Primitive streak mesoderm-like cell lines expressing *Pax-3* and *Hox* gene autoinducing activities

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**SUMMARY**

Differentiating P19 embryonal carcinoma (EC) cells transiently express an endogenous activity capable of inducing *Pax-3* expression in adjacent P19 stem cells (Pruitt, *Development* 116, 573-583, 1992). In the present study, expression of this activity in mesodermal cell lineages is demonstrated. First, expression of the mesodermal marker *Brachyury* correlates with expression of *Pax-3*-inducing activity. Second, the ability of leukemia inhibitory factor (LIF) to block mesoderm differentiation at two different points is demonstrated and correlated with the inhibition of *Pax-3*-inducing activity. Finally, two mesodermal cell lines that express *Pax-3*-inducing activity were derived from P19 EC cells. Each of these lines expresses high levels of the mesodermal marker *Brachyury* and high levels of *Oct-3/4* (which is down-regulated at early times during mesoderm differentiation) suggesting that these lines are early mesodermal derivatives.

Unlike EC or embryonic stem cell lines, each of the two mesodermal derivatives autoinduces *Hox* gene expression on aggregation even in the presence of LIF. Following aggregation, anterior-specific genes are expressed more rapidly than more posterior genes. These observations directly demonstrate the ability of murine mesodermal derivatives to autoinduce *Hox* gene expression in the absence of signals from other cell lineages. Similar to the *Pax-3*-inducing activity, signals from mesodermal cell lines were sufficient to induce HOX expression in adjacent P19 stem cells in cell mixing assays. These observations are consistent with the previous suggestion (Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumberg, B., Bittner, D. and De Robertis, E. M. (1992) *Cell* 69, 1097-1106) that signals responsible for anterior-posterior organizer activity are localized to the anterior primitive streak mesoderm of the mouse embryo.

Key words: *Pax-3*, *Hox* genes, mesoderm, P19 embryonal carcinoma, embryonic induction

**INTRODUCTION**

Many of the experimental observations on which the understanding of inductive interactions during vertebrate development are based were originally made using amphibian embryos (reviewed by Slack and Tannahill, 1992). In amphibians, induction of dorsal mesoderm and specification of the anterior-posterior axis begin at the time of gastrulation, in Spemann’s organizer region, in response to signals from dorsovegetal blastomeres. Initially, the organizer has a posterior specification; however, the first invaginating mesodermal cells acquire an extreme anterior specification. As the dorsal mesoderm invaginates, the invaginating cells progressively acquire more posterior specifications. A property of even the most anterior dorsal mesoderm is the expression of neural-inducing activity.

Several genes have been isolated that are expressed specifically in dorsal mesodermal lineages of both amphibian and mouse gastrula and are likely to have a role in defining the properties of this tissue. The *Brachyury* gene (Herrmann et al., 1990) is expressed throughout the primitive streak mesoderm in the mouse starting at approximately 6.5 days post-coitum (d.p.c.; Wilkinson et al., 1990) and is required for axial mesoderm formation, particularly notochord (Herrmann, 1991). Abnormal development of the neural tube occurs in regions where notochord formation is disrupted in *Brachyury* T homozygous mutants consistent with an essential role for notochord in neural induction in the mouse (Chesley, 1935).

The homeobox gene *goosecoid* (Blumberg et al., 1991) is expressed specifically in the organizer region of *Xenopus* embryos at the dorsal lip of the blastopore and migrates with the sheet of invaginating dorsal mesodermal cells. Additionally, injection of *goosecoid* mRNA into ventral mesoderm is sufficient to induce a complete secondary body axis suggesting that this gene has a central role establishment of Spemann’s organizer activity (Cho et al., 1991). *Goosecoid* mRNA is also expressed in the mesoderm of the mouse and is first detected at the site where primitive streak formation first occurs (Blum et al., 1992). As the primitive streak develops, *goosecoid* expression occurs specifically in the anterior region of the streak over a short period (approximately 6.4 to 6.9 d.p.c.). Further, this region of the mouse embryo has also been shown to have organizer-like activity when transplanted into *Xenopus* embryos. Based on these observations, it has been suggested that expression of *goosecoid* in the mouse gastrula reflects the localization of the murine equivalent to Spemann’s organizer of amphibians (Blum et al., 1992). If this is the case, anterior
primitive streak mesoderm of the mouse might be expected to express both a neural-inducing activity and activities capable of inducing genes responsible for specifying aspects of the anterior-posterior body axis.

Differentiating P19 EC cells express endogenous activities capable of inducing both Pax-3 and neuroectoderm in adjacent P19 stem cells (Pruitt, 1992). These activities are closely associated and may be expressed by the same cell type. In the mouse embryo, Pax-3 is first expressed in the dorsal neural tube at early times during its differentiation and in the adjacent segmented dermomyotome (Goulding et al., 1991). The proximity of the neuroectodermal and mesodermal cells, which express Pax-3 in the embryo, is consistent with a single source, possibly the dorsal mesoderm, for the activity that induces Pax-3 expression in each of these lineages. The initial objective of the present work was to determine the relationship between mesoderm differentiation and the expression of Pax-3-inducing activity in differentiating P19 cells. Results of these studies demonstrate that one source, and possibly the only source, of Pax-3-inducing activity in these cells is a mesodermal derivative. Further, during the course of these studies, mesodermal cell lines that express high levels of Pax-3-inducing activity were isolated. Characterization of these cell lines demonstrates that they also express markers specific to anterior primitive streak mesoderm and activities capable of autoinducing Hox gene expression.

