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Cell cycle analysis of facial mesenchyme in the chick embryo

II. Label dilution studies and developmental fate of slow cycling cells

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SUMMARY

The spatial distribution and developmental fate of quiescent and/or slow cycling cell populations of the primary palate were studied employing label-dilution techniques. 3½-day-old chick embryos were labelled sequentially for 12 h with [^{3}H]thymidine and then chased with cold thymidine. The embryos were reincubated to continue development and were subsequently sacrificed at intervals from the end of labelling at 4 days to 14 days of incubation (10 days after labelling) and processed for autoradiography. Retention of label was used as the assay for identification of quiescent and/or slow-cycling cells. Grain density over the nuclei of labelled cells was determined in the maxillary process and the roof of the stomodeum. Cells with a label density at later time points comparable to that found in cells immediately after the labelling period were defined as label-retaining cells, i.e. those which had become quiescent or had significantly altered generation times. The location and developmental fate of these cell populations were confirmed using slides containing adjacent sections stained with Nuclear Fast Red, Alcian Blue, and Tartrazine. The results demonstrated an association between label-retaining cells and chondrogenic differentiation in the roof of the stomodeum. Subpopulations of label-retaining cells (quiescent and/or slow cycling) which we believe to be prechondroblasts appeared in the chondrogenic region of the roof of the stomodeum prior to, or coincident with, cartilage formation. The retention of label, as evidenced by comparison of nuclear grain counts at the end of the labelling period with subsequent time points, indicated that a cell cycle block may have occurred in the prechondroblastic cell population. The block lasted until these cells expressed the chondrogenic phenotype, after which they resumed cell division.

INTRODUCTION

Prior studies of primary palate development in the chick embryo demonstrated that cell proliferation rates of mesenchyme declined earlier and/or more rapidly in regions adjacent to the facial primordia (e.g. the roof of the stomodeum) than in the facial processes themselves, resulting in a relative enlargement of structures such as the maxillary and nasal processes (Minkoff & Kuntz, 1977, 1978; Minkoff, 1980).

In an accompanying study, the decrease in cell proliferation rates and differences in rates of decline could be explained by the exit of cells from rapidly

proliferating pools and the appearance of subpopulations of cells with longer generation times, a more heterogeneous distribution of generation times and, possibly, the appearance of quiescent, or non-cycling cells (Minkoff, 1982, 1984). The possibility that the decline (or cessation) of cell division in mesenchymal cell subpopulations during facial process formation may be associated with expression of a differentiated phenotype was considered and served as a working hypothesis for this study. An association between the appearance of subpopulations of slow-cycling cells and the differentiation of cartilaginous structures in the facial region of the chick embryo was noted earlier (unpublished observations). For these reasons this study was designed to follow the developmental fate and spatial distribution of slow-cycling cells.

Autoradiographic techniques analogous to those used to determine the time of origin of neurons were employed (Yurkewicz, Lauder, Marchi & Giacobini, 1981). Retention of label in cells was monitored and associated with the expression of a differentiated phenotype in an effort to identify and trace the lineage of slow-cycling cell populations.

MATERIALS AND METHODS

Labelling

White Rock chick embryos were incubated, windowed and labelled with [^{3}H]thymidine (20 Ci/mmole, 1 mCi/ml concentration, New England Nuclear Co.). The label was diluted to 2·0 μCi/0·1 ml final concentration with balanced salt solution and was injected into the amniotic fluid surrounding the head of the embryo. 3½-day-old embryos were labelled every 3 h over a 12 h period with doses ranging from 0·5 μCi to 1·0 μCi/ml. At approximately 3·5 h after the last injection the embryos received a chase of cold thymidine (1·2 mg in 0·5 ml) to dilute any remaining label in the extraembryonic fluids. Embryos were sacrificed at time points immediately after the chase and at 24 h intervals for 10 days thereafter.

Histology and autoradiography

Embryos were fixed by immersion in Bouin's solution, washed, dehydrated and embedded in paraffin (Humason, 1972). Each embryo head was sectioned in the frontal plane through the entire length of the maxillary process with the plane of sectioning perpendicular to the long axis as described previously (Minkoff & Kuntz, 1978). Serial sections (7 μm) were cut and mounted so that equivalent sets of slides were produced containing adjacent consecutive sections. Slides were dipped in NTB-2 Nuclear Track emulsion (Eastman-Kodak), exposed for 3½ weeks, developed and stained with Harris' haematoxylin and eosin. All slides were processed together (i.e. dipping, exposure times, development, etc.) to ensure uniformity (Rogers, 1979).

