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Cell lineage and pattern formation in the earthworm embryo

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Summary

The pattern of segmental contribution of teloblasts in the earthworm embryo was examined by injection of the lineage tracer HRP and by studying the timing and symmetry of the first few divisions in each teloblast bandlet. The changing spatial relationship of the ectoteloblasts during development was also used to assess segmental contributions. A mathematical method for determining the cell cycle time of each teloblast is presented. The teloblasts and their immediate progeny, the blast cells, were found to undergo unique and highly stereotyped patterns of division which lead to equally stereotyped patterns of contribution in the segments apparent in the later embryo.

Key words: earthworm, embryo, teloblasts, pattern formation, lineage, segments.

Introduction

Earthworms have a variable number of body segments and are able to regenerate those that are injured or lost (Morgan, 1901). This contrasts with leeches, which have a fixed segment number and cannot replace lost segments (Needham, 1952; Sawyer, 1986). The embryos of both these annelids develop from a spirally cleaving egg, typically associated with a deterministic mode of development, whereby early divisions assign cells unique fates. It might be expected, therefore, that the regenerative ability of the earthworm would be confined to the adult animal and arise after the completion of embryogenesis. However, an experiment, in which all the ectoteloblasts (a set of cells that give rise to most of the ectodermal tissue of the embryo) were destroyed by ultraviolet (UV) radiation in the earthworm embryo (Devries, 1974), suggests that the progeny of these cells can be replaced by cells derived from other lineages in the embryo. In the leech, similar experiments (Weisblat and Blair, 1982; Blair, 1983; Blair and Weisblat, 1984), in which individually identified ectoteloblasts were destroyed show that the progeny of these cells cannot be replaced (except in the case of the ectodermal progeny and then only by the proliferation of the progeny of other ectoteloblasts). I have explored these apparently contradictory findings first by establishing that the teloblasts in the leech and the earthworm are equivalent not only in their derivation, (Fernandez, 1980; Devries, 1973*a*), but in their pattern of contribution to the segments of the developing embryo, and second by examining the consequences of selectively ablating individually identified ectoteloblasts in the earthworm embryo.

This paper describes the earthworm embryo at the time at which ablation experiments were performed. Development up to this stage (6–7·5 days) has been described by Devries (1973*a*,*b*). The earthworm embryo gastrulates on day 4 of development and by day 6 eight ectoteloblasts lie in a characteristic arrangement on its ventral surface (see Fig. 1A–D). Teloblasts are stem cells, undergoing repeated asymmetric divisions to produce a chain of small blast cells. For the sake of comparison, I refer to the teloblasts in the earthworm using the nomenclature devised by Fernandez (1980) to describe the teloblasts in the leech *T. rude*. The teloblasts in the earthworm embryo are derived from two precursor cells (Dervies, 1973*a*) in an identical sequence to that observed in the leech by Fernandez (1980). In the earthworm, the N teloblast is born first, on day 3 of development, on the dorsal surface and migrates, along with the OPQ precursor cell, to the ventral surface during gastrulation (Devries, 1973*a*). Towards the end of day 3, the Q teloblast is produced by the OPQ precursor cell. This division places the Q teloblast lateral to the N teloblast and the OP precursor cell, which divides finally on day 4 to give the O and P teloblasts. These cells lie between and slightly posterior to the N and Q teloblasts (Devries, 1973*a*).

The segmental contribution of the teloblasts was assessed by examining the pattern of labelled cells following injection of horse radish peroxidase (HRP) into the N teloblast and by observing the pattern of divisions in each bandlet. These patterns were revealed using conventional histochemical stains and by a brief exposure to BUdR (which is incorporated into cells synthesizing DNA and can be identified immunocytochemically). The cell cycle time for each teloblast (determined by taking advantage of the same immunocytochemical technique, see appendix 1), helped to interpret the changing spatial arrangement of the ectoteloblasts and allowed me to calculate the number of

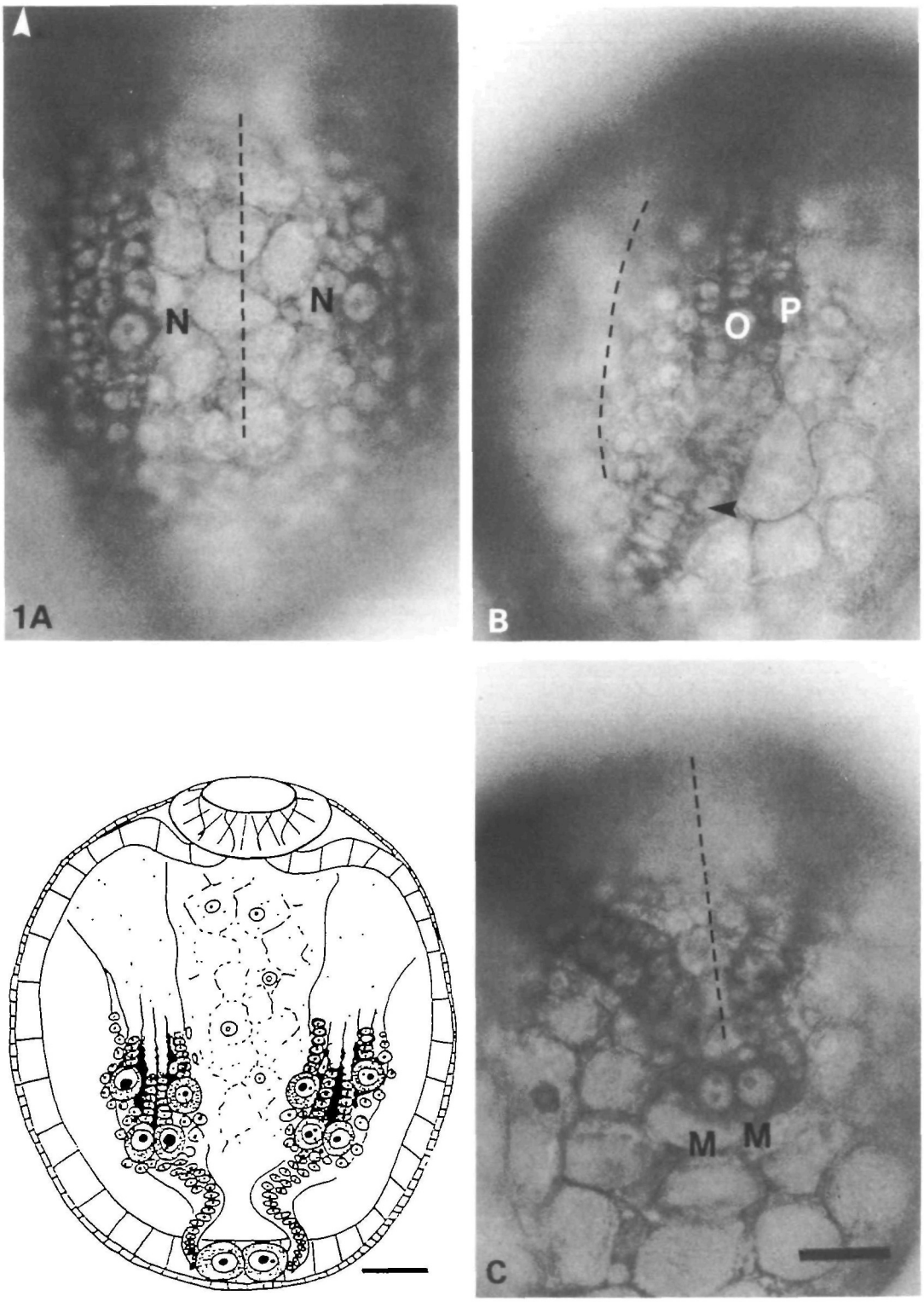

Fig. 1. Whole 6-day-old embryo stained with toluidine blue, showing the positions of the teloblasts. (A) N teloblasts on the ventral surface. (B) Rolling the embryo forward and to one side reveals the O and P teloblasts. One of the m bandlets can be seen at the point of the first division in this bandlet (arrowhead). (C) Rolling the embryo back to the centre and forward again reveals the M teloblasts on the dorsal surface. Their bandlets reach round the embryo to its ventral surface where they run beneath the ectoteloblast bandlets. Anterior is up, dotted lines indicate midline and scale bar = 50 μm. (D) Diagram showing the position of all the teloblasts in an earthworm embryo about 6 days old. Anterior is up and scale bar = 40 μm.

blast cells produced at the time of an ablation. This further helped to predict the segment in which ablation effects would first be seen (see companion paper).

