

Cell proliferation in the gastrulating chick embryo: a study using BrdU incorporation and PCNA localization

E. J. Sanders, M. Varedi and A. S. French

Department of Physiology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

SUMMARY

Cell proliferation in the gastrulating chick embryo was assessed using two independent techniques which mark cells in S phase of the mitotic cycle: nuclear incorporation of bromodeoxyuridine (BrdU) detected immunocytochemically and immunolocalization of proliferating cell nuclear antigen (PCNA). Computer-reconstructed maps were produced showing the distribution of labelled nuclei in the primitive streak and the cell layers. These distributions were also normalized to take into account regional differences in cell density across the embryo. Results from a 2 hour pulse of BrdU indicated that although cells at caudal levels of the primitive streak showed the highest incorporation, this region showed a similar proportion of labelled cells to the surrounding caudal regions of the epiblast and mesoderm when normalized for cell density. The entire caudal third of the embryo showed the highest proportion of cells in S phase. Cells of Hensen's node showed a relatively low rate of incorporation and, although the chordamesoderm cells showed many labelled nuclei, this appeared to be a reflection of a high cell density in this region. Combining this result with results from a 4 hour pulse of BrdU permitted mapping of cell generation time across the entire embryo. Generation times ranged from a low value of approximately 2 hours at caudal levels of both the epiblast and mesoderm, to an upper value of approximately 10 hours in the rostral regions of the primitive streak, in the mid-lateral levels of the epiblast and in the chordamesoderm rostral to Hensen's node.

Cells at caudal regions of the primitive streak showed a generation time of approximately 5 hours. Taking into account that cells are generally considered to be continuously moving through the primitive streak, we conclude that cell division, as judged by generation time, is greatly reduced during transit through this region, despite the presence there of cells in S phase and M phase. Immunocytochemical localization of PCNA-positive nuclei gave generally similar distributions to those obtained with BrdU incorporation, confirming that this endogenous molecule is a useful S-phase marker during early embryogenesis. Mid-levels and caudal levels of the primitive streak showed the highest numbers of positive nuclei, and the highest proportion of labelling after cell density was accounted for. As with BrdU incorporation, the highest proportions of PCNA-positive nuclei were found towards the caudal regions of the epiblast and mesoderm. These results suggest that the differential growth of the caudal region of the embryo at this time is a direct consequence of elevated levels of cell proliferation in this region. However, no evidence was obtained to indicate that the primitive streak cells, or the cells of the emerging mesoderm, are highly proliferative, and we therefore conclude that the morphogenesis of the early mesoderm does not rely on elevated rates of cell division.

Key words: chick embryo, gastrulation, mesoderm, cell proliferation, PCNA, BrdU

INTRODUCTION

Embryonic morphogenesis is usually considered to result from cell movements and rearrangements that are driven by intrinsic or programmed changes in cell behaviour, integrated with developmentally regulated changes in the extracellular environment. However, it has also been recognised that cell proliferation may contribute to morphogenetic processes by the mechanical forces resulting from increased pressure of cell numbers. For example, in the early chick (Spratt, 1963) and mouse (Snow, 1977) embryos, localized zones of high cell proliferation in the epiblast layer have

been thought to be important in determining several phases of early development. This includes initiating the processes leading to the formation of the primitive lower layer of cells, or hypoblast (Spratt, 1963), and acting as a primary force driving the cell movements of gastrulation (Wetzel, 1929, pp. 201 and 203). Also, in the mouse embryo, morphogenesis of the early mesoderm has been suggested to rely on the presence of local proliferation zones, rather than extensive cell migration (Poelmann, 1981). However, such proposals have always been controversial (Pasteels, 1943, p. 23; Emanuelsson, 1961, p. 216), largely because they are very difficult to verify unequivocally in living systems, and

also because it has been difficult to reach a consensus on regional differences in proliferative rates owing to conflicting data. In the specific case of hypoblast formation, the notion has apparently been disproved (Mitrani, 1984).

The early development of amniotes is characterized by the conversion of a two-layered embryo, consisting of epithelial epiblast and endoblast layers, into a three-layered structure. This process of gastrulation is achieved by movements of cells in the upper epiblast layer and their ingression through a medial structure, the primitive streak. Cells emerge from the primitive streak and contribute to the newly formed third layer of cells, the mesoderm (Bellairs, 1986; Sanders, 1986; 1989). Because the geometry of this system is relatively simple, and the number of interacting cell populations is limited, the gastrulating chick embryo has often been used as a model to test the concept that cell proliferation can drive morphogenesis. This idea has been tested repeatedly over the last 70 years, most commonly by estimation of the mitotic index in various regions of the early embryo (Shultz, 1922; Derrick, 1937; Woodard, 1948; Bellairs, 1957; Emanuelsson, 1961; Dondua et al., 1966; Ozato, 1969; Stern, 1979). Results from this approach have been contradictory, even for the restricted area of the primitive streak (summarized by Bellairs, 1971, and by Stern, 1979). The most recent study (Stern, 1979), suggested that the primitive streak of colchicine-arrested embryos at developmental stage 4 (Hamburger and Hamilton, 1951) is a region of elevated mitotic index, particularly at the caudal end, as is the region rostral to Hensen's node, although the node itself does not contain a highly proliferative population of cells. These findings were broadly consistent with those of Derrick (1937), Emanuelsson (1961), Dondua et al. (1966) and Ozato (1969).

Mitotic index (MI), calculated as the percentage of observably dividing nuclei, gives an estimate of the proportion of cells in the M phase of the cell cycle at a given time. However, MI cannot be correlated with the rate of cell division unless the duration of the M phase is also known (Woodard, 1948; Emanuelsson, 1961; Dondua et al., 1966; Ozato, 1969). A high MI may only reflect a high degree of synchronization in a given region. These uncertainties, combined with the problem of simply recognizing metaphase nuclei, severely limit the usefulness of MI as a measurement of rates of cell proliferation in different regions of the embryo. The most common alternate method of estimating cell proliferation rate has been to incorporate [^3H]thymidine into cells during S phase by pulsing embryos with the label for a fixed period of time. Ozato (1969) used this method with gastrulating chick embryos, compared the results with the MI method, and concluded that the labelling index (percentage of labelled nuclei) did not correlate regionally with the MI. Indeed, along the length of the primitive streak, the labelling index and MI were found to be inversely related. However, incorporation techniques are only valuable when the duration of S phase is known, because it then becomes possible to estimate cell cycle time. Ozato (1969) did not consider this parameter, and indeed accurate measurement of the length of S phase of cells in early amniote embryos has been elusive, but estimates of 6-8 hours are most frequent (Cameron, 1964; Mitchison, 1971), and Poelmann (1981) has estimated 6.3 hours for

the early mouse embryo. Primmitt et al. (1989), using a combination of mitotic index and thymidine incorporation methods on slightly later stages of chick development, estimated S phase to be 2.3 hours in the cells of the segmental plate mesoderm, and MacAuley et al. (1993) estimated 2.5-3.5 hours for some regions of the gastrulating mouse.

Perhaps the most useful indicator of proliferative activity is the generation or doubling time, i. e. the length of the cell cycle. A regional analysis of this parameter would provide a direct picture of patterns and levels of proliferation across the embryo in different tissues, but this has been difficult to achieve. Various estimates of cell generation time, usually obtained from MI measurements, are available for the gastrulating chick and mouse embryos, ranging from 2-3 hours (Solomon, 1957; Snow, 1977) to 8-10 hours (Woodard, 1948; Emanuelsson, 1961; Dondua et al., 1966; Poelmann, 1981). Most recently, MacAuley et al. (1993) have calculated generation times of 3-7.5 hours in the gastrulating mouse embryo. Primmitt et al. (1989), using the methods mentioned above, estimated a cell cycle time of 9.5 hours for the segmental plate mesoderm cells at a slightly later stage of development. However, there is no comprehensive description of the patterns of variation in generation time across the gastrulating embryo.