Cell and grain counting

Grain counts over cell nuclei in the central regions of both the maxillary process and the roof of the stomodeum were obtained using an ocular grid with an oil-immersion objective at a magnification of ×1250. Approximately 100 cells in consecutive grid squares were counted to obtain the mean and distribution of grain counts/cell for each area. In the 7-day embryos grain counts were obtained in chondrogenic regions as well as the surrounding non-chondrogenic area (delineated using Alcian-Blue-stained sections). Grain counts in the older embryos (8 day, 9 day, 10 day, . . . 14 day) were obtained in the chondrogenic region of the nasal septum and in the surrounding perichondrium. The initial grain density in the 4-day embryos was used to evaluate the degree of dilution of label in older embryos. Slow-cycling cells were identified by residual high grain counts during the course of the experiment and were defined as those having grain counts comparable to the mean grain count per cell in the 4-day embryos.

Since preliminary studies indicated that slow cycling cells were present in chondrogenic regions, a slide from each embryo was stained with Nuclear Fast Red, Alcian Blue (pH 2·2), and Tartrazine (Sams & Davies, 1967) primarily for the identification of cartilage and bone. The stained sections and adjacent autoradiograms were compared to assess the developmental fate of cell populations. A third slide from each embryo was stained with Harris' haematoxylin and eosin and the mitotic figures observed in 100 consecutive grid squares (12 000 μm^2) in the central region of the cartilaginous nasal septum were counted.

Background counts were obtained in a tissue-free area immediately adjacent to the tissue sections. The number of cells in 150 grid squares provided an average value for the number of cells per grid square. By dividing the background count per grid square for each slide by the average number of cells per grid square the background count per cell was determined for each slide. The mean grain count per cell in the regions counted was then adjusted for background.

RESULTS

Qualitative observations

Autoradiograms of the 4-day embryos showed uniformly and comparably heavy grain distributions in all regions (Fig. 1A). The 6-day embryos showed a general reduction in intensity of labelling and it was now possible to distinguish a higher intensity of labelling over the roof of the stomodeum relative to the developing maxillary processes (Fig. 1B). The nasal septal cartilage and scleral cartilages could be identified in the Alcian-Blue-stained slides of 6-day embryos. In 7-day embryos, although label intensity continued to decrease throughout the embryo, high-grain densities were observed over the nasal cartilages which were now clearly visible in the Alcian-Blue-stained sections (Fig. 1C). In the 8-day and

