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ROLE OF THE BRACHYURY T GENE IN P19 EMBRYONAL CARCINOMA CELL DIFFERENTIATION

by

Gaël Vidricaire

A thesis submitted to

the school of Graduate studies and Research

in partial fulfillment for the degree

of

Master of Science

Department of Biochemistry

University of Ottawa

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Abstract

Brachyury T is a gene required for mesoderm formation and is expressed early during gastrulation in presumptive mesoderm. We found that this gene is expressed transiently in P19 cells destined to differentiate into cells of the mesodermal lineages. The expression of Brachyury in P19 cells peaks two days after initiation of differentiation and is induced by cell aggregation. The formation of compact cell aggregates and the expression of Brachyury are both dependent on extracellular calcium. Activin A, bone morphogenetic proteins and cAMP all induced Brachyury expression. By contrast, leukaemia inhibitory factor significantly reduced Brachyury expression in aggregated P19 cells and inhibited differentiation. Overexpression of Brachyury in P19 cells leads to their spontaneous differentiation with low levels of Brachyury resulting in the development of skeletal muscle while higher levels result in the formation of neurons.

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List of abbreviations

BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
CREB	cAMP response element binding protein
DMSO	dimethyl sulfoxide
EC	embryonal carcinoma
FGF	fibroblast growth factor
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
h	hour
HLH	helix-loop-helix
IBMX	3-isobutyl-methylxanthine
ICM	inner cell mass
Id	inhibitor of DNA binding
kb	kilobase
LIF	leukaemia inhibitory factor
M	molar
MBT	mid-blastula transition
MIF	mesoderm inducing factor
min	minute

mRNA	messenger ribonucleic acid
Pgk	phosphoglycerate kinase
PBS	phosphate buffered saline
p.c.	postcoitum
RA	retinoic acid
RAR	retinoic acid receptor
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
TGFβ	transforming growth factor beta
UTR	untranslated region
Wnt	Wingless/int-1
Xbra	Xenopus Brachyury

Chapter One

Introduction

Understanding how a single cell, resulting from the fusion of a male and a female gamete, gives rise to multiple cells and ultimately to a complete organism has been the subject of intense studies over the past decades. The interest in this phenomenon is two fold: a clear understanding of normal developmental processes is a fascinating subject in itself but this knowledge is also essential for discovering mechanisms of oncogenesis; tumour formation arises as a consequence of disturbing the balance between cell proliferation and differentiation.

The study presented in this thesis was directed towards gaining information on the mechanism of murine early embryonic development.

1.1 Features of vertebrate development:

Embryonic development is initiated following fertilization when male and female gametes fuse. Subsequently during cleavage, the embryo becomes a morula as the zygote subdivides by a series of mitotic divisions into many smaller cells called blastomeres. Within the embryo, a cavity is formed, the blastocoel, and the cells surrounding the cavity become organized into an epithelium; the embryo is then generally termed a blastula. Gastrulation begins when the blastula becomes a three layered structure

composed by the primary germ layers, the endoderm, the mesoderm and the ectoderm. During gastrulation, the blastomeres undergo extensive migrations and rearrangement wherein by invagination, a large area of cells on the outside of the embryo are brought to lie inside and as a result two germ layers are established, the endoderm and the mesoderm. At the same time, the remainder of the outer layer spreads to take the place of the cells that have turned inward forming the ectoderm. Hence, the outermost region composed is by the ectoderm, the inner most layer is formed by the endoderm, and in between lies the mesoderm. Following gastrulation, interactions within and between the three germ layers occur to produce the organs; the ectoderm generates epithelium and the nervous system (brain and spinal cord); the endoderm gives rise to the lining of the digestive tube and its associated organs; the mesoderm produces several organs (heart, kidney, liver), connective tissues (bone, muscles, tendons) and the blood cells (Gilbert, 1988; Oppenheimer and Lefevre, 1989; Alberts et al., 1983).

1.1.1 Mammalian development:

In mammalian embryonic development, the embryo goes through a morula stage, after which the cells become more compacted and the blastocoel is formed. At this stage the embryo is called a blastocyst; the outer cells enclosing the cavity forms the trophoectoderm, while the accumulation of cells inside the trophoectoderm at one pole constitutes the inner cell mass (ICM). The trophoectoderm gives rise to extra-embryonic structures involved in implantation of the embryo into the uterus.

Gastrulation begins when the ICM separates into two layers; the top epiblast layer

and the bottom hypoblast layer. The hypoblast, also named primitive endoderm, gives rise to extra-embryonic structures and is also involved in specifying the overlying epiblast cells. The epiblast generates the embryo proper; at the posterior end of the epiblast, lateral cells accumulate to form the primitive streak. Next, down the midline of the primitive streak, cells produce an indentation called the primitive groove. The primitive streak elongates as prospective endodermal and mesodermal cells migrate from the surface, through the primitive groove and travel between the epiblast and the hypoblast layers. Thus, the primitive streak gives rise to the endoderm and mesoderm germ layers while the remaining epiblast generates the ectoderm layer (Gilbert, 1988; Oppenheimer and Lefevre, 1989; Alberts et al., 1983)

1.1.2 Amphibian embryonic development:

Although amphibian and mammalian embryos generate completely different organisms, the steps undertaken by both species during early embryonic development are similar. The Xenopus embryo has been extensively studied over the past decades in the attempt to elucidate mechanisms of early embryonic development. An overview of amphibian embryonic development is introduced first in order to better understand recent studies on amphibian development presented subsequently.

The Xenopus ovum is separated in two hemispheres, the animal pole in the upper half of the egg and the vegetal pole in the lower part. During embryonic development, cells originating from the animal pole will form the ectoderm, while cells arising in the vegetal pole will constitute the endoderm. Mesoderm on the other hand will appear from

animal cells at the marginal (below the equator) zone.

Upon fertilization, embryonic development is initiated; cleavage, morula and the blastula stages are achieved. One of the first events to be triggered following fertilization is establishment of the dorso-ventral axis. Sperm entry in the animal pole triggers rotation of the cortical cytoplasm over the inner cytoplasm toward the sperm entry point. The rotation causes the animal-vegetal polarity of the egg surface to be offset relative to the animal-vegetal polarity of the internal cytoplasm. Thus, the sperm entry site marks the future ventral pole, whereas the opposite hemisphere forms the presumptive dorsal side. The area on the dorsal side of the marginal zone opposite to the sperm entry site is now termed the gray crescent. In addition, the cytoplasmic rearrangement slowly renders dorso-vegetal cells to be activated such that in turn they enable overlying cells in the gray crescent to initiate gastrulation.

Gastrulation starts in the gray crescent as dorso-vegetal cells invaginate to form an indent in the exterior of the egg named the blastopore. Subsequently, dorso-vegetal cells migrate through the blastopore into the interior of the embryo forming part of the endoderm. Then dorso-marginal zone cells invaginate through the blastopore between the endoderm and the outer layer to produce posterior mesoderm including the notochord, a precursor of the vertebral column involved in initiating differentiation of the nervous system. Subsequently, the blastopore moves laterally and ventrally for lateral and ventral endodermal and mesodermal precursor cells to migrate inward. They respectively complete the endoderm layer in addition to generating lateral and ventral mesoderm

(muscle, blood cells and mesenchyme). During this entire process, the animal cells spread gradually to take the place of the cells that have turned inward and eventually cover the entire external surface to generate the ectoderm (Gilbert, 1988; Oppenheimer and Lefefevre, 1989; Alberts et al., 1983).

1.2 Embryonic induction:

In the course of development, differences between cells and organization of cells into tissues are triggered by embryonic inductions. They are defined as an interaction between an inducing tissue with a responding tissue such that the responding tissue undergoes change in its direction of differentiation (Gurdon, 1987). Inductive interactions involve a signal that is generated by the inducing tissue and received by the responding tissue via receptors. The activated receptors mobilize an intracellular signalling pathway that results in modification of expression of specific target genes (reviewed in Jessel et al., 1992).

1.2.1 Mesoderm induction:

One of the first embryonic induction events to occur in development is mesoderm induction. This phenomenon has been most extensively studied in Amphibia (Smith, 1989).

Mesoderm emerges in the Xenopus embryo from an inductive interaction between cells from the vegetal and animal hemispheres. Vegetal cells signal the overlying animal cells in the marginal zone to become mesoderm. It has been shown that animal pole cells

cultured in isolation form epidermis while vegetal pole cells alone form endoderm (Nakaruma and Takasaki, 1967; Nukarama et al., 1970). Combinations of cells from the two poles generate mesoderm from the animal cells (Nieuwkoop, 1969). In addition, it was observed that the type of mesoderm formed in animal-vegetal combinations depends on the origin of the vegetal cells; ventral vegetal cells induce ventral mesoderm (blood and mesenchyme) whereas dorsal ventral cells induce dorsal mesoderm (notochord and muscle). Thus, vegetal cells contain information that not only determines the fate of animal cells into mesoderm but also imparts dorsa-ventral polarity. The dorsalizing vegetal cells were named Nieuwkoop centre or blastula organizer (Boterenbrood and Nieuwkoop, 1973). Cells derived from the Nieuwkoop center do not themselves produce mesoderm but rather provide necessary information to induce the surrounding cells to become mesoderm (Gimlish and Cooke, 1983).

However, these in vitro results do not account for the fact that in vivo ventral vegetal cells generate not only blood cells but also muscle. Spemann and Mangold made the observation that at the start of gastrulation the cells of the dorsal blastopore lip can induce head structure and an entire secondary axis when transplanted to the ventral marginal zone of an early gastrula host. In addition, it has been shown that the second axis is formed by cells descended from and induced by the transplanted cells (Gimlish and Cooke, 1983). This suggested that certain cells in prospective mesoderm are able to determine the fate of their surrounding cells; this region was named Spemann's organizer or gastrula organizer (Spemann and Mangold, 1924).

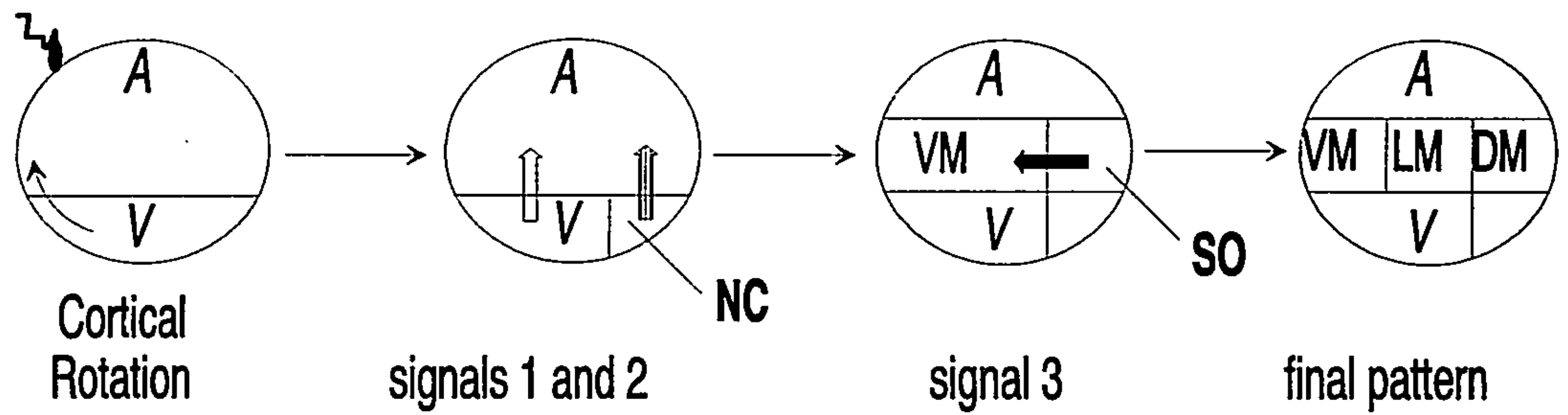
Based on this information a model to describe mesoderm induction has been proposed (fig. 1A). Mesoderm is specified by three signals. Postfertilization cortical rotation activates dorsal vegetal cells (the Nieuwkoop center). A first mesoderm-inducing signal is sent by these activated cells to overlying dorsal cells corresponding to the Spemann's organizer. A second signal is released from the ventral and lateral vegetal poles to the marginal zone to create ventral-type mesoderm (i.e. blood cells). Finally, the newly activated Spemann's organizer gives rise to the notochord and head mesoderm (thus establishing antero-posterior polarity) while a third signal originating from the Spemann's organizer, acts within the prospective mesoderm germ layer to convert it i.e. dorsalize adjacent ventral mesoderm cells to intermediate mesoderm cell types such as muscle (reviewed in: Smith, 1989; Lemaire, 1992; Moon and Christian, 1992; Kimelman et al., 1992). Thus, The function of the Spemann's organizer includes the patterning of lateral mesoderm, initiation of the morphogenetic movements of gastrulation and the induction of neural tissue from overlying ectodermal cells via the notochord.

1.3 Mesoderm inducing factors:

Discovering the nature of the signals involved in mesoderm determination i.e. "mesoderm inducing factors" (MIFs), has been the subject of intense studies. In the Xenopus embryo, the first signal(s) triggering mesoderm is most likely maternally encoded since mesoderm induction starts shortly before midblastula transition (MBT). MBT is the time at the end of blastula stage when, in the Xenopus embryo, zygotic

Fig. 1A **Three-signal model for early development of Xenopus embryos.**

Postfertilization cortical rotation activates vegetal (*V*) cells on the dorsal side, giving rise to the Nieuwkoop center (NC). A signal is released from the vegetal (*V*) cells on the ventral side to convert overlying animal (*A*) cells into ventral mesoderm (VM). A second signal is sent by the NC to produce the Spemann's organizer (SO). Finally, a third signal is sent by the SO which converts the ventral mesoderm into different types of lateral mesoderm (LM). The SO itself gives rise to dorsal mesoderm (DM). The ventral side is on the left of the diagram whereas the dorsal side is on the right.


A
V
Cortical
Rotation
A
V
NC
signals 1 and 2
A
VM
V
SO
signal 3
A
VM
LM
DM
V
final pattern

transcription is first seen. In the Xenopus embryo, several peptide growth factors including transforming growth factor β (TGFβ), fibroblast growth factor (FGF), Wingless/int-1 (Wnt) and noggin have been shown to mimic different aspects of mesoderm induction. Thus, they are believed to be good candidates for mesoderm formation. In mammals, some of these factors are also expressed during embryonic development but their role has not been ascertained. In mice, zygotic transcription starts after the second cleavage.

1.3.1 Assays of mesoderm induction:

The mesoderm-inducing capacity of these factors has been tested first *in vitro* on cells derived from the animal portion of the early Xenopus blastula. As mentioned before, when cultured in isolation these cells form epidermis; however, when placed in contact with MIFs they differentiate into various mesodermal cells.

In this assay, basic FGF (bFGF) induces ventral mesodermal tissues and at higher concentrations dorsal mesoderm except the notochord (Slack et al., 1987). Within the TGFβ family, activin A and B have the most potent mesoderm-inducing activity on isolated Xenopus animal cells; they induce anterior mesoderm including the notochord, muscle, neuronal tissues and eyes. The neuronal tissues and eyes, however are probably the result of a secondary induction (Thomsen et al., 1990; Sokol and Melton, 1991). Bone morphogenic proteins (BMP) 2 to 7 are also members of the TGFβ family. BMP-4 when added to isolated Xenopus animal cells, preferentially induces ventral mesoderm and its action is dominant over that of activin when used jointly (Jones et al., 1992).

1.3.2 Ectopic expression of MIFs:

Overexpression of activin in the ventral vegetal side of a Xenopus embryo leads to incomplete secondary axis that lacks anterior structures. Also, activin fails to fully restore ventralized embryos (Smith and Harland, 1991). Thus, activin is not able to fully account for dorsal mesoderm formation *in vivo*.

It has been shown that ectopic expression of BMP-4 in Xenopus embryos causes ventralization of the embryos. These embryos have excess ventral mesoderm and completely lack dorsal structures including dorsal mesoderm and a neural tube. Gastrulating movements are also inhibited (Dale et al., 1992).

Wnt genes encode secreted glycoproteins believed to be mediators of cell-cell signalling events during pattern formation (reviewed by: Nusse and Varmus, 1992). Injection of Wnt-1 or Wnt-8 mRNA into one cell stage Xenopus embryos leads to duplicated anterior structures (McMahon and Moon, 1989). Injection of these Wnt mRNAs into vegetal cells triggers a complete second axis and injection in vegetal cells of ventralized embryos restores dorso-anterior development. In these assays, if Wnt mRNAs are injected into marginal blastomeres, these Wnt-injected blastomeres act as a Spemann organizer. On the other hand, if injected in ventral blastomeres, the Wnt-injected blastomeres act as a Nieuwkoop center which in turn induces a second Spemann organizer (Sokol et al., 1991; Smith and Harland, 1991).

1.3.3 Endogenous mesoderm inducers:

Acidic FGF (aFGF) and bFGF are believed to be involved in mesoderm induction

in vivo since their expression has been detected in the amphibian egg and early embryo at the appropriate time and localization to induce mesoderm (Kimelman et al., 1988; Slack and Isaacs, 1989). Moreover, Xenopus embryos expressing a dominant negative FGF receptor develop abnormal trunk and tail mesoderm, indicating that FGF transmits a signal required for the development of posterior mesoderm (Amaya et al., 1991).

In mouse, activin B is first found in the blastocyst while activin A is expressed at the gastrula stage (van den Eijnden et al., 1992). The Xenopus embryo shows a similar pattern of expression since activin B first appears at the MBT while activin A is expressed at the late gastrula stage (Thomsen et al., 1990). Moreover, maternally-derived activin-like activity was found in early Xenopus embryos (Asashima et al., 1991). Activin signalling has been shown to be necessary for mesoderm induction *in vivo*; disruption of activin signalling by a dominant negative activin receptor in the Xenopus embryo prevents formation of the notochord and muscle. Gastrulation does not proceed and the dorsal axis is not formed. In addition, as the FGF pathway is not disrupted in these embryos, the endogenous FGF would be expected to induce ventral or posterior mesoderm. In fact, many of these embryos do not contain any type of mesoderm indicating that during normal development, FGF may need to collaborate with activin to induce mesoderm in the marginal zone (Hemmati-Brivanlou and Melton, 1992).

