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IDENTIFICATION OF ADENYLATE CYCLASE-COUPLED β-ADRENERGIC RECEPTORS IN THE DEVELOPING MAMMALIAN PALATE

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A direct radioligand binding technique utilizing a β-adrenergic antagonist [^{3}H]Dihydroalprenolol ([^{3}H]DHA) was employed in the identification and characterization of fetal palatal β-adrenergic receptors. [^{3}H]DHA binding was saturable (Bmax 16 fmol/mg protein) with high affinity and an apparent equilibrium dissociation constant (K_D) of 1.5 nM. Binding of [^{3}H]DHA was displaced by the competitive β-adrenergic antagonist propranolol in a concentration-dependent manner. Dissociation kinetic studies demonstrated almost complete reversibility of radioligand binding within 60 min. The functionality of these β-adrenergic receptors was demonstrated by showing that fetal palatal mesenchymal cells responded to catecholamine agonists with dose-dependent accumulations of intracellular cAMP. This effect could be entirely blocked by the β-antagonist, propranolol. The relative potency order of catecholamines in eliciting an elevation of cellular cAMP was characteristic of a β_2-adrenergic receptor-mediated response: (-) isoproterenol > (-) epinephrine > (-) norepinephrine. In addition, this response was found to be stereospecific with (-) isoproterenol being significantly more potent than (+) isoproterenol. Both the [^{3}H]DHA binding characteristics and the catecholamine sensitivity of fetal palatal tissue support the presence of adenylate cyclase-coupled β-adrenergic receptors in the developing mammalian secondary palate.

Cleft palate, one of the most prevalent birth defects, occurs with a frequency of 1 in 600 live births in the United States (1). Etiologic investigations of palatal clefting suggest that no simple causative mechanism exists (2). Rather, such congenital malformations are usually multifactorial in origin, resulting from an interaction between genetic and environmental factors (3). Normal development of the palate depends upon a complex series of morphogenetic movements and a precise ontogenetic sequence of biochemical events, such that an alteration in any one of these integrated processes could lead to a structural abnormality. Meaningful investigations of the pathogenesis of cleft palate therefore necessitate a thorough understanding of normal palate development at the morphologic and biochemical levels. While the exact nature of the control mechanisms operative in palate morphogenesis is still

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largely unknown, a variety of hormones, hormone-like substances, and growth factors have been implicated as modulators of normal development (4, 5). Evidence currently suggests that adenosine 3':5'-monophosphate (cAMP), a known mediator of hormonal effects, may play a role in normal development of the palate (4). This study deals with the identification and characterization of a β-adrenergic receptor system which may be involved in modulation of cyclic AMP levels and hormonal responsiveness during palatal growth and differentiation.

Development of the mammalian secondary palate proceeds via a series of morphogenetic movements whereby bilateral palatal processes, derived from the maxillary mesenchyme, reorientate from a vertical position alongside the tongue to a horizontal position above the tongue. Each process consists of mesenchymal cells embedded in an extracellular matrix enclosed within a several cell-layered epithelium. Reorientation of the palatal processes brings the apposing medial edge epithelia into contact, and formation of the definitive secondary palate is accomplished following epithelial adherence, degeneration and union of the mesenchyme from adjacent palatal processes (6).

Published descriptions of the morphology of palatal development have been legion. Investigations of the biochemical regulatory mechanisms operative during palatogenesis have been far less extensive. Recent investigations into the nature of these control mechanisms indicate that normal development of the palate may depend upon the presence and interaction of a variety of hormones, growth factors, prostaglandins and cyclic nucleotides (4, 5). Cyclic AMP's role as a mediator of cellular differentiation has been well documented in a variety of developing tissues (7-10). Evidence suggests that cAMP may act in a similar capacity during growth and differentiation of the secondary palate (4). One of the several hormonal regulators of palatal cAMP levels under investigation is catecholamines which have been implicated as modulators of development in other systems (11-13).