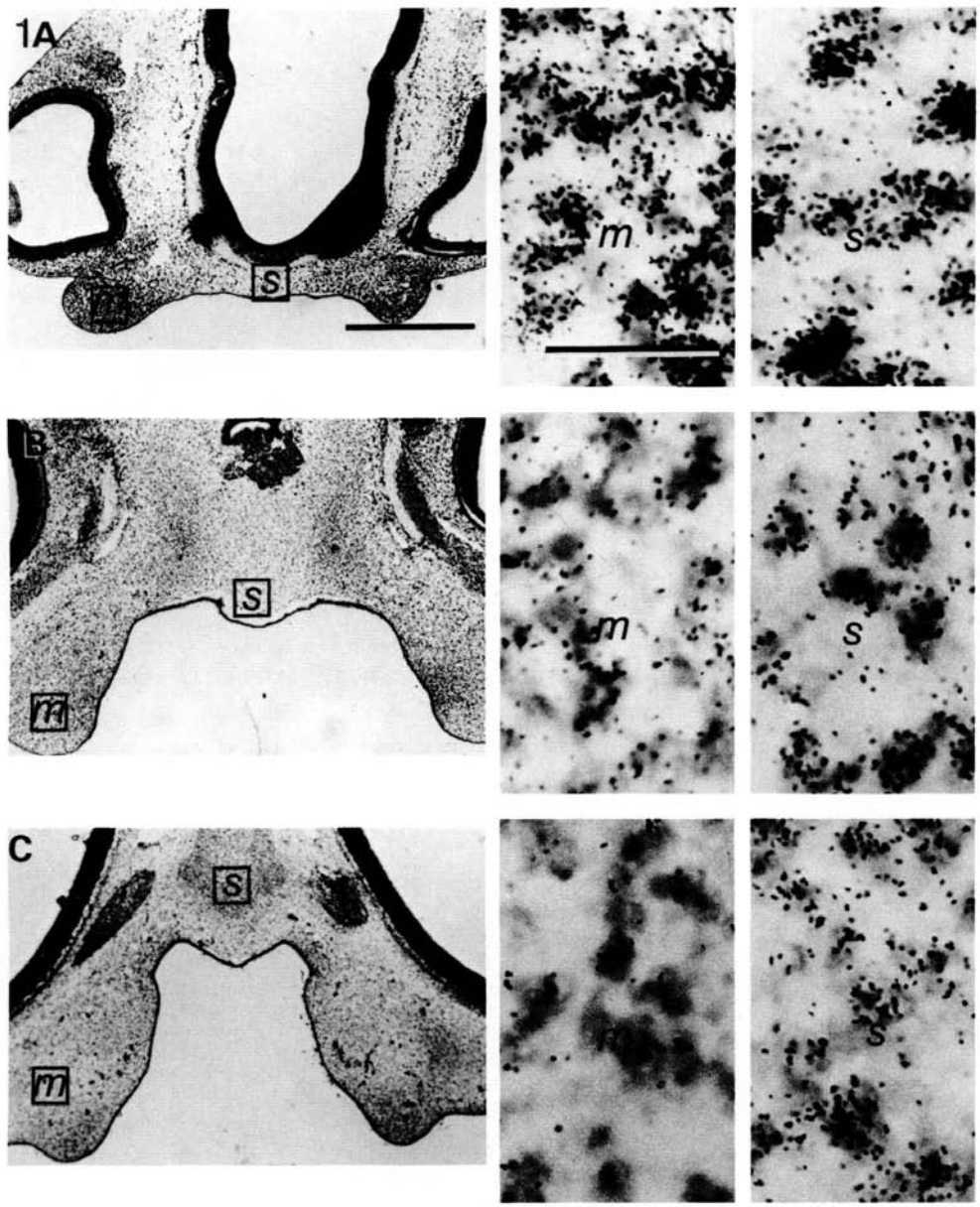


Fig. 1. Representative sections through the maxillary processes (*m*) and the roof of the stomodeum (*s*) of 4-day chick embryos at the end of the labelling period (A), 6-day embryos (B) 48 h after labelling and 7-day embryos (C) 72 h after labelling. Photomicrographs at the right of the sections indicate the intensity of labelling in each of the indicated regions.

9-day embryos, the nasal cartilages continued to maintain higher grain counts relative to adjacent regions. A distinct perichondrium could be observed surrounding the nasal septal cartilage with a label intensity higher than that in the

adjacent cartilage. Little change in the intensity of labelling was noted in the 11-day to 14-day embryos except in the nasal cartilages, which now showed a marked decrease in label intensity.

Quantitative observations

Mean grain counts for the maxillary process and roof of the stomodeum of 4-day embryos were similar (Fig. 2 and 3 and Table 1). In contrast, by 24 h after labelling (5 day) the mean grain counts per cell were consistently greater in the roof of the stomodeum than in the maxillary process and these differences persisted at 48 h (6 day) and 72 h (7 day) (Table 1 and Figs 2, 4, and 5).

The grain counts in the 7-day embryos also revealed differences in label intensity between the chondrogenic and non-chondrogenic regions of the roof of the stomodeum. Grain density declined in the non-chondrogenic regions between 48 and 72 h after labelling. No decrease, however, was seen from 48 h to 72 h in label intensity of cells in the chondrogenic region of the roof of the stomodeum; the label intensity remained at 60–70 % of the original grain count (Table 1 and Figs 2, 5, and 6).

Grain counts were obtained in older embryos (8 day, 9 day, . . . 14 day) over approximately 50 cells in the chondrogenic region of the roof of the stomodeum as well as the perichondrium surrounding the cartilage (Table 1). In the 8-day embryo the mean grain count per cell in the perichondrium remained at approximately the same level as in the chondrogenic regions at 6 days and 7 days; from 9 to 14 days, grain counts in the perichondrium

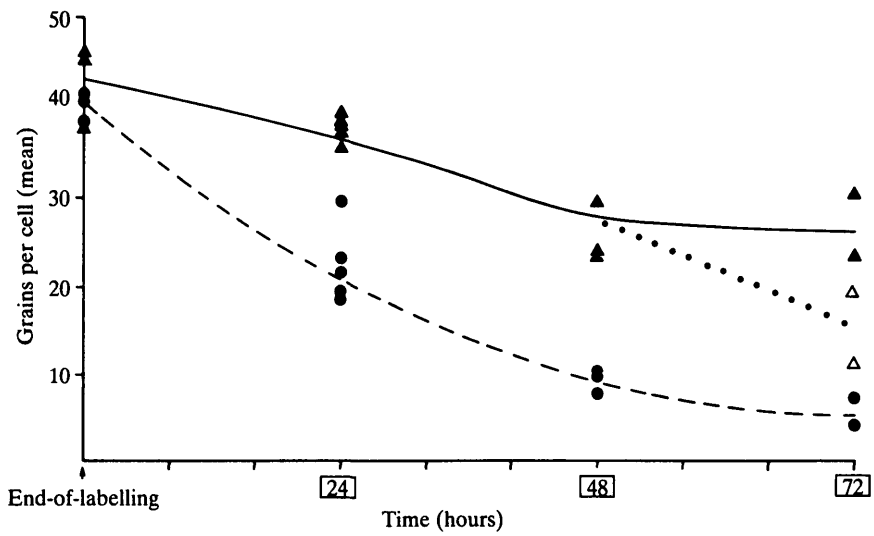


Fig. 2. Grain counts per cell (mean) in the maxillary process (●) and in the roof of the stomodeum (▲) at the end of the labelling period and at 24, 48, and 72 h after labelling. Grain counts per cell in the roof of the stomodeum at 72 h after labelling were further delineated by chondrogenic (▲) and non-chondrogenic regions (△).