In the present work, we have brought some new techniques to this old problem. We used the bromodeoxyuridine (BrdU)-incorporation procedure, which is analogous to [^3H]thymidine incorporation (Böswald et al., 1990), followed by immunoperoxidase detection of the label (Gratzner, 1982). This allowed us to use computer-reconstruction methods to create a map of the gastrulating chick embryo from serial sections, plotting the incidence of labelled nuclei, the proportions of nuclei that are labelled and, by using two different timed pulses of BrdU, mapping the generation time of cells across the entire embryo for the first time. We conclude that although the primitive streak contains a large population of dividing cells, particularly at the caudal end, the high cell density in this region lowers the overall probability of finding a dividing cell in any given region. Furthermore, when generation times are calculated, it becomes apparent that the rostral regions of the streak, including Hensen's node, are dividing more slowly (generation time 7-10 hours) in comparison with caudal regions (approximately 5 hours). In both epiblast and mesoderm, the highest rates of division occur in the caudal regions of the embryo, while the lowest rates are rostral to Hensen's node.

In addition, we localized the proliferating cell nuclear antigen (PCNA) which is a highly conserved protein expressed only in cells committed to DNA synthesis and which is an auxiliary protein of DNA polymerase (Bravo et al., 1987; Prelich et al., 1987; Baserga, 1991). PCNA is detectable immunocytochemically only during late G₁ and S phases and may be used as a naturally occurring marker for cell proliferation (Galand and Degraef, 1989; Thaete et al., 1989; Hall and Woods, 1990). PCNA localization therefore provides an alternate assessment of cell proliferation rates independent of, but analogous to, results obtained with BrdU incorporation. For this purpose, we used monoclonal antibody PC10 (Waseem and Lane, 1990) immunocytochemically on serial sections through the entire gastrulat-

ing embryo. We found a distribution of labelled cells similar to that obtained with BrdU incorporation. In particular, labelling was highest in the primitive streak and, when cell density was taken into account, the probability of finding labelled cells was highest laterally and caudally in the epiblast and mesoderm.

MATERIALS AND METHODS

Bromodeoxyuridine incorporation

Fertile White Leghorn hen's eggs were incubated until the embryos had reached stage 5 of Hamburger and Hamilton (1951). The embryos were then cultured according to the method of New (1955), by explanting them ventral surface uppermost on their vitelline membranes stretched over a 25 mm internal diameter glass ring. Embryos at the appropriate stage were exposed to 5-bromo-2'-deoxy-uridine (BrdU, Sigma Chemical Co.) at a concentration of 10 µg/ml, for either 2 hours or 4 hours at 37°C, by placing approximately 0.5 ml on top of the embryo and the same volume underneath. After the requisite length of time, the embryos were fixed with 4% paraformaldehyde overnight, embedded in paraffin wax and sectioned at 8 µm thickness. Only embryos sectioned in their entirety through the area pellucida were processed further; this entailed collecting, staining and analyzing between 150 and 200 sections for each embryo. After dewaxing and dehydrating the serial sections, the slides were immersed in 1% hydrogen peroxide in 50% methanol for 30 minutes in order to quench endogenous peroxidase activity. This step was critical for the reduction of background staining. All incubations were carried out at room temperature in a humid chamber, making sure at each step that every section was exposed to the reagent. The sections were washed in phosphate-buffered saline (PBS) for 20 minutes and then incubated with 4% bovine serum albumen (BSA) with 0.05% Tween 20 for 20 minutes to block non-specific antibody binding. The primary antibody used to detect incorporated BrdU was a mouse monoclonal antibody preparation, containing a nuclease, obtained from Amersham International plc. The presence of the nuclease produces single-stranded DNA which is accessible to the antibody without the need for chemical denaturation. The antibody was applied undiluted to the sections for 1 hour at room temperature followed by washing for 20 minutes with PBS containing 4% BSA. The secondary antibody was biotinylated goat anti-mouse IgG (Zymed Laboratories Inc.) used at a dilution of 1:50 in PBS plus BSA for 20 minutes. Binding was detected with the biotin-avidin technique using the 'Vectastain ABC Kit' (Vector Laboratories Inc.) and the diaminobenzidine (DAB) substrate kit from Zymed Laboratories Inc. These pre-prepared kits gave the reproducibility and reliability required to stain the large number of sections necessary for reconstruction of each complete embryo. After washing with PBS, each slide was dehydrated and mounted. Slides were then examined for completeness of the serially sectioned embryo, integrity of the individual sections, contrast and general quality of the immunocytochemical staining, and the uniformity of the staining. Only embryos that satisfied all of these criteria were selected for computer reconstruction of the distribution of stained nuclei. A total of four complete embryos that had been exposed to BrdU for 2 hours were digitized as described below. Because the process of digitizing individual labelled nuclei was time-consuming, the sample size was, of necessity, small.

Four embryos exposed to BrdU for 4 hours were also sectioned and processed for immunocytochemistry as above and used to estimate the proportion of labelled cells in selected regions of the embryo. The regional data from the embryos pulsed for 4 hours was averaged and transposed directly onto the map constructed from the 2 hour pulse. In combination with the mapped data for

the 2 hour pulse, the data from the 4 hour pulse was used to reconstruct a map of the rate of cell division across the embryo, using the procedures detailed below (Eqs. 1 and 2).

Immunocytochemistry for proliferating cell nuclear antigen (PCNA)

Embryos at stage 5 were removed from the egg and rinsed clean of yolk with Tyrode's saline. The embryos were kept flat while fixing in 4% paraformaldehyde for 4 hours at room temperature. Preliminary experiments using methacarn fixation (Puchtler et al., 1970) did not give satisfactory immunocytochemical labelling, and this fixative was therefore abandoned. Paraffin wax serial sections (8 µm) were treated with 1% hydrogen peroxide in 50% methanol for 30 minutes, washed with PBS containing 4% fetal bovine serum and immersed in a mouse monoclonal antibody to PCNA diluted 1:20 with PBS plus serum. The antibody used was from the PC10 clone (Waseem and Lane, 1990) obtained from Novocastra Laboratories, Newcastle-upon-Tyne, UK, or Dako, Glostrup, Denmark. Treatment was carried out at room temperature for 1 hour, and care was again taken to ensure that all sections were exposed to all the reagents applied. After rinsing with PBS plus serum, sections were incubated with biotinylated goat anti-mouse IgG at a dilution of 1:10 for 1 hour. Antibody binding was detected with the ABC and DAB kits as described above. Complete series of sections were examined according to the criteria outlined earlier, and four embryos were digitized.

Immunocytochemical double labelling

For simultaneously labelling incorporated BrdU and endogenous PCNA, a fluorescence technique was used. Sections of embryos that had been exposed to BrdU were dewaxed, rehydrated and incubated with 4% goat serum containing 0.05% Tween 20 for 20 minutes. The anti-PCNA antibody, at a dilution of 1:20 in PBS plus goat serum, was then applied for 1 hour, followed by washing in PBS plus serum for 20 minutes. This antibody was visualized with rhodamine-labelled goat anti-mouse IgG (Sigma Chemical Co.), at a dilution of 1:100 in PBS plus serum. After washing in PBS plus serum, the sections were exposed to undiluted anti-BrdU monoclonal antibody for 1 hour, followed by a wash in PBS plus serum, and then fluorescein-labelled goat anti-mouse IgG (Sigma Chemical Co.), at a dilution of 1:100 in PBS plus serum, for 1 hour. Finally, the sections were washed in PBS and mounted in Mowiol. Although both secondary antibodies were against mouse IgG, the labelling of each of the primary antibodies was shown to be specific by double controls, in which a different primary antibody was replaced by PBS. In the controls, only one wavelength of fluorescence was detected, corresponding to the primary antibody present. This specificity was ensured by the blocking effect of the goat serum, which was present in all steps of the procedure. When both primary antibodies were omitted, no fluorescence was observed.

