

## Epithelial Cell Death and Cyclic AMP Increase During Palatal Development

(terminal differentiation/DNA/glycoprotein)

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**ABSTRACT** Cyclic AMP levels increase abruptly in the secondary palatal shelf (mesenchyme plus a two- to three-cell-layered epithelium) between day 15 and 16 of gestation in the rat embryo. The addition of dibutyryl cyclic AMP to the media in which immature palatal shelves (day 14) are cultured causes a precocious decrease in DNA synthesis, but increased glycoprotein synthesis and epithelial adhesiveness. These changes are most evident in the medial-edge epithelial cells and resemble the developmental events that occur normally on day 15 and 16 during formation of the secondary palate. The initiation of epithelial cell death and adhesiveness in the medial edge of the palatal shelf may be mediated through an increase in cyclic AMP.

By the fifteenth day of development, the palatal shelves of the rat have grown from the maxillary processes and lie in a vertical position alongside the tongue. The palatal shelves, composed primarily of mesenchyme, but surrounded by an epithelium, undergo a marked alteration in location and shape as they rotate to a horizontal position above the tongue. During this same period, the presumptive adhering surfaces (medial-edge epithelium) acquire a glycoprotein coat (1, 2), cease DNA synthesis (3, 4), and begin autolysis in a manner similar to the programmed cell death observed in the formation of other structures, such as the digits and wing buds (5-7). Finally, the opposing medial-edge epithelial surfaces adhere, fuse, and form a continuous mesenchymal layer as intervening basement membrane and epithelial elements disappear.

At least some of the events referred to above can occur when competent shelves are cultured *in vitro* on various substrates (8, 9). Although rotation does not occur when isolated shelves are cultured, surface glycoproteins appear (1, 2), medial epithelial cells cease division and begin autolysis (9); those portions of the shelves in apposition adhere and fuse in a manner remarkably similar to the *in vivo* events. The rapidity and completeness with which isolated palates undergo these steps depend on the maturity of the donor embryo (8). Rat palatal shelves removed on day 15 and 16 are more competent for fusion than those removed on day 14.

While the signal that initiates these developmental changes *in vivo* in the palatal shelves is as yet unknown, we show in this study that tissue levels of cyclic AMP undergo a marked increase during this important period. Further, the addition of dibutyryl cAMP (Bt<sub>2</sub>cAMP) to cultured palates accelerates such developmental events as glycoprotein synthesis, adhesion, and the programmed cell death of medial epithelial

cells. These results suggest that the increase in cAMP levels plays a role in activating the developmental program in immature palatal shelves.

### MATERIALS AND METHODS

Mature female Sprague-Dawley rats, weighing approximately 250 g, were caged overnight with fertile males. Gestation was estimated to begin on the demonstration of sperm in vaginal smears taken the following morning, which was designated as day zero.

Pregnant animals were anesthetized with chloroform on either day 14, 15, or 16 of gestation, and the embryos (including placenta and membranes) were placed in phosphate-buffered saline at 4°. The palatal shelves were subsequently dissected, trimmed, and either completely submerged in 1 ml of growth medium (Eagle's basal plus 10% fetal calf serum) or placed nasal surface down on an ultra-thin Millipore filter in an organ culture dish with the same medium (9, 10). The palatal shelves were cultured for specified times in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°.

The synthesis of DNA or glycoproteins by the palates *in vitro* was determined by following the incorporation into palatal shelves of labeled precursors which were added to the culture medium for specified times. [<sup>3</sup>H]Thymidine (57.2 Ci/mmol), [<sup>3</sup>H]glucosamine (7.3 Ci/mmol) and [<sup>3</sup>H]fucose



FIG. 1. Levels of cAMP present in palatal shelves at various times during development. Values are the mean of four determinations  $\pm$  SEM. Each determination represents six palatal shelves pooled from three littermates at the gestational age indicated. Gestational age is given in days and hours between day 14 (+16 hr) and day 16 (+8 hr). Midnight preceding a positive vaginal smear was taken as zero hour.

Abbreviations: cAMP, adenosine 3':5'-cyclic monophosphate; Bt<sub>2</sub>cAMP, N<sup>6</sup>,O<sup>2'</sup>-dibutyryl adenosine cyclic monophosphate.

