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Differential cytokeratin gene expression reveals early dorsal–ventral regionalization in chick mesoderm

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Summary

The induction and spatial patterning of early mesoderm are known to be critical events in the establishment of the vertebrate body plan. However, it has been difficult to define precisely the steps by which mesoderm is initially subdivided into functionally discrete regions. Here we present evidence for a sharply defined distinction between presumptive dorsal and presumptive ventral regions in early chick mesoderm. Northern blot and *in situ* hybridization analyses reveal that transcripts corresponding to CKse1, a cytokeratin gene expressed during early development, are present at high levels in the presumptive ventral mesoderm, but are greatly reduced or undetectable in the future dorsal region of mesoderm, where the formation of axial structures occurs later in development. This distinction is present even while the mesoderm layer is being formed, and persists during the extensive cellular movements and tissue remodelling associated with morphogenesis. These results point to an early step in which two fundamentally distinct states are established along the presumptive dorsal–ventral axis in the mesoderm, and suggest that determination in this germ layer occurs in a hierarchical manner, rather than by direct specification of individual types of histological differentiation. The differential expression of CKse1 represents the earliest molecular index of dorsoventral regionalization detected thus far in the mesoderm.

Key words: chick embryo, mesoderm regionalization, cytokeratin gene expression.

Introduction

The formation of vertebrate mesoderm has recently been the subject of intensive scrutiny because this germ layer plays a central role in the establishment of embryonic pattern (Slack, 1983). Even at very early stages, the mesoderm appears not to be homogeneous but instead possesses region-specific characteristics along the dorsal–ventral axis of the embryo. This regional specialization of early mesoderm has particularly important implications for the formation of the basic vertebrate body plan, as recognized by Spemann and Mangold (1924), who first demonstrated the unique ability of the dorsal lip mesoderm to direct the formation of a second set of axial structures when placed in a ventral location. In addition to this striking regional difference in inductive ability, the mesoderm becomes subdivided with respect to the types of phenotypic differentiation that are specified at different positions along the dorsoventral axis (Slack and Forman, 1980; Dale and Slack, 1987). Such differences can be detected by removing portions of the prospective mesoderm at very early stages, before any sign of histological differentiation, and examining their development in isolation. In the amphibian embryo, isolated cultures of prospective dorsal mesoderm differentiate primarily into notochord and muscle (the most dorsal mesodermal derivatives), but very little mesothelium or blood (ventral mesoderm). Isolates removed from progressively more ventral locations form much more mesothelium and blood, with the amount of muscle or notochord becoming greatly reduced, particularly in the most ventral isolates (Dale and Slack, 1987).

While the above data argue that regional specialization occurs along the dorsal–ventral axis in the early mesoderm, it has been difficult to define the steps by which cell diversity is first established in this germ layer. One possibility is that local, possibly graded signals could establish a series of positional states along the dorsal–ventral axis. Individual states could then specify the various types of histological differentiation, such as blood, mesothelium, muscle or notochord. Alternately, the mesoderm might be subdivided in a progressive, hierarchical manner starting perhaps with fundamentally distinct dorsal and ventral states. Cells within the dorsal and ventral regions could then interact to

generate various intermediate grades of mesoderm. This latter idea is supported by data indicating that interactions within the mesoderm can influence the final phenotypic outcome of cells in this germ layer (Dale and Slack, 1987). However, there is considerable overlap in the types of differentiation obtained in induction and explant studies, and no direct means of detecting regional specialization has been available for the period before the activation of cell type-specific genes. Consequently, it has not been possible to establish the existence or location of a boundary delineating fundamentally distinct regions of dorsal and ventral mesoderm.

Here we present evidence for an early and sharply defined distinction between presumptive dorsal and ventral regions of chick mesoderm. Previously, we isolated a chick cDNA clone (CKse1) on the basis of differential hybridization within the ectoderm of the early embryo (Charlebois *et al.* 1990). This cDNA clone encodes a putative type II cytokeratin which exhibits approximately 75% identity with a number of keratins characteristic of embryonic and simple epithelia, and is most closely related to cytokeratin number 7 of the human catalog (Moll *et al.* 1982; Glass *et al.* 1985). CKse1 was found to be expressed at high levels in body ectoderm beginning at neurulation, but transcript levels were seen to be greatly reduced in ectoderm of the head and in neural ectoderm (Charlebois *et al.* 1990). In the present work we report on our finding that this gene is expressed differentially in the mesoderm as well. The pattern of CKse1 gene expression in this germ layer appears to delineate two distinct states that are defined with respect to cellular position along the future dorsal–ventral axis.