Computer reconstruction of nuclear distributions

Immunocytochemically stained sections of embryos that had been exposed to BrdU for 2 hours were viewed with a compound microscope (Olympus BH2) using a 10× objective. The position of each stained nucleus was digitized with a SummaSketch II graphics tablet (Summagraphics). An aperture of 0.5 mm, illuminated by a green light-emitting diode, was fixed to the cursor of the graphics tablet and viewed together with the sections through a drawing tube. The effective size of the aperture when marking a nucleus was 4 µm, and the corresponding resolution of the graphics tablet was 0.5 µm. Nuclei from cells of the epiblast, primitive streak and mesoderm plus endoderm were marked separately and identified as such in the data files. It was necessary to combine the mesoderm nuclei with those of the very thin layer of endoderm cells, because the available resolution did not always allow an

unequivocal distinction to be made between these two populations. However, for purposes of interpretation this layer represented the mesoderm, since the contribution made by the endoderm was both relatively small and uniform (see Figs 1A-D and 6A,B). It was not possible to reconstruct the most caudal regions of the embryos, where the distinction between the layers was also difficult.

Alignment of digitized sections was carried out automatically by custom-written software. For each section, the operator marked the outer edges of the area pellucida and, when appropriate, the centre of the primitive streak. During alignment, each section was first rotated to have the two or three marked positions in a horizontal line, and then moved horizontally to place the centre of the primitive streak at the mid-line. For sections lacking a central mark, the position half way between the two outer markers was used instead.

For further data processing, marked nuclear positions were counted into squares of 64 μm by 64 μm to produce a distribution map. An integer multiple of 8 μm was chosen to prevent counting artefacts from the 8 μm section thickness. 64 μm offered the best compromise between resolution and accuracy. To normalize the density of stained nuclei by the total cell density, counts were made of the total numbers of cells in 8 μm sections, taken at 64 μm distances through the embryo. The same sections that had previously been stained with BrdU or PCNA and digitized were therefore unmounted and additionally stained with diaminodiphenylindole (DAPI, Sigma Chemical Co.) to stain all nuclei. Slides were incubated for 2 minutes in DAPI at a concentration of 0.0675 $\mu\text{g}/\text{ml}$ in PBS with 1% BSA and viewed with a Leitz Diavert fluorescence microscope. Sections were photographed using T-Max 400 film and the images printed. Nuclei were counted by eye using the 64 $\mu\text{m} \times 64 \mu\text{m}$ grid and multiplying the final number in each grid square by 8 to account for the ratio between the section thickness and the grid dimensions.

Nuclei that had previously been stained with DAB for BrdU or PCNA did not fluoresce with DAPI. Therefore, the total number of cells in each element of the grid was calculated from the sum of the two separate counts. The probability of finding a nucleus stained with BrdU or DAPI was then obtained by dividing the BrdU or PCNA counts by the total nuclei counts.

The mean diameter of nuclei in the section plane was estimated from electron micrographs to be 6 μm . Therefore, the theoretical probability of a single nucleus being observed on two consecutive sections of 8 μm thickness was: 6 $\mu\text{m}/8 \mu\text{m}=0.75$. However, because small grazing sections of nuclei were unlikely to be observed, we assumed that the effective nuclear diameter for this calculation was only 4 μm , reducing the probability of double marking to 0.5. Counts of BrdU- or PCNA-stained nuclei and total nuclei were adjusted by this factor. However, because identical sections and similar procedures were used for all counting, the probabilities, and therefore also the generation times, were not affected by double marking and did not require this correction.

For final viewing, the data in the maps were smoothed once by an algorithm that calculated the mean value of each grid element and its nearest neighbours. Data for the primitive streak region were then placed in the appropriate positions on the map of epiblast data. Grid elements at the boundary of the epiblast and primitive streak regions were unreliable because of overlap within the 64 μm grid dimension. These grid elements were not included in the smoothing operation or the final images. The maps were coloured using an artificial scale with 10 steps, and visualized and photographed using a high resolution computer graphics screen. Using these methods, we constructed maps of: nuclei labelled with BrdU for 2 hours; total cell number; probabilities of finding labelled cells, normalized for cell density; and generation time, using the 2 and 4 hour pulse times and the equations detailed below.

THEORY

We assume that BrdU stained every nucleus that was in S phase during the BrdU application period. If the mean duration of S phase in the region being stained was T_S and BrdU was applied to the embryo for a time T_B , then the total time during which staining could occur was T_S+T_B . If the generation time in the region was T_G , and the cells were randomly distributed through the cell cycle, then the probability, P , of a nucleus being stained by BrdU was:

$$P = \frac{T_S+T_B}{T_G} \quad (1)$$

This equation has three unknowns, T_S , T_B , and T_G . In the majority of experiments, the BrdU was applied for 2 hours, so that $T_B=T_{B1}=2$ hours and $P=P_1$. Another experiment was conducted with $T_B=T_{B2}=4$ hours, so that a different probability, $P=P_2$, was obtained from equation (1). Substituting these two sets of parameters into equation (1) and re-arranging gives:

$$T_G = \frac{T_{B1}-T_{B2}}{P_1-P_2} \quad (2)$$

from which T_G and the rate of division per hour, $1/T_G$, were calculated.

It should be noted that the numerator of equation (2) contains the difference between the two different periods of application of BrdU. Any time required for the BrdU to be taken up into the intracellular pool, before incorporation, would have to be subtracted from the values of T_{B1} and T_{B2} . However, taking the difference between the two terms eliminates any uptake time from the equation.

The assumption that cells were randomly distributed through the cell cycle is not strictly true because each dividing cell produces two daughters, skewing the distribution towards younger cells (Cook and James, 1964). However, if S phase occurs approximately in the middle of the cell cycle, the probability of finding a cell in S phase is close to unity. In the absence of evidence to the contrary, we assumed a value of unity in these calculations. Although the length of S phase would theoretically be available from equation (1) and the calculated values of P and T_G , in practice, the estimated value would include the period required for the saturation of the intracellular pool, as mentioned above. Since this time is unknown, it was not possible to produce reliable estimates of T_S .

RESULTS

Bromodeoxyuridine incorporation

Examination of serial sections through stage 5 embryos pulsed with BrdU for 2 hours clearly showed regional variations in levels of incorporation (Fig. 1A-D). Although inspection of the staining characteristics of individual sections did not necessarily predict the overall patterns that emerged after reconstructing serial sections, it was readily apparent that high labelling was present in the epiblast and mesoderm rostral to Hensen's node (Fig. 1A) and at the caudal end of the primitive streak (Fig. 1D). By contrast, in the node itself (where epiblast and mesoderm cells were not distinguishable from one another), Fig. 1B, and in the rostral part of the primitive streak, Fig. 1C, labelling was noticeably low. The uncertain distinction between the nuclei from cells in the more ventral regions of the mesoderm