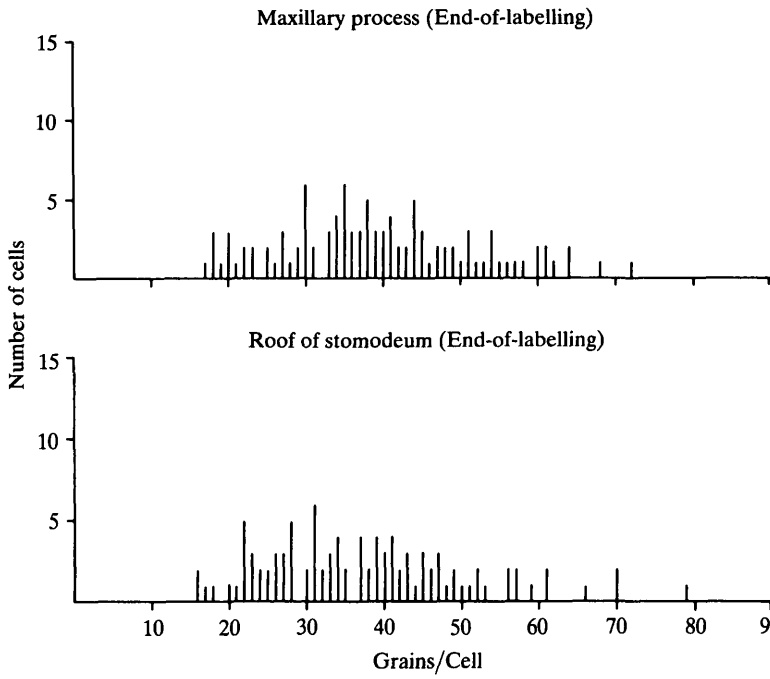


Fig. 3

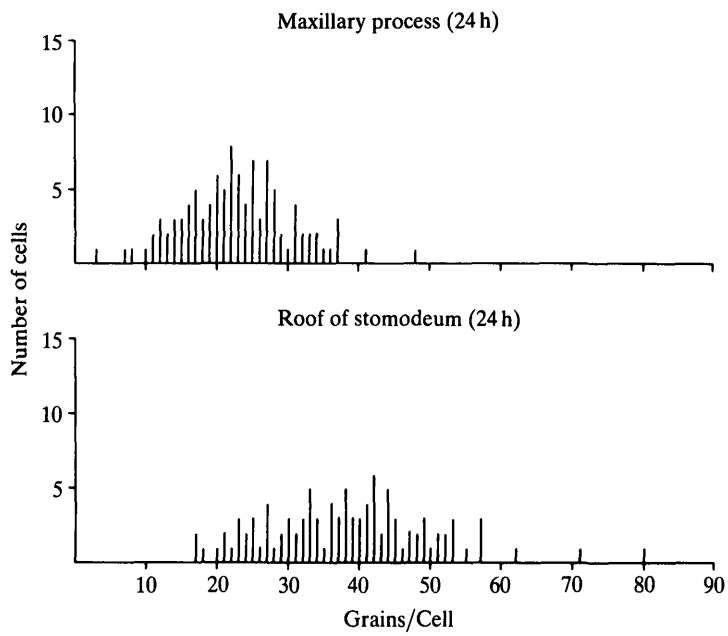


Fig. 4

Figs 3–6. Representative illustrations of the distribution of grain counts per cell in the maxillary process and the roof of the stomodeum of embryos at 4 days at the end of labelling (Fig. 3), at 5 days, 24 h after labelling (Fig. 4), at 6 days, 48 h after labelling (Fig. 5), at 7 days, 72 h after labelling (Fig. 6). Each figure represents data from one embryo.

