Involvement of Mitogen-Activated Protein Kinase and Casein Kinase 2 in Regulation of Cell Proliferation During Development of the Secondary Palate in Quail.

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Oral Biology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December 1996

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Date **Dec 20/1996**
ABSTRACT: A study was undertaken to investigate: (1) the kinetics of cell proliferation; (2) the activity of two protein kinases, MAPK and CK2, that are implicated in the regulation of cell proliferation; (3) the effect of a growth factor, EGF, on the proliferation of cultured quail palate mesenchymal cells (QPMC); and (4) the activity of MAPK and CK2 following EGF treatment of cultured QPMC.

Initially, an in vivo/in vitro comparison of developing quail was performed to validate the whole embryo culture technique. The data showed that, on the basis of CRL and developmental staging method, the in vitro (whole embryo culture) embryos developed in a manner similar to their in vivo counterparts. The whole embryo culture was then used to analyze the cell proliferation kinetics by $^3$H-thymidine autoradiography during quail palate morphogenesis. The results indicated that the rate of cell proliferation declined with the advancing morphogenesis of quail palate.

Subsequently, embryonic palates were dissected at 24 hour intervals between days 5 and 9 of incubation and homogenized. Isolated proteins from the homogenate were fractionated by FPLC, and analyzed by phosphotransferase assays, scintillation counting, and Western blotting to detect the presence and activation of two second-messenger independent protein kinases, MAPK and CK2, implicated in the regulation of cell proliferation. The results showed that phosphotransferase activity towards MBP showed a negative, whereas that towards phosvitin showed a positive correlation with proliferation of QPMC. Western blotting indicated that both MAPK and CK2 were present during quail palate development.

Finally, growth behaviour of QPMC in primary cell culture was analyzed. The results showed that the number of QPMC increased faster in EGF-treated than in control cultures. EGF stimulated phosphotransferase activity towards MBP and phosvitin, and Western blotting indicated that both MAPK and CK2 were activated. It was suggested that both MAPK and CK2 may be involved in growth factor regulated cell proliferation during the palate development.
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LIST OF ABBREVIATIONS

AP buffer - Alkaline Phosphatase buffer
ATP - Adenosine triphosphate
BCIP - 5-bromo-4-chloro3-indolylphosphate
cAMP - cyclic adenosine phosphate
cAPK - cAMP dependent protein kinase
cGMP - cyclic guanine monophosphate
cGPK - cGMP dependent protein kinase
CK2 - Casein Kinase 2
CK2-NT - CK2 amino terminus
CRE - cAMP response element
CREB - CRE binding protein
CRL - Crown - rump length
db-cAMP - dibuturyl cAMP
DMEM - Dulbecco’s Modified Eagle Medium
ECM - Extracellular matrix
EDTA - Ethylene diamine tetra acetic acid
EGF - Epidermal growth factor
EGF-R - EGF receptor
ERK - Extracellular regulated kinase
ERK-CT - ERK carboxyl terminus
FCS - Fetal calf serum
FGF - Fibroblast growth serum
FPLC - Fast Protein Liquid Chromatography
GAG - Glycosaminoglycan
GTP - Guanine triphosphate
IGF - Insulin growth factor
JNK - c-jun N-terminal kinase
MAPK - Mitogen-activated protein kinase
MEE - Medial edge epithelium
NBT - Nitro blue tetrazolium
NGF - Nerve growth factor
ODC - Ornithine decarboxylase
PDE - Phosphodiesterase
PDGF - Platelet-derived growth factor
PGs - Prostaglandins
PLA2 - Phospholipase A2
PKA - Protein kinase A (cAMP dependent protein kinase)
PKC - Protein kinase C
PMA - Phorbol ester
QPMC - Quail palatal mesenchymal cells
SAPK - Stress activated protein kinase
SDS-PAGE - Sodium docecyl sulfate-polyacrylamide gel electrophoresis
TBS - Tris buffer saline
TGF - Transforming growth factor
T-TBS - Tween 20-Tris buffer saline
ACKNOWLEDGEMENTS

This thesis represents the essence of my dreams and goals, a step along the path I am trying to forge for my life. The process by which it took place, both intellectually in my thoughts, and emotionally, in my heart, although founded and therefore determined by my family, were ultimately brought into the current state of differentiation by my mentor, Dr. Ravindra Shah. He has relentlessly given his love, attention, patience, and support for the process of learning. Ultimately, the techniques, methods, even the science, in this endeavor may be forgotten or even proven wrong, but the spark of knowledge that was nurtured, caressed, fed, and balanced by my mentor cannot be diminished. The flame that has blossomed is forever grateful to the kind spirit who had the foresight and vision to commit so much energy, time, and wisdom to the learning process. Throughout my period of study, he has reinvented the world for me, his countless remarkable and thought-provoking discussions have brought me to a new plane of awareness, where honor and integrity coexist with passion and fortitude.

I am forever grateful to my mother, father, and sister Michelle, who always pushed me to explore my limits and never settle for the ordinary; they raised me with unconditional love.

I greatly appreciate all my committee members for their excellent critiques and advice, which vastly improved this document.

My time in the lab was always very precious to me, and I am tremendously lucky that this time was blessed with the uncompromising friendship of Alan Young and Mehrnaz Izadnegahdar, my fellow graduate students whose advice was always available. Without their companionship and help, this work would not have been possible.

I sincerely thank Dr. J. Sanghera and Dr. S. L. Pelech for the invaluable knowledge and expertise that they provided to me, which served as the framework for the biochemical approaches used in this study.

I wish to acknowledge NSERC of Canada whose financial support made this study possible.
INTRODUCTION

Vertebrate Palate Morphogenesis

The vertebrate secondary palate is formed intraorally by the growth of bilaterally symmetrical projections (shelves) from the maxillary processes of the first branchial arch. The growing shelves consist of mesenchyme covered by an epithelium. A review of the literature shows that, although the development of palate has only been studied in a few species of vertebrates, palate morphogenesis is a complex but class-specific process, which occurs late in organogenesis and occupies a relatively long period during gestation (Greene and Pratt, 1976; Shah, 1984; Ferguson, 1988; Shah et al., 1990).

In fish and amphibia, the palatal shelves emerge from the inferior surface of the maxillary processes and grow vertically (LeCluyse et al., 1985; Shah et al., 1990). Subsequently, in contrast to other vertebrates, there is no advancement in morphogenesis, and the fish and amphibian palate remains open throughout ontogeny.

In the alligator, the only reptile studied thus far, the secondary palatal shelves grow ad initium, from the medial edge of the maxillary processes in a horizontal direction towards the midline. Thereafter, the opposing palatal shelves unite to separate the oral and nasal passages (Ferguson, 1981). On the other hand, although the information on the embryogenesis of palate in other reptiles is not available, it has been noted that in lizard, the palate is completely cleft; and in snake, the palate is partially cleft (Shah, 1984). In both species, the palatal shelves are horizontal.

The avian palatal shelves, like the alligator, initiate from the medial edge of the maxillary processes and grow in a horizontal direction towards one another superior to the developing tongue (Shah and Crawford, 1980; Koch and Smiley, 1981; Greene et al., 1983; Shah et al., 1985a, 1987, 1988). Subsequently, the palatal shelves do not fuse, thus leaving a
natural cleft of the secondary palate throughout avian ontogeny (Bellairs and Jenkin, 1960; Romanoff, 1960; Shah and Crawford, 1980).

Much of the information on secondary palate formation is derived from studies on mammals. In mammals, the embryogenesis of the secondary palate involves three sequential events: 1) growth of the shelves from the roof of the oronasal passage in a vertical direction toward the floor, along the sides of the developing tongue, 2) reorientation of the shelves from a vertical to a horizontal plane superior to the tongue, 3) closure of the opposing horizontal shelves, resulting in the separation of the oral and nasal passages (Walker and Fraser, 1956; Fulton, 1957; Wood and Kraus, 1962; Coleman, 1965; Anderson and Matthiessen, 1967; Shah and Travill, 1976a).

Thus, it seems that, from an evolutionary standpoint, birds may have retained some of the features of reptilian palate morphogenesis (i.e., initial development in a horizontal direction), whereas mammals may have conserved some features from early vertebrates (i.e., initial vertical development as in fish and amphibia) as well as reptiles (i.e., closure of palate).

**Biological Phenomena Involved During Vertebrate Palate Morphogenesis**

Several fundamental biological phenomena are involved in the formation of the vertebrate palate. These phenomena include cell proliferation, cell migration, synthesis of extracellular matrix molecules (ECM), epithelial-mesenchymal interactions, programmed cell death and/or epithelial-mesenchymal transformation (Shah, 1984; Ferguson, 1988; Shuler, 1995). Hence, investigations of these phenomena during the morphogenesis of palate are essential to enhance the understanding of the complex development of the vertebrate secondary palate.

Studies examining the origin of the cells that form the maxillary process of the first branchial arch suggest that a considerable proportion of the cells that contribute to the
mesenchyme of the arch are neural crest in origin (Hall and Horstadius, 1988; Couly et al., 1993). By employing various isotopes, vital dyes or quail-chick chimeras, several investigators have traced the migration of neural crest cells from their origin from the neural ectoderm to the maxillary arch (Johnston, 1966; Nichols, 1986; Morriss-Kay and Tan, 1987; Fukiishi and Morriss-Kay, 1992; Serbedzija et al., 1992; Hanken and Thorogood, 1993). Furthermore, ablation of cranial neural crest in chick results in clefts of the primary and secondary palate (Been and Lieuw Kie Song, 1978; vanLimborgh et al., 1983) thus indicating that cleft palate can result from the insufficient growth of the maxillary process mesenchyme caused by an initial deficiency of neural crest cells. Currently, it is widely believed that neural crest derived cells of the branchial arch would subsequently form the bulk of the mesenchyme of the developing vertebrate head, including that of the secondary palate (Gans and Northcutt, 1983; Osumi-Yamashita and Eto, 1990; Couly et al., 1993).

During the morphogenesis of the mammalian secondary palate, cell proliferation is a major component of the early growth of the palatal shelves (Shah et al., 1989a, b, c; 1994b, 1995). Earlier studies, although focused on shelf reorientation in mammals, observed an increasing rate of cell proliferation during the vertical development of palate but did not pursue investigations relating to it (Mott et al., 1969; Jelinek and Dostal, 1973). Also, in most teratological studies investigating cleft palate induction, chemicals were usually administered either prior to or during the period of initial palate development (Burdett et al., 1988; Shah et al., 1989a; 1991). Recently, teratogen-sensitive peak(s) of DNA synthesis and cell proliferation have been identified during the early development of the secondary palate in mammals and birds (Burdett et al., 1988; Shah et al., 1989a,b,c, 1994b). In addition, the majority of studies on human embryos and fetuses with cleft palate, as well as children born with cleft palate document hypoplastic vertical shelves (Vij and Kanagasutheram, 1971; Blocksema et al., 1973; Latham, 1973; Maher, 1977), are suggestive of a growth deficiency.
The results of these diverse studies in animals and human underscore the significance of the involvement of cell proliferation during early vertebrate palate formation.

In addition to cell proliferation, the synthesis of ECM is also important for the normal development of the secondary palate. Histochemical, autoradiographic and biochemical studies have demonstrated that several ECM molecules [sulphated and nonsulphated glycosaminoglycans (GAG), collagen, fibronectin, etc.] are synthesized during mammalian, avian and fish palatal growth (Larsson, 1962; Jacobs, 1964; Nanda, 1971; Pratt et al., 1973, Jacobson and Shah, 1981, Brinkley and Vickerman, 1982; Brinkley and Morris-Wiman, 1984, Foreman et al., 1991; Benkhaial et al., 1993; Benkhaial and Shah, 1994; Singh et al., 1994; Young et al., 1994; Ohsaki et al., 1995; Shah et al., 1995) and contribute to the shelf size (Young et al., 1994). In mammals, the accumulation of these ECM molecules is essential for the reorientation of the shelves from a vertical to a horizontal plane (Larsson, 1962; Jacobs, 1964; Brinkley and Morris-Wiman, 1984; Benkhaial and Shah, 1994; Young et al., 1994). Also, a disturbance in the synthesis of extracellular matrices have been correlated with teratogen-induced cleft palate in mammals and birds (Larsson 1962; Jacobs, 1964; Shapira, 1969; Pratt et al., 1973; Jacobson and Shah, 1981; Sasaki and Kurisu, 1983; BenKhaial et al., 1993; BenKhaial and Shah, 1994; Young et al., 1994). These observations indicate a crucial role played by the matrix molecules during normal palate morphogenesis.

The precise role played by epithelial-mesenchymal interaction during vertebrate palate morphogenesis is not clear. During the closure of palate in mammals, programmed cell death appears to be critical in the elimination of the medial edge epithelia (MEE) to allow the mesenchymal union (Mato et al., 1966; Smiley, 1970; Chaudhry and Shah, 1973; Schupbach and Schroeder, 1983; Mori et al., 1994; Taniguchi et al., 1995). It has been suggested that one of the factors involved in specifying epithelial differentiation into nasal, oral or medial edge types during palate morphogenesis is the influence exerted by the mesenchyme on the overlying
epithelium (Shah, 1984; Ferguson et al., 1984). Thus, the programmed cell death of the medial edge epithelia during palate formation in mammals, or its absence during palate formation in birds, would be dependent on epithelial-mesenchymal interactions (Ferguson et al., 1984; Shah, 1984).

Recently, however, it has been proposed that the mammalian medial edge epithelium may undergo transformation into mesenchyme rather than elimination by programmed cell death (Fitchett and Hay, 1989, Griffith and Hay, 1992, Shuler, 1995; Yano et al., 1996).

Overall, however, it seems clear that the morphogenesis of vertebrate secondary palate is dependent on temporally regulated biological phenomena, which are expressed in an orderly manner to specify the function and fate of the palatal epithelia and mesenchyme.

**Palate Morphogenesis in Quail**

The avian palate is naturally cleft (Romanoff, 1960; Shah and Crawford, 1980). The embryogenesis of palate in various species of birds studied so far is remarkably similar (chick, quail, duck, pigeon; Shah and Crawford, 1980; Koch and Smiley, 1981; Greene et al., 1983; Shah et al., 1985a, 1987, Shah and Cheng; 1988). Depending on species, it occurs between Hamburger and Hamilton's (HH) developmental stages 26-37 (i.e., incubation days 5-11). Furthermore, the embryogenesis of avian palate appears to be less complex than that of mammal, as evidenced by the observation that the bird palatal shelves develop horizontally from the start and do not fuse. Thus, it lacks the mammalian type reorientation and fusion stages. The morphological differences in the direction of initial growth of the palatal shelves and the lack of programmed cell death strongly suggest that regulation and behaviour of both mesenchymal and epithelial cells in the developing palate of the bird and mammal may be different. Hence, characterization of the cellular, biochemical and molecular events during growth and differentiation of the bird palate may provide insightful information in understanding not only the comparative biological aspects of vertebrate palatogenesis, but may
also potentially clarify crucial aspects of cleft palate formation in mammals.

The morphogenesis of the secondary palate in Japanese quail (*Coturnix japonica*) occurs between days 5 and 8 of incubation [Hamilton and Hamburger (stages 26-35), 1951]. A previous study has shown that the growth of the palatal shelves begins from the medial aspects of each of the maxillary processes on day 5 of incubation, and then proceed towards the midline (Shah et al., 1985a). Histologically, a bilaminar epithelium envelops the shelf mesenchyme. The superficial epithelial cells are flat, whereas the deeper cells are cuboidal. The mesenchymal cells are spherical or elongated. Both the epithelial and mesenchymal cells contain large oval nuclei surrounded by scant cytoplasm. Between days 6 and 7 of incubation, as the shelves grow in size, the deeper cells of the medial edge epithelium become columnar and contain oval or irregular nuclei. On day 8 of incubation, the palatal shelves approximate one another only in the anterior third of the mouth. In the posterior two-thirds of the mouth, the palatal shelves never approximate and a wide gap persists between them. The epithelium on the medial edges of the shelves differentiates into 6-10 layers of cells. It adopts a parakeratinized morphology, in which the basal cells are columnar with centrally located round nuclei; intermediate layers of cells are polygonal with irregular nuclei; and the superficial 4-6 layers of cells are flat with elongated nuclei (Shah et al., 1985a). On the other hand, the epithelium on the medial edges of the developing chick palatal shelves differentiate into an orthokeratinized type (Shah and Crawford, 1980; Koch and Smiley, 1981), whereas in duck and pigeon non-keratinized type (Shah et al., 1987, 1988). Also, most importantly, in comparison to mammals where the MEE disappears, the avian MEE persists (Shah and Crawford, 1980; Koch and Smiley, 1981; Shah et al., 1985a,b, 1987, 1988; Shah and Cheng, 1988), and thus may contribute to the clefting of the palate.

