Staging of ovine embryos and expression of the T-box genes \textit{Brachyury} and \textit{Eomesodermin} around gastrulation

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Abstract

The high rates of embryonic mortalities which follow \textit{in vitro} production of ruminant embryos have emphasized the need for increased knowledge of early development. It is likely that early failures in embryonic development and placenta formation involve abnormal differentiation of mesoderm. The aim of this study was to investigate the pattern of expression of two T-box genes known to control the gastrulation process, \textit{Brachyury} and \textit{Eomesodermin}, by whole-mount \textit{in situ} hybridization. To allow a more precise comparison of both expression patterns between embryos, we describe a new staging of pre-implanted ovine embryos by gross morphology and histology from pre-gastrulation stages to the beginning of neurulation. In pre-streak embryos primitive mesoderm cells delaminated in between the primitive endoderm and the epiblast. At that stage, no expression of \textit{Brachyury} or \textit{Eomesodermin} could be detected in the embryos. Early expression of both T-genes was observed by the early-streak stages in epiblast cells located close to the presumptive posterior pole of the embryos. Later on, during gastrulation both genes followed a pattern of expression similar to the ones described in other mammals. These observations suggest that other genes, which remain to be identified, are responsible for extra-embryonic mesoderm differentiation in ruminant embryos.


Introduction

Gastrulation is the process during which the three germ layers and the body axes of the embryo are established. In mammals, gastrulation mechanisms have been studied mainly in the mouse. Little is known about gastrulation mechanisms in the ruminant embryo. Some aspects of the process have been reported in sheep (Green & Winters 1945) and in the cow (Winters et al. 1942, Greenstein & Foley 1958, Fléchon 1978, Betteridge et al. 1980). By days 9–10, the primitive endoderm cells (also referred to as the hypoblast) differentiate from the inner cell mass (ICM) and form a confluent layer which lines the inner face of the trophoblast. After rupture of the Rauber layer, the trophoblast overlying the ICM, the epiblast cells of the embryonic disc are in direct contact with the uterine environment. By days 14–16 in the cow, primitive mesoderm cells intercalate in between the epiblast and the primitive endoderm, then migrate towards the extra-embryonic areas and start to split to form the yolk sac by fusion with the primitive endoderm and the chorion by fusion with the trophoblast (Maddox-Hytte1 et al. 2003). Three to 4 days later, the primitive streak has formed and the amniotic folds begin to enclose the embryo. The whole process occurs before implantation, which starts by days 15 and 19 in the sheep and cow respectively (Guillomot, 1995). In ruminants, the choriovitelline placenta is transient since the yolk sac regresses and is replaced by the growing allantois giving rise to a definitive choioallantoic placenta. However, a vitelline vascularization occurs, probably ensuring the early exchanges between the embryo and the maternal endometrium. In the sheep and cow, failure of placental development is a common feature after nuclear transfer cloning (Hill et al. 2000, Chavatte-Palmer et al. 2002). Most of these failures are attributed to an inadequate transition of the choriovitelline to the allanto-chorionic placentae (De Sousa et al. 2001). The same authors observed that, although pathologies associated with cloning affect fetal tissues as well as the placenta, they have a common origin, namely the mesoderm. Thus in ruminants, the formation of the extra-embryonic mesoderm, which constitutes part of the placental chorion, is of importance in the establishment of pregnancy.

Numerous studies have identified a number of factors involved in gastrulation and mesoderm formation. The
members of the T-box gene family, which encode transcription factors, are highly conserved and play important roles in the development of both vertebrate and invertebrate embryos (Hermann & Kispert 1994, Papaioannou & Silver 1998, Smith 1999). At least two T-box genes, Brachyury (bra) and Eomesodermin (Eomes), have been shown to play major roles in the primitive streak formation and mesoderm patterning in the mouse (Hermann et al. 1990, Wilkinson et al. 1990, Ciruna & Rossant 1999, Hancock et al. 1999), Xenopus (Ryan et al. 1996) and chicken (Bulfone et al. 1999). bra is one of the first morphogenetic mutants identified in the mouse. T-heterozygotes have short tails while in homozygous embryos the anterior part of the body develops but the trunk fails to develop and the embryos die in utero on day 11 of pregnancy (Herrmann 1991). Mouse embryos lacking Eomes arrest soon after implantation and fail to form organized embryonic or extra-embryonic structures (Russ et al. 2000). Studies in Xenopus have shown that Eomes is expressed 1–2 h before any other known mesodermal genes and might control bra expression before gastrulation (Ryan et al. 1996). Recently, a bovine bra cDNA fragment has been cloned and it has been observed that the pattern of expression in bovine embryos is similar to the ones described in the mouse during gastrulation (Hue et al. 2001). To further understand the process of gastrulation in ruminant embryos, we investigated the profiles of bra and Eomes expression in ovine embryos in relation to the differentiation of primitive mesoderm and primitive streak formation.