**MATERIALS AND METHODS**

**Tissue culture, mixing assays and immunofluorescence**

The P19S18O1A1 (McBurney et al., 1982) and EH3 (Pruitt, 1992) cell lines have been described previously and were cultured and treated with 1.0% DMSO, $1 \times 10^{-6}$ M RA, or $1 \times 10^{-6}$ M RA as aggregates as described in Pruitt and Natoli (1992). GCLB cells were derived from P19S18O1A1 cells as described below and cultured and treated with RA under similar conditions. LIF was prepared and used at a concentration of 100 units/ml and RA was prepared and used at concentrations described in the text as described in Pruitt and Natoli (1992). Mixing assays using the EH3 cell line as a reporter for Pax-3-inducing activity in the presence of 100 unit/ml LIF were performed as described in Pruitt (1992). For immunofluorescence studies, mixed aggregates were fixed by treatment with methanol at $-20^\circ$C and stained using either a 1/200 dilution of rabbit anti-HOXC-3(3.1) antisera (Awgulewitsch and Jacobs, 1990) or a 1/200 dilution of rabbit anti-HOXB-5(2.1) antisera (Wall et al., 1992) plus a 1/200 dilution of mouse anti-β-galactosidase antisera (Sigma) and primary antibodies were detected using 1/200 dilutions of goat anti-rabbit-TRITC (Sigma) and goat anti-mouse-FITC (BM) conjugates using techniques essentially as described previously (Pruitt and Natoli, 1992).
Northern and Southern blot analysis

For northern blot analysis, RNAs were extracted, electrophoresed on 1.4% agarose-formaldehyde gels, transferred to nylon membranes and probed using the method of Church and Gilbert (1984) as described in Pruitt and Natoli (1992). Hybridizations were performed at 68°C. For Southern blot analysis, Brachyury-transfected cell lines were electrophoresed on 0.85% agarose gels, transferred to nitrocellulose filters and probed using a formamide-dextran sulfate hybridization method as described in Pruitt and Reeder (1984). Autoradiographic exposures were performed using intensifying screens at −70°C for times indicated in the Figure Legends.

Probe fragments used for northern blot analysis were: Brachyury, a 1.8 kb EcoRI fragment from the plasmid pSK75 (Herrmann et al., 1990); Oct-3/4, a 450 bp EcoRI to HindIII fragment from the plasmid pG2F9 (Rosner et al., 1990); Xenopus laevis goosecoid, a 1.1 kb EcoRI to XhoI fragment from the plasmid pH7 (Cho et al., 1991); mouse goosecoid, a 0.9 kb PstI to XhoI fragment from the plasmid pmGSC-2 (Blum et al., 1992); RAR-β, a 1.3 kb SmaI to BamHI fragment from the plasmid pmKAR-β (Zelent et al., 1989); Hoxa-1(1.6), a 600 bp HpaI fragment from the plasmid pHox1.6 (Baron et al., 1987); Hoxa-3(1.5), a 478 bp PstI fragment from the plasmid pGHHB (Fainsod et al., 1987); Hoxc-8(3.1), a 320 bp XhoI fragment from the plasmid pHox1.6 (Baron et al., 1991); Smad, a 0.48 kb BamHI to PvuI fragment from the plasmid pSMad (Smith et al., 1992); Mos-I, a 2.2 kb EcoRI fragment from the plasmid pBM-Mos (Candia et al., 1992) and triosephosphate isomerase (TPI), a 320 bp EcoRI fragment from the plasmid pMo-EA (Awgulewitsch et al., 1986); Pax-3, a 1.8 kb EcoRI to Sall fragment from the plasmid pBS-Pax-3 (Goulding et al., 1991); Mos-1, a 1.3 kb BamHI fragment from the plasmid pC1 (Pruitt et al., 1990). The nodal-specific probe was prepared by rt-PCR using primers and conditions described in Zhou et al. (1993) except that mRNA was derived from GCLB cells.

Plasmid construction, transfections and isolation of cell lines

pcDXlgooseoid was constructed by insertion of a 1.1 kb XhoI to KpnI fragment from the plasmid pH7, containing the Xenopus gooseoid gene, into the XbaI and KpnI polylinker sites of pcDpoly (Pruitt, 1988).

Stable transfection of P19 EC stem cells was performed by a calcium-phosphate-mediated transfection method (Wigler et al., 1979) using a 10:1 ratio of pcDXlgooseoid or pcDpoly to pSV-neoXh. Transfected cells were trypsinized and replated to 400 µg/ml G418 (Sigma) in either the presence or absence of 100 units/ml LIF and selected for a period of two weeks prior to isolation of colonies. Following demonstration of Pax-3-inducing activity in GCLB cells as described above, these cells were recloned prior to use in additional experiments. Transient transfections were performed using similar methods except that pPgk-CAT was substituted for pSV-neoXh. Chloramphenicol-acetyl transferase assays were performed as described previously (Gorman et al., 1982).