BMPs are highly homologous to the decapentaplegic (dpp) gene of Drosophila which is involved in dorso-ventral body patterning and imaginal disk formation. BMPs are also homologous to Vg1, a maternal mRNA localized in the presumptive endoderm

of the Xenopus embryo before gastrulation. BMP-4 mRNA in the Xenopus embryo is derived from a maternal pool and is first expressed by the zygote at the onset of gastrulation (Koster et al., 1991). In mammals, BMP-2, -4 and -6 have been localized in the developing embryo (reviewed by Rosen and Thies, 1992). For example, BMP-4 is expressed by 8.5 days postcoitum in posterior and ventral mesoderm and endoderm tissues of the primitive streak (Jones at al., 1991). It has been postulated that BMP-4 is not involved in the initial determination of mesoderm but rather in specifying posterior and ventral polarity of mesoderm tissues during gastrulation (Jones et al., 1991). BMP may serve as a cell competence modifier in gastrula and as such establish proper antero-posterior patterning. Also, BMP may induce and/or maintain tissues in the state of ventroposterior differentiation.

In the mouse, the Wnt genes are believed to be involved in regulating major development events since they have a plethoric pattern of expression throughout embryonic development (reviewed by Nusse and Varmus, 1992). In the Xenopus embryo none of the Wnt genes is expressed at the right time or at right place to be the endogenous inducer. Wnt may also act as a mesoderm modifier by cooperating with other MIFs. In support of this hypothesis, before MBT, ectopically expressed Wnt dorsalizes the response of isolated Xenopus animal cells to bFGF whereas after MBT it ventralizes the differentiation induced by activin (Christian et al., 1992). Thus, during gastrulation Wnt is believed to modify cell competence so that MIFs induction would be synergized or antagonized.

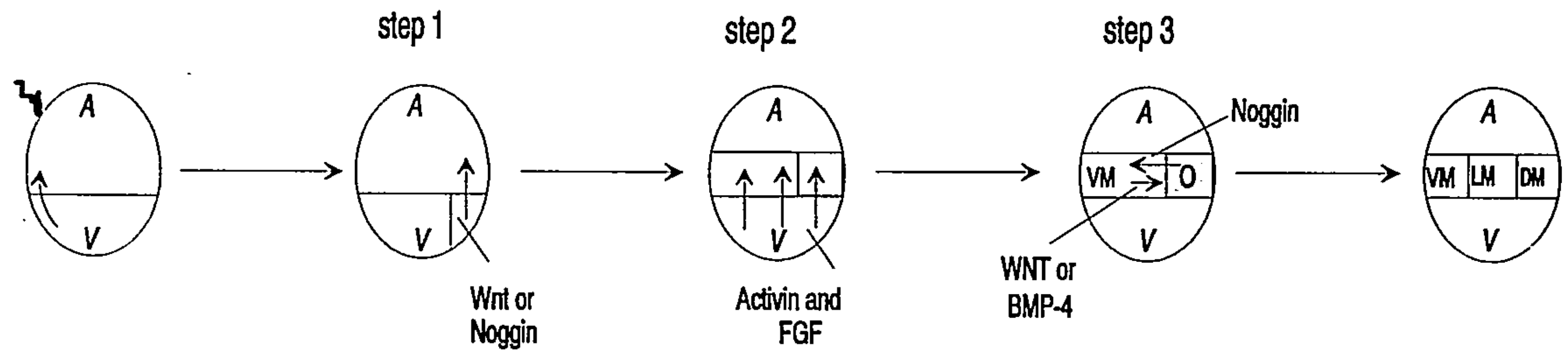
Noggin encodes a novel polypeptide identified in the Xenopus embryo. It is capable of inducing dorsal development when injected in Xenopus embryos. It has an activity similar to Wnt-8 in promoting formation of a vegetal dorsalizing center (Nieuwkoop's center). However, unlike Wnt, noggin expression is consistent with this function since it is found both maternally and in the dorsal vegetal cells of the blastula. Moreover, noggin may also act later since it is expressed in the dorsal lip during gastrulation and later in the notochord (Smith and Harland, 1992).

1.3.4 A model of mesoderm induction:

In light of what has been presented above, mesoderm induction appears to be more complicated than first anticipated in the "three signal model". The process in fact involves not only MIFs but also competence modifiers acting in concert. Taking into consideration these observations, one revised model of mesoderm induction in Xenopus has been proposed by Christian and Moon (Kimelman et al., 1992; Moon and Christian, 1992) (fig. 1B). In the first step, postfertilization cortical rotation activates a maternal Wnt or noggin like-signal in vegetal cells of the prospective dorsal side. This signal is sent to overlying marginal cells to prepattern their competence such that in response to MIFs, they are biased to form dorsal mesoderm. In the second step, occurring before MBT, maternal FGF and activin are released from vegetal cells and broadly distributed in overlying marginal cells. Consequently, ventral mesoderm is induced in the marginal zone of the prospective ventral side; whereas, dorsal mesoderm is induced in the zone of overlap between Wnt/noggin-induced cells and these MIFs i.e. on the prospective

Fig.1B **Mesoderm induction as proposed by Christian and Moon.**

In a first step, postfertilization cortical rotation activates WNT or noggin in vegetal (*V*) cells. In a second step, FGF and activin are released from vegetal cells and broadly distributed in overlying marginal cells. In a third step, the gastrula organizer (O) patterns further adjacent cells. (*A*), animal pole; VM, ventral mesoderm; LM, lateral mesoderm; DM, dorsal mesoderm.


A
V
step 1
A
V
Wnt or
Noggin
step 2
A
V
Activin and
FGF
step 3
A
VM
O
V
Noggin
WNT or
BMP-4
A
VM
LM
DM
V

dorsal side. The newly induced dorsal mesoderm gives rise to the gastrula organizer. In a third step starting post-MBT, the gastrula organizer generates the notochord and patterns further adjacent ventral mesodermal cells to more dorsal-type mesoderm; endogenous noggin may act as the dorsalizing factor while Wnt and/or BMP-4 may counteract this dorsalizing signal in cells outside the organizer field.

1.4 **The Brachyury T gene:**

The Brachyury T gene has recently been identified as an important gene in mesoderm specification. The murine Brachyury T gene is localized in the t-complex which is a large region of chromosome 17 that includes several unrelated genes. The t-complex was identified via a wide array of mutant forms of that complex called t haplotypes. They are naturally occurring dominant and recessive mutations some of which have profound effects on embryonic development, sperm production and function, and genetic recombination (reviewed by Bennett, 1975; Silver, 1981; Silver, 1985).

In this thesis: + means wild type allele, t refers to mice bearing a t-haplotype i.e bearing a mutant allele of the t-complex, and T is a t-haplotype where the t-complex is mutated in the Brachyury T gene.

t haplotypes were first detected in mice bearing a dominant mutation in the Brachyury T gene (Dobrovolskaia-Zavadskaia, 1927). Indeed, heterozygous T/+ animals have short tails due to haplo-insufficiency, while T/t animals are tailless due to an interaction between the T allele and the tct (t-complex-tail) factor of the t haplotype (tct

is most likely an allele of T with less than normal activity) (Dobrovolskaia-Zavadskaia and Kobozieff, 1932; Justice and Bode, 1988). Heterozygous +/t animals on the other hand, have normal tails and are not visibly distinguishable from wild type (+/+) animals (reviewed by Silver, 1985; Willison, 1990).

Interest in the Brachyury T gene itself came from the fact that loss of function of the Brachyury T alleles results in a recessive embryonic lethal phenotype. In homozygous (T/T) embryos insufficient mesoderm is generated, whereas the number of ectodermal cells is increased. The primitive streak is disturbed and the posterior region of the embryo is entirely missing probably as a result of a failure of primitive streak regression. The most affected part of the mesoderm in these embryos is the notochord; the notochordal plate forms initially but it degenerates and no notochord is established. In the absence of a notochord, the somites and neural tube are abnormal. Finally, the T/T embryos fail to form the allantois, a derivative of the mesoderm, and die at 10 days of gestation (reviewed in Herrmann et al., 1990).

Brachyury T gene is highly conserved among vertebrates. It encodes a 2.1 kilobase transcript and the predicted gene product is not related to any known protein. It does not contain a signal peptide or a membrane spanning region suggesting that it has an intracellular or possibly nuclear localization (Herrmann et al., 1990).

Brachyury T gene expression is first detected in the mouse in early gastrulation stage embryos and subsequently becomes restricted in the notochord. No expression can be found after 17.5 day postcoitum or in any adult tissues. At 7 days of development T-

transcripts are first found next to the primitive streak in ectodermal cells destined to form mesoderm as well as in mesodermal cells next to the primitive streak but not in more lateral mesoderm. Between 8.5 and 9.5 days, the expression is found again in early mesoderm and primitive ectoderm near the primitive streak; the expression persists in the notochordal plate of 8.5-day embryos and in the definitive notochord of 9.5-day embryos but the expression is down-regulated in mesoderm destined to form the somites and is absent in the allantois. After gastrulation, Brachyury T mRNA is found only in the notochord, around which cartilaginous cells condense and differentiate to form the vertebral column. The notochord degenerates and forms mucosal cells in the vertebral discs such that Brachyury T transcripts are found in 17.5-day embryos along the length of the spinal column. This suggests that Brachuyry T plays a direct role in the formation of the mesoderm and morphogenesis of the notochord since the tissues expressing Brachyury T gene are those absent in T/T homozygote embryos. The defects in the somites and the neural tube also seen in the T-mutants are probably a reflection of the inductive interaction of the notochord on these tissues (Wilkinson et al., 1990).

The Xenopus homolog of the murine Brachyury T gene has been cloned and named Xbra (Smith et al., 1991). Interestingly, the expression pattern of Brachyury during Xenopus development is very similar to that of the mouse, supporting the possibility that mesoderm formation occurs through similar mechanisms. At the early gastrula stage, Xbra transcripts are present in presumptive mesodermal cells in the marginal zone. The expression is highest around the blastopore lip and transcript levels

decline around the end of gastrulation but persists in the notochord (Smith et al., 1991).

The expression of Xbra is activated when cells of the animal pole from Xenopus embryos are exposed to vegetal pole cells. Moreover, activin and bFGF induce the expression of Xbra from isolated animal cells and this induced expression appears to be an immediate-early response (i.e. not depending on protein synthesis) suggesting that Brachyury gene is a direct target of mesoderm induction (Smith et al., 1991).

A better understanding of the role of Brachyury T in mesoderm formation came from the following observation: injection of Xbra mRNA in the animal pole of one-cell stage Xenopus embryos caused the ectopic formation of mesoderm of posterior character, whereas these embryos lacked dorso-anterior mesoderm (Cunliffe and Smith, 1992). Thus, Brachyury T appears to be involved in the specification of mesoderm of ventro-posterior character.

Brachyury T is believed to accomplish two functions: act cell autonomously to establish the notochord and play a critical role upstream of a signalling cascade to specify mesodermal cells (such as the allantois) which themselves do not express Brachyury T (Rashbass et al., 1991). It has been shown that T/T $\leftrightarrow$ +/+ chimeras mimic the T/T phenotype such that mesodermal cells specified by Brachyury T (ex. the allantois) could not be rescued by the normal cells. This indicates that the signalling involving Brachyury T could not be rescued by the normal cells and thus important threshold effects operate (Rashbass et al., 1991).

Very little is known about the signal transduction pathway leading to Brachyury

T expression. It has been shown that a dominant inhibitory ras mutant blocks the mesoderm-inducing activity of FGF and activin as well as the endogenous inducing activity of prospective endoderm cells, suggesting that $p21^{ras}$ may play a central role in the transduction of MIFs signalling (Whitman and Melton, 1992).

1.5 Embryonal carcinoma cells as a model system for the study of embryogenesis.

The stem cells of teratocarcinoma, which show remarkable similarities to the cells of the early embryo, can be isolated and grown in culture (Kahan and Ephrussi, 1970; Martin and Evens, 1974; Jakob et al., 1973). These established embryonal carcinoma cell lines provide a model to study mammalian embryonic processes of cellular determination and differentiation since they circumvent many of the difficulties of working with embryonic material.

1.5.1 Teratocarcinoma:

Mouse teratocarcinoma are malignant tumours composed of stem cells and a wide array of disorganized differentiated cells (Stevens and Hummel, 1957). The pluripotent stem cells proliferate in the undifferentiated state and are responsible for the malignant properties of teratocarcinomas such as progressive growth and transplantability (Martin, 1980). Also, the stem cells can produce derivatives of all three primary germ layers; consequently, these stem cells were named embryonal carcinoma (EC) cells (Martin, 1980). Some cells undergo differentiation in the tumours giving rise to the random array of differentiated tissues. These differentiated tissues are non-tumorigenic (Pierce, 1967).

In cases where all the stem cells cease to proliferate because they differentiate or die, the tumours become benign and are known as teratomas (Martin, 1980).

Teratocarcinoma can arise spontaneously from primordial germ cells in testis or from the disorganized growth of eggs in ovaries (the latter are rarely malignant) (Stevens, 1962; Stevens and Varnum, 1974). In a similar fashion, they can be experimentally produced by grafting genital ridges (primordial gonads) from 12 day old mouse embryos into the testis of an adult mouse (Stevens, 1964; Stevens, 1966; Stevens, 1970b) or by transplanting 1-7 day old mouse embryos to extra-uterine sites (in testis or kidney for example) (Stevens, 1968; Stevens, 1970a; Solter et al., 1970).

Differentiated teratoma cells, when transplanted subcutaneously, do not produce tumours; however, a single EC cell can give rise to a teratocarcinoma containing a wide variety of differentiated tissues (Kleinsmith and Pierce, 1964). The pluripotency as well as morphological and biochemical characteristics of EC cells are indications of their fundamental similarity to normal early embryonic cells. For example, EC cells can participate in the formation of a normal embryo. When EC cells are injected into recipient blastocysts and these blastocysts are placed into foster mothers, the resulting embryos are tumour-free and chimeric, in that some tissues are derived from the EC cells as distinguished by genetic markers (Brinster, 1974; Mintz and Illmensee, 1975). Hence, EC cells have the capacity to respond to developmental signals and to behave like normal embryonic cells; in this regard they are believed to be resemble cells of the inner cell mass (ICM) (Martin, 1975).

1.5.2 Isolation and *in vitro* differentiation of embryonal carcinoma cell lines:

Clonal lines of EC cells from teratocarcinoma can be isolated and grown in culture. EC cell lines have stable and near euploid karyotypes. They generally retain their pluripotency when kept in exponential growth phase in culture (Martin, 1975).

EC cell lines can be induced to differentiate *in vitro* by exposing the cells to drugs and/or by changing the culture conditions. The mode of induction chosen depends on the inherent predisposition of the EC cell line to differentiate (Rudnicki and McBurney, 1987).

Spontaneous differentiation can be achieved by culturing the cells at low density or at high density. High density can be achieved by maintaining the cells at confluence or by allowing the cells to aggregate into spheres of cells that are not able to adhere to the culture dish. Under these conditions, EC cells form an extra-embryonic endoderm like-layer that surrounds the undifferentiated EC cells. If the aggregates are subsequently plated onto an adherent surface, they form many cell types such as epithelium, cartilage, neurons, and muscle (Martin, 1980).

Spontaneous differentiation occurs asynchronously and the cell types which develop are highly heterogenous. Some of these problems can be avoided using drugs to trigger differentiation. EC cells can be induced to differentiate using either retinoic acid and its derivatives or bipolar compounds such as dimethyl sulfoxide (DMSO) and hexamethylene bis-acetamide (HMBA); different cell lines will respond differently to the drugs (Rudnicki and McBurney, 1987).

1.5.3 The P19 embryonal carcinoma cell line:

The P19 embryonal carcinoma cell line was derived from a primary teratocarcinoma obtained by grafting a 7.5 day C3H/He strain mouse embryo into the testis of an adult mouse (McBurney and Rogers, 1982). P19 cells have a normal karyotype and participate in the formation of a large variety of cell types when injected into blastocysts (Rossant and McBurney, 1982).

1.5.4 P19 cell differentiation into neurons:

Aggregation of P19 cells in the absence of drugs triggers little differentiation. Some visceral endoderm cells form on the periphery of the aggregates (Smith et al., 1987). When monolayer cultures of P19 cells are treated 48 h with RA, virtually all the cells differentiate into fibroblast-like cells (Jones-Villeneuve et al., 1982). However, when P19 cells are treated with concentrations of RA over $1X10^{-7}$ M while aggregated, they differentiate into ectodermal cell types including neurons, glia and fibroblast-like cells (Jones-Villeneuve et al., 1982). The neurons in these cultures are small post-mitotic cells with long branching processes that become abundant in culture by 5-6 days after RA treatment. These cells contain neurofilament proteins and tetanus toxin binding sites (Jones-Villeneuve et al., 1982). The glia cells contain glial fibrillar acidic protein and appear around day 9-10 (Jones-Villeneuve et al., 1983). The neurons possess choline acetyl transferase activity and synthesize and store acetyl choline. They also have high-affinity uptake sites for γ-amino butyric acid (GABA) and synthesize cathecolamines. The neurons eventually mature and neuronal processes differentiate into axons and

dendrites which form synaptic junctions, closely resembling embryonic brain tissue (McBurney et al., 1988).

1.5.5 P19 cell differentiation into muscle:

When aggregated in the presence of 0.5-1% DMSO for at least 4 days, P19 cells differentiate into striated muscle (cardiac and skeletal muscle) and smooth muscle cells (McBurney et al., 1982; Rudnicki et al., 1990a). The cardiac muscle cells often exhibit spontaneous contractile activity and develop within 5 to 6 days following treatment. These cells possess properties of embryonic cardiac muscle cells. They are mononucleated and contain myofibrils characterized by occasional Z-bands and a hexagonal arrangement of thick and thin filaments. In addition, intercalated disks form between adjacent cells (Edwards et al., 1983; Smith et al., 1987). These cells express cardiac-specific isoforms of myosin heavy and light chains as well as actin (Rudnicki et al., 1990b). Skeletal muscle cells on the other hand, appear on day 7 to 8 (Edwards et al., 1983). They are bipolar multinucleated cells expressing striated-specific myosin isoform heavy and light chains and skeletal-specific actin isoforms (McBurney et al., 1982; Rudnicki et al., 1990b). The smooth muscle cells appear within 2-3 days after exposure to DMSO and have been identified based on their expression of cytokeratins and smooth muscle-specific actin isoforms. This indicates that they may resemble fetal myofibroblast or myoepithelial cells (Rudnicki et al., 1990a).

1.5.6 Mechanism of P19 cell differentiation:

The mechanism of differentiation remains unclear; however, observations made

on aggregation of the cells and on the drugs used in the assays are informative. Retinoids are naturally occurring compounds believed to play an active role in pattern formation during development. They are believed to act by interacting with specific nuclear receptor proteins (RAR) to modify gene expression (Oro et al., 1989). DMSO on the other hand, is an organic compound not synthesized by eukaryotes.