Materials and methods

Embryos and tissue dissections

Fertile White Leghorn chicken eggs (Truslow Farms, Chestertown, MD) were incubated at 38°C, 60% relative humidity, and staged according to Hamburger and Hamilton (1951). For the isolation of presumptive dorsal and ventral mesoderm, the trunk region (defined as the portion of the body between the first and last somite; see Fig. 1A) of stage 11–13 embryos was placed in 2.25% trypsin (Gibco, 1:250), 0.75% pancreatin (Difco) in phosphate-buffered saline (PBS) on ice for 15–30 min, and transferred to 20% fetal bovine serum (Sigma; heat inactivated at 56°C) in PBS for dissection. Endoderm and ectoderm were removed and discarded, and mesoderm was separated into presumptive dorsal and ventral components by making a longitudinal cut at the ventrolateral margin of the somites (see Fig. 1B). Tissue fragments were rinsed in PBS, and frozen in a dry ice/ethanol bath pending RNA isolation, which was performed as described elsewhere (Charlebois *et al.* 1990). Embryos used for *in situ* hybridization were trimmed at the outer margin of the area pellucida (stages 4–6) or within 3–4 somite widths of the midline (stage 10) prior to fixation.

Northern blot analysis

Approximately 4.5 μg total RNA from dorsal and ventral tissues was electrophoresed in a 1.2% agarose, 6% formaldehyde gel, transferred to nylon membrane, and UV-crosslinked at 1200 $\mu W\,cm^{-2}$ for 2 min. Equivalency of gel loading was checked by ethidium bromide staining of rRNA bands. Filters were hybridized to 4.5×10^6 cts $min^{-1}\,ml^{-1}$ of ^{32}P-labelled antisense RNA transcribed from pCKse1 (see Charlebois *et al.* 1990), in 50% formamide, 0.05% ficoll, 0.05% polyvinylpyrollidone, 0.1% sodium dodecyl sulfate (SDS), 5×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate), 100 μg ml^{-1} salmon sperm DNA at 62°C. Blots were washed in 2×SSC, 5% SDS, at room temperature, followed by 0.1×SSC, 1% SDS at 67°C. Exposures were carried out using X-Omat AR film at −70°C with intensifying screens. Autoradiograms were scanned using a BioImage Visage 2000 computerized image analysis system.

In situ *hybridization*

Embryos were fixed in 4% paraformaldehyde, dehydrated and embedded in Paraplast. 8 μm sections were prepared, dried on slides, paraffin was removed and sections were treated with proteinase K and acetic anhydride prior to hybridization. [^{35}S]RNA (antisense) probes were prepared by transcription of pCKse1 using T7 RNA polymerase, and subjected to partial hydrolysis with 0.2 M $NaHCO_3/Na_2CO_3$ (pH 10). Hybridization was carried out for 16 h at 61°C in 50% formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 8.0), 5 mM EDTA, 0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrollidone, 10% dextran sulfate, 10 mM dithiothreitol to which [^{35}S]RNA was added to a final concentration of approximately 1.3×10^7 cts $min^{-1}\,ml^{-1}$. Slides were washed in 4×SSC, 10 mM dithiothreitol at room temperature and treated with RNase A (20 μg ml^{-1}) at 37°C for 30 min. Slides were then washed in 50% formamide, 2×SSC at 50°C for 45 min, followed by 0.1×SSC at 50°C for 45 min. Dried slides were dipped in Kodak NTB-2 emulsion and exposed for approximately 3 days at 4°C. Control sections were hybridized under identical conditions to a sense probe prepared from the 3′ *Pst*I fragment of a chick δ-crystallin cDNA clone (Bhat and Piatigorsky, 1979). No specific hybridization to this control probe was observed; only a very low and uniform level of signal could be seen throughout these sections (Charlebois, 1988).

Results

In the course of carrying out preliminary *in situ* hybridization studies to determine the regional distribution of CKse1 transcripts in early ectoderm, we observed that in the trunk region of stage 10 embryos (stages are according to Hamburger and Hamilton, 1951, for primitive streak and later stage embryos), CKse1 was also expressed in the mesoderm and endoderm (Charlebois *et al.* 1990). However, CKse1 transcripts were seen only in the more lateral regions of mesoderm and did not appear to be detectable in the mesoderm immediately flanking the midline. The three primary germ layers at this stage are still largely discrete, laying flat on the large yolk mass in the order (from the yolk) endoderm, mesoderm, ectoderm (see Fig. 1B). During the formation of the lateral body folds, all three germ layers fold downward and inward, undercutting the body of the embryo proper. Thus the most medial portion of the embryo constitutes the future dorsal side; the more lateral regions on either side are destined to become ventrally located (for