RESULTS

Expression of Pax-3-inducing activity correlates with expression of the mesodermal marker Brachyury

Expression of endogenous activities capable of inducing both Pax-3 expression and neuroectoderm differentiation in adjacent cells has been demonstrated previously in differentiating P19 cells treated with either 1% dimethylsulfoxide (DMSO) or 1×10⁻⁶ M retinoic acid (RA; Pruitt, 1992). To determine if mesodermal cell derivatives are present at appropriate times to account for expression of Pax-3-inducing activity, the expression of Brachyury transcripts was assayed at various times following treatment of P19 cell aggregates under each of these conditions (Fig. 1). Brachyury transcripts are first detected on day 3 following DMSO treatment. Expression is transient, peaking on day 4, and is nearly undetectable by day 12. A similar pattern of expression is seen in cells treated with 1×10⁻⁶ M RA except that the induction is more rapid and peaks by day 2. Since Brachyury expression is specifically restricted to primitive streak mesoderm when first detected on day 6.5 of mouse development (Herrmann, 1991), these results suggest that primitive streak mesoderm-like cells are present transiently in differentiating P19 EC cell cultures following treatment with either DMSO or RA. Additionally, the temporal expression of Brachyury transcripts correlates with that of the Pax-3 and neuroectoderm-inducing activities characterized previously (Pruitt, 1992). In DMSO-treated cells, expression of these activities is absent on day 2, peaks by day 4 and declines subsequently. In RA-treated cells, induction of the activities occurs more rapidly and is already present by day 2. These observations are consistent with the possibility that primitive streak mesoderm-like cells present in these cultures express the Pax-3- and neuroectoderm-inducing activities.

Effect of leukemia inhibitory factor on mesodermal differentiation in DMSO and RA-treated P19 cell cultures

Previous studies have demonstrated that leukemia inhibitory

Fig. 2. Expression of Pax-3-inducing activity in a Xenopus gooseoid transfected cell line. (A) Phase-contrast photomicrograph of a mixed aggregate containing EH3 cells and GCLB cells following staining for β-galactosidase activity. Photograph was taken magnification of ×100. (B) Bright-field photomicrograph of the field shown in A at the same magnification.
factor (LIF) blocks differentiation of the mesodermal lineages formed following treatment of P19 EC cells with DMSO (Pruitt and Natoli, 1992). Additionally, when LIF is present during either DMSO or RA treatment of P19 cell cultures, these cultures fail to express Pax-3-inducing activity consistent with the possibility that mesodermal lineages express the activity (Pruitt, 1992). The effect of LIF on Brachyury expression was determined for each of these conditions (Fig. 1). In the case of DMSO plus LIF-treated cultures, Brachyury expression is nearly completely inhibited. In contrast, when LIF is present during RA treatment, Brachyury expression is not inhibited and the peak level of expression occurs at 2 days following RA treatment in each case. However, down-regulation of Brachyury expression is much slower in RA-treated cultures when LIF is present.

In mouse embryos, Oct-3/4 is expressed in primitive ectoderm and is down-regulated during differentiation in both mesodermal and neuroectodermal lineages (Scholer et al., 1990). Down-regulation in the mesoderm occurs at approximately the time that Brachyury expression is induced on day 6.5. Oct-3/4 is also down-regulated in P19 EC cells by approximately 4 fold following treatment with 1% DMSO and approximately 10 fold following treatment with 1×10⁻⁶ M RA (Fig. 1, quantitations were made by densitometry and corrected for loading differences using TPI as a reference). The residual levels of Oct-3/4 expression at later times under each of these conditions are likely to reflect the continued presence of undifferentiated stem cells and, consistent with this explanation, 1×10⁻⁶ M RA treatment results in differentiation of a higher proportion of P19 cells than does 1% DMSO treatment (McBurney et al., 1982). The effect of LIF on Oct-3/4 expression in DMSO- and RA-treated cultures is also shown in Fig. 1. Consistent with the failure to induce Brachyury expression when LIF is present in DMSO-treated cultures, Oct-3/4 expression is not down-regulated. In RA-treated cultures in the presence of LIF, Oct-3/4 expression is slightly down-regulated (less than a factor of 2) but not as completely as in the absence of RA alone. Decreased Oct-3/4 expression under this condition may reflect the ability of 1×10⁻⁶ M RA to induce differentiation of neuroectodermal lineages even in the presence of LIF (Pruitt and Natoli, 1992). These observations suggest that LIF blocks progression along the mesoderm differentiation pathway in DMSO-treated cultures at a point prior to the induction of Brachyury and in RA-treated cultures at a point subsequent to the induction of Brachyury but prior to either induction of the Pax-3-inducing activity or down-regulation of Brachyury or Oct-3/4 expression.