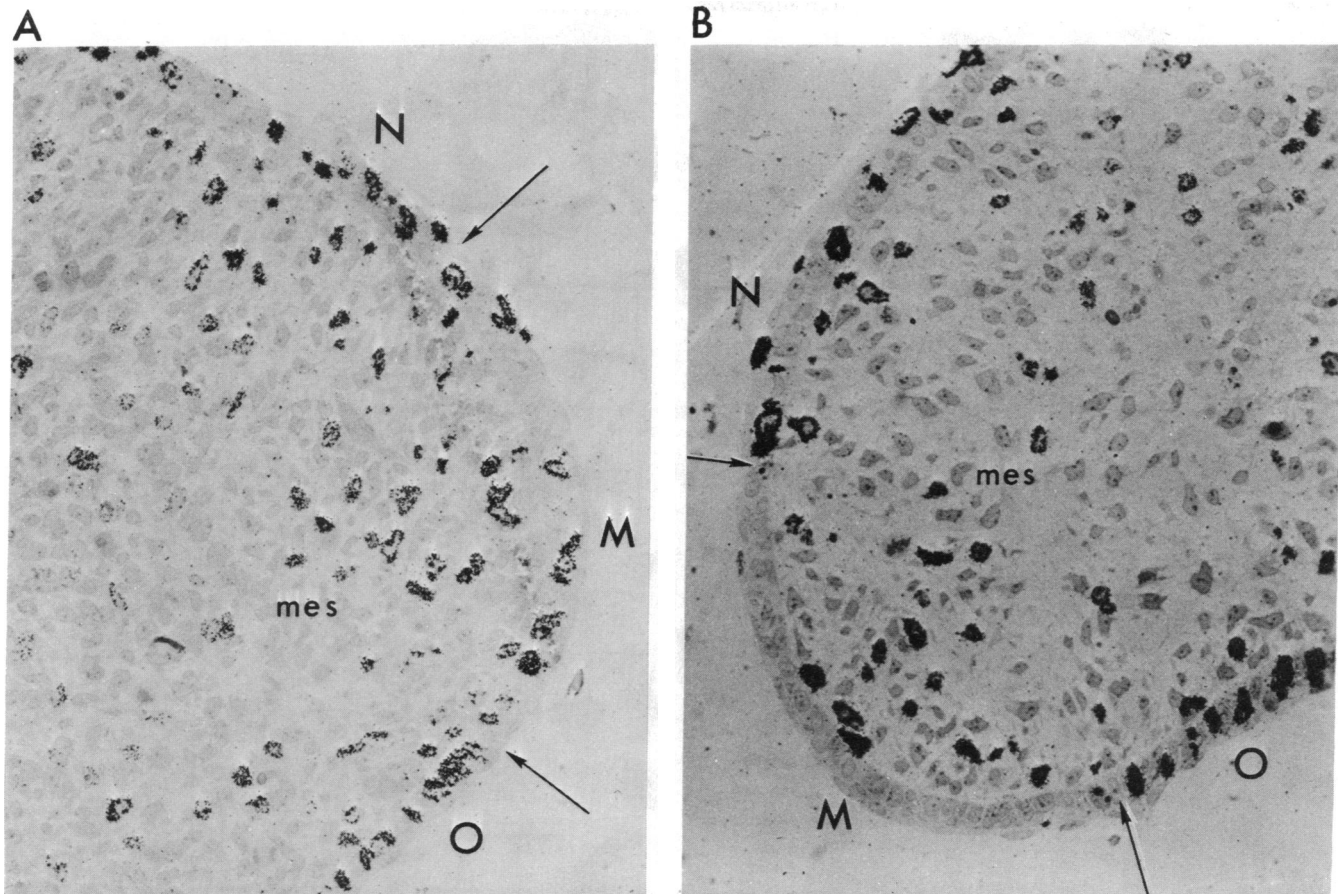


FIG. 2. Autoradiograms of palatal shelves cultured for 4 hr submerged in growth medium containing [ $^3\text{H}$ ]thymidine at  $40 \mu\text{Ci/ml}$ . (A) Day 14 (+12 hr) ( $\times 350$ ) and (B) Day 15 (+12 hr) ( $\times 350$ ). The various areas of the epithelium are: M, medial edge; O, oral and N, nasal epithelium; mes, mesenchyme.

(13.4 Ci/mmol) were purchased from the New England Nuclear Corp., Boston, Mass. Labeled shelves were rinsed in the buffered saline and placed in 5% trichloroacetic acid, followed by homogenization, centrifugation, and resuspension of the pellet in 5% trichloroacetic acid at  $4^\circ$ . After recentrifugation, the radioactivity in the acid-soluble and insoluble fractions was determined by standard methods of liquid scintillation spectrometry.