Reproductive biologists generally rely upon ‘days post-insemination’ to stage pre-implanted ruminant embryos. However, this method is not precise enough to overcome the large distribution of the developmental status of the embryos, even within the same litter. A precise staging of the embryos is essential to compare the differential expression of gene markers during this time window, for example. A more precise staging of embryos might allow comparative studies between closely related ruminant species too. Thus, it has become necessary to establish a new staging of pre-implanted ruminant embryos by a combination of morphological landmarks and differentiation features related to gastrulation. In the first part of the present study, we describe a staging of gastrulating ovine embryos by morphological criteria and in the second part we report the results of whole-mount in situ hybridization (ISH) on the expression of the T-genes bra and Eomes before and during gastrulation. Our results indicate that the differentiation of the primitive mesoderm occurs ahead of primitive streak formation and before expression of bra and Eomes, which start at the early-streak (ES) stage. Thus, no time lag was observed between bra and Eomes expression. However, during gastrulation, both genes followed the patterns of expression that have been described in other species.

Materials and Methods

Animals

All the animals used in this study were managed according to the French legislation on welfare and care of experimental animals. Oestrus-synchronized ewes of the Pre-Alpes breed were mated (day 0) and slaughtered between days 11 and 16 post coitus (p.c.). The uteri were dissected and the embryos were collected by perfusion of the uterine horns with PBS. The conceptuses were measured, photographed under a binocular microscope and classified according to their rate of development (Table 1). For fixation, the ovoid conceptuses were kept intact whereas in tubular (1–2 cm long) and fully elongated conceptuses (≥10 mm), the embryonic area was isolated from the rest of the trophoblastic vesicle. The tissues were fixed by immersion in 4% paraformaldehyde for 2–3 h at room temperature, rinsed in 0.1% Tween-20 phosphate buffer (PBTr), dehydrated in methanol series and stored in pure methanol at −40°C until use.

Probes

A bovine bra cDNA fragment was amplified by RT-PCR on RNA from whole day-16 conceptuses, using primers designed from amino acid comparisons between human and mouse T proteins (Hue et al. 2001). Mouse Eomes cDNA was a generous gift from J Rossant (S Lunenfeld Research Institute, Canada). Digoxigenin (DIG)-labelled antisense and sense RNA were produced by in vitro transcription using T7 or SP6 RNA polymerase, respectively.

Table 1 Staging and major features of the ovine embryos during the peri-implantation period.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Days p.c.</th>
<th>Conceptus shape</th>
<th>Embryo shape</th>
<th>Embryonic layers</th>
<th>Placental annexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-1</td>
<td>11–13</td>
<td>Spherical</td>
<td>Round</td>
<td>Epiblast, hypoblast</td>
<td>—</td>
</tr>
<tr>
<td>PS-2</td>
<td>12–13</td>
<td>Ovoid ≤ 0.5 cm</td>
<td>Oblong</td>
<td>Epiblast, hypoblast, primitive mesoderm</td>
<td>—</td>
</tr>
<tr>
<td>PS-3</td>
<td>12–13</td>
<td>Tubular 1–3 cm</td>
<td>Pear-shaped</td>
<td>—</td>
<td>Yolk sac</td>
</tr>
<tr>
<td>ES</td>
<td>13–14</td>
<td>Elongated ≥ 10 cm</td>
<td>Primitive streak</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MS</td>
<td>13–14</td>
<td>—</td>
<td>Node</td>
<td>—</td>
<td>Amniotic folds</td>
</tr>
<tr>
<td>LS</td>
<td>14–15</td>
<td>—</td>
<td>Notochord</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NP</td>
<td>15</td>
<td>Implanted</td>
<td>1–3 somite pairs</td>
<td>—</td>
<td>Amnion</td>
</tr>
<tr>
<td>HF</td>
<td>15–16</td>
<td>—</td>
<td>Head folds</td>
<td>—</td>
<td>Allantois bud</td>
</tr>
</tbody>
</table>

