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Onset of the segmentation clock in the chick embryo: evidence for oscillations in the somite precursors in the primitive streak

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SUMMARY

Vertebrate somitogenesis is associated with a molecular oscillator, the segmentation clock, which is defined by the periodic expression of genes related to the Notch pathway such as *hairy1* and *hairy2* or *lunatic fringe* (referred to as the cyclic genes) in the presomitic mesoderm (PSM). Whereas earlier studies describing the periodic expression of these genes have essentially focussed on later stages of somitogenesis, we have analysed the onset of the dynamic expression of these genes during chick gastrulation until formation of the first somite. We observed that the onset of the dynamic expression of the cyclic genes in chick correlated with ingression of the paraxial mesoderm territory from the epiblast into the primitive streak. Production of the paraxial mesoderm from the primitive streak is a continuous process starting with head mesoderm formation, while the streak is still extending rostrally, followed by somitic mesoderm production when the streak begins its regression. We show that head mesoderm formation is associated with only two pulses of cyclic gene expression. Because such pulses are associated with segment production at the body level, it suggests the existence of, at most, two segments in the head mesoderm. This is in marked contrast to classical models of head segmentation that propose the existence of more than five segments. Furthermore, oscillations of the cyclic genes are seen in the rostral primitive streak, which contains stem cells from which the entire paraxial mesoderm originates. This indicates that the number of oscillations experienced by somitic cells is correlated with their position along the AP axis.

Key words: Chick, Segmentation clock, Segmentation, Cyclic gene expression, *hairy*, *lunatic fringe*

INTRODUCTION

In the vertebrate body, segments are conspicuous at the level of the vertebral column, the associated muscles and peripheral nervous system. This segmental pattern is laid down during embryogenesis through the sequential production of the mesodermal somites. The process of somitogenesis is reminiscent of segment production from a growth zone, as seen in annelids and short germband insects (McGrew and Pourquié, 1998). Somites arise bilaterally as epithelial spheres, which sequentially bud off from the rostral extremity of the presomitic mesoderm (PSM). Somitogenesis occurs in a very coordinated manner, with a new pair of somites produced every 90 minutes in the chick embryo (Packard and Jacobson, 1976).

In chick, the somitic series initiates just caudal to the otic vesicle (Hinsch and Hamilton, 1956; Huang et al., 1997). Anterior to this somitic series, the paraxial mesoderm is termed the head or cephalic mesoderm. This mesoderm is laid down before, and is continuous with, the somitic series with which it shares some characteristics. Like the somites, the major derivatives of head mesoderm are some bones and skeletal muscle fibres of the face and of the branchial arches (Couly et al., 1993; Couly et al., 1992; Noden, 1986). During embryonic development, head mesoderm precursors are transiently found in the rostral streak, in the same territory as the somite precursors (Nicolet, 1971; Psychoyos and Stern, 1996). Therefore, no discontinuity in the mode of production of the two territories in the streak is observed. Head mesoderm, however, exhibits several characteristics distinct from somitic mesoderm. For example, development of the head mesoderm derived muscles is subject to a different genetic control than that of trunk muscles (Hacker and Guthrie, 1998; Tajbakhsh et al., 1997). Furthermore, unlike somitic tissue, head mesoderm does not give rise to any dermis, which at the head level is provided by the neural crest (Couly and Le Douarin, 1988).

Somitogenesis has been shown to involve a molecular oscillator called the ‘segmentation clock’, which acts in presomitic cells (Palmeirim et al., 1997). This molecular clock has been identified in fish, chick and mouse and controls the periodic expression of ‘cyclic genes’, which are, so far, all related to the Notch pathway. The cyclic genes include vertebrate hairy homologues, such as c-*hairy1* and c-*hairy2*, *HES1*, *HES7* or *Her1*, the glycosyl transferase *lunatic fringe* (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et

al., 1998), and the Notch ligand *DeltaC* (Jiang et al., 2000). Expression of these genes appears as a dynamic wave, which sweeps across the whole PSM once during each somite formation. One proposed role for the segmentation clock is to modify periodically the activation of the Notch pathway in order to generate the somite boundaries (Pourquie, 1999).

So far, all studies describing the dynamic expression of the cycling genes have been performed in embryos already containing several formed somites. Nothing is known about the onset of the oscillations during embryogenesis. We have undertaken a detailed study of the expression pattern of the c-*hairy1*, c-*hairy2* and *lunatic fringe* mRNAs from gastrulation to the beginning of somite formation in the chick embryo. We observed a cyclic expression of these genes as soon as the paraxial mesoderm territory (which includes the head and somitic mesoderm territories) ingresses from the epiblast into the primitive streak. Oscillations of c-*hairy2* and *lunatic fringe* expression are detected in the whole presumptive territory of the paraxial mesoderm in the rostral primitive streak which includes the pool of somitic stem cells from which the paraxial mesoderm originates (Nicolas et al., 1996; Stern et al., 1992). Therefore, somitic cells do not only experience 12 pulses of *hairy* and *lunatic fringe* expression prior to their segmentation as originally proposed (Palmeirim et al., 1997), but rather the number of cycles may correlate with the future regional level of the cells along the anteroposterior (AP) body axis. This observation strongly suggests that the segmentation clock might be linked to the AP patterning system of the axis. Furthermore, during its formation, head mesoderm undergoes only two pulses of *hairy* and *lunatic fringe* expression. Therefore, as most head segmentation models describe at least five head segments, our data do not support a link between such mesodermal head segments and the segmentation clock. This suggests that metamery of the paraxial mesoderm of the head and of the body rely on different molecular mechanisms.

MATERIALS AND METHODS

Eggs and embryos

Fertilised chick (*Gallus gallus*, JA57, Institut de Sélection Animale, Lyon, France) eggs were obtained from commercial sources. Eggs were incubated for up to 24 hours in a humidified atmosphere at 38°C. The embryos were staged according to the developmental table of Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992). Intermediate stages between stages 3 and 4 were determined according to Psychoyos and Stern (Psychoyos and Stern, 1996). Embryos were also classified by age after in situ hybridisation with the c-*hairy2* and *lunatic fringe* probes. To that end, the ratio of primitive streak length over that of the axial mesoderm cranial to the node was used as a staging criteria. Length of the structures was measured using a Leica MZ6 stereomicroscope equipped with a graticule.

Whole-mount in situ hybridisation and histology

The c-*hairy1*, c-*hairy2* and *lunatic fringe* probes were produced as described in (Jouve et al., 2000; McGrew et al., 1998; Palmeirim et al., 1997). Embryos and explants were fixed overnight at 4°C in 4% formaldehyde-2mM EGTA, rinsed in phosphate-buffered saline (PBS), dehydrated through a methanol series and stored in 100% methanol at –20°C. Whole-mount in situ hybridisation was performed according to the procedure described previously (Henrique et al., 1995). Embryos were photographed as wholemounts in PBT (PBS, 0.1% Tween 20) using a Leica MZ10 stereomicroscope.