Some labeled shelves were fixed and embedded in paraffin or plastic, and sections cut at  $8 \mu\text{m}$  or  $1 \mu\text{m}$  were processed for autoradiography by standard methods. In some experiments,  $N^6, O^{2'}$ -dibutyryl adenosine  $3':5'$ -cyclic monophosphate was added to the media with or without theophylline (Sigma). The ether-extracted  $\text{Bt}_2\text{cAMP}$  was the gift of Dr. G. Johnson, National Cancer Institute.

Prior to cAMP assay, excised shelves were placed in 5% trichloroacetic acid at  $4^\circ$ , homogenized and centrifuged. The protein content of the insoluble fraction was assayed (11), and after ether extraction and lyophilization of the soluble fraction, cAMP levels were assayed by the method of Gilman (12).

## RESULTS

### cAMP, adhesiveness, and glycoprotein synthesis

Preliminary experiments established that the level of cAMP increased substantially in embryonic palatal shelves from day 15 to 16 (Fig. 1). A 2-fold increase was observed between day

15 (+16 hr) and day 16 (+8 hr). During this same period, the levels of cAMP in the forepaw region remained constant at approximately 20 pmol/mg of protein. In the rat, fusion of the shelves begins at day 16 (+8 hr). In part, this increase in cAMP levels coincides with decreased mitotic activity observed in the mesenchyme (13), but it seemed possible that cAMP levels may have increased in the epithelial cells at day 14 but could not be detected in analyses made on the whole tissue. For this reason, we studied the action of  $\text{Bt}_2\text{cAMP}$  on immature (day 14) palates during culture; three processes, adhesion, glycoprotein synthesis, and DNA synthesis, were monitored.

We and others have noted that shelves cultured with their medial surfaces in apposition will adhere, presumably as an early step in fusion. However, when immature palates (day 14) are cultured, no adhesion is noticed over a 48 hr period and the epithelial surfaces of the shelves pull apart due to dehydration shrinkage during routine processing for histology (8). In contrast to this, in preliminary experiments approximately half of the day 14 palates cultured with  $\text{Bt}_2\text{cAMP}$  were found to adhere after 48 hr in culture.

Recent studies have indicated that the ability to adhere is associated with the production of glycoproteins on the surface of epithelial cells destined for extinction in the areas that are to undergo fusion (1, 2). For this reason, we measured the incorporation of two glycoprotein precursors, [ $^3\text{H}$ ]fucose and [ $^3\text{H}$ ]glucosamine, in shelves incubated with or without  $\text{Bt}_2\text{cAMP}$ .