Materials and methods

Embryos of the earthworm *Eisenia foetida (unicolor)* were obtained from a breeding colony maintained in the laboratory, (for culture method see T. J. Barrett, 1949). This culture was kept at between 18 and 22°C and produced an average of thirty cocoons a day. Each cocoon contains on average three or four embryos, although up to twenty were found in a single cocoon. Cocoons were removed from the culture daily, washed in tap water, rinsed briefly in a weak solution of bleach and rinsed again in distilled water before being placed in a Petri dish of earthworm saline (see below) for inspection. The bleach treatment not only helped to sterilise the cocoons but also increased their transparency so that the number and size of embryos in a cocoon could be seen without opening it.

At birth the cocoon is white and has a mucousy, jelly-like consistency. After an hour the outer layers of this secretion take on an amber hue, while the jelly-like consistency is preserved inside. Embryos from cocoons timed from within an hour of birth were stopped at regular intervals and the early embryonic events observed were consistent with those described by Devries (1968 and 1973*a*,*b*). However, removing cocoons at hourly intervals greatly reduced breeding and large numbers of embryos were more easily obtained by collecting cocoons after longer intervals. Embryos were then staged as they reached a later, distinctive point in development.

At 5 days embryos viewed through the cocoon measure between 0·16 and 0·2 mm in diameter and have a characteristic movement. Shortly after gastrulation on the fourth day of development (Devries, 1973*a*,*b*), the embryo begins to rotate slowly in the vertical plane (Wilson, 1899). This is caused by ciliated cells that surround the stomodeum and lie along the ventral midline. This movement is later masked, on the seventh day, by the active ingestion of albumen by the stomodeum. In cocoons in which embryos were at slightly different stages, the most advanced embryo was selected. Once the embryos had been staged they were kept in a dish of saline in an incubator set at 22°C.

Embryos were released from their cocoons into saline by holding the cocoon lightly with tweezers and cutting along its length with iridectomy scissors, starting at the plug end. The saline consisted of 1·46 g NaCl, 0·44 g K_2SO_4, 9·66 g $Na_2SO_4 \cdot 10H_2O$, 0·12 g $MgSO_4 \cdot 7H_2O$, 1·47 g $CaCl_2 \cdot H_2O$, 0·6 g Tris, 0·9 g glucose per litre, plus 4·35 ml 1 N-HCl, after Gunther (1976) and was routinely supplemented with 10 ml per litre antibiotic–antimycotic medium, (Sigma cell culture reagent no. A9909), for use as the basis of culture media following teloblast injection.

Dissection and staining

Prior to dissection, the embryos were fixed briefly in 1% glutaraldehyde in 0·05 M-phosphate buffer for 5 to 10 min. This was long enough to fix the tissue of the embryo (a thin sheet only two to three cells thick), while the albumen inside remained fluid. The albumen was then sucked out following a wash in phosphate buffer. Dissected embryos were stained either as intact spheres or after flattening onto a coverslip. Two histochemical stains, toluidine blue and orcein, and an immunocytochemical method that labelled cells synthesizing DNA were used. Embryos were stained with toluidine blue after Altman and Bell (1973), except that they were fixed briefly prior to staining as an aid to dissection. Orcein (prepared after Unna, 1904) stains chromatin preferentially, a deep red, while cytoplasm acquires only a pale pink hue. This stain was used to pick out mitotic figures, while toluidine blue stains both cytoplasm and nucleus. Following exposure to either stain, embryos were dehydrated, cleared in xylene and mounted in Canada balsam.

The sequence of divisions in each type of bandlet revealed by these two histological stains was confirmed by the pattern of cells undergoing DNA synthesis in each bandlet as revealed by the incorporation of bromodeoxyuridine (BUdR). BUdR is an analogue of thymidine and is incorporated in its place during DNA synthesis and its presence was revealed immuno-

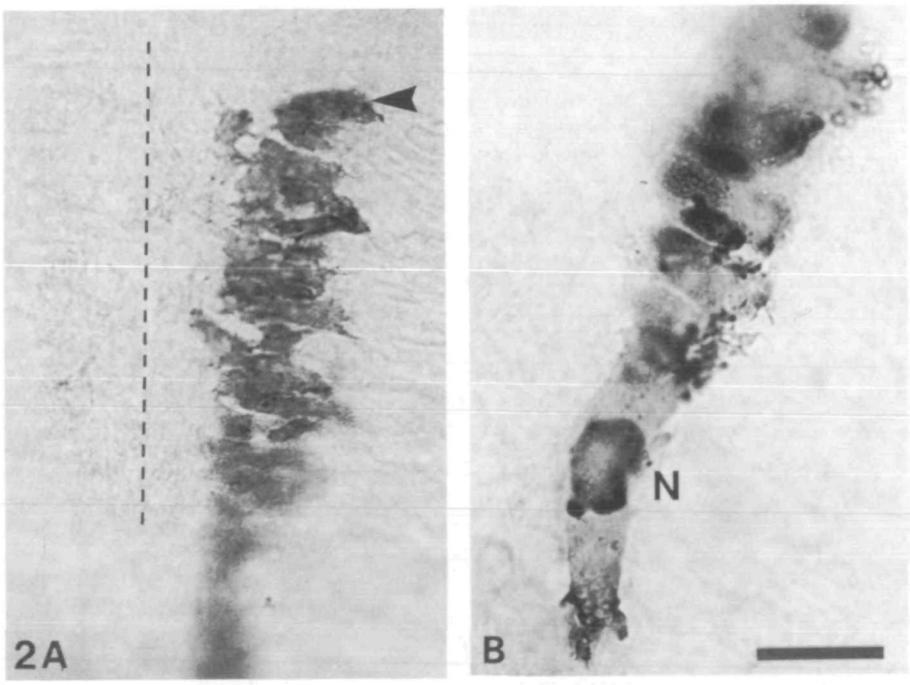

Fig. 2. (A) Anteriormost labelled cells in the n bandlet 3 days after injection of HRP into the N teloblast (not in figure). The midline is indicated by a dotted line. (B) N teloblast (N) and progeny 3 days after injection with HRP. See text for details. Anterior is up and scale bar = 20 μm.

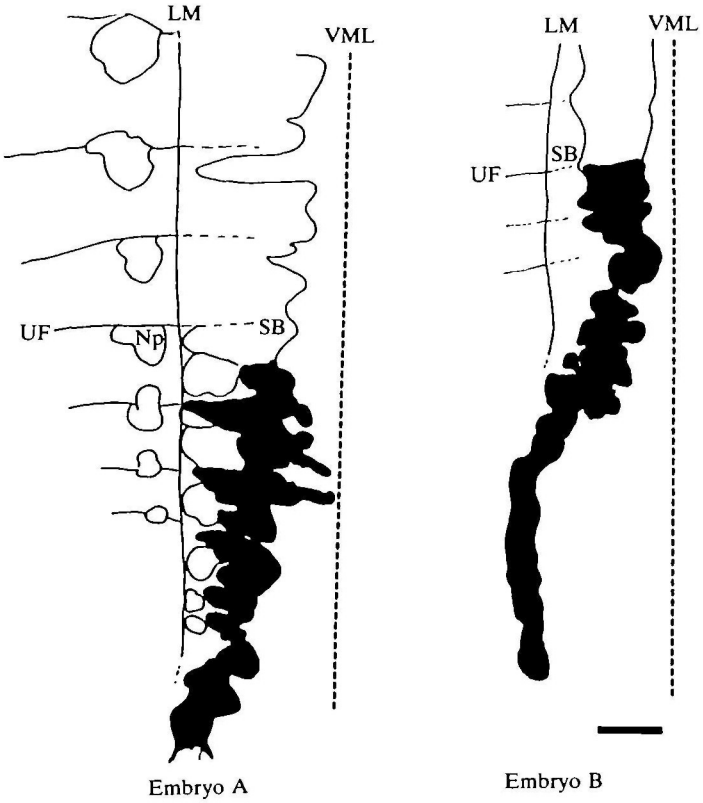

Fig. 3. (A) Camera-lucida drawings of an n bandlet in two embryos. (B) 5 days after the left N teloblast was injected with HRP. The position of the anteriormost clone in the n bandlet can coincide or fall between a segment boundary (SB). This is defined by the early differentiating nephridia (Np) and underlying fibres (UF). Weak counterstain in B meant that the segment boundary was defined by UF only. These fibres may correspond to the primary circular muscles in the leech embryo (Torrence and Stuart, 1986) or to developing septal structures. LM, longitudinal muscles, define the lateral extent of the hemiganglion rudiment, VML, ventral midline. Anterior is up and scale bar = 20 μm.