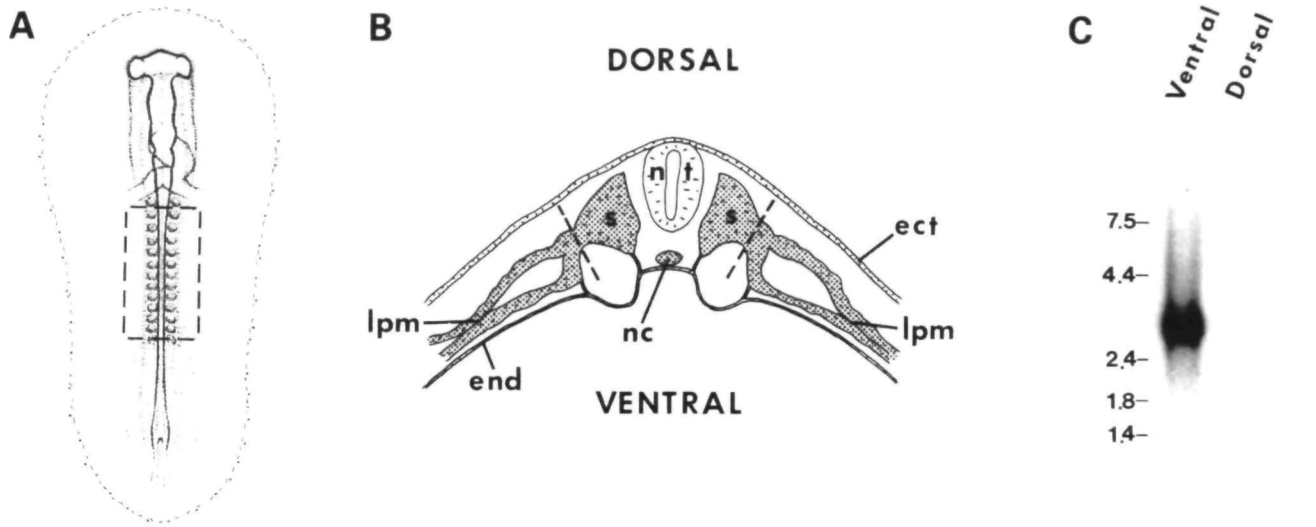


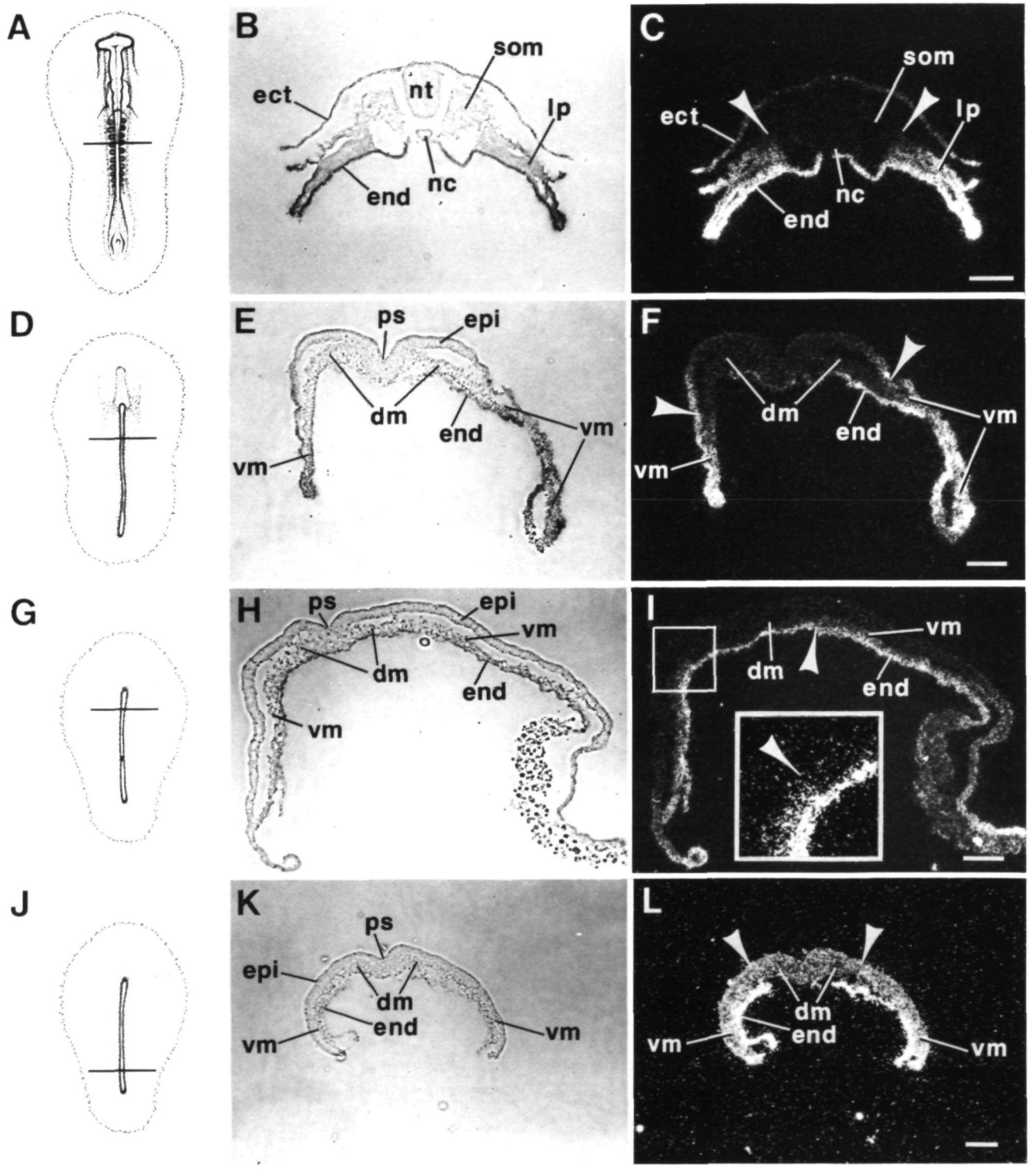
Fig. 1. Differential cytokeratin gene expression in chick mesoderm. (A) Diagramatic representation of a stage 11 (38–40 h; Hamburger and Hamilton, 1951) chick embryo, showing the trunk region dissected for mesoderm isolation. (B) Cross-sectional illustration of the trunk region depicted in (A). The shaded region lying between the ectoderm (ect) and endoderm (end) constitutes the mesoderm layer. Broken lines through the mesoderm indicate the position of the cuts made to separate dorsal mesoderm from ventral mesoderm. Abbreviations: nt, neural tube; s, somite; nc, notochord; lpm, lateral plate mesoderm; ect, ectoderm; end, endoderm. (C) Northern blot analysis of cytokeratin gene expression in dorsal and ventral mesoderm regions shown in B. Migration of marker RNAs of known length are indicated in kilobases at left.