PS, pre-streak; ES, early-streak; MS, mid-streak; LS, late-streak; NP, neural plate; HF, head-folds.
Figure 1 PS-1 (a, b), PS-2 (c–f), PS-3 (g) and ES (h) embryos. (a, c, e, g and h) Whole embryos observed under the stereomicroscope. Whole-mount ISH with the *bra* (a, e) and *Eomes* (c) probes; no staining is detected in the embryonic discs. (a) The embryonic disc (arrow) protrudes at the surface of the spherical trophoblastic vesicle. (c, e) At one pole of the embryos, the dense cellular areas (*) are due to mesoderm cell accumulation as shown on transverse sections (d) and (f). (e) Extra-embryonic mesoderm (arrow) extends away from the embryonic disc. (b, d and f) Transverse sections through the areas indicated by the dotted arrows on (a, c and f). On each figure of histological sections, the oblique bars mark the limits between the trophoblast and the embryonic disc. pe, primitive endoderm, m, mesoderm.
transcription using RNA DIG-labelling kits (Roche, Mannheim, Germany).

**Whole-mount ISH**

Whole-mount ISH was performed as previously published (Hue et al. 2001). After rehydration in PBT, the samples were incubated in 10 μg/ml proteinase K for 20 min at 37°C, post-fixed in 0.2% glutaraldehyde, incubated in prehybridization buffer for 1 h at 70°C, then overnight at the same temperature in the hybridization buffer containing 2 ng/μl of DIG-labelled probes. After post-hybridization washes under stringent conditions, hybrid detection was performed with a DIG-RNA detection kit using anti-DIG alkaline phosphatase-conjugated antibody and NBT/BCIP staining (Roche). Some samples were also treated in an ISH automate (Insitu Pro, Intavis, Germany) according to a protocol adapted from Nguyen et al. (2001). In that case, for both probes prehybridization and hybridization incubations and post-hybridization washes were conducted at 65 and 55°C respectively. Since no labelling differences resulted from these two protocols, the results presented here refer to either one, indifferently. The whole-mount embryos were photographed under a binocular microscope then embedded in paraffin or in plastic resin Technovit (Heuser, Wherheim, Germany). Serial sections of the embryos were observed with or without counterstaining.

**Results**

**Staging of pre-implanted ovine embryos**

Table 1 summarizes the criteria used to identify and classify the embryos under the stereomicroscope according to their developmental stages. The developmental criteria were the following: the shape and length of the trophoblastic vesicle, the shape of the embryonic disc, occurrence of a mesodermal layer that could be observed by transparency through the trophoblast, the appearance of the primitive streak, the folding of the amnion, extension of the neural plate (NP) and somitogenesis. Gross morphological observations were completed by examination of histological sections. To avoid redundancy in illustrations, some figures of typical embryos at a given stage are illustrated with the results on *bra* and *Eomes* expression.

To account for conceptus elongation, embryo shape and early occurrence of the primitive mesoderm, the pre-streak (PS) stage was subdivided into three sub-stages. A typical PS stage 1 (PS-1) embryo is shown in Fig. 1a. The conceptus was spherical with a diameter not larger than 2 mm. The rounded embryonic disc appeared as a dense cellular mass bulging at the surface of the trophoblastic vesicle. On histological sections, the primitive endoderm (also referred to as the hypoblast) bordered the whole inner face of the blastocoelic cavity (Fig. 1b). By the PS-2 stage, the conceptuses presented early signs of elongation with an ovoid shape and a diameter ranging from 3 to 5 mm. The embryonic disc was still round with only slight signs of polarity. At one pole of the embryo a thickening of the primitive mesoderm was visible beneath the embryo (Fig. 1c). Serial transverse sections of these embryos confirmed the presence of mesoderm cells migrating in between the epiblast and the primitive endoderm at a single pole (Fig. 1d). In late PS-2 embryos, the extra-embryonic mesoderm spread out around the extra-embryonic area. The pole from which the mesoderm originated was laterally oriented to the elongation axis of the conceptus (Fig. 1e). As shown on the histological sections, a great density of mesoderm cells migrated from that pole (Fig. 1f). At PS-3, the conceptuses were tubular with a size ranging from 1 to 3 cm long. One of the main changes at PS-3 was a transition from a round to an oval embryonic disc. The long axis of the embryos was perpendicular to the long axis of the elongating conceptus (Fig. 1g and Fig. 2). We considered that the more parallel was the embryonic axis with the conceptus ones the more developed was the embryo. The stage variation between embryo collections is shown in Table 2. The PS-1 embryos were collected on days 11, 12 and 13 p.c. (100, 33 and 5% of the embryos respectively). PS-2 embryos were found on days 12 and 13 p.c. (44 and 20% of the embryos respectively) and the PS-3 embryos represented 22 and 25% of the embryos collected on days 12 and 13 respectively.