Intracellular cAMP levels are controlled in part by hormone binding to specific receptors and the subsequent activation of adenylate cyclase. Catecholamine-sensitive adenylate cyclase activity has been demonstrated in the

developing palate in vivo with maximal enzyme activity during the period of palatal cellular differentiation (14, 15). Moreover, fetal palatal mesenchymal cells in vitro respond to the potent β-adrenergic agonist, isoproterenol, with a dose-dependent elevation of cAMP (16). These findings suggest the presence of a β-adrenergic receptor system in the developing palate where such receptors have not been previously demonstrated. We report here the existence of β-adrenergic receptors in fetal palatal tissue and demonstate its responsiveness to a variety of catecholamines.

MATERIALS AND METHODS

β-Adrenergic Receptor Binding Assay

A direct radioligand binding method, utilizing tritiated dihydroalprenolol ([^{3}H]DHA, New England Nuclear), was employed in the identification of binding sites (18). [^{3}H]DHA, a β-adrenergic antagonist with substantial specific radioactivity (90 Ci/mmol), binds receptor sites with high affinity and biological specificity. This method has been successfully employed in the characterization of β-adrenergic receptors in a variety of tissues (19). [^{3}H]DHA binding was routinely determined in crude homogenates of day 13 fetal mouse palatal tissue. Day 13 of gestation was chosen since it is during the period of palate epithelial and mesenchymal differentiation.

Pregnant female mice (day 13 of gestation) were killed by cervical dislocation. Fetuses were dissected from uteri in phosphate buffered saline and the extraembryonic membranes removed. Palatal processes, with some adjoining maxillary mesenchyme, were dissected from the fetuses, pooled in assay buffer (50 mM TRIS-HCl, pH 8.0) and a cell-free crude homogenate was formed. [^{3}H]DHA binding was determined by incubation of 100 ul of homogenate (50 ug protein) with varied concentrations of [^{3}H]DHA in a total volume of 200 ul for 10 min at 37°C, unless otherwise noted in figure legends. In order to separate bound from free [^{3}H]DHA, the binding reaction was terminated by the rapid addition of 3 ml cold buffer and filtration under suction through Whatman GF/C filters. The filters were subsequently washed with 15 ml buffer, transferred to vials containing scintillation fluid and the amount of bound radioactivity on each filter determined by scintillation spectrometry. Specific binding of radioligand is generally defined as total binding minus nonspecific binding. Nonspecific binding of [^{3}]DHA was determined by parallel incubations in the presence of 5×10^{-7} M (+) propranolol.

Tissue Culture

Mature male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were housed together overnight and the presence of a vaginal plug the following morning (day 0 of gestation) was considered evidence of mating. Pregnant females (day 13 of gestation) were killed by cervical dislocation. Fetuses were dissected from uteri in sterile calcium-magnesium-free phosphate buffered saline (CMF-PBS). Extraembryonic membranes were removed and fetuses rinsed in CMF-PBS. Maxillary processes were dissected from the fetuses, pooled and minced. Tissue was dissociated with 0.25% trypsin (GIBCO 1.250), 0.1% EDTA in CMF-PBS for 10 min at 37°C with constant agitation. Trypsin was inhibited by addition of cold (4°C) Eagle's minimum essential medium (MEM)

containing 10% fetal bovine serum (FBS). Primary cultures were established by seeding cells into 35 mm tissue culture dishes (Falcon) at a density of 2.5×10^4 cells/cm^2. Cells were grown in MEM with Earle's salts and 25 mM HEPES buffer, supplemented with 300 mM glutamine, fetal bovine serum (10%), streptomycin (200 ug/ml) and penicillin (200 units/ml). Cultures were maintained at 37°C in an atmosphere of 5% CO_2/95% air.

Catecholamine Stimulation and Cyclic AMP Determination

Primary cultures of fetal palatal mesenchymal cells were grown to subconfluency prior to stimulation with a variety of catecholamines. Serum-containing medium was removed from the cultures and the cells were rinsed twice with MEM. Cells were then incubated for 5 min at 37°C in serum-free medium containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2 mM) and individual catecholamines at a final concentration of 1×10^{-7}M to 1×10^{-3}M. Following removal of the catecholamine-containing medium, the cell monolayers were scraped from the dish and sonicated in cold 10% trichloroacetic acid (TCA). The acid-soluble fraction was extracted with water-saturated ether, lyophilized to dryness, resuspended in acetate buffer (50 mM, pH 6.2), and levels of cyclic AMP determined by radioimmunoassay ([^{125}I] cAMP RIA Kit, New England Nuclear). The acid-insoluble fraction was hydrolyzed in 10% TCA for 20 min at 90°C, centrifuged and the DNA content of supernatants determined spectrofluorometrically (17).

