated with ultraviolet light at 120
ml using a GS Gene linker UV chamber (Biorad, Mississauga,
Ontario). Hybridizations to random-primed 32P-labelled DNAs were
as described (Maniatis et al., 1982). Filters were washed several times
in 2x SSC and 0.1% SDS at 22°C for 30 minutes, followed by several
changes of 0.2x SSC and 0.1% SDS at 65°C for 45 minutes. Signals
were visualized by autoradiography using Kodak XAR-5 films.

To strip the filters before rehybridizing, they were incubated for 30
minutes in 100°C water containing 0.1% SDS.

The Brachyury probe was a BamHI-SalI 1.8 kbp cDNA fragment
(Herrmann et al., 1990). The actin probe was a 0.6 kbp PstI fragment
containing the last exon of the human cardiac actin gene (Rudnicki et
al., 1988). The α-tubulin probe was a full-length mouse cDNA
(Lemishka et al., 1981).

RESULTS

Transient Brachyury expression in differentiating P19 cells

When P19 cells are aggregated and exposed to DMSO, they
differentiate into a variety of mesodermal and endodermal cell
types including cardiac and skeletal muscle (McBurney et al.,
1982; Edwards et al., 1983; Smith et al., 1987). We investi-
gated the expression of the Brachyury gene in differentiating
P19 cells by northern blot analysis. Brachyury mRNA was
present at very low to undetectable levels in undifferentiated
P19 cells and dramatically increased in abundance to peak at
day 2 before declining to indetectable levels by day 5 (Fig. 1A).
On day 5, cardiac muscle first appeared as indicated by the
presence of the sarcomeric isoform of actin found in striated
muscle (Fig. 1B) (Rudnicki et al., 1990).

P19 cells differentiate into different cell types depending on the
concentration of RA to which they are exposed; low levels
(10-100 nM) induce development of mesodermal cell types
while higher concentrations (>100 nM) induce formation of
neuroectodermal cells (Edwards and McBurney, 1983). In P19
cells cultured on plastic surfaces and exposed to RA, Brachyury
expression was restricted to cultures exposed to
intermediate concentrations of RA that induce mesoder-
mal differentiation (Fig. 2).

RAC65 cells (Jones-Villeneuve et al., 1983) are a mutant
cell clone of P19 cells that fail to differentiate in response to RA
and that contain a mutation in the alpha RA receptor (RARα)
(Pratt et al., 1990). These cells differentiate normally when
exposed to DMSO. Brachyury was transiently expressed
during DMSO-induced differentiation of RAC65 cells but no

Fig. 1. Brachyury expression during DMSO-induced differentiation of P19 cells. Total RNA was extracted from P19 cells at daily
intervals following induction of differentiation with 0.8% DMSO and examined by northern blot analysis. (A) The RNA blot (15
µg/lane) was first hybridized with a Brachyury cDNA probe then
stripped and (B) rehybridized with an actin cDNA probe as described
in Material and methods. Lanes 1-7: days of differentiation
numbered consecutively after the first day of aggregation and DMSO
treatment. Lane 8: P19 cells aggregated 2 days without drug. The
actin probe hybridizes to mRNAs encoding cytoskeletal (β and γ) at
2.1 kbp and striated muscle (α) actins at 1.7 kbp.
Brachyury expression in P19 cells

Expression was detected in these cells exposed to RA at any of the concentrations tested (data not shown).

D3 cells (Edwards et al., 1983) are another mutant P19 cell line that fail to differentiate in DMSO but do differentiate into neuroectodermal cells when exposed to high RA concentrations. Brachyury mRNA levels were very low in untreated D3 cells and became elevated following aggregation in the presence of DMSO (Fig. 3). Densitometric scans of northern blots from a number of different experiments indicated that the level of Brachyury expression in D3 cells was between 30 and 100% that seen in P19 cells. In contrast to the situation in P19 cells, expression of Brachyury mRNA in D3 cells did not decline but remained elevated for as long as the cells were cultured as aggregates. Thus, the differentiation of aggregated cells appeared to correlate with the loss of Brachyury expression. Southern blots of DNA from D3 cells probed for the Brachyury gene indicated no evidence for rearrangement affecting this gene.

F9 embryonal carcinoma cells are able to differentiate only into cells resembling those derived from extraembryonic endoderm. We did not detect the Brachyury transcript in aggregated F9 cells (data not shown).