The concentrations of the drugs used affect the nature of the cell types which develop; 0.5%, 1% and 2% DMSO are required for induction into predominantly cardiac, skeletal and neuronal cells respectively (Edwards et al., 1983a). Treatment with RA concentrations in the order of 10^{-9}, 10^{-8} and 10^{-7} M are required to obtain the equivalent cell types (Edwards and McBurney, 1983). In addition, aggregation of the cells affects the nature of differentiation; RA-treated cell monolayers differentiate into fibroblast-like cells (Jones-Villeneuve et al., 1982) whereas DMSO has no effect on cell monolayers (McBurney et al., 1982). Thus, both aggregation and exposure to drugs are mandatory to trigger differentiation into muscle and neurons.

Aggregation has been postulated to achieve high cell density whereby cell-cell interactions and cell cooperation would occur (Campione-Piccardo et al., 1985). Since DMSO has an effect on differentiation only when cells are aggregated, DMSO may induce differentiation by enhancing the cooperative response of adjacent cells within an aggregate. In this regard, it has been shown that an extra-embryonic endoderm cell layer must develop in the periphery of each aggregate in order to achieve muscle development. DMSO may be causally involved in the formation of this epithelial cell layer since within

3-4 days of DMSO exposure, this endoderm layer forms. Subsequently 2-3 days later, muscle develops within the aggregate interior (Stevens et al., 1987). Thus, the temporal and spatial relationship between the epithelial and muscle cells suggests that the former cell type may be involved in the formation of the latter.

1.5.7 Mutant P19 clonal cell lines:

D3 and RAC65 cells are cloned mutant lines of P19 cells. In the attempt to elucidate the intracellular mechanism of DMSO action, D3 cells were isolated based on the fact that they do not differentiate when aggregated in the presence of that drug. Upon treatment with DMSO or low concentrations of RA, they remain morphologically undifferentiated, retain expression of the EC-specific antigen SSEA-1 and do not develop beating muscle or skeletal muscle. However, D3 cells differentiate normally into neurons when exposed to high RA concentrations (Edwards et al., 1983). RAC65 cells do not respond to RA but differentiate into muscle when exposed to DMSO (Jones-Villeneuve et al., 1983). The mutation in D3 cells remains unknown. RAC65 cells appear to bear a mutation in the RAR-α (Pratt et al., 1990).

1.6 Area of investigation:

The Brachyury T gene has been shown to be essential for mesoderm formation. The mechanism controlling Brachyury T expression as well as the mechanism whereby Brachyury T exerts its effect remain unknown. The P19 embryonal carcinoma cell line, because it can be induced to differentiate into mesodermal derivatives, provides an

excellent model to achieve a better understanding of the regulation and the function of the Brachyury T gene during mesoderm formation.

The goal of this study was to establish whether Brachyury T plays a role in P19 cell mesoderm differentiation and to investigate its regulated expression. The expression of Brachyury T during P19 cell differentiation was analyzed and was found to be restricted to cells destined for mesodermal lineages. Interestingly, aggregation of the cells was sufficient to trigger Brachyury T expression. Efforts were made towards identifying peptide growth factors and intracellular transduction pathways leading to Brachyury T expression. Activin, BMPs and cAMP activated Brachyury T expression but this induction was not sufficient to induce P19 cell differentiation. In order to gain information on the role of Brachyury T, ectopic expression of that gene was performed in D3 and P19 cells. D3 cells could not be induced to differentiate but ectopic expression of Brachyury T in P19 cells induced spontaneous differentiation into striated muscle and neuroectodermal tissues according to the level of Brachyury T expressed.

Chapter Two

Material and Methods

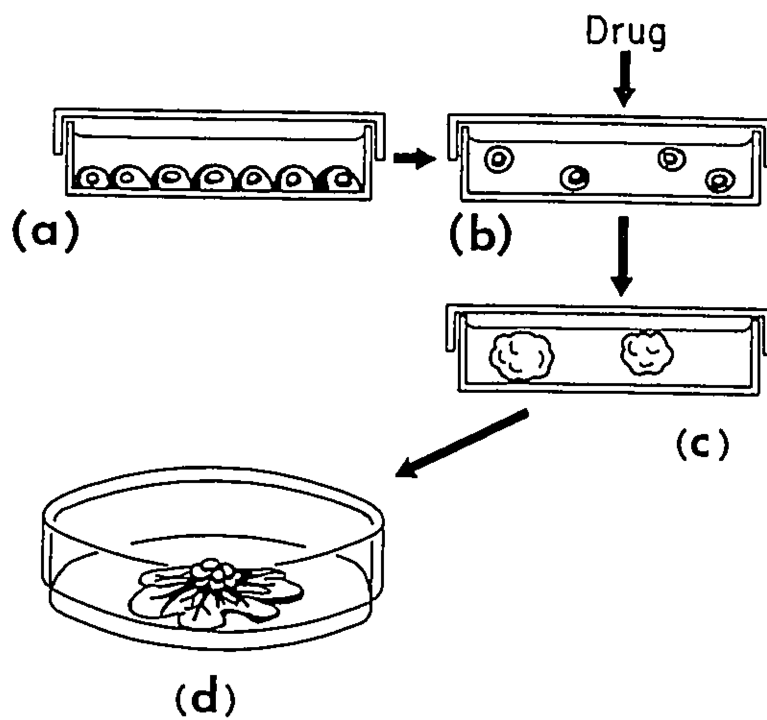
2.1 Cell culture and differentiation:

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 h 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, each cell line was cultured for 4 days in petri-grade dishes. Since the cell do not adhere to the plastic of these dishes, they remain in suspension and aggregate to form spheres of cells. 0.8% dimethyl sulfoxide (DMSO) was added to the culture for the 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) (figure 1C). 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).

Fig.1C **Formation of aggregates in suspension cultures.**

Differentiation is facilitated by the formation of aggregates. (a) EC cells are dispersed then transferred into bacteriological grade petri dishes. (b) The cells are unable to attach to the surface of the petri dish and aggregate with each other. (c) Chemicals such as DMSO, when added to aggregates, induce the differentiation of cells within the aggregate. (d) By transferring the aggregates to tissue culture dishes the product of differentiation can be more easily viewed because the cells migrate onto the plastic surface. (taken from Rudnicki and McBurney, 1987).



Drug
(a)
(b)
(c)
(d)

2.2 **DNA transfection:**

P19 and D3 cell lines were transfected with plasmid DNA by the calcium phosphate DNA precipitation method (Chen et al. 1987). The cells were co-transfected with plasmids carrying the Brachyury cDNA and hygromycin B resistance gene both under the control of the mouse phophoglycerate kinase (Pgk-1) promoter (Adra et al., 1987, McBurney et al., 1991). Cultures of 10^6 cells were exposed for 8 h to 14 μg of plasmid DNA precipitated at room temperature for 10 min in 0.25 M $CaCl_2$ and 2X BES-buffered saline (BBS). Permanently transformed clones were established: the cells were cultured for 48 h before seeding at $4X10^5$ cells per 100 mm dish in the presence of 0.4 mg/ml of Hygromycin B and 0.1 mM β-mercaptoethanol (BME). This medium was changed every 2 days. After 14 days of selection, the hygromycin B resistant-colonies were individually picked and expanded into cell lines in the presence of 0.2 mg/ml of hygromycin B.

2.3 **Growth in low calcium media:**

Calf serum was dialysed for 36 h at 4°C against phosphate buffered saline (PBS) using a dialysis membrane having a molecular weight cutoff of 6000-8000 and then filter-sterilyzed using a Nalgene 0.2 μ filtering device. Cells were aggregated for 2 days in Joklik's medium (Gibco, Burlington, Ontario) supplemented with 10% dialysed calf serum and calcium chloride ranging from 0 to 4.5 mM.

2.4 Antibodies and immunofluorescence:

Immunofluorescence experiments were performed as described elsewhere (Rudnicki and McBurney, 1987). Briefly cells grown on coverslips were quickly washed 3 times with PBS, fixed by immersion in methanol at -20°C for 5 min, air-dried for 5 min and rehydrated in PBS for 20 min at room temperature (RT). Muscle myosin proteins were detected by staining the fixed cells at RT for 1 h with the mouse monoclonal antibody, MF20 (Bader et al., 1982). The cells were then washed 3 times for 5 min with PBS and incubated at RT for 1 h with a flurescein isothiocyanate (FITC) conjugated goat anti-mouse IgG secondary antibody (Tago, Markham, Ontario) diluted in PBS in a 1:50 ratio. Following 3 washes of 5 min in PBS, the coverslips were mounted inverted in 50% glycerol containing 0.1% paraphenylene diamine buffered in PBS. Stained cells were examined with a Leitz microscope equipped with epifluorescent optics.

For staining with the monoclonal mouse antibody NF160 (Boehringer Mannheim, Laval, Québec), the cells were washed 3 times for 5 min in PBS and fixed for 40 min at RT in LANA'S fixative (3% depolymerized paraformaldehyde, 0.16 M dibasic sodium phosphate pH 7.1, 14% saturated picric acid) and washed 3 times for 5 min in PBS. The cells were incubated 1 h at RT with NF160 antibody diluted in PBS in a 1:4 ratio. The cells were then washed 3 times for 5 min and stained with the secondary antibody and mounted as described above.

To detect the cell surface SSEA-1 antigen, live staining of the cells grown on

coverslips was performed. The cells were washed 3 times 5 min in serum free medium, incubated at 4°C 1 h with the mouse monoclonal antibody AEC3A1-C3 (Harris et al., 1984). The cells were washed again 3 times 5 min in serum free medium and stained with the secondary antibody as described above to the exception that the incubation was done at 4°C. The cells were washed 3 times 5 min in serum free medium and fixed in 4% formaldehyde in PBS for 20 min at RT. The cells were washed 3 times 5 min and mounted as described above.

2.5 Plasmid isolation and construction:

All the plasmids used were propagated in *Escherichia coli* strain DH5α and were isolated by the sodium dodecyl sulfate (SDS) lysis method (Birnboim, 1983; Marko et al., 1982).

To construct Brachyury T expression vectors (pGV-1 and pGV-2), *SmaI*-digested pKJ1 F was ligated to the 1.8 kilobase *EcoRI* Brachyury T fragment derived from pBluescript (SK) (Hermann et al., 1990). This fragment had been previously purified by extraction from melted agarose gel fragments using a GENECLEAN kit (Bio 101, Lajolla, California). The Brachyury T fragment encompasses the complete coding region but lacks 108 base pairs (bp) from its 5' untranslated region (UTR) and 300 bp from its 3' UTR. The orientation of Brachyury T cDNA in the resulting plasmids was determined by restriction enzyme mapping.

2.6 RNA isolation and Northern blot analysis

Total RNA was prepared from cells by the lithium/urea method of Auffray and Rougeon (Auffray et al., 1980). Poly A^+ selection was carried out by passage of total RNA over oligo-d(T) cellulose, following the method of Jacobson (Jacobson, 1987). Aliquots of 15 μg of total RNA or 10 μg of poly A^+ RNA were ethanol precipitated and resuspended into 20 μl sample buffer (50% formamide, 10% formaldehyde and 20 mM 3-[N-Morpholino]propane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). After heating at 65°C for 10 min and cooling on ice for 5 min, the RNA samples were loaded on a gel with 1/10 volume of loading buffer (0.1% xylene cyanol, 30% glycerol, 0.05 mg/ml ethidium bromide) and electrophoresed at 60 volts for 6 h in 0.9% agarose gels containing 1X RNA gel buffer (20 mM 3-[N-Morpholino]propane-sulfonic acid, 1mM EDTA, 5mM sodium acetate, pH 7.0) and 10% formaldehyde. The electrophoretically separated RNAs were transferred by capillary blotting for 16 h to Hybond-N membranes (Amersham, Oakville, Ontario), the membranes were then treated with ultraviolet light at 120 mJ using a GS Gene linker uv chamber (Biorad, Mississauga, Ontario). Prehybridizations were carried out for 16 to 24 h, at 42°C in 50% formamide, 5X Denhardt's solution, 5X SSPE, 1% SDS and 250 μg/ml denatured herring sperm DNA. Hybridizations were done using a random-primed ^{32}P-labelled DNA insert at $5X10^6$ cpm/ml at 42°C for 16 to 24 h. The ^{32}P-labellings were done using Dupont's multiprime labelling kit (NEN, Boston, Mass.). Filters were washed in several washes of 2X SSC and 0.1% SDS at RT for 30 min, followed by several changes of 0.2X SSC

and 0.1% SDS at 65°C for 45 min. Finally, hybridizations were visualized by autoradiography, by exposing the filters to Kodak XAR-5 films. To re-hybridize filters, incubation in 100°C water containing 0.1% SDS, 30 min 2 times was performed to remove the previous probe. Prehibridization and hybridization would then follow as described above.

2.7 **DNA probes:**

The Brachyury T DNA probe was a *BamHI-SalI* 1.8 kb cDNA fragment excised from *BamHI-SalI* sites of pBluescript (SK) (Hermann et al., 1990). The actin probe was a 0.6 kb *PstI* fragment containing the last exon of the human cardiac actin gene (Rudnicki et al 1988). The α-tubulin probe was used to control RNA loading and was a full-length mouse cDNA (Lemishka et al 1981). NF68 probe was a 1.6 kb *EcoRI* mouse cDNA fragment (Liesi et al., 1986). Oct-3 was a 400 bp *AvaI* mouse cDNA fragment (Okamoto et al., 1990). Id-1 probe was a mouse full length cDNA (Benezra et al., 1990). The PKA subunits DNA probes were the following: Cα was from the 3' end a 0.5 kb *EcoRI-HindIII* mouse fragment (Uhler et al., 1986). Cβ was a *EcoRI-HindIII* 395 bp mouse fragment from the 5' end including 195 bp of non-coding region (Showers and Maurer, 1986). RIα was a 1516 bp *EcoRI-SmaI* mouse fragment (Lee et al., 1988). RIIα was a *SalI-HindIII* 240 bp cDNA mouse fragment (Scott et al., 1987) and RIIβ was a *EcoRI-HindIII* 1198 bp rat cDNA fragment including 300 bp of non-coding sequence (Jahnsen et al., 1986). CREB probe was a 1040 bp *EcoRI* cDNA fragment from Human

hypothalamic killer CREB (Yamamoto et al., 1988). c-kit probe was a *PvuII* 928 bp mouse cDNA fragment (Yarden et al., 1987).

2.8 **Drugs:**

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 at in the Xenopus animal cap assay 1:200 dilution and was used in our assays at 1:200 in culture medium.

BMP-2, 3 and 5 were respectively obtained at 7.5 μM, 1.1 μM and 8.3 μM at 90% purity; whereas, BMP-4 was 75% pure and its molarity was 7.3 μM. In the 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 plastic ware. Siliconization was done following the procedure described by Maniatis (Maniatis, 1982). Leukemia inhibitory factor (LIF) was obtained at 10^6 Units/ml with a specific activity of 10^5 Units/μg from GIBCO BRL (Burlington, Ontario) whereas 3-isobutyl-1-methylxanthine (IBMX) and forskolin were purshased from Sigma (St-Louis, Missouri).

Chapter Three

Results

3.1 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). Since the Brachyury gene is known to be necessary for mesoderm development in the mouse, we investigated the expression of this gene in differentiating P19 cells by Northern blot analysis. As shown in fig. 2A, Brachyury mRNA was detected at low levels in undifferentiated P19 cells and dramatically increased in abundance early during differentiation induction peaking at day 2 before declining to undetectable levels by day 5. On day 5, cardiac muscle first appeared as indicated by the presence of the sarcomeric isoform of actin found in striated muscle (fig. 2b) (Rudnicki et al., 1990). As shown in lane 8 of fig. 2, Brachyury expression was triggered in P19 cells aggregated for two days in the absence of DMSO. Further discussion of this data will be presented in section 3.3.

Retinoic acid (RA) like DMSO is an effective inducer of P19 cell differentiation. P19 cells can differentiate into different spectra of cell types depending on the concentration of RA to which they are exposed; low levels of RA (from 10 to 100 nM) induce development of mesodermal cell types, whereas high concentrations

Fig. 2 **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 (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with 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 sizes of the transcripts were: Brachyury T, 2.1 kb; actin probe hybridizes to both cytoskeletal (β and γ) at 2.1 kb and striated muscle (α) actins at 1.7 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