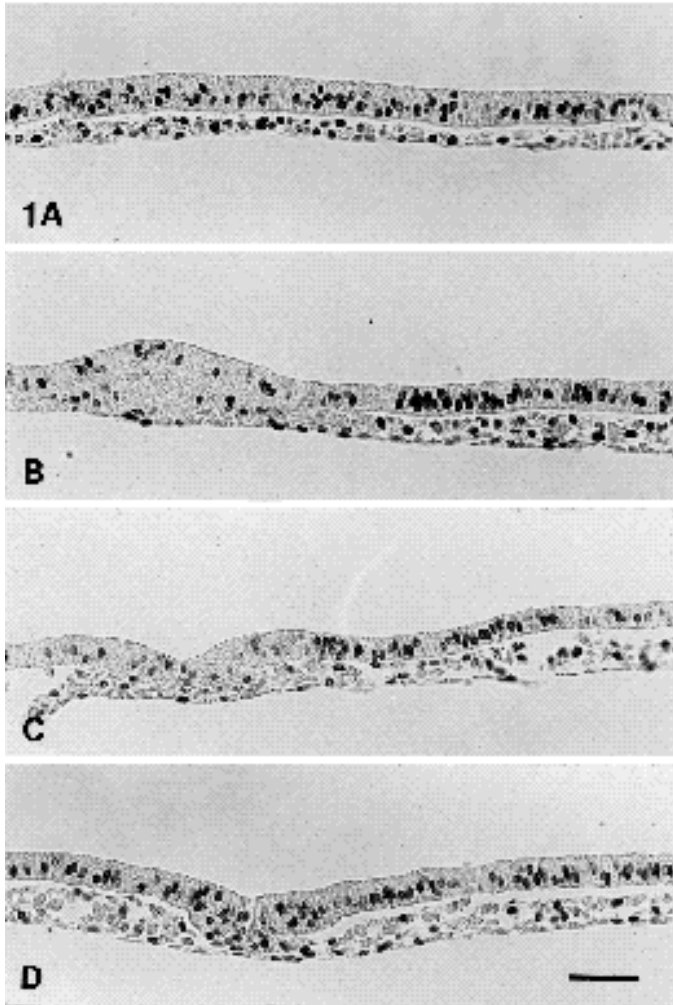


Fig. 1. Sections at different rostrocaudal levels through a stage 5 embryo that had been exposed to BrdU for 2 hours, and immunostained using a monoclonal antibody to BrdU. The sections are taken from: (A) the rostral region of the embryo; (B) Hensen's node; (C) the rostral part of the primitive streak; (D) the caudal part of the primitive streak. Note the differing labelling frequency at various levels of the streak. $\times 200$. Bar, 50 μm .

layer and the very thin endoderm layer necessitated the combining of these two cell populations during digitization.

The positions of the labelled nuclei in the epiblast and mesoderm/endoderm layers were digitized from serial sections, and the resulting distributions were plotted (Fig. 2A,B). However, full appreciation of the distribution of labelled nuclei across the embryo was only apparent after conversion of these data to fixed-resolution maps (Fig. 4A,B). Inspection of these maps from the four embryos showed similar distributions in all of them. The primitive streak (Fig. 4A) was characterized by a progressively increasing incidence of labelled nuclei in the rostrocaudal direction, with Hensen's node showing relatively few labelled nuclei. In the epiblast (Fig. 4A), the highest incidence of labelling was found in an arc in the rostral and lateral regions of the area pellucida, although the region immediately rostral to Hensen's node was an area of some-

what lower labelling (see Figs 2A, 4A). By contrast, in the mesoderm/endoderm (Fig. 4B) a relatively high incidence was found rostral to Hensen's node and bilaterally in the caudal regions of the embryo close to the primitive streak. That the high level of labelling in the caudal region was due to incorporation into mesoderm rather than endoderm cells, was clear from the position of the labelled nuclei well within the mesodermal layer (see Fig. 1D).

Estimates of total cell numbers were made by examining the same embryos after staining all nuclei with DAPI (Fig. 3). Since nuclei previously labelled with DAB did not stain with DAPI, maps of total cell numbers were constructed by combining the DAPI counts with the BrdU label counts. These maps showed that the entire primitive streak (Fig. 4C) was an area of relatively high cell density. In the epiblast, rostral areas were more dense than caudal ones, while in the mesoderm/endoderm (Fig. 4D) the highest cell density occurred rostral to Hensen's node and to a lesser extent surrounding the primitive streak.

When the BrdU counts were divided by the total nuclei counts, it was possible to construct maps showing the probability of finding a stained nucleus (ie: a nucleus in S phase) in any region of the embryo (Fig. 5A,B). These maps showed that for the primitive streak, the probability was highest (approximately 0.5) caudally and lowest rostrally, including Hensen's node, while in the epiblast and mesoderm/endoderm the probability was highest bilaterally at the caudal end of the area pellucida.

Cell generation time across the embryo was computed using the above results in combination with the results of the 4 hour pulse of BrdU, as described above. These maps (Fig. 5C,D) showed a range of generation times between approximately 2 hours and 10 hours. The longer generation times were found in the rostral regions of the primitive streak including Hensen's node, mid-lateral regions of the epiblast, and anterior to Hensen's node in the mesoderm. The shortest generation times were found in the most caudal regions of the embryo, in both the epiblast and the mesoderm.

PCNA localization

Immunocytochemical localization of PCNA also indicated regional variations in the incidence of labelled nuclei, with the rostral regions of the primitive streak showing fewer labelled cells than the caudal region (Fig. 6A,B). This was consistent with the results from BrdU labelling, although the total numbers of labelled cells were higher in the case of PCNA. The distribution map for epiblast and primitive streak (Fig. 7A) confirmed the high level of labelling in the primitive streak and showed the highest levels in the epiblast in regions closest to the streak. In the mesoderm/endoderm (Fig. 7B), the result was similar to that for BrdU, again with higher numbers of positive cells, showing high labelling caudally and laterally in the area pellucida. When the total number of cells was plotted for this embryo (Fig. 7C,D), the primitive streak showed high cell density (compare with BrdU incorporation, Fig. 4C), and the epiblast showed a generally similar pattern to the results from the BrdU-incorporated embryos, although cell numbers were slightly higher in this example. However, the mesoderm/endoderm map for this particular embryo (Fig.

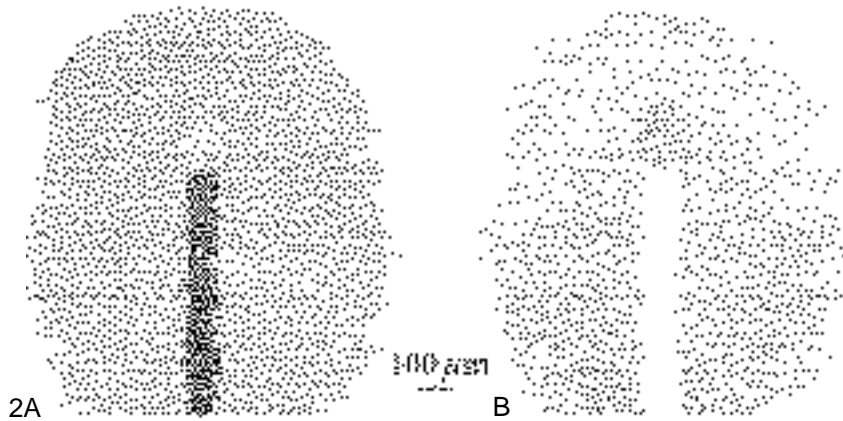


Fig. 2. The distributions of labelled nuclei in the area pellucida in: (A) the epiblast and primitive streak, and (B) the mesoderm and endoderm. Labelled nuclei from each section, visualized as shown in Fig. 1, were manually digitized into a data file and reconstructed. In this particular sample, 160 sections were digitized. The entries in the primitive streak are shown with larger points in order to distinguish them from the epiblast.

7D) did not show the elevated cell numbers rostral to Hensen's node seen in some examples (compare Fig. 4D). When the probability of finding a nucleus labelled for PCNA was computed (Fig. 8A,B), the result was remarkably similar to that obtained for BrdU-labelling (compare with Fig. 5A,B). In the primitive streak, a higher probability was found in the caudal region, though not as marked as with BrdU-incorporation, while in the epiblast and mesoderm/endoderm higher probabilities were found in the lateral and caudal regions of the area pellucida.

Immunocytochemical double labelling

Sections that were labelled simultaneously for incorporated BrdU (2 hour pulse) and endogenous PCNA (Fig. 9A,B) showed that BrdU-positive nuclei were also PCNA-positive. Although we did not systematically study all regions of the embryo, it was clear that more nuclei were PCNA-positive than were BrdU-positive.