For cryosections, embryos stained in whole mount were embedded in gelatine-sucrose as described elsewhere (Chedotal et al., 1996) and serial 25 μm sections were cut using a Leica cryostat. Some stained embryos were also embedded in albumin gelatine as described elsewhere (Chedotal et al., 1996) and sectioned at 50 μm using a Leica Vibratome. Sections were observed using a Leica DM equipped with Nomarski optics.

In vitro culture of chick explants

Chick embryos ranging from stage HH3 to HH7 were used throughout this study. Different types of explants were cultured on albumin agar plates. These plates were prepared using albumin collected from eggs incubated for 2 days, and stirred for 15 minutes, then a solution of NaCl was added to obtain a final concentration of 150 mM. This albumin-NaCl solution was added to an 0.6% agar solution at 1:1 ratio. This solution was pre warmed at 50°C, before being poured into 35 mm culture dishes (Sundin and Eichele, 1992). Different types of explants were precisely delimited, excised and disposed directly on albumin agar plate with their dorsal side up. Explants were then cultured for different time periods (from 1 hour to 4.5 hours) in a humidified atmosphere at 38°C and then fixed to be processed for in situ hybridisation. Using this technique, half-embryos were observed to develop as in New cultures up to the 10-15 somite stage.

In the first series of experiments, embryos were divided into two halves by cutting across the germ layers in the middle of the primitive streak. One half was immediately fixed. The other was cultured as described above.

In the second series of experiments, embryos were also divided sagittally, and in one of the two halves, the caudal part was removed and fixed immediately. Both halves, the entire and the truncated one, were incubated for the same period of time.

RESULTS

Analysis of c-*hairy1*, c-*hairy2* and *lunatic fringe* expression prior to somite formation

We have examined the expression of c-*hairy1*, c-*hairy2* and *lunatic fringe* mRNA during the stages ranging from primitive streak formation (HH2) to the appearance of the first morphological somite (HH7). Large series of embryos between stage HH2 and stage HH7 were collected (n=258 for c-*hairy2*; n=381 for *lunatic fringe*; n=147 for c-*hairy1*) and analysed by whole-mount in situ hybridisation. The embryos were then classified by age using morphological criteria such as the ratio between the length of axial mesoderm cranial to the node (notochord plus prechordal mesoderm) versus primitive streak. The embryos were then categorised by expression pattern. Histological analysis of the embryos was also performed to determine the germ layers in which the expression was located. The three genes are expressed in dynamic overlapping domains in the paraxial mesoderm and its prospective territory in the rostral primitive streak. Expression of these genes will be discussed in relation to the major events concerning paraxial mesoderm formation.

The expression profiles of c-*hairy1*, c-*hairy2* and *lunatic fringe* are similar in the paraxial mesoderm and its prospective territory in the streak. To demonstrate this, we bisected the primitive streak along the AP axis in embryos from stage HH3 to HH4. Both halves were then fixed immediately, one was probed for c-*hairy2* or c-*hairy1* expression, and the other for *lunatic fringe*. The same expression pattern was observed in