Table 1. *Cell cycle times and the lengths of the phases of the cell cycle for each teloblast*

Teloblast	M	N	O	P	Q	Rounded average (h)
Cell cycle time (min)	177	154	136	153	163	2·75
S-phase (S)	44	23	21	29	26	0·5
Mitosis (M)	39	26	29	23	37	0·5
G_1 and G_2	94	105	86	101	99	1·75

cytochemically with a monoclonal antibody (Becton Dickinson) against BUdR, Gratzner (1982).

This technique was also used to determine the cell cycle times of the teloblasts (see below and appendix 1). Embryos between 6 and 7·5 days old were placed in saline containing 15 μg ml^{-1} of BUdR (Sigma), at 22°C (at very much higher concentrations BUdR appears to block the cell cycle, Devries, 1983). As the age of the embryos ranged over a period of

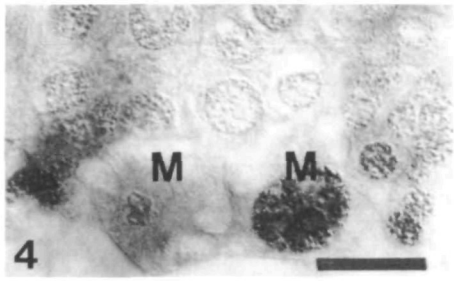

Fig. 4. M teloblasts (M) after a 30 min exposure to BUdR. These two cells lie on either side of the midline. The dividing teloblast on the left is unlabelled, indicating that the time between the end of s phase and mitosis (G_2) is longer than 30 min. Anterior is up and scale bar = 20 μm.

36 h the teloblasts and blast cells in these embryos could be in any phase of the cell cycle and therefore constituted a random population.

20 embryos were removed from BUdR after 15, 30, 60, 90 min for determination of the teloblast cell cycle times, while lots of 10 embryos were left for longer periods of 4, 6, 8 and 10 h and these were used to confirm cell division patterns in the bandlets. All embryos were washed briefly in cold (4°C) saline, fixed for 30 min in Carnoy's fixative and then rehydrated to phosphate-buffered saline (PBS) with 0·3% Triton X-100 (PBS-TX). Immunocytochemistry was carried as in Truman and Bate (1988) and the peroxidase label conjugated to the secondary antibody was revealed using diaminobenzidine (Sigma) as a substrate according to the method of Watson and Burrows (1981).

After being reacted, the embryos were washed in PBS without TX and dissected. The embryos were then dehyrated, cleared in xylene and mounted in Canada balsam. In all embryos the labelling was confined to the nucleus, confirming its exclusive association with the cells' DNA. All slides in this study, prepared using all three staining techniques, were examined using ×40 and ×63 oil immersion lenses mounted on a Zeiss microscope fitted with Nomarski optics.

Injection of lineage tracer

One teloblast was injected in each embryo. To do this the embryo was placed in a pool of saline, in a small depression made in a slide coated with Sylgard resin and viewed using bright-field optics through a ×40 water-immersion lens mounted on a fixed-stage Zeiss microscope. The teloblast to be injected was identified by position (see earlier description) and impaled with a glass microelectrode filled with 4% HRP (Sigma type VI) in 0·2 M-Tris buffer (pH 7·4) containing 0·5 mM-KCl. Some teloblasts were injected with 4·6% peroxidase rhodamine isothiocyanate (HRP-RITC, Sigma P5031). Both HRP and HRP-RITC were injected iontophoretically according to the method of Watson and Burrows (1985), except that the injection period was reduced to 3–4 min as longer periods tended to kill the teloblast.

The N teloblast was successfully injected with HRP in four embryos. Two were fixed and developed 3 days after injection (Fig. 2A,B) and two were examined 5 days after injection (Fig. 3A,B). Both embryos cultured for 3 days generated a string of labelled progeny. The predicted number of blast cells produced by the N teloblast in 3 days (given a cell cycle time of approximately 2·75 h, see below), is 26, the oldest of which will have begun to undergo divisions themselves. This pattern was observed (Fig. 2A), however, (probably due to the

trauma of injection) one N teloblast (Fig. 2B), produced fewer cells than predicted.

Embryos examined 5 days after N teloblast injection were counterstained with toluidine blue. This allowed the positions of segment boundaries as defined by the early differentiating nephridia and muscle fibres to be identified.

Culture of embryos

Following injection, embryos were cultured in a mixture of earthworm saline and the albumenous contents of four cocoons, after André (1963). Each embryo was cultured separately in a small quantity of this solution sealed beneath a layer of liquid paraffin (Merck Art. 7162) in a watch glass. This was then placed in an incubator at 22°C.

Nomenclature

The blast cells in each of the five different bandlets were named using a modification of the system devised by Zackson (1984). Each cell is given a name based on its lineage history. The blast cells are named according to their teloblast of origin. Only one kind of blast cell is produced by each of the O and P teloblasts and their progeny are designated as o_1 and p_1. The n (and possibly q) bandlet has two types of blast cell designated n_1 and n_2 and q_1 (and possibly q_2). In the leech, the blast cells of the O and P teloblasts are simply designated o and p, while in the n and q bandlets the type of blast cell that divides early is designated n_f and q_f (f = frueh, early) and the other which divides late is designated n_s and q_s (s = spaet, late). I have used numerals in addition and instead to distinguish the blast cells in the earthworm from those in the leech.

Descendants of the blast cells are named by placing letters to the right of the numeral. When a blast cell or one of its progeny divides, a single lower case letter indicative of the daughter cells' position relative to its sister cell immediately after division is appended to the name of its mother. Thus, for anterior/posterior division of a cell the letter a or p is added to the daughter's name. For example, o_1 divides to produce o_{1p} and o_{1a}. The only medial/lateral division was observed in the m bandlet. Thus m_{1m} and m_{1l} are produced by the division of m_1. In the leech, however, the terms medial and lateral cannot be used (Zackson, 1984). None of the early divisions in the bandlets occurred in the dorsoventral axis.

Results

Lineage tracing

The strings of small labelled cells produced by the N teloblasts, cultured for 2 days after injection of HRP, demonstrate that the teloblasts are stem cells; undergoing repeated asymmetric divisions (Fig. 2A,B). The irregular shape of the labelled blast cells made it difficult to confirm the precise configuration of cells in