Calcium-mediated aggregation triggers Brachyury expression

The expression of Brachyury was induced very weakly in P19 cells cultured on plastic surfaces in the presence of DMSO whereas expression was induced to high levels in cells aggregated in the absence of DMSO (Fig. 1A, lane 8; Fig. 3, lane 3). DMSO did not enhance the level of Brachyury expression in P19 cell aggregates.

Aggregation of embryonal carcinoma cells is mediated by calcium-dependent adhesion molecules (Yoshida and Takeichi, 1982). P19 cells cultured in medium with reduced calcium concentrations formed small poorly compacted aggregates of cells (Fig. 4). Induction of Brachyury expression was reduced in these cultures (Fig. 5). The calcium chelator, EGTA, also blocked the induction of Brachyury expression in aggregated P19 cells if present at concentrations higher than that of calcium in normal medium (Fig. 3, lanes 1 and 2). The calcium ionophore A23187 did not induce Brachyury expression in P19 cells growing on solid surfaces and the calcium channel blocker, verapamil, did not prevent aggregation-induced expression of Brachyury (data not shown). Thus, the expression of Brachyury is likely induced indirectly by calcium-mediated cell aggregation.

Modulation of Brachyury expression by extracellular factors

Activin efficiently induces mesodermal differentiation (Thomsen et al., 1990) and Brachyury expression (Smith et al., 1991) in Xenopus animal caps. Activin A induced Brachyury expression in P19 cells cultured on solid surfaces (Fig. 6). Expression was evident as early as 1 hour after exposure to activin but the level of Brachyury transcript never rose as high as in aggregated cells. In addition to activin A, we also tested other members of the TGFβ gene family, the bone morphogenetic proteins (BMP) -2, -3, -4 and -5. All induced Brachyury expression (Fig. 7). Basic fibroblast growth factor (bFGF) also induced Brachyury expression but to a much lower level than that seen with activin and BMPs (data not shown).

Although activin and BMPs induced elevated levels of Brachyury mRNA these agents did not induce cell differentiation. In fact, activin has been reported to inhibit the differentiation of P19 cells (Hashimoto et al., 1990; Van den Eijnden-van Raaij et al., 1991).
Agents that elevate the intracellular levels of cyclic AMP also induced the expression of Brachyury expression in P19 cells cultured on plastic surfaces (Fig. 8A). Cells were treated with forskolin and IBMX to elevate cAMP levels (IBMX is a phosphodiesterase inhibitor and forskolin activates adenylate cyclase). In addition, IBMX and forskolin enhanced the expression of Brachyury in P19 cells aggregated for 24 hour (Fig. 8C).

Although leukemia inhibitory factor (LIF) is not essential for embryogenesis (Stewart et al., 1993), its overexpression inhibits murine gastrulation (Conquet et al., 1992). LIF prevents endoderm and mesoderm differentiation in P19 cells (Pruitt and Natoli, 1992). P19 cells aggregated for two days in...
Brachyury expression in P19 cells

the presence of LIF expressed lower levels of Brachyury mRNA than cells aggregated in the absence of LIF (Fig. 9). Hence, LIF inhibition of P19 cell differentiation could be due to reduced Brachyury expression.

Goosecoid expression

The mouse homeobox gene, goosecoid, is expressed in the anterior mesoderm of the primitive streak (Gaunt et al., 1993) and its Xenopus homologue appears essential for cell migration (Niehrs et al., 1993). When P19 cells were aggregated and treated with DMSO, they transiently expressed goosecoid with kinetics similar to those seen for Brachyury (Fig. 10).

DISCUSSION

P19 cell cultures develop into mesodermal cell types in culture following aggregation and treatment with DMSO. This differentiation was accompanied by transient bursts of expression from both the Brachyury and goosecoid genes, which preceded the appearance of markers of such mesodermal cell types as cardiac muscle. This result provides further evidence that similar molecular mechanisms are responsible for embryonic development and for differentiation of EC cells in culture (McBurney, 1993). Brachyury expression was induced by activin and BMPs. These experiments point to parallels in behaviour between Xenopus animal cap cells and EC cells, in
both systems Brachyury and goosecoid expression are associated with differentiation into mesodermal lineages.