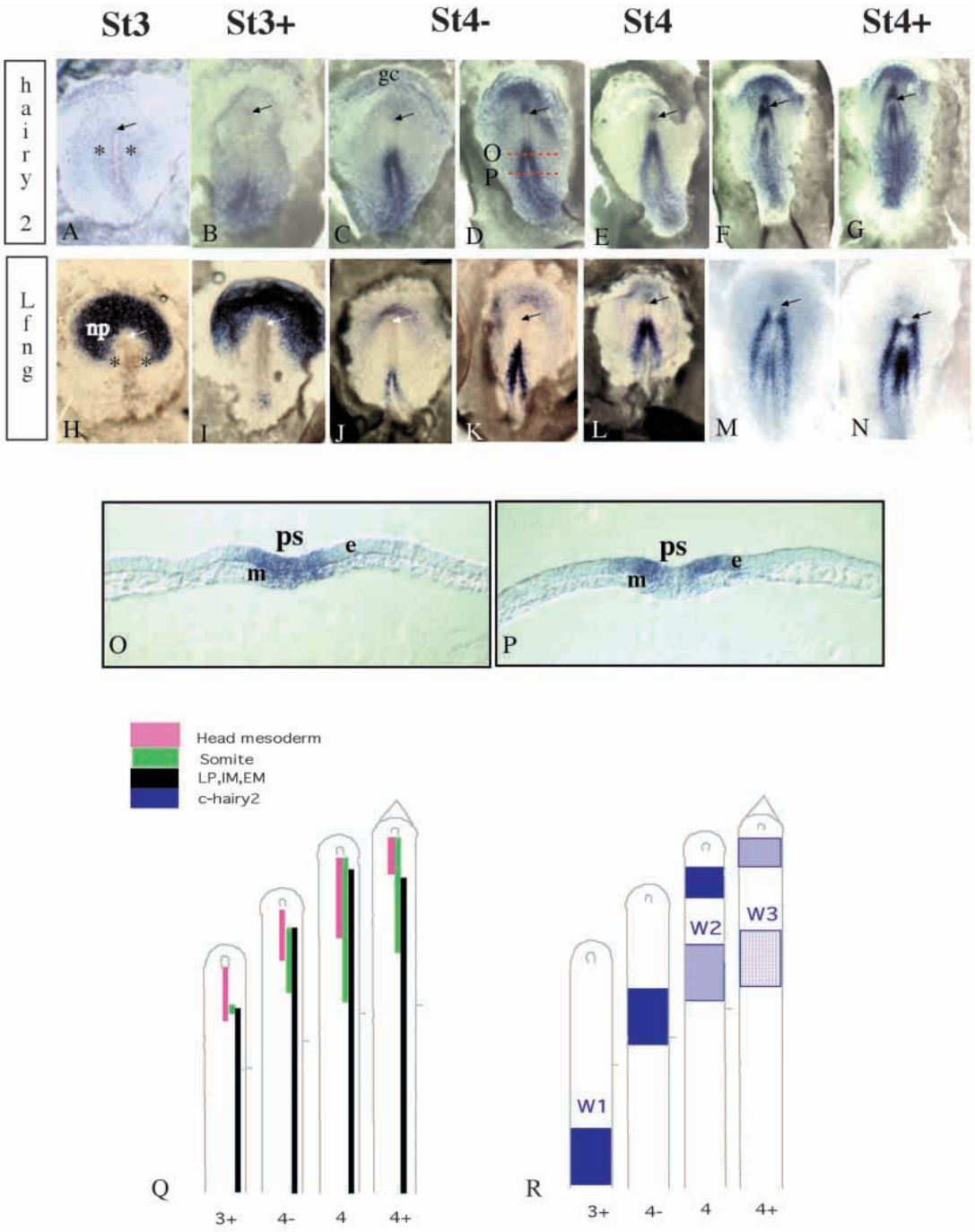

Fig. 1. Onset of c-*hairy2* and *lunatic fringe* RNA expression in paraxial mesoderm precursors. (A-G) Expression of c-*hairy2*; (H-N) expression of *lunatic fringe*. (O,P) Transverse sections from the embryo seen in D. The arrow indicates the level of Hensen's node. (A,H) Stage HH3: expression of c-*hairy2* and *lunatic fringe* is not detected in the presumptive territory of the paraxial mesoderm in the epiblast lateral to the primitive streak (asterisks). Transient expression of *lunatic fringe* (H,I) is detected in the neural plate (np). (B,I) Stage HH3+: expression is observed in the caudal primitive streak. (C-E,J-L). In progressively older embryos, expression of the c-*hairy2* (C-E) and *lunatic fringe* (J-L) mRNA appears as a chevron, which sweeps caudorostrally along the primitive streak. (F,M) At stage HH4, the chevron of c-*hairy2* and *lunatic fringe* expression ends its rostral progression. Its rostral tip coincides with the axial prechordal mesoderm, which maintains c-*hairy2* (F,G) but not *lunatic fringe* (M,N) expression. (F,G,M,N) Subsequently a second chevron of c-*hairy2* and *lunatic fringe* expression is initiated in the mid-primitive streak. This chevron also undergoes an anterior progression that terminates at the level of Hensen's node. (O) The centre of the chevron appears to include the primitive streak (ps), and the immediately adjacent cells of the epiblast (e) and already ingressed mesoderm (m), while the wings of the chevrons are comprised solely of epiblast cells (P). (Q) Fate map of the precursors of the head and somitic mesoderm in the streak, adapted from Psychoyos and Stern (Psychoyos and Stern, 1996), compared with the expression domains of c-*hairy2* or *lunatic fringe* in the primitive streak (R). The two chevron-like waves (W1, W2) sweep across the rostral primitive streak, which at these stages contains the precursors of the head and somitic mesoderm. EM, extra-embryonic mesoderm; GC, germinal crescent; IM, intermediate mesoderm; LP, lateral plate.

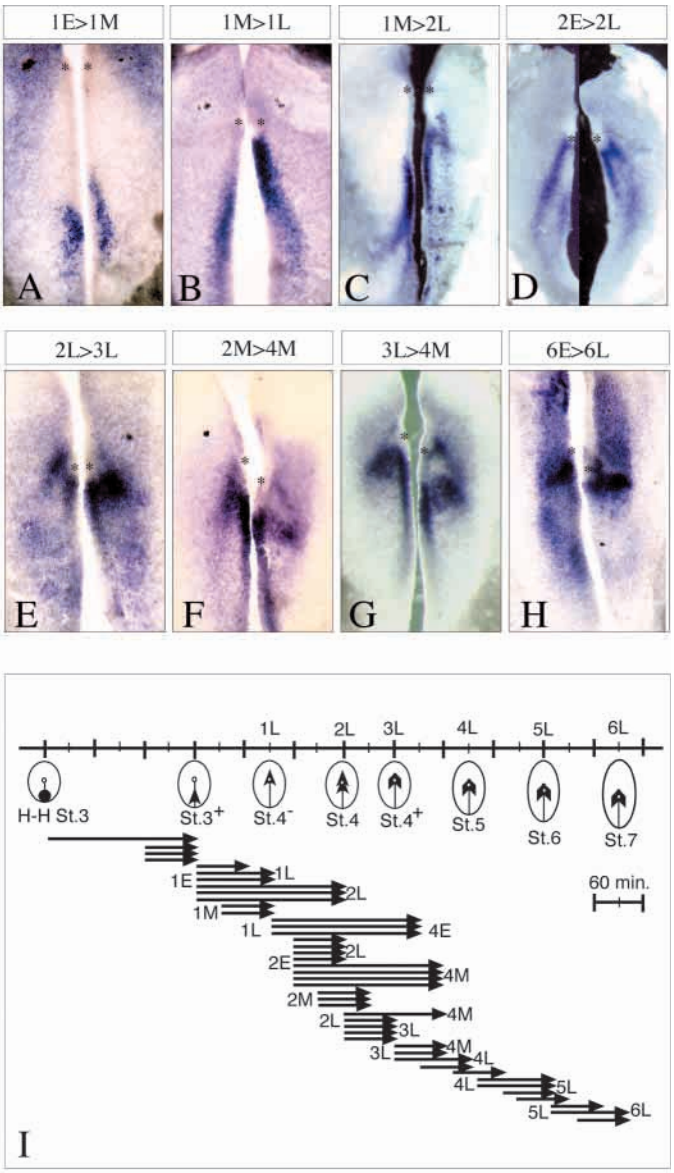

Fig. 2. (A-H) Evidence for periodic waves of *lunatic fringe* expression before formation of the first pair of somites. Existence of a dynamic wave of expression of *lunatic fringe* in the paraxial mesoderm was demonstrated by performing half-embryo cultures. (A-H) Embryos from stage HH3+ to stage HH6 were bisected along the midline, and one half was fixed immediately (left half in each panel), while the other half (right half in each panel) was cultured for various time. Three phases were distinguished on the basis of the AP location of the streak expression domain. These phases were termed E (early), when the domain was located at its most caudal position in the streak, and M (intermediate) and L (late), when it became located progressively more rostral. Asterisks indicate the position of the Hensen's node. (A) Stage HH3+ half embryo (left) showing caudal expression of *lunatic fringe* (1E, early phase of first chevron). After 3 hours in culture (right explant), expression has reached the mid-streak level (1M, intermediate phase of the first chevron). (B) Stage HH4– half embryo (left) with chevron-like expression of *lunatic fringe* located at the mid-streak level (1M). After 1 hour in culture, the chevron migrated rostrally and reached Hensen's node level (right explant, 1L; late phase of first chevron). (C) Stage HH 4– half embryo (left, 1M). After 3 hours in culture, two chevrons of *lunatic fringe* expression are observed at the level of the Hensen's node, indicating a late phase of second chevron migration (2L) (right explant). (D) Stage HH4 half embryo showing an early phase of second chevron migration (2E, left). After 1 hour in culture, the second chevron has reached the level of the Hensen's node (right, 2L). (E) Stage HH4+ half embryo showing a late stage of the second chevron migration (left, 2L). After 1 hour in culture, the expression domain of *lunatic fringe* now appears as a stripe at the level of the node (right, 3L: late phase of third wave). Note the expression in the rostral primitive streak adjacent to the Hensen's node. (F) Stage HH4+ half embryo (left, 2M). When cultured for 3 hours, the streak expression domain is separated from the Hensen's node by a negative domain. Expression of *lunatic fringe* now appears as an intermediate stage of the fourth wave (4M: intermediate phase of fourth wave). Note the non-expressing domain between the streak-positive domain and the Hensen's node. (G) Stage HH5 half embryo showing the stripe lateral to the Hensen's node characteristic of the late stage (left, 3L). After a 1 hour culture period, the expression now corresponds to the intermediate stage of the fourth wave (right, 4M). (H) Stage HH6 half embryo showing the caudal expression domain in the primitive streak and the lateral mesodermal domain characteristic of the early stage of the sixth wave (6E, left). After 1 hour in culture, expression appears as a stripe adjacent to the Hensen's node, indicating a late stage of the 6th wave (6L, right). (I) Schematic representation of the kinetics of the early waves of *lunatic fringe* expression based on the results of half-embryo cultures. Each arrow corresponds to a half embryo-culture in which the length of the arrow indicates the culture period of the right half (a small unit in the scale indicates 30 minutes). *lunatic fringe* expression pattern before and after culture are indicated on both sides of the arrows.

the paraxial mesoderm and its presumptive territory in both halves for each of the three genes (data not shown).