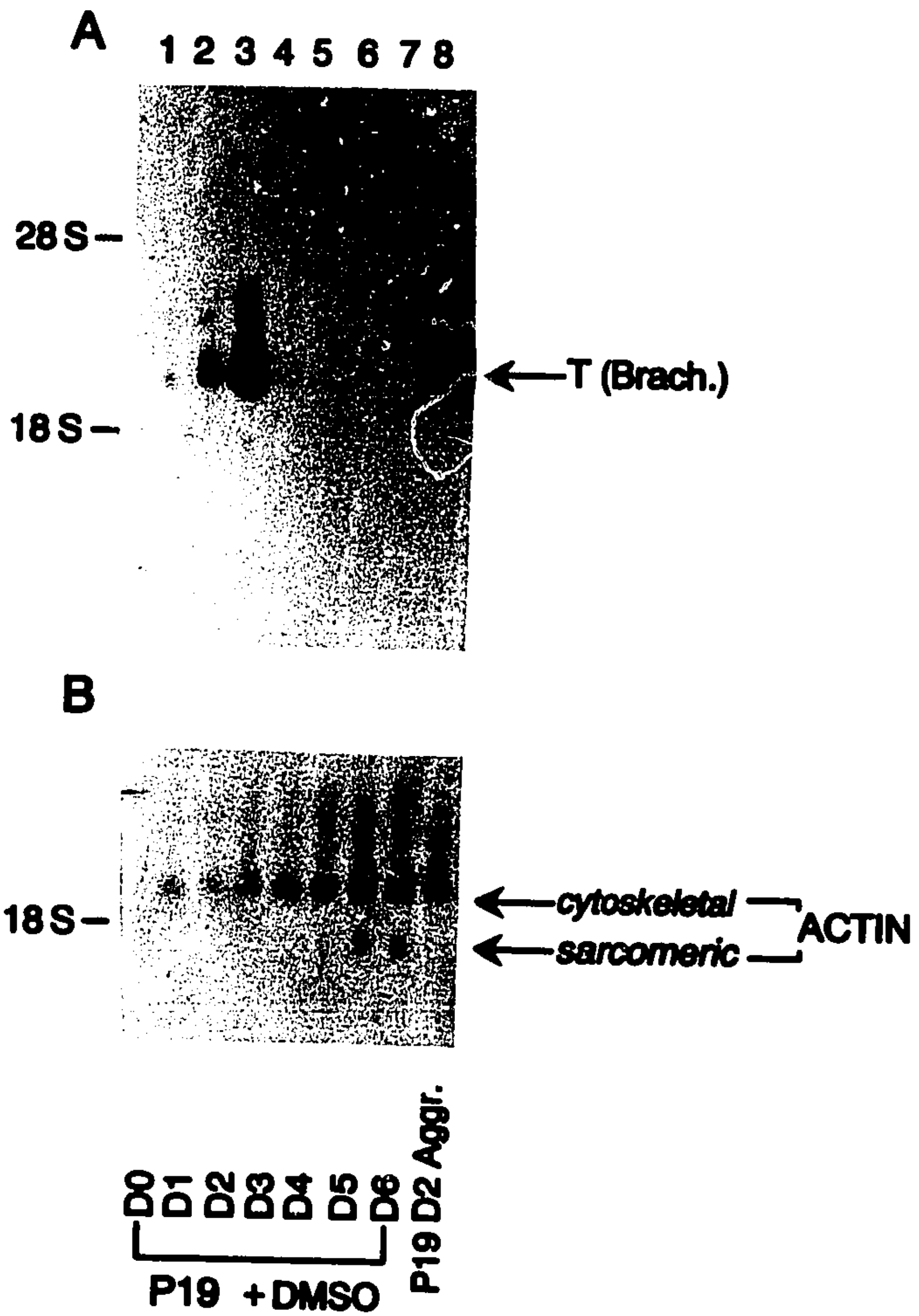
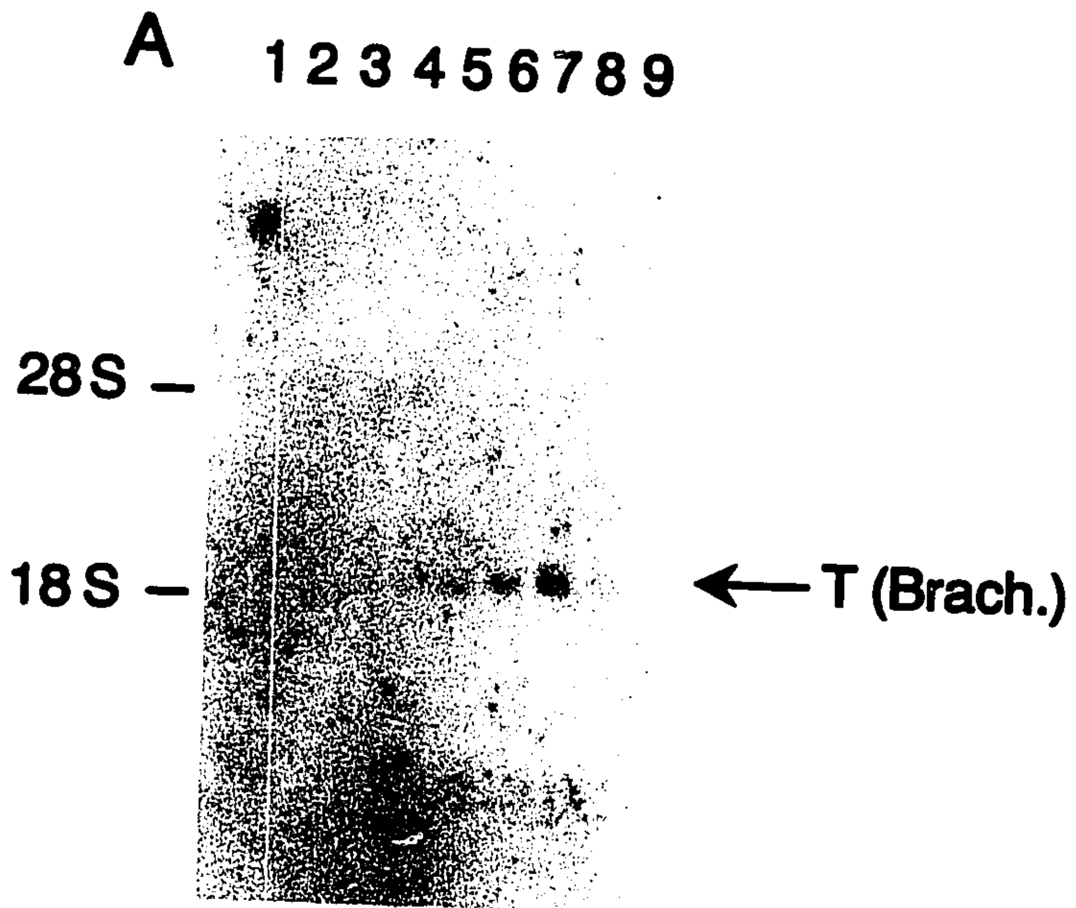
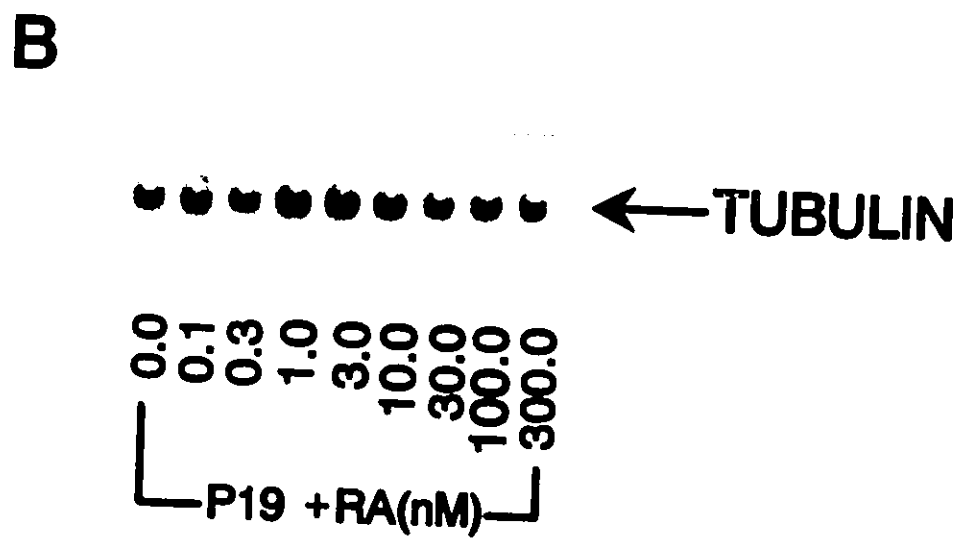

A
1 2 3 4 5 6 7 8
28 S
18 S
T (Brach.)
B
18 S
cytoskeletal
sarcomeric
ACTIN
D0
D1
D2
D3
D4
D5
D6
P19 +DMSO
P19 D2 Aggr.

Fig. 3 Brachyury expression in retinoic acid-treated P19 cells.

Total RNA was analyzed from P19 cells grown on solid surface 48 h following treatment with RA concentrations indicated at the bottom. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury probe then stripped and (B) re-hybridized with an α-tubulin probe as described in Material and methods. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively. cells (Edward and McBurney, 1983).


A
1 2 3 4 5 6 7 8 9
28 S
18 S
T (Brach.)


B
TUBULIN
0.0 0.1 0.3 1.0 3.0 10.0 30.0 100.0 300.0
P19 +RA(nM)

(above 0.1 μM) induce neuroectodermal cells (Edward and McBurney, 1983). To determine if Brachyury expression is restricted to cell destined to differentiate into mesoderm, P19cells were exposed for 2 days to different RA concentrations and the expression of Brachyury was found to be restricted to cultures exposed to these concentrations of RA effective in inducing mesodermal differentiation (fig. 3). Tubulin was probed to control for the RNA loading.

3.2 Brachyury mRNA expression in mutant P19 cells.

RAC65 cells (Jones-Villeneuve et al., 1982) are a mutant clone of P19 cells that fail to differentiate in response to RA and that contain a mutation in the RA receptor alpha (RARα) (Pratt et al., 1990). These cells differentiate normally when exposed to DMSO. Brachyury was transiently expressed during DMSO-induced differentiation of RAC65 cells (fig. 4) although its expression was induced more slowly (peaking at 3 days) and was sustained for longer (up to day 5). DMSO-treated RAC65 cells appear to develop a higher proportion of cardiac and skeletal muscle than P19 cells (Smith, 1986), consistent with the more sustained expression of Brachyury in these cultures.

RAC65 cells fail to differentiate when exposed to RA (Jones-Villeuve et al., 1982). No induction of Brachyury expression was detected in these cells exposed to RA at any of the concentrations tested (fig. 5).

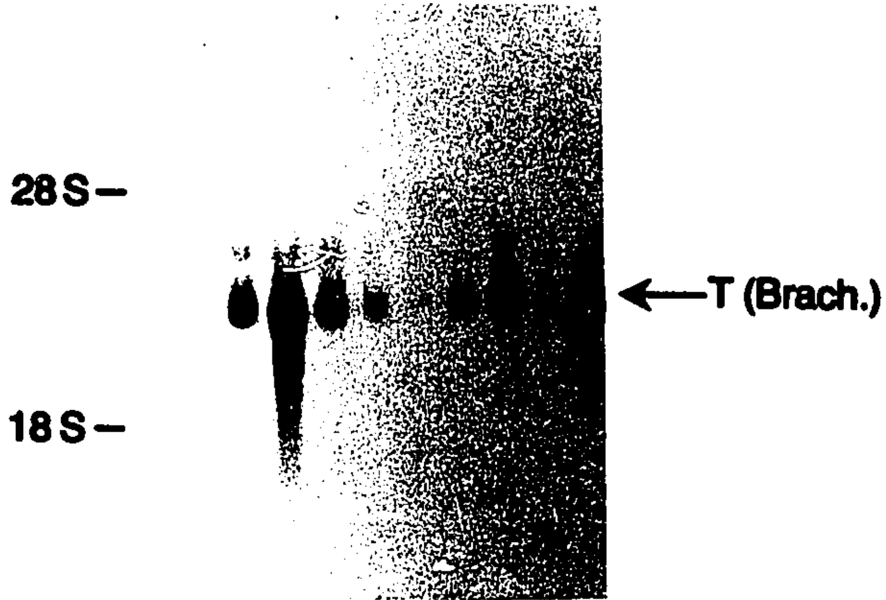
D3 cells are a mutant P19 cell line (Edwards et al., 1983) that fail to differentiate in DMSO but do differentiate into neuroectodermal cells when exposed to high RA

Fig. 4 **Brachyury expression during DMSO-induced differentiation of RAC65 cells.**

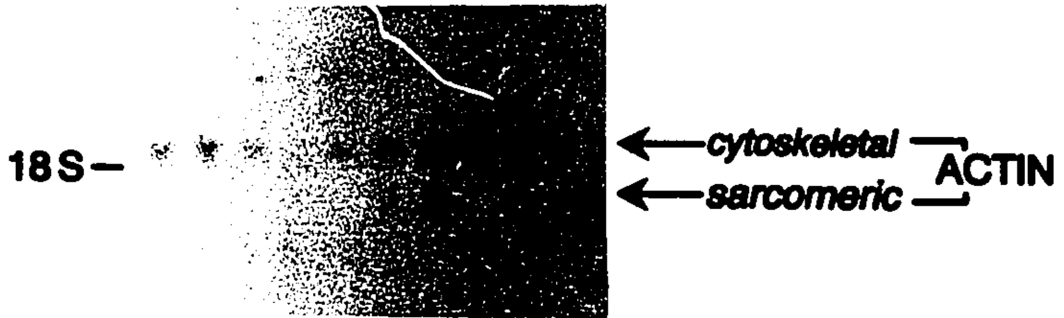
Total RNA was extracted from RAC65 cells at daily intervals following induction of differentiation with 0.8% DMSO and analyzed by Northern blot hybridization. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with with an actin cDNA probe as described in Material and methods. Lanes 1-7: days of differentiation numbered consecutively after the first day of treatment. Lanes 8-9, RAC65 cells aggregated 1 and 2 days without drug; lane 10, untreated P19 cells; lane 11, P19 cells aggregated 2 days without drug. The sizes of the transcripts were: Brachyury T, 2.1 kb; actin probe hybridizes to both cytoskeletal (β and γ) at 2.1 kb and striated muscle (α) actins at 1.7 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A

1 2 3 4 5 6 7 8 9 10 11



B



D0 D1 D2 D3 D4 D5 D8

RAC65 +DMSO

RAC65 D1 Aggr.

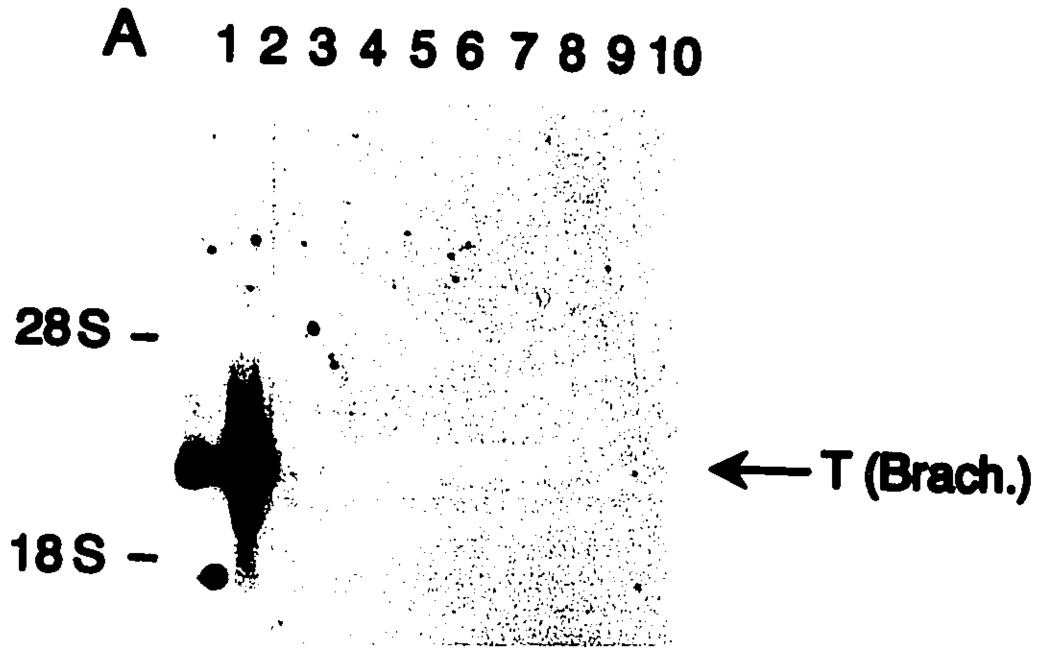
RAC65 D2 Aggr.

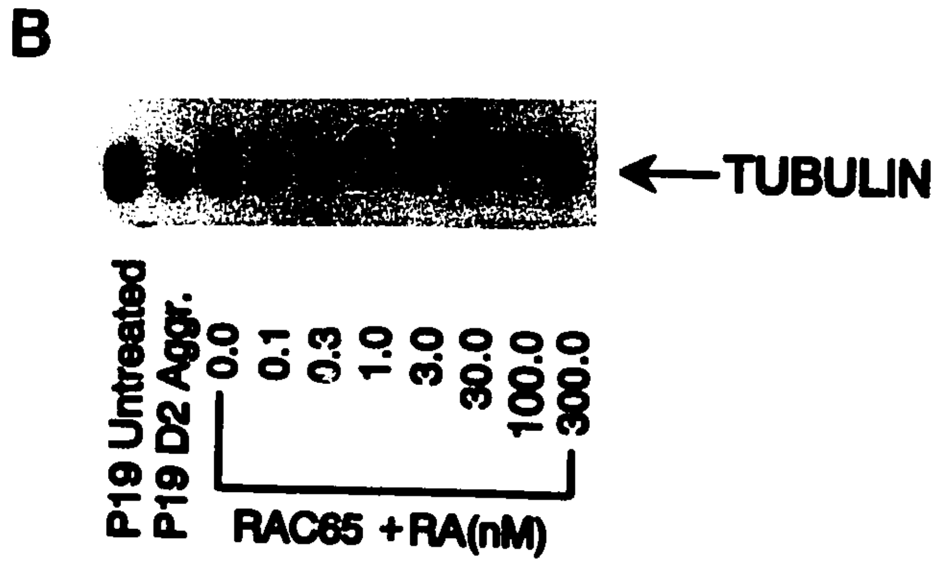
P19 Untreated

P19 D2 Aggr.

Fig. 5 **Brachyury expression in retinoic acid-treated RAC65 cells.**

Total RNA was extracted from RAC65 cells grown on solid surface 48 h following treatment with various RA concentrations and analyzed by Northern blot hybridazation. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with a α-tubulin cDNA probe as described in Material and methods. Lane 1, untreated P19 cells; lane 2, P19 cells aggregated 2 days without drug; lanes 3-10, RAC65 cells treated with 0 to 300.0 nM RA. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.


A
1 2 3 4 5 6 7 8 9 10
28S –
18S –
T (Brach.)


B
TUBULIN
P19 Untreated
P19 D2 Aggr.
0.0
0.1
0.3
1.0
3.0
30.0
100.0
300.0
RAC65 +RA(nM)

concentrations. Brachyury mRNA levels remained low throughout the DMSO time course as compared to P19 cells aggregated for 2 days (fig. 6A, lanes 1 to 7 vs lane 8), and they fail to express the sarcomeric isoform of actin indicating their failure to differentiate intomuscle (fig. 6B, lane 1 to 7). Furthermore, the lack of Brachyury mRNA induction is not restricted to DMSO since when D3 cells are exposed to various RA concentrations (fig. 7), Brachyury mRNA levels remained undetectable in these RA-treated cultures. D3 cells fail to differentiate into mesodermal cells in DMSO and RA treatments, this correlates with an absence in Brachyury mRNA expression.

We looked by Southern blotting, for gross rearrangements affecting the Brachyury gene in D3 cells but none was detected (data not shown). Also, D3 cells do not express significantly more Brachyury mRNA when aggregated in the absence of any drug (data not shown). Hence, it appears that D3 cells' phenotype is likely due at least in part to an event upstream of Brachyury causing a failure to induce its expression.