RESULTS

A number of criteria must be satisfied to demonstrate that radioligand binding represents binding to true physiological receptors. Such criteria include saturability of binding sites, high affinity, reversible ligand binding, pharmacologic and sterospecificity and biological potency. Specific binding of [^{3}H]DHA to palatal tissue homogenates was saturable at approximately 5 nM indicating a finite number of specific binding sites (Fig. 1). By contrast, nonspecific binding was nonsaturable and increased linearly with increasing [^{3}H]DHA concentrations. Scatchard analysis (20) of this saturation curve (Fig. 1, inset) yields an apparent equilibrium dissociation constant (K_D) of 1.5 nM indicative of high affinity binding sites. Binding site density was estimated at 16.0 fmol/mg protein. Saturability of binding was confirmed in a competition binding assay (Fig. 2). The β-adrenergic antagonist propranolol effectively competed with [^{3}H]DHA for occupancy of a limited number of binding sites, as evidenced by a dose-dependent decrease in specifically bound radioligand. The biphasic nature of this curve is suggestive of competition for palatal tissue binding sites of differing affinities. This is supported by binding studies over a wider range of [^{3}H]DHA concentrations (0-140 nM) which demonstrate a

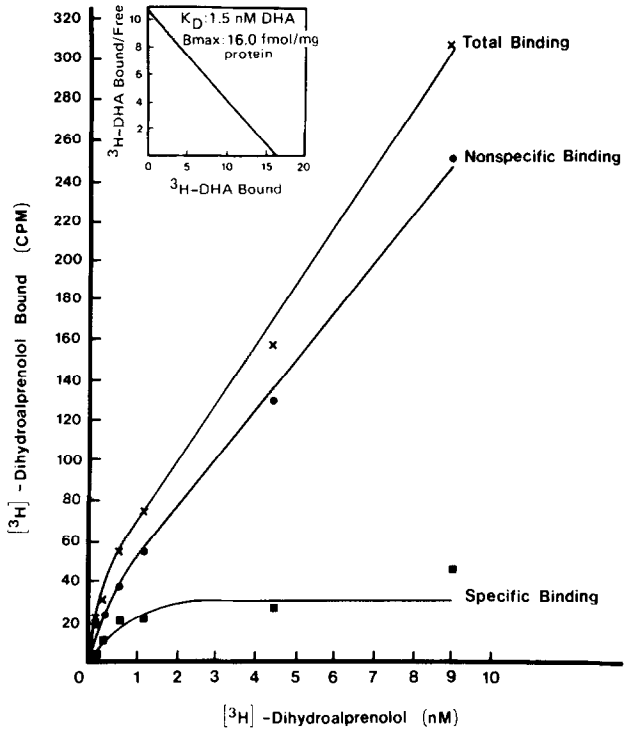

Fig. 1 (-) [^{3}H]Dihydroalprenolol binding to day 13 fetal mouse palatal tissue homogenates as a function of [^{3}H]DHA concentration. Aliquots of homogenate (50 ug protein/100 ul) were incubated with increasing concentrations of [^{3}H]DHA (0-10 nM) in the absence or presence of 0.5 uM (+) propranolol for 10 min at 37°. Bound and free [^{3}H]DHA were separated by vacuum filtration over glass-fiber filters. Specific binding was determined from the difference between total binding (in absence of propranolol) and nonspecific binding (in presence of propranolol). Scatchard analysis of saturation binding data yielded a Bmax (maximum number of [^{3}H]DHA binding sites) of 16.0 fmol/mg protein and a K_D (equilibrium binding constant) of 1.5 nM [^{3}H]DHA.

second region of saturability indicating a second class of moderate affinity (K_D 20.8 nM) binding sites (Bmax 31 fmol/mg protein) (data not shown).

Cellular hormonal responsiveness depends upon the presence of available receptors. This necessitates that the interaction of a hormone with its receptor be a reversible process. Specific binding of [^{3}H]DHA to palatal tissue homogenates was almost totally reversible within 60 min (Fig. 3). The addition of the competitive antagonist propranolol resulted in a time-dependent dissociation of [^{3}H]DHA from bound sites.