ES embryos were characterized by the change of the obloneg to a pyriform one (Fig. 1h and Fig. 3). ES embryos were observed on elongated conceptuses more than 3 cm long. In some of the ES embryos, but not all, the ante-posterior axis was parallel to the elongation axis of the conceptus. The ES embryos were found on days 13 and 14 p.c. (40 and 29% respectively).

Figure 2 Early expression of *bra* (a) and *Eomes* (b) at PS-3 stage. Whole-mount ISH showing expression of both genes at one pole of the embryos. Note the elongated yolk sac (arrow) beneath the trophoblast in (a).
Table 2 Stage variation between embryo collections from days 11 to 16 of pregnancy.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Day 11</th>
<th>Day 12</th>
<th>Day 13</th>
<th>Day 14</th>
<th>Day 15</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-1</td>
<td>100% (5/5)</td>
<td>33% (3/9)</td>
<td>5% (1/20)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS-2</td>
<td>—</td>
<td>44% (4/9)</td>
<td>20% (4/20)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS-3</td>
<td>—</td>
<td>22% (2/9)</td>
<td>25% (5/20)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ES</td>
<td>—</td>
<td>—</td>
<td>40% (8/20)</td>
<td>29% (9/31)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MS</td>
<td>—</td>
<td>—</td>
<td>10% (2/20)</td>
<td>26% (8/31)</td>
<td>33% (12/31)</td>
<td>—</td>
</tr>
<tr>
<td>LS</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>39% (12/31)</td>
<td>—</td>
<td>38% (8/21)</td>
</tr>
<tr>
<td>NP</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6% (2/31)</td>
<td>29% (6/21)</td>
<td>—</td>
</tr>
<tr>
<td>HF</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>100% (5/5)</td>
</tr>
</tbody>
</table>

PS, pre-streak; ES, early-streak; MS, mid-streak; LS, late-streak; NP, neural plate; HF, head-folds.

Figure 3 Expression of *bra* (a–c) and *Eomes* (d–f) at ES stages. Whole-mount ISH showing expression in the differentiating primitive streak from the posterior pole (PP), in ES-1 embryos (a, d) and in ES-2 embryos (b, e). (a, d) dorsal views. (b) Front view from the anterior pole. Note the expression of *bra* in the node (N) and a lack of staining in the anterior tip of the primitive streak (*). (c, f) Transverse sections through the areas shown by dotted arrows in (a) and (e) respectively. Oblique bars mark the limits between the embryo and the trophoblast. pe, primitive endoderm; ee, embryonic endoderm; m, mesoderm; YS, yolk sac cavity.
At mid-streak (MS) the conceptuses were filamentous with lengths ranging from 10 to 15 cm. The volume of the yolk sac had greatly increased. The embryos were pear-shaped showing a rounded anterior edge and a constricted posterior pole covered by the amnion folds (Fig. 4a and e). The primitive streak was apparent as a groove and extended more than two-thirds from the posterior to the anterior pole. The ante-posterior axis of the embryo was parallel to the elongation axis of the conceptus. MS embryos represented 10% of the embryos and 26% of those collected on days 13 p.c. and 14 p.c. respectively.

At the late-streak (LS) stage, the amnion folds surrounded the embryo and covered the posterior pole. The primitive streak had regressed and occupied around half of the length of the embryo (Fig. 5a). In late MS embryos, the pre-cephalic vesicles are apparent (Fig. 5f). LS embryos were found on days 14 and 15 p.c. (39 and 33% respectively).