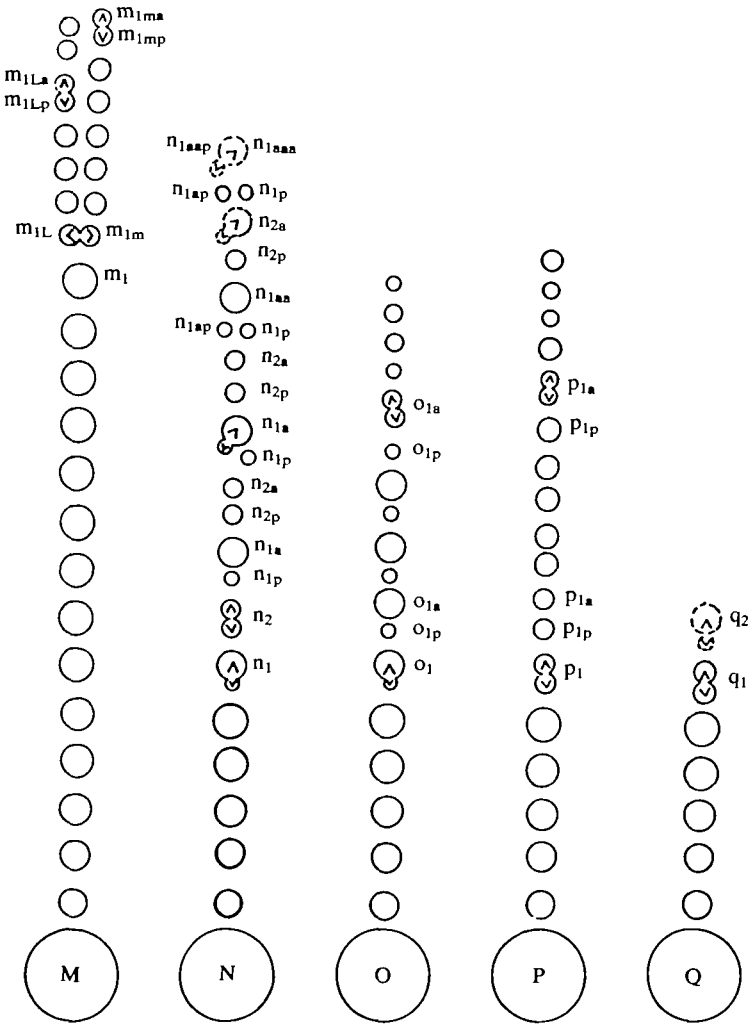

Fig. 5. Summary of sequence and symmetry of first few divisions in each type of bandlet. Criteria used to establish the pattern of divisions in the bandlets. Given that each blast cell in a bandlet is 2·75 h older than its immediate posterior neighbour and provided that all blast cells subsequently undergo the same sequence of divisions, the sequence of divisions in a bandlet can be inferred from the progressive caudorostral change in the blast cell pattern in the bandlet. A minimum of twenty embryos ($n = 40$ for each type of bandlet) were examined and at least six examples of each division were observed. In bandlets in which mitotic figures were not seen the predicted arrangement of cells that would result from the division observed was found in at least 85% of cases. Dashed outlines indicate uncertain divisions (the n_{1aa} cell was observed dividing asymmetrically on two only occasions. The division of the n_{2a} cell was observed in four embryos and the configuration of cells in this part of the bandlet was visible in only 66% of embryos. Evidence for q_2 is given in Fig. 8). The cells constituting a bandlet were defined by their position, size and staining properties. All divisions are interstitial (increase the length of the bandlet), except for the first division in the m bandlet. There is only one type of blast cell in the m, o and p bandlets, while the n (and possibly the q) bandlets have two kinds of blast cell, identified here by different symmetries of their first divisions. Nomenclature used is described in Materials and methods. Growth between divisions is symbolised by using progressively larger circles. Teloblast size is standardised. The drawing is not to scale.

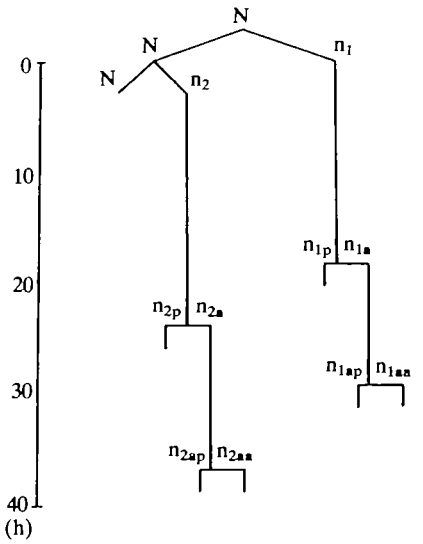

Fig. 6. Lineage tree of the first four divisions in the n bandlet. Time is measured from the birth of a blast cell, here and in Figs 8, 13 (except for n_2 and q_2 which are timed from the birth of n_1 and q_1, respectively, their time scale is thus shifted by 2·75 h). Blast cells are born at intervals of 2·75 h and divisions take place sometime during the 2·75 h in which a blast cell is in a particular position. For example, a division observed in the 7th blast cell from its parent teloblast may divide between 16·5 and 19·25 h after its birth. For simplicity a rounded average of the two times is taken as the time from birth to the division observed, which in this example is 18 h.

the bandlet (see below), however, groups of cells were seen in the anteriormost part of the bandlet shown in Fig. 2A.

After 5 days these groups are more discrete. In embryo A (Fig. 3A) the anterior group of labelled cells occupies only a portion of the segment in which it is found, while in embryo B (Fig. 3B) the margin of the most anterior group of cells coincides with a segment border (defined in Fig. 3,SB). One explanation for these different positions is that there are two types of n blast cell, one that contributes to the anterior part of a hemisegment and one that contributes to the posterior (see below).

The rudiments of the hemiganglia in the earthworm embryo are outlined by the longitudinal muscles, which define their lateral extent, while the segmental limits of these rudiments can be assigned by the position of the nephridia and underlying fibres (see Fig. 3). It is clear in embryo A that not all the cells in each rudiment are labelled with HRP. A patch of unlabelled cells in the lateral-posterior portion of the rudiment is clearly visible in at least three segments in this embryo. This suggests that the cells in the n bandlet are not the sole contributors to each hemiganglion rudiment.

Determination of the cell cycle time in each type of teloblast

To determine the cell cycle times for each teloblast a series of embryos between 6 and 7·5 days old were exposed to BUdR for set periods of time (see Materials and methods) and graphs plotting the percentage of labelled teloblasts (of a particular type) against time immersed in BUdR were constructed. Standard linear regression statistics were then used to find the best line through a set of points for each teloblast. Values taken at any two points on this line were then substituted into a simultaneous equation which was solved for T, the total cell cycle time, and then for S, the length of s phase (see appendix 1).

The rate of entry into s phase was found to be the same for all five types of teloblast. Table 1 lists values for T and s derived from the simultaneous equations. The teloblasts' cell cycle time ranges from 136 to 177·5 min. This compares reasonably well with direct observations made of the cell cycle time of the N teloblast, which was found to be approximately 3 h.

The values of S phase for the teloblasts range from 20 to 44 min. The length of mitosis in each teloblast was found by determining the mitotic index for each teloblast (M = 0·22, N = 0·17, O = 0·21, P = 0·15 and

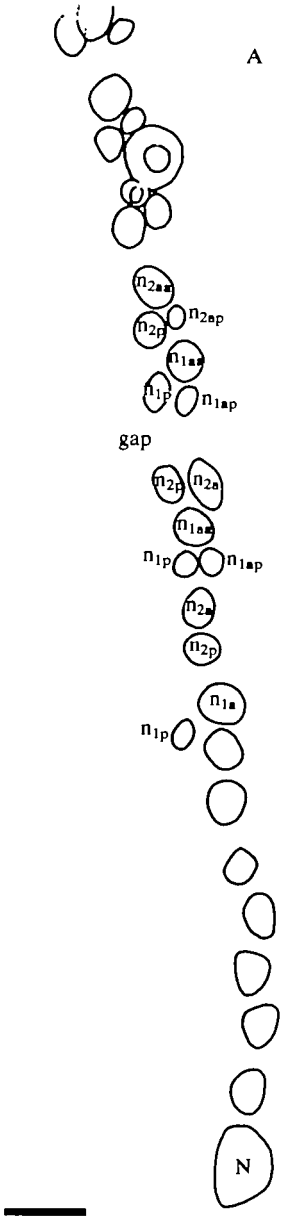

Fig. 7. Camera-lucida drawing of the N teloblast and its progeny. Only the nuclei of these cells are shown. Anterior to the n_{2aa} cell (although sometimes anterior to the n_{2a} cell) a gap is observed between this cell and the next anterior group of cells. Anterior to this first gap, repeated groups of cells separated by gaps are seen. The n bandlet no longer takes the form of a continuous ribbon of cells and this pattern seems to mark the beginning of segmentation in this bandlet. The first gap falls such that the n_2 blast cell clone is anterior in the group and the n_1 blast cell clone is posterior. This arrangement appears to define the sequence of repeat units in the n bandlet as n_2 and n_1, rather than n_1 and n_2, reading from anterior to posterior. Scale bar = 20 μm.