By contrast, expression of the three genes differs in the other embryonic tissues. For example, c-*hairy1* is strongly expressed in the forming neural plate, making expression in the underlying paraxial mesoderm difficult to observe in whole-mount embryos (data not shown). This led us to focus our analysis upon the c-*hairy2* and *lunatic fringe* genes, in which mesodermal expression could easily be observed using whole-mount in situ hybridisation.

Onset of the dynamic expression of the cyclic genes in the prospective paraxial mesoderm correlates with ingression of its precursors into the primitive streak

Between stages HH2 and HH3, the presumptive territory of the paraxial mesoderm has been mapped to the epiblast lateral to the primitive streak (Bortier and Vakaet, 1992; Hatada and Stern, 1994). At these stages, no expression of either c-*hairy2* or *lunatic fringe* is detected in this area (Fig. 1A,H asterisks and data not shown). The strong expression of *lunatic fringe* that persists up to stage HH4 is seen in the neural plate (Fig. 1H,I).

Expression of these two genes is observed at stage HH3+ in the caudal primitive streak and, for c-*hairy2*, in an adjacent zone of the epiblast (Fig. 1B,I). Between stages HH3+ and HH4–, c-*hairy2* and *lunatic fringe* expression appears as a chevron, which undergoes an apparent 'zipper-like' movement anteriorwards. During this progression, it first crosses primitive streak territories fated to give rise to extra-embryonic mesoderm (see Fig. 1B-D,I-K,Q,R). Examination of transverse

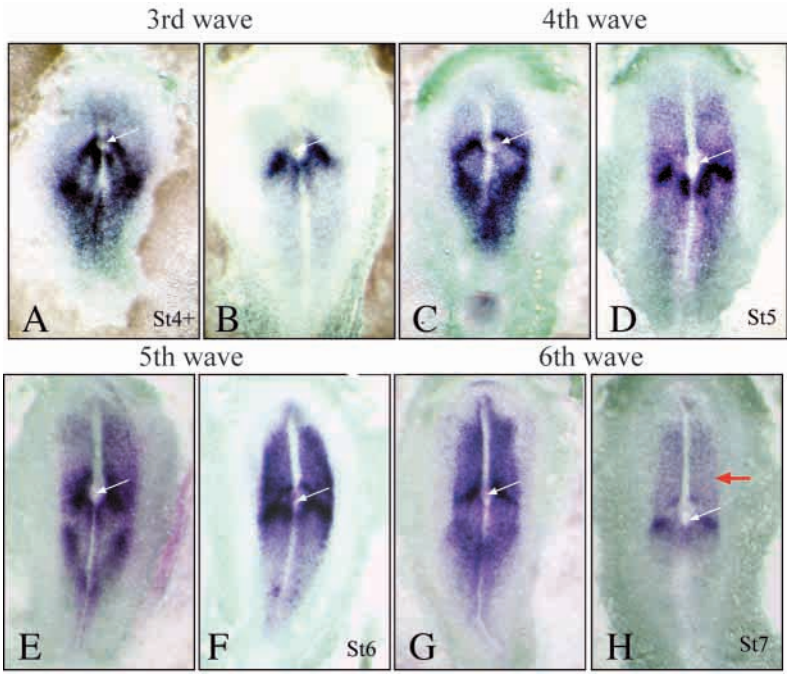

Fig. 3. Waves of *lunatic fringe* expression before formation of the first somite. (A-H) *lunatic fringe* expression sequence in developing embryos from stage HH4+ to stage HH7. After stage HH4+, *lunatic fringe* becomes expressed in a sequence resembling that observed in 15-20 somite stage embryos (McGrew et al., 1998). (A) Third wave: expression first appears in a caudal domain of the rostral primitive streak and laterally in two adjacent domains in the newly ingressed mesoderm. Remnants of the first two chevrons can been seen rostral and caudal to Hensen's node (white arrow). (B) Slightly later, the expression domain has moved anteriorwards and appears as two broad stripes lateral and then anterior to Hensen's node. Subsequently, three similar waves of expression of *lunatic fringe* (C-F) are observed before the formation of the first somite (G,H). The red arrow indicates the level of the caudal boundary of the first somite. White arrows indicate the level of Hensen's node.

sections of embryos of this stage shows that the chevron centre corresponds to expression by the primitive streak and a narrow lateral strip of adjacent epiblast and mesoderm (Fig. 1D,O), whereas the more caudal lateral regions of the chevron correspond to expression in epiblast cells (Fig. 1D,P). In addition to this expression in the epiblast and the streak, c-*hairy2* is also expressed in the hypoblast in the germinal crescent (Fig. 1C-G).

The chevron of expression reaches the anterior part of the primitive streak at the pit of Hensen's node, at stage HH4 when presumptive paraxial mesoderm cells (head and somite mesoderm) are detected in this region of the primitive streak (Psychoyos and Stern, 1996) (Fig. 1F,M,Q,R). At this stage, a second chevron of expression appears in the mid-primitive streak region, i.e. in the presumptive territory of the paraxial mesoderm (Fig. 1F,G,M,N). Analysis of transverse sections reveals that this chevron is located in the same tissue layers as the first one (data not shown). Its centre is formed by cells of the primitive streak and immediately adjacent mesoderm and epiblast, while its lateral extensions are exclusively in the epiblast.

During the final stages of primitive streak extension (stage HH4/4+), the first chevron continues its anterior movement and reaches the territory of the forming axial prechordal mesoderm, which becomes positive for c-*hairy2*, but not for *lunatic fringe* (Fig. 1F,G,M,N). The second chevron continues its anterior progression until it finally reaches the Hensen's node region at stage HH4+ (Fig. 1G and data not shown).

To further confirm this expression sequence, we cultured bilaterally bisected avian embryos in vitro (n=101). One half was fixed immediately and the other half was cultured on an albumin agar plate for different periods of time. Both halves were then hybridised with the probe for *lunatic fringe*, and the expression patterns on the two sides were compared (Fig. 2A-D). This analysis confirms the chevron progression shown in Fig. 1. It also indicates that, in vitro, the first chevron takes approximately 5 hours to complete its progression, whereas the second chevron achieves its migration along the rostral streak in about an hour (Fig. 2I).

Therefore the onset of c-*hairy2* and *lunatic fringe* expression in paraxial mesoderm precursors correlates with the time of their ingression from the epiblast into the primitive streak. During the stages when head mesoderm is produced by the primitive streak (between stages HH3 to HH4+), the entire presumptive territory of the paraxial mesoderm, including the head mesoderm, is swept by two chevron-like waves of expression of c-*hairy2* and *lunatic fringe* (Fig. 1Q,R). No cyclic expression of these genes is later detected in the head mesoderm territory, indicating that this tissue experiences only two oscillations of the cyclic genes.