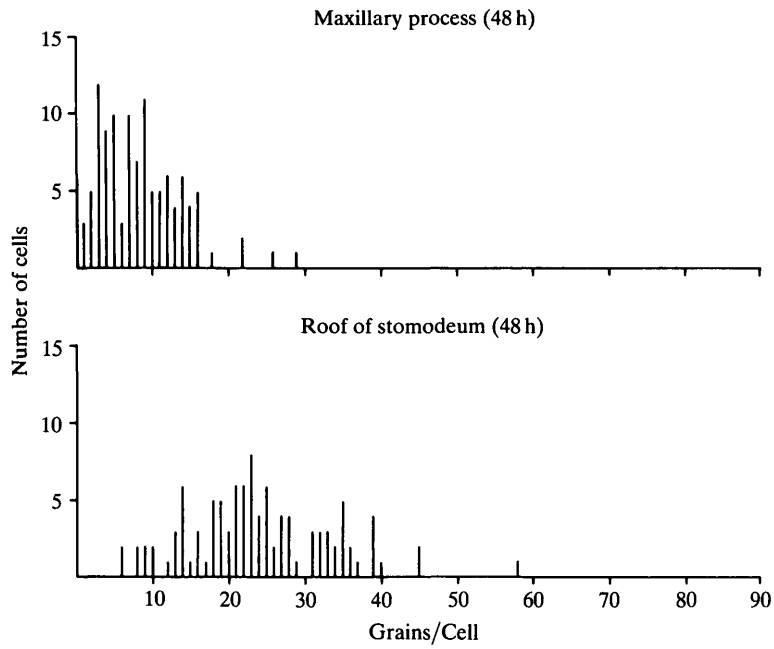

Maxillary process (48 h)
15
10
5
Number of cells
Roof of stomodeum (48 h)
15
10
5
10
20
30
40
50
60
70
80
90
Grains/Cell

Fig. 5

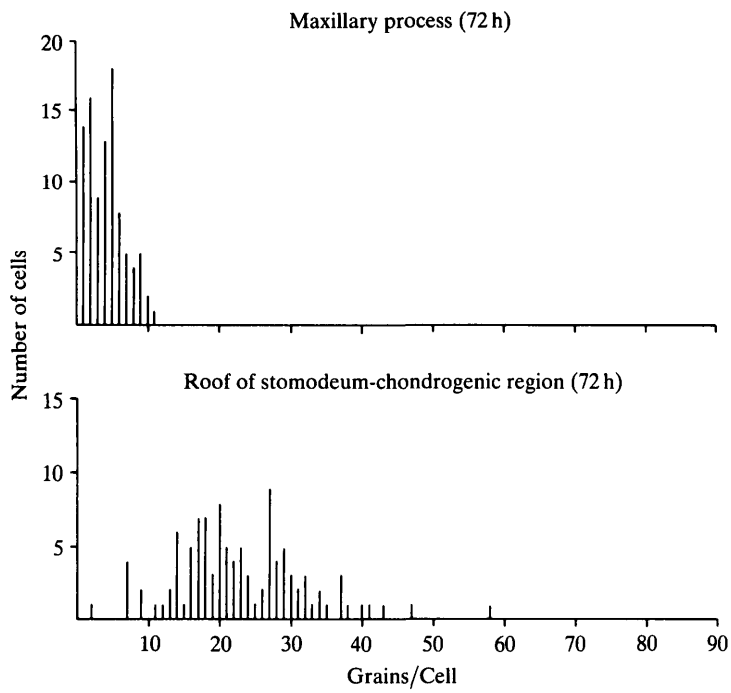

Maxillary process (72 h)
20
15
10
5
Number of cells
Roof of stomodeum-chondrogenic region (72 h)
15
10
5
10
20
30
40
50
60
70
80
90
Grains/Cell

Fig. 6

Table 1. *Mean grain counts per cell in the maxillary process and the roof of the stomodeum (adjusted for background)*

Age of embryo	Time beyond labelling	Mean Grain Counts Per Cell: Maxillary process	Roof of stomodeum		
4 day	End-of-labelling	38·8	38·1		
4 day	End-of-labelling	40·7	45·3		
4 day	End-of-labelling	41·7	45·6		
5 day	24 h	23·3	38·8		
5 day	24 h	29·6	39·3		
5 day	24 h	18·5	36·0		
5 day	24 h	21·8	38·0		
5 day	24 h	19·4	36·8		
6 day	48 h	8·0	23·6		
6 day	48 h	9·5	23·8		
6 day	48 h	9·8	29·4		
			Nonchondrogenic	Chondrogenic	
7 day	72 h	4·2	11·2	23·5	
7 day	72 h	7·1	19·6	30·4	
				Cartilage	Perichondrium
8 day	4 day			19·7	28·2
9 day	5 day			6·7	14·9
10 day	6 day			9·6	20·9
11 day	7 day			7·3	16·0
12 day	8 day			2·0	8·4
13 day	9 day			1·7	4·1
14 day	10 day			0·7	3·3

declined. A decline in grain counts in the cartilage regions also resumed, but at an earlier time, i.e. at 8 days. Grain counts, however, remained higher in the perichondrium than in cartilage throughout the observation period from 8 to 14 days (Table 1).

As a control on the autoradiographic study, mitotic figures were counted on slides stained with Harris' haematoxylin in the chondrogenic regions of the roof of the stomodeum. Few mitotic figures were found in the younger embryos. Means for the 4, 5, 6, and 7-day embryos ranged from 0 to 1·5 mitotic figures per 12 000 μm^2. In the 8-day through 14-day embryos, however, a sharp increase in the number of mitotic figures was observed. The mean for 8 days was 5·0 mitotic figures per 12 000 μm^2 and for 9–14 days from 1·0 to 3·0 mitotic figures per 12 000 μm^2.