There are only a few studies on the biochemical aspects of avian palate morphogenesis (Greene et al., 1983; Foreman et al., 1991; Benkhaial et al., 1993; Shah et al., 1994, Young et
During palatogenesis in quail, the volume of the shelf increases approximately 3 fold between days 5 and 9 of incubation, indicating that biochemical growth processes at the cellular and extracellular levels are indeed crucial for advancing palate development. The rate of DNA synthesis, indicative of the rate of cell proliferation, is high in the developing quail palate on day 5 of incubation and decreases gradually thereafter (Shah et al., 1994a). Subsequently, a burst of RNA synthesis occurs on day 7 of incubation, but the rate of protein synthesis remains fairly stable during the period of quail palatogenesis (Shah et al., 1994a). Of the proteins synthesized (indicative of differentiation), the rate of collagen synthesis increases five-fold between days 6 and 8 of incubation but drops subsequently (Benkhaial et al., 1993). In addition, although the overall level of GAG synthesis remains constant in developing quail palate between days 6-9 of incubation (Young et al., 1994), an observation consistent with that made in chick by Foreman et al (1991), sulphated GAG accumulated with the advancing morphogenesis of avian secondary palate (Foreman et al., 1991; Young et al., 1994). The levels of cAMP, which in mammals shows temporal changes and are associated with the fusion process (Pratt and Martin, 1975; Greene and Pratt, 1979), remain low and constant during chick (Greene et al. 1983), quail (Shah and Cheng, 1988) and duck (Shah et al., 1987) palate development.

**Regulation of Palate Morphogenesis**

While considerable attention has been paid in the recent past on the role of various biological phenomena involved during palate development, some attention was also directed towards their regulation. It has been suggested that interactions between extracellular molecules such as growth factors, hormones, prostaglandins and neurotransmitters may be important for regulation of the complex biological phenomena observed during palate development (Pratt, 1980, 1983, Greene 1989; Greene et al., 1991). Furthermore, this would involve a coordinated expression of receptors of extracellular molecules, and interaction
between various signal transduction pathways that communicate extracellular messages to the nucleus.

Using immunohistochemical and in situ techniques, many growth factors have been localized in the developing palate of mammal. These include epidermal growth factor (EGF), transforming growth factor-α and -β₁,₂,₃ (TGFα, TGF-β₁,₂,₃), insulin-like growth factor I and II (IGF I, IGF II), platelet derived growth factor AA and BB (PDGF-AA and PDGF-BB), and basic and acidic fibroblast growth factor (bFGF and aFGF) (Heine et al., 1987; Abbott et al., 1988, 1994; Abbott and Birnbaum, 1990, Abbott et al., 1992; Fitzpatrick et al., 1990; Pelton et al., 1990; Abbott and Pratt, 1991; Dixon et al., 1991; Williams et al., 1991; Gehris and Greene, 1992; Ferguson et al., 1992; Sharpe et al., 1992a; Brunet et al., 1993; Citterio and Gaillard, 1994; Qiu and Ferguson, 1995). These growth factors have been implicated in the regulation of cell proliferation, ECM biosynthesis, epithelial-mesenchymal interactions and cell differentiation during palate morphogenesis (Ferguson, 1988, Greene et al., 1991).

Of all the growth factors localized and tested in the developing palate, by far, EGF has received the most attention. Several studies have shown that palatal epithelial and mesenchymal cells in tissue or cell culture respond to EGF or TGF-α, (Nexo et al., 1980; Pratt, 1980; Pratt et al., 1984; Adamson et al., 1981; Adamson and Meek, 1984; Grove and Pratt, 1984; Silver et al., 1984; Greene and Lloyd, 1985; Turley et al., 1985; Pratt, 1987; Gawel-Thompson and Greene, 1989; Abbott and Pratt, 1991; Dixon and Ferguson, 1992; Foreman et al., 1991, Chepenik et al., 1994). The primary cultures of palatal mesenchymal cells respond to EGF or TGF-α by synthesizing various ECM molecules (especially collagen, tenascin, and chondroitin sulphate proteoglycan). The palatal mesenchymal cells can also be stimulated to divide in the presence of EGF (Gawel-Thompson and Greene, 1989). In contrast, the palatal medial edge epithelium responds to exogenous EGF by differentiating into a keratinized epithelium, which is
analogous to the oral epithelium, rather than proceeding through programmed cell death/cell transformation as implicated in vivo (Hassell, 1975; Hassell and Pratt, 1977; Pratt et al., 1980; Brunet et al., 1993). Also, a high number of EGF receptors have been localized in both the mesenchyme and epithelium of the developing mammalian palate (Nexo et al., 1980; Dixon et al., 1991; Sharpe et al., 1992a; Brunet et al., 1993). The EGF receptor expression or ligand binding ability can be modulated by other growth factors. For example, TGF-\(\beta_1\) pretreatment causes a marked reduction in \(^{125}\text{I}-\text{EGF}\) binding to embryonic palatal mesenchymal cells in culture (Sharpe et al., 1992a). Such an effect of TGF-\(\beta_1\) on ligand binding to the EGF receptor may indicate that the interaction of growth factors effects on palatal cells can regulate their proliferative and differentiative states, and consequently modulate the morphogenesis of palate. Also, TGF\(\alpha\), which expresses its effect through the EGF receptor, is localized in the mesenchyme during the vertical stage of mammalian palate development (Dixon et al., 1991), thus providing further support for a potential involvement of the EGF receptor during palate morphogenesis.

A spatio-temporal expression pattern of TGF-\(\beta_1\), \(\beta_2\), and \(\beta_3\) have been observed during the development of mammalian palate (Abbott and Birnbaum, 1990; Fitzpatrick et al., 1990, 1992; Pelton et al., 1990, 1991; Gehris et al., 1991, 1994; Williams et al., 1991). TGF-\(\beta_1\) is localized predominately in the MEE especially during epithelial seam formation and degeneration (Fitzpatrick et al., 1990; Pelton et al., 1990), whereas TGF-\(\beta_2\) is immunodetected in the mesenchyme immediately below the MEE prior to and during palatal shelf fusion (Fitzpatrick et al., 1990; Gehris et al., 1991). In organ culture, TGF-\(\beta_1\) and \(\beta_2\) peptides accelerate palatal fusion (Shuler et al., 1991, 1992; Gehris and Greene, 1992).
Treatment of murine embryonic palatal mesenchymal cells with TGF-β₁ causes an increase in the synthesis of type III collagen and decreases collagenase and stromelysin activity (D'Angelo et al., 1994) indicating that TGF-β₁ may play a role as a stimulator of ECM synthesis during palate development. Furthermore, the effects of TGF-β₁ appear to be species specific: following treatment with TGF-β₁, human palatal cells continue to proliferate, whereas mouse palatal cell proliferation is inhibited (Linask et al., 1991). Human embryonic palatal mesenchymal cells possess only type I TGF-β receptors, whereas the mouse cells have both type I and type II TGF-β receptors (Linask et al., 1991).

TGF-β₃ mRNAs are localized predominately in the MEE (Fitzpatrick et al., 1990; Pelton et al., 1990; Gehris et al., 1994). In TGF-β₃ knockout mice, palatal shelves make contact but subsequently do not fuse (Proetzel et al., 1995). Similarly, antisense oligomers or neutralizing antibodies to TGF-β₃ also block the fusion process (Shuler et al., 1991, 1992; Gehris and Greene, 1992). These experimental results show that TGF-β₃ may be essential for normal closure of palatal shelves during embryonic development. Furthermore, regulation of TGF-β₃ expression in murine embryonic palatal cells has been shown to be upregulated by treatment with TGF-β₁, β₂, cAMP and retinoic acid but downregulated by EGF treatment (Gehris et al., 1994). Thus, the regulation of TGF-β₃ expression by other growth factors further raises the complexity of the regulatory mechanisms involved during palate morphogenesis.

Both platelet derived growth factors AA and BB (PDGF-AA and PDGF-BB), and their receptors, have been localized to the embryonic murine palate (Qui and Ferguson, 1995).
Prior to reorientation, both PDGF-AA and its α receptor are localized throughout the palate except for the oral epithelium; after reorientation and during fusion, they are intensely localized to the nasal epithelium and MEE. In contrast to the strong expression of the PDGF-AA receptor, the PDGF-BB receptor is weakly localized in the developing palate.

The distribution patterns of IGF-I and II mRNA's, and peptides, have also been established in the developing palate of mouse (Ferguson et al., 1992). Immunohistochemical observations show weak staining of IGF-I throughout the palatal mesenchyme, with a decrease in staining from the vertical to the horizontal stages of palate development. On the other hand, IGF-II mRNA is absent in the vertical shelf. Significant expression of IGF-II mRNA, however, was observed in the mesenchymal cells of the horizontal shelf, prior to fusion. In contrast to the mRNA expression, IGF-II protein is predominately detected in the nasal and medial edge epithelia, but little in the palatal mesenchymal cells prior to fusion. The patterns of IGF-II mRNA expression and localization of the IGF-II peptide were interpreted by Ferguson (1992) to be of possible importance in mesenchymal-epithelial signaling during palate development.

Fibroblast growth factor has also been shown to regulate cellular behaviour of developing palate in organ culture. In vitro treatment with FGF stimulates both collagen and GAG synthesis (Foreman et al., 1991; Sharpe et al., 1993) and supports proliferation of palate mesenchymal cells (Sharpe et al., 1993). Also, FGF affect EGF binding to the palatal mesenchymal cells: after 4 hours of FGF treatment, there is a decrease in EGF binding to the palatal cells, whereas after 24 hours of FGF treatment, there is an increase in EGF binding to the palatal cells (Sharpe et al., 1992a).

Prostaglandins (PGs) are biologically active compounds capable of regulating cellular growth and differentiation. Various PGs and their receptors have been localized in the developing palate (Chepenik and Greene, 1981; Alam et al., 1982; Greene and Lloyd, 1985; Jones and Greene, 1986). Both PGE2 and PGF2 show intense immunocytochemical localization
in both the mesenchyme and epithelium of mouse vertical palate, but are absent in the horizontal shelf mesenchyme, indicating a potential role for the PGs in regulation of palatal morphogenesis (Jones and Greene, 1986). The addition of PGE2 to palatal mesenchymal cell cultures, at levels that cause a rise in cAMP inhibits reentry of mesenchymal cells into the cell cycle after serum stimulation (Greene et al., 1981a, b; Pisano et al., 1986), and stimulates dose-dependent increase in GAG synthesis (Greene et al., 1982). The stimulation of GAG synthesis in mesenchymal cells can also be induced by the addition of db-cAMP to the culture media (Greene et al., 1982). These data further indicate that prostaglandins may be involved in the regulation of proliferation and differentiation of palatal mesenchyme.

Alterations in catecholamine levels have been identified in developing palate (Zimmerman et al, 1981; Pisano et al., 1986). Waterman et al (1976, 1977) observed that norepinephrine and epinephrine were capable of inducing increased activation of adenylate cyclase during in vivo and in vitro hamster palate growth. β-adrenergic receptors have also been identified in developing palatal tissue, which upon stimulation, cause a dose-dependent increase in accumulation of intracellular cAMP (Garbarino and Greene, 1984; Greene and Garbarino, 1984). Also, experiments in murine embryonic palatal cell cultures have shown that isoproterenol treatment delays the re-entry of cells into the cell cycle after serum stimulation following starvation (Pisano et al., 1986). These observations suggest a putative regulatory role for the catecholamines and their receptors during palate formation.

Whereas some effort was directed towards investigating the regulation of biological phenomena during mammalian palate formation, studies on the regulation of avian palate development are relatively few. It has been shown that cAMP levels remain inversely proportional to DNA synthesis during avian palate morphogenesis (Greene et al., 1983; Shah et al., 1985, 1987; Shah and Cheng, 1988). There is some evidence in the literature that proliferation and differentiation of avian palatal cells can be modified by growth factors.
Foreman and associates (1991) indicated that TGFβ1, but not FGF, TGFα, EGF or PDGF, caused an increase in glycosaminoglycan synthesis. Recently, it was observed that FGF stimulated but TGFβ1 inhibited proliferation of cultured quail palate mesenchymal cells (Izadnegahdar et al., 1995; Shah et al., 1996). During embryonic development, however, quail palate cells do not express TGF-β1 mRNA (Shah et al., 1996). Clearly, as in mammals, further information on the involvement of various growth factors, and the mechanisms by which they regulate proliferation and differentiation of palatal cells in birds during development, is necessary for further elucidation of avian palate morphogenesis.

In summary, there appears to be a coordinated, complex and co-operative orchestration of various growth factors, catecholamines and prostaglandin's effects, which may be critical in the regulation of various biological phenomena during vertebrate palate morphogenesis.

**Signal Transduction Mechanisms**

Normal development of an organ is profoundly influenced by cell-cell communication. The communication allows respondent cells in a developing organ to receive and process external messages to regulate proliferation, migration and differentiation, which are ultimately involved in establishing the complex structure of an organ. Cell communication can be mediated in several ways: by direct cell-cell contact, cell-ECM interactions, or synthesis and secretion of signaling molecules such as hormones, growth factors, neurotransmitters, that may act in an endocrine, paracrine, or autocrine manner. During the process of cell-cell communication, the secreted signaling molecule (extracellular ligand) binds to a receptor present on the target cells. The binding of the ligand to a membrane receptor induces a conformational change of the receptor, which leads to an intracellular signal transduction cascade(s) linking events at the cell surface to those in the cytoplasm and/or nucleus.

Although the overall design of a signaling cascade, which consists of a series of proteins
(Morgan, 1989; Hardie, 1995), are evolutionarily conserved (Ahn et al., 1992; Dickson and Hafen, 1994; Marshall, 1994), their functional relevance may vary according to the ontogenetic history of the cell. Signaling cascades are mediated by seven membrane spanning G protein-linked receptors, ion channel-linked receptors, and enzyme-linked receptors associated with catalytic functions (Alberts et al., 1994), which allow for rapidly switching the activities of cellular proteins from one state to another (Alberts et al., 1994), and in turn induce changes in the cell machinery regulating cell proliferation and differentiation.

**Protein Kinases**

Protein kinases are a group of cellular enzymes involved in the relay of signals from the cell surface to intracellular targets (Hunter, 1995): The protein kinases, along with protein phosphatases, play critical roles in regulating and coordinating gene expression; growth, proliferation, differentiation, motility and death of cells; and cellular metabolism (Pelech et al., 1990; Ralph et al., 1990; Freeman and Donogue, 1991; Charbonneau and Tonks, 1992; Hunter and Karin, 1992; Kozma and Thomas, 1992; Lau and Baylink, 1993, Maller, 1993; Mumby and Walter, 1993; Hill and Treisman, 1995). Hundreds of different protein kinases exists in eukaryotic cells (Hunter, 1991). These protein kinases are organized in signaling pathways or cascades (Pelech et al., 1990). A signaling cascade initiates from phosphorylation at the level of a cell surface receptor (caused by ligand binding to receptor), which then leads to phosphorylation of a sequence of proteins until a key metabolic enzyme is phosphorylated and activated (or inactivated), or that of a transcription factor to modulate gene expression (Hunter and Karin, 1992; Jackson, 1992; Karin and Smeal, 1992; Edwards, 1994; Boulikas, 1995). The amplification, feedback, cross-talk, and branching of cascading molecules of signaling pathways can differentially regulate the cell's decision to divide or differentiate, or otherwise change its physiological behaviour (Hunter, 1995). In general, the efficacy of cellular proteins to act as substrates for protein kinases depends not only on the cellular localization of the kinase
relative to the putative substrate, but also on the structural modifications of the substrate protein, and characteristics of the structures of the protein substrates as result of allostERIC interactions (Walsh and VanPatten, 1994).

For protein kinases to function as molecular switches, protein phosphatases, which catalyze the removal of the phosphates from amino acid functional groups, are necessary (Krebs, 1986; Charbonneau and Tonks, 1992). There are many unique protein phosphatases, perhaps as many as there are kinases (Charbonneau and Tonks, 1992; Hunter, 1995). The regulation of their activity can be modulated in the same way as protein kinases, i.e., by phosphorylation/dephosphorylation.

Phosphorylation/dephosphorylation is one of the crucial events in regulation of various cellular functions, and plays a pivotal role in integration of multicellular systems in an organism (Krebs, 1986; Taylor et al., 1992). Phosphorylation involves transfer of the $\gamma$-phosphate of ATP (or GTP, in some cases) to an amino acid residue, for example, serine, threonine, tyrosine, lysine, histidine, etc., in a substrate molecule (Cohen, 1983), which can cause conformational changes in the tertiary structure of the molecule, to change its functional properties (Roach, 1991; Johnson et al., 1996).

In the past, various attempts have been made to classify protein kinases; for example, by phylogenetic trees derived from an alignment of kinase catalytic domain amino acid sequences (Hanks and Hunter, 1995), or by ways in which groups of kinases are activated (Krebs, 1986), or by functional characteristics of kinases such as involvement in the cell cycle (Pelech et al., 1990). In addition, based on the manner in which kinase pathways are regulated, two categories of protein kinases, second-messenger independent or second-messenger dependent, have also been proposed (Krebs, 1986; Sommercorn et al., 1987; Ahn et al., 1992). Within each category of protein kinases (in eukaryotes), there are at least three recognized classes, based on amino acid substrate specificity. These include tyrosine protein...
kinases (generally found on the cell membrane), serine/threonine protein kinases (majority found in the cytosol), and the newly discovered dual-specificity protein kinases, which can add a phosphate to either serine/threonine or tyrosine amino acid functional groups (found in the cytosol). Generally, all protein kinases share very similar catalytic domain primary structures, which range from 250-300 amino acid residues, corresponding to 30 kDa (Hanks et al., 1988), with substrate specificity resulting from differences within short stretches of amino acids in the catalytic domains.