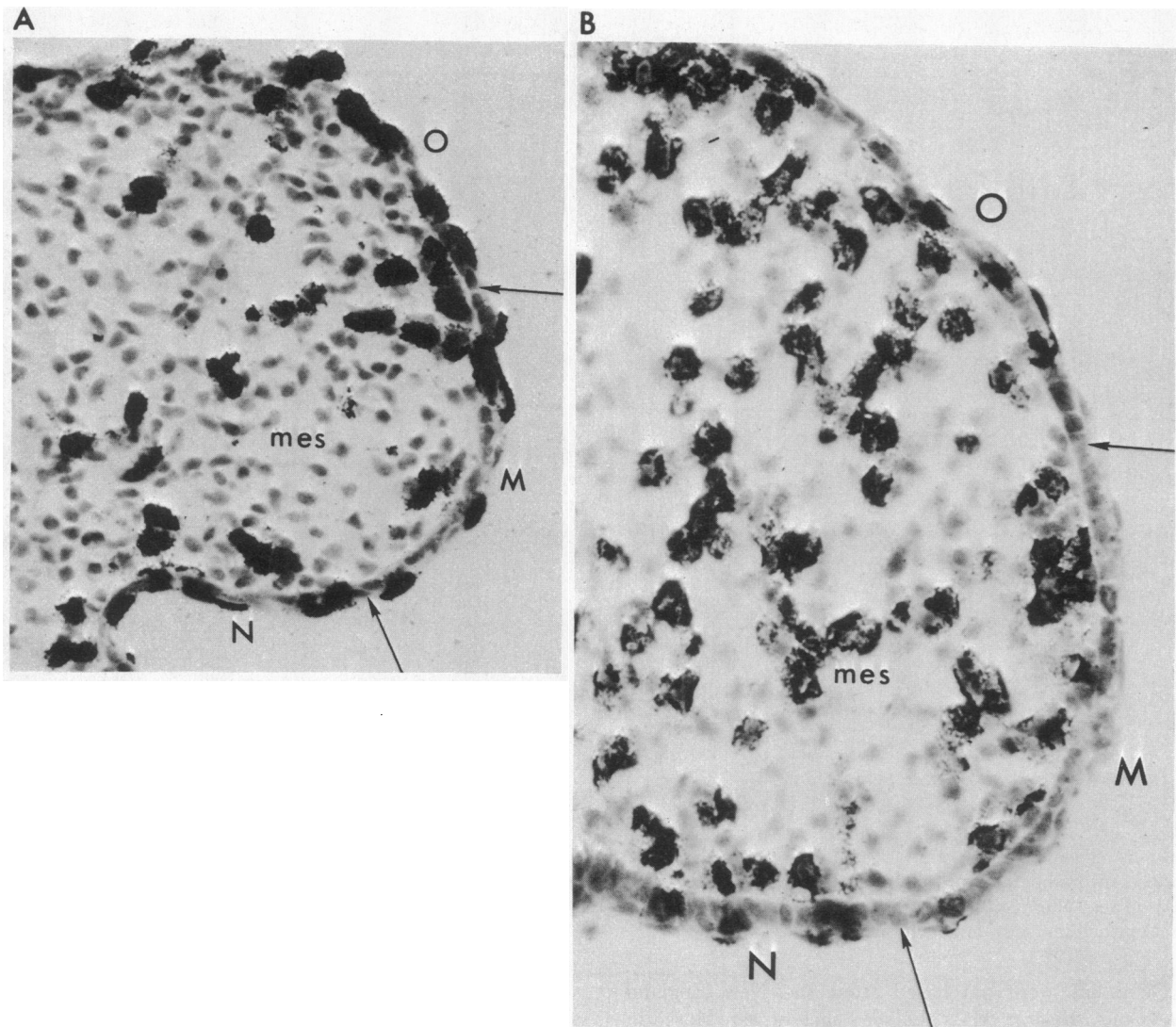


FIG. 3. Autoradiograms of day 14 (+12 hr) palatal shelves cultured on Millipore filters (see text) for 18 hr with or without  $Bt_2cAMP$ . The shelves were then submerged in culture medium containing [ $^3H$ ]thymidine at  $20 \mu Ci/ml$  for a 4 hr pulse. (A) Day 14 (+12 hr) control shelf ( $\times 460$ ) and (B) day 14 (+12 hr) shelf incubated with  $Bt_2cAMP$  at  $0.6 mM$  ( $\times 550$ ). The various areas of the epithelium are: M, medial edge; O, oral and N, nasal epithelium; mes, mesenchyme.

cAMP. Shelves incubated with  $Bt_2cAMP$  incorporated significantly more [ $^3H$ ]fucose or [ $^3H$ ]glucosamine into acid-precipitable macromolecules than do control shelves (Table 1). No differences in the uptake of [ $^3H$ ]fucose or [ $^3H$ ]glucosamine were noted, suggesting that the differences with  $Bt_2cAMP$  were related to synthesis of macromolecules. These differences may be related to the changes in shelf adhesiveness noted earlier and the increased synthesis of glycoprotein by medial epithelial cells (1, 2). Preliminary experiments suggest that the incorporation of labeled glucosamine and fucose in the presence of cyclic AMP is particularly enhanced in the palatal epithelium.

#### DNA synthesis

DNA synthesis was monitored by measuring the incorporation of [ $^3H$ ]thymidine into trichloroacetic acid-insoluble macromolecules and by autoradiography following the incubation of palatal shelves with [ $^3H$ ]thymidine. No label was incorporated into medial-edge epithelial cells in day 15 (+12 hr) shelves, whereas the label was generally distributed through-

out the mesenchyme as well as the nasal and oral epithelial cells (Fig. 2B). Cells on all surfaces as well as mesenchyme were labeled when immature palates (day 14) were incubated under the same conditions (Fig. 2A). These studies confirm those of earlier workers in that the cells in the medial epithelial surface cease DNA synthesis between days 15 and 16 (3, 4).

The effect of  $Bt_2cAMP$  on DNA synthesis was studied in order to determine the response of embryonic palatal cells to elevated levels of cAMP. When shelves were incubated with  $Bt_2cAMP$ , DNA synthesis was depressed approximately 30%, and addition of theophylline did not alter this inhibition. These studies suggest that only a portion of DNA synthesis in the shelves is sensitive to added  $Bt_2cAMP$ . Autoradiographic studies suggested that the major effect of  $Bt_2cAMP$  on DNA synthesis was on the cells in the medial edge (Fig. 3B). Incubation with  $Bt_2cAMP$  specifically inhibited the incorporation of [ $^3H$ ]thymidine into the medial epithelial cells without any obvious effect on the epithelial cells comprising the nasal or oral surfaces or on the cells in the mesenchyme. The extent of repression of DNA synthesis on day 14 with  $Bt_2cAMP$  is