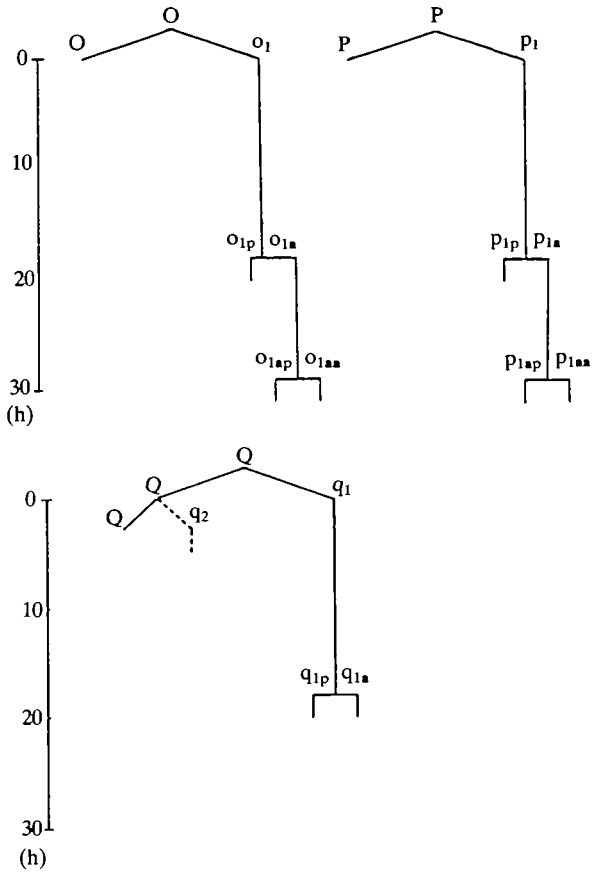

Fig. 8. Lineage trees of the o, p and q bandlets. The second division in the p bandlet was seen in only three bandlets, however, the arrangement of cells in 75 % the p bandlets was consistent with this pattern of division. There is some evidence for a q_2 blast cell. One mitotic figure was observed immediately anterior to the q_1 division, although its symmetry was unclear. Further, the rate of contribution of the Q teloblast compared with that of the O and P teloblasts suggests that two q blast cells are incorporated for every one o and p blast cell (see text).

$Q = 0{\cdot}23$, where n was over 100 for each of the five teloblasts). The mitotic index was then multiplied by the value obtained for T for the respective teloblasts and values for the length of mitosis ranged from 23 to 39 min.

The values for the lengths of G_1 plus G_2 were obtained by subtracting the values for s phase and mitosis from the value for T for each teloblast. The length of G_1 plus G_2 ranged from 85 to 105 min. An indication of the length of G_2 was found by observing, in two embryos, the duration of pulse after which mitotic teloblasts had labelled chromosomes. After 30 min dividing teloblasts were unlabelled (Fig. 4) but after an hour such teloblasts had labelled chromosomes. This observation suggests that G_2 lasts between 30 and 60 min.

If all five teloblasts are considered examples of a homogenous cell population, then the average cell cycle time for a teloblast is approximately 2·75 h with mitosis and s phase each lasting approximately 30 min and the remaining 1 h 45 min spent in G_1 and G_2.

Pattern of blast cell divisions

The pattern of divisions observed in each bandlet and the criteria for establishing this pattern are presented in Fig. 5.

n bandlet divisions

The n bandlet is separated from the o bandlet laterally by ectodermal cells, and from the contralateral n bandlet by ventromedial ciliated cells. The first four divisions in this bandlet were identified and are shown in the lineage tree presented in Fig. 6. Two kinds of n blast cells were observed. Because the n bandlet is easily distinguished from other bandlets it was also possible to observe groups of cells formed in the older part of this bandlet (Fig. 7).

The number of cells in each group and the gaps between them increase anteriorly. In a 7-day-old embryo each of these groups appears to correspond to a segmental unit as defined by the position of nephridial rudiments and a set of fibres that lie beneath the ectodermal layer (see Fig. 3). This suggests that each group represents the primordium of a hemiganglion.

o and p bandlet divisions

The first two divisions were identified in the o and p bandlets (Fig. 8). Unlike the pattern observed in the n bandlet the arrangement anterior to the first division in the o bandlet is a simple repeat of a small cell and a large cell (Figs 5, 9). This pattern suggests that there is only one type of o blast cell. Similarly in the p bandlet the configuration of cells anterior to the first division suggests that there is only one type of p blast cell (Figs 5, 8, 9, 10).

q bandlet divisions

The provisional epithelium, which covers the dorsal surface of the embryo, merges with the q bandlet so that it is difficult to discern the arrangement of cells beyond the first division (Figs 5, 8, 11). There is some evidence for a second type of q blast cell (Fig. 8).

m bandlet divisions

The m bandlets are the easiest to define as they form their own layer within the embryo lying between an inner sheet of endoderm cells and a covering epithelium (Fig. 1B,C). The first three divisions in this bandlet were identified (Figs 5, 12, 13).

Pattern of DNA synthesis in the bandlets

This study was undertaken to confirm the sequence and timing of the divisions described above. It was also hoped that the patterns observed would reveal whether different blast cell types spend different times in the same phases of the cell cycle. s phase was determined by a brief exposure to BUdR, as described in Fig. 14. The number of ectoteloblast bandlets analysed was 45 and the number of m bandlets was 12.

Pattern of DNA synthesis in the ectoteloblast bandlets

The pattern of labelling was the same in all the ectoteloblast bandlets and is summarised in a histo-

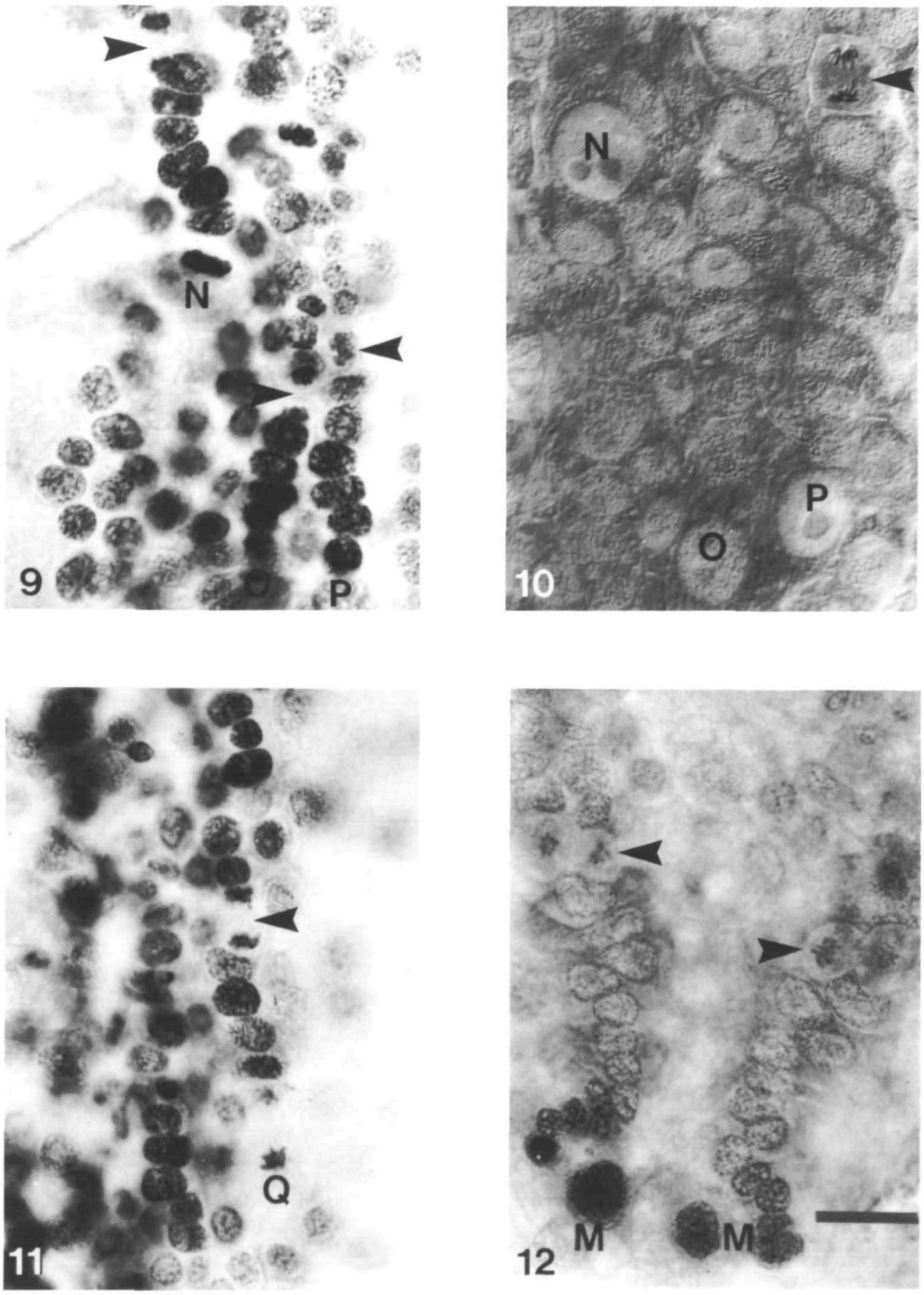

Fig. 9. N, O and P teloblasts in an embryo following a 10 h exposure to BUdR. The arrowheads indicate the first division in each bandlet.