Isolation of a cell line expressing Pax-3-inducing activity

Expression of the homeobox gene goosecoid in ventral mesoderm is sufficient to induce a complete secondary axis in Xenopus (Cho et al., 1991). Additionally, expression of this gene is localized to the earliest anterior mesoderm in the mouse (Blum et al., 1992). Based on these observations, the ability of goosecoid expression to direct differentiation of P19 stem cells towards mesodermal lineages was tested by stably transfecting P19 stem cells with a Xenopus goosecoid expression vector. These experiments resulted in the isolation of a mesodermal cell line that expresses Pax-3-inducing activity. However, additional experiments described below suggest that this isolation may have been fortuitous since the cell line does not carry the transfected Xenopus goosecoid gene and additional experiments have shown that ectopic expression of the Xenopus goosecoid gene is neither sufficient nor required to establish this phenotype (data not shown). Nonetheless, isolation of this line demonstrates that a mesodermal derivative is at least one source of the Pax-3-inducing activity and its isolation and characterization are described here. Six stable lines of P19 cells transfected with the expression vector containing the Xenopus goosecoid gene (pcD-Xlgoosecoid), and four additional control lines transfected with the vector alone (pcDpoly), were established. Each of these cell lines was tested for Pax-3-inducing activity using a cell mixing assay. This assay takes advantage of a reporter cell line (EH3) described previously in which the endogenous Pax-3 gene is marked by fusion to lacZ (Pruitt, 1992). These cells are derived from P19 EC stem cells and, like the parental line, are pluripotent. Results of this assay demonstrated that one of the cell lines (GCLB) transfected with pcD-Xlgoosecoid shows a high level of Pax-3-inducing activity. Photomicrographs of one of the mixed aggregates are shown in Fig. 2A and B and demonstrate that the pattern of β-galactosidase expression is consistent with induction of Pax-3 expression only at the boundaries between GCLB and EH3 cells. The other pcD-Xlgoosecoid transfected and control lines show no activity in this assay.

Cells expressing Pax-3-inducing activity also express markers characteristic of primitive streak mesoderm

The morphology of GCLB cells is altered, and stage-specific embryonic antigen-1 (SSEA-1)-specific immunofluorescence is reduced relative to P19 or EH3 stem cells (data not shown) suggesting that these cells have undergone a differentiation event. Further, unlike P19 stem cells, GCLB cells are not pluripotent and do not give rise to neuroectodermal lineages when treated with 1×10⁻⁶ M RA (data not shown). Finally, in both monolayer and aggregate cultures, either in the presence or absence of LIF, GCLB cells express high levels of

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**Fig. 3.** GCLB cells are an early mesodermal derivative. RNA was extracted from EH3 (E) stem cells and GCLB (G) cells following growth as either monolayer (M) or aggregate (A, harvested on day 6 of aggregation) cultures in the presence of 100 units/ml LIF and 20 μg/sample was analyzed for the expression of Brachyury (exposed for 18 hours), Oct-3/4 (exposed for 6 hours), Pax-3 (3.3 and 3.6 kb, exposed for 10 days), and TPI (exposed for 2 days) transcripts as indicated in the figure.
Brachyury transcripts demonstrating that this line is a mesodermal derivative (Fig. 3). The level of Brachyury expression in GCLB cells is approximately 3-4 fold higher than the peak levels of expression observed in differentiated cultures of P19 or EH3 cells treated with either RA or DMSO (data not shown). The expression of Oct-3/4 at levels equivalent to those expressed by undifferentiated stem cells is also detected in GCLB cells under the same conditions (Fig. 3). Expression of both Brachyury and Oct-3/4 suggests that GCLB cells are equivalent in many of their properties to early primitive streak mesoderm of the mouse embryo.

In addition to its expression in the dorsal neural tube, Pax-3 is expressed mesodermally in the adjacent segmented dermomyotome in the mouse embryo (Goulding et al., 1991). Further, Pax-3 expression has been demonstrated in P19 cells following treatment with DMSO (Pruitt, 1992), a condition where neuroectodermal lineages are not formed efficiently (McBurney et al., 1982). These observations demonstrate that mesodermal lineages can express Pax-3. Since GCLB cells express Pax-3-inducing activity, it was determined if these cells also express the Pax-3 gene itself for both monolayer and aggregate cultures in the presence of LIF (Fig. 3). Pax-3 transcripts are not detected in monolayer cultures. However, when GCLB cells are aggregated, a low level of Pax-3 transcription is detected. One explanation for this observation is that the Pax-3-inducing activity expressed by GCLB cells acts by autoinduction to induce Pax-3 expression in these cells but this induction requires growth of the cells as aggregates to allow either the close proximity of cells or restricted diffusion of a soluble factor.

**Induction of Hox gene expression in primitive streak mesoderm-like cells by an endogenous activity and comparison to induction by RA**

To determine if the primitive streak mesoderm-like cell line GCLB expresses activities capable of inducing Hox gene expression, the expression levels of the anterior gene Hoxa-1(1.6), the intermediate gene Hoxa-3(1.5), and the posterior gene Hoxc-8(3.1) were determined for monolayer and aggregate cultures of GCLB cells grown in the presence of LIF and compared to the EH3 stem cell line grown under similar conditions (Fig. 4). Both the revised nomenclature for vertebrate Hox genes proposed in Scott (1992) and earlier nomenclatures (in parentheses) are used here. Similar to the case for Pax-3, expression from each of the Hox genes is detected at higher levels in aggregate than monolayer cultures. A low level of the anterior gene Hoxa-1(1.6) is detected in monolayer cultures and is increased by a factor of approximately 4 in aggregate cultures. Expression of neither the intermediate gene Hoxa-3(1.5) nor the posterior gene Hoxc-8(3.1) is detected in monolayer cultures but each is induced in aggregates. The expression of each of these genes at different times following aggregation was also determined (data not shown) and demonstrated that Hoxa-1(1.6) reaches maximum levels by days 1-2, Hoxa-3(1.5) expression is induced between days 3 and 4, and Hoxc-8(3.1) expression is induced between days 4 and 6 of growth as aggregates. The levels of expression of these genes observed on day 6 do not increase further through at least day 8 even though studies described below demonstrate that the more posterior genes are expressed at less than the maximum levels obtainable by RA treatment.