**Second-Messenger Dependent Protein Kinases**

The classical mechanism of signal transduction involves the generation of second messengers that employ G proteins for transfer and amplification of information (Morgan, 1989). Several second-messenger dependent protein kinases have been identified, including cAMP-dependent protein kinase, Protein Kinase C (PKC), and cGMP-dependent protein kinase. These kinases are activated by small intracellular molecules or ions such as cAMP, cGMP, Ca$^{2+}$, inositol 1,4,5-triphosphate, and 1,2-diacylglycerol (Alberts, 1994).

CAMP-dependent protein kinase, also called PKA or cAPK, is a tetramer holoenzyme, consisting of 2 regulatory subunits and 2 catalytic subunits (Taylor and Radzio-Andzelm, 1994). The regulatory subunits prevent catalytic activity when bound to the catalytic subunits. cAMP binds to regulatory subunits of PKA, releasing the catalytic subunits, allowing phosphorylation of various cytoplasmic and nuclear proteins (Walsh and VanPatten, 1994). The substrates for PKA differ in various cell types, thereby contributing to cell-type specificities of cellular responses to cAMP (Walsh and Van Patten, 1994). The catalytic subunits can translocate to the nucleus where they phosphorylate a transcription factor called cAMP-response element binding (CREB) protein, which then binds to a cis acting DNA sequence, called cAMP response element (CRE), and stimulates gene transcription (Lalli and Sassone-Corsi, 1994).
Protein kinase C (PKC), one of the major mediators of extracellularly generated signals, comprises a family formed by at least twelve structurally distinct isozymes, which are selectively expressed in different tissues (Asaoka et al., 1992; Posada and Cooper, 1992; Jaken, 1996; Perletti and Monti, 1996). Most members of the PKC family are activated by the second-messenger, diacylglycerol (DAG) (Nishizuka, 1995). The various isotypes are unique in primary structure, expression pattern, subcellular localization, activation in vitro and in responsiveness to extracellular signals (Dekker and Parker, 1994; Jaken, 1996). Depending on their state of activation, PKC isoforms are found on the cell membrane, cytosol or nucleus (Olson et al., 1993). For long term physiological responses such as cell proliferation and differentiation, a sustained (hours), rather than a transient (minutes), activation of PKC is necessary (Asaoka et al., 1992). Activated PKC phosphorylates a broad range of cellular proteins including receptors, ion channels, contractile and cytoskeletal proteins, nuclear proteins and several proto-oncogenes products (Nishizuka, 1992; Olson et al., 1993; Mahoney and Huang, 1994). In addition, PKC can also activate the mitogen activated protein kinase (MAPK) cascade, which provides a mechanism for signaling to the nucleus (Pelech et al., 1990; Hii et al., 1995; vanBiesen et al., 1995).

The least studied second-messenger dependent kinase is the cGMP dependent protein kinase (cGPK). There are two forms of cGPK in mammals. Type 1 is formed of a homodimer of 150 kDa, whereas type 2 is a monomeric protein of 86 kDa (Hofmann et al., 1992). The physiological targets of cGPK are largely unknown, but are thought to include proteins regulating intracellular calcium and cGMP phosphodiesterase activities.

Second-Messenger Independent Protein Kinases

Second-messenger independent protein kinases represent a large class of protein kinases. These kinases are activated through receptor-mediated signaling pathways, and use phosphorylation and/or guanine-nucleotide binding proteins as links to communicate with
cytosolic and nuclear proteins (Pelech, 1993). Among the best known second-messenger independent protein kinases are the Mitogen-activated protein kinases (MAPK), the Cyclin-dependent protein kinases (CDKs), and Casein kinase 2 (CK2). These protein kinases, as their name suggests, act largely independent of second-messengers, and are activated either by membrane receptor proteins, or through adaptor proteins acting downstream of receptors. The following review focuses only on MAPK and CK2, which are analyzed in the present study on quail palate development.

**Mitogen Activated Protein Kinase**

The MAPKs are one of the most well studied family of second-messenger independent protein kinases. MAPK was discovered by Ray and Sturgill (1987) as 42-44 kDa proteins that phosphorylated myelin basic protein in insulin stimulated 3T3 cells. Alternative names for MAPK, such as extracellularly regulated kinase (ERK), myelin basic protein (MBP) kinase, or microtubule associated protein kinase (MAP-2 Kinase), have been used in the past. More than 20 MAPK homologs have been identified in various organisms including yeast, *Caenorhabditis elegans*, *Drosophila Melanogaster*, and mammals (Mordret, 1993). Currently, three functionally distinct MAPK’s have been recognized in vertebrates (Hunter, 1995): ERK, c-jun N-terminal kinase (JNK) or stress activated protein kinases (SAPK), and the p38 subgroups. The MAPK homolog in each signaling pathway is activated by dual phosphorylation on threonine and tyrosine residues (Pelech et al., 1993). In mammalian cells, usually two closely related MAPK isotypes, ERK1 (44 kDa) and ERK2 (42 kDa), are expressed. There is 90% homology between these two isotypes and thus a functional redundancy is thought to exist (Seger and Krebs, 1995).

The MAPK signaling cascade initiates from a receptor protein tyrosine kinase (i.e., a growth factor receptor), which, upon ligand binding, autophosphorylates its cytosolic domain on a tyrosine residue (Malarkey et al., 1995). The cytosolic domain of the receptor is then
recognized by an adaptor protein, Grb2, which binds to specific phosphotyrosine residues via a SH2 containing region (Lowenstein et al., 1992; Panayotou and Waterfield, 1993). Subsequently, a guanine nucleotide exchange factor, Sos, recognizes and binds to Grb2 via a SH3 domain in Grb2 (Boguski and McCormick, 1993). Sos then binds p21 Ras, a GTPase, which binds and activates Raf protein kinase (Hall, 1994, Marshall, 1995). Raf, a serine/threonine protein kinase, phosphorylates and activates another serine/threonine protein kinase, MAPK kinase (MAPKK, also called MEK, a dual-specificity enzyme) which phosphorylates MAPK (Mordret, 1993; Marquardt et al., 1994, Seger and Krebs, 1995). MAPK then phosphorylates several other cytoplasmic or nuclear targets, such as other protein kinases (S6K, p90rsk, MAPKAP-K2, c-Raf-I, MAPKK), nuclear proteins (Elk-1, c-myc, c-jun, c-fos, lamin A), and cell surface receptors, EGF-R and cPLA2 (Sturgill and Wu, 1991; Alvarez et al., 1991; Takishima et al., 1991; Stokoe et al., 1992; Blenis, 1993; Marais et al., 1993; Davis, 1993; Lin et al., 1993). The activation of these targets leads to subsequent phosphorylation of important regulatory proteins and changes in gene transcription (Ahn, 1993; Pelech et al., 1993; Ruderman, 1993; Davis, 1994).

In addition to the tyrosine kinase membrane receptor linked pathway, MAP kinases have also been suggested to be activated by several converging signaling pathways (Figure 1) involving input from PKC, phospholipase C, PKA, p34<sup>cdc2</sup> and G proteins (Crews and Erickson, 1993, Pelech, 1993; Pelech et al, 1993; Hii et al., 1995). The details of PKC-, PKA-, phospholipase C- or p34<sup>cdc2</sup>-mediated pathways of MAPK activation, unlike that involving Ras activation by tyrosine kinase (and certain G-protein coupled receptors), are not well defined. Some evidence, however, is available to suggest that Ras and PKC can function in a distinct as well as collaborative manner to activate MAPK. Additionally, Raf can also be a direct target for
Figure 1. The MAPK signaling cascade and interaction with other signaling proteins. Modified from Pelech, 1993.
PKC (Marquardt et al., 1994). The MAPK signaling cascade may also be tightly regulated through the activity of structural proteins, which form scaffolds between signaling molecules, and thus coordinate and prevent crosstalk between the functionally distinct MAPK pathways (Faux and Scott, 1996). For example, STE 5 in yeast (Choi et al., 1994) serves to bring the MEKK, MEK and MAPK homologs into close association. Also, an important aspect of MAPK regulation relates to the duration of its activation, i.e., transient versus sustained, specifying cell proliferation versus cell differentiation signals, respectively, in response to receptor tyrosine kinase activation (Marshall, 1995). Various agents, such as growth factors and hormones, which have been shown to regulate cell growth and differentiation in various cells/tissues, also stimulate MAPK activity (Ray and Sturgill, 1988; Tsao et al., 1990; Downen et al., 1993). These agents include insulin, EGF, IGF-I and II, PDGF, NGF, progesterone, and 1-methyladenine (Mordret, 1993; Seger and Krebs, 1995). Also, non-mitogenic stimuli, such as thrombin, bradykinin, electric, UV radiation, osmotic and heat shock, and mechanical stretch, can activate MAPKs (Seger and Krebs, 1995; Davis, 1994).

The mechanisms by which MAPKs are inactivated are poorly understood. There are at least three known MAPK phosphatases that inactivate phosphorylated MAPK by dephosphorylating either of the two phosphorylated residues, threonine or tyrosine (Nebreda, 1994). The tyrosyl phosphatase CD45, the dual specificity phosphatase MPK-1, and the seryl/threonyl phosphatase PP2A are all capable of dephosphorylating ERK1 and ERK2 (Anderson et al., 1990; Alessi et al., 1993; Sun et al., 1993).

Several possible roles for MAPK have been suggested. These include regulation of cellular activities such as induction and maintenance of proliferation and differentiation, and thus cell cycle regulation seen in embryonic and adult cells, as well as those occurring during pathological processes, such as inflammation and oncogenesis. In addition to being activated by numerous extracellular agents, the effects of MAPK and MAPKK mutants on NIH-3T3 cells
further indicate a direct role in the regulation of cell proliferation (Seger and Krebs, 1995; Pages et al., 1994).

MAPK is also thought to play a role in the cell cycle, as its duration of activity has been correlated with its mitogenicity, and it has been shown to be required for progression through the $G_0/G_1$ to S-phase transition in fibroblasts (Meloche et al., 1992; Pages et al., 1993; Brondello et al., 1995).

Recently, MAPK has been also suggested to play a role in the regulation of maturation of oocytes and induction of mesoderm in *Xenopus* (Hartley et al., 1994; LaBonne et al, 1995), development of cell fate specification in *C. elegans* vulva (Lackner et al., 1994; Wu and Han, 1994), and determination during *Drosophila* eye development (Biggs et al., 1994, Brunner et al., 1994; O'Neil1 et al., 1994). These studies indicate that MAPK may be involved in regulation of a variety of biological processes during embryonic development. Information on the involvement of MAPK during morphogenesis of organs/structures in vertebrates, is, however, hitherto unavailable.

Ultimately, the mechanism by which MAPKs regulates various biological processes involves the activation of specific transcription factors (Treisman, 1996). For example, during the induction of immediate early genes, a complex of serum response factor and ternary complex factor must first form in order to bind the DNA and stimulate transcription of genes such as *c-fos* (Treisman, 1994, 1996). It has been shown that phosphorylation of the carboxyl terminus of Elk-1, a transcription factor of the ternary complex family, is mediated by MAPK (Marais et al., 1993). Mutation of the carboxyl phosphorylation sites of Elk-1 leads to altered binding specificity and decreased ability to transactivate *c-fos* expression (Hill et al., 1993). EGF and serum can cause an increase of Elk-1 phosphorylation which is correlated to subsequent *sre* promoter activity (Zinck et al., 1993) to regulate expression of immediate
early genes.

The other two MAPK pathways, i.e., JNK/SAPK and p38, unlike the ERKs, are not activated in response to mitogens but are potentially activated by environmental stress and inflammatory cytokines (Davis, 1994; Kyriakis et al., 1994; Cano and Mahadevan, 1995).

**Casein Kinase 2**

Casein kinase 2 (CK2) is a second-messenger independent protein kinase first isolated by Hathaway and Traugh in 1979. CK2 is a ubiquitous multifunctional serine/threonine kinase found in all eukaryotic organisms (Krebs et al., 1988; Pinna, 1990, 1994; Pinna et al., 1990, 1995; Meisner and Czech, 1991; Tuazon and Traugh, 1991; Issinger, 1993; Litchfield and Luscher, 1993; Litchfield et al., 1994; Allende and Allende, 1995). Vertebrate CK2 is localized mainly in the cytoplasm, but has also been found in the nucleus, mitochondria, and microsomal fraction. It consists of a tetrameric subunit holoenzyme with an $\alpha$, $\alpha'$, and two $\beta$ subunits, of 42, 38, and 28 kDa respectively. The $\alpha$ and $\alpha'$ subunits are catalytic, whereas the two $\beta$ subunits are regulatory, and increase the activity of the enzyme when bound. The holoenzyme can be found with a configuration of $\alpha'$ $\alpha$, or $\alpha'\alpha'$. cDNA sequences of these subunits show that they are encoded by separate genes, which are highly conserved evolutionarily, suggesting critical importance in cell growth and survival (Tuazon and Traugh, 1991; Ahmed et al., 1993). Genetic mutations involving both the $\alpha$ and $\alpha'$ subunits of CK2 are lethal in yeast (Padmanabha et al., 1990), which can be rescued with a functional $\alpha$ subunit from *Drosophila* (Birnbaum and Glover, 1991).

In contrast to most other protein kinases, in which the regulatory subunits or regulatory domains inhibit the catalytic activity, it has been suggested that the regulatory subunit ($\beta$) of CK2 stimulates the catalytic activity (Tuazon and Traugh, 1991). CK2 is further distinguished from most other protein kinases by its effective utilization of both ATP and GTP as phosphate donors and the lack of cycling between active and inactive forms (Tuazon
and Traugh, 1991). Also, in comparison with other protein kinases, the catalytic subunit of CK2 contains two unique amino acids, Val-66 and Trp-176 in subdomains II and VII, respectively. These two amino acids are conserved in more than 95% of other protein kinases as alanine and phenylalanine in the corresponding positions in subdomains II and VII, respectively (Hanks et al. 1988; Benner and Gerloff, 1991; Hanks and Quinn, 1991).

CK2 has been shown to be activated by several agents, indicating that the enzyme plays an important role in signal transduction (Summercorn et al., 1987; Carrol and Marshak, 1989). These agents include insulin (Diggle et al., 1991; Maeda et al., 1991; Villa-Moruzzi and Crabb, 1991), EGF (Ackerman and Osheroff, 1989; Ackerman et al., 1990), serum (Caroll and Marshak, 1989), bombesin (Agostinis et al., 1992), and phorbol esters (Caroll et al., 1988; DeBenedette and Snow, 1991). It was suggested that, since the enzyme phosphorylates a large number of substrates, CK2 activation would affect metabolic pathways, nuclear transport, and transcription/translation processes (Meissner and Czech, 1991; Litchfield and Lascher, 1993). Substrates for CK2 include enzymes involved in nucleic acid synthesis such as RNA polymerase I and II, DNA topoisomerase I and II; transcription factors including nuclear oncogenes products and tumor suppressor factors such as c-jun, c-myc, and p53; signal transduction proteins such as p34cdc2, PKC, and the regulatory subunit of PKA; protein synthesis factors such as elF3 and elF2; and cytoskeleton and structural proteins such as α-tubulin and myosin heavy chain (Allende and Allende, 1995). Phosphorylation of many of these substrate proteins are thought to be important in the progression of the cell cycle. For example, recent observations have shown that microinjected antibodies against the β subunit of CK2 inhibit nuclear accumulation of CK2 and prevent cell cycle progression from the G1 to S phase (Lorenz et al., 1993; Pepperkok et al., 1994). Also, the addition of antisense oligonucleotides directed against α, α' or β subunits of CK2 inhibit quiescent cells from reaching the S phase of the cell cycle (Pepperkok et al., 1991). Additionally, CK2 activity was
shown to be enhanced in tumors (Prowald et al., 1984; Schneider et al., 1986; McManaway et al., 1987; Daya-Makin et al., 1994). Further, CK2 mRNA and protein has been shown to be expressed during mouse embryogenesis (Schneider et al., 1986; Perez et al, 1987; Hu and Rubin, 1990; Mestres et al., 1994) and during chick embryonic development (Maridor et al., 1991). These observations provide the basis for the ubiquitous and multifunctional roles for CK2 in the regulation of various biological phenomena.

**Signaling During Palate Morphogenesis**

The involvement of a few components of intracellular signaling cascades, and their putative regulators have been identified in palatal cells during development. Pratt and Martin (1975), Greene and Pratt, (1979) and Shah et al (1985b) observed that cAMP levels peaked at the time of terminal differentiation of the MEE during mammalian palate development. Co-treatment of palatal shelves with cAMP partially attenuated the inhibitory effect of EGF on the terminal differentiation of the palatal MEE (Pratt and Martin, 1975; Hassell and Pratt, 1977). Subsequently, it was indicated that cAMP and its downstream effector kinase, PKA, may be involved in the regulation of mammalian palatal MEE differentiation (Linask and Greene, 1989).