3.3 Calcium mediated aggregation triggers Brachyury expression.

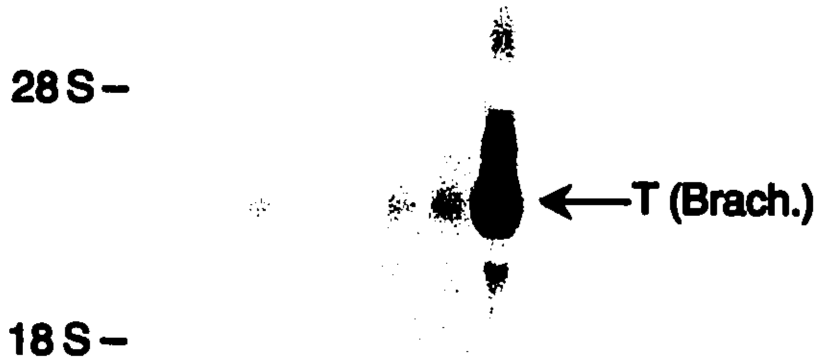
P19 cells differentiate into mesoderm only after aggregation and exposure to DMSO (McBurney et al., 1982; Smith et al., 1987). The expression of Brachyury in P19 and RAC65 cells is induced in cells aggregated for two days even in the absence of DMSO indicating that aggregation is sufficient to trigger Brachyury expression (fig. 2A, lane 8 and fig. 4A, lanes 8-9). Data collected on the expression of Brachyury in P19 cells indicated that expression was transient regardless of the presence of DMSO in the

Fig. 6 **Time course of Brachyury expression in DMSO-treated D3 cells.**

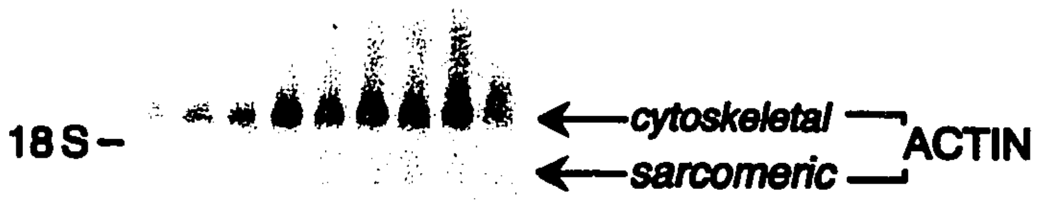
Total RNA was extracted from D3 cells at daily intervals following initiation of treatment with 0.8% DMSO and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with with an actin cDNA probe as described in Material and methods. Lanes 1-7: days of differentiation numbered consecutively after the first day of treatment. Lane 8: P19 cells aggregated 2 days without DMSO. The sizes of the transcripts were: Brachyury, 2.1 kb; actin probe hybridizes to both cytoskeletal (β and γ) at 2.1 kb and striated muscle (α) actins at 1.7 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A

1 2 3 4 5 6 7 8



B



D0 D1 D2 D3 D4 D5 D6 P19 D2 Aggr.

D3 +DMSO

Fig. 7 **Brachyury expression in retinoic acid-treated D3 cells.**

Total RNA was extracted from D3 cells grown on solid surface 48 h following treatment with various RA concentrations and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with an α-tubulin probe as described in Material and methods. Lane 1, untreated P19 cells; lane 2, P19 cells aggregated 2 days without drug; lane 3, P19 cells aggregated two days with 0.8% DMSO; lanes 4-12, D3 cells treated with 0 to 300.0 nM RA. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A 1 2 3 4 5 6 7 8 9 10 11 12

28 S —

18 S —

⟵ T (Brach.)

B

⟵ TUBULIN

Untreated D2 Aggr. D2 DMSO | 0.0 0.1 0.3 1.0 3.0 10.0 30.0 100.0 300.0

P19 | D3 + RA(nM)

cell cultures (fig. 13 lane 4, fig. 15 lanes 4 and 5 and data not shown). Moreover, the expression of Brachyury was only slightly enhanced by DMSO in P19 cells cultured on solid surface and DMSO did not enhance the level of Brachyury expression in P19 cell aggregates (data not shown).

Aggregation of embryonal carcinoma cells (EC) like P19 cells is dependent on calcium (Takeichi et al., 1981; Yoshida and Takeichi, 1982). To evaluate the relationship between cell aggregation and induction of Brachyury expression, P19 cells were cultured in medium with reduced calcium concentrations. These cells formed small aggregates of cells which were poorly compacted (fig. 9).In parrallel, Brachyury was not induced in these cells cultured in reduced calcium concentrations (fig. 8). The calcium chelator EGTA at 2 mM added to normal medium containing 1.8 mM ca^{2+} also blocked Brachyury expression and P19 cell aggregation (data not shown). Thus supporting the association between calcium-dependent cell aggregation and activation of Brachyury expression.

Moreover, preliminary experiments indicated that the calcium ionophore A23187 did not induce Brachyury in P19 cells growing on solid surface and that the calcium channel blocker, verapamil, did not prevent aggregation nor the expression of Brachyury (data not shown). These two last experiments confirm that calcium is not a mediator of Brachyury expression but rather expression of Brachyury gene is likely induced indirectly by calcium-mediated cell aggregation.

3.4 Modulation of Brachyury expression by soluble factors.

In Xenopus laevis, activin and other growth factors are believed to induce mesoderm during embryonic development possibly by triggering expression of Brachyury (Whitman and Melton, 1992; Hermmati-Brivanlou and Melton, 1992). We tested the effect of activin A on Brachyury expression in P19 cells cultured on solid surface and found induced Brachyury expression (fig. 10). Induction of Brachyury was evident as early as 1 h after exposure to activin (lane 3), became very intense after 24 h (lane 5) and declined by 48 h (data not shown).

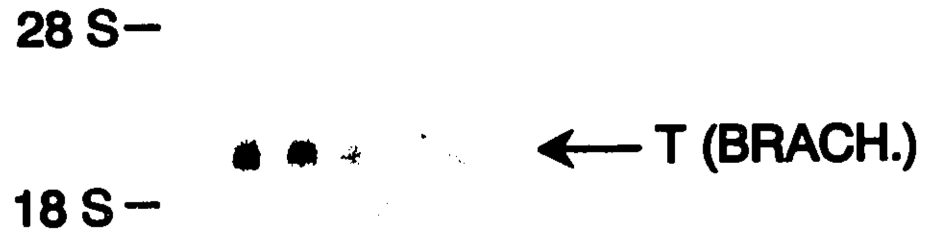
In addition to activin A, we also tested other members of the TGFβ gene family, the bone morphogenetic proteins (BMP) 2, 3, 4, 5. All were able to induce Brachyury expression as shown in fig. 11A and 11B. BMP-2 and 3 were assessed at 24 h (lanes 3 and 4); whereas, BMP-4 and 5 were assessed for different periods of times. BMP-4 and 5 enhanced Brachyury expression starting at 6 h (fig 11A, lanes 6 and 9). This increase lasted up to 24 h with BMP-5 and it declined by 48 h (fig. 11c, lanes 4-5) but it was still high at that point with BMP-4 (fig. 11C, lanes 2-3).

Activin A and BMP-2 were tested to see whether they could induce P19 cells to differentiate. Both failed (data not shown). Hence, induced Brachyury expression to the extent achieved by aggregation or by activin or BMP-2 in P19 cells is not sufficient to induce cell differentiation.

Fig. 8 **Brachyury expression in P19 cells grown in low calcium concentrations.**

Total RNA was extracted from P19 cells grown 48 h in calcium free medium supplemented with 0.45 μM to 4.5 mM $CaCl_2$ and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with an α-tubulin cDNA probe as described in Material and methods. Lane 1, untreated P19 cells; lane 2, P19 cells aggregated 2 days in MEMα medium supplemented with 10% calf serum; lanes 3-7, P19 cells grown 2 days in Joklik's medium supplemented with 10% dialysed calf serum and 4.5 mM to 0.45 μM $CaCl_2$. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A 1 2 3 4 5 6 7



B

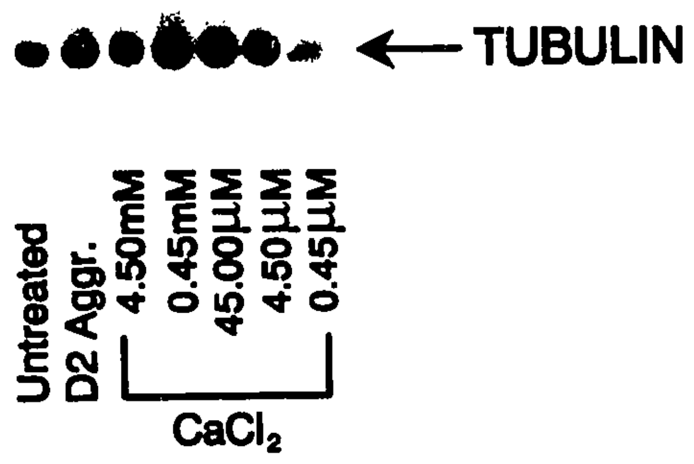
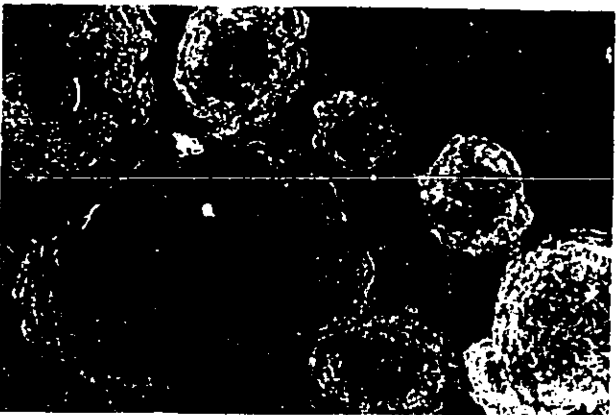


Fig. 9 **P19 cells grown in low calcium concentrations.**

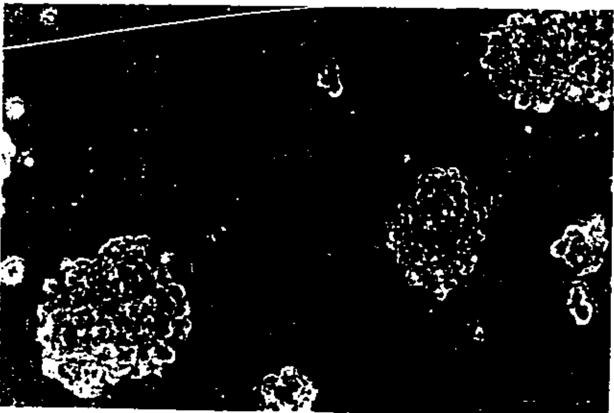
Phase-contrast fields of P19 cells cultured on bacterial grade dishes for 48 h in: panel A, MEMα medium supplemented with 10% calf serum; panel B, Joklik's medium supplemented with 10% dialysed calf serum and 4.5 mM $CaCl_2$; panel C, 0.45 mM $CaCl_2$; panel D, 45 μM $CaCl_2$. Bar represents 50 μm.

A

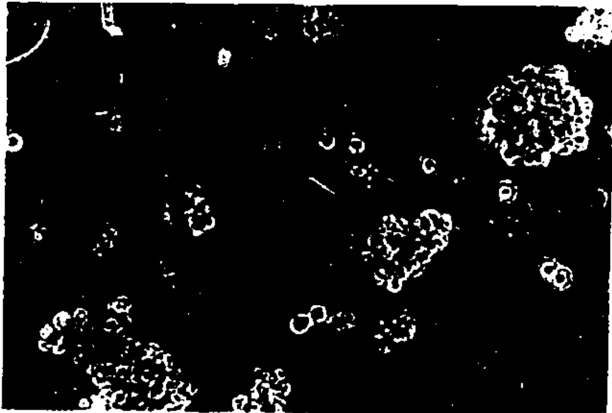


B

C



D

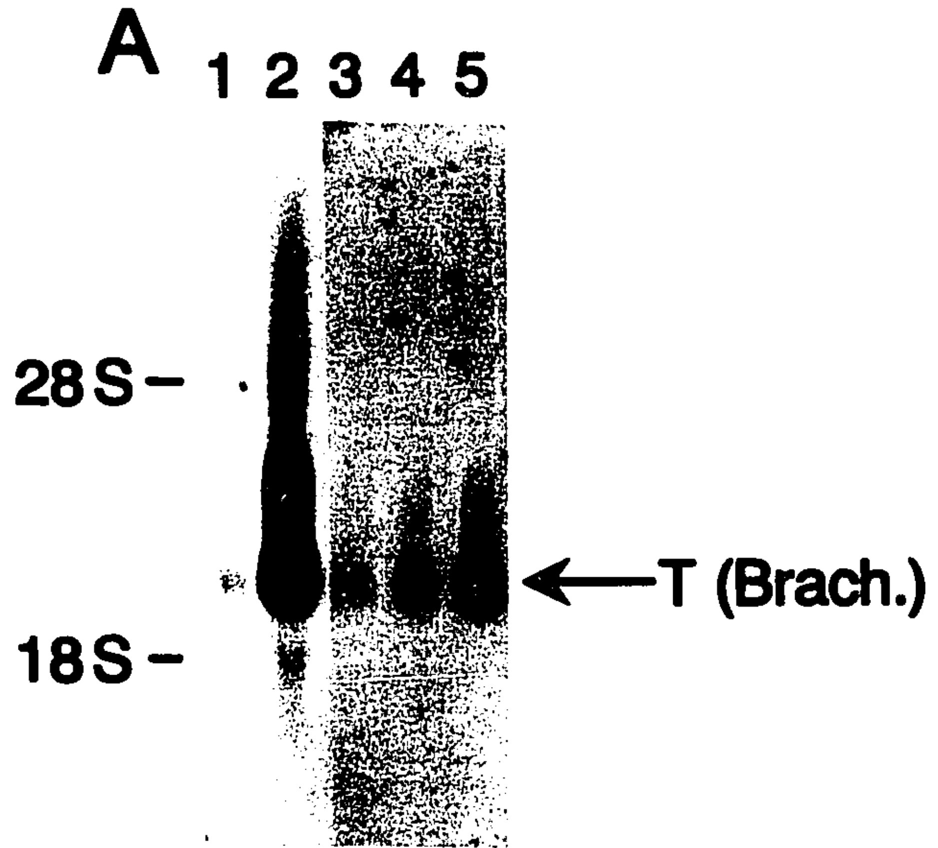


Leukaemia inhibitory factor (LIF) is not essential for embryogenesis (Stewart et al., 1992) but its overexpression inhibits murine gastrulation (Conquet et al., 1992). LIF also prevents endoderm and mesoderm differentiation in P19 cells treated with either DMSO or RA (Pruitt and Natoli, 1992). P19 cells aggregated for two days in the presence of LIF expressed significantly lower levels of Brachyury mRNA than cells aggregated in the absence of LIF (fig. 12). Hence, LIF inhibition of P19 cells differentiation could be due in part by a reduced Brachyury expression.

Very little is known about the mechanism leading to Brachyury expression. Numerous differentiation processes are affected by cAMP (reviewed by Cho-Chang, 1989). Treatment of cells with IBMX and forskolin is known to elevate intracellular cAMP levels (IBMX is a phosphodiesterase inhibitor and forskolin activates adenylate cyclase). These drugs induced Brachyury expression in P19 cell growing on solid surfaces (fig. 13A). In addition, IBMX and forskolin enhanced the expression of Brachyury in P19 cells aggregated for 24 h (fig. 13C). IBMX and forskolin failed to induce P19 cell differentiation (data not shown), indicating that triggering the expression of Brachyury is not sufficient to achieve differentiation, although it is believed to be a necessary step.

Fig. 10 **Activin induces Brachyury expression in P19 cells.**

Total RNA was extracted from P19 cells grown on a solid surface in the presence of activin A and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe and then stripped and (B) re-hybridized with an α-tubulin cDNA probe as described in Material and methods. Lane 1, untreated P19 cells; lane 2, P19 cells aggregated 2 days without drug; lane 3, P19 cells grown with 1:200 dilution activin A for 1h; lane 4, 6.5 h; lane 5, 24 h. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.


A
1 2 3 4 5
28 S
18 S
T (Brach.)

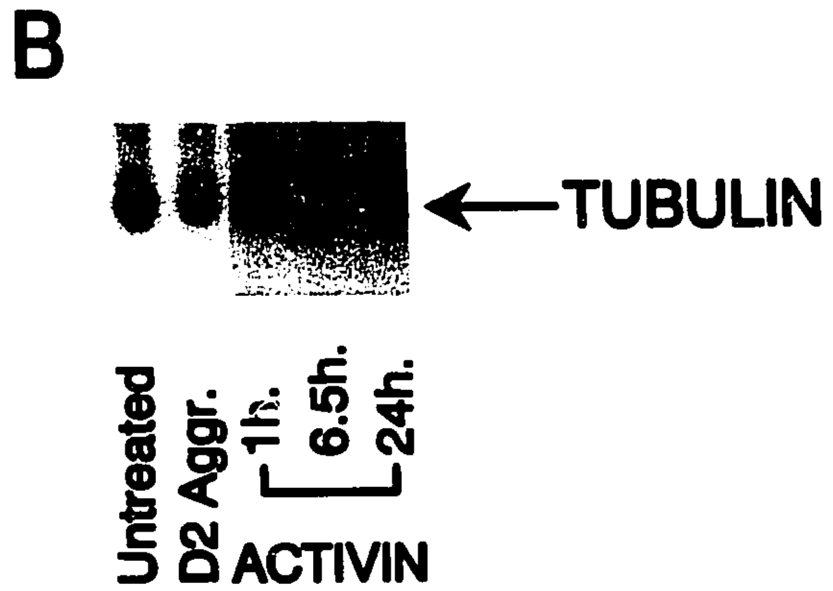

B
TUBULIN
Untreated
D2 Aggr.
1h.
6.5h.
24h.
ACTIVIN

Fig. 11 Bone morphogenetic proteins activate Brachyury expression in P19 cells.

Total RNA was extracted from P19 cells grown on solid surfaces in the presence of BMP-2 to 5 and analyzed by Northern blotting. (A-B) The RNA blots (15μg/lane) were first hybridized with a Brachyury cDNA probe then stripped and (C-D) re-hybridized with an α-tubulin cDNA probe as described in Material and methods. Panels A and B, lane 1, untreated P19 cells; lane 2, P19 cells aggregated 2 days without drug; lane 3, P19 cells treated 24h with 1.0 nM BMP-2; lane 4, 24h with 0.7 nM BMP-3; lanes 5-7, 1h, 6h, 24h with 1.0 nM BMP-4; lanes 8-10, 1h, 6h, 24h with 1.0 nM BMP-5. Panels C and D, lane 1, untreated P19 cells; lanes 2-3, P19 cells treated 24h and 48h with 1.0 nM BMP-4; lanes 4-5, 24h and 48h with 1.0 nM BMP-5; lane 6, P19 cells aggregated 2 days without drug. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A
1 2 3 4 5 6 7 8 9 10
C
1 2 3 4 5 6
28S
18S
T (Brach.)
B
D
TUBULIN
Untreated
D2 Aggr.
BMP-2 24h.
BMP-3 24h.
BMP-4 1h. 6h. 24h.
BMP-5 1h. 6h. 24h.
Untreated
BMP-4 24h.
BMP-4 48h.
BMP-5 24h.
BMP-5 48h.
D2 Aggr.