To determine if GCLB cells express signals capable of inducing Hox gene expression in adjacent stem cells, mixed aggregates between GCLB cells and the Pax-3/LacZ marked EH3 stem cell line were assayed for co-expression of β-galactosidase and either HOXC-8(3.1) or HOXB-5(2.1) using double immunofluorescence. Results for HOXC-8(3.1) are shown in Fig. 5 where the mixed aggregate, shown using bright-field optics in panel A, was stained for β-galactosidase in panel B and HOXC-8(3.1) in panel C and demonstrates that β-galactosidase-expressing EH3 cells are present in the HOXC-8(3.1) expression domains, which are typically present near the centers of the aggregates. β-galactosidase-expressing EH3 cells were also detected in regions of mixed aggregates expressing HOXB-5(2.1) (data not shown). No expression of β-galactosidase, HOXC-8(3.1) or HOXB-5(2.1) was detected in aggregates of EH3 cells in the absence of GCLB cells. To determine if the same cells express both β-galactosidase and HOXC-8(3.1) in mixed aggregates, aggregates were trypsinized and individual cells were assayed at higher magnification (panel D, β-galactosidase, and panel E, HOXC-8, 3.1). These studies demonstrate that approximately 60% of the β-galactosidase-expressing cells also express HOXC-8(3.1) and a higher proportion (over 80%) co-express HOXB-5(2.1). These results demonstrate that GCLB cells express an activity capable of inducing Hox gene expression in adjacent stem cells and, as suggested for induction of Pax-3 expression, Hox gene expression in GCLB cell aggregates may result from autostimulation by a Hox gene inducing activity.

To determine the efficiency of Hox gene expression induced by the endogenous activity, the level of Hox gene expression in aggregate cultures grown in the presence of increasing concentrations of RA was determined and compared to the level of expression of Hox genes in P19 stem cells grown under similar conditions (Fig. 6). Additionally, expression of the early RA response gene retinoic acid receptor β (RAR-β) was assayed. Similar to previous reports for other EC stem cell lines, P19 stem cells show little expression of Hoxa-1(1.6), a-3(1.5), or c-8(3.1) in the absence of RA. RA treatment induces expression of each of these genes. In contrast, in aggregate cultures of GCLB cells, the anterior gene Hoxa-1(1.6) is expressed at high levels even in the absence of exogenously added RA and its expression is not induced further by RA treatment. Expression of the posterior gene Hoxc-8(3.1) is low, but detectable, in the absence of RA and is induced by increasing RA concentrations. The level of Hoxc-8(3.1) expression in untreated aggregates is approximately 10% of its
maximum level in cells treated with $1 \times 10^{-6}$ M RA. *Hoxa-3*(1.5), which is expressed in the embryo at positions intermediate to those of *Hoxa-1*(1.6) and *Hoxc-8*(3.1), is also constitutively expressed in GCLB cell aggregates. However, only the smaller of the three major transcripts of 2.7, 3.3 and 3.5 kb that are observed following treatment with RA is expressed in aggregated GCLB cells. The level of expression of the 2.7 kb transcript in aggregated GCLB cells is approximately 30% of its maximum level in cells treated with $1 \times 10^{-6}$ M RA.

**Mesodermal cell lines expressing Pax-3 and Hox gene inducing activities can be isolated from chemically induced P19 EC cell cultures**

To test the possibility that cells at early stages of mesodermal differentiation could be isolated by growth at low density, where additional signals leading to the fully differentiated mesodermal phenotypes normally found following differentiation as aggregates would not be present, P19 EC cell aggregates where chemically induced to differentiate by treatment with either DMSO or RA in the absence of LIF for either 4 or 2 days respectively and replated as single cells at low density in the absence of chemical inducers but in the presence of LIF. Ten clonal cell lines, five from DMSO and five from RA-treated cultures, were established. One of these lines, LD3, which was derived from DMSO-treated cells expresses both *Pax-3*-inducing activity in the EH3 cell mixing assay (Fig. 7) and *Brachyury* transcripts (Fig. 8). None of the additional lines express either of these markers.
Parallel analysis of the expression of additional mesodermal markers and Hox genes in GCLB and LD3 monolayer and aggregate cultures (Fig. 8) demonstrates that the two cell lines have very similar properties. In addition to expression of both Brachyury transcripts and Pax-3-inducing activity in the EH3 cell mixing assay, each of the cell lines expresses a high level of Oct-3/4 transcripts. Additionally, both GCLB and LD3 monolayer cultures express elevated levels of the gene nodal (Zhou et al., 1993). Nodal is required for mesoderm formation and is detected at high levels in the node that forms at the anterior end of the primitive streak just prior to migration of the primitive streak.