The interaction of hormones with cellular receptors generally results in a biological response. Such cellular responsiveness differentiates receptor-ligand from "acceptor"-ligand interactions. The primary biochemical event in

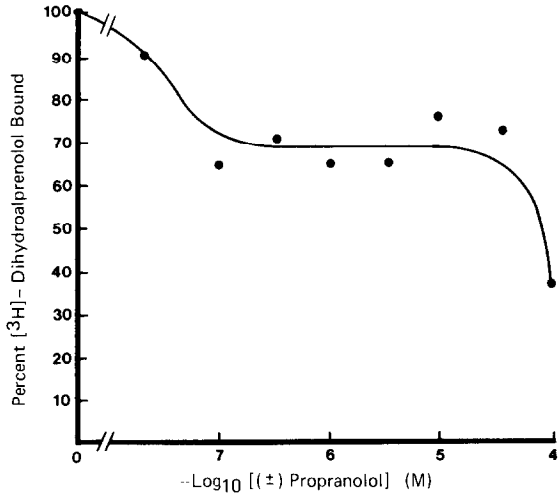

Fig. 2 Competition between [^{3}H]DHA and propranolol (a β-adrenergic antagonist) for binding to day 13 fetal mouse palatal tissue homogenates. Aliquots of homogenate (50 ug protein/100 ul) were incubated with [^{3}H]DHA (6.9 nM) in the absence or presence of increasing concentrations of (±) propranolol for 10 min at 37°. Total binding of [^{3}H]DHA at each concentration of propranolol is expressed as a percent of binding in the absence of competitor.

β-adrenergic receptor-mediated responses is the activation of adenylate cyclase and the generation of cAMP (21). The responsiveness of fetal palatal tissue

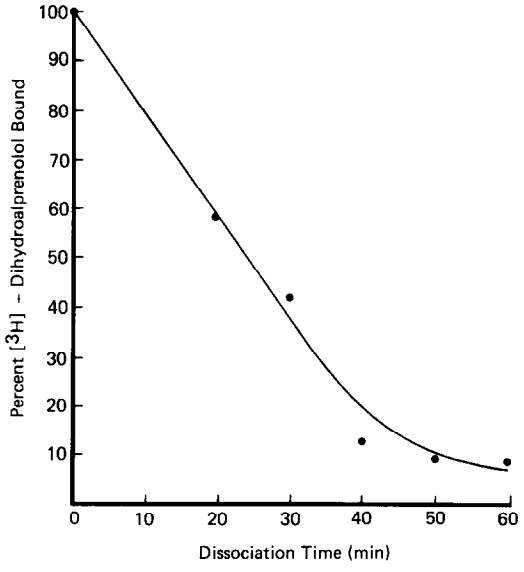

Fig. 3 Reversibility of [^{3}H]DHA binding to day 13 fetal mouse palatal tissue homogenates. Aliquots of homogenates (50 ug/100 ul) were incubated with [^{3}H]DHA (6.9 nM) for 10 min at 37°. At time 0 (time immediately following initial 10 min incubation) binding was reversed by the addition of excess propranolol (2.0 uM) and incubation continued at 37°. At the indicated times, binding was terminated by dilution and filtration of the incubation mixtures. [^{3}H]DHA specifically bound at each time point is expressed as a percent of specific binding at time 0.