Activation of the Brachyury gene in P19 cells accompanied cell aggregation and required extracellular calcium. Aggregation and compaction of EC cells is mediated by E-cadherin (Yoshida and Takeichi, 1982). How aggregation results in Brachyury induction is not clear. Homotypic binding of E-cadherins might be the source of the intracellular signal that results in Brachyury activation. Components of the extracellular matrix such as laminin, fibronectin and collagens are present in aggregates so calcium-dependent binding of integrins to these molecules could also initiate an intracellular signal; however, plating cells onto laminin- or fibronectin-coated surfaces did not induce expression of Brachyury (data not shown). Alternatively, the close juxtaposition of membranes in compacted aggregates might allow for the interaction between cell surface-associated receptors and ligands.

Brachyury expression was induced by various members of the TGFβ family including activin and BMPs. Embryonal carcinoma cells have shown to express mRNAs encoding activin (Albano et al., 1993) and BMP-4 (Rogers et al., 1992). Both proteins are able to induce Brachyury expression in P19 cells. It is possible that the induction of Brachyury expression in cell aggregates might be mediated by activin or BMPs synthesized by P19 cells. Members of the TGFβ family are often secreted in inactive form. Perhaps the close cell contact afforded by aggregation enhances activation of latent BMPs secreted by P19 cells. A newly discovered member of the TGFβ family, nodal, is known to be expressed in early mouse embryos at the time of gastrulation (Zhou et al., 1993) so this molecule might be the one responsible for the induction of Brachyury in embryos.

P19 cells are known to express the activin receptor (Kondo et al., 1989; Nakamura et al., 1992) and activin is a survival and growth factor for these cells (Schubert et al., 1990; Hashimoto et al., 1990). The activin receptor is a serine-threonine-tyrosine kinase (Mathews and Vale, 1991; Attisano et al., 1992; Mathews et al., 1992; Nakamura et al., 1992). Activation of protein kinase A by elevating intracellular levels of cyclic AMP also induced the expression of Brachyury mRNA suggesting that the induction of Brachyury expression might be regulated in large part by protein phosphorylation.

The expression of Brachyury can be repressed as well as enhanced by soluble factors. LIF reduced the level of Brachyury expression in aggregated cultures and has also been shown to suppress mesodermal development in embryos (Conquet et al., 1992), embryonic stem cells (Shen and Leder, 1992) and P19 cells (Pruitt and Natoli, 1992).

Although cell aggregation induced expression of Brachyury mRNA, differentiation does not subsequently occur unless the aggregates are exposed to DMSO (McBurney et al., 1982). Since DMSO did not appear to have any effect on the level of Brachyury mRNA, it seems likely that DMSO enhances the cell’s response to the Brachyury protein. DMSO is known to induce the release of calcium from intracellular stores (Morley and Whitfield, 1993) suggesting that this drug might be responsible for indirectly activating calcium-dependent protein kinases or protein phosphatases that modify the Brachyury protein and alter its activity. Calcium metabolism is frequently affected by growth factors and factors secreted by some cell lines (Mummery et al., 1991) are able to induce differentiation of aggregated P19 cells.

The D3 mutant of P19 cells is a clone selected for its inability to differentiate in DMSO-treated cell aggregates (Edwards et al., 1983). Brachyury mRNA is induced in these cells following cell aggregation suggesting that the site of the mutation(s) in D3 cells resides in genes whose protein products are required downstream of the Brachyury protein.

In Xenopus, Brachyury expression appears to be sufficient for the development of mesoderm (Cunliffe and Smith, 1992). However, mouse ES cells lacking the Brachyury gene are able to differentiate normally into mesodermal cell types (Beddington et al., 1992) and Brachyury expression in P19 cells in aggregated cells is not sufficient to induce their differentiation.
The Brachyury protein in mouse may be important for the migration of primary mesoderm from posterior regions of the primitive streak (Beddington et al., 1992).

The mechanism of Brachyury protein action remains unclear. The zebrafish (Schulte-Merker et al., 1992) and murine (Belcourt and McBurney, unpublished data) Brachyury proteins appear to be nuclear and recent work indicates that it is a sequence-specific DNA-binding protein (Kispert and Herrmann, 1993). The P19 cell cultures should provide a system useful for investigating the mechanism by which Brachyury protein affects cell fate and maturation.

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