![Fig. 6. Comparison of constitutive and RA inducible Hox gene expression in P19 EC and GCLB cells. RNA was extracted from P19 and GCLB cells treated as aggregates with no (0), 1×10^{-7} M (−7), or 1×10^{-6} M (−6) RA in the absence (−) or presence (+) of 100 units/ml LIF for 4 days followed by replating to tissue culture dishes in the absence of RA but under the original LIF condition for an additional 2 days. RNAs were isolated from cells at 3 and 6 days of culture under these conditions and 20 µg/sample was probed for RAR-β (RNA from 3 day cultures; 3 day exposure), Hoxa-1 (Hox 1.6; RNA from 6 day cultures; 2.2 kb transcript; 10 day exposure), Hoxa-3 (Hox 1.5; RNA from 6 day cultures; 2.7, 3.3, and 3.5 kb transcripts, minor transcripts of 1.7 and 2.2 kb are also detected in 1×10^{-6} M RA-treated cells as described previously, Fainsod et al., 1987; 10 day exposure); F, Hoxc-8 (Hox 3.1; RNA from 6 day cultures; 2.7 kb transcript, 4 day exposure); and E, TPI (18 hour exposure) transcripts.]
the paraxial mesoderm when assayed by in situ analysis. Nodal is also detectable in prestreak and early streak embryos when assayed using PCR analysis and the lower levels of expression of this gene in P19 stem cells is consistent with this observation. Similar to GCLB cells, LD3 cells also autoinduce expression of Pax-3, Hoxa-1(1.6), Hoxa-3(1.5) (data not shown) and Hoxc-8(3.1) when grown as aggregate cultures. GCLB and LD3 cells show quantitative differences in the levels of expression of several markers. GCLB cells express equivalent, or slightly greater, levels of Pax-3-inducing activity when assayed using either the EH3 cell mixing assay or autoinduction of Pax-3 transcripts in aggregate cultures. However, LD3 cells exhibit approximately 3 fold higher levels of Nodal expression over that observed in GCLB cells in monolayer cultures. Additionally, LD3 cells express approximately 5-10 fold higher levels of the posterior-specific gene Hoxc-8(3.1) in aggregate cultures over that observed in GCLB cell aggregates although the anterior-specific gene Hoxa-1(1.6) is induced to approximately equivalent levels in aggregates of each cell line. The level of Hoxc-8(3.1) expression in LD3 cell aggregates is approximately equivalent to that obtainable following treatment of P19 stem cells with 1×10^{-6} M RA. However, LD3 cells do not express elevated levels of the early RA response gene RAR-β relative to GCLB cells demonstrating that elevated levels of endogenous RA synthesis, or accumulation, in LD3 cells are unlikely to be responsible for the enhanced induction of Hoxc-8(3.1) in this cell line. Unlike GCLB cells, which express only a 2.7 kb Hoxa-3(1.5) transcript, aggregation of LD3 cells results in expression of each of the isoforms induced by RA treatment. Finally, although Brachyury expression is detectable in both GCLB and LD3 cells, GCLB cells express approximately 3 fold higher levels of this transcript in monolayer cultures than do LD3 cells. However, on aggregation the level of expression in LD3 cells increases to that observed in either GCLB cell monolayer or aggregate cultures. Two qualitative differences are also observed between GCLB and LD3 cells. First, on aggregation, LD3 cells express the posterior mesodermal marker Mox-1 (Candia et al., 1992) which is not detected in GCLB cell aggregates. Second, LD3 cells express the mesodermal marker Snal (Smith et al., 1992; Nieto et al., 1992) at equivalent levels in monolayer and aggregate cultures while this marker is undetectable in GCLB cells under either condition. Similar to Brachyury, Snal is expressed in axial mesoderm. However, unlike Brachyury, Snal expression continues to be observed in mesoderm migrating laterally away from the streak. Additionally, in situ analysis suggests that Snal expression may occur subsequent to Brachyury expression during migration of primitive ectoderm into the primitive streak. The differences between the GCLB and LD3 cell lines could simply be a consequence of clonal variation. However, it is also possible that the two lines represent slightly different stages, or types, of axial mesoderm. In this case, differences in the expression of nodal, Mox-1 and Snal and the efficiency of Hoxc8(3.1) induction may reflect differences occurring in different mesoderm cell populations of the embryo.

**DISCUSSION**

**Pax-3-inducing activity is expressed in mesodermal lineages**

The initial question addressed in this study was whether the previously demonstrated Pax-3-inducing activity that is expressed during differentiation of P19 EC cells following chemical treatments could be localized to the mesodermal cell lineages formed in these cultures. Several observations support this possibility. First, expression of the pan-mesodermal marker Brachyury correlates well with the expression of Pax-3-inducing activity during differentiation of both DMSO and
RA-treated cultures. Second, the ability of LIF to block different stages in the progression of mesoderm differentiation in DMSO- and RA-treated cultures correlates with its ability to inhibit expression of \( \text{Pax-3} \)-inducing activity under each of these conditions. Finally, two clonally isolated mesodermal cell lines (but neither the P19 or EH3 stem cell lines nor 18 other non-mesodermal clonally isolated cell lines derived from P19 cells following either transfection or brief chemical inductions) express high levels of \( \text{Pax-3} \)-inducing activity directly demonstrating that mesodermal lineages are at least one source, and possibly the only source, of the activity during P19 cell differentiation.

**LIF inhibits mesoderm differentiation at two discrete steps**

The effect of LIF on differentiation of P19 EC cells following treatment with either DMSO or RA has been studied previously (Pruitt and Natoli, 1992). In that study LIF was shown to inhibit differentiation of the mesodermal lineages formed following DMSO treatment but many of the neuroectodermal lineages formed following RA treatment were unaffected. The inhibition of \( \text{Brachyury} \) expression by LIF in DMSO- but not RA-treated cultures is consistent with the differential effects of LIF on these induction pathways.