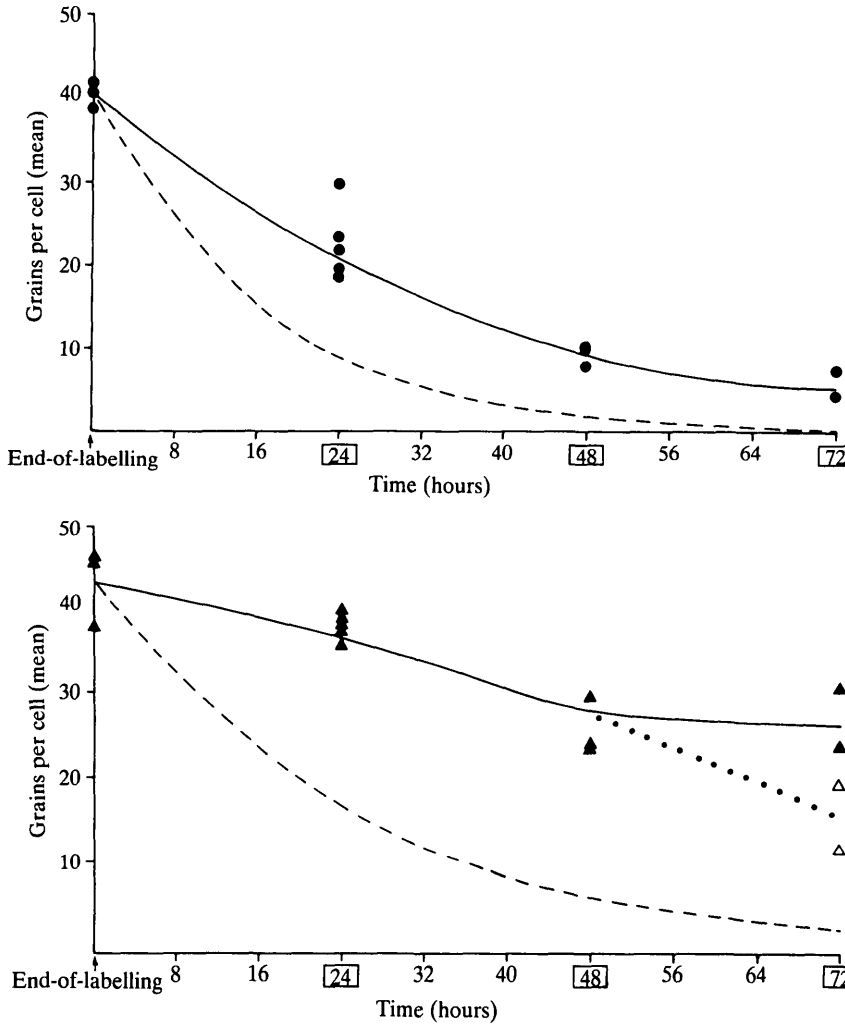
DISCUSSION

Correlation of autoradiograms with Alcian-Blue-stained sections revealed that the cell populations which had been described as slow cycling and/or quiescent ultimately appeared in the chondrogenic regions of the roof of the stomodeum. (Non-chondrogenic regions of the roof of the stomodeum appeared to have labelling levels intermediate between that of the chondrogenic regions and of the mesenchyme of the maxillary process.) Chondrogenic regions at 48 h after labelling retained 60–70 % of the label initially present; more significantly, there was no decrease in labelling intensity in these regions between 48 and 72 h after labelling. In addition, label intensity remained at these levels in the perichondrium for at least another 24 h (between day 8 and 9). These data suggest that a cell cycle block occurs at this time in those cells undergoing chondrogenesis.

Data based on mitotic counts support these observations. Values for the first 72 h after labelling range from 0 to 1·5 mitotic figures/12 000 μm^2 in the chondrogenic region of the roof of the stomodeum. However, a sharp increase in the number of mitoses was noted between 7 and 8 days in the chondrogenic region. Correlated with this was a marked decline in grain counts for these same regions between days 7 and 8. Cell division resumes at this time and, therefore, the cell cycle block appears to be transient. These events appear to be delayed by at least one day in the perichondrium.

Comparison of predicted *versus* observed differences in label retention using previously determined generation times support the proposal of a cell cycle block in chondrogenic cells of the roof of the stomodeum. Assuming consistent generation times in the maxillary process and the roof of the stomodeum of 11 h and 18 h, respectively (Minkoff, 1984), grain counts shown in Figs 7 and 8 would have been predicted from the loss of label due to halving of the grain counts over the cell nuclei with each cell division using the 4-day mean grain count per nucleus of 40 in the maxillary process and 43 in the roof of the stomodeum. As shown in Figs 7 and 8 for both the maxillary process and the roof of the stomodeum, mean grain counts per cell were observed to be greater than those predicted from previously determined cell cycle times. Label loss from the maxillary process however more closely paralleled the predicted values than label loss in the roof of the stomodeum.