TABLE 1. Effect of  $Bt_2cAMP$  on palatal macromolecular synthesis *in vitro*

	Incorporation (cpm/mg of protein)		% Change acid- insoluble
	Acid- soluble	Acid- insoluble	
<i>Day 14</i>			
[ <sup>3</sup> H]Thymidine (40 $\mu$ Ci/ml)			
Control	683 $\pm$ 66	3980 $\pm$ 244	—
$Bt_2cAMP$	612 $\pm$ 24	2862 $\pm$ 182	-28%
$Bt_2cAMP$ + theophylline	648 $\pm$ 25	2800 $\pm$ 227	-29%
<i>Day 15</i>			
[ <sup>3</sup> H]Thymidine (20 $\mu$ Ci/ml)			
Control	135 $\pm$ 10	913 $\pm$ 70	—
$Bt_2cAMP$	139 $\pm$ 8	769 $\pm$ 35	-16%
[ <sup>3</sup> H]Fucose (40 $\mu$ Ci/ml)			
Control	137 $\pm$ 10	22 $\pm$ 2	—
$Bt_2cAMP$	136 $\pm$ 10	30 $\pm$ 1	+27%
[ <sup>3</sup> H]Glucosamine (20 $\mu$ Ci/ml)			
Control	387 $\pm$ 22	109 $\pm$ 8	—
$Bt_2cAMP$	385 $\pm$ 18	131 $\pm$ 7	+16%

Palatal shelves were excised at the indicated times and incubated submerged with or without 0.6 mM  $Bt_2cAMP$  and 1 mM theophylline for 1 hr at 37° (see *text*). Radioactive precursors were then added and the shelves were incubated for a further 3 hr, rinsed in phosphate-buffered saline, and processed as described in the *text*. Values are given as the mean of three determinations  $\pm$  SEM.

similar to the pattern obtained when day 15 (+12 hr) shelves were incubated alone (Fig. 2B).

### DISCUSSION

Formation of the secondary palate during development requires the precise execution of a series of morphological changes that bring the medial-edge epithelia of the palatal shelves into contact. During this same period, certain biochemical changes in the epithelium are required in order for the contacting shelves to adhere and midline epithelium to autolyze. These biochemical changes include a cessation in DNA synthesis with a concomitant increase in glycoprotein synthesis and epithelial adhesiveness. Cessation of DNA synthesis prior to epithelial contact is restricted to the presumptive adhesion surface (medial edge) and not the adjacent oral and nasal epithelium. Since these medial-edge epithelial cells eventually die (day 17), the cessation of DNA synthesis on day 15 is the first indication of epithelial cell death. This cell death cannot be explained on the basis of nutritional deficiencies, since the underlying mesenchymal cells are well nourished and actively dividing.

On day 15 of gestation, the medial-edge epithelial cells rapidly cease their mitotic activity. In the present study, the levels of cAMP determined in the whole palatal shelf begin to increase on day 15 but remain constant in the embryonic forepaw during this time. It was not possible to determine the distribution of cAMP between the mesenchyme and epi-

thelium using the techniques employed in this study. Perhaps an increase in the level of cAMP occurs even earlier in the epithelial cells, but was not detected in analyses of the whole palatal shelf. Recently (19), it has been shown that cAMP levels increase dramatically *in vitro* 5–6 hr prior to the initiation of myoblast fusion and cessation of DNA synthesis.

It was also shown in the present study that  $Bt_2cAMP$  can precociously induce a decreased DNA synthesis, as well as increased glycoprotein synthesis in the immature day 14 palatal shelf *in vitro*. This suggests that increased levels of cAMP in the medial-edge epithelium may mediate epithelial cell death. Many studies have shown that the levels of cAMP are inversely related to the rate of cell division, with high levels present in nondividing cells (14–16). Activation of cell death in the palatal shelves could be brought about by the production of a substance which in turn brings about elevation of cAMP levels. This substance most likely would be of mesenchymal origin, since Pourtois (8) has shown that an epithelial-mesenchymal interaction is necessary for palatal fusion. Here we would postulate that the sensitive cells (medial-edge epithelium) contain a receptor protein for cAMP, whereas resistant cells (oral and nasal epithelium as well as mesenchyme) lack this receptor. Daniels *et al.* (17) have presented evidence for such a difference to explain the resistance of certain lymphocytes to the normally lethal action of cAMP on lymphocytes. Increased levels of cAMP have also been implicated in the programmed death of certain plant cells (18). It is possible that the elevation of cAMP levels is a common event associated with the elimination of a variety of cells during development.

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