DISCUSSION

Bromodeoxyuridine incorporation

Our maps of BrdU incorporation showed that, at stage 5 of development, high rates of incorporation could be found in the caudal region of the primitive streak. In the epiblast, highest incorporation occurred rostrally (Fig. 4A) while, in the mesoderm/endoderm, labelling was highest in caudal

regions and rostral to Hensen's node (Fig. 4B). When these data were normalized for total cell numbers (Fig. 5A,B), it was found that the probability of finding a cell in S phase was highest in the caudal parts of the embryo; in both epiblast and mesoderm, and also in the primitive streak. The high level of incorporation in the caudal streak was due in part simply to the high cell density of the region. By contrast, the regions rostral to Hensen's node displayed a low proportion of cells in S phase, despite the presence there of a relatively high cell density. In the case of the epiblast, the probability of finding a cell in S phase was highest caudally (Fig. 5A), although the cell density was relatively high in rostral regions (Fig. 4C). Similarly, in the mesoderm, cell density was highest surrounding the rostral levels of the primitive streak (Fig. 4D), but again the highest proportion of cells in S phase was present at caudal levels (Fig. 5B). These results show the risk of equating high levels of incorporation with high rates of cell division, without taking cell density into account.

Unlike previous studies at this stage of development, our results, using two different timed pulses of BrdU, also allowed us to map generation times across the embryo. From these maps (Fig. 5C,D), it is clear that the rostral regions of the primitive streak, including Hensen's node are regions of relatively long generation times, of the order of 10 hours. The length of time that cells spend in the primitive streak is not known, however the majority of them are presumably not there for 10 hours. It is therefore difficult

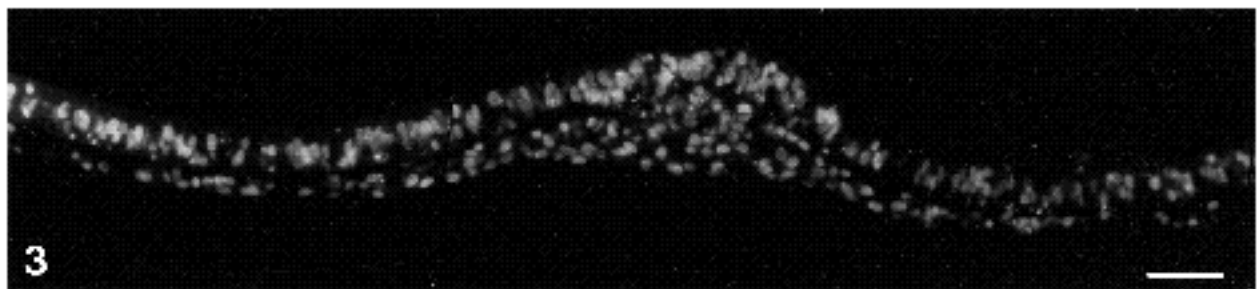


Fig. 3. A montage of fluorescence micrographs of a section through Hensen's node immunostained for BrdU and then subsequently stained with DAPI. Only the DAPI-stained nuclei are visible. The nuclei that were positive for BrdU (compare Fig. 1B for a BrdU-stained section at a similar level) did not fluoresce. $\times 200$. Bar, 50 μm .

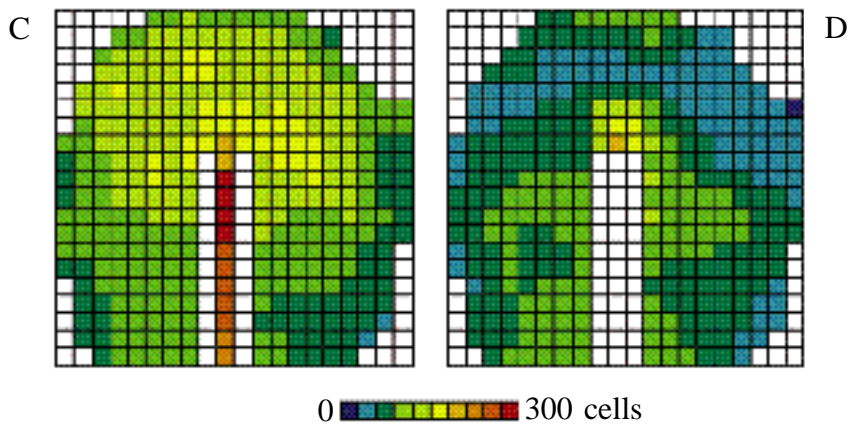
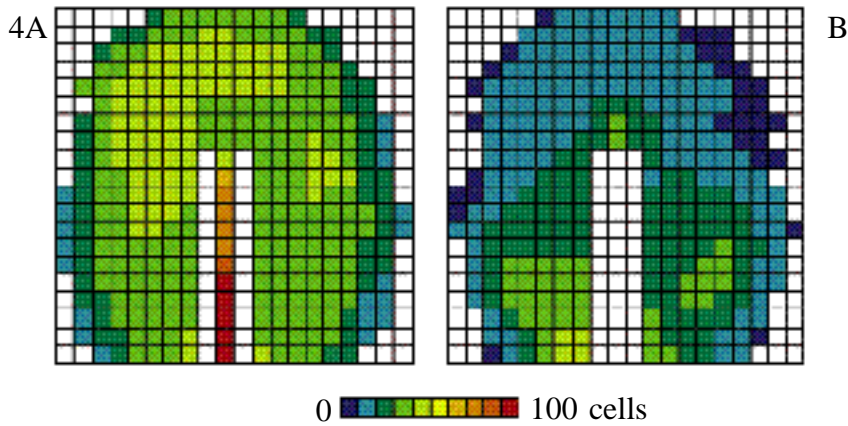


Fig. 4. Artificially coloured computer reconstruction of the area pellucida of an individual stage 5 embryo. The distributions of BrdU-labelled nuclei in the epiblast and primitive streak (A) and the mesoderm and endoderm combined (B). The distributions of total nuclei in the epiblast and primitive streak (C), and the mesoderm and endoderm (D) of the same embryo.

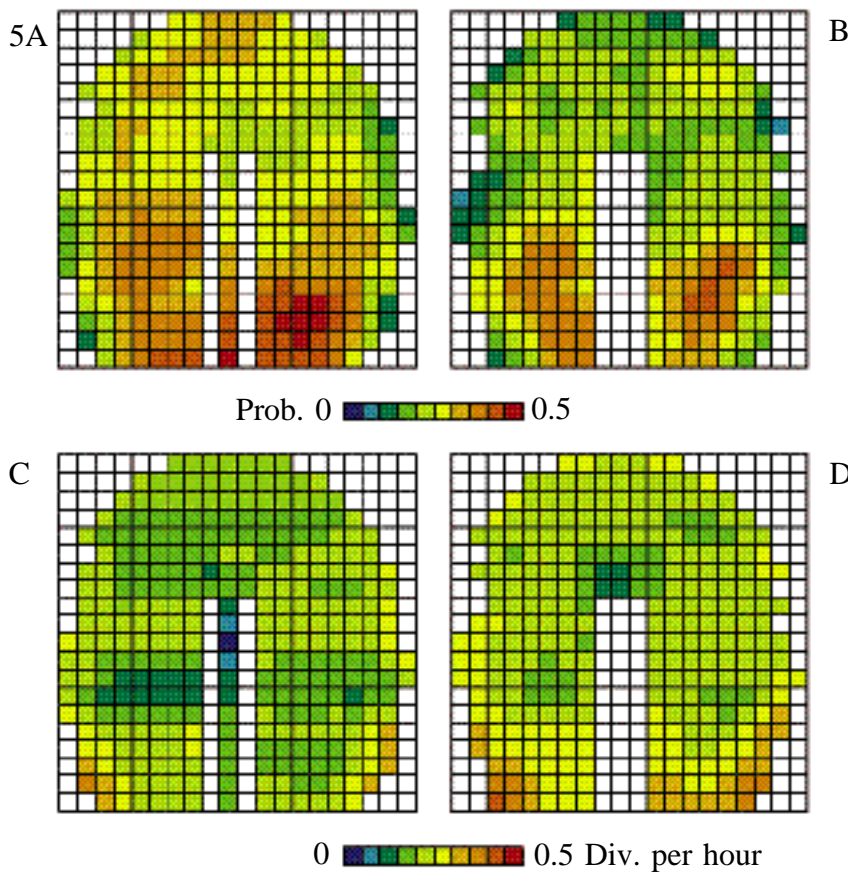


Fig. 5. The same embryo illustrated in Fig. 4, reconstructed to show the probability of finding BrdU-labelled nuclei, i. e. normalized for total cell numbers, in regions of the epiblast and primitive streak (A), and the mesoderm and endoderm combined (B). The rate of cell proliferation is shown for the epiblast and primitive streak (C), and mesoderm and endoderm (D).