review, see Balinsky, 1981). Our initial observation therefore suggested that cytokeratin gene expression might be regulated along the future dorsal–ventral axis in the mesoderm.

To pursue this observation further, we performed Northern blots to confirm the identity and distribution of CKse1 transcripts in dissected regions of mesoderm. Mesoderm from the trunk region (illustrated in Fig. 1A) of 40 h embryos (stage 10–12) was isolated and dissected into two regions based on visible morphological criteria: a cut was made parallel to the midline at the ventrolateral edge of the somites, such that the more medial (presumptive dorsal) fragment containing notochord and somitic mesoderm was separated from the (presumptive ventral) mesoderm of the lateral plate (Fig. 1B). These fragments were then analyzed for the presence of cytokeratin RNA using antisense RNA transcribed from pCKse1 as a probe. Hybridization was only barely detectable in the medial mesoderm, whereas a very strong signal was observed in the more lateral fragment (Fig. 1C). The transcripts detected were indistinguishable in size from the major, 2.9 kb band previously detected by the CKse1 probe in the ectoderm (Charlebois *et al.* 1990). Densitometric scanning of autoradiograms indicated that CKse1 transcripts are well over 200-fold more abundant in the lateral (presumptive ventral) mesoderm.

In order to obtain more precise spatial and temporal information concerning the mesodermal distribution of CKse1 transcripts, *in situ* hybridization to sectioned embryos was carried out (Fig. 2). Fig. 2A illustrates the plane of a section cut through the somite region of a stage 10 (36 h) embryo; this section is shown under phase contrast in Fig. 2B. The lateral mesoderm seen at the margins moves ventrally during formation of the lateral body folds, and gives rise to a variety of phenotypes characteristic of ventral mesoderm, including mesothelium and blood (Patten, 1951; Romanoff, 1960). Between the lateral plate and the more medial mesodermal structures (notochord and somites; i.e. future dorsal mesoderm) lies a thin strip of intermediate mesoderm, destined to form nephrotome and venous vasculature (Meier, 1980). The pattern of *in situ* hybridization of antisense CKse1 RNA to this section is shown under darkfield illumination in Fig. 2C. As expected, CKse1 RNA is detected in the trunk ectoderm (expression in this tissue was the basis for its isolation), and very high levels of hybridization are also seen in the endoderm. Within the mesoderm, consistent with the RNA blot result shown above, strong hybridization is seen in the lateral plate mesoderm. This signal, which is maximal in the most lateral regions of the mesoderm, disappears abruptly at the boundary between the lateral plate and the intermediate mesoderm. No hybridization can be discerned in any of the more dorsal structures, i.e. the notochord, somites, or intermediate mesoderm. A similar pattern of differential cytokeratin gene expression is seen in sections at various levels along the anterior–posterior axis.

To investigate the developmental time course of differential cytokeratin gene expression in the mesoderm, we examined *in situ* hybridization of the CKse1 probe to transcripts at earlier stages. At stage 6 (23–25 h; see Fig. 2, D–F), somites have not yet formed and the notochordal rudiment is present only anterior to the primitive streak. When sections through the primitive streak are examined at this stage, it is evident that mesoderm immediately on either side of the



primitive streak (presumptive dorsal region) exhibits little or no detectable CKse1 hybridization. In contrast, much stronger hybridization is seen at more lateral (presumptive ventral) positions in the mesoderm, which lies between epiblast and endodermal layers that exhibit very high levels of CKse1 hybridization. The expression pattern in the mesoderm indicates that the dorsal/ventral difference in CKse1 expression precedes, and therefore cannot be dependent on, the overt morphological organization of this germ layer (e.g. formation of somites, notochord, lateral plate).