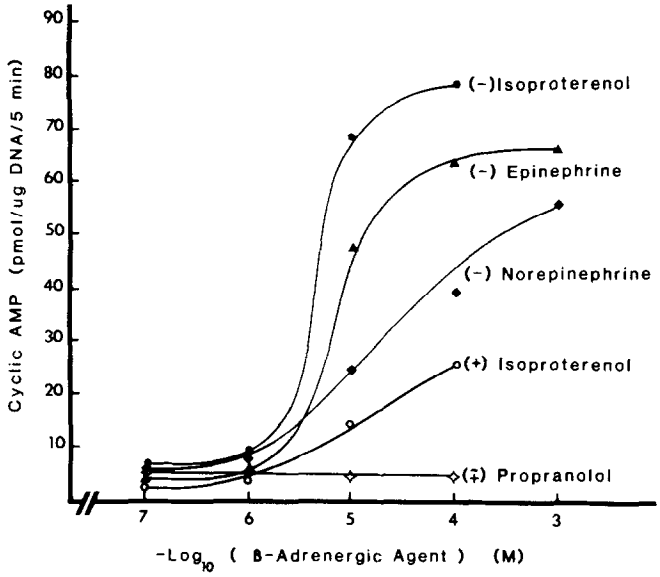

Fig. 4 Potency series of adenylate cyclase activation and the generation of cAMP by β-adrenergic catecholamines. Fetal palatal mesenchymal cells were grown to subconfluency and incubated with increasing concentrations of various catecholamines. Incubations were carried out in serum-free medium with the addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2 mM) for 5 min at 37°C. Levels of cAMP were determined by radioimmunoassay and expressed as pmol cAMP/ug DNA/5 min. Basal activity averaged 3.5 pmol cAMP/ug DNA.

in vitro to various catecholamines was investigated by measuring the accumulation of cAMP (Fig. 4). The β-agonists, isoproterenol, epinephrine and norepinephrine, elicited dose-dependent elevations of cAMP levels. The β-antagonist propranolol was unable to evoke this response. Epinephrine-stimulated accumulation of cAMP could be completely blocked by the simultaneous addition of 5×10^{-5} M propranolol, indicating that catecholamine-stimulated cAMP accumulation was mediated primarily by β-adrenergic receptors. The order of potency of agonists in eliciting an accumulation of cAMP was characteristic of a β_2-adrenergic receptor: (-)isoproterenol > (-)epinephrine > (-)norepinephrine. Furthermore, the (-) isomer of isoproterenol was far more potent than the (+) isomer in eliciting a response, demonstrating stereospecificity of palatal β-receptors.

DISCUSSION

This study demonstrates the presence of [^{3}H]Dihydroalprenolol binding sites in fetal palatal tissue, having the characteristics of β-adrenergic receptors. [^{3}H]DHA binding was saturable, reversible, and of high affinity.

Fetal palatal tissue responded to catecholamines with the stereo- and pharmacologic specificity characteristic of a β_2-adrenergic receptor-mediated response. These results support the hypothesis that the developing mammalian secondary palate contains functionally-coupled β-adrenergic receptors and allows one to speculate that this hormone-receptor system might function in normal palate development.

Hormones, being a very diverse class of bioregulatory substances, are known to modulate a variety of developmental events (22). During embryonic and fetal development, differentiating cells and tissues acquire a unique repertoire of hormone receptors with a resultant differential responsiveness to hormonal stimuli. This variable responsiveness is thought to be critical in determining the cell's normal developmental and differentiative fate (23). Catecholamine hormones appear during early vertebrate ontogeny (24, 25) and have been suggested to play a role in both morphogenesis and growth (11, 26). The effects of catecholamines in the developing vertebrate organism may be mediated by the regulation of cyclic nucleotide levels through β-adrenergic mechanisms. Such a notion is supported by the demonstration of functionally coupled β-adrenergic receptors and catecholamine sensitive adenylate cyclase activity in a variety of developing tissues (27-30).

A number of factors intrinsic and extrinsic to the palatal processes have been implicated in the control of palatogenesis (31). Both hormones and growth factors, such as glucocorticoids and EGF, are involved in normal and abnormal palate morphogenesis (32, 33). The effects of these agents are mediated by specific receptor proteins present in the palate during critical stages of development. Locally derived factors, such as prostaglandins and cyclic nucleotides have also been suggested to modulate normal development of the palate (4, 16).

While cyclic AMP has been shown to play a key role in normal secondary palate development (16, 34-37), agents which regulate palatal cyclic AMP levels have not been precisely defined. A functionally-coupled β-adrenergic receptor system, as demonstrated, may mediate the effects of catecholamines on palatal

cyclic AMP levels and function in cyclic AMP-dependent palatal growth and differentiation.