Fig. 6. Sections through a stage 5 embryo immunocytochemically stained for PCNA. These sections were taken through rostral (A) and middle (B) levels of the primitive streak, and illustrate the varying labelling frequency in the streak at different levels. $\times 200$. Bar, 50 μm .

to consider a generation time of this length for this continuously moving population of cells. Nevertheless, it must be concluded from our results that the rate of division of these cells is greatly reduced as they accumulate in, and transit, the rostral streak. This may also be true for the more caudal regions of the streak, where generation times approached

4-5 hours. The reduced rate of division as cells enter the primitive streak may correlate with the release from their basement membrane that occurs just prior to their entry into this region, and the change in the integrin-mediated affinity for extracellular matrix components as the cells undergo their phenotypic transformation. The mesoderm cells imme-

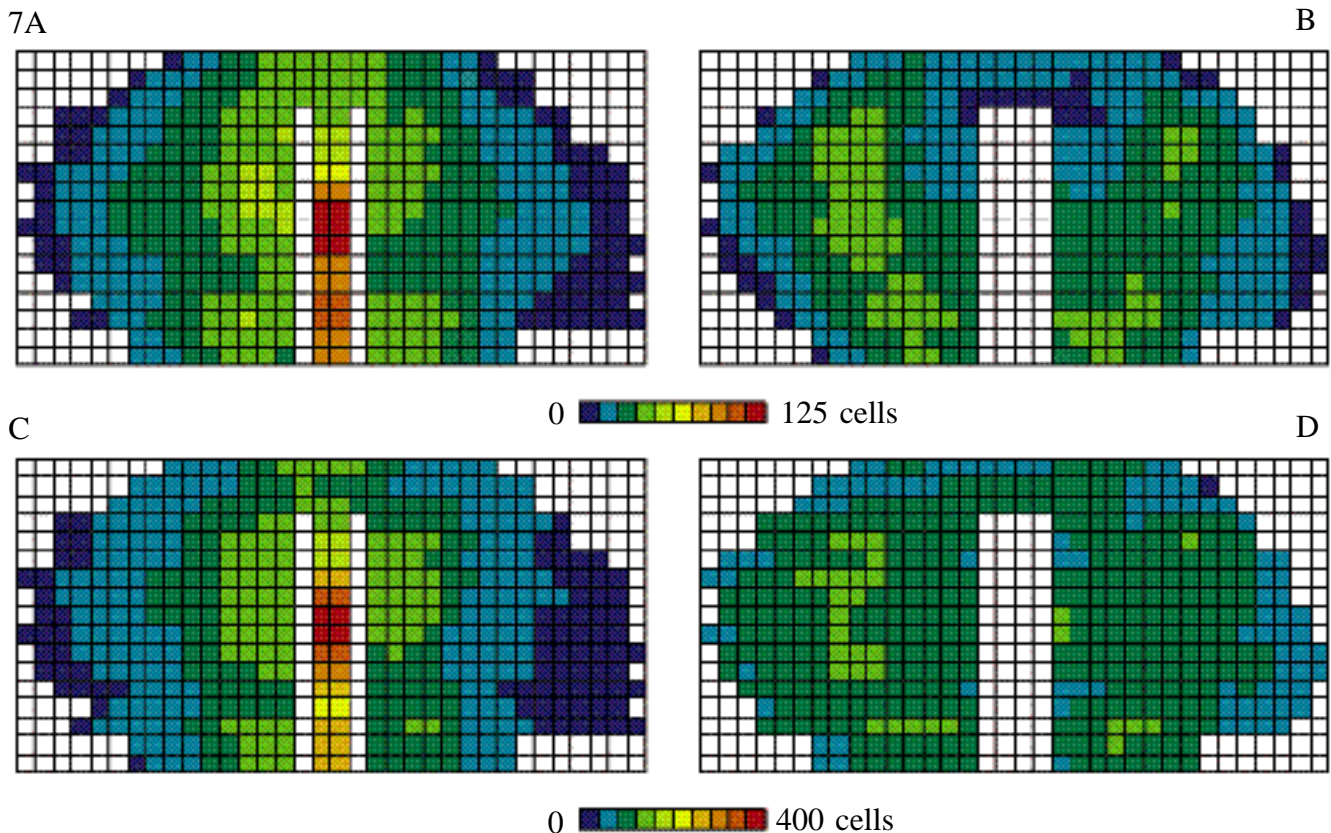
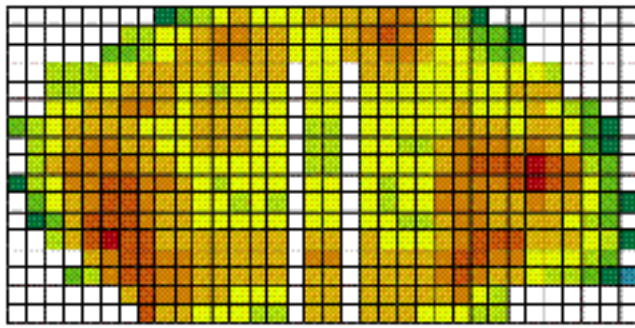
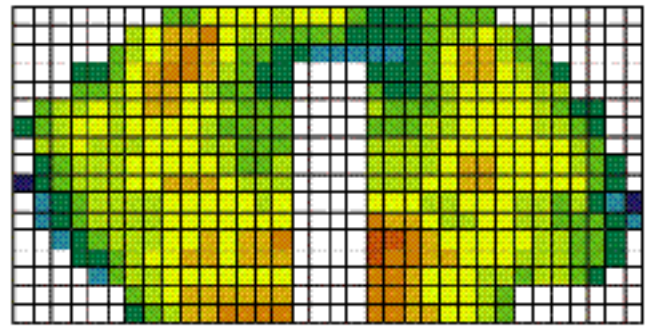


Fig. 7. Artificially coloured computer reconstruction of the area pellucida of an individual stage 5 embryo. The distributions of PCNA-positive nuclei in the epiblast and primitive streak (A), and the mesoderm and endoderm combined (B). The distributions of total nuclei in the epiblast and primitive streak (A), and the mesoderm and endoderm combined (B) of the same embryo.

8A



B



Prob. 0 0.6

Fig. 8. The same embryo illustrated in Fig. 7, reconstructed to show the probability of finding PCNA-positive nuclei, i. e. normalized for total cell numbers, in regions of the epiblast and primitive streak (A), and mesoderm and endoderm (B).

diately rostral to Hensen's node, where the notochord is being laid down, also gave relatively long generation times. By contrast, generation times as short as 2-3 hours were found in the most caudal regions of both the epiblast and mesoderm, suggesting that this is where the greatest differential growth in the embryo is occurring, despite the high BrdU-incorporation rates found in the primitive streak. Our range of figures for generation time, from approximately 2-10 hours, is comparable to the overall estimations made previously for this stage of development (see for example: Dondua et al., 1966 and Poelmann, 1981, who estimated 8-10 hours). In the mouse embryo, Snow (1977) estimated 2-3 hours in a region of high proliferative activity, while MacAuley et al. (1993) found values of 3-3.5 hours in the primitive streak and 7-7.5 hours in the mesoderm and ectoderm. Inconsistencies in the estimations between the different studies may well be due to the differing ages of the embryos used. Here, we provide detailed distributions of the regional variations in the chick.