Sections through the anterior half of the primitive streak in stage 4 (18–19 h; see Fig. 2, G–I) embryos reveal that a distinct boundary of CKse1 gene expression exists in the mesoderm prior to even the most subtle evidence of organization yet detected in this germ layer. The primitive streak delineates the portion of the embryo undergoing gastrulation, where prospec-

Fig. 2. *In situ* hybridization of pCKse1 antisense RNA to sectioned embryos. For each example shown, the illustration at left (A,D,G,J) shows the position and orientation of the section photographed under phase contrast (B,E,H,K) and darkfield illumination (C,F,I,L). Illustrations are oriented with the anterior of the embryo toward the top; sections are shown with the dorsal surface at the top. Arrowheads indicate position where transition in CKse1 gene expression is seen between presumptive dorsal and ventral regions of mesoderm. (A–C) Section through the somite region of a stage 10 embryo. Hybridization is seen throughout the ectoderm and is very intense in the endoderm. In the mesoderm, hybridization can be seen only in the lateral plate mesoderm and is undetectable in intermediate mesoderm, somites and notochord. (D–F) Section through the primitive streak of a stage 6 embryo. The overall level of CKse1 expression in the mesoderm is somewhat lower than that seen in the endoderm and epiblast. The mesoderm on either side of the primitive streak is CKse1-negative, but CKse1 RNA is readily detected in the presumptive ventral mesoderm. (G–I) Section through the primitive streak of a stage 4 embryo. In the epiblast, strong hybridization is only seen at the very outer margins of the area pellucida. The endoderm layer exhibits high levels of CKse1 RNA throughout. In the mesoderm (which is adherent to the endoderm in this section), CKse1 RNA is seen in the presumptive ventral region but is not detected in the presumptive dorsal area closest to the midline. The boxed region in I is shown at higher magnification in inset, to facilitate visualization of the boundary of CKse1 hybridization. (J–L) Section through the posterior portion of the primitive streak of a stage 4 embryo. The endoderm exhibits very strong hybridization (a portion of this layer is missing at the midline), while CKse1 RNA is detectable only at the outer margins of the epiblast. In the mesoderm, CKse1 expression is low in the presumptive dorsal region, but becomes elevated in more ventral locations. Abbreviations: dm, dorsal mesoderm; ect, ectoderm; end, endoderm; epi, epiblast; lp, lateral plate mesoderm; nc, notochord; nt, neural tube; ps, primitive streak; s, somite; vm, ventral mesoderm. Scale bars represent 100 μm.

tive mesoderm and endoderm cells are actively ingressing from the upper, epiblast layer, leading to the formation of the three basic germinal layers (Bellairs, 1982). At stage 4, the 'definitive streak stage', the streak is of maximum length; as development proceeds, the streak regresses posteriorly, so that ingression ceases first in the anterior but continues for many hours, restricted to a progressively more posterior location. The first subtle evidence of morphological organization appears to take place in mesoderm just after ingression is complete. The condensation of axial mesoderm into notochord can first be observed just anterior to the regressing streak. Flanking the axial mesoderm, tandem circular domains of paraxial mesoderm, known as somitomeres, have been observed in stereo pairs of scanning electron micrographs (Meier, 1979). In Fig. 2, G–I, a section is shown through the mid-anterior streak, where ingression is still occurring; this is posterior to where even these most subtle measures of mesodermal organization have been observed in definitive streak stage embryos (Meier, 1981; Triplett and Meier, 1982; Packard and Meier, 1983). In the mesoderm at this stage, the level of CKse1 RNA appears to be lower than at later stages, and the hybridization signal is less intense than in the thin layer of endoderm with which it is closely associated (see Fig. 2, H–I). Close inspection of the mesoderm demonstrates nonetheless that a well-defined transition in CKse1 gene expression occurs along the presumptive dorsal–ventral axis: expression is seen in the lateral mesoderm (presumptive ventral region), but is undetectable in the medial (presumptive dorsal) region of mesoderm (Fig. 2I). The region containing the boundary of CKse1 gene expression is enlarged in the inset, Fig. 2I. The only detectable signal above and to the right of the arrowhead (i.e. in the medial region) corresponds to the heavily labelled endoderm. No signal can be seen in the mesoderm in this medial region. In contrast, to the left and below the arrow (lateral region), in addition to the strong endodermal signal, a band of hybridization is seen over presumptive ventral mesoderm. This signal is not due to autoradiographic grain scattering from the endoderm, as no such grains are observed to the right or beneath the endoderm layer. The distinct difference in CKse1 expression along the presumptive dorsal–ventral axis is seen in serial sections throughout the anterior half of the stage 4 primitive streak (see Fig. 3).