Onset of somitic mesoderm production correlates with a change in cyclic gene expression

When the second chevron reaches the level of Hensen's node at stage HH4+, a new domain of c-*hairy2* and *lunatic fringe* expression appears in the mid-streak region and in two lateral domains of adjacent mesoderm (Fig. 3A). This expression domain moves anteriorly while narrowing, until it reaches the level of Hensen's node (stage HH4+/5−) where it remains transiently as two bilateral stripes of expression in the paraxial mesoderm lateral to the node (Fig. 3B). According to fate maps, these two stripes of expression delimit the rostral most extent of the PSM at that stage and thus map the level of the presumptive first somite (Psychoyos and Stern, 1996) (C. J., T. I. and O. P., unpublished).

At the end of this wave progression, a new expression domain of *lunatic fringe* and c-*hairy2* appears in the caudal part of the presumptive paraxial mesoderm territory in the mid-streak region, and in two lateral domains of mesoderm adjacent to the streak-positive domain (Fig. 3C and data not shown). This expression pattern follows the same anteriorwards progression and ends up as two stripes lateral to Hensen's node (stage HH5) (Fig. 3D). One more wave of c-*hairy2* and *lunatic*

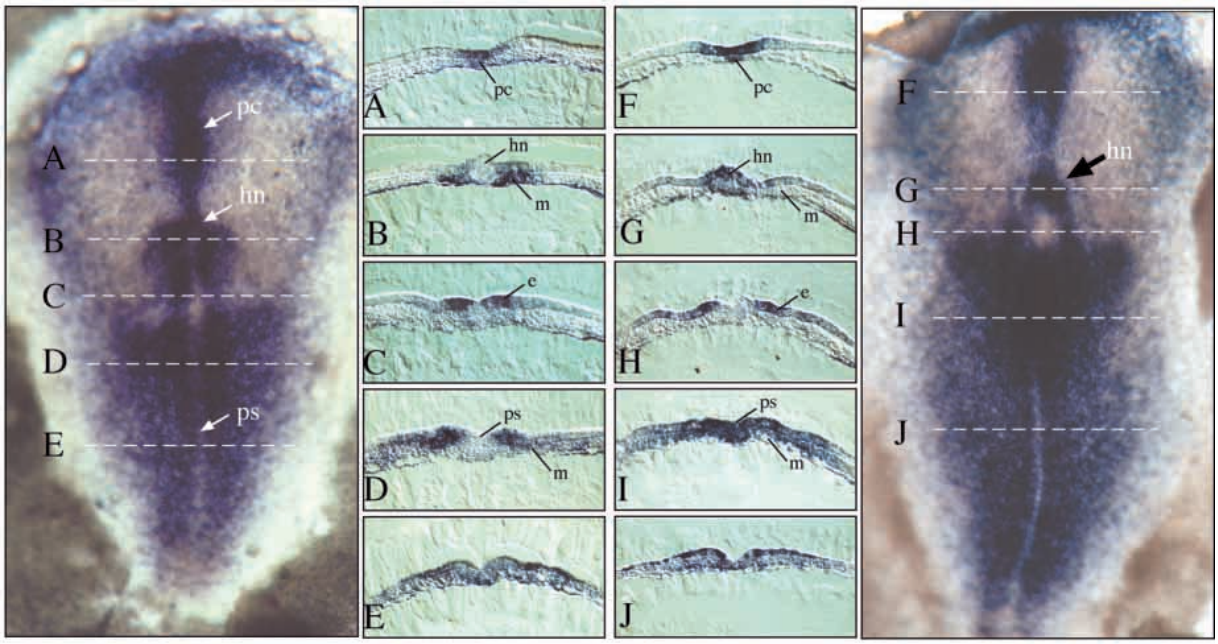

Fig. 4. Somitic progenitors in the primitive streak undergo pulses of c-*hairy2* expression. Right and left panels show two stage 4+ embryos hybridised in wholemount with the c-*hairy2* probe. The right embryo is slightly older than the left one, as is seen by the slightly extended length of axial mesoderm. The left embryo corresponds to the end of the third wave, while the right embryo marks the beginning of the fourth wave (see Fig. 6 and Fig. 3B,C). (A-E) Transverse cryosections of the left embryo at the levels shown on the wholemount. Mesodermal expression is observed in the prechordal mesoderm and notochord and in the overlying neural midline (A and not shown). At the level of Hensen's node, the expression is detected only in the paraxial mesoderm precursors, in two stripes lateral to the node (B), which probably correspond to the prospective first somite. The other major site of expression is the epiblast (C,D) and the caudal mesoderm fated to give rise to extra-embryonic mesoderm (E). (F-J) Transverse sections of the right embryo. In contrast to the left embryo, no expression is detected in the mesoderm that flanks Hensen's node, while Hensen's node itself expresses the c-*hairy2* message (G). In this embryo, the only expression detected in the paraxial mesoderm precursors includes the primitive streak and the adjacent mesoderm in a territory located caudal to Hensen's node (I). As in the younger embryo, strong expression of c-*hairy2* is also detected in the ventral neural plate and underlying axial mesoderm (F), in the epiblast (H-J) and in the mesoderm ingressing at the level of the caudal primitive streak (J). At this stage, expression of c-*hairy2* message in the paraxial mesoderm is detected as a broad caudal domain in the newly ingressed presomitic mesoderm. This data demonstrates that pulses of expression occur in the territory containing the precursors of the paraxial mesoderm, i.e. the rostral primitive streak (compare D with I). hn, Hensen's node; pc, prechordal mesoderm; ps, primitive streak.

fringe expression (stage HH6) (Fig. 3E,F and data not shown) is observed before the first somitic boundary is visible (stage HH7) (Fig. 3G,H). Therefore, when the production of presomitic material by the primitive streak begins, the dynamic expression pattern of the cyclic genes starts to resemble the wave-like expression described in 15-20 somite stage embryos by Palmeirim et al. (Palmeirim et al., 1997).

This dynamic expression sequence was confirmed using the half-embryo culture system described above (n=74). The expression in the primitive streak and in the two lateral domains of mesoderm takes 1 hour to progress anteriorly to the most anterior region of the primitive streak to form the two lateral stripes around Hensen's node (Fig. 2E-H,I). Under these experimental conditions, at stage HH5, one complete wave is achieved in 1.5 hours.

Surprisingly, expression of c-*hairy2* and *lunatic fringe* does not appear simultaneously in the whole presumptive territory of the paraxial mesoderm in the primitive streak. Rather, it is initiated in the caudal half of the rostral primitive streak and then moves rostrally to the rostral-most part of the streak and Hensen's node (Fig. 2G,H and Fig. 3). Thus, the waves of cyclic gene expression undergo an anterior to posterior progression in the streak, reminiscent of the behaviour of the first two chevrons.

Cyclic gene expression oscillates in the paraxial mesoderm progenitors in the rostral primitive streak

This complex expression profile of c-*hairy2* and *lunatic fringe* was further analysed in detail in transverse sections (Fig. 4). At the stage when c-*hairy2* expression appears as two lateral mesodermal stripes flanking Hensen's node (stage HH4+), the node itself, the primitive streak and the ingressed mesoderm immediately lateral to the streak are negative for c-*hairy2* (Fig. 4B-D). Indeed, the c-*hairy2* expression domain observed in whole-mount embryos is mainly localised to the epiblast (Fig. 4A-E). At this stage c-*hairy2* expression in the paraxial mesoderm is essentially restricted to these two rostral stripes (Fig. 4B), and no expression is observed in the rostral primitive streak. A similar expression pattern in the streak and the paraxial mesoderm is observed for *lunatic fringe* at the same stage (Fig. 3B,D,F,H).