Fig. 10. First division in the p bandlet (arrowhead) revealed in a whole mount stained with orcein. In this particular p bandlet, this division occurs in the eighth blast cell from the P teloblast (P), although it was seen most often in the sixth or seventh blast cell from its parent. Orcein staining causes the cells to swell so that they are slightly larger than those in Figs 9, 11.

Fig. 11. Q teloblast (Q) in division and the first division (symmetrical) in the q bandlet (arrowhead), following an 8 h exposure to BUdR.

Fig. 12. First division in the m bandlet (arrowheads), seen here taking place simultaneously in each bandlet in the fourteenth blast cell from the M teloblast (M) the position of the m_1 blast cell division is variable (ranging from 12 to 18, and is found most often at 15 or 16), following exposure to BUdR for 30 min. The M teloblasts lie either side of the midline. Anterior is up and scale bar = 20 μm.

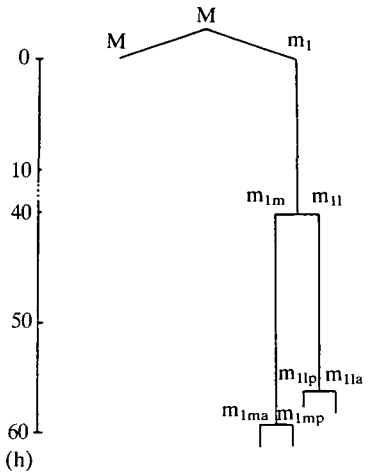

Fig. 13. Lineage tree of the divisions in the m bandlet. The cell cycle of the m_1 blast cell is approximately twice as long as that of the ectoteloblast blast cells. The progeny of m_{11} usually come to lie over those of its sibling, m_{1m}, creating a twist and giving the bandlet its third dimension.

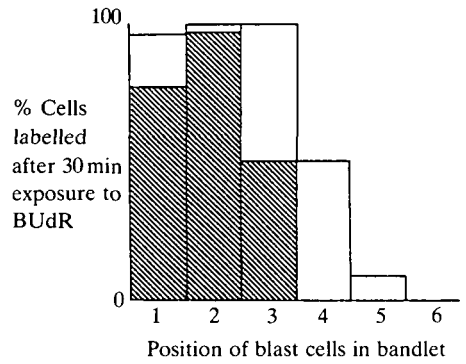

Fig. 14. Histogram showing % of cells labelled in the first 6 positions in the ectoteloblast bandlets. % of strongly labelled cells in stripes and % lightly labelled cells white (see text). Calculation of s phase: Given that the blast cells in any one type of bandlet undergo the same sequence of divisions and spend the same amount of time in each phase of the cell cycle (with the exception of the n and possibly the q bandlets), the phase of the cell cycle seen in successive blast cells represents a series of moments (seen at 2·75 h intervals) in the cell cycle of a single blast cell. The time spent in s phase by a single blast cell can thus be found by studying the pattern of labelled blast cells in a bandlet after a brief exposure (30 min) to BUdR (which is incorporated into cells synthesizing DNA). As each blast cell represents 2·75 h an estimate of the initial s phase of the blast cells can be made by counting the number of labelled blast cells prior to the first division and multiplying by 2·75.

gram, Fig. 14. This shows that the first blast cell is labelled 96% of the time, indeed in 63% of cases the teloblast was unlabelled while the first blast cell was labelled. This means that blast cells enter s phase before their parent teloblast and shows that G_1 is very short, approximately 6 min.

The histogram shows that BUdR is incorporated rapidly at the onset of s phase, reaching a peak in the second blast cell and is incorporated at a reduced rate for at least the same period of time (upto the fourth blast cell) (Fig. 15). This suggests an s phase of between 8·25 and 11 h, a rounded average of 10 h. M phase was found to be approximately 30 min (using the mitotic index of the cell in position 6), leaving a G_2 of 7 to 8 h.

The pattern of labelled cells in the ectoteloblast bandlets seen after a longer exposure of 2 h ($n = 40$) (Fig. 16) was consistent with that observed after 30 min (Fig. 15). In particular, the pattern of labelled cells in the n bandlet was the same as that seen in the other ectoteloblast bandlets, suggesting that n_1 and n_2 cells spend the same amount of time in s phase. However, as the n_2 blast cell divides only 2·75 to 5·5 h after the n_1 blast cell (and on three occasions these two divisions were seen simultaneously) longer time spent in s phase by the n_2 cell may have been too short to distinguish from background staining. The longer cell cycle time of the n_2 cell, therefore, may be accounted for by extra time spent in either s or G_2 phases of the cycle.

Pattern of DNA synthesis in the m bandlet

The pattern of labelling in the m bandlet after a 30 min exposure to BUdR is summarised in Fig. 17. G_1 was the same as in the ectoteloblast bandlets. However, s phase was found to be approximately 5·5 h longer. Further, the pattern of labelling in the m bandlet following a 2 h exposure revealed light labelling up to the seventh m_1 blast cell in eight bandlets and in one embryo, with particularly low background, up to the twelfth and thirteenth blast cells from the M teloblast (Fig. 18).

This observation was borne out by the pattern of labelling observed after 6, 8 and 10 h exposures to BUdR ($n = 6$, 8, and 4 bandlets respectively), and suggests that an intense period of DNA synthesis in the first 12 h (approximately a third of a 40 h cell cycle) is followed by a decreasing level of synthesis for a further 20 h. It may be that a 30 min exposure is too short to label cells synthesizing DNA at a very low rate, although the level of background may have contributed to this effect as well. M phase (calculated from the mitotic index of the fifteenth m blast cell) was approximately 40 min, leaving a G_2 of 7–8 h, the same as in the ectoteloblast blast cells.

N and Q teloblasts contribute twice as many blast cells to each segment as the O and P teloblasts

The eight ectoteloblasts lie in a characteristic, mirror-image formation on the ventral surface of the 5-day-old embryo, with the two ectoteloblast groups separated by a median band of ciliated cells. The N teloblast is the most median, next to the ciliated cells. The O and P teloblasts sit as a pair two or three cell lengths posterior to the N teloblast. The Q teloblast is the most lateral and at 5 days is usually parallel, though occasionally a few blast cell lengths anterior to the N teloblast (Fig. 19). The relative positions of the ectoteloblasts observed at 5 days are consistent with those dictated by their birth sequence (described in the introduction).

A teloblast cell cycle time of 2·75 h corresponds to the production of nine blast cells a day. As the N teloblast is born on day 3 (Devries, 1973*a*), by 5 days it has