Although the sharp boundary of CKse1 expression between presumptive dorsal and ventral mesoderm extends posterior to any morphological differentiation in the mesoderm of stage 4 embryos, it is interesting to look at even more posterior regions of these embryos, where the overt regionalization of mesoderm (which takes place in an anterior-to-posterior manner) lags behind. In this part of the embryo, there is still differential CKse1 gene expression (Fig. 2, J–L), but the boundary between medial and lateral regions is not as sharply defined. In the section shown, no expression is seen in the epiblast layer (except at the very lateral edges), while strong hybridization is again seen in the endoderm (pulled slightly away from the mesoblast in several places, and missing at the midline). In the mesoderm in the posterior, no distinct boundary of CKse1 expression can be discerned. Instead, the level of CKse1 hybridization is seen to diminish more gradually in a lateral-to-medial direction, eventually becoming indistinguishable from the background signal (at the position of the arrows) in the region flanking the primitive streak. Counts of exposed silver grains in the lateral mesoblast (near the point labelled vm in Fig. 2L) are some 2.8-fold higher than in the medial region (near the point labelled dm in Fig. 2L). The basic pattern of CKse1 hybridization detected in the stage 4 mesoderm is summarized in Fig. 3.

Discussion

The differential detection of cytokeratin gene transcripts in the newly forming mesoderm suggests that CKse1 expression serves as an early molecular index of

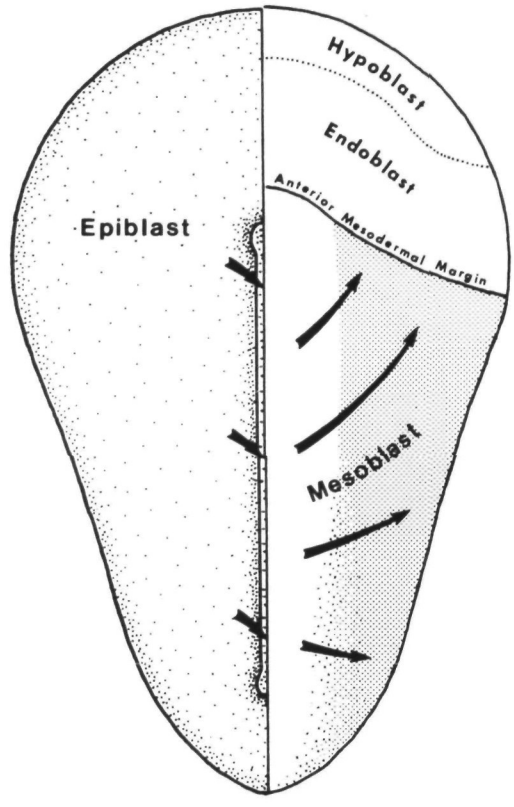


Fig. 3. Cellular movements of presumptive mesoderm in the definitive streak stage embryo. Anterior of the embryo is at top of the figure; the illustration is shown with the epiblast (upper layer) intact at left, but removed at right to reveal the underlying mesoderm (and hypoblast/endoblast at anterior, beyond the limits of mesodermal migration). Arrows are included to show the general properties of mesodermal movement (Rosenquist, 1966; reviewed by Balinsky, 1981). Prospective mesoderm cells initially lie within the upper, epiblast layer. During gastrulation, cells that are destined to ingress migrate medially such that they converge on the center of active ingression, the primitive streak. Presumptive mesoderm cells ingress at the primitive streak and populate the space between the epiblast and the hypoblast/endoblast. Once beneath the epiblast, these cells spread laterally, moving away from the streak. Shading at right illustrates the region in which CKse1 gene expression can be detected. As indicated by the degree of shading, the boundary of CKse1 hybridization at the posterior of the embryo is somewhat less sharp than at more anterior positions (see text).

dorsal–ventral regionalization within this germ layer. In amphibian embryos, the expression of the myogenic gene MyoD1 and subsequent accumulation of muscle-specific α-actin transcripts have been found to be early markers of mesoderm destined to differentiate into muscle (Hopwood *et al.* 1989; Mohun *et al.* 1984). The expression of these genes is specific to the forming somites and at early stages is probably restricted to their precursor mesoderm cells (Hopwood *et al.* 1989). Similar results have been obtained in avian embryos, where the expression of several contractile protein genes as well as a quail MyoD1 homologue (*qmf1*) was seen specifically in the more mature rostral somites (Charles de la Brousse and Emerson, 1990). This study also showed that *qmf1*, but not several contractile protein genes, is expressed in the dorsal medial lip of the dermatome, suggesting that this region of the somite is a site of myogenic lineage determination. In contrast, the differential expression of CKse1 is established prior to any morphological evidence of mesodermal organization and seems not to be a reflection of any individual type of histological differentiation. Fate mapping experiments (Rosenquist, 1966) have shown that several different mesodermal cell types are derived from the dorsal region (CKse1-negative), and from the ventral region (CKse1-positive). Throughout the period in which the mesoderm changes from a morphologically unorganized mesenchyme to become discretely organized into notochord, somites, intermediate mesoderm and lateral plate, the regional distinction in cytokeratin gene expression is maintained.