Although \( \text{Brachyury} \) expression demonstrates that an initial stage of mesoderm differentiation occurs in RA-treated P19 cell cultures in the presence of LIF, several observations suggest that LIF blocks progression of mesoderm differentiation at a later stage in these cultures. First, the rapid down-regulation of \( \text{Brachyury} \) expression observed in RA-treated cultures fails to occur when LIF is present. LIF also partially inhibits the down-regulation of \( \text{Oct-3/4} \) expression in these cultures. These observations are consistent with the existence of an early mesodermal intermediate, which expresses both \( \text{Brachyury} \) and \( \text{Oct-3/4} \) at high levels, and suggest that LIF may prevent progression of differentiation of these cells to stages where expression from these genes is down-regulated. Failure of RA-induced cultures to express endogenous \( \text{Pax-3} \)-inducing activity or \( \text{wnt-1} \) in the presence of LIF (Pruitt, 1992) suggests that LIF also blocks progression of mesoderm differentiation in these cultures prior to the stage at which either of these activities is present. The observation that both GCLB and LD3 cells express endogenous \( \text{Pax-3} \)-inducing activity in the presence of LIF, even though RA-treated P19 stem cells do not, demonstrates that these cell lines have differentiated past the point at which LIF acts during differentiation in RA-treated P19 cell cultures.

The effects of LIF, and the properties of the GCLB and LD3 cell lines, allow the identification of discrete stages in the differentiation of mesoderm in P19 cell cultures. An early mesoderm lineage is defined by \( \text{Brachyury} \) expression and it is assumed that the DMSO and RA pathways converge at this point. The more rapid expression of \( \text{Brachyury} \) in RA-treated cultures, relative to DMSO-treated cultures, suggests that RA may be acting by a more direct mechanism. However, even in RA-treated cultures, \( \text{Brachyury} \) expression does not appear for 2 days following treatment suggesting that induction of the early mesodermal lineage by RA may also be indirect. A later mesodermal lineage is defined by expression of the endogenous \( \text{Pax-3} \)-inducing activity described previously (Pruitt, 1992). Early and late mesoderm are distinguished by the ability of LIF to block the induction of this activity in RA-treated cultures. In both DMSO- and RA-treated P19 cell aggregates, \( \text{Brachyury} \) and \( \text{Oct-3/4} \) expression are eventually down-regulated, which may reflect the further differentiation of the later mesoderm derivative into the fully differentiated mesodermal cell types formed by these cultures at later times.

GCLB and LD3 cells express the \( \text{Pax-3} \)-inducing activity suggesting that these cells are equivalent to the later mesoderm lineages present in chemically treated P19 cell cultures. Expression of high levels of both \( \text{Brachyury} \) and \( \text{Oct-3/4} \) in these cell lines is also consistent with this possibility. Differences between these lines, particularly the expression of \( \text{Sna I} \) in LD3 cells, but not GCLB cells, suggest the possibility that additional intermediate stages in mesoderm differentiation, which can be propagated as stable intermediates, may occur at least in P19 EC cultures in vitro.

**GCLB and LD3 cells have properties of anterior primitive streak mesoderm**

Since \( \text{Brachyury} \) expression is restricted specifically to axial mesoderm lineages during embryogenesis (Wilkinson et al., 1990; Herrmann 1991), the high level of \( \text{Brachyury} \) expression in GCLB and LD3 cells demonstrates that these are mesodermal cell lines. However, \( \text{Brachyury} \) expression initiates at very early times during mesoderm differentiation (first detected at approximately 6.5 days in the early primitive streak and primitive ectoderm migrating into the streak) and its expression occurs throughout the axial mesoderm (with higher levels in the notochord) through at least 12 days of development (Wilkinson et al., 1990; Herrmann, 1991). Thus, expression of \( \text{Brachyury} \) in GCLB and LD3 cells does not indicate the specific stage of mesodermal differentiation that these cell lines represent.

Expression of \( \text{Pax-3} \)-inducing activity in GCLB and LD3 cells suggests that they represent an early stage of mesoderm differentiation. \( \text{Pax-3} \) expression first occurs in the developing neural tube at approximately 8.5 days of development in the mouse and closely parallels formation of the neural plate (Goulding et al., 1991). Further, although expression of neuroectoderm-inducing activity is not addressed in the present study, expression of \( \text{Pax-3} \)-inducing activity in differentiating P19 cells has been shown to be closely associated with a neural-inducing activity (Pruitt, 1992). It is known from studies in amphibians that one of the earliest properties that defines anterior mesoderm is the expression of neural-inducing activity (reviewed in Slack and Tannahill, 1992). These observations suggested the possibility that GCLB and LD3 cells represent a very early stage of mesoderm differentiation equivalent to the primitive streak in the mouse embryo.