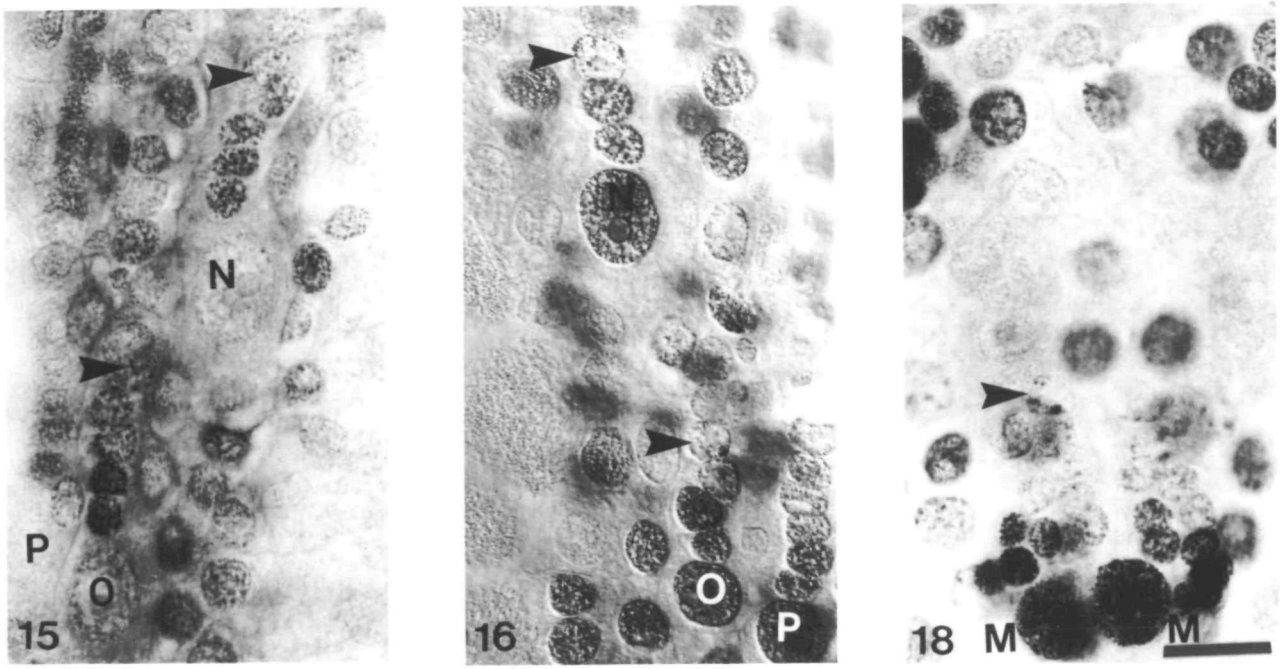

Fig. 15. N and O teloblasts and their bandlets following a 30 min exposure to BUdR. The N teloblast is unlabelled while the O teloblast is lightly labelled. In these two bandlets the first two blast cells are strongly labelled, while the third and fourth cells are lightly labelled and the fifth blast cell is still more lightly labelled (arrowheads).
Fig. 16. N and O teloblasts after a 2 h exposure to BUdR. The first two blast cells in each bandlet are strongly labelled, while the third (upper arrowhead) and subsequent two blast cells (lower arrowhead) are more lightly labelled. This pattern is similar to that seen after a 30 min exposure (see text). Anterior is up and scale bar = 20 μm in Figs 15, 16.
Fig. 18. M teloblasts after a 2 h exposure to BUdR. The nucleus of twelfth blast cell (arrowhead) in the bandlet on the left is lightly labelled. Anterior is up and scale bar = 20 μm.

produced eighteen blast cells; the Q teloblast, which is born towards the end of day 3 (Devries 1973*a*), has produced approximately eleven blast cells, while the O and P teloblasts, which are born on day 4 of development, have each produced nine blast cells.

Thus, at 5 days the N teloblast has produced twice as many blast cells as the O and P teloblasts and is in addition anterior to them by three or four blast cell lengths. This suggests that the n bandlet extends further anterior than the o and p bandlets, perhaps reflecting its participation in the development of head structures. Further, in older embryos the distance between the O and P teloblasts and the N and Q teloblasts increases, so that by 6 days they are separated by six or seven blast cell lengths and by 7 days by twelve to fifteen blast cells or blast cell clones (Fig. 19).

Ostensibly, this increasing separation may simply be a consequence of the expansion of the developing embryo. However, given that all four types of ectoteloblast have the same cell cycle time and divide at a constant rate throughout development, this increasing separation may also reflect a difference in the number of blast cells contributed to each segment by each type of teloblast. As judged by the pattern of divisions (see above) there is one type of o and one type of p blast cell, while the n and q lineages each have two kinds of blast

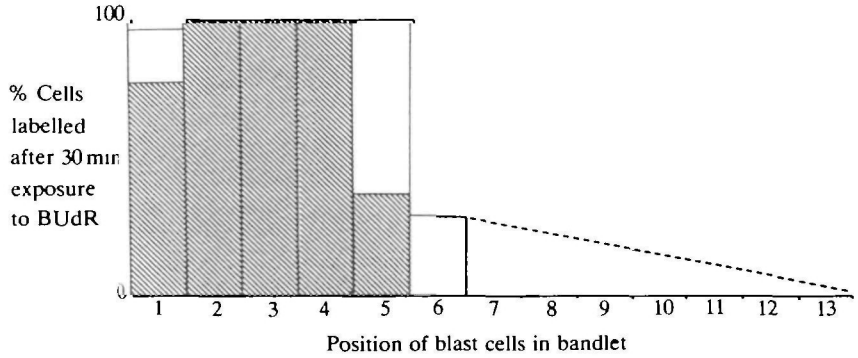

Fig. 17. Histogram showing the pattern of labelling in the m bandlet after a 30 min exposure to BUdR. No labelling was seen beyond the sixth blast cell from the teloblast. However, a 2 h exposure revealed very light labelling up to the thirteenth blast cell. This is indicated by the dashed line to the thirteenth position (see text for details).

cell. These observations are also supported by the distribution of labelled cells observed following HRP injection of the N teloblast.

If two n and two q blast cells are incorporated for every one o and p blast cell, the distance between these two sets of teloblasts should increase in a linear fashion by 4·5 blast cell diameters a day (given that the divisions within each bandlet result in equal elongation of each bandlet). As the N teloblasts are born 1 day earlier than the O and P teloblasts, the difference in the number of blast cells contributing to each segment is not at first manifest. However, by the day 7 the distance between the two sets of teloblasts has greatly increased: n blast cells, which have yet to divide are positioned adjacent to o blast cells, which have already undergone several divisions, while posterior to the N teloblast a chain of approximately fifteen o blast cells (or blast cell clones) without corresponding n blast cells is seen (Fig. 19).

This arrangement appears to explain the discovery of two sites at which ablation effects begin in embryos in which the N, O and P teloblasts were ablated simultaneously (see companion paper). The number of segments that separated these two sites ranged from 11 to 20, which roughly corresponds to the number of 'extra' o and p blast cells posterior to the N teloblast in embryos between 6 and 7·5 days of development (the age at which ablations were performed).

Discussion

Earthworm teloblasts and blast cells were found to have unique and stereotyped patterns of division. Occasional inconsistencies, such as the division of an n_2 blast cell before that of the posterior n_1 blast cell in an otherwise normal bandlet, were found and these suggest that the division of a blast cell does not depend on interaction with other cells in its bandlet. Autonomous mechanisms are also manifest at the level of the bandlets: ablation experiments reveal that the absence of the n bandlet does not affect the division pattern of the o_1 blast cells in the neighbouring o bandlet (see companion paper).

An almost identical set of divisions to those described here have been observed in the bandlets of the leech embryo, (Weisblat *et al.* 1980; Zackson, 1984; Schimmerling, (1986); Bissen and Weisblat, 1989): the only difference being the symmetry of the first (and possibly second) divisions in the q bandlet. Blast cells in leech bandlets display the same occasional inconsistencies