3.5 Overexpression of Brachyury in P19 cells but not in D3 cells induces spontaneous differentiation.

We tested the effect of overexpressing Brachyury mRNA in P19 cells. Pgk-1 regulatory sequences were used to drive Brachyury cDNA. This gene was transfected into P19 cells along with a Pgk-hygromycin B construct. The Pgk promoter was cloned in Dr. McBurney's laboratory. It has very strong promotor activity found ubiquitously (Adra at al., 1987; McBurney et al., 1991). 24 hygromycin B resistant colonies were picked and individually propagated. Cells from 5 of these clones differentiated spontaneously. Some clones such as P19T(S)-2 formed muscle while other clones such as P19T(S)-1 differentiated into fibroblast and neuronal-like cells (see below). P19 cells were also co-transfected with a plasmid carrying the Pgk-1 driving Brachyury cDNA in the anti-sense orientation. Again 24 hygromycin B resistant colonies were picked and propagated but none differentiated spontaneously. Fig. 14 shows phase contrast photomicrographs of three representative clones. P19T(AS)-1 carries the anti-sense construct and resembles the parental P19 cells (fig. 14A). P19T(S)-1 and -2 carry the sense construct and these cultures contain cells with differentiated morphology. Neurons are visible in the lower left corner of panel C.

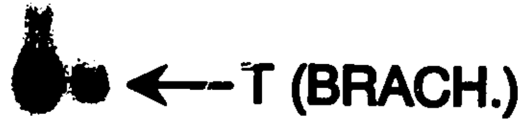
Cells from the three clones were cultured for 4 days as aggregates in medium

Fig. 12 **Leukaemia inhibitory factor blocks Brachyury expression in aggregated P19 cells.**

Total RNA was extracted and analyzed by Northern blotting from untreated P19 cells, lane 1; P19 cells aggregated 48 h without drug, lane 2; aggregated for two days in the presence of 10 ng/ml LIF, lane 3. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then stripped and (B) re-hybridized with an α-tubulin cDNA probe as described in Material and methods. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A 1 2 3

28 S –



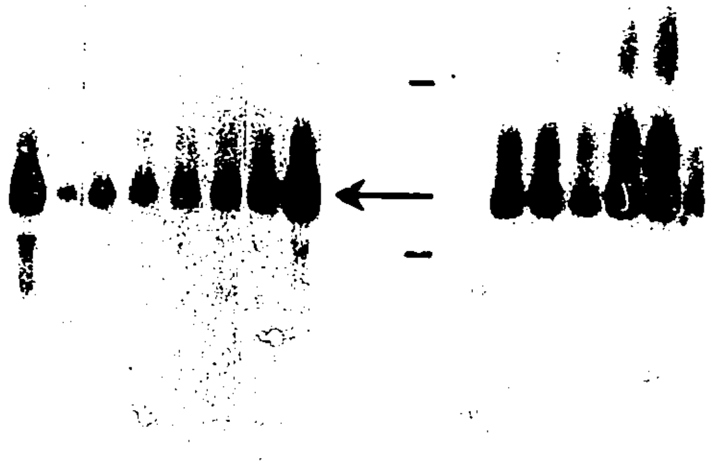
18 S –

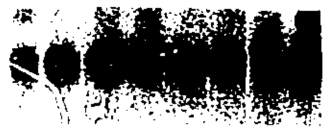
B

Untreated
D2 Aggr. - LIF
D2 Aggr. + LIF

Fig. 13 **cAMP activates Brachyury expression in P19 cells.**

Total RNA was extracted from P19 cells grown on solid surfaces (panels A and B) or aggregated (panels C and D) in the presence of 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μM forskolin and analyzed by Northern blotting. The RNA blots (15μg/lane) were first hybridized with a Brachyury cDNA probe then stripped and re-hybridized with an α-tubulin cDNA probe as described in Material and methods. Panels A-B, lane 1, P19 cells aggregated 2 days without drug; lanes 2-8, P19 cells grown on a solid surface with I and F for 0, 0.5, 1.5, 4, 9, 24 and 48h. Panels C-D, lane 1, untreated P19 cells; lanes 2-4, P19 cells aggregated for 1, 2 and 3 days without drug; lanes, 5-7 P19 cells aggregated 1, 2 and 3 days with I and F. The sizes of the transcripts were: Brachyury, 2.1 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively. +I/F indicates the presence and -I/F the absence of IBMX and forskolin.





A
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7
28 S -
18 S -
T (BRACH.)
B
D
TUBULIN
0.0h.
0.5h.
1.5h.
4h.
9h.
24h.
48h.
+ I/F
Untreated
D1 Aggr.
D2 Aggr.
D3 Aggr.
D1 Aggr.
D2 Aggr.
D3 Aggr.
- I/F
+ I/F

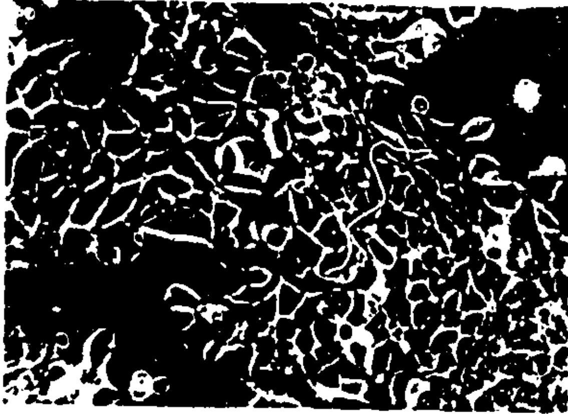
with or without DMSO. They were then plated in drug-free medium and harvested two days latter. P19 cells normally remain undifferentiated when cultured under theseconditions in the absence of DMSO but differentiate into mesodermal and endodermal cells when exposed to DMSO.

The P19T(AS)-1 clone behaved like the parental P19 cells. Cells aggregated in the absence of DMSO remained morphologically undifferentiated and expressed two markers of EC cells, the cell surface antigen SSEA-1 (fig. 16A) and the transcript encoding oct-3 (fig. 15D). DMSO-treated cultures differentiated, as indicated by the lost of expression of oct-3 (fig. 15D), and formed large amounts of cardiac muscle that could be stained with an antibody to sarcomeric myosin (fig. 16B) and that contained sarcomeric actin mRNA (fig. 15B, lane 4). The antisense Brachyury mRNA was not detected in P19T(AS)-1, the signal seen in the Northern blot derives from the endogenous gene (fig. 15A, lanes 3,4,5). The P19T(S)-1 clone expressed abundant Brachyury mRNA from the transfected gene (fig. 15A, lanes 6,7,8). This transcript was somewhat shorter than that from the endogenous gene owing to the removal of some untranslated sequences from the 5' and 3' ends of the cDNA. P19T(S)-1 cells growing attached to plastic surfaces appeared comprised of a mixture of cell morphologies including EC cells. The presence of oct-3 mRNA (fig. 15D, lane 6) confirms the presence of such undifferentiated cells. No such undifferentiated cells were present in P19T(S)-1 cultures that had been aggregated in presence or absence of DMSO. These aggregated cultures contained large numbers of neurons that stained with antibodies to the 160 kd

Fig. 14 **Clones of P19 cells transfected with Brachyury expression constructs.**

Phase-contrast photomicrographs of P19 clones transfected with the Pgk-Brachyury recombinant gene as they were propagated into cell lines. Panel A, P19T(AS)-1; panel B, P19T(S)-2; panel C, P19T(S)-1. Bar represents 50 μm. The anti-sense (AS) clone carries the Pgk-antisense Brachyury cDNA recombinant gene. The sense (S) clones carry the Pgk-sense Brachyury cDNA recombinant gene.

A



B

C

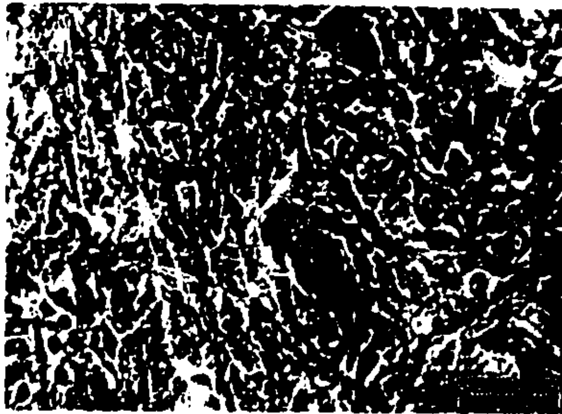


Fig. 15 Northern blot analysis of P19(Brachyury T) transformants.

Total RNA was extracted from P19T(AS)-1, P19T(S)-1 and P19T(S)-2 and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then consecutively stripped and re-hybridized with an actin cDNA probe (B); a neurofilament 68 (NF68) cDNA probe (C); an oct-3 cDNA probe (D); an α-tubulin cDNA probe (E) as described in Material and methods. Lane 1, untreated P19 cells; lane 2, P19 cells aggregated 2 days without drug; lanes 3, 6, 9, untreated P19T(AS)-1, P19T(S)-1, P19T(S)-2 cells; P19T(AS)-1, P19T(S)-1, P19T(S)-2 cells 7 days after initiation of the differentiation regime (as described in Material and methods) in the absence (lanes 4, 7, 10) or presence of 0.8% DMSO (lanes 5, 8, 11). The sizes of the transcripts were: Brachyury, 2.1 kb; actin probe hybridizes to both cytoskeletal (β and γ) at 2.1 kb and striated muscle (α) actins at 1.7 kb; NF68, 4.0 and 2.5 kb; oct-3, 1.5 kb; α-tubulin, 1.9kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

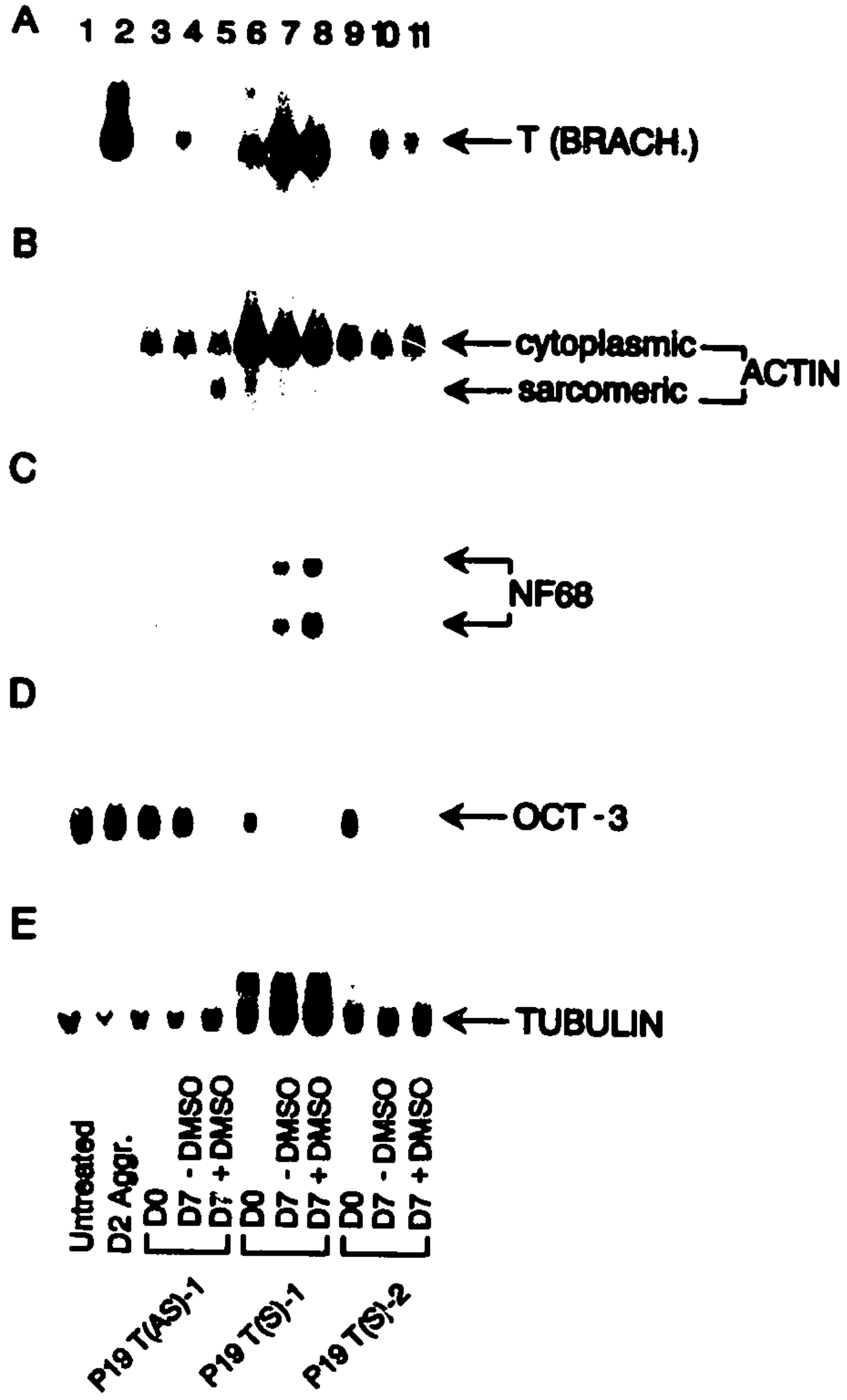
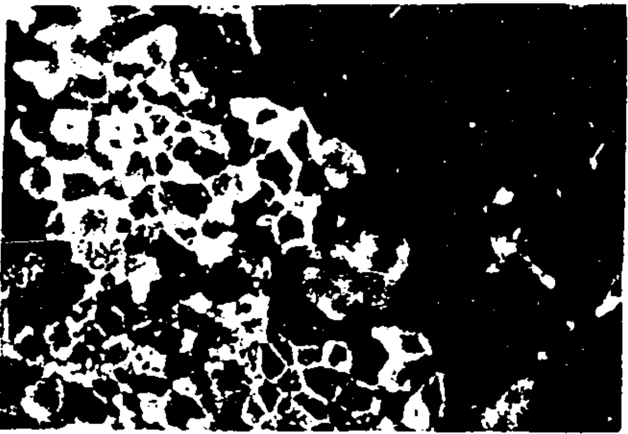
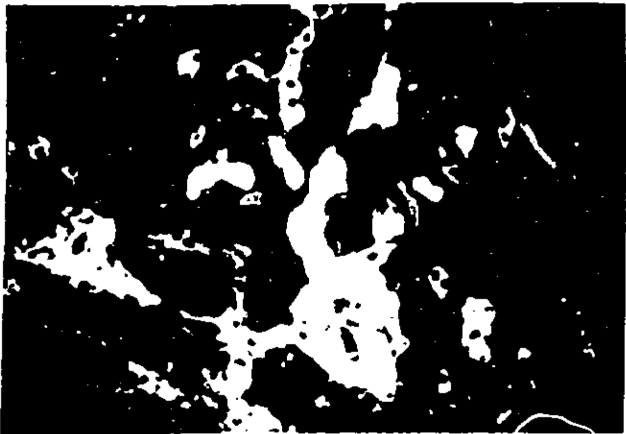

A
1 2 3 4 5 6 7 8 9 10 11
T (BRACH.)
B
cytoplasmic
sarcomeric
ACTIN
C
NF68
D
OCT-3
E
TUBULIN
Untreated
D2 Aggr.
D0
D7 - DMSO
D7 + DMSO
D0
D7 - DMSO
D7 + DMSO
D0
D7 - DMSO
D7 + DMSO
P19 T(AS)-1
P19 T(S)-1
P19 T(S)-2

Fig. 16 **Immunostaining of P19(Brachuyry T) transformants.**

P19T(AS)-1 cells 7 days after initiation of the differentiation regime in the absence of DMSO (A); stained with mouse monoclonal AEC-3A1-C3 antibody; with DMSO stained with mouse monoclonal antibody MF20 (B). (C) P19 T(S)-1 7 days after initiation of differentiation regime in the absence of DMSO stained with anti-neurofilament 160 (NF160), both cell bodies and processes are stained. (D) P19T(S)-2 7 days after initiation regime in the absence of DMSO stained with mouse monoclonal antibody MF20. Secondary antibody was fluorescein-conjugated anti-mouse IgG. AEC-3A1-C3 recognizes the cell surface antigen SSEA-1. MF20 recognizes sarcomeric myosin. Bar represents 50 μm.


A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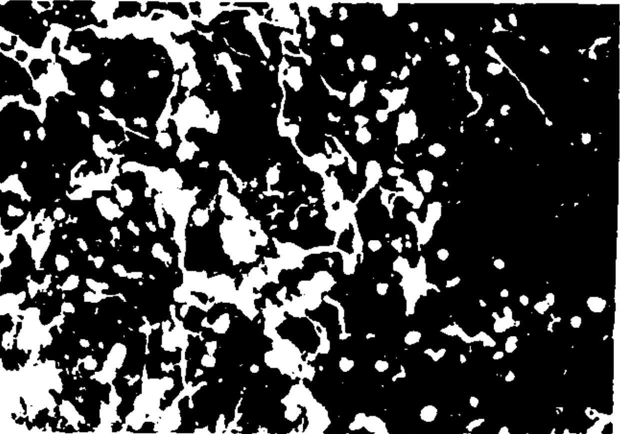
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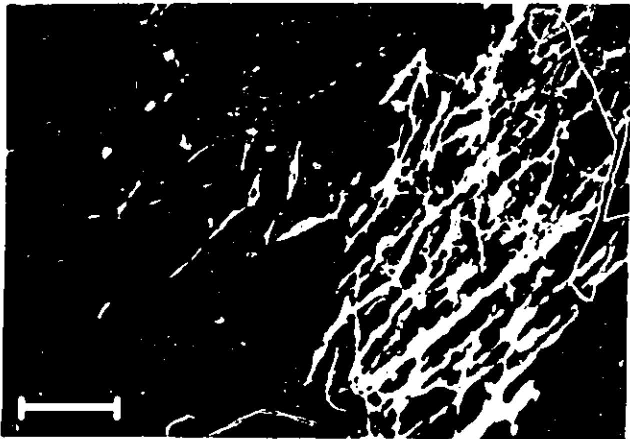

D

neurofilament protein (fig. 16C). In addition, they contained high levels of mRNA encoding the 68 kd neurofilament subunit (fig. 15C, lanes 7 and 8).

The P19T(S)-2 clone expressed lower levels of Brachyury mRNA than the P19T(S)-1 clone (fig. 15A, lanes 9-11). Aggregation of P19T(S)-2 cells appeared to induce differentiation as evidenced by the lower oct-3 mRNA in these cultures (fig. 15D, lanes 10 and 11) but sarcomeric α actin mRNA was not detected. These cultures of aggregated cells contained very little cardiac muscle but did contain bipolar skeletal muscle cells that reacted with antibody to sarcomeric muscle myosin (fig. 16D).