The formation of the mesoderm layer appears to occur by virtue of perhaps the earliest inductive event during embryogenesis (Dale *et al.* 1985; Smith *et al.* 1985; reviewed by Woodland, 1989). The most extensive progress toward a mechanistic understanding of this event has been made in amphibian embryos, where recombination experiments have shown that prospective endoderm cells at the vegetal pole can direct animal pole cells (which would otherwise form epidermis) to differentiate into mesodermal derivatives (Nieuwkoop, 1969; Nakamura *et al.* 1970; Dale *et al.* 1985; Gurdon *et al.* 1985). There are distinct regional differences in the inductive specificity of the prospective endoderm (Dale and Slack, 1987): the most dorsal quadrant induces 'dorsal-type' mesoderm (notochord and muscle) while the rest is equipotent in inducing 'intermediate-type' (muscle, mesothelium, mesenchyme and blood) or 'ventral-type' mesoderm (mesothelium, mesenchyme and blood). Recent studies have demonstrated that members of growth factor families are capable of inductive effects similar to those obtained with vegetal cells (Woodland, 1989). Different factors in different combinations and/or concentrations induce different types of mesodermal differentiation in culture. There is actually little evidence for a period in which amphibian 'mesoderm' exists as a truly homogeneous tissue, and such studies point to the possibility that this germ layer possesses regional character even as it is induced (Dale *et al.* 1985; Smith *et al.* 1985).

In the chick embryo, the processes of mesoderm induction and regional specialization are not as well understood. The embryonic mesoderm has its cellular origin in the epiblast and, along with the presumptive gut endoderm, must ingress through the primitive streak to form the three-layered embryo. There is evidence that some commitment to mesoderm formation occurs as early as the blastula stage, before primitive streak formation. Epiblasts that are stripped of underlying hypoblast prior to the appearance of the primitive streak (stage XIII of Eyal-Giladi and Kochav, 1976) and cultured either intact (Azar and Eyal-Giladi, 1979) or after dissociation (Mitrani and Eyal-Giladi,

1982) are able to differentiate into non-axial mesoderm such as blood and mesenchyme. Whether all mesoderm precursors in the epiblast are capable of this type of differentiation is unclear. Recently, the epiblast of stage XIII (Eyal-Giladi and Kochav, 1976) embryos has been shown to contain two distinct cell populations that differ in their reactivity to the monoclonal antibody HNK-1 (Stern and Canning, 1990). The cell population recognized by HNK-1, which is distributed randomly throughout the epiblast, is destined to converge to form the primitive streak and give rise to the mesoderm and endoderm (Stern and Canning, 1990). Thus, before the formation of the primitive streak, HNK-1-positive cells in the epiblast may already be committed to ingress through the streak and become mesoderm and/or endoderm. Our results suggest, however, that the mesodermal precursors that are HNK-1-positive in the epiblast have not yet undergone the dorsal–ventral regionalization reflected by differential CKse1 expression, since CKse1 RNA is not seen in the presumptive embryonic cells of the epiblast until the time of neurulation (Henry *et al.* submitted; see also Fig. 2). It is possible that early regional properties of chick mesoderm, such as the decision to express CKse1 or to form axial structures, are only established during or after gastrulation.

The differential distribution of CKse1 transcripts provides the first direct evidence that, even as the mesoderm layer is being formed during gastrulation, it is subdivided into sharply defined dorsal and ventral elements. Previously, Dale and Slack (1987) proposed on the basis of explant and induction studies in *Xenopus* that the first specialization within the prospective mesoderm involves the establishment of only two states: a dorsal type and a ventral type. If simple states of dorsal and ventral specification do indeed exist, one might predict that these two states are sharply distinguished by some feature(s) which is common among cells within each (dorsal or ventral) subdivision. This would be in spite of the fact that several different terminal differentiation pathways will ultimately arise from each subdivision. Our current findings are consistent with such a simple, two-state model in which dorsal and ventral states of mesoderm are distinguishable with respect to cytokeratin gene expression.

What kind of biological difference(s) might be specified in the fundamental subdivision of the chick mesoderm into two distinct states? Many studies have demonstrated that the properties established for the Spemann organizer in amphibians are applicable to avian embryos (reviewed by Hara, 1978). Thus, 'organizer' activity in the chick mesoderm appears to be localized to the prospective dorsal region in the vicinity of Hensen's node and the anterior portions of the primitive streak. It is therefore likely that one aspect of early dorsoventral specialization in the mesoderm involves the establishment of region-specific inductive properties necessary for the formation of the body axis. A second biological difference between dorsal and ventral components may relate to their capacity to respond to inductive stimuli. Hornbruch *et al.* (1979) grafted Hensen's node from quail embryos to various dorsoventral positions of definitive streak (stage 4) chick embryos, and analyzed the origin of somites formed in the resultant secondary axes. Grafts to the prospective dorsal region ($\leq$200–300 μm from the primitive streak) lead to the formation of supernumerary somites of host origin. In contrast, when grafts were placed into prospective ventral locations (500–600 μm from the host primitive streak), there were somites derived from the transplanted tissue but none from the host. One interpretation of these results (Hornbruch *et al.* 1979) is that the mesoderm in the prospective ventral region is not competent to respond to the inductive signals from the graft, whereas more dorsal mesoderm can respond and produce supernumerary somites. Therefore, differential CKse1 expression may reflect a difference in the ability of dorsal and ventral mesoderm to respond to such inductive signals.