By contrast, at a slightly later time point, when the mesodermal domains of expression lateral to the primitive streak are observed (Fig. 4F-J), cells of the primitive streak and the laterally adjacent epiblast and mesoderm express c-*hairy2*

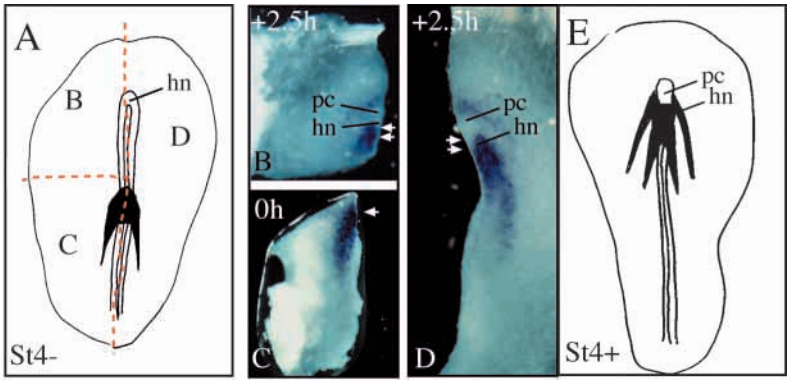

Fig. 5. Dynamic expression of *lunatic fringe* is independent of propagatory signals spreading anteriorwards along the primitive streak. (A) The experiment testing for signal propagation along the primitive streak. Embryos were divided sagittally, and in one of the two halves the caudal part was removed and fixed immediately (C). Both halves, the entire (D) and the truncated one (B), were incubated for the same period of time. The positions of the cuts are marked by the broken red lines. The black chevron on the primitive streak corresponds to the expression pattern of *lunatic fringe* in the embryo shown in (B-D) prior to culture. (E) The expression pattern of *lunatic fringe*, showing the two chevrons of expression (in black) in the embryo shown in (B-D) after culture. The same expression pattern is observed in ablated and unoperated halves, even after extended culture (B-D). Therefore, progression of *lunatic fringe* expression along the primitive streak does not rely on a posteriorly derived signal spreading through the primitive streak. hn, Hensen's node; pc, prechordal mesoderm.

and *lunatic fringe* mRNA (Fig. 4I, compare with Fig. 4D) and *lunatic fringe* (Fig. 3A,C,E,G and data not shown). Weaker expression in Hensen's node is detected and the mesoderm lateral to the node is negative at this stage (Fig. 4G, compare with Fig. 4B).

Waves of expression in the primitive streak are independent of a propagatory signal

Cell-labelling experiments indicate that the anteriorwards progression of the chevrons of cyclic genes expression along the primitive streak is unlikely to result from massive forward cell movements (Psychoyos and Stern, 1996) (C. J. and O. P., unpublished). Another possibility is that chevron migration along the streak is caused by the propagation of a signal originating in the posterior part of the embryo spreading and activating expression of the *hairy* and *lunatic fringe* genes in successively more anterior cells. We have tested this possibility by creating a physical discontinuity in the streak. We divided embryos at stage HH3+/4– sagittally, and in one of the two halves the caudal part was removed and fixed immediately to see where the gene was expressed at the onset of the experiment (n=192). Both halves, the entire and the truncated one, were cultured for the same period of time. Then the three parts were hybridised with the probe for *lunatic fringe*. The same expression pattern was observed in truncated and control halves, even after extended culture (Fig. 5A-E). Therefore, progression of *lunatic fringe* expression along the primitive streak does not rely on a posteriorly derived signal being propagated along the primitive streak.

DISCUSSION

Onset of the segmentation clock correlates with ingression of the paraxial mesoderm territory within the primitive streak

During primitive streak formation in the chick embryo, the prospective paraxial mesoderm territory includes both head mesoderm and somitic territories, and is located in the epiblast lateral to the streak (Bortier and Vakaet, 1992; Hatada and Stern, 1994). It is only during the final phase of primitive streak elongation (stage HH3+/4) that precursors of the paraxial mesoderm ingress within the rostral primitive streak. These precursors are thought to become resident in the rostral primitive streak as a population of stem cells from which the whole somitic territory will arise during primitive streak and then tail bud regression (Nicolas et al., 1996; Stern et al., 1992).

Precursors of the head mesoderm are already detected in the rostral streak at stage HH3+ and some of these remain up to stage HH4+, whereas precursors of the somitic paraxial mesoderm are found in the streak slightly later, i.e. mostly from stage HH4– onwards (see Fig. 1Q) (Nicolet, 1971; Psychoyos and Stern, 1996). After the head mesoderm progenitors have left the streak, from stage 4+/5 on, cells leaving the primitive streak will participate in somite formation. In contrast to the head mesoderm (Fig. 6, pink) which undergoes a striking anteriorwards extension, the somitic mesoderm (Fig. 6, green) appears to be laid down in an anterior to posterior fashion, correlating with the onset of the regression movements of the streak.

c-*hairy2* and *lunatic fringe* expression are not detected in the presumptive territory of the paraxial mesoderm in the epiblast before its ingression into the primitive streak. At this stage, the cyclic genes are detected only in the posterior-most part of the primitive streak in a territory fated to give rise to extra-embryonic mesoderm. During primitive streak extension between stages HH3 to 4, the expression pattern of the two genes appears as a chevron moving anteriorly and finally crossing the presumptive territory of the paraxial mesoderm during stage HH4–/4 (Figs 1, 6). Hence, paraxial mesoderm progenitors of the streak experience the first pulse of cyclic gene expression by stage HH4. Thus, the pulses of c-*hairy2* and *lunatic fringe* expression indicative of the onset of the clock in prospective paraxial mesoderm cells (head and somitic) are initiated after their ingression into the streak. Interestingly, other tissues that transit through the primitive streak, such as the heart, gut and notochord, will also experience one pulse of cycling gene expression.