Expression of additional markers in GCLB and LD3 cells is consistent with this possibility. The gene \( \text{Oct-3/4} \), although not restricted specifically to mesodermal lineages, is expressed at the highest levels in primitive ectoderm of very early embryos and is down-regulated during differentiation (Scholer et al., 1990). Down-regulation occurs at very early times during mesoderm differentiation and is approximately coincident with the up-regulation of \( \text{Brachyury} \) in the very early primitive streak on day 6.5 of development (Herrman, 1991). In GCLB and LD3 cells, \( \text{Oct-3/4} \) is expressed at levels equivalent to those occurring in the primitive ectoderm-like P19 and EH3 stem cells. The observation that GCLB and LD3 cells express
high levels of both Brachury and Oct-3/4 suggest that these cells represent very early mesodermal cell types, possibly equivalent to a transition stage between primitive ectoderm and the first cells of the primitive streak. Additionally, monolayer cultures of GCLB and LD3 cells express a low level of the anterior-specific gene *Hoxa-1*(1.6) but not more posterior-specific *Hox* genes, and elevated levels of *Nodal*, which is expressed at the high levels in the node that forms at the anterior end of the primitive streak. These observations are consistent with the possibility that these cells are equivalent to very early anterior primitive streak stage mesoderm.

**Anterior primitive streak mesoderm-like cells express an endogenous activity capable of inducing *Hox* gene expression**

The possibility that the anterior primitive streak mesoderm of the mouse embryo is equivalent in inducing activities to Spemann’s organizer of amphibians has been suggested (Blum et al., 1992). If this is the case, anterior primitive streak mesoderm should express signals capable of inducing genes that mark specific anterior-posterior positions along the body axis of the mouse. Other cell types present in the embryo at this stage of development, primitive ectoderm or extraembryonic endoderm, would not be expected to exhibit this property. Expression of *Hox* genes in several EC cell lines, which like P19 cells resemble primitive ectoderm of the embryo, has been studied (reviewed in Boncinelli et al., 1991). Little or no *Hox* gene expression is detected in stem cells of these lines, consistent with a lack of expression of activities responsible for inducing *Hox* gene expression along the anterior-posterior axis of the embryo. However, induction of differentiation by RA treatment is sufficient to induce *Hox* gene expression in these lines. In general, *Hox* genes expressed in the most anterior regions of the embryo respond more rapidly and at lower doses of RA than those expressed in more posterior regions. The observations reported here for the effect of RA on P19 EC stem cells are consistent with these earlier studies.

The primitive streak mesoderm-like cell lines GCLB and LD3 show a very different pattern of *Hox* gene expression. In monolayer cultures, the anterior-specific gene *Hoxa-1*(1.6) is expressed at low levels while transcripts encoding more posterior *Hox* genes are not detected. On aggregation, expression of *Hoxa-1*(1.6) is induced to maximum levels, and expression of the more posterior genes *Hoxa-8*(3.1) and *Hoxc-8*(3.1) is induced, even in the absence of exogenous RA treatment. Expression of the more posterior-specific *Hox* genes is delayed relative to anterior-specific genes and could indicate that a cascade of differentiation events is occurring in aggregates. It is possible that different mechanisms are responsible for induction of each of the different *Hox* genes studied here. Nonetheless, the term autoinduction is used to describe the ability of isolated mesodermal lineages to up-regulate expression from their own *Hox* genes in the absence of exogenous signals or non-mesodermal cell lineages. That the induction of *Hox* genes in these aggregates results from autostimulation by an extracellular factor is further suggested by the observation that EH3 stem cells can also be induced to express HOXC-8(3.1) and HOXB-5(2.1) when mixed with GCLB cells. In GCLB cells, reduced levels of expression of the posterior *Hox* genes relative to anterior genes seems likely to result from expression of the posterior genes in a more restricted set of cells, mimicking the restriction of posterior *Hox* gene expression in the embryo. Localization of HOXB-5 and HOXC-8 by immunofluorescence confirms this possibility for at least these genes (data not shown).

**Comparison of mesoderm differentiation in P19 EC cells with mouse and *Xenopus* embryos**

Early observations from transplantation studies in amphibians demonstrated that the first dorsal mesoderm to form has an anterior specification (reviewed in Slack and Tannahill, 1992). Progress has been made in identifying genes responsible for differentiation of anterior dorsal mesoderm in amphibians. In particular, injection of goosecoid mRNA into ventral mesoderm of *Xenopus* blastocysts is sufficient to induce a complete secondary dorsal axis (Cho et al., 1991). In the mouse, goosecoid is expressed in anterior primitive streak mesoderm and this tissue is capable of organizer activity when transplanted to *Xenopus* embryos suggesting that an anterior-posterior organizing activity is also localized to anterior mesoderm of the mouse (Blum et al., 1992). Consistent with this possibility, signals responsible for expression of the engrailed-1 and -2 genes in the neuroectoderm have been localized to anterior mesendoderm using an in vitro explant assay and it has been suggested that the mesodermal component of the mesendoderm is expressing the inducing activity (Ang and Rossant, 1993). Results presented here directly demonstrate the expression of an activity capable of inducing high levels of the anterior gene *Hoxa-1*(1.6) and progressively reduced levels of the more posterior genes *Hoxa-3*(1.5) and *Hoxc-8*(3.1) in clonally isolated mesodermal cell lines. This observation demonstrates that early mesodermal lineages, in isolation from any other cell type, are capable of autoinducing genes that are restricted in their expression along the anterior-posterior axis of the mouse embryo and suggest that, in the mouse, once anterior mesoderm is differentiated it has an intrinsic capacity to induce differentiation of more posterior mesoderm. These results raise the possibility that the ability to establish a rudimentary anterior-posterior axis is an intrinsic property of anterior primitive streak mesoderm in mammals is consistent with the previous suggestion (Blum et al., 1992) that anterior primitive streak mesoderm of the mouse has organizer activity.

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