On the other hand, in mammalian palatal mesenchymal cells, cAMP appears to regulate both the entry of cells into the S phase of the cell cycle (Pisano et al., 1986) and GAG synthesis (Greene et al., 1982). In addition, preincubation of murine embryonic palatal mesenchymal cells with EGF can enhance the level of prostaglandin-induced cAMP (Greene and Lloyd, 1985). Consequently, the modulation of PKA activity by the increased cAMP concentration can delay mesenchymal cell entry into the cell cycle as well as stimulate GAG synthesis (Greene et al, 1982; Pisano and Greene, 1986). The differing effects of cAMP on the MEE and mesenchymal cells further suggest that other factors may be involved in regulation of the pleiotropic roles of cAMP and PKA on cell proliferation and differentiation during palate morphogenesis.
Chepenik and Haystead (1989) observed that in murine embryonic palatal mesenchymal cells, EGF modulate phosphorylation of a PKA-independent 80 kDa protein whose pl, phosphopeptide map, and phosphoamino acid pattern matches those of a 80 kDa protein phosphorylated in response to PMA treatment, a stimulator of PKC. On the basis of these observations, they proposed that PKC may be involved in the regulation of palate mesenchymal cells. In a separate study investigating protein phosphorylation induced by EGF and PMA treatment of murine embryonic palatal mesenchymal cells, Chepenik and Grunwald (1988) observed phosphorylation of this 80 kDa protein, whose function was unclear. Interestingly, further evaluation of their autoradiograph (Figure 2 in Chepenik and Grunwald, 1988) reveals that there are also phosphoproteins induced by EGF and PMA at 44 and 42 kDa, which may represent putative isotypes of MAPK. Indeed, in cultures of hamster embryonic palatal mesenchymal cells, EGF activates both the 42 and 44 kDa isotypes of MAPK (Shah et al., 1995), suggesting that these kinases may be involved in the EGF mediated regulation of palatal cell proliferation and differentiation.
PURPOSE OF THE STUDY

The preceding analysis of the literature indicates that, in the past, efforts were directed toward evaluating the biological aspects of vertebrate palate development mainly at the tissue, cellular and biochemical levels. Although much of the information on vertebrate palate development is derived from studies on mammals, significant data has been gathered from examination of non-mammalian species. Recent studies have indicated that a co-ordinated, complex and co-operative orchestration of various growth factors may be critical in the regulation of various biological phenomena (cell proliferation, ECM synthesis, epithelial-mesenchymal interaction, programmed cell death/cell transformation, etc.) during vertebrate palate morphogenesis. Studies on normal and teratogen-induced cleft palate have led to a concept that cell proliferation is one of the crucial factors for the advancement of palate morphogenesis. An understanding of how the growth factors regulate cell proliferation during palate development is, however, unclear. Hence, the present study was undertaken to investigate: (1) the kinetics of cell proliferation; (2) the activity of two protein kinases, MAPK and CK2, that are implicated in the regulation of cell proliferation; (3) the effect of a growth factor, EGF, on the proliferation of cultured QPMC; and (4) the effect of EGF on the activity of MAPK and CK2 in cultured QPMC.
MATERIALS AND METHODS.

Quail Maintenance and Procurement.

Random bred, wild type Japanese quail (Coturnix japonica) eggs were obtained from the Quail Genetic Resource Center, the University of British Columbia. Fertile eggs were incubated in a Robbin’s incubator (Model I.H.A., Montello Industrial, Denver, Colorado, USA) in an environment of 37.5°C and 50% relative humidity at the Quail Genetic Stock Center. The fertile eggs were used for the following: (1) for whole embryo culture and tritiated-thymidine autoradiography; (2) to obtain quail palatal shelves between days 5 and 9 of incubation for the analysis of protein kinases during in vivo palate development; and (3) to prepare primary cultures of quail palate mesenchymal cells (QPMC) for in vitro analysis of protein kinases.

Whole Embryo Culture

For the whole embryo cultures, the method described by Dunn et al (1981) was followed. The quail eggs were cleaned with 70% alcohol on day 3 of incubation, and the embryos were removed under sterile conditions. In order to obtain the intact embryo and the associated vitelline membranes, a hole, approximately 2 centimetres in diameter, was made through the rounded pole of the eggshell, which violated the shell membrane but not the extra-embryonic membranes. Care was taken to ensure that the edges of the hole were smooth. The egg was inverted and another hole (1-2 mm) created at the opposite pole. Caution was taken to prevent perforation of the extra-embryonic membranes at the second puncture site. The extra-embryonic membranes, along with the yolk and embryo were then allowed to slide out of the egg intact into a cellophane basket (Figure 2). The basket, into which the embryo, yolk, and membranes were transferred for the culture procedure, is composed of a draped sheet of Safeway saran wrap (Safeway brand), which is suspended on an inverted 250 ml tripour beaker that has had its bottom removed. The three pouring lips of the beaker form a tripod on
Figure 2. Shell-less whole embryo culture. The quail embryo was removed from the shell on day 3 of incubation. Care was taken to maintain the integrity of the extraembryonic membranes. The embryo was placed in a cellophane basket suspended on a beaker with the bottom removed.
the incubator tray to allow free circulation of air under the cellophane basket. Since the Safeway brand saran wrap has been shown to be porous, the embryo has access to air from above and below, facilitating air exchange for normal embryonic circulation (Dunn et al., 1981). Prior to use, the tripour beakers were sterilized, the saran wrap was added and then the baskets were positioned under a ultraviolet light for 2 hours to disinfect the saran wrap. The shell-less culture thus prepared allows the embryo to grow and survive normally to the term without any supplemental nutrients (Dunn et al., 1981).

The quail embryos in shell-less culture conditions (i.e., *in vitro*) were incubated at 37.5°C and 5% CO₂ under humidified conditions as described by Dunn et al (1981). In order to assess whether the embryogenesis in shell-less culture conditions resembled that *in vivo*, 10 cultured embryos were obtained on days 2, 3, 4, 5, and 6 of culture (total of 50 embryos), weighed, measured for crown-rump length (CRL) and staged accordingly to the criteria for the development of the chick, described by Hamilton and Hamburger (HH; 1951). A corresponding group of *in vivo* developing quail embryos, i.e., days 5, 6, 7, 8, and 9 of incubation, which is the period of palate morphogenesis in quail (Shah et al., 1985a), were similarly processed. [Previously, Graham and Meier (1975) observed a high correlation between the age of quail and the HH stage of its development, which was subsequently verified for studies on palate development (Shah et al., 1985a, 1994; Shah and Cheng, 1988)].

**Tritiated-Thymidine (³H-TdR) Light Microscopic Autoradiography.**

In order to examine the proliferative behaviour of cells in the developing palate, *in vitro* embryos were labelled on day 2, 3, 4, 5, and 6 of culture with 20 μCi of ³H-TdR in aqueous solution (ICN, Montreal, Canada; Specific Activity 50.8 Ci/mmol) for 1 hour to label cells in the S phase of cell cycle. The ³H-TdR exposed embryos were then removed from the culture environment, decapitated and the heads rinsed three times in 0.2 M phosphate buffered saline
(pH 7.2) to remove any unbound $^3$H-TdR. The heads were immersed in Bouin's fixative for 48 hours, dehydrated in an ascending series of alcohol (30-100%), processed through 3 changes of xylene and embedded in paraffin to obtain frontal sections. Six $\mu$m serial sections were mounted on gelatin coated glass slides, deparaffinized in xylene, and rehydrated through a descending series of alcohol (100-30%-water). The slides were then dipped in Kodak NTB-2 emulsion solution (diluted 1:1 with distilled water) at 42°C, dried, and kept in a dark, dry (packed with Drierite material, Sigma) airtight box at 0-4°C for 8 weeks. The slides were developed with Kodak D-19 and fixed with Kodak fixer. Subsequently, the slides were stained with Haematoxylin and Eosin and coverslipped with permount.

The proliferation indices of the mesenchymal cells were determined from the developing palatal shelves of different ages. $^3$H-TdR labelled cells were counted from three sections from each embryo, simultaneously ensuring that the adjacent section was not used in the analysis, to determine the average number of $^3$H-TdR cells for each embryo. Sections only from the anterior third of the developing secondary palate were used in the analysis because the morphogenesis of quail secondary palate begins anteriorly and progresses posteriorly (Shah et al., 1985a). A total of 15 embryos were counted between days 5 and 9 (HH stages 26-35) to determine the percent mesenchymal cells undergoing proliferation. The proliferation index was defined as the number of labelled cells expressed as a percent of the total cells. A cell was considered labelled when the number of silver grains was 5 or more above the background level. The boundaries of the developing palatal shelf was determined by points where the nasal and oral epithelia of the palatal shelf changed direction.

In order to further determine whether the dividing cells in the developing shelf were distributed randomly or were localized in a particular area, the palate sections were divided into four segments with the aid of a 10x10 marked glass grid inserted into the eyepiece of the
microscope. These segments were constructed from a line dividing: (1) the medial (M) half (towards the growing tip of the shelf) and the lateral (L) half (away from the growing tip of the shelf), or (2) an upper (U) half (away from the tongue) and a lower (L) half (adjacent to the tongue). The mesenchymal cell proliferation index was determined in each of the four segments (U-M, U-L, L-M, L-L). The data were evaluated by Freidman two-way analysis of variance, nonparametric Wilcoxon test or student's t-test (Zar, 1984).

**Analysis of Protein Kinases During In vivo Palate Development.**

**Sample Preparation**

*In vivo* developing quail embryos were obtained at 24 hour intervals between days 5 and 9 of incubation. At each time, secondary palatal shelves were dissected from 70-80 embryos, washed and then pooled into 0.5 ml homogenizing buffer [75 mM β-glycerophosphate, 20 mM MOPS (pH 7.2), 15 mM EGTA, 2 mM EDTA, 1 mM Na$_2$VO$_4$, 1 mM dithiothreitol(DTT)], and sonicated for 30 seconds at 0-4°C. The homogenate was centrifuged at 200,000 g for 15 minutes in a Beckman TL-100 ultracentrifuge at 0-4°C, and the supernatant was immediately frozen at -70°C. A 10 μl aliquot of supernatant was used for protein determination (Bradford, 1976) and bovine serum albumin was used as a standard.

**Fast Protein Liquid Chromatography**

For Fast Protein Liquid Chromatography (FPLC), 1.5 mg protein in 2 ml MonoQ buffer (10 mM MOPS, 25 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 2 mM Na$_2$VO$_4$, 1 mM DTT) was loaded onto a MonoQ column (Pharmacia) at 0-4°C. (A MonoQ column is an anionic exchanger that binds negatively charged proteins and then releases them as the buffer salt concentration increases to a level at which the affinity of the column for the protein is balanced or disrupted by the salt solution. FPLC allows for the separation of cellular proteins based on their charge). The column was eluted with a 10 ml linear 0-0.8 M NaCl gradient in
buffer [25 mM β-glycerophosphate, 10 mM MOPS (pH 7.2), 5 mM EGTA, 2 mM EDTA, 1 mM Na$_2$VO$_4$ and 2 mM DTT] at a flow rate of 0.8 ml/minute. A total of 55 fractions of 250 µl were collected.

**Phosphotransferase Assays**

*In vitro* phosphotransferase activity in MonoQ-chromatographed extracts that were derived from the quail palatal tissue on ensuing days of palate development were analyzed. The fractionated samples were assayed for phosphotransferase activity either towards phosvitin (CK2 substrate) or myelin basic protein (MBP; MAPK substrate). The assay cocktail, in which the assays were performed, was prepared by adding the appropriate substrate (1 µg/µl; Sigma) to a cocktail [25 mM β-glycerophosphate; 10 mM MOPS (pH 7.2); 15 mM MgCl$_2$; 2 mM EGTA; 2 mM EDTA; 1 mM Na$_2$VO$_4$; and 1 mM DTT]. A 5 µl aliquot of each fraction was separately mixed with 15 µl of the assay cocktail, and then 5 µl of γ-$^{32}$P-ATP (final concentration of 50 µM γ-$^{32}$P-ATP; 1500 cpm/pmol) was added for a total volume of 25 µl. The assays were performed for 10 minutes at 30°C to allow the phosphotransferase reaction. At the end of the reaction period, 20 µl of the reaction product from each assay were individually spotted on to 2 cm$^2$ pieces of Whatman P81 phosphocellulose paper (The P81 binds proteins but not unbound γ-$^{32}$P-ATP, which can be washed off). The spotted papers were washed overnight with several changes of 1% (v/v) phosphoric acid and then transferred into 6 ml plastic scintillation vials with 0.5 ml of BCS scintillation fluid (Amersham) and quantitated for radioactivity in a Wallac 1410 Counter (LKB). γ-$^{32}$P activity of the reaction product was counted as CPM and the data were standardized to pmol/min/ml and plotted to determine phosphotransferase activity.

Since MBP may also be recognized by kinases other then MAPK, such as PKC, additional phosphorylation assays were initiated to further clarify MAPK activity in the fractionated
samples (Cicirelli et al., 1988; Pelech et al., 1988). Separate phosphotransferase assays were carried out with R3, a PKC inhibitor (analogue of staurosporine) to determine the activity of PKC in the original assays, or S5, a MAP kinase specific substrate derived from amino acid sequence in MBP recognized by MAPK (Clark-Lewis et al., 1991) which is specifically phosphorylated by MAPK. These supplemental assays were performed in fractions 25-32 only, since these are the fractions in which the MAPK elutes (Sanghera et al., 1992). Also, since there was a phosphotransferase activity towards MBP in the developing palate on days 6, 7, and 8 the assays with R3 and S5 were conducted only on these days. For the R3 assays, 5 µl of R3 (5 µg/µl) was added to the total assay cocktail including MBP such that the total volume of the assay was increased to 30 µl after γ-32P-ATP addition. For the MAP kinase specific substrate, MBP was replaced with 5 µl of the substrate (1 mg/ml) S5 peptide in the original assay cocktail.

**Western Blotting**

Western blotting of the appropriate fractions containing significant phosphotransferase activity was performed using 1.5 mm thick 11% separating polyacrylamide gel and 4% stacking gel. 80 µl of each fractionated sample (2 fractions pooled, 40 µl each) was mixed with 40 µl sample buffer [125 mM Tris/HCl (pH 6.8), 4% SDS, 0.01% bromophenol blue, 10% mercaptoethanol and 20% glycerol], and incubated for five minutes in boiling water before transferring a total volume of 120 µl to the well on the gel. The samples were electrophoresed overnight at 10 mA/gel until the tracking dye reached the bottom of the gel. Following electrophoresis, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 minutes and then placed together with a nitrocellulose membrane (Scheicher and Schuell) to form a sandwich. Electrophoresed proteins were transferred onto the nitrocellulose membrane for 3 hours at 300 mA in a Hoefer transfer apparatus maintained at 0-4 °C. The membrane was blocked with Tris-buffered saline (TBS;
20 mM Tris, 0.5 M NaCl, pH 7.5) containing 3% bovine serum albumin for 1 hour at 20°C. Subsequently, the membrane was washed twice with TBS containing 0.05% Tween 20 (T-TBS) for 5 minutes, and incubated overnight with the primary antibody at 20°C. The next morning, the membrane was washed twice with T-TBS and incubated with the secondary antibody for two hours at 20°C. Next, the membrane was rinsed three times with T-TBS, followed by TBS, and then with alkaline phosphatase buffer (AP buffer; 0.1 M NaHCO₃, 10 mM MgCl₂, pH 9.8). Finally, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma)/ nitro blue tetrazolium (NBT; Sigma) color development solution (1.5% BCIP in 340 µl 10% dimethyl formamide and 3% NBT in 680 µl 70% dimethylformamide subsequently added to 100 ml of AP buffer). The color was developed between 15 minutes to 2 hours at 20°C in the dark, and the reaction was stopped by rinsing the membrane with water.

**Primary Antibodies**

**MAPK antibodies**

The methods for the production of the MAPK antibodies are described by Sanghera and associates (1992). The MAPK polyclonal antibodies, raised in rabbit, are specific for amino acid sequences of rat erk1 subdomain III (R1-ERK-III) and its C-terminus (R2-ERK-CT) (Boulton and Cobb, 1991). GEGA, a polyclonal antibody from rabbit, is directed against the ATP-binding site of sea star p44mpk (Posada et al., 1991). 4G10, a monoclonal antibody from mouse (UpState Biotechnology, Cat. # 05-321) is directed against phosphorylated tyrosine residues (Sanghera et al., 1992).

**CK2 antibodies**

The methods for the production of the CK2 antibodies are described by Charlton and co-workers (1992). Briefly, CK2-III was raised against synthetic peptides of subdomain III of the human CK2 α subunit and CK2-NT was raised against synthetic peptides based on the
N-terminus of Drosophila CK2 α subunit.

Secondary Antibodies

Goat anti-mouse or goat anti-rabbit secondary antibodies (Bio-rad) were linked to alkaline phosphatase.

In vitro Analysis of Protein Kinases in Quail Palate Mesenchymal Cells (QPMC).