The slightly higher grain counts in the maxillary process could be attributed to an increase in mean generation times. It is also possible that the cold thymidine chase may not have adequately diluted the radioactive label permitting additional incorporation beyond the initial labelling period. Label retention observed in the roof of the stomodeum, however, is too exaggerated to attribute to minor cell cycle variations. The discrepancy between the predicted loss of label and the observed loss is more adequately explained by the appearance of subpopulations of cells with greatly extended cell cycle times or of a cell cycle block during the observation period.



Figs 7–8. Comparison between the observed loss of label (solid lines) with that predicted (broken lines) from previously determined generation times of mesenchymal cells of 11 h in the maxillary process (Fig. 7) and of 18 h in the roof of the stomodeum (Fig. 8) (from data of Minkoff, 1984).

A consistent difference was noted between cartilage cells and perichondrial cells in the retention of label during the observation period from 8 days to 14 days. Apparently the cells of the perichondrium maintain longer generation times throughout the development of the septal cartilage. In addition, no decline in grain counts is seen until day 9 and may reflect a more prolonged cell cycle block in the cells of the perichondrium.

The emergence of subpopulations of quiescent cells in the roof of the stomodeum occurs at approximately the time that chondrogenesis begins in the roof of the stomodeum. Cells in this region, therefore, may have undergone a cell cycle block prior to overt differentiation since cartilage formation was noted by

Alcian Blue staining to begin between 24 and 48 h after labelling. Studies currently in progress indicate that sulphated mucopolysaccharides appear in the extracellular matrix of the chondrogenic regions of the roof of the stomodeum between stage $24\frac{1}{2}$ and 25 (Minkoff & Petrone, unpublished observations). From the data of this and previous studies, therefore, it would appear that the cell cycle block occurs prior to expression of the differentiated phenotype, perhaps during commitment of mesenchymal cells to chondrogenesis. (See Minkoff & Kuntz, 1978, p. 69; labelling indices in the roof of the stomodeum reach a minimum at approximately stage 25.)

An association between a decline in labelling and mitotic indices with the formation of chondrogenic regions *in vivo* had been observed in the developing nasal and anterior cranial base cartilages of the rat embryo (Diewert, 1980) and in the developing limb bud of the chick embryo (Janners & Searls, 1970; Ede, Flint & Teague, 1975). Janners & Searls noted that, in the chondrogenic region of the limb bud, labelling indices declined to minimal values 10 h before cells exhibited histologic characteristics or became developmentally stabilized as cartilage.

In vitro studies have also demonstrated that the determination of mesenchymal cells for chondrogenesis may be linked to the cessation of cell division (Flickinger, 1974; Solursh & Reiter, 1975). Employing organ culture of varying sized explants of chondrogenic limb-bud mesenchyme from 4-day-old chick embryos, Flickinger (1974) showed that the differentiation of cartilage appeared to be linked to an early restriction of cell division.

Employing a cell culture system, Solursh & Reiter (1975) demonstrated that when undetermined mesenchymal limb-bud cells were temporarily prevented from passing through the cell cycle, either by maintenance of cells on a Petri dish or through the use of high concentrations of cyclic AMP, cartilage colonies were subsequently formed. Since non-physiological blocking agents did not result in cartilage colony formation, cells appeared to have been blocked at a particular point in the cell cycle.

The coupling of growth arrest and differentiation has also been proposed for adipocytes (Scott, Florine, Wille & Yun, 1982*a*; Scott *et al.* 1982*b*), myoblasts (Nadal-Ginard, 1978), haematopoietic cells (Lajtha *et al.* 1978) as well as other cell types. Scott *et al.* (1982*a*,*b*) have demonstrated that the differentiation of 3T3 proadipocytes was preceded by growth arrest at a point in G_1 which was distinct from other G_1 growth arrest points induced by serum or nutrient deprivation. They have suggested that cell cycle growth control models based upon density-dependent phenomena or growth factor or nutrient deprivation (e.g., Pardee, 1974; Smith & Martin, 1973; Pledger, Stiles, Antoniades & Scher, 1977) may be inadequate to explain *in vivo* physiological regulation of growth, unless the coupling of growth arrest and differentiation is also considered (Scott & Florine, 1982).

Our data, based upon analysis of an *in vivo* system are consistent with this

view. The generation times of mesenchymal cells of the primary palate destined to become chondroblasts lengthened, the distributions of generation times became more heterogeneous, and the growth fraction declined (Minkoff, 1984). Label-dilution studies indicated that the cell cycle of cells within the chondrogenic region of the roof of the stomodeum may have undergone a transient block prior to expression of the chondrogenic phenotype. Whether these data, however, represent arrest at a specific point in the cell cycle, as defined by Pardee, Scott and others, and the arrest is coupled to the process of differentiation, or whether the data represent a gradual uninterrupted traverse of the cell cycle secondary to other differentiative events remains to be determined.