In discussing the rates of proliferation in the epiblast and primitive streak, we are hampered by a lack of information on the nature and timing of cell traffic through the streak. For example, it is possible that cells ingressing through the streak do so preferentially at particular phases of the cell cycle. If this were so, then it would not be necessary to assume that cells rapidly change their cycle times as they enter the streak region. It is also possible that the streak contains several separate populations of cells, which may be synchronized and cycling at different rates, in a similar manner to the 'mitotic domains' that appear to be occupied by distinct subsets of cells in the early zebrafish embryo, and which are related to lineage segregations (Kane et al., 1992).

It is noteworthy that the rostral portion of the primitive streak, which shows the highest cell density in the embryo, is also the region showing the lowest rate of cell division. The accumulation of cells in this region must therefore be due entirely to the ingression movements of the epiblast cells, with little or no contribution from cell proliferation. By the same token, the low rate of cell division in the primitive streak, and the lack of any marked increase among the

mesoderm cells after emergence from the streak, suggest that the pressure of cell numbers due to high proliferation is not a major factor in driving the outward migration of the mesoderm cells. In fact, we found a fairly uniform generation time among the mesoderm cells, of 3.5-5 hours, decreasing to 2-2.5 hours caudally. However, the cells of the mesoderm layer were generally proliferating at a slightly higher rate than those of the overlying epiblast.

We have assumed that S phase is constant across the entire embryo, and, for reasons indicated earlier, we are unable to calculate accurate estimates of this period. Nevertheless, it is clear that with generation times of the order of 2 hours in some regions, S phase must be correspond-

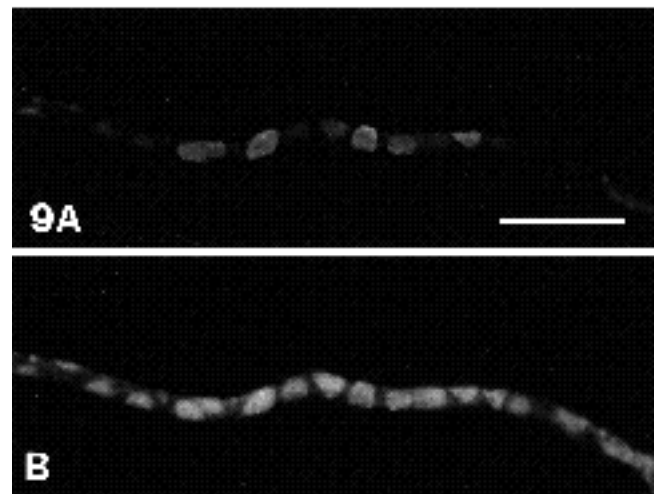


Fig. 9. Section through an extreme lateral region of the epiblast of a stage 5 embryo which had been exposed to BrdU for 2 hours. The section has been doubly immunofluorescence stained to show: (A) BrdU-positive nuclei, using an antibody labelled with fluorescein, and (B) PCNA-positive nuclei, using an antibody labelled with rhodamine. The section was taken from the lateral regions of the embryo where the epiblast is very thin, thus allowing clear comparison of labelled nuclei. All nuclei staining positive for BrdU are also positive for PCNA. $\times 380$. Bar, 50 μm .

ingly small. Previous estimates of 6-8 hours for embryonic cells (Cameron, 1964; Mitchison, 1971; Poelmann, 1981) appear to be high, except possibly among cells in the rostral levels of the primitive streak. The estimates of Primmett et al. (1989) of 2.2-2.3 hours for segmental plate and somite cells, and of MacAuley et al. (1993) of 2.5-3 hours in the mouse primitive streak, seem much more likely.

Previous findings, which largely relied on measurement of the mitotic index (Dondua et al., 1966; Stern, 1979) inferred high rates of cell proliferation in the caudal region of the streak and epiblast. Those studies also showed a high mitotic index in the mesoderm rostral to Hensen's node where chordamesoderm differentiation is just commencing. We also found that these regions show a high level of BrdU incorporation relative to surrounding tissues (Fig. 4A,B), but that this elevation is due to the presence there of a high cell density (Fig. 4C,D), not to an increased proportion of dividing cells (Fig. 5B). By contrast, the caudal regions of the embryo showed high levels of incorporation despite a more modest cell density. Ozato (1969), on the other hand, found a high mitotic index in the *rostral* streak and Hensen's node, but a low labelling index with [³H]thymidine in this region. The latter is confirmed by our BrdU-incorporation results. Similarly, Ozato's result showing high incorporation in the cells of the chordamesoderm is confirmed, indicating a synchrony of division in this region after the cells emerge from the primitive streak rostrally.

PCNA localization

Proliferating cell nuclear antigen (PCNA) is, like BrdU incorporation, an S phase marker that is generally considered to provide an estimation of the level of cell proliferation (Galand and Degraef, 1989; Hall and Woods, 1990; Theunissen et al., 1992), albeit a somewhat high estimation (Francis et al., 1992). Although usually thought to be expressed through late G₁ phase in addition to S phase (Thaete et al., 1989), evidence generally supports the view that PCNA immunoreactivity correlates well with S phase (Hall and Woods, 1990) and indeed the only other work with embryonic tissue suggests that in *Xenopus* embryos, PCNA is detectable *only* during S phase (Leibovici et al., 1992). Our results with nuclei double labelled for incorporated BrdU and endogenous PCNA indicated that the former generally expressed PCNA, but that PCNA-positive nuclei had not always incorporated BrdU.

Therefore, it is important that these two totally independent techniques, BrdU incorporation and PCNA immunoreactivity, gave broadly similar results with respect to regional variations in proliferative activity, although there were always more PCNA-positive nuclei than BrdU-positive nuclei. Thus, in the primitive streak (Fig. 7A), labelling was lower in Hensen's node and at rostral levels in comparison with more caudal levels (compare Fig. 7A with Fig. 4A), and in the mesoderm, higher labelling was present in the lateral and caudal regions of the embryo (compare Fig. 7B with Fig. 4B). Similarly, when the proportion of cells in S phase was considered for the primitive streak, epiblast and mesoderm, high probabilities were found in more caudal regions of the embryo (compare Fig. 5A with Fig. 8A, and Fig. 5B with Fig. 8B). However, results from BrdU incorporation and PCNA localization were not entirely in

agreement. Notably, we never found the equivalent to the relatively heavy BrdU incorporation in the mesoderm in regions rostral to Hensen's node (compare Fig. 4B with Fig. 7B). Indeed, this region showed conspicuously low labelling for PCNA, suggesting that here, at least, PCNA did not mark cells in S phase. Such a finding is similar to that of Connolly and Bogdanffy (1993) and compromises the general opinion that PCNA is of comparable reliability to BrdU incorporation as an S phase marker (Leibovici et al., 1992). This supports the need for caution in interpreting PCNA distributions (Hall and Woods, 1990; Francis et al., 1992).

Our results provide some direct evidence in support of the concept of 'centres of growth' proposed by Spratt (1963), who postulated from circumstantial evidence that differential rapid cell proliferation in the caudal regions of the chick embryo could provide the motive force required for early morphogenetic movements. Spratt reached this conclusion indirectly, using earlier stages than those used here, based on cell-marking experiments and by observing the differential growth of isolated parts of the blastoderm. Our estimates of generation time support this idea in the caudal epiblast and mesoderm, and suggest that this differential growth is responsible for the elongation of the caudal part of the area pellucida that characterizes this period of development. Clearly, our results provide only a snapshot of the embryo during this phase of development, and for a true picture of cell proliferation throughout the entire process of gastrulation, and its role in morphogenesis, it will be necessary to carry out this type of analysis at both earlier and later stages than described here.