Primary Culture of QPMC

All techniques and protocols were performed under sterile conditions. For primary culture of QPMC, the secondary palatal shelves from embryos, obtained on day 7 of incubation, were dissected and rinsed in ice-cold (0-4°C) Calcium Magnesium free/Phosphate Buffered Saline (CMF/PBS). The dissected palatal shelves were then pooled, minced finely with a razor-blade, and incubated in 3 ml of trypsin/EDTA solution [0.025% trypsin and 0.27 mM EDTA (GIBCO; Cat. No. 610-5305AG) in CMF/PBS] in a 15 ml plastic culture tube for 10 minutes at 37°C. The tube was continuously shaken with a gentle motion during the incubation. Subsequently, the sample was centrifuged at 90g for 3 minutes at room temperature to pellet the cells, and the supernatant was discarded. The action of trypsin was inhibited by suspending and washing the pellet in 6 ml of ice-cold complete media (CM; Dulbecco's Modified Eagle Medium (DMEM) (Stem Cell Technologies, Vancouver, Cat. No. HBM 2200) + 10% Fetal Calf Serum (FCS; GIBCO, Cat. No. 26140-038). High Glucose (4500 mg/l D-glucose) DMEM was supplemented with 1 mM sodium pyruvate, 25 mM Hepes and antibiotics (60 mg/l penicilllin and 100 mg/l streptomycin). The cells were then centrifuged at 33g for 15 minutes at room temperature. The supernatant was discarded and the cells were resuspended in 3 ml CM. In order to determine the total number of cells in the suspension, trypan blue exclusion method was used. 50 μl of the cell suspension was mixed with 40 μl of 0.2 % trypan blue and 410 μl
of CM, and vortexed. One drop of the mixture was placed on the haemocytometer and live cells were counted. The total number of cells in the suspension was calculated as the average number of cells/haemocytometer grid x 10 (i.e., 50 μl of cell suspension in 500 μl of mixture) x 10^4 (conversion factor for haemocytometer grid to determine number of cells per ml) x 2.95 ml (total volume of suspension). Cells were then suspended in an appropriate volume of CM to obtain 2.5 x 10^5 cells/ml/plate and seeded into 35 mm sterile plastic culture plates (Falcon, Cat. No. 3001). Cultures were maintained at 37°C with 5% CO₂ and 100% relative humidity.

The culture media was changed on day 1 of plating, and every second day thereafter.

**Growth Behaviour of QPMC in Primary Culture.**

In order to analyze the growth behaviour of QPMC in primary culture, cells were counted on days 1, 3, 5, 7, 10 and 15 post plating. To count the cells, the media from the culture plate was discarded, and the plate was incubated with 1 ml trypsin (1 mg/ml) in the water-bath at 37°C for 3-5 minutes. Subsequently, the cells were detached by gentle pipetting with a pasteur pipette and transferred into a glass tube containing 0.5 ml of CM. To each plate, 0.5 ml of CM was added and the remaining attached cells, if any, were scraped off the plate with a plastic scraper, "Cell Lifter" (COSTAR, Cat. No. 3008). The cell with CM from the plate were added to the suspension in the glass tube. After pipetting up and down a few times, 95 μl of cell-suspension and 5 μl of 0.2% trypan blue were mixed and vortexed in an eppendorf tube. One drop of suspension was placed on the haemocytometer and the live cells were counted. The total number of the cells was determined as the average cell number on the haemocytometer grid x 10^4 (conversion factor for haemocytometer grid to determine number of cells per ml) x 2 ml (total volume of suspension). At each time, three plates were counted to determine the mean of one experiment. Each experiment was repeated three times. The mean and standard deviation were determined.
EGF Treatment of Cell Culture

In order to analyze the effect of EGF on the rate of proliferation of QPMCs, the cultured cells were maintained in CM for 3 days post-plating. They were then washed three times with serum-free DMEM and maintained in serum-free DMEM for 24 hours for synchronization. The plates were again washed three times with 1 ml serum-free media and then treated with EGF (20 ng/ml; GIBCO; Cat # 3247SA) in serum-less media or CM alone. Previously, Tyler and Pratt (1980), Grove and Pratt (1984), Silver et al (1984), and Pisano and Greene (1987) have used 20 ng/ml EGF in their growth response studies on cultured mammalian palate mesenchymal cells. After 24 hours, the plates were washed with CM, and maintained in the fresh CM. Growth-curves for serum treated (control) and EGF treated cells were obtained, as described in the preceding section.

Growth Factor Stimulation of MAPK Activity

Protein kinase assays were performed on cultured QPMC treated with EGF or complete media alone as a negative control, to determine if the MAPK or CK2 signalling pathways were activated by EGF. Pre-confluent cultures were rinsed and serum starved for 24 hours as described above. After starvation, the cultures were treated with either serumless media or with EGF for 4 or 8 minutes since maximal MAPK activity occurred during this period, as indicated by a time-course study (Figure 17). In other systems, including cultured mammalian palate mesenchymal cells (Shah et al., 1995), MAPK activity also peaks after 4-8 minutes of treatment with EGF (Chao et al., 1992). At the conclusion of the treatment period, the media was drained off, and 1 ml homogenizing buffer (0-4°C) was added to the culture plate. The cells were scraped off the culture dishes with a plastic scraper “Cell Lifter” (COSTAR, Cat. No. 3008), and the cell suspension was collected in a 50 ml plastic culture tube. Next, the suspension was centrifuged at 90g for 5 minutes to pellet the cells, which were resuspended in 0.5 ml homogenizing buffer. After collection of the cells in 0.5 ml homogenizing buffer,
subsequent protocols for protein isolation, total protein determination by Bradford method, FPLC, phosphotransferase assays, scintillation counting, standardization to pmol/min/ml, and Western Blotting were similar to that described for the in vivo collected samples, and are, therefore, not repeated.
RESULTS

Whole Embryo Culture

Figures 3 outlines the data on growth characteristics [weight and crown rump length (CRL)] of in vivo and in vitro developing embryos during the period of quail palate development. Between days 5 through 9 of incubation, embryos developing in vivo showed a gradual increase in weight, from 160 mg on day 5 of incubation to 1150 mg on day 9 of incubation (Figure 3a). Similarly, the embryonic weight in vitro climbed steadily from 155 mg on day 2 of culture to 840 mg on day 6 of culture (i.e., equivalent of day 5 and 9 of incubation in vivo, respectively). Further analysis of the data in figure 3a revealed that on days 5 and 6 of incubation, equivalent of days 2 and 3 of culture, the embryonic weight in both groups was similar (p<0.05). On days 7, 8, and 9 of incubation, or days 4, 5, and 6 of culture, however, the cultured embryos were lighter than the corresponding in vivo developing embryos (p<0.05).

During in vivo development, the CRL of the embryos increased gradually from 2.0 cm on day 5 to 2.75 cm on day 9 of incubation (Figure 3b). Similarly, during days 2 through 6 of shell-less culture, the embryonic CRL increased from 1.55 cm to 2.6 cm (Figure 3b). When the two conditions, i.e., in vivo and in vitro development, were compared for changes in embryonic CRL, there was no significant difference.

To further investigate more precisely whether any morphological differences were apparent between the in vivo and in vitro developing embryos, both groups of embryos were staged according to HH staging method (Hamburger and Hamilton, 1951). In both groups, the HH developmental stage progressed from stage 26-27 on day 5 (day 2 in vitro) to stage 34 on day 8 (day 5 in vitro) and stage 35 on day 9 (day 6 in vitro) of incubation indicating that there were no developmental staging differences between the two groups. Based on these findings, in
Figure 3.
Comparison between the weight (a) or crown-rump length (CRL) (b) of the *in vivo* versus *in vitro* quail embryos during the period of secondary palate development. The embryos were removed from the shell or from shell-less culture on the respective day of incubation, then weighed and measured for CRL. (*P*<0.05; Student's t-test).
the subsequent experiments, the embryos were grouped according to the HH developmental stages.

The data on the viability of the in vitro developing quail embryos are summarized in figure 4. Initially, the survival rate of the embryos was poor on days 1 and 2 of culture (equivalent to days 4 and 5 of incubation); a total of 42% embryos died by the end of day 2 of culture. Subsequently, on days 3 and onwards of culture, the viability of the developing quail embryos was similar to that seen in vivo (p<0.05).

Tritiated-Thymidine (³H-TdR) Light Microscopic Autoradiography

The spatio-temporal distribution of ³H-TdR labelled cells, at different times during quail palate development, is shown in figure 5a-c. It is clear from the figures that there are spatio-temporal changes in the pattern and quantity of labelled cells during palate development. Furthermore, the figures demonstrate that the labelled cells appear to be distributed randomly throughout the mesenchyme and epithelium.

The data on mesenchymal cell proliferation indices during quail palate formation are summarized in figure 6. One may deduce from the figure that approximately one-third of the mesenchymal cells were dividing on days 5 and 6 of incubation (HH stages 27-29). Subsequently, as the palate morphogenesis advances, the rate of proliferation declined, and on day 9 of incubation (HH stages 34-36) it was reduced by approximately a third of that seen on day 5 of incubation (p<0.01).

The spatio-temporal distribution of dividing cells is summarized in figure 7. The data shows that on day 5 of incubation, i.e., at the time of appearance of the palatal primordia, fewer dividing mesenchymal cells were present in the LO-ME segment than in the other three segments (U-ME=U-LA=LO-LA) where they were equally distributed (p<0.01). On day 6 of incubation, the percent dividing cells remained unchanged in the U-ME and LO-LA segments. It,
Figure 4. Viability of quail embryos in shell-less culture. Embryos were isolated on day 3 of incubation, then cultured for six days, equivalent to days 4 through 9 of incubation in vivo. Percent non-viable refers to the percent of embryos dying as a fraction of the total embryos cultured. In vivo incubation days have been used on the horizontal axis for comparison with later results.
Figure 5a. Frontal section of the developing quail palate showing distribution of $^3$H-thymidine labelled cells. Palatal primorida on day 5 of incubation (culture day 2; HH stage 27). The labelled cells appear as dark dots. x 190

Figure 5b. Frontal section of the developing quail palate showing distribution of $^3$H-thymidine labelled cells. Horizontal palatal shelf on day 6 of incubation (culture day 3; HH stage 29). The labelled cells appear as dark dots. x 190

Figure 5c. Frontal section of the developing quail palate showing distribution of $^3$H-thymidine labelled cells. Approximating palatal shelves on day 9 of incubation (culture day 5; HH stage 35). The labelled cells appear as dark dots. x 190
Figure 6.
Profile of the rate of mesenchymal cell proliferation in the developing palate of quail at different times during incubation (culture). *In vivo* incubation days have been used on the horizontal axis for comparison with later results.

Figure 7.
Profile of the rate of mesenchymal cell proliferation in different segments of the developing palate of quail at different times during incubation (culture). *In vivo* incubation days have been used on the horizontal axis for comparison with later results.
however, approximately doubled in LO-ME and decreased by 28% in the U-LA segments. The rank order of proliferation rate was LO-ME, LO-LA, U-ME and U-LA. On day 7 of incubation, the distribution pattern of dividing cells changes dramatically. Although in all four segments the percent dividing cells were decreased, the dramatic differences were only seen in the LO-ME and both lateral segments (p<0.01). The rank order of the proliferation rate was U-ME, LO-LA=U-LA, and LO-ME. Subsequently, on day 8 of incubation, when quail palatal shelves approximate (Shah et al., 1985a), the percentage of labelled cells decreased further in the U-ME segment (p<0.01). Consequently, the dividing cells were distributed equally among all four segments. On day 9 of incubation, the percent labelled cells, although increased in all four segments, were distributed equally in all segments (p<0.01).

Analysis of Protein Kinases During in vivo Palate Development

Mitogen Activated Protein Kinase (MAPK)

Phosphotransferase Assays

The results of phosphorylation assays using myelin basic protein as a substrate, performed between days 5 and 9 of incubation, are outlined in figure 8. MAPK typically elutes from the MonoQ column in fractions 25 through 32 under the fractionation parameters used in the present study (Sanghera et al., 1992). Hence, the fractions were used to compare the enzyme activity at various times during palate morphogenesis. Between days 5 and 6 of incubation, when the horizontally growing palatal shelves showed a high rate of cell proliferation (Figure 6), phosphotransferase activity was low (Figure 8). Subsequently, on days 7 and 8 of incubation, the phosphotransferase activity increased. In comparison to day 5, the enzyme activity was four-fold higher on day 8 of incubation, at a time when the palatal shelves approximated and the proliferation declined (Figure 6). The MBP phosphotransferase activity then plummeted on day 9, when it was 50% of that observed on day 5 of incubation.

Additional phosphorylation assays were performed in fractions 25 through 32,
Figure 8. Phosphotransferase activity towards MBP in the developing quail secondary palate in vivo. Secondary palates were dissected on days 5 through 9 of incubation and processed for phosphotransferase assays, using MBP as a substrate, as described in the Materials and Methods.
obtained on days 6-8 of incubation, using a specific MAPK substrate (S5) or by adding a PKC inhibitor (R3) to the MBP phosphotransferase assays. The results showed that, in comparison to MBP (Figure 9a), the levels of phosphotransferase activity were greatly reduced when S5 was used as a substrate (Figure 9c), suggesting that activated MAPK was present in the developing quail palate, albeit in a low amount. The phosphotransferase activity towards S5, however, increased with advancing palate morphogenesis, showing a trend similar to that seen in the MBP phosphotransferase assays. On the other hand, the addition of R3 to the MBP phosphorylation assays showed a decreasing phosphotransferase activity between days 6, 7, and 8 of incubation (Figure 9b), which may indicate a temporal increase in active PKC (in the fractions tested) as palate development progressed.

In general, in the present study, since MAPK was confirmed in fractions 26-31 by Western blotting (see below), the area under the curve of phosphotransferase assays of these fractions were summated (Figure 10. The experiments were repeated three times. The data are representative of a given experiment). The results showed that phosphotransferase activities towards both MBP and S5 increased temporally as palate development progressed. When the protein kinase C inhibitor was added to the fractions in the presence of MBP, the phosphotransferase activity towards MBP dropped between days 6 through 8 of incubation, suggesting a temporal increase in the activity of protein kinase C in the developing palate.

**Western Blotting:** Since several protein kinases have the ability to phosphorylate MBP (Cicirelli et al., 1988; Pelech et al., 1988), Western blot analysis was performed using R2-ERK-CT or R1-ERK-III, polyclonal antibodies against MAPK. The presence of MAPK was determined in the fractions that possessed phosphotransferase activity towards MBP (Figure 11a,b). A strong immunoreactivity against a 42 kDa MAPK isoform was observed in fractions 26 through 31 on all five days of quail palate development. Additionally, MAPK was investigated using GEGA (to detect the ATP-binding domain) and 4G10 (to determine the level of tyrosine
Figure 9. The phosphorylation assays were performed on those fractions in which MAPK typically elutes from a MonoQ column under the fractionation parameters used in the present study. Figure 9a shows the phosphorylation assays towards MBP. Figure 9b shows the phosphotransferase activity towards MBP in the presence of a PKC inhibitor (R3). Figure 9c shows the phosphotransferase activity towards S5, a MAPK specific substrate.
Figure 10. Summated phosphotransferase activities during quail secondary palate development. The total phosphotransferase activity in fractions 25 through 32, where MAPK immunoreactivity was observed in Western Blots, was determined by taking the area under the curves from figure 9. Only days 6, 7, and 8 were analyzed since on these days, the phosphotransferase activity towards MBP was the highest.
Figure 11a. Detection of MAPK during *in vivo* development of quail palate using R2-ERK-CT antibody (indicated by the arrows). Western blots of quail palatal shelves obtained on days 5 through 9 of incubation. Samples were fractionated on a MonoQ column, and fractions 20-33 were subjected to SDS-PAGE. Lane 1 through 7 = pooled fractions 20+21(1); 22+23(2); 24+25(3); 26+27(4); 28+29(5); 30+31(6); and 32+33(7), respectively. For each lane, 40 μl of each of the fractions (total 80 μl), and 40 μl sample buffer were mixed and boiled for 5 minutes prior to loading a total volume of 120 μl onto the gel. The molecular weights (kDa) of the marker proteins are shown on the left.
Figure 11b. Detection of MAPK during in vivo development of quail palate using R1-ERK-III antibody (indicated by the arrows). Western blots of quail palatal shelves obtained on days 5 through 9 of incubation. Samples were fractionated on a MonoQ column, and fractions 20-33 were subjected to SDS-PAGE. Lane 1 through 7 = pooled fractions 20+21(1); 22+23(2); 24+25(3); 26+27(4); 28+29(5); 30+31(6); and 32+33(7), respectively. For each lane, 40 µl of each of the fractions (total 80 µl), and 40 µl sample buffer were mixed and boiled for 5 minutes prior to loading a total volume of 120 µl onto the gel. The molecular weights (kDa) of the marker proteins are shown on the left.
Figure 11c. Detection of MAPK during in vivo development of quail palate using GEGA antibody (indicated by the arrows). Western blots of quail palatal shelves obtained on days 5 through 9 of incubation. Samples were fractionated on a MonoQ column, and fractions 20-33 were subjected to SDS-PAGE. Lane 1 through 7 = pooled fractions 20+21(1); 22+23(2); 24+25(3); 26+27(4); 28+29(5); 30+31(6); and 32+33(7), respectively. For each lane, 40 µl of each of the fractions (total 80 µl), and 40 µl sample buffer were mixed and boiled for 5 minutes prior to loading a total volume of 120 µl onto the gel. The molecular weights (kDa) of the marker proteins are shown on the left.
**Figure 11d.** Detection of MAPK during *in vivo* development of quail palate using 4G10 antibody (indicated by the arrows). Western blots of quail palatal shelves obtained on days 5 through 9 of incubation.