The number of pulses of expression in paraxial mesoderm cells correlates with their position along the AP axis

Previous studies of c-*hairy1*, c-*hairy2* and *lunatic fringe* expression have not addressed the status of the clock in the presumptive territory of somites in the streak and the tail bud, i.e. before entry of these cells into the PSM. In this study, we

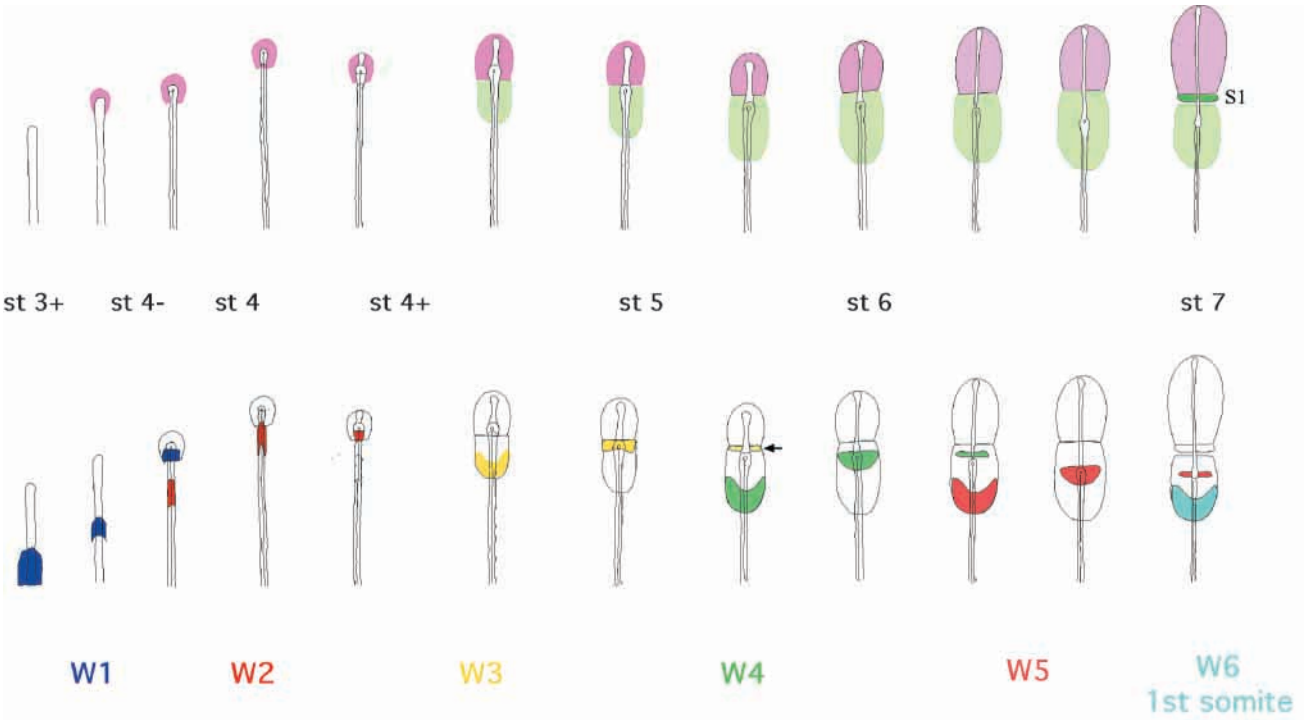

Fig. 6. Onset of cyclic gene expression in the paraxial mesoderm in relation to head and somitic mesoderm development. The top row shows a diagrammatic representation of primitive streak elongation and regression, and the forming axial and paraxial mesoderm of embryos between stage HH3+ and HH7. Head mesoderm (pink) is produced by the primitive streak between stage HH4– and 5 and it then undergoes a striking anteriorwards elongation when the primitive streak regresses. Presomitic mesoderm (light-green domain) starts to be formed from stage HH4–/5 and its production progresses together with primitive streak regression movements. Formation of somites (dark green) starts from stage 6 onwards. Bottom row shows the waves of cyclic gene expression prior to somite formation. The expression domains of c-*hairy2* (or *lunatic fringe*) in the paraxial mesoderm and its precursors in the streak are presented in colour, each wave being represented by a different colour. W1 and W2 correspond to the first two chevrons described in Fig. 1. Subsequently, waves of expression sweep across the rostral primitive streak and the newly formed PSM. Altogether, a dynamic expression sequence in the paraxial mesoderm and its precursors is reiterated five times before the formation of the first morphological somite. The black arrow marks the position of the first somite precursors.

have observed that once the presumptive territory of the paraxial mesoderm becomes resident in the rostral primitive streak, it starts to undergo pulses of gene expression similar to those observed in the PSM of older embryos.

Single-cell injection studies in the chick embryo and a retrospective clonal analysis performed in the mouse embryo have led to the hypothesis that somites are populated by the descendants of a small number of stem cells located in the rostral primitive streak (Nicolas et al., 1996; Stern, 1992). These stem cells remain resident in the streak and later in the tail bud, and generate all the cells that contribute to the somites. Our observations from histological sections of the primitive streak demonstrate that the streak cells undergo pulses of cyclic gene expression (Fig. 4 and data not shown, see also Fig. 3). Therefore, if the somitic stem cells are located in the streak, they should be continually experiencing pulses of cyclic gene expression.

According to this model, the number of oscillations undergone by the PSM descendants of the stem cells in the primitive streak will be directly correlated with their future position along the AP body axis. We observed that the production of the first somite is preceded by the two chevron-like waves of cyclic gene expression associated with the production of head mesoderm. If the whole paraxial mesoderm including both head and somitic mesoderm is derived from the same stem cell pool, then cells of somite number x will have undergone $x+2$ oscillations in the primitive streak corresponding to the number of formed somites plus the first two waves. Such a counting mechanism might play a fundamental role in AP regionalisation of the body axis. These results are in agreement with recent studies that suggest a link between the segmentation clock and spatiotemporal activation of Hox genes (Dubrulle et al., 2001; Zakany et al., 2001).

Asynchronous oscillations in the precursors of the medial and lateral paraxial mesoderm

In the PSM of two-day old embryos, the wave of cyclic gene expression occurs in a caudorostral direction and results in cells of progressively more anterior character activating these genes during one cycle (Palmeirim et al., 1997). We show that the first two waves of cyclic gene expression, which appear as chevrons migrating along the primitive streak, also undergo a caudal-to-rostral progression in the embryo. However, given the fate of the embryonic territories in the streak, this progression does not cause cells of progressively more anterior character but of progressively more axial character to activate these genes: the chevrons progressively cross the prospective extra-embryonic, lateral plate and paraxial mesoderm to end their migration in the most axial territories, i.e. the prechordal mesoderm and the notochord.

This dynamic behaviour in the streak appears to be maintained during later oscillations of the cycling genes, as later waves are also characterised by a streak-expressing domain that moves rostrally (Fig. 3 and data not shown). In addition to this streak domain, from the third wave onwards, dynamic expression is seen in the descendants of these streak precursors that form the PSM. This mesodermal expression domain moves rostrally to finally form two stripes of expression lateral to Hensen's node. This wave-like expression in cells of progressively more anterior character is similar to the one originally described for the PSM (Fig. 3) (Palmeirim et al., 1997). Apart for the first wave, oscillations in the precursors of the rostral streak and in the PSM, occur with the same periodicity in the streak and in the PSM.

Somites can be subdivided into a medial and a lateral moiety on the basis of their origin during gastrulation (Psychoyos and Stern, 1996; Selleck and Stern, 1991) and of their fate (Ordahl and Le Douarin, 1992). DiI injection experiments have shown that at these stages, the presumptive territory of the medial part of the prospective somites is found in more anterior territories of the streak associated with the Hensen's node, whereas the lateral part of the somites is found more caudally in the rostral streak. Thus, the dynamic expression pattern we observe in the streak suggests a differential regulation of the cyclic genes in the precursors of the medial and lateral somitic halves. Surprisingly, this differential expression in the streak is not maintained, as no difference in cycling gene expression is seen in the precursors of the lateral and medial somites in the PSM. Thus, the rostrocaudal dynamics of the expression domain of the cyclic genes in the streak suggest a desynchronisation of the pulses between the progenitors of the medial and lateral somitic domains.