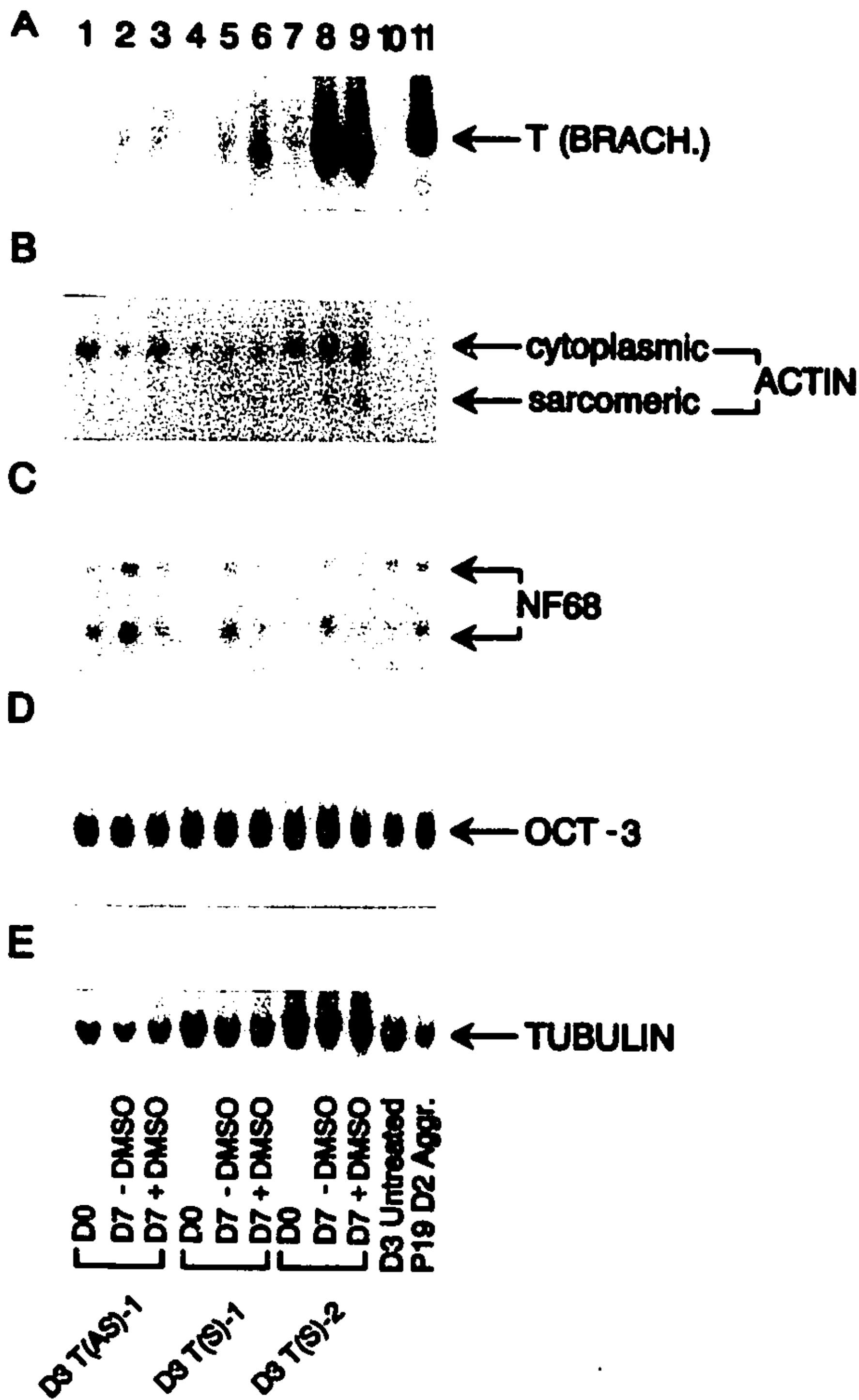
We interpret the above results as indicating that expression of Brachyury mRNA from the Pgk-1 promotor induces spontaneous differentiation of P19 cells and that the cell types that develop depend on the level of Brachyury mRNA. High levels of Brachyury mRNA yield neurons while lower levels yield muscle. This same dose relationship was found for the chemical inducers of P19 cell differentiation such as RA (Edwards, 1982), DMSO and 6-thioguanine (Edwards, 1983).

D3 cells were also transfected with the recombinant sense and anti-sense Brachyury genes. 24 colonies from each transfection were picked and individually propagated. None differentiated spontaneously. The clones D3T(S)-1 and D3T(S)-2) carried the sense vector while D3T(AS)-1 carried the antisense gene. No antisense transcript was detected in D3T(AS)-1. D3T(S)-1 expressed low levels and D3T(S)-2 expressed high levels of the sense transcript encoding Brachyury. Aggregated cells did not appear to differentiate in the presence or absence of DMSO as the oct-3 transcript

remained high and the sarcomeric α actin mRNA was not detected (fig. 17). The Pgk-driven Brachyury mRNA was more abundant in the clones after they had been aggregated (fig. 15 and 17). Since the Pgk-1 mRNA does not change in abundance during differentiation, this increase in Pgk-Brachyury mRNA is likely due to a post-transcriptional event such as mRNA stabilization.

Fig. 17 **Northern blot analysis of D3(Brachyury T) transformants.**

Total RNA was extracted from D3T(AS)-1, D3T(S)-1 and D3T(S)-2 and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a Brachyury cDNA probe then consecutively stripped and re-hybridized with (B) an actin cDNA probe (C) a neurofilament 68 (NF68) cDNA probe (D) an oct-3 cDNA probe (E) an α-tubulin cDNA probe as described in Material and methods. Lanes 1, 4, 7, untreated D3T(AS)-1, D3T(S)-1, D3T(S)-2 cells; D3T(AS)-1, D3T(S)-1, D3T(S)-2 cells 7 days after initiation of the differentiation regime (as described in Material and methods) in the absence (lanes 2, 5, 8) or presence of 0.8% DMSO (lanes 3, 6, 9); lane 10, untreated D3 cells; lane 11, P19 cells aggregated 2 days without drug. The sizes of the transcripts were: Brachyury, 2.1 kb; actin probe hybridizes to both cytoskeletal (β and γ) at 2.1 kb and striated muscle (α) actins at 1.7 kb; NF68, 2.5 and 4.0 kb; oct-3, 1.5 kb; α-tubulin, 1.9kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.


A
1 2 3 4 5 6 7 8 9 10 11
T (BRACH.)
B
cytoplasmic
sarcomeric
ACTIN
C
NF68
D
OCT -3
E
TUBULIN
D0
D7 - DMSO
D7 + DMSO
D0
D7 - DMSO
D7 + DMSO
D0
D7 - DMSO
D7 + DMSO
D3 Untreated
P19 D2 Aggr.
D3 T(AS)-1
D3 T(S)-1
D3 T(S)-2

Chapter Four

Discussion

4.1 Brachyury expression during P19 cell differentiation.

This study was aimed at unveiling events that occur at the onset of P19 cell differentiation into mesoderm. In the mouse embryo,

Brachyury is necessary for mesoderm formation during gastrulation (Herrmann et al., 1990). The causal relationship between mesoderm formation and Brachyury expression is clear from the failure of T/T embryos to develop mesoderm. P19 cells can be induced to differentiate into mesodermal cell types when aggregated and treated with DMSO (McBurney et al., 1982; Rudnicki et al., 1990). Brachyury is expressed at high levels early in DMSO-treated P19 cells and disappears as the cells morphologically differentiate. This pattern of expression resembles that seen in the mouse embryo suggesting that the mechanisms responsible for mesoderm differentiation are similar in the two developing systems.

During embryonic development, Brachyury establishes the mesoderm germ layer and subsequently expression of mesoderm specific genes are activated (Herrmann et al, 1990; Wilkinson et al. 1990). In differentiating P19 cells, Brachyury expression is followed by the expression of markers of differentiation (such as α-actin) and the disappearance of markers of the undifferentiated state such as oct-3. Although, oct-3

Both the formation of tightly compacted aggregates and the induction of Brachyury are dependent on extracellular calcium. P19 cells express the calcium-dependent adhesion molecules E-cadherin which are believed to mediate P19 cell aggregation (Mummery et al., 1990). Increasing evidence indicates that cell adhesion molecules such as E-cadherin participate in embryonic development not only as adhesion molecules but also as signal transducers possibly by activating specific protein tyrosine kinases (reviewed by Singer, 1992). Hence, it is possible that E-cadherins are directly involved in the signal transduction pathway leading to Brachyury expression.

4.3 **cAMP-mediated Brachyury expression.**

Elevated intracellular levels of cAMP enhanced Brachyury expression. Hence, this second messenger could be an intracellular mediator of Brachyury activation. In support of this hypothesis, cAMP plays an active role in various differentiation processes. For example, cAMP analogs have been shown to prevent proliferation, reverse transformation and induce differentiation of a broad spectrum of human and rodent cancer cell lines (reviewed by Cho-Chang, 1989). Furthermore, cAMP plays a role in neuronal induction in the Xenopus embryo (Otte et al., 1989).

4.4 **Activin and BMPs induce Brachyury expression.**

In the Xenopus embryo, activin and BMPs are believed to play an active role in mesoderm formation possibly by the induction of Brachyury expression (Dale et al., 1992; Hemmati-Brivanlou and Melton, 1992; Smith et al., 1991). In mammals, however,

it has not been ascertained that they play similar roles. Activin A and BMPs-2 to 5 activate Brachyury expression in P19 cells suggesting that these factors may have the capacity to trigger early mesoderm development in mammalian embryos as well. These factors may have physiological significance since activin and BMP transcripts are found in murine embryos at times early enough to be responsible for Brachyury expression (van den Eijnden-van Raaij et al., 1992; Rosen and Thies, 1992).

BMP-4 sustained Brachyury expression longer than the other BMPs. BMP-4 is the member of the BMP family showing the highest homology (75%) to the dpp gene of Drosophila, and may therefore be the most highly conserved member of this gene family (Rosen and Thies, 1992).

Brachyury is necessary for posterior mesoderm formation; mice heterozygotes for the gain-of-function T allele (called the T^c allele) have severe malformations in the axial skeleton, rib fusions and lack of the tail (Searle, 1966). Also, ectopic expression of Brachyury in Xenopus embryos triggers excess formation of mesoderm of posterior character (Cunliff and Smith, 1992). BMPs possess potent osteoinducing activity *in vitro*. BMPs-2, 3 and 4 are found in early murine embryos in areas undergoing skeleton but their role in embryonic development remains unknown. (Rosen and Thies, 1992). Hence, it is tempting to speculate that BMPs play a role early during embryonic development by activating Brachyury expression in cells destined to form posterior mesoderm.

4.5 LIF inhibits P19 cell differentiation and Brachyury expression.

Previously LIF was shown in P19 cells to prevent endoderm and mesoderm pathways induced either by DMSO or RA but did not affect the ectoderm lineage (Pruitt and Natoli, 1992). LIF significantly reduced Brachyury expression in aggregated P19 cells. Hence, the inhibition of Brachyury expression by LIF may be responsible for inhibition of mesoderm differentiation. Murine embryos ectopicly expressing LIF were incapable of undergoing gastrulation, lacked differentiated mesoderm, and were devoid of Brachyury transcript (Conquet et al., 1992). Moreover, LIF selectively blocks ES (embryonic stem) cell differentiation *in vitro* into primitive ectoderm but allows formation of endodermal differentiation (Shen and Leder, 1992). The physiological role of LIF is uncertain because embryos devoid of functional LIF protein develop normally (Stewart et al., 1992). However, LIF may participate in maintaining proliferation and/or survival of primitive ectoderm cells. LIF may also maintain primitive ectodermal cells undifferentiated for appropriate decision-making during embryonic development.

4.6 A model of P19 cell differentiation.

The data presented in this report, taking into consideration current litterature, can be incorporated in the process of P19 cell differentiation into muscle as follows. Cell adhesion molecules mediate aggregation and directly trigger Brachyury expression or indirectly activate Brachyury through a mesoderm inducing like-factor(s) activated upon aggregation. In this perspective, the F9 cell line of embryonal carcinoma cells has been

shown to express the mRNA encoding BMP-4 (Rogers et al., 1992). It is possible that the induction of Brachyury expression by cell aggregation might be mediated by the BMPs synthesized by the P19 cells.

Although, cell aggregation induced expression of Brachyury mRNA, differentiation does not subsequently occur unless the aggregates are exposed to DMSO (McBurney et al., 1982). Preliminary results (Morley, Vanderhyden, and McBurney, personal communication) seem to indicate that treatments of P19 cells with DMSO result in the release of Ca^{2+} from intracellular stores. It was postulated that DMSO may act by activating Ca^{2+} or calmodulin-dependent protein kinases or phosphatases that modify specific proteins and alter their activity. In this perspective, DMSO may modify specific yet unknown protein(s) that act in concert with the Brachyury protein. It is also possible that DMSO directly modifies the Brachyury protein and as such, DMSO would enhance the cell's response to the Brachyury protein.

It is known that P19 cells become irreversibly committed to differentiation into muscle after two days of aggregation and exposure to DMSO (Edwards et al., 1983). Interestingly, this coincides with a transient Brachyury expression. Hence, it is possible that the Brachyury protein once modified by DMSO, plays an important role in committing P19 cells to differentiation. In support of this hypothesis, the expression of a marker of undifferentiated P19 cells, oct-3 is down-regulated by day three. Following commitment to differentiation, muscle specific genes are activated by specific yet unknown proteins. Preliminary data indicate that Brachyury protein may be localized in

the nucleus suggesting that mechanism of Brachyury has to do with regulation of gene transcription.

During P19 cell differentiation, epithelial cells appear outside of each aggregate and subsequently muscle cells are formed within the interior (Smith et al, 1987). It is believed that the epithelial layer is necessary for the formation of muscle cells. Perhaps the epithelium layer secretes factors that induce differentiation of the underlying cells (Smith et al., 1987). Supporting this hypothesis, a factor secreted by certain endoderm-like cells have been shown to induce differentiation of aggregated P19 cells (van den Eijnden-van Raaij et al., 1991). Brachyury is not expressed in the somites but the expression of Brachyury is required for the specification of the somites. Therefore, during P19 cell differentiation, we expect Brachyury to be expressed in the outer layer of an aggregate. As such, Brachyury expressing cells would be involved in specifying muscle formation in the aggregate interior while the Brachyury expressing cells are fated to become mesenchymal cells.

4.7 Ectopic expression of Brachyury leads to spontaneous differentiation.

Ectopic expression of Brachyury in P19 cells was sufficient to trigger spontaneous differentiation into skeletal muscle and neurons. Neuronal cells are produced in P19 clones expressing high Brachyury levels. In a developing embryo, Brachyury is expressed at highest levels in the notochord and the notochord is involved in inducing the neural plate (precursor of the nervous system). Thus, the development of neurons may be due

to the inductive effect of notochord like-cells in these cultures. It will be important to determine whether P19(Brachyury) clones express markers found in the notochord.

These cultures might induce neuronal cells by secreting neuronal inducing factors. Towards answering that question, conditioned media from P19(Brachyury) clones may induce differentiation of P19 cells.

4.8 Future directions.

P19 cells provide an excellent cell system to study the role of Brachyury gene in mesoderm formation. Several important experiments can be done. Knowledge of the subcellular localization of Brachyury protein is required to unveil its function. Raising antibodies to Brachyury protein will enable us to answer that question. Moreover, we will be able to assess the localization of Brachyury within an aggregate in the attempt to learn whether Brachyury is found in the outer or in the inner layers. Also, we will be able to see if the localization is modified upon DMSO treatment. If Brachyury protein interacts with other proteins, such protein complexes might be detected by immunoprecipitation.

On the other hand, we are in a good position to unveil the chain of events from undifferentiated P19 cells to primary mesoderm induction and ultimately to muscle determination by identifying gene products triggering Brachyury expression via the cloning of its promoter as well as genes activated by the Brachyury gene.

Appendix A.

Id-1 is transiently expressed during P19 cell differentiation into mesoderm.

A.1 Introduction:

In mammals, myogenesis i.e. skeletal muscle formation is believed to be activated in part by the MyoD family of proteins which includes MyoD, myogenin, myf-5 and MRF-4. These proteins possess a basic region which is believed to mediate DNA binding and a helix-loop-helix (HLH) domain though to be the dimerization domain. The HLH domain consists of two segments that can form amphiphatic alpha helices connected by a non-conserved loop. The HLH domain is related to the myc genes and so, MyoD belongs to the myc superfamily of proteins. This family includes several gene products such as AS-C, twist, hairy, and (daughterless involved in sex determination and neurogenesis in Drosophila) as well as two proteins that are ubiquitously expressed, in mammals E12 and E47 (reviewed by Olson, 1990; Weintraub et al., 1991).

The HLH domain has been shown to mediate oligomerization by HLH-containing proteins. Homodimers as well as heterodimers between MyoD proteins and E12 or E47 are formed and are believed to be crucial in muscle formation. Once oligomerization has occurred, the protein complex via their basic domains, bind specific control regions called E boxes in muscle specific genes such as muscle creatine kinase and activate the expression of these genes. It has been demonstrated that combined as a heterodimer (E12 or E47 with MyoD), this protein complex binds with greater affinity to the E box than

either one as a homodimer. Hence, it has been postulated that the specificity and modulation of transcriptional control by HLH proteins is a function of combinatorial interactions (reviewed by Olson, 1991; Weintraub et al., 1991).

A novel family of HLH proteins has recently been identified called Id for inhibitor of DNA binding (Benezra et al., 1990). Three members of the Id family have been identified so far in the mouse: Id-1, Id-2 (Sun et al., 1991) and Id-3 (Christy et al., 1991). These proteins are believed to be negative regulators of HLH proteins. Id proteins have a HLH domain but lack a basic domain. They have been shown to form heterodimers with E12 or E47 and also but with less affinity with MyoD proteins. Since Id lacks a basic domain, the heterodimers they form with E12 or E47 are not able to bind DNA and consequently, these protein interactions prevent E12 or E47 from forming active heterodimers with MyoD; thus, muscle gene expression is inhibited.

During myogenesis, Id is expressed in myoblasts but not in myotubes (Benezra et al., 1990; Sun et al., 1991), and it has been postulated that Id myoblasts inhibits the expression of the terminal differentiation program until the appropriate stimulus is received by the cells. It appears then that during muscle development Id expression constitutes another important level of control but very little is known about the regulation of Id expression.

A.2 Results:

We investigated Id-1 mRNA expression in P19 cells treated with DMSO to gain

information on its pattern of expression. As shown in fig. 18, Id-1 mRNA was assessed by Northern blotting analysis on P19 cells treated with DMSO. Id-1 mRNA expression was detected at low levels in untreated P19 cells (D0) and in cells treated for one day with DMSO. Id-1 mRNA expression was enhanced by day 2 to reach its maximum by day 4. As soon as muscle specific actin isoform expression starts on day 5, the expression of Id-1 declined. Id-1 mRNA expression is not activated when the cells are aggregated in the absence of DMSO.