Samples were fractionated on a MonoQ column, and fractions 20-33 were subjected to SDS-PAGE. Lane 1 through 6 = pooled fractions 20+21(1); 22+23(2); 24+25(3); 26+27(4); 28+29(5); and 30+31(6), respectively. For each lane, 40 μl of each of the fractions (total 80 μl), 40 μl sample buffer were mixed and boiled for 5 minutes prior to loading a total volume of 120 μl onto the gel. The molecular weights (kDa) of the marker proteins are shown on the left.
phosphorylation) antibodies (Figure 11c,d). GEGA antibody detected a signal at 42 kDa on all five days of palate development. Western blotting with the 4G10 antibody showed weak bands of 42 kDa in fractions 26-31 on days 5 through 9 of incubation (Figure 11d) indicating that an activated form of MAPK was present during quail palate morphogenesis.

To further investigate the temporal expression of the 42 kDa MAPK isoform, Western immunoblotting was performed using crude homogenate unfractionated samples obtained during the period of palate morphogenesis using the R2-ERK-CT, GEGA, and 4G10 antibodies (Figure 12). The results showed that although the enzyme activity displayed temporal changes, the level of protein between days 5 and 9 of incubation remained constant. This would suggest a post-translational regulation of MAPK activity.

To analyze the biological significance of the enzyme activity during quail palate development, the summated phosphotransferase activities towards MBP at different days of palate development (days 5 through 8 of incubation) were correlated with the rate of cell proliferation, as measured by tritiated-thymidine light microscopic autoradiography (Figure 6). A negative correlation was found between the phosphotransferase activity and the proliferation index ($r = -0.889$) indicating that the MAPK activity may or may not be associated with cell proliferation during quail palate development.

**Casein Kinase 2 (CK2)**

**Phosphotransferase Assays**

Figure 13 shows the results of phosphotransferase activity towards phosvitin, a substrate for CK2, during morphogenesis of quail palate. The major phosphotransferase activity was found in fractions 37-40, which is consistent for the elution of CK2 through a MonoQ column in a 0-0.8 linear NaCl gradient (Sanghera et al., 1992). The phosphotransferase activity was high on days 5 and 6 of incubation, and subsequently declined on days 7, 8 and 9 of incubation indicating that the CK2 enzyme activity was decreasing with advancing palate...
**Figure 12.** Detection of MAPK during *in vivo* development of quail palate (indicated by the arrows). Western blots of crude proteins obtained from quail palatal shelves between days 5 and 9 of incubation, using R2-ERK-CT, R1-ERK-III, and 4G10 antibodies. Samples of 100 ug crude protein were subjected to SDS-PAGE. The molecular weights (kDa) of marker proteins are shown on the left of each gel.
Figure 13. Phosphotransferase activity in the developing quail secondary palate in vivo. Secondary palates were dissected on days 5 through 9 of incubation and processed for phosphotransferase assays, using phosvitin as a substrate, as described in the Materials and Methods.
Western Blotting

To determine whether the phosphotransferase activity in fractions 37-40 towards phosvitin was specifically due to CK2, a polyclonal antibody directed against subdomain III of CK2-α (CK2-III) was used for Western blot analysis. Figure 14 show the Western blot, obtained from fractions 35-43, treated with CK-2-III antibody between days 5 and 9 of incubation, and demonstrates the presence of a strong 42 kDa band, which is the molecular weight of the α subunit of CK2 (Litchfield and Lascher, 1993). Additionally, a weak band is also present in fraction 39-40 at 38 kDa on days 7 and 8 of incubation, which corresponds to the α' subunit of CK2 (Litchfield and Luscher, 1993). Thus, CK2 appears to be responsible for the major peak in phosphorylation towards phosvitin during palate development.

Figure 15 shows immunoblots of fractionated samples between days 5 and 9 of incubation showing the presence of CK2 using the CK2-NT antibody, a polyclonal antibody directed against the amino terminus of the catalytic domain of the α subunit. The CK2-NT antibody detected α subunit at 42 kDa in the fractions that showed high phosphotransferase activity towards phosvitin.

To demonstrate that the decline in CK2 phosphotransferase activity was not due to a change in CK2 protein during development, immunoblots prepared with crude homogenate of palatal tissue obtained between days 5 and 9 of development were analyzed with CK2-III antibody (Figure 16). Strong 42 kDa bands, reflecting CK2 α, were present on all days of palate development, suggesting that although the activity of enzyme CK2 changed, the CK2 protein levels remained unchanged during quail palate development. This would indicate that the activity of CK2 enzyme observed during quail palate development would be regulated at a post-translational level rather than through changes in CK2.

To analyze the biological significance of the decreasing phosphorylation activity of
Figure 14. Detection of CK2 during *in vivo* development of quail palate using CK2-III antibody (indicated by the arrows). Quail palatal shelves were obtained between days 5 and 9 of incubation. Samples were fractionated on a MonoQ column, and fractions 35-43 were subjected to SDS-PAGE. Lane 1 through 7 = fractions 35(1), 37(2), 38(3), 39(4), 40(5), 41(6), and 43(7). For each lane, 80 μl of the fractions was mixed with 40 μl sample buffer and boiled for 5 minutes prior to loading a total volume of 120 μl onto the gel. The molecular weights (kDa) of the marker proteins are shown on the left.
Figure 15. Detection of CK2 during in vivo development of quail palate using CK2-NT antibody (indicated by the arrows). Quail palatal shelves were obtained between days 5 and 9 of incubation. Samples were fractionated on a MonoQ column, and fractions 35-43 were subjected to SDS-PAGE. Lane 1 through 7 = fractions 35(1), 37(2), 38(3), 39(4), 40(5), 41(6), and 43(7). For each lane, 80 µl of the fractions was mixed with 40 µl sample buffer and boiled for 5 minutes prior to loading a total volume of 120 µl onto the gel. The molecular weights (kDa) of the marker proteins are shown on the left.
Figure 16. Western blot of crude proteins obtained from quail palatal shelves between days 5 and 9 of incubation using CK-2-III antibody (indicated by the arrow). Samples of 50 ug crude protein were subjected to SDS-PAGE. The molecular weights (kDa) of marker proteins are shown on the left.
phosvitin during quail palate development, the kinase activity was compared with the proliferation index, as measured by tritiated-thymidine light microscopic autoradiography (Figure 6). The phosphotransferase activity in the fractions which contained CK2 enzyme as determined from the Western blotting, were summated for each day of palate development (days 5 through 8 of incubation). The correlation coefficient between the enzyme activity and cell proliferation index and DNA synthesis was determined. A high positive correlation \( r=0.967 \) was observed between the phosphotransferase activity and the cell proliferation index.

**Analysis of Protein Kinases in Quail Palate Mesenchymal Cell (QPMC) Cultures**

**Proliferative Behaviour of QPMC in Primary Culture**

*In vitro* analysis of protein kinases was initiated by establishing a primary culture of quail palatal mesenchymal cells obtained on day 7 of incubation. Figure 17 outlines the growth behaviour of QPMC in primary culture maintained in media containing 10% fetal bovine serum. After a small growth during the initial 24 hours of culture, the number of QPMC underwent gradual growth, reaching approximately 2 million cells per plate, indicating a 8 fold growth, on day 15 of culture.

**Effects of EGF Treatment on Proliferative Behaviour of QPMC in Primary Culture**

Following the addition of EGF (20 ng/ml) to the culture medium (Figure 18), the cell number were comparable to control until day 5 after EGF treatment. Subsequently, the EGF exposed cultures grew faster, in comparison to control, and were 50% higher on day 10 of incubation.

**Effects of EGF on MAPK Activity in QPMC**

**Phosphotransferase Assays**

In the initial study, the phosphotransferase activity towards MBP was analyzed in the homogenate, obtained from cultured QPMC following different durations of exposure to EGF.
Figure 17. Growth behavior of QPMC in vitro. QPMC were plated at an initial density of 250,000 cells per 35 mm culture plate and maintained in DMEM with 10% fetal calf serum. Media was changed every second day. Using trypan blue exclusion method, cells were counted on days 1, 3, 5, 7, 10, and 15 after plating as described in Materials and Methods.
Figure 18. QPMC in primary culture were treated with EGF for 24 hours after a serum-free period of 24 hours. Control cells were starved also for 24 hours, and then maintained in 10% serum for the duration of the culture period. Media was changed every second day. A growth curve was obtained using trypan blue exclusion method on days 1, 3, 5, 10, and 15 after EGF treatment. (*P<0.05; Student's t-test)
The time-course data (Figure 19. The experiments were repeated three times. The data are representative of a given experiment.) showed that, with 10 μl cytosolic aliquot of sample, the peak activation of phosphotransferase activity towards MBP occurred at 8 minutes after EGF addition to the serum-starved culture. With a 5 μl sample, no specific changes in phosphotransferase activity towards MBP was observed. Based on the results of the time-course activation of MBP, 4 and 8 minutes of EGF treatment were employed for further studies to investigate MAPK activation.

The effects of EGF on phosphotransferase activity towards MBP in QPMC are shown in figure 20. Control, serum-starved cultures showed a low level of phosphotransferase activity towards MBP in the fractions containing MAPK. At 4 minutes of EGF treatment, the phosphotransferase activity had doubled in the fractions where MAPK elutes, and at 8 minutes the phosphotransferase activity had increased four-fold over control values in the fractions where MAPK elutes.

**Western Blotting**

The results of the Western blotting to determine the presence of MAPK in QPMC are shown in figure 21. The MAPK specific antibody (R2-ERK-CT) detected a protein with an apparent molecular weight of 42 kDa in fractions 26-31 in which the phosphotransferase activity toward MBP was observed (Figure 21). A similar intensity of immunoreactivity was seen in the control, 4 minute and 8 minute EGF treated samples indicating that the differences in kinase activity between the different durations of EGF stimulation were not due to variations in the amount of MAPK present, but to post-translational modifications induced by the signaling pathway downstream of the EGF receptor. To examine the activation of MAPK, the fractions were probed with an antibody raised against phosphorylated tyrosine (4G10) as shown in figure 22. No change was observed in the phosphotyrosine status of the 42 kDa isoform of MAPK before or after treatment with EGF.
Figure 19. QPMC were treated with EGF (20 ng/ml) for the times indicated, then assessed for phosphotransferase activity towards MBP.
Figure 20. The effects of EGF treatment on phosphotransferase activity towards MBP in samples obtained from serum-starved primary cell cultures of QPMC after 0, 4, and 8 minutes of treatment.
Figure 21. Detection of MAPK in cultured QPMC following EGF treatment using R2-ERK-CT antibody (indicated by the arrows). Western blots of QPMC obtained before and 4 and 8 minutes after EGF treatment. Samples were fractionated on a MonoQ column, and fractions 20-33 were subjected to SDS-PAGE. Lane 1 through 7 = fractions 20+21(1), 22+23(2), 24+25(3), 26+27(4), 28+29(5), 30+31(6), and 32+33(7), respectively. For each lane, 40 μl of each of the fractions was pooled and mixed with 40 μl sample buffer, boiled for 5 minutes prior to loading a total volume of 120 μl. The molecular weights (kDa) of the marker proteins are shown on the left.
Figure 22. Detection of MAPK in cultured QPMC following EGF treatment using 4G10 antibody (indicated by the arrows). Western blots of QPMC obtained before and 8 minutes after EGF treatment. Samples were fractionated on a MonoQ column, and fractions 20-33 were subjected to SDS-PAGE. Lane 1 through 7 = fractions 20+21(1), 22+23(2), 24+25(3), 26+27(4), 28+29(5), 30+31(6), and 32+33(7), respectively. For each lane, 40 μl of each of the fractions was pooled and mixed with 40 μl sample buffer, boiled for 5 minutes prior to loading a total volume of 120 μl. The molecular weights (kDa) of the marker proteins are shown on the left.
Effects on EGF on CK2 Activity in QPMC

Phosphotransferase Assays

The activity of CK2 in the MonoQ fractions obtained from the QPMC following EGF treatment was assessed by the ability of the samples to phosphorylate phosvitin. The data showed that four and eight minute of EGF treatment of QPMC cultures was sufficient to stimulate phosphotransferase activity towards phosvitin in MonoQ fractions 38-40 (Figure 23). In comparison to control, the phosphotransferase activity towards phosvitin in EGF treated samples increased two-fold in both the 4 and 8 minute stimulated samples.

Western Blotting

To determine whether the fractions showing phosphotransferase activity (38-40) towards phosvitin were indeed due to CK2, fractions 35-43 were probed with a polyclonal antibody raised against subdomain III of CK2 (Figure 24). The results show that both the CK2 $\alpha$ (42 kDa) and $\alpha'$ (38 kDa) subunits were present in fractions 37 through 43 in the control, as well as in 4 minute and 8 minute EGF treated QPMC, i.e., in the same fractions that show the peaks phosphotransferase activity.
Figure 23. The effect of EGF treatment on phosphotransferase activity towards phosvitin in samples obtained from serum-starved primary cell cultures of QPMC after 0, 4, and 8 minutes of treatment.
Figure 24. Detection of CK2 in cultured QPMC following EGF treatment using CK2-III antibody (indicated by the arrows). Western blots of QPMC obtained at different durations after EGF treatment. Samples were fractionated on a MonoQ column, and fractions 35-43 were subjected to SDS-PAGE. Lane 1 through 7 = fractions 35(1), 37(2), 38(3), 39(4), 40(5), 41(6), and 43(7), respectively. For each lane, 80 μl of each of the fractions were mixed with 40 μl sample buffer and boiled for 5 minutes prior to loading a total volume of 120 μl. The molecular weights (kDa) of the marker proteins are shown on the left.
DISCUSSION

Several experimental approaches were used in the present study to investigate the cell proliferation during quail palate development. Initially, an in vivo/in vitro comparison of developing quail was performed to validate the whole embryo culture technique; once the technique was validated, the in vitro model was used to analyze the cell proliferation kinetics during quail palate morphogenesis. Further, a primary cell culture technique was implemented to study the regulation of cell proliferation in quail palatal mesenchymal cells. Finally, the regulation of activity of two second-messenger independent protein kinases, MAPK and CK2, both in vivo and in vitro, were analyzed because these kinases are involved in the regulation of cell proliferation.

Because of the variations in the developmental rates among embryos, numerous criteria have been employed in the past to characterize the general prenatal development of an organism or morphogenesis of a specific structure. For example, in fish, external features were employed to stage the embryo and development of palate (Armstrong and Child, 1965; Shah et al., 1990, 1994). In mammals, weight, CRL, chronological age, and/or numerical ratings based on the external morphological features of the embryos were used to evaluate prenatal development of palate (Grunberg, 1943; Walker and Fraser, 1956; Dostal and Jelinek, 1972; Shah and Travill, 1976a; Ferguson, 1977). In birds, the most commonly used method to characterize general embryonic development has been the criteria developed by Hamburger and Hamilton (1951). In the present study, two growth parameters (weight, CRL) and developmental staging criteria (Hamburger and Hamilton, 1951) were used to compare the in vivo and the in vitro embryos. The analysis of the data showed that, at least on the basis of CRL and HH staging method, the in vitro (whole embryo cultures) embryos developed in a manner similar to their in vivo counterparts. The only significant difference between the in vivo and in
vitro experimental conditions was observed in the weight measurements. During the latter half of the period of observation in the present study, the in vitro embryos weighed less than their in vivo counterparts. Dunn et al (1981) noted that because the embryos were directly exposed to the external environment in the incubator, the weight difference may be due to dehydration of the embryos or temperature fluctuations in the incubator. Earlier, Tuan (1980) also reported a weight difference between the two growth conditions: the in vitro embryos attained 60 percent weight of the in ovo controls. Further, unlike the in vivo procedure, where eggs are rotated during incubation, the in vitro cultures would provide a static environment to the embryos, which could affect the diffusion of nutrient molecules, and thus contribute to the weight differences as development progressed. Additionally, following the exhaustion of ion supplies from the yolk, which is a major source of calcium ions during in vivo development, the embryos under in vitro circumstances could be affected by the reduced availability of calcium during progressive development, as indicated by Koide and Tuan (1989) and Tuan and Nguyen (1987). The data of the present study, however, clearly shows that the reduction in the weight of the in vitro developing embryos did not affect the CRL or HH developmental staging. Hence, the whole embryo culture techniques was employed to study the cell proliferation kinetics during quail palate formation.