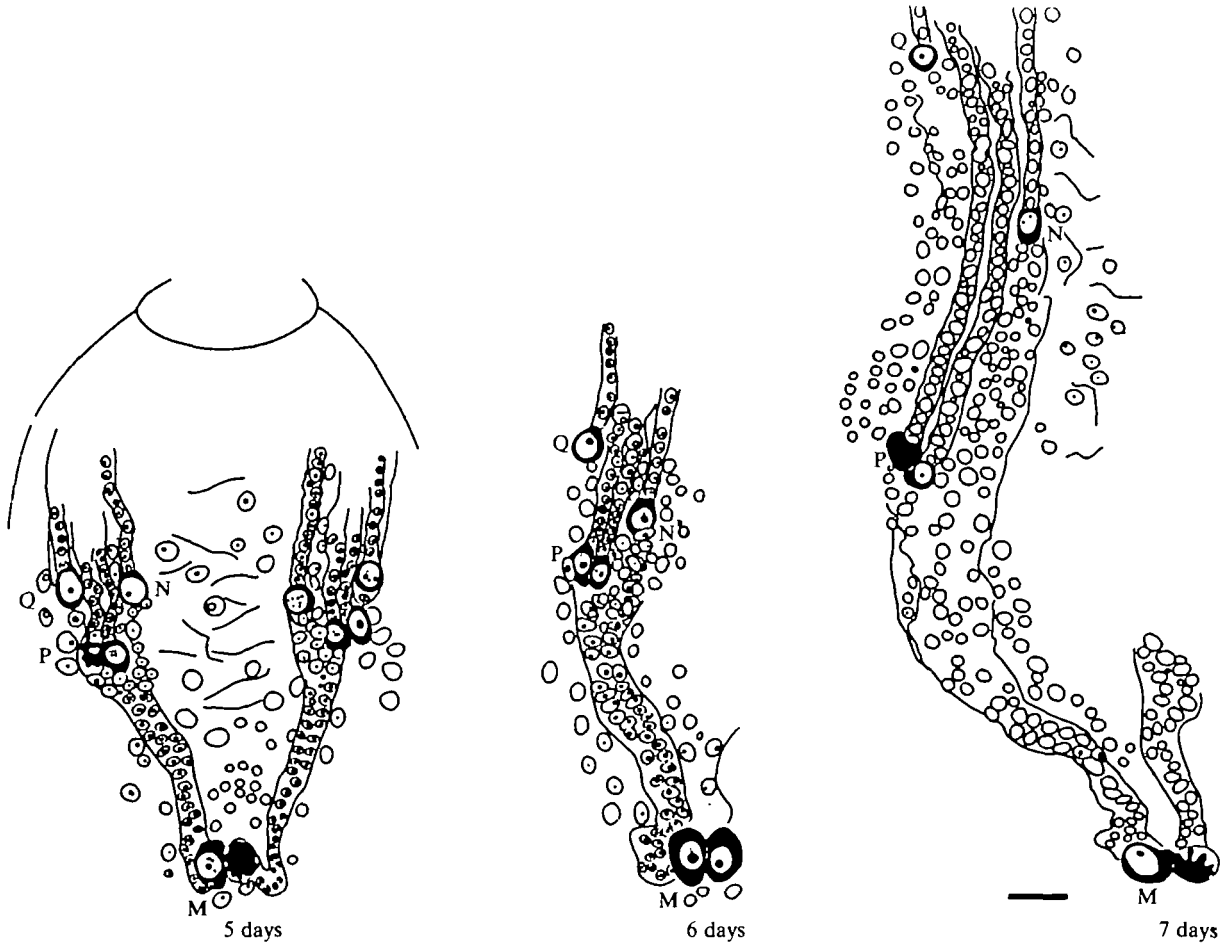

Fig. 19. Camera lucida drawings showing the change in spatial relationship of the ectoteloblasts as the embryo develops. The N and Q teloblasts are found at increasingly anterior positions relative to the O and P teloblasts. As the teloblasts have the same cell cycle time this may reflect the different numbers of blast cells contributed by each teloblast to each hemisegment. Scale bar = 50 μm.

(Zackson, 1984) and the importance of interactions between bandlets in determining the pattern of division of cells in neighbouring bandlets in the leech embryo has been examined by extensive ablation experiments (Zackson, 1984; Shankland and Weisblat, 1984; Ho and Weisblat, 1987).

The pattern of labelling following HRP injection of the N teloblast was similar to that observed in the leech embryo after injection of the N teloblast (Weisblat *et al.* 1980; Shankland and Weisblat, 1985). In the leech the two different patterns of contribution have been shown to correspond directly with the two types of n blast cell. Further, n blast cells are also not the sole contributors to the hemiganglion rudiment in the leech (Weisblat *et al.* 1980; Kramer and Weisblat, 1985). The lateral position of the unlabelled cells in the hemiganglion rudiment is consistent with the migration of progeny from the more laterally placed o, p and q cell lines.

A possible mechanism for generating the two types of n blast cell has been proposed (Bissen and Weisblat, 1987), in which feedback from a blast cell to its parent affects the type of blast cell produced next. However, it remains to be demonstrated that n blast cells are in fact different from birth, as this model predicts. One way to investigate this is to kill n blast cells soon after they are born. However, problems inherent in the glossiphoniid leech embryo make it difficult to interpret such experiments (Bissen and Weisblat, pers.com.). The earthworm, on the otherhand, appears more amenable, having teloblasts and bandlets firmly embedded in an ectodermal sheet.

Some differences between these annelid embryos appear to be associated with the possession of either small, non-yolk-containing teloblasts, which grow between divisions, or large yolk-filled teloblasts, which cleave at each division. The small teloblasts in the earthworm embryo have a cell cycle of about 3 h, while the cycle of teloblasts in the glossiphoniid leech *H. triserialis* lasts only an hour (Wordeman, 1983). This difference reflects the time spent in G_1 and G_2 by the teloblasts in the earthworm (about 2 h) and the negligible time spent in these phases by the teloblasts in the leech (Bissen and Weisblat, 1989). Interestingly, not all leeches have large, yolk-filled teloblasts, and the small teloblasts of the hirudinid leeches (Fernandez and Stent, 1982), might be expected to have a longer cell cycle time than the teloblasts in the glossiphoniid leech.

One consequence of the growth of earthworm teloblasts after each division is the dilution of tracer injected into these cells. This factor, coupled with the small size of teloblasts and their sensitivity to positive current (used to inject HRP), limited the longevity and the amount of the tracer injected into the teloblast and so confined this study to the earliest part of the n lineage.

Using autoradiographic and immunocytochemical methods Bissen and Weisblat (1989), have shown that the seven types of blast cell in the leech (m, nf, ns, o, p, qf and qs) spend the same amount of time in s phase (4 h) and that differences in the cell cycle length (which range from 11 to 33 h) arise from differences in the length of G_2. In contrast, in this study the length of G_2 was found to be the same in each type of blast cell and, if the cell cycle varied, it was due to an increase in the length of time spent in s phase. This was the case for the m_1 blast cell, which has a very long cell cycle, although it is not clear whether the difference in cell cycle time between the n_1 and n_2 blast cells was due to a longer s phase or G_2.

The more rapid posterior displacement of the O and P teloblasts in contrast with that of the N and Q teloblasts supports the proposition that the progeny of O and P are incorporated at a lower rate than those of the N and Q. This observation is consistent with the incorporation of two n and q blast cells for every one o and p blast cell. In the glossiphoniid leech, injection of fluorescent lineage tracer into the N or Q teloblast at the same time as into the M or O/P teloblasts has shown that the cells in the n and q bandlets come into register with consegmental cells in the m, o and p bandlets by sliding past cells in these bandlets that are destined for more posterior segments (Shankland and Weisblat, 1985). In the earthworm it may be that a similar sliding forward of the n and q bandlets accounts for the changing spatial relationship of the ectoteloblasts.

The three strands of this investigation, the pattern of labelled cells following HRP injection of the N teloblast, the pattern of divisions in the bandlets and the changing spatial relationship of the ectoteloblasts, together suggest that each segmental unit in the early earthworm embryo is contributed to by 2n, 2q, 1o, 1p and 1m blast cells. This pattern of contribution is strikingly similar to that observed in the leech embryo (Weisblat *et al.* 1980, 1984; Weisblat and Shankland, 1985), and underlines the apparent importance of fixed cell lineages in the assignment of cell fate in annelid embryos.

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Appendix 1

Determination of the cell cycle time of the teloblasts: a mathematical method

Cell cycle times are often determined by substituting values into the equation which relates the length of a particular phase in the cycle (e.g. s phase) and the proportion of cells in that phase at any given moment to the total length of the cell cycle (T):

$$\text{length of s phase (S)} = \%\text{ of cells in s phase (S)} \times \text{T}.$$

However, instead of obtaining a value for the proportion of cells in s phase at any one instant, the proportion of cells shown to be in s after pulses of different durations was recorded. The equation was

thus modified to take into account the length of the pulse applied and pulses of different durations could then be compared in the form of simultaneous equations which were solved for T and then for s. The equation was also further modified to take into account the time taken to incorporate new molecules into replicating DNA. The window in the cycle revealed by the pulse is equal to the length of the pulse plus the length of s phase (as a cell which at the onset of the experiment is the length of the pulse before s phase will have just entered s phase by the end of the pulse). The shortest pulse after which cells in culture have been seen to have incorporated BUdR is 3 min, Dolbeare *et al.* (1983). As the teloblasts are at the surface of the embryo at the stage of development when they are exposed to the BUdR, it is assumed that there is a delay of the same duration (3 min) before cells can be seen to have incorporated the molecule. If a cell enters s phase for only 3 min or is in the last 3 min of s phase, it will not be labelled. Thus a correction factor of 6 min (3 min either end of s) was included in the equation which now takes the form:

$$s + (\text{pulse length} - 6\,\text{min}) = \%\ s \text{ labelled in pulse} \times T.$$

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