A.3 Discussion:

In differentiating P19 cells there is a rise and fall in Id-1 expression. Little expression is evident in cultures expressing muscle specific genes. These observations correlate with what has been documented during murine gastrulation (Olson, 1990; Weintraub, 1991). The fact that Id-1 mRNA expression is transient constitutes an interesting observation since it implies that the expression of Id-1 must be developmentally regulated. Id may help coordinate a range of developmental decisions to control commitment to differentiation and/or timing of differentation.

Fig. 18 **Id-1 expression in DMSO-induced differentiating P19 cells.**

Total RNA was extracted from P19 cells at daily intervals following induction of differentiation with 0.8% DMSO and analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with an Id-1 DNA probe then stripped and (B) re-hybridized with with an actin DNA probe as described in Material and methods. Lanes 1-7: days of differentiation numbered consecutively after the first day of treatment. Lane 8: P19 cells aggregated 2 days without drug. The sizes of the transcripts were: Id-1, 1.1 kb; actin probe hybridizes to both cytoskeletal (β and γ) at 2.1 kb and striated muscle (α) actins at 1.7 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

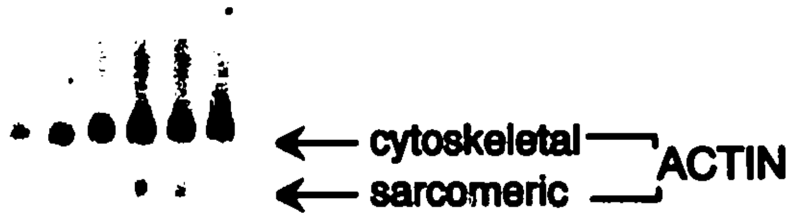
A 1 2 3 4 5 6 7 8

28 S —

18 S —

←ID-1

B



D0 D1 D2 D3 D4 D5 D6 D2 Aggr.

P19 + DMSO

Appendix B.

The expression of protein kinase A subunit remains constant during P19 cell differentiation into muscle.

B.1 Introduction:

The second messenger cAMP is a mediator of various hormonal signals present in many cells and tissues. As reviewed by Roesler and Vandenbark, (1988) and Cho-Chung (1990), the primary mediator of cAMP action in eukaryotic cells is cAMP-dependent protein kinase also named protein kinase A (PKA). PKA is composed of two genetically distinct catalytic (C) subunits and regulatory (R) subunits. There are two classes of PKA designated type I and type II which contain distinct R subunits (I or II) but share a common C subunit. There are also an alpha and a beta form for each subunit (i.e. RIα, RIβ, RIIα, RIIβ, Cα, Cβ). The R subunits contain two cAMP binding domains. On binding to the R subunits, cAMP induces conformational changes that dissociate holoenzyme R_2C_2 into an $R_2(cAMP)_4$ dimer and two free C subunits that are catalytically active. Regulation of cellular processes is then affected through the phosphorylation of specific target proteins such as CREB (cAMP response element binding protein) and their subsequent binding to CRE (cAMP response element)-containing genes (Roesler et al., 1988; Cho-Chung, 1990).

Other investigators (reviewed by Nagamine and Reich, 1985; Constantinou et al., 1985) believe that dissociation of PKA by cAMP activates not only the C subunits but

also the R subunits. In this model the R subunits are capable of modulating transcription by interacting with regulatory DNA sequences.

Differential expression of PKA isozymes has been observed during cell development, differentiation and neoplastic transformation. It is generally believed that PKA type I is associated with cell growth and transformation whereas PKA type II is implicated in cell differentiation (Cho-Chung, 1990).

B.2 Results:

Based on the information presented above and on the fact that cAMP affects Brachyury T expression, we investigated the expression of PKA subunits as well as CREB during P19 cell differentiation into mesoderm in order to gain more information on the involvement of the cAMP/PKA pathway during mesoderm induction.

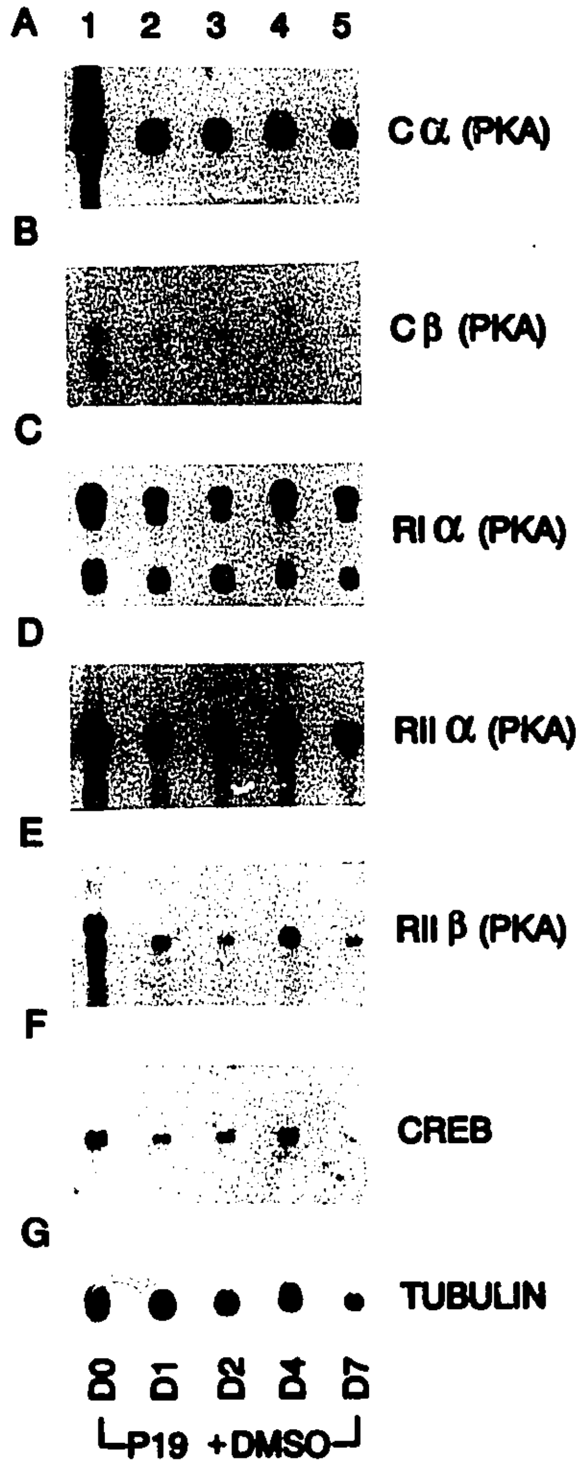
As shown in fig. 19, Northern blotting analysis was performed on RNA from differentiating P19 cells. The mRNA levels of all the PKA subunits assessed as well as of CREB remain constant throughout the differentiation regime. The second band found in P19 D0 (lane 1) for Cα (panel A), Cβ (panel B), RIIα (panel D) and RIIβ (panel E) is artifactual and was not seen in other experiments.

B.3 Discussion:

P19 cells contain abundant cα, RIα and RIIα transcripts. We cannot make any conclusion on the role of PKA subunits during mesoderm induction based on their pattern

Fig. 19 **CREB and PKA subunit expression in differentiation of P19 cells.**

Poly A^+-selected RNA was prepared from P19 cells following induction of differentiation with 1% DMSO and analyzed by Northern blotting. (A) The RNA blot (10μg/lane) was first hybridized with a Cα DNA probe and consecutively stripped and re-hybridized with: (B) Cβ DNA probe; (C) RIα DNA probe; (D) RIIα DNA probe; (E) RIIβ DNA probe; (F) CREB DNA probe; (F) α-tubuline DNA probe as described in Material and methods. Lanes 1-5: days of differentiation numbered after the first day of treatment.


A
1
2
3
4
5
Cα (PKA)
B
Cβ (PKA)
C
RIα (PKA)
D
RIIα (PKA)
E
RIIβ (PKA)
F
CREB
G
TUBULIN
D0
D1
D2
D4
D7
P19 +DMSO

of mRNA expression since they remain constant. However, we cannot exclude simply on these observations that PKA is not involved in this process. Other levels of controlare present that could differentially modify the cAMP/PKA pathway activity: the transcription, the translation and the enzyme activity of all the proteins involved in the cAMP/PKA pathway are all possible levels of control that ultimately could affect the activity of this pathway. In this regard, it is possible that modulation of the PKA subunit mRNA is only very brief and/or restricted to a subpopulation of the differentiating cells. By the method we used, subtle differences could not have been detected.

Appendix C.

c-kit mRNA levels are transiently increased during P19 cell differentiation into muscle.

C.1 Introduction:

The development of multicellular organisms requires mechanisms for the determination of cell fate; more and more evidence suggests that cell-cell interactions play a major role in this process as well as in the differentiation of specific cell types during embryogenesis. Recently, it has become apparent that association of extracellular ligands with receptors that have intrinsic protein-tyrosine kinase activity regulates cell fate during mammalian development (reviewed by Pawson and Bernstein, 1990). Evidence for the roles played by receptor tyrosine kinase (RTK)-mediated signalling pathways in mammalian development are provided in part by strains of mice bearing mutant alleles at *Dominant white spotting (W)* (Little, 1915) or *Steel (Sl)* (Sarvella and Russell, 1956) loci. Mutations at either the W or Sl locus result in a broad spectrum of defects affecting both development and in the blood-forming system in adult life (Russell, 1979). The most severe W or Sl mutations produce homozygotes that are characterized by a partial or complete lack of hair pigmentation, a severe macrocytic anaemia that can be responsible for perinatal death, mast cell deficiency and sterility. W or Sl heterozygotes have varying degrees of white spotting, and occasionally anaemia and reduced fertility. These cellular defects in W mutants are due to an inability of

haematopoietic stem cells, melanoblasts and primordial germ cells to proliferate, migrate and/or survive during development (Russell, 1979).

It has been shown that in W mutants the defect in all three lineages is cell-autonomous, indicating that the W gene product is expressed and required by early stem cells within these three lineage. On the other hand, the Sl lesions perturb some aspect of the microenvironment that supports the growth and differentiation of these three lineages (reviewed by Pawson and Bernstein, 1990). Hence, the identical phenotypes induced by W or Sl mutations and the complementary sites of action of these mutations (in the stem cells or their microenvironment) provide evidence that the signalling pathway involved in this phenomenon is essential in mediating appropriate cell-cell interactions in development.

The W locus related-mutations are attributed to loss of function mutations in the c-kit receptor tyrosine kinase that result in diminished or undetectable levels of c-kit-associated kinase activity, while Sl mutants have been found to bear mutations in a kit ligand also called steel or mast cell growth factor, stem cell factor or Kit ligand (reviewed by Reith et al., 1990).

In F9 teratocarcinoma cells it has been shown that c-kit is expressed when the cells are induced to differentiate by RA in conjunction with db-cAMP into parietal endoderm indicating that the expression of c-kit is regulated by the level of intracellular cAMP in differentiated F9 cells induced by RA (Nishina et al., 1992).

C.2 Results:

We investigated the expression of c-kit gene in DMSO-induced differentiation of P19 cells in order to better understand the function of this gene in the differentiation cascade. In P19 cells differentiating into mesodermal, c-kit mRNA was transiently elevated on day 3 as observed by Northern blotting analysis (fig. 20). c-kit mRNA was triggered when the cells are simply aggregated for two days in the absence of DMSO (data not shown). Also, P19 cells stably transfected with the Brachyury expression vector maintained c-kit expression seven days after initiation of the differentiation regime done with or without DMSO (data not shown). There was no correlation between the levels of c-kit expression and the levels of Brachyury mRNA expressed by each P19(Brachyury) clone. However, there was a marked increase in c-kit expression in the P19(Brachyury) clones that were spontaneously differentiating into neuroectodermal cells, but not in the clones spontaneously differentiating into mesodermal cells (data not shown).

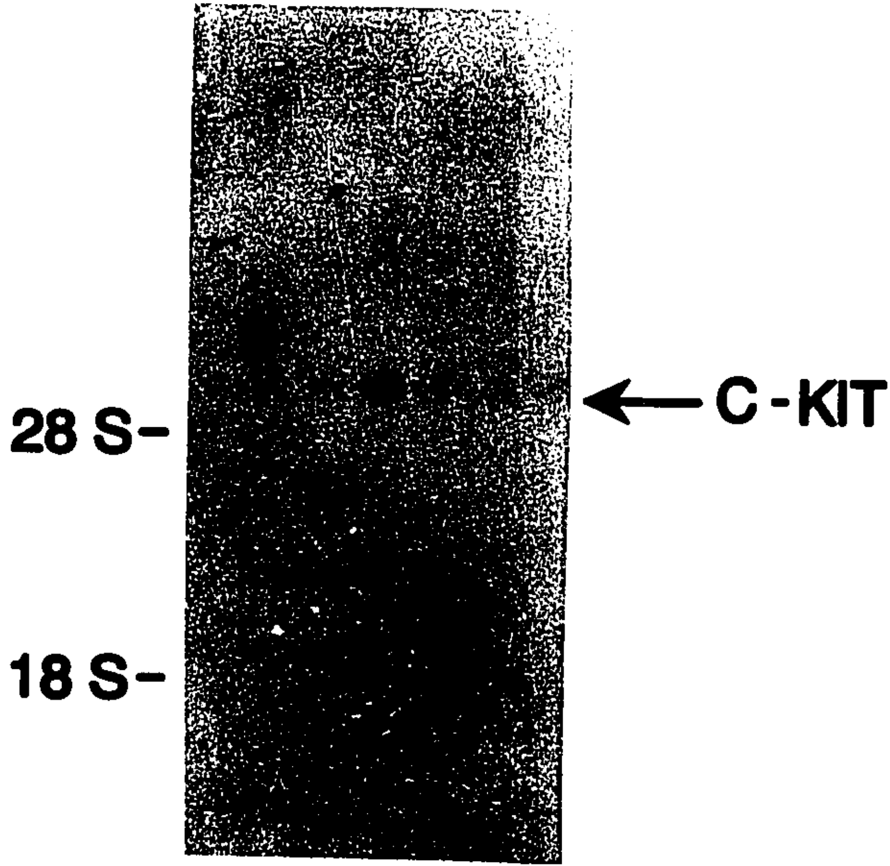
C.3 Discussion:

c-kit was transiently expressed during P19 cells differentiation into mesoderm. Although Brachyury T and c-kit mRNAs were coincidently elevated on day two in the absence of DMSO, in the presence of DMSO the expression of c-kit was delayed to day 3. Therefore, it would be interesting to learn whether c-kit expression is maintained in aggregated P19 cells after three days in the absence of DMSO to better understand the relation between DMSO, Brachyury T and c-kit during differentiation.

Fig. 20 **c-kit 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 analyzed by Northern blotting. (A) The RNA blot (15μg/lane) was first hybridized with a c-kit cDNA probe then stripped and (B) re-hybridized with an α-tubulin cDNA probe as described in Material and methods. Lanes 1-7: days of differentiation numbered consecutively after the first day of treatment. The sizes of the transcripts were: c-kit, 5.8 kb; α-tubulin, 1.9 kb. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

A 1 2 3 4 5 6 7



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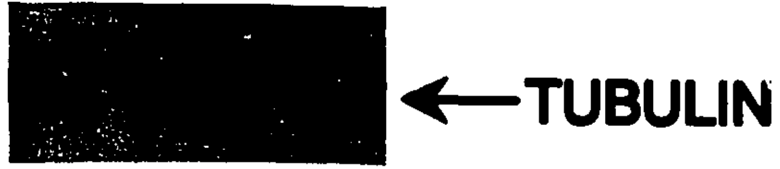

D0 D1 D2 D3 D4 D5 D6

P19 + DMSO

The function of c-kit in P19 cell differentiation remains unknown, it is possible to hypothesize that the c-kit gene product is involved in inducing proliferation, migration and/or differentiation of the cells within an aggregate. The first step towards answering that question would be to learn whether steel is also expressed in differentiating P19 cells.

Appendix D.

Solutions were made up as follows:

Denhardt's solution (50X):

Ficoll	5g
polyvinylpyrrolidone	5g
BSA	5g
water	to 500 ml

Filter-sterilized and store at -20°C.

SSC (20X):

NaCl	175.3g
sodium citrate	8.2g
water	to 1 l

pH adjusted to 7.0 with 10 N NaOH and sterilized by autoclaving.

SSPE (20X):

NaCl	174g
$NaH_2PO_4 \cdot H_2$	27.6g
EDTA	7.4g
water	to 1 l

pH adjusted to 7.4 with 10 N NaOH and sterilized by autoclaving.

Phosphate buffered saline (PBS) (10X):

NaCl	40g
KCl	1g
KH_2PO_4	1g
Na_2HPO_4	5.75g
water	to 500 ml

BES-buffered saline (BBS) (2X):

BES	1.07g
NaCL	1.64g
Na_2HPO_4	0.02g
water	to 100 ml

pH adjusted to 6.95 with 10 N NaOH, filtered-sterilized and stored at -20°C.

RNA gel buffer (10X):

MOPS	46.26g
$NaOAc\text{-}3 \cdot H_2O$	6.81g
EDTA	4.16g
water	to 1 l

pH adjusted to 7.0 with acetic acid, sterilized by autoclaving.

Formamide (deionized):

formamide	1l
Amberlite monobed resin	150g

stired 60 min at RT and filtered twice through Whatman #1 filter paper. Stored at -20°C.

Forskolin (2 mM):

forskolin	12.1 mg
DMSO	to 50 μl

mixed with 14.95 ml ETOH/PBS 50:50 (vol/vol) and filter-sterilized.

3-isobutyl-1-methylxanthine (IBMX) (30 mM):

IBMX	74.8 mg

DMSO to 500 μl

mixed with 9.50 ml ETOH/PBS 50:50 (vol/vol) and filter-sterilized.

3 M lithium chloride / 8 M urea:

LiCl	127.2 g
urea	480g
water	to 1 l

filter sterilize through Whatman #1 paper, add 1 ml diethylpyrocarbonate (DEPC) and let at RT 16 h. Autoclave to destroy the DEPC and store at 4°C.

Lana's fixative:

1) Depolymerized paraformaldehyde solution:

paraformaldehyde	40g
water	300 ml at 58°C

add several drops of 1 N NaOH until the solution clears.

2) Phosphate buffer:

dibasic sodium phophate	17.75g
water	to 250 ml

pH adjusted to 7.1 with 0.5 M monobasic sodium phosphate.

3) Lana's solution:

phosphate buffer	320 ml
saturated picric acid	140 ml

Add the depolymerized paraformaldehyde solution and bring the volume to 1 l with water. Filter through Whatman #1 paper and adjust pH to 6.9 with 1 N HCl.

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