A declining pattern of cell proliferation (DNA synthesis) has been noted in various developing embryonic structures such as facial primordia, eye, limb, and pharyngeal plate in birds and mammals (Ede, 1971; Searls and Janner, 1971; Minkoff and Kuntz, 1977, 1978; Summerbell, 1977; Truby, 1983; Bailey et al., 1988; Miller et al., 1993), and thus seems to be a general feature of advancing embryogenesis. In the developing palate of quail, initially, the rate of cell proliferation, as measured by tritiated-thymidine autoradiography, was high. Subsequently, it declined as palate morphogenesis advanced. These observations parallel the biochemically determined declining rates of DNA synthesis described earlier during quail palate formation.
development (Shah et al., 1994). In mammals, the rate of cell proliferation was also high at the
time of primordial (vertical) formation of the palatal shelves, and declined as the vertical
palate morphogenesis advanced (Shah et al., 1994b). During reorientation and fusion of the
mammalian palatal shelves, however, the proliferation rates remained unchanged (Mott et al.,
1969; Jelinek and Dostal, 1974; Nanda and Romeo, 1975; Cleaton-Jones, 1976; Singh and
Moxham, 1993). On the other hand, the profile of DNA synthesis reflecting cell proliferation
kinetics, was different during palate development in fish. In fish, the rate of DNA synthesis was
low during initial palate formation, and it increased only after the palatal form was established
(Shah et al., 1995). Thus, it appear that there are class-specific differences in the timing of
palatal cell proliferation among vertebrates, which may be indicative of differing phylogenetic
and ontogenetic programs acquired during evolution. In birds and mammals, even though the
direction of initial shelf formation and the termination of palate morphogenesis are different, it
is plausible that the similarities in the trends of cell proliferation observed during advancing
palatogenesis may be reflective of utilization of putatively similar cellular mechanisms that
guides acquisition of the shape and volume during palate morphogenesis. In fish, where the
presumptive mesenchyme of the developing palatal shelves was already well differentiated prior
to the outgrowth of the palatal bulges (Shah et al., 1990, 1995), cell proliferation did not seem
to contribute significantly to the volume of the palate during its formation. This would further
suggest that, during the vertebrate palate evolution, differing cellular developmental programs
may also have been acquired to regulate the eventual outcome of palate morphogenesis.
Evolutionarily, in contrast to fish, the ability of the mammalian and avian palatal mesenchymal
cells to maintain their relatively undifferentiated and highly proliferative state for a prolonged
period of time, may provide sufficient flexibility during development to achieve the final form
of the palate.

The data of the present study further demonstrated spatio-temporal differences in the
pattern of proliferation of mesenchymal cells indicating that, as in mammals (Shah et al., 1994), the role of the proliferating cells during quail palate development may also be very complex. Segmental (spatial) analysis revealed that, as the quail palate morphogenesis advanced, the proliferation pattern in different areas of the shelves changed. Overall, during the period of quail palate morphogenesis when the proliferation was high, the lower (oral) half of the mesenchyme showed a greater amount of cell division than the upper (nasal) half. In contrast, in mammals, the upper region of the developing palate had the most rapidly proliferating pools of cells (Shah et al., 1994b). Whether these varying patterns of rapidly proliferating pools of cells in mammals and birds are related to the contrasting morphogenetic patterns of the direction of palatal growth (bird palate in a horizontal direction; mammalian palate in a vertical direction), or in eventual cellular differentiation pathways (lack of programmed cell death of cells in birds or its presence in mammals) is unknown. It, however, appears that the changing pattern of cell proliferation may be indicative of differences in both the initiation and the duration of the cell cycle in each segment, which, in turn, may contribute to formation of a heterogeneous pool of cells in the developing palatal shelves of both birds and mammals.

The overall temporal similarities in the pattern of cell proliferation may indicate that various putative factors or molecules regulating proliferation, and thus possibly the entry or exit of cells through the cell cycle, during palate morphogenesis may be similar in both the mammal and bird. It has been suggested in the literature that the regulation of the cell proliferation involves the activation of several intracellular signaling pathways (Pelech, 1993; Edwards, 1994; Sager and Krebs, 1995). Hence, the temporal activity of two signaling molecules, MAPK and CK2, was analyzed during the in vivo transition of the quail palatal mesenchymal cells from initially high proliferative activity (present study; proliferation phase) to low proliferative activity, which was accompanied by an increase in the synthesis of
specific ECM molecules (Introduction, page 7; differentiation phase).

The *in vivo* data of the present study showed a temporally regulated activation of a 42 kDa (ERK2) MAPK isoform in the developing palate of quail. In contrast to quail palate, both 42 and 44 kDa MAPK isoforms were present in the developing mammalian palate (Young et al., 1994). In other developing systems, various MAPK isoforms have also been identified. Biggs and co-workers (1994) identified a 43 kDa MAPK isoform (DmErkA) during Drosophila eye development. Gotoh et al. (1991) described a 42 kDa MAPK isoform (xeERK2) in *Xenopus* embryogenesis, whereas Lackner and associates (1994) showed the presence of a 42 kDa isoform (MPK-1) associated with *C. Elegans* vulva development. The precise implication(s) of the differences between the presence or absence of different isoforms of MAPK in different vertebrates, and specifically in the developing palate of the birds and mammal are unclear. It has been suggested that they might serve different functions as a consequence of alternative spliced isoforms that display distinct subcellular localization (Boulton et al., 1990). A differential activation of p42 and p44 has been described in platelets (Papkoff et al., 1994), Rat-1 fibroblasts (Anderson and Milligan, 1994), HeLa cells (Heider and Widmer, 1995), porcine granulosa cells (Keel et al., 1995), and ras/mos transformed NIH/3T3 cells (Zhao et al., 1995). Furthermore, in NIH/3T3 cells, p44 (ERK1) and p42 (ERK2) were shown to differ in their ability to activate the transcription factors Elk-1 and c-myc, respectively, both of which are involved in regulation of cell proliferation (Chuang and Ng, 1994). Since p44 (ERK1) is absent in bird palate, it would be interesting to see if quail p42 (ERK2) has the ability to phosphorylate and activate Elk-1, which is only activated by p44 (ERK1) in NIH/3T3 fibroblasts (Chuang and Ng, 1994). Additionally, it is well known that morphogenesis of palate is class-specific. Several events of mammalian palate formation, i.e., formation of vertical shelves, and their reorientation and fusion (Greene and Pratt, 1976; Shah, 1984) are absent in birds (Shah and Crawford, 1980; Koch and Smiley, 1981). It is possible that the
differences in the pattern of morphogenesis between bird and mammal may also be reflected in
the complexity of the various intracellular signaling cascades. Although it is not known whether
the presence of p42 and p44 isoforms of MAPK in mammals relates to a necessity for a larger
repertoire of signal transduction pathways, the fact that the 44 kDa isoform was not found
during avian palate development may be important from a biochemical perspective since the
different isoforms may be regulated differently (Anderson and Miligan, 1994; Afshari et al.,
1993; Oliver et al., 1995), and may have different substrate specificities (Chuang and Ng,
1994). The proposition, however, has been recently challenged by Pages et al (1995), who
indicated that the p42 and p44 MAPK isoforms may be redundant.

In the Introduction section of this thesis, on the basis of the analysis of the literature, it
was postulated that MAPK activation may play a role in the proliferation of cells. The data of the
present study, however, shows a negative correlation between the temporal pattern of cell
proliferation and phosphotransferase activity towards MBP. This may indicate that: 1) either
MAPK may or may not be playing a direct role in stimulating cell proliferation, or 2) MAPK
may be involved in regulating cell differentiation during palate development, or 3) the
complexities associated with heterogeneous pool of cells during the in vivo development of palate
may mask the true role of MAPK in regulating cell proliferation, i.e., the activity of MAPK in a
heterogeneous population of palatal cells may be overshadowed by the multiplicity of biological
processes that occurs with advancing palatogenesis. Hence, a primary culture system was
established to further discriminate the potential role and regulation of MAPK in the developing
quail palate.

The primary cell culture data of the present study showed that QPMC were capable of
sustained growth under in vitro circumstances. Further, EGF was capable of regulating in vitro
proliferation of QPMC. In comparison to controls, the addition of EGF to serum starved primary
cultures resulted in an acceleration of cell proliferation. Earlier, it had been shown that EGF
promotes proliferation of mammalian palatal mesenchymal cells (Yoneda and Pratt, 1981; Gawel-Thompson and Greene, 1989; D'Angelo and Greene, 1991; Dixon and Ferguson, 1992; Chepenik et al., 1994). In addition, EGF has been shown to enhance proliferation of cultured cells from other avian tissues including those from chick otic vesicles, chick limb bud mesoderm, and quail trunk neural crest (Erickson and Turley, 1987; Aona and Ide, 1988; Repesa et al., 1988).

There are no reports in the literature to show that EGF mRNA/protein/receptors are present in the avian palate mesenchyme, although EGF and its receptors have been identified in the developing palate of the mammal (Abbott and Pratt, 1991; Dixon et al., 1991; Abbott and Birnbaum, 1990; Brunet et al., 1993). In addition to EGF, several other growth factors, such as FGF, TGFα, TGF-β, IGF-I, and PDGF have been implicated in the regulation of mesenchymal cell proliferation during mammalian palate formation (Dixon et al., 1993b; Sharpe et al., 1993) or in the development of various embryonic tissues in birds (Goldin and Opperman, 1980; Nag et al., 1985; Erickson and Turley, 1987; Aona and Ide, 1988; Repesa et al., 1988; Stewart et al., 1989; Lau, 1993). It is not yet known, however, whether these growth factors are involved in the regulation of QPMC proliferation or are indeed present in the developing palate of quail. Since null mutations in many of these growth factors show few abnormalities during organogenesis, it is possible that either the regulatory activity of a missing growth factor may be compensated by other growth factors (Derynck, 1986; Torado et al., 1980), or there may be complex interactions between growth factors, their receptors and/or their respective intracellular signaling pathways during morphogenesis of an organ/structure. Cooperation among various growth factors have been observed during the growth, proliferation, and differentiation of many different types of cells. For example, bFGF and TGFβ1 have been shown to invoke synergistic effects during chondrogenesis in cultured mouse periotic mesenchymal cells (Frenz et al., 1994), the production of developmentally competent bovine
blastocysts (Larson et al., 1992), alteration of phenotype in chick sternal chondrocytes (Horton et al., 1989), and the modulation of collagen type II synthesis in chondrocytes (Bradham et al., 1994). Also, in cultured smooth muscle cells, multiple growth factors (including EGF, bFGF, PDGF-BB) can modulate the delayed growth response observed after TGFβ treatment (Stouffer and Owens, 1994). Furthermore, depending upon cell types, various combinations of growth factor(s) have been shown to promote synergistic effects on DNA synthesis (Drago et al., 1991; Dennison et al., 1994; Ip et al., 1994; O'Keefe et al., 1994; Kells et al., 1995; Nilsson et al., 1995; Okazaki et al., 1995; Thommes et al., 1996). In addition, some growth factors possess isoforms which can act as a functional surrogate for the missing growth factor. For example, TGFα performs many embryological functions, while EGF performs many adult functions (Derynck, 1986; Torado et al., 1980; Twardzik, 1985). Both interactions between growth factors and structural/functional redundancies of a particular growth factor, or effect through a common receptor, may allow a greater level of flexibility in the system during development. Hence, hitherto unavailable information on the presence or absence of various growth factors, including their receptors, in the developing palate of quail would be of crucial significance in understanding their possible cooperative role(s), and in analyzing the mechanisms that regulate various biological phenomena, including cell proliferation, during the advancing morphogenesis of palate.

The result of the present study also showed that EGF activated the MAPK pathway in QPMC. Initially, the time course study indicated that MBP phosphorylation activity peaked between 4-8 minutes. Similar time-course activation for phosphotransferase activity towards MBP following EGF exposure have been observed in various other cells (Ahn et al., 1991; Chao et al., 1992; Lamy et al., 1993; Mitev et al., 1995). Western immunoblot data showed that, as in in vivo, only a 42 kDa MAPK isoform was present in EGF-exposed QPMC. In a recent study, EGF-induced MAPK activation has been shown to be associated with proliferation of
cultured mesenchymal cells of the developing mammalian palate (Shah et al., 1995). Hence, it is reasonable to suggest that EGF-induced activation of MAPK may be associated with the proliferation of QPMC, and would thus corroborate the proposal made in the literature on the potential role for MAPK in the regulation of the cell cycle, and thus in cell proliferation (Gotoh et al., 1991; Peter et al., 1992; Lenormand et al., 1993; Pages et al., 1993; Wu et al., 1993; Fujimura, 1994; Brondello et al., 1995). It has been suggested that EGF stimulated MAPK is activated at G0/G1 transition and/or M phase of the cell cycle to support the proliferation of cells (Pelech et al., 1990). In cultured QPMC, because EGF supports cell proliferation, and activates MAPK, it is reasonable to propose that EGF receptors must be available on QPMC for regulation of the MAPK cascade. However, since it is not yet clear whether the EGF is produced by QPMC in vivo or in vitro, or that in vivo EGF affects QPMC in an autocrine/paracrine manner, the precise significance of in vitro correlation between MAPK activation and cell proliferation remains to be defined.

How can the activity of MAPK increase after a stimulation with a mitogen that results in proliferation in vitro, and also become activated in vivo not only during the proliferation but also during the differentiation phase of the palatal mesenchymal cells? It has been suggested that in PC12 cells a transient activation of MAPK may support proliferation, whereas sustained activation of MAPK would support differentiation (Qui et al., 1991; Traverse et al., 1992, 1994; Nguyen et al., 1993; Cowley et al., 1994; Marshall, 1995; Schramek et al., 1996). A prolonged activation of MAPK may allow its translocation to the nuclear compartment where it may activate or inhibit specific transcription factors (Marshall, 1995; Schramek et al., 1996). The proposition provides possible mechanisms by which threshold effects resulting from different receptor and/or ligand concentrations in a tissue may specify the duration of MAPK activation, to influence cellular decisions during development. The regulation of threshold effects via changes in ligand and receptor concentration may produce qualitative
differences in gene expression (Green et al., 1992), which, in turn, may be reflected in the role of MAPK in the intracellular integration of extracellular messages. On the other hand, it has been shown that in fibroblasts, prolonged activation of MAPK is associated with proliferation, the opposite of the result with the PC12 cells (Meloche et al., 1992; Mansour et al., 1994; Cowley et al., 1994). Alternatively, it has been suggested that cell proliferation may occur in absence of MAPK activity (Sakurai et al., 1994). For example, in *Xenopus* oocytes, MAPK becomes inactivated upon fertilization, due to the greatly increased transcription and activity of XCL 100 (a MAPK phosphatase), and remains inactive for the rest of embryogenesis (Hartley et al., 1994). In Saccharomyces cerevisiae, a MAPK homolog is required for G1 arrest of the cell cycle, to inactivate a G1 cyclin necessary for cell cycle progression (Fujimura, 1994). MAPK can be activated in epithelial cells by TGFβ1 and TGFβ2, resulting in inhibition of proliferation through G1 (Hartsough and Mulder, 1995). Also, in *Xenopus* egg extracts, MAPK may stimulate microtubule depolymerization and prevent spindle assembly, to arrest mitosis (Minshull et al., 1994). Thus, it seems that the signaling role of MAPK in the regulation of cell proliferation or differentiation may depend on the cell type, the nature of the stimuli, duration of exposure to a stimuli, and effects of phosphatases and various other regulatory proteins that affect the entry, and exit of cells through various phases of the cell cycle. Information on all these variables would be useful in delineating the putative role of MAPK in the regulation of QPMC proliferation during development. Taken together, however, the *in vivo* and *in vitro* data of the present study clearly suggests an important role for MAPK during the morphogenesis of the quail secondary palate.

MAPK can be upregulated by other protein kinases (Mordret, 1993; Pelech, 1993; Blumer and Johnson, 1994; Cano and Mahadevan, 1995). For example, during the *in vivo* assays in the present study, a peptide R3 (a PKC inhibitor) was used to ascertain the presence
of PKC in fractions containing MAPK. The data indicated that PKC may be upregulated during advancing palatogenesis in quail. PKC has been shown to be a downstream target of Ras during oocyte maturation in Xenopus (Dominguez et al., 1992). More recently, a pathway progressing from TPA through PKC/raf/MEK to MAPK has been described in vitro (Marquardt et al., 1994). In Cos cells, PKC has been shown to activate MAPK (Berra et al., 1995). In addition, during secondary palate formation in mammals, Chepenik and Grunwald (1988) demonstrated the EGF and PMA-induced activation of a PKC like molecule, which was independent of PKA. It seems that MAPK and PKC are activated simultaneously in EGF treated cultures of avian and mammalian palate mesenchymal cells (Chepenik and Grunwald, 1988; Shah et al., 1995; present study). Whether the PKC and MAPK signaling pathways interact (cross-talk) to regulate proliferation of palatal mesenchymal cells during development is not known and should be investigated.

In contrast to PKC, cAMP-dependent protein kinase (PKA) has been intensively investigated during mammalian palate development. During the latter stages of mammalian palate development, the endogenous levels of cAMP (Greene and Pratt, 1979; Shah et al., 1985b), basal levels of PKA activity, levels of regulatory subunits of PKA, and phosphorylation of CREB transcription factor increases transiently (Greene and Pratt, 1979; Greene et al., 1989; Linask and Greene, 1989; Weston and Greene, 1995). In contrast, cAMP levels remain steady during avian palate morphogenesis (Shah et al., 1987, 1988). Additionally, cultured mammalian palatal mesenchymal cells respond to cAMP with proliferation and synthesis of glycosaminoglycans (Greene et al., 1982; Pisano and Green, 1986). A recent study (Xing et al., 1996) showed that MAPK also phosphorylates and activates CREB kinase, which in turn phosphorylates and activates CREB, suggesting that biological phenomena (i.e., synthesis of glycosaminoglycans) previously thought to be regulated by PKA may also involve the MAPK signaling cascade. Since the data of the present study in quail, as well as those from mammal (Young et al., 1994), show that MAPK was activated during palate development, it is plausible
that the CREB phosphorylation may be partially due to MAPK, as well as PKA. Interestingly, an increase in PKA activity may lead to an attenuation of MAPK activity (Sevetson et al., 1993). In the mammalian palate, where the PKA and cAMP levels are high (references cited above), it is possible that PKA may inhibit MAPK, allowing the cells to differentiate. In such a scenario, MAPK may be supporting cell proliferation and when PKA is activated the possible resulting inhibition of MAPK activity could result in the loss of the required signals to sustain cell proliferation. Currently, there are no data in the literature on the involvement of PKA during avian palate development. Such information may be of crucial significance not only in determining the role of MAPK, but also in understanding the role of PKA during the morphogenesis of avian palate.