At the NP stage, the amnion folds had fused to form the amniotic cavity and the embryo is fully enclosed within the chorionic sac. Due to the enlargement of the anterior pole, the primitive streak is reduced to the posterior half

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**Figure 4** Expression of *bra* (a–d) and *Eomes* (e, f) at the MS stage. (a, e) Whole-mount ISH. *Eomes* expression is confined to the mid-part of the primitive streak (e). (b–d) Serial transverse sections through the node (b), the mid-part of the primitive streak (c) and at the posterior pole (d) as indicated by the dotted arrows in (a). (f) Sagittal section through the primitive streak of the embryo shown in (e). PP, posterior pole; ee embryonic endoderm; m, mesoderm; short arrows, amniotic folds.
Figure 5 LS expression of *bra* (a–e) and *Eomes* (f–i). (a) Whole-mount ISH showing *bra* expression alongside the primitive streak, in the notochord (b) and in the node (c). (b–e) Transverse sections through the planes indicated by the dotted arrows. Note the decrease of staining in the anterior tip of the primitive streak (d). (f) No expression of *Eomes* is detected on whole-mount observation. (g–i) Transverse sections through the pre-cephalic vesicles and the notochord (*) (g), the node (h) and the caudal region (i). PP, posterior pole; ee, embryonic endoderm; m, mesoderm; ac, amniotic cavity; G, hindgut; short arrow, trophoblast.
of the embryo (Fig. 6a). NP embryos were collected at day 15 p.c. (29% of day-15 embryos). Implantation on the uterine epithelium begins at this stage.

Head fold (HF) embryos presented well-defined HFs, the neural groove was formed and somite pairs were apparent (Fig. 7a and b). In late HF embryos the allantois bud could be seen at the caudal extremity close to the allantois bud (arrow). hf, head folds.

Figure 6 Expression of bra at the neural-plate stage. (a) Whole-mount ISH showing bra expression alongside the notochord and in the posterior half part of the primitive streak. (b, c) Transverse sections through the notochord (b) and through the caudal area (c). m, mesoderm; ac, amniotic cavity; G, primitive gut.

Figure 7 Expression of bra at the early and late HF stages (a and b respectively). In the late HF stage, embryo expression is decreased except at the caudal extremity close to the allantois bud (arrow). hf, head folds.
Expression of bra and Eomes

To assess the specificity of the hybridization signals, the embryos were hybridized with *bra* and *Eomes* sense probes. In all the cases the samples were negatively stained (not shown).

In PS-1 and PS-2 embryos, expression of *bra* and *Eomes* was not detected (Fig. 1a, c and e).

The earliest expression of both *bra* and *Eomes* genes was detected in PS-3 embryos. Specific hybridization signals were observed as a clump of epiblast cells located at the one pole assumed to be the posterior pole of the embryo (Fig. 2a and b). In some embryos, this positive spot of cells was laterally orientated to the elongation axis of the conceptus (Fig. 2b). On transverse sections, the hybridization signal was localized both in the epiblast cells and in the mesoderm migrating below the embryonic disc (not shown).

Expression of both T-genes increased at the ES stage and followed a post-anterior gradient along the primitive streak (Fig. 3a, d and e). However, a different pattern of expression of the two genes was noticed: *bra* expression was strong in the posterior part of the primitive streak and in the node with a negative area in the anterior margin of the primitive streak (Fig. 3b). On histological sections of hybridized embryos, *bra* expression was restricted to the epiblast cells of the primitive streak and to the nascent mesoderm cells but neither in the primitive endoderm nor in the mesoderm migrating away from the primitive streak in the extra-embryonic regions (Fig. 3c). *Eomes* was more diffusely expressed in the epiblast lateral to the primitive streak while the posterior marginal zone of the embryo was not stained (Fig. 3e). As shown on transverse sections, *Eomes* expression was observed in the nascent and distal mesoderm migrating beneath the epiblast layer, in the newly formed embryonic endoderm (Fig. 3f) but not in the node. No expression was detected in the trophoblast.

At MS, expression of *bra* increased as shown by the strong staining intensity in the epiblast cells (Fig. 4a). As confirmed on transverse sections of the embryos, the node was still positive and only the mesoderm delaminating from the primitive streak expressed *bra* while the distal mesoderm remained negative (Fig. 4b–d). Expression of *Eomes* began to decrease and was restricted to the mid-part of the primitive streak. The caudal extremity of the primitive streak remained negative (Fig. 4e). The embryonic endoderm and the distal mesoderm, which had migrated away from the primitive streak, were still positive (Fig. 4f).