During chick gastrulation, there is extensive migration of presumptive mesoderm cells (depicted in Fig. 3), and this must be considered in any model trying to explain how differential expression of CKse1 is set up in this germ layer. These cells migrate from initial positions in the upper, epiblast layer, by first moving medially in the epiblast and ingressing through the primitive streak, and then by spreading out in a ventrolateral direction to occupy the layer beneath the epiblast (Rosenquist, 1966). This migration process is actively occurring at the definitive streak stage (stage 4) and continues for several hours afterward. Most significantly, many cells that are within the more medial, CKse1-negative region at stage 4 will continue to migrate to more lateral positions at subsequent stages. Throughout this period, however, the distinction in CKse1 gene expression between presumptive dorsal and ventral regions is maintained. This observation suggests that the developmental state of migrating mesoderm cells is determined by their position along the future dorsal–ventral axis: as cells move laterally and cross a morphologically indistinguishable boundary, a marked increase in CKse1 gene expression takes place.

The position-dependent expression of CKse1 in the mesoderm suggests that early regionalization might specify positional identities which are fundamentally distinct in the prospective dorsal and ventral regions. Interactions between these two regions could then be responsible for generating finer grades of specialization during the subsequent development of the vertebrate mesoderm, as suggested by experiments with amphibian embryos (Dale and Slack, 1987). This type of progressive specification has been elegantly shown to occur in *Drosophila*, where the body plan is laid out in a hierarchical manner. Initially, maternally encoded determinants specify in broad outline the anterior–posterior and dorsal–ventral axes. These maternal gene products then act to generate spatially restricted patterns of zygotic gene expression, thereby initiating pathways of genetic instructions to successively subdivide the embryo into smaller and smaller domains (reviewed by Ingham, 1988). Currently, we have little

information about the steps involved in the regional subdivision of the vertebrate embryo. Our results support the idea (Dale *et al.* 1985) that dorsal–ventral regionalization of the mesoderm could involve a fundamental positional coding which is established early in a hierarchy of decisions in this germ layer. From this perspective, it may not be surprising that the dorsal/ventral boundary of CKse1 gene expression is not as distinct in the posterior region of the mesoderm. Coordinate with primitive streak regression, the morphological organization of mesoderm takes place in an anterior-to-posterior fashion. Therefore, more posterior mesoderm undergoes dorsal/ventral specialization later in development. In *Drosophila*, progressive sharpening of expression boundaries is seen for a number of genes involved in the hierarchy of controls leading to the establishment of embryonic pattern (for reviews, see Carroll, 1990; Ingham, 1988).

These findings now emphasize the need to address the causative influences that underly the early establishment of dorsal and ventral states. Our data concerning the position-dependent regulation of CKse1 gene expression suggest several possible mechanisms that should be experimentally testable. One hypothesis might hold that a signal, perhaps emanating from a medial 'dorsalizing center', serves to confer a dorsal phenotype upon cells exposed to it in concentrations above a certain threshold; cells moving to distances where the putative signal is at subthreshold concentrations would become defined as 'ventral-type'. An alternative hypothesis is that region-specific influences of either the underlying hypoblast/endoblast layer or the overlying epiblast layer have specific dorsal and ventral inductive effects on the associated mesoderm. With regard to these hypotheses, Azar and Eyal-Giladi (1979) have shown that once the primitive streak has substantially formed (by stage 3+), the hypoblast and marginal zone are no longer needed to support the normal regionalization of the mesoderm that results in the formation of notochord, somites and lateral plate. Also, Mitrani and Shimoni (1990) have obtained organized axial structures in isolated epiblasts cultured in the presence of conditioned media from the *Xenopus* XTC cell line (Pudney *et al.* 1973; see Smith, 1987). These results suggest that once the primitive streak is established, there is no longer a need for the underlying hypoblast to supply localized positional cues. Instead, regional properties of the mesoderm may instead be fixed either with reference to the epiblast or to the streak itself. The epiblast is known to possess some polarity with respect to axis formation (Mitrani and Eyal-Giladi, 1981). The availability of CKse1 to assess directly the regional character of the mesoderm should facilitate transplant and explant experiments designed to sort out the influences important in establishing dorsal–ventral specialization in this germ layer.

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