Another potential way by which MAPK may be activated is through an indirect mechanism involving the extracellular matrix. Recent evidence suggests that the extracellular matrix itself can regulate signal transduction pathways. For example, in addition to the classical mechanism of integrin modulation of cytoskeletal organization, it is now recognized that many integrin mediated changes in cell growth and differentiation may be the result of integrin-mediated signal transduction (Julioan and Haskill, 1993). Several reports have shown that integrin binding to substrates causes changes in intracellular patterns of tyrosine phosphorylation (Guan et al., 1991; Kornberg et al., 1991; Lipfert et al., 1992). Also, Morino et al (1995) observed an activation of p42 and p44 MAPK isoforms via β1 integrin crosslinking. This becomes important when put in context of palate development in quail, where it has been shown that extracellular matrix accumulates with advancing morphogenesis (Young et al., 1994), and may perhaps modulate the activity of MAPK and other signaling pathways through integrin mediated signaling.

However, the main pathway for the growth factor regulated tyrosine kinase receptor to transduce its effect appears to be through the Ras\Raf\MEK\MAPK signaling cascade, which
subsequently activates transcription factors involved in the cell cycle (Mordret, 1993). Since, with the exception of a study by Shah et al (1996), showing an absence of TGFβ1 in the developing quail palate, there are no studies in the literature that have examined whether any growth factors are present during avian palatogenesis, the mechanism by which MAPK is activated during in vivo quail palate development still requires elucidation.

In addition to MAPK, other protein kinases may also be involved in the regulation of palate development. Indeed, phosphotransferase assays and Western immunoblots analysis in the present study showed that the in vivo CK2 phosphotransferase activity changed during morphogenesis of the quail palate. Unlike MAPK, the CK2 activity was high during early palate formation (day 5 and 6 of incubation), and then decreased as palate development progressed (days 7 through 9 of incubation). A similar profile of CK2 activity was also observed during mammalian palate development (Young et al., 1996). The elution profile of phosvitin phosphotransferase activity in the MonoQ fractionated sample corresponded to that reported earlier by Sanghera and associates (1992). In addition, Western blot data of these fractions confirm the identity of CK2 α and α' with molecular masses of 42 and 38 kDa, which corresponded to CK2 in other species (Kandror et al., 1989; Charlton et al., 1992). The results further showed that the relative intensity of the bands on the Western blots was the same through all 5 days of incubation, suggesting that the decrease in CK2 activity during palate development did not originate from an altered rate of transcription or protein degradation as palate formation progressed. In addition, the data of the present study show that, as in mammals (Shah et al., 1994b), a large number of mesenchymal cells withdrew from the proliferative pool with the advancing morphogenesis of palate in quail. The CK2 activity correlated well with cell proliferation activity during quail palate morphogenesis. A similar correlation between CK2 activity and cell proliferation was also noted during palate morphogenesis in mammals (Young et al., 1996). These data suggest the involvement of CK2 in the regulation of cell
proliferation during development of the quail secondary palate.

Only a few studies have been reported on the involvement of CK2 in developing systems (Schneider et al., 1986; Perez et al., 1987; Hu and Rubin, 1990, 1991; Maridor et al., 1991; Mestres et al., 1994). These studies show that CK2 activity is high during early embryogenesis (i.e., when cells are proliferating rapidly) and declined with advancing development. The observation of declining CK2 activity during advancing morphogenesis of vertebrate palate (Young et al., 1996, present study) are thus consistent with the general observations made during embryogenesis. Also, there are numerous investigations that demonstrate an association of CK2 activity with cellular proliferation. For example, Lorenz et al (1993) observed that the concentration of CK2 was two to three times greater in exponentially dividing cells than quiescent cells. Pepperkok and co-investigators (1994) noted that progression of the cell cycle required the presence and specific localization of a functional level of CK2 at the transition of $G_0/G_1$, early $G_1$ and $G_1/S$ phases. Additionally, levels of CK2 enzyme activity are elevated in a variety of tumor cell populations (Prowald et al., 1984; Schneider et al., 1986) and in rapidly proliferating tissues (Perez et al., 1987; Munstermann et al., 1990). Indeed, Schneider and Issinger (1989) suggested that CK2 may be a "proliferation marker". The observations of the present study, as well as those made in embryonic mammalian palate (Young et al., 1996), that the activity of CK2 correlated with cell proliferation as quail palate development progressed, may also be viewed in this light. As in other systems, it is plausible that the activation of CK2 may also be related to the regulation of the cell cycle in the proliferating palatal mesenchymal cells of quail (as well as mammals). This possibility is reasonable since CK2 phosphorylates a number of important proteins involved in transcriptional control, which are vital for progression of the cell cycle (Meisner and Czech, 1991). For instance, phosphorylation of CK2 by cdc2, a protein crucial for $G_2/M$ phase transition, results in a 2-3 fold increase in CK2
activity (Mulner-Lorillon et al., 1990). In addition, meiosis in *Xenopus* oocytes is accompanied by a 10-fold increase in CK2 activity (Kandror et al., 1989). Also, when both the α and α' CK2 subunits are knocked out in *Saccharomyces cerevisiae*, the cells grow in size but cannot divide, and subsequent introduction of the CK2 α subunit from Drosophila allows for cells to progress through the cell cycle (Padmanabha et al., 1990). These results imply a crucial role for CK2 in the regulation of cell proliferation, and the cell cycle.

The results of the present study further showed that CK2 was activated after exposure of QPMC to EGF. There was a two-fold increase in phosphotransferase activity towards phosvitin following EGF treatment. Western blotting with a CK2 antibody showed that this phosphotransferase activity was indeed due to CK2. Others have shown, in a variety of biological systems, that CK2 activity is rapidly and transiently elevated in response to external signals such as EGF, insulin, or insulin-like growth factor (Sommercorn et al., 1987; Klarlund and Czech, 1988; Ackerman et al., 1990), PMA (Carroll et al., 1988), and hormones (Sommercorn et al., 1987, Klarlund and Czeck, 1988). Recently, Litchfield et al (1994) suggested that growth factor-induced regulation of CK2 activity may differ from that of other protein kinases. They stimulated WI.38 or A431 cells with different growth factors and could not obtain a stimulation of CK2 activity. One may point out, however, that Litchfield et al (1994) measured the CK2 activity only in crude (unfractionated) protein samples that also contain various other proteins and phosphatases, which may interfere with the phosphorylation of the substrate by CK2. It has been shown that CK2 requires isolation from possible inhibitors before its activation can be measured (Klarlund and Czech, 1988; Ackerman and Osheroff, 1989). Indeed, in the present study, in initial time course experiments with EGF, no activation of CK2 could be elicited (data not shown). Only after MonoQ elution did we demonstrate a substantial increase in CK2 activity.

The precise mechanism by which CK2 activity may be regulated during palate
development is unclear. Pisano and Greene (1987) observed that EGF stimulates the activity of ornithine decarboxylase (ODC), which in turn, stimulates glycosaminoglycan (GAG) synthesis in palatal cells. Subsequently, Gawel-Thompson and Greene (1989) showed that exogenous polyamines inhibited ODC activity in palatal cells. The activity of CK2 is, however, stimulated by polyamines and inhibited by heparin and sulphated GAGs (Hathaway et al., 1983). Since an increase in synthesis and/or accumulation of specific GAGs occurs at a later time during palate formation in both mammals and birds (Brinkley and Morris-Wiman, 1984; Foreman et al., 1991; Young et al., 1994), it is plausible that a reduced activity of CK2, which can phosphorylate and inhibit ODC (Tuazon and Traugh, 1991; Allende and Allende, 1995), may indeed also facilitate the synthesis/accumulation of GAG at later stages of morphogenesis of palate in quail. In this setting, the GAGs would themselves stimulate their own production, in a feedback loop, inhibiting CK2 activity, thus releasing the block on ODC activity and subsequent GAG synthesis. Further, CK2 has been shown to phosphorylate the regulatory subunit of cAMP dependent protein kinase, PKA (Carmichael et al., 1982); and regulation of PKA seems to be important for increased synthesis of GAG observed during the later part of palate development (Greene et al., 1982, Greene et al., 1991). It is possible that the activities of both CK2 and PKA may be regulated in a way so as to coordinate various growth and differentiation events during palate development.

Although the precise role of CK2 in regulation of cellular activities is unclear, it has been suggested that CK2 may be involved, either directly or indirectly, in the regulation of the cell cycle. For example, CK2 can phosphorylate cdc2 on the region that binds to cyclin; this may prevent the association between cdc2 and its cyclin counterpart, and inhibit the G1/S phase transition (Marshak and Russo, 1994). In an earlier study, activity of cdc2 was localized in the developing palate of quail (Hehn et al., 1995). Whether CK2 phosphorylates cdc2 during
quail palate morphogenesis, however, is not yet known. Furthermore, CK2 can also be phosphorylated and activated in vitro by purified p34cdc2, which is involved in regulating progression of cells through the cell cycle (Mulner-Lorillon, 1990; Meggio et al., 1995). More recently, Gotz et al. (1996) noted an association between cyclin-dependent kinase (CDK) inhibitor p21 and the β subunit of CK2. Taken as a whole, these observations suggest that CK2 may act in concert with CDK to regulate the cell cycle. In addition, CK2 activity can be inhibited by cAMP, thus indicating a opposite role for PKA in signal transduction via CK2 (Caroll et al., 1988). These interrelated phosphorylations between PKA, CK2, CDKs, and cyclin dependent inhibitors (i.e., p21), suggests the existence of a complex network of cell cycle regulatory proteins, which may modulate cell proliferation in a biological system.

**Signaling Mechanisms During Palate Development: A Synthesis**

The present study has considered the involvement of two second messenger independent protein kinases, MAPK and CK2, during the morphogenesis of secondary palate in quail. Both these kinases have been implicated in coordination of cell growth, proliferation and differentiation. To further clarify the manner in which different protein kinases may interrelate to coordinate various cellular activities during palate morphogenesis, an attempt is made to integrate the observations of the present study with those reported in the literature into a general scheme of signaling mechanisms that may regulate some of the biological behaviour of palatal cells during vertebrate palate development.

Figure 25 summarizes information on signaling molecules, much of which is derived from studies in mammals (Greene and Lloyd, 1985; Chepenik and Grunwald, 1988; Chepenik and Haystead, 1989; Greene et al., 1991; Chepenik et al., 1994, Young et al., 1996) and birds (present study) during palate morphogenesis. The interconnections between the signaling molecules, known to be involved in palate development, are shown in blue. In addition,
Figure 25. Potential cross-talk between signaling pathways in embryonic palatal mesenchymal cell. References cited in text.
on the basis of general literature on signaling mechanisms, hypothetical connections between the signaling molecules that could regulate the behaviour of palatal cells have been noted in red. All the extracellular molecules displayed in the figure 25 have been localized in the developing vertebrate palate. In order to maintain simplicity and clarity, putative spatio-temporal interconnections between molecules involved in the regulation of various biological events of palate morphogenesis have not been included. Also, only the behaviour of mesenchymal cells have been analyzed. Since it is generally acknowledged in the literature that the structure and function of the signaling molecules indicated in figure 25 are evolutionarily conserved (Hunter, 1987; Hanks et al., 1988; Taylor et al., 1992; Hanks and Hunter, 1995), it is speculated that they may also be involved in the regulation of palate formation in all vertebrates.

The most well studied regulatory protein in the embryonic palatal cells is PKA, which has been shown to be expressed as two isozymes, and whose expression patterns changed during development. It was observed that exogenous cAMP accelerated differentiation of the mammalian palatal MEE (Pratt and Martin, 1975) as well as mesenchymal cells (Greene et al., 1982). Subsequently, it was shown that catecholamine treatment of cultured palatal mesenchymal cells, which increases cAMP concentrations, resulted in delay of entry into the S phase of the cell cycle (Pisano et al., 1986). One can speculate as to a possible connection between the increased cAMP concentration and MAPK activity, since an increase in cAMP has been found to block activation of Raf-1 via a PKA-dependent Raf-1 phosphorylation. The PKA-dependent phosphorylation of Raf-1 reduces the affinity of Raf-1 to interact with Ras (Wu et al., 1993). Several reports have substantiated the inhibitory effect of PKA on the MAPK pathway (Burgering et al., 1993; Cook and McCormick, 1993; Seetson et al., 1993, Graves et al., 1993, Hordijk et al., 1994). An experiment to test both the activity of PKA and MAPK in palatal cells would help clarify the connection. A second link for the Ras and cAMP mediated signaling pathways is introduced by the recent observation that MAPK has been shown to
phosphorylate cAMP specific phosphodiesterase (PDE) (Lenhart et al., 1996), although a functional significance to this phosphorylation has not yet been established. It is important to note that the phosphodiesterase requires hierarchical phosphorylation of an additional site near the MAPK site, the amino acid sequence at this site confers that of CK2 specificity (Lenhard et al., 1996). At a downstream level, PKA is capable of regulating the activity of CREB. Interestingly, CREB activity can also be stimulated by the MAPK pathway (Xing et al., 1996). CREB may work coordinately with Elk-1, a transcription factor phosphorylated and activated by MAPK, to stimulate the transcription of the immediate early gene, c-fos, which subsequently upregulates the transcription of many genes involved in the cell cycle (Herschman, 1991; Edwards, 1994). Greene et al (1995) have recently identified an increase in the phosphorylation of CREB with advancing palate development, which correlates to the trend in MAPK activity observed in the present study. Whether or not MAPK is responsible for this phosphorylation requires further investigation. A further twist to this complex regulation is that p90\textsuperscript{rsk}, a protein kinase downstream of MAPK, binds to the coactivator CREB-binding protein, and is necessary for induction of Ras-responsive genes (Janknecht and Hunter, 1996).

A recently elucidated route for MAPK activation is via G-protein coupled receptors in Ras-dependent and Ras-independent mechanisms (Crespo et al., 1994; Faure et al., 1994; Koch et al., 1994; van Biesen et al., 1995). These route are potentially important, since palatal cells respond to catecholamines (isoproterenol, norepinephrine, and epinephrine) with increases in adenylate cyclase activity (Garbarino et al., 1984), which is probably mediated by G-protein interactions with the adrenergic receptors. G proteins can induce a variety of cellular responses, including: induction of immediate early genes, regulation of cell-specific genes, stimulation of DNA synthesis, cellular transformation, and cellular hypertrophy (Post and Brown, 1996). Based on the likelihood of important signal transduction pathways originating from these heterotrimeric G proteins, there is a need for work in this area in palate
research.

MAPK has also been shown to regulate the activity of phospholipase A2 (PLA2), via phosphorylation of PLA2 on Ser 505. Chepenik et al (1994) has detected PLA2 in murine embryonic palatal mesenchymal cells in culture, where its activation appears to be modulated by EGF. The activity of PLA2, in turn, results in the synthesis of prostaglandins, which have been found to stimulate an increase in cAMP in palatal mesenchymal cells. Thus MAPK, by modulating PLA2 activity, may indirectly modulate the activation of PKA.

Chepenik and Haystead (1989) have identified a molecule that may be PKC in embryonic palate, and they proposed a role for it in palate development. PKC has been shown to modulate the activity of both the MAPK and PKA signaling pathways (Marquardt et al., 1994; Berra et al., 1995).

The TGF-β family has been localized and studied thoroughly during in vivo and in vitro mammalian palate development. Recently, several studies have suggested that TGF-β1 may regulate the activity of MAPK by an unknown pathway (Yan et al., 1994; Hartsough and Mulder, 1995; Berrou et al., 1996). Berrou et al (1996) found that TGF-β1 inhibited MAPK induction by bFGF in smooth muscle cells. It is promising to suggest that the observed attenuation of cell proliferation in palatal mesenchymal cells in culture after TGF-β1 treatment (Linask et al., 1991) may work indirectly through an affect on the MAPK pathway, whose activity may be required for continued cell proliferation. However, since TGF-β1 is absent in quail palate (Shah et al., 1996), its putative biological role may only relate to mammalian palate development.

CK2 appears to have an important role in modulating cell proliferation (Lorenz et al., 1993; Pepperkok et al., 1994). Our findings concur and suggest a role for CK2 in regulating
palatal cell proliferation. Among the potential substrates for CK2 are cdc2 and CREB, as well as PDI, thus linking up CK2 with different components of the PKA and MAPK signaling pathways. In the present study, both CK2 and MAPK activity were stimulated by EGF treatment, however, the duration and subsequent function of these protein kinases in embryonic palatal mesenchymal cells requires elucidation.
REFERENCES