In LS embryos, *bra* expression was still high in the epiblast lining the primitive streak, in the node and in the notochord process (Fig. 5a–e). No expression of *Eomes* was detected in the primitive streak at this stage (Fig. 5f).

However, on the histological sections through the pre-cephalic vesicles, a faint expression of *Eomes* was observed in the embryonic mesoderm and endoderm whereas the notochord and the node remained negative (Fig. 5g and h). In the caudal extremity of the embryo, no expression of *Eomes* was detected (Fig. 5i).

At the NP stage, *bra* was still strongly expressed in the epiblast of the remaining primitive streak, in the notochord and in the mesoderm within and immediately adjacent to the primitive streak (Fig. 6a–c).

At the HF stage, expression of *bra* was high in the caudal part of the primitive streak (Fig. 7a). In late HF embryos, *bra* expression declined to become barely detected in the caudal region close to the allantoid bud (Fig. 7b). At both NP and HF stages, no expression of *Eomes* could be detected.

Discussion

Precise staging of embryos around implantation is essential to be able to compare the different studies undertaken during this period of pregnancy. As shown here, considerable variation occurs in the developmental state of ovine embryos collected on the same day p.c. and even from a single litter. Thus, staging of individual embryos should be based on their morphology rather than on the day of gestation. We present here a set of morphological landmarks for classifying pre- and gastrulating ovine embryos to provide a more precise method when reporting embryonic stages with the stereomicroscope.

The observations reported here demonstrate that in ovine embryos, the primitive mesoderm differentiates at the PS-3 stage, that is before any morphological signs of primitive streak formation. Interestingly, the aggregates of differentiating mesoderm cells were observed at a single pole of the rounded embryonic disc, before any sign of morphological ante-posterior polarity. Moreover, the occurrence of the mesoderm preceded the expression of known inductive genes, *bra* and *Eomes*, which were first expressed at the PS-3 stage. Taken all together, these features clearly distinguish the sheep embryo from other mammalian embryos studied so far. In the mouse (Hermann et al. 1990, Wilkinson et al. 1990) and in the rabbit (Viebahn et al. 2002), expression of *bra* starts at the PS stage and is concomitant with mesoderm differentiation. The results presented here suggest that other, still unknown, inducing genes are involved in mesoderm differentiation and axis patterning of the ruminant embryo. As previously described in bovine (Hue et al. 2001), rabbit (Viebahn et al. 2002) and chick embryos (Knezevic et al. 1997), whole-mount ISH of ovine embryos revealed a reduced expression in the anterior part of the primitive streak at the beginning of node differentiation. This pattern of *bra* expression might correspond to differences in cell fates along the primitive streak as seen in the mouse and chicken (Lawson et al. 1991, Garcia-Martinez et al. 1993, Tam et al. 1997). Other genes show a graded expression along the primitive streak, such as Evx1 (Dush & Martin 1992), Mesp1 (Saga et al. 1996), LCFR-F1 (Farmer et al. 1997), mrg1 (Dunwoodie et al. 1998), caudal (Epstein et al. 1997) and plp2 (Milde et al. 2001). The gradient of
bra expression is probably under anteroposterior regulatory control through the action of fibroblast growth factors (Griffin et al. 1995, Clements et al. 1996).

In mammals, the expression of Eomes has been investigated in the mouse only. Eomes was first detected in the extra-embryonic epiblast prior to gastrulation and in the embryo proper at the ES stage, thereafter expression declines to be renewed in the forebrain and limbs at days 12.5–14.5 (Buñone et al. 1999, Ciruna & Rossant 1999, Hancock et al. 1999). Overall, Eomes expression in the gastrulating ovine embryo fits the cellular pattern of expression described in the mouse. The main difference was the absence of expression in the trophoblast whatever the stage analysed. This discrepancy with the murine embryo reflects the differences in implantation type between rodents and ruminants. In the latter, the trophoblast is not invasive and implantation is superficial. In Xenopus, it has been shown that Eomes expression precedes that of other mesodermal genes including bra and induces its expression (Ryan et al. 1996). At the PS stage no time lag between bra and Eomes expression was observed in the ovine embryos. Our results suggest that other unknown mesoderm-inducing factors act up-stream of these two genes in ruminant embryos before the beginning of the gastrulation proper.

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