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REFERENCES

1. Pruzansky, S. (1973) J. Am. Dent. Assoc. 87, 1048-1054.
2. Kalter, H. (1965) Teratology: Principles and Techniques, pp. 57-64, University of Chicago Press, Chicago.
3. Slavkin, H.C. (1979) Ear, Nose and Throat J. 58, 7-20.
4. Greene, R.M. (1983) Handbook of Experimental Pharmacology, Vol. 65, pp. 79-92, Springer-Verlag, Berlin, Heidelberg.
5. Pratt, R.M. (1980) Development in Mammals, Vol. 4, pp. 203-231, Elsevier North Holland.
6. Greene, R.M., and Pratt, R.M. (1976) J. Embryol. Exp. Morph. 36, 225-246.
7. Zalin, R.J., and Montague, W. (1974) Cell 2, 103-108.
8. Rickenberg, H.V. (1978) Differentiation and Development, pp. 149-167, Academic Press, New York.
9. Nijjar, M.S. (1979) Biochem et Biophys. Acta. 586, 464-472.
10. Elias, L., and Wood, A. (1983) Exp. Cell Res. 145, 447-451.
11. Lawrence, I.E., and Burden, H.W. (1973) Am. J. Anat. 137, 199-208.
12. Curtis, D.H., and Zalin, R.J. (1981) Science 214, 1355-1357.
13. Pairault, J., Lasnier, F., and Landat, M.H. (1982) Eur. J. Biochem. 129, 351-358.
14. Waterman, R.E., Palmer, G.C., Palmer, S.J., and Palmer, S.M. (1976) Anat. Rec. 185, 125-138.
15. Palmer, G.C., Palmer, S.J., Waterman, R.E., and Palmer, S.M. (1980) Ped. Pharm. 1, 45-54.
16. Greene, R.M., Lloyd, M.R. and Nicolaou, K.C. (1981) J. Craniofacial Genet. and Devel. Biol. 1, 261-272.
17. Kissane, J.M., and Robins, E. (1958) J. Biol. Chem. 233, 184-188.
18. Lefkowitz, R.J. (1975) Biochem. Pharm. 24, 1651-1660.
19. Lefkowitz, R.J. (1978) Fed. Proc. 27, 123-129.
20. Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660-673.
21. Lefkowitz, R.J., Caron, M.G., Michel, T., Stadel, J.M. (1982) Fed. Proc. 41, 2664-2670.
22. Turkington, R.W. (1971) Developmental Aspects of the Cell Cycle. pp. 315-355, Academic Press, New York.
23. Sato, G., Reid, L. (1978) International Review of Biochemistry, Vol. 20 pp. 219-251, University Park Press, Baltimore.
24. Burden, H.W., and Lawrence, I.E. (1973) Am. J. Anat. 136, 251-257.
25. Schlumpf, M., and Lichtensteiger, W. (1979) Anat. Embryol. 156, 177-187.
26. McMahon, D. (1974) Science 185, 1012-1021.
27. Giannopoulos, G. (1980) Biochem. Biophys. Res. Comm. 95, 388-394.

28. Sytkowski, A.J., Kessler, C.J., and D'Albis, J.N. (1981) Biochem. Biophys. Res. Comm. 101, 1221-1227.
29. Schonberg, M., Morris, S.A., Krichevsky, A., and Bilezikian, J.P. (1983) Cell Diff. 12, 321-327.
30. Chen, F.M., Yamamura, I., and Roeske, W.R. (1982) J. Pharm. Exp. Ther. 222, 7-13.
31. Shah, R.J. (1979) Advances in the Study of Birth Defects, Vol. 1, pp. 69-84, University Park Press, Baltimore.
32. Salomon, D.S., and Pratt, R.M. (1979) Differentiation 13, 141-154.
33. Nexø, E., Hollenberg, M., Figueroa, A., and R.M. Pratt (1980) Proc. Natl. Acad. Sci. 77, 2782-2785.
34. Erickson, R.P., Butley, M.S., and Sing, C.F. (1979) J. Immunogenetics 6, 253-262.
35. Greene, R.M. and Pratt, R.M. (1979) J. Histochem. and Cytochem. 27, 924-931.
36. Greene, R.M., Shanfeld, J.L., Davidovitch, Z., and Pratt, R.M. (1980) J. Embryol. Exp. Morph. 60, 271-281.
37. Greene, R.M., MacAndrew, V.I. and Lloyd, M.R. (1982) Biochem. Biophys. Res. Comm. 107, 232-238.