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REFERENCES

DIEWERT, V. M. (1980). Differential changes in cartilage cell proliferation and cell density in the rat craniofacial complex during secondary palate development. *Anat. Rec.* **198**, 219–228.

EDE, D. A., FLINT, O. P. & TEAGUE, P. (1975). Cell proliferation in the developing wing-bud of normal and *talpid*^{3} mutant chick embryos. *J. Embryol. exp. Morph.* **34**, 589–607.

FLICKINGER, R. A. (1974). Muscle and cartilage differentiation in small and large explants from the chick embryo limb bud. *Devl Biol.* **41**, 202–208.

HUMASON, G. L. (1972). *Animal Tissue Techniques*. San Francisco: W. H. Freeman and Company.

JANNERS, M. Y. & SEARLS, R. L. (1970). Changes in rate of cellular proliferation during the differentiation of cartilage and muscle in the mesenchyme of the embryonic chick wing. *Devl Biol.* **23**, 136–165, 1970.

LAJTHA, L. G., LORD, B. I., DEXTER, T. M., WRIGHT, E. G. & ALLEN, T. D. (1978). Interrelationship of differentiation and proliferation control in hemopoietic stem cells. In *Cell Differentiation and Neoplasia* (ed. G. F. Saunders), pp. 179–193. New York: Raven Press.

MINKOFF, R. (1980). Cell proliferation and migration during primary palate development. In *Current Research Trends in Prenatal Craniofacial Development*, (eds R. M. Pratt & R. L. Christiansen), pp. 119–132. New York: Elsevier.

MINKOFF, R. (1982). Cell cycle analysis of mesenchyme during primary palate formation. *J. dent. Res.* **61**, 244.

MINKOFF, R. (1984). Cell cycle analysis of facial mesenchyme in the chick embryo: I. Labeled mitoses and continuous labeling studies. *J. Embryol. exp. Morph.* **81**, 49–59.

MINKOFF, R. & KUNTZ, A. J. (1977). Cell proliferation during morphogenetic change; analysis of frontonasal morphogenesis in the chick embryo employing DNA labeling indices. *J. Embryol. exp. Morph.* **40**, 101–113.

MINKOFF, R. & KUNTZ, A. J. (1978). Cell proliferation and cell density of mesenchyme in the maxillary process and adjacent regions during facial development in the chick embryo. *J. Embryol. exp. Morph.* **46**, 65–74.

NADAL-GINARD, B. (1978). Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* **15**, 855–864.

PARDEE, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proc. natn. Acad. Sci., U.S.A.* **71**, 1286–1290.

PLEDGER, W. J., STILES, C. D., ANTONIADES, H. N. & SCHER, C. D. (1977). Induction of DNA synthesis in BALB/c 3T3 cells by serum components: Reevaluation of the commitment process. *Proc. natn. Acad. Sci., U.S.A.* **74**, 4481–4485.

ROGERS, A. W. (1979). *Techniques of Autogradiography*. Amsterdam: Elsevier Publishing Company.

SAMS, A. & DAVIES, F. M. (1967). Commercial varieties of nuclear fast red; their behavior in staining after autoradiography. *Stain Techn.* **42**, 269–276.

SCOTT, R. E. & FLORINE, D. L. (1982). Cell cycle models for the aberrant coupling of growth arrest and differentiation in hyperplasia, metaplasia and neoplasia. *Am. J. Path.* **107**, 342–348.

SCOTT, R. E., FLORINE, D. L., WILLE, J. J., JR. & YUN, K. (1982*a*). Coupling of growth arrest and differentiation at a distinct state in the G_1 phase of the cell cycle: G_D. *Proc. natn. Acad. Sci., U.S.A.* **79**, 845–849.

SCOTT, R. E., HOERL, B. J., WILLE, J. J., JR., FLORINE, D. L., KRAWISZ, B. R. & YUN, K. (1982*b*). Coupling of proadipocyte growth arrest and differentiation. II. A cell cycle model for the physiological control of cell proliferation. *J. Cell Biol.* **94**, 400–405.

SMITH, J. A. & MARTIN, L. (1973). Do cells cycle? *Proc. natn. Acad. Sci., U.S.A.* **70**, 1263–1267.

SOLURSH, M. & REITER, R. S. (1975). Determination of limb bud chondrocytes during a transient block of the cell cycle. *Cell Differentiation* **4**, 131–137.

YURKEWICZ, L., LAUDER, J. M., MARCHI, M. & GIACOBINI, E. (1981). ^{3}H-thymidine long survival autoradiography as a method for dating the time of neuronal origin in the chick embryo: The locus coeruleus and cerebellar purkinje cells. *J. Comparative Neurol.* **203**, 257–267.

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