We are grateful to Sita Prasad and Ninghe Hu for assistance with these experiments and to the Medical Research Council of Canada for operating grants to E. J. S. and A. S. F.

REFERENCES

- Baserga, R. (1991). Growth regulation of the PCNA gene. *J. Cell Sci.* **98**, 433-436.
- Bellairs, R. (1957). Studies on the development of the foregut in the chick embryo. IV. Mesodermal inductions and mitosis. *J. Embryol. Exp. Morph.* **5**, 340-350.
- Bellairs, R. (1971). *Developmental Processes in Higher Vertebrates*. London: Logos Press Ltd.
- Bellairs, R. (1986). The primitive streak. *Anat. Embryol.* **174**, 1-14.
- Böswald, M., Harasim, S. and Maurer-Schultze, B. (1990). Tracer dose and availability time of thymidine and bromodeoxyuridine in cell kinetic studies. *Cell Tiss. Kinet.* **23**, 169-181.
- Bravo, R., Frank, R., Blundell, P. A. and Macdonald-Bravo, H. (1987). Cyclin/PCNA is the auxiliary protein of DNA polymerase- δ . *Nature* **326**, 515-517.
- Cameron, I. L. (1964). Is the duration of DNA synthesis in somatic cells of mammals and birds a constant? *J. Cell Biol.* **20**, 185-188.
- Connolly, K. M. and Bogdanffy, M. S. (1993). Evaluation of proliferating cell nuclear antigen (PCNA) as an endogenous marker of cell proliferation in rat liver: a dual-stain comparison with 5-bromo-2-deoxyuridine. *J. Histochem. Cytochem.* **41**, 1-6.
- Cook, J. R. and James, T. W. (1964). Age distribution of cells in logarithmically growing cell populations. In *Synchrony in Cell Division and Growth* (ed. E. Zeuthen), pp. 485-495. New York: John Wiley and Sons Inc.
- Derrick, G. E. (1937). An analysis of the early development of the chick by means of the mitotic index. *J. Morphol.* **61**, 257-284.
- Dondua, A. K., Efremov, V. I., Krichinskaya, E. B. and Nikolaeva, I. P.

- (1966). Mitotic index, duration of mitosis and proliferation activity in the early phases of the development of the chick embryo. *Acta Biol. Hung.* **17**, 127-143.
- Emanuelsson, H.** (1961). Mitotic activity in chick embryos at the primitive streak stage. *Acta Physiol. Scand.* **52**, 211-233.
- Francis, S. E., Holt, C. M., Gadson, P. A., Taylor, T. and Angelini, G. D.** (1992). Another cautionary note on the use of PCNA. *J. Pathol.* **166**, 418.
- Galand, P. and Degraef, C.** (1989). Cyclin/PCNA immunostaining as an alternative to tritiated thymidine pulse labelling for marking S phase cells in paraffin sections from animal and human tissues. *Cell Tiss. Kinet.* **22**, 383-392.
- Gratzner, H. G.** (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218**, 474-475.
- Hall, P. A. and Woods, A. L.** (1990). Immunohistochemical markers of cell proliferation: achievements, problems and prospects. *Cell Tiss. Kinet.* **23**, 505-522.
- Hamburger, V. and Hamilton, H. L.** (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.
- Kane, D. A., Warga, R. M. and Kimmel, C. B.** (1992). Mitotic domains in the early embryo of the zebrafish. *Nature* **360**, 735-737.
- Leibovici, M., Monod, G., Géraudie, J., Bravo, R. and Méchali, M.** (1992). Nuclear distribution of PCNA during embryonic development in *Xenopus laevis*: a reinvestigation of early cell cycles. *J. Cell Sci.* **102**, 63-69.
- MacAuley, A., Werb, Z. and Mirkes, P.** (1993). Characterization of the unusually rapid cell cycles during rat gastrulation. *Development* **117**, 873-883.
- Mitchison, J. M.** (1971). *The Biology of the Cell Cycle*. Cambridge: Cambridge University Press.
- Mitrani, E.** (1984). Mitosis in the formation and function of the primary hypoblast of the chick. *Roux's Arch. Dev. Biol.* **193**, 402-405.
- New, D. A. T.** (1955). A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morph.* **3**, 320-331.
- Ozato, K.** (1969). Cell cycle in the primitive streak and the notochord of early chick embryos. *Embryologia* **10**, 297-311.
- Pasteels, J.** (1943). Proliférations et croissance dans la gastrulation et la formation de la queue des Vertébrés. *Arch. Biol.* **54**, 1-51.
- Poelmann, R. E.** (1981). The formation of the embryonic mesoderm in the early post-implantation mouse embryo. *Anat. Embryol.* **162**, 29-40.
- Prelich, G., Tan, C.-K., Kostura, M., Mathews, M. B., So, A. G., Downey, K. M. and Stillman, B.** (1987). Functional identity of proliferating cell nuclear antigen and a DNA polymerase- auxiliary protein. *Nature* **326**, 517-520.
- Primmett, D. R. N., Norris, W. E., Carlson, G. J., Keynes, R. J. and Stern, C. D.** (1989). Periodic segmental anomalies induced by heat shock in the chick embryo are associated with the cell cycle. *Development* **105**, 119-130.
- Puchtler, H., Waldrop, F. S., Meloan, S. N., Terry, M. S. and Conner, H. M.** (1970). Methacarn (methanol-Carnoy) fixation. Practical and theoretical considerations. *Histochemie* **21**, 97-116.
- Sanders, E. J.** (1986). Mesoderm migration in the early chick embryo. In *Developmental Biology: A Comprehensive Synthesis* (ed. L. W. Browder), pp. 449-480. New York: Plenum Pub. Corp.
- Sanders, E. J.** (1989). *The Cell Surface in Embryogenesis and Carcinogenesis*. Caldwell, New Jersey: The Telford Press.
- Shultz, A. F.** (1922). The mitotic index of the chick. *Proc. Oklahoma Acad. Sci.* **52**, 45-48.
- Snow, M. H. L.** (1977). Gastrulation in the mouse: growth and regionalization of the epiblast. *J. Embryol. Exp. Morph.* **42**, 293-303.
- Solomon, J. B.** (1957). Increase of deoxyribonucleic acid and cell number during morphogenesis of the early chick embryo. *Biochim. Biophys. Acta* **23**, 24-27.
- Spratt, N. T.** (1963). Role of the substratum, supracellular continuity, and differential growth in morphogenetic cell movements. *Dev. Biol.* **7**, 51-63.
- Stern, C. D.** (1979). A re-examination of mitotic activity in the early chick embryo. *Anat. Embryol.* **156**, 319-329.
- Thaete, L. G., Ahnen, D. J. and Malkinson, A. M.** (1989). Proliferating cell nuclear antigen (PCNA/cyclin) immunocytochemistry as a labeling index in mouse lung tissues. *Cell Tiss. Res.* **256**, 167-173.
- Theunissen, P. H. M. H., Leers, M. P. G. and Bollen, E. C. M.** (1992). Proliferating cell nuclear antigen (PCNA) expression in formalin-fixed tissue of non-small cell lung carcinoma. *Histopathology* **20**, 251-255.
- Waseem, N. H. and Lane, D. P.** (1990). Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J. Cell Sci.* **96**, 121-129.
- Wetzel, R.** (1929). Untersuchungen am Hühnchen. Die Entwicklung des Keims während der Ersten beiden Bruttage. *Arch. Entwicklungsmech. Organ.* **119**, 188-321.
- Woodard, T. M.** (1948). The mitotic index in the chick embryo. *Am. Naturalist* **82**, 129-136.

(Accepted 25 February 1993)