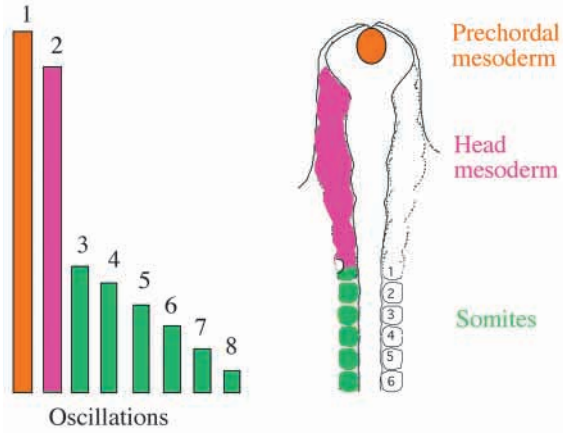

Fig. 7. The fate of the paraxial mesoderm with respect to head segmentation. As shown on the left, only two pulses of cyclic gene expression are detected for the whole mesodermal territory anterior to the first somite. The prechordal mesoderm shares with the paraxial mesoderm the ability to give rise to skeletal muscle derivatives. Moreover, the prechordal mesoderm precursors experience the first chevron of cyclic genes in the streak and they subsequently maintain this expression. We propose that the first pulse of cyclic gene expression (W1) marks the production of the prechordal territory, while the second one (W2) marks the production of the whole paraxial head mesoderm.

Analysis of the first pulses of expression of the cyclic genes suggests the existence of only two head 'segments'

In the vertebrate head, segmented structures include the brain, the cranial nerves, the branchial arches and clefts, the visceral pouches, and the aortic arches (Kimmel et al., 1988). Muscles and bones of the branchial arches, which derive from head mesoderm and neural crest, respectively, obey this segmental arrangement (Kontges and Lumsden, 1996). In the brain, segments are defined either on morphological grounds or by criteria such as gene expression. These brain segments are known as neuromeres, and are comprised of prosomeres anteriorly and rhombomeres in the hindbrain (Lumsden and Krumlauf, 1996; Rubenstein et al., 1994). The mechanisms implicated in hindbrain segmentation are clearly different from those of somitogenesis and are reminiscent of those used during fly segmentation. The branchial arches, their associated muscles and bones, and the neural structures that innervate them are generally thought to belong to the same segmentation series as the rhombomeres (Kontges and Lumsden, 1996).

How does head segmentation relate to body segmentation? One possibility is that the somitic series extends into the head (Goodrich, 1930; Neal, 1918). This hypothesis implies that, as in invertebrates, anterior segments are modified segments of an original common metameric series. An argument in favour of such an idea comes from the fact that in the cephalochordate, Amphioxus, somites extend to the anterior tip of the head, reflecting perhaps an ancestral condition of primitive vertebrates (Holland et al., 1997). Moreover, in elasmobranch fishes, classical anatomical descriptions report the existence of head somites located anterior to the otic vesicle (Balfour, 1878; Goodrich, 1930). It was thus proposed that the paraxial mesoderm of the head (the cephalic or head mesoderm) is segmented into head somites (Gilland and Baker, 1993).

A second conceptually similar hypothesis proposed the existence of mesodermal head segments, known as somitomeres in several vertebrate species (Meier, 1981). Under the scanning electron microscope, these segments appear as a series of concentrically organised cells. The number of these mesodermal head segments varies, depending on the studies and on the species considered. For example, Meier reported the existence of seven preotic somitomeres in chick embryos (Meier, 1981), while frogs were reported as having only four (Jacobson, 1988). Existence of the somitomeres is, however, highly controversial (Freund et al., 1996; Kuratani et al., 1999).

When the number of proposed mesodermal head segments is compared with that of ectodermal head segments, such as branchial arches or neuromeres, no simple correlation is observed. In answer to this discrepancy, the existence of two independent segmentation series has been proposed. The first concerns the formation of somites from the mesoderm and the second includes ectodermal derivatives such as the branchial arches and the associated cranial nerves and brain structures (Gans and Northcutt, 1983; Stern, 1990). A third possibility is that the whole head mesoderm corresponds to the first modified segment of the somitic series. This mesoderm would undergo a secondary segmentation imposed by the ectoderm of the branchial arches. Finally, a fourth viewpoint holds that the vertebrate head mesoderm is not in fact segmented and is part of a new structure that has evolved rostral to the somitic series. Such a proposal was put forward in the 'New Head hypothesis' proposed by Gans and Northcutt (Gans and Northcutt, 1983).

In summary, although the issue has been intensively studied since the last century, no consensus on the existence or otherwise of vertebrate head somites has yet been reached.

If the head mesoderm did belong to the same metameric series as the somites, then its segmentation might be expected to be regulated by the same molecular machinery. Therefore, a reasonable expectation would be that the clock linked to segmentation might also operate during formation of the head mesoderm. As one oscillation of the clock, monitored as one wave of gene expression, corresponds to the production of one somite (Palmeirim et al., 1997), then such a correlation might also be expected for presumptive head segments. As segmentation proceeds in an anterior-to-posterior fashion, examination of the first oscillation cycles of gene expression should be informative with respect to the number of segments that form in the head mesoderm.

Production of head mesoderm by the primitive streak is completed by stage HH5, after which somitic mesoderm begins to be produced (Nicolet, 1971; Psychoyos and Stern, 1996). Our observations of the c-*hairy2* and *lunatic fringe* expression patterns in the paraxial mesoderm and its precursors indicate that between the time the head mesoderm territory ingresses within the streak (stage HH3) and the time it has left the streak (stage HH5), some cells will have experienced one pulse, and the last to leave will have experienced two pulses of gene expression. Therefore, it suggests that head mesoderm only experiences two pulses of cycling gene expression. This argues for the existence of only two segments in the head, which is at odds with head segmentation models (such as the somitomere model that proposes the existence of many more segments). Alternatively, it is also possible that head segments form independently of the segmentation clock or that the clock is active but drives a different set of, as yet unidentified, cycling genes.

The first pulse of cyclic genes expression correlates with the production of axial prechordal mesoderm, which gives rise to extrinsic ocular muscles (Couly et al., 1992; Wachtler et al., 1984). This tissue maintains c-*hairy2* but not *lunatic fringe* expression. Prechordal mesoderm, which forms at the rostral most tip of the notochord, shares with the paraxial mesoderm the ability to give rise to skeletal muscle, and could thus represent the first segment in the series (Fig. 7). The second pulse of cyclic gene expression coincides with the generation of the whole head mesoderm. In this case, the whole head mesoderm would represent the second segment in the series (Fig. 7). Evidence for such a subdivision of the anterior paraxial mesoderm in these two domains has been provided in lamprey and chick embryos (Adelman, 1922; Kuratani et al., 1999). According to this model, the first somite that lies caudal to the otic vesicle would represent